Phenylpropanoids and furanocoumarins as antibacterial and antimalarial constituents of the Bhutanese medicinal plant Pleurospermum amabile

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Publication Details

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Abstract
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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/2190
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Pleurospermum amabile

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With the objective of determining safety and verifying the traditional uses of the Bhutanese medicinal plant, Pleurospermum amabile Craib & W. W. Smith, we investigated its crude extracts and the isolated phytochemicals for their biological activities. Four phenylpropanoids [(E)-isomyristicin (1), (E)-isopiol (2), methyl eugenol (3) and (E)-isoelemicin (4)] and six furanocoumarins [psoralen (5), bergapten (6), isoimperatorin (7), isopimpinellin (8), oxypeucedanin hydrate (9) and oxypeucedanin methylate (10)] were isolated from this plant. Among the test samples, compound 10 showed weak antibacterial activity against Bacillus subtilis and best antimalarial activity against the Plasmodium falciparum strains, TM4/8.2 (chloroquine and antifolate sensitive) and K1CB1 (multidrug resistant). None of the test samples showed cytotoxicity. This study generated scientific data that support the traditional medical uses of the plant.

Keywords: Pleurospermum amabile, Bhutanese medicinal plant, Antibacterial, Antimalarial, Furanocoumarins, Phenylpropanoids.

The Pleurospermum belong to the family Apiaceae (Umbelliferae) and about 30-50 species are reported from Eastern Europe, north Asia and the Himalayan region [1a-b]. Phytochemicals including essential oils, coumarins, flavonoids, terpenoids, saponins, steroids, fatty acids, esters, glycosides and triterpene saponins were reported from the few species that were studied [2a-b]. Pharmacologically, the crude extracts, essential oils and the phytochemicals were reported to possess various therapeutic activities including, anticance,r anti-inflammatory, antimalarial, anti-trypanosomal, antibacterial, analgesic, apoptosis inducing, and inhibition of hyperlipidemia and hypercholesterolemia [1a, 2a, 3a-b].

Traditionally, most of these species have been used as vegetables, medicinal plants and herbal perfumes. For example, in Korea, the aerial portion of P. kamtschaticum is used as an edible mountain vegetable and for treating colds, arthritis, atherosclerosis and impotence [4]. The root of P. angelicoides is used in Himalayan and Chinese folk medicines for treating dysentery and as an antipyretic and a diaphoretic agent [5]. Similarly, P. densiflorum is used by the Kumaon Himalayan people (Indian) as one of the best herbal perfumes in the region and is known for its long lasting pleasant smell [6].

In Bhutan, out of the eight reported species of Pleurospermum [7], P. amabile Craib & W. W. Smith is used in Bhutanese traditional medicine (BTM) for treating dyspepsia, poisoning and fever, and is also often used as a substitute for musk, which is obtained from the Himalayan musk deer (endangered and prohibited) [8]. P. amabile grows to a height of 15-50 cm, with a stout root and a solitary stem in open scrub, alpine turf and the semi-stable scree of the high altitude Himalayan mountains (3950 to 4700 masl) of Bhutan [7,8].

We have previously studied the essential oil component of this plant and found interesting bioactivity [1a]. This interesting preliminary finding encouraged us to further investigate the chemical components of the methanol extract of this plant for the first time. The MeOH extract was sequentially fractionated with n-hexane, petroleum spirit, CH2Cl2, CHCl3, EtOAc and n-butanol to obtain...
their respective fractionated extracts. These extracts were purified repeatedly using silica gel column chromatography and preparative thin layer chromatography (PTLC). Finally, four phenylpropanoids and six furanocoumarins were identified by comparing the LR-ESI-MS, LR-EL-MS, 1H NMR and 13C NMR spectroscopic data of the isolated compounds with those of the relevant literature, as detailed in the experimental section. The compounds are (Figure 1): (E)-isomyristicin (1), (E)-isoapiol (2), methyl eugenol (3), (E)-isoelemicin (4), psoralen (5), bergapten (6) (major compound of a plant), isomperatorin (7), isopimpinellin (8), oxyypeucedanin hydrate (9) and oxyypeucedanin methanolate (10).

We investigated these compounds (1-10) and various crude extracts for their antimicrobial, antimalarial and cytotoxicity activities. Some biological activities reported earlier by us [1a, 9] for this plant are reproduced in Table 1 for easy comparisons. The antimicrobial assay was performed using seven bacterial strains and one fungal strain in a modified Agar Well Diffusion method (AWD) and the broth microdilution methods in 96 well plates that determined the minimum inhibitory concentrations (MIC). Unlike the previously reported activity of crude extracts and compounds 1-2 [1a] (Table 1), the crude petroleum spirit, EtOAc and n-ButOH extracts and the pure compounds 3-8 studied here did not show any interesting antimicrobial activities. However, compound 10 showed weak activity against the Gram-positive bacterium, Bacillus subtilis with a minimum inhibition zone (MIZ) of 9 mm and a MIC value of 125 µg/mL (Table 1). Compound 6 (bergapten), isolated from Heracleum maximum, was reported to be moderately active against Mycobacterium tuberculosis with a MIC value of 200 µg/mL and an IC50 value of 27 µg/mL [10].

Among the three crude extracts (petroleum spirit, EtOAc and n-ButOH extracts) and seven pure compounds (3 and 5-10) studied here, the petroleum spirit and EtOAc crude extracts, and compounds 7-10 exhibited moderate to significant antiparasitic activity against a wild type chloroquine and antifolate sensitive strain, TM4/8.2 and the multidrug resistant strain, K1CB1 of Plasmodium falciparum (Table 1). The petroleum spirit and EtOAc crude extracts showed moderate activities with IC50 values in the range of 24.3-37.9 µg/mL. The varying activities of the crude extracts and the EO described in Table 1 (including previous data, but inactive fractions not shown) suggested that the more potent chemical components are present in the CHCl3 and CH2Cl2 extracts when compared with the other extracts.

Out of ten compounds (1-10) isolated from these active extracts, oxyypeucedanin methanolate (10) isolated from the CHCl3 extract exhibited the highest and the most significant antimalarial activity against a wild type chloroquine and antifolate sensitive strain, TM4/8.2 and the multidrug resistant strain, K1CB1 of Plasmodium falciparum (Table 1). The petroleum spirit and EtOAc crude extracts showed moderate activities with IC50 values in the range of 24.3-37.9 µg/mL. The varying activities of the crude extracts and the EO described in Table 1 (including previous data, but inactive fractions not shown) suggested that the more potent chemical components are present in the CHCl3 and CH2Cl2 extracts when compared with the other extracts.

While none of the test samples that we studied showed any major cytotoxicity or anticancer activity against human oral carcinoma KB cells, compounds 7 and 9-10 that were isolated from different plant species have been reported to possess antiproliferative activity by inhibiting the growth of nude mouse transplantable human gastric adenocarcinoma (MK-1), human uterus carcinoma (HeLa) and murine melanoma (B16F10) cells [11]. Compounds 5 (against HL-60 and K562) and 7 (against L1210, HL-60, K562, B16F10) were also reported to exhibit moderate cytotoxicity against various tumor cell lines [12].

In summary, the petroleum spirit and EtOAc crude extracts exhibited moderate antiparasitic activity against a wild type chloroquine and antifolate sensitive strain, TM4/8.2 and the multidrug resistant strain, K1CB1 of Plasmodium falciparum (Table 1). The petroleum spirit and EtOAc crude extracts showed moderate activities with IC50 values in the range of 24.3-37.9 µg/mL. The varying activities of the crude extracts and the EO described in Table 1 (including previous data, but inactive fractions not shown) suggested that the more potent chemical components are present in the CHCl3 and CH2Cl2 extracts when compared with the other extracts.

### Table 1: Antibacterial, antimalarial and cytotoxicity activities of crude extracts and compounds 1-10 isolated from *P. amabili*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Antibacterial (MIC in µm)</th>
<th>Antiplasmodial (IC50 in µg/mL)</th>
<th>Cytotoxicity (IC50 in µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>K1CB1</td>
<td>B16F10</td>
</tr>
<tr>
<td>CH2Cl2</td>
<td>14</td>
<td>12.1 ± 0.4</td>
<td>&gt;25</td>
</tr>
<tr>
<td>CHCl3</td>
<td>6</td>
<td>7.8 ± 1.8</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Pet. spirit</td>
<td>2</td>
<td>27.6 ± 4.3</td>
<td>&gt;30.4</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td>5</td>
<td>24.3 ± 3.2</td>
<td>&gt;18.6</td>
</tr>
<tr>
<td>n-Butanol extract</td>
<td>5</td>
<td>79.0 ± 6.4</td>
<td>&gt;18.6</td>
</tr>
<tr>
<td>Essential oil (EO)</td>
<td>5</td>
<td>72.3 ± 2.1</td>
<td>&gt;18.6</td>
</tr>
<tr>
<td>O xyypeucedanin hydrate</td>
<td>5</td>
<td>&gt;18.6</td>
<td>&gt;18.6</td>
</tr>
<tr>
<td>Oxyypeucedanin methanolate</td>
<td>5</td>
<td>&gt;10.8</td>
<td>&gt;18.6</td>
</tr>
<tr>
<td>Chlorouquinol</td>
<td>5</td>
<td>0.01 0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Cycloquinol</td>
<td>5</td>
<td>0.09 0.81</td>
<td>0.09</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>5</td>
<td>0.02 7.70</td>
<td>0.09</td>
</tr>
<tr>
<td>Ellipticin</td>
<td>5</td>
<td>0.09 0.56</td>
<td>0.09</td>
</tr>
</tbody>
</table>


### Experimental

**Plant materials:** The aerial components of *P. amabili* Craib & W. W. Smith (voucher number 29) were collected from Lingzhi (4200 m above sea level) in July 2009 and a herbarium specimen was deposited at Manjong Sorig Pharmaceuticals, Ministry of Health, Thimphu, Bhutan. The dried plant material (2 kg) was extracted with methanol (190 g of MeOH obtained).

**Isolation and identification of compounds from the MeOH extract:** The equipment used, and the isolation and characterization techniques carried out were as described by us previously [13a-b]. 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 and discussed as follows. The MeOH extract was dissolved in MeOH/water (1:9) and fractionated first with n-hexane and petroleum spirit to obtain their respective extracts. The remaining aqueous portion was acidified with HCl (5%) and fractionated with CH2Cl2 to obtain the acidic fractions.
extract. The aqueous portion was again basified with NaOH to pH 9-12 and then fractionated with CHCl3 to obtain a basic CHCl3 extract followed by EtOAc and n-butanol giving EtOAc and BuOH extracts, respectively. The remaining aqueous layer was dried to obtain the H2O extract. Each of these extracts (50-100 mg) was used for determining their biological activities. The initial purification was focused on the most active CHCl3 extract (13.3 g) using silica gel CC and a gradient eluent solvent system of MeOH-CHCl3 (500 mL, v/v ratios 0:100, 5:95, 10-90, 15:85, 50:50), which yielded 13 fractions, CHCl3-F1 to CHCl3-F13. Separation of fraction CHCl3-F5 using PTLC plates with CH2Cl2 (100%) as an isocratic solvent system, yielded compounds 1-4, which were identified as (E)-isomyristicin (1), (E)-isoapiolin (2), methyl eugenol (3) and (E)-isoelemicin (4). Fraction CHCl3-F7 was separated using the same method and solvent system as above and furnished psoralen (5), bergapten (6) (as major compound) and isoinperatorin (7). Fraction CHCl3-F10, upon separation using preparative TLC plates with MeOH-CHCl3 (1:19) as the solvent system, yielded isopimpinellin (8), oxyypeucedanin hydrate (9) and oxyypeucedanin methanolate (10). From the petroleum spirit, CH2Cl2, and EtOAc extracts, the same compounds were obtained. The n-hexane, n-BuOH and H2O extracts were not studied here.

Identification of phenylpropanoids (1-4): (E)-isomyristicin (1) showed a [M+H+] peak at m/z 193, and an ion fragmentation pattern that matched that of isomyristicin in the MS library system (NIST08.LIB, Entry # 15372, CAS: 607-91-0). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those reported for isomyristicin [14a-b]. 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those reported for bergapten [10, 18]. Isoimperatorin (7) was isolated and recrystallized from MeOH/CHCl3. Its LR-ESI-MS indicated a [M+H+] peak at m/z 271, and its LR-ESI-MS fragmentation pattern matched that of isoinperatorin in the MS library system (NIST08.LIB, Entry # 90508, CAS: 482-45-1). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isoinperatorin reported in the literature [19a-b]. Isopimpinellin (8) was isolated as white solid. The EI-MS, 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isoimperatorin reported in the literature [20]. Oxyypeucedanin hydrate (9) was isolated as light yellowish needles from MeOH/CHCl3. Its LR-ESI-MS showed a [M+H+] peak at m/z 305 and the LR-ESI-MS demonstrated an ion fragmentation pattern which matched that of oxyypeucedanin hydrate in the MS library system (NIST08.LIB, Entry # 114683, CAS: 24724-52-5). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isoimperatorin reported in the literature [11]. Oxyypeucedanin methanolate (10) was isolated as a white solid. Its LR-ESI-MS indicated a [M+H+] peak at m/z 319, and the LR-ESI-MS established the ion fragmentation pattern as m/z 318 [M+], 286, 215, 202 (base peak), 185, 174, 157, 145, 118, 99, 85, 73, 59, and 51. The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of oxyypeucedanin methanolate reported in the literature [11].

Identification of furanocoumarins (5-10): Psoralen (5) was isolated and recrystallized as white needles from MeOH/CHCl3. Its LR-ESI-MS showed a [M+H+] peak at m/z 187, and the LR-ESI-MS exhibited an ion fragmentation pattern which matched that of psoralen in the MS library system (NIST08.LIB, Entry # 34463, CAS: 66-97-7). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those reported for psoralen [17]. Bergapten (6) was isolated and recrystallized as clear crystals from MeOH/CHCl3. Its LR-ESI-MS displayed a [M+H+] peak at m/z 217, and its LR-ESI-MS matched that of bergapten in the MS library system (NIST08.LIB, Entry # 53002, CAS: 484-20-8). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those reported for bergapten [10, 18]. Isomitercurin (7) was isolated and recrystallized from MeOH/CHCl3. Its LR-ESI-MS showed a [M+H+] peak at m/z 271, and its LR-ESI-MS fragmentation pattern matched that of isomitercurin in the MS library system (NIST08.LIB, Entry # 90508, CAS: 482-45-1). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isomitercurin reported in the literature [19a-b]. Isopimpinellin (8) was isolated as white solid. The EI-MS, 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isoimperatorin reported in the literature [20]. Oxyypeucedanin hydrate (9) was isolated as light yellowish needles from MeOH/CHCl3. Its LR-ESI-MS showed a [M+H+] peak at m/z 305 and the LR-ESI-MS demonstrated an ion fragmentation pattern which matched that of oxyypeucedanin hydrate in the MS library system (NIST08.LIB, Entry # 114683, CAS: 24724-52-5). The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of isoimperatorin reported in the literature [11]. Oxyypeucedanin methanolate (10) was isolated as a white solid. Its LR-ESI-MS indicated a [M+H+] peak at m/z 319, and the LR-ESI-MS established the ion fragmentation pattern as m/z 318 [M+], 286, 215, 202 (base peak), 185, 174, 157, 145, 118, 99, 85, 73, 59, and 51. The 1H NMR and 13C NMR spectroscopic data of this compound agreed well with those of oxyypeucedanin methanolate reported in the literature [11].

Bioassay methods: The antimicrobial, antiplasmodial and cytotoxicity tests were carried out in the same ways as we have reported earlier [9, 13a-b]. For the antimicrobial agar well diffusion and broth microdilution bioassay, Escherichia coli (ATCC 25922), Bacillus subtilis (ATCC 6633), Staphylococcus aureus (ATCC 6538), mitecinil resistant S. aureus (MRSA), (DMST 20651), S. epidermidis (ATCC 12228), Vibrio cholerae (ATCC 2873) and Candida albicans (ATCC 10231) were used as test strains. Amphotericin B (Sigma-Aldrich, USA) was used as a positive control for antifungal testing against Candida albicans (not shown in Table 1 as the samples were found to be inactive). Vancomycin (Edicin, Slovenia) and amoxicillin (GPO, Thailand) were used as the positive controls for antibacterial assays. The K1CB1 (a multidrug resistant) and TM4.8.2 (a wild type chloroquine and antifolate sensitive) strains of Plasmodium falciparum were used for the modified microdilution radioisotope antiplasmodial bioassay. This assay used chloroquine (Sigma-Aldrich), cycloguanil (Sigma-Aldrich) and pyrimethamine (Sigma-Aldrich) as positive controls. For the sulforhodamine B (SRB) cytotoxicity assay, normal vero cells from kidney of African green monkey, Cecopithecus aethiops and the human oral carcinoma KB cells were used. This assay used cycloguanil (Sigma-Aldrich, USA) as reference drugs. All the experiments were performed 3 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.

Acknowledgments - We appreciate the support of: a) Ms Norbu Drolma, Ms. Tshring Zam, Mr Samten and Mr Ugyen Dhendup (Head) of Manjong Sorig Pharmaceuticals and Mr Dorji Wangchuk (Director) of the National Institute of Traditional Medicine for providing the materials and necessary administrative services; and b) Roonglawan Rattanajak of BIOTECH, Thailand for antiplasmodial assistance. Mr P. Wangchuk is an Endeavour Award Holder of Australia.