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

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Abstract

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)-isoapiol (2), methyl eugenol (3) and (E)-isoelemicin (4)] and six furanocoumarins [psoralen (5), bergapten (6), isoimperatorin (7), isopimpinellin (8), oxypeucedanin hydrate (9) and oxypeucedanin methanolate (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.

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Phenylpropanoids and Furanocoumarins as Antibacterial and Antimalarial Constituents of the Bhutanese Medicinal Plant *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*)-isoapiol (**2**), methyl eugenol (**3**) and (*E*)-isoelemicin (**4**)] and six furanocoumarins [psoralen (**5**), bergapten (**6**), isoimperatorin (**7**), isopimpinellin (**8**), oxypeucedanin hydrate (**9**) and oxypeucedanin methanolate (**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, anticancer, 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*

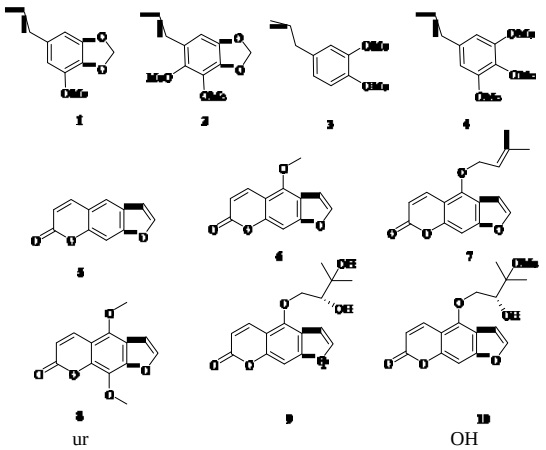

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Figure 1: Struct es of compounds (**1**-**10**) isolated from the Me extract of *P. amabile.*

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 screes 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, CH_2Cl_2, $CHCl_3$, 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-EI-MS, ^{1}H NMR and ^{13}C 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), isoimperatorin (**7**), isopimpinellin (**8**), oxypeucedanin hydrate (**9**) and oxypeucedanin 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*-BuOH 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 IC_{50} value of 27 µg/mL [10].

Among the three crude extracts (petroleum spirit, EtOAc and *n*-BuOH 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 antiplasmodial 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 IC_{50} 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 CH_2Cl_2 and $CHCl_3$ extracts when compared with the other extracts.

Out of ten compounds (**1**-**10**) isolated from these active extracts, oxypeucedanin methanolate (**10**) isolated from the $CHCl_3$ extract exhibited the highest and the most significant antimalarial activity against both *P. falciparum* strains, TM4/8.2 and K1CB1 with IC_{50} values of 7.4 µg/mL and 6.7 µg/mL, respectively. Such activities were similar to that of its parent crude $CHCl_3$ extracts, but threefold better than its closely related compound **9** (oxypeucedanin hydrate) (Table 1). Compounds **7**-**9** also showed weak to moderate antiplasmodial activity with IC_{50} values in a similar range of 15.6-24.9 µg/mL. Compound **5** showed selectivity against the K1CB1 strain with an IC_{50} value of 16.4 µg/mL, but it was not active against the TM4/8.2 strain. Interestingly, two major compounds of this plant, (*E*)-isomyristicin (**1**) and bergapten (**6**), did not exhibit any biological activity.

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].

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

Samples	Antibacterial (MIZ in mm)	Antiplasmodial (IC_{50} in µg/mL)		Cytotoxicity (IC_{50} in µg/mL)	
	B. subtilis	TM4/8.2	K1CB1	Vero	KB
CH_2Cl_2 extract[a]	14	12.1 ± 0.4	10.9 ± 2.3	>25	>25
$CHCl_3$ extract[a]	6	7.8 ± 1.8	7.3 ± 2.6	>25	>25
Pet. spirit extract*	–	27.6 ± 4.3	37.9 ± 14.1	>50	>50
Ethyl acetate extract	–	24.3 ± 3.2	25.2 ± 6.5	>100	>100
n-Butanol extract	–	>100	>100	>100	>100
Essential oil (EO)[b]	5	79.0 ±4.6	72.3 ± 2.1	>100	>100
1[b]	5	>100	>100	>100	>100
2[b]	6	52.9 ± 2.9	69.9 ± 2.0	>100	>100
3	–	>17.8	>17.8	>17.8	>17.8
4[b]	–	>20	>20	>20	>20
5	–	>18.6	16.4 ± 0.3	>18.6	>18.6
6	–	>10.8	>10.8	>10.8	>10.8
7	–	19.1 ± 0.9	18.5 ± 0.7	>27.0	>27.0
8	–	19.5 ± 2.3	15.6 ± 2.5	>24.6	>24.6
9	–	24.9 ± 2.0	18.7 ± 2.3	>30.4	>30.4
10	9	7.4 ± 1.3	6.7 ± 1.1	>31.8	>31.8
Amoxicillin[c]	8				
Chloroquine[d]		0.01	0.09		
Cycloguanil[d]		0.009	0.81		
Pyrimethamine[d]		0.02	7.70		
Ellipticine[e]				0.09	
Doxorubicin[e]					0.56

[a] Original activity taken from [9]. [b] Original activity taken from [1a]. [c]Positive controls for antibacterial activity. [d] Positive controls for antiplasmodial activity. [e] Positive controls for cytotoxicity activity. *Petroleum spirit extract. –: Not Active.

In summary, the petroleum spirit and EtOAc crude extracts exhibited moderate antiplasmodial activity against a wild type chloroquine and antifolate sensitive strain, TM4/8.2 and the multidrug resistant strain, K1CB1 of *Plasmodium falciparum*. These activities were lower than the $CHCl_3$ and CH_2Cl_2 which we reported earlier. Focusing our isolation of phytochemicals on these active fractions (Table 1), ten compounds (**1**-**10**) were isolated with isomyristicin (**1**) and bergapten (**6**) as the major constituents. Among the ten compounds that were bio-assayed, compound **10** exhibited the highest activity against the same *P. falciparum* strains mentioned above. These biological activities were found to be in conformity with the traditional uses of *P. amabile* in BTM formulations and, therefore, provide scientific insight to this plant.

Experimental

Plant materials: The aerial components of *P. amabile* 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, $CDCl_3$) 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 CH_2Cl_2 to obtain the acidic

extract. The aqueous portion was again basified with NH_4OH to pH 9-12 and then fractionated with $CHCl_3$ to obtain a basic $CHCl_3$ extract followed by EtOAc and *n*-butanol giving EtOAc and BuOH extracts, respectively. The remaining aqueous layer was dried to obtain the H_2O extract. Each of these extracts (50-100 mg) was used for determining their biological activities. The initial purification was focused on the most active $CHCl_3$ extract (13.3 g) using silica gel CC and a gradient eluent solvent system of MeOH-CH_2Cl_2 (500 mL, v/v ratios 0:100, 5:95, 10:90, 15:85, 50:50), which yielded 13 fractions, $CHCl_3$-F1 to $CHCl_3$-F13). Separation of fraction $CHCl_3$-5 using PTLC plates with CH_2Cl_2 (100%) as an isocratic solvent system, yielded compounds **1**-**4**, which were identified as (*E*)-isomyristicin (**1**), (*E*)-isoapiol (**2**), methyl eugenol (**3**) and (*E*)-isoelemicin (**4**). Fraction $CHCl_3$-F7 was separated using the same method and solvent system as above and furnished psoralen (**5**), bergapten (**6**) (as major compound) and isoimperatorin (**7**). Fraction $CHCl_3$-F10, upon separation using preparative TLC plates with MeOH-$CHCl_3$ (1:19) as the solvent system, yielded isopimpinellin (**8**), oxypeucedanin hydrate (**9**) and oxypeucedanin methanolate (**10**). From the petroleum spirit, CH_2Cl_2, and EtOAc extracts, the same compounds were obtained. The *n*-hexane, *n*-BuOH and H_2O 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 ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those reported for isomyristicin [14a-b]. (*E*)-isoapiol (**2**) was isolated as an oil, which showed a [M+H$^+$] peak at *m/z* 222 in the LR-ESI-MS. The LR-EI-MS exhibited fragmentation ions at *m/z* 222 (base peak), 207, 191, 177, 163, 149, 134, 121, 106, 91, 77, 65, 53, and 39. The ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of isoapiol reported in the literature [15]. Methyl eugenol (**3**) was isolated as colorless oil. Its LR-ESI-MS showed a [M+H$^+$] peak and fragmentation ions that matched those of eugenol methyl ether in the MS library system (NIST08s.LIB, Entry # 13416, CAS: 93-15-2). The ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of methyl eugenol reported in the literature [16]. (*E*)-isoelemicin (**4**) was isolated as colorless oil. Its LR-ESI-MS showed a [M+H$^+$] peak at *m/z* 209 and a [M$^+$] peak at *m/z* 208. The ^{1}H NMR spectroscopic data of this compound agreed with those of (*E*)-isoelemicin reported in the literature [14b].

Identification of furanocoumarins (5-10): Psoralen (**5**) was isolated and recrystallized as white needles from MeOH/$CHCl_3$. Its LR-ESI-MS showed a [M+H$^+$] peak at *m/z* 187, and the LR-EI-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 ^{1}H NMR and ^{13}C 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/$CHCl_3$. Its LR-ESI-MS displayed a [M+H$^+$] peak at *m/z* 217, and its LR-EI-MS matched that of bergapten in the MS library system (NIST08.LIB, Entry # 53002, CAS: 484-20-8). The ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those reported for bergapten [10, 18]. Isoimperatorin (**7**) was isolated and recrystallized from MeOH/$CHCl_3$. Its LR-ESI-MS indicated a [M+H$^+$] peak at *m/z* 271, and its LR-EI-MS fragmentation pattern matched that of isoimperatorin in the MS library system (NIST08.LIB, Entry # 90508, CAS: 482-45-1). The ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of isoimperatorin reported in the literature [19a-b]. Isopimpinellin (**8**) was isolated as white solid. The EI-MS, ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of isopimpinellin reported in the literature [20]. Oxypeucedanin hydrate (**9**) was isolated as light yellowish needles from MeOH/$CHCl_3$. Its LR-ESI-MS showed a [M+H$^+$] peak at *m/z* 305 and the LR-EI-MS demonstrated an ion fragmentation pattern which matched that of oxypeucedanin hydrate in the MS library system (NIST08.LIB, Entry # 114683, CAS: 24724-52-5). The ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of isopimpinellin reported in the literature [11]. Oxypeucedanin methanolate (**10**) was isolated as a white solid. Its LR-ESI-MS indicated a [M+H$^+$] peak at *m/z* 319, and the LR-EI-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 ^{1}H NMR and ^{13}C NMR spectroscopic data of this compound agreed well with those of oxypeucedanin 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), methicillin resistant *S. aureus* (*MRSA*), (DMST 20651), *S. epidermidis* (ATCC 12228), *Vibrio cholerae* (DMST 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 (RSB) 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 ellipticine (Sigma-Aldrich, USA) and doxorubicin (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.

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