2013

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

Phurpa Wangchuk
University of Wollongong, pw54@uowmail.edu.au

Paul A. Keller
University of Wollongong, keller@uow.edu.au

Stephen G. Pyne
University of Wollongong, spyne@uow.edu.au

Malai Taweechotipatr
Srinakharinwirot University

Sumalee Kamchonwongpaisan
BIOTEC, Thailand

Publication Details

Wangchuk, P., Keller, P. A., Pyne, S. G., Taweechotipatr, M. & Kamchonwongpaisan, S. (2013). GC/GC-MS analysis, isolation and identification of bioactive essential oil components from the Bhutanese medicinal plant, Pleurospermum amabile. Natural Product Communications: an international journal for communications and reviews, 8 (9), 1305-1308.

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

Abstract
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)-isoapiol (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
identification, isolation, analysis, ms, gc, plant, amabile, medicinal, pleurospermum, bhutanese, components, oil, essential, bioactive, CMMB

Publication Details
Wangchuk, P., Keller, P. A., Pyne, S. G., Taweechotipatr, M. & Kamchonwongpaisan, S. (2013). GC/GC-MS analysis, isolation and identification of bioactive essential oil components from the Bhutanese medicinal plant, Pleurospermum amabile. Natural Product Communications: an international journal for communications and reviews, 8 (9), 1305-1308.

GC/GC-MS Analysis, Isolation and Identification of Bioactive Essential Oil Components from the Bhutanese Medicinal Plant, *Pleurospermum amabile*

Phurpa Wangchuk[a,b], Paul A. Keller[a], Stephen G. Pyne[a*], Malai Taweechotipatr[c] and Sumalee Kamchonwongpaisan[d]

[a] *School of Chemistry, University of Wollongong, Wollongong, NSW, 2522, Australia*

[b] *Manjong Sorig Pharmaceuticals, Ministry of Health, Thimphu, Bhutan*

[c] *Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok, 10110, Thailand*

[d] *Medical Molecular Biology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand Science Park, Pathumthani, 12120, Thailand*

spyne@uow.edu.au

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*)-isoapiol (**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 (ethnomedicine), 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. lindleyani*, 73 compounds were identified with 1-propoxy-2-propanol, myristicine, *cis*-asarone, *n*-hexane, apiol, 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, piperitenal, (*Z*)-2-decenaldehyde and 2,4,5-trimethyl benzaldehyde [6]. From the EO of *P. wrightianum*, about 49 compounds were identified with (*E*)-9-octadecenoic acid and 1,3,5,7-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*)-isoapiol (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%), myrtenyl 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*)-isoapiol (**2**) [12], methyl eugenol (**3**) [13-14], and (*E*)-isoelemicin (**4**) [15] (Figure 1).

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

GC Peak No.	% oil	Compound name	KI
1	0.7	*α*-Pinene	934[a]
2	0.3	*β*-Pinene	977[a]
3	2.3	Myrcene	991[a]

4	0.4	*α*-Phellandrene	1005[a]
5	0.2	Isoamyl isobutyrate	1013[b]
6	17.0	Limonene	1030[a]
7	0.5	(*Z*)-*β*-Ocimene	1038[a]
8	0.6	(*E*)-*β*-Ocimene	1048[c]
9	0.6	Terpinolene	1090[c]
10	0.6	Linalool	1100[c]
11	0.4	Isoamyl valerate	1106[a]
12	0.2	2-Methylbutyl isovalerate	1109[c]
13	0.2	Fenchol	1115[a]
14	0.2	Valeric acid, 3-methylbut-2-enyl-ester	1149[b]
15	0.2	Terpinen-4-ol	1179[a]
16	0.2	*α*-Terpineol	1189[a]
17	0.8	Myrtenol	1193[a]
18	0.6	Fenchyl acetate	1223[c]
19	1.2	*β*-Citronellol	1229[a]
20	0.2	2,4-Dimethoxytolouene	1240[b]
21	1.6	Geraniol	1256[a]
22	0.6	Citronellyl formate	1276[a]
23	0.3	Pinocarvyl acetate	1293[a]
24	1.1	Myrtenyl acetate	1329[b]
25	1.0	Citronellyl acetate	1354[a]
26	0.5	Geranyl acetate	1384[c]
27	0.2	2-methyl-5,7-dimethylene-1,8-nonadiene	1388[b]
28	0.2	Benzyl 2-methylbutanoate	1396[b]
29	**3.8**	**Methyl eugenol**	**1406[d]**
30	2.2	*β*-Caryophyllene	1427[c]
31	0.8	Aromadendrene	1462[a]
32	0.3	Geranyl *n*-propionate	1475[a]
33	0.2	Citronellyl isobutyrate	1484[a]
34	0.3	Germacrene D	1489[a]
35	1.2	*β*-Selinene	1500[c]
36	0.5	*α*-Bisabolene	1507[a]
37	1.3	Geranyl isobutyrate	1514[a]
38	1.3	Myristicin	1526[a]
39	3.9	*β*-Sesquiphellandrene	1529[b]
40	2.3	(*Z*)-Isomyristicin	1575[e]
41	0.2	Germacrene D-4-ol	1584[a]
42	0.7	Caryophyllene oxide	1587[a]
43	0.7	Geranyl isovalerate	1605[b]
44	2.4	Geranyl pentanoate	1611[c]
45	**32.5**	**(*E*)-Isomyristicin**	**1624[f]**
46	1.4	Dillapiole	1630[i]
47	0.3	*α*-Cadinol	1640[a]
48	**1.3**	**(*E*)-Isoelemicin**	**1654[g]**
49	0.5	Apiole	1665[i]
50	1.2	Valeranone	1684[a]
51	**7.6**	**(*E*)-Isoapiol**	**1722[h]**
52	0.2	Hexadecanal	1840[c]

[*] Retention time of the compounds based on GC-FID peaks (see Figure 1) and the isolated compounds were highlighted in bold face.
[a] Identified by NIST and NISTREP mass spectra library and agrees with [16].
[b] Identified tentatively by NIST and NISTREP mass spectra library.
[c] Identified by NIST and NISTREP mass spectra library and agrees with [5].
[d] Identified by its KI, ^{1}H and ^{13}C-NMR spectra comparisons with literature [13-14].
[e] Identified by NIST and NISTREP mass spectra library and agrees with [10].
[f] Identified by NIST and NISTREP mass spectra library and [10] and the comparison of its NMR spectra with [11].
[g] Identified by ^{1}H and ^{13}C-NMR spectra comparison with [15].
[h] Identified by ^{1}H and ^{13}C-NMR spectra comparison with [12].
[I] Identified by comparing the calculated KI with [17].

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 CH_2Cl_2 and $CHCl_3$ extracts exhibited significant antibacterial and antiplasmodial 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 CH_2Cl_2 and $CHCl_3$ 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* antiplasmodial activity against the *P. falciparum* strains: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain) and K1CB1 (multidrug resistant strain) with IC_{50} values of 79.0 µg/mL and 72.3 µg/mL, respectively. These activities were lower than those exhibited by the CH_2Cl_2 and $CHCl_3$ extracts (Table 2). Such correlations, including the antimicrobial activities, suggested that the more potent chemical components are present in the CH_2Cl_2 and $CHCl_3$ extracts rather than in EO. In comparison to the antiplasmodial activity of the mother EO, compound **2** showed improved activity with IC_{50} values of 52.9 µg/mL and 69.9 µg/mL against TM4/8.2 and K1CB1, respectively. Compounds **1** did not show any antiplasmodial activity and compound **4** had poor solubility which restricted the determination of its accurate IC_{50} 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.*

Samples	Antibacterial (MIZ in mm)		Antiplasmodial (IC_{50} in µg/mL)		Cytotoxicity (IC_{50} in µg/mL)	
	B. subtilis	MRSA	TM4/8.2	K1CB1	Vero	KB
CH_2Cl_2 extract	14[a]	12[a]	12.1±0.4[a]	10.9±2.3[a]	>25[a]	>25[a]
$CHCl_3$ extract	6[a]	NT	7.8±1.8[a]	7.3±2.6[a]	>25[a]	>25[a]
Essential oil (EO)	5	NA	79.0±4.6	72.3±2.1	>100	>100
(*E*)-Isomyristicin (**1**)	5	NA	>100	>100	>100	>100
(*E*)-Isoapiol (**2**)	6	NA	52.9± 2.9	69.9±2.0	>100	>100
(*E*)-Isoelemicin (**4**)	NA	NA	>20	>20	>20	>20
Amoxicillin[b]	8					
Vancomycin[b]		13.5				
Chloroquine[c]			0.010	0.089		
Cycloguanil[c]			0.009	0.810		
Pyrimethamine[c]			0.020	7.700		
Ellipticine[d]					0.093	
Doxorubicin[d]						0.56

NT: Not Tested, NA: Not Active.
[a] Original activity taken from [9].
[b] Positive controls for antibacterial activity.
[c] Positive controls for antiplasmodial 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*)-isomyristicin (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* antiplasmodial 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 screes of high altitude Himalayan mountains (3950 to 4700 meters above sea level) in Bhutan [18-19]. It grows to 15-50 cm tall with stout root, solitary stem, sheathed broad leaves, white to dark purple flower 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 $MgSO_4$. The EO was stored at 0–5°C until analysed.

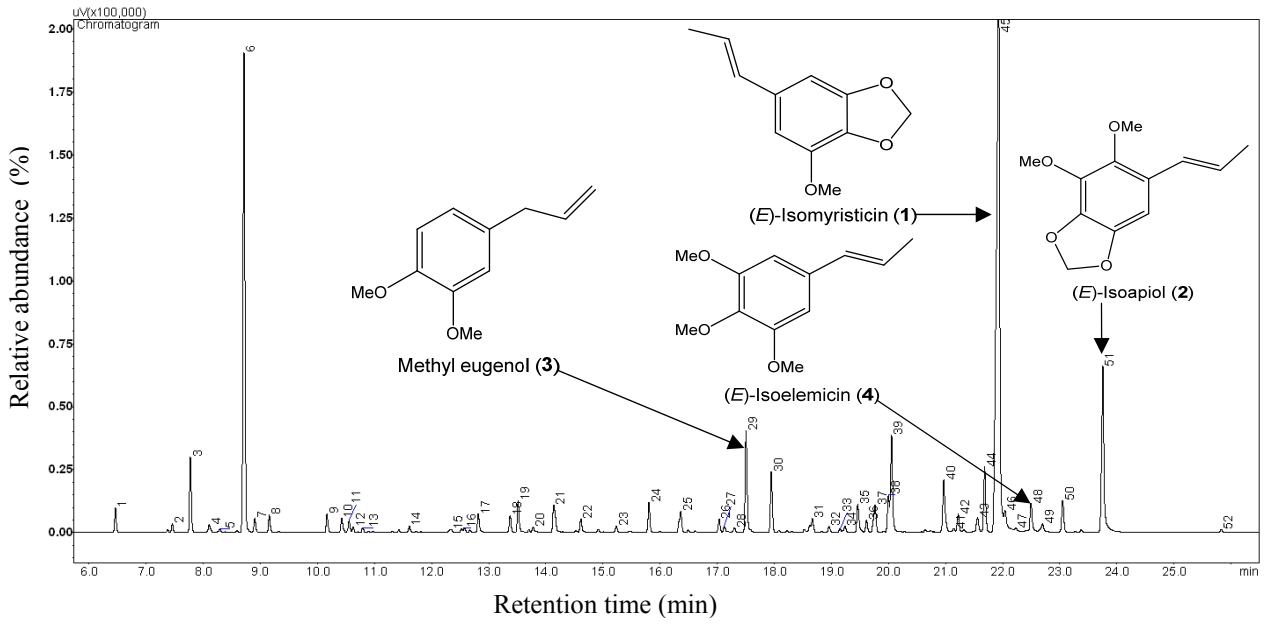

Figure 1: GC-FID peaks for % oil and the structures of the isolated compounds (**1**-**4**) from the EO of *P. amabile*

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, $CDCl_3$) 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 $CHCl_3$-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% H_2O:90% MeOH) yielded compound **1** (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% $CHCl_3$) on a normal phase silica gel column furnished (*E*)-isoapiol (**2**) (30.7 mg, Fig. 1) and methyl eugenol (**3**) (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-Aldrich) were used as positive controls in the antiplasmodial assays. For the antimicrobial assay, the test organisms including *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. 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, *Cecopithecus 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.

References

[1] Bakkali F, Averbeck S, Averbeck D, Idaomar M. (**2008**) Biological effects of essential oils-A review. *Food and Chemical Toxicology*, ***46***, 446-475.

[2] Romeilah RM, Fayed SA, Mahmoud GI. (**2010**) Chemical compositions, antiviral and antioxidant activities of seven essential oils. *Journal of Applied Sciences Research*, ***6***, 50-62.

[3] Zehui P,Watson MF. (**2005**) *Pleurospermum* Hoffmann, Gen. Pl. Umbell. Viii.1814. In *Flora of China*, ***14***, 40-51.

[4] Ajiaikebaier A, Lu Q. (**2002**) Chemical constituents from the essential oil of Herba Pleurospermi lindleyani. *Tianran Chanwu Yanjiu Yu Kaifa*, ***14***, 46-49.

[5] Radulovic NS, Dordevic ND, Palic RM. (**2010**) Volatiles of *Pleurospermum austriacum* (L.) Hoffm. (Apiaceae). *Journal of the Serbian Chemical Society*, ***75***, 1653-1660.

[6] Li T, Wang T. (**2001**) Chemical constituents of volatile oil of *Pleurospermum hookeri*. *Zhongcaoyao*, ***32***, 780-781.

[7] Liu Z, Tian X. (**2004**) Analysis of volatile components in *Pleurospermum wrightianum* Boissieu by gas chromatography/mass spectrometry (GC/MS). *Xibei Zhiwu Xuebao*, ***24***, 693-697.

[8] Liu Q, Gao Y, Zhang Z, Chen Z. (**1991**) Chemical constituents of volatile oil from *Pleurospermum giraldii*. *Zhongguo Yaoxue Zazhi (Beijing, China)*, ***26***, 593-594.

[9] Wangchuk P, Keller PA, Pyne SG, Taweechotipatr M, Tonsomboon A, Rattanajak R, Kamchonwongpaisan S. (**2011**) Evaluation of an ethnopharmacologically selected Bhutanese medicinal plants for their major classes of phytochemicals and biological activities. *Journal of Ethnopharmacology*, ***137***, 730-742.

[10] Tzakou O, Lempesis K, Loukis A. (**2010**) Essential oil composition of the endemic species *Thamnosciadium junceum* (Sm.) Hartvig. *Journal of Essential Oil Research*, ***22***, 257-258.

[11] Antunes AM, Palma FMSB, Santos SMBP. (**1995**) Isomyristicin: ^{1}H and ^{13}C-NMR assignments. *Fitoterapia*, ***LXVI***, 554.

[12] Jakupovic J, Eid F. (**1987**) Benzoxanthenone derivatives from *Polemannia montana*. *Phytochemistry*, ***26***, 2427-2429.

[13] Jirovetz L, Buchbauer G, Stoyanova AS, Georgiev EV, Damianova ST. (**2003**) Composition, quality control and antimicrobial activity of the essential oil of long-time stored dill (*Anethum graveolens* L.) seeds from Bulgaria. *Journal of Agriculture and Food Chemistry*, ***51***, 3854-3857.

[14] Miyazawa M, Kohno G. (**2005**) Suppression of chemical mutagen-induced SOS response by allylbenzen from *Asiasarum heterotropoides* in the *Salmonella typhimurium* TA1535/PSK 1002 umu test. *Natural Product Research*, ***19***, 29-36.

[15] Enqiquez RG, Chavez MA, Jauregui F. (**1980**) Propenylbenzenes from *Guatteria gaumeri*. *Phytochemistry*, ***19***, 2014.

[16] Adams RP. (**1995**) *Identification of Essential Oil Components by Gas Chromatography/ Mass Spectroscopy*. Allured Publishing Corporation, Illinois, USA.

[17] Siani AC, Ramos MFS, Guimaraes AC, Susunaga GS, Zoghbi MDGB. (**1999**) Volatile constituents from oleoresin of *Protium heptaphyllum* (Aubl.) March. *Journal of Essential Oil Research*, ***11***, 72-74

[18] Grierson AJC, Long DG. (**1999**) *Pleurospermum* Hoffmann. In *Flora of Bhutan: Including a Record of Plants from Sikkim*. Royal Botanic Garden, Edinburgh, ***2*** (2), pp.451-457.

[19] Wangchuk P, Samten, Ugyen. (**2009**) *High Altitude Medicinal Plants of Bhutan: An Illustrated Guide for Practical Use* (pp. 102). Pharmaceutical and Research Unit, Ministry of Health, Thimphu, Bhutan.

[20] Wangchuk P, Keller PA, Pyne SG, Korth J, Samten, Taweechotipatr M, Rattanajak R, Kamchonwongpaisan S. (**2013**) Antimicrobial, antimalarial and cytoxicity activities of constituents of a Bhutanese variety of *Ajania nubigena*. *Natural Product Communications*, ***8***, 733-736.

[21] Wangchuk P, Keller PA, Pyne SG, Willis AC, Kamchonwongpaisan S. (**2012**) Antimalarial alkaloids from a Bhutanese traditional medicinal plant *Corydalis dubia*. *Journal of Ethnopharmacology*, ***143***, 310-313.

Hwang, Y.S., Chung, W.Y., Kim, J., Park, H.J., Kim, E.C., Park, K.K. 2011. Buddlejasaponin IV induces cell cycle arrest at G2/M phase and apoptosis in immortalized human oral keratinocytes. Phytotherapy Research 25, 1503-1510.

Jung, H.J., Nam, J.H., Park, H.J., Lee, K.T., Park, K.K., Kim, W.B., Choi, J. 2007. The MeOH extract of *Pleurospermum kamtschaticum* and its active component buddlejasaponin (IV) inhibits intrinsic and extrinsic hyperlipidemia and hypercholesterolemia in the rat. Journal of Ethnopharmacology 112, 255-261.