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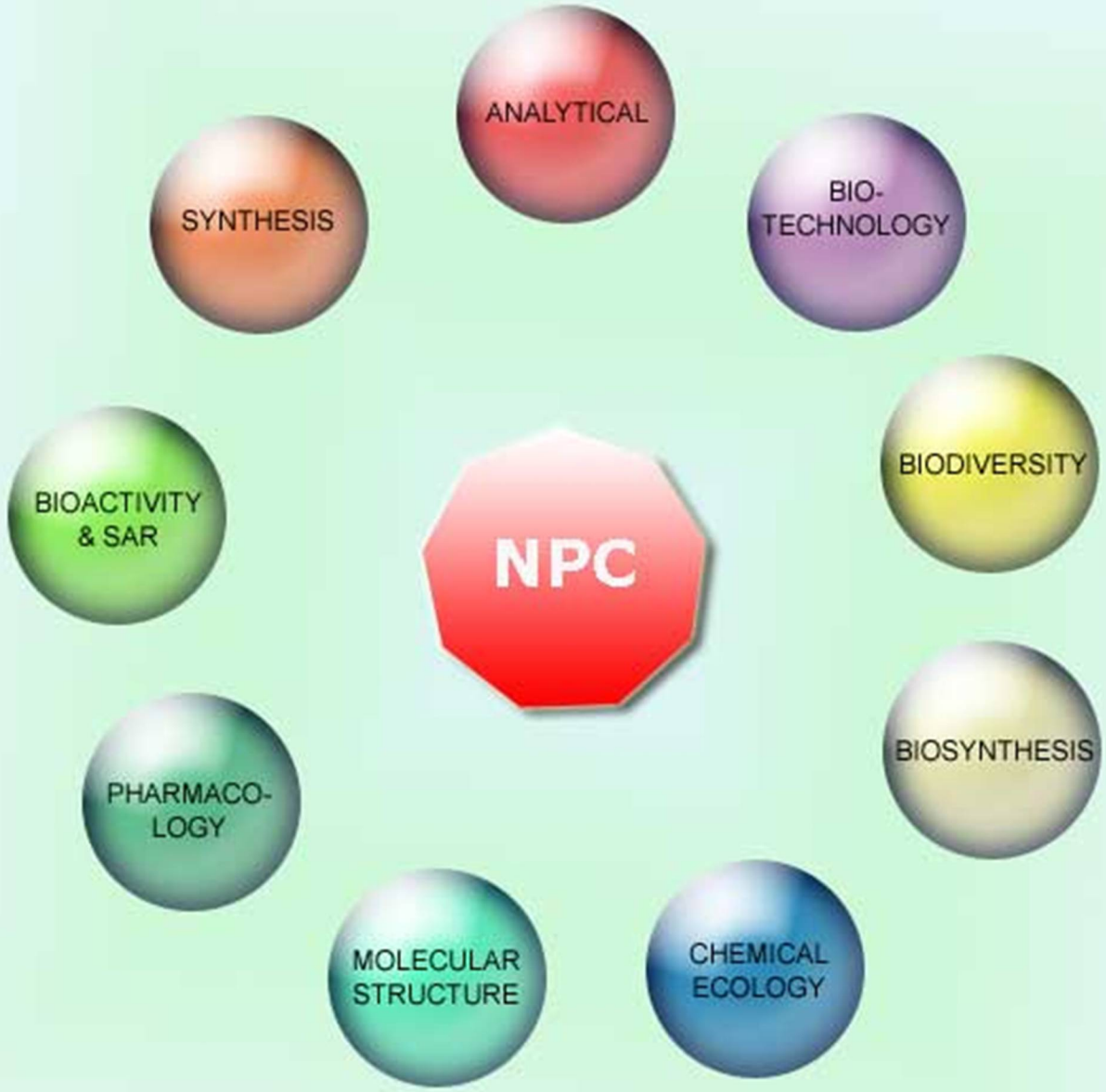

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Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence

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The anticonvulsant activity of the racemate and enantiomers of linalool have been evaluated. Pretreatment of the mice with (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool increased the latency of convulsions significantly in the PTZ model. Only *rac*-linalool had an effect at the dose of 200 mg/kg. The enantiomers and their racemic mixture were effective in inhibiting the convulsant effect of PTZ at the dose of 300 mg/kg. The linalools presented pharmacological activity close to that of diazepam. In the PIC seizure model, (*R*)-(-)-linalool and *rac*-linalool presented activity at the dose of 200 mg/kg, but the *rac*-linalool was more potent than (*R*)-(-)-linalool; (*S*)-(+)-linalool had no effect at this dose. On the other hand, at the dose of 300 mg/kg this enantiomer was effective, but less potent than (*R*)-(-)-linalool and *rac*-linalool. In the MES model, linalools decreased the convulsion time of the mice in the doses of 200 and 300 mg/kg. *rac*-Linalool presented maximum effect at 300 mg/kg. Surprisingly, it increased significantly the convulsion time at a dose of 100 mg/kg. Using the parameter of tonic hind convulsions, only (*R*)-(-)-linalool produced protection from tonic extension at the dose of 200 mg/kg. When the (+)- and (-)-enantiomers, and *rac*-linalool were administered at the dose of 300 mg/kg they were also effective in preventing tonic convulsions induced by transcorneal electroshock in the animals. The (+)- and (-)-forms were equipotent and the *rac*-linalool was more effective than phenytoin. We have demonstrated that the two enantiomers have similar qualitative anticonvulsant activity, but show different potencies.

Keywords: epileptic seizure, anticonvulsant activity, terpenes, essential oils, structure-activity relationship.

In folk medicine as well as in phytotherapy, essential oils have been utilized as therapeutic agents to treat many diseases. They are used, for example, as sedatives, relaxants, and anticonvulsants [1,2]. Many essential oils possess a great variety of pharmacological activities, such as anxiolytic [3], anticonvulsant[2], and antinociceptive [4]. Evidence for the effects of their components on animal behavior has been supplied. Compounds such as linalool [5], α-terpineol [6], and citronellol [7] have anticonvulsant activity, while (+)-limonene [8] and (+)-pulegone [9] show analgesic properties, and isopulegol present an anxiolytic-like effect [10]. The monoterpene derivatives also have been shown to have several effects on the central nervous system (CNS), including antinociceptive [11], sedative [12], and antidepressant [13] activity.

A significant number of herbal medications and dietary supplements are used for treating patients with neurological and psychiatric complaints. Some of these products may be anticonvulsant and thus of possible benefit to patients with epilepsy. There are many studies that report the antiseizure activity of a nonallopathic preparation with animal models of epilepsy. Several of these medications are prepared with plants that contain linalool [14].

Linalool (3,7-dimethyl-1,6-octadien-3-ol), one of the most common terpene alcohols (found in rosewood, linaloe, and lavender), exists in two enantiomeric forms [1,14]. (*R*)-(-)-linalool (licareol) occurs in many essential oils, such as orange blossom flower and lavender flower and has a fine fresh floral odor-character. (*S*)-(+)-Linalool (coriandrol) occurs in coriander fruits and has a more herbal, floral odor-note [15-17]. Linalool is a fragrance ingredient used in many fragrance products as well as in non-cosmetic materials, such as household cleaners and detergents [18].

Pharmacological assessment of chiral compounds in an early research phase can lead to the selection of a single isomer for development. This selection process can

maximize the potential for specific activity and minimize the potential for side-effects. For various reasons, however, many racemates have been developed and for these compounds the pharmacological picture is considerably more complex than that of the single enantiomer. Therefore, in the present paper, we studied the anticonvulsant properties of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool in three animal models of convulsion exploring differences between the enantiomers and racemic mixture with respect to their pharmacological effects.

Because the linalool enantiomers are common in many plant species, and are used in cosmetic, non-cosmetic, and pharmaceutical preparations, as well as in the food industry [19-21], it is interesting and important to know the effects and the enantioselectivity of the convulsion receptors in relation to these monoterpenes. Chiral recognition by receptors and enzymes is well demonstrated in biochemical, pharmaceutical, and chemosensory research. We report in this comparative study the findings of the central effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on the behavioral parameters of these anticonvulsant drugs in mice.

We first investigated the anticonvulsant activity involved in the PTZ-induced seizures model. The administration of PTZ caused clonic convulsions in mice. As shown in Figure 1, pretreatment of the mice with (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool increased the latency of convulsions significantly. Only *rac*-linalool ($p<0.05$) had an effect at the dose of 200 mg/kg [$F(3.27) = 3.747$; $P = 0.02$]. This indicates that the largest potency of the *rac*-linalool is determined by the synergistic action of its constituents. When the enantiomers and their racemic mixture were administered at the dose of 300 mg/kg they were also effective in inhibiting the convulsant effect of PTZ [$F(3.23) = 14.55$; $P < 0.0001$]. The linalools presented pharmacological activity close to that of diazepam ($p<0.001$), a standard anticonvulsant drug. PTZ is the prototype agent in the class of systemic convulsants, and is used as a screening test for anticonvulsants. Generally, compounds with anticonvulsant activity in petit mal epilepsy are effective in the PTZ-induced seizure model [22-24]. So (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool may be useful in petit mal epilepsy.

The anticonvulsant activity was also evaluated in the PIC seizure model. (*R*)-(-)-Linalool and *rac*-linalool presented activity at the dose of 200 mg/kg (Figure 2), but the *rac*-linalool ($p<0.001$) was more potent than (*R*)-(-)-linalool ($p<0.05$). (*S*)-(+)-Linalool had no effect at this dose. On the other hand, at the dose of 300 mg/kg [$F(3.28) = 27.23$; $P < 0.0001$], this enantiomer was effective ($p<0.01$) but less potent than (*R*)-(-)-linalool

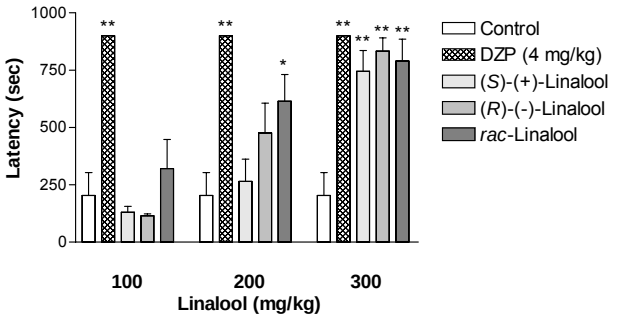

Figure 1: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on PTZ-induced seizure in mice. Values are the latency of convulsions. Values are presented as mean ± S.E.M. for 8 mice; *$p<0.05$, **$p<0.01$, when compared with vehicle (control), one-way ANOVA, followed by Tukey's test.

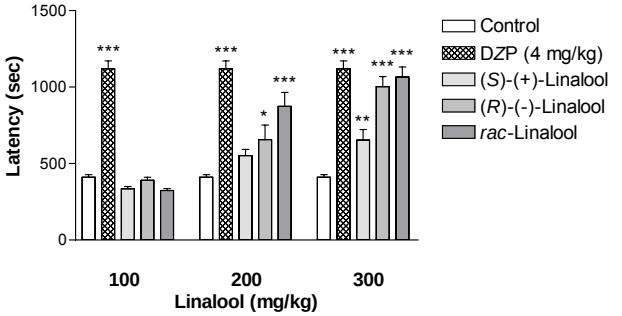

Figure 2: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on PIC-induced seizure in mice. Values are the latency of convulsions. Values are presented as mean ± S.E.M. for 8 mice; *$p<0.05$, **$p<0.01$, ***$p<0.001$, when compared with vehicle (control), one-way ANOVA, followed by Tukey's test.

($p<0.001$) and *rac*-linalool ($p<0.001$). Again, a synergistic action was observed. The present result also shows that the chiral center at carbon 3 in the linalool molecule is important in the interaction with the receptor. The molecule with the *R*-configuration hydroxy group at carbon 3 is clearly more bioactive than the molecule with the *S* configuration. PIC seizure is a popular systemic convulsant model and is known to be a GABA antagonist exerting its effect by binding to the picrotoxin binding site, which is closely related to the chloride ionophore in the $GABA_A$ receptor complex. Classical anticonvulsants such as carbamazepine, phenytoin and DZP have a protective effect against PIC-induced seizures [25].

In another model, MES seizure, (*S*)-(+)-linalool ($p<0.001$), (*R*)-(-)-linalool ($p<0.001$), and *rac*-linalool ($p<0.001$) decreased the convulsion time of the mice in the doses of 200 [$F(3.28) = 21.40$; $P < 0.0001$] and 300 mg/kg; *rac*-Linalool presented maximum effect at 300 mg/kg. Surprisingly, it also increased significantly the convulsion time at the dose of 100 mg/kg [$F(3,28) = 100.5$; $P < 0.0001$] (Figure 3). Using the parameter of tonic hind convulsions, only (*R*)-(-)-linalool produced protection from tonic extension of about 62.5% ($p<0.05$) at the dose of 200 mg/kg.

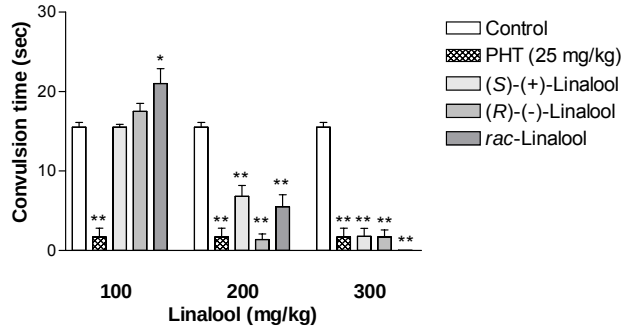

Figure 3: Effect of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on the convulsion time induced by electroconvulsive shock.. The bars indicate mean ± S.E.M. (n = 8). Statistically significant differences *p<0.05, **p<0.001 with respect to control according to one-way ANOVA, followed by Tukey's test.

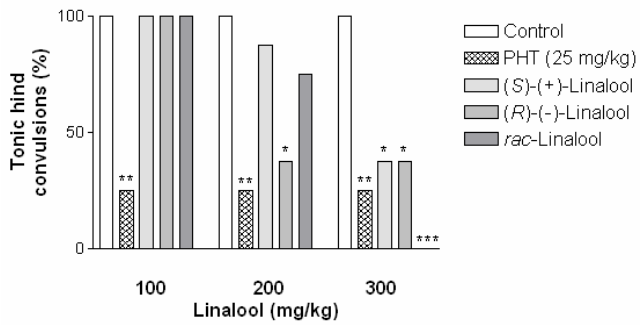

Figure 4: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on tonic convulsions induced by electroconvulsive shock. The bars indicate percentage values. Statistically significant differences at *p<0.05, **p<0.01, ***p<0.001 (Fisher's exact test).

When the (+)- and (-)-enantiomers, and *rac*-linalool were administered at the dose of 300 mg/kg they were also effective in preventing tonic convulsions induced by transcorneal electroshock in the animals, producing 62.5% (p<0.05), 62.5% (p<0.05), and 100% (p<0.001) protection, respectively. The (+)- and (-)-forms were equipotent and the *rac*-linalool was more effective than phenytoin [75.0% (p<0.01)], a standard anticonvulsant drug (Figure 4). Again the influence of the stereogenic center at carbon 3 and the synergistic action was shown. The MES method is arguably the best-studied and most useful animal model of seizures. Drugs able to inhibit MES seizures in mice are considered to be candidate therapies for primary and secondarily generalized tonic-clonic epilepsies [22].

Chirality has been used to see whether more specific agents can be found for subsets of GABA-receptors. A higher affinity for these receptors results in a better pharmacological profile of the drug. The PTZ and PIC seizure models showed the involvement of the GABA-receptor in the anticonvulsant activity of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool [26]. It has been reported that linalool also acts on glutamatergic transmission. This monoterpene significantly reduces potassium-stimulated glutamate release, as well as glutamate uptake, but not interfering with basal glutamate release [27]. It also inhibited and delayed the behavioral expression of PTZ-kindling, but did not modify the PTZ-kindling-induced increase in L-[3H]glutamate binding [28]. Therefore, linalool acts through different mechanisms of action to inhibit the action of various convulsant agents.

The pharmacological differences between linalool enantiomers have been observed on physiological parameters of stress [29] and sedative effect in humans [17]. The chiral influence of other optically active monoterpenes on behavior experimental models also was demonstrated [9,30]. Interestingly, the combined results showed that (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool inhibit not only the action of pentylenetetrazol and picrotoxin (chemical-convulsions), but also protect the mice against MES-induced seizures. This feature may be of interest when one thinks of a good candidate for a drug designed to cause neuroprotection in response to various pro-convulsive agents. The MES and PTZ procedures are of predictive relevance regarding the clinical spectrum of activity of the investigated compounds [31].

In conclusion, we have found that (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool may be effective in blocking generalized tonic-clonic partial and generalized clonic seizures. Our data also demonstrate that the two enantiomers have similar qualitative anticonvulsant activity, but differ in their potencies. The administration of a single enantiomer appears to offer no advantage over the racemic mixture.

Experimental

Chemicals: Pentylenetetrazol (PTZ), phenytoin (PHT), picrotoxin (PIC), Tween 80 and diazepam (DZP) were purchased from Sigma Chemical Co. (USA). (*R*)-(-)-Linalool $[\alpha]_D^{23}$ = -16.2° (*c* 4.2, $CHCl_3$) and *rac*-linalool were purchased from Sigma Aldrich Chemical Co. (USA) and Dierberger (Brazil), respectively. (*S*)-(+)-Linalool (1.23 g) was isolated by column chromatography on silica gel (*n*-hexane/ethyl acetate, 9 : 1 v/v) from *Coriandrum sativum* L. oil (3.15 g) [17]. (*S*)-(+)-Linalool $[\alpha]_D^{23}$ = +13.6° (*c* 2.7, $CHCl_3$) was identified on the basis of chromatographic (TLC) behavior, optical rotation, and spectroscopic data (^{1}H and ^{13}C NMR and IR). These assignments agreed with literature values [32] and data of (*R*)-(-)-linalool. All drugs were injected intraperitoneally (ip).

Animals: Adult, male, 3 month old, albino Swiss mice weighing 24–30 g, were used throughout this study. The animals were randomly housed in appropriate cages at 21±1°C on a 12 h light cycle with free access to food (Purina – Brazil) and tap water. This study used 360

animals that were placed in groups, each of eight animals. All animals were acclimatized before the experiments and the behavioral observations were conducted between 08:00 and 13:00 h. Experimental protocols and procedures were approved by the Laboratório de Tecnologia Farmacêutica Animal Care and Use Committee (CEPA/LTF-UFPB #1205/06).

Statistical analysis: The behavioral data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's Test. The incidence of clonic or tonic convulsions were evaluated by Fisher's exact test. Differences were considered to be statistically significant when $p<0.05$.

Pentylenetetrazol (PTZ)-induced convulsions: Mice were divided into 5 groups (n = 8). The control and positive control groups received 5% Tween 80 and diazepam (4 mg/kg), respectively. The remaining groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool at doses of 100, 200 and 300 mg/kg. Thirty min after drug administration, the mice were treated with PTZ (ip) at a dose of 60 mg/kg and observed for at least 15 min to detect the occurrence of the first episode of forelimb clonus [33].

Picrotoxin (PIC)-induced convulsion: Animals were divided into 5 groups (n = 8). The first group served as a control and received saline with one drop of Tween 80, while the second group was given diazepam (4 mg/kg, ip). The remaining groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool (100, 200 and 300 mg/kg, ip). After 30 min of administration, the mice were challenged with picrotoxin at a dose of 8 mg/kg (ip). Immediately after the picrotoxin injection, mice were individually placed in plastic boxes and observed for the onset of clonic seizures [34].

Maximal electroshock (MES)-induced convulsion: The maximal electroshock (MES) protocol to produce convulsions characterized by tonic hindlimb extension was used [33]. Electroconvulsive shock (130V, 150 Hz, for 0.5 s) was delivered through auricular electrodes (ECT UNIT 7801 – Ugo Basile). Mice were divided into 5 groups (n = 8). The first group served as a control and received saline with one drop of Tween 80, while the second group was treated with phenytoin (25 mg/kg, ip) and the other groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool (100, 200 and 300 mg/kg, ip). After 30 min all groups received the electroconvulsive shock. The convulsion time (s) and percentage of animals showing tonic convulsions, characterized by the presence of tonic hind limb extension, was carefully observed. The animals that did not exhibit tonic hindlimb extension were considered protected [35]. Phenytoin and saline were used as positive and negative controls, respectively.

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Kinetic Analysis of Genipin Degradation in Aqueous Solution

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Degradation of genipin (GP), a low toxicity natural protein crosslinking agent, in aqueous solution was monitored by HPLC at various pH levels. Degradation of GP was consistent with a mechanism consisting of a first order reaction with a reversible first step. Formation of the intermediate was slowest at more neutral pHs while formation of the irreversible product was correlated to increasing alkalinity. Degradation at all pHs was enhanced by the presence of phosphate ions. Degradation of GP most likely proceeds via the reversible opening of the dihydropyran ring by water followed by irreversible polymerization of the intermediate. Degraded solutions containing no detectable GP or intermediate, however, are still capable of crosslinking proteins.

Keywords: Genipin, crosslinking, degradation, kinetics.

Genipin (GP), whose structure is depicted in Figure 1, is the aglycone of geniposide [1,2], an iridoid glycoside that is a major component of the fruit of the gardenia plant, *Gardenia jasmindides* Ellis. GP is a potent and yet relatively non-toxic [3,4] crosslinker of proteins [5] and has elicited a great deal of interest in areas as diverse as tissue engineering [4,6], biomechanics [7,8], forensics [9] and leather tanning [10]. In addition, GP may be a promising therapeutic for the treatment of type-2 diabetes [11]. Although a complete chemical synthesis of GP has been reported [12], the process is not commercially viable and GP is still produced from plant-derived geniposide by enzymatic deglycosylation [13].

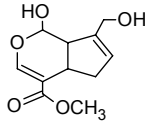

Figure 1: Structure of Genipin.

In order for GP to be utilized in both pharmacological and non-pharmacological applications, some information on the stability of GP in aqueous solution would be valuable as a basis for the design of both products with acceptable shelf lives and crosslinking reaction schemes. Although it has been reported that GP is particularly labile under both acidic [1] and alkaline conditions [14,15], its degradation kinetics have never been studied in detail. This paper reports on the stability of GP in aqueous solution.

When GP was incubated at 24°C and pH 9 it degraded rapidly (Figure 2), but did not appear to obey the first order rate law since the correlation coefficient obtained from an exponential fit (Figure 2, broken line) was only 0.9952. The first order equation approximated the data obtained at later time points but deviated substantially in the earlier samplings.

A number of possible reaction mechanisms might better describe this data, including that of a sequential reaction with a reversible first step as depicted in Equation 1.

$$GP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} GP^* \xrightarrow{k_3} X \qquad (1)$$

In this reaction GP is converted into a hypothetical intermediate (GP*) which can either reform GP or be degraded to a second product (X) in an irreversible step. The kinetics of such a reaction are governed by three kinetic constants; k_1 for the conversion of GP to GP*, k_2 for the reversion of GP* to GP and k_3 for the breakdown of GP* to X.

This reaction is described mathematically by equations 2-6, and thus the concentration of the three molecules in this reaction scheme can be determined using equations

2, 3 and 4 respectively [16], which express the concentration of each molecule at time t with respect to the starting GP concentration ($[GP]_0$) and the three rate constants.

$$[GP]_t = [GP]_0 \left(\left[\frac{\gamma_1 - k_2 - k_3}{\gamma_1 - \gamma_2} \right] e^{-\gamma_1 \cdot t} + \left[\frac{k_2 + k_3 - \gamma_2}{\gamma_1 - \gamma_2} \right] e^{-\gamma_2 \cdot t} \right) \quad (2)$$

$$[GP^*]_t = \frac{k_1 [GP]_0}{\gamma_1 - \gamma_2} \left(e^{-\gamma_2 \cdot t} - e^{-\gamma_1 \cdot t} \right) \quad (3)$$

$$[X]_t = k_1 k_3 [GP]_0 \left(\left[\frac{-1}{\gamma_2 (\gamma_1 - \gamma_2)} \right] e^{-\gamma_2 \cdot t} + \left[\frac{1}{\gamma_1 (\gamma_1 - \gamma_2)} \right] e^{-\gamma_1 \cdot t} + \frac{1}{\gamma_1 \gamma_2} \right) \quad (4)$$

Where:

$$\gamma_1^2 - (k_1 + k_2 + k_3)\gamma_1 + k_1 k_3 = 0 \quad (5)$$

$$\gamma_2 = \frac{k_1 k_3}{\gamma_1} \quad (6)$$

To determine rate constant values, data was fitted iteratively to Equation 2 using Octave software. This produced an accurate fit (R^2=0.99994) with values of 0.52, 1.24 and 0.99 hr^{-1} for k_1, k_2 and k_3 respectively (Figure 2, solid line), suggesting that Equation 2 is a more accurate description of GP degradation than simple first-order decay. A wavelength scan of the degraded GP sample did not reveal the presence of new light-absorbing species in the 200-900 nm range and so subsequent experiments were limited to measuring the disappearance of GP and not the appearance of GP* or X.

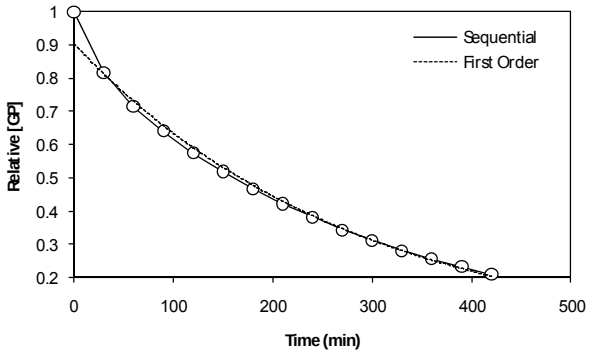

Figure 2: Degradation of GP at pH 9. GP was incubated at 24°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted exponentially (First Order) or to Equation 2 (Sequential).

GP degradation was strongly influenced by pH and was more rapid under alkaline conditions (Figure 3). Interestingly, however, while stability increased with increasing acidity from pH 9 to 6, stability at pH 4 and 5 appeared to be comparable to that at pH 7. These experiments were repeated several times at each pH and the average value of each rate constant and the half-life ($t_{½}$) of GP under each condition were determined (Table 1). These data suggested that this effect was due to a relative increase in the k_1 rate constant at pH 4 and 5 relative to pH 6 and that the apparent decrease in stability observed under the more acidic conditions was due to a more rapid establishment of the GP/GP* equilibrium and not to irreversible degradation. In fact, the k_3 constant decreased substantially at pH 4 and 5, suggesting that irreversible degradation might indeed be slower under the more acidic conditions.

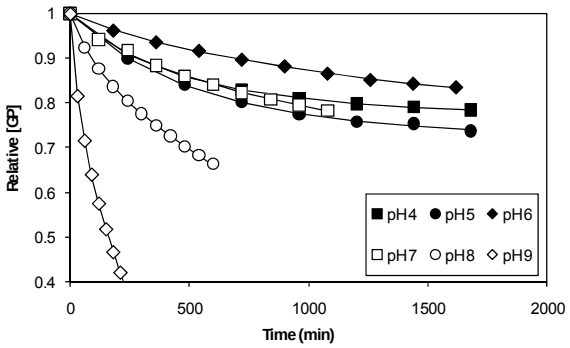

Figure 3: Degradation of GP at various pHs. GP solutions at various pHs were incubated at 24°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted using Equation 2.

Table 1: Summary of experimentally determined rate constants (in $hour^{-1}$) and calculated half-lives (in hours).

pH	n	k_1	k_2	k_3	$t_{½}$	$t^*_{½}$
4	4	0.033	0.126	0.005	443.2	650.7
5	4	0.039	0.113	0.006	293.1	494.4
6	3	0.017	0.116	0.046	139.8	162.7
7	4	0.029	0.138	0.056	77.9	97.1
8	3	0.105	0.468	0.273	16.9	20.8
9	3	0.491	1.406	1.196	2.9	3.8

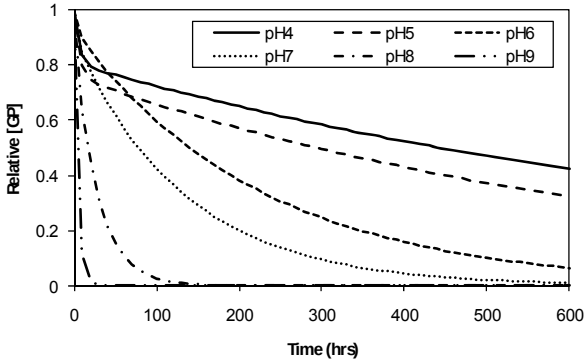

Figure 4: Calculated long-term GP degradation curves. Using experimentally determined rate constants (Table 1), the degradation of GP at various pHs was modeled and plotted using Equation 2.

The experimentally determined values for the three constants were used to plot theoretical curves for the degradation of GP at the various pHs (Figure 4). This indeed showed that at longer time scales GP was more stable at pH 4 and 5, due to the reduction in the magnitude of k_3. This also demonstrates that the concentration of GP at any given time point does not truly reflect the stability of GP since GP* can reform GP instead of being permanently converted to X. It is therefore the sum of [GP] and [GP*] that determines the amount of irreversible degradation that has occurred.

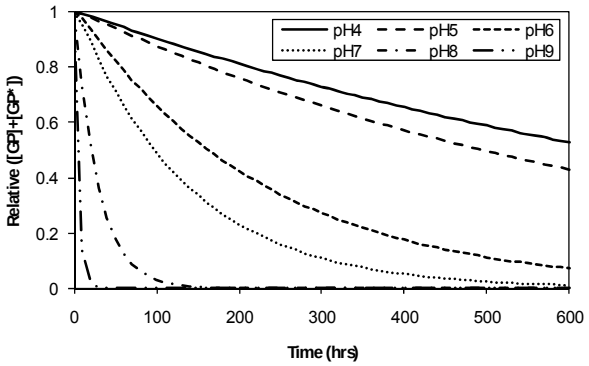

Figure 5: Calculated long-term permanent GP degradation curves. Since GP* is not a permanent breakdown product of GP, the actual breakdown was calculated by determining the loss of both GP and GP* over time using experimentally determined rate constants (Table 1) and Equations 2 and 3.

The concentration of GP + GP* was calculated at various time points to obtain curves (Figure 5) and half-lives (Table 1) that describe the true irreversible degradation of GP and demonstrate a clear and direct correlation between pH and GP stability from pH 4 to 9.

It was previously shown that GP crosslinking is enhanced by the presence of phosphate ions [17]. It was therefore investigated whether they could also affect stability. GP was incubated in the presence or absence of phosphate at pH 7, 8 or 9 and its degradation monitored. In it's presence degradation was still consistent with the mechanism of Equation 1 (R^2 > 0.999 in all cases), but was more rapid in all cases. In general, phosphate increased the magnitude of each rate constant by approximately 1.5 to 3-fold, resulting in decreases in half-lives of 45-70% (Table 2). There was no apparent correlation between the changes in the rate constants or half-lives and pH.

Table 2: Summary of experimentally determined rate constants ($hour^{-1}$) and calculated half lives (in hours) in the presence of phosphate ions.

pH	k_1	k_2	k_3	$t_{½}$	$t^*_{½}$
7	0.10	0.38	0.12	26.3	35.6
8	0.24	0.83	0.34	9.2	12.4
9	0.91	2.03	2.50	1.3	1.7

As expected, GP stability increased when a sample was frozen and stored at -20°C (Figure 6). The degradation was still consistent with the mechanism of Equation 1, although the fit was not as close as in previous experiments (R^2 = 0.998). This was probably due to experimental errors from the use of an external calibration standard to quantify GP concentration at each time point since this experiment was conducted over a period of 4 months and not in a single run. The values for k_1, k_2 and k_3 determined from this experiment were 0.667, 1.110, and 4.849x10-3 hr^{-1} respectively under these conditions. When compared to the average

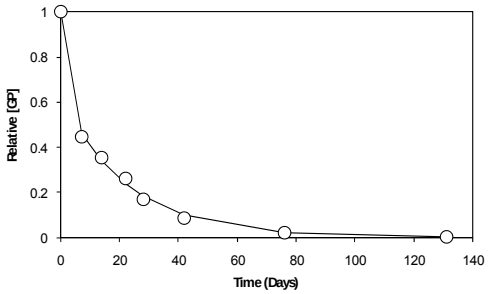

Figure 6: Degradation of GP at -20°C. GP was incubated at -20°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted to Equation 2.

values obtained from identical samples stored at 24°C (Table 2), the k_1 and k_2 rate constants decreased by only 0.36- and 0.82-fold respectively at -20°C. The k_3 constant, however, appeared to be substantially more sensitive to temperature and was reduced 515-fold. These decreases in rate constant increased the apparent half-life of GP under these conditions from 1.3 hours for an equivalent sample stored at 24°C to 124 hours, a 96-fold increase. The actual GP half life (i.e. GP+GP*), however, increased 221-fold from 1.7 hours at 24°C to 382 hours.

GP reaction with amines is thought to occur via a nucleophilic attack by the free N-electron pair on the olefinic carbon of the dihydropyran ring [18,19], resulting in ring opening and reclosure to incorporate the amine nitrogen. Crosslinking then proceeds via polymerization. It has also been suggested that since GP contains a cyclic hemi-acetal in its dihydropyran ring it may be susceptible to reversible attack and opening by water [20] to form aldehydes (Figure 7). Breaking of the dihydropyran ring in alkaline solution has been confirmed experimentally by Mi and colleagues, who also demonstrated that ring-opened GP is capable of polymerization by aldol condensation [14]. However, while Mi *et al* reported that ring opening and subsequent polymerization occurs only at very high pH levels (i.e. 13.6), our data suggests otherwise. Closer inspection of their data, in fact, shows that the absorbance at 240nm of GP solutions prepared at different pHs follows the order 7.4>5>1.2>9, i.e. similar to the "apparent" GP stability observed here. Possibly the degradation of GP at lower pH was previously overlooked due to the extremely high degradation/polymerization rate observed at pH 13.6.

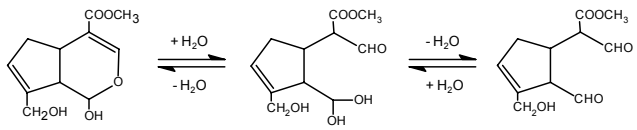

Figure 7: The proposed reversible ring-opening reactions of genipin in aqueous solution.

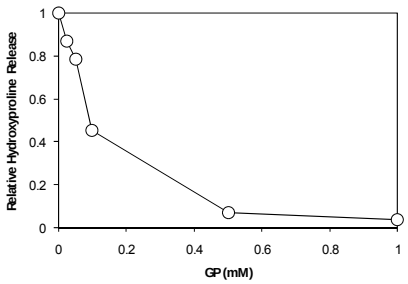

Figure 8: Protein crosslinking by GP. Samples of bovine annulus tissue were incubated with various concentrations of GP and then subjected to proteolysis. The extent of proteolysis, and hence crosslinking, was monitored by measuring the release of hydroxyproline-containing peptides from the insoluble tissue matrix.

While this paper [14] does not report the incubation times used before spectral analysis, the authors do state: "Because polymerization of genipin at strong base (pH 13.6) was quick (the color of aqueous genipin changed from transparent to brownish immediately), its intermediate reaction could only be examined at a lower temperature." It is therefore possible that the intermediates and products of degradation were not observed previously because their formation rate at lower pHs was too slow to allow their detection upon subsequent analysis. In fact, given sufficient time, the "browning" of GP solutions was observed at all pHs reported here, including at pH4 at -20^{o}C after 6 months (data not shown**).** The data therefore, suggest that GP degradation by reversible ring opening and subsequent polymerization is a phenomenon that occurs at all pH levels, albeit with dramatically different kinetics.

While the chemical identities of both GP* and X have not been determined, in light of these previous studies, it seems likely that GP* represents the open-ring structure of GP while X represents the first irreversible product of the polymerization process. It should be noted that our data are also consistent with a mechanism where GP is converted to GP* via multiple reversible steps through one or more additional intermediates. In such an instance, our k_1 and k_2 rate constants would describe a simplification of a more complex equilibrium, but would nevertheless be valid.

Interestingly, the GP sample that had been stored at -20^{o}C for 131 days was still capable of crosslinking proteins even though it contained no detectable GP (Figure 6). The resistance of insoluble protein matrices to enzymatic proteolysis increases with the extent of crosslinking [21], due to the increased number of scission events required to release soluble peptide fragments and a decrease in the steric accessibility of the protease to its substrate. Such a protease protection assay was used to indirectly measure the ability of GP to crosslink the insoluble collagenous tissue of the annulus fibrosis of bovine spinal discs.

Treatment of tissue with a fresh solution of 0.25 mM GP resulted in a 93% decrease in the proteolysis of the tissue. The 131-day sample originally containing a 10 mM solution of GP was diluted 40-fold to produce the equivalent of a 0.25 mM solution. While this sample contained no GP by HPLC it was still capable of decreasing proteolysis by 67%.

Since the concentration of GP in the 131-day sample was below the detection limits of our assay, the levels of both GP and GP* were calculated using Equations 2 and 3 and the experimentally determined values for the three rate constants. The GP content of the sample was 21 μM while the GP* concentration was 12 μM, giving a total of 33 μM. Since the sample was diluted 40-fold prior to use, the final concentration of GP and the reversible intermediate in the crosslinking reaction was therefore less than 1 μM. The crosslinking activity of low concentrations of fresh GP was then determined under the same conditions (Figure 8) and, at the lowest concentration tested (25 μM), GP was only able to retard proteolysis by 13% compared to the 67% by the aged, <1 μM sample. This suggests that X and/or further downstream products are still capable of crosslinking proteins leading to the speculation that X corresponds to polymers of ring-opened genipin reported previously [14], and crosslinking is due to the reaction of aldehydes present in the polymer termini with the protein.

Experimental

Genipin was purchased from Challenge Bioproducts Co., Ltd. (Taiwan). All other reagents were purchased from Sigma.

HPLC Analysis: Analysis of GP was an adaptation of previous methods [3,22,23] and conducted using a Hewlett Packard Series 1050 HPLC and an Agilent Extend C-18 column fitted with a guard cartridge. The column was equilibrated in 40% methanol/0.1% perchloric acid and run isocratically in the same buffer.

For short-term kinetic studies, 50 μL of a solution of freshly prepared 1 mM GP in buffer was injected onto the column. The sample was incubated at 24^{o}C and further 50 μL aliquots injected and analyzed at regular intervals thereafter. The magnitude of the intervals was determined by the rate of GP degradation under the given conditions. The samples were run for 10 minutes at 1 mL/min and absorbance was monitored at 240nm. GP eluted at approximate 6.7 minutes. Peak integration was performed using Chemstation version A.09 software at default settings. Data were normalized by dividing by the peak area of the t=0 sample.

Buffers were selected to contain no amines, which could react with GP and thus complicate results. All buffers used in the pH studies were 100 mM sodium acetate (pH 4), sodium cacodylate (pH 5), MES (pH 6), MOPS (pH 7), and EPPS (pH 8 and 9). In experiments containing phosphates, the appropriate buffers were supplemented with 100 mM sodium triphosphate.

A long term -20°C stability experiment was conducted with a 10 mM solution of GP in 100 mM EPPS/ 100 mM sodium triphosphate, pH 9. A sample of the solution was diluted 10-fold with water and analyzed as described above while the remainder was divided into aliquots and frozen at -20°C. An aliquot was thawed, diluted and analyzed at regular intervals over 4 months. In addition, before each GP analysis, a 50 µL aliquot of freshly prepared 0.5 mM uracil in water was also analyzed using the GP method as a calibration standard. The peak area of each GP peak was normalized by dividing by the area of the uracil peak produced on the same day of analysis and then by the normalized peak area of the t=0 sample.

Half-lives were calculated iteratively using an algorithm programmed into custom Visual Basic script to determine the time at which the concentration of either GP or GP+GP* equaled 0.5.

Tissue Crosslinking: Crosslinking and analysis of bovine annulus tissue by GP was conducted as described previously [24]. Crosslinking was conducted on 20-25 mg of homogenized bovine annulus fibrosis per duplicate sample at 37°C for 1 hour with shaking in the presence of 0.5 mL of crosslinking solution. Crosslinking solutions consisted of either various concentrations of fresh GP in 100 mM EPPS/100 mM sodium triphosphate pH 9 or a 1/40 dilution into the same buffer of degraded 10 mM GP.

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Microbial Transformation of Marine Halogenated Sesquiterpenes

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The sesquiterpene pacifenol is one of the main constituents of the red alga *Laurencia claviformis.* Earlier work on the semisynthetic derivatives of pacifenol afforded a series of halogenated sesquiterpenes. The aim of the present work was to obtain new hydroxylated derivatives of halogenated sesquiterpenes by means of microbial transformation using *Aspergillus níger*, *Gibberella fujikuroi* and *Mucor plumbeus*. The best results were obtained with *M. plumbeus*. The microbiological transformation by *M. plumbeus* of pacifenol, and two semisynthetic derivatives, is described. The structures of the new compounds obtained were determined by spectroscopic means.

Keywords: *Laurencia claviformis*, *Mucor plumbeus*, biotransformation, pacifenol, pacifidiene.

Biotransformation is an important tool in the structural modification of organic compounds, especially natural products, due to its significant regio and stereoselectivies [1-3].

Filamentous fungi have frequently been used to catalyze selective hydroxylation reactions that are usually difficult to achieve by chemical means [2]. This study looks at the microbial hydroxylation of pacifenol and its derivatives **2** and **3**, using the fungal microorganisms *Aspergillus níger*, *Gibberella fujikuroi* and *Mucor plumbeus*. From screening experiments, *M. plumbeus* was selected as the best yielding microorganism and incubations with this (6 days, 27°C, 0.5 g/L) afforded, after extraction, compounds **4** – **9**.

Pacifenol (**1**), from the red alga *Laurencia claviformis* [4], when treated with *p*-toluenesulfonic acid, yielded pacifidiene (**2**). However, when pacifenol was treated with sodium hydride, compound **3** was obtained [5].

A previous study described the microbiological transformation of pacifenol by the fungus *Penicillium brevicompactum* [6]. Continuing these studies, we report here results obtained from incubations with *M. plumbeus* of pacifenol and two semi-synthetic pacifenol derivatives (**2** and **3**).

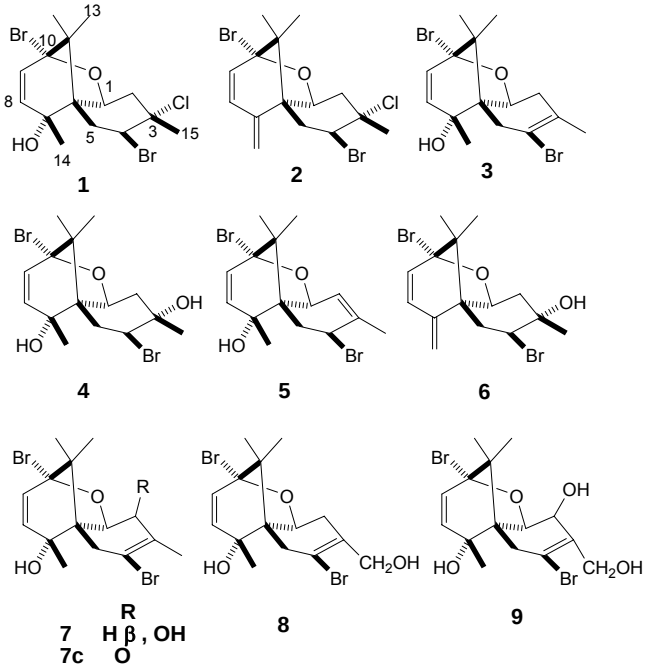

Figure 1: Compounds obtained from the biotransformation from pacifenol **1** and the semisynthetic derivatives **2** and **3**.

The fermentations were carried out for a period of 6 days. The combined broth and mycelium were extracted with ethyl acetate, and then separated into neutral and acid fractions.

In the biotransformation of pacifenol with *M. plumbeus* two metabolites that were not present in a control fermentation were detected by TLC. The ethyl acetate extract of the medium was purified by chromatography to yield the biotransformation products **4** and **5**.

Metabolit**e 4,** obtained in good yield, was identified by comparing the physical and spectroscopic data with those of the product obtained in the biotransformation of pacifenol with *P. brevicompactum* [6].

Comparison of the ^{1}H NMR spectra of compound **5** and the substrate **1** showed the disappearance of a double doublet due to H-2 in compound **1** and the appearance of a broad doublet at δ_H 5.81, corresponding to the presence of a tri-substituted double bond in compound **5**; also, the signal of the methyl (Me-12) geminal to a chlorine atom at δ_H 1.72 in **1** was shifted downfield to δ_H 1.85 in **5**.

The position of the tri-substituted double bond was inferred from the following correlations observed in the 2D experiments and COSY and TOCSY. The HMBC interaction of the H-1 resonance at δ_H 4.76 (C-1, δ_C 75.0) with the olefinic carbon at δ_C 136.3, and also the correlation between Me-12 at δ_H 1.85 with the olefinic carbons at δ_C 136.3 and 126.7 ppm, and with the methine carbon bearing the bromine atom at δ_C 67.9, indicate the presence of the C_2-C_3 double bond. Furthermore, a COSY experiment revealed correlation between a broad doublet at δ_H 5.81 and a methyl at 1.85 ppm. A TOCSY spectrum, when the signal at δ_H 5.81 is irradiated, showed correlations with the signal at δ_H 4.76 and with the methyl at 1.85 ppm. Consequently, structure **5** was assigned to this compound.

The biotransformation of pacifidiene (**2**) with *M. plumbeus* gave the metabolites **1** and **6**. The molecular formula, $C_{15}H_{20}Br_2O_2$, of compound **6** was established based on ^{13}C NMR, DEPT and MS [*m/z*: 392]. This formula suggests that oxygen was introduced in **2** in place of the chlorine atom. Comparison of the ^{1}H NMR spectra of **2** and **6** showed only minor differences in the chemical shift corresponding to the geminal methyl to the heteroatom (chlorine in **2**, δ_H 1.73 and oxygen in **6**, δ_H 1.26).

As in the compound obtained from the biotransformation of pacifenol with *P. brevicompactum*, the hydroxyl group at C-3 is axial (exo face of the molecule) [6]. So, compound **6** is shown to be 3-hydroxydechloropacifidiene.

From the microbiological transformation of compound **3**, two products, **7** and **8**, were obtained, the structures of which were established unambiguously by NMR spectroscopy. Another compound (**9**) was obtained, but only in small quantities such that only a ^{1}H NMR spectrum was recorded.

The ^{1}H NMR spectrum of compound **7** exhibited a downfield CH signal at δ_H 4.23 (brs, H-2). This observation suggested the introduction of an OH group at one of the CH_2 groups, i.e C-2 or C-5. The assignments of all the protons were accomplished by interpretation of the HMQC spectrum. The position for the newly introduced OH group at C-2 was inferred on the basis of HMBC couplings of the protons resonating at δ_H 1.93 (Me-15) and 4.40 (H-C-1) with the newly hydroxylated methine C-atom at δ_C 75.3 (H-C(2)) . The orientation of the OH group at C-2 was inferred from the NOESY correlations of Hβ–C(2) with H β–C(5), Me-15 and with Me-13; Hα–C(1) has correlation only with H α-C(5). Also, there are correlations between the methyl at δ 0.98 (Me-13) with Me–15 (δ 1.93) and Hβ–C(5); between Me-12 with Hα–C(5) and Me-14 (δ 1.34). Other correlations observed are between Me-14 with Hβ–C(5) and Hα–C(5); between H-9 with Me-12 and between H-8 with Me-12 and Me-14; Hα–C(5) with Me-14 and Hα–C(1); and H β–C(5) with Me-13, Hβ –C(2) and Me-14. The stereochemistry of pacifenol was assigned by X-ray-diffraction, and as compound **3** is a synthetic derivative of pacifenol, the stereochemistry of this compound is assumed to be the same as that of pacifenol. Furthermore, the coupling constant between H-1 and H-2 ($J_{1,2}$=1.9Hz) indicates that the H-2 must be β and, therefore, the hydroxyl group must be pseudo-equatorial at the exoface of the molecule, which is appropriate for this hydroxylation.

In order to confirm the spectroscopic data, a DFT/GIAO approach has been used to calculate the ^{1}H and ^{13}C chemical shifts. This methodology has been used for NMR assignments in several natural products [7-9]. The combined approach of extensive spectroscopic analysis and quantum mechanical methods has been used for the reassignment of structures [10], and can be very helpful to either confirm or discard both rigid and flexible molecular structures [11,12]. Recently Bassarello *et al*. [13] have used this methodology to derive the stereo structures of unknown compounds by comparing the experimental NMR spectroscopic data with the corresponding calculated spectra for all the possible stereo isomers. In addition, quantum mechanical calculations of proton-proton and proton-carbon *J* coupling constants have been proposed as useful tools to assign the relative configurations of chiral organic compounds. This approach provides results that were in good agreement with the experimental data [14,15]. When heavy atoms

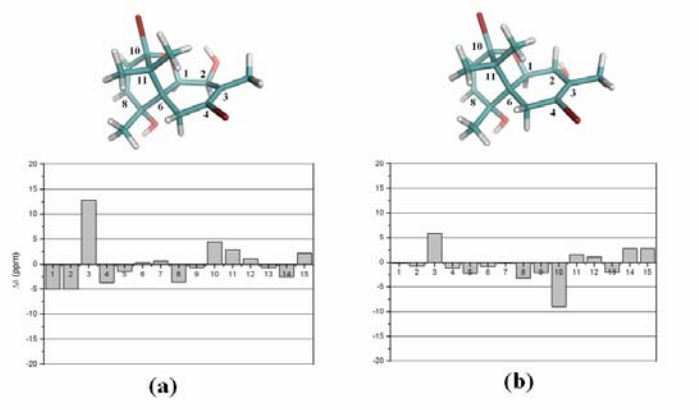

Figure 2: Deviation from calculated and observed ^{13}C NMR chemical shift for both configurations in C2 for configurations (a) and (b) of compound **7**.

are present in the structure, some spin–orbit (SO) coupling may be operative [16]. This effect has been discussed by Braddock *et al.* [10]. These authors report that the use of the extended basis set do not produce a great change in the chemical shift. They recommended that an average correction of ca. -3 ppm for bromine attached to a sp^3 carbon [17] was adequate to empirically reproduce the experimental chemical shift.

Proton-proton $J_{H,H}$ coupling constants were calculated at the mPW1PW91/6-31G(d,p) level of theory for compound **7** and optimized at the mPW1PW91/6-31G(d) level. The resultant structure for compound **7** is shown in Figure 2. The differences between theoretical and experimental values $\Delta\delta = |\delta_{exp}-\delta_{calc}|$ show a measure of the dispersion between the theoretical and experimental chemical shift values for compound **7b**. The coupling constants for protons H-1 and H-2 were calculated for both configurations. The values for $^3J_{H\text{-}H}$ for the protons at C-2 and C-1 are 8.21 Hz for isomer (a) and 1.92 Hz for isomer (b), respectively.

In summary, our results show that GIAO/DFT calculations on the optimized structure at the mPW1PW91/6-31G(d,p) level of theory provide excellent results that are in agreement with experimental values for ^{13}C chemical shifts and in fair agreement with experimental proton–proton $^3J_{H,H}$ coupling constants. The theoretical results confirm and support the experimentally derived assignments of compound **7**.

Compound **7** was treated with Jones' reagent to obtain compound **7c**, the ^1H NMR spectrum of which was very similar to that of compound **7**, with the disappearance of the H-atom geminal to the OH group at δ 4.23, and the appearance in the ^{13}C NMR spectrum of a signal due to an oxo group at δ_C 191.5. The assignments of all the H-C atoms were accomplished by interpretation of the HMQC spectrum. The position of the carbonyl group was inferred on the basis of HMBC couplings, i.e. the H-C-1 proton at δ_H 4.75 correlates with C-6 (δ 54.1), C-7 (δ 75.0) and C-2 (δ 191.5). Me-15 (δ 2.00) correlates with C-3 (δ 135.4), with C-4 (δ 146.5) and with C-2 (δ 191.5)

The least polar compound isolated (**8**) had a molecular formula $C_{15}H_{20}O_3Br_2$, and possessed one more oxygen atom than substrate **3**. Its ^1H NMR spectrum was very similar to that of **3** except that the signal of a methyl group (Me-15) at δ_H 2.01 had been replaced by that of a hydroxymethylene group, two signals, forming an AB system, at δ_H 4.32 and 4.27 (J = 16 Hz). Thus, structure **8** was assigned to this compound.

The most polar substance isolated in the incubation of **3** was the triol **9**. The two novel alcoholic groups introduced in the molecule were located at C-2 and C-15, in accordance with the ^1H NMR spectrum. Thus the resonance of H-2 was similar to that of compound **7** and the resonance for the hydroxymethylene in C-15 was similar to that of compound **8**. The quantity available was insufficient to obtain a ^{13}C NMR spectrum.

Experimental

General experimental procedures: IR spectra were obtained using a Perkin-Elmer Spectrum BX FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 400.13 and 100 MHz, respectively, with a Bruker AMX2-400 spectrometer. Chemical shifts are given in δ (ppm). Mass spectra were taken at 70 eV (probe) with a Micromass Autospec spectrometer. Semipreparative HPLC was carried out with a Beckman System Gold 125P. Dry column chromatography was performed on Merck 0.02-0.063 mm silica gel.

Computational details: Geometry optimization was performed using the mPW1PW91[18] exchange-correlation function, together with the standard 6-31G(d) basis set [19]. The ^1H and ^{13}C NMR chemical shifts were calculated using the GIAO (Gauge Invariant Atomic Orbitals) method [20] at this level of theory. Relative chemical shift and coupling constants were estimated by using the corresponding TMS shielding calculated at the same level of theory. Spin-spin coupling calculations were performed taking into account the contributions of the following interactions: Fermi contact (FC), paramagnetic spin-orbit (PSO), diamagnetic spin-orbit (DSO), and spin-dipole (SD). All calculations were carried out with the Gaussian 03 suite of programs [21].

Substrate **1** was isolated from *Laurencia claviformis,* an alga that grows on the coast of Easter Island. Substrate

2 was obtained by treating compound **1** with *p*-toluene sulfonic acid and compound **3** was obtained after the reaction of pacifenol with NaH in THF [5].

Incubation and isolation procedures: *Mucor plumbeus* was grown in shaking culture at 25°C for 2 days in 65-75 conical flasks (250 mL), each containing sterile medium (50 mL) [6]. The substrate was dissolved in EtOH (13-15 mL) and distributed equally between the flasks, and the incubation was allowed to continue for 6 days. The broth was separated from the mycelium by filtration, and both were extracted with EtOAc. The extracts were combined and chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient. Some mixtures were resolved by HPLC on an Ultrasphere silica gel 5 μm column (1× 25 cm), eluting with mixtures of isocratic *n*-hexane-EtOAc at 3 mL/min.

Biotransformation of pacifenol (1): The incubation of **1** (300 mg) afforded, from the neutral fraction, starting material **1** (45 mg), **4** (100 mg), and **5** (27 mg).

Compound 5

^{1}H NMR (δ, 400 MHz): 6.09 (1H, d, *J* = 9.9 Hz, H-9), 5.81 (1H, brd, *J* = 2.3 Hz, H-2), 5.48 (1H, d, *J* = 9.9 Hz, H-8), 4.76 (1H, d, *J* = 2.3 Hz, H-1), 4.21 (1H, dd, *J* = 5.0, 4.7 Hz, H-4), 2.07 (1H, dd, *J* = 4.7, 15.3 Hz, H-5), 2.03 (1H, dd, *J* = 5.0, 15.3 Hz, H-5'), 1.85 (3H, brs, Me-15), 1.44 (3H, s, Me-14), 1.11 (3H, s, Me-12), 1.09 (3H, s, Me-13).

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 75.0 (CH_2, C-1), 136.3 (CH_2, C-2), 126.7 (C, C-3), 67.9 (CH, C-4), 29.5 (CH_2, C-5), 55.4 (C, C-6), 77.2 (C, C-7), 134.0 (CH, C-8), 132.5 (CH, C-9), 100.3 (C, C-10), 51.9 (C, C-11), 24.7 (CH_3, C-12), 21.9 (CH_3, C-13), 25.1 (CH_3, C-14), 20.7 (CH_3, C-15).

EIMS (70 eV) *m/z* (%, rel.int): 392 $[M - H_2O]^+$(20), 313 $[M - H_2O - {}^{79}Br]^+$(100), 312 $[M - H_2O - Br]^+$(20), 311 $[M - H_2O - {}^{81}Br]^+$(90), 295(10).

Biotransformation of pacifidiene (2): The incubation of **2** (300 mg) afforded, from the neutral fraction, starting material **2** (60 mg), **6** (86 mg), and **1** (27 mg).

Compound 6

^{1}H NMR (δ, 400 MHz): 5.87 (1H, d, *J* = 9.9 Hz, H-9), 5.58 (1H, d, *J* = 9.9 Hz, H-8), 5.1 and 5.0 (1H each, brs, H-14 and H-14'), 4.48 (1H, d, *J* = 6.1 Hz, H-4), 4.07 (1H, dd, *J* = 4.7, 14.2 Hz, H-1), 2.66 (1H, dd, *J* = 4.7, 14.0 Hz, H-2), 2.20 (1H, dd, *J* = 6.1, 11.8 Hz, H-5a), 1.72 (1H, d, *J* = 11.8 Hz, H-5e), 1.26 (3H, brs, Me-15), 1.15 (3H, s, Me-12), 1.03 (3H, s, Me-13)

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 77.9 (CH, C-1), 43.1 (CH_2, C-2), 75.2 (C, C-3), 61.5 (CH, C-4). 32.8 (CH_2, C-5), 53.8 (C, C-6), 147.0 (C, C-7), 133.0 (CH, C-8), 130.1(CH, C-9), 100.3 (C, C-10), 50.5 (C, C-11), 23.3 (CH_3, C-12), 20.1 (CH_3, C-13), 114.7 (CH_2, C-14), 29.7 (CH_3, C-15).

MS (EI, 70 eV) *m/z* (%, rel.int): 392 $[M - H_2O]^+$ (20), 313 $[M - H_2O - {}^{79}Br]^+$ (100), 312 $[M - H_2O - Br]^+$ (20), 311 $[M - H_2O - {}^{81}Br]^+$ (90), 295 (10).

Biotransformation of compound 3: The incubation of **3** (260 mg) afforded, from the neutral fraction, starting material **3** (50 mg), **7** (120 mg), **8** (35 mg) and **9** (1 mg).

Compound 7

^{1}H NMR (δ, 400 MHz): 6.06 (1H, d, *J* = 9.8 Hz, H-9), 5.42 (1H, d, *J* = 9.8 Hz, H-8), 4.40 (1H, d, *J* = 1.9 Hz, H-1), 4.23 (1H, brs, H-2), 2.93 (1H, d, *J* =18 Hz, H-5), 2.48 (1H, d, *J* = 18 Hz, H-5'), 1.93 (3H, s, Me-15), 1.34 (3H, s, Me-14), 1.08 (3H, s, Me-12), 0.98 (3H, s, Me-13).

^{13}C NMR (δ, 100 MHz, $CDCl_3$): 83.1 (CH, C-1), 75.3 (CH_2, C-2), 121.7 (C, C-3), 132.5 (C, C-4). 35.3 (CH_2, C-5), 54.0 (C, C-6), 75.1 (C, C-7), 133.8 (CH, C-8), 132.8(CH, C-9), 99.4 (C, C-10), 52.4 (C, C-11), 21.3 (CH_3, C-12), 25.1 (CH_3, C-13), 24.3 (CH_3, C-14), 20.1 (CH_3, C-15).

MS (EI, 70 eV): *m/z* (%, rel.int.) = 409.96 $[M^+ + 2]$ (0.45), 407.96 $[M]^+$ (1.2), 405.96 $[M - 2]^+$ (0.7), 391.95 $[M + 2\text{-}H_2O]^+$ (2.1), 389.97 $[M - H_2O]^+$ (3.0), 387.97 $[M^+ - 2\text{-}H_2O]^+$ (1.7), 329.06 $[M - {}^{79}Br]^+$ (18.7), 327.06 $[M - {}^{81}Br]^+$ (18.9), 311.04 [390-^{79}Br] (28.6), 309.05 [390-^{81}Br] (26.5), 283.05 (18.5), 281.05 (12.9), 265.04 (4.7), 229.13 (11.3), 219.14 (11.2), 212.99 (10.1), 211.01 (12.0), 201.13 (19.8), 201.01 (56.0), 200.0 (26.3), 199.01 (61.7), 198.0 (24.7), 196.99 (9.1), 187.11 (13.7), 186.1 (15.0), 184.12 (19.6), 173.13 (13.9), 149.09 (11.7), 149.02 (10.7), 147.07 (14.8), 143.08 (10.5), 141.06 (9.4), 135.08 (17.7), 134.96 (10.3), 120.08 (100), 119.07 (46.6), 105.06 (29.1) 91.05 (65.2), 85.02 (45.1).

Compound 7c: To a solution of compound **7** (30 mg, 0.074 mmol) in acetone (3 mL) cooled to 0ºC was added Jones' reagent (2.67 g CrO_3, 2.3 mL H_2SO_4, dilute to 10 mL with water), dropwise, until disappearance of the starting material, as monitored by TLC. The reaction was quenched by the addition of iso-propanol, followed by saturated $NaHCO_3$. The solution was extracted 3 times with EtOAc and the combined organic layers were washed with water, dried over $MgSO_4$, and filtered. The solvent was removed *in vacuo* and the residue was chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient obtaining **7c**.

^{1}H NMR (δ, 400 MHz): 6.17 (1H, d, *J* = 9.8 Hz, H-9), 5.40 (1H, d, *J* = 9.8 Hz, H-8), 4.75 (1H, s, H-1), 3.33 (1H , ddd, *J* = 2.5, 5.1, 19.6 Hz, H-5), 2.87 (1H,

dd, *J* = 1.7, 19.6 Hz, H-5'), 2.00 (3H, s, Me-15), 1.45 (3H, s, Me-14), 1.14 (3H, s, Me-12), 0.98 (3H, s, Me-13),
^{13}C NMR (δ, 100 MHz, $CDCl_3$): 77.7 (CH, C-1), 191.5 (C, C-2), 135.4 (C, C-3), 146.5 (C, C-4). 36.7 (CH_2, C-5), 54.1 (C, C-6), 75.0 (C, C-7), 134.3 (CH, C-8), 132.8 (CH, C-9), 98.2 (C, C-10), 53.0 (C, C-11), 20.7 (CH_3, C-12), 24.7 (CH_3, C-13), 24.9 (CH_3, C-14), 15.7 (CH_3, C-15).

Compound 8

^{1}H NMR (δ, 400 MHz): 6.11 (1H, d, *J* = 9.8 Hz, H-9), 5.44 (1H, d, *J* = 9.8 Hz, H-8), 4.69 (1H, dd, *J* = 4.0, 8.0 Hz, H-1), 4.32 (1H , d, *J* = 16 Hz, H-15), 4.27 (1H , d, *J* = 16 Hz, H-15'), 2.97 (1H, d, *J* = 18.0 Hz, H-5), 2.95 (2H, dd, *J* = 8.0, 10.0 Hz, H-2), 2.65 (1H, d *J* = 18.0 Hz, H-5'), 2.63 (2H, dd, *J* = 4.0, 10.0 Hz, H-2'), 1.44 (3H, s, Me-14), 1.16 (3H, s, Me-12), 1.15 (3H, s, Me-13).
^{13}C NMR (δ, 100 MHz, $CDCl_3$): 74.7 (CH, C-1), 35.1 (C, C-2), 119.1 (C, C-3), 132.8 (C, C-4). 36.7 (CH_2, C-5), 53.7 (C, C-6), 76.3 (C, C-7), 133.7 (CH, C-8), 133.2 (CH, C-9), 100.2 (C, C-10), 52.8 (C, C-11), 22.3 (CH_3, C-12), 24.8 (CH_3, C-13), 25.1 (CH_3, C-14), 65.8 (CH_2, C-15).

MS (EI, 70 eV): *m/z* (%, rel.int.) = 391.95 $[M + 2\text{-}H_2O]^+$ (8.9), 389.95 $[M\text{-}H_2O]^+$ (17.7), 387.95 $[M^+ - 2\text{-}H_2O]^+$ (9.2), 329.05 $[M\text{-}^{79}Br]^+$ (2.2), 327.06 $[M\text{-}^{81}Br]^+$ (2.9), 311.04 [390-^{79}Br] (41.4), 309.05 [390-^{81}Br] (41.0), 229.03 (16.5), 227.00 (15.6), 214.99 (14.3), 212.99 (19.7), 211.00 (10.0), 201.00 (15.8), 200.00 (53.3), 199.00 (20.9), 198.00 (50.9), 196.99 (10.2), 190.95 (19.8), 188.95 (19.3), 184.12 (22.1), 183.11 (15.5), 171.11 (14.2), 169.10 (16.9), 149.01 (55.1), 147.07 (9.9), 143.08 (13.5), 141.06 (10.4), 135.07 (14.3), 134.06 (22.1), 133.09 (35.4), 132.08 (19.8), 121.09 (19.7), 120.08 (26.1), 119.07 (51.0), 115.05 (23.1), 91.05 (88.3), 57.07 (100).

Compound 9

^{1}H NMR (δ, 400 MHz): 6.02 (1H, d, *J* = 9.8 Hz, H-9), 5.41 (1H, d, *J* = 9.8 Hz, H-8), 4.66 (1H, brs, H-1), 4.53 (1H, d, *J* = 12.7 Hz, H-15), 4.47 (1H, brs, H-2), 4.35 (1H, d, *J* =12.7 Hz, H-15'), 2.97 (1H, brd, *J* = 18.2 Hz, H-5), 2.60 (1H, d, *J* = 18.2 Hz, H-5'), 1.39 (3H, s, Me-14), 1.08 (3H, s, Me-12), 1.02 (3H, s, Me-13).

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Two New Guaianolides from *Amberboa ramosa*

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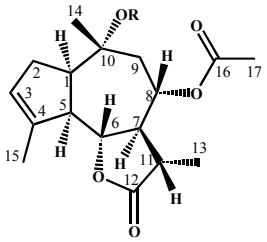
Two new sesquiterpene lactones named amberbins A (**1**) and B (**2**), have been isolated from the ethyl acetate soluble fraction of *Amberboa ramosa*, together with jaseocidine (**3**), crysoeriol (**4**) and 3*β*,8*α*-dihydroxy-11*α*-methyl-1*α*H,5*α*H,6*β*H, 7*α*H,11*β*H-guai-10(14), 4(15)-dien-6,12-olide (**5**). The structures of the isolated compounds have been elucidated on the basis of their spectroscopic data.

Keywords: *Amberboa ramosa*, guaianolides, amberbins A and B.

The genus *Amberboa* belongs to the family Compositeae and comprises six species. *A. ramosa* is an annual herbaceous plant, mainly found in Pakistan and India, with tonic, aperient, febrifuge, deobstruent, cytotoxic and antibacterial properties [1]. The butyrylcholinesterase inhibitory activity of the chloroform soluble fraction has also been reported [2]. A literature survey revealed that triterpenoids, flavonoids, steroids and sesquiterpene lactones have previously been reported from this species [1,3]. The chemotaxanomic and ethnopharmacological significance of the genus *Amberboa* prompted us to reinvestigate the constituents of *A. ramosa*. As a result, we herein report the isolation and structural elucidation of two new sesquiterpene lactones named as amberbins A (**1**) and B (**2**), respectively. In addition, the previously reported compounds, jaseocidine (**3**) [2], crysoeriol (**4**) [4] and 3*β*,8*α*-dihydroxy-11*α*-methyl-1*α*H,5*α*H,6*β*H, 7*α*H,11*β*H-guai-10(14), 4(15)-dien-6,12-olide (**5**) [2] have been isolated and characterized.

Amberbin A (**1**) was isolated as white crystals. The HREI-MS exhibited an $[M^+]$ peak at *m/z* 308.3681 corresponding to the molecular formula $C_{17}H_{24}O_5$ (calcd. for $C_{17}H_{24}O_5$, 308.3693), which indicated six degrees of unsaturation. Further prominent peaks at *m/z* 290, 265, 250 and 247 represented the losses of $[M\text{-}H_2O]^+$, $[M\text{-}COCH_3]^+$, $[M\text{-}COCH_3\text{-}CH_3]^+$ and $[M\text{-}COCH_3\text{-}H_2O]^+$, respectively. The IR spectrum showed characteristic absorption of hydroxyl (3447 cm^{-1}), γ-lactone (1755 cm^{-1}), ester (1735 cm^{-1}) and C=C (1656 cm^{-1}). The UV absorption maxima at 197 and 205 nm were typical of a γ-lactone [5]. Further spectral data showed close agreement with a guaianolide-type sesquiterpene [3,6-7].



1. R = H
2. R = β-D-glucose

Figure 1: Structures of amberbins A (**1**) and B (**2**).

The ^1H NMR spectrum showed signal for a trisubstituted double bond at δ_H 5.08. It also showed signals for oxymethine protons at δ_H 5.08 (ddd, J = 8.3, 6.0, 5.5 Hz) and 4.33 (dd, J = 10.0, 9.0 Hz). The latter was assigned to the proton geminal to the lactone oxygen atom. The larger coupling constant allowed us to assign *β* and axial orientation, which is characteristic of all guaianolides of the genera *Amberboa* and *Ixeris* [8]. The spectrum showed ^1H-^1H correlations with the vicinal protons at δ_H 2.78 (dd, J = 9.0, 8.5 Hz) and δ_H 2.59 (ddd, J = 10.0, 9.5, 8.5 Hz), which could subsequently be assigned to H-5 and H-7, respectively. The larger coupling constants suggested a *trans*-diaxial disposition among H-5, H-6 (*β*) and H-7, providing conclusive evidence for an *α* orientation of both H-5 and H-7 [8].

Table 1: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for amberbins A (**1**) and B (**2**) (CD_3OD, δ in ppm).

Positions	**1** δC	δH multiplicity (*J* in Hz)	Positions	**2** δC	δH multiplicity (*J* in Hz)
1	55.5	2.71 ddd (9.0, 8.5, 8.0)	1	53.0	2.81 ddd (9.0, 8.5, 8.0)
2	34.0	2.26 dd (8.0, 7.0) 2.37 dd (9.0, 5.0)	2	34.0	2.36 dd (8.0, 7.0) 2.22 dd (9.0, 5.0)
3	127.1	5.50 m	3	126.8	5.46 m
4	143.8	-	4	144.0	-
5	52.7	2.78 dd (9.0, 8.5)	5	55.0	2.95 dd (9.0, 8.5)
6	82.1	4.33 dd (10.0, 9.0)	6	82.3	4.20 dd (10.0, 9.0)
7	54.4	2.59 ddd (10.0, 9.5, 8.3)	7	52.3	2.87 ddd (10.0, 9.5, 8.3)
8	75.5	5.08 ddd (8.3, 6.0, 5.5)	8	75.4	5.08 ddd (8.3, 6.0, 5.5)
9	44.2	1.77 dd (12.3, 5.5) 2.34 dd (12.3, 6.0)	9	42.8	1.87 dd (12.3, 5.5) 2.39 dd (12.3, 6.0)
10	73.4	-	10	81.1	-
11	42.64	2.34 dq (9.5, 5.8)	11	42.1	2.52 dq (9.5, 5.8)
12	172.1	-	12	172.1	-
13	15.8	1.23 d (6.9)	13	15.7	1.23 d (6.9)
14	29.5	1.16 s	14	26.1	1.26 s
15	21.2	2.06 s	15	17.7	1.8 s
16	180.8	-	16	181.0	-
17	17.4	1.83 s	17	21.3	2.06 s
			1′	98.4	4.54 d (7.5)
			2′	78.2	3.38 m
			3′	71.8	3.28 m
			4′	75.5	3.17 m
			5′	77.7	3.23 m
			6′	62.9	3.82 dd (2.1, 12.0) 3.6 dd (6.0, 12.0)

The coupling pattern of the proton signals for H-1 and H-9 supported the guaianolide structure [9]. The entire sequence of protons attached to the guaianolide skeleton was established by ^{1}H-^{1}H COSY and spin decoupling experiments. Irradiation of the H-5 proton at δ_H 2.78 simplified the double doublet of H-6 at δ_H 4.33 into a doublet and the doublet of double doublets of H-1 into a double doublet. Irradiation of H-7 at δ_H 2.59 simplified the doublet of quartets at δ_H 2.34 into a quartet. Irradiation of H-11 at δ_H 2.34 simplified the doublet of the methyl group at δ_H 1.23 into a singlet, confirming the presence of a methyl group at C-11. Irradiation of H-3 at δ_H 5.50 turned the double doublets at δ_H 2.26 (H-2α) and 2.37 (H-2β) into doublets. The signal at δ_H 2.06 could be assigned to methyl protons at C-4 and further confirmed by ^{2}J and ^{3}J correlations with C-4 (δ_C 143.8), C-3 (δ_C 127.1) and C-5 (δ_C 52.7). The *O*-acetyl group was assigned to C-8 on the basis of the HMBC spectrum, in which H-8 at δ 5.08 showed an interaction with C-16 (δ_C 180.8). The hydroxyl group could be assigned to C-10 based on its downfield shift compared with the unsubstituted guaianolides and supported by HMBC, in which the methyl protons at C-14 (δ_C 1.16) showed ^{2}J and ^{3}J correlations with C-10 (δ_C 73.4), C-9 (δ_C 44.2) and C-1 (δ_C 55.5).

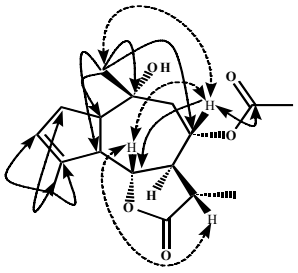


Figure-2. Important HMBC (⟷) and NOESY (⟷) correlations of amberbins A (**1**) and B (**2**).

The ^{13}C NMR and DEPT spectra showed 17 signals comprising four methyl, two methylene, seven methine and four quaternary carbons. The low frequency region showed four signals at δ_C 180.8, 172.1, 143.8 and 127.1, which could be assigned to *O*-acetyl, lactone ester and trisubstituted olefinic carbons. One oxygenated quaternary and two oxygenated methine carbons resonated at δ_C 73.4, 82.1 and 75.5, respectively. The position of substituents could be confirmed by HMQC, HMBC and COSY experiments.

The relative stereochemistry at various chiral centers of amberbin A was assigned through NOESY experiments [10-11] [Figure-2], which revealed *trans*/anti/*cis*-fusion of the *α*-methyl-*γ*-lactone moiety, the seven membered ring at C-7 and the five membered ring at C-5 and C-1. Interaction was shown between H-1 at δ_H 2.71, H-5 at δ_H 2.78 and H-7 at δ_H 2.59, confirming a 1,5-*cis*-fused guaianoloide. H-1 also showed interaction with H-2*α*. The *α*-orientation of the acetate group at C-8 could also be deduced through NOESY interactions between H-8 at δ_H 5.08, H-6 at δ_H 4.33 and H-11 at δ_H 2.34.The methyl protons attached to C-10 showed correlation with H-8 at δ_H 4.33 revealing its *β*-orientaion. On the basis of this evidence, the structure of amberbin A (**1**) could be assigned as 8*α*-acetoxy-10*α*-hydroxy-1*α*H,5*α*H,6*β*H,7*α*H,11*β*H,11*α*-methylguaia-3-enolide (Figure 1).

Amberin B (**2**) was isolated as white crystals. The HRESI-MS exhibited a pseudomolecular $[M-H]^-$ peak at *m/z* 469.2068 corresponding to the molecular formula $C_{23}H_{33}O_{10}$ (calcd. for $C_{23}H_{33}O_{10}$; 469.2074). The EIMS showed a $[M-162]^+$ peak at *m/z* 308, followed by further fragments at *m/z* 265 $[M-162-COCH_3]^+$ and 250 $[M-162-COCH_3-Me]^+$. The IR and UV spectra were similar to those of **1.** The ^{13}C NMR and DEPT spectra were also similar to those of **1**, except for the additional signals of a hexose moiety at δ_C 98.4 (C-1'), 78.2 (C-2'), 71.8 (C-3'), 75.5 (C-4'), 77.7 (C-5') & 62.9 (C-6'), as well as the downfield shift of C-10 by 7.7 ppm revealing its presence at C-10. The ^{1}H NMR spectrum was also similar to that of **1** except for the presence of additional signals due to a hexose moiety, including the anomeric proton at δ_H 4.54 (d, *J* = 7.5 Hz), the

oxymethine protons between δ_H 3.17-3.38 and oxymethylene protons at δ_H 3.82 (dd, J = 2.1, 12.0 Hz) and 3.60 (dd, J = 6.0, 12.0 Hz). The position of the hexose moiety at C-10 could further be confirmed by HMBC experiments showing ^3J correlation of the anomeric proton at δ_H 4.54 with C-10 (δ_C 81.1). The larger coupling constant of the anomeric proton allowed us to assign a *β*-configuration to the sugar moiety. Acid hydrolysis provided D-glucose, which could be identified through the sign of its optical rotation and retention time of its TMS ether by GC. The NOESY correlations were exactly the same as those of **1**. Thus, the structure of amberbin B could be assigned as 8*α*-acetoxy-10*α*-*O*-(*β*-D-glucopyranosyl)-1*α*H,5*α*H, 6*β*H,7*α*H,11*β*H,11*α*-methylguaia-3-enolide (**2**).

Experimental

General: Melting points were determined on a Buchi melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 polarimeter. The IR spectra were recorded on a JASCO 302-A spectrophotometer with KBr discs. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer. EIMS and HREI-MS spectra were recorded on JEOL JMS-HX-110 and Varian MAT-311-A mass spectrometers. The HRESI-MS was recorded on a Jeol JMS 600H instrument. The NMR spectra were recorded on a Bruker Avance DRX 400 NMR spectrometer. Chemical shifts are given on the δ scale and referenced to TMS at 0 ppm for proton and carbon. Coupling constants (*J*) are in Hertz. Silica gel 230–400 mesh (E. Merck, Darmstadt, Germany) was used for column chromatography.

Plant material: The whole plant of *Amberboa ramosa* Jafri (Compositae) was collected in June 2002, from Karachi (Pakistan) and identified by Dr Surraiya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (no. KU 312 b) has been deposited.

Extraction and isolation: The shade dried plant material (22 kg) was extracted with methanol at room temperature 3 times. The solvent was evaporated under reduced pressure and the residue (217 g) was suspended in H_2O, then partitioned with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH.

Column chromatography of the EtOAc soluble fraction (90 g) over silica gel and elution with *n*-hexane/EtOAc in increasing order of polarity afforded 4 major fractions A [*n*-hexane/EtOAc (8:2)], B [*n*-hexane/ EtOAc (6.5:3.5)], C [*n*-hexane/EtOAc (5.5:4.5)] and D [*n*-hexane/EtOAc (4:6)], respectively.

Fraction B was chromatographed over silica gel, eluting with mixtures of *n*-hexane/EtOAc in increasing order of polarity to afford sub-fractions B_A [*n*-hexane/EtOAc (7:3)], B_B [*n*-hexane/EtOAc (6:4)] and B_C [*n*-hexane/ EtOAc (6.5:3.5)]. Fraction B_A on further chromatography over silica gel and elution with *n*-hexane/EtOAc (6.5:3.5) yielded compound **3** (9 mg). Chromatography of fraction B_C and elution with *n*-hexane/EtOAc (6:4) furnished compound **4** (11.5 mg).

Fraction C was chromatographed over silica gel and eluted with mixtures of *n*-hexane/EtOAc to provide two sub-fractions C_A [*n*-hexane/EtOAc (4.5:5.5)] and C_B [*n*-hexane/EtOAc (4:6)]. Fraction C_B on further chromatography over silica gel and elution with *n*-hexane/EtOAc (7:3) afforded compound **5** (10 mg).

Fraction D was chromatographed over silica gel and eluted with mixtures of EtOAc/MeOH in increasing order of polarity to obtain compound **1** [EtOAc/MeOH (9:1)] (11 mg) and compound **2** [EtOAc/MeOH (7:3)] (13 mg), respectively.

Amberbin A [**8*α*-acetoxy-10*α*-hydroxy-1*α*H,5*α*H, 6*β*H,7*α*H,11*β*H,11*α*-methylguaia-3-enolide**] (**1**)

White crystals.
MP: 130-132°C.
$[\alpha]_D^{20}$: +33 (*c* 0.02, $CHCl_3$).
IR (KBr) ν_{max}: 3447, 1775, 1735, 1656 cm^{-1}.
UV (MeOH) λ_{max}: 197 and 205 nm.
^{1}H NMR: Table 1.
^{13}C NMR: Table 1.
HMBC: Figure 2.
EIMS (70 ev) *m/z* (rel. int. %) 308 $[M]^+$ (12).
HREI-MS *m/z* [M^+] calcd. for $C_{17}H_{24}O_5$; 308.3693: found 308.3698.

Amberbin B [**8*α*-acetoxy-10*α*-*O*-(*β*-D-glucopyranosyl) -1*α*H,5*α*H,6*β*H,7*α*H,11*β*H,11*α*-methylguaia-3-enolide**] (**2**)

White crystals.
MP: 137-139°C.
$[\alpha]^{20}_D$: +51.1 (*c* 0.02, $CHCl_3$).
IR (KBr) ν_{max}: 1740, 3600, 1779 and 1649 cm^{-1}.
^{1}H NMR: Table 1.
^{13}C NMR: Table 1.
HRESI-MS: *m/z* $[M-H]^-$ calcd. for $C_{23}H_{33}O_{10}$; 469.2074: found 469.2068.

Acid hydrolysis of amberbin B (2): Amberbin B (**2**) (2 mg) in MeOH (3 mL) containing 1 N HCl (3 mL) was refluxed for 4 h, concentrated under reduced pressure, diluted with water (7 mL) and extracted with ethyl acetate. The residue recovered from the organic phase was a mixture of aglycone products, which could

not be worked up due to paucity of material. The aqueous phase was concentrated and D-glucose was identified by its optical rotation $[\alpha]^{20}_{D}$ = +50.6 and by comparing retention times of its TMS ether (α-anomer 3.7 min, β-anomer 5.1 min) with a standard sample by gas chromatography (GC). Preparation of the TMS ether and its subsequent GC was carried out according to the reported protocol [12].

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Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from *Canella winterana*

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The hexane extract from the leaves of *Canella winterana* exhibited strong activity against the chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10) *in vitro* (IC_{50} 2.53 µg/mL). Bioassay guided fractionation of this extract has led to the isolation of 5 drimane-type sesquiterpenoids: 9-epideoxymuzigadial, 9-deoxymuzigadial, muzigadial, 3-*β*-acetoxypolygodial and the newly isolated hemiacetal, named muzigodiol, with IC_{50}-values of 1.01, 2.19, 0.31, 2.77 and 7.43 µg/mL, respectively. The first four compounds were tested for their cytotoxicity using Chinese Hamster Ovarian (CHO) cells, where they showed IC_{50}-values of 1.82, 33.69, 1.18, and 58.31 µg/mL, respectively. A structure-activity relationship is discussed.

Keywords: *Canella winterana*, antimalarial, antiplasmodial, cytotoxicity, drimane sesquiterpenes.

Malaria is one of the most important health problems in tropical and subtropical regions of the world [1a]. One of the main factors contributing to the escalating prevalence and distribution of malaria is the emergence and spread of drug-resistant parasites highlighting a need for the discovery and development of novel, affordable antimalarial treatments.

Drug discovery based on results from *in vitro* antiplasmodial bioassays, i.e., bioassay-guided isolation, has revealed many active constituents from plant origins [1b]. *Canella* is one of five genera (*Canella, Cinnamosa, Pleodendron, Warburgia* and *Capsicocordendron* or *Cinnamodendron*) of the family Canellaceae. All plants belonging to this family are characterized by their high content of drimane-type sesquiterpenoid dialdehydes, which are considered chemosystematic markers of the Canellaceae [1c]. One of the species belonging to this family, *Cinnamosma fragrans* Baill, is traditionally used in the east and central parts of Madagascar for the treatment of malaria symptoms [2]

As a part of a large project seeking new antimalarial lead compounds, we investigated the antiplasmodial activity of *Canella winterana* (L.*)* Gaertin leaves. *C. winterana* is a small tree that grows in the subtropical areas of the Florida Keys, and throughout the Caribbean region. The bark is used as a spice similar to true cinnamon, giving rise to the common name "wild cinnamon" [3]. The plant is known as a rich source of drimane sesquiterpenoids with unsaturated 1, 4-dialdehyde functionality. Most of these compounds have a very hot taste and are known for their antifeedant [4a], molluscicidal [4b] and antimicrobial activities [4c]. Several drimane sesquiterpene compounds have been reported for this plant [5a-5c]. *C. winterana* was originally screened by Spencer and co-workers during the World War II era [6a] in an effort to identify candidates with antimalarial activity. Their results showed that water extracts from stems showed very high quinine equivalent activity when administered orally to *Plasmodium gallinaceum* sporozoite infected chicks. However, further characterization of the active principles was not conducted.

In order to determine the antiplasmodial principles in *C. winterana* leaves, we followed an isolation scheme which, at every step, monitored and assessed the antiplasmodial activity of the resulting fractions. Dried powdered leaves were extracted sequentially with

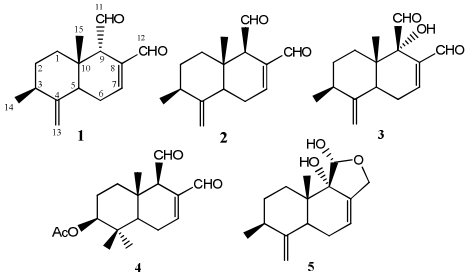

Figure 1: Structures of compounds **1-5**.

n-hexane, ethyl acetate and methanol. The *n*-hexane and ethyl acetate extracts were found to be active with IC_{50} values of 2.53 and 5.30 µg/mL, respectively. HPLC and TLC of both *n*-hexane and ethyl acetate extracts indicated close similarity of their main components (data not shown). The *n*-hexane extract was subjected to further fractionation, which led to the isolation of five structurally related compounds of the drimane sesquiterpene class of compounds (**1**-**5**, Figure 1).

Compounds **1** and **2** were identified as 9-epideoxy-muzigadial and its stereoisomer, 9-deoxymuzigadial, respectively. Both compounds have been previously reported for *C. winterana* [5b,5c]. Compound **3**, which represented the major compound in the *n*-hexane extract (~ 8.5%), was identified as muzigadial. Muzigadial was first isolated from *Warburgia ugandensis* [4a], and then from *C. winterana* and fully identified through X-ray crystallography and given another name "canellal" [5a]. Compound **4** was identified as 3β-acetoxypolygodial [5c]. Although compounds **1**-**4** have been previously reported in *C. winterana* for their antimicrobial and phytotoxic properties [5a,6b], this is the first report to assign their antiplasmodial activity. Compound **5** was purified by repeated TLC to afford white crystalline material. The ^{1}H and ^{13}C NMR spectra, unlike compounds **1**-**4**, lacked the downfield signals for the aldehydic functionality. This led us to suspect cyclization to form the hemiacetal. The study of the ^{1}H, ^{13}C, COSY, NOSEY, HMQC and HMBC NMR spectra and with the aid of the HR MS, **5** was identified as the hemiacetal derivative of muzigadial and given the name muzigodiol (**5**, Figure 1). This is the first time that this compound has been isolated from either *C. winterana* or any other natural resource. The hemiacetal of muzigadial was previously prepared by fermentation of muzigadial with certain microbial cultures. In the same study, the same compound was prepared synthetically by the reaction of muzigadial with diisobutylaluminum hydride [6c].

Although the acetals are reported as natural products, they were often considered artifacts. This usually occurs by reactions of bioactive compounds when extracted with alcohols [6d]. Since we did not use methanol for extraction, and the leaves were collected fresh and directly freeze-dried, we confirm that the hemiacetal form is a natural metabolite of this plant.

Table 1: IC_{50}-values with standard deviations of compounds **1-5** isolated from *C. winterana* and tested *in vitro* for antiplasmodial activity and cytotoxicity.

Compound	D10 IC_{50} (µg/mL) (n=3)	CHO IC_{50} (µg/mL) (n=3)	SI
1	1.01 ± 0.06	1.82 ± 0.11	1.8
2	2.19 ± 0.08	33.96 ± 4.18	15.5
3	0.31 ± 0.02	1.18 ± 0.09	3.8
4	2.77 ± 0.15	58.31± 0.21	20.7
5	7.43 ± 0.60	Not tested	-
Chloroquine (n=4)	18.58 ± 0.33 ng/mL	-	-
Emetine (n=9)	-	0.08 (n=3)	

D10: *P. falciparum* strain D10, CHO: Chinese Hamster Ovarian cell line, n = Number of replicates, SI (Selectivity index) = IC_{50} CHO/ IC_{50} D10.

Results of the *in vitro* antiplasmodial activity of the five purified sesquiterpenoids **1**-**5** (Table 1) showed that muzigadial (**3**) was the most potent sesquiterpene against the chloroquine-sensitive strain of *P. falciparum* (D10) with an IC_{50}–value of 0.31 µg. 9-Deoxy-muzigadial (**2**) and 9-epideoxymuzigadial (**1**) showed antiplasmodial activity of 2.19 and 1.01 µg/mL, respectively. Comparing the structures of compounds **1**-**3** (Figure 1), we could see that muzigadial (**3**) has an additional hydroxyl group at position 9. This indicated that the hydroxyl substitution at this position increased the antiplasmodial activity of muzigadial by about 7 and 3 fold compared with its deoxy compounds **1** & **2**, respectively. Muzigadial has been reported as one of the strongest antimicrobial drimane sesquiterpenes isolated from the *Warburgia* genus [4c].

Interestingly, it was found that 9-epideoxymuzigadial (**1**) showed higher activity than its isomer 9-deoxymuzi-gadial (**2**) (~2 fold), indicating that the α-orientation of the aldehyde group in 9-deoxy drimane sesquiterpenes is favorable for antiplasmodial activity. 3β-Acetoxy-polygodial (**4**) showed antiplasmodial activity with an IC_{50} of 2.77 µg/mL which is comparable with 9-deoxymuzigadial (IC_{50} 2.19 µg/mL). Compound **4** has the same configuration of the aldehydic groups as compound **2**, but they differ in ring A methyl group arrangement, and the β-acetoxy substitution at C-3 (see Figure 1), which indicated that the substitution and arrangement of positions 3 and 4 in ring A did not affect the antiplasmodial activity. This finding supports the reported literature for the phytotoxic activity of sesquiterpenes from *C. winterana* [6b].

Muzigodiol (**5**), the hemiacetal cyclic form of muzigadial was the least active antiplasmodial compound with an IC_{50} of 7.43 µg/mL. The diminished activity compared with muzigadial indicated the importance of the 1, 4 dialdehydic groups for the

antiplasmodial activity. This finding was different from the published results for the phytotoxic activity of sesquiterpenes from *C. winterana*, where they found that compounds with masked aldehydic groups still retained the tested activity [6b].

The isolated sesquiterpenes **1**-**5** were also tested for cytotoxicity to test their selective antiplasmodial activity reflected by their selectivity index (IC_{50} CHO/ IC_{50} D10) (see Table 1). Muzigadial showed a high cytotoxicity (IC_{50} 1.18 µg/mL, SI 3.8), indicating non-specific activity. Interestingly, the 9-deoxymuzigadial (**2**) was much less toxic than its stereoisomer 9-epideoxymuzigadial (**1**) (IC_{50} 33.96 and 1.82 µg/mL, SI 15.5 and 1.8, respectively). 3-β-Acetoxypolygodial (**4**) was the least toxic compound to the CHO cells (IC_{50} 58.31 µg/mL, SI 20.7), indicating that the 3β-acetoxy substitution with 4 dimethyl arrangement has a significant effect in reducing the toxicity of this dialdehyde sesquiterpene.

The stereochemistry and the presence of a substituent may modulate the reactivity and bioactivity of the unsaturated dialdehydes considerably. It was reported that polygodial, a drimane sesquiterpene analogous to 9-deoxymuzigadial with 4 dimethyl groups, is known to react with primary amines under biomimetic conditions to form pyrroles, a reaction that has been proposed to be responsible for the bioactivity of polygodial. It was found that its 9-epimer was less bioactive, and did not undergo the same reaction because the formation of a pyrrole is less favored due to the larger distance between the aldehyde carbons [7a].

We conclude that the presence of these very reactive compounds is responsible for the biological activities of this plant and related plants containing the same type of compounds. The major component identified in this plant was muzigadial (**3**). Although muzigadial showed very strong *in vitro* antiplasmodial activity, its potential for development as an antimalarial drug is limited due to its inherent cytotoxicity and lack of selectivity. This is often the case with antimalarial compounds identified from plants [1b]. 9-Deoxymuzigadial (**2**) and 3β-acetoxypolygodial (**4**) showed less antiplasmodial activity than muzigadial, but they were less toxic to the CHO cells. The three compounds (**2**-**4**) represent hits that could potentially be subjected to more detailed analysis. These compounds could also be used as scaffolds to generate leads with enhanced antiplasmodial activity, reduced cytotoxicity and improved bioavailability using different medicinal chemistry approaches to the dialdehyde reactive sites of the molecule.

Experimental

Plant material: Leaves from *C. winterana* were collected from Coral Gables, Fairchild Tropical Botanical Garden, Florida in October 2008 and identified by Dr Lena Struwe (Rutgers University). A voucher specimen (RG # 6) was deposited in the Chrysler Herbarium (CHRB) at Rutgers University, New Brunswick, NJ. The leaves were freeze-dried and stored at room temperature until extraction.

Extraction and bioassay-guided fractionation: Dried powdered leaves (200 g) were successively extracted with *n*-hexane (3 X 1L), ethyl acetate (3 x 1L) and methanol (3 x 1L) at room temperature. The solvents were evaporated to afford 13.4 g, 4.75 g and 17.5 g, respectively. The *n*-hexane extract (11.5 g) was subjected to flash chromatography using silica gel G 60 (Merck, 70-230 mesh, 2.5 x 30 cm). Gradient elution was performed using *n*-hexane with increasing amounts of ethyl acetate from 10 to 100%. Twelve fractions, 500 mL each, were collected and bioassayed for their antiplasmodial activity. Active fractions were subjected to further fractionation using flash chromatography and elution with varying solvent gradients of *n*-hexane-ethyl acetate. Separation was monitored by TLC (benzene-ethyl acetate 8:2, visualization with vanillin/H_2SO_4), as well as HPLC-UV. Fractionation was guided by antiplasmodial testing to concentrate on isolating and purifying only compounds which showed activity. Fraction 2 (5.13 g), eluted with 20% ethyl acetate in *n*-hexane, was further fractionated to afford 9-epideoxy-muzigadial (**1**, 120 mg) [5c] and 9-deoxymuzigadial (**2**, 200 mg) [5b]. Fraction 3 (0.5 g), eluted with 30% ethyl acetate in *n*-hexane was further fractionated using CC and crystallization (diethyl ether) to afford another quantity of 9-deoxymuzigadial (**2**, 150 mg), muzigadial (**3**, 90 mg) [5c] and 3β-acetoxypolygodial (**4**, 150 mg) [5c] in pure form. Fraction 4 (2.4 g) was subjected to crystallization from *n*-hexane-diethyl ether. Crystals were washed with ice cold diethyl ether to afford a larger quantity of muzigadial (**3**, 890 mg). Fraction 5 (0.58 g) was fractionated using CC and purified by preparative TLC to afford additional quantity of **4**, (50 mg), and muzigodiol (**5**, 22 mg) [6b].

In vitro *antiplasmodial activity:* A chloroquine-sensitive strain (D10) of *Plasmodium falciparum* was continuously cultured according to a modified method [7b], and parasite lactate dehydrogenase (pLDH) activity was used to measure parasite viability [8a]. The *in vitro* assays were performed as previously described [8b]. Chloroquine diphosphate (Sigma) served as the positive control and was made up in double distilled water and serially diluted in medium to the required concentrations. Crude extracts, fractions and purified compounds were prepared to 2 mg/mL stock solution in

10% DMSO. Stock solutions were stored at -20ºC. Further dilutions were prepared on the day of the experiment. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% of the parasite growth (IC_{50}-value). Test samples were tested at a starting concentration of 100 µg/mL, which was then serially diluted 2-fold in complete medium to give 10 concentrations, with the lowest one being 0.2 µg/mL. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 1000 ng/mL. Muzigadial was tested at a starting concentration of 10 µg/mL. The highest concentration of solvent to which the parasites were exposed had no measurable effect on the parasite viability. Test samples were tested in triplicate on one occasion. The IC_{50}-values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

In vitro *cytotoxicity assay:* Active fractions and pure compounds were tested for *in vitro* cytotoxicity against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [8c]. The CHO cells were cultured in Dulbecos Modified Eagles Medium (DMEM): Hams F-12 medium (1:1) supplemented with 10% heat-inactivated fetal calf serum (FCS) and gentamycin (0.05 µg/mL). Sample preparation was the same as for antiplasmodial testing. The highest concentration of solvent to which the cells were exposed had no measurable effect on cell viability. Emetine dihydrochloride (Sigma) was used as the positive control in all cases. The initial test concentration of emetine was 100 µg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/mL. The same dilution technique was applied to all test samples with an initial concentration of 100 µg/mL to give 5 concentrations, with the lowest being 0.01 µg/ mL. The concentration of test samples that inhibited 50% of the cells (IC_{50} values) was obtained from dose–response curves, using a non-linear dose–response curve fitting analyses via GraphPad Prism v. 4 software.

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Three New 18-Oxygenated *ent*-Kaurane Diterpenoids from *Isodon leucophyllus*

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Three new 18-oxygenated *ent*-kaurane diterpenoids, isoleuconins A-C (**1**–**3**) and ten known diterpenoids were isolated from the aerial parts of *Isodon leucophyllus*. The structures were elucidated by 1D and 2D NMR spectroscopic analysis. All of the compounds were evaluated for their cytotoxicity. Rabdokunmin A (**13**) showed significant cytotoxicity against HT-29 cells, with an IC_{50} value of 6.2 μM.

Keywords: *Isodon leucophyllus*, Labiatae, *ent*-kaurane, diterpenoid, cytotoxicity.

Isodon leucophyllus (Dunn) Kudo (Labiatae/Lamiaceae) is a small shrub mainly distributed in the western districts of Sichuan Province and the north-western regions of Yunnan Province, People's Republic of China [1]. Previous research reported the isolation of 28 diterpenoids (C-20 nonoxygenated and 7, 20-epoxy *ent*-kaurane), 6 flavones and one derivative of ionone [2a-2e]. In continuation of our research for new diterpenoids with antitumor activities, we have reinvestigated the aerial parts of *I. leucophyllus*, collected in Shangri-La County, Yunnan Province. As a result, along with 10 known diterpenoids (rabdoloxin A (**4**) [3a], isoscoparin I (**5**) [3b], 4-*epi*-henryine (**6**) [3c], rabdokunmin C (**7**) [3d], rabdokunmin E (**8**) [3d], rabdoinflexin B (**9**) [3e], excisanin A (**10**) [3f], phyllostachysin H (**11**) [3g], rabdoloxin B (**12**) [3a, and rabdokunmin A (**13**) [3d]), three new 18-oxygenated *ent*-kaurane diterpenoids [isoleuconins A-C (**1**–**3**)] were isolated from *I. leucophyllus*.

Isoleuconin A (**1**), exhibited a pseudomolecular ion peak (*m/z* 389.1947 $[M+Na]^+$, calcd 389.1940), corresponding to the molecular formula $C_{20}H_{30}O_6$, with six degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene groups in according with the absorptions at 3373, 1697 and 898 cm^{-1}, respectively. Analyses of the ^1H, ^{13}C and DEPT NMR data (Table 1) provided evidence that **1** possessed one *exo*-methylene group, one carbonyl group, two tertiary methyls, five methylenes (including one oxygenated), seven methines (including four oxygenated), and four quaternary carbons. On the basis of the characteristic signals of three methines (δ_C 44.4, 62.1, and 53.6 assigned to C-5, 9, and 13), three quaternary carbons (δ_C 37.3, 51.4, 39.5 assigned to C-4, 8 and 10), two methyls (δ_C 17.4, 18.0 assigned to C-19 and 20), and an oxygenated methylene (δ_C 70.1, assigned to C-18), together with the *exo*-methylene group (δ_C 149.2 s, 113.6 t, elucidated as C-16 and 17), we presumed that **1** should be an *ent*-kaur-16-ene diterpenoid.

Chemical shift values of some typical carbon signals of **1** were similar to those of the known compound **4** [3a]. The main difference between them was that the conjugated carbonyl group at C-15 of **4** was reduced to a secondary hydroxyl group in **1**. HMBC correlations H-15/C-8, H-15/C-9, H-15/C-7, and H-15/C-16, and the related ^1H-^1H COSY correlations H-15/H_2-17 confirmed the above deduction. ROESY correlations H-15/H-13α, H-15/H-7β indicated that the hydroxyl group located at C-15 adopted a β-orientation, as shown in Figure 1. The signal for C-9 (δ_C 70.1) in **4** shifted upfield to δ_C 62.1 (C-9) in **1**, caused by the γ-steric compression effect between HO-15 and H-9β. That could also support the view that HO-15 in **1** was β-oriented. Consequently, compound **1** was elucidated as 7α,12α,14β,15β,18-pentahydroxy-*ent*-kaur-16-en-11-one, to which the name isoleuconin A was given.

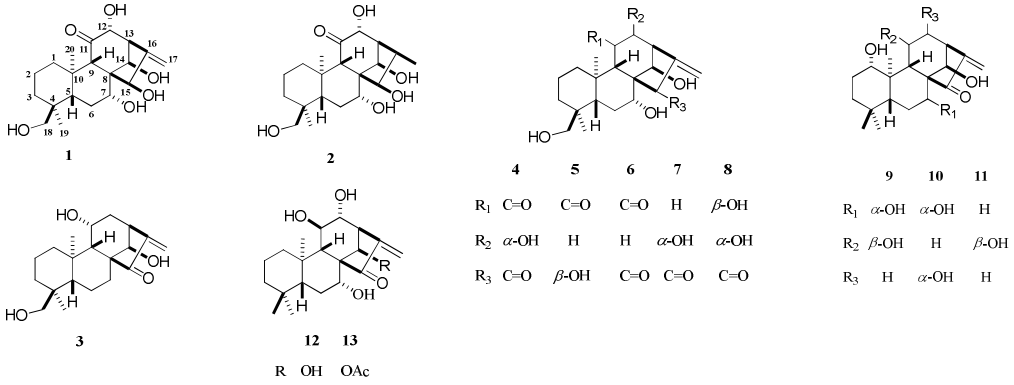

Table 1: ^{1}H and ^{13}C NMR spectroscopic data of isoleuconins A–C (**1**–**3**) (δ in ppm, *J* in Hz, recorded at 400 MHz and 100 MHz, respectively).

No.	**1**[a]		**2**[b]		**3**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1*α*	1.48[c] (d, 13.2)	39.1 t	2.2[c] (m)	40.9 t	1.91 (d, 13.0)	39.5 t
1*β*	0.97[c] (m)		1.59 (m)		1.07[c] (m)	
2*α*	1.40 (m)	17.3 t	1.71 (m)	18.5 t	1.70[c] (m)	18.5 t
2*β*	1.35[c] (m)		1.50 (m)		1.50[c] (m)	
3*α*	1.02[c] (m)	34.0 t	1.32 (d, 12.7)	35.6 t	1.39 (m)	35.7 t
3*β*	1.32[c] (m)		1.82[c] (m)		1.81[c] (m)	
4		37.3 s		38.3 s		38.1 s
5*β*	1.17 (s, overlap)	44.4 d	1.96 (d, 12.0)	46.7 d	1.80[c] (m)	49.0 d
6*α*	1.61 (m)	28.5 t	2.22[c] (m)	30.8 t	1.45[c] (m)	18.5 t
6*β*	1.68 (m)		2.49 (d, 9.4)		1.85[c] (m)	
7*α*		73.2 d		76.4 d	2.61 (d, 13.8)	26.7 t
7*β*	3.60 (dd, 4.0, 11.2)		4.53 (m)		2.21 (d, 13.8)	
8		51.4 s		54.7 s		57.5 s
9*β*	2.15 (s)	62.1 d	3.30 (s)	64.5 d	2.18 (s)	66.9 d
10		39.5 s		40.1 s		38.9 s
11*β*		210.3 s		211.4 s	4.28 (s)	64.9 d
12*α*		78.4 d		74.9 d	2.34 (m)	41.7 d
12*β*	3.48 (d, 3.1)		4.50 (s)		2.43 (m)	
13*α*	2.69 (br. d)	53.6 d	2.85 (d, 5.6)	54.6 d	3.36 (s)	46.4 d
14*α*	5.07 (br. s)	71.2 d	6.10 (s)	74.3 d	4.90 (s)	73.4 d
15	4.94 (br. s)	72.9 d	5.57 (m)	73.8 d		209.2 s
16*α*		149.2 s	3.35[c] (m)	35.0 d		150.6 s
17a	5.10 (br.s)	113.6 t	1.22 (d, 7.7)	11.3 q	5.35 (s)	112.8 t
17b	5.24 (br.s)				6.22 (s)	
18a	2.81 (d_{AB}, 11.3)	70.1 t	3.34[c] (m)	71.3 t	3.32 (m)	71.4 t
18b	3.26 (d_{AB}, 11.3)		3.66 (d, 10.3)		3.62 (m)	
19*α*	0.59 (s)	17.4 q	0.85 (s)	18.2 q	0.80 (s)	18.0 q
20*α*	1.13 (s)	18.0 q	1.78 (s)	19.3 q	1.08[c] (s)	18.5 q

a: in $CDCl_3$+CD_3OD, **b:** in C_5D_5N, **c:** revealed by HSQC correlations.

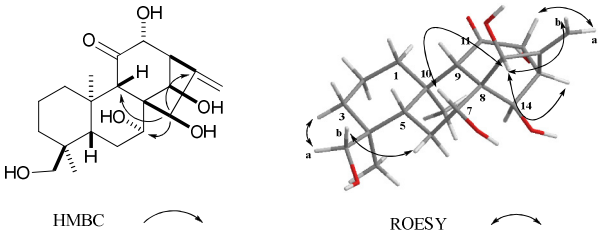

Figure 1: Key HMBC and ROSEY correlations for **1**.

Isoleuconin B (**2**) was obtained as a grey amorphous powder. The HR-ESI-MS gave a pseudomolecular ion peak (*m/z* 391.2109 $[M+Na]^+$, calcd 391.2096), corresponding to the molecular formula $C_{20}H_{32}O_6$,with five degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl and carbonyl groups with absorptions at 3386 and 1698 cm^{-1}, respectively. The absorption for *exo*-methylene disappeared in the IR spectrum of **2**. Typical carbon signals of three methines (δ_C 46.7, 64.5, 54.6 assigned as C-5, 9, and 13), three quaternary carbons (δ_C 38.3, 54.7, 40.1 assigned as C-4, 8 and 10), two methyls (δ_C 18.2, 19.3 assigned as C-19 and 20), and an oxygenated methylene (δ_C 71.3, assigned as C-18) indicated that **2** also should be an *ent*- kaurane diterpenoid. Carefully comparing the ^{13}C NMR data of compound **2** with those of **1**, we found that the *exo*-methylene of compound **1** was reduced to a methyl (δ_C 11.3, q, assigned as C-17) in compound **2**. HMBC correlations H_3-17/C-16, H_3-17/C-13, and H_3-17/C-15, and ^{1}H-^{1}H COSY correlations H_3-17/H-16, H-15/H-16, and H-16/H-13 proved the above elucidation. ROESY correlations H_3-17/H-12*β* and H-16*α*/H-13*α* revealed that the methyl located at C-16 adopted the *β*-orientation. Thus, the structure of compound **2** was established as 7*α*,12*α*,14*β*,15*β*,18-pentahydroxy-16*β*-methyl-*ent*-kaur-11-one, to which the name isoleuconin B was assigned.

The molecular formula for isoleuconin C (**3**) was established as $C_{20}H_{30}O_4$ on the basis of HR-ESI-MS data (*m/z* 357.2040 [M+Na]+, calcd 357.2041). The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene from absorptions at 3371, 1713, 1648, and 928 cm^{-1}, respectively. Analyses of the ^{1}H-, ^{13}C- and DEPT-NMR data (Table 1) provided evidence that **3** possessed one conjugated carbonyl group (δ_C 209.2 assigned to C-15), one *exo*-methylene (δ_C 150.6,

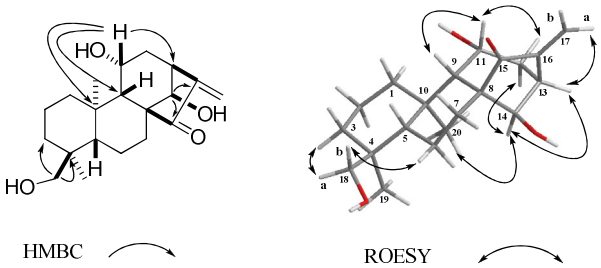

Figure 2: Key HMBC and ROSEY correlations for **3**.

112.8, assigned to C-16 and 17), two oxygenated methines (δ_C 64.9, 73.4, assigned to C-11 and 14), one oxygenated methylene (δ_C 71.4 assigned to C-18) and other typical carbon signals of an *ent*-kaurane diterpenoid. HMBC correlations H_2-17/C-16, H_2-17/C-13, and H_2-17/C-15 indicated that the *exo*-methylene was located at C-16 and conjugated with the carbonyl group (C-15). HMBC correlations H-11/C-8, H-11/C-10, and H-11/C-13, and H-14/C-16, H-14/C-15 disclosed that C-11 and C-14 were substituted by two hydroxyl groups, which was confirmed by ^{1}H-^{1}H COSY correlations H-11/H-12/H-13/H-14 and H-11/HO-11, H-14/HO-14. ROESY correlations H-14/H-20, H-14/H-13α, and H-14/H-12α revealed that H-14 adopted an *α*-orientation, as shown in Figure 2. ROESY correlations H-11/H-9*β* and H-11/H-12*β* revealed that H-11 adopted a *β*-orientation. HMBC correlations H-18/C-3 and H-18/C-4 indicated that C-18 was substituted by one hydroxyl group. Like compound **1**, this substitution also could be proved by the upfield shifted carbon signals (δ_C 35.7, C-3; δ_C 49.0, C-5) caused by the *γ*-steric compression effect between HO-18 and H-3*β* and H-5*β* in **3** compared with those signals (δ_C 41.8, C-3; δ_C 53.2, C-5) of compound **12**. Therefore, the structure of compound **3** was determined as 11*α*,14*β*,18-trihydroxy-*ent*-kaur-16-en-15-one, and named as isoleuconin C.

Compounds **1**–**13** were evaluated for their cytotoxic activities against SK-OV-3, BEL-7402 and HT-29 cell lines, using the sulforhodamine B (SRB) method with adriamycin as the positive control [3h]. As may be seen from Table 2, compound **3** exhibited weak activities against SK-OV-3 and BEL-7402 cell lines, while compounds **1** and **2** were found to be inactive against all of these cells. Their analogue **4** exhibited weak inhibitory effects against two kinds of cell lines; more importantly, another analogue, **6**, showed significant activity against these cell lines. Carefully comparing the structure of **6** with that of **4**, we found that the *α*-hydroxyl group C-12 of compound **4** disappeared in compound **6**. The above results disclosed that in the diterpenoids with a structure like **1**, the *α*-hydroxy group located at C-12 weakened the cytotoxicity of the *ent*-kaurane diterpenoid. On the other hand, in diterpenoid with a structure like **12** and **13**, esterification with the hydroxyl group located at C-14 could

Table 2: Cytotoxicity bioassay result [a] for compounds **1-13**.

Compd	SK-OV-3	BEL-7402	HT-29
1	>100	>100	>100
2	>100	>100	>100
3	38.0	40.8	82.8
4	38.9	98.9	35.7
5	>100	>100	>100
6	8.6	23.5	6.6
7	55.3	71.3	74.7
8	>100	>100	>100
9	40.0	46.7	29.7
10	>100	30.2	6.2
11	17.1	24.0	17.0
12	9.9	32.8	6.6
13	7.4	26.2	6.2
ADR	0.17	0.067	0.092

[a] Results are expressed as IC_{50} values in μM. Cell lines: SK-OV-3 (human ovarian cancer cell line); BEL-7402 (human lung cancer cell line); HT-29 (human colon cancer cell line).

improve the cytotoxicity. The above results further proved that the cyclopentanone conjugated with an *exo*-methylene is the active center of *ent*-kauranoids. [3i]

Experimental

General: Optical rotations, Perkin-Elmer Model 241 polarimeter; UV, Shimadzu UV-2401 PC UV-VIS spectrophotometer; IR, Bio-Rad Fts-135 spectrophotometer; MS, VG Auto spec-3000 spectrometer or Finnigan MAT 90; NMR, Bruker AV-400 or DRX-500 instrument.

Plant material: Aerial parts of *Isodon leucophyllus* (Dunn) Kudo were collected and air dried in Shangri-La county of Yunnan Province in August, 2004. The identity of the plant material was verified by Prof. Xi-Wen Li, and a voucher specimen was deposited in the Herbarium of the Kunming Institute of Botany.

Extraction and isolation: Powdered aerial parts of *I. leucophyllus* (1.8 kg) were extracted with 70% aq. acetone (3× 6 L) at RT for 3 days each time. The extract was evaporated *in vacuo* to remove acetone, then partitioned between H_2O and EtOAc. The EtOAc extract (78 g) was decolored with MCI gel, and then chromatographed over a silica gel column (650g, 100-200 mesh, Qingdao marine chemical factory), eluted with a gradient solvent system [$CHCl_3$–CH_3COCH_3 (1:0, 9:1, 8:2, 7:3, 2:1, 1:1, 0:1)] to afford fractions A–G, monitoring by TLC (volume of each collection was 1000 mL). Fraction E (2:1, 5 g) was submitted to chromatography over a RP-18 column (100 g, 40-63 μm, Merck Company) eluted with 30%→100% MeOH–H_2O to give fractions E1–E7, monitoring by TLC (volume of each collection was 250 mL). Fraction E4 (590 mg) was chromatographed over a silica gel column (200-300 mesh, 20 g) eluted with a gradient solvent system of $CHCl_3$–CH_3OH (60:1→10:1) to afford a mixture of 2 diterpenoids (volume of each collection was 50 mL), which were purified by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 9.4 ×250 mm,72% MeOH–H_2O, λ_{max} = 210 nm) to yield compound **1** (46 mg) and compound **2** (14 mg). Fraction E2 (105 mg) was subjected to a silica gel CC, eluting

with a gradient solvent system [$CHCl_3$–CH_3OH (40:1→5:1)] to afford a mixture of diterpenoids, which was purified by semi-preparative HPLC (45% MeOH–H2O, λ_{max} = 210 nm) to obtain compound **5** (45 mg). Fraction C (8:2, 11 g) was submitted to CC over a RP-18 column (200 g, 40-63 μm, Merck Company, 30%→100% MeOH–H_2O) to give fractions C1–C5, monitoring by TLC (volume of each collection was 250 mL). In the sixth, seventh and eighth bottles of elution solvent belonging to fraction C1, compound **4** (3.1 g) was separated as needle crystals. The mother liquid of compound **4** was subjected to RP-18 CC (100 g, 40-63μm, Merck Company, 30%→100% MeOH–H_2O) to obtain compounds **6** (434 mg) and **13** (78 mg). Fraction C2 (2.3 g) was subjected to silica gel CC (200-300 mesh, 40g) eluting with a gradient solvent system of light petroleum–CH_3COCH_3 (1:0 to 0:1, volume of each collection was 50 mL). Compound **9** (509 mg) was separated out as needle crystals from the first bottle of fraction C2. Compound **7** (3.1 mg) and compound **8** (1.7 mg) were isolated by semi-preparative HPLC (40% MeOH–H_2O, 45% MeOH–H_2O, λ_{max} = 230 nm) from the fourth and fifth bottle of elution solvent. The tenth bottle (23 mg) of the above elution was subjected to RP-18 CC (5 g, 40-63 μm, Merck Company, 30%→100% MeOH–H_2O) to obtain a mixture mainly contained two diterpenoids. The mixture was separated by semi-preparative HPLC (24% ACN–H_2O, λ_{max} = 230 nm) to obtain compound **10** (5 mg) and one unpurified diterpenoid, and then purified by semi-preparative HPLC (38% MeOH–H_2O, λ_{max} = 230 nm) to obtain compound **3** (3 mg). Compound **11** (4.0 mg) was isolated from fraction F (1:1) after continued CC on RP-18, and then purified by semi-preparative HPLC (42% MeOH–H_2O, λ_{max} = 238 nm).Compound **12** (2.4 g) separated as granular crystals from the EtOAc extract when dissolved in acetone.

Isoleuconin A (**1**)

Grey tablet-like crystals
$[\alpha]_D^{23.6}$: +52.0 (*c* = 12.60 mg/mL, MeOH).
IR (KBr): 3373, 2930, 2873, 1697, 1455, 898, cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 204 (3.66).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{30}O_6Na$ (calcd. 389.1940); found: 389.1947.

Isoleuconin B (**2**)

Grey powder
$[\alpha]_D^{23.8}$: +12.0 (*c* = 5.82 mg/mL, MeOH).
IR (KBr): 3386, 2931, 2874, 1697, 1453 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 203 (3.23).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{32}O_6Na$ (calcd. 391.2096); found: 391.2109.

Isoleuconin C (**3**)

White powder
$[\alpha]_D^{18.9}$: -76.0 (*c* = 0.79 mg/mL, MeOH).
IR (KBr): 3443, 3371, 2935, 2855, 1713, 1648, 928 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 236 (3.81).
^1H and ^{13}C NMR: Table 1.
HR-ESI MS: *m/z* $[M+Na]^+$, calculated for $C_{20}H_{30}O_4Na$ (calcd. 357.2041); found: 357.2040.

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Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber *Cucumaria okhotensis*: Stimulation of Activity of Mouse Peritoneal Macrophages

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Six monosulfated triterpene glycosides, frondoside A_1 (**1**), okhotoside B_1 (**2**), okhotoside A_1-1 (**3**), frondoside A (**4**), okhotoside A_2-1 (**5**) and cucumarioside A_2-5 (**6**), isolated from *Cucumaria okhotensis* Levin et Stepanov, stimulate spreading and lysosomal activity of mouse macrophages and ROS-formation in the macrophages. The highest macrophage spreading and stimulation of their lysosomal activity was induced by glycosides **1**, **4** and **6.** All glycosides similarly stimulate ROS formation in macrophages, but glycoside **2** caused minimal stimulation.

Keywords: triterpene glycosides, *Cucumaria okhotensis*, macrophages, ROS formation, spreading, lysosomal activity.

Triterpene glycosides of sea cucumbers possess a wide spectrum of biological activity caused by their membranolytic action [1]. During recent decades their immonomodulatory activity in subtoxic doses has attracted attention. Monosulfated glycosides from the sea cucumbers *Cucumaria japonica* [2–5] and *C. frondosa* [6] stimulate mammalian cell immunity while their activity is effective in doses significantly less than the cytotoxic ones against immune cells [7].

Recently it was found that populations of *C. japonica* in the northern parts of the Sea of Japan and Sea of Okhotsk, including the shallow waters of the Kamchatka Peninsula and North Kuril Islands, really are a series of endemic species that differ from *C. japonica* by several features, including composition of triterpene glycosides [8,9]. Because monosulfated glycosides from *C. japonica* are used for the preparation of immunostimulants [5], it is of interest to investigate the immunostimulatory activity of the glycosides isolated recently from the newly described *C. okhotensis* Levin et Stepanov, found near the south-western shore of Kamchatka Peninsula [10].

The structures of the studied monosulfated glycosides from *C. okhotensis* are presented in Figure 1. Glycosides **1** and **4** were also isolated from *C. frondosa* [11,12], and glycoside **6** from *C. conicospermium* [13]. Because the immunomodulatory action of frondoside A (**4**) has been studied previously [6], we used it as a positive control. To study immunomodulatory activity we chose spreading and lysosomal activity of mouse macrophages, and the formation of reactive oxygen species (ROS) in them.

Macrophage adhesion onto an extracellular matrix, followed by their spreading, is necessary to follow their functional activity. These indexes reflect the initial stage of phagocytosis and macrophage ability for phagocytosis. It is accompanied by changes in macrophage geometric parameters, including cell area and perimeter. The cell may elongate and change shape because of the formation of lamellae and fillopodia [14,15].

All the studied glycosides induced an increase of all these indexes on the fourth day after intraperitoneal injection. Macrophages of mice administrated with frondoside A (**4**) had larger geometrical parameters and their shape was more differentiated because of an increase in spreading, elongation, and formation of lamellae and fillopodia compared with macrophages of mice from the control group.

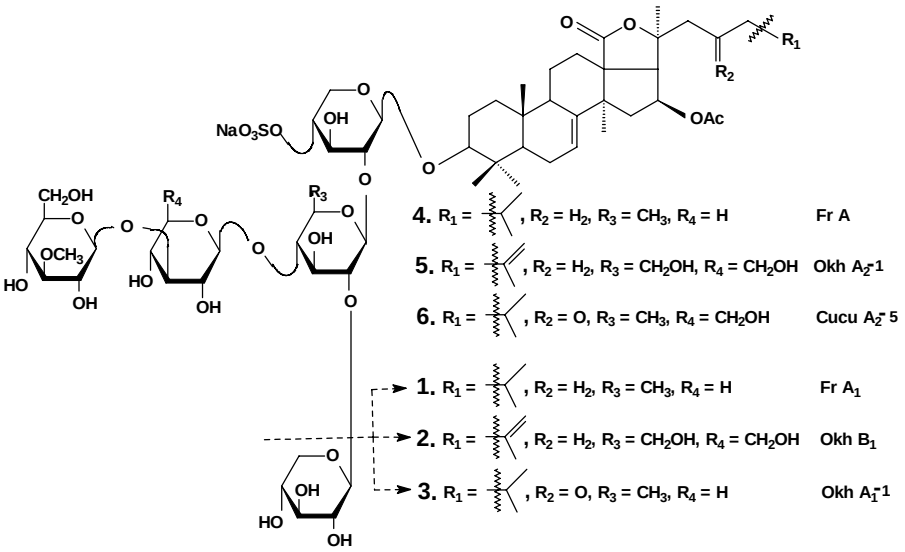

Figure 1: Chemical structures of monosulfated triterpene glycosides from *Cucumaria okhotensis*: 1 – frondoside A_1 [9,11]; 2 – okhotoside B_1 [9]; 3 – okhotoside A_1-1 [8]; 4 – frondoside A [9,12]; 5 – okhotoside A_2-1 [8]; 6 – cucumarioside A_2-5 [9,13].

Table 1: Effect of triterpene glycosides on peritoneal macrophage spreading. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages that were then stained with the fluorescent probe 5-carboxyfluorescein diacetate and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

Substance	Geometric parameters of macrophages, pixels			
	Cell area	Perimeter	Maximal chord	Shape
Control	92.163 ± 13.111	80.698 ± 9.484	11.050 ± 1.094	0.189 ± 0.016
Fr A_1 (**1**)	239.315 ± 25.444*	104.849 ±7.578*	21.284 ± 1.465*	0.245 ± 0.012*
Okh B_1 (**2**)	107.989 ± 15.989	120.333 ± 8.621*	14.774 ±0.863*	0.075 ± 0.003*
Okh A_1-1 (**3**)	154.146 ± 18.282*	142.343 ± 12.40*	19.214 ± 1.452*	0.106 ± 0.007*
Fr A (**4**)	250.125 ± 33.284*	128.659 ± 12.240*	22.472 ± 1.611*	0.210 ± 0.011*
Okh A_2-1 (**5**)	157.138 ± 22.937*	88.400 ± 8.956	17.493 ± 1.064*	0.285 ± 0.011*
Cucu A_2-5 (**6**)	229.608 ± 39.828*	125.411 ± 16.941*	22.592 ± 2.453*	0.206 ± 0.014

Almost all the studied glycosides induced statistically significant increases in cell area, perimeter, maximal chord and integral index of cell shape at an injection dose of 0.2 μg/mouse. Quantitative estimation of the influence of the glycosides on macrophage spreading is presented in Table 1. The most effective glycosides were frondoside A_1 (**1**), frondoside A (**4**) and cucumarioside A_2-5 (**6**). These substances activate spreading 2–2.5 fold greater than the control. Frondoside A was more active than frondoside A_1 and cucumarioside A_2-5.

Lysomal activity is one of the important markers of physiological and biochemical macrophage status. Almost all the studied glycosides induced a statistically significant increase in lysosomal activity of peritoneal macrophages on the fourth day after a single intraperitoneal injection of 0.2 μg/mouse. The most effective glycosides were frondoside A_1 (**1**), frondoside A (**4**) and cucumarioside A_2-5 (**6**). These compounds activated lysosomal activity approximately1.5–2.5 times greater than that of control cells (Figure 2). The most active glycoside was frondoside A, whereas the activity of glycoside **6** was similar to that of glycoside **1**.

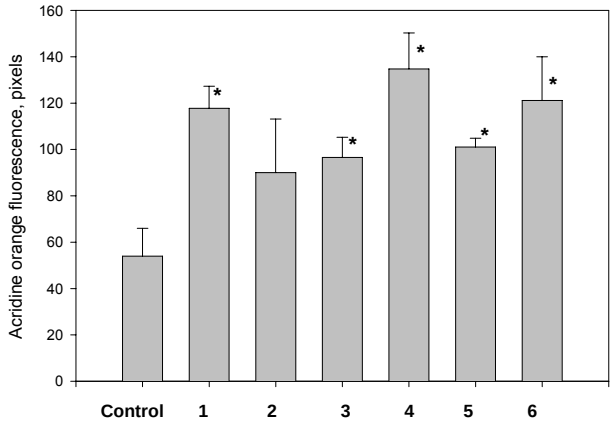

Figure 2: Influence of triterpene glycosides on lysosomal activity of mouse peritoneal macrophages. Glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages. The lysosomes were stained with acridine orange and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

The ability to generate ROS, singlet oxygen, hydrogen peroxide and the products of their interaction with each other, and NO, the so called "oxidative burst", is

important for the ability of macrophages to kill ingested microorganisms.

Almost all the studied glycosides induced statistically significant increases of ROS formation in peritoneal macrophages isolated from mice on the fourth day after a single intraperitoneal injection at a dose of 0.2 μg/mouse (Figure 3). All the glycosides activated lysosomal activity 1.3–1.8 folds greater than control cells. Okhotoside B_1 (**2**) showed minimal activity.

Hence all the studied monosulfated glycosides isolated from *Cucumaria okhotensis* showed immunostimulatory activity of mouse macrophages, as indicated by an increase in macrophage spreading, their lysosomal activity and ROS-formation. This indicates that *C. okhotensis* can be used as a source for immunostimulant preparations.

The activity of the studied glycosides was changed depending on their structure, but not significantly. Glycosides **4** and **5** having a fifth terminal monosaccharide unit (xylose) were more active than their corresponding tetrasaccharide analogs **1** and **2**.

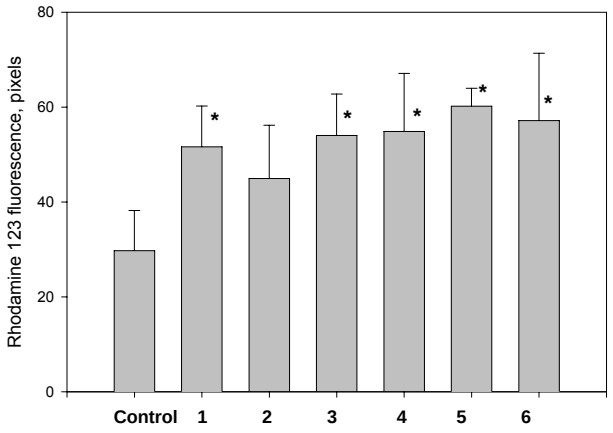

Figure 3: Influence of triterpene glycosides on the ROS-formation in peritoneal mouse macrophages. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 μg/mouse. On the fourth day the mice were killed by pervisceral dislocation followed by isolation of macrophages. The cells were stained with dihydrorhodamine 123 probe and analyzed. The data are presented as m ± se (n=100); *– $p < 0.05$.

Experimental

Triterpene glycosides: The glycosides were isolated from *Cucumaria okhotensis* using common procedures of hydrophobic chromatography of the extracts on teflon powder Polichrom-1, followed by chromatography on a Si gel column and HPLC [8,9]. Structures and purity of individual isolated frondoside A_1 (**1**), okhotoside B_1 (**2**), okhotoside A_1-1 (**3**), frondoside A (**4**), okhotoside A_2-1 (**5**) and cucumarioside A_2-5 (**6**) were confirmed by ^{13}C NMR spectra [8,9]. Individual substances were dissolved in sterile distilled water at a concentration of 1 mg/mL (initial concentration). Forty μL initial glycoside solution were added to 960 μL of distilled water to produce a solution of 40 μg/mL. Ten μL of this solution was added to 990 μL of distilled water to obtain the final concentration of 0.4 μg /mL. The final concentration was used for injection into mice.

Determination of immunomodulatory activity: CBA mice (female, 20 g) were used for *in vivo* testing. The glycosides were intraperitoneally injected (0.5 mL of a glycoside solution in distilled water) at a final glycoside dose of 0.2 μg/mouse. The control mice were injected with distilled water. On the fourth day after injection the mice were killed by pervisceral dislocation and peritoneal macrophages were isolated by standard procedures. The estimation of immunomodulatory activity was carried out by staining and localization of lysosome in macrohages, determination of reactive oxygen species (ROS) formation in macrophages, and determination of macrophage spreading on an extracellular matrix. Molecular fluorescent probes followed by cell imaging analysis were used. Mice peritoneal liquor (250 μL) containing macrophages was placed onto a microscope cover slip and incubated for 1 h at 37°C. After macrophage adhesion, the cover slip was washed twice with phosphate buffered saline (PBS, pH 7.5). Then 250 μL of a solution of the fluorescent probe was added to the cell monolayer in drops and the cells were incubated at 37°C. To determine macrophage lysosomal activity, a solution of acridine orange (Calbiochem, 100 μg/mL in PBS) was applied and the cells were incubated for 30 min. To estimate ROS formation, a solution of dihydrorhodamine 123 (Sigma, 100 ηg/mL in PBS containing 0.5 mM of sodium azide) was used. The cells were incubated for 10 min. To estimate macrophage spreading, a solution of 5-carboxyfluorescein diacetate (Molecular Probes, 50 μg/mL in PBS) was applied. The cells were incubated for 60 min. The cell monolayer was washed twice with PBS. Cover slips were placed on the stage of a fluorescent scanning device composed on the base of an inverted microscope Axiovert 200 (Zeiss, Germany). A luminiscent 75 W Optosource xenon arc lamp and a DAC-controlled monochromator Optoscan (Cairn Research Ltd., UK) were used as light sources for inducing fluorescence at λex = 488 ηm; a HQ FITC filter-block (Chroma Technology Corp., USA) and Fluar 40×/1.3 oil objective lens (Zeiss, Germany) were used to visualize cell fluorescence. Cell fluorescence images were recorded using a Hamamatsu Orca-ER C4742-95 digital monochrome video camera (Hamamatsu Photonics K.K., Japan) and an IBM-compatible computer having a Fireware data interface.

The intensity of fluorescence of 100 randomly chosen cell images was estimated using an AQM Advance 6 computer program (Kinetic Imaging Ltd., UK) and calculated along with average intensity of fluorescence for each cell, in pixels. Geometric cell parameters (area, perimeter, maximal chord and shape) were also estimated. Each experiment was repeated in triplicate. Average value, standard error, standard deviation and p-values were calculated and plotted on the chart using a SigmaPlot 3.02 computer program (Jandel Scientific, USA).

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Three New Aaptamines from the Marine Sponge *Aaptos* sp. and Their Proapoptotic Properties

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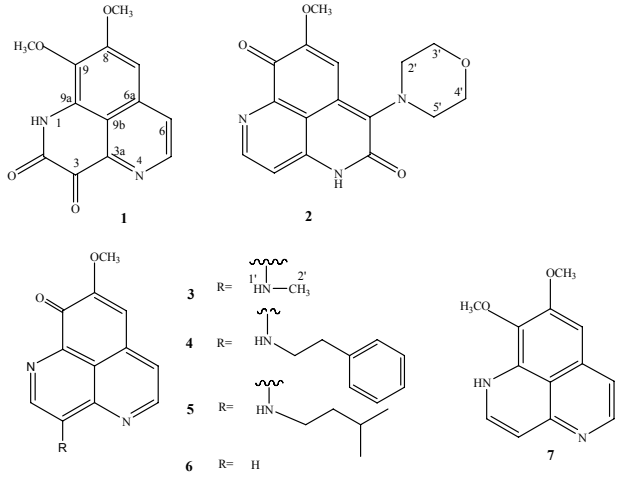
Three new aaptamine-type alkaloids, 2,3-dihydro-2,3-dioxoaaptamine (**1**), 6-(*N*-morpholinyl)-4,5-dihydro-5-oxo-demethyl(oxy)aaptamine (**2**) and 3-(methylamino)demethyl(oxy)aaptamine (**3**), along with known aaptamines were isolated from the sponge *Aaptos* sp. Their structures were determined on the basis of detailed analysis of their 1D and 2D NMR spectroscopic and mass spectral data. The isolated compounds induced apoptosis in human leukemia THP-1 cells.

Keywords: aaptamine, benzo[*de*][1,6]-naphthyridine alkaloids, marine sponge, apoptosis, NMR.

The genus *Aaptos* comprises about 20 species of sponges that are mostly known as a rich source of benzo[*de*][1,6]-naphthyridine alkaloids (aaptamines) possessing antioxidant [1], antiviral [2], antimicrobial [3], antifungal [4], antiparasitic [5], cytotoxic [3a,3b,6], and other activities [7]. The various and interesting bio-activities of these compounds have raised a high interest in their syntheses and the preparation of derivatives [7]. During our search for anticancer metabolites from marine organisms [8-10], we recently isolated a new aaptamine derivative [10] from the Vietnamese sponge *Aaptos* sp. In this paper we report the results of a study on another collection of a sponge belonging to the same genus which has resulted in the isolation of three new benzo[*de*][1,6]-naphthyridine alkaloids, namely 2,3-dihydro-2,3-dioxoaaptamine (**1**), 6-(*N*-morpholinyl)-4,5-dihidro-5-oxo-demethyl(oxy) aaptamine (**2**) and 3-(methylamino)demethyl(oxy)aaptamine (**3**), along with the known 3-(phenethylamino)demethyl-(oxy)aaptamine (**4**) [11], 3-(isopentylamino)-demethyl(oxy)aaptamine (**5**) [11], demethyl(oxy)-aaptamine (**6**) [3a] and aaptamine (**7**) [12].

Compound **1** was obtained as a yellow amorphous solid, the molecular formula for which was established as $C_{13}H_{10}N_2O_4$ from the $[M + Na]^+$ ion at *m/z* 281.0544 (calcd for $C_{13}H_{10}N_2O_4Na$, 281.0546) and $[M + H]^+$ ion at *m/z* 259.0727 (calcd for $C_{13}H_{11}N_2O_4$, 259.0726) in the HRESIMS (positive ion mode). The ^{1}H NMR spectrum of **1** exhibited two doublets of coupled protons at δ 8.73 and 8.07 (each 1H, d, *J*=5.4 Hz), one isolated



singlet at δ 7.30 and singlets for two methoxyl groups at δ 4.04 and 4.09 (each 3H), which are characteristic for aaptamines [3b]. The signal of the exchangeable proton at δ 11.4 (1H, br.s) in the ^{1}H NMR spectrum recorded in DMSO-d_6, together with the IR absorption at 3375 cm^{-1} suggested the presence of a NH group.

Careful analysis of the ^{13}C NMR data, and the HSQC, HMBC and 1D NOE correlations (Table 1) allowed us to establish the modified aaptamine core and attribute proton signals to the 5, 6 and 7 positions. Among all the carbon signals of **1**, two quaternary oxygenated carbons at δ 178.8 and 158.4 did not show any HMBC correlation with protons. These signals have been assigned to the C-3 keto group and C-2 amide carbonyl by comparison of ^{13}C NMR data of N-1 – C-3 fragment

Table 1: ^{1}H and ^{13}C NMR spectroscopic data for **1** and **2** in CD_3OD.

	1						**2**					
Position	δ_H[a] (mult, *J* in Hz)	δ_C[b]	HMBC (H→C)			1D NOE	δ_H[a] (mult, *J* in Hz)	δ_C[b]	HMBC (H→C)			1D NOE
			J^2	J^3	J^4				J^2	J^3	J^4	
1	11.4 br.s[c]											
2		158.4					8.61 d (5.4)	149.1	3	3a, 9a	9b	H3
3		178.8					7.34 d (5.4)	112.1	2	9b	9a	H2
3a		145.7						145.1[d]	3			
4							10.18 br.s[c]					
5	8.73 d (5.4)	146.1	6	3a, 6a	9b	H6		162.4				
6	8.07 d (5.4)	126.4	5, 6a	7, 9b	9a	H5, H7		143.9				
6a		136.9						122.5				
7	7.30 s	102.3	8	6, 9, 9b	9a	H6, 8-OMe	7.18 s	109.2	8	6, 9, 9b	9a	H2', 8-OMe
8		158.9						154.8				
9		139.4						178.9				
9a		125.8						142.6[d]				
9b		116.9						116.5				
1'												
2'							3.70 m	54.3	3'	6, 5'		H3', H7
3'							3.88 m	68.9	2'	4'		H7, H2'
4'							3.88 m	68.9	5'	3'		H5'
5'							3.70 m	54.3	4'	6, 2'		H3'
8-OMe	4.09 s	57.0		8		H7	3.97 s	56.5	8			H7
9-OMe	4.04 s	62.2		9								

[a] Measured at 700 MHz, [b]at 175 MHz. Assignments were confirmed from HSQC, HMBC and NOE data.[c] In DMSO-d_6, [d] Interchangeable signals.

with literature data for known bis indol alkaloids containing an unusual α-keto enamide functionality [13] and aaptanone, having a rare oxygenated 1,6-naphthyridine core [14]. This suggestion was in agreement with IR data (two carbonyl bands at 1697 and 1714 cm^{-1}) and an ESI MS/MS experiment, where MS/MS fragmentation of the $[M+H]^+$ ion gave a daughter ion at *m/z* 187 indicative of the loss of a $C_2H_2NO_2$ fragment. Thus the structure of **1** was determined as 2,3-dihydro-2,3-dioxoaaptamine, the first naturally occurring 2,3-disubstituted aaptamine alkaloid. Earlier, compound **1** was obtained during attempts at the synthesis of aaptamine [15].

The molecular formula of **2** was determined as $C_{16}H_{15}N_3O_4$ from the HRESIMS ions for $[M+H]^+$ at *m/z* 314.1129 (calcd for $C_{16}H_{16}N_3O_4$, 314.1135) and $[M + Na]^+$ at *m/z* 336.0949 (calcd for $C_{16}H_{15}N_3O_4Na$, 336.0954). In the ^{1}H NMR spectrum, signals observed at δ 8.61 and 7.34 (each 1H, d, *J*=5.4 Hz), along with the singlet at 7.18 (1H, s), were characteristic of the aaptamine alkaloids, suggesting the benzo[*de*][1,6]-naphthyridine skeleton. Signals for one methoxyl group at δ 3.97 (3H, s) in the ^{1}H NMR spectrum and a carbonyl group at δ 178.9 in the ^{13}C NMR spectrum were similar to the characteristic 8-OMe and C-9 carbonyl signals observed in demethyl(oxy)aaptamine [3a]. Detailed analysis of the 1D and 2D NMR spectra (Table 1) allowed us to establish a modified demethyl(oxy)-aaptamine core substituted at C-5 and C-6, and confirm the position of aromatic protons at C-2, C-3 and C-7. In addition, signals for four methylene groups at δ 3.70 (4H, m) and 3.88 (4H, m) in the ^{1}H NMR spectrum and δ 54.3 and 68.9 in the ^{13}C NMR spectrum resembled signals for the morpholinyl fragment in 3-(*N*-morpholinyl)-9-demethyl(oxy)aaptamine [10]. The ^{1}H-^{1}H COSY correlations from H_2-2' to H_2-3' and from H_2-4' to H_2-5', as well as HMBC correlations H-2'/C-3', C-5' and H-3'/C-2', C-4' confirmed the presence of a morpholinyl fragment in the structure. Furthermore, HMBC correlations from H-2' and H-5'to C-6 and NOE interactions between H-2', H-3' and H-7 indicated attachment of this fragment to the benzo[*de*][1,6]-naphthyridine part at C-6. The ^{1}H NMR spectrum recorded in DMSO-d_6 shows a signal for an exchangeable proton at δ 10.18 (1H, br.s). The quaternary carbon at δ 162.4 did not show any correlations in the HMBC spectrum and was assigned to the amide carbonyl carbon. This supposition was in agreement with the IR absorption band at 1716 cm^{-1} and literature data [14]. Thus, the structure of **2** was determined as 6-(*N*-morpholinyl)-4, 5-dihydro-5-oxo-demethyl(oxy)aaptamine.

Only few substituted morpholine derivatives have so far been isolated from marine sources [16]. The first morpholinyl fragment in an aaptamine-type alkaloid was recently reported in another collection of *Aaptos* sp [10].

Alkaloid **3** was obtained as an orange amorphous solid and its molecular formula was established to be $C_{13}H_{11}N_3O_2$ from the $[M + Na]^+$ ion at *m/z* 264.0768 (calcd for $C_{13}H_{11}N_3O_2Na$, 264.0743) in the HRESIMS (positive ion mode). ^{1}H and ^{13}C NMR data of **3** (Table 2) were similar to those of **4** and **5** **[**11**]** showing the same C-3 substituted benzo[*de*][1,6]-naphthyridine moiety. The difference was in the NMR signals of a substituent. A methylamino group at C-3 was deduced from signals at δ 3.22 (3H, s) in the ^{1}H NMR (CD_3OD) and δ 29.6 in the ^{13}C NMR spectra. The 1H multiplet at δ 7.0 in the ^{1}H NMR spectrum recorded in $CDCl_3$ and its 1H-1H COSY correlation with H-2', as well as the IR absorption band at 3400 cm^{-1} confirmed the presence of NH. Again, the J^3 HMBC correlation from H_3-2' to

Table 2: ^{1}H and ^{13}C NMR spectroscopic data for **3** in CD_3OD.

Position	δ_H[a] (*J* in Hz)	δ_C[b]	HMBC (H→C)			1D NOE
			J^2	J^3	J^4	
2	8.30 s	130.5	3	3a, 9a	9b	
3		147.9				
3a		137.1				
5	8.83 d (4.5)	152.3	6	3a, 6a	9b	H6
6	7.70 d (4.9)	124.0	5	7, 9b	3a, 9a	H5, H7
6a		137.5				
7	6.99 s	108.6	8	6, 9, 9b	9a	H6, 8-OMe
8		158.8				
9		177.2				
9a		134.1				
9b		119.2				
1'	7.00m[c]					
N-Me	3.22 s	29.6		3	2	
8-OMe	3.97 s	56.7	8		7	H7

[a] Measured at 700 MHz, [b]at 175 MHz. Assignments were confirmed by HSQC, HMBC and NOE data. [c] In $CDCl_3$.

C-3 confirmed the connection of the methylamino substituent to the benzo[*de*][1,6]-naphthyridine moiety at C-3. Based on these data, the structure of **3** was assigned as 3-(methylamino)-demethyl(oxy)aaptamine.

We have studied the apoptosis inducing activity of alkaloids **1**-**7** and isoaaptamine. The substances were found to induce a dose-dependent apoptosis of human leukemia THP-1 cells. Isoaaptamine was shown to be the most active inducer of apoptosis (34% of early and 62% of late apoptosis at 12.5 μM concentration), whereas alkaloids **2** and **5** were the least active (89% and 80% of total apoptosis at 320 and 400 μM concentration, respectively). By comparison of the activity of compounds **3** (40% of early and 56% of late apoptosis at 208 μM concentration) and **5** (38% of early and 42% of late apoptosis at 400 μM concentration), it was concluded that the apoptosis inducing activity of the substances studied decreased with an increase of the side chain length at position 3.

Experimental

General: NMR, Bruker Avance III-700 spectrometer; IR, Bruker Vector 22 spectrophotometer; UV, UV-1601 spectrophotometer (Shimadzu); HREIMS, AMD-604S (Intectra, Germany) mass spectrometer. Low pressure column liquid chromatography was performed using Polichrom-1 (powder Teflon, Biolar, Latvia) and silica gel KSK (50-100 μm, Sorbpolimer, Russia). HPLC was performed using a Shimadzu LC-10 AD instrument equipped with an UV-Vis detector and a Develosil ODS-UG-5A (250×4.6 mm) column.

Animal material: The *Aaptos* sp. was collected by scuba during the scientific cruise of R/V "Academic Oparin" in June 2007, in Vang Fong Bay, Vietnam, at a depth of 5-10 m, and identified by Dr Krasokhin V.B. A voucher specimen (PIBOC #O34-064) is kept in the marine invertebrate collection of the Pacific Institute of Bioorganic Chemistry (Vladivostok, Russia).

Extraction and isolation: Animal material (400 g, dry weight) was cut and extracted with EtOH immediately after collection (3 x 3 L). The EtOH extract, after evaporation *in vacuo*, was redissolved in EtOH-H_2O (5:1) and extracted with *n*-hexane. The EtOH-H_2O layer after evaporation was subjected to CC on Polychrom-1 using H_2O with increasing amounts of EtOH as eluents. The fractions eluted with 10% EtOH (5 g) were further chromatographed on a silica gel column using chloroform with increasing amounts of EtOH as eluent to obtain 3 fractions: 1a (50 mg), 1b (250 mg) and 1c (330 mg), containing aaptamine-type alkaloids. Reversed-phase chromatography (Develosil ODS-UG-5, CH_3CN/H_2O, 20%→60%, 25 min, 1 mL/min, 280 nm) of fraction 1a afforded the new compounds **1** (1 mg), **2** (0.5 mg) and **3** (1 mg), together with the known 3-(phenethylamino)demethyl(oxy)aaptamine (6 mg, 0.0015% dry weight) and 3-(isopentylamino)demethyl (oxy)aaptamine (5 mg, 0.0012% dry weight). Repeated chromatography of fractions 1b and 1c using silica gel CC ($CHCl_3$/EtOH, 7:1) resulted in the isolation of 9-demethyl(oxy)aaptamine (70 mg, 0.018% dry weight), and aaptamine (80 mg, 0.02% dry weight).

2,3-Dihydro-2,3-dioxoaaptamine (**1**)

0.001 g (0.00025% dry weight), crimson amorphous solid.
IR ($CHCl_3$): 3375 (br), 1714, 1697, 1602, 1305 cm^{-1}.
UV/Vis λ_{max} (EtOH) nm (log ε): 280 (3.7), 376 (3.07), 447 (3.03).
^{1}H and ^{13}C NMR (CD_3OD): Table 1.
HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{11}N_2O_4$ 259.0726; found: 259.0727.

6-(*N*-Morpholinyl)-4,5-dihydro-5-oxo-demethyl(oxy) aaptamine (**2**)

0.0005 g (0.00012% dry weight), yellow amorphous solid.
IR ($CHCl_3$): 3400 (br), 1716, 1652, 1602 cm^{-1}.
UV/Vis λ_{max} (EtOH) nm (log ε): 241 (1.55), 286 (1.27), 487 (1.20).
^{1}H and ^{13}C NMR (CD_3OD): Table 1.
HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{16}H_{16}N_3O_4$, *m/z* 314.1135; found: 314.1129.

3-(Methylamino)demethyl(oxy)aaptamine (**3**)

0.001 g (0.00025% dry weight), orange amorphous solid.
IR ($CHCl_3$): 3400, 1683, 1274 cm^{-1}.
UV/Vis λ_{max} (EtOH) nm (log ε): 246 (3.84), 280 (3.64), 411 (3.39), 497 (3.63) .
^{1}H and ^{13}C NMR (CD_3OD): Table 2.
HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{13}H_{11}N_3O_2Na$, *m/z* 264.0743; found: 264.0768.

Cell culture: Human monocytic leukemia THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C and 5% CO_2 in RPMI containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

Apoptosis assay: Early and late apoptosis induced by aaptamine and its derivatives in THP-1 cells was analyzed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA) and PE Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, the onset of early and late apoptosis was analyzed using Phycoerythrin (PE), Annexin V and 7-amino-Actinomycin (7-AAD) double staining. THP-1 cells in a 6-well plate, 2×10^5cells/well, in 10% RPMI were treated with various concentrations of the substances for 24 h. After incubation, cells were washed with PBS by centrifugation at 1000 rpm (170 rcf) for 5 min, and processed for detection of apoptosis. A total of 1×10^5-2×10^5 cells were resuspended in 500 μL of 1× binding buffer. Then, 5 μL of PE Annexin V and 5 μL of 7-AAD were added, and the cells were incubated at room temperature for 15 min in the dark and analyzed by flow cytometry.

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Isolation and Characterization of Crotosparsamide, a New Cyclic Nonapeptide from *Croton sparsiflorus*

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Crotosparsamide (**1**), a new cyclic nonapeptide, has been isolated from the *n*-butanol soluble sub-fraction of *Croton sparsiflorus* along with *p*-hydroxy methylcinnamate and kaempferol, which are reported for the first time from this species. Their structures were determined by chemical and spectral studies including ESIMS, and 1D and 2D NMR spectroscopic data.

Keywords: *Croton sparsiflorus*, Euphorbiaceae, cyclic peptide, crotosparsamide.

Cyclic peptides have been isolated from marine and micro organisms [1] as well as from higher plants [2] of the families Caryophyllaceae [3,4], Labiatae [5,6], Linaceae [7], Annonaceae [8], Amaranthaceae [9] and Euphorbiaceae [10,11]. Plant cyclopeptides [12,13] are cyclic compounds formed with the peptide bonds of 2-37 protein or non-protein amino acids, mainly L-amino acids. From higher plants, cyclolinopeptide A was the first Caryophyllaceae-type cyclopeptide isolated from the seeds of *Linum usitatissimum.* The structure was determined in 1959 [7]. The cyclopeptides are divided into two classes, five subclasses and eight types [2] on the basis of their structure and distribution in plants. Highly functionalized natural cyclic peptides are being used as therapeutic agents. Many of these bioactive cyclic peptides contain tryptophan residues such as diazonamides, complestatin and celogentins. These peptides are extensively modified through incorporation of an oxygen functionality into the amino acid residues and/or oxidative cross-linking of the indole group of the tryptophan side chain [14].

The aim of our research was to isolate and identify bioactive and structurally novel compounds from *Croton* species. The genus *Croton* is an important genus of Euphorbiaceae comprising 1300 species growing as trees, shrubs and herbs in tropical and subtropical regions of both hemispheres [15]. *C. sparsiflorus* (syn. *C. bonplandianus*) is a shrub growing in sandy clay soil in Asia and South America [16]. A literature survey revealed that a number of alkaloids have so far been reported from this plant [17,18]. The chemotaxonomic and ethnopharmacological importance of the genus *Croton* prompted us to carry out further phytochemical studies on *C. sparsiflorus*. As a result, we herein report the isolation and structural elucidation of a new cyclic peptide named, crotosparsamide (**1**) (Figure 1), along with *p*-hydroxy methylcinnamate (**2**) and kaempferol (**3**), reported for the first time from this species.

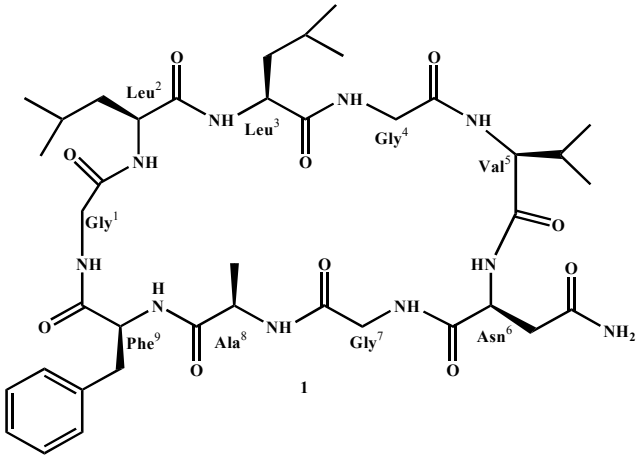

Figure 1: Structure of crotosparsamide (**1**).

The 80% ethanolic extract of the whole plant of *C. sparsiflorus* was suspended in water and extracted successively with *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and water. Column chromatographic techniques applied to the *n*-butanol soluble fraction led to the isolation of a cyclic nonapeptide, which was named crotosparsamide (**1**). The ethyl acetate soluble

Table 1: ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) spectroscopic data of crotosparsamide (**1**).

Residue	δ_C	δ_H [multi, (Hz)]	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC	^{1}H-^{1}H NOESY
Gly[1]					
C=O	172.2				
C^{α}	44.3	4.02[a] (d, 8.5 Hz)	3.58	174.7, 172.2	3.58
		3.58[a] (d, 8.5 Hz)	4.02	174.7, 172.2	4.02
Leu[2]					
C=O	174.8				
C^{α}	53.1	4.64 (d, 8.5 Hz)	1.77, 1.41	174.8, 172.2 41.7	1.71, 1.41
C^{β}	41.7	1.77 (m)	4.64, 1.71,1.41	174.8, 53.1, 25.1, 24.0	1.41, 1.00, 0.98
		1.41 (m)	4.64, 1.71,1.41	174.8, 53.1, 25.1, 23.7	4.64, 1.77, 0.98
C^{γ}	25.1	1.71(h, 6.0 Hz)	1.77, 1.0, 0.98	41.7, 24.0, 23.7	4.64, 1.00, 0.98
C^{δ}	24.0	1.00 (d, 6.0 Hz)	1.71	41.7, 25.1	1.77, 1.71, 0.98
$C^{\delta'}$	23.7	0.98 (d, 6.0 Hz)	1.71	41.7, 25.1, 24.0	1.77, 1.41, 1.00
Leu[3]					
C=O	174.6				
C^{α}	51.9	4.62 (d, 8.0 Hz)	1.95, 1.37	174.8, 174.6, 40.7, 25.8	1.60, 1.37
C^{β}	40.7	1.95 (m)	4.62, 1.60, 1.37	174.6, 51.9, 25.8	1.37, 0.96
		1.37(m)	4.62, 1.60, 1.37	174.6, 51.9, 25.8	4.62, 1.95, 0.92
C^{γ}	25.8	1.60 (h, 6.0 Hz)	1.95, 0.96, 0.92	40.7, 21.9, 21.6	4.62, 0.96, 0.92
C^{δ}	21.6	0.92 (d, 6.0 Hz)	1.60	40.7, 25.8, 21.9	1.60, 0.92
$C^{\delta'}$	21.9	0.96 (d, 6.0 Hz)	1.60	40.7, 25.8	1.95, 1.60, 0.92
Gly[4]					
C=O	170.9				
C^{α}	43.8	3.90 (d, 8.5 Hz)	3.78	174.6, 170.9	3.78
		3.78 (d, 8.5 Hz)	3.90	174.6, 170.9	3.90
Val[5]					
C=O	175.2				
C^{α}	63.8	3.65 (d, 7.5 Hz)	2.06	175.2, 170.9, 30.4, 19.9, 19.3	2.06, 1.06, 0.99
C^{β}	30.4	2.06 (m)	3.65, 1.06, 0.99	175.2, 63.8, 19.9, 19.3	3.65, 1.06, 0.99
C^{γ}	19.9	1.06 (d, 6.5 Hz)	2.06	63.8, 30.4, 19.3	3.65, 2.06
$C^{\gamma'}$	19.3	0.99 (d, 6.5 Hz)	2.06	63.8, 30.4, 19.9	3.65, 2.06
Asn[6]					
C=O	173.7				
C^{α}	52.6	4.52 (t, 7.5 Hz)	3.00, 2.87	175.2, 174.4, 173.7, 36.6	3.00, 2.87
C^{β}	36.6	3.00 (dd, 7.5, 8.5 Hz)	4.52, 2.87	175.4, 52.6	7.51, 4.52, 2.87
		2.87 (dd, 7.5, 8.5 Hz)	4.52, 3.00	175.4, 52.6	7.05, 4.52, 3.00
$^{\gamma}$C=O	175.4				
NH_2		7.51	7.05, 3.00, 2.87	175.4, 36.6	7.05, 3.00
		7.05	7.51, 3.00, 2.87	175.4, 36.6	7.51, 2.87
Gly[7]					
C=O	171.6				
C^{α}	44.1	4.03[a] (d, 8.5 Hz)	3.59	173.7, 171.6	3.59
		4.59[a] (d, 8.5 Hz)	4.03	173.7, 171.6	4.03
Ala[8]					
C=O	173.4				
C^{α}	50.8	3.96(q, 7.5Hz)	1.31	173.4, 171.6, 16.6	1.31
C^{β}	16.6	1.31 (d, 7.5 Hz)	3.96	173.4, 50.8	3.96
Phe[9]					
C=O	174.7				
C^{α}	58.4	4.29 (t, 8.0 Hz)	3.18	174.7, 173.3, 37.4, 138.1	3.18
C^{β}	37.4	3.18 (d, 8.0 Hz)	4.29	174.7, 138.1, 130.4, 58.4	4.29
Ph: C-1	138.1				
C-2,C-6	130.4	7.30 (d, 6.0 Hz)	7.26	138.1, 129.6, 128.0, 37.4	7.26
C-3,C-5	129.6	7.26 (m)	7.30, 7.22	130.4,128.0, 138.1	7.30, 7.22
C-4	128.0	7.22 (m)	7.26	130.4, 129.6	7.26

[a] Signals partially obscured.

fraction provided *p*-hydroxy methylcinnamate (**2**), and kaempferol (**3**).

Crotosparsamide (**1**) was obtained as white crystals (MeOH), mp 309°C, $[\alpha]^{25}_{D}$ -48 (*c* 0.02, MeOH). The UV spectrum (MeOH) showed bands at 204 and 193 nm, while the IR spectrum showed absorptions at 3306 (HN), 1665 (amide), and 1606-1400 cm^{-1} (aromatic moiety). The HR-ESI-MS showed the $[M+H]^+$ peak at *m/z* 829.4521 consistent with the formula $C_{39}H_{61}O_{10}N_{10}$. The ^{1}H NMR spectrum showed two N-H signals at δ 7.51 and 7.05, which were coupled with each other and each gave HMBC correlations to a methylene carbon signal at δ 36.6. This indicated the presence of an $–NH_2$ group. The ^{13}C NMR spectrum showed 10 carbonyl signals (δ 170.9-175.4) and 9 carbon resonances in the region of δ 43.8–63.8. The DEPT ^{13}C NMR spectrum revealed that six of them represent methine and three methylene carbons. All these data suggested that **1** was a peptide consisting of nine amino acids, out of which three are glycine and another asparagine. The ^{1}H and ^{13}C NMR spectra of **1** also showed signals indicating the presence of a mono-substituted benzene ring, suggesting the presence of a

phenylalanine moiety. The ^{1}H-^{1}H COSY spectrum along with HMQC and HMBC spectra allowed the determination of the amino acids leucine, valine and alanine besides glycine, asparagine and phenylalanine. Since the molecular formula showed 15 double bond equivalents, it was evident that compound **1** is a cyclic nonapeptide. The sequence of amino acids was determined by the extensive use of a combination of ^{1}H-^{1}H COSY, NOESY and HMBC. In the HMBC correlations, all the α-carbons showed $^{1,3}J$ correlations with the carbonyls of the adjacent amino acid, as shown in Table 1. The sequence of amino acids in **1** was further confirmed by the evidence of peptide fragments in the ESI-MS-MS at *m/z* 275 (Phe-Ala-Gly), *m/z* 489 (Phe-Ala-Gly-Asn-Val), *m/z* 545 (Phe-Ala-Gly-Asn-Val-Gly), *m/z* 659 (Phe-Ala-Gly-Asn-Val-Gly-Leu), and *m/z* 771 (Phe-Ala-Gly-Asn-Val-Gly-Leu-Leu). On the basis of the above evidence the amino acid sequence in **1** was determined as cyclo (-Gly^{1}-Leu^{2}-Leu^{3}-Gly^{4}-Val^{5}-Asn^{6}-Gly^{7}-Ala^{8}-Phe^{9}-) (Figure 1). The absolute configurations of the amino acids of **1** were shown to be all L, by comparison of the chromatograms from GC analysis of suitably derivatized L- and D-amino acid standards with those obtained from the acid hydrolyzate of the peptide [19]. This finding is consistent with the observation that all cyclic peptides isolated to date from the Euphorbiaceae family consist exclusively of L-amino acids [10,11].

The known compounds *p*-hydroxy methylcinnamate (**2**) and kaempferol (**3**) were also isolated from the ethyl acetate soluble fraction and identified by comparison of physical and NMR data with those in the literature [20,21].

Experimental

General experiment procedures: Column chromatography was carried out using silica gel (230-400 mesh, E. Merck, Darmstadt, Germany), Diaion HP-20 ion exchange resin (Nippon Rensui Co., Mitsubishi Chemical Corporation, Tokyo, Japan) and Sephadex LH-20 (25-100 μ, Amersham Biosciences Limited, Stockholm, Sweden). TLC was performed using precoated silica gel F_{254} plates and detection was achieved at 254 and 366 nm, and by spraying with ceric sulfate in 10% H_2SO_4 and ninhydrin [2]. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer, while the IR spectra were recorded as KBr pellets on a Jasco 302-A spectrometer. Optical rotation was recorded on a Jasco P-2000 polarimeter. Mass spectra were measured in electron spray ionization mode (LRESIMS, HRESIMS and ESI-MS-MS) on QSTAR XL spectrometers and ions are given in *m/z* (%). Melting points were determined on a Gallenkemp apparatus and are uncorrected. The ^{1}H, ^{13}C and 2D (^{1}H-^{1}H COSY, HMQC, HMBC, NOESY) NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD using standard pulse sequence. Chemical shifts are reported in ppm (δ), relative to tetramethylsilane used as an internal standard, and scalar coupling are reported in Hz. GC analyses were carried out using a Shimadzu GC-17A Gas Chromatograph.

Plant material: The whole plant of *Croton sparsiflorus* Morong (18 kg) was collected in 2005 from Karachi district, province of Sindh, and identified by Prof. Dr Surraiya Kahtoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (No. 4309 KUH) has been deposited in the herbarium.

Extraction and isolation: The freshly collected whole plant material of *C. sparsiflorus* (18 Kg) was shade dried, cut into small pieces, and extracted with 80% ethanol (3 x 20 L, 10 days each) at room temperature. The combined ethanolic extract was evaporated under reduced pressure at rt. to yield a residue (300 g), which was suspended in water and successively extracted with *n*-hexane (50 g), CH_2Cl_2 (10 g), EtOAc (6 g), *n*-BuOH (14 g) and water (220 g). The *n*-butanol soluble fraction was subjected to CC over diaion HP-20, eluting with water, water-methanol and methanol in decreasing order of polarity. The fraction obtained with water-methanol (1:1) was re-chromatographed over Sephadex LH-20 and eluted with mixture of $CHCl_3$-MeOH in increasing order of polarity. The fractions eluted with 15% $CHCl_3$/ MeOH provided a semi-pure compound, which was re-chromatographed over silica gel and eluted with CH_2Cl_2: MeOH (9.0:1.0) to afford crotosparsamide (**1**) (12 mg).

The ethyl acetate soluble fraction was subjected to CC over silica gel and divided into 8 sub-fractions by eluting with *n*-hexane, *n*-hexane-CH_2Cl_2, and CH_2Cl_2-MeOH in increasing order of polarity. The fractions eluted with *n*-hexane: CH_2Cl_2 (2.0:8.0) were re-chromatographed over silica gel and eluted with the same solvent system to afford compound **2** (10 mg). The fractions eluted with CH_2Cl_2 were triturated with dry acetone, and the residue was re-chromatographed over silica gel, eluting with CH_2Cl_2: MeOH (9.9:0.1) to afford compound **3** (20 mg) as a yellow amorphous powder.

Crotosparsamide (1)

White crystals (MeOH).
MP: 309^{o}C.
$[\alpha]^{25}_{D}$: -48 (*c* 0.02, MeOH).
UV λ_{max}^{MeOH} nm (log ε): 204 (1.9), 193 (6.0).

IR (KBr) ν_{max} cm^{-1}: 3306, 1665, 1606, 1540, 1248.
^1H and ^{13}C NMR: Table 1.
LRESIMS *m/z*: 829 $[M+H]^+$.
HRESIMS *m/z*: 829.4521 (calcd for $C_{39}H_{61}O_{10}N_{10}$, 829.4500).

Acid hydrolysis of 1: A solution (1 mg) of **1** was heated with 6 N HCl (2 mL) at 110°C for 10 h. The solution was concentrated to dryness and the residue esterified with 4 N HCl and methanol by heating at 100°C for 20 min. After cooling, the residue was dissolved in 200 µL of trifluoroacetic anhydride/ethyl trifluoroacetate (1:1) and heated for 15 min at 115°C. The obtained solution was concentrated to dryness, the residue dissolved in toluene and subjected to gas chromatography (Chirasil-L-Val capillary column) with H_2 as carrier gas at a flow rate maintained at 1.5 mL/min and an oven temperature increasing from 60°C to 180°C at a rate of 4°C/min. The retention times were noted of the peaks corresponding to the eluted amino acid derivatives. Similarly, derivatized D- and L-amino acid standards were subjected to GC analysis under identical conditions and the retention times obtained for the derivatives were as follows: D- and L-alanine: 4.65 and 5.40 min; D- and L-valine: 8.34 and 9.24 min; D- and L-leucine: 11.11 and 12.58 min; D- and L-asparagine: 14.51 and 14.71 min; and D- and L-phenylalanine: 21.15 and 21.75 min. Comparison of data indicated that all the amino acids of the peptide have the L-configuration.

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Two New Lavandulyl Flavonoids from *Sophora flavescens*

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Two novel lavandulyl flavonoids, (2*S*)-7-methoxyl-4'',5''-dihydroxynorkurarinone (**1**) and (2*S*)-6''-hydroxynorkurarinone-7-*O*-β-D-galactoside (**2**), were isolated from the rhizome of *Sophora flavescens*. Their structures were elucidated by spectral methods, including 2D NMR spectroscopy. Both compounds showed cytotoxic activity against Hela cells, with **2** being more active than **1**.

Keywords: *Sophora flavescens* Ait, lavandulyl flavanone, (2*S*)-7-methoxyl-4'',5''-dihydroxy norkurarinone, (2*S*)-6''-hydroxy-norkurarinone-7-*O*-β-D-galactoside, cytotoxicity.

The root of *Sophora flavescens* Ait (family Papilionaceae) is a well-known Chinese herbal medicine (Chinese name "Ku-Shen") used to treat gastric disturbance and eczema, and as an antifebrile and anthelmintic [1]. The major constituents are quinolizidine alkaloids and flavonoids [2-7]. Pharmacological research was focused on the lavandulyl flavonoids, which have significant cytotoxicity [8] and are glycosidase inhibitors [9]. This paper reports the isolation and characterization of two minor lavandulyl flavanones from the MeOH extract of *S. flavescens*.

Compound **1** was obtained as yellow powder. Its HR-ESIMS provided a molecular ion $[M-H]^-$ at *m/z* 471.1957 (calcd. 471.2019), suggesting a molecular formula of $C_{26}H_{32}O_8$. In the ^{1}H NMR spectrum, four aromatic protons, and three methyl and one methoxy groups were observed. The aromatic protons at δ 6.46 (d, *J* = 2.5 Hz), 6.43 (dd, *J* = 2.5, 9.0Hz) and 7.38 (d, *J* = 9.0Hz) formed an ABX spin system. In addition, 5-OH was observed at δ 12.17, due to the hydrogen-bonding effect. The ^{13}C NMR spectrum of **1** indicated five oxygenated aromatic carbons at δ 162.4, 162.7, 161.9, 156.9 and 156.2, and a carbonyl carbon at δ 198.0. The splitting patterns of the B ring in the ^{1}H NMR spectrum and the chemical shifts in the ^{13}C NMR spectrum indicated that two oxygen-bearing groups were located on the B-ring of **1**. In the HMBC

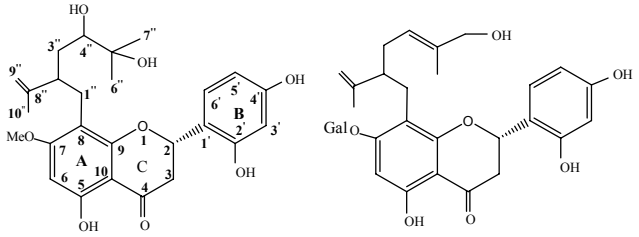

Figure 1: The structures of compounds **1** and **2**.

spectrum, H-5' (δ 6.43) correlated with C-1' (δ 117.6), C-6' (δ 128.3) and C-4' (δ 156.2), while the carbon at δ 156.9 (C-2') correlated with the aromatic protons at δ 6.46 (d, *J* = 2.5 Hz) and δ 7.38 (d, *J* = 9.0 Hz), suggesting oxygen groups at C-2' and C-4'. The methoxy group was located at C-7, due to the HMBC correlation of its protons (δ 3.10) with C-7. The H-1'' protons at δ 2.59 and δ 2.67 showed long range correlations with the carbon signals at δ107.4 (C-8) and δ161.9 (C-9). Furthermore, the aromatic proton at δ 6.00 correlated with C-7 (δ 162.7) and C-5 (δ 162.4), suggesting that a lavandulyl skeleton is located at C-8 of the A ring. In addition, the HMBC correlations of the carbon at δ 74.5 with the six protons at δ 1.03 (Me-6'' and 7'') and δ 3.22 (H-4''), and HMBC correlations of C-4'' (δ 77.4) with H-6''/H-7'', H-3'' and H-2'', implied that hydroxyl groups were present at C-4'' and C-5'' of the lavandulyl group. The circular dichroism (CD) spectrum of **1** showed a negative absorption at

Table 1: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of **1** and **2** (DMSO-d_6).

No.	Compound 1		Compound 2	
	H	C	H	C
2	5.64 (dd, *J* = 3.0, 13.0 Hz)	75.1	5.85 (dd, *J* = 3.0, 13.0 Hz)	75.0
3	2.76 (m) 3.04 (m)	42.4	2.82 (m) 2.92 (m)	43.2
4	---	198.0	---	197.7
5	12.17 (s)	162.4	12.2 (s)	162.7
6	6.00 (s)	95.9	6.00 (s)	96.0
7	---	162.7	---	165.3
8	---	107.4	---	107.5
9	---	161.9	---	161.9
10	---	102.9	---	102.9
1'	---	119.6	---	120.8
2'	---	156.9	---	159.0
3'	6.46 (d, *J* = 2.5 Hz)	103.3	6.77 (d, *J* = 2.5 Hz)	104.3
4'	---	156.2	---	155.9
5'	6.43 (dd, *J* = 2.5, 9.0 Hz)	109.6	6.64 (dd, *J* = 2.5,8.5 Hz)	110.1
6'	7.38 (d, *J* = 9.0 Hz)	128.3	7.49 (d, *J* = 8.5 Hz)	127.7
1''	2.67 m 2.59 m	28.3	2.62 m 2.51 m	27.6
2''	2.89 m	44.1	2.49 m	47.4
3''	1.36 m 1.55 m	34.0	2.13 m 1.96 m	31.1
4''	3.22 m	77.4	5.24 (brt, *J* = 6.0, 6.5 Hz)	124.2
5''	---	74.5	---	136.2
6''	1.03 (s)	20.1	3.79 (brs)	68.2
7''	1.03 (s)	20.0	1.45 (s)	13.6
8''	---	148.6	---	148.7
9''	4.67 brs 4.65 brs	111.6	4.60 brs	111.1
10''	1.65 s	18.6	1.66 s	19.1
7-OMe	3.10 s	48.8	---	---
Glu-1			4.82 (d, *J* = 7.0 Hz)	102.8
2			3.45 (m)	74.3
3			3.50 (m)	77.5
4			3.41 (m)	71.0
5			---	77.4
6			3.89 (m) 3.71 (m)	62.4

286 nm and a positive absorption at 310 nm, due to the n→π* transition of flavanone, indicating that the absolute configuration of C-2 was *S*. Based on the above analysis, **1** was characterized as (2*S*)-7-methoxy-4'',5''-dihydroxynorkurarinone.

Compound **2** was obtained as yellow powder. The molecular formula of **2** was determined to be $C_{31}H_{38}O_{12}$ from the high-resolution ESI-MS *pseudo*-molecular ion $[M-H]^-$ at *m/z* 601.2189 (calcd. 601.2285). This evidence suggested that a sugar residue may be linked to a flavonoid moiety. Similar to compound **1**, the ^{1}H NMR spectrum showed aromatic proton signals at δ 6.77 (d, *J* = 2.5 Hz), 6.64 (dd, *J* = 2.5, 8.5Hz) and 7.49 (d, *J* = 8.5Hz) forming an ABX spin system, suggesting that two oxygen atoms were attached to C-2' and C-4' of **2**. The hydrogen-bonded 5-OH of **2** was observed at δ 12.2. In the HMBC spectrum, the proton at δ 2.62 (H-1'') correlated with the carbon signals of C-2'' (δ 47.4), C-8 (δ 107.5) and C-9 (δ 161.9), indicating that a group with a lavandulyl skeleton is located at C-8 of the A ring. The HMBC correlation of the proton at δ 3.79 with the carbon signals at δ 136.2 (C-5'') and δ 13.6 (C-7'') suggested that a hydroxyl group is present at C-6'' of the lavandulyl skeleton. The carbon signals at δ 102.8, 74.3, 77.5, 71.0, 77.4 and 62.4 indicated the presence of a hexose [6,7]. By acid hydrolysis and thin-layer chromatography using reference samples, the sugar was identified as D-galactose. In the HMBC spectrum, the proton at δ 4.82 correlated with the carbon signal at δ 165.3 (C-7), showing that the β-D-galactopyranose moiety was at C-7. In the CD spectrum, a positive absorption at 310 nm, and a negative absorption at 291 nm were observed, indicating that the absolute configuration of C-2 was also *S*. Based on the above data, compound **2** was characterized as (2*S*)-6''-hydroxynorkurarinone-7-*O*-β-D-galactopyranoside.

Compounds **1** and **2** showed cytotoxic activity against Hela cell with IC_{50} values of 51.2 and 9.5 μmol/L, respectively, which implied that the introduction of a sugar into the skeleton of a lavandulyl flavonoid increases the cytotoxicity.

Experimental

General experimental procedure: IR spectra were recorded in KBr pellets on a NEXUS-470 FTIR (Nicolet) spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were measured with an INOVA-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 with TMS as internal standard. Optical rotations were obtained with a Perkin-Elmer 243B polarimeter. TLC analyses were performed on silica gel G. All chemicals were purchased from Beijing Chemical Factory. Silica gels were produced by Qingdao Haiyang Chemical Group Co., China.

Extraction and isolation: The dried roots of *S. flavescens* Ait were collected from Hebei Province, China. A voucher specimen was identified by Prof. X.L. Xin, and deposited at the School of Science, Dalian Nationalities University, China. The dried roots (5 Kg) were extracted by refluxing with MeOH 3 times. After removal of MeOH, water (1 L) was added to the residue and partitioned with equal volumes of light petroleum, EtOAc and *n*-BuOH, successively, three times with

each solvent. The EtOAc layer (90 g) was concentrated *in vacuo* and the residue subjected to silica gel CC, eluting with $CHCl_3/CH_3OH$ (100:1-1:1) in a gradient manner. Eight fractions were obtained; fraction V (5.2 g) was purified over Sephadex LH-20 and by preparative HPLC (Ultimate 3000 HPLC system, YMC-ODS column (5um, Ø10×250mm)) with CH_3OH-H_2O, 45:55 (v/v) to afford compounds **1** (6 mg) and **2** (5 mg), respectively.

Acid hydrolysis: Acid hydrolysis of compound **2** was carried out by refluxing 2 mg in 5 mL of 6% aqueous HCl for 3 h. The reaction mixture was extracted with 30 mL EtOAc to obtain the aglycone, which was separated by TLC using EtOAc–H_2O–MeOH–HOAc (13: 3: 3: 4); sugars were detected using thymol in H_2SO_4 (0.5 g thymol in 95 mL EtOH and 5 mL H_2SO_4), followed by heating at 120°C for 10 min.

(2*S*)-7-Methoxyl-4'', 5''-dihydroxy norkurarinone (1)

Yellow powder (MeOH).
$[\alpha]_D^{22}$: -16 (*c* 0.3, MeOH).
CD (MeOH): $[\theta]_{310}$ +8051, $[\theta]_{286}$ -18056.
IR (KBr) ν_{max} (cm^{-1}): 3300, 2950, 1750, 1625, 1170.
^1H NMR and ^{13}C NMR: Table 1.
HR-ESIMS: *m/z* = 471.1957 $[M-H]^-$ (calcd. 471.2019).

(2*S*)-6''-Hydroxynorkurarinone-7-*O*-β-D-galactoside (2)

Yellow powder (MeOH).
$[\alpha]_D^{22}$: -58 (*c* 0.2, MeOH).
CD (MeOH): $[\theta]_{310}$ +5606, $[\theta]_{291}$ -13218.
IR (KBr) ν_{max} (cm^{-1}): 3375, 2930, 1700, 1653, 1170.
^1H NMR and ^{13}C NMR: Table **1**.
HR-ESIMS: *m/z*= 601.2189 $[M-H]^-$ (calcd. 601.2285).

Bioassay: Hela cells were maintained in RPMI-1640 medium with 10% (v/v) fetal bovine serum and cultured in 96 well microtiter plates. Appropriate dilutions of **1** and **2** were added and the cells cultured at 37°C in 5% CO_2 for 72 h. The MTT method was used to evaluate the survival rates of the cancer cells [12]. The concentration of test compound that gave 50% inhibition of cell growth was expressed as the IC_{50} value. Results were expressed as the mean value of triplicate determinations.

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Biotransformation of Naringenin to Eriodictyol by *Saccharomyces cerevisiea* Functionally Expressing Flavonoid 3' Hydroxylase

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To increase the biological activities of flavonoids and to enhance their stability and solubility by functionalization reactions (polymerization, esterification, alkylation, glycosylation and acylation), an increase in the number of hydroxyl groups in these molecules is needed. Hydroxylation reactions may be achieved using either chemical or enzymatic methods, the latter being more highly specific than the former. In our study, the flavonoid 3' hydroxylase (F3'H) from *Gerbera hybrid*, functionally expressed in *Saccharomyces cerevisiae*, was used to hydroxylate naringenin (the first flavonoid core synthesized in plants). Furthermore, we studied factors that may affect naringenin hydroxylation by recombinant cell-like yeast growth on selective or rich media and plasmid stability.

The whole recombinant cells hydroxylated naringenin at position 3' to give eriodictyol. In a selective media, the yeast failed to grow to high cell densities (maximum 5 g/L), but the plasmid stability was nearly 90 %, and naringenin hydroxylation reached 100 %. In a rich complex media, the biomass reached 10 g/L, but the yield of naringenin hydroxylation reached only 71 %, and the plasmid stability decreased. When yeast functionally expressing F3'H from *Gerbera hybrid* was used, in a selective media, 200 mg/L of eriodictyol from naringenin was produced.

Keywords: flavonoid, F3'H from *Gerbera hybrid*, hydroxylation, plasmid stability.

Flavonoids are secondary metabolites found in plants [1], in which their roles are as diverse as their structures, contributing to plant protection from environmental aggression [2,3]. In addition, flavonoids have potential benefits to human health [4], but the major hurdles of their formulation are their low solubility and stability. In order to take advantage of their beneficial properties, functionalization reactions (polymerisation, esterification, alkylation, glycosylation and acylation) have been suggested by several authors as a promising route. However, these reactions need free hydroxyl groups in aglycons or in glycosides to react with functional groups. Through hydroxylation, the basic flavonoid backbone can serve as useful starting materials for the development of new drugs. However, the ability to generate either regioselective or stereoselective compounds through chemical hydroxylation presents a major obstacle for the development of new pharmaceuticals. Fortunately, biological hydroxylation using either enzymes or transgenic microbes has the ability to overcome this problem. Enzymatic hydroxylation reactions have been achieved, in particular with either flavonoid hydroxylase (FH) directly extracted from plants or from recombinant *Escherichia coli* or *Saccharomyces cerevisiae* functionally (Table 1) expressing FHs [5,6,12]. In the present study, the 3' hydroxylation of naringenin was evaluated using the microsomal fraction and whole recombinant yeast expressing the F3'H from *Gerbera hybrid*. Also we investigate the effect of media

Table 1: Origin of flavonoid hydroxylation enzyme functionally expressed in recombinant cells.

Flavanone 3 hydroxylase	Flavonoid 3'hydroxylase	Flavonoid 3'5' hydroxylase
Malus domestica cv. M9 [6] *Ammi majus, Anethum graveolens, Pimpinella anisum* [7] *Petunia hybrida* [8-10] *Petroselinum crispum* cv. Italian Giant plants [11]	*Brassica napus* [12] *Callistephus chinensis* line '01', *Osteospermum hybrid* line '082', *Gerbera hybrid* line 'D1', *Hieracium pilosella* [5], Sorghum [13] *Torenia hybrida* [14], Gentian [15] *Arabidopsis thaliana* [16], *Petunia hybrida* [17]	*Callistephus chinensis* line '01', *Osteospermum hybrid* cv. 'Bamba', *Pericallis cruenta* cv. 'Blue Bicolor' [5] *Verbena temari*, *Butterfly pea* [18] *Catharanthus roseus, Petunia hybrida* [19]

Table 2: Effect of microsome extraction pressure on naringenin bioconversion yield and protein quantity.

	Manual extraction	French press extraction				
		500 bars	750 bars	1000 bars	1500 bars	2000 bars
Protein (g/L)	0.25	0.34	0.78	0.89	1.05	1.25
Naringenin bioconversion yield (%)*	100	66	90	97	83	85

*: Relative to the activity of microsomes extracted manually (100%). The quantity of proteins used for the reaction of naringenin hydroxylation was standardized to 0.25 g/L

culture on naringenin bioconversion, yeast growth and plasmid stability.

Indeed, the functional expression of *Gerbera hybrid* F3'H has been previously studied by Seitz *et al*. [5]; the ORF of isolated cDNA is constituted by 1539 base pairs; the encoded protein has a specific activity of 5.63 pkat/mg. The recombinant activity has been explored using the microsomal fraction to convert naringenin to eriodictyol [5]. Generally, the preparation of the microsomal fraction is done manually. In order to facilitate the microsome extraction procedure, we have used a modified extraction method based on the use of a constant disruption system. As can be seen in Table 2, the increase of extraction pressure was accompanied with an increase of protein yield and a decrease in naringenin bioconversion yield. A pressure of 750 and 1000 bars gave nearly the same bioconversion yield as that obtained with manual extraction, but the protein content of the microsomal fraction was more important. Whereas 500, 1500 and 2000 bars gave no amelioration of naringenin bioconversion, this may be attributed to insufficient extraction pressure (500 bars) or to the deleterious effect of pressure on enzyme structure and activity (1500 and 2000 bars). Manual extraction gave the best ratio for bioconversion/ protein quantity, but the use of a constant disruption system is easier and a more comfortable tool, since it permits the characterization of the naringenin bioconversion reaction and enzyme constants.

Whole cells and purified enzymes are both used as biocatalysts, but the former presents the advantages of simplicity, convenient manipulation and low costs, while enzyme purification is tedious and expensive. Moreover, the purification of enzymes could lead to significant loss of activity and cofactors such as NADPH, usually required for enzymes like F3'H [5,12-17]. Consequently, in order to probe the whole cell activity, 1 g/L of recombinant yeast was incubated with naringenin as substrate. In the course of the incubation,

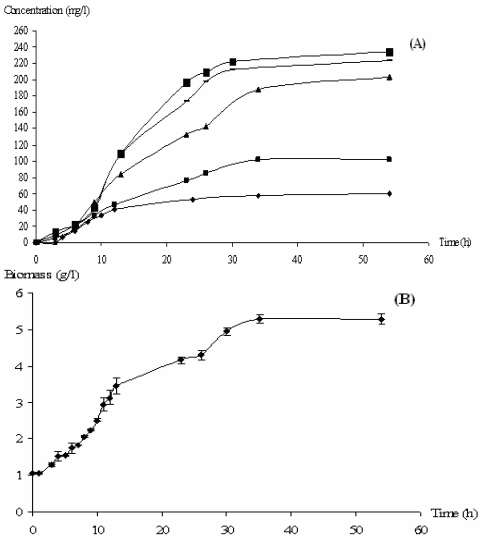

Figure 1 (A): Eriodictyol production by recombinant *S. cerevisiea* expressing the F3'H of *G. hybrid* in the presence of various concentrations of naringenin. (■): 2025 mg/L, (-): 675 mg/L, (▲): 225 mg/L, (■): 100 mg/L, (♦): 50 mg/L. **(B):** Growth curve of the recombinant *S. cerevisiea* expressing the F3'H of *G. hybrid* (means of growth curves of the recombinant yeast strain with different concentrations of naringenin (0, 50, 100, 225, 675 and 2025 mg/L) with standard deviation).

the initial naringenin concentration (100 mg/L) started to decrease and a new product appeared (data not shown). The new product was identified as eriodictyol (LC/MS). Thus, the whole recombinant yeast can be used to hydroxylate naringenin.

In order to investigate the maximum bioconversion ability of *S. cerevisiae* strain harbouring F3'H, the naringenin was added to the culture media at final concentrations of 50, 100, 225, 675 and 2025 mg/L; product formation and residual substrate concentrations against incubation time were plotted (Figure 1). We analysed the yeast growth and the naringenin biotransformation kinetics with *S. cerevisiae* expressing F3'H. In *S. cerevisiae* strain harbouring F3'H, recombinant protein production was induced by galactose in the YPL medium, as described in the experimental section. The growth of the recombinant

strain was not affected by the naringenin concentration (Figure 1).The bioconversion kinetic and growth were slow for the first 10 hours; the biomass was doubled, and 33 to 50 mg/L of naringenin was converted to eriodictyol. Within 10 to 33 hours of the exponential growth phase, the biomass was multiplied by 5 (when compared with the initial biomass) and the growth was associated with the bioconversion kinetic, both of which progress faster than during the first 10 hours after induction. Nearly 200 mg/L of eriodictyol were synthesized from 225, 675 and 2025 mg/L of naringenin. Within 33 to 54 h, the stationary growth phase was attained and only an extra 5 mg/L of eriodictyol, at a maximum, was formed (Figure 1).

It is clear that increasing naringenin concentrations enhanced the production yield and bioconversion kinetic but there is a limit of saturation (of substrate bioconversion). It is not necessary to increase naringenin concentration over 225 mg/L because the bioconversion yield decreases 3 and 9 folds when compared to a total bioconversion (for 675 and 2025 mg/L of naringenin, respectively).

The bioconversion yield is influenced by the initial naringenin concentration and also by the culture media composition. The latter dictates the growth rate of the recombinant yeast, the cell yield, the plasmid stability and naringenin bioconversion. Consequently, we tested, in parallel, the effect of culture conditions on the factors cited above.

Two stage culture modes were used: (i) a growth phase to form the initial biomass and (ii) an induction phase. We tested two culture media; a minimal medium without uracil (to select recombinant yeast harboring plasmid) and a rich medium. We combined these two media, as indicated in Table 3. As can be seen in Table 3, independently of the media used to prepare the inoculums, when the induction was done in YPL rich medium, the biomass production was nearly 10 g/L compared with 5 g/L in SC minimal medium. Despite the use of the same quantity of carbon source and the same initial biomass (adjusted to 1 g/L), biomass production was not the same, indicating that minimal medium presents a limitation in its saline, vitamin or nitrogen composition.

Within 33 hours of induction, it was notable that SC-SC and YPL-SC two stage culture modes gave the same maximum bioconversion yield (100%), but in SC-YPL and YPL-YPL culture mode the bioconversion yield did not exceed 71% (Table 3). This indicates the importance of induction media in the conversion reaction. Indeed, SC minimal media exert a selection pressure to stabilize the plasmid in cells, and consequently, the F3H expression is maintained to a high level allowing the production of the maximum amount of eriodictyol (100 mg/L of naringenin were totally converted to eriodictyol). As can be shown in Figure 2, after 30 hours of induction, only 58 % of cells in YPL medium contain plasmid, while in SC medium, 90 % of the yeast contains plasmid. We noticed that during the exponential phase, between 6 and 24 hours, the fraction of plasmid free cells increased. This phenomenon may be due to the acceleration of cell division. However, in the stationary phase, this fraction decreased, probably because the media components become limiting and only cells containing the recombinant plasmid can survive uracil limitation.

Over-viewing our work, we have successfully produced eriodictyol, a 3'hydroxylated flavonoid from naringenin, by using a whole cell activity. Recombinant *S. cerevisiea* harboring F3'H from *Gerbera hybrid* was used as a selective single step for high-yield hydroxylation to replace a series of chemical synthetic reactions that suffers from drawbacks of simultaneous formation of diverse by-products and low isolated yield.

Contrary to the works reported in Table 1, we have used another easy method for microsome (containing the F3'H) extraction, to test enzymatic activity. The use of a cell disruption system at 750 and 1000 bars gave nearly the same naringenin bioconversion yield as that of the classic manual extraction. Moreover, in the present study, we demonstrated that it is not necessary to extract enzyme to achieve the hydroxylation reaction. The whole recombinant *S. cerevisiea* cell expressing flavonoid hydroxylases can hydroxylate naringenin and

Table 3: Effect of culture mode on growth of the recombinant yeast strain and eriodictyol production.SC: selective medium, YPL: rich medium.

Medium used to two stage culture mode		Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)
Inoculum media	Induction and bioconversion media	**Time after induction of recombinant hydroxylase synthesis (h)**									
		0		5.5		11		22		33	
SC min	SC min	1	0	1.5	17.5	2.1	47.6	4.4	90.4	5.1	100
YPL		1	0	1.1	30.7	2.2	85.3	4.7	94	4.8	100
SC min	YPL	1	0	1.5	12	2.5	36.8	8	71.2	8.3	71
YPL		1	0	1.7	20	3.7	61.1	8.9	70.4	9.9	71

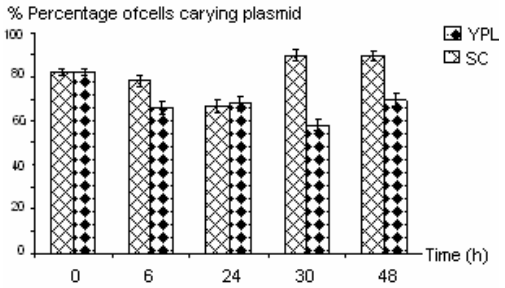

Figure 2: Effect of culture media on the percentage of plasmid containing cells. SC: selective medium, YPL: rich medium.

other flavonoids (like apigenin ;data not shown)). As substrate concentration increased, the naringenin bioconversion yield decreased and goes much slower. This may be ascribed to a substrate saturation phenomenon, low substrate solubility in aqueous medium, low substrate penetration to cells, and limitation in medium composition... Special techniques, such as two-phase biocatalysis and cell immobilization and/or permeabilization could be used to increase the concentration tolerance of this reaction [20].

Naringenin bioconversion to eriodictyol depends on the production of the recombinant F3'H enzyme. Synthesis of the cloned gene product is influenced by genetic and environmental factors, including plasmid stability, cell growth rate and medium composition. We have tested four two stage culture modes using two culture media: the SC minimal and YPL rich medium. We found that the naringenin bioconversion yield depends on the media used to induce the recombinant F3'H production, independently of the medium used to form the initial yeast biomass. SC minimal medium used as an inductor gave the best bioconversion/biomass ratio; 20 mg of eriodictyol per g of recombinant yeast (dry weight), when compared with the YPL rich complex medium. The induction in this latter case gave a ratio of 7.1 mg of eriodictyol per g of recombinant yeast. In addition, culture in the minimal medium offers the advantage of maintaining high plasmid stability. Thus, the decrease of naringenin bioconversion, in rich media, may be correlated to plasmid instability. It is notable (Figure 2) that the fraction of plasmid-carrying cells decreased during the exponential growth phase, but increased during the stationary phase. This phenomenon may be ascribed to the death rate difference between the plasmid-free and plasmid carrying cells caused by starvation in the stationary phase. Indeed, the high segregational instability of recombinant *S. cerevisiae* is primarily caused by asymmetric cell division, which can lead to the formation of 9 to 43 buds per cell [21]. In contrast to binary cell division in *E. coli*, budding results in an unequal distribution of plasmids among daughter cells, leading to an increased probability of plasmid-free cell emergence, especially in the exponential growth phase. In theory, plasmid-free cells respond to environmental changes much quicker than plasmid carrying cells. Plasmid-free cells may grow faster than plasmid carrying cells when there is plenty of growth substrate, but they also may lyse or die faster upon depletion of growth substrate (uracil). Thus, pulse addition of growth substrate at an appropriate time interval, with a significant starvation period between two consecutive feedings during feedbatch fermentation may have positive effects on stabilizing plasmid and enhancing protein production [22]. Other various operating strategies have been proposed to address the problem of competitive plasmid instability in recombinant yeast. These include cycling growth rate changes, cycling of substrate levels, cycling dissolved oxygen tension, dilution rate cycling, and autoselection systems [23].

Our work represents the first report of using recombinant yeast whole cell activity to achieve a single flavonoid hydroxylation step. Yan *et al* [24] have reported the use of whole cells expressing multiple enzymes to produce eriodictyol from caffeic acid. Their recombinant system produced 6.5 mg/L of eriodictyol from 180 mg/L of caffeic acid. In the present work, we produced 200 mg/L of eriodictyol from 225 mg/L of naringenin. Consequently, eriodictyol formation is 24 orders of magnitude higher than that reported [24]. This work opens up alternative routes for the production of additional hydroxylated flavonoid structures from other non-hydroxylated flavonoid precursors, using recombinant *S. cerevisiea* cells and by engineering substrate specificity of the F3'H by using site directed mutagenesis, laboratory evolution or chimeragenesis and engineering improved redox partners [25].Generally, the relatively poor stability of P450 monooxygenases, such as F3'H, still impede the technical application of these enzymes. Immobilization of enzyme or recombinant permeabilized yeast has proven useful for *in vitro* biotransformations [26]. A combination of chemical, physical and biological approaches will contribute to the production of polyhydroxylated flavonoid structures needed for functionalization reactions, like glycosylation [4,27], acylation [4], methylation [4,28], and sulfation [4,29].

Experimental

Plasmid and cell transformation: The F3'H from *Gerbera hybrid* was generously given by Pr. Christian Seitz (Technical University Munich, Germany) in yeast plasmid pYES2.1/V5-His-TOPOZ® [5]. The *Saccharo-myces cerevisiea* INVSc1 strain was purchased from Invitrogen, (Paisley, UK). The yeast transformation was carried out according to the manufacture's prescription. Chemicals used in this study were purchased from Sigma Chemicals (St. Louis, (USA).

Hydroxylation reaction by microsomes extracted from *S. cerevisiea* expressing Gerbera hybrid F3'H: Microsomes were prepared as described by Pompon *et al*. [30]. Ten mL of the minimal growth medium (SC minimal composed of 20 g glucose, 1.92 g yeast synthetic drop-out media without uracil, and 6.7 g yeast nitrogen base without amino acids in 1L of distilled water) were inoculated with the recombinant yeast and grown to stationary phase. Cells were harvested by centrifugation (3000 rpm for 5 min), then used to inoculate 250 mL of rich induction medium composed of 20 g/L galactose, 10 g/L peptone and 10 g/L yeast extract (YPL) and incubated overnight at 28°C, with shaking at 200 rpm. Cells were harvested by centrifugation at 7000 *g* for 10 min at 4°C. The cell pellet was washed with TEK buffer (l0 mM Tris-HCL (pH=7.5), 1 mM EDTA and 100 mM KCl). After centrifugation, cells were suspended in 50 mL TESB buffer (50 mM Tris-HCl (pH=7.5), 1 mM EDTA, 0.6 mM sorbitol, 1% BSA and 20 mM β-mercaptoethanol). The cell suspension was passed through either a constant cell disruption system or disrupted manually, as described [30]. Five extraction pressures were applied: 500, 750, 1000, 1500 and 2000 bars. The suspension was centrifuged at 10000 *g* for 15 min at 4°C. The supernatant was recovered and the microsomes precipitated with $MgCl_2$ (125 mM) for 30 min at 4°C. Microsomes were then recovered by centrifugation for 20 min at 15000 rpm at 4°C and suspended in 3 mL cold TEG (50 mM Tris-HCL (pH=7.5), 1 mM EDTA, 30% glycerol). Protein quantification was determined by the bicinchoninic acid method [31].

Determination of naringenin hydroxylation product by whole cells of *S. cerevisiea* expressing F3'H: Ten mL of culture grown to stationary phase in SC minimal medium was used to inoculate 100 mL of a rich induction medium. Naringenin was added at a concentration of 100 mg/L and the mixture was incubated on a shaker at 28°C for 55 h. 750 µL of supernatant was collected and mixed with 250 µL of acetonitrile. Identification of the hydroxylation products was monitored using HPLC and LC/MS analysis.

Effect of substrate concentration on bioconversion kinetics with *S. cerevisiea* expressing F3'H: Thirty mL of culture grown to stationary phase in SC minimal medium was used to inoculate 250 mL of SC minimal medium. Cells were harvested by centrifugation at 3000 rpm for 5 min before their transfer to SC medium, with different concentrations (50, 100, 225, 675 and 2025 mg/L) of the substrate. The mixture was incubated on a shaker at 28°C for 55 h. 750 µL of the supernatant was collected and mixed with 250 µL of acetonitrile. Quantification of the metabolites and parent material was monitored using HPLC.

Determination of the appropriate culture modes for growth of the recombinant *Saccharomyces cerevisiea* and naringenin bioconversion: A single colony of recombinant yeast strain was placed in 30 mL of (20%) glucose supplemented SC minimal media or in YPL rich medium and incubated at 28°C till stationary phase. The culture was centrifuged for 3 min at 5000 rpm and the pellet suspended in (20%) galactose supplemented fresh minimal (SC) or complex media (YPL).This gives four types of culture, as seen in Table 3. Naringenin was added to a final concentration of 100 mg/L and its transformation was assessed for 34 h.

Analytical method: Cell density was measured by the dry cell weight method. For determining dry cell weight, 10 mL of sample was spun in a pre-weighed centrifuge tube, at 5000 rpm for 5 min. The cell pellet was washed with deionized water and dried at 100°C for 24 h. The tube was weighed along with the dried pellet and used to calculate the dry weight of the sample. Total cell concentration was determined by measuring the optical density at 600 nm (OD_{600nm}). One unit of OD_{600nm} was found to be equivalent to about 0.45 g/L dried cell weight. The percentage of plasmid-containing cells was determined by the replica-plating technique. The percentage of plasmid stability was calculated as the ratio of the number of plasmid-containing cells forming the clones in selective plates (SC min) to the total number of cells growing on non selective YPL medium (including plamid-free and plasmid-containing cells). Cell samples were diluted to make the colony counts within the range of 30-300. All plate counts were taken from the average of at least 3 replicates.

HPLC analysis: A Thermo Finnigan (San Jose, CA, USA) HPLC apparatus equipped with an U.V. 6000 LP detector was used. For analytical scale, the mobile phase consisted of water and acetonitrile and was programmed as follows; 10% acetonitrile from 0 min to12 min, then a linear gradient was used starting from 40% acetonitrile to 60% at 17 min, followed by 8 min of a constant eluant of 10% acetonitrile. The flow rate was 1 mL/min; detection wavelength 296 nm; column temperature 28°C.

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Two New 3-*C*-Carboxylated Flavones from the Rhizomes of *Caragana conferta*

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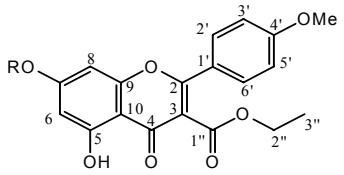
Confertins A (**1**) and B (**2**), new 3-*C*-carboxylated flavones, have been isolated from the ethyl acetate soluble fraction of the rhizomes of *Caragana conferta.* Their structures have been assigned on the basis of spectroscopic studies.

Keywords: *Caragana conferta*, 3-*C*-carboxylated flavones, confetins A and B.

The genus *Caragana* belongs to the family Fabaceae and comprises over 80 species out of which ten have so far been identified in Pakistan [1]. Some of these are used for the treatment of hypertension, irregular menstruation, and fatigue. The extracts and compounds from the genus have antitumor, antivirus, anti-inflammation, hypertensive, sedative, acetylcholinesterase inhibitory, and immunosuppressant activities [2].

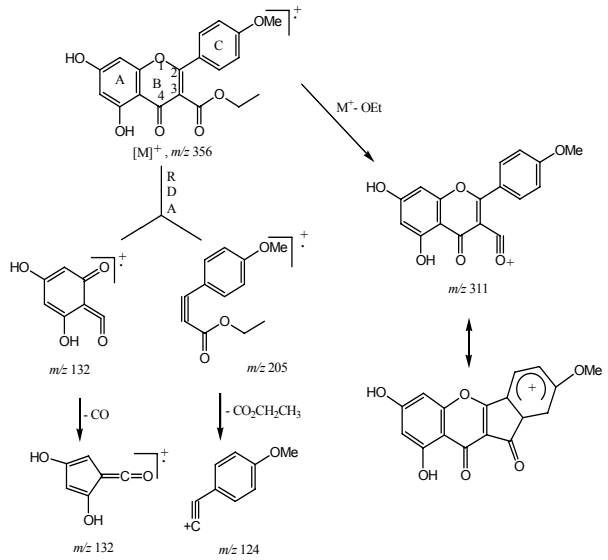


Compound 1. R = H
Compound 2. R = $COCH_3$

C. conferta is mainly found in the Ziarat, Gilgit and Kashmir valleys of Pakistan at an altitude of 7000-12000 feet above sea level [1]. A literature survey revealed that one isoflavone and two 4-hydroxyisoflavones have so far been reported from this species [3,4]. The ethnopharmacologial and chemotaxonomic importance of *Caragana* species prompted us to carry out phytochemical studies on the rhizomes of *C. conferta*. Herein we report the isolation and structural elucidation of two new 3-*C*-carboxylated flavones named as confertin A (**1**) and confertin B (**2**), respectively.

Confertin A (**1**) was obtained as light yellow needles, mp 184°C. The infrared (IR) spectrum showed absorption bands for hydroxyl (3450 cm^{-1}), conjugated ester (1700 cm^{-1}), conjugated ketone C=O (1660 cm^{-1}), aromatic (1545 cm^{-1}) and OCH_3 (1250 cm^{-1}) functionalities. The UV spectrum showed absorptions bands at 265, 295 and 330 nm, which are characteristic of a C-3-substituted flavone-type skeleton [5]. Addition of $AlCl_3$ and NaOAc resulted in bathochromic shifts of 30 nm and 12 nm, respectively, indicating a free phenolic group at both C-7 and C-5, respectively [6]. The high resolution electron impact mass spectrum (HREI-MS) of **1** exhibited the $[M]^+$ peak at *m/z* 356.0513 (Calcd. for $C_{19}H_{16}O_7$: 356.0584) with twelve degrees of unsaturation.

The broad-band decoupled (BB) and DEPT ^{13}C-NMR spectra of **1** showed 19 signals, comprising two methyl, six methine, one methylene and ten quaternary carbons. The signals at δ 182.5 (C-4) and 162.4 (C-1") were due to the conjugated carbonyl and ester moieties, while the conjugated olefinic carbons resonated at δ 152.0 and 123.5, respectively. The methoxyl group gave a signal at δ 55.7, while oxygenated aromatic carbons appeared at δ 166.7, 163.3 and 161.2. In the HREI-MS the peak at *m/z* 311.2661 ($C_{17}H_{11}O_6$) resulted from the loss of an ethoxyl group. The retro Diels-Alder (RDA) fragments A^+ at *m/z* 152.1041 ($C_7H_4O_4$) and B^+ at *m/z* 205.2221 ($C_{12}H_{13}O_3$) revealed the presence of two hydroxyl groups in ring A and one methoxyl and ethyl ester groups in rings B and C. The daughter ion fragments at *m/z* 124.0941 ($C_6H_4O_3$) and *m/z* 132.1591 (C_9H_9O) resulted from the loss of CO and an ethoxyl carbonyl group from the fragments A^+ and B^+, respectively (Scheme 1) [7]; the latter ion peak provided evidence



Scheme 1

for the presence of an ethoxy carbonyl group at C-3. Further evidence was provided by the carbon signals at δ 123.5 (C-3), δ 152.0 (C-2) and δ 182.5 (C-4), and the absence of a proton signal for H-3 in the ^{1}H and ^{13}C NMR spectra, confirming the presence of an ethyl ester group at C-3.

The ^{1}H NMR spectrum showed a chelated hydroxyl group at δ 12.40 as a singlet and meta coupled protons of ring-A at δ 6.23 (d, *J* = 1.6 Hz) and 6.34 (d, *J* = 1.6 Hz), thereby confirming the presence of hydroxyl groups at C-5 and C-7, respectively. Protons for a methoxyl group resonated at δ 3.75. Its presence at C-4' was confirmed by the protons of ring-B showing an AA' BB' pattern at δ 6.94 (d, *J* = 8.8 Hz) and 7.20 (d, *J* = 8.4 Hz). The presence of an ethoxyl group was revealed by a typical two-proton quartet at δ 4.20 and a three-proton triplet at δ 1.03. The position of the methoxyl group at C-4' was further confirmed by HMBC correlations in which the methoxyl protons at δ 3.75 showed ^{3}J correlations with C-4' (δ 55.7). Irradiation of these protons at δ 3.75 caused strong NOE enhancement of neighboring protons at C-3 and C-5. The NMR assignments were facilitated by ^{1}H-^{1}H COSY and HMQC experiments. The positions of the substituents were further authenticated through HMBC

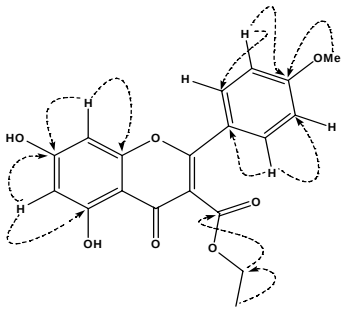


Figure- 2. Important HMBC correlations of confertin A (1)

Table 1: ^{1}H- and ^{13}C-NMR data ($CDCl_3$) of compounds **1** and **2** at 400 and 100 MHz, respectively; δ in ppm, *J* in Hz. Assignments were confirmed by COSY, HMQC and HMBC experiments.

Position	**1**		**2**	
	δ_H (*J* = Hz)	δ_C	δ_H (*J* = Hz)	δ_C
2	-	152.0	-	149.2
3	-	123.5	-	110.2
4	-	182.5	-	175.7
5	-	163.3	-	150.7
6	6.23 (*d*, *J* = 1.6)	105.0	6.48 (*d*, *J* = 2.0)	109.7
7	-	166.7	-	162.7
8	6.34 (*d*, *J* = 1.6)	94.9	6.72 (*d*, *J* = 2.0)	101.1
9	-	158.5	-	158.1
10	-	100.5	-	123.2
1"	-	162.4	-	161.8
2"	4.13 (q)	63.4	4.07 (q)	62.3
3"	1.03 (*t*)	13.8	0.92 (*t*)	13.4
1'	-	125.0	-	126.0
2'	6.94 (*d*, *J* = 8.8)	132.1	7.09 (*d*, *J* = 8.4)	131.0
3'	7.20 (*d*, *J* = 8.4)	114.5	6.87 (*d*, *J* = 8.8)	113.6
4'	-	161.2	-	159.6
5'	7.20 (*d*, *J* = 8.4)	114.5	6.87 (*d*, *J* = 8.8)	113.6
6'	6.94 (*d*, *J* = 8.8)	132.1	7.09 (*d*, *J* = 8.4)	131.0
4'-OCH_3	3.75 (s)	55.7	3.75 (s)	55.2
7-CO	-	-	-	169.9
7-CH_3	-	-	2.30 (s)	21.0

[a]) Arbitrary C-atom numbering.

correlations (Figure 2). The structure of confertin A (**1**) could, therefore, be assigned as ethyl 5, 7-dihydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-3-carboxylate.

Confertin B (**2**) was also obtained as light yellow needles, mp 186°C. The IR and UV spectra were similar to those of **1**. However, a bathochromic shift of 28 nm was observed with $AlCl_3$, but no such shift was observed with NaOAc, indicating the absence of a phenolic group at C-7. The HREI-MS showed an $[M]^+$ ion at *m/z* 398.3630 corresponding to the molecular formula $C_{21}H_{18}O_8$ (Calcd. for $C_{21}H_{18}O_8$: 398.3670).

The BB and DEPT ^{13}C NMR spectra of compound **2** showed twenty-one carbon signals comprising three methyl, six methine, one methylene and eleven quaternary carbons. The spectrum was similar to that of **1** with additional signals for an acetyl group (δ 169.9 and δ 21.0). The ^{1}H NMR spectrum was also similar to that of **1**, with an additional signal for an acetyl group at δ 2.30 (COC*H*$_3$). The signal for the chelated hydroxyl group at C-5 was observed at δ 12.3 as a singlet. Compound **2** is therefore, the acetylated derivative of **1**. The UV spectrum indicated acetylation of the phenolic group at C-7. The HMBC correlations were in complete agreement with the assigned structure of confertin B (**2**) as ethyl 7-(acetyloxy)-5-hydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-3-carboxylate.

This is the first report of the natural occurrence of 3-*C*-carboxylated flavones, although these type of compounds have previously been synthesized [8].

Experimental

General: Melting points were determined on a Gallenkamp apparatus and are uncorrected. ^{1}H- and ^{13}C-NMR spectra and two-dimensional correlation spectroscopy (COSY, NOSEY, HMQC, and HMBC) were recorded on a Bruker AV-400 spectrometer (400 MHz for ^{1}H- and 300 MHz for ^{13}C-NMR) in $CDCl_3$ with TMS as internal standard. Chemical shifts (δ) are shown in ppm relative to TMS. The UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. The IR spectra were recorded on a JASCO 302-A spectrometer in $CHCl_3$. Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60G F_{254} plates (20 x 20 cm, 0.2 mm layer thickness, E. Merck, Darmstadt, Germany) and visualized under UV light (254 nm) and also by spraying with ceric sulfate reagent. Silica gel 230-400 mesh (E. Merck. Darmstadt, Germany) was used for column chromatography. The HREI-MS were recorded on a JEOL JMS-HX-110 mass spectrometer.

Plant material: Rhizomes of *Caragana conferta* Benth were collected from Gilgit valley (Pakistan) and identified by a Senior Scientist of the National Agriculture Research Center (NARC), Islamabad Pakistan. A voucher specimen has been deposited in the herbarium of the Department of Botany, University of Karachi (voucher no 319).

Extraction and isolation: The air-dried powdered rhizomes (12 kg) were extracted with MeOH (3 x 25 L) at room temperature. The combined extract was evaporated to yield the residue (250 g), which was divided into *n*-hexane (50 g), ethyl acetate (EtOAc) (120 g) and water (80 g) soluble sub-fractions. The EtOAc fraction was subjected to column chromatography (CC) over silica gel, eluting with mixtures of *n*-hexane and EtOAc in increasing order of polarity to obtain 3 major fractions, A, B and C. Fraction A obtained from *n*-hexane/EtOAc (4:6) was again chromatographed over silica gel using *n*-hexane/EtOAc (8:2) as eluent to afford two successive fractions A_A and A_B. CC of the sub fraction A_A gave the compound **1** (20 mg) through elution with *n*-hexane/EtOAc (5:5), while elution with *n*-hexane/EtOAc (6:4) provided compound **2** (11 mg). Fractions B and C could not be worked up due to paucity of material.

Confertin A (**1**)

Light yellow needles.
MP: 184°C
IR (KBr) ν_{max} cm^{-1}: 3450 (OH), 1700 (ester), 1660 (conjugated ketone), 1545 (aromatic C=C), and 1250 cm^{-1} (OCH_3).
UV ($CHCl_3$) λ_{max} (log ε): 330 (4.43), 295 (3.99) and 265 nm (4.73).
^{1}H and ^{13}C NMR: Table 1
HREI-MS: *m/z* 356.0584 ($C_{19}H_{16}O_7$), 311.2661 ($C_{17}H_{11}O_6$), 205.2221 ($C_{12}H_{13}O_3$), 152.1041 ($C_7H_4O_4$), 132.1591 (C_9H_9O), 124.0941 ($C_6H_4O_3$).

Confertin B (**2**)

Light yellow needles.
MP: 186°C.
IR (KBr) ν_{max} cm^{-1}: 3440, 1700-1720 (ester), 1660 (conjugated ketone), 1540 (aromatic C=C), and 1252 cm^{-1} (OCH_3).
UV λ_{max} (log ε): 332 (4.12), 294 (3.85) and 267 nm (4.50).
^{1}H and ^{13}C NMR: Table 1.
HREI-MS: *m/z* 398.3630 (Calcd. for $C_{21}H_{18}O_8$: 398.3670).

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Kaempferol Glycosides in the Flowers of Carnation and their Contribution to the Creamy White Flower Color

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Three flavonol glycosides were isolated from the flowers of carnation cultivars 'White Wink' and 'Honey Moon'. They were identified from their UV, MS, ^{1}H and ^{13}C NMR spectra as kaempferol 3-*O*-neohesperidoside, kaempferol 3-*O*-sophoroside and kaempferol 3-*O*-glucosyl-(1→2)-[rhamnosyl-(1→6)-glucoside]. Referring to previous reports, flavonols occurring in carnation flowers are characterized as kaempferol 3-*O*-glucosides with additional sugars binding at the 2 and/or 6-positions of the glucose. The kaempferol glycoside contents of a nearly pure white flower and some creamy white flower lines were compared. Although the major glycoside was different in each line, the total kaempferol contents of the creamy white lines were from 5.9 to 20.9 times higher than the pure white line. Thus, in carnations, kaempferol glycosides surely contribute to the creamy tone of white flowers.

Keywords: carnation, creamy white flower, flavonols, kaempferol 3-*O*-glycosides.

Anthocyanin flower pigments of carnations have been reported for some pink, red, red-purple and mauve cultivars. Pelargonidin 3-*O*-glycoside was found in salmon and red cultivars, pelargonidin 3,5-di-*O*-glycoside in pink, cyanidin 3-*O*-glycoside in lavender and crimson, and cyanidin 3,5-di-*O*-glycoside in lavender and magenta ones [1,2]. In 1986, Terahara *et al.* reported that the major anthocyanins in pink and red carnations were pelargonidin and cyanidin 3-*O*-glucosides acylated with malic acid [3], and isolated pelargonidin and cyanidin 3-*O*-(6′′-*O*-malyl-β-glucopyranosides) from the red cultivar 'Scania' and the purplish-red one 'Nina' [4]. Recently, cyclic 5-3 malyl pelargonidin and cyanidin were also identified from deeper colored cultivars [5,6]. The yellow color in the petals of carnations is due to the accumulation of a chalcone, chalcononaringein 2′-*O*-glucoside (i.e., isosalipurposide) [7-9]. Some flavonoids also have co-pigmentation effects, which influence coloration by interaction with anthocyanins. Information of flavonoid structures is important to understand the coloration mechanism in carnation flowers. Recently, one *C*-glycosylflavone, isovitexin 7-*O*-(6′′-malylglucoside) and two flavonols, kaempferol 3-*O*-glucosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] and kaempferol 3-*O*-(6′′-malylglucosyl)-(1→2)-[rhamnosyl-(1→6)-glucoside] were isolated from the petals of transgenic carnation cultivars 'Moondust' and 'Moon shadow' [10]. Of their flavonoids, it was proved that isovitexin 7-*O*-(6′′-malylglucoside) exhibits the strongest co-pigment effect.

In previous studies, only cyanic cultivars were investigated. As the first step of our study, acyanic cultivars were investigated seeking compounds whose occurrence in carnation flowers has not yet been reported. We selected a white flower cultivar 'White Wink' and a cream one 'Honey Moon' and detected three kaempferol 3-*O*-glycosides. In addition, the flavonoid contents of some white flower lines were compared to determine the contribution of kaempferol glycosides, including those identified here, to the creamy tone coloration of carnation flowers.

In this survey, three flavonoids (**1** - **3**) were obtained as pale yellow powders from the petals of *D. caryophyllus* 'Honey Moon' and 'White Wink'. Acid hydrolysis of these liberated kaempferol as the aglycone, and glucose and rhamnose (**1** and **3**), and glucose alone (**2**) as the sugars. In the UV spectra, the bathochromic shift of band I by addition of NaOMe, with an increase in the intensity relative to the spectrum of the MeOH solution, showed the presence of free 4′-hydroxyl and substituted 3-hydroxyl groups [11]. Moreover, though band I bathochromically shifted on the addition of $AlCl_3$, there was no hypsochromic shift on addition of HCl, showing the presence of a monohydroxyl group in ring B, and a free 5-hydroxyl group. The appearance of an accompanying peak on NaOMe addition, and a bathochromic shift of band II on addition of NaOAc showed the presence of a free 7-hydroxyl group. Thus, it was proved that the sugars are attached at the 3-hydroxyl position of their glycosides. Finally, flavonoids **1** and **2** were identified as kaempferol 3-*O*-neohesperidoside and kaempferol 3-*O*-sophoroside by ESIMS, and ^{1}H and ^{13}C NMR, respectively. In flavonoid **3**, the shifts of 3-*O*-glucosyl C-2 (δ 82.9) and C-6 (δ 66.5) were recognized from the ^{13}C NMR spectra. The attachment of rhamnose to the 6-position and another glucose to the 2-position of 3-*O*-glucose were determined by the presence of HMBC and/or NOESY signals. Thus, flavonoids **3** was completely identified as kaempferol 3-*O*-β-glucopyranosyl-(1→2)-*O*-[α-rhamnopyranosyl-(1→6)-*O*-β-glucopyranoside] (kaempferol 3-*O*-2^{G}-glucosylrutinoside).

The kaempferol glycoside content of a nearly pure white line 'MB3' was compared with those of five common white cultivars or lines, 'White Sim', 'Bridal White', 'Annabel', 'Milky Way' and 'BD2349-08' (Table 1). Three kaempferol glycosides, i.e. 3-*O*-neohesperidoside (**1**), 3-*O*-sophoroside (**2**) and 3-*O*-(2^{G}-glucosylrutinoside) (**3**) were detected in the flowers of the cultivars except for 'White Sim', which contained flavonoids **1** and **3** alone. The fourth kaempferol glycoside (**4**), which was not isolated from the flowers of 'White Wink' and 'Honey Moon', was found in all the cultivars except for 'White Sim'. However, it could not be characterized. Although the major glycoside was different in each of the cultivars, the total kaempferol contents of the five creamy white cultivars or line were from 5.9 (BD2349-08) to 20.9 (White Sim) times higher than that of the nearly pure white line 'MB3'.

Kaempferol 3-*O*-neohesperidoside has been isolated from the leaves of *Nerisyrenia linearifolia* and *N. gracilis* (Cruciferae) [12]. Recently, kaempferol 3-*O*-sophoroside has been isolated from the purple flowers of *Gladiolus* cultivar 'Ariake' as a co-pigment, together with kaempferol 3-*O*-rutinoside and quercetin 3-*O*-rutinoside [13]. Kaempferol 3-*O*-2^{G}-glucosylrutinoside has been reported from *Hosta ventricola* (Liliaceae) [14]. In carnation, they have been reported from the flowers [15,16], roots and stems [17] of common cultivars. The basic structure of these glycosides was characterized as kaempferol 3-*O*-glucoside, with additional sugar moieties at the 2 and/or 6-positions of the 3-*O*-glucose. White carnation cultivars have been divided into three flower flavonoid types, i.e. flavonoid-lacking cultivars, naringenin accumulating cultivars, and kaempferol accumulating cultivars [18]. Moreover, it was shown that the flavonoid biosynthesis pathway is blocked before chalcone synthesis in flavonoid-lacking cultivars, whereas it is blocked at the flavanone-3-hydroxylase step in naringenin accumulating cultivars [19]. However, flavonoid identification had been not performed at the glycosylation level. In this experiment, it was shown that four kaempferol glycosides are involved, i.e. 3-*O*-neohesperidoside, 3-*O*-sophoroside, 3-*O*-(2^{G}-glucosylrutinoside) and an unknown glycoside. Although quercetin glycosides produce pale yellow colors, in the case of yellow *Clematis* species and cultivars, kaempferol glycosides do not apparently act as yellow pigments [20, 21]. In carnation, however, creamy white flowers contained over five times higher levels of kaempferol glycosides than a nearly pure white flower. The presence of such amounts of kaempferol glycosides surely contributes to the creamy tone in the white flowers of carnations.

Table 1: Quantitative HPLC analysis of the flowers of six white carnation cultivars and lines.

Cultivars/lines	Colors	**1***	**2**	**3**	**4**	Total
MB3	pure white	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.3	0.1 ± 0.1	0.9 (1.0)
White Sim	white	□	10.4 ± 0.4	8.4 ± 0.3	□	18.8 (20.9)
Bridal White	white	2.5 ± 0.2	3.1 ± 0.1	2.4 ± 0.1	4.1 ± 0.2	12.1 (13.4)
Annabel	white	0.3 ± 0.0	0.5 ± 0.0	4.3 ± 0.4	1.5 ± 0.1	6.6 (7.3)
Milky Way	white	1.3 ± 0.1	0.7 ± 0.0	4.5 ± 0.3	2.0 ± 0.6	8.5 (9.4)
BD2349-08	white	0.9 ± 0.1	0. 5± 0.0	2.5 ± 0.1	1.4 ± 0.1	5.3 (5.9)

Each 1 g fresh petals was extracted with 6 mL 10% HOAc in MeOH.

1 = kaempferol 3-*O*-neohesperidoside, **2** = kaempferol 3-*O*-sophoroside, **3** = kaempferol 3-*O*-(2^{G}-glucosylrutinoside) and **4** = unknown kaempferol glycoside.

* Calculated from the absorption coefficient of authentic quercetin 3-*O*-rutioside (μmol/g fresh weight).

( ) = Relative amounts of total flavonoids as peak area of pure white line 'MB3' is 1.00.

Experimental

General: Prep. HPLC was performed on a Wakosil II 5C18 AR Prep (I.D. 20×250 mm) with a flow-rate of 9 mL/min and by monitoring absorbance at 360 nm. The solvent system used included linear gradient elution for 25 min using 25-50% of solvent B (0.5% TFA, 10% formic acid, 40% MeCN) in solvent A (0.5% TFA, 10% formic acid). Paper chromatography was performed using solvent systems: BAW (*n*-BuOH/HOAc/H_2O = 4:1:5, upper phase), BEW (*n*-BuOH/EtOH/H_2O = 4:1:2.2), 15% HOAc and 5% HOAc for flavonol glycosides; BAW, BEW and Forestal (HOAc/HCl/H_2O = 30:3:10) for flavonol aglycones; and BBPW (*n*-BuOH/benzene/pyridine/H_2O = 5:1:3:3) and BTPW (*n*-BuOH/toluene/pyridine/H_2O = 5:1:3:3) for glycosidic sugars. UV spectral analysis and acid hydrolysis were performed according to published procedures [11]. The ESIMS were analyzed on a TSQ system (Thermo Quest, San Jose, CA, USA). 1D and 2D NMR spectra were measured on a Bruker AVANCE 800.

Plant materials: The flowers of two carnation cultivars 'White Wink' and 'Honey Moon' were used for flavonoid isolation. They were cultivated in Japan by Tobacco Inc., Co. Ltd., Oyama, Tochigi, Japan. A nearly white line 'MB3' and four white cultivars or lines, 'Bridal White', 'Annabel', 'Milky Way' and 'BD2349-08' were cultivated by Kirin Agribio Co., Ltd. The other white cultivar 'White Sim' was cultivated by the National Institute of Floricultural Science, National Agriculture and Bio-oriented Research Organization, Tsukuba, Ibaraki, Japan. Live specimens are growing in the nurseries mentioned above.

Extraction and isolation: Dry petals (each 200 g) of 'Honey Moon' and 'White wink' were extracted with 80% MeOH for 2 days at room temperature. After concentration *in vacuo*, the residue was dissolved in a small volume of 10% MeOH and applied to a Sephadex LH-20 column and eluted with 70% EtOH. The fractions containing the flavonoids were concentrated and purified by prep. HPLC. The isolated flavonoids were concentrated to dryness and freeze dried.

Kaempferol 3-*O*-neohesperidoside (1)

Although UV and ^{1}H NMR spectroscopic data of flavonoid **1** have been published [12], ^{13}C NMR data have not been reported previously.

^{13}C NMR (150 MHz, DMSO-d_6 + TFA-*d* = 9:1): (kaempferol) δ 156.9 (C-2), 133.3 (C-3), 177.9 (C-4), 160.3 (C-5), 98.9 (C-6), 164.5 (C-7), 94.1 (C-8), 156.7 (C-9), 104.6 (C-10), 121.5 (C-1′), 131.2 (C-2′), 115.5 (C-3′), 159.4 (C-4′), 115.5 (C-5′), 131.2 (C-6′); (3-glucose) δ 99.1 (C-1), 78.1 (C-2), 77.7 (C-3), 69.7 (C-4), 77.9 (C-5), 61.2 (C-6); (rhamnose) δ 101.1 (C-1), 71.0 (C-2), 70.6 (C-3), 72.3 (C-4), 68.5 (C-5), 17.7 (C-6).

ESIMS: *m/z* 611.1604 $[M+H]^+$ (base, calcd. for $C_{27}H_{30}O_{16}$, 611.1607); *m/z* 633.1426 $[M+Na]^+$ (base, calcd. for $C_{27}H_{30}O_{16}Na$, 633.1426).

Kaempferol 3-*O*-sophoroside (2)

UV, ^{1}H and ^{13}C NMR spectroscopic data have been reported by Budzianowski [14].

Kaempferol 3-*O*-β-glucopyranosyl-(1→2)-[rhamnopyranosyl-(1→6)-glucopyranoside] (3)

PC (Rf): 0.26 (BAW), 0.36 (BEW), 0.66 (15%HOAc), 0.60 (5%HOAc); UV (365 nm) – dark purple, UV/NH_3 – dark greenish yellow.

UV λmax nm (MeOH): 267, 345. +NaOMe: 281, 325, 404 (inc.). +$AlCl_3$: 274, 304, 353, 396. +$AlCl_3$/HCl: 275, 302, 346, 393. +NaOAc: 274, 307, 384. +NaOAc/H_3BO_3: 266, 352.

^{1}H NMR (800 MHz, DMSO-d_6 + TFA-*d* = 9:1): δ 7.99 (2H, dd, *J* = 2.0 and 8.9 Hz, H-2′,6′), 6.90 (2H, dd, *J* = 2.1 and 8.9 Hz, H-3′,5′), 6.40 (1H, d, *J* = 2.1 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.55 (1H, d, *J* = 7.1 Hz, 3-glucosyl H-1), 4.59 (1H, d, *J* = 7.9 Hz, *t*-glucosyl H-1), 4.32 (1H, d, *J* = 1.3 Hz, rhamnosyl H-1), 0.93 (3H, d, *J* = 6.2 Hz, rhamnosyl Me).

^{13}C NMR (150 MHz, DMSO-d_6 + TFA-*d* = 9:1): (kaempferol) δ 156.9 (C-2), 133.4 (C-3), 178.0 (C-4), 161.6 (C-5), 98.8 (C-6), 164.4 (C-7), 94.2 (C-8), 157.0 (C-9), 104.6 (C-10), 121.5 (C-1′), 131.5 (C-2′), 115.7 (C-3′), 160.3 (C-4′), 115.7 (C-5′), 131.5 (C-6′); (3-glucose) δ 99.1 (C-1), 82.9 (C-2), 76.9 (C-3), 70.0 (C-4), 76.1 (C-5), 66.5 (C-6); (*t*-glucose) δ 104.5 (C-1), 74.8 (C-2), 77.0 (C-3), 70.0 (C-4), 77.5 (C-5), 61.3 (C-6); (rhamnose) δ 100.9 (C-1), 70.8 (C-2), 71.0 (C-3), 72.3 (C-4), 68.7 (C-5), 18.0 (C-6).

Analysis of flavonoid contents: White petals of 'Bridal White', 'Annabel', 'Milky Way' 'BD2349-08' and 'White Sim' were extracted with 10% HOAc in MeOH. The extracts were analyzed by HPLC using an HP1100 system with photodiode array detector (Agilent Technologies-Yokokawa Analytical Systems, Tokyo, Japan) and an Inertsil ODS-2 column (4.6 mm x 250 mm, GL science, Tokyo, Japan) combined with an Inertsil ODS-2 guard column at 40ºC with a flow rate of 0.8 mL/min. Absorption spectra were monitored at 240-580 nm. A linear gradient of 10-50% of solvent B (1.5% H_3PO_4, 40% MeCN, 50% HOAc) in solvent A (1.5% H_3PO_4) was run for 40 mins. Flavonoids were identified and quantified based on absorption spectra and the absorption at 360 nm.

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Factors Influencing Glabridin Stability

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Glabridin, a polyphenolic isoflavan of *Glycyrrhiza glabra*, has shown a variety of pharmaceutical properties. We have previously studied the isolation of glabridin using macroporous resin and found that it is partially degraded, giving a dark color. To illustrate the degradation of glabridin, the present work studied the stability of glabridin under various conditions. Licorice extract containing about 20% glabridin, obtained from *G. glabra* by silica gel column chromatography, was used in the stability study. Seven different factors (temperature, illumination, humidity, pH, solvent, oxygen, and oxidant) were studied and content changes were determined through HPLC analysis. Except for oxygen, all the above factors had an effect on the stability of glabridin, with illumination being the main one. Moreover, the interactions between temperature and pH, temperature and humidity, and illumination and pH can promote the degradation of glabridin. In conclusion, we suggest that a dark, dry and airtight environment provides the optimized condition for the long-term storage of glabridin.

Keywords: Glabridin, stability, illumination, solvent.

Glabridin is a polyphenolic isoflavan found in the hydrophobic extract of *Glycyrrhiza glabra* [1]. Recent studies have shown that glabridin has a wide range of pharmacological effects, including antioxidant, anti-microbial, anti-inflammatory, and anti-gastric ulcer activities, as well as inhibitory effects on the formation of melanin and low density lipoprotein [2–5]. Due to its many applications, interest in glabridin has intensified. However, its isolation is complicated because of its low content and high decomposition rate in the preparation process [6,7]. Up to now, there are no reports referring to the stability of glabridin. Temperature, illumination, humidity, oxidant, oxygen, pH and solvent are known to be effective factors which may influence the stability of various compounds. Thus, in our study, licorice extract containing a high proportion of glabridin was first obtained from *G. glabra.* This was then used to determine the effects of the seven parameters on the stability of glabridin, from which a rational protocol for its preparation and preservation was proposed.

An extract of licorice root was treated by silica gel chromatography [6,7]. The glabridin concentration of the resulting brown product was then determined by an HPLC method [8]. Under optimized chromatographic conditions, the peak of glabridin could easily be distinguished from other co-eluting compounds.

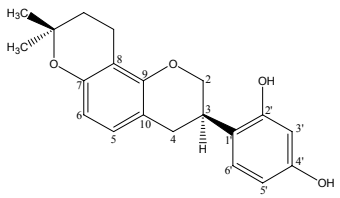

Figure 1: Structure of glabridin.

The calibration curve was obtained through the least-square method, which is widely used in linear analysis. The established linear regression equation was: Y=1154.9X-33.101, where Y is the peak area of glabridin and X the concentration of each injection. The correlation coefficient (R^2) was 0.9967 for glabridin. $S_{y,x}$ was 13.202, while b was 1154.9. Thus, the LOD and LOQ within the linearity of 0.9967 were 0.034 mg/mL and 0.114 mg/mL, respectively.

Intra-day and inter-day precisions were measured by repeated injections of standard glabridin on the same and three different days [8]. The relative standard deviation (RSD) values for both situations were 2.02% and 2.48%, respectively, which were considered to be acceptable. Linear analyses at four other wavelengths (210, 254, 310 and 377 nm) were also carried out to investigate the specificity of the HPLC method. The concentration of glabridin was 0.72, 0.72, 0.73, 0.75

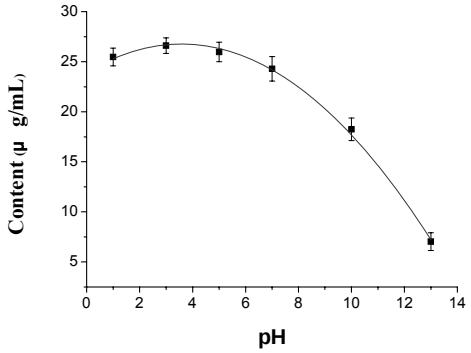

Figure 2: Degradation of glabridin at different pHs.

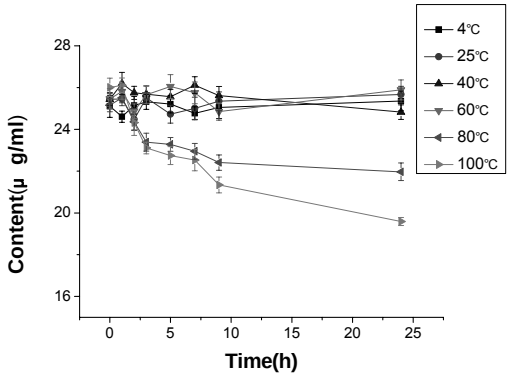

Figure 3: Degradation of glabridin at different temperatures.

and 0.67 mg/mL at the above wavelengths, respectively, with a RSD value of 3.7%, which indicated that the established HPLC method could be used for the stability study of glabridin.

The effects of temperature, illumination, humidity, oxidant, oxygen, pH and solvent on the chemical stability of glabridin were assessed. The stability of glabridin at pH values of 1.0, 3.0, 5.0, 7.0, 10.0 and 13.0 were evaluated. In acid and neutral solutions the compound was mainly stable, whereas in alkaline solutions it underwent severe degradation within 24 hours. At pH 13.0, degradation was 71.0% (Figure 2). Friedman [9] reported that the susceptibility of plant phenolic compounds to pH strongly depended on their structure. Glabridin, with two hydroxyl substituents at positions 2' and 4', can exhibit a low degree of ionization. Under alkaline conditions, a neutralization reaction may be aroused leading to the degradation of glabridin and other flavones [9].

Thermal stability of glabridin (pH 8.0) was studied at 4, 25, 40, 60, 80 and 100°C. The interaction between pH and temperature was carried out as well. Glabridin exhibited great stability below 60°C at a specific pH, while it started to degrade moderately after 5 hours above 60°C (Figure 3). The results were in agreement with earlier studies on other kinds of isoflavonoid [10]. Meanwhile, the close relationship between the pH of the solvent and the stability of glabridin at high temperature is shown in Figure 4. Glabridin at pH 5 remained almost stable, whereas at pH 1, 3, 7 and 10 it degraded significantly at 100°C. Stintzing [11] found that the thermal degradation of isoflavonoids was highly dependent on their substitution pattern and solvent pH. A molecule with hydroxyl groups at positions 7 and 4' was more stable under high temperature compared with ones with other substituents at these positions. Glabridin, with a hydroxyl substituent at position 4' and one side ring on ring A, showed stability under high temperature in our study. The effect of the side ring remains to be demonstrated.

The optical stability of glabridin was examined in the dark, natural light and ultraviolet light (Figure 5). Glabridin remained almost unchanged in the dark after 24 h, while it was remarkably unstable in both natural and ultraviolet light after 8 h, the contents being reduced by 26.3% and 54.1%, respectively. It was obvious that the light degradation of glabridin was closely correlated with the exposure time to light and the type of light source. We speculated that the light degradation was promoted by the absorbed energy from photons. Since the energy of ultraviolet light was more powerful than that of natural light, glabridin degraded more in the former conditions [12]. Interaction between pH and illumination was also carried out. Glabridin in acid (pH 1) and alkaline (pH 13) solutions was more unstable than when in neutral solution (pH 7), indicating the synergistic effect between the pH of the solvent and light.

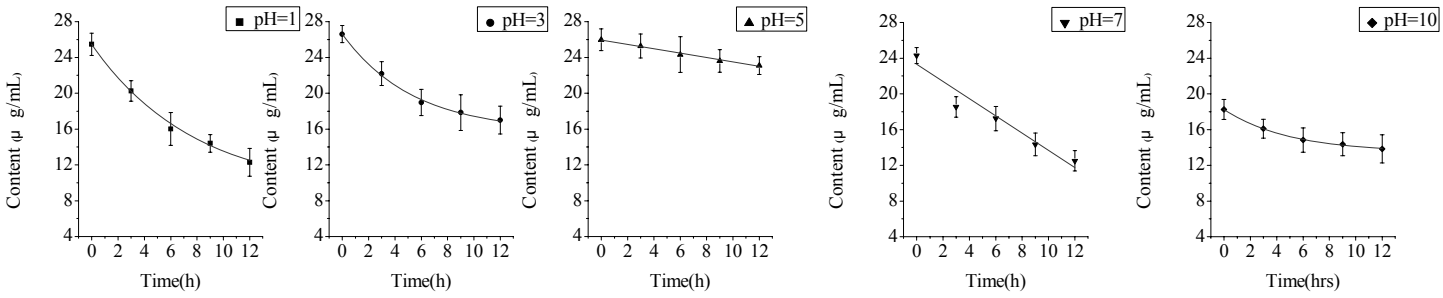

Figure 4: Degradation of glabridin at different pHs at 100°C.

Table 1: Average content of glabridin in seven solvents during 45 days.

	Methanol	Ethanol	Acetone	Ethyl acetate	Methylene dichloride	Chloroform	Propylene glycol
Content (μg/mL)	30.0±0.6	29.9±1.0	31.5±3.7	31.6±3.9	8.7±1.6	8.9±0.6	30.0±0.9
RSD	2.3%	4.3%	3.2%	4.1%	26.2%	8.9%	3.6%

Table 2: Degradation rate of glabridin at different pHs under ultraviolet light.

Degradation rate	pH 1	pH 7	pH 13	Stock solution
8 h	16.4%	13.1%	16.8%	13.1%
16 h	50.6%	28.0%	49.7%	19.1%

Table 3: Degradation of glabridin at different relative humidities and different temperatures.

Degradation rate	75% at 60°C	75% at 25°C	90% at 60°C	90% at 25°C
5^{th} day	14.2%	5.9%	13.7%	8.3%
10^{th} day	22.9%	12.7%	22.2%	14.9%

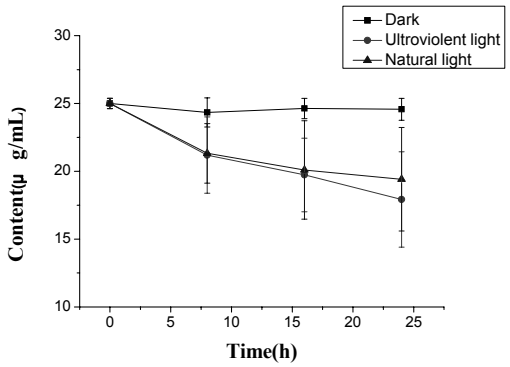

Figure 5: Degradation of glabridin under light.

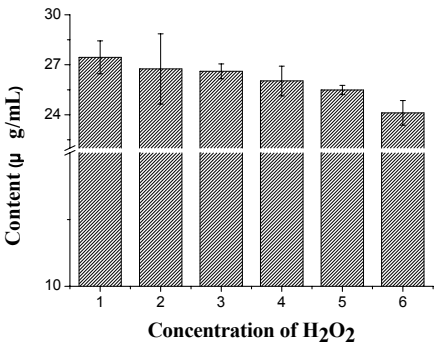

Figure 6: Degradation of glabridin with different concentrations of H_2O_2 (1: Contrast; 2: Stock solution with water; 3: 5% H_2O_2; 4:10% H_2O_2; 5: 20% H_2O_2; 6: 30% H_2O_2).

Glabridin examined in seven different organic solvents demonstrated varying stability (Table 1). It was apparent that the stability was dependent on the properties of the solvents. Methanol was the most suitable for glabridin, followed by acetone under the same conditions. On the contrary, glabridin was equally unstable in methylene dichloride and chloroform. The reason why the solvent could affect the stability of phenolic compounds was because of its ionizability, relative volatility and dissolved oxygen. In the present study, the solvents were first degassed to create low-oxygen conditions. The solvents can be divided into two groups, protonic and nonprotonic [13]. An analogous dissociation to water was observed in organic solvents and was described as the pKa value. For glabridin dissolved in protonic solvents with higher pKa values, the solvation effect between solvents and glabridin was easily initiated. Nevertheless, when dissolved in nonprotonic solvents with lower pKa values, solvation energies were too negative to start a solvation effect. It was concluded that glabridin with two hydroxyl substituents on the benzene ring was more unstable in protonic solvents because of the solvation effect [13], which was inconsistent with our results. We presumed that it was the relative volatility of the solvents rather than the ionizability that caused the degradation of glabridin, which remains to be confirmed in a further study.

The effect of oxygen and H_2O_2 was carried out at room temperature. Glabridin displayed different stabilities under these two conditions. As shown in Figure 6, the stability of glabridin in H_2O_2 was concentration dependent, while the content of glabridin remained unchanged after exposure to air in the dark (data not shown). Glabridin was reported to possess the ability to scavenge oxygen free radicals, which may explain its content change in H_2O_2. We supposed that the stability of glabridin was relevant to the type of free radical [14].

The degradation of glabridin was positively related to relative humidity and treatment time (Figure 7). The interaction between humidity and temperature indicated that a high temperature can promote the degradation of glabridin at the same relative humidity. In our oxidant study, glabridin in CH_3OH/H_2O (80/20) also degraded after several days. We have mentioned that the stability of glabridin was in part correlated with the ionizability of the solvent. From the results above, it seemed that water was one of the factors that had an impact on the stability of glabridin for its strong ionizing ability. Thus we suggest that glabridin should be kept in a dry environment during storage.

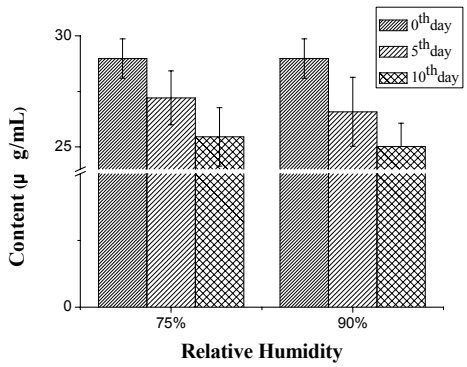

Figure 7: Degradation of glabridin at different humidities.

In conclusion, the results from this study show that the stability of glabridin in licorice extract depends on illumination, temperature, pH, solvent, humidity and oxidant, of which illumination is the main factor. Interaction effects between temperature and pH, illumination and pH, and temperature and humidity increased the degradation rate of glabridin. To minimize degradation, we suggest that pure glabridin and licorice extract should be stored in a dark, dry and airtight environment. A suitable solvent is recommended, high temperature should be avoided, and pH should be carefully controlled in the isolation process. However, it is worth noting that oxygen can promote a variety of reactions. Our studies showed that the direct impact of oxygen on the stability of glabridin was weak, but the interactions it may cause with other factors still need to be investigated. Thus the presence of oxygen should be avoided both in the preservation and isolation of glabridin to reduce its degradation.

Experimental

Apparatus: HPLC determinations were performed on Agilent 1200 Series (Agilent Technologies Inc., America) equipment, consisting of an Iso pump, UV detector and Man Injection. UV Penetration Reflection Analysis Instrument (Beijing Institute of New Technology, China) was used for photolysis treatment. Thermo analysis was performed on a 101A-1BY Infrared Radiation Electric Oven (Hangzhou LanTian Chemical Instrument Plant, China). A 320-S Acidometer (Mettler Toledo, Switzerland) was used in the adjustment of the pKa of the test solutions.

Chemicals: Glabridin (>97%) was purchased from National Vaccine & Serum Institute of China. The solvents used for extraction and chromatographic isolation were of analytical grade. Ultrapure water was prepared using the MRR-2(D) ultrapure water production system (Hangzhou Repure Ltd. China). Other solvents were of high-performance liquid chromatography (HPLC) grade.

Isolation of glabridin: Powdered roots of *Glycyrrhiza glabra* (provided by Xinjiang Production and Construction Corps) were extracted in acetone (1:5, w/v) at room temperature for 24 h. After solvent evaporation, the brown crude extract was extracted in chloroform (1:3, w/v) to obtain the hydrophobic constituents. This fraction (after evaporation) was separated on a silica gel column with CH_2Cl_2, 2% CH_2Cl_2/CH_3OH, 5% CH_2Cl_2/ CH_3OH and 10% CH_2Cl_2/CH_3OH. The fractions collected were first examined through HPLC and the fraction of 5% CH_2Cl_2/CH_3OH containing glabridin was subsequently used for the stability study.

HPLC analysis: HPLC separation was performed on a Kromasil C18 reverse-phase column (5 μm particle size, 150 x 4.6 mm i.d.) with a flow rate of 1.0 mL/min and column temperature of 25°C. A mixture of methanol–water containing 0.2% acetic acid (70/30, v/v) was used as the mobile phase, which was filtered through a 0.45 μm membrane and degassed by sonication. The column effluent was monitored at 282 nm with UV detection. Prior to HPLC analysis, all the standards and extracts were filtered through a 0.45 μm syringe membrane filter.

Calibration curve: The linearity of the HPLC method was established by triplicate injections of standard glabridin in the range of 0.125–0.625 mg/mL. The linear regression curve was obtained using the least-squares method with the peak area (*Y*-axis) of glabridin as the dependent variable and the concentration (*X*-axis) of each injection as the independent variable.

Validation of the HPLC method: The precision was measured by means of intra-day and inter-day repeatability. Intra-day precision was obtained by 6 injections of standard glabridin on the same day, while inter-day precision was established by 3 repeated injections on days 1, 2 and 3 at the same concentration. The specificity of the method was evaluated through linear analysis at 5 different wavelengths (210, 254, 282, 310 and 377 nm). A linear regression curve was obtained through the least-squares method. The concentration of glabridin was subsequently determined at the above 5 wavelengths. The RSD value of concentration was used to evaluate the purity of the glabridin peak. Limit of detection (LOD) and limit of quantification (LOQ) were determined through the following equation: LOD=3$S_{y,\ x}/b$ and LOQ=10$S_{y,\ x}/b$, where $S_{y,x}$ is the standard deviation of the Y value distribution and b is the slope of regression equation.

Statistical analysis: All data were recorded as means ± standard deviation of either duplicate or triplicate measurements. Analyses of variance (ANOVA) and

significance differences between the means were performed through SPSS 7.0 for Windows. Relative standard deviation (RSD) was also calculated to evaluate the stability.

Chemical stability evaluations: In this study, seven factors involved in glabridin stability were studied. The 5% CH_2Cl_2/CH_3OH eluent was evaporated to obtain a brown solid extract, which was dissolved in methanol to a final concentration of 0.5 mg/mL (stock solution) and used in the following experiments.

The thermal stability of glabridin was studied at 4, 25, 40, 60, 80 and 100°C. Capped glass bottles with 10 mg extract were stored in an oven (fridge for 4°C) at different temperatures for 1, 2, 3, 5, 7, 9 and 24 h, respectively. The extract was then dissolved in 5 mL methanol and filtered through a 0.45 μm syringe membrane filter before HPLC analysis. Each temperature group was repeated 3 times.

For the optical stability study, an aliquot (10 mL) of the stock solution was placed in an open container and treated in the dark, natural light and ultraviolet light, respectively, for 8, 16 and 24 h [12]. The gross weight of each sample was determined at the beginning. Before HPLC analysis, the gross weight of each sample was adjusted to its initial value to avoid the influence of solvent evaporation. The analysis of each group was repeated 3 times.

H_2O_2 of varying concentration (5%, 10%, 20% and 30%) was used in the study of the oxidant effect on stability of glabridin. Sample solutions were prepared by mixing a stock solution with different concentrations of H_2O_2 (4:1, v/v) and storing in a well-capped bottle to avoid solvent evaporation. Meanwhile, water and methanol were added to the stock solution (1:4, v/v) to contrast with groups 1 and 2, respectively. All samples were kept in the dark at room temperature for 14 days. Each group was repeated 3 times.

The effect of pH on chemical stability of glabridin was studied at 6 different levels (1.0, 3.0, 5.0, 7.0, 10.0 and 13.0) at room temperature [9]. Sodium hydroxide solution, sodium chloride solution and hydrochloric acid solution were prepared and then mixed with stock solution (500 μg/mL) at a ratio of 9:1. Sample solutions were kept in the dark at room temperature for 24 h. Each group was repeated 2 times.

The solvent effect on the stability of glabridin was carried out in 7 different organic solvents (methanol, ethanol, acetone, ethyl acetate, chloroform, methylene dichloride and propylene glycol). Licorice extract (25 mg) was dissolved in 100 mL of solvent, degassed and kept hermetically sealed at room temperature in the dark. An aliquot (20 μL) of the sample solution was periodically removed and subjected to HPLC analysis on days 0, 7, 12, 19, 25, 32, 35 and 42.

The effect of humidity was studied at relative humidities of 75% and 90%. A constant humidity environment was established in desiccators with super-saturated solutions. In our study, NaCl and KNO_3 were used to set up the constant humidity environment of 75% and 90%, respectively. Licorice extract (10 mg) was transferred into an open container and placed in the desiccators. The treated extracts were then dissolved in 5 mL methanol and subjected to HPLC analysis on days 5 and 10. Each group was repeated twice.

A control group with stock solution was kept hermetically sealed in the dark. The experimental group with stock solution was exposed to air in the dark. After 24 h, the contents of glabridin in both groups were determined. The effect of oxygen was characterized by the content change.

Moreover, the interaction effects between temperature and pH, illumination and pH, and temperature and humidity were studied. The effect of pH on thermal stability was studied at 5 different pHs (1.0, 3.0, 5.0, 7.0 and 10.0) at 100°C [9]. The content of glabridin was determined after 3, 6, 9 and 12 h. The optical stability of glabridin at different pHs (1.0, 7.0 and 13.0) was studied under UV light [12]. Stock solution (10 mL) was filled into an open container and treated under UV light for 8 and 16 h, respectively. The effect of humidity on thermal stability was carried out at relative humidities of 75% and 90% at 60°C. Licorice extract (10 mg) was transferred into an open container and placed in the desiccators. The treated extracts were then dissolved in 5 mL methanol and subjected to HPLC analysis on days 5 and 10. Each group was repeated twice.

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Effect of Different Strains of *Agrobacterium rhizogenes* and Nature of Explants on *Plumbago indica* Hairy Root Culture with Special Emphasis on Root Biomass and Plumbagin Production

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The aim of the present study was to determine the effect of three strains of *Agrobacterium rhizogenes* (ATCC 15834, A_4 and LBA 9402) and the nature of explants (leaf and stem) on hairy root induction, growth and plumbagin production in *Plumbago indica*. The first appearance of hairy roots, the transformation frequency, dry root biomass and plumbagin accumulation were found to be maximum in hairy roots induced in leaf explants infected with *A. rhizogenes* ATCC 15834 as compared with the other two bacterial strains. The hairy roots generated from stem explants infected with all three strains were not found to be productive in terms of the selected parameters. Finally, the insertion of the *rolB* gene of *A. rhizogenes* ATCC 15834 in hairy roots of *P. indica* derived from leaf explants was confirmed by PCR analysis.

Keywords: *Agrobacterium rhizogenes*, hairy root, *Plumbago indica*, plumbagin.

Plumbago indica Linn. (Family: Plumbaginaceae) is a rich source of a therapeutically active, root specific metabolite, plumbagin. This has been reported to possess filaricidal, anticancer, antimicrobial, and antifertility activity [1-4]. During the past few decades, several attempts have been made to scale up production of plumbagin *in vitro* by suspension culture [5] and cell culture [6], but low product yield coupled with instability necessitates improvement for better commercial exploitation. Thus, it is necessary to scale up the production of plumbagin without hampering the natural flora through modern biotechnological applications.

Hairy root culture offers a valuable source of root derived phytochemicals [7]. *Agrobacterium rhizogenes*, a Gram negative soil grown bacterium, is the causative agent of hairy root formation in dicotyledonous plants. Hairy root cultures are capable of unlimited growth and produce superior yields of root specific plant secondary metabolites. In the present study, it was aimed to determine the effect of three different strains of *A. rhizogenes* (ATCC 15834, A_4 and LBA 9402) and the nature of the explants (leaf and stem) on hairy root induction, growth and plumbagin accumulation.

Three strains of *A. rhizogenes* were utilized to infect leaf and stem explants of *P. indica.* A virulent strain of *A. rhizogenes* was selected on the basis of transformation frequency, appearance of hairy roots in minimum time, root biomass and plumbagin accumulation. The transformation frequency was found to be highest when *P. indica* leaf explant was infected with *A. rhizogenese* ATCC 15834 (Figure 1). Similarly, the appearance of hairy roots was best observed (6.6 ± 0.5th day) when *P. indica* leaf explants were infected with *A. rhizogenese* ATCC 15834 (Figure 1). The highest dry root biomass and plumbagin contents were found when *P. indica* leaf explant was infected with *A. rhizogenese* ATCC 15834 (Figure 2).

A. rhizogenes ATCC 15834-induced hairy roots of *P. indica*, emerging from leaf lamina at the exponential phase, are shown in Figures 3 A-B.

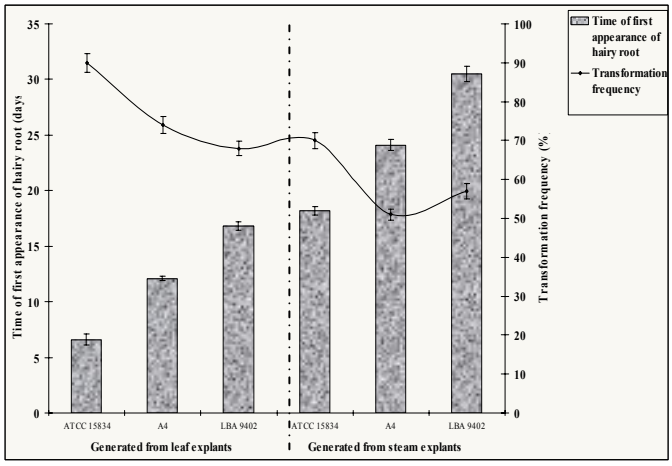

Figure 1: Effect of different strains of *A. rhizogenes* on the hairy root formation from *P. indica* hairy roots derived from leaf and stem explants.

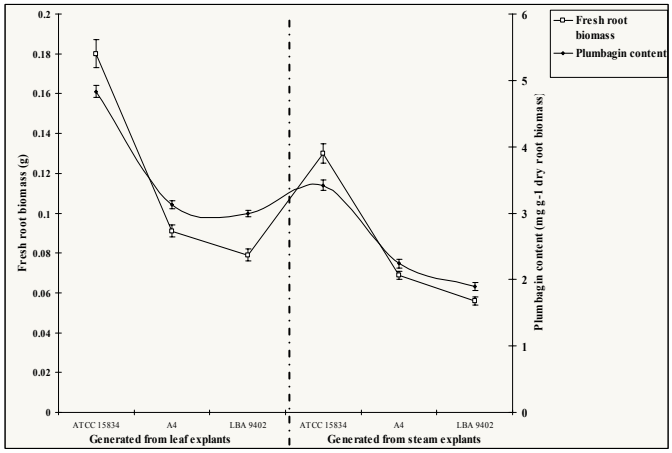

Figure 2: Effect of different strains of *A. rhizogenes* on the hairy root biomass and plumbagin production from *P. indica* hairy roots derived from leaf and stem explants.

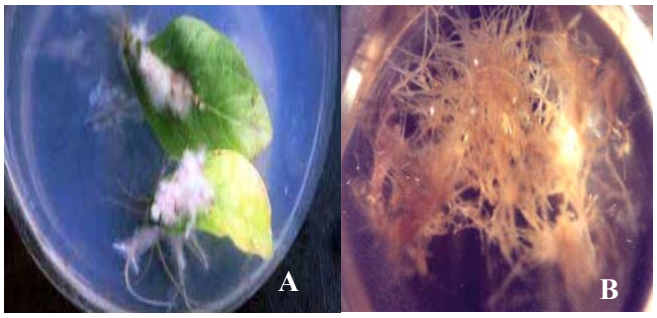

Figure 3: *A. rhizogenes* ATCC 15834 induced *P. indica* hairy roots emerging from leaf lamina (A) and at exponential phase of growth medium (B).

The best results in term of hairy root generation, root biomass and plumbagin content, were generated from leaf explants of *P. indica* infected with *A. rhizogenes* ATCC 15834. These were, therefore, subjected to polymerase chain reaction (PCR) analysis for confirmation of *rolB* insertion. The presence of the*rolB* gene (*A. rhizogenese* ATCC 15834 strain) in the genomic DNA from randomly selected hairy root clones derived from leaf and stem explants was tested by PCR amplification using *rolB* forward and reverse primers. All the selected hairy root clones showed amplification of the 780 bp fragment (*rolB*) and the DNA isolated from non-transformed roots gave no such amplification.

In the present study, the effect of different strains of *A. rhizogenes* (ATCC 15834, A_4 and LBA 9402) and the nature of the explants (leaf and stem) on hairy root induction, growth and plumbagin production in *P. indica* have been investigated. The strain specificity observed in the present study agrees with the hypothesis that the ability for infection of a given species by different *A. rhizogenes* strains is different [8,9]. It was also observed that the nature of the explants had a significant effect on hairy root culture and metabolite production. It is believed that transformation by *A. rhizogenes* is species specific and strongly depends on the bacterial strains and culture conditions [10,11]. The effectiveness of ATCC 15834 in hairy root culture and secondary metabolite production has been previously documented for other species [7,12]. Due to the better performance of ATCC 15834 than the other two strains, it will be used for subsequent experiments.

Experimental

Bacterial culture: Three different strains of *A. rhizogenes* viz. ATCC 15834 (pRi 15834), A_4 (pRi A_4) and LBA 9402 (pRi 1855) were used in the experiments. *A. rhizogenes* ATCC 15834 (pRi 15834) strain was maintained in solid *Agrobacterium* broth (AB) medium [13]. *A. rhizogenes* A_4 (pRi A_4) and LBA 9402 (pRi 1855) strains were maintained in Yeast-Mannitol Broth (YMB) medium [14].

Induction and establishment of hairy roots: Bacterial (*A. rhizogenes* strains viz. ATCC 15834, A_4 and LB 9402) infection was made artificially to the stem and leaf explants of *in vitro* grown axenic *P. indica* plants by wounding the surface tissue with the needle of a loaded syringe, showing absorbance (A_{600}) 0.5.

Frequency of successful transformation and time (days) taken for first appearance of hairy roots from leaf and stem explants with the 3 *A. rhizogenes* strains were recorded. Developing fresh hairy roots arising from the infected explants were cut at the tips and transferred to the MS solid medium (gelled with 0.8% agar) containing 3% sucrose supplemented with cefotaxime (cefotaxime sodium, ALKEM, India) to eliminate bacteria from the culture. Vigorously growing hairy roots free from bacterial contamination were selected from the young root tips and were transferred to 250 mL Erlenmeyer flasks containing 50 mL liquid Murashige, Skoog (MS) medium [15], pH 5.6, with 3% sucrose, on an orbital shaker at 70 rpm and maintained

under darkness at 25 ± 2°C. Dry root biomass (g) was measured by drying a weighed amount of fresh root, obtained during the exponential phase of root culture, in an oven at 60°C for 4 h.

Quantification of plumbagin by high performance liquid chromatography: Standard plumbagin (Sigma Aldrich, USA) solution for HPLC was prepared by dissolving in HPLC methanol (1µg μL^{-1}) and kept at 4^0C as a stock solution. Before use, a working solution was freshly prepared (50 µg mL^{-1}). Hairy root tissue was crushed with 50% (v/v) methanol and the homogenized root material was centrifuged at 10000 *g* for 20 mins. The supernatant was collected and filtered. The filtered sample was transferred to a Phenomenex™ (Torrance, USA) C_{18} column (Luna, 4 µm, 250 x 4.6 mm) using a JASCO HPLC (Tokyo, Japan) system equipped with a PU-2080 Plus™ pump and a PU-2075 Plus™ UV-VIS detector. For the mobile phase, an isocratic linear solvent system of acetonitrile and water (80: 20, v/v) with a flow rate of 1 mL min^{-1} for 15 min was used to elute the plumbagin. The chromatogram was monitored at a wave length of 410 nm and analyzed on a Microsoft ™ Windows 2000™ platform with DataApex Clarity™ software (Prague, Czech Republic). Identification of plumbagin was performed on the basis of retention time and chromatographic behavior in comparison with an authentic standard.

Confirmation of rolB gene in hairy roots: The transformed root nature of two randomly selected hairy root clones derived from leaf explants of *P. indica* infected with *A. rhizogenes* ATCC 15834 was checked by detecting the *rol* B gene in their genomes by PCR analysis. For extraction of genomic DNA from hairy root clones and non-transformed *in vitro* root clones (negative control) the procedure of mini preparation of plant genomic DNA was used [16]. Plasmid DNA from *A. rhizogenes* ATCC 15834 was isolated by the alkaline lyses method [17], which served as a positive control. The primer sequences (Bangalore Genie, India) used to amplify a 780 bp fragment of the *rolB* gene of the isolated DNA samples were: 5′-ATG GAT CCG AAA TTG CTATTC CTT CCA CGA-3′- Forward primer and 3′-TTA GGC TTC TTT CTT CAG GTT TAC TGCAGC-5′ - Reverse primer.

A 25 µL volume of reaction mixture used consisted of 20 ng (2 µL) of template DNA, 100 ng (1 µL) of forward and reverse primer; 1 µL (2.5 mM) dNTPs; 0.5 µL (0.5 U) Taq DNA polymerase; 2.5 µL 10X Taq buffer, and 17 µL H_2O. PCR amplification was programmed at an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min, followed by final extension for 10 min at 72°C. Amplified products were separated by electrophoresis analyzed along with a 100 bp DNA ladder (Bangalore Genie, India) on a 1.5% agarose gel stained with ethidium bromide with 1% Tris borate EDTA (TBA) as running buffer.

Data analysis: All sets of experiments were carried out in triplicate and the data were analyzed statistically using SPSS software (10.0.5, 1999, SPSS Inc). Variability in data was expressed as the mean ± standard errors.

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Fujianmycin C, A Bioactive Angucyclinone from a Marine Derived *Streptomyces* sp. B6219 [1]

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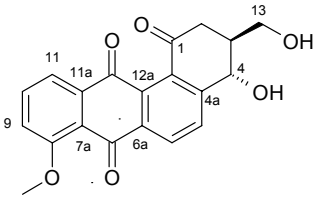
From a marine-derived streptomycete, a new bioactive angucyclinone, fujianmycin C (**1**), has been isolated along with five known, metabolites fujianmycins A (**2**) and B (**3**), ochromycinone (**4**), ochromycinone methyl ether (**5**), and tetrangulol methyl ether (**6**). The structure elucidation of fujianmycin C (**1**) was performed by detailed analysis of data such as ^{1}H, ^{13}C, $^{1}H,^{1}H$ COSY, HSQC, HMBC and NOESY spectra. Fujianmycin C (**1**) exhibited antibacterial activity against *Streptomyces viridochromogenes* (Tü57).

Keywords: marine streptomycetes, angucyclinones, fujianmycin C.

Fujianmycins [2] are members of the angucyclinone family. Currently, more than 40 derivatives of the latter are known. They are the third class of quinone antibiotics after the tetracyclines and anthracyclines [3]. Most of them are found as free aglycones, and all are oxygenated at C-4. While the related linear anthracyclinones usually have low pharmaceutical activities, the angucyclinones show pronounced antibacterial activity, such as inhibiting prolyl-endopeptidases of *Flavobacterium meningosepticum* non-competitively (IC_{50} = 8.9 μM) [4], and enhancing the cytotoxicity of colchicine. Of special interest is the inhibition of mycobacteria [5] and of *Helicobacter pylori* [6,7]. Both angucyclines/ angucyclinones and anthracyclines have been isolated from various streptomycete strains.

Extracts obtained from the marine derived *Streptomyces* sp. B6219 exhibited antibacterial activity against *Streptomyces viridochromogenes* (Tü57), *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli.* An unselective antitumor activity against six cancer cell lines was found with average IC_{50} and IC_{70} values of 17.0 and 36.0 μg/mL, respectively [8].

The strain B6219 was cultivated on M_2^+ medium (50% seawater) and incubated on a linear shaker for 8 days. Both culture filtrate and mycelium were extracted with either XAD-16 resin or ethyl acetate, respectively.



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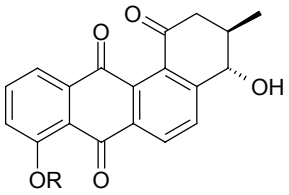

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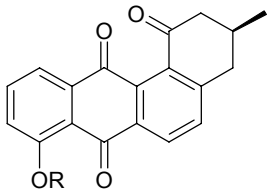

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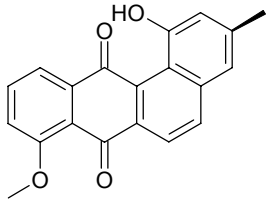

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Separation by chromatographic techniques (see experimental part) afforded fujianmycins A (**2**) and B (**3**), ochromycinone (**4**), ochromycinone methyl ether (**5**), tetrangulol methyl ether (**6**) and a new member of the angucyclinone family, fujianmycin C (**1**).

Fujianmycin C (**1**) and the other compounds were obtained as yellow needle crystals. On TLC they showed a UV absorbing band, which turned to yellow

with anisaldehyde/sulfuric acid spray reagent. Compounds **2** and **4** turned to violet with 2N NaOH indicating a *peri*-hydroxy quinone moiety, while the other compounds did not react. Due to this behavior and their similar UV/vis and NMR spectra, a group of related quinones was expected.

In the ^{1}H NMR spectrum of **1**, two doublets and a triplet of three adjacent aromatic protons and an AB system of two further protons in the *ortho* position in another ring appeared; the patterns were confirmed by COSY data. In the aliphatic region, a methoxy singlet at δ 3.95, two methylene groups at δ 3.82/3.75 (CH_2-13) and 2.99/2.77 (CH_2-2) and two methine signals at δ 4.75 (H-4) and δ 2.37 (H-3) were visible. The ^{13}C NMR spectrum displayed 20 signals, a ketone carbonyl, two quinone carbonyls, twelve sp^2 and five sp^3 carbon signals.

The CI mass spectrum afforded a *pseudo*molecular ion at *m/z* 353 $[M + H]^+$, and the HRESIMS of **1** delivered the formula $C_{20}H_{16}O_6$. A quinone nucleus was predicted based of the UV/vis data, the presence of carbonyl signals at δ 182.7 and 185.4 and the positive test for *peri*-hydroxy groups in the related components **2** and **4**.

The triplet of H-10 showed HMBC correlations with C-8 and 11a. The doublet at δ 7.66 (H-11) displayed a correlation with a quinone carbonyl and must be in a *peri*-position relative to the latter (C-8 or C-11). Further correlations (Figure 1) confirmed the methoxy group in the same ring, so that it must occupy the opposite *peri*-position (C-11 or C-8). Proton H-6 correlated with the second quinone carbonyl (C-7) and must be in a *peri*-position of a second ring on the same side as the methoxy group. Further HMBC and COSY correlations completed the angucyclinone skeleton, as drawn in Figure 1. Compound **1** is the new 13-hydroxyfujianmycin B and was named fujianmycin C. To date, it seems to be the second hydroxymethyl-angucyclinone after YM-181741 [9,10].

The relative configuration at C-3 and C-4 was supposed to be the same *trans* orientation as for fujianmycins A (**2**) and B (**3**). Force field calculations resulted in a *bis*-axial orientation of the hydrogens H-3 and H-4, which required a coupling constant of $J_{H\text{-}3,4}$ ~ 10 Hz. This agrees well with the experimental value of 9.3 Hz for H-4. The small NOE between 3-H and 4-H seemed to indicate their *cis* orientation, but this was also observed for fujianmycin B (**3**) and was therefore neglected. As the observed optical rotation of **1** (+18°, MeOH) was nearly the same as for **2** (+17°, $CHCl_3$) and as their biosyntheses are certainly closely related, a (3*S*,4*S*) configuration can be assumed.

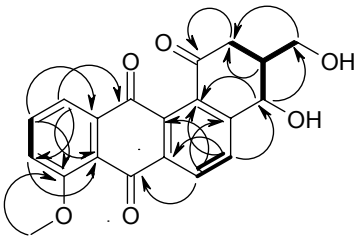

Figure 1: Selected H,H COSY (▬) and HMBC (→) correlations of **1**.

Fujianmycin C (**1**) showed weak antibacterial activity against *Streptomyces viridochromogenes* Tü 57, causing inhibition zones of 14 mm at 40 µg/ disk, but was not cytotoxic.

Further components were identified as fujianmycins A (**2**) [11] and B (**3**), ochromycinone (**4**) [12], ochromycinone methyl ether (**5**), and tetrangulol methyl ether (**6**) [13] by interpretation of their NMR spectroscopic data by means of AntiBase [14]. These compounds were responsible for the cytotoxicity of the crude extract and contributed also to its antibacterial properties. It is obvious that all these compounds have a close biosynthetic relationship.

Experimental

General experimental procedures: see ref. [15]. R_f values were measured on Polygram SIL G/UV_{254} TLC cards from Macherey-Nagel & Co. (Düren, Germany) with CH_2Cl_2/5% MeOH.

Taxonomy of *Streptomyces* sp. B6219: Strain B6219 was derived from sediment of a mangrove forest in Galapagos and was isolated on casein peptone agar [16] containing 50% of natural seawater. The reference culture of B6219 is kept on yeast extract-malt extract agar in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven.

The almost complete 16S rRNA gene sequence of strain B6219 was deposited in GenBank under the accession no. HQ419059. The sequence is 99 % similar to that of the type strain *Streptomyces griseorubiginosus* (accession no. AJ781339).

The substrate mycelium is brown. Spores are light grey. Spore chains are straight to flexuous (*Rectiflexibiles*). The surface of spores is smooth to warty. Melanin pigment is produced on peptone-yeast extract-iron agar and tyrosine agar [17]. Optimum growth temperature is at about 30°C. The strain neither reproduces at 4°C nor at 45°C. The strain does not develop in media with 7% or higher seawater salinity. Starch and chitin are degraded, casein is not hydrolyzed [18]. The strain is

nitrate reductase positive. For a photograph, see supplementary material Figure S1.

Fermentation, extraction and isolation: The marine derived *Streptomyces* sp. B6219 was cultivated on M_2^+ medium (10 g malt extract, 4 g glucose, 4 g yeast extract per L, 50% seawater) for 8 days and extracted with ethyl acetate (mycelium) and XAD-16 resin (filtrate), respectively, as described for related strains [19]. The resulting yellow brownish combined crude extract (6.43 g) was defatted using cyclohexane and chromatographed on a silica gel column using CH_2Cl_2/MeOH (gradient 0 to 10 % MeOH) to deliver 4 fractions FI-FIV. Fraction FII was purified on Sephadex LH-20 using MeOH to afford 2 sub-fractions. FIIa was subsequently purified using PTLC (CH_2Cl_2/5% MeOH) and again Sephadex LH-20 using MeOH to afford fujianmycin C (**1**, 1.90 mg, R_f = 0.38), while FIIb after purification on Sephadex LH-20 afforded fujianmycin A (**2**, 6.4 mg, R_f = 0.43) and ochromycinone (**4**, 1.41 mg, R_f = 0.36). Separation of FIII on silica gel (CH_2Cl_2/MeOH gradient 0 to 10 % MeOH) and Sephadex LH-20 delivered 2 sub-fractions: FIIIa afforded on PTLC (CH_2Cl_2/5% MeOH) fujianmycin B (**3**, 8.0 mg, R_f = 0.33; spectra see supplementary material), while FIIIb was purified on Sephadex LH-20 to deliver ochromycinone methyl ether (**5**, 1.4 mg, R_f = 0.40). FIV delivered, in a similar way, tetrangulol methyl ether (**6**, 1.0 mg, R_f = 0.30).

Fujianmycin C (**1**)

Yellow needles

R_f : 0.38 (CH_2Cl_2-MeOH, 95:5).

$[\alpha]_D^{20}$: +18° (*c* 0.1, CH_3OH).

UV/VIS: λ_{max} (log ε) = (MeOH): 225 (4.41), 256 (4.46), 375 (3.87).

^{1}H NMR (300 MHz, CD_3OD): δ 8.33 (1H, d, *J* = 8.2 Hz, H-6), 8.04 (1H, d, *J* = 8.2 Hz, H-5), 7.79 (1H, t, *J* = 8.4, 7.6 Hz, H-10), 7.66 (1H, d, *J* = 7.6 Hz, H-11), 7.51 (1H, d, *J* = 8.5 Hz, H-9), 4.75 (1H, d, *J* = 9.3 Hz, H-4), 3.95 (3H, s, 8-OCH_3), 3.82, 3.95 (2H, ABX, *J* = 10.9, 5.5, 4.6 Hz, CH_2-13), 2.99, 2.77 (2H, ABX, *J* = 16.2, 6.3, 10.4 Hz, CH_2-2), 2.37 (1H, m, H-3).

^{13}C NMR (125 MHz, CD_3OD): δ 200.0 (C_q-1), 185.4 (C_q-12), 182.7 (C_q-7), 161.5 (C_q-8), 153.6 (C_q-4a), 138.6 (C_q-11a), 137.0 (CH-10), 136.7 (C_q-12b), 135.3 (C_q-12a), 135.1 (C_q-6a), 132.3 (CH-5), 130.7 (CH-6), 121.5 (C_q-7a), 120.2 (CH-11), 119.1 (CH-9), 69.4 (CH-4), 63.3 (CH_2-13), 56.9 (8-OCH_3), 46.4 (CH-3), 41.0 (CH_2-2).

CIMS: *m/z* (%) 353 $[M + H]^+$ (100).

HRESIMS: *m/z* 353 $[M + H]^+$ calcd for $C_{20}H_{17}O_6$: 353.10196; found: 353.10205.

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Dioscorealide B from the Traditional Thai Medicine Hua-Khao-Yen Induces Apoptosis in MCF-7 Human Breast Cancer Cells via Modulation of Bax, Bak and Bcl-2 Protein Expression

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Dioscorealide B is a pharmacologically active compound from the rhizome of the Thai medicinal plant *Dioscorea membranacea*. Here, we demonstrated that *in vitro* treatment of dioscorealide B resulted in a cytotoxic effect on MCF-7 human breast cancer cells (IC_{50} = 2.82 μM). To determine whether this compound induces apoptosis in MCF-7, the Annexin V assay was performed. The data showed that the number of apoptotic cells were increased 7–12 folds over that of the control cells after treatment with various concentrations of dioscorealide B (3, 6 and 12 μM) for 24 hours. Dioscorealide B-induced apoptosis was associated with modulation of the multidomain Bcl-2 family members Bax, Bak and Bcl-2. After treatment with 3 μM dioscorealide B, acceleration of the level of proapoptotic proteins Bax and Bak were observed at 6 hours and 12 hours, respectively, while the decrease in the expression of antiapoptotic protein Bcl-2 was observed 3 hours after the treatment. These effects of dioscorealide B might result in the activation of caspase-8, -9 and -7, which lead to apoptosis in MCF-7 cells. Taken together, the results of this study provide evidence that dioscorealide B possesses an antitumor property against human breast cancer cells and thus provide the molecular basis for the further development of dioscorealide B as a novel chemotherapeutic agent for breast cancer treatment.

Keywords: Dioscorealide B, apoptosis, Bcl-2, MCF-7.

Breast cancer is the most common cancer in women worldwide, comprising 16% of all female cancers. It is estimated that 519,000 women died in 2004 due to this disease, and although it is thought to be a problem of the developed world, a majority (69%) of all breast cancer deaths occurs in developing countries [1]. The American Cancer Society estimated that 192,370 new cases of invasive breast cancer would be diagnosed in women in the U.S. and about 40,170 breast cancer deaths were expected in 2009 [2]. These statistics accentuate the immediate need for improvements in detection, diagnosis, and treatment of this disease. Breast cancer is currently controlled through surgery and/or radiotherapy, and is frequently supported by adjuvant chemo- or hormonal therapies. However, these classical treatments are impeded by unwanted side effects and, most importantly, the development of tumor resistance. The medicinal use of naturally occurring substances or natural agents then becomes an alternative for the patients suffering from cancer [3,4].

Dioscorealide B (Figure 1) is a pharmacologically active compound from the ethanol extract of *Dioscorea membranacea* Pierre (Dioscoreaceae), a plant known in Thailand as "Hua-Khao-Yen", which is used in Thai traditional medicines as an ingredient in several

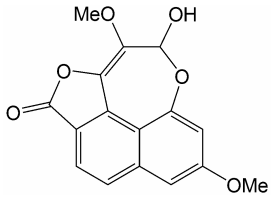


Figure 1: Chemical structure of dioscorealide B

preparations, including those used in the treatments of lymphopathy, dermopathy, venereal diseases, leprosy, bacterial infections and cancers [5–7]. Previous study revealed that dioscorealide B, one of the isolated compounds from *D. membranacea*, has cytotoxic activity against MCF-7 human breast cancer cells and has been shown to serve as an anti-proliferative and selective cytotoxic agent. The compound selectively inhibited the proliferation of breast cancer cell line (MCF-7), without being significantly cytotoxic towards non-malignant cells (SVK) [8]. In the present study, some mechanisms related to apoptosis, which might underlie the antiproliferation activity of dioscorealide B, have been investigated.

In this study, the cytotoxic effect of dioscorealide B on MCF-7 was determined by SRB assay. Dioscorealide B showed a cytotoxic effect on MCF-7 with an IC_{50} value of 2.82 ± 0.36 μM (Figure 2), whereas vinblastine, the positive control, had a stronger cytotoxic effect with anIC_{50} value of 1.65 ± 0.13 nM. This result confirmed that dioscorealide B was able to inhibit *in vitro* growth of MCF-7.

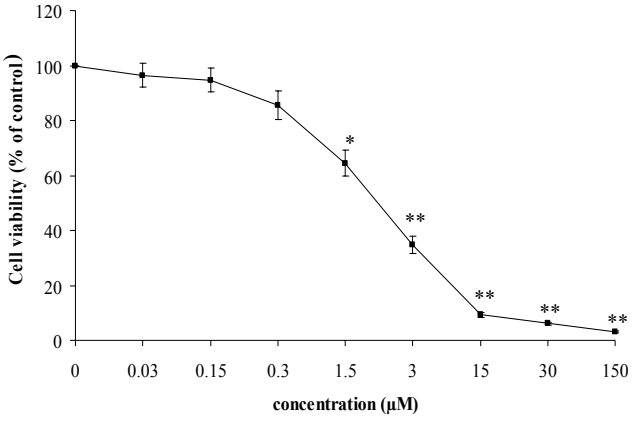
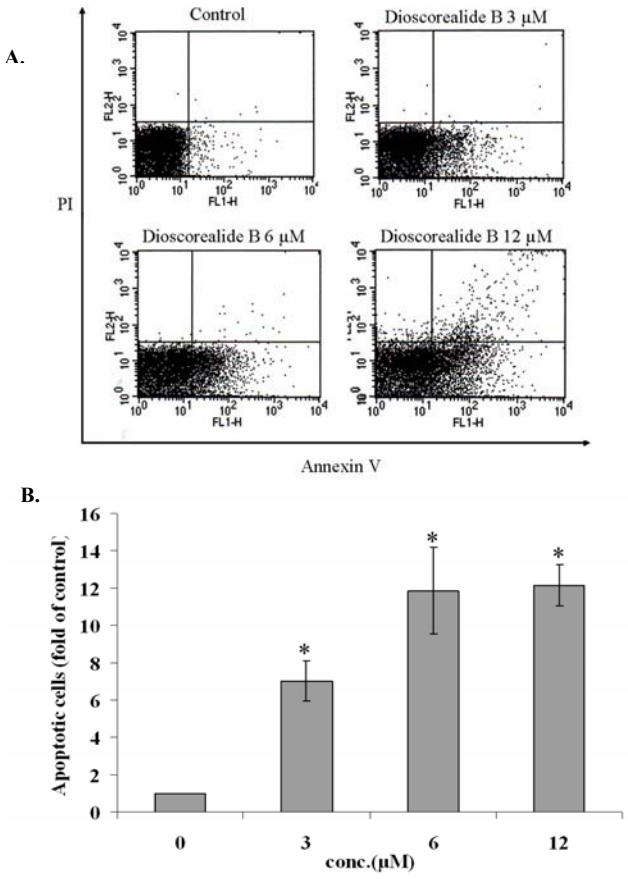


Figure 2: The antiproliferative effect of dioscorealide B on MCF-7 cells. MCF-7 cells were incubated with various concentrations of dioscorealide B. After 72 hours, cell proliferation was determined by SRB assay. Results are expressed as percent of viable cells in the studied group as compared with those in the control group. (* $p < 0.05$ and ** $p < 0.01$)

Cancer is an aberrant net accumulation of atypical cells which can arise from an excess of proliferation, an insufficiency of apoptosis, or a combination of the two [9]. Increased frequency of apoptosis could result in cell loss in tumors and promote tumor regression. In cancer treatment, a major recent focus is on activating the apoptotic program in the cell [10], and there is evidence that many anticancer agents cause tumor cell death via the mechanisms of apoptosis [11–13]. Thus, we extended our study to examine whether the anti-tumor property against breast cancer cells is mediated through apoptosis induction after dioscorealide B treatment. MCF-7 cells were stained with Annexin V-FITC and PI, and then subsequently analyzed by flow cytometry. This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in the early apoptotic cells. MCF-7 cells were challenged with dioscorealide B at 3, 6 and 12 µM for 24 h. We found that dioscorealide B significantly induced apoptosis in a dose-dependent manner compared with control cells. The numbers of apoptotic cells in MCF-7 were increased to 7.02, 11.87 and 12.17 fold after 3, 6 and 12 µM dioscorealide B treatment, respectively (Figure 3B).



I annexin-V fluorescein isothiocyanate and propidium iodide double staining (An/PI). Breast cancer cells were treated with either dioscorealide B or vehicle (control) for 24 h. Apoptotic cells were detected as An+/PI- cells, while viable cells were An-/PI cells and necrotic cells were PI+. Panel 3 A, representative dot plots after five days of dioscorealide B exposure. Panel 3 B, the fold increase of apoptotic cells compared with control was determined from three experiments (mean ± SD). Statistical analysis on log transformed data; asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

Caspases are members of a family of cysteine proteases that play a crucial role in the apoptotic pathway [14,15]. There are two major mechanisms that initiate the caspase cascade: the extrinsic, involving caspase-8; and the intrinsic pathway, involving caspase-9 as the apical caspase, which leads to the proteolytic activation of effector caspases, including caspase-3 and -7, which cleave the cellular substrate, resulting in cell death [16,17]. The extrinsic pathway is triggered by the interaction between a death ligand, such as TNF or FasL, and its cognate receptor, TNF-R or Fas (CD95), inducing the trimerization of the receptors, which recruit adaptor proteins such as FADD and procaspase-8, leading to the activation of caspase-8 [18,19]. The intrinsic pathway is regulated by the Bcl-2 family of proteins, including proapoptotic members such as Bax and Bak, and anti-apoptotic members such as Bcl-2 and Bcl-X_L [20-22]. In response to apoptotic signals, proapoptotic Bcl-2 family members translocate to and alter the permeability of the mitochondrial membrane [23]. These proteins are thought to either form channels in the outer mitochondrial membrane or to alter the activity of existing channels, leading to changes in the mitochondrial membrane potential and cytochrome c release. Cytochrome c then interacts with Apaf-1 and procaspase-9, which in turn activates caspase-9 [24,25].

To gain insights into the mechanism of apoptosis induction in MCF-7, the effect of dioscorealide B treatment on levels of Bcl-2 family proteins by western blotting was studied and the results are shown in Figure 4. Following treatment with 3 μM of dioscorealide B, the formation of anti-apoptotic protein Bcl-2 was down-regulated after three hours, whereas the expression of proapoptotic protein Bax was upregulated after six hours. The dioscorealide B treatment also caused an increase in the protein level of Bak, as observed at twelve hours.

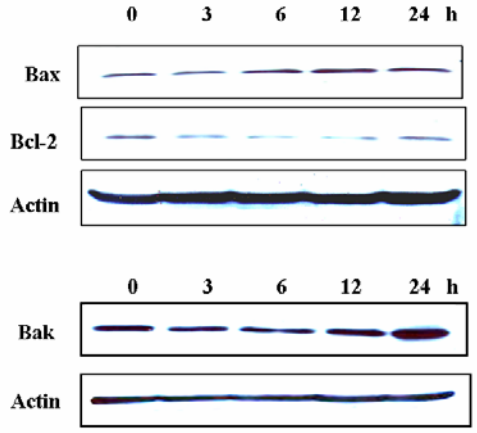


Figure 4: Immunoblotting for Bax, Bak and Bcl-2 using lysates from MCF-7 cells treated with 3 μM dioscorealide B for the indicated time periods. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein loading.

Next, we examined whether the caspases are involved in dioscorealide B-induced apoptosis. Immunoblot analyses of lysates obtained from MCF-7 cells treated with dioscorealide B at 3 μM were examined and results are shown in Figure 5. The cleaved form of caspase-7 increased after three hours and procaspase-7 was totally cleaved after twenty-four hours of treatment, suggesting that this compound induces the activation of caspase-7. Dioscorealide B increased the activity of caspase-7 activity in a dose- and time-dependent manner, as demonstrated in Figures 6 and 7. After incubation with 3 μM of dioscorealide B, the caspase-7 activity significantly increased to 230% at six hours. The effect of dioscorealide B on caspase-8 and caspase-9 activities is shown in Figure 8. Our findings revealed that activation of caspase-9 significantly increased after treatment of the cells with dioscorealide B, starting from three hours of exposure time, but caspase-8 activity showed a significant elevation after only one hour of treatment.

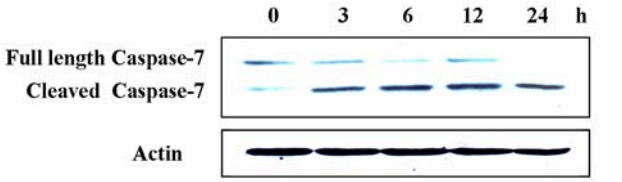


Figure 5: Western Blot analysis of apoptotic proteins in dioscorealide B treated cells. Proteins from MCF-7 cells treated with 3 μM of dioscorealide B for the indicated times were resolved on 12% SDS-PAGE and subjected to Western Blotting analysis with antibodies against caspase-7 and actin.

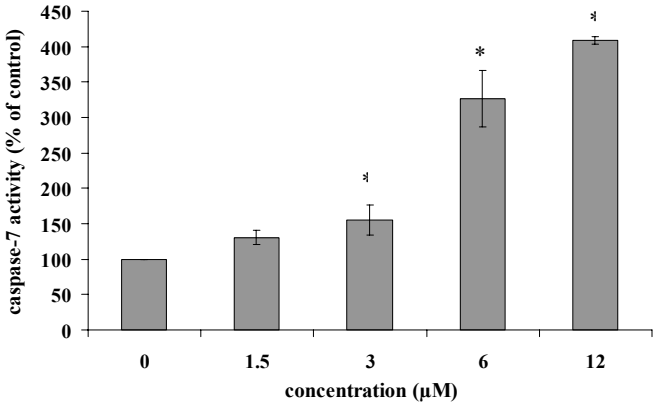


Figure 6: The effect of induction of caspase-7 activity by dioscorealide B in MCF-7 breast cancer carcinomas**.** MCF-7 cells were treated with 1.5, 3, 6 and 12 μM of dioscorealide B for 3 h. The data represent the mean value of 3 replications from 2 independent experiments.

In a further experiment, the effect of caspase inhibitors on dioscorealide B-induced apoptosis was studied. In MCF-7, cells were pretreated with either 50 μM of the caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK for 3 h prior to treatment with 3 μM of dioscorealide B. As shown in

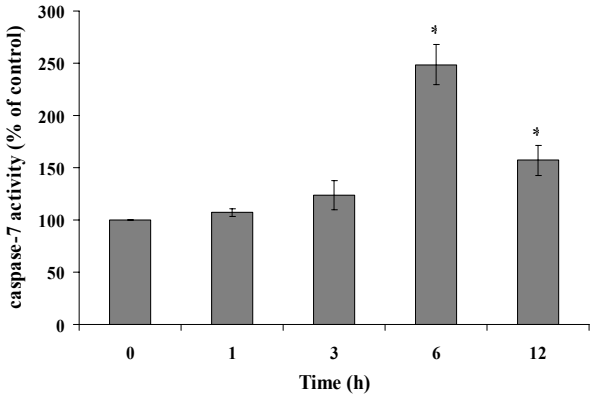


Figure 7: The activation of caspase-7 in MCF-7 by dioscorealide B. MCF-7 cells were incubated with 3 μM of dioscorealide B for the indicated times. The data represent the mean value of 3 replications from 2 independent experiments. Asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

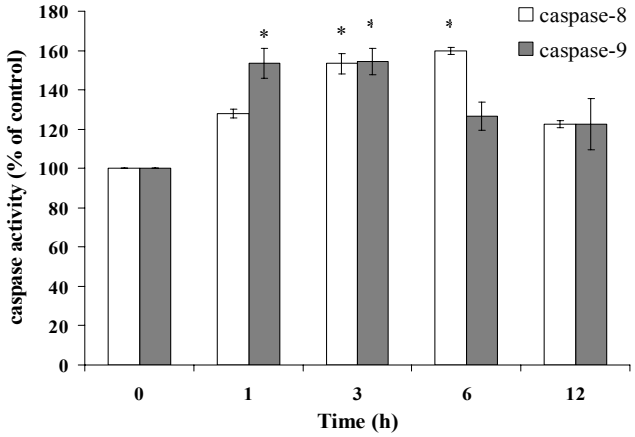


Figure 8: The activation of caspase-8 and caspase-9 in MCF-7 by dioscorealide B. MCF-7 cells were incubated with 3 μM of dioscorealide B for the indicated times. Asterisks indicate ANOVA significance $p < 0.05$ versus corresponding control.

Figure 9, pretreatment of MCF-7 cells with either the caspase-8 inhibitor or the caspase-9 inhibitor significantly decreased the caspase-7 activity. These results suggested that this bioactive compound might be involved in both intrinsic and extrinsic apoptotic pathways. This observation further supports the fact that induction of apoptosis in MCF-7 cells by dioscorealide B is mediated through reduction of antiapoptotic protein Bcl-2 expression and induction of proapoptotic protein Bax and Bak expression, leading to the activation of caspase-9 and -7, respectively. However, the mechanism of diosocorealide B in the extrinsic pathway needs to be refined. The role of the TNF superfamily such as Fas, Fas ligand, TNF-R1 and TNF-α might be investigated in the further study. In this study, we found that dioscorealide B caused an increase in both caspase-8 and -9 activities. Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, might be involved in dioscorealide B-induced apoptosis. Bid is a specific proximal substrate of caspase-8 in the Fas apoptotic

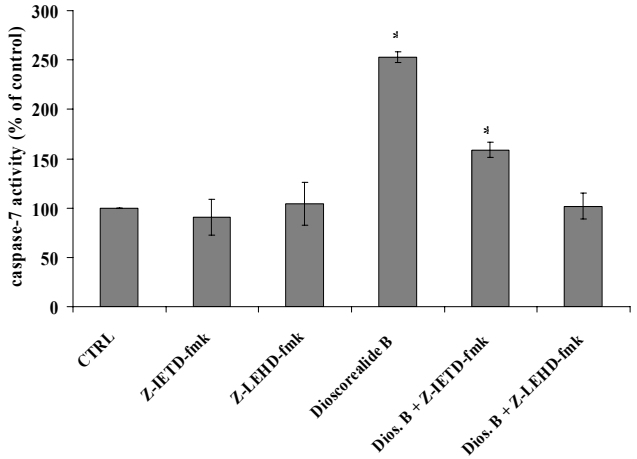


Figure 9: The effect of caspase-8 and -9 inhibitor on dioscorealide B-induced apoptosis. MCF-7 cells were pre-incubated with Z-IETD-FMK (50 μM) for 3 h before challenge with 3 μM dioscorealide B. After 6 h of treatment, caspase-7 activity was measured by caspase-Glo® assay. Each value is the mean ± SD of 3 determinations. The asterisk indicates a significant difference between control and dioscorealide B-treated cells, as analyzed by ANOVA $p < 0.05$.

signaling pathway. While full-length BID is localized in cytosol, truncated BID (tBID) translocates to mitochondria and, therefore, transduces apoptotic signals from the cytoplasmic membrane to mitochondria. tBID induces first the clustering of mitochondria around the nuclei and release of cytochrome c independent of caspase activity, and then the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion [26].

In conclusion, for the first time, the mechanisms of dioscorealide B against breast cancer were elucidated. The results of the present study indicated that dioscorealide B treatment decreased the expression of antiapoptotic protein Bcl-2 and increased the expression of proapoptotic proteins Bax and Bak, which leads to the activation of caspase-9 and -7, respectively. These studies thus provide the molecular basis for the further development of dioscorealide B as a novel chemotherapeutic agent for breast cancer treatment.

Experimental

Materials: Reagents were purchased from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin from Gibco BRL (Gaithersburg, MD, USA), anti-□-actin antibody from Sigma–Aldrich Co. (St. Louis, MO, USA); antibodies against Bak and caspase-7 from from Cell Signaling (Beverly, MA, USA); antibody against Bcl-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Bax antibody from US biological (Massachusetts, USA), Annexin V-FITC and propidium iodide (PI) from BD

Pharmingen (Franklin Lake, NJ, USA), Caspase-Glo® assay from Promega (Madison, WI), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) from Calbiochem (Darmstadt, Germany).

Plant materials: The rhizomes of *D. membranacea* Pierre (Dioscoreaceae) were collected from Pa-tue, Chumporn, Thailand. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKP A062041305) is kept. Specimens are also kept in the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla,Thailand.

Isolation of dioscorealide B: Dioscorealide B was isolated following the method previously described and agreed in all respects as regards reported chromatographic and spectral data [8].

Cell culture conditions: MCF-7 human breast cancer cell line was kindly provided by Dr P. Twentyman and Dr P. Rabbitts of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK. Cells were cultured in monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO_2.

SRB assay: The cytotoxicity assay was carried out using the sulforhodamine B (SRB) assay [8,27]. Briefly, 3,000 of MCF-7 cells were plated per well in 96-well culture plates kept in an incubator at 37°C. After overnight incubation, the cells were treated without or with dioscorealide B 0.03, 0.15, 0.3, 1.5, 3, 15, 30, 150 µM, with 6 replications. The cells were incubated for 72 h and then the medium was removed and washed. The survival percentage was measured colorimetrically using the SRB assay, and IC_{50} values were calculated by means of the Prism program. Cells incubated with regular cell culture media with 0.2% DMSO were used as a negative control and vinblastine sulfate as a positive control.

Annexin V– FITC assay: Induction of apoptosis by dioscorealide B treatment was measured by an Annexin V–FITC apoptosis detection kit, following the manufacture's instructions. Briefly, MCF-7 cells (1×10^6 cells) were seeded in 12-well plates and treated with dioscorealide B at 3, 6 and 12 µM. After 24 h, cells were collected, washed with cold PBS twice, gently resuspended in 100 µl of staining solution (containing annexin V, fluorescein and propidium iodide in a HEPES buffer). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Annexin V binds to those cells that express phosphotidylserine on the outer layer of the cell, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and propidium iodide).

Caspase-7, -8 and -9 activity assay: The Caspase-Glo®-3/7, -8 and -9 assays were used to measure caspase-3/7, caspase-8 and caspase-9 activity. Briefly, 10^4 cells were cultured in 96-well plates and treated with dioscorealide B (3, 6 or 12 µM). After the incubation period, caspase-Glo® reagent was added to each well according to the manufacturer's instructions. Plates were mixed on a plate shaker for 30 secs and incubated at room temperature for 1 h. Luminescence was measured using a luminometer. The assay was performed in triplicate.

Western blotting: After treatment, cells were washed with ice-cold PBS and lysed with SDS lysis buffer. The protein concentration of the supernatant was determined by the Bradford method. The lysates were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and then transferred to a PVDF membrane. After the membrane was blocked in Tris–buffer saline (TBST) containing 0.05% Tween 20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies specific for Bax, Bak, Bcl-2 and caspase-7. After washing 3 times with TBST for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA).

Statistics: Data were expressed as means ± SEM. Statistical comparisons of the results were made using analysis of variance (ANOVA) and a *P* value less than 0.05 was considered significant.

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Inhibition of Protein Tyrosine Phosphatase 1β by Hispidin Derivatives Isolated from the Fruiting Body of *Phellinus linteus*

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Protein tyrosine phosphatase 1β (PTP1β) acts as a negative regulator of insulin signaling. Selective inhibition of PTP1β has served as a potential drug target for the treatment of type 2 diabetes mellitus. We evaluated the inhibitory effect of *Phellinus linteus* against PTP1β as part of our ongoing search for natural therapeutic and preventive agents for diabetes mellitus. Fractions of the *P. linteus* extract were found to exhibit significant inhibitory activities against PTP1β. In an attempt to identify bioactive components, we isolated, from the most active ethyl acetate fraction, five hispidin derivatives (phelligridimer A, davallialactone, hypholomine B, interfungins A, and inoscavin A) and four phenolic compounds (protocatechuic acid, protocatechualdehyde, caffeic acid, and ellagic acid). The chemical structures of these compounds were elucidated from spectroscopic evidence and by comparison with published data. All the compounds strongly inhibited PTP1β activity in an *in vitro* assay; their IC_{50} values ranged from 9.0 ± 0.01 to 58.2 ± 0.3 μM. Our results indicated that the hispidin skeleton may be an important moiety for inhibitory activity of the above compounds against PTP1β. Thus, hispidin derivatives could be a potent new class of natural PTP1β inhibitors.

Keywords: *Phellinus linteus*, hispidin, protein tyrosine phosphatase 1β, diabetes.

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important signaling molecules, such as members of the MAP kinase family. Among them, protein tyrosine phosphatase 1β (PTP1β) plays a critical role in the signal transduction of both the insulin and leptin pathway [1,2]. This role has been demonstrated for the dephosphorylated insulin receptor and other proteins in intact cells and thus, PTP1β can act as a negative regulator of the insulin signaling pathway [3]. Also, the deletion of the PTP1β gene in mice has been reported to induce marked insulin sensitivity and prolonged the autophosphorylation of the insulin receptor [4]. PTP-1β inhibitors would increase insulin sensitivity by blocking the PTP1β-mediated negative regulation of the insulin signaling pathway [5]. Therefore, PTP1B has emerged as a novel target for the treatment of diabetes and obesity, with numerous compounds having been developed as PTP1β inhibitors [6]. Small molecule inhibitors of PTP-1β, such as oxalamides, benzoic acid, and phenoxyacetic acids were reported [7]. Of them, only ertiprotafib progressed to clinical trials prior to discontinuation at phase II due to insufficient efficacy, coupled with unwanted side effects [8]. Although several types of PTP1β inhibitors have been reported, they have low selectivity and poor pharmacokinetic properties; therefore, new types of PTP1β inhibitors with improved pharmacological properties are still being sought.

Phellinus linteus (Berkeley & M. A. Curtis), an orange colored mushroom in the family Hymenochaetaceae,

Table 1: Inhibitory effects on PTP1β of MeOH extract and its fractions isolated from the fruiting body of *P. linteus*.

Fractions	PTP1β inhibitory activity[a] (μg/mL)
MeOH Extract	23.4 ± 1.9
n-hexane fr.	-
Methylene chloride fr.	-
Ethylacetate fr.	17.2 ± 0.8
n-BuOH fr.	35.9 ± 2.1
Water fr.	-

[a] IC_{50} values were determined by regression analyses and are expressed as mean ± SD of 3 replicates.

has been used in oriental countries for centuries to prevent ailments such as gastrointestinal dysfunction, diarrhea, hemorrhage, inflammation, and cancer [9]. Although they produce a large and diverse variety of secondary metabolites, polysaccharides have been considered to be responsible for their biological effect. β-Glucans derived from *P. linteus* act as an effective immunomodulator, exhibit a wide range of antitumor activity and have been shown to prevent metastasis [10]. Also, polysaccharide of *P. linteus* has been reported to be effective for diabetes in non-obese diabetic mouse [11].

A recent investigation of the chemical constituents of the genera *Inonotus* and *Phellinus* resulted in isolation of hispidin derivatives with a novel carbon skeleton, such as phellifuropyranone A [12], phellinusfurans A and B [13], phellinone [14], phellinsin A [15], inoscavins A–D and methylinoscavin A-D [16,17], phelligridins A–G [18–20] and interfungins A–C [21]. However, there have been few studies on the biological activities of these compounds. In our recent study, hispidin derivatives from *P. linteus* exerted potent antioxidant effects, as well as marked inhibitory effects on rat lens aldose reductase and advanced glycation end product formation, corresponding to high values of total phenolic content and total flavonoid content [22,23].

While screening for PTP1β inhibitors from natural resources, the MeOH extract of the fruiting body of *P. linteus* was found to inhibit PTP1β activity at a concentration of 16.3 μg/mL; this led us to investigate the PTP1β inhibitory components from this plant. In this paper, we report the isolation and structural elucidation of active principles with an inhibitory activity against PTP1β.

To identify the active principles, we performed bioassay-guided fractionation and purification by using repeated column chromatography, which resulted in 5 hispidin derivatives and 4 phenolic compounds (Figure 1). The isolated compounds were identified by analysis of their NMR and MS data as phelligridimer A (**1**), davallialactone (**2**), hypholomine B (**3**), interfungin A (**4**) and inoscavin A (**5**), as well as hispidin (**6**), protocatechuic acid (**7**), protocatechualdehyde (**8**), caffeic acid (**9**), and ellagic acid (**10**), In addition, these

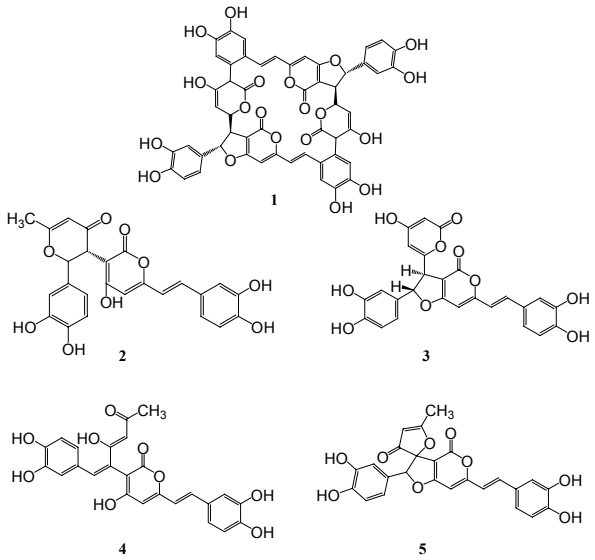

Figure 1: Chemical structures of the compounds isolated from *P. linteus*. **1**: phelligridimer A; **2**: davallialactone; **3**: hypholomine B; **4**: interfungin A; **5**: inoscavin A.

data were compared with those found in the literature [24–27]. The inhibitory activities of the isolated compounds against PTP1β were measured using *p*-nitrophenyl phosphate (*p*NPP) as the substrate; the results are summarized in Table 2. The known PTP1β inhibitor, ursolic acid (IC_{50} = 3.9 ± 0.3 μM), was used as a positive control. The isolated compounds **1–10** dose-dependently inhibited the PTP1β activity with IC_{50} values ranging from 9.0 ± 0.6 to 58.2 ± 3.4 μM.

The hispidin derivatives **1**–**6** were found to show greater inhibitory activities against PTP1β than the phenolic compounds **7**–**10**. Of the hispidin derivatives, compounds **1** and **3** with a hispidin moiety exhibited a greater inhibitory activity against PTP1β than compounds **2**, **4**, and **5**, which have a hispolon moiety. As shown in Table 2, the inhibitory effects of the hispidin derivatives were enhanced when a hispidin dimer or tetramer was formed. Among the hispidin derivatives, compound **1**, a hispidin tetramer, exhibited the highest inhibitory activity against PTP1β with an IC_{50} of 8.9 ± 0.6 μM. This result indicates that compound **1** is approximately 2- to 3-fold more effective than other hispidin derivatives. Compounds **1**–**6** are highly oxygenated and functionalized aromatic compounds that possess the unique basic structural unit, namely, 6-[2-(3,4-dihydroxyphenyl)ethenyl]-4-hydroxy-2H-pyran-2-one (hispidin, **6**). It has been reported that compound **3**, which has an unusual structure with 2,3-dihydro-4-H-furo[3,2-c]pyran-4-one, may be biosynthesized by oxidative coupling of two hispidin mediates, while compound **1** may be sequentially or simultaneously formed by oxidative coupling of four hispidin mediates and/or by oxidative coupling of two hypholomine B molecules [24].

Table 2: Inhibitory effects of compounds isolated from the fruiting body of *P. linteus* on PTP1β.

Compounds	Concentration (μM)	Inhibition (%)	IC_{50} (μM)
Phelligridimer A (**1**)	10	56	8.9 ± 0.6
	5	29	
	1	9	
Davallialactone (**2**)	25	51	24.2 ± 1.0
	10	26	
	2.5	11	
Hypholomine B (**3**)	25	91	12.9 ± 0.9
	10	42	
	2.5	12	
Interfungins A (**4**)	25	59	20.1 ± 1.7
	10	32	
	2.5	12	
Inoscavin A (**5**)	50	95	27.3 ± 2.1
	25	46	
	10	15	
Hispidin (**6**)	50	80	33.6 ± 3.8
	25	32	
	10	10	
Protocatechuic acid (**7**)	100	88	52.9 ± 3.6
	50	48	
	10	15	
Protocatechualdehyde (**8**)	100	82	58.6 ± 3.4
	50	42	
	10	14	
Caffeic acid (**9**)	100	93	44.5 ± 2.5
	50	52	
	10	25	
Ellagic acid (**10**)	50	88	26.2 ± 1.9
	25	49	
	10	23	
Ursolic acid[b]	5	65	3.9 ± 0.3
	2.5	30	
	1	10	

[a]IC_{50} values were determined by regression analyses and are expressed as mean ± SD of 3 replicates. [b] Positive control.

Compounds **2** and **5** are known to be biosynthesized by rearrangement of compound **4**, which is biosynthesized by the condensation of hispidin and 6-(3,4-dihydroxyphenyl)-4-hydroxy-3,5-hexadiene-2-one (hispolon) [21]. This suggests that a dihydrofuro[3,2-c]pyran-4-one moiety is formed by a cyclization linkage between the A and B rings via an oxygen-bridge that might increase the inhibitory activity against PTP1β. Phelligridins H and I have an unprecedented carbon skeleton (pyrano-[4,3-*c*]isochromen-4-one) as the structural element. A previous study reported inhibitory activity of these 2 derivatives from *P. igniarius* against PTP1β [20]. Although the structure-activity relationships of the hispidin derivatives were not thoroughly investigated, the dihydrofuro[3,2-c]pyran-4-one moiety appeared to correlate with the inhibitory activity against PTP1β. The hispolon moiety might be responsible for a loss of PTP1β activity. Thus, the inhibitory activities of the hispidin derivatives might provide valuable information regarding the structure -activity relationship for the development of novel PTP1β inhibitors.

Experimental

Plant materials: The fresh fruiting bodies of *P. linteus* were provided by Samsung Herb Medicine Co., Ltd., Chuncheon, Korea and the voucher specimen (No. RIC-021) was deposited at the Regional Innovation Center, Hallym University, Republic of Korea.

Chemicals and equipment: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX 400 spectrometer. Sephadex LH 20 (GE Healthcare Bio-Science AB, Sweden) and cosmosil 75C18-OPN (Nacalai tesque, Kyoto, Japan) were used as column packing material. PTP1β (human, recombinant) drug discovery kit was purchased from BIOMOL® International Inc. (USA).

Isolation and identification: The lyophilized fruiting bodies of *P. linteus* (1 kg) were extracted 3 times with MeOH for 5 h. After removal of MeOH under reduced pressure, a dark brown residue (50 g, 5%) was obtained. This was suspended in water and then partitioned sequentially with *n*-hexane (8 g, 0.8%), methylene chloride (4.9 g, 0.5%), ethylacetate (EtOAc, 22 g, 2.2%), *n*-butanol (14.1 g, 1.4%), and water (1 g, 0.1%). Sequential fractionation indicated that the EtOAc-soluble fraction showed PTP1β inhibitory activity. Therefore, this fraction was chromatographed over Sephadex LH 20 eluting with MeOH. Among the 8 pools (Fr.1-Fr.8), combined by their HPLC profiles, Fr. 7 was found to have the highest activity. Mixed frs. 5, 7, and 8 was subjected to Lichroprep® RP-18 CC using a stepwise gradient of AcCN-H_2O (1:4 to 4.5:5.5) to afford compounds **1**-**6**. Fractions 1-3 were each purified by reversed phase HPLC using an isocratic solvent system of 15% AcCN to isolate compounds **7**-**9**, respectively. Compound **10** was obtained as crude crystals from Fr. 4, which was further purified by HPLC using an isocratic solvent system of 20% AcCN. The structures of **1**-**10** were identified by comparison of observed spectroscopic data with published values.

Assay for PTP1β inhibitory activity: The PTP 1β tyrosine phosphatase drug discovery kit is a colorimetric, non-radioactive assay designed to measure the phosphatase activity of purified PTP1β [28]. The enzyme activity was measured by using IR5 phosphopeptide (Insulin Receptor B residues 1142-1153, pY-114) as a substrate. To each 96-well (final volume: 125 μL) was added 75 μM IR5 substrate and PTP1β (2.5 ng/well) in a buffer containing 100 mM MES (pH 6.0), 0.3 M NaCl, 2 mM EDTA, 2 mM dithiothreitol (DTT) and 0.1% NP-40 with or without test compounds. Following incubation at 37°C for 30 min, the reaction was terminated with BIOMOL RED™ reagent. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 620 nm. The non-enzymatic hydrolysis of IR5 substrate was corrected by measuring the increase in absorbance at 620 nm obtained in the absence of PTP1β enzyme.

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A New Azafluorenone from the Roots of *Polyalthia cerasoides* and its Biological Activity

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Chromatographic separation of the ethyl acetate extract of roots of *Polyalthia cerasoides* has led to the isolation of the new compound, 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone. This compound exhibited potent cytotoxic activities with IC_{50} values in the range of 2.64-3.58 μg.mL^{-1} for A549, GLC4 and GLC4/Adr cells, but was not recognized by ABCC1/MRP1 protein. The compound also showed very strong inhibition of *M. tuberculosis* using a broth microdilution method, with an MIC value of 0.78 μg.mL^{-1}, which was equal to that of ofloxacin, one of the four antibiotic drugs used as a positive control.

Keywords: *Polyalthia cerasoides*, Annonaceae, azafluorenone, antimycobacterial activity, cytotoxicity.

Polyalthia cerasoides (Roxb.) Bedd. (Annonaceae) is a medium-sized tree up to 5-15 meters tall, occurring mainly in Asiatic and Oceanic areas such as Burma (Myanmar), South China, India, Indochina, Thailand, and Laos (Khammouan) [1a,1b]. Previous phytochemical studies of various parts of *P. cerasoides* have resulted in the characterization of a benzofuran derivative [2a], oxoprotoberberine alkaloids [2b], dimeric aporphine alkaloids, a sesquiterpene, an isoquinoline alkaloid and triynoic acid [2c], and phytosterols [2d]. The constituents of Polyalthia species have shown remarkable antibacterial [2e], antifungal [3a], cytotoxic [3b], and antimalarial [2e] properties. In traditional Thai medicine, *P. cerasoides* is used for the treatment of tuberculosis. Based on already known preliminary data from bioassay-guided fractionation, we undertook further cytotoxicity studies of the crude *n*-hexane, ethyl acetate, acetone and methanol extracts. We present here the results obtained from testing the ethyl acetate extract for cytotoxic activity. From this extract, a new azafluorenone was isolated and examined for its antimycobacterial activity.

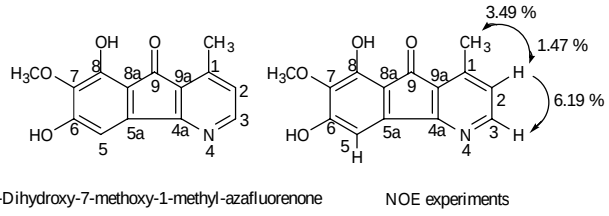

Figure 1: Chemical structure and NOE experiments.

The new azafluorenone was isolated as yellow needle crystals from ethanol (mp 200.9-202.7ºC). In the ESIMS, the molecular weight was indicated by the HRAPCIMS peak at m/z 257 [M^+], in combination with the measurement of the $[M+H]^+$ ion at m/z 258.0764 (cald. 258.0761 for $C_{14}H_{12}NO_4$). The presence of a hydroxyl group was suggested in the mass spectrum by a fragment ion base peak at *m/z* 239 (100) due to the direct loss of an 18 a.m.u. water unit from the $C_{14}H_{12}NO_4^+$ ion. In addition, the fragment ions at *m/z* 242 (M^+-Me) and 224 (M^+-Me-H_2O) in the mass spectrum indicated the presence of an OMe group in the position next to the OH group. As a unique group of alkaloids, azafluorenones

Table 1: ^{13}C NMR and ^{1}H NMR spectroscopic data of the azafluorenone and ^{1}H-^{13}C and ^{1}H-^{1}H correlations exhibited in the 2D NMR spectra in $CDCl_3$.

Carbon	δ ^{13}C (DEPT)	δ ^{1}H (*J* Hz)	HMBC correlation	COSY correlation
1	147.41 (C)	-	H-1CH_3, H-3	-
2	124.16 (CH)	6.80 (1H, d, 5.53)	H-1CH_3, H-2, H-3	H-3, H-1CH_3
3	150.80 (CH)	8.16 (1H, d, 5.53)	H-2	H-2
4	-	-	-	-
4a	166.35 (C)	-	H-3, H-5	-
5	104.30 (CH)	6.87 (1H, s))	-	-
5a	125.35 (C)	-	-	-
6	151.89 (C)	-	H-5	-
7	139.13 (C)	-	H-7OCH_3, H-5	-
8	145.92 (C)	-	-	-
8a	119.75 (C)	-	H-5	-
9	191.58 (C=O)	-	H-1CH_3	-
9a	129.72 (C)	-	H-1CH_3, H-2	-
1-CH_3	17.09 (CH_3)	2.56 (3H, s)	-	H-2
7-OCH_3	60.94 (OCH_3)	4.13 (3H, s)	-	-

show a characteristic UV spectrum; the substituted onychines (1-methyl-4-azafluorenone) give two absorption bands in the UV spectrum (MeOH); band I at 207 nm (2.35) and band II at 269 nm (2.48) that exhibit $\pi\rightarrow\pi^*$ of an aromatic unit together with a double bond, and $n\rightarrow\pi^*$ from a conjugated carbonyl chromosphore. In the IR spectrum, absorption bands attributable to hydroxyl (3526 cm^{-1} and 3329 cm^{-1}) and the carbonyl of an unsaturated ketone (1701 cm^{-1}) were observed. In addition, a C-O stretching vibration occurs at 1350 cm^{-1} indicating an ether moiety.

The ^{1}H NMR spectrum displayed only three unique signals: three aromatic [δ_H 6.80, d, J = 5.53 Hz; 6.87, s, and 8.16, d, J = 5.53 Hz)], one methoxy resonance (δ_H 4.13, s) and one C-methyl signal (δ_H 2.56, s). All the direct proton-carbon connectivities were assigned following HMQC analysis. Using COSY, ^{1}H-^{1}H coupling constants and HMBC correlations, a trisubstitued pyridine moiety was readily established. HMBC correlations from protons of the methyl group at δ_H 2.56 (1-Me) to three carbons of the pyridine unit [δ_C 147.41(C-1), 129.72 (C-9a), and 124.16 (C-2)] indicated its *para*-position relative to the pyridine ring system. An additional weak HMBC correlation from this methyl group to a carbon at δ_C 191.58 (C-9) suggested that the carbonyl functionality was attached *ortho* to the methyl moiety. Moreover, three carbon signals of the pyridine ring at δ_C 166.35 (C-4a), 147.41 (C-1) and 124.1(C-2) were HMBC correlated with the proton signals at δ_H 8.16 (H-3), 6.87 (H-5) and 8.16 (H-2). The one aromatic proton at δ_H 6.87 (s) exhibited HMBC correlations to three quaternary carbons [δ_C 151.89 (C-6), 139.13 (C-7), and 119.75 (C-8a)], three of which emerged to be connected to an oxygen heteroatom on account of their ^{13}C chemical shifts. The HMBC spectrum of this compound, which provided reciprocity from δ_H 4.13 (7-OMe) to the carbon at δ_C 139.13, clearly showed that the methoxy group was located on an aromatic ring. An additional strong HMBC correlation from proton δ_H 6.87 (5-H) to a carbon at δ_C 139.13 suggested that a methoxy group was located at the C-7 position. Moreover, a series of NOE experiments were carried out in order to assign the methoxy group at position 7. Enhancements were observed in the pyridine ring, but not in the phenolic ring (Figure 1). Based on the above data, the compound was identified as 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone, an onychine analogue not previously reported.

The inhibition effects of *P. cerasoides* root extracts were investigated using the MTT assay. The IC_{50} value was determined against three human lung cancer cell lines. Of the four extracts, the ethyl acetate one exhibited the highest antiproliferative activity with IC_{50} values of 2.7, 4.3 and 3.4 $\mu g.mL^{-1}$ for A549, GLC4 and GLC4/Adr cells, respectively (Table 2). Strong growth inhibitory activity was also found for the *n*-hexane and acetone extracts with IC_{50} values in the range from 3.5 to 11.4 $\mu g.mL^{-1}$; the methanol extract was only weakly active. The ethyl acetate extract was, therefore, considered for subsequently purification and the new active compound, 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone, was isolated. This compound showed inhibitory activity against the three cancer cell lines equal to or higher than that of the ethyl acetate extract. The IC_{50} values of the crude extracts and the pure compound showed similar values against both GLC4 and GLC4/Adr cells. It is proposed that neither the crude extracts nor the pure compound are recognized by ABCC1/MRP1 protein.

The new compound was also examined for its efficacy against *M. tuberculosis* using a broth microdilution method. It demonstrated very strong inhibition, with a MIC value of 0.78 μg/mL, equal to that of ofloxacin, one of four antibiotic drugs used as positive controls. This antimycobacterial activity is consistent with a previous report for compounds isolated from *P. cerasoides* roots [2c]. Three compounds isolated from seeds exhibited antiproliferative action against the CACO-2 cell line, and two phytosterols showed antimutagenic activity [2d]. It has been reported that some extracts of this plant have antimalarial [2c] and antioxidant potential [4a].

Table 2: Antiproliferative, anti-*Mycobacterium tuberculosis*, and $H_{37}Ra$ effects.

P. cerasoides (roots)	%IC_{50} (μg.mL^{-1})						MIC (μg.mL^{-1})
	A549		GLC4		GLC4/Adr		$H_{37}Ra$
	Mean	SD	Mean	SD	Mean	SD	
n-Hexane extract	11.4	0.3	4.9	4.2	3.5	2.3	-
Ethyl acetate extract	2.7	0.8	4.3	3.4	3.4	1.6	-
Acetone extract	10.7	1.3	7.4	4.7	7.3	1.8	-
Methanol extract	14.4	0.4	45.5	16.0	28.7	5.7	-
6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone	2.6	0.2	2.9	0.7	3.6	1.0	0.78

Experimental

General experimental procedures: IR, Shimadzu 8900 FTIR spectrophotometer; Melting point, Büchi 322 micromelting point apparatus; NMR (1D and 2D), Brüker DPX 400 spectrometer; ESIMS (positive mode), Finnigan LC-Q Advantage Thermoquest spectrometer equipped with Xcalibur software; HRMS, Finnigan INCOS 50 and Brüker Daltonics (micro TOF); CC, Silica gel 60 (Merck, 70-230 mesh); TLC analysis on Si gel GF_{254} precoated plates with UV detection.

Plant material: Roots of *P. cerasoides* were collected in Amnartcharoen Province, Thailand, in March 2009. A voucher specimen (BKF no. 151499) has been deposited at the Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Extraction and isolation: Air-dried powdered roots (5 kg) were successively defatted with *n*-hexane, WP0455 (18 L x 3 days x 7 times) and then sequentially extracted at room temperature with ethyl acetate, WP0456 (18 L x 3 days x 7 times), acetone, WP0457 (18 L x 3 days x 6 times), and methanol, WP0458 (18 L x 3 days x 6 times), followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to afford 14.1, 45.6, 144.0 and 359.5 g of residues, respectively. Based on the bioassay results (carried out by our collaborators from Chiang Mai University of Thailand), the ethyl acetate extract was chosen for further study. This (45.6 g) was subjected to a coarse separation by silica gel CC (400 g), eluting with various proportions of ethyl acetate–*n*-hexane, followed by an increasing amount of methanol in ethyl acetate, and finally with methanol. Fractions (1000 mL each) were collected and combined on the basis of TLC behavior. The solvents were evaporated to dryness to afford 7 fractions ($F_1 – F_7$). Fraction F_3 (2.27 g), eluted by 10% ethyl acetate-*n*-hexane, was obtained as a semisolid. Further separation was achieved by CC over silica gel (100 g), eluting with *n*-hexane, followed by an increasing amount of methanol in ethyl acetate, and finally with methanol. Fractions were collected and combined before the solvents were removed under reducing pressure to afford subfractions $A_1 – A_2$. Subfraction A_2 (0.87 g), was separated by CC over silica gel to yield a yellow solid subfraction B_2 (0.38 g). This was recrystallized from ethanol to give purified yellow needles (0.38 g) of azafluorenone.

6, 8-Dihydroxy-7-methoxy-1-methyl-azafluorenone

MP: 200.9-202.7ºC.
IR (KBr): 3329, 1701, 1350 cm^{-1}.
^{1}H and ^{13}C NMR ($CDCl_3$): Table 1.
HRAPCIMS: *m/z* 257 [M^+].

Cell lines and culture conditions: The crude extracts and the pure compound were tested on 3 cancer cell lines {non small cell lung cancer (A549), adriamycin-sensitive small cell lung cancer (GLC4), and adriamycin-resistant small cell lung cancer (GLC4/Adr) expressing ABCC1/MRP1 [4b,4c]}. All were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 37ºC incubator under 5% CO_2.

Anti-proliferation assay: The cellular viability of cell A549 was determined using the sulforhodamine B (SRB) method. The sulforhodamine B assay is used for cell density determination, based on the measurement of cellular protein content. The cellular viability of cell lines GLC4 and GLC4/Adr were determined by using the conventional MTT-colorimetric method. Cells were incubated under cell culture conditions with various concentrations of pure compound up to 62.5 μg.mL^{-1} and crude extracts up to 250 μg.mL^{-1} for 72 h; the cells without either crude extracts or pure compound served as a blank. At 72 h, 50 μL of MTT (2.5 mg.mL^{-1}) was added to each well. The culture plates were gently shaken and incubated for 4 h. MTT in solution was converted to blue formazan crystals by mitochondrial succinate dehydrogenase of the living cells. The formazan crystals formed within the cells were solubilized with 50 μL DMSO and shaken well. The obtical density (OD) of the blue formazan chromophore was determined at 550 nm in an automated plate reader [4d]. The cytotoxic parameter was expressed as the concentration of compound in which the cellular proliferation was inhibited by 50% (%IC_{50}). The percentage of cell-growth inhibition (%IC) was calculated using the formula:

$$\%\,IC = \frac{(C72 - S72)}{(C72 - C0)} \times 100$$

C0 is the OD value representing the initial cell amount of non treated cells (control), C72 is the OD value representing the cell amount of the control at 72 h and S72 is the OD value representing the cell amount of the treated cells at 72 h

***Anti*-Mycobacterium tuberculosis, *$H_{37}Ra$*:** Green fluorescent protein (GFP) expressing *Mycobacterium tuberculosis* strain H37Ra was established by Changsen *et al*. [4e]. $H_{37}Ra$ *gfp* was cultivated on 7H10 agar containing 30 μg.mL^{-1} kanamycin at 37^{o}C for either 4 weeks or until growth was observed. Starter cultures were prepared by fully looping 2-3 single colonies into 7H9 broth supplemented with 0.2% v/v glycerol, 0.1 % w/v casitone, 0.05% v/v Tween 80, 10% v/v Middlebrook OADC enrichment solution (BD Biosciences) and 30 μg.mL^{-1} kanamycin. The mixture was then incubated at 37^{o}C in a 200 rpm shaker incubator until the optical density (OD) at 550 nm was between 0.5 and 1. For batch cultivation, the starter cultures were transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37^{o}C in a 200 rpm shaker incubator until the OD550 nm was approximately 0.5 to 1. The cells were pelleted, washed and suspended in PBS buffer, and then sonicated 8 times for 15 secs each. The sonicated samples were then aliquoted and frozen at -80°C for up to 2 to 3 months prior to use. Titer stocks were determined by the colony forming unit (cfu) assay and the seeding density for anti-TB assay was optimized by serial dilutions. The dilution that grew at logarithmic phase on day 7 was used as an optimal bacterial seeding density. For assay in a 384-well format, the seeding was approximately $2x10^4$ to $1x10^5$ cfu/mL/well. The assay was performed in duplicate, each well containing 5 μL of test sample serially diluted in 5% dimethyl sulfoxide, followed by 45 μL of cell suspension prepared as described above. Plates were incubated at 37°C for 7 days and the fluorescence signals were measured using a SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom-reading mode at the excitation and emission wavelengths of 485 nm and 535 nm. Fluorescence signals on day zero were used as background, which was used to subtract the signals on day 7. The percentage growth inhibition was calculated from the mean of the fluorescence unit of cells treated with sample (FUT) and untreated cells (FUC), according to the following equation:

$$\% \text{ Inhibition} = [1- (FUT / FUC)] \times 100$$

The lowest drug concentration that inhibits cell growth by 90% is reported as the Minimum Inhibitory Concentration (MIC). Rifampicin, streptomycin, isoniazid and ofloxacin were used as positive controls, and 0.5% DMSO as a negative control.

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Evaluation of Antiviral Activities of Curcumin Derivatives against HSV-1 in Vero Cell Line

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Antiviral drug resistance is one of the most common problems in medicine, and, therefore, finding new antiviral agents, especially from natural resources, seems to be necessary. This study was designed to assay the antiviral activity of curcumin and its new derivatives like gallium-curcumin and Cu-curcumin on replication of HSV-1 in cell culture. The research was performed as an *in vitro* study in which the antiviral activity of different concentrations of three substances including curcumin, Gallium-curcumin and Cu-curcumin were tested on HSV-1. The cytotoxicity of the tested compounds was also evaluated on the Vero cell line.

The CC_{50} values for curcumin, gallium-curcumin and Cu-curcumin were 484.2 µg/mL, 255.8 µg/mL and 326.6 µg/mL, respectively, and the respective IC_{50} values 33.0 µg/mL, 13.9 µg/mL and 23.1 µg/mL. The calculated SI values were 14.6, 18.4 and 14.1, respectively. The results showed that curcumin and its new derivatives have remarkable antiviral effects on HSV-1 in cell culture.

Keywords: Herpes simplex virus type 1 (HSV-1), curcumin, gallium-curcumin, Cu-curcumin, cell culture.

Viral diseases have always been a major health problem and scientists have continually tried to find new antiviral compounds. Cold sores, one of these viral diseases, are caused by herpes simplex virus type 1 (HSV-1), which is a DNA virus of the herpesviridae family [1].

Most complications caused by HSV-1 are self-limited, but HSV-1 can establish lifelong latent infection in sensory ganglia and some factors will cause the reactivation of the virus [2]. Herpes simplex virus-1 infection is common worldwide, with 45% to 98% of the world population being infected in different populations [1,2]. Due to the high prevalence of HSV-1 infections, several antiviral drugs have been developed for the treatment of HSV-1 infections, but many of them show severe side effects and are unable to cure the infections completely. Nearly all clinically effective antiviral drugs are nucleoside analogues. However, following a long period of their use, drug resistance has emerged [3]. Therefore, finding novel anti HSV-1 agents with low side effects is necessary. It has been suggested that natural products from plants can exhibit anti HSV-1 activities. Such natural products need to be isolated and screened for their potential to act as antiviral compounds [4]. One of these natural antiviral agents is curcumin, a major antioxidant compound and a principal constituent of the spice turmeric (*Curcuma longa*) [5], a native plant from south India and Indonesia. This spice has been used for hundreds of years for flavoring and coloring of many kinds of foods and is also used as a food preservative [6,7].

Regarding the use of turmeric in Indian and Chinese traditional medicine many scientists are interested to reveal the therapeutic and biological functions of this compound. Curcumin can affect the metabolism of cells and organisms [8]. Also, it has anti-tumor, antioxidant,

Table 1: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of curcumin. Each value represents the mean of four replicate assays

Concentration (μg/mL)	CPE Inhibition (%)
12	0
18	10
24	30
30	40
36	55
42	75
48	90
54	100

antiinflammatory, antiviral and anti-infectious properties [6,9-12]. Although various antiviral effects of curcumin have been reported, more studies are necessary to develop it as an alternative antiviral drug for HSV-1 treatment or for providing a template for the synthesis of new anti HSV-1 agents [13].

In this study, we evaluated the *in vitro* anti-HSV-1 activity of curcumin and its novel derivatives gallium-curcumin and Cu-curcumin. Based on our knowledge, there has been no study of the antiviral activity of these last two compounds.

The cytotoxicities of curcumin, gallium-curcumin and Cu-curcumin on Vero cells were determined by calculation of their CC_{50} values as 484.2, 255.8 and 326.6 μg/mL, respectively. Regarding the collected data for the antiviral activity of curcumin (Table 1), gallium-curcumin (Table 2), and Cu-curcumin (Table 3) against HSV-1 in cell culture, the IC_{50} values of these compounds, calculated using STATA software, were 33.0, 13.9 and 23.1 μg/mL, respectively. As shown in Tables 1, 2 and 3, 12 μg/mL of curcumin, 4 μg/mL gallium-curcumin and 8 μg/mL of Cu-curcumin could not prevent CPE presentation, which is related to HSV-1 replication in cell culture. However, 54 μg/mL of curcumin, 30 μg/mL of gallium-curcumin and 42 μg/mL of Cu-curcumin totally prevented viral CPE presentation.

Table 2: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of gallium-curcumin. Each value represents the mean of four replicate assays.

Concentration (μg/mL)	CPE Inhibition (%)
4	0
8	30
12	55
18	75
24	90
30	100

Based on our knowledge, no research has been reported up to now on the activity of the new derivatives of curcumin, Cu-curcumin and gallium-curcumin against viruses, especially HSV-1.

Antibacterial activities of curcumin and curcuminoids have been reported [14]. Curcumin has also been shown to inhibit the growth of several types of viruses and malignant cells [15]. Curcumin can inhibit the expression of immediate early genes of HSV-1 [8], and also inhibit the activity of HIV-1 integrase, which is necessary for replication of this virus [16].

Table 3: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of Cu-curcumin. Each value represents the mean of four replicate assays.

Concentration (μg/mL)	CPE Inhibition (%)
8	0
12	10
18	40
24	60
30	75
36	90
42	100

Various derivatives related to curcumin were synthesized and tested as inhibitors of the replication cycle of some viruses [17]. Synthetic modification of antiviral agents in order to improve either their antiviral activity or pharmacological properties is of interest [18]. Therefore, two new complexes, Cu-curcumin and gallium-curcumin, were chosen for the present study.

In this study, the CC_{50} values obtained showed that the cytotoxicity of curcumin is less than those of its new derivatives gallium- and copper-curcumins. Based on the differences in the CC_{50} values of gallium-curcumin and Cu-curcumin it could be concluded that the gallium compound was more cytotoxic than the copper one to Vero cells.

In most studies, DMSO showed antiviral and cytotoxic effects *in vitro* on different cell types [19]. Thus we tested the probable DMSO cytotoxic and virucidal effects in the current study. The concentration of DMSO used in our study was less than 2%, which is the lowest concentration for an antiviral effect; therefore we ignored the effect of DMSO in our study.

Based on the data obtained, it could be concluded that curcumin and its new derivatives exhibited *in vitro* anti-HSV-1 activities. Such agents could be developed either as anti HSV-1 compounds or provide a template for the synthesis of new anti HSV-1 agents. Our results can be considered as an early step in elucidating the molecular basis of the antiviral activities of curcumin. Future research is necessary to determine the possible *in vivo* anti-HSV-1 activity of curcumin and its related derivatives. Also, the mechanism(s) of action of these compounds should be revealed in future studies.

Experimental

Cell and virus: The African green monkey kidney cell line (Vero) was used as an appropriate cell line for

HSV-1 propagation. The cells were cultured using Dulbecco minimum essential medium (Gibco) containing 10% fetal bovine serum (Gibco). The antiviral activity assay was carried out on the KOS strain of HSV-1. The virus was propagated in Vero cells and the titer of propagated viral stock was firmed as TCID 50 mL^{-1} by using Karber's method. After titration, viral stock was dispensed in sterile tubes, which were stored at -70°C until the date of use.

Preparation of curcumin and its derivatives: Curcumin was purchased from Sigma, and the curcumin derivatives were prepared as previously described [20]. Dimethyl sulfoxide (DMSO) was used as the solvent for curcumin and its derivatives.

Cytotoxicity assay: Cytotoxicity values of all compounds were determined by culturing Vero cells for 96 h in the presence of increasing amounts of each substance. Three wells for each concentration of each compound were used. Viable cells were determined by the trypan blue exclusion test. Results were plotted as a dose response curve and 50% cell growth inhibitory concentration (CC_{50}) was obtained by using STATA modeling software.

Antiviral activity assay: The cytopathic inhibiton assay was used to determine the semi-quantitative antiviral activity of each test compound. Briefly, Vero cells were grown in a 96-well cell culture microplate (2×10^3 cells/well). The cultured plates were incubated at 37°C in the presence of 5% CO_2 until the cells showed 80% confluency. Subsequently, the culture medium was removed from each well and 100 $TCID_{50}$ of virus suspension and different concentrations of curcumin and its derivatives from minimal to maximal non-cytotoxic concentrations were added to each well of the cell culture microplate. For each concentration of curcumin and its derivatives 4 wells were chosen.

For the virus control, 100 $TCID_{50}$ with the highest amount of DMSO which did not show cytotoxicity were added to 4 wells. Also, in each microplate, 4 wells were treated with DMSO without virus as a negative control for virus. In addition, 4 wells of each row were treated with the highest level of each curcumin based compound which did not previously exhibit cytotoxicity.

The plates were incubated at 37°C in a humidified CO_2 atmosphere (5% CO_2) and were investigated every day for cytopathic effect (CPE) presentation up to 5 days post infection.

The degree of inhibition was expressed as percent yield of virus control (% virus control = CPE experimental group/CPE virus control × 100). The concentration of each compound which reduced 50% of CPE presentation with respect to virus control was estimated from graphic plots defined as 50% inhibited concentration (IC_{50}) expressed in μg per mL by using STATA modeling software. The selectivity index (SI) was measured from the ratio of CC_{50}/IC_{50} [21,22].

Statistical analysis: The STATA statistical analysis package was used for curve plotting in order to calculate IC_{50} and CC_{50} values.

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Hyloglyceride and Hylodiglyceride: Two New Glyceride Derivatives from *Hylodendron gabunensis*

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Phytochemical investigation of *Hylodendron gabunensis* resulted in the isolation of two new glyceride derivatives, hyloglyceride (**1**) and hylodiglyceride (**2**). The structures of the two new compounds were determined by comprehensive analysis of their 1D and 2D NMR spectroscopic, and HREIMS data. One known compound was also isolated and identified as β-sitosterol.

Keywords: *Hylodendron gabunensis*, Fabaceae, glyceride derivatives, hyloglyceride, hylodiglyceride.

The African continent is one of the continents endowed with the richest biodiversity in the world, with an avalanche of plants used as foods, herbs and health foods, and for therapeutic purposes [1]. In the course of phytochemical studies of medicinal plants from Africa [2], we investigated *Hylodendron gabunensis* Taub. (Fabaceae). Previous studies of this species resulted in the isolation of two apiitol derivatives [3]. We now report on the structure elucidation of two new glyceride derivatives, namely, hyloglyceride (**1**) and hylodiglyceride (**2**) (Figure 1), together with the known compound β-sitosterol [4] from *H. gabunensis*. Glycerides and diglyceride derivatives have been reported from plants, endophytic fungi and marine organisms [5].

Hyloglyceride (**1**) was obtained as white powder. Its molecular formula was assigned as $C_{34}H_{68}NO_4$ on the basis of the ion at *m/z* 540.5110 $[M]^+$ in the HREIMS, and ^1H and ^{13}C NMR spectral analyses. The IR spectrum revealed absorption bands for an hydroxyl group at 3420 cm^{-1} and C=O at 1733 cm^{-1}. The ^{13}C (DEPT) and ^1H NMR spectra of **1** showed signals for a fatty acid moiety [δ 14.0 (q), 22.6 (t), 24.8 (t), 29.2 (t), 32.8 (t), 34.1 (t), 174.3 (s), δ 0.85 (3H, t, J = 6.4 Hz), 1.27 (br s, n x CH_2), 1.60 (2H, m), 2.33 (2H, t, J = 7.5 Hz)], two oxymethylene [δ_C 63.2, 65.1; δ_H 4.40 (1H, dd, J = 11.2, 3.5 Hz), 4.37 (1H, dd, J = 11.2, 5.5 Hz), 3.70 (1H, dd, J = 11.2, 3.5 Hz), 3.60 (1H, dd, J = 11.2, 3.5 Hz)], and one oxymethine [δ 70.2, δ 3.92 (1H, m)].

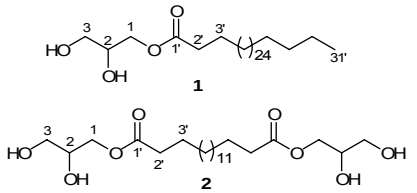

Figure 1: Structure of hyloglyceride (**1**) and hylodiglyceride (**2**) isolated from *H. gabunensis*.

These data suggested that **1** was a fatty acid-1-glyceride [5]; this was supported by the HMBC spectrum, which showed correlations of H-1 and H-3a with C-2, and H-1, H-2 with C-3, indicating the presence of an asymmetrically substituted glycerol moiety. Furthermore, correlations of H-1, H-2', and H-3' with C-1' indicated that the acyl of the fatty acid moiety was attached to C-1. According to the molecular weight obtained from the HREIMS, the fatty acid moiety should be hentriacontanoic acid. Based on the literature which reported that glyceride analogue exhibiting +ve optical rotation had an absolute configuration of 2*S*, [5a,6] the structure of **1** was elucidated as (2*S*)-1-*O*-hentriacontanoyl glycerol (Figure 1).

Hylodiglyceride (**2**) was found to have a molecular formula of $C_{23}H_{44}O_8$ from the molecular ion peak at *m/z* 448.3030 $[M]^+$ in the HREIMS. The IR spectrum revealed absorption bands for an hydroxyl group at 3420 cm^{-1} and C=O at 1730 cm^{-1}. The ^1H and ^{13}C NMR spectra of **2** were similar to those of compound **1** (see

Experimental), with differences observed in the fatty acid. A methyl group seen in **1** {δ_C 14.0, t; δ_H 0.85 (t, J = 6.4 Hz} is missing in **2** and replaced by another glycerol moiety, as observed from the ^{1}H and ^{13}C NMR spectra. The ^{1}H and ^{13}C NMR spectra showed peaks for half of the molecule. According to the HREIMS, the di fatty acid moiety should be heptadecanedioic acid. Based on these findings, hylodiglyceride was established as **2** (Figure 1).

Experimental

General experimental procedures: Optical rotation was recorded on a Perkin–Elmer 241 MC polarimeter at the sodium $_D$-line. IR spectra were obtained from a Nicolet-510P spectrophotometer; ν_{max} in cm^{-1}. EIMS and HREIMS were carried out using a MAT 8200 and Micromass LCT mass spectrometers. The ^{1}H NMR spectra were recorded on a Bruker AMX-500 instrument using TMS as an internal reference.

Plant material: The bark of *H. gabunensis* was collected at Mont Elounden 11- Yaounde, in the central region of Cameroon, in December 2007, and identified by Dr Nole Tsabang, Ministry of Scientific Research. A voucher specimen (N° 9336) has been deposited at the National Herbarium, Yaounde, Cameroon

Extraction and isolation: Dried and powdered bark (12.0 kg) of *H. gabunensis* was extracted with EtOAc at room temperature for 48 h and then filtered. The filtrate was concentrated under vacuum to give 200 g of crude residue. The ethyl acetate-fraction (200 g) was then subjected to CC (silica gel, *n*-hexane, *n*-hexane-EtOAc, EtOAc and EtOAc-MeOH, in order of increasing polarity), yielding 7 fractions (F_1 to F_7). Fraction F_7 was eluted with a mixture of CH_2Cl_2, CH_2Cl_2-MeOH (order of increasing polarity) yielding hylodiglyceride (**2**, 9.0 mg). Fraction F_5 [*n*-hexane-EtOAc (8.0:2.0)] was similarly subjected to CC, yielded hyloglyceride (**1**, 11.0 mg). Finally, fraction F_2 gave β-sitosterol (7 mg) [*n*-hexane-EtOAc (8.5:1.5)].

Hyloglyceride (**1**)

Colorless powder.
MP: 64-66°C.
$[\alpha]_D^{20}$: +6.2 (*c* 0.25, CH_2Cl_2).
IR ν_{max} (CH_2Cl_2): 3420, 2920, 2850, 1733, 1469, 1389, 1180 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): δ 0.85 (3H, t, J = 6.4 Hz, Me-31'), 1.27 (54H, br s, H-4' to H-30'), 1.60 (2H, m, H-3'), 2.33 (2H, t, J = 7.5 Hz, H-2'), 3.60 (1H, dd, J = 11.2, 3.5 Hz), 3.70 (1H, dd, J = 11.2, 3.5 Hz), 4.37 (1H, dd, J = 11.2, 5.5 Hz), 3.92 (1H, m), 4.40 (1H, dd, J = 11.2, 3.5 Hz).
^{13}C NMR (125 MHz, $CDCl_3$): δ 14.0 (CH_3, C-31'), 22.6 (CH_2, C-29'), 24.8 (CH_2, C-3'), 29.2 (CH_2, C-4' to C-28'), 32.8 (CH_2, C-30'), 34.1 (CH_2, C-2'), 63.3 (CH_2, C-3), 65.2 (CH_2, C-1), 70.2 (CH_2, C-2), 174.3 (C, C-1').
HREIMS: *m/z* 540.5110 $[M]^+$ (calcd. 540.5118 for $C_{34}H_{68}NO_4$).

Hylodiglyceride (**2**)

Colorless powder.
MP: 87°C.
$[\alpha]_D^{20}$: +10.2 (*c* 0.20, CH_2Cl_2).
IR ν_{max} (CH_2Cl_2): 3420, 2920, 2850, 1730, 1469, 1389, 1180 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (br s, H-4' to H-8'), 1.60 (2H, m, H-3'), 2.33 (2H, t, J = 7.5 Hz, H-2'), 3.60 (1H, dd, J = 11.2, 3.5 Hz), 3.70 (1H, dd, J = 11.2, 3.5 Hz), 4.37 (1H, dd, J = 11.2, 5.5 Hz), 3.92 (1H, m), 4.40 (1H, dd, J = 11.2, 3.5 Hz).
^{13}C NMR (125 MHz, $CDCl_3$): δ 24.9 (CH_2, C-3'), 29.7 (CH_2, C-4' to C-8'), 34.2 (CH_2, C-2'), 63.3 (CH_2, C-3), 65.2 (CH_2, C-1), 70.3 (CH_2, C-2), 174.3 (C, C-1').
HREIMS: *m/z* 448.3030 $[M]^+$ (calcd. 448.3036 for $C_{23}H_{44}O_8$).

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Chemical Composition and Bioactivities of the Marine Alga *Isochrysis galbana* from Taiwan

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The present study investigated the chemical composition of *Isochrysis galbana* Parke, a marine microalga which is widely used as a feedstock in aquaculture. From gas chromatography/mass spectrometric analysis the mono-sugar compositions of *I. galbana* were 2.1% fucose, 2.5% rhamnose, 2.7% arabinose, 8.5% xylose, 15.7% mannose, 32.7% galactose and 35.8% glucose. The polysaccharides of *I. galbana* were able to induce prointerleukin-1β (pro-IL-1β) protein expression within murine macrophages. Furthermore, five kinds of chlorophyll and one sterol were separated from the ethanolic extracts, including pheophorbide-a, ethyl pheophorbide-a, 10*S*-10-hydroxypheophytin-a, 10*R*-10-hydroxypheophytin-a, (13^2-*R*)-pheophytin-a, and brassicasterol. In addition, the major soluble components of the ethanol/*n*-hexane extract were 9-octadecenoic acid (*E*) (38.4%), hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), and octadecanoic acid (7.2%), but only a few polyunsaturated fatty acids were found, such as 9,12,15-octadecatrienoic acid (1.9%), 9,12-octadecadienoic acid (*Z,Z*) (3.4%), and docosahexaenoic acid (0.2%). This is the first occasion that polysaccharides from *I. galbana* have been demonstrated to exert immunomodulatory properties by the induction of IL-1 within macrophages.

Keywords: *Isochrysis galbana*, extracts, chemical compositions, polysaccharides, bioactivity, pro-IL-1β.

Marine microalgal biomasses play an important role as primary producers in the animal food chain. *Isochrysis galbana* Parke, a golden-brown flagellate marine microalga, is widely used as an aquaculture feed for young fish and in bivalve hatcheries because it is rich in polyunsaturated fatty acids (PUFA) [1]. Many studies have focused on the relationship between fatty acids and algal growth [2-4]. In addition, because of the high content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), *I. galbana* is recognized for its beneficial effects on human health, and is considered to be a good substitute for fish oil in the human diet. The purpose of the present study was to analyze the chemical composition of *I. galbana*, including *n*-hexane/ethanol extracts; ethanol extracts; total carbon, hydrogen, oxygen, nitrogen and sulfur; and total polysaccharides.

First we analyzed the ratio of carbon, hydrogen, oxygen, nitrogen, sulfur and ash in *I. galbana* cultured in the laboratory. Based on dry weight, the values obtained were: C (56.3%), H (7.5%), O (20.3%), N (6.25%), S (1.0%) and ash (8.6%). *I. galbana* also yielded a high content of *n*-hexane/ethanol (1/1) and ethanol extractive (21.7% and 38.2%, respectively).

Sixteen fatty acids were identified. These are given in Table 1, where all compounds are listed in order of their elution from the DB-5HT column. The major fatty acid was 9-octadecenoic acid (*E*) (38.4% of total), followed by hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), octadecanoic acid (7.2%), and 9-octadecenoic acid (*Z*) (3.2%). The composition of fatty acids in *I. galbana* found in this study differs from the results of

Table 1: Chemical composition of *I. galbana* ethanol/*n*-hexane extractive.

Compound ID	RT [a]	Conc. (%)	Identification [b]
Tetradecanoic acid, methyl ester	14.51	15.7	MS, KI, ST
Pentadecanoic acid, methyl ester	16.52	0.8	MS, KI, ST
11,14,17-Eicosatrienoic acid, methyl ester	17.76	0.1	MS, KI
(*Z*)-9-Hexadecenoic acid, methyl ester	18.04	2.5	MS, KI
Hexadecanoic acid, methyl ester	18.50	23.3	MS, KI, ST
Heptadecanoic acid, methyl ester	20.53	0.2	MS, KI, ST
9,12,15-Octadecatrienoic acid, methyl ester,	21.22	1.9	MS, KI, ST
9,12-Octadecadienoic acid (*Z,Z*)-, methyl ester	21.54	3.4	MS, KI
9-Octadecenoic acid (*E*)-, methyl ester	21.66	38.4	MS, KI, ST
9-Octadecenoic acid (*Z*)-, methyl ester	21.77	3.2	MS, KI,ST
Octadecanoic acid, methyl ester	22.16	7.2	MS, KI, ST
Arachidonic acid, ethyl ester	24.30	0.09	MS, KI
Heneicosanoic acid, methyl ester	27.14	0.1	MS, KI
4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-*Z*)-	27.40	0.2	MS, KI
Docosanoic acid, methyl ester	28.67	2.8	MS, KI
Total saturated		50.1	
Total monoenoic		44.1	
Total PUFA		5.8	

[a] Retention time on a DB-5 HT column with reference to *n*-alkanes.
[b] MS, NIST and Wiley library spectra, and the literature; RI, retention index; ST, authentic standard compounds.

an earlier study, which showed higher DHA (22:6n-3) (7.91%) [5].

Also, some differences were found from the study by Lin *et al.*, who determined DHA to be a major fatty acid at every growth phase [4]. It is interesting that we found a few fatty acids, such as pentadecanoic acid, heptadecanoic acid, and heneicosanoic acid, which had not been previously found [4]. This could be a result of different growth conditions.

Few existing studies have focused on the variety of pigments from *I. galbana*. Herein we have separated and identified the pigments from 1.65 g of an ethanolic extract of *I. galbana*. At least five chlorophyll compounds (Figure 1) were found including (13^2-*R*)-pheophytin-a (1.5 mg; RT = 8.03 min), pheophorbide-a (1 mg; RT = 15.82 min), 10R-10-hydroxypheophytin-a (3 mg; RT = 22.54 min), 10S-10-hydroxypheophytin-a (1 mg; RT = 23.51 min), and ethyl pheophorbide-a (4 mg; RT = 34.92 min). We also found brassicasterol (2 mg; RT = 15.10 min).

In 1981, Volkman *et al*. [6] were the first to report that 24-methyl-22-dehydrocholesterol is the dominant sterol in *I. galbana* [6]. Our experimental results were similar. Park *et al*. [7] demonstrated that autotrophically grown *I. galbana* contains three major sterols (24-oxocholesterol acetate, ergost-5-en-3-ol, and cholest-5-en-24-1, 3-(acetyloxy)-,3-ol), with 24-methylcholesta-5,22-dien- 3-ol as a minor sterol [7]. They found that the total sterol content clearly decreased during dark culture, with such decreases being particularly evident in two major sterols, 24-oxocholesterol acetate and ergost-5-en-3-ol.

Our study showed that the monosaccharide composition of a polysaccharide (IP) from *I. galbana* was 2.07%

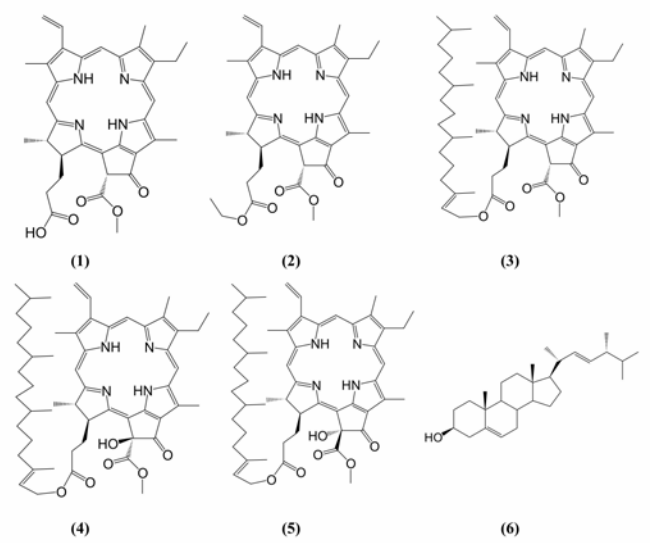

Figure 1: The chemical structure of (1) pheophorbide-a; (2) ethyl pheophorbide-a; (3) (13^2-*R*)-pheophytin-a; (4) 10*R*-10-hydroxypheophytin-a; (5) 10*S*-10-hydroxypheophytin-a; (6) brassicasterol.

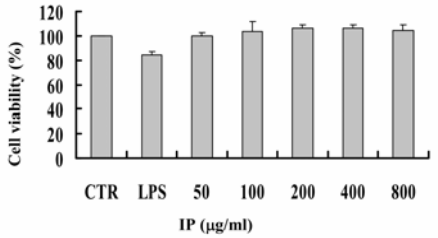

Figure 2: Effect of *Isochrysis* polysaccharide (IP) on cell viability. J774A.1 macrophages (5×10^3/well) were treated with IP or DMSO (control) for 24 h, followed by incubation with MTT reagent. Absorbance (A550-A690) was measured by spectrophotometry. Data are expressed as mean ± SE from three separate experiments.

fucose, 2.50% rhamnose, 2.72% arabinose, 8.49% xylose, 15.70% mannose, 32.73% galactose and 35.79% glucose. No cytotoxic effect was observed after J774A.1 cells were treated with various concentrations of IP for a period of 24 hours, as measured by MTT assay (Figure 2).

It is well known that IL-1β is secreted mainly from activated macrophages; this could activate other immune cells and modulate immune responses. An IP-induced IL-1β precursor, pro-IL-1β, was detected in whole cell lysates after IP stimulation by means of Western-blotting analysis. In this time course study, the expression of pro-IL-1β protein within IP-stimulated cells was detected at six hours post-stimulation. In addition, the expression of pro-IL-1β increased with increasing IP concentrations in a dose-dependent manner. Such results demonstrate that IP stimulates pro-IL-1β expression, a result that would appear to be similar to the ability of polysaccharides isolated from the algae *Rhizoclonium riparium* (Roth) Harvey and *Chlorella pyrenoidosa* Chick to stimulate pro-IL-1β expression within murine macrophages [8,9].

Fabregas *et al*. found that endocellular extracts of *I. galbana* clearly inhibited viral hemorrhagic septicemia virus (VHSV) replication at a dose of 20 μg/mL, and that *I. galbana* contained sulfated soluble exopolysaccharides [10].

Experimental

Algal culture and collection: *I. galbana* used in this study was obtained from the Tungkang Biotechnology Research Center, Fisheries Research Institute, Republic of China. Purified *I. galbana* CCMP 1324 (0.9 L, 680 nm, OD 1.10) was inoculated and cultured in Walne's medium [9]. This included nutrient, vitamin and trace metal solutions in a 10 L PET tank containing 8.1 L seawater autoclaved at 120°C for 20 min, to which was added 9.0 mL of nutrient solution and 0.9 mL of vitamin solution. The culture medium was agitated gently by bubbling air with a flow rate of 4.7 L/min and a culture time of 7 d. Continuous illumination at an irradiance of 5900 lx was provided by fluorescent lamps. The culture medium was then centrifuged (Himac CR22-GII, Hitachi, Japan) continuously at 12000 rpm at 25ºC. After lyophilization the yield of alga was 0.1 g/L (dry weight) culture medium.

Total C, H, O, N, S: Total carbon, hydrogen, oxygen, nitrogen and sulfur contents were determined by CHN elemental analysis. Freeze-dried samples (15 g each) were combusted in a 2400 CHN/O elemental analyzer (Perkin-Elmer, Waltham MA, USA) [11].

Extraction and composition of* n-*hexane/ethanol extracts of I. galbana: Ten grams of sample was extracted in a Soxhlet apparatus with *n*-hexane: ethanol (95% v/v) (50:50) for 48 h. The solution was rotary-evaporated at 65 ºC to provide a hydro-ethanolic extractive (HAE) (2.175 g). The HAE (0.5 g) was trimethylsilylated with Sylon HTP (HMDS/TMCS/ pyridine, 3:1:9) trimethylsilylation reagent (Supelco, Bellefonte PA, USA). The final derivatives were kept in *n*-hexane for gas chromatography–mass spectrometric (GC-MS) analysis. A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5HT fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness; Agilent Technologies, Santa Clara CA, USA) and a FID detector were used for quantitative determination of the components. The oven temperature was programmed as follows: 100ºC for 2 min, rising to 275ºC at 5ºC/min; injector temperature, 270ºC; carrier gas, He with a flow rate of 1 mL/min; detector temperature, 250ºC; split ratio 50.1:1. One μL sample was injected. Identification of the oil components was based on their retention indices and MS results. The GC analysis parameters listed above and the MS were obtained (full scan mode; scan time, 0.3 s; mass range, MHz 30–500) in the electron impact (EI) mode at 70 eV.

Extraction, purification and identification of ethanolic extracts of I. galbana: Dry alga (5 g) was treated with ethanol (95% v/v for 10 d, repeated 3 times) at room temperature. Then the extract was concentrated to provide the ethanolic extract (AE). AE (1.65 g) was applied to a silica gel column (Si 60) and eluted with acetone/*n*-hexane to give 43 sub-fractions. Each eluted fraction was 150 mL. The chlorophyll compounds were purified by preparative HPLC (KNAUER RI detector 2400, pump 100; KNAUER, Germany) on a Merck (Germany) Hibar Fertigsaule RT column Si 60 (25 cm length, 1 cm i.d., 5.0 μm). The separation conditions were as follows: flow rate 4 mL/min; mobile phase, acetone/*n*-hexane = 1/9. The sterol was separated by a Phenomenex Luna silica (2) column (25 cm length, 1 cm i.d., 5.0 μm) under the following conditions: flow rate 4 mL/min; mobile phase, acetone/*n*-hexane = 1/15. The structures of the compounds were confirmed by comparison of physical and spectral data (including optical rotation, EIMS, ^{1}H NMR) with previously reported values.

Extraction of polysaccharides from I. galbana: Five grams dry alga was extracted with *n*-hexane/ethanol. The extractive was ground into a fine powder, and then suspended in 100 mL distilled water. After autoclaving at 121°C for 30 min, the extract was filtered through a 0.2 μm membrane. The extract was then vacuum-concentrated at 50°C, giving a final volume of 30 mL to which 5 volumes of 95% ethanol was added slowly at 4°C. Then the mixture was centrifuged to produce a precipitate of ca. 1.910 g, dry wt. Sixty mg of the precipitate was treated further with 3 mg proteinase K for removal of the peptide part, and dialyzed against H_2O (Spectra/Por® membrane, molecular weight cutoff 1,000 Da), resulting in 17.5 mg polysaccharide [*I. galbana* (IP)].

Sugar composition analysis: Sugar composition was determined by GC-MS. The polysaccharide content of *I. galbana* was determined by methanolysis with 0.5 M methanolic HCl at 80°C for 16 h, and trimethylsilylation with Sylon HTP. The final trimethylsilylated (TMS) derivatives were kept in *n*-hexane for GC-MS analysis [12]. Carbohydrate analysis was done with inositol as the internal standard; integrated peak area was used to establish the relative amounts of the constituents. Compounds were identified by comparing of their mass spectrometric fragmentation patterns with those of authentic standards, and the quantity of compounds was obtained by integrating the peak area of the spectra.

Microculture tetrazolium (MTT) assay for cell viability: J774A.1 macrophages were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were incubated with IP for 24 h. Cell viability was determined using colorimetric MTT assays.

Cell cultures: Murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC) (Rockville MD, USA). All cells were propagated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Life Technologies, Carlsbad CA, USA), and cultured in a 37°C, 5% CO_2 incubator [8,9].

Western blotting: Whole cell lysates were separated by 12% SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was then incubated with anti-IL-1β antibody at room temperature for 2 h. After washing 3 times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system (DuPont NEN® Research Products, Boston MA, USA) according to the manufacturer's instructions [8,9].

Statistical analysis: All values are given as mean ± SE. Data analysis involved one-way ANOVA with subsequent Scheffé test.

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An Efficient Protocol for High-frequency Direct Multiple Shoot Regeneration from Internodes of Peppermint (*Mentha* x *piperita*)

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A simple, repeatable and efficient protocol for direct multiple shoot regeneration from internodal explants has been defined in peppermint (*Mentha* x *piperita* var. Indus). *In vitro* regenerated shoots of peppermint were excised into 4 to 8 mm long internodes and cultured on Murashige and Skoog's medium supplemented with different cytokinins. In the hormonal assay, 3.0 mg L^{-1} zeatin or 6-isopentenyl adenine independently supplemented to half strength MS medium exhibited multiple shoot regeneration, while thiaduzorn (0.1-3.0 mg L^{-1}) showed no morphogenetic effect. A maximum of 85% *in vitro* cultured explants showed multiple shoot formation with an average of 7 shoots per explant on MS medium supplemented with zeatin. Multiple shoots were initiated within three weeks of cultivation. Internodes with regenerated multiple shoots were transferred to half - strength MS medium without supplementing with any plant growth hormone for shoot elongation and rhizogenesis. Rooted plants acclimatized and grew to maturity under glasshouse conditions. The plantlets developed were phenotypically identical to the parent plant and exhibited 96 % survival.

Keywords: *Mentha* x *piperita*, plant growth hormones, internodes, multiple shoot regeneration, cytokinins.

Peppermint (*Mentha piperita* L.), an allopolyploid (2n=72) and a natural hybrid of *M. aquatica* x *M. spicata* is cultivated in India and subtropical regions. Its essential oil is valued commercially as an additive to food products, cosmetics and pharmaceuticals [1]. *M. piperita* var. Indus [2] produces high menthofuran (27.2%) and pulegone (15.4%) levels in its essential oil. In the present investigation, we report a simple and efficient method of direct multiple shoot regeneration and formation of complete plantlets from internodal segments of *M. piperita* var. Indus without the production of a callus phase.

Effect of cytokinins on multiple shoot induction: Multiple shoot induction in internodal segments was observed within 3 weeks on half strength MS medium, while a comparatively low regeneration response was observed in full strength MS medium. Of the three cytokinins (Z, 2-iP and TDZ) tested (0.1-3.0 mg L^{-1}), Z at higher levels (>1.0 mg L^{-1}) and 2-iP at all levels (0.1-3.0 mg L^{-1}) tested exhibited the highest (85%) multiple shoot regeneration response (Table 1) and an average of 7 shoots/explant were obtained with Z (3.0 mg L^{-1}), while a comparatively low response (55%) was observed with 2-iP (3.0 mg L^{-1}). None of the explants showed regeneration in medium supplemented with

Table 1: Effect of cytokinins on morphogenetic response in *M. piperita*.

Cytokinin (mgl^{-1})	Morphogenetic response (%)		
	Z	TDZ	2-iP
0.0	–	–	–
0.1	–	–	R (25)
0.5	–	R (35)	R (50)
1.0	MS (25)	–	R (40)
2.0	MS (35)	–	MS (35)
3.0	MS (85)	–	MS (50)

MS - multiple shoots; R - rooting; – no response ; figure in parentheses denotes percentage of cultures showing morphogenetic response

TDZ at any of the levels tested. This is contrary to the earlier reports where regeneration of plantlets in the presence of TDZ has been reported [3-5]. Coconut water (25%), along with either TDZ or BA or 2-iP were evaluated for their effect on organogenesis. Amongst these cytokinins, TDZ was found to be the most effective for inducing shoot formation in peppermint (*Mentha* x *piperita*) leaf explants [4]. These results are in contrast to our present study in which none of the concentrations of TDZ tested induced shoot regeneration. This indicated that the TDZ levels tested alone may not be able to induce multiple shoot induction. Occasionally TDZ and 2-iP at certain low levels also showed root initiation in cultured explants, but shoot regeneration was not observed along with the rooting (Table 1). Leaf disks and petioles of *M. citrata*

Ehrh., *M. piperita* cv. Black mitcham, *M. spicata* L. and *M. gracilis* Sole ex Baker also exhibited high multiple shoot regeneration responses with 2iP [6], as has also been observed in the present study. Coconut water in the MS medium supplemented with 4.5 µM TDZ has been found to affect adventitious shoot formation in callus of *Mentha x gracilis* [3]. Excision of the growing shoots at each sub-culture passage further enhanced the elongation of dormant buds formed during the initial shoot formation.

Shoot elongation and root induction: The half strength MS medium was found to be more effective for rapid and healthy growth of explants. Shoot elongation and rhizogenesis were much more rapid and significant in half strength MS medium in contrast to MS full strength. Although Z and 2-iP stimulated multiple shoot formation, they slowed down the development and elongation of shoots. Simultaneous shoot elongation and rooting was observed in nodal explants and therefore, single shoots separated from multiple shoot clumps were sub-cultured in growth hormone free half strength MS medium. The observed effects of shoot elongation and root initiation on medium devoid of hormones support the reported work [7]. About 90-95% of the single shoots elongated and formed roots within three weeks.

Recovery of plantlets: Of the plantlets transferred to glasshouse conditions, 96% showed survival and grew to maturity. No phenotypic variation was observed among the control (sucker grown parent plants) and *in vitro* raised plants. This study thus demonstrated a simple and efficient protocol for direct multiple shoot regeneration and production of true-to-type plants that could be adapted for study of transgenic *M. piperita*.

Experimental

Establishment of aseptic cultures: Nodal segments were collected from healthy young plants from the National Gene Bank at CIMAP, Lucknow. These were surface sterilized and inoculated on half strength MS medium [8] under reported culture conditions [9].

Shoot induction and multiplication: Internodal segments (4 to 8 mm in size) from pre-cultured mother stocks were placed on half strength MS medium supplemented either with zeatin (Z), thidiazuron (TDZ) or N6-2(2-isopentyl) adenine (2-iP) at 0.1, 0.5, 1.0, 2.0 and 3.0 mg L^{-1} levels. Full and half strength MS basal media were tested to define optimal concentration of cytokinins for multiple shoot induction. Ten replicates of each level of all the 3 cytokinins were used and the experiment was repeated twice. Multiple shoots were separated and sub-cultured on fresh cytokinin free half strength MS medium for further shoot elongation and rooting.

Acclimatization of plantlets: The rooted shoots measuring 4-6 cm in length with 5–7 leaves were taken from the culture vessel, washed gently under running tap water and kept in a culture tube containing water for 5-7 days. After this hardening phase, plants were transferred to plastic trays containing soil and vermicompost mixture (2:1) and acclimatized in a glass house under normal day length conditions.

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Essential Oil Yield and Chemical Composition Changes During Leaf Ontogeny of Palmarosa (*Cymbopogon martinii* var. *motia*)

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Changes in leaf biomass yield, essential oil yield, and chemical composition were investigated during leaf ontogeny of palmarosa {*Cymbopogon martinii* (Roxb.) Wats. var. *motia* Burk., family Poaceae}. Eleven leaves representing different developmental stages, serially numbered from the apex to the base of the plant were utilized for the study. Leaf biomass yield increased up to the eighth leaf. Essential oil recovery increased up to the third leaf; thereafter it decreased. Minimum essential oil recovery was observed in the eleventh leaf. Essential oil yield/leaf increased up to the sixth leaf. Essential oil yield and concentrations of linalool, α-terpineol, geranyl isobutyrate and geraniol were relatively higher in the essential oils of mature, older leaves. Essential oil recovery, and percentages of myrcene, β-caryophyllene, geranyl acetate, (*E*,*Z*) farnesol and geranyl hexanoate were higher in the essential oils of young, expanding leaves.

Keywords: palmarosa, *Cymbopogon martinii* var. *motia*, leaf ontogeny, leaf biomass yield, essential oil yield, essential oil composition, geraniol.

Palmarosa {*Cymbopogon martinii* (Roxb.) Wats. var. *motia* Burk., family Poaceae} essential oil finds extensive use in perfumery (soaps, cosmetics, rose-like perfumes), flavoring (tobacco, food products, non-alcoholic beverages), aromatherapy, and in medicine (stiff joints, lumbago, skin diseases) [1]. The major constituents of the essential oil are linalool, geranyl acetate and geraniol. Leaves and inflorescences are the chief sources of essential oil. Leaf sheath [1], seeds [2], and stalks [3] also possess essential oil. In the aromatic crops lemongrass (*Cymbopogon flexuosus*) [4], citronella (*Cymbopogon winterianus*) [5], rose-scented geranium (*Pelargonium* species) [6], sage (*Salvia officinalis*) [7], clove (*Eugenia caryophyllata*) [8], and peppermint (*Mentha* x *piperita*) [9] changes in essential oil yield and composition were reported during leaf ontogeny. In the present study, we describe in detail, the changes in leaf growth, biomass yield, essential oil recovery, essential oil yield, and essential oil composition during palmarosa leaf development.

Leaf area increased significantly up to the sixth leaf due to increases in leaf length and width (Table 1). Earlier formed, older leaves reached smaller sizes than the latter formed young leaves; a similar pattern was observed in rose-scented geranium [6]. Leaf biomass yield increased significantly up to the eighth leaf due to increase in leaf size. There was a significant decrease in biomass yield of the eleventh leaf in comparison with the ninth leaf, due to its smaller size and loss of moisture. In spite of the smaller leaf sizes, the biomass yields of the ninth and tenth leaves were at par with that of the eighth leaf. Decrease in biomass yield with advancement of leaf age was reported in lemongrass [4], and citronella [5].

The essential oil recovery increased significantly up to the third leaf, declined up to the seventh leaf, but with no further significant decrease up to the tenth leaf. The eleventh leaf recorded the lowest essential oil recovery. Young, expanding leaves exhibited higher recovery due to high biosynthetic activity [5,9,10], rapid formation of oil glands, and accumulation of essential oil in them [9,10]. The relationship between oil gland density, and leaf oil recovery was demonstrated in rose-scented geranium [6], and mints [11,12]. Crop responses to the relationship between leaf age, and essential oil recovery varied. In lemongrass, and citronella, the essential oil recovery increased in young, expanding leaves, and decreased with leaf age [4,5]. In rose-scented geranium,

Table 1: Length, width, area, yield, essential oil recovery, and essential oil yield of palmarosa leaves.

Leaf number	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf yield (mg/leaf)	Oil recovery (mL/100 g)	Oil yield (µL/leaf)
1	8.92	0.34	3.03	29.0	1.26	0.36
2	12.75	0.59	7.52	54.0	1.44	0.78
3	16.96	0.90	15.26	88.8	1.64	1.46
4	22.41	1.16	25.99	172.2	1.47	2.53
5	28.96	1.31	37.94	267.3	1.35	3.61
6	34.50	1.24	42.78	376.8	1.28	4.82
7	34.99	1.21	42.34	450.7	1.10	4.96
8	34.69	1.04	36.08	513.0	0.99	5.08
9	33.87	0.85	28.79	501.3	0.98	4.91
10	29.65	0.74	21.94	493.8	0.91	4.49
11	21.32	0.55	11.73	468.5	0.82	3.84
LSD[a] (*P*=0.05)	2.85	0.09	4.25	26.0	0.17	0.53

[a]LSD = least significant difference

Table 2: Percentage composition of essential oils of palmarosa leaves.

Leaf number	Myrcene	Linalool	β-Caryophyllene	α-Terpineol	Geranyl acetate	Geranyl isobutyrate	Geraniol	(*E*,*Z*) Farnesol	Geranyl hexanoate
1	0.18	1.04	0.60	0.26	3.76[a]	0.09	85.24[b]	2.75[a]	2.38[a]
2	0.12	0.99[b]	0.75	0.31	3.32	0.10	86.31	2.73	1.75
3	0.12	1.34	0.78[a]	0.20[b]	2.48	0.09	88.22	2.55	1.54
4	0.14	1.30	0.56	0.24	1.52	0.08[b]	90.05	2.42	1.52
5	0.14	1.72	0.48	0.27	1.21	0.17	91.30	2.33	1.35
6	0.32[a]	1.96[a]	0.43	0.35	0.90	0.16	91.44	2.30	1.33
7	0.20	1.86	0.53	0.45	0.59	0.16	92.18	2.23	1.26
8	0.13	1.64	0.53	0.56[a]	0.42	0.18	92.09	2.24	1.24
9	0.11[b]	1.67	0.51	0.38	0.40	0.16	92.60	2.21	1.18
10	0.12	1.52	0.36[b]	0.27	0.39	0.19[a]	93.25[a]	2.15	1.08
11	0.13	1.64	0.38	0.42	0.33[b]	0.18	92.03	2.14[b]	1.07[b]
LSD[c] (*P*=0.05)	0.02	0.20	0.16	0.17	0.21	0.03	0.81	0.06	0.11

[a]Highest concentrations recorded in the leaves of different ages; [b]Lowest percentages observed in the leaves of different ages; [c]LSD = least significant difference.

the essential oil recovery decreased with leaf age [6], whereas in peppermint, after an initial increase in the young leaves, it remained constant with increasing leaf age [9].

Nine constituents of the essential oils, accounting for 96.3-99.5%, were identified and are listed in Table 2. All the leaves produced essential oils of good quality with high levels of geraniol, the principal component of palmarosa essential oil, for which it is internationally traded. The leaves were, however, distinct in possessing the maximum (first, third, sixth, eighth, and tenth leaves), and the minimum (first, second, third, fourth, ninth, tenth, and eleventh leaves) concentrations of individual compounds. The percentages of myrcene, β-caryophyllene, geranyl acetate, (*E*,*Z*) farnesol, and geranyl hexanoate were comparatively higher in young leaves, and declined with leaf age. Mature, older leaves had relatively greater amounts of linalool, α-terpineol, geranyl isobutyrate, and geraniol. The accumulation of specific essential oil constituents in leaves of different ages is a function of relative abundance and activity of the enzymes responsible for their synthesis [10,13-15]. The relationship between levels and activities of the enzymes and accumulation of specific constituents in leaves of different ages has been demonstrated in lemongrass [13]. Essential oil composition variation in leaves of different ages was reported in lemongrass [4], citronella [5], rose-scented geranium [6], sage [7], clove [8], and mints [9,11,16]. To the best of our knowledge, this is the first report on chemical profile changes in palmarosa leaf essential oils during leaf ontogeny.

Experimental

Leaf sample collection: Palmarosa cultivar Trishna was cultivated in the research farm of the Central Institute of Medicinal and Aromatic Plants Research Center, Hyderabad, India, following standard cultivation practices. The rainy season encourages luxuriant growth and produces high biomass and essential oil yields of palmarosa [17]. The present study was conducted during the rainy season when the crop was ready for harvest. Uniformly growing, healthy palmarosa plants were selected at random. Leaves were numbered serially (from one to eleven) from the apex proceeding downwards to the base of the plant. The first leaf on the inflorescence was the youngest, and the eleventh leaf, the oldest. Two hundred leaves of each developmental stage were collected in 3 replicates (11 leaves X 200 each X 3 replications = 6600 leaves in total). The fresh weights were recorded separately for all the leaves and presented as biomass yield. A separate set of 100 leaves each was collected for leaf area estimation. Leaf lengths and leaf widths were measured. Leaf areas (length X width) were computed.

Essential oil isolation: Leaf samples, in triplicate (11 leaves X 3 replications = 33 samples) were distilled in a Clevenger-type glass apparatus for 3 h. The essential oil samples were dried over anhydrous sodium sulfate and stored in a refrigerator in sealed vials until analysis. Essential oil recovery (mL/100 g biomass) and yield (biomass yield X essential oil recovery) were calculated.

GC analysis: GC analyses were carried out using a Varian Star 3400CX GC fitted with a flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a Supelcowax-10 capillary column (30 m X 0.25 mm X 0.25 μm film thickness) coated with carbowax 20 M (polyethylene glycol). Nitrogen was the carrier gas at 1 mL/min flow rate. The column temperature program was: 80ºC (2 min) to 220ºC (5 min) at 7ºC/min ramp rate. The injector and the detector temperatures were 200ºC and 240ºC, respectively. Samples (0.1 μL) were injected with a 1:50 split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C_8-C_{23}). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction.

GC/MS analysis: GC-MS analyses were performed on a Hewlett–Packard 5890 GC coupled to a HP-5970 mass selective detector (MSD) and quadrupole EI mass analyzer. A HP-1 column (coated with methyl silicone) (25 m X 0.25 mm X 0.25 μm film thickness) was used as the stationary phase. Helium was the carrier gas at 1 mL/min flow rate. Temperature was programmed from 60^o to 220ºC at 5ºC/min ramp rate. The injector and the GC-MS interface temperatures were maintained at 250ºC and 280ºC, respectively. Mass spectra were recorded over 40–400 amu range at one span/s with 70 eV ionization energy and EI mode of ionization. The ion source and the detector temperatures were maintained at 250ºC and 150ºC, respectively. The samples (0.1 μL) were injected with a 1:50 split ratio.

Identification of constituents: Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds run under identical conditions, by comparison of retention indices with published literature [18,19], and by comparison of MS with those reported in the literature [20,21], and stored in NIST and Wiley libraries.

Statistical analysis: The data were statistically analyzed employing analysis of variance technique [22]. The significance of differences between treatment variance and error variance was tested with variance (F) ratio. Least significant difference (LSD) values at 5% probability level (P=0.05) were calculated by multiplying standard error of difference (SEd) values with tabulated t values.

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Essential Oil Composition of Four Endemic *Ferulago* Species Growing in Turkey

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The essential oils from aerial parts of *Ferulago pachyloba* (Fenzl) Boiss., *F. platycarpa* Boiss. & Bal., *F. isaurica* Peşmen, and *F. longistylis* Boiss. (Apiaceae) were obtained by hydrodistillation and analyzed by GC and GC-MS. The highest oil yield (1.50%) was obtained from *F. pachyloba* followed by *F. longistylis* (0.16%), *F. isaurica* (0.08%) and *F. platycarpa* (0.07%). Fifty-three compounds were identified in the oil of *F. pachyloba* with (*Z*)-β-ocimene (25.7%) and α-pinene (9.8%) as main constituents; sixty-seven in the oil of *F. platycarpa* with 2,3,6-trimethylbenzaldehyde (29.8%) and *cis*-chrysanthenyl acetate (24.2%) as main components; seventy-eight in the oil of *F. isaurica* with nonacosane (25.5%) and hexadecanoic acid (14.8%) as main constituents; and fifty-nine in the oil of *F. longistylis* with 2,3,6-trimethylbenzaldehyde (32.7%) and bornyl acetate (12.6%) as main components. Quantitative and qualitative differences in the oil compositions of these four species were observed.

Keywords: *Ferulago pachyloba*, *F. platycarpa*, *F. isaurica*, *F. longistylis*, Apiaceae, essential oil composition, GC analysis, GC-MS analysis.

Ferulago W. Koch. is a perennial genus of the Apiaceae family represented by nearly forty species, thirty-two of which exist in the flora of Turkey, seventeen being endemic. This suggests that the gene centre for this genus is Anatolia [1-7]. The species of this genus are known as kuzukemirdi, kuzukişnişi, kuzubaşı, çakşırotu, cağşır, asaotu and kişniş in different regions of Turkey.

Ferulago species have been used since antiquity for the treatment of intestinal worms, hemorrhoids and as a sedative, tonic and digestive. Moreover, they are used against ulcers, snake bites, spleen diseases and headache [8]. It has also been reported that gums obtained from the incision of the roots of some species are used as seasoning and as a carminative [9]. However, the plants are mainly known as aphrodisiacs and as a preferred fodder to increase animal productivity [10].

Ferulago species have been reported to contain flavonoids, quinones, coumarin esters, sesquiterpenes, coumarins, furanocoumarins and aromatic compounds [11-17]. The genus is rich in essential oil and several species have been studied; *F. angulata* (Schlecht.) Boiss., *F. asparagifolia* Boiss., *F. galbanifera* (Mill.) W. D. J. Koch, *F. humilis* Boiss., *F. sandrasica* Pesmen et Quézel, *F. aucheri* Boiss., *F. confusa* Velen, *F. idaea* Özhatay et E. Akalın, *F. macrosciadia* Boiss. et Bal., *F. mughlae* Pesmen, *F. silaifolia* (Boiss.) Boiss, *F. bernardii* L. Tomkovich & M. Pimenov. *F. carduchorum* Boiss. et Hausskn., *F. contracta* Boiss. et Hausskn, *F. isaurica* Pesmen, *F. syriaca* Boiss., *F. nodosa* (L.) Boiss., *F. thyrsiflora* (Sm.) W. D. J. Koch, *F. phialocarpa* Rech. f. et Riedl, *F. sylvatica* (Besser) Reichb., *F. thirkeana* (Boiss.) Boiss., *F. trachycarpa* Boiss., *F. longistylis* Boiss. The major constituents of the oils of these foregoing species were β-ocimene, α-pinene, α- and β-phellandrene, limonene, myrcene and *p*-cymene [18]. The aim of this paper is to present and compare the chemical composition of the essential oils of four endemic *Ferulago* species; *F. pachyloba*, *F. platycarpa*, *F. isaurica* and *F. longistylis* growing in Turkey. Gas chromatographic (GC) and gas chromatographic-mass spectroscopic (GC-MS) analysis helped us to establish the composition and the relationship of the essential oil constituents. To the best of our knowledge, this is the first report on the chemical analysis of *F. pachyloba* and *F. platycarpa.* The identified constituents are presented in Table 2.

Table 1: Collection data for the investigated *Ferulago* species.

Code	*Ferulago* species	Specimen Herbarium Number	Collection place, and altitude	Collection date	Oil Yield[¥] (%)
A	*F. pachyloba*	GAZI[Φ]	Niğde, Aladağlar, around Demirkazık, rocky slopes, 2600 m	August 2007	1.5
B	*F. platycarpa*	AEF* 23173	Nevşehir, Üçhisar, Gemil Mount, northern slopes, 1450 m	July 2004	0.07
C	*F. isaurica*	AEF 22957	Alanya – Antalya road, 6 km after Derince turn, rocky slopes, 1110 m	September 2002	0.08
D	*F. longistylis*	AEF 23795	Erzincan, Sakaltutan Pass, roadsides, 2000 m	July 2006	0.16

[Φ] Herbarium of Gazi University Faculty of Arts and Sciences
* Herbarium of Ankara University Faculty of Pharmacy
[¥] Yields are given on moisture free basis

A total of fifty-three compounds representing 96.6% of the oil were identified in the essential oil of *F. pachyloba*. (*Z*)-β-ocimene, α-pinene, sabinene and δ-cadinene were the major components, amounting to 25.7%, 9.8%, 6.3% and 5.6%, respectively. The analysis of *F. platycarpa* resulted in the identification of sixty-seven volatile compounds representing 94.1% of the oil. 2,3,6-Trimethylbenzaldehyde at 29.8% was the most abundant compound in the volatile oil, followed by *cis*-chrysanthenyl acetate (24.2%), nonacosane (7.7%) and α-pinene (4.2%). Seventy-eight compounds were characterized in the oil of *F. isaurica* representing 86.3% of the oil. The major constituents were nonacosane (25.5%), hexadecanoic acid (14.8%), bornyl acetate (5.3%) and terpinen-4-ol (4.6%). Fifty-nine compounds, representing 92.5% of the oil, were identified in the oil of *F. longistylis* with 2,3,6-trimethylbenzaldehyde (32.7%), bornyl acetate (12.6%), *p*-cymene (11.9%) and *cis*-chrysanthenyl acetate (4.2%), as main components.

Considering the different groups of compounds, monoterpene hydrocarbons contributed most to the oils obtained from *F. pachyloba* (57.7%) and *F. longistylis* (22.4%), whereas oxygenated monoterpenes formed the main portion of the oils of *F. platycarpa* (33.1%) and *F. isaurica* (18.0%). Comparison of the main constituents of these four species (Table 2) shows that each species has a different set of dominant compounds. However, previous studies of the oils of *Ferulago* species [18] revealed that the three compounds detected in high percentages in this study, namely (*Z*)-β-ocimene, α-pinene and *p*-cymene have also been detected as major components in many other species. Erdurak *et al*. reported the essential oil contents of the fruit and root of *F. isaurica* [19] in 2006. The oils obtained from the different parts of this species did not show much qualitative resemblance. The major constituents detected, namely α-pinene, limonene, and myrcene in the fruit, and terpinolene and myrcene in the root were not identified in the oil from the aerial parts of *F. isaurica*. Analysis of the fruit oil of *F. longistylis* conducted in 2008 showed 2,3,6-trimethylbenzaldehyde and bornyl acetate as the major compounds, in accordance with the results obtained for the aerial parts of the same species in this study [18].

Chemical profiling of the volatiles may be useful in taxonomical classification. The results presented in this study confirm some specific features of the oil composition of *Ferulago* species in Turkey and contribute to a better knowledge of this genus.

Experimental

Plant materials: Aerial parts of 4 *Ferulago* species were collected by the authors from their natural habitats in different localities of Turkey by random sampling from a single established population, as shown in Table 1; the plants were identified by Prof. Dr. Hayri Duman and voucher specimens were deposited in GAZI and in AEF.

Essential oil isolation: Air dried aerial parts of plants (50 g) were subjected hydrodistillation for 3 h using a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia [20]. The obtained oils were dried over anhydrous sodium sulfate and stored in sealed vials at +4°C in the dark until analyzed and tested. All oils were pleasant smelling, transparent with a faint yellow color.

GC/MS analysis: GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. The mass range was from *m/z* 35 to 450.

GC analysis: The GC analysis was carried out using an Agilent 6890N GC system. The FID detector temperature was 300°C. To obtain the same elution order with GC/MS, simultaneous autoinjection was used on a duplicate column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Table 2: Composition of the essential oils of A: *Ferulago pachyloba*, B: *F. platycarpa*, C: *F. isaurica*, and D: *F. longistylis*.

RRI	Compound	A (%)	B (%)	C (%)	D (%)
1032	α-Pinene	9.8	4.2	-	3.5
1035	α-Thujene	0.3	0.1	-	0.1
1076	Camphene	0.8	0.2	-	0.2
1118	β-Pinene	0.6	0.3	-	0.2
1132	Sabinene	6.3	1.6	-	0.4
1159	δ-3-Carene	3.0	-	-	-
1174	Myrcene	1.9	1.7	-	1.4
1176	α-Phellandrene	0.5	-	-	-
1195	Dehydro-1,8-cineole	0.1	-	tr	-
1203	Limonene	2.2	0.6	0.9	0.5
1218	β-Phellandrene	0.2	0.1	-	0.1
1246	(*Z*)-β-Ocimene	25.7	0.2	-	2.3
1255	γ-Terpinene	3.1	0.3	0.3	1.4
1266	(*E*)-β-Ocimene	1.9	0.4	-	0.4
1280	*p*-Cymene	1.2	1.5	0.2	11.9
1286	Isoterpinolene	0.2	-	-	-
1290	Terpinolene	1.2	-	0.2	-
1294	1,2,4-Trimethyl benzene	-	0.8	-	1.8
1355	1,2,3-Trimethyl benzene	-	0.3	-	0.7
1382	*cis*-Alloocimene	0.2	-	-	-
1429	Perillen	-	0.1	-	-
1439	γ-Campholene aldehyde	-	0.1	-	0.1
1441	(*E*)-2-Octenal	-	0.1	-	-
1446	2,6-Dimethyl-1,3(*E*),5(*Z*),7-octatetraene	0.1	-	-	-
1452	α,*p*-Dimethylstyrene	-	0.1	0.2	0.2
1476	(*Z*)-β-Ocimene epoxide	-	tr	-	0.1
1479	δ–Elemene	-	0.1	-	-
1492	Cyclosativene	-	0.1	tr	-
1497	α-Copaene	0.2	0.2	0.3	tr
1499	α-Campholene aldehyde	-	0.6	0.1	0.7
1528	α-Bourbonene	-	-	0.1	-
1535	β-Bourbonene	0.1	0.1	0.5	0.2
1553	Linalool	-		0.7	-
1562	Octanol	-	0.1	tr	-
1571	*trans*-*p*-Menth-2-en-1-ol	-	0.1	0.3	tr
1582	*cis*-Chrysanthenyl acetate	-	24.2	-	4.2
1586	Pinocarvone	0.1	-	-	-
1589	β-Ylangene	-	-	0.1	-
1591	Bornyl acetate	0.9	2.1	5.3	12.6
1597	β-Copaene	tr	-	0.1	-
1600	β–Elemene	0.6	0.2	1.2	-
1611	Terpinen-4-ol	-	1.3	4.6	0.4
1612	β-Caryophyllene	4.4	-	-	0.3
1639	*trans*-*p*-Mentha-2,8-dien-1-ol	-	-	0.3	-
1639	Cadina-3,5-diene	0.1	-	-	-
1645	*cis*-Verbenyl acetate	-	-	-	0.3
1648	Myrtenal	-	0.3	0.1	0.2
1650	γ–Elemene	-	-	-	-
1661	*trans*-Pinocarvyl acetate	0.3	0.9	-	1.0
1670	*trans*-Pinocarveol	-	0.7	0.4	0.4
1678	*cis*-*p*-Mentha-2,8-dien-1-ol	-	-	0.1	-
1683	*trans*-Verbenol	-	0.7	0.2	-
1687	α-Humulene	0.6	-	0.2	-
1700	*p*-Mentha-1,8-dien-4-ol (=Limonen-4-ol)	-	-	0.8	-
1704	Myrtenyl acetate	0.1	-	-	-
1704	γ-Muurolene	0.2	-	-	-
1706	α-Terpineol	-	-	0.5	-
1719	Borneol	-	0.1	0.3	0.3
1725	Verbenone	-	0.1	tr	0.1
1726	Germacrene D	1.9	-	1.7	-
1738	*p*-Mentha-1,5-dien-8-ol	-	-	-	0.7
1740	α–Muurolene	0.8	-	-	-
1741	β–Bisabolene	-	-	-	-
1742	β–Selinene	-	0.3	-	-
1744	α-Selinene	-	tr	0.2	-
1751	Carvone	-	0.1	0.7	-
1755	Bicyclogermacrene	1.2	-	-	-
1758	*cis*-Piperitol	-	tr	0.2	-
1763	Naphthalene	-	-	0.2	0.3
1764	*cis*-Chrysanthenol	-	0.7	-	-
1773	δ-Cadinene	5.6	-	0.3	-
1776	γ-Cadinene	1.2	-	0.2	-
1785	7-*epi*-α-Selinene	0.2	-	-	-
1786	*ar*-Curcumene	-	0.1	-	0.4
1797	*p*-Methyl acetophenone	-	-	0.1	-
1799	Cadina-1,4-diene (=Cubenene)	0.1	-	-	-
1804	Myrtenol	-	0.2	0.1	-
1811	*trans*-*p*-Mentha-1(7),8-dien-2-ol	-	-	0.3	-
1827	(*E,E*)-2,4-Decadienal	-	0.1	0.1	0.1
1838	(*E*)-β-Damascenone	-	-	0.1	-
1845	*trans*-Carveol	-	0.4	0.9	0.3
1857	Geraniol	-	-	0.3	-
1864	*p*-Cymen-8-ol	-	0.3	0.8	0.4
1868	(*E*)-Geranyl acetone	-	0.1	0.4	0.1
1882	*cis*-Carveol	-	-	0.2	-
1896	*cis*-*p*-Mentha-1(7),8-diene-2-ol	-	-	0.1	-
1900	*epi*-Cubebol	0.5	-	-	-
1925	2,3,4-Trimethyl benzaldehyde	-	1.5	-	3.1
1941	α-Calacorene	0.1	-	0.1	-
1945	1,5-Epoxy-salvial(4)14-ene	-	-	-	0.1
1957	Cubebol	0.6	-	-	-
1958	(*E*)-β-Ionone	-	-	0.1	-
1973	Dodecanol	-	0.1	-	-
2008	Caryophyllene oxide	0.5	-	1.7	0.2
2019	2,3,6-Trimethylbenzaldehyde	-	29.8	0.7	32.7
2037	Salvial-4(14)-en-1-one	-	-	0.3	0.1
2050	(*E*)-Nerolidol	-	-	0.5	-
2069	Germacrene D-4β-ol	5.3	-	-	-
2071	Humulene epoxide-II	-	0.1	0.2	0.1
2073	*p*-Mentha-1,4-dien-7-ol	-	-	0.1	-
2080	Cubenol	0.2	-	-	-
2088	1-*epi*-Cubenol	0.2	-	-	-
2100	Heneicosane	-	0.1	-	-
2103	Guaiol	0.6	-	-	-
2122	Hedycaryol	1.9	-	-	-
2130	Salviadienol	-	-	0.3	0.2
2131	Hexahydrofarnesyl acetone	-	0.3	0.8	-
2144	Spathulenol	1.1	0.7	0.9	-
2148	(*Z*)-3-Hexen-1-yl benzoate	-	-	-	0.9
2179	Tetradecanol	-	0.2	0.3	-
2187	T-Cadinol	1.8	-	-	-
2200	Docosane	-	0.1	-	-
2209	T-Muurolol	1.5	-	-	-
2219	Dimyrcene II-a	-	0.1	0.7	-
2226	Methyl hexadecanoate	-	tr	0.3	-
2250	α-Eudesmol	0.2	-	-	-
2255	α-Cadinol	4.2	-	tr	-
2269	Guaia-6,10(14)-dien-4β-ol	-	12	0.3	-
2269	Dimyrcene II-b	-	-	0.4	-
2278	Torilenol	-	tr	0.4	-
2296	Myristicine	-	-	3.4	-
2300	Tricosane	-	0.2	0.6	0.1
2324	Caryophylla-2(12),6(13)-dien-5α-ol (=Caryophylladienol II)	-	-	0.5	0.3
2369	Eudesma-4(15),7-dien-1β-ol	-	-	1.1	0.3
2384	Hexadecanol	-	-	-	0.2
2384	Farnesyl acetone	-	-	1.0	-
2392	Caryophylla-2(12),6-dien-5β-ol (=Caryophyllenol II)	-	-	-	0.4
2500	Pentacosane	-	0.7	0.7	0.2
2622	Phytol	tr	0.4	3.9	0.2
2655	Benzyl benzoate	-	-	0.6	0.7
2670	Tetradecanoic acid	-	0.1	0.6	tr
2700	Heptacosane	-	-	0.6	tr
2822	Pentadecanoic acid	-	tr	tr	tr
2900	Nonacosane	-	7.7	25.5	2.7
2931	Hexadecanoic acid	-	3.9	14.8	1.7
	Monoterpene Hydrocarbons	**57.7**	**11.2**	**1.4**	**22.4**
	Oxygenated Monoterpenes	**2.9**	**33.1**	**18**	**21.9**
	Sesquiterpene Hydrocarbons	**17.3**	**1.1**	**5.0**	**0.9**
	Oxygenated Sesquiterpenes	**18.6**	**2.0**	**7.2**	**1.7**
	Diterpenes	**-**	**0.5**	**5. 0**	**0.2**
	Fatty acid	**-**	**4.0**	**15.4**	**1.7**
	Others	**0.1**	**42.2**	**34.3**	**43.7**
	Identified compounds	**53. 0**	**67. 0**	**78. 0**	**59. 0**
	Total	**96.6**	**94.1**	**86.3**	**92.5**

Identification of components: Identification of the essential oil components was carried out either by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) with a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, Adams Library, MassFinder 2.1 Library) [21,22], and in-house "Başer Library of Essential Oil Constituents" built up from genuine compounds and components of known oils, as well as MS literature data [23-25], was used for the identification.

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Essential Oils of *Daucus carota* subsp. *carota* of Tunisia Obtained by Supercritical Carbon Dioxide Extraction

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The essential oils and supercritical CO_2 extracts of wild *Daucus carota* L. subsp. *carota* from two different sites in Tunisia were investigated. The main components of the essential oil of the flowering and mature umbels with seeds from Sejnane were eudesm-7(11)-en-4-ol (8.2 – 8.5%), carotol (3.5 - 5.2%), sabinene (12.0 -14.5%), α-selinene (7.4 – 8.6) and 11-α-(H)-himachal-4-en-1-β-ol (12.7 – 17.4%), whereas the oils from Tunis were predominantly composed of elemicin (31.5 – 35.3%) and carotol (48.0 – 55.7%). The antimicrobial activity of the essential oils were assayed by using the broth dilution method on *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 43300, and clinical strains of *Candida albicans* and *C. tropicalis* 1011 RM. The MIC values obtained were all > 2.5% (v/v).

Keywords: *Daucus carota* L. subsp. *carota*, essential oil, supercritical CO_2, antibacterial activity, antifungal activity.

Daucus carota L. subsp. *carota* (Apiaceae) is native to Europe, Asia and Africa. Traditional medicine uses *D. carota* extracts for hepatic and renal insufficiency as well as for skin disorders, for example burns and furunculous [1]. Extracts of the wild plants are known to be antioxidative and iron-chelative. Ahmed *et al*., from the roots of wild *D. carota* subsp. *carota*, isolated three new sesquiterpene daucane derivatives and four known compounds that showed antifungal activity [2]. Also, the methanol extract of the seeds showed antibacterial activity [3]

Carrot seed essential oil is widely used as a flavor ingredient in many foods and as a fragrance in perfumes, cosmetics and soaps. Claimed biological properties of the oil include antimicrobial, hepato-cellular regenerator, general tonic and stimulant, cholesterol regulator, and cicatrisant. The essential oil from the aerial parts of the plant showed particular activity against Gram-positive bacteria, but the activity of the oils from different organs of the plant were not the same, decreasing in the sequence, mature umbel oils > herb oil > flowering umbel oils [4]. The part of the plant, the stage of development as well as the geographical origin can influence significantly the composition of the oils obtained from this species. From literature data it can be seen that leaf, stem, and blooming umbel oils are dominated by monoterpenes and/or sesquiterpenes [5-6]. Oils isolated from umbels or seeds were dominated by sesquiterpenes and phenylpropanoids, β-bisabolene and β-asarone or by (*E*)-methylisoeugenol accompanied by α-pinene and elemicin.

Considerable variations were also found depending on geographical area, especially in the composition of the seed oils. Góra *et al*. [7] and Staniszewska and Kula, [8] showed that oils from Poland were dominated by either sabinene and α-pinene or sabinene, α-pinene and geranyl acetate. Similar results were reported by Mockute and Nivinskiene [9] for Lithuanian carrot seed essential oil. However, carrot seed oil from Turkey was

reported to contain a high content of carotol (67%) and daucene (9%) [10]. Also Glisic *et al*. [1] found in the supercritical extract from seed of carrot from Serbia, carotol as the principal component (30%). For commercial carrot seed oil, which includes oils of all the subspecies of *D. carota*, four compositions can be distinguished. Three of these are dominated by sabinene (32–60%) and either geranyl acetate (32–77%) or carotol (23–77.5%), and the fourth contains these compounds with an identical ratio [6]. The aim of the present work was to investigate the compositions of the volatile oils of *D. carota* subsp. *carota* growing wild in Tunisia at two different sites (Sejnane and Tunis). Carbon dioxide supercritical extracts (SFE) were also produced and their compositions investigated.

D. carota subsp. *carota* essential oil of ripe umbels (with mature seeds) was obtained in yields of 1.0% and 2.0% for HD and SFE, respectively. Thirty-six identified compounds made up 93.2-97.3% of the oil (Table 1).

The oil from mature umbels with seeds from Sejnane (sample1) was composed of sesquiterpene hydrocarbons (36.1 – 37.2%) and oxygenated sesquiterpenes (29.2 – 35.9%). The main components were carotol (3.5 – 5.2%), β-bisabolene (5.5-7.6), α-selinene (7.4-8.6%), eudesm-7(11)-en-4-ol (8.2 – 8.5%), sabinene (12.0 - 14.5%) and 11-α-(H)-himachal-4-en-1-β-ol (12.7 – 17.4%). However, the oil from the mature umbels with seeds from the second site (sample 2) was of the carotol chemotype (48.0-55.7%), with the second main constituent being elemicin (31.5 - 35.3%). This oil is predominantly composed of oxygenated sesquiterpenes (48–55.7%) and phenylpropanoids (31.5- 35.3%). This latter sample did not contain any oxygenated monoterpenes.

It is interesting to note that phenylpropanoids represented 31.5% of the oil in sample 2, but only 4.7 % of sample 1 (data of HD extract). Similar results were reported by Maxia *et al*. [18], who found that the umbels with seed oils obtained from plants of *D. carota* ssp. *carota* from Italy contained 15.2% of phenylpropanoids, which was nearly five times that found in oils obtained from plants growing in Portugal (3.4%). 11α-H-himachal-4-ene-1β-ol and eudesm-7(11)-en-4-ol, the main compounds identified in the extracts from site 1 were not identified in the extracts obtained from site 2. The same components were found in the oil from Sardinia [18]. These differences could have several reasons, such as climate, soil or other geographical factors [19], but also to genetic differences probably responsible for the chemical variability in the carrot oil.

Table1: Retention index (RI) and chromatographic area percentages of compounds found in *Daucus carota* subsp. *carota* essential oils from site 1 (A1=HD and B1=SFE) and site 2 (A2=HD and B2=SFE).

RI	Compound	A1	B1	A2	B2
937	α-Pinene [a,b,c]	1.0	0.5	1.4	0.4
977	Sabinene [a,b,c]	14.5	12.0	1.7	0.5
980	β-Pinene [a,b,c]	2.7	2.0	1.0	0.3
992	Myrcene [a,b,c]	0.6	0.2	0.2	-
1019	α-Terpinene [a,b]	0.6	-	0.2	-
1027	*p*-Cymene [a,b,c]	0.5	-	-	-
1031	Limonene [a,b,c]	0.4	0.1	0.4	0.5
1061	γ-Terpinene [a,b,c]	0.7	-	-	-
1141	*cis*-Sabinol [a,b]	0.5	-	-	-
1178	Terpinen-4-ol [a,b,c]	1.2	0.2	-	-
1191	α-Terpineol [a,b]	0.4	-	-	-
1286	Bornyl-acetate [a,b,c]	0.5	-	-	-
1351	α-Longipinene [a,b]	1.3	1.4	1.3	0.8
1376	α-Copaene [a,b]	0.2	1.9	0.2	-
1380	β-Cubebene [a,b]	0.2	0.3	0.1	-
1391	β-Elemene [a,b]	0.8	0.9	0.2	-
1404	Methyleugenol [a,b,c]	0.7	0.5	-	-
1415	*cis*-α-Bergamotene [a,b]	0.3	-	-	-
1418	(*E*)-Caryophyllene [a,b,c]	0.4	0.4	2.2	1.4
1437	*trans*-α-Bergamotene [a,b]	3.8	0.2	0.2	-
1445	(*Z*)-β-Farnesene [a,b]	4.5	5.0	2.6	1.6
1453	α-Humulene [a,b,c]	1.3	1.5	-	-
1458	(*E*)-β-Farnesene [a,b]	2.9	3.5	-	-
1480	γ-Muurolene [a,b]	1.2	2.0	-	-
1485	Bicyclogermacrene [a,b]	2.4	0.2	-	-
1493	δ-Selinene [a,b]	1.0	1.2	-	-
1498	α-Selinene [a,b]	7.4	8.6	-	-
1509	β-Bisabolene [a,b]	5.5	7.6	-	-
1513	Elemecin [a,b]	1.4	1.6	31.5	35.3
1524	*E*(α)-Bisabolene [a,b]	1.8	1.9	0.4	0.5
1556	Germenene B [a,b]	0.6	0.6	0.3	0.3
1559	Carotol [a,b]	3.5	5.2	48.0	55.7
1630	11-α-(H)-Himacal-4-en-1-β-ol [a,b]	12.7	17.4	-	-
1653	α-Cadinol [a,b]	1.8	2.3	-	-
1682	α-Asarone [a,b]	1.6	2.6	-	-
1694	Eudesm-7(11)-en-4-ol [a,b]	8.2	8.5	-	-
	Monoterpene hydrocarbons	21.0	14.8	4.9	1.7
	Oxygen containing monoterpenes	2.6	0.2	-	-
	Sesquiterpene hydrocarbons	36.1	37.2	7.5	4.6
	Oxygen containing sesquiterpenes	29.2	35.9	48.0	55.7
	Phenylpropanoids	4.7	5.1	31.5	35.3
	Total identified	**93.6**	**93.2**	**91.9**	**97.3**

Identification has been realized by comparing mass spectra (a), retention indices (b), and injection of authentic compound (c).

Extracts obtained by SFE revealed quantitative differences in their composition when compared with those of distilled essential oils. For example, SFE extract contains lower amounts of monoterpenes (15 vs. 23.6% and 1.7 vs. 4.9%) and higher amounts of phenyl-propanoids (5.1 vs. 4.7% and 35.3 vs. 31.5%).

Hydrodistillation induces migration of volatile compounds from the inside of the secretory structures to the surface, followed by their subsequent evaporation. Therefore, low molecular weight compounds are easily taken from the vegetable matrix while supercritical CO_2 emulates an organic solvent, improving the extraction of high molecular weight compounds. No differences were noted in the compositions of SFE extracts with changing extraction times.

The MIC and MCC values of the *D. carota* oil, both samples, obtained by the microdilution method were > 2.5 (v/v) against all the test microorganisms. The values for the Italian and Portuguese carrot oils varied depending on the fungal species investigated. *C. neoformans* and dermatophyte strains showed more sensitivity to these oils than *Candida* and *Aspergillus* strains [9]. On the other hand, Jabrane *et al.* [20] found that flower and root oils of Tunisian *D. carota* ssp. *maritimus* had a significant and broad spectrum of activity against Gram-positive and Gram-negative bacteria, the flower oil being more effective than the root oil against *E. coli* (ESLb). Conversely, the root oil was found to be more active than the flower oil towards *S. aureus* (reference and environmental strains). An essential oil is usually a complex mixture of different components and so it is difficult to reduce the antimicrobial effect of the total oil to either one or a few active principles. It cannot be ignored that, in addition to the main compounds, minor compounds could be making a significant contribution to the oil activity [21].

Experimental

Plant material: Umbels of *Daucus carota* L. subsp. *carota* were collected from two different sites in Tunisia from two different bioclimatic zones: Sejnane (humid) (samples **1**) and Tunis (sub-humid) (samples **2**). Voucher specimens (numbers: COI00033068 and CAG 625) were deposited at the Herbarium of the Department of Botany of the University of Tunis. Vegetal material was air-dried in a hot air-drier at 40°C with forced ventilation for 2 days. Before utilization, the plant material was ground with a Malavasi mill (Bologna, Italy) taking care to avoid overheating.

Hydrodistillation: Hydrodistillation (HD) was performed in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [11] for 4 h.

SFE extraction: Supercritical CO_2 (purity 99% - Air Liquide Italia, Cagliari, Italy) extractions were performed according Marongiu *et al*. [12] in a laboratory apparatus equipped with a 320 cm^3 extraction vessel and two separator vessels of 300 and 200 cm^3, respectively connected in series. Experiments were carried out at 90 bar and 40°C in the extraction section. In the first separator, the temperature was set at -10°C and the pressure at the same value as that of the extraction section. The second separator was set at 15 bar and 10°C. Extractions were carried out in a semi batch mode, with batch charging of vegetable matter and continuous flow of solvent. About 180 g of material were charged in each run.

GC/MS analysis: An Agilent Technologies Inc. gas chromatograph (Santa Clara, CA, USA) model 6890N was employed for analysis of the essential oils. It was equipped with a split-splitless injector, an autosampler Agilent model 7683 and an Agilent HP5 fused silica column; 5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 μm. GC conditions used were: programmed heating from 60 to 280°C at 3°C/min, followed by 30 min under isothermal conditions. The injector was maintained at 250°C. Helium was the carrier gas at 1.0 mL/min; the sample (1 μL) was injected in the split mode (1:20). The GC was fitted with a quadrupole mass spectrometer (MS, Agilent model 5973 detector). MS conditions were as follows: ionization energy 70 eV, electronic impact ion source temperature 200°C, quadrupole temperature 100°C, scan rate 1.6 scan/sec, mass range 50-500 u. The software adopted to handle MS and chromatograms was a ChemStation NIST 02 [13] and LIBR (TP) [14]. Mass Spectra Libraries were used as references. Samples were run in chloroform with a dilution ratio of 1:100. Compounds were identified by matching their MS and retention index with those reported in the literature [15]. Moreover, whenever possible, identification was confirmed by injection of an authentic sample of the compound. A quantitative analysis of each oil component (expressed in percentages) was carried out by peak area normalization measurement. The response factors were estimates using standard compounds having the same molecular weight as the compound families that constitute the essential oil (hydrocarbon and oxygenated monoterpenes, hydrocarbon and oxygenated sesquiterpenes).

Antimicrobial activity: The organisms tested in this study were as follow: *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 43300), *Candida albicans* (clinical strain) and *C. tropicalis* (1011 RM) (clinical strain). Bacteria were cultured overnight in Luria-Bertani Broth (LB) and fungi in Sabouraud Dextrose Agar plates. Minimal inhibitory concentration (MIC) values were determined as the lowest essential oil concentration that inhibits visible growth of the isolates after 24-48 h incubation at 37°C. It was measured with the broth dilution method (microdilution using 96-well microplates) [16,17]. Nine different concentrations of each essential oil from 2.5%, v/v to 0.001%, v/v with 10% Tween 80 were used. The bacterial and fungal cultures were diluted with LB broth and RPMI medium, respectively, to obtain $1.0x10^8$ CFU/mL (0.5 MacFarland).

Minimal cidal concentration (MCC) values were determined as the lowest essential oil concentration that kills both bacteria and fungi. It was measured with the

broth dilution method starting from the MIC as the lowest concentration to the maximum one (2.5% v/v). Positive and negative controls were also included.

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Oil Constituents of *Artemisia nilagirica* var. *septentrionalis* Growing at Different Altitudes

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Oils of *Artemisia nilagirica* var. *septentrionalis* plants growing at different altitudes in Himachal Pradesh, India were hydro-distilled and analyzed by GC-GC/MS. The major constituents of the oil show variation with changes in altitude. At lower, middle and higher altitudes, the major constituents of the oil were caryophyllene oxide (28.6%), borneol (35.8%) and camphor (46.9%), respectively. The percentages of α-humulene and *trans*-β-guaiene also increased, but the percentage of sabinene, *trans*-sabinene hydrate, 4-terpineol, caryophyllene oxide and humulene epoxide-II decreased with an increase in altitude. The characteristic compounds observed in the plants from lower altitudes were 2-hexene-1-ol, β-thujone, thujanol, myrtenol and linalyl acetate, while the higher altitude plants were characterized by the presence of α-pinene, β-pinene, limonene, linalool, γ-gurijunene, germacrene-D and farnesol.

Keywords: *Artemisia nilagirica* var. *septentrionalis*, Asteraceae, essential oil constituents.

Artemisia nilagirica var. *septentrionalis* Pamp., syn *A. vulgaris* L. (Family Asteraceae), plants are distributed mostly in the Western Himalayas. The plants produce a light yellow oil. [1]. *A. nilagirica* is used in traditional medicine as a tonic and in the treatment of bronchial troubles [2]. An ointment of *A. nilagirica* oil has been reported as an antifungal agent against dermatomycosis [3,4]. An insecticidal principle, capillin, has also been isolated from the petroleum extract of *A. nilagirica* [5].

The essential oil of *A. nilagirica* is reported to contain camphor, β-eudesmol, 1,8-cineole and borneol [6]. Recently, the composition of the essential oil of *A. nilagirica* var. *septentrionalis* has been reported [7]. In the present study, the oil obtained from *A. nilagirica* var. *septentrionalis* plants growing at different altitudes in Himachal Pradesh were analyzed by GC-GC/MS and thirty-four constituents were identified. In the Shimla, Mandi and Manali samples, the oil yield was 0.25%, 0.56% and 1.0%, respectively. The identified constituents in the oils totaled 23, 17 and 24, constituting 76%, 83% and 93% of the total oil, respectively. At the lower altitudes of Mandi, caryophyllene oxide (28.6%) was the major constituent of the oil followed by methanoazulene (10.9%) and borneol (4.5%). At the middle altitude of Manali, borneol (35.8%) became the major constituent of the oil followed by methanoazulene (14.7%) and caryophyllene oxide (13.4%). However, at the higher altitude of Shimla, the major oil constituents were quite different. In this case camphor (46.9%) was the major constituent followed by β-caryophyllene (13.3%) and α-humulene (9.7%). The characteristic constituents identified in the oil of plants collected from Mandi were 2-hexene-1-ol, β-thujone, thujanol, myrtenol and linalyl acetate, which were not identified in the oils from the other places. Oil obtained from the plants collected from Manali had only three characteristic constituents, which were α-phellandrene, β-phellandrene and α-thujone. The Shimla materials contained a maximum of seven characteristic constituents in its oil, which were α-pinene, β-pinene, limonene, linalool, γ-gurjunene, germacrene D and farnesol. However, eleven constituents were observed to be common in all the examined samples; these were sabinene, *trans*-sabinene hydrate, camphor, borneol, β-elemene, β-caryophyllene, α-humulene, *trans*-β-guaiene, caryophyllene oxide, methanoazulene and humulene epoxide-II.

It is interesting to note that some of the compounds, such as α-humulene and *trans* β-guaiene, increase in quantity with an increase of altitude, while constituents like sabinene, *trans*-sabinene hydrate, 4-terpineol, caryophyllene oxide and humulene epoxide-II increase in quantity with a decrease in altitude. Although the oil of *A. nilagirica* var. *septentrionalis* has been analyzed during different growth stages in the plants that were

Table 1: Effect of altitude on the oil constituents of *Artemisia nilagirica* var. *septentrionalis*.

Constituents	RI	% oil constituents (dry weight)		
		Mandi (1044 m)	Manali (2050 m)	Shimla (2210 m)
2-Hexene-1-ol	854	2.6	-	-
α-Pinene	939	-	-	0.6
Camphene	953	0.2	-	0.5
Sabinene	967	4.4	0.1	0.1
β-Pinene	981	-	-	1.1
β-Myrcene	987	-	0.3	0.1
α-Phellandrene	1105	-	0.7	-
Limonene	1021	-	-	0.9
p-Cymene	1026	0.7	-	0.2
β-Phellandrene	1031	-	0.2	-
1,8-Cineole	1035	2.7	-	0.5
cis-Sabinene hydrate	1063	1.3	6.8	-
α-Thujone	1089	-	0.1	-
Linalool	1099	-	-	0.2
trans-Sabinene hydrate	1100	1.1	4.0	0.5
Chrysanthenone	1106	0.9	-	0.7
β-Thujone	1114	0.5	-	-
β-Fencyl alcohol	1117	0.4	-	1.3
Camphor	1144	2.0	0.2	46.9
Thujanol	1166	2.0	-	-
Borneol	1168	4.5	35.8	0.6
4-Terpineol	1175	2.2	0.8	-
Myrtenol	1194	0.7	-	-
Linalyl acetate	1257	1.3	-	-
β-Elemene	1390	3.0	1.1	1.5
β-Caryophyllene	1420	2.3	0.8	13.3
α-Humulene	1456	0.4	0.4	9.7
γ-Gurjunene	1473	-	-	3.6
Germacrene D	1484	-	-	2.6
trans β-Guaiene	1500	0.6	2.1	2.8
Caryophyllene oxide	1579	28.6	13.4	0.5
Methanoazulene	1587	10.9	14.7	2.8
Humulene epoxide II	1606	2.6	1.2	1.2
Farnesol	1697	-	-	0.7
Oil yield		**0.56%**	**1.0%**	**0.25%**

domesticated under sub-tropical north Indian conditions [7], the present study suggests that the yield of oil is low at lower altitude, but increases when the plants grow at higher altitudes. Interestingly, several constituents like *p*-cymene, β-fenchyl alcohol, γ-gurjunene, *trans*-β-guaiene, humulene epoxide-II and farnesol, which were observed in the natural plants, were not observed in the domesticated plants. In sub-tropical conditions, the domesticated plants also developed constituents like γ-terpinene, bornyl acetate, δ-cadinene and β-eudesmol, which were not observed in the naturally growing plants. However, the major oil constituents in the vegetative plants remained camphor and β-caryophyllene, but the quantity of camphor reduced drastically in the domesticated plants, while that of β-caryophyllene remained almost the same [7].

Experimental

***Plant material*:** Leaves from mature plants of *Artemisia nilagirica* var. *septentrionalis*, before flowering, were collected from different altitudes in Himachal Pradesh such as Shimla (2210 m), Mandi (1044 m) and Manali (2050 m) in June 2005. Plants were deposited in the CIMAP Herbarium, Lucknow. Collected samples were dried in the shade and 100 g of accurately weighed leaves were subjected to hydro-distillation in a Clevenger type apparatus for 5 h to obtain light yellow oil.

***GC-GC/MS analysis*:** GC of essential oils was carried out using a Perkin Elmer AUTO-XL GC, capillary column PE-5 (50 m x 0.32 mm x 0.25 μ film thickness) with the oven temperature programmed from 100°-280°C at 3°/min with an initial hold of 2 min. Injector and detector temperatures were maintained at 220°C and 300°C, respectively. Hydrogen at 10 psi was used as a carrier gas, with a split ratio of 1:50. The GC/ MS analysis was carried out in EI mode using a Perkin Elmer Auto XL/ Tubo Mass instrument with the same column and under identical analytical conditions. Helium at 10 psi was used as a carrier gas with a split ratio 1:20. Mass spectral data were analyzed using NIST and Wiley library information.

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Volatile Oil Composition of *Pogostemon heyneanus* and Comparison of its Composition with Patchouli Oil

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The volatile oil of the leaves of *Pogostemon heyneanus* Benth. (Lamiaceae) was analyzed by GC and GC-MS. Twenty-six components representing 96.0% of the oil were identified. The major components of the oil were acetophenone (51.0%), β-pinene (5.3%), (*E*)-nerolidol (5.4%), and patchouli alcohol (14.0%). Comparison of the compositions of the oils of *P. heyneanus* and *P. cablin* (Blanco) Benth. (Patchouli oil) showed wide variation between them. Though 13 sesquiterpenes and oxygenated sesquiterpenes were detected in both oils, their concentrations in the oils differed widely. Acetophenone, benzoyl acetone and (*E*)-nerolidol present in the oil of *P. heyneanus* were not detected in patchouli oil.

Keywords: *Pogostemon heyneanus*, *Pogostemon cablin*, essential oil composition, acetophenone, patchouli alcohol.

Pogostemon Desf. (Lamiaceae) is an aromatic genus, globally represented by 97 species and in India, it has 54 species [1]. *P. heyneanus* Benth., otherwise called 'Indian Patchouli', is reported to occur in India, Indonesia, Malaysia and Sri Lanka [2,3]. However, it is believed to be indigenous to southern India and Sri Lanka [3]. The plant is a strongly aromatic, sparsely pubescent shrub which grows wild in moist, partially shady places in semi-evergreen and evergreen forests. Several years ago, this species was cultivated in a limited area in Java and the essential oil was distilled. Hence, it is also known as 'Java patchouli'. As the odor of the oil was quite different from that of true patchouli oil *i.e. P. cablin* oil, the production of the oil was discontinued [4].

While there are several reports on the composition of the essential oil of patchouli *i.e. P. cablin* [5-16], there are only three records of the composition of the oil of *P. heyneanus* [17-19]. Venturella [17] investigated the essential oil of *P. heyneanus* var. *patchouli* Pellet. grown in the Palermo Botanical Garden and reported β-pinene, limonene, borneol, β-patchoulene, α-guaiene, norpatchoulenol and patchoulol as the major components of the oil. Maia and Andrade [18] reported that the oil of *P. heyneanus* from the Amazon contained, as its major constituents, patchouli alcohol, α-guaiene, aromadendrene and δ-guaiene (α-bulnesene). Recently, Souza-Filho *et al* [19] analyzed the essential oil of *P. heyneanus* and reported patchuoli alcohol, α-bulnesene, α-guaiene, seychellene, α-patchoulene as the principal components. We have analyzed by GC and GC-MS, the essential oils of *P. heyneanus* collected from the Courtallum hills of the southern Western Ghats in Tamil Nadu, India and true patchouli (*P. cablin*), which was procured from the Central Institute of Medicinal and Aromatic Plants, Resource Centre, Bangalore. The compositions of these two oils are compared in this paper.

Hydrodistillation of the dried leaves of *P. heyneanus* yielded 0.8% of a yellow colored essential oil. GC analysis showed more than fifty peaks. GC-MS analysis and GC retention indices of the peaks enabled the identification of 26 components representing 96.0% of the oil. The identified compounds are listed in Table 1

in the order of elution from a GC column. The major components of the oil were acetophenone (51.0%), β-pinene (5.3%), (*E*)-nerolidol (5.4%), and patchouli alcohol (14.0%). The oil contained monoterpenes (11.8%), oxygenated monoterpenes (1.2%), sesquiterpenes (8.4%), oxygenated sesquiterpenes (20.3%), benzenoid compounds (54.1%) and aliphatic compounds (0.2%). As *P. heyneanus* is often confused with *P. cablin* and is often referred to as Indian or Java patchouli, the composition of the oil of *P. heyneanus* was compared with that of patchouli oil. The major components of patchouli oil are α-guaiene, seychellene, α-patchoulene, α-bulnesene and patchouli alcohol. The minor components, such as norpatchoulenol, nortetrapatchoulol and α-cedrenal, and the main component patchouli alcohol were reported to be responsible for the characteristic odor of patchouli oil [13]. Though some constituents, mainly sesquiterpenes and oxygenated sesquiterpenes, were found in both the oils, there are considerable and significant differences between the two oils. The compounds, namely, acetophenone, benzoyl acetone and (*E*)-nerolidol, found in the essential oil of *P. heyneanus* were not detected in that of *P. cablin*. Patchouli oil contained higher amounts of patchouli alcohol, α-guaiene, seychellene, α-patchoulene, aciphyllene, α-bulnesene, caryophyllene oxide and pogostol than the oil of *P. heyneanus*. The compounds, namely, cycloseychellene, δ-patchoulene, norpatchoulenol, nortetrapatchoulol, patchoulenone, (*E*,*E*)-farnesol and pogostone found in patchouli oil were not detected in the oil of *P. heyneanus*. Patchouli oil has a pleasant and lasting aroma, whereas that of *P. heyneanus* has a strong pungent odor. Thus, the oil of *P. heyneanus* is quite different from patchouli oil in composition and odor.

The oil of *P. heyneanus* in the present study is quite different in composition from that of the earlier studies. Venturella [17] reported the essential oil composition of *P. heyneanus* var. *patchouli*, but this name is synonymous with *P. cablin*, as mentioned in Flora Malesiana [3]. Hence, the species investigated by Venturella was the true patchouli plant and not *P. heyneanus*. Although several compounds reported in the oil of this plant were also present in the oil of *P. cablin*, a high concentration of monoterpenes, and a low concentration of the sesquiterpenes, α-bulnesene and patchouli alcohol showed that the composition of this oil was somewhat abnormal. Another noteworthy feature was the presence of a high concentration of norpatchoulenol (9.0%).

The composition of the oil of *P. heyneanus* from the Amazon was similar to that of patchouli oil, with large amounts of patchouli alcohol, α-guaiene, aromadendrene and δ-guaiene. Aromadendrene is, however, not reported to be a constituent of patchouli oil. Furthermore, the wild occurrence of *P. heyneanus* in the Amazon has not been reported and the species investigated by Maia and Andrade [18] may be *P. cablin*, as they mentioned it as patchouli plant. Souza-Filho *et al* [19] analyzed the essential oil of *P. heyneanus* from Brazil and the composition of this oil was similar to that of the oil of *P. cablin*. Furthermore, the Brazilian vernacular name '*oriza*', given to *P. heyneanus* in this study, actually refers to *P. cablin* [20]. Hence the *Pogostemon* species investigated by Souza-Filho *et al* [19] may have been *P. cablin*. Thus, the composition of the oil of *P. heyneanus* in the present study is quite different from the earlier reports. The high concentration of the aromatic ketone, acetophenone in the oil is noteworthy. Earlier, this compound was reported as a minor compound in the oils of *Cistus ladaniferus* L. (Labdanum oil) [21,22],

Table 1: Composition of the essential oils of *Pogostemon heyneanus* and *P. cablin*.

Compounds	RI*	Area %		Detection
		Pogostemon heyneanus	***Pogostemon cablin***	
α-Pinene	940	2.4	tr	GC, MS
Camphene	953	tr	--	GC, MS
1-Octen-3-ol	975	--	0.1	GC, MS
3-Octanone	976	0.2	--	GC, MS
β-Pinene	979	5.3	tr	GC, MS
Limonene	1030	4.0	tr	GC, MS
Acetophenone	1068	51.0	--	GC, MS
Terpinolene	1090	0.1	--	GC, MS
Linalool	1098	0.2	tr	GC, MS
α-Terpineol	1189	1.0	--	GC, MS
Methyl salicylate	1191	--	tr	GC, MS
δ-Elemene	1341	--	0.8	GC, MS
β-Patchoulene	1385	--	1.8	GC, MS
β-Elemene	1388	--	0.8	GC, MS
Benzoyl acetone [ti]	1391	3.1	--	MS
Cyperene	1395	tr	--	GC, MS
Cycloseychellene [ti]	1411	--	0.5	MS
(*E*)-Caryophyllene	1420	2.0	3.0	GC, MS
(*E*)-α-Bergamotene	1435	tr	--	GC, MS
α-Guaiene	1441	1.7	9.6	GC, MS
Seychellene	1452	1.5	5.8	GC, MS
α-Humulene	1458	0.3	0.6	GC, MS
α-Patchoulene	1465	1.5	} 6.1	GC, MS
γ-Patchoulene		--		MS
Germacrene D	1482	tr	0.1	GC, MS
δ-Patchoulene [ti]	1490	--	0.4	MS
Aciphyllene	1500	0.4	2.4	GC, MS
α-Bulnesene	1507	0.7	13.3	GC, MS
7-*epi*-α-Selinene	1524	0.1	tr	GC, MS
α-Guaiene epoxide	1540	--	tr	GC, MS
(*E*)-Nerolidol	1557	5.4	--	GC, MS
Norpatchoulenol	1567	--	1.2	GC, MS
α-Bulnesene epoxide	1576	0.1	0.5	GC, MS
Caryophyllene oxide	1588	0.3	1.1	GC, MS
Nortetrapatchoulol	1600	--	0.2	GC, MS
Pogostol	1667	0.7	6.2	GC, MS
Patchouli alcohol	1679	14.0	38.3	GC, MS
Pogostone	1702	--	2.1	GC, MS
(*E*,*E*)-Farnesol	1722	--	0.3	GC, MS
Patchoulenone [ti]	1728	--	0.4	MS
		96.0	**95.6**	

* RI = Retention index on OV-5 Column; tr = < 0.1%; ti = Tentative identification.

Trifolium repens L. [23], *Elsholzia ciliata* (Thunb.) Hyl. [24], *Garcinia dulcis* Kurz. [25], *Rhodiola rosea* L. [26] and in a few *Hypericum* species [27,28].

Of the 97 species in the genus *Pogostemon* distributed throughout the world, so far, *P. cablin* is the only species commercially exploited and cultivated for producing patchouli oil, which is widely used in perfumery. Recently, the essential oils of two other *Pogostemon* species namely, *P. benghalensis* (*P. plectranthoides*) [29] and *P. travancoricus* [30] were investigated and these oils were found to be rich in sesquiterpenes and oxygenated sesquiterpenes. However, the oil of *P. heyneanus* is unique in having as its main constituent acetophenone, which was not reported in the oils of the above mentioned *Pogostemon* species.

Experimental

Plant material: Leaf material of *P. heyneanus* was collected from Courtallum hills, Tirunelveli district, Tamil Nadu, India. The voucher specimens (*R. Murugan* 33) have been deposited in the Herbaria of Madras Christian College (MCCH), Chennai and Foundation for Revitalisation of Local Health Traditions (FRLH), Bangalore, India. The plant was identified by the first author. The identity of the plant specimen was confirmed by matching it with the Type specimen [*Macrae* 1532 (K-isotype)].

Extraction of oil: The leaf sample of *P. heyneanus*, after drying in the shade for 2 weeks, was hydrodistilled in a Clevenger type apparatus for 8 h. The pale yellow oil was collected, dried over anhydrous sodium sulfate and stored in a refrigerator. The oil content was 0.8% (dry weight basis). The true patchouli oil was procured from the Central Institute of Medicinal and Aromatic Plants, Resource Centre, Bangalore.

Analysis of oil: GC analysis of the essential oil samples was carried out on a Shimadzu GC-2014 Gas Chromatograph using a non-polar, OV-5 column (30 m x 0.25 mm x 0.25 µm film thickness, coated with 5% diphenyl - 95% dimethyl polysiloxane). Helium was used as carrier gas at a linear velocity of 30 cm/sec. and a pressure of 93.6 kPa. The flow rate was 1 mL/min. Temperature programme: 60^0C - 240^0C at the rate of 3^0C/min. Samples of 1 µL dissolved in *n*-hexane were injected. Split ratio used was 1:60. A Shimadzu GC-MS (17A / QP5050) was used, fitted with a CP-Sil 5 CB column (30 m x 0.25 mm x 1 µm film thickness coated with 100% dimethyl polysiloxane). Helium was used as carrier gas at a linear velocity of 45 cm/sec. and a pressure of 64.5 kPa. The flow rate was 1 mL/min. Temperature programme: 60^0C - 250^0C at the rate of 5^0C/min. Samples of 1 µL dissolved in *n*-hexane were injected. Split ratio used was 1:60. MS were recorded at 70eV with a mass range of m/z 40-500. Identification of the components was achieved by comparison of the retention indices (RI) of the GC peaks with those of compounds reported in the literature [6,31,32] and by comparison of the MS of the peaks with those of compounds reported in the literature [9,31,32]. Peak area percentages were obtained from GC-FID responses without the use of an internal standard of correction factors.

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Chemical Composition of Volatile Oils of *Aquilaria malaccensis* (Thymelaeaceae) from Malaysia

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This paper is dedicated to Professor KHC Başer on occasion of his 60th birthday.

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Volatile oils of *Aquilaria malaccensis* Benth. (Thymelaeaceae) from Malaysia were obtained by hydrodistillation and subjected to detailed GC-FID and GC/MS analyses to determine possible similarities and differences in their chemical composition in comparison with the commercial oil. A total of thirty-one compounds were identified compared with twenty-nine identified in the commercial oil. The major compounds identified were 4-phenyl-2-butanone (32.1%), jinkoh-eremol (6.5%) and α-guaiene (5.8%), while the major compounds in the commercial oil were α-guaiene (10.3%), caryophellene oxide (8.6%), and eudesmol (3.2%). The results of the present study showed that more than nine sesquiterpene hydrocarbons were present, which is more than previously reported. Analysis also showed that the number of oxygenated sesquiterpenes in this study were much less than previously reported. Among the compounds detected were α-guaiene, β-agarofuran, α-bulnesene, jinkoh-eremol, kusunol, selina-3,11-dien-9-one, oxo-agarospirol and guaia-1(10),11-dien-15,2-olide.

Keywords: *Aquilaria malaccensis* Benth., volatile oil, sesquiterpene, gas chromatography-flame ionization detector, gas chromatography-mass spectrometry.

Agarwood originates from north-eastern India, Bhutan and parts of south-east Asia, especially Vietnam, Cambodia, Philippines, Malaysia, Indonesia, and Papua New Guinea. Agarwood has had a very long history of use in folk medicine as incense and as an aromatic oil. Agarwood oil, also known as oud, aloeswood oil, eagleswood, kiara and gaharu, is a natural oil obtained from the fragrant resin of several species of *Aquilaria* (Thymelaeaceae) including *A. malaccensis* Benth. (syn. *Aquilaria agallocha*) and *A. crassna. A. malaccensis* is included in Appendix II of the Convention on International Trade in Endangered Species (CITES) and is listed as endangered by the Malaysian government [1-3].

In Malaysia, rural villagers and indigenous peoples depend on *A. malaccensis* harvesting to supplement household income. The species has been introduced as a potential income-generating crop to be planted alongside vegetable farms in agroforestry programs during the Ninth Malaysian Plan (2006-2010). The Peninsular Malaysia Forestry Department is striving to develop a uniform grading system for the fragrant resin and volatile oil which until now is being carried out by most traders by visual inspection and smell.

Agarwood oil has been reported to contain sesquiterpenoids of the eremophilane, spirovetivane, eudesmane, nor-guaiane and prezizaane types, and 2-(2-phenylethyl) chromone derivatives [4-11]. In the present study, we report on the chemical composition of the volatile oils of *A. malaccensis* from Malaysia obtained by hydrodistillation and subjected to detailed GC-FID and GC/MS analyses. GC-FID and GC-MS are the most common analytical methods to-date for compound identification in volatile oils [12]. The chemical composition of the laboratory prepared and commercially obtained oils are compared with the compounds previously reported in agarwood volatile oils [10,11].

The agarwood volatile oil obtained via hydrodistillation (0.2% yield based on dry weight) was dark green. The commercial oil, however, was brown. The general chemical profiles of the oil samples, their chemical composition and retention indices are summarized in

Table 1: Chemical composition of volatile oils of agarwood.

Compounds	Hydrodistilled			Nor Azah *et al.* [12]	Ishihara *et al.* [11]			Identification
	DB-1	Lab.	Commercial		Sample I	Sample II	Sample III	
Monoterpene hydrocarbons								
Benzaldehyde	935	3.3						RI,MS
2-Hydroxy-benzaldehyde	1003	0.6						RI,MS
Acetophenone	1066	0.7						RI,MS
4-Phenyl-2-butanone	1210	32.1	3.4	5.8				RI,MS
Sesquiterpene hydrocarbons								
β-Maaliene	1414	0.4	0.7					RI,MS
α-Guaiene	1440	5.8	10.3	0.7	0.1	0.1		RI,MS
Aromadendrene	1443		0.6					RI,MS
γ-Gurjunene	1472	0.7	1					RI,MS
β-Agarofuran	1474		0.5	2.0	0.3	0.2	0.1	RI,MS
β-Selinene	1486	4.9						RI,MS
α-Muurolene	1496	3.4	0.7					RI,MS
γ-Guaiene	1499	1.5	0.8					RI,MS
α-Bulnesene	1503	1.3	1		0.2	0.2		RI,MS
Oxygenated sesquiterpenes								
α-Elemol	1530		3.3					RI,MS
nor-Ketoagarofuran	1555				0.1	0.1	0.1	RI,MS
Epoxybulnesene	1572				0.1			RI,MS
Caryophellene oxide	1600	0.9	8.6					RI
Guaiol	1603	0.6	1.2					RI
Humulene epoxide II	1606	1.7	2.3					RI
1,5-Epoxy-nor-ketoguaiene	1614	1.1	0.6		0.1			RI,MS
10-*Epi*-γ-eudesmol	1619	1.6	0.8	9.0				RI,MS
Agarospirol	1631	0.9	1.4	5.5			0.1	RI,MS
Epi-α-cadinol	1640		2.9					RI
Jinkoh-eremol	1643	6.5	0.5	7.7	0.4	0.7	0.8	RI
Kusunol	1650	1	0.6		1.7	1	1	RI
α-Eudesmol	1652	0.7	0.9					RI,MS
Bulnesol	1664	1.5	0.6					RI, MS
Dehydrojinkoh-eremol	1673	1.4	1.2				0.1	RI,MS
Epi-α-bisabolol	1678	1.5	1					RI
α-Bisabolol	1683	1.8	0.5					RI
Selina-3,11-dien-9-one	1687	1.3	0.5		0.9	2.1	0.2	RI.MS
Rotundone	1703	0.5			0.2	0.1		RI,MS
Selina-3,11-dien-9-ol	1721				1.2	2.8	0.4	RI,MS
Selina-4,11-dien-14-oic acid	1728				0.6			RI,MS
Selina-3,11-dien-9-al	1735				0.7	0.6	0.8	RI,MS
9,11-Eremophiladien-8-one	1740				1.1	1.2	0.3	RI,MS
Selina-3,11-dien-14-ol	1750				1.2	1.5	0.4	RI,MS
Guaia-1(10),11-dien-9-one	1752				0.1			RI,MS
Selina-4,11-dien-14-al	1758				0.8	0.6	0.4	RI,MS
Guaia-1(10),11-dien-15-ol	1770				0.6	1.2		RI,MS
Selina,3,11-dien-14-oic acid	1775				0.9	0.2		RI,MS
Sinenofuranol	1776						0.2	RI,MS
Dihydrokaranone	1799				1	0.7	0.2	RI,MS
Guaia-1(10), 11-dien-15-al	1806	1.7			3.4	2.5	0.4	RI,MS
Guaia-1(10), 11-dien-15-oic acid	1811	0.6			4.7			RI,MS
Karanone	1812		1.1		0.1	0.1		RI,MS
Oxo-agarospirol	1822	0.3	0.8		1.6	1.4	5.3	RI,MS
Eudesmol	1880	2.1	3.2					RI, MS
2-Hydroxyguaia-1(10),11-dien-15-oic acid	1932				1.8	0.3		RI,MS
n-Hexadecanoic acid	1950	4.2						RI
9-Hydroxyselina-4,11-dien-14-oic acid	1948				0.8			RI,MS
Guaia-1(10),11-dien-15,2-olide	2019		0.5		0.5	0.5	0.2	RI,MS
Others								
2-(2-Phenylethyl) chromone	2296				16.1	17.2	23.6	RI,MS
2(2-(4-Methoxyphenyl)ethyl) chromone	2545				21.2	24.5	33	RI,MS
6-Methoxy-2-(2-4-methoxy-phenyl) chromone	2949				2.0	3.2	3.7	RI,MS

[1]Component are listed in order of their relative content > 0.1 %; [2]RI, linear retention indices were determined relative to the retention times on a DB-1 column of a homologous series of C_8-C_{20} *n*-alkanes.[3]Identification: MS, by comparison of the MS with those of the NIST98 library (>90% matching from the library).

Table 1. Over twenty-five compounds were identified in both the laboratory and commercial samples. The laboratory sample was found to contain 42.4% monoterpene compounds, 20.8% hydrocarbon sesquiterpenes and 36.8% oxygenated sesquiterpenes. The commercial oil contained less monoterpene (5.0 %) compared with the laboratory sample, but more sesquiterpene hydrocarbons (22.9%) and oxygenated sesquiterpenes at 72.1%. One possible explanation for the lower content of monoterpenes in the commercial oil could be vaporization during the prolonged extraction time of 3 days. The percentage of sesquiterpene hydrocarbons was essentially equal for both the laboratory sample and the commercial oil.

The major compounds identified in the laboratory sample were 4-phenyl-2-butanone (32.1%), jinkoh-eremol (6.5%), and α-guaiene (5.8%). The commercial oil contained several major compounds, namely α-guaiene (10.3%) caryophellene oxide (8.6%) and eudesmol (3.2%).

Previously reported data [10] on solvent extracted agarwood oil from Vietnam, which ranged in color from green to brown and purple and labelled I, II and III, respectively, are included for comparison. Also included are findings from a recent report indicating 4-phenyl-2-butanone, β-agarofuran, α-agarofuran, agarospirol and 10-*epi*-γ-eudesmol, which were detected in hydrodistilled oils from Peninsular Malaysia [11].

Based on the data obtained from the present study and previous reports [10,11], several compounds in *A. malaccensis* oil can be classified as marker compounds, namely α-guaiene, β-agarofuran, α-bulnesene, jinkoh-eremol, kusunol, selina-3,11-dien-9-one, oxo-agarospirol and guaia-1(10),11-dien-15,2-olide.

Experimental

Plant material: *Aquilaria malaccensis* infected wood from Kelantan Forest (Malaysia) and a sample of commercial oil were procured in September 2008 from Mazlan Mohamed, an agarwood trader certified by the Forestry Department, Malaysia.

Isolation procedure: Fresh, air dried woods were dried further in an oven at 40°C for 24 h. This was followed by 3 days of soaking in water prior to hydrodistillation in a Pyrex glass Clevenger type apparatus for 12 h. The oils were taken up in *n*-hexane, dried over anhydrous sodium sulfate, purged with nitrogen gas (N_2), and then stored at 4°C in glass amber vials prior to analysis. The commercial oil was isolated by the trader from 25 kg air dried wood chips in a large scale Clevenger-type steel apparatus.

GC-FID and GC-MS analyses: Chemical analyses of agarwood oil were undertaken by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). An Agilent 7890 gas chromatograph coupled to a 5973 quadruple mass spectrometer equipped with a DB-1 capillary column (30m x 0.25 mm, film thickness 0.25 μm) and a selective mass detector was used for GC-MS detection; an electron ionization system was set with an ionization energy of 70eV. Helium was the carrier gas, set at a flow rate of 1.0 mL/min. Injector and ion source temperatures were both set at 230°C. Injection volume was 1 μL (split ratio 20:1) and the oven temperature was programmed from 50°C to 230°C at 3°C/min.

Identification of components: The components were identified on the basis of comparison of their retention indices and mass spectra with published data [13], and by matching with the National Institute of Standards Technology (NIST) libraries. Retention indices were calculated using a homologous series of *n*-alkanes (C8-C22).

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Chemical Composition and Phytotoxic Effects of Essential Oils from Four *Teucrium* Species

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The essential oils of four *Teucrium* species were studied and 131 components, in all, were identified. All oils were rich in sesquiterpenes (50.0-61.9%). Caryophyllene and caryophyllene oxide were the main components of *Teucrium arduini*; germacrene D, δ-cadinene and γ-cadinene predominated in *Teucrium maghrebinum*. Carvacrol and caryophyllene predominated in *Teucrium polium* ssp. *capitatum*, while carvacrol, caryophyllene oxide and caryophyllene were the most abundant components in *Teucrium montbretii* ssp. *heliotropiifolium*. The germination of radish and garden cress was less sensitive to the four essential oils. The radicle elongation, above all, of radish was significantly inhibited by all oils, in particular by the essential oil of *T. arduini*, at the highest doses tested. Among the main components of the oils, monoterpenes resulted the more active compounds.

Keywords: *Teucrium arduini*, *Teucrium maghrebinum*, *Teucrium polium* ssp. *capitatum*, *Teucrium montbretii* ssp. *heliotropiifolium*, essential oils, monoterpenes, radicle elongation, radish, garden cress.

Teucrium is a genus of perennial plants which belongs to the Lamiaceae family: is represented by more than 340 species, distributed mainly in the Mediterranean basin [1]. An unusual feature of this genus compared with other members of Lamiaceae is that the flowers completely lack the upper lip of the corolla. As in other Lamiaceae, the aerial organs of *Teucrium* spp. are covered by an indumentum of glandular and non-glandular trichomes. Plants of this genus are well known in traditional medicine as antispasmodics, diuretics, antidiabetics [2], tonics, diaphoretics, analgesics [3], antiphlogistics, antirheumatics, antiseptics, antihelmintics, carminatives, flavouring [4] and as well as antipyretic and stimulant agents [5].

The genus *Teucrium* is one of the richest sources of diterpenes with a neoclerodane skeleton. More than 220 diterpenes have been described to date, many of which are particularly interesting because of their ecological role as antifeedants against different species of insects and of their role in the medicinal properties of the plants [6]. So far, essential oils have been also reported from the aerial parts of several *Teucrium* species [7-10].

Teucrium arduini L. is an endemic Illyric Balcan species; it is a semi-woody, branchy, erect or ascending, dwarf shrub, 10–40 cm (rarely up to 60 cm) high. Whitish flowers form simple, very dense, up to 16 cm long inflorescences. *T. arduini* grows on calcareous rocky slopes at altitudes between 0 and 1600 m a.s.l. Previous papers reported the biological activities of leaf and flower infusions of this species [11] and the composition of the essential oil [12,13].

T. maghrebinum Greuter et Burdet is a species growing in Algeria and Morocco. The plant is called locally "kayatat el gerah" and is used in traditional medicine to treat burns and fevers, as well as an antimicrobial agent [14]. The chemical composition of its essential oil has been reported [8].

Teucrium montbretii Benth. ssp. *heliotropiifolium* (Barbey) Davis is an evergreen dwarf (5–20 cm high) semishrub–chasmophyte with lignified branches, opposite and ovate leaves. The different subspecies of *T. montbretii* show different qualitative contents of neoclerodane diterpenoids [6]; the essential oil of this species was also analyzed [9].

Table 1: Essential oil composition of *T arduini* (***Ar***), *T. maghrebinum* (***Ma***), *T. montbretii* ssp. *heliotropiifolium* (***Mo***) and *T. polium* ssp. *capitatum* (***Po***).

Component	**KI**[a]	**KI**[b]	**Identification**[c]	***Ar*%**[d]	***Ma*%**[d]	***Mo*%**[d]	***Po*%**[d]
Monoterpene hydrocarbons					**8.9**		**1.2**
α-Thujene	930	1014	LRI, MS		0.2		0.2
α-Pinene	938	1075	LRI, MS, Co-GC		1.6		0.2
Camphene	953	1076	LRI, MS, Co-GC		0.3		
Sabinene	973	1132	LRI, MS				0.3
β-Pinene	978	1118	LRI, MS, Co-GC		1.9		
Myrcene	993	1174	LRI, MS, Co-GC		0.5		t
α-Terpinene	1012	1189	LRI, MS, Co-GC				
p-Cymene	1025	1278	LRI, MS, Co-GC				0.5
Limonene	1030	1203	LRI, MS, Co-GC		4.4		
(*Z*)-β-Ocimene	1038	1245	LRI, MS				
Oxygenated monoterpenes				**2.8**	**6.7**	**3.1**	**15.0**
cis-Linalool oxide, furanoid	1062	1450	LRI, MS			0.1	0.5
cis-Sabinene hydrate	1063	1555	LRI, MS				0.2
trans-Linalool oxide, furanoid	1076	1478	LRI, MS			0.1	0.1
trans-Sabinene hydrate	1093	1474	LRI, MS				0.1
Linalool	1098	1553	LRI, MS, Co-GC	1.6	1.3	2.7	1.0
α-Campholenal	1128	1497	LRI, MS		0.4		
cis-Sabinol	1135	1789	LRI, MS				1.7
Nopinone	1136	1597	LRI, MS		0.1		0.2
cis-Verbenol	1144	1667	LRI, MS		0.4		2.0
Sabina ketone	1155	1652	LRI, MS				0.4
Pinocarvone	1165	1587	LRI, MS		0.6		0.1
Borneol	1167	1719	LRI, MS, Co-GC				0.1
Umbellulone	1175	1656	LRI, MS				0.3
Terpineol-4	1176	1611	LRI, MS, Co-GC		0.4		0.5
α-Terpineol	1189	1706	LRI, MS, Co-GC		0.6	0.2	
Myrtenal	1193	1648	LRI, MS, Co-GC		t		t
Myrtenol	1196	1812	LRI, MS				1.2
cis-Verbenone	1204	1723	LRI, MS		0.3		4.0
trans-Carveol	1217	1845	LRI, MS		0.1		
β-Cyclocitral	1223	1629	LRI, MS		t		0.1
cis-Carveol	1226	1878	LRI, MS				0.6
Cumin aldehyde	1232	1804	LRI, MS				0.1
Carvone	1241	1750	LRI, MS		2.5		0.3
p-Menth-9-en-1-ol	1291	1945	LRI, MS	1.2			
p-Cymen-7-ol	1293	2067	LRI, MS				1.5
Sesquiterpene hydrocarbons				**30.8**	**51.5**	**29.4**	**33.9**
δ-Elemene	1335	1476	LRI, MS		0.6		
α-Longipinene	1351		LRI, MS				0.1
α-Cubebene	1352	1466	LRI, MS	t	0.8	0.4	
Cyclosativene	1363	1492	LRI, MS				
α-Ylangene	1372	1493	LRI, MS			0.3	
α-Copaene	1377	1503	LRI, MS	0.6	0.8	0.6	1.0
β-Cubebene	1382	1547	LRI, MS	0.7	2.5		0.1
β-Bourbonene	1385	1535	LRI, MS	1.2	1.0	1.8	0.5
β-Elemene	1387	1598	LRI, MS	0.2			0.2
α-Elemene	1396		LRI, MS			0.2	
α-Gurjunene	1408	1529	LRI, MS				1.2
α-Funebrene	1409	1510	LRI, MS				0.2
Caryophyllene	1415	1612	LRI, MS, Co-GC	10.0	4.9	8.2	10.1
Aromadendrene	1422	1628	LRI, MS	0.2	0.6		0.2
β-Gurjunene (Calarene)	1423	1632	LRI, MS		0.3		0.3
epi-Bicyclosesquiphellandrene	1424		LRI, MS	0.6			0.7
β-Cedrene	1430	1638	LRI, MS			0.9	
γ-Elemene	1436	1650	LRI, MS	0.8	0.1		0.1
β-Humulene	1437	1674	LRI, MS		t		0.3
(*E*)-β-Farnesene	1452	1673	LRI, MS	t	0.3		0.1
α-Humulene	1455	1689	LRI, MS	3.1	0.9	2.8	3.4
allo-Aromadendrene	1463	1661	LRI, MS	1.9	0.5	2.8	0.4
Germacrene D	1477	1726	LRI, MS	5.8	14.3	3.7	3.9
γ-Muurolene	1478	1704	LRI, MS	1.0	0.3	1.0	
(*E*)-β-Ionone	1482	1957	LRI, MS, Co-GC	1.4	0.5		
ar-Curcumene	1483	1784	LRI, MS				0.2
α-Amorphene	1487	1679	LRI, MS	0.2	0.5	0.5	2.5
cis-β-Guaiene	1490	1694	LRI, MS				1.5
Valencene	1494	1741	LRI, MS			0.5	0.2
Bicyclosesquiphellandrene	1491	1626	LRI, MS	0.2		1.8	0.1
Bicyclogermacrene	1492	1756	LRI, MS	1.9			0.7
α-Muurolene	1503	1740	LRI, MS	0.5		0.3	0.1
γ-Cadinene	1515	1776	LRI, MS	0.5	7.5		0.1
δ-Cadinene	1526	1773	LRI, MS		13.5	2.7	3.1

Table1 (*contd.*)

Cadina-1,4-diene	1538	1799	LRI, MS		0.9		0.2
α-Calacorene	1541	1942	LRI, MS		0.3	0.2	0.2
Germacrene B	1554	1856	LRI, MS	t			0.6
Cadalene	1677	2256	LRI, MS		0.4	0.7	1.6
Oxygenated sesquiterpenes				**21.4**	**10.4**	**20.6**	**21.3**
1-*endo*-Bourbonanol	1520		LRI, MS			0.5	
Ledol	1565	2057	LRI, MS	0.8		1.7	
(*E*)-Nerolidol	1566	2050	LRI, MS		1.4		
Longipinanol	1572				t		
Germacrene D 4-ol	1577	2069	LRI, MS				3.0
Spathulenol	1578	2150	LRI, MS, Co-GC	5.8	1.8		0.1
Caryophyllene oxide	1580	2008	LRI, MS, Co-GC	7.7	4.0	8.8	5.0
Globulol	1588	2098	LRI, MS				0.2
Viridiflorol	1591	2104	LRI, MS	0.7		2.0	
Widdrol	1600		LRI, MS			1.4	0.1
Humulene epoxide II	1605	2071	LRI, MS			0.9	1.3
Cedrenol	1606	2133	LRI, MS	4.8		0.1	0.1
Torreyol	1645		LRI, MS				6.5
α-Cadinol	1649	2255	LRI, MS		1.9	0.7	4.0
Caryophyllenol II	1650	2396	LRI, MS			3.2	0.1
(*E*)-Isoelemicin	1659	2403	LRI, MS	1.6			0.2
Patchoulol	1664		LRI, MS			0.5	
Germacrone	1685		LRI, MS		t		
α-Bisabolol	1686	2219	LRI, MS	t	0.8		0.3
(*Z,E*)-Farnesol	1689	2276	LRI, MS		0.5		0.2
Vulgarol B	1691		LRI, MS			0.8	
cis(*Z*)-α-Bisabolene-epoxide	1698		LRI, MS				0.2
Phenolic compounds				**2.5**	**3.6**	**14.3**	**10.3**
Thymol	1290	2198	LRI, MS, Co-GC	0.2			0.1
Carvacrol	1297	2239	LRI, MS, Co-GC			13.5	9.6
4-Vinyl guaiacol	1312	2180	LRI, MS	1.8	2.0		0.1
Eugenol	1353	2186	LRI, MS, Co-GC	0.5	1.6	0.8	0.5
Hydrocarbons				**10.2**	**4.3**	**6.8**	**6.5**
α-Ionene	1208		LRI, MS			0.2	
Heptadecane	1700	1700	LRI, MS, Co-GC		0.2		0.1
Pentacosane	2500	2500	LRI, MS	1.0	0.8	1.9	0.7
Hexacosane	2600	2600	LRI, MS	0.2	0.2		
Heptacosane	2700	2700	LRI, MS	4.0	1.5	1.7	1.9
Octacosane	2800	2800	LRI, MS	0.6	0.1	0.5	
Nonacosane	2900	2900	LRI, MS	2.8	1.0	1.2	2.5
Triacontane	3000	3000	LRI, MS	0.4	t		0.1
Hentriacontane	3100	3100	LRI, MS	1.2	0.5	1.3	1.2
Fatty acids				**9.7**	**2.6**	**13.1**	**0.6**
Hexadecanoic acid	1957	2931	LRI, MS, Co-GC	9.3	1.8	10.7	0.1
Heptadecanoic acid	2054	2975	LRI, MS, Co-GC	0.1			
(*Z*)-9-Octadecenoic acid	2115		LRI, MS, Co-GC	0.1			
(*Z,Z*)-9,12-Octadecadienoic acid	2122	3157	LRI, MS, Co-GC		0.6	2.4	0.5
Octadecanoic acid	2172	3402	LRI, MS, Co-GC	0.2	0.2		
Others				**1.2**	**1.3**	**2.9**	**1.0**
1-Octen-3-ol	977	1425	LRI, MS	0.5	0.3	0.1	0.1
Octan-3-ol	992	1394	LRI, MS, Co-GC		0.4		
2-Pentylfuran	1002	1244	LRI, MS	t			
Dihydroedulan I	1296		LRI, MS	0.4	0.1		
Dihydroactinidiolide	1486	2354	LRI, MS		0.5	1.6	
13-*epi*-Manoyl oxide	1963	2388	LRI, MS			0.7	
Manoyl oxide	1994		LRI, MS			0.5	0.1
Kaurene	2048	2399	LRI, MS				0.8
Squalene	2829		LRI, MS	0.3			
Carbonylic compounds				**7.6**	**4.7**	**3.2**	**2.6**
(*E*)-2-Hexenal	854	1231	LRI, MS		t		
Benzaldehyde	963	1543	LRI, MS, Co-GC		0.2		
1-Octen-3-one	975	1312	LRI, MS		0.3		
Acetophenone	1036	1645	LRI, MS			0.1	
Phenylacetaldehyde	1048	1663	LRI, MS, Co-GC	0.1	0.4	0.1	0.1
p-Methoxyacetophenone	1302	1797	LRI, MS, Co-GC	2.3			1.5
(*E*)-β-Damascenone	1380	1835	LRI, MS	1.1	1.0	0.4	0.2
(*E*)-α-Ionone	1419		LRI, MS, Co-GC	0.3			
Hexahydrofarnesylacetone	1845	2131	LRI, MS	3.8	2.8	2.6	0.8
Total amount of compounds				**86.2**	**94**	**93.4**	**92.4**

[a]: Kovats retention index on HP-5 MS column; [b]: Kovats retention index on HP Innowax; [c]: LRI linear retention index; MS identification based on comparison of mass spectra; Co-GC retention time identical to authentic compounds; [d]: t = trace<0.05.

Teucrium polium L. ssp. *capitatum* (L.) Arcangeli (syn. *Teucrium capitatum* L.) is widespread diffused in the dry and stony places of Greece and almost all Mediterranean countries [15]. It is a perennial and prostrate species with white-tobright pink flowers, crenate leaves and ramified indumentum. This plant is used in folk medicine to treat diabetes and intestinal troubles. Aerial part extracts were known for their anti-inflammatory, antibacterial, antihypertensive, hypoglycemic, hypolipidemic, anorexic and antioxidant activities [9]. The species has been reported for its neo-clerodane diterpenoids and essential oils [16].

In continuation of our studies on the possible phytotoxic activity of essential oils from plants collected in the Mediterranean area [9,17-18], we analyzed the chemical composition of the essential oils of four different species of *Teucrium* and carried out *in vitro* experiments in order to verify the possible effects of the essential oils and of their main components on germination and initial radicle elongation of *Raphanus sativus* L. (radish) and *Lepidium sativum* L. (garden cress).

In the four oils, 131 compounds in all were identified (Table 1): 53 for oil of *T. arduini* (86.2% of the total oil), 71 for *T. maghrebinum* (94.0% of the oil), 51 for *T. montbretii* (93.4% of the oil) and 89 for *T. polium* (92.4% of the oil). The components are listed in Table 1 according to their retention indices on HP-5 column and are classified on the basis of their chemical structures into 9 classes.

The oil of *T. arduini* comprised mainly sesquiterpenes (52.2%), particularly sesquiterpene hydrocarbons (30.8%). In particular, 21 sesquiterpene hydrocarbons were present in the oil, with a prevalence of caryophyllene (10.0%), germacrene D (5.8%) and α-humulene (3.1%). In particular, 7 oxygenated sesquiterpenoids were present in the oil, being the main constituents caryophyllene oxide (7.7%), spathulenol (5.8%) and cedrenol (4.8%). Hydrocarbons were quite abundant (10.2%) and were constituted by almost heptacosane (4.0%) and nonacosane (2.8%). Fatty acids (9.7%) were represented by hexadecanoic acid (9.3%), while hexahydrofarnesylacetone (3.8%) was the main constituent among carbonylic compounds (7.6%). Blazevic and coworkers [12] and Kovacevic and coworkers [13] analyzed the essential oil composition of this species: in the both cases, β-caryophyllene and germacrene D were the main compounds, as in the our samples.

In the oil of *T. maghrebinum* the most abundant compounds were germacrene D (14.3%), δ-cadinene (13.5%) and γ-cadinene (7.5%). On the whole, the oil was constituted mainly by sesquiterpenes (61.9%) and monoterpenes (15.6%). Sesquiterpene hydrocarbons (51.5%) prevailed over oxygen containing sesquiterpenes (10.4%). Twenty-three sesquiterpene hydrocarbons were present in the oil, with a prevalence of germacrene D (14.3%), δ-cadinene (13.5%), γ-cadinene (7.5%) and caryophyllene (4.9%). Among 8 oxygen containing sesquiterpenes, the most abundant was caryophyllene oxide (4.0%). Among monoterpenes, 6 hydrocarbons accounted for the 8.9% of the total oil, with limonene (4.4%) as the main compound, while carvone (2.5%) prevailed among the 12 oxygen containing monoterpenes. The composition of essential oil of *T. maghrebinum*, growing in Algeria, was studied by Formisano and coworkers [8]: germacrene D, δ-cadinene and γ-cadinene were the most abundant compounds.

The oils from *T. montbretii* and *T. polium* from Greece were previously analyzed by us [9]; for the present study the aerial parts of the plants have been collected again in the same place and the GC and GC-MS analyses have been repeated on the new samples. Table 1 shows that results obtained in the present study are similar to those of the previously studies [9], even if the percentages of some components are slightly different.

In the essential oil of *T. montbretii*, the main fraction was constituted by sesquiterpenes (50.0%). Among these, sesquiterpene hydrocarbons (29.4%) prevailed on oxygen containing sesquiterpenes (20.6%). In the first fraction caryophyllene (8.2%), germacrene D (3.7%), *allo*-aromadendrene (2.8%) and α-humulene (2.8%) predominated, while among the 11 oxygen containing sesquiterpenes the most abundant compounds were caryophyllene oxide (8.8%) and caryophyllenol II (3.2%). The phenols were quite abundant (14.3%) and were constituted almost entirely by carvacrol (13.5%). Fatty acids (13.1%) were mainly represented by hexadecanoic acid (10.7%), while linalool (2.7%) was the main constituent of oxygenate monoterpenes (3.1%). The composition of the oil of *T. montbretii* confirmed literature data [9], in which the main fraction was constituted by sesquiterpenes.

As in the other oils studied, in the oil of *T. polium* sesquiterpenes constituted also the main fraction and accounted for the 55.2% of the total oil with a prevalence of sesquiterpene hydrocarbons (33.9%) over oxygen containing sesquiterpenes (21.3%). Among the 30 sesquiterpene hydrocarbons, caryophyllene (10.1%), germacrene D (3.9%), α-humulene (3.4%), δ-cadinene (3.1%) and α-amorphene (2.5%) were the most abundant. In the other fraction, torreyol (6.5%), caryophyllene oxide (5.0%) and α-cadinol (4.0%) prevailed. Monoterpenes contributed for the 16.2% of the oil with a predominance of oxygen containing

monoterpenes (15.0%), particularly *cis*-verbenone (4.0%) and *cis*-verbenol (2.0%).

The phenolic compounds (10.3%) were represented almost entirely by carvacrol (9.6%). The chemical analysis of the essential oil of *T. polium* was also reported by Antunes and coworkers [19] and Cozzani and coworkers [20] with different compositions.

The four essential oils were evaluated for their phytotoxic activity against germination and initial radicle elongation (Table 2) of radish and garden cress, two species usually utilized in biological assays [21]. Also the main components of these oils were assayed for the same activity (Table 3).

The oils affected the germination and the radicle elongation of radish and garden cress in a distinct way. Radicle elongation seemed to be more affected in comparison to germination. The germination of radish did not appeared sensitive to the four essential oils: only the essential oil of *T. polium*, at a dose of 1.25 µg/mL, significantly inhibited the germination of radish. The germination of garden cress did not appeared sensitive to the four essential oils, too: only the essential oil of *T. arduini*, at the highest dose tested, significantly inhibited the germination of garden cress. The radicle elongation of radish was significantly inhibited by the all oils: particularly, the essential oil of *T. arduini*, at the highest doses tested, inhibited the radicle elongation of radish, and in minor measure, of garden cress. The essential oil of *T. montbretii* inhibited only the radicle elongation of radish, at the dose of 1.25 µg/mL.

The main components, both monoterpenes and sesquiterpenes, were also assayed against germination and radicle elongation of two seeds at four concentrations (10^{-3}-10^{-6}). Monoterpenes resulted the more active compounds: in fact, limonene was the most active compound, at the highest doses assayed, against both germination and radicle elongation of radish; also carvacrol is very active in inhibiting radicle elongation of garden cress.

Among sesquiterpenes, *allo*-aromadendrene, at 10^{-4} M, inhibited, in a significative way, only the germination of garden cress; caryophyllene, at the lowest dose, inhibited significantly only the germination of radish. On the other hand, caryophyllene oxide promoted the germination of this seed. Radicle elongation of the seeds was never affected.

In previous papers, Kordali and coworkers [22] reported that limonene and β-pinene inhibited seed germination of *Chenopodium album*. Also linalool completely inhibited seed germination of *Cassia occidentalis*. Kordali and coworkers also reported phytotoxic activity of carvacrol [23].

The different degrees of biological activity could be related to the composition of the essential oils: in particular, the presence of monoterpenoids [18] could explain the biological activity. Although the mechanisms of essential oil action against germination is still unclear, it reported that volatile oils, monoterpenoids and also sesquiterpenoids inhibit cell division and induce structural breaks and decomposition in roots [18,24]. In a previous paper, we demonstrated that essential oils of different species of *Salvia*, rich in sesquiterpenoid compounds, showed a good antigerminative activity, both *in vitro* and *in vivo* [18]. So, both monoterpenoids and sesquiterpenoids appear to be involved in allelopathic effects: some monoterpenoids, as citronellol, linalool, α-pinene and limonene, are potent inhibitors of seed germination and radicle elongation; moreover, sesquiterpenoid compounds, as β-maaliene, α-isocomene, β-isocomene, δ-cadinene, 5-hydroxy-calamenene and 5-methoxy-calamenene were recently shown to inhibit the seedling growth of associated native vegetation, and thus possibly help in successful invasion in the introduced sites [18,25].

Experimental

Plant material: Aerial parts of *T. arduini* and *T. maghrebinum* were cultivated in the Orto Botanico "G. E. Ghirardi", University of Milano, Toscolano (Garda Lake, Italy). Seeds were provided by the Jardin des Plantes, Paris. Plant materials were collected in June 2009, at the full flowering stage. Aerial parts of *T. montbretii* ssp. *heliotropiifolium* were collected in June 2009, at Spoa, on the Karpathos Island of Greece. Aerial parts of *T. polium* ssp. *capitatum* were collected in June 2009, at Gournes, about 15 km east of Heraklion, Crete.

Chemicals: *allo*-Aromadendrene, β-pinene**,** carvacrol**,** caryophyllene, caryophyllene oxide, limonene and linalool were purchased from Sigma-Aldrich Co. (Milan, Italy).

Isolation of the essential oils: The air-dried samples were ground in a Waring blender and then subjected to hydrodistillation for 3 h using *n*-hexane as a solvent. The extracts were dried over anhydrous sodium sulphate and then stored in sealed vials, at 20°C, ready for the GC and GC-MS analyses. The hydrodistillation yielded 0.5%, 0.9%, 0.7% and 0.8% of pale yellow oil (on a dry mass basis) for *T. arduini*, *T. maghrebinum*, *T. polium* ssp. *capitatum* and *T. montbretii* ssp. *heliotropiifolium* respectively.

Table 2: Biological activities of essential oils of *T. arduini* (*Ar*), *T. maghrebinum* (*Ma*), *T. montbretii* ssp. *heliotropiifolium* (*Mo*) and *T. polium* ssp. *capitatum* (*Po*) against germination and initial radicle elongation of radish and garden cress, 120 h after sowing. Results are shown as mean ± standard deviation (SD) of three experiments.

Raphanus sativus								
	Germinated seeds				**Radicle elongation (cm)**			
Doses	***Ar***	***Ma***	***Mo***	***Po***	***Ar***	***Ma***	***Mo***	***Po***
Control	6.2±1.2	6.2±1.2	6.2±1.2	6.2±1.2	0.5±0.3	0.5±0.3	0.5±0.3	0.5±0.3
2.5 µg/mL	6.0±1.0	3.7±3.2	6.7 ± 0.6	5.7±0.6	0.3±0.2*	0.4±0.2	0.4 ± 0.3	0.4±0.2
1.25 µg/mL	5.3±1.5	5.7±1.2	6.7 ± 1.2	3.7±0.6*	0.3±0.2*	0.4±0.2	0.4 ± 0.2*	0.4±0.2
0.625 µg/mL	4.7±0.6	7.0±1.0	5.0 ± 0.0	5.7±1.5	0.3±0.2*	0.5±0.3	0.4 ± 0.2	0.3±0.2*
0.25 µg/mL	6.3±1.2	5.3±0.6	4.7 ± 3.1	4.7±0.6	0.4±0.2	0.4±0.2	0.5 ± 0.3	0.6±0.3
0.125 µg/mL	4.0±1.7	5.0±1.0	5.3 ± 1.2	4.3±1.2	0.4±0.1	0.4±0.3	0.4 ± 0.2	0.5±0.3
0.06 µg/mL	4.3±1.2	6.3±0.6	5.0 ± 1.0	5.0±1.0	0.3±0.2	0.2±0.2***	0.4 ± 0.2	0.5±0.2
Lepidium sativum								
	Germinated seeds				**Radicle elongation (cm)**			
Doses	***Ar***	***Ma***	***Mo***	***Po***	***Ar***	***Ma***	***Mo***	***Po***
Control	9.2±0.8	9.2±0.8	9.2±0.8	9.2±0.8	2.2±1.2	2.2±1.2	2.2±1.2	2.2±1.2
2.5 µg/mL	7.7±0.6*	9.3±0.6	9.3±0.6	9.3±0.6	1.7±1.0*	2.3±1.0	2.2±0.9	2.5±0.9
1.25 µg/mL	8.0±1.0	9.3±0.6	9.7±0.6	9.0±1.7	2.4±1.0	1.9±0.9	2.2±1.1	1.9±1.1
0.625 µg/mL	8.0±2.0	9.3±0.6	9.3±0.6	9.3±0.6	2.3±1.2	2.4±1.0	2.5±0.9	2.2±1.0
0.25 µg/mL	10.0±0.0	8.3±0.6	9.0±1.0	8.3±1.2	2.2±1.1	2.1±1.1	2.4±1.3	1.8±0.9*
0.125 µg/mL	9.0±1.0	8.0±0.0	9.0±1.0	9.7±0.6	2.3±0.9	1.9±1.0	2.2±0.9	2.7±1.2
0.06 µg/mL	8.7±2.3	9.3±0.6	9.0±1.0	9.3±1.2	2.4±1.1	1.7±1.2*	2.1±1.1	2.3±1.1

Note: *p < 0.05; ** p<0.01; ***p<0.001 *vs.* control.

Table 3: Biological activities of main constituents of essential oils of *T. arduini*, *T. maghrebinum*, *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* against germination and initial radicle elongation of radish and garden cress, 120 h after sowing. Results are shown as mean ± standard deviation (SD) of three experiments.

	Raphanus sativus							
	Germinated seed				**Radicle elongation (cm)**			
	[10^{-3}]	**[10^{-4}]**	**[10^{-5}]**	**[10^{-6}]**	**[10^{-3}]**	**[10^{-4}]**	**[10^{-5}]**	**[10^{-6}]**
Control	9.0±0.6	9.0±0.6	9.0±0.6	9.0±0.6	7.8±2.0	7.8±2.0	7.8±2.0	7.8±2.0
β-Pinene	8.7±0.0	8.0±0.0	9.4±1.4	8.0±2.1	5.6±0.6	6.5±0.9	5.6±0.6	6.1±0.8
Limonene	5.1±1.4**	5.8±2.8*	6.2±4.9	8.3±2.1	3.9±0.4**	3.9±0.5**	8.2±1.1	6.1±0.7
Linalool	7.2±1.4	7.6±2.1	7.0±2.1	8.7±2.8	6.7±1.1	6.5±0.7	5.2±0.7	6.7±0.6
Carvacrol	7.6±0.7	9.4±1.4	9.1±2.1	8.3±3.5	7.8±1.0	8.7±1.3	8.7±1.3	9.5±1.6
allo-Aromadendrene	9.0±0.0	9.0±0.0	9.7±0.6	9.7±0.6	8.6±4.5	9.2±5.0	8.6±5.4	8.5±4.5
Caryophyllene	9.7±0.6	8.3±0.6	9.0±1.0	7.7±0.6*	9.4±4.7	10.9±4.6	9.1±5.0	7.8±6.3
Caryophyllene oxide	7.7±1.5	9.0±1.0	10.0±0.0*	10.0±0.0*	8.7±5.1	9.1±5.1	8.5±5.3	7.6±4.9
	Lepidium sativum							
	Germinated seed				**Radicle elongation (cm)**			
	[10^{-3}]	**[10^{-4}]**	**[10^{-5}]**	**[10^{-6}]**	**[10^{-3}]**	**[10^{-4}]**	**[10^{-5}]**	**[10^{-6}]**
Control	9.2±0.4	9.2±0.4	9.2±0.4	9.2±0.4	8.1±1.1	8.1±1.1	8.1±1.1	8.1±1.1
β-Pinene	9.6±0.0	9.6±0.0	9.6±0.0	9.6±0.0	8.5±0.8	8.5±0.8	7.2±0.8	7.7±0.8
Limonene	7.3±3.5*	9.6±0.0	9.3±0.7	8.3±1.4*	7.7±0.8	5.5±0.9	6.4±0.6	5.1±0.8
Linalool	8.9±0.0	8.6±0.7	8.3±1.4	9.3±0.7	6.8±0.7	8.5±0.9	6.0±0.7	7.2±0.8
Carvacrol	8.6±0.7	8.9±0.0	9.6±0.0	8.5±1.4	2.5±0.3***	7.2±0.8	7.7±0.8	8.9±0.7
allo-Aromadendrene	9.3±1.2	8.3±0.6*	9.0±0.0	9.7±0.5	8.2±2.6	8.3±3.4	7.0±3.8	7.7±3.4
Caryophyllene	9.7±0.6	9.5±0.7	8.3±1.5	9.7±0.6	6.2±2.9	7.0±3.4	7.9±3.7	8.8±2.8
Caryophyllene oxide	9.0±0.0	8.7±0.6	8.5±2.1	9.7±0.6	7.3±2.4	7.2±3.6	7.9±1.4	7.5±2.8

Note: *p < 0.05; ** p<0.01; ***p<0.001 *vs.* control.

GC and GC/MS analyses: GC analyses were carried out on a Hewlett Packard Sigma 115 gas chromatograph equipped with FID and a HP 5MS fused silica capillary column (30 m x 0.25 mm i.d.; film thickness: 0.25 µm). Column temperature: 40°C, with 5 min initial hold, and then to 260°C at 2°C/min, 260°C (20 min); injection mode splitless (1 µL of a 1:1000 *n*-pentane solution). Injector and detector temperatures were 250°C and 290°C, respectively. Analysis was also run by using a fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm, i.d.; 0.25 µm film thickness). In both cases, carrier gas was He, with flow rate of 1 mL/min. GC-MS analyses were performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica DB-5 capillary column (30 m x 0.25 mm i.d.; 0.33 µm film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy 70 eV; electron multiplier voltage 2,000 V. Mass spectra were scanned in the range 40-500 amu, scan time 5 scans/s. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C.

Identification of components: Most constituents were identified by gas chromatography by comparison of their linear retention indices (LRI) with either those of the literature [26,27] or with those of authentic

compounds available in our laboratories. The linear retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{28}) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature [26,28] and a home made library. Components relative concentrations were obtained by peak area normalization. No response factors were calculated.

Biological assay: A bioassay based on germination and subsequent radicle growth was used to study phytotoxic effects of the essential oils of *T arduini*, *T. maghrebinum*, *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* on seeds of *Raphanus sativus* L. cv. "Saxa" (radish), and *Lepidium sativum* L. (cress). Seeds of *L. sativum* and *R. sativus* were purchased from Blumen srl, Piacenza, Italy. The seeds were surface-sterilized in 95% ethanol for 15 s and sown in Petri dishes (Ø=90 mm), containing five layers of Whatman filter paper, impregnated with 7 mL of distilled water (control) or 7 mL of tested solution of the essential oil at the different assayed doses. The germination conditions were 20 ± 1 °C, with natural photoperiod. The essential oils, in water–acetone mixture (99.5:0.5), were assayed at the doses of 2.5, 1.25, 0.625, 0.25, 0.125 and 0.062 μg/mL. The pure compounds, dissolved in water–acetone mixture (99.5:0.5), were assayed at the concentrations of 10^{-6} M, 10^{-5} M, 10^{-4} M, and 10^{-3} M. Controls performed with water–acetone mixture alone showed no appreciable differences in comparison with controls in water alone. Seed germination was observed directly in Petri dishes, each 24 h. Seed was considered germinated when the protrusion of the radicle became evident [29]. After 120 h (on the fifth day), the effects on radical elongation were measured in centimeters. Each determination was repeated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean ± SD of both germination and radical elongation. The Student's t test of independence was applied [30].

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Chemical Constituents and Larvicidal Activity of *Hymenaea courbaril* Fruit Peel

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The chemical compositions of the essential oils from the peel of ripe and unripe fruits of *Hymenaea courbaril* L., obtained by hydrodistillation, were analyzed by GC and GC-MS. The main constituents of the essential oil from the peel of the ripe fruits were the sesquiterpenes α-copaene (11.1%), spathulenol (10.1%) and β-selinene (8.2%), while germacrene-D (31.9%), β-caryophyllene (27.1%) and bicyclogermacrene (6.5%) were the major compounds in the oil from unripe fruits. The essential oils were tested against *Aedes aegypti* larvae and showed LC_{50} values of 14.8 ± 0.4 μg/mL and 28.4 ± 0.3 μg/mL for the ripe and unripe fruit peel oils, respectively. From the peel of the ripe fruits, the diterpenes zanzibaric acid and isoozic acid were isolated, along with the sesquiterpene caryolane-1,9β-diol. To the best of our knowledge, this is the first report of this sesquiterpene in the genus. The structures of all compounds isolated were identified on the basis of their spectral data (IR, MS, 1D- and 2D-NMR) and by comparison with literature spectral data.

Keywords: *Hymenaea courbaril*, Caesalpinoideae, sesquiterpenes, diterpenes, *Aedes aegypti*.

The genus *Hymenaea* (Caesalpinoideae) comprises 14 species [1] and is widely distributed from Central to South America, mainly in the Amazon basin [2]. *H. courbaril* L., known popularly as "jatobá", is useful for its timber and has been employed in folk medicine as an anodyne, antiseptic, astringent, expectorant, laxative, purgative, sedative, stimulant, tonic, and vermifuge [3]. *Enantio*-labdanoic and *enantio*-halimane type diterpenes have been isolated from *H. courbaril* [2,4-12], but there is no report of essential oils.

Interest in the control of *Aedes aegypti* lies in the fact that it acts as a vector of dengue and dengue hemorrhagic fever. Dengue is an endemic disease in most of the countries of the Americas and, over the past twenty years, has shown sustained cycles of outbreaks, every 3 to 5 years, and outbreaks are re-emerging in Brazil [13]. There are no effective vaccines, and vector control is the only way to minimize the transmission of the virus. Plant products have been used against the vectors, and plant-derived products can act as larvicides [14]. Essential oils from plants like *Lippia* species [15] and *Croton zehntneri* [16], and various extracts and compounds [17,18] have been documented for larvicidal activity towards *Aedes aegypti*.

Table 1: Volatile components identified in the essential oils from ripe (oil A) and unripe fruit peel (oil B) of *H. courbaril*.

Compound	RI	Oil A (%)	Oil B (%)
δ-Elemene	1333	-	0.1
α-Cubebene	1342	1.7	0.4
α-Ylangene	1364	0.5	0.1
α-Copaene	1371	11.1	4.2
β-Elemene	1384	5.0	-
7-*epi*-Sesquithujene	1386	-	0.8
Cyperene	1401	-	0.1
β-Caryophyllene	1414	2.0	27.1
β-Copaene	1424	0.6	0.1
α-*trans*-Bergamotene	1431	-	0.9
Aromadrendrene	1432	1.6	-
(*Z*)-β-Farnesene	1439	-	1.2
cis-Muurola-3,5-diene	1447	-	0.1
α-Humulene	1450	0.3	4.2
Allo-Aromadrendene	1454	0.6	0.5
γ-Muurolene	1469	7.9	-
Amorpha-4,7(11)-diene	1475	0.3	
trans-Cadina-1(6),4-diene	1475	-	2.3
Germacrene-D	1482	-	31.9
β-Selinene	1488	8.2	0.6
δ-Selinene	1490	5.7	-
trans-Muurola-4(14),5-diene	1491	-	0.8
Bicyclogermacrene	1494	-	6.5
γ-Cadinene	1507	2.7	1.1
δ-Amorphene	1512	5.3	0.3
trans-Calamenene	1515	1.1	-
δ-Cadinene	1516	-	3.3
trans-Cadina-1,4-diene	1530	-	0.2
α-Cadinene	1534	-	0.2
α-Calacorene	1535	1.1	-
Germacrene-B	1553	1.8	1.7
Spathulenol	1572	10.1	0.9
Caryophyllene oxide	1577	6.9	2.1
β-Copaen-4α-ol	1583	1.2	-
Globulol	1583	-	0.2
Salvial-4(14)-en-1-one	1587	1.8	-
Humulene epoxide II	1604	1.2	0.3
1-*epi*-Cubenol	1622	1.3	0.2
Camphoric acid	1631	0.8	-
epi-α--Muurolol	1637	1.1	-
α--Muurolol	1641	0.7	-
Selin-11-en--4α-ol	1649	1.8	-
α-Cadinol	1653	-	0.8
(*Z*)-14-Hydroxy-caryophyllene	1664	0.4	-
Mustakone	1668	0.9	-
Amorpha-4,9-dien-2-ol	1684	0.4	-
Levomenol	1887	-	0.1
Oxygenated sesquiterpenes		28.6	4.6
Non-oxygenated sesquiterpenes		57.5	88.7
Total		**86.1**	**93.3**

As part of a continuous research program on plants from northeast Brazil, this work describes the isolation of the diterpenes zanzibaric acid and isoozic acid, and the sesquiterpene caryolane-1,9β-diol from the peel of the ripe fruits of *H. courbaril*, and the chemical composition of the essential oils from the peel of both ripe and unripe fruits of *H. courbaril*, as well as the evaluation of these oils for their larvicidal activity against *A. aegypti*.

Successive chromatographic treatments of the *n*-hexane extract of the air-dried, ripe, fruit peel of *H. courbaril* afforded the *ent*-labdane diterpene zanzibaric acid [19] and the sesquiterpene caryolane-1,9β-diol [20]; from the ethyl acetate extract, the diterpene isoozic acid was isolated [21].

The chemical composition of the essential oils from the peel of the ripe and unripe fruits of *H. courbaril*, including retention index (RI) values listed in order of elution from the DB-5MS column, and the percentage relative to each constituent is presented in Table 1. From the two analyzed oil samples, a total of 47 compounds were identified, all of which were sesquiterpenes, representing 86.1% and 93.3% of the oils. α-Copaene (11.1%), spathulenol (10.1%), β-selinene (8.2%), γ-muurolene (7.9%) and caryophyllene oxide (6.9%) were the major constituents identified in the essential oil from ripe fruit peel (oil A), while germacrene D (31.9%), β-caryophyllene (27.1%), bicyclogermacrene (6.5%), α-humulene (4.2%), and α-copaene (4.2%) were the prevalent compounds in the essential oil from unripe fruit peel (oil B).

The larvicidal potential of the essential oils was evaluated against *A. aegypti* larvae. The essential oil from the ripe fruit peel (oil A) showed stronger larvicidal activity against third-instar *A. aegypti* larvae (LC_{50} 14.8 $\pm$ 0.4 μg/mL) than the oil from unripe fruit peel (oil B; LC_{50} 28.4 $\pm$ 0.3 μg/mL); this can be justified by the higher concentration of oxygenated sesquiterpenes (28.6%), mainly spathulenol [22], present in the essential oil from mature fruit peel (oil A). *O,O'*-(Thiodi-4,1-phenylene)bis(*O,O*-dimethyl phosphorothioate (Temephos®) was used as a positive control (LC_{50} 1.4 $\pm$ 0.2 μg/mL).

This study showed the efficacy of the essential oils from the peels of ripe and unripe fruits of *H. courbaril* against *A. aegypti* larvae. Furthermore, it may represent a contribution to mosquito control. The isolation of caryolane-1,9β-diol represents the first report of this sesquiterpene in the genus.

Experimental

Plant material: Fruits of *H. courbaril* were collected in 2007 in Crato County, State of Ceará, northeast Brazil. A voucher specimem (#EAC41026) is deposited at the Herbário Prisco Bezerra, Departamento de Biologia, Universidade Federal do Ceará, Brazil.

Extraction of essential oils: The ripe (636 g) and unripe fruit peels (940 g) of *H. courbaril* were triturated and subjected to hydrodistillation in a Clevenger-type apparatus for 2 h to afford 0.27 g (0.042%) and 0.67 g

(0.071%) of pale yellow oils, respectively. The yields (w/w) were calculated based on the fresh weight of the plant materials. The isolated oils, after drying over anhydrous sodium sulfate (0.20 g) and filtration, were stored in sealed glass vials and maintained under refrigeration before analysis.

Analytical conditions: The essential oils were analyzed by GC/MS on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system equipped with a AOC-20i autosampler under the following conditions: J&W Scientific DB-5MS fused silica capillary column (30 m x 0.25 mm i.d., x 0.25 μm film thickness, composed of 5%-phenyl-95%-methylpolysiloxane) operating in EI mode at 70 eV. Helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL/min. The injection volume was 0.5 μL (split ratio of 1:100), the injector temperature 250°C and the ion-source temperature 280°C. The oven temperature was programmed from 50°C (isothermal for 1.5 min), with an increase of 4°C/min to 200°C, then 10°C/min to 300°C, ending with a 10 min isothermal period at 300°C. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and fragments from 40 to 500 Da.

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) instrument, under the following operational conditions: capillary ZB-5M5 column (5%-phenyl-arylene-95%- methylpolysiloxane fused silica capillary column 30 m x 0.25 mm i.d. x 0.25 μm film thickness), under the same conditions reported for the GC-MS. Quantification of each constituent was estimated by area normalization (%). Compound concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Identification of individual components of the essential oils was performed by computerized matching of the acquired MS with those stored in NIST21 and NIST22 mass spectral library of the GC/MS data system. Retention indices (RI) for all compounds were determined according to literature [23] for each constituent, as previously described [24].

Larvicidal bioassay: Aliquots of the essential oils tested (12.5 to 500 μg/mL) were placed in a beaker (50 mL) and dissolved in DMSO/H_2O 1.5% (20 mL). Fifty instar III larvae of *Aedes aegypti* were delivered to each beaker. After 24 h at room temperature, the number of dead larvae was counted and the lethal percentage calculated. A control using DMSO/H_2O 1.5% was carried out in parallel. For each sample, 3 independent experiments were run [25].

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Caryophyllene Oxide-rich Essential Oils of Lithuanian *Artemisia campestris* ssp. *campestris* and Their Toxicity

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The chemical composition of the essential oils of aerial parts of *Artemisia campestris* ssp. *campestris,* collected from ten different locations in Lithuania is detailed in this paper. The major component in all the oils was caryophyllene oxide (8.5-38.8%), whereas compounds with the caryophyllane skeleton ranged from 10.2 to 44.5%. Other representative constituents were germacrene D (≤15.0%), humulene epoxide II (≤8.1%), β-ylangene (≤7.7%), spathulenol (≤6.8%), β-elemene (≤6.8%), β-caryophyllene (≤6.2%), junenol (≤6.1%) and α- or β-pinene (≤5.5%). Eighty-seven compounds were identified, comprising 73.6-92.3% of the oils. The chemical composition was highly variable depending on the sample location.

Toxicity of *A. campestris* oils was determined using the brine shrimp (*Artemia* sp.) assay. LC_{50} values ranging to 20 μg/mL were obtained for three of the oils after 24 hours of exposure. Data of this test revealed that *A. campestris* ssp. *campestris* essential oils with dominant caryophyllene oxide are notably toxic.

Keywords: *Artemisia campestri*s ssp. *campestris*, essential oil, toxicity, brine shrimp lethality test, β-caryophyllene, caryophyllene oxide, β-elemene, β-ylangene, humulene epoxide II, junenol.

Artemisia (with up to 500 species) is one of the largest and most widely distributed genus of the Asteraceae. *A. campestris* (tribe Anthemideae, *Dracunculus* section), commonly known as field wormwood or field sagewort, is a perennial plant that is found in temperate regions throughout the northern hemisphere. Field wormwood, common for most parts of Europe, prefers open sites in light (sandy) and medium (loamy) soils. The species is polymorphous and is divided into several subspecies and forms. The plant is known as a medicinal herb with anthelmintic, antiseptic, cholagogue, deobstruent, emmenagogue, stomachic and tonic properties.

Flavonoids and terpenoids are the main secondary metabolites of the genus *Artemisia* [1]. Previous studies of the chemical composition of essential oils of *A. campestris* from different countries gave contrasting results (Table 1, [2-13]). However, the authors worked on plant material from various locations with many environmental variables, and in some of these studies, the plant parts used were not clearly specified and/or the subspecies was not indicated. Moreover, even for the same subspecies, i.e. *A. campestris* ssp. *glutinosa*, oil compositions varied from one study to another [2-5,12]. In addition, variation in the essential oil composition may occur depending on the phenological stage. This parameter was not always specified in the previous reports.

Furthermore, the literature shows the lack of research concerning the ethno-pharmacology, volatile oil composition and antibacterial activity of *A. campestris* from Lithuania. Thus the aim of this study was to determine the chemical composition and toxicity of the essential oils from the aerial parts of *A. campestris* ssp. *campestris* from various Lithuanian locations. All samples were collected at the full flowering to compare oil compositions of plants at the same phenological stage.

Table 1: Major constituents (%) of *Artemisia campestris* essential oils from various countries, according to the literature.

A. campestris subspecies	Organ	Extract	Main components, (%)	Place of origin	Reference
ssp. *glutinosa*	not specified	EO	*ar*-curcumene, caryophyllene oxide, *p*-cymene, β-pinene and germacrene D	Italy	2
ssp. *glutinosa*	flowers and leaves		β-pinene (6.9-57.2), germacrene D (0.4-28.6), bicyclogermacrene (1.0-14.5) and myrcene (1.7-11.2)	Italy	3
ssp. *glutinosa*	aerial parts	EO	γ-terpinene (≤46.5), capillene (33.1), 1-phenyl-2,4-pentadiyne (≤29.7) and spathulenol (≤11.3)	France	4
ssp. *glutinosa* even if ns	aerial parts	EO	(*Z*,*E*)-farnesol (10.3), cedrol (5.4) and verbenone (3.8)	Algeria	5
ssp. *campestris* and *borealis*	aerial parts	EO	α-pinene (≤16.5), β-pinene (≤10.7), caryophyllene oxide (≤18.2), spathulenol (≤18.7), *epi*-cubenol (≤14.2) and 1,8-cineole (≤19.2)	North west Italy	1, 6
ssp. *maritima Archangelis**	aerial parts ?	EO	β-pinene (17.8), cadin-4-en-7-ol (16.4), γ-terpinene (8.7), (*Z*)-β-ocimene (7.4), aromadendrene (6.7)	Portugal	7
ns	leaf	EO	β-pinene (24.0-49.8), α-pinene (5.9-12.5), *p*-cymene (3.4-9.4), limonene (4.9-9.3), spathulenol (1.2-8.9), γ-terpinene (2.0-6.5), eudesmol (1.0-6.4) and (*Z*)-β-ocimene (0.2-5.5)	Southern Tunisia	8
ns		vapor	α-pinene (41.0), β-pinene (29.7), limonene (6.4) and sabinene (4.5)	Southern Ural	9
ns	leaf	EO	β-pinene (24.2-27.9), *p*-cymene (17.4-22.3) and α-pinene (4.1-11.0)	South eastern Tunisia	10
ns	aerial parts	EO	spathulenol (9.2), β-pinene (9.1), α-pinene (3.4), limonene (2.5), germacrene D (3.3), 4-hydroxy-9-*epi*-β-caryophyllene (3.0)	Serbia	11
var. *glutinosa* Gay ex Bess	aerial parts	EO	β-pinene (41.0), *p*-cymene (9.9), α-terpinene (7.9), limonene (6.5) and myrcene (4.1)	Southern Tunisia	12
ns	flowers, leaves and stems, separately	EO	α-pinene (23.9, 23.0, 29.2) and spathulenol (23.9, 15.8, 29.2) in the flower, leaf and stem oils, respectively; bicyclogermacrene (12.0) in the flower and β-pinene (12.6) in the leaf oil	Iran	13

EO-essential oils, ns-name of subspecies not indicated, * also referred to as *Artemisia crithmifolia* L.

1. Chemical composition of Lithuanian *Artemisia campestris* essential oils: The composition of the essential oils of *A. campestris* collected from 10 populations in different parts of Lithuania (east, west and south of the country) and rich in caryophyllene oxide is presented in Table 2. The major component in all the oils was caryophyllene oxide (8.5-38.8%), while the amount of compounds with a caryophyllane skeleton (caryophyllene, its oxide and caryophylla-4(12),8(13)-dien-5α-ol) ranged from 10.2 to 44.5%.

In four samples (A, E, F and K) out of ten, germacrene D (9.4-15.0%) was the second main constituent. In the other six oils, four compounds were identified as the second principal component, i.e. caryophyllene (5.7-6.2% in oil samples H and J), β-pinene (4.9% in sample C), spathulenol (5.7-6.8% in B and D) and humulene epoxide II (8.1% in G).

The third most dominant components were found to be α- or β-pinene (4.5-5.5% in A, C and D), β-ylangene (7.7% in sample E), germacrene D (4.9-7.5% in G, H and J), humulene epoxide II (5.3% in B), junenol (6.1% in F) and β-elemene (6.8% in K). Caryophyllene oxide, germacrene D, α- or β-pinene and spathulenol have been previously determined in appreciable amounts in field wormwood oils from other countries (Table 1), while humulene epoxide II, junenol, β-elemene and β-ylangene have not been mentioned before among the main constituents of these oils. Bicyclogermacrene was only determined in minor amounts in this study, while it was a major constituent (up to 14.5%) in *A. campestris* oils from Italy and Iran [3,13].

Eighty-seven identified constituents (one of them tentatively) comprised 73.6-92.3% of the total oils. Chemical analysis showed a multi-component composition of the essential oils of Lithuanian field wormwood. The main fraction was sesquiterpenoids (49.9-79.3%), where amounts of sesquiterpene hydrocarbons and oxygenated sesquiterpenes varied from 16.1 to 51.2% and from 25.0 to 52.6%, respectively. Monoterpene hydrocarbons accounted for only 5.4-19.7%.

2. Toxic activity of *A. campestris* essential oils against *Artemia* sp. nauplii: Three oils obtained from the studied plant materials were chosen for tests of toxic activity. The test showed that lethality (LC_{50}) of brine shrimp larvae was 15-20 μg/mL (16.8, 19.5 and 14.9 μg/mL for samples A, H and K, respectively). The essential oils of *A. campestris* containing 12.1%, 22.1% and 10.2% of caryophyllene oxide, and 9.4%, 5.8% and 9.9% of germacrene D, respectively were toxic enough to kill the shrimps, despite the fact that the extremely toxic ketones (such as α- and β-thujone or artemisia

Table 2: Main constituents (with quantity over 5%) of essential oils, rich in caryophyllene oxide, of *Artemisia campestris* from Lithuania (2003-2007).

Compound	RI [a]	A	B	C	D	E	F	G	H	J	K	Interval
α-Pinene	939	5.3	t	4.5	4.0	6.8	1.1	5.6	1.3	1.5	0.7	t-6.8
β-Pinene	975	3.9	4.3	4.9	5.5	2.3	0.5	0.3	0.2	t	0.3	t-5.5
cis-Pinane	986	1.0	3.6	0.8	1.5	1.2	1.5	6.0	3.3	3.2	1.9	0.8-6.0
β-Elemene	1391	3.2	t	0.3	0.9	0.7	1.3		0.7	0.1	6.8	0-6.8
β-Caryophyllene	1419	3.0	2.0	2.7	1.7	1.6	2.8	6.1	6.2	5.7	4.9	1.6-6.2
β-Ylangene	1420	1.8	1.0	1.5	0.7	7.7	3.1		1.0		6.0	0-7.7
γ-Curcumene+Acoradiene	1480		5.7			4.6	0.8	5.1	1.5	3.1		0-5.7
Germacrene D	1485	9.4	3.5	3.8	3.7	10.1	15.0	7.5	5.8	4.9	9.9	3.5-15.0
Spathulenol	1578	0.9	6.8	4.5	5.7							0-6.8
Caryophyllene oxide	1583	12.1	19.7	14.5	8.5	16.0	18.7	22.0	22.1	38.8	10.2	8.5-38.8
Humulene epoxide II	1608	3.7	5.3	0.7	2.5	2.9	5.2	8.1	5.6	4.1	2.4	0.7-8.1
Junenol	1619	1.6		1.4			6.1	2.9	0.8		4.6	0-6.1
Sum of main constituents (%)		45.9	51.9	39.6	34.7	53.9	56.1	63.6	48.5	61.4	47.7	34.7-63.6
Total ([b] including all compounds with quantity ≤5.0%)		75.2	73.6	79.3	80.1	81.2	85.4	92.3	84.3	81.0	92.0	73.6-92.3
Compounds with caryophyllane skeleton [b]		15.4	21.7	17.2	11.8	17.6	21.5	30.0	28.3	44.5	15.1	11.8-44.5
Monoterpene hydrocarbons [b]		17.1	9.7	15.6	19.7	15.9	5.4	13.3	10.8	8.1	6.8	5.4-19.7
Sesquiterpene hydrocarbons [b]		27.2	16.1	24.7	21.1	34.1	33.0	31.4	25.7	18.4	51.2	16.1-51.2
Oxygenated sesquiterpenes [b]		25.0	42.2	33.2	28.8	26.6	40.8	39.3	40.4	52.6	28.1	25.0-52.6

A-K indicate harvesting localities, in eastern Lithuania: Trakai district, Streva (A, 2003) and Vilnius district, Vievis (B, 2007); in western Lithuania: Palanga city, Sventoji (C, 2005), Klaipeda district, Plikiai (D, 2005), Palanga city, Butinge (E, 2007), Silute district, Pagegiai (F, 2007), Klaipeda district, Karkle (G, 2007), Kretinga city (H, 2007), Klaipeda city (J, 2007) and in south Lithuania: Druskininkai city, Latezeris (K, 2007).

[a]RI- retention index on nonpolar column DB-5; t-traces (≤0.05%).

[b]Compounds with quantity ≤5.0%:

0-0.5%: α-thujene, camphene, sabinene, *p*-cymene, 6-camphenone, *allo*-ocimene, *neo-allo*-ocimene, *trans*-verbenol, camphor, verbenone, α-cubebene, citronellyl acetate, neryl acetate, geranyl acetate, β-bourbonene, aromadendrene, *cis*-muurola-4(14),4-diene, α-cadinene, nonadecane and eicosane;

up to 1.5%: α-terpinene, β-phellandrene, (*Z*)-β-ocimene, benzene acetaldehyde, (*E*)-β-ocimene, γ-terpinene, terpinolene, linalool, α-campholenal, *trans*-pinocarveol, terpinen-4-ol, α-terpineol, myrtenol, 4(*Z*)-decen-1-ol, *trans*-sabinyl acetate, terpinyl acetate, *ar*-curcumene, bicyclogermacrene, γ-cadinene, *trans*-cadina-1(2),4-diene, α-calacorene, β-copaen-4-α-ol, *trans*-β-elemenone, *epi*-α-muurolol and phytol;

lower than 3.0%: 1,3-dimethyl benzene, myrcene, γ-terpineol, δ-elemene, α-copaene, (*E*)-β-ionone, α-zingiberene, *trans*-β-guaiene, α-muurolene, (*E*, *E*)-α-farnesene, δ-cadinene, (*E*)-nerolidol, caryophylla-4(12),8(13)-dien-5α-ol, germacra-4(15),5,10(14)-trien-1-α-ol and (*6R*, *7R*)-bisabolone;

up to 5.0%: limonene, bornyl acetate, β-copaene, (*Z*)-β-farnesene, α-humulene, (*E*)-β-farnesene, β-selinene, *trans*-muurola-4(14),5-diene, (*Z*)-nerolidol, unknown 1, salvial-4(14)-en-1-one, *epi*-α-cadinol, α-cadinol and 3-thujopsanone.

Unknown 1(RI-1580): M^+ 220, 107(100), 135(69), 91(57), 41(37), 79(34), 69 (21), 119(21), 204(16).

ketone) characteristic for *Artemisia* species, were not detected in the investigated oils. The strong toxicity of *A. campestris* evaluated by us can justify why the plant is used in Lithuanian folk medicine, but not as a food or spice.

According to the literature data [14-16], essential oils of other plant species possessing caryophyllene oxide as a major constituent are toxic. Volatile oils of *Acroptilon repens*, containing 36.6% of caryopyllene oxide and 10% of caryophyllene inhibited the growth of Gram-positive bacteria [14]. This sesquiterpene oxide is toxic to ants and inhibits growth of ant-associated fungi [15]. The toxicity of essential oils isolated from *Artemisia* species and containing caryophyllene oxide as a major component showed a high mortality to granary weevil [16]. However, not only compounds present in the greatest proportions are responsible for the total oil activity. The influence of the less abundant constituents and synergetic effects might also be considered.

Experimental

Plant material and oil isolation: The aerial parts (~20 cm, 15-100g) of individuals from various *A. campestris* ssp. *campestris* populations were collected at full flowering stage in July-August (2003, 2005 and 2007) from ten different localities in Lithuania. Voucher specimens were deposited in the herbaria of the Institute of Botany (BILAS), Vilnius and Vytautas Magnus University, and Kaunas Botanical Garden, and their numbers are: A-68906, B-68919, C-68913, D-68914, E-SS 657, F-SS 658, G-SS 659, H-68918, J-68911 and K-SS 660. Plant material was dried at room temperature (20-25°C). Essential oils of the air-dried aerial parts (leaves and inflorescences) were prepared by hydrodistillation for 2 h using a Clevenger-type apparatus and a mixture of *n*-pentane and diethyl ether (1:1) as a collecting solvent. Pure oils of yellow-grey color were obtained from 80-100g of dry material. Yields ranged from 0.03 to 0.08 %, v/w on a dry mass basis.

GC-MS analysis: Analyses were performed using an HP 5890 chromatograph interfaced to an HP 5971 mass spectrometer (ionization voltage 70 eV, scan time 0.6 s, scan range 35-400 Da) and equipped with a capillary column DB-5 (50 m × 0.32 mm i. d., film thickness 0.25 μm). The oven temperature was held at 60°C for 2 min, then programmed from 60 to 160°C at a rate of 5°C/min, held for 1 min, then increased to 250°C at a

rate 10°C/min and finally isothermal at 250°C for 3 min, using He as a carrier gas (1.0 mL/min), split 1:40. Injector and detector temperatures were 250°C. Qualitative analysis was based on a comparison of retention times, indexes and MS with the corresponding data in the literature [17], by co-injection of some terpene references, and from computer MS libraries (Wiley and NBS 54K).

Toxicity test: Toxicity of 3 of the *A. campestris* oils (A, H and K, plant material from different parts of Lithuania) was tested *in vivo*, using brine shrimp *Artemia* sp. (larvae) [18]. The eggs of the shrimps hatch within 48 h to provide larvae (nauplii) in sea water (31g/L sea salt) at 20-25°C. Then, different concentrations of field wormwood essential oils dissolved in dimethyl sulfoxide (DMSO) were added, and survivors were counted after 24 h. Lethality (LC_{50}) of nauplii was calculated (n=4, with 95% confidence interval). A control test was done with DMSO.

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Comparison of Antibacterial Activity of Natural and Hydroformylated Essential Oil of *Thymus capitatus* Growing Wild in North Sardinia with Commercial *Thymus* Essential Oils

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Thymus capitatus growing wild in Sardinia showed different essential oil composition if grown surrounding Cagliari than in north Sardinia. Here we verify the composition and antimicrobial activity of the oil to make it suitable for the cosmetic and confectionery industries. With the aim of improving the scent and the antimicrobical activity of *T. capitatus* essential oil, a hydroformylation reaction was carried out to transform the unsaturated components of the oil into the corresponding aldehydes. The essential oil of *T. capitatus* exhibited a significant antibacterial activity (MIC 0.125-0.5 mg/mL), and was also found effective on *C. albicans* (MIC 0.125 mg/mL). After hydroformylation, several new irregular terpenoid aldehydes were detected. The perfume of the new terpenic-like aldehydes is very agreeable and, therefore, the acceptability of the aroma is remarkably improved, but the antimicrobial activity was not increased.

Keywords: *Thymus capitatus*, essential oil, hydroformylation, antibacterial activity, carvacrol.

The family *Lamiaceae* shows an intraspecific biodiversity resulting in the heterogeneous composition of the essential oil produced by various species, which is based on genetic polymorphism, but which is also influenced by environmental conditions. In the genus *Thymus*, for example, many species present intraspecific chemotypes [1]. Wild *Thymus* species growing in Sardinia include only two chemotypes, thymol and carvacrol [2-5], which are present only with two species: *T. catharinae* Camarda (ex *T. herba-barona* Loisel) and *T. capitatus* (L.) Hoffmanns & Link [6,7].

T. capitatus (synonyms *Satureja capitata* L., *Coridothymus capitatus* Rchb. and *Thymbra capitata* (L.) Cav.) is a characteristic species of *Lamiaceae* growing wild in the Mediterranean basin. In Italy, it is present in the South of the Italian peninsula (Abruzzo, Molise, Campania, Puglia, Basilicata, Calabria) and in the two biggest islands, Sicily and Sardinia. In Sardinia, *T. capitatus* is present only in a few calcareous northern areas (in few villages around Sassari) and in southern zones surrounding Cagliari. The composition and activity of its essential oil have been studied by several researchers [8-12]. *T. capitatus* essential oil has been used since ancient times in pharmaceutics, for food flavoring, cosmetics and perfumery [13].

Thymus species have been studied for their composition and biological activity, but few studies on *T. capitatus* growing wild in Sardinia are present in the literature, but they indicate that the essential oil from *T. capitatus* growing wild around Cagliari has an essential oil composition [3] that is very different from that of plants growing in north Sardinia [2]. The aim of the present study was the characterization of *T. capitatus* essential oil in order to determine whether its composition and antimicrobial activity made it suitable for the cosmetic, fragrance and confectionery industries**.** Moreover, the essential oil underwent a hydroformylation reaction catalyzed by rhodium complexes to transform unsaturated terpenes into oxygenated compounds (aldehydes), with the aim of giving the essential oil a more pleasant smell and improving its antimicrobial activity.

Essential oil of *T. capitatus* obtained from plants growing wild in a large station located in north Sardinia was analyzed using GC and GC/MS; 36 constituents were identified, with a percentage of identification near to 100%. To be sure that the essential oil composition of the plants from this station remained constant with time, we monitored the essential oil composition for 3 years before the present analyses (Table 1). As shown in Table 1, the major constituent of the oil is carvacrol. Between 2005-2007 there were no substantial differences, at least in the main constituents, but big differences were found in samples collected in 2008. The first three collections were made at the time of maximum blooming (May-June in normal climatic conditions), while the 2008 harvest was made at the beginning of July, during a very dry summer following a prolonged period of drought.

The biggest detectable differences were the presence of 3.8% α-thujene, 1.5% α–pinene, the doubling of the β–myrcene content, and the presence of 8.6% *p*-cymene. Remarkable was the increase of γ-terpinene (6.9%) and the dramatic decrease in carvacrol, that varied from 83% to 57%. These differences may partially be due to the major concentration of α-pinene and *p*-cymene; the different environmental conditions were in favor of the presence of compounds like α-thujene and 1-octen-3-ol.

It is known that *T. capitatus* essential oil is highly effective against bacteria and fungi [2]. With the aim of increasing the efficiency of the antimicrobial activity and to improve the odor of *Thymus* oil we submitted it to a hydroformylation reaction. Because of the unsaturated terpenes present in the oil, the hydroformylation process should furnish a modified oil containing new terpenic-like aldehydes capable of increasing the antimicrobial performance. Table 2 compares the original *T. capitatus* essential oil with the hydroformylated one. After hydroformylation, we found several new irregular terpenoid aldehydes in the modified oil (Table 2). As a consequence of these modifications, the acceptability of the oil's smell was remarkably enhanced due to the new terpenic-like aldehydes; however, the antimicrobial characteristics of the oil did not improve.

The original essential oil was hydroformylated. The modified essential oil was washed and hydrodistilled to obtain the new, pure, modified essential oil. Comparing the analytical data of the original and modified oils, it was evident that the most important variations were: α–thujene, which was present in good percentage in the original oil, but lost about 2% as a result of hydroformylation; compounds like camphene, *trans*-pinene, 1-octen-3-ol, α-phellandrene, β-myrcene,

Table 1: Quantitative and qualitative composition of *Thymus capitatus* essential oil.

Constituents	K.I.	*T.capitatus* (2005)	*T.capitatus* (2006)	*T.capitatus* (2007)	*T.capitatus* (2008)
α-Thujene	931	--	--	--	3.8
α-Pinene	939	--	--	--	1.5
Camphene	953	tr	--	--	0.3
Heptanol	969	tr	1.7	1.6	--
1-Octen-3-ol	978	--	--	--	1.3
β-Pinene	980	0.1	--	--	--
3-Octanone	988	0.6	0.6	0.7	--
β-Myrcene	991	1.6	1.6	1.7	3.7
3-Octanol	993	0.2	0.4	0.4	0.4
α-Phellandrene	1005	0.2	0.3	0.3	0.5
Δ^3-Carene	1011	0.1	--	--	0.2
Δ^4-Carene	--	0.7	0.8	0.8	2.2
p-Cymene	1026	4.5	4.9	5.0	8.6
β-Phellandrene	1031	--	--	--	1.0
1-8-Cineole	1033	0.4	0.2	0.2	tr
α-Ocimene	1050	--	--	--	0.1
γ-Terpinene	1062	2.6	3.4	3.2	6.9
cis-Sabinene-hydrate	1068	--	--	--	0.1
Terpinolene	1088	--	--	--	0.3
trans-Sabinene-hydrate	1097	0.5	0.7	0.7	--
Linalool	1098	1.3	1.2	1.2	1.9
Exo-Fenchol	1117	--	--	--	0.1
Borneol	1165	0.1	--	--	0.7
Nonanol	1171	--	--	--	0.7
Terpinen-4-ol	1177	0.5	1.3	1.2	2.1
Dihydro carveol	1192	--	--	--	0.5
Nerol	1228	--	--	--	0.1
Neral	1240	0.2	--	--	--
Carvone	1242	tr	--	--	0.2
Thymol	1290	0.1	--	--	tr
Carvacrol	1298	83.5	81.2	81.0	57.0
β-Caryophyllene	1418	1.6	1.5	1.4	3.5
α-Caryophyllene	1454	--	--	--	0.2
β-Bisabolene	1509	--	--	--	0.1
Caryophyllene oxide	1581	0.3	--	--	0.5
undentified		0.1	0.2	0.5	1.0
Total		99.2	100	98.7	97.2

β-caryophyllene and linalool were detected only in the original essential oil because these compounds were hydroformylated and transformed into the corresponding aldehydes; 3-octanol and Δ^3-carene were present in all samples and did not vary significantly in their concentrations; and carvacrol, which showed little variation, probably due to the experimental steps. On the other hand, there are some compounds that are present only in the hydroformylated oils, as for instance the terpinen-4-ol isomer: 4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol and 3-7-dimethyl-7-octenal. Most of them derive from hydroformylation, but also some isomerization reactions were detected during the oil modification process.

The antimicrobial activity screening was carried out on the *Thymus* essential oils and their main components, respectively, in comparison with chlorhexidine gluconate. The essential oil of *T. capitatus* exhibited a significant antibacterial activity (MIC 0.125-0.5 mg/mL), qualitatively similar to but higher than those of oils of other species of *Thymus*; Gram-positive strains

Table 2: Constituents of original essential oil of *Thymus capitatus* and hydroformylated redistilled oil.

Constituents	K.I.	*T. capitatus* (2008) (original oil)	*T. capitatus* (2008) hydroformylated (redistilled)
α–Thujene	931	3.8	1.5
α–Pinene	939	1.5	1.9
Camphene	953	0.3	0.1
2,6-dimethyl-3-Octene	nd	--	0.1
3,7-dimethyl 2-Octene	nd	--	0.1
trans-Pinene	973	0.1	--
1-Octen-3-ol	978	1.3	--
β-Pinene	980	--	0.2
isolimonene	983	--	0.1
p-Menth-3-ene	986	--	1.6
β-Myrcene	991	3.7	--
3-Octanol	993	0.3	0.4
2,6-Dimethyl-2-*trans*-6-Octadiene	nd	--	0.3
α-Phellandrene	1005	0.5	--
Δ^3-Carene	1011	0.2	--
Δ^4-Carene	n.d.	2.2	2.4
o-Cymene	1022	8.6	--
β-Phellandrene	1031	1.0	--
α–Ocimene	1050	0.1	--
γ-Terpinene	1062	6.9	6.9
cis- Sabinene-hydrate	1068	0.1	--
Terpinolene	1088	0.3	0.3
Linalool	1098	1.8	--
exo-Fenchol	1117	0.1	--
Citronellal	1153	--	0.4
Borneol	1165	0.7	0.9
Terpinen-4-ol	1177	2.1	2.3
4-Methyl-(1-methylethyl)-3-Cyclohexen-1-ol	n.d	--	3.5
Dihydrocarveol	1192	0.5	0.2
3-7 dimethyl-7-Octanal	n.d	--	1.7
Nerol	1228	0.1	--
Carvone	1242	0.2	--
Thymol	1290	tr	0.8
Carvacrol	1298	57.0	54.7
Carvacrol acetate	1371	--	0.2
γ–Caryophyllene	1404	--	0.5
β-Caryophyllene	1418	3.5	0.1
α-Caryophyllene	1454	0.2	--
α– *neo*-Clovene	1454	--	1.2
β-Bisabolene	1509	0.1	--
α-Caryophyllene oxide	1581	0.5	0.7
Hexadecene	1593		0.2
1-Cyclohexylheptene	1637		0.1
Heptadecane	1700	--	0.5
undentified		1.0	1.9
Total		99.5	85.8

were more sensitive that the Gram-negative bacteria examined. *T. capitatus* oil was also found effective on *C. albicans*, with a MIC of 0.125 mg/mL. Under the same experimental conditions, chlorhexidine gluconate showed remarkable antimicrobial activity (MIC 0.001-0.016 mg/mL) against all strains tested. Hydroformylation decreased the antimicrobial activity of *T. capitatus* oil; the MICs of the hydroformylated product were 0.25 mg/mL for *S. aureus* and *S. epidermidis*, 0.5 mg/mL for *E.coli* and >0.5 mg/mL for *Ps. aeruginosa*. As regards the antimicrobial activity of the main oil components, carvacrol showed good inhibitory activity against bacterial strains and *Candida* (MIC values 0.062-0.500 mg/mL), whereas linalool, terpinen-4-ol and β-myrcene did not exhibit any antimicrobial activity at concentrations up to 0.500 mg/mL.

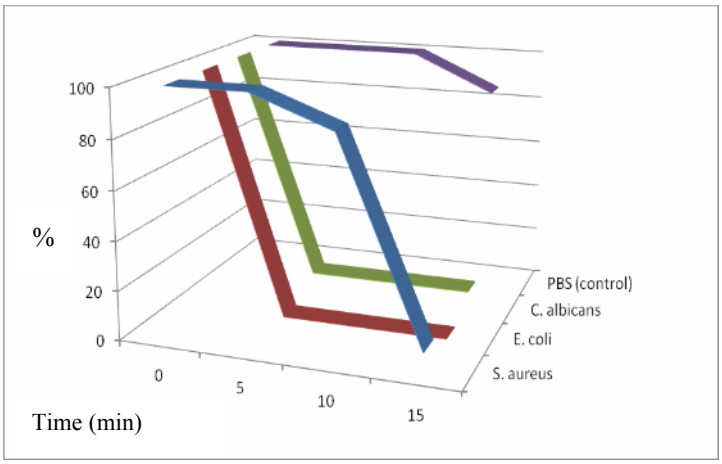

Figure 1: Percentages of surviving microorganisms after different exposure times and inhibiting concentrations of essential oil of *T. capitatus*. The percentages are the averages of three experiments.

Figure 1 shows the inactivation kinetics of the killing of standardized inocula of *S. aureus*, *E. coli* and *C. albicans* by inhibitory concentrations of *T. capitatus* oil (0.250 mg/mL for *E. coli*, and 0.125 mg/mL for *S. aureus* and *C. albicans*).

Experimental

Plant materials: Samples of wild *Thymus capitatus* (L.) Hoffmanns. & Link) were collected in 4 consecutive years, in San Michele in Plaiano, one of the largest stations existing around Sassari (north Sardinia). The plants were identified by M. Usai. Voucher specimens have been deposited at the Herbarium S.A.S.S.A. (cumulative identification number: 1078) of the Department of Scienze del Farmaco, University of Sassari. The aerial parts of the plants were randomly collected in June during the blooming period.

Oil distillation and yield: Fresh plant material (5 Kg) was separately hydrodistilled using a Clevenger-type apparatus, according to the Italian Official Pharmacopoeia X [14]. The obtained oil was light yellow. Three replicate samples were distilled simultaneously. Oil floating to the water surface was collected by draining away water, dried over anhydrous sodium sulfate and stored at –20°C (under nitrogen atmosphere) until analyzed. The yield was up to 1.1%.

GC oil analyses: Three replicates of each sample were analyzed by using a Hewlett-Packard Model 5890A GC equipped with a flame ionization detector and fitted with a 60 m x 0.25 mm thickness 0.25μm AT-5 fused silica capillary column (Alltech). Injection port and detector temperatures were 280°C. The column temperature was programmed from 50°C to 135°C at 5°C/min (1 min), 5°C/min to 225°C (5 min), 5°C/min to 260°C and then held for 10 min. The samples (0.2 μL each), analyzed without dilution (using 2,6-dimethyl-

phenol as internal standard), were injected using a split/splitless automatic injector HP 7673 and with helium as carrier gas. Measurements of peak areas were performed with a HP workstation; the threshold was set at 0, peak width at 0.02. The quantification of each compound was expressed as the absolute weight percentage using internal standard and response factors. The detector response factors (RFs) were determined for key components relative to 2,6-dimethylphenol and assigned to other components on the basis of functional group and/or structural similarity, since oxygenated compounds have lower detectability by F.I.D. than hydrocarbons [15] The standards purity was checked by GC. Several response factor solutions were prepared that consisted of only 4 or 5 components (plus 2,6-dimethylphenol) to prevent interference due to trace impurities.

GC/MS: GC/MS analyses were carried out with a Hewlett Packard G1800B-GCD system using the same conditions and column described above. The column was connected with the ion source of the mass spectrometer. Mass units were monitored from 10 to 450 at 70 eV. The identification of the components was made by comparison of their retention time with respect to *n*-alkanes (C6-C22). The MS and retention indices (RI) were compared with those of commercial (NIST 98 and WILEY) and home-made library MS built up from authentic samples (analytical standards from Aldrich and Fluka; purity > 97%) and MS literature data [16-21]. The percentage composition of the oil was obtained by the normalization method from the GC peak areas, using correction factors.

Evaluation of antibacterial activity: The antibacterial activity of the essential oils (*Thymus capitatus*, both unmodified and hydroformylated, *T. vulgaris* and *T. serpillum*) and standard compounds (carvacrol, linalool, β-myrcene and terpinen-4-ol) was determined as minimum inhibitory concentration (MIC) against a panel of bacterial strains, by using chlorhexidine gluconate as reference antimicrobial substance. Microorganisms examined included both Gram-positive (*Staphylococcus aureus* ATCC 25923, CultiLoops® Oxoid, Basingstoke, UK; *S. epidermidis* 155U and *Propionibacterium acnes* ATCC 6919, supplied by LCG Promochem, Middlesex, UK) and Gram-negative strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853; CultiLoops® Oxoid). MICs were determined by using an agar dilution technique, except for *P. acnes*, which was examined by a broth dilution technique. Oils and standards were dissolved at 10% w/v in PEG 200 (Sigma); preliminary tests with this vehicle were performed to ensure that no micro-organism inhibition occurred at the used concentrations. For the agar dilution technique, two-fold serial dilutions of the solutions (0.5 mg/mL to 0.0031 mg/mL) were prepared in triplicate in molten Mueller Hinton Agar (Oxoid) at 45°C and poured into 50 mm Petri dishes. Each dish was inoculated with about 1 x 10^4 bacteria, applied as a spot of about 5 mm in diameter; after 18-24 h of incubation at 35°C, MICs were recorded as the lowest concentrations which completely inhibited bacterial growth. MICs against *P. acnes* were determined by preparing two-fold serial dilutions (0.5 mg/mL to 0.0031 mg/mL) in triplicate in Brain Heart Infusion (Oxoid) and pouring them into microtiter 96-well plates; each well was inoculated with about 1 x 10^4 bacteria. MICs were recorded after 72 h of anaerobic incubation (Gas Generating Kit, Anaerobic System BR 038B, Oxoid) as the lowest concentrations which completely inhibited bacterial growth.

Evaluation of antifungal activity: The above mentioned essential oils and standard compounds were also tested against *Candida albicans* ATCC 10231 in an agar dilution test similar to that one previously described for bacteria, by using Sabouraud Dextrose Agar (Oxoid). Agar plates were inoculated with about 10^4 yeast cells from an overnight culture and incubated at 25°C. After 18-24 h, MICs were recorded as the lowest concentrations able to inhibit completely fungal growth.

Killing time test: The exposure times required by inhibitory concentrations of *T. capitatus* essential oil to kill an appropriate microbial inoculum were determined according to a previously described technique [5]. Assays were carried out on *E. coli* ATCC 25922 and *C. albicans* ATCC 10231. Briefly, microorganisms in the logarithmic phase of growth were centrifuged at 1200 *g* for 15 min, washed in phosphate buffered saline (PBS, pH 7.3) and resuspended in an appropriate volume of essential oil in the same buffer (at a concentration of oil corresponding to the MIC for the tested microorganism) at a density of $5x10^5$-$1x10^6$ c.f.u./mL. A control suspension (bacterial in PBS at the same density) was included in each experiment. At time zero and after 5, 10 and 15 mins, 0.5 mL of each suspension was removed, subjected to serial tenfold dilutions in PBS and inoculated onto either MHA or SDA plates. The number of viable microorganisms at each time was evaluated by counting colonies after incubation for 24 h at 35°C, and was expressed as the percentage of the control.

Hydroformylation of Thymus capitatus essential oil: [22-24]. All chemicals were purchased from commercial sources and used as received, unless otherwise indicated. Dicarbonylrhodiumacetilacetonate [$Rh(CO)_2(acac)$] 98% (acacH = acetylacetonate) and triphenylphosphine (PPh_3) were purchased from Sigma-

Aldrich. Hydroformylation experiments were carried out in homemade autoclaves with magnetic stirring. To prevent direct contact with stainless steel, the reaction solution was kept in a glass vessel and the autoclave cap was glass-covered. The reaction products were identified by GC/MS.

In a typical experiment, $Rh(CO)_2(acac)$ (0.02 mmol), PPh_3 (0.04 mmol), and essential oil (3.7 g) were transferred under nitrogen into the autoclave, which was pressurized to 50 bar total pressure (CO/H_2 = 1/1), heated (80°C) and stirred with a magnetic stirrer. After carrying out the reaction for 24 h and cooling at room temperature, the excess CO and H_2 were slowly vented. The essential oil was redistilled in steam to avoid contamination from the catalyst and analyzed using a Hewlett Packard gas-chromatograph, Mod. G1800 B GCD System, equipped with an Alltech ATTM-5 column (60 m x 0.25 mm x 0.25 μm) and a MS EI detector operating at 70 eV. The components were identified as previously reported and by accurate interpretation of MS.

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Composition and Chemical Variability of the Leaf Oil from Corsican *Juniperus thurifera*. Integrated Analysis by GC(RI), GC-MS and ^{13}C NMR

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The composition of 16 samples of leaf oil from Corsican *Juniperus thurifera* was investigated by integrated techniques, GC, GC-MS and ^{13}C NMR. K-means partitioning and PCA analysis of the data allowed the definition of a main group (14 samples) dominated by limonene (mean = 52.2%, SD = 6.4) and α-pinene (mean = 7.2%, SD = 3.8). Limonene and β-elemol (up to 19.7%) were identified as the major components of two atypic samples.

Keywords: *Juniperus thurifera*, limonene, β-elemol, PCA, ^{13}C NMR, Corsica.

The genus *Juniperus*, consisting of 67 species and 34 varieties, is divided into three sections: *Caryocedrus* (one species: *J. drupacea* Labill.), *Juniperus* (= *Oxycedrus* Spach with 10 species) and *Sabina* (Miller) Spach (56 species). *J. thurifera* L., a member of the *Sabina* section, is constituted of two varieties: var. *thurifera* (from southern Europe, Spain and France) and var. *africana* (from North Africa, Morocco). RAPDs showed that the Moroccan plants (var. *africana*) were barely distinct from var. *thurifera* [1]. According to Adams [1], *J. thurifera* var *thurifera* has several synonyms, of which *J. hispanica* Mill., *J. sabinoides* Endl., *J. bonatiana* Vis., *J. cinerea* Carriere, *J. thurifera* L. var *gallica* Coincy, *J. gallica* (Coincy) Rouy, *Sabina foetidissima* (Willd.) Antoine, *S. thurifera* (L.) Antoine, and *S. pseudothurifera* Antoine are retained.

J. thurifera var *thurifera* is a dioecious species. It is a pyramidal tree, up to 20 m high, which grows in the mountains of central Spain, the Spanish and French Pyrenees and French Alps [1]. In Corsica, it is found on both faces of Monte Cinto, the highest mountain of the island, in Niolu and Asco valley [2,3].

The essential oil of *J. thurifera* is obtained by hydrodistillation of leaves, berries and wood. A few studies have reported on the isolation and characterization of sesquiterpenes from the wood oil [4,5]. However, the chemical composition of the essential oils from leaves, berries and wood has been studied less frequently. Only one paper reported the composition of a sample of wood oil, dominated by sesquiterpenes: cedrol (41%), thujopsene (20%), widdrol (16%) and α-cedrene (12%) [6]. Two berry oil samples were characterized by a high content of limonene (88.5% and 84.3%) [7,8]. Concerning the leaf oil, Adams [9, 10] studied the composition of various samples from Morocco, Spain and France. Limonene dominated the composition of the samples from Spain (28.8-61.8%), the Pyrenees (53.0 and 75.1%) and France (French Alps, 52.1%; Corsica, 60.6%). Some samples contained appreciable amounts of linalool (up to 13.4%) and linalyl acetate (up to 13.7%). In contrast, Morrocan oils contained sabinene as the major component in three samples (37.4-45.8%). The composition of the fourth sample was dominated by α-pinene (17.1%), elemol (14.7%) and sabinene (12.2%). It could be pointed out that δ-2-carene was totally absent in the samples of the variety *Africana*, while it accounted for 0.9-4.4% in the samples of the variety *thurifera*.

The aim of this study was to characterize *J. thurifera* var *thurifera* growing wild in a restricted area in Corsica through the composition of its leaf oil. Leaves were collected from 16 individual trees growing in two locations covering the geographic range of *J. thurifera* in Corsica. The yield of the essential oil isolated by water distillation and calculated from fresh material, varied substantially from sample to sample (0.16-0.89%).

Table 1: Volatile components of the leaf oil from Corsican *Juniperus thurifera* L: average (A) and standard deviation (SD) for samples of the main group, atypical composition of samples 3 and 6.

	Compound*	RI		Main group			Sample		Identification
		BP-1	BP-20	A % (S.D)	Min %	Max %	No. 3 %	No. 6 %	mode
1	α-Pinene	930	1020	7.2 (3.8)	1.9	15.6	2.1	2.4	RI, MS ^{13}C NMR
2	Sabinene	963	1108	0.3 (0.1)	0.0	0.5	0.1	3.3	RI, MS *^{13}C NMR*
3	β-Pinene	969	1105	0.5 (0.3)	0.1	1.0	0.2	0.2	RI, MS *^{13}C NMR*
4	Myrcene	979	1155	3.0 (0.3)	2.5	3.4	1.0	2.2	RI, MS ^{13}C NMR
5	δ-2-Carene	994	1125	2.0 (0.9)	0.6	4.3	1.0	1.8	RI, MS ^{13}C NMR
6	δ-3-Carene	1004	1142	4.9 (2.9)	0.2	9.6	3.4	2.2	RI, MS ^{13}C NMR
7	*p*-Cymene	1010	1263	0.1 (0.0)	0.1	0.2	0.1	0.1	RI, MS
8	Limonene	1022	1201	52.2 (6.4)	43.7	67.0	29.7	40.6	RI, MS ^{13}C NMR
9	Terpinolene	1077	1275	2.3 (1.2)	0.9	4.9	0.3	0.9	RI, MS ^{13}C NMR
10	Linalool	1081	1544	5.2 (3.7)	0.8	12.8	2.0	2.4	RI, MS ^{13}C NMR
11	1,2-*trans*-Limonene oxide	1120	1456	0.2 (0.2)	0.0	0.7	0.1	0.1	RI, MS *^{13}C NMR*
12	Borneol	1148	1698	0.2 (0.2)	0.0	0.6	-	0.4	RI, MS
13	*p*-Cymene-8-ol	1158	1803	0.4 (0.2)	0.1	0.9	0.3	0.1	RI, MS
14	Terpinen-4-ol	1161	1598	0.3 (0.1)	0.2	0.5	0.1	1.5	RI, MS *^{13}C NMR*
15	α-Terpineol	1170	1694	0.7 (0.4)	0.1	1.3	0.8	0.9	RI, MS ^{13}C NMR
16	Myrtenol	1180	1789	0.2 (0.0)	0.1	0.2	0.2	-	RI, MS
17	*trans*-Carveol	1196	1830	0.3 (0.1)	0.1	0.4	0.3	0.5	RI, MS *^{13}C NMR*
18	Citronellol	1208	1761	0.2 (0.2)	0.0	0.9	0.3	-	RI, MS ^{13}C NMR
19	Pulegone	1215	1647	0.2 (0.1)	0.0	0.4	0.4	-	RI, MS *^{13}C NMR*
20	Piperitone	1225	1729	2.6 (2.1)	0.4	8.5	1.1	1.7	RI, MS ^{13}C NMR
21	Geraniol	1231	1844	0.4 (0.2)	0.1	0.6	0.5	0.4	RI, MS *^{13}C NMR*
22	Linalyl acetate	1240	1553	1.3 (0.9)	0.3	2.9	1.0	1.2	RI, MS ^{13}C NMR
23	Bornyl acetate	1269	1575	0.2 (0.2)	0.1	0.7	0.2	0.2	RI, MS *^{13}C NMR*
24	*α*-Terpinyl acetate	1333	1681	0.9 (0.4)	0.3	1.6	1.1	1.0	RI, MS ^{13}C NMR
25	Neryl acetate	1341	1725	0.1 (0.1)	0.0	0.3	0.2	0.2	RI, MS *^{13}C NMR*
26	Geranyl acetate	1359	1748	0.2 (0.1)	0.0	0.5	0.4	0.3	RI, MS *^{13}C NMR*
27	*trans*-*α*–Bergamotene	1417	1580	0.1 (0.1)	0.0	0.4	0.2	0.1	RI, MS
28	Bicyclogermacrene	1491	1727	0.1 (0.1)	0.0	0.3	0.2	0.2	RI, MS
29	γ–Cadinene	1505	1752	0.2 (0.2)	0.0	0.6	0.3	0.2	RI, MS
30	δ-Cadinene	1513	1740	0.5 (0.3)	0.0	0.9	0.5	1.0	RI, MS *^{13}C NMR*
31	β–Elemol	1535	2079	2.5 (2.6)	0.5	10.2	19.7	13.6	RI, MS ^{13}C NMR
32	Cedrol	1597	2021	0.6 (0.8)	0.0	2.8	6.9	0.7	RI, MS ^{13}C NMR
33	γ-Eudesmol	1620	2163	0.3 (0.1)	0.2	0.9	1.8	0.9	RI, MS ^{13}C NMR
34	β-Eudesmol	1638	2225	0.6 (0.3)	0.0	2.5	3.8	3.0	RI, MS ^{13}C NMR
35	α-Eudesmol	1641	2216	0.8 (0.4)	0.2	2.8	2.2	0.3	RI, MS ^{13}C NMR

*Order of elution and percentages are given from apolar column. ^{13}C NMR (italics) indicate that the corresponding component has been identified by NMR in a chromatography fraction.

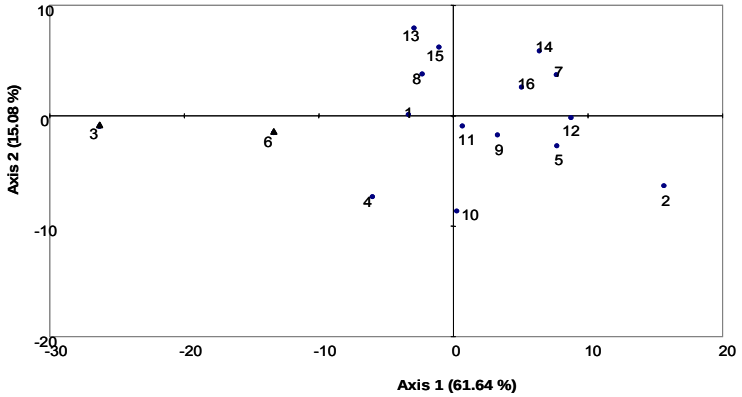

Figure 1: PCA of 16 leaf oil samples of Corsican *J. thurifera*.

Thirty-five components (26 monoterpenes and 9 sesquiterpenes), accounting for 84.2-99.4% of the whole composition of the samples, were identified (Table 1) using a combination of analytical techniques: GC, GC-MS and ^{13}C NMR spectroscopy. The composition of the samples varied substantially. Limonene (29.7-67.0%), β–elemol (0.5-19.7%), α-pinene (1.9-15.6%), linalol (0.8-12.8%), δ-3-carene (0.2-9.6%), and piperitone (0.4-8.5%) were the major constituents. Other monoterpenes, terpinolene (0.3-4.9%), δ-2-carene (0.6-4.3%), myrcene (1.0-3.4%), linalyl acetate (0.3-2.9%), α-terpinyl acetate (0.3-1.6%), terpineol-4 (0.1-1.5%) and α-terpineol (0.1-1.3%), as well as two sesquiterpenes, α-eudesmol (0.2-2.8%) and γ-eudesmol (0.2-1.8%) were present in moderate amounts. The contents of cedrol and β-eudesmol reached 6.9% and 3.8%, respectively, in one sample.

The 16 compositions were submitted to statistical analysis. Principal Components Analysis (PCA)

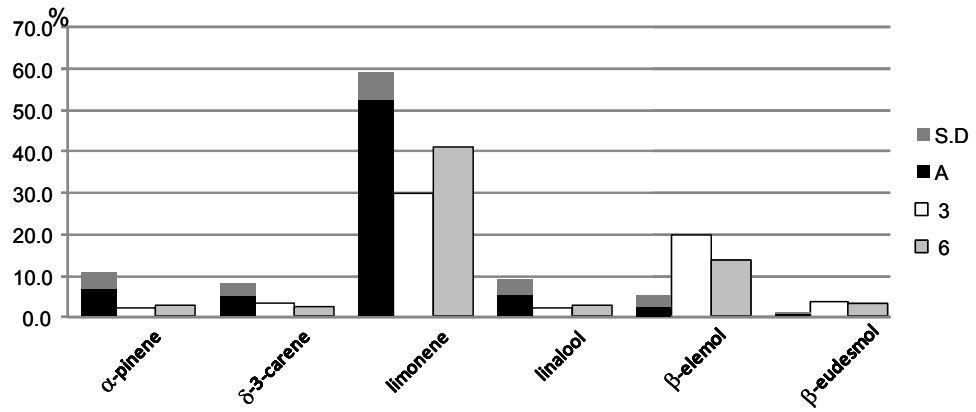

Figure 2: Major components of the main group (A = average, SD = standard deviation) and of the two atypic samples 3 and 6.

(Figure 1) and k-means partitioning were performed on all the terpene data, with individual compounds expressed as a percentage. Although the compositions of the individual samples varied substantially, it was not possible to distinguish groups within the essential oil samples. Therefore, we constituted one major group (14 samples) and differentiated two atypical compositions (Figure 2). For that reason, in Table 1 we reported the mean composition of the 14 samples of the main group, as well as the highest and the lowest values for each component, and the compositions of the two atypic samples n° 3 and n° 6.

All the leaf oil samples of *J. thurifera* var. *thurifera* from Corsica of the main group exhibited limonene as the major component, with a mean value of 52.2% (SD = 6.4) (Table 1, Figure 2). The similarity to oil samples from Spain, the Pyrenees and French Alps is particularly observed for some compositions which afforded α-pinene as the second major component. The sample from Corsica reported by Adams [10] belongs to that group. However, several samples contained fair amounts of δ-3-carene (up to 9.6%) and β-elemol (0.5-5.7% and 10.2% in sample 4), both of these components being scarce in the *J. thurifera* var. *thurifera* leaf oils reported in the literature (0.0-0.8% and 0.3-3.2%). The samples of that group differed drastically from the sabinene-rich oils from Morocco [9,10].

The content of limonene in the two atypical samples was less (40.6 and 29.7%). Conversely, in those samples, β-elemol accounted for 13.6 and 19.7% and the content of cedrol reached 6.9%. It could be pointed out that similar contents of β-elemol and cedrol (14.7 and 4.4%, respectively), were reported in one sample of *J. thurifera* var. a*fricana* from Morocco, which also contained α-pinene (17.1%) and sabinene (12.2%) as major components [10].

Experimental

Plant material: Leaves from 16 mature trees of *J. thurifera* were collected from 2 locations in Corsica: Asco (1-6) and Niolu (7-16) during March to April 2007.

Essential oil isolation and fractionation: Leaves of individual trees (55-393 g) were hydrodistilled in a Clevenger-type apparatus for 4 h. In order to carry out a detailed analysis, one oil sample (1.3 g) was fractionated by flash chromatography (column: 18 mm id; silica gel, 200-500 μm, 26 g) and 4 fractions (F1-F4) were eluted with a mixture of solvents of increasing polarity (*n*-pentane:diethyl oxide, 100:0 to 50:50): *n*-pentane, F1, (200 mL, 456 mg); *n*-pentane: Et_2O, 95:5, F2 (200 mL, 52 mg), *n*-pentane:Et_2O, 90:10, F3 (200 mL, 20 mg), *n*-pentane:Et_2O, 50:50, F4 (200 mL, 188 mg). The 4 fractions were analysed by GC(RI) and ^{13}C NMR.

Analytical GC: GC analyses were carried out using a Perkin-Elmer Autosystem GC apparatus equipped with 2 flame ionisation detectors, and fused-silica capillary columns (50 m x 0.22 mm i.d., film thickness 0.25 μm), BP-1 (polydimethylsiloxane) and BP-20 (polyethyleneglycol). Oven temperature was programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min); detector temperature, 250°C; injector temperature, 250°C (injection mode, split, 1/60); carrier gas, helium (0.8 mL/min). Injected volume: 0.5μL of a solution of 50 μL of the mixture (oil or chromatographic fraction) diluted in 350 μL of CCl_4.

GC-MS analysis: GC-MS analyses were carried out using a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem XL, equipped with a fused-silica capillary column (60 m x 0.22 mm i.d., film thickness 0.25 μm), Rtx-1 (polydimethylsiloxane). Carrier gas, helium at 1 mL/min; split, 1/80; injection volume, 0.2 μL; injector temperature, 250°C; oven temperature programmed

from 60°C to 230°C at 2°C/min and then held isothermal (45 min). Ion source temperature, 150°C; energy ionisation, 70 eV; EI MS were acquired over the mass range 35-350 Da.

^{13}C NMR analysis: ^{13}C NMR spectra of 5 selected samples and the chromatographic fractions were recorded on a Bruker 400 Avance Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with a 5 mm probe, in deuterochloroform, with all shifts referred to internal tetramethylsilane (TMS). ^{13}C NMR spectra were recorded with the following parameters: pulse width = 4 μs (flip angle 45°); acquisition time = 2.7 s for 128K data table with a spectral width of 25,000 Hz (250 ppm); CPD mode decoupling; digital resolution = 0.183 Hz/pt. The number of accumulated scans was 3,000 for each sample (around 50 mg of the oil or chromatographic fraction in 0.5 mL $CDCl_3$).

Identification of components: All 16 samples were submitted to GC(RI) analysis. Among them, 2 samples (N° 11, 15) selected on the basis of their chromatographic profile, were analysed by GC-MS. Five samples (N° 1, 3, 4, 7, 8) were analyzed by ^{13}C NMR spectroscopy. Identification of the components was based: (i) on comparison of their GC retention indices (RI) on polar and apolar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation with those of authentic compounds, and literature data [11]; (ii) on computer matching against laboratory-made (CPN Lab, University of Corsica) and commercial mass spectral libraries [12-15] and (iii) by ^{13}C NMR spectroscopy, following the methodology developed and computerised in our laboratories, using home-made software and spectral data library [16-18]. Most of the components were identified by comparison of their spectral data (MS and ^{13}C NMR) with those of reference compounds compiled in our laboratory-made spectral data library. In order to confirm the identification of minor components suggested by MS analysis, all the chromatographic fractions obtained from one oil sample (Experimental, second paragraph) were analysed by GC(RI) and ^{13}C NMR spectroscopy.

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Combined Analysis by GC (RI), GC-MS and ^{13}C NMR of the Supercritical Fluid Extract of *Abies alba* Twigs

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Two samples (leaves and twigs) of *Abies alba* Miller from Corsica were extracted using supercritical CO_2 and their chemical compositions were compared with those of the essential oils obtained from the same batch of plant material. In total 45 components were identified using combined analysis by GC (RI), GC-MS and ^{13}C NMR. It was observed that the contents of monoterpenes (mainly represented by limonene, α-pinene and camphene) were significantly lower in the supercritical fluid extract (SFE) than in the essential oil (EO). Conversely, the proportions of sesquiterpenes were much higher in CO_2 extracts than in essential oils (around 30% *vs* 4%). *Cis*-abienol, a diterpene alcohol, was identified only in SFE, and the proportions of this constituent (7.5% and 17.3%) were determined using quantitative ^{13}C NMR since it was under estimated using the standard conditions of GC.

Keywords: *Abies alba* Miller, essential oil, supercritical fluid extract, ^{13}C NMR spectroscopy, gas chromatography, mass spectrometry, *cis*-abienol.

The silver fir (*Abies alba* Miller, family Pinaceae) is widely distributed in many European mountain forests (for example in France, Germany and Poland). In Corsica, the south-western limit of its area of distribution, it constitutes pure fir forests as well as mixed forests with other conifers, particularly *Pinus nigra* ssp. *laricio* [1]. Twigs of *A. alba* produce by either vapour or water distillation an essential oil (EO) whose chemical composition is well documented. Most of the studies concerned plants from the centre and south of Europe (Poland [2], Germany [3], Austria [4], France [5], Montenegro [6], Serbia [7] and Greece [8]). All the investigated samples were characterised by the predominence of limonene (34-55%, Germany [3], Austria [4], France [5], Greece [8]), or β-pinene (20-33%, Montenegro [6], Serbia [7]). Other important components were camphene (15-17%) [4,6,7], α-pinene (11-17%) [5-7] and bornyl acetate (9-14%) [6,7].

Recently, we analysed 53 oil samples from twigs of *A. alba* collected in all the Corsican forests where the tree is growing wild [9]. The results were submitted to chemometric analysis (K-mean's clustering and Principal Component Analysis) and two groups were distinguished within the oil samples depending on the content of limonene [9].

Extraction by means of carbon dioxide, in the supercritical state, is a good technique for the production of flavors and fragrances from vegetable matter. The extensive use of compressed carbon dioxide to extract either volatiles or aroma substances destined for human nutrition and for the pharmaceutical industry is due to its chemical and physical properties. It is safe, non-toxic, non-combustible, inexpensive and its critical temperature and pressure are not high (31.06 °C; 7.38 MPa) [10]. The aim of the present work was to determine the yield and the chemical composition of the supercritical fluid extract (SFE) of twigs of *A. alba* and to compare them with those of the essential oil obtained by hydrodistillation of the same batch material.

Two samples, each belonging to a cluster previously defined [9], were submitted to supercritical CO_2 extraction and hydrodistillation and their chemical compositions were compared (Table 1). The four oil

Table 1: Compounds identified in the supercritical CO_2 extracts and the essential oils of leaves and twigs of *Abies alba*.

				Sample 1		Sample 2		
				SFE	EO	SFE	EO	
	Constituents	**Ir $_a$**	**Ir $_p$**	**%**	**%**	**%**	**%**	**Identification**
1	Santene	884	984	tr	3.6	0.1	3.0	RI, MS, ^{13}C NMR
2	Tricyclene	925	1020	0.3	2.1	0.5	2.1	RI, MS, ^{13}C NMR
3	α-Pinene	935	1032	3.4	18.0	2.6	11.4	RI, MS, ^{13}C NMR
4	Camphene	948	1079	1.9	13.7	3.9	15.7	RI, MS, ^{13}C NMR
5	β-Pinene	975	1120	0.4	1.3	2.7	8.1	RI, MS, ^{13}C NMR
6	Myrcène	984	1170	0.4	1.2	0.3	0.9	RI, MS, ^{13}C NMR
7	α-Phellandrene	998	1175	-	-	-	0.2	RI, MS
8	*p*-Cymene	1013	1270	-	0.1	-	0.1	RI, MS
9	Limonene*	1025	1209	17.7	43.5	5.6	15.6	RI, MS, ^{13}C NMR
10	β-Phellandrene*	1025	1219	0.1	0.2	7.0	14.4	RI, MS, ^{13}C NMR
11	γ-Terpinene	1049	1254	-	0.1	-	0.1	RI, MS
12	Terpinolene	1081	1291	0.1	0.4	0.1	0.5	RI, MS, ^{13}C NMR
13	Linalool	1083	1555	-	-	-	0.4	RI, MS
14	Citronellal	1134	1489	-	0.2	-	0.2	RI, MS
15	Borneol	1153	1713	-	0.1	0.9	1.0	RI, MS, ^{13}C NMR
16	α-Terpineol	1173	1709	-	0.7	-	1.2	RI, MS, ^{13}C NMR
17	Decanal	1188	1498	-	-	0.1	0.1	RI, MS
18	Citronellol	1212	-	-	0.3	0.1	0.2	RI, MS
19	Geraniol	1237	1858	-	0.1	0.3	0.2	RI, MS
20	Linalyl acetate	1243	1564	-	-	0.4	0.1	RI, MS
21	Bornyl acetate	1274	1589	1	1.4	8.1	9.9	RI, MS, ^{13}C NMR
22	Citronellyl acetate	1337	1670	0.7	1.2	1.1	1.1	RI, MS, ^{13}C NMR
23	α-Longipinene	1359	1475	2.3	0.5	2.3	-	RI, MS, ^{13}C NMR
24	Geranyl acetate	1364	1766	-	0.2	1.7	2.0	RI, MS, ^{13}C NMR
25	Dodecanal	1392	1718	-	0.2	1.2	0.4	RI, MS, ^{13}C NMR
26	Longifolene	1413	1575	1.3	0.4	1.7	-	RI, MS, ^{13}C NMR
27	(*E*)-β-Caryophyllene	1425	1604	5.6	1.5	6.3	0.8	RI, MS, ^{13}C NMR
28	Himachala-2,4-diene	1430	1611	2.6	-	2.4	-	RI, MS, ^{13}C NMR
29	α-Himachalene	1455	1650	0.7	-	0.9	-	RI, MS, ^{13}C NMR
30	α-Humulene	1458	1678	2.2	0.7	2.1	0.4	RI, MS, ^{13}C NMR
31	γ-Curcumene	1472	1685	0.4	-	1.0	-	RI, MS, ^{13}C NMR
32	γ-Himachalene	1477	1706	0.8	-	0.2		RI, MS, ^{13}C NMR
33	Germacrene D	1479	-	-	-	-	0.2	RI, MS
34	γ-Humulene	1485	1732	2.0	-	1.6	0.1	RI, MS, ^{13}C NMR
35	β-Selinene	1489	1726	3.8	-	5.2	-	RI, MS, ^{13}C NMR
37	α-Selinene	1498	1732	0.7	-	1.1	-	RI, MS, ^{13}C NMR
38	β-Himachalene	1503	1732	1.3	-	0.9	0.1	RI, MS, ^{13}C NMR
36	γ-Cadinene	1506	1766	1.4	-	0.3	-	RI, MS, ^{13}C NMR
39	β-Sesquiphellandrene	1511	-	-	0.8	-	0.1	RI, MS, ^{13}C NMR
40	δ-Cadinene	1517	1766	1.8	-	0.7	0.1	RI, MS, ^{13}C NMR
41	*ar*-Himachalene	1530	1729	0.4	-	0.7	-	RI, MS, ^{13}C NMR
42	Longiborneol	1594	2162	-	0.2	2.6	0.7	RI, MS, ^{13}C NMR
43	τ-Cadinol	1639	-	-	-	-	0.3	RI, MS
44	Manoyl oxide	1999	-	-	tr	0.2	-	RI, MS
45	*cis*-Abienol	2112	-	17.3#	-	7.5#	-	RI, MS, ^{13}C NMR
	Total			70.4	92.7	74.2	91.9	
	Monoterpenes			25.8	88.4	35.2	88.4	
	Sesquiterpenes			27.3	4.1	30.0	3.0	
	Diterpenes			17.3	-	7.7	-	
	Acyclic non terpenic compounds			-	0.2	1.3	0.5	

[a] Order of elution from apolar column (BP-1). The percentages are those obtained from the apolar column corrected after quantification of *cis*-abienol using ^{13}C NMR (correcting factors 0.875 for sample 1 and 0.947 for sample 2). *: percentages measured on polar column. #: percentage measured by ^{13}C NMR.

samples were analyzed by GC, in combination with retention indices, GC-MS and ^{13}C NMR spectroscopy. As previously reported [9], qualitative analysis of both EO was easily achieved by combination of GC (RI), GC-MS and ^{13}C NMR and quantitative analysis was performed by GC (FID). Conversely, in the SFE, GC-MS and ^{13}C NMR revealed the occurrence of *cis*-abienol, a diterpene alcohol, bearing the labdane skeleton (Figure 1). The quantitative determination of that alcohol was more accurately achieved using ^{13}C NMR spectroscopy.

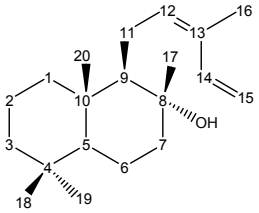
Quantitative determination of *cis*-abienol by ^{13}C NMR spectroscopy. Concerning *cis*-abienol, we observed a bad correlation between the mean intensity of the carbon signals of this compound in both ^{13}C NMR spectra and the percentages measured by GC, the content of the compound being substantially under estimated. In order to overcome this problem, we carried out a quantitative determination of *cis*-abienol using ^{13}C NMR spectroscopy. Quantification of *cis*-abienol in the SFE was achieved using standard quantitative conditions with respect to a fixed amount of diglyme chosen as internal standard. The pulse sequence in the inverse gated decoupling technique used a pulse angle of 90° and a total recycling time (T_R) of 20s, corresponding to 5 x T_1 of the longest T_1 of the protonated carbons of *cis*-abienol and of diglyme. Using this experimental procedure, the content of *cis*-abienol in SFE was estimated at 17.3% and 7.5%, respectively, instead of 12.5% and 5.3% evaluated by GC.

Composition of the SFE of *Abies alba* and comparison with EO. Individual components of the SFE and EO of *A. alba,* their retention indices on two columns of different polarities, their relative percentages and the mode of identification are reported in Table 1.

Taking into account the quantitative determination of *cis*-abienol by ^{13}C NMR, the percentages of the other constituents of the SFE, measured by GC (FID), were modified applying a correcting factor of 0.875 and 0.947 for SFE samples 1 and 2, respectively. The two essential oils exhibited quite different compositions; each sample belonged to one of the two groups previously defined [9].

Indeed, in the first oil sample, the main constituent was limonene (43.5%) accompanied by α-pinene (18.0%) and camphene (13.7%), while the second oil sample contained various monoterpene hydrocarbons in appreciable amounts [camphene (15.7%), limonene (15.6%), β-phellandrene (14.4%), α-pinene (11.4%) and bornyl acetate (9.9%)].

Figure 1: Structure and spectroscopic data (chemical shifts $\delta^{13}C$ and longitudinal relaxation times T_1) of *cis*-abienol.



C	$\delta^{13}C$	T_1
1	40.08	1.3
2	18.58	1.3
3	**41.81**	1.2
4	33.27	1.2
5	56.06	2.6
6	20.25	1.2
7	43.95	1.3
8	74.24	4.8
9	62.16	1.7
10	38.93	3.7
11	23.09	1.7
12	133.83	3.5
13	130.83	4.9
14	133.57	2.6
15	113.74	2.7
16	19.89	3.1
17	24.44	2.3
18	33.46	1.7
19	21.55	1.9
20	15.42	3.4

Comparison of the compositions of the SFE and EO led to the observation of noticeable differences:

i) in both SFE samples, the proportions of monoterpene hydrocarbons, such as limonene (17.5 and 5.6%), α-pinene (3.4 and 2.6%) and camphene (1.9% and 3.9%), are significantly less important than in the EO. Moreover, in sample 2, β-phellandrene accounted for 7.0% of the SFE *vs* 14.4% in the EO.

ii) The proportions of the sesquiterpene hydrocarbons, such as α-longipinene, longifolene, (E)-β-caryophyllene, and α-humulene varied from 1.3% to 6.3% in the two SFE, while they accounted for less than 1.5% in the EO. Moreover, 11 sesquiterpene hydrocarbons, belonging mainly to the selinane, cadinane and himachalane families, were found only in the SFE, with proportions ranging from 0.2% to 5.2%. Therefore, the identified sesquiterpenes accounted for 27.3% and 30.0% in the SFE of sample 1 and sample 2 *vs* 4.1% and 3.0% in the corresponding EO.

iii) Finally, *cis*-abienol (Figure 1) was found only in the two SFE, in which it accounted for 17.3% and 7.5%.

Experimental

Plant material and extractions: The twigs of *Abies alba* Mill. were collected from 2 trees in the forest of Aitone (Corsica) in July 2006. Hydrodistillation and supercritical CO_2 extraction were carried out from the same batches of air-dried material using a laboratory apparatus described in previous papers [9,11,12]. Hydrodistillation yielded 0.65% and 0.74% for samples 1 and 2, respectively, and supercritical CO_2 extraction yielded 0.30% for both samples.

Spectroscopic analysis: GC (FID), GC-MS and ^{13}C NMR analyses were performed as previously reported [9]. Quantitative determination of *cis*-abienol was carried out by ^{13}C NMR using the standard inverse gated decoupling sequence with the following

parameters: pulse width = 8 μs (flip angle 90°); acquisition time = 2.7 s for 128K data table with a spectral width of 25000 Hz (250 ppm); total recycling time T_R=20 s.

The longitudinal relaxation delays of the ^{13}C nuclei (T_1 values) were determined by the inversion-recovery method, using the standard sequence: 180°-τ-90°-D_1, with a relaxation delay D_1 of 20s. Each delay of inversion (τ) was thus taken into account for the computation of the corresponding T_1 using the function $I_p=I_0+p.e^{-\tau/T1}$

Identification of components: Identification of the individual components was based : (i) by comparison of their GC retention indices (RI) on polar and apolar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation with those of authentic compounds or literature data [13]; (ii) on computer matching against laboratory-made (Laboratoire CNP, University of Corsica) and commercial mass spectral libraries [14a-14c] and by comparison of spectra with literature data [13,15,16a], and (iii) by ^{13}C NMR spectroscopy, following the methodology developed and computerised in our laboratories, using home-made software and spectral data library [16b,16c].

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Eugenol: A Natural Compound with Versatile Pharmacological Actions

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Eugenol, the major constituent of clove oil, has been widely used for its anesthetic and analgesic action in dentistry. Eugenol exhibits pharmacological effects on almost all systems and our aim is to review the research work that has identified these pharmacological actions. Eugenol possesses significant antioxidant, anti-inflammatory and cardiovascular properties, in addition to analgesic and local anesthetic activity. The metabolism and pharmacokinetics of the compound in humans have been studied. Eugenol has also been used as a penetration enhancer. The compound is a very promising candidate for versatile applications, and the design of new drugs based on the pharmacological effects of eugenol could be beneficial.

Keywords: eugenol, clove oil, pharmacological actions.

Clove (*Syzygium aromaticum*, Myrtaceae) oil contains eugenol, an oily liquid that has proven analgesic and antiseptic properties [1]. Eugenol ($C_{10}H_{12}O_2$; 2-methoxy-4-(2-propenyl) phenol), is an allyl chain-substituted guaiacol. It is a clear, pale yellow, oily liquid which is slightly soluble in water and soluble in organic solvents. In dental plasters, fillings, and cements either clove oil or eugenol have been used for their topical analgesic properties. In addition to these indications it is used as a local antiseptic and anesthetic. Both clove and eugenol are also used in the fragrance and flavoring industries [2]. In the last few decades, a large number of pharmacological studies on the effects of eugenol on the immune, reproductive, cardiovascular, gastric, central nervous, and urinary systems have been reported, as well as the effects on blood biochemistry [3]. Clove essential oil possesses anti-inflammatory, cytotoxic, insect repellent and anesthetic properties in addition to its established antimicrobial, antioxidant, antifungal and antiviral activities [2].

Analgesic activity: Kurian and coworkers studied the antinociceptive ability of eugenol (100 mg/kg) in several mouse models of pain and found that the effect was more pronounced in the inflammatory phase than the neurogenic phase [4]. Eugenol can alleviate neuropathic pain [5]. Guenette *et al.*, in their study in male Sprague-Dawley rats, showed that eugenol, at a dose of 40 mg/kg, is capable of prolonging reaction time to thermal stimuli [6]. All these results suggested the possible use of eugenol as an analgesic agent.

Antioxidant effect: The potential use of eugenol as a therapeutic antioxidant has been demonstrated by the studies of Nagababu and coworkers [7]. In their study, the formation of thiobarbituric acid reactive substances (TBARS) was decreased by eugenol at concentrations of 4-15 μM. Tests with compounds that are reactive with the hydroxyl radical in the presence of eugenol showed that it competes with these reactive compounds for this radical and thus could protect skin damage by UV-light [8]. The antioxidant effect of eugenol-grafted chitosan hydrogel has also been reported [9]. Eugenol can cause significant suppression of lipid peroxidation and low density lipoprotein oxidation. Eugenol-like compounds significantly reduce copper-dependent oxidation of low density lipoprotein, and lipid peroxidation and autooxidation of Fe^{2+} [10]. Electron spin resonance spectroscopic studies by Fujisawa and coworkers showed that eugenol (18.50 mU per 100 μM sol) significantly scavenged reactive oxygen species [11]. Ogata *et al.* found that the antioxidant action of eugenol (62.5 – 250 μM) is mediated through the

inhibition of lipid peroxidation at the level of initiation [12]. Vidhya and coworkers investigated the short and long term antioxidant activities of eugenol (1000 mg/ kg) on rat intestine and observed that the compound helps in the removal of toxic materials from the intestine as it caused the induction of glutathione-S-transferases [13]. Antioxidants and free radical scavengers like eugenol have also been monitored as agents for preventing the progress of Parkinson disease. A study demonstrated that eugenol (0.1 - 1.0 μM/kg) inhibits lipid peroxidation and thereby suppresses 6-hydroxydopamine-induced dopamine depression thereby suggesting its potential use in Parkinson disease [14]. The antioxidant activity of eugenol is comparable with that of butylated hydroxyanisole and butylated hydroxytoluene [15]. Although many *in vitro* studies have been carried out on the antioxidant activity of eugenol, there have been insufficient *in vivo* studies.

Protective effect: Eugenol modulates both the NMDA receptor and superoxide radical and thus seems to provide protection against ischemic injury. Eugenol can attenuate NMDA induced acute neurotoxicity and decrease xanthine/xanthine oxidase produced oxidative neuronal injury [16]. Eugenol (10.7 mg/kg/day) has been found to show an *in vivo* antioxidant property and a better inducing effect on phase II enzymes which counteract the metabolizing enzyme reduction caused by carbon tetrachloride [17]. *In vitro* studies on liver microsomal monooxygenase activities and carbon tetrachloride (CCl_4)-induced lipid peroxidation have shown that eugenol inhibits both [18]. The *in vivo* studies by the same researchers also showed a protective effect of eugenol at doses of 5 and 25 mg/kg against CCI_4 induced hepatotoxicity. The methanolic extract of leaves of *Ocimum suave* (Lamiaceae), which contains eugenol as a major constituent [19], has shown gastric cytoprotective and anti-ulcer effects in experimental Wistar rats [20]. Eugenol pre-treatment (10-100 mg/kg) reduces both the number of gastric ulcers and the gravity of lesions induced by ulcerogenic agents like platelet activating factor and ethanol [21]. The mechanism of the gastroprotective action of eugenol has been established by the studies of Morsy and Fouad [22]. Gastric mucosal lesions, gastric acid outputs and pepsin activity in male Sprague-Dawley rats were markedly reduced by a single oral dose of eugenol (100 mg/kg), used as a pretreatment one hour before indomethacin injection. This pretreatment resulted in a marked increase in mucin concentration, suppression of the rise in gastric mucosal malondialdehyde and total nitrite, and attenuation of the decrease in reduced glutathione. It was further confirmed that the protective action of eugenol is through opening of ATP-sensitive potassium channels and not through the transient receptor potential vanilloid 1. The DNA-protective activity of eugenol has been observed by Yogalakshmi and coworkers in thioacetamide-induced liver injury in rats at a dose of 10.7 mg/kg/day [23]. Eugenol imparts a dose dependent protection against nicotine-induced superoxide mediated oxidative damage at a concentration range of 1-20 μg/mL [24].

Anesthetic action: A randomized, single-blind study was carried out in 73 subjects to evaluate the topical anesthetic action of clove oil in comparison with benzocaine [1]. The pain scores between the clove gel and the benzocaine groups were not significantly different, but were significantly lower than those of the placebo groups. The study showed that clove gel is a cheap and easily available topical anesthetic which could be used as an alternative to benzocaine gel in dentistry. A reversible, dose dependent anesthesia has also been reported after eugenol administration (5 – 60 mg/kg, iv) in male Sprague–Dawley rats [5].A clinical study was conducted in 100 adult patients to study the effect of eugenol as an intravenous anesthetic. However, they concluded that it is not advisable to use eugenol except in special circumstances as the incidence of development of venous thrombosis around the site of injection was high [25]. The possible use of eugenol as a local anesthetic has also been demonstrated by the studies of Park and coworkers, who suggested its suitability for non-dental anesthesia as well [26].

Antibacterial, antifungal and antiviral activities: The activity has been studied of *Eugenia aromaticum* on several microorganisms and parasites, including pathogenic bacteria, and herpes simplex and hepatitis C viruses [27]. Burt and coworkers studied the antibacterial activity of clove bud oil and found it to be effective [28]. Eugenol is active against *Neisseria gonorrhoeae*, with a minimum inhibitory concentration of 85-256 mg/L [29]. Moreover, eugenol shows synergistic effects with antibiotics against Gram-negative bacteria [30]. Mytle and coworkers have demonstrated the ability of clove oil to inhibit the growth *Listeria monocytogenes*. A 1% v/w concentration was sufficient for the inhibition of growth at 5^oC and 15^oC [31]. Eugenol was found to be active against sessile cells in *Candida albicans* biofilms [32]. The compound showed fungicidal activity *in vitro* for exponentially growing *Candida albicans*. The mechanism of this action was found to be through envelope damage [33]. In the *in vivo* study, treatment with eugenol caused a significant reduction in the colony count number in an immunosuppressed rat model of oral candidiasis in comparison with the untreated control group [34]. Chami and coworkers also evaluated eugenol in an immunosuppressed rat model of vaginal candidiasis for its efficacy in the prophylaxis

and treatment of this condition [35]. Eugenol has been demonstrated to have activity against *Penicillium* (100 mg/L), *Aspergillus* (100-140 mg/L) and *Fusarium* species (140-140 mg/L) also [36].

Eugenol can cause damage to viral envelopes of freshly formed virons and can cause inhibition of viral replication at the initial stage [37]. The anti-viral activity of eugenol, clove flower bud extract and clove essential oil against *Herpes simplex* virus has been carried out [37]. Direct inactivation of viruses and inhibition of intracellular and extracellular viruses after replication were observed with eugenol. In another study for antiviral activity against HSV-1 and HSV-2 viruses [38], 50% inhibitory concentration values were 25.6 μg/mL and 16.2 μg/mL for HSV-1 and HSV-2, respectively. Eugenol demonstrated a synergistic action with acyclovir against *in vitro* replication of herpes virus. Topical eugenol therapy was observed to suppress herpes virus induced keratitis in mouse.

Anticonvulsant activity: Zelger and coworkers investigated the anticonvulsant activity of phenyleugenol, benzyleugenol and phenylethyleugenol, which are synthetic derivatives of eugenol [39]. All three compounds showed significant anticonvulsant activity in the maximal electroshock seizure test. Phenyleugenol and benzyleugenol were found to have a high therapeutic index. All these studies indicated that eugenol could be taken as a lead molecule in the development of novel anticonvulsant drugs.

Anti-inflammatory action: Eugenol inhibits cycloxygenase and thus inhibits prostaglandin H synthase [40]. This can be the result of its competition with arachidonic acid. Eugenol is able to resist the release of proinflammatory mediators like interleukin-1β, tumor necrosis factor-α and prostaglandin E2 from macrophages and is thus useful for acute inflamed dental pulps and apical periodontitis as an anti-inflammatory agent [41]. Eugenol can also inhibit cycloxygenase-2 in macrophages. Eugenol caused reduction of both paw and joint swelling in arthritis induced male Sprague-Dawley rats at a dose of 33 mg/kg [42]. An *in vivo* study by Rodrigues and coworkers on the effect of an hydroalcoholic extract of clove on pro-inflammatory cytokines production by macrophages of mice showed that clove oil (200 mg/kg) caused cytokine inhibition due to the presence of eugenol, which imparts an anti-inflammatory activity [43]. The *in vivo* anti-inflammatory activity of eugenol in lipopolysaccharide induced lung injury has been demonstrated on administration of 160 mg/kg, ip [44].

Penetration enhancement: The utility of clove oil (82.6% eugenol) as a penetration enhancer has been demonstrated by Shen and coworkers [45]. Permeation enhancement was found to be significant both *in vivo* and *in vitro*. The *in vivo* effect observed was comparatively weaker than that observed *in vitro*. A marked increase in drug flux was observed with clove oil in *in vivo* percutaneous absorption studies in rabbit. All the results suggested the potential ability of clove oil, of which eugenol is the major active component, as a penetration enhancer in transdermal drug delivery systems [45]. *In vitro* evaluation of penetration enhancers, including eugenol, has been conducted by Mutalik and coworkers using mouse skin [46]. In their study, eugenol was also found to increase skin retention and solubility of glibenclamide and glipizide. Zhao and coworkers studied the *in vitro* penetration enhancement effect of eugenol on percutaneous absorption of a drug through porcine epidermis [47]. The study showed that eugenol was able to enhance markedly the permeability coefficient of tamoxifen with respect to the control group. The study also found that lipid extraction and increased partitioning of the tamoxifen to the stratum corneum is responsible for the observed permeability enhancement action of eugenol.

Cardiovascular actions: Eugenol (0.2 – 20 μmol) produces dose-dependent, reversible vasodilator responses that are partially dependent on the endothelium [48]. Sensch and coworkers showed that eugenol (60 – 600 μmol/L) exerted negative inotropic effects in heart muscle of Guinea-pig [49]. The effect was found to be comparable with that of nifedipine, a calcium channel blocker. Eugenol (0.006 – 6 mM) causes hypotensive effects in hypertensive rats due to vascular relaxation [50]. The blocking of voltage-sensitive and receptor-operated channels by eugenol is responsible for its smooth muscle relaxant effect and these actions are mediated through endothelial-generated nitric oxide [51]. Choudhary and coworkers observed eugenol to counteract isoproterenol-induced cardiac hypertrophy [52]. They conducted their study in male Wister rats using a dose of 1 mg/kg twice daily. They showed that serum calcineurin activity *in vitro* was suppressed by eugenol, and isoproterenol-induced oxidative stress and apoptosis were reduced. Eugenol was also found to increase cardiac calcineurin and protein kinase C activity in ventricular tissue to normal values.

Anticancer activity: Significant induction of activity of glutathione S-transferase by eugenol was observed in liver and small intestine [53]. Miyazawa and coworkers showed that due to the presence of a C-4 hydroxy group in their structure, *trans*-isoeugenol and eugenol had significant suppressive effects on the SOS-inducing activity of chemical mutagens such as furylfuramide [54]. The antimutagenic effects of a eugenol derivative,

dehydroeugenol, have also been demonstrated by the same authors. Studies have demonstrated that eugenol provides protection from chemical induced skin cancer [55, 56].

Other pharmacological actions: An antigenotoxic effect of eugenol was studied in humans by Rompelberg and coworkers [57], but no evidence was observed for these effects. Tajuddin and coworkers investigated the effect of a 50% ethanolic extract of clove on sexual function improvement in male Swiss mice (500 mg/kg) and observed an enhanced sexual behavior of the mice [58].

Molecular mechanisms and biochemical changes: Eugenol showed inhibition of high-voltage-activated calcium channel currents in both capsaicin-sensitive and capsaicin-insensitive dental primary afferent neurons [59]. However, the action of eugenol was unaffected by capsazepine. This suggested that the action is not mediated by transient receptor potential vanilloid 1 activation. N-type calcium currents were inhibited by eugenol in the cell line C2D7. The study showed that high-voltage-activated calcium channel current inhibition in dental primary afferent neurons by eugenol is responsible for its dental analgesic action. The mechanism of anesthetic action of eugenol by inhibition of sodium channel currents has been established by the whole-cell patch-clamp method in rat dental primary afferent neurons [60]. Capsaicin-sensitive and capsaicin-insensitive neurons are both inhibited by eugenol and its irritable action is possibly due to the inhibition of voltage-gated K^+ currents [61]. Yang and coworkers showed that eugenol produces its effects in the sensory nerve endings in the teeth through vanilloid receptor 1, at least partially [62].

The analgesic effect of eugenol is probably due to the inhibition of Ca(V)2.3 calcium channels. A study by Chung and coworkers showed that the mechanism of inhibition of Ca(V)2.3 calcium channels by eugenol is not involved through transient receptor potential vanilloid 1 and thus appeared to be distinctly different from that of capsaicin [63]. Both capsaicin receptor mediated and capsaicin receptor independent pathways are involved in the activation of Ca^{2+} channels by eugenol [64]. Eugenol has the ability to inhibit pro-inflammatory mediators like nitric oxide synthase, lipoxygenase and cyclooxygenase. Eugenol exerts its analgesia by inhibition of Na^+ currents and the inhibition is independent of the stimulus frequency [65]. Inotropic effects of eugenol have been investigated in rat left ventricular papillary muscles by Damiani and coworkers [66]. The contraction force was depressed without affecting the contractile machinery. Complete blockade of inward Ca^{2+} current was observed with 0.5 mM of eugenol. Xu and coworkers explained that the transient receptor potential vanilloid 3 is expressed by eugenol. It is a warm-sensitive Ca^{2+}-permeable cation channel in the skin, tongue and nose [67].

Eugenol has been reported to potentiate the GABA response. The compound's ability to potentiate GABAA receptors can modulate neural transmission in the brain just like, for example, benzodiazepine and barbiturate [68]. Salah and coworkers showed that eugenol causes effects such as relaxation of rat ileal strip, reduced intestinal transit in rats, and the potentiation of the diarrhea inducing effect of castor oil [69]. They suggested that all these effects are mediated through Ca^{2+} channel modulation. Eugenol accelerates inactivation of the Ca^{2+} current in isolated canine and human ventricular cardiomyocytes [70].

Metabolism and pharmacokinetics: Fischer and coworkers investigated the metabolism of eugenol in male and female healthy volunteers [71]. The study revealed that eugenol is rapidly absorbed and metabolized after oral administration. It is almost completely excreted in the urine within 24 hours. Only less than 0.1% of the administered dose was excreted unmetabolized in urine. Eugenol was found in the urine in the form of conjugates and metabolites. Among the metabolite conjugates, 55% consisted of eugenol-glucuronide and sulfate. The epoxide-diol pathway, allylic oxidation, synthesis of a thiophenol and a substituted propionic acid, and migration of the double bond were also found to be other routes of eugenol metabolism in humans. A study was made by Guénette and coworkers of the pharmacokinetic parameters of eugenol using non-compartmental analysis after gavage administration in male Sprague–Dawley rats in a dose of 40 mg/kg [6]. Plasma $T_{1/2}$ of eugenol was found to be 14.0 hours and blood $T_{1/2}$ was 18.3 hours. Glucuronide and sulfate conjugates of eugenol in urine after eugenol administration were also identified in male Sprague–Dawley rats [5].

Toxicity: The prooxidant activity of eugenol may be responsible for its toxicity [72]. An *in vitro* study by Medeiros and coworkers demonstrated that the binding of lysine to eugenol might lead to protein inactivation and consequently to its toxicity [73]. The insecticidal activity of eugenol also has been reported [2].

A summary is given in Table 1 of the various research carried out to demonstrate the different pharmacological activities of eugenol and clove oil. Further research is required to collect more information about the pharmacological effects of eugenol, and also to find new areas of therapeutic applications. A detailed knowledge of eugenol pharmacology could be utilized

Table 1: Pharmacological studies of eugenol and clove oil.

Activity studied	Experimental	Effect (Dose/Concentration)	Ref.
EUGENOL			
Analgesic	Mice	Significant antinociceptive effect (100 mg/kg)	[4]
	Male Sprague-Dawley rats	Prolonged reaction time (40 mg/kg)	[6]
Antioxidant	Microsomal mixed function oxidase mediated peroxidation	Decreased TBARS, inhibition of oxygen uptake, monooxygenase activity inhibition (4-15 μM)	[7]
	LDL oxidation	Suppressed oxidation (1.5 μM)	[10]
	Lipid peroxidation	Significant suppression (0.05 – 0.15 mM)	[10]
	Reactive oxygen scavenging activity	Significant activity (18.50 mU/100 μM sol)	[11]
	Free radical reaction	Inhibited lipid peroxidation at propagation step (62.5 – 250 μM)	[12]
	Rat intestine	Induced glutathione-*S*-transferases (1000 mg/kg)	[13]
	Lipid peroxidation	Inhibited (0.1 -1.0 μM/kg)	[14]
	DPPH scavenging activity	Activity similar to BHA & BHT (20 μg/mL)	[15]
	Hydroxyl radical scavenging	Activity greater than quercetin (0.6 μg/mL)	[15]
Protective	NMDA neurotoxicity	Prevented acute neuronal swelling and reduced neuronal death (100- 300 μM)	[16]
	Carbon tetrachloride intoxicated rat liver	Counteracted metabolic enzyme reduction (10.7 mg/kg/day)	[17]
	Liver monoxygenase activity & Carbon tetrachloride induced lipid peroxidation	Inhibition (5 & 25 mg/kg)	[18]
	Induced gastric lesions in rat	Reduced number and severity of ulcers observed (10-100 mg/kg)	[21]
	Induced ulcer in male Sprague-Dawley rats	Gastroprotective action (100 mg/kg)	[22]
	Adult male Wistar rats	Curtailed thioacetamide (TA)-induced hepatic injury (10.7 mg/kg/day)	[23]
	Nicotine-induced oxidative damage	Dose dependent protection (1-20 μg/mL)	[24]
Anesthetic	Randomized, single-blind study	Activity similar to benzocaine	[1]
	Male Sprague-Dawley rats	Reversible & dose dependent activity (5- 60 mg/kg)	[5]
Antibacterial, Antifungal & Antiviral	*Candida albicans* biofilms	Active against sessile cells (20-2000 mg/L)	[32]
	Candida albicans	Antifungal activity by envelope damage (0.2 %)	[33]
	Oral candidiasis in immunosuppressed rats	Significant reduction in the colony counts (0.5 mL, 24 mM)	[34]
	Vaginal candidiasis in immunosuppressed rats	Reduction in the colony counts of *Candida albicans* (20 mg/kg/day)	[35]
	Penicillium species	Growth inhibition (100 mg/L)	[36]
	Aspergillus species	Growth inhibition (100-140 mg/L)	[36]
	Fusarium species	Growth inhibition (140-150 mg/L)	[36]
	Anti-herpes simplex virus	Active against HSV-1 and HSV-2 viruses (16.2 and 25.6 μg/mL)	[38]
Anticonvulsant	Maximal electroshock seizure test	Synthetic eugenol derivatives showed significant activity	[39]
Anti-inflammatory	Prostaglandin H synthase inhibition	Inhibited cycloxygenase (100-200 μM)	[40]
	Human macrophages	Resisted the release of proinflammatory mediators & inhibited cycloxygenase-2	[41]
	Male Sprague-Dawley rats	Reduced both paw and joint swelling in induced arthritis (33 mg/kg)	[42]
	Mice	Reduced lipopolysaccharide induced lung inflammation (160 mg/kg, ip)	[44]
Penetration enhancer	*In vitro* evaluation in mouse skin	Increased drug flux (5 %)	[46]
	In vitro penetration through porcine epidermis	Significant enhancement of permeability coefficient of drug (5 %)	[47]
Cardiovascular	Rat mesenteric vascular bed	Dose-dependent, reversible vasodilatation (0.2 – 20 μmol)	[48]
	Guinea-pig heart muscle	Potassium current inhibition (60 – 600 μmol/L)	[49]
	Hypertensive rats	Vascular relaxation (0.006 – 6 mM)	[50]
	Smooth muscle relaxant effect	Action mediated through endothelial-generated nitric oxide (300 μM)	[51]
	Isoproterenol-induced cardiac hypertrophy in	Oxidative stress and apoptosis were reduced (1 mg/kg twice daily)	[52]
Anticancer	Mice	Protection against chemically induced skin cancer (30 μL)	[55]
	Swiss mice	Restriction of skin carcinogenesis at dysplastic stage (1.25 mg/kg)	[56]
CLOVE OIL			
Antioxidant	DPPH scavenging activity	Activity similar to BHA & BHT (0.5 μg/mL)	[15]
	Hydroxyl radical scavenging	Active more than quercetin (0.2 μg/mL)	[15]
	Lipid peroxidation	Active more than BHT	[15]
Anti-inflammatory	Mice	Caused cytokine inhibition (200 mg/kg)	[43]
Penetration enhancer	Excised rabbit abdominal skin	Significant increase in drug flux (1-3 %)	[45]
	In vivo percutaneous absorption in rabbit	Increase in drug flux (1-3 %)	[45]
Anticancer	Antimutagenic activity against MNNG, 4NQO, AfB_1 and Trp-P-1.	Suppressive effect on mutagens (ethyl acetate extract)	[54]
Aphrodisiac	Male Swiss mice	Improved sexual function (500 mg/kg of 50 % ethanolic extract)	[58]

to consider eugenol as a lead molecule for the development of new drugs [74] with enhanced therapeutic efficacy.

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