2013

GC/GC-MS analysis, isolation and identification of bioactive essential oil components from the Bhutanese medicinal plant, Pleurospermum amabile

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Publication Details

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Abstract
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Keywords
identification, isolation, analysis, ms, gc, plant, amabile, medicinal, pleurospermum, bhutanese, components, oil, essential, bioactive, CMMB

Disciplines
Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

This journal article is available at Research Online: http://ro.uow.edu.au/smhpapers/1253
GC/GC-MS Analysis, Isolation and Identification of Bioactive Essential Oil Components from the Bhutanese Medicinal Plant, Pleurospermum amabile

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We have hydrodistilled the essential oil (EO) from the aerial parts of the Bhutanese medicinal plant, Pleurospermum amabile using a Clevenger apparatus and evaluated this EO by GC/GC-MS and NMR analysis followed by testing for bioactivity. The GC-MS analysis identified 52 compounds with (E)-isomyristicin as a major component (32.2%). Repeated purification yielded four compounds; (E)-isomyristicin (1), (E)-isoelemicin (2), methyl eugenol (3) and (E)-isoelemicin (4). Compound 2 and the mother EO showed the best antiplasmodial activity against the Plasmodium falciparum strains, TM4/8.2 (chloroquine and antifolate sensitive) and K1CB1 (multidrug resistant). They exhibited mild antibacterial activity against Bacillus subtilis. None of the test samples showed cytotoxicity.

Keywords: Pleurospermum amabile, medicinal plant, essential oil, (E)-isomyristicin, antibacterial, antimalarial, multidrug resistant strain, cytotoxicity.

Essential oils (EOs) are secondary metabolites of aromatic plants with strong odors and volatile constituents composed of terpenoids (mono-, sesqui- and di-terpenes), alcohols, ketones, aldehydes, alkanes, and phenylpropanoids. They are responsible for the protection of plants against microbes, insects, and herbivores [1]. Since antiquity, mankind has used EOs and the EO-containing plants as medicinals (ethnmedicine), perfumeries, incense, fragrances and embalmments and in culinary and the preservation of foods [2].

While many plants from various genera have been studied and some even commercially explored for EOs, the genus Pleurospermum (family Apiaceae) has been rarely investigated. Out of 30-50 species of Pleurospermum reported from eastern Europe, north Asia and the Himalayan region [3], only five species; P. lindleyanum [4], P. austriacum [5], P. hookeri [6], P. wrightianum [7], and P. giraldii [8] have been investigated for EOs. From the EO of P. lindleyanum, 73 compounds were identified with 1-propoxy-2-propanol, myristicene, cis-asarone, n-hexane, apiole, dimethyl ether, acetic acid, ethyl ester, spathulenol, 4-trimethylbenzene methanol, (E)-methyl isoeugenol and β-phellandrene as the main constituents [4]. Apparently, 1-propoxy-2-propanol, dimethyl ether, and n-hexane appear to be solvent contaminant or the artifacts. From the EO of P. austriacum, 205 compounds were identified with germacrene D, β-caryophyllene, β-farnesene, β-phellandrene, δ-cadinene, epi-cubebol, bicyclogermacrene, humulene, α-cadinol and hexadecanoic acid as some of the major components [5]. Out of 72 GC peaks detected, 51 compounds were identified from the EO of P. hookeri and the major ones were palmitic acid, decanoic acid, ligustilide, piperteralen, (Z)-2-decenaldehyde and 2,4,5-trimethyl benzaldehyde [6]. From the EO of P. wrightianum, about 49 compounds were identified with (E)-9-octadececenic acid and 1,5,7-cyclooctatetraene as the major parts of oil [7]. Out of 45 compounds identified from the EO of P. giraldii, L-carvone and limonene were found to be the major components [8].

In this study, we have analyzed a Bhutanese Himalayan medicinal plant, P. amabile (synonym Hymenidium amabile) for its essential oil (EO) components and biological activities for the first time. The aerial components of this plant are used in Bhutanese traditional medicine (BTM) for treating dyspepsia, poisoning (antidote) and fever (febrifuge) that correlates to the symptoms of microbial infections and malaria [9]. The hydrodistillation of the dried powdered aerial plant material (250 g dry weight) yielded 0.7 % EO. Fifty two component peaks were detected and identified by GC/GC-MS analysis including MS library matching and Kovats retention indices (KI) comparison techniques (Table 1).

The percent contents of the EO were determined on the basis of their FID responses upon GC (Figure 1). (E)-isomyristicin (1) was the major component (32.5%) of the EO (Figure 1, Table 1) followed by limonene (17.0%), (E)-isoelemicin (7.6%), β-sesquiphellandrene (3.9%), methyl eugenol (3.8%), geranyl pentanoate (2.4%), (Z)-isomyristicin (2.3%), myrcene (2.3%), β-caryophyllene (2.2%), geraniol (1.6%), (E)-isoelemicin (1.3%), geranyl isobutyrate (1.3%), myristicin (1.3%), valeranone (1.2%), β-citronellol (1.2%), β-selinene (1.2%), myrtanyl acetate (1.1%) and citronellyl acetate (1%).

Although slightly different column and protocol conditions were used in the EOs analysis, many EO compounds of P. amabile including limonene, β-caryophyllene, germacrene D, α-cadinol, α-bisabolene, α-pinene, myrcene, α-phellandrene, β-selinene, geranyl acetate, β-ocimene, and few others (see Table 1 and literature for details) were found common to the EO of P. austriacum [5].

Subsequent purification of the EO of P. amabile using column chromatography and preparative TLC plates resulted in the isolation of four compounds which were identified by KI and NMR spectroscopy data analysis as (E)-isomyristicin (1) [10-11], (E)-isoelemicin (2) [12], methyl eugenol (3) [13-14], and (E)-isoelemicin (4) [15] (Figure 1).

Table 1: Chemical compositions of EO from P. amabile.

<table>
<thead>
<tr>
<th>GC Peak No.</th>
<th>% oil</th>
<th>Compound name</th>
<th>KI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7</td>
<td>α-Pinene</td>
<td>934a</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>β-Pinene</td>
<td>977a</td>
</tr>
<tr>
<td>3</td>
<td>2.3</td>
<td>Myrcene</td>
<td>991a</td>
</tr>
</tbody>
</table>
Given the traditional use of *P. amabile* to treat fever and various disorders bearing relevance to microbial infections and a malaria and the evidence that its CH₂Cl₂ and CHCl₃ extracts exhibited significant antibacterial and antimalarial activity (Table 2) [9], we investigated the mother EO and three major compounds (1, 2 and 4) isolated above for their antimicrobial, antimalarial and cytotoxicity activities (Table 2). The mother EO and compounds 1 and 2 showed moderate antibacterial activity against *B. subtilis* with minimum inhibition zones (MIZ) ranging from 5-6 mm (Table 2). However, unlike the CH₂Cl₂ and CHCl₃ extracts (from previous study, Table 2), the EO and its compounds (1, 2 and 4) did not show any activity against MRSA and other microbial strains tested here. The mother EO also exhibited moderate *in vitro* antimalarial activity against the *P. falciparum* strains: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain) and K1CB1 (multidrug resistant strain) with IC₅₀ values of 79.0 µg/mL and 72.3 µg/mL, respectively. These activities were lower than those exhibited by the CH₂Cl₂ and CHCl₃ extracts (Table 2). Such correlations, including the antimicrobial activities, suggested that the more potent chemical components are present in the CH₂Cl₂ and CHCl₃ extracts rather than in EO. In comparison to the antimalarial activity of the mother EO, compound 2 showed improved activity with IC₅₀ values of 52.9 µg/mL and 69.9 µg/mL against TM4/8.2 and K1CB1, respectively. Compounds 1 did not show any antimalarial activity and compound 4 had poor solubility which restricted the determination of its accurate IC₅₀ values for its highest test concentrations at 20 µg/mL. None of the test samples in this study showed any major cytotoxicity which thereby supports the assumption that the use of *P. amabile* in BTM is safe.

**Table 2:** Antibacterial, antimalarial and cytotoxicity activities of EO and compounds 1, 2 and 4 isolated from *P. amabile*.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Antibacterial (MIZ in mm)</th>
<th>Antiplasmodial (IC₅₀ in µg/mL)</th>
<th>Cytotoxicity (IC₅₀ in µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>B. subtilis</em></td>
<td>MRSA</td>
<td>TM4/8.2</td>
</tr>
<tr>
<td>CH₂Cl₂ extract</td>
<td>14</td>
<td>12</td>
<td>12.1±0.4</td>
</tr>
<tr>
<td>CHCl₃ extract</td>
<td>6</td>
<td>NT</td>
<td>7.8±1.5</td>
</tr>
<tr>
<td>Essential oil (EO)</td>
<td>5</td>
<td>NA</td>
<td>79.0±4.6</td>
</tr>
<tr>
<td>(E)-Isomericin (1)</td>
<td>5</td>
<td>NA</td>
<td>&gt;100</td>
</tr>
<tr>
<td>(E)-Isoapiol (2)</td>
<td>6</td>
<td>NA</td>
<td>52.9±2.9</td>
</tr>
<tr>
<td>(E)-Isoleucin (4)</td>
<td>NA</td>
<td>NA</td>
<td>20</td>
</tr>
<tr>
<td>Vancocin</td>
<td>8</td>
<td>NA</td>
<td>13.5</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>0.010</td>
<td>0.089</td>
<td></td>
</tr>
<tr>
<td>Cyclonocillin</td>
<td>0.009</td>
<td>0.810</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>0.020</td>
<td>7.700</td>
<td></td>
</tr>
<tr>
<td>Etopisome</td>
<td>0.093</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOxorubicin</td>
<td>0.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NT: Not Tested, NA: Not Active.

a Original activity taken from [9].
b Positive controls for antibacterial activity.
c Positive controls for antimalarial activity.
d Positive controls for cytotoxicity activity.

In conclusion, this study found that *P. amabile*, which is one of the important medicinal ingredients of the Bhutanese traditional medicine, has EO (0.7% oil w/w) with (E)-isomericin (32.5% oil) as the major component. The EO and compounds 1 and 2 inhibited the growth of only *B. subtilis* and no other strains. The EO and compound 2 also demonstrated moderate *in vitro* antimalarial activity without mammalian cell toxicity. This *in vitro* bioactivity of the EO supports the reported biological activities [9] of the crude extracts of the plant which further verifies the safety record of *P. amabile* used in BTM for treating various aforementioned disorders. However, *in vivo* experiments will be required to further justify the use of EO for treating humans.

**Experimental**

*Plant material and essential oil: *P. amabile* is an endangered species inhabiting the open scrub, alpine turf and the semi-stable scree of high altitude Himalayan mountains (3950 to 4700 meters above sea level) in Bhutan [18-19]. It grows to 15-50 cm tall with white flowers and ovoid-oblong fruit [3,18]. For this study, the aerial components of *P. amabile* were collected from Lingzhi (Altitude: 4200 m) in Thimphu district in July 2009. A herbarium specimen (voucher number 29) was deposited at the herbarium of the Manjong Sorig Pharmaceuticals, Ministry of Health, Thimphu, Bhutan. The air-dried plant material (250 g) was powdered and hydro-distilled using a Clevenger apparatus for 3 hours to obtain the pale green pleasantly aromatic EO (1.8 mL). The EO collected was dried over MgSO₄. The EO was stored at 0-5°C until analysed.

**References**

Analysis of EO using GC and GC-MS: The EO was analysed for its chemical composition using GC and GC-MS systems and equipment as described by us previously [20]. The GC analysis was performed on a Shimadzu GC-2010 Plus gas chromatograph. Hydrogen was used as carrier gas and the separation was achieved using a Restek fused silica capillary column (Rxi-5MS: 30 m × 0.25 mm i.d., 0.25 μm film thickness). Injector and detector temperature were set at 260 °C and 300 °C, respectively. The starting oven temperature was programmed at 40 °C with an increasing temperature of 6 °C/min until it reached 290 °C. KI were obtained by GC-FID analysis of an aliquot of the EOs spiked with a commercially available n-alkane mixture (C9 to C21). The GC-MS analysis was performed using Shimadzu QP5050A GC-MS system (electron impact (EI) mode at 70 eV). The column and the GC-MS chromatographic conditions were same as that for GC with the exception that the He was used as carrier gas. The EO constituents were identified by comparing mass spectra with NIST and NISTREP mass spectra library of GC-MS data system and further confirmed by comparing their KI with those reported [5,10,16-17].

Isolation of compounds from EO: The equipment, general protocols and isolation techniques were carried out as described by us previously [21]. The known compounds were identified through MS library matching techniques (NIST and NISTREP mass spectra library) and then confirmed through comparison of their MS and NMR spectra (500 MHz, CDCl3) with those reported (see footnote of Table 1 for references).

The EO (383 mg) was column chromatographed on a silica gel (120 g, 200-300 mesh) eluting with a gradient solvent system of CHCl3-petroleum spirit (v/v ratio of 0:100, 5:95, 10:90, 15:85, 20:80, 30:70, 50:50, 70:30, 100:0) to obtain nine fractions, PAoil.1-9. Further separation of PAoil.1 using reversed phase preparative silica gel plates in highly polar solvent system (10% petroleum spirit:90% MeOH) yielded compound I (185.3 mg) (major constituent of oil, Fig. 1) which was identified as (E)-isomyristicin through NMR spectral data analysis. Eluting fraction PAoil.5 with isocratic or fixed ratio solvent system (10% petroleum spirit:90% CHCl3) on a normal phase silica gel column furnished (E)-isoelemicin (4) (44.7 mg, Fig. 1). Separation of fraction PAoil.8 on a preparative silica plate using the solvent system of 30% diethyl ether:96% petroleum spirit, yielded (E)-isoelemicin (4) (3.4 mg, Figure 1).

Bioassay methods: Antimicrobial, antiplasmodial and cytotoxicity bioassays were carried out using the standard protocols reported by us previously [9]. The test strains of Plasmodium falciparum used for the antiplasmodial bioassay were K1CB1, a multidrug resistant strain; and TM4/8.2, a wild type chloroquine and antifolate sensitive strain. Chloroquine (Sigma-Aldrich), cycloguanil (Sigma-Aldrich) and pyrimethamine (Sigma-Alrdich) were used as positive controls in the antiplasmodial assays. For the antimicrobial assay, the test organisms including Escherichia coli (ATCC 25922), Bacilli subtilis (ATCC 6633), Staphylococcus aureus (ATCC 6538), methicillin resistant S. aureus (MRSA), (DMST 20651), S. epidermidis (ATCC 12228), Vibrio cholerae (DMST 2873) and Candida albicans (ATCC 10231) were used. Amphotericin B (Sigma-Aldrich, USA) was used as a positive control for antifungal testing against Candida albicans (not shown in Table 2 as the samples were found to be inactive). Vancomycin (Edicin, Slovenia) and amoxicillin (GPO, Thailand) were used as the positive controls for antibacterial assays. For the cytotoxicity assay, normal vero cells from kidney of African green monkey, Cebopithecus aethiops and the human oral carcinoma KB cells were used. Ellipticine (Sigma-Aldrich, USA) and doxorubicin (Sigma-Aldrich, USA) were used as reference drugs for cytotoxicity activities. All the experiments were performed three times in duplicate (3x2) and DMSO (0.1%) and distilled water were used as controls to rule out the solvent effects on the bioassay results of the test samples.

Acknowledgements We acknowledge the support of: a) Endeavour Award, Australia for providing scholarship to PW; b) Mr. Samten, Pharmacognosist of Manjong Sorig Pharmaceuticals for providing the essential oil; c) Dr. John Korth of University of Wollongong for GC/GC-MS assistance; d) Roonglawan Rattanajak of BIOTECH, Thailand for antiplasmodial assistance. SK is an international Research Scholar of the Howard Hughes Medical Institute, USA.
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