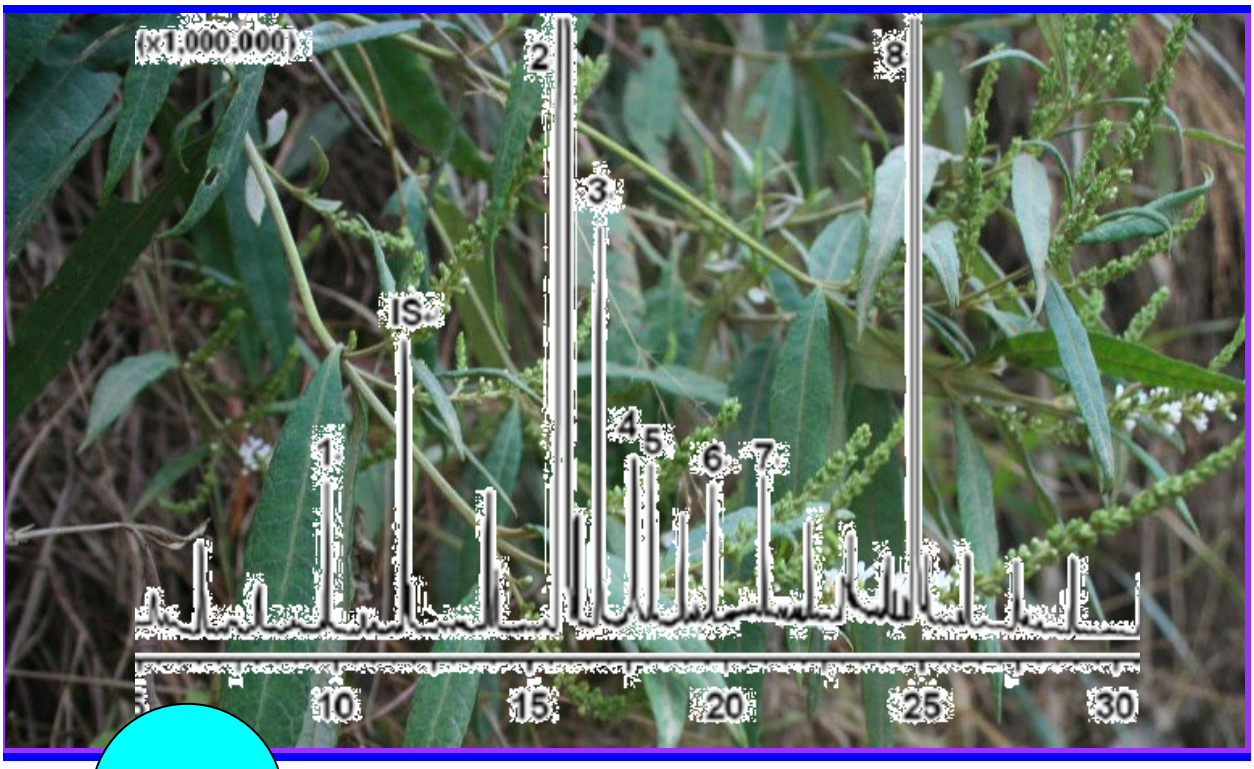

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GC/MS analysis, antimicrobial and *in vitro* anti-cholinesterase activities of the essential oil from *Buddleja asiatica*

Farman Ali Khan[1], Abdul Khaliq Jan[1], Nasir Mehmood Khan[1], Nayab Ali Khan[2] and Shahnaz Khan[2]

[1]*Department of Chemistry, Shaheed Benazir Bhutto University, Sheringal, Dir 18000, KP, Pakistan;*
[2]*Department of Chemistry, University of Science and Technology, Bannu 28100, KPK, Pakistan.*

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Abstract

Buddleja asiatica essential oil from the leaves by hydrodistillation was subjected to gas chromatography/mass spectrometery analysis which revealed the presence of 17 constituents out of which 14 were identified as: four monoterpenes hydrocarbons, four oxygenated monoterpenes, one hydrocarbon sesquiterpenes and five oxygenated sesquiterpenes. The major constituent being found was 1,8-cineole (38.1%) while β-sinensal, 1, 10-seco-1-hydroxy-calamenen-10-one and α-phellandrene were found to be in 11.8%, 10.2% and 5.8%, respectively. The essential oil exhibited 66% strong antibacterial activity against *Shigella boydii* while in fungicidal assay, it revealed an outstanding 79% inhibition against *Aspergillus flavus*. The essential oil showed outstanding acetylcholinesterase (IC_{50} 5.2 µM) and butyrylcholinesterase inhibitory effect (IC_{50} 27.9 µM) as compared to standard drugs respectively.

Introduction

The genus *Buddleja* comprises of more than 100 species and belongs to family *Scrophulariaceae* (Hegnauer, 1962; Fathy et al., 2006). *Buddleja* plants are widely distributed throughout the world and only four species are found in Pakistan, i.e., *B. asiatica*, *B. crispa*, *B. davidii* and *B. lindleyana* (Abdullah, 1974). The ethnopharmacology of *Buddleja* species summarise major traditional medicinal uses such as anti-inflammatory, analgesic, antipyretic, anticataratic, antihepatotoxic, hypotencive, hypoglycaemic, neuroprotective, antimicrobial, mollusicidal and amebocidal activities (El-Domiatya et al., 2009). They have been used in the treatment of cancer (Hu et al., 2001) and as a cure of articular rheumatism in the Chinese traditional medicine (Dictionary of Chinese Traditional Medicine, 1977). The whole plant *B. asiatica* has been used medicinally in skin complaints, to treat head tumour and malaria (Pande et al., 2007; Hartwell, 1970; Reis and Von, 1973). The essential oil of the leaves has been reported to posses *in vitro* anti-fungal activities (Garg and Dengre, 1992). The extracts of *B. asiatica* also showed strong cyclo-oxygenase (COX) inhibitory activities using elicited rat peritoneal leukocytes (Liao et al., 1999). Earlier, we reported the antimicrobial, antispasmodic, Ca^{++} antagonist and cholinesterase inhibitory potential of the plant *B. asiatica*, followed by the GC/MS analysis of the fixed oils (Ali et al., 2011; Ali et al., 2011; Ali et al., 2013; Ali et al., 2013). In this article, we report the GC/MS analysis, antimicrobial activities and cholinesterase inhibitory activities of the essential oil obtained from the leaves of *B. asiatica*. Fourteen of the constituents were identified out of the total seventeen. The mass spectral data and retention times of the constituents were analyzed by the data system library and confirmed by comparison of their mass spectra using NIST mass spectral search program or Kovat's Retention Index (RI).

Materials and Methods

Plant material

The whole plant of *B. asiatica* was collected from Banda

Piran, Siran valley (34° N/73° E, 2900 meters above the sea level), District Mansehra, in October, 2007. It was identified by Prof. Manzoor Ahmad, Plant Taxonomist, Department of Botany, Government Degree College, Abbotabad, Pakistan, where a voucher specimen has been deposited in the herbarium (accession No. B-0015).

Volatile oil extraction

Fresh leaves (100 g) of *B. asiatica* were suspended in 5 L water and subjected to hydro-distillation for 4 hours using a clevenger-type apparatus for essential oil extraction. The resulted extracted oil (1%) was separated from water by extracting three times with ethyl acetate (300 mL) and was dried by filtration over anhydrous sodium sulfate. The oil was placed in sealed tubes and kept at 4°C (Shojaaddini et al., 2008).

Sample Preparation

One milligram of the sample was dissolved in 1 mL dichloromethane, mixed well and allowed to stand for 10 min. Alongside the samples, a method blank was also prepared and analyzed.

GC/MS analysis

An Agilent 7890A Series GC connected to a 5975C Inert XL mass selective detector (combined) was used to carry out GC/MS analysis. Splitless injector and the interface were maintained at 300°C and 340°C respectively. Helium gas was used as carrier gas (at constant inlet pressure). The oven temperature was programmed from 50°C to 350°C (2-10 min) at 10°C/min. The GC contained an HP-5MS 5% phenyl methyl siloxane phase fused silica column with a diameter of 15 m X 0.25 mm, 0.25 µm and was directly inserted into the ion source. A full scan at 70 eV from m/z 50 to 800 was carried out to obtain the electron impact (EI) spectra.

Antibacterial activity

B. asiatica essential oil was screened for their antibacterial assay against selected bacterial strains including; *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6663), *Staphylococcus aureus* (ATCC 25923), *Shigella boydii* (ATCC 35966), *Shigella flexneri* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella typhi* (ATCC 19430) according to agar–well diffusion method (Rahman et al., 2011). Briefly, a loop full of 24 hours old culture containing approximately 10^4-10^6 CFU was spread on the surface of Mueller-Hinton Agar plates. The stock solutions of *B. asiatica* (1 mg/mL) were prepared in dimethylsulfoxide, followed by digging wells in the medium and adding 100 µL dilutions in the respective wells. Imepinem was used as positive control and reference compound.

Antifungal activity

B. asiatica essential oil was screened for their antifungal bioassay using agar well diffusion method (Duraipandiyan and Lgnacimuthu, 2009) against *Aspergillus flavus* (ATTC 32611), *Microsporum canis* (ATTC 11622), *Ttichophyton longifusus* (ATTC 22397), *Fusarium solani* (ATTC 11712), *Candida glaberata* (ATTC 90030) *and Candida albicans* (ATTC 2091). Briefly, the stock solutions of *B. asiatica* essential oil prepared in dimethylsulfoxide. Sterile sabouraud's dextrose agar medium (5 mL) was placed in a test tube and inoculated with the sample solutions (400 µg /mL) kept in slanting position at room temperature overnight. The fungal culture was then inoculated on the slant. The samples were incubated for 7 days at 29°C and growth inhibition was observed. Amphotericin-B and miconazole were used as the standard drugs.

Cholinesterase inhibition assay and determination of IC_{50} values

Acetylcholinesterase (AChE, EC 3.1.1.7), butyrylcholinesterase (BChE, horse-serum E.C 3.1.1.8) along with, acetylthiocholine iodide, butyrylthiocholine chloride, galanthamine and DTNB (5, 5´-dithiobis [2-nitrobenzoic-acid]) were purchased from Sigma. AChE and BChE inhibiting activities were measured according to modified spectroscopic method (Shojaaddini et al., 2008; Kavanagh, 1963; Ellman et al., 1961). All the protocol and assay conditions were the same as mentioned in the literature (Rocha et al., 1993). All other chemicals used in this assay were of analytical grade.

Briefly, 0.2 mM DTNB in 62 mM sodium phosphate buffer (pH 8.0, 880 µL), AChE/ BChE solution (40 µL) and test sample solution (40 µL) were mixed and incubated for 15 min (25°C). To this mixture, 40 µL of acetylthiocholine or butyrylthiocholine were added to initiate the reaction. Hydrolysis of acetylthiocholine and butyrylthiocholine were monitored at a wavelength of 412 nm (15 min), by the formation of 5-thio-2-nitrobenzoate anion (yellow colour), due to the enzymatic hydrolysis of acetylthiocholine/butyrylthiocholine, respectively. All the reactions were performed in triplicate in a BMS spectrophotometer (USA). The IC_{50} values were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values while acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates in AChE and BChE assays.

Results

The IR spectrum of *B. asiatica* essential oil in chloroform showed major absorption at 3410 cm^{-1} and 1077 cm^{-1} which indicated hydroxyl group. The absorption appeared at 3008 cm^{-1}, 886 cm^{-1} and 741 cm^{-1} were assigned for aromatic functional group. The absorption at 2924 cm^{-1} was indicative of aliphatic while absorption at 1681 cm^{-1} was due to the presence of carbonyl

functionalities.

The GC chromatogram obtained for leaves essential oil showed total of 17 peaks which appeared at varies retention indices/times. Both combined GC and MS studies resulted in the identification of 14 compounds. The scan numbers, retention time, compound names and their percentage abundances/quantities as well as other related results have been provided in Table I. The mass spectral data and retention times of the constituents were analyzed by the data system library and confirmed by comparison of their mass spectra using NIST mass spectral search program or Kovat's Retention Index (RI).

Antibacterial activity of the *B. asiatica* essential oil was performed against even human pathogens including *E. coli, B. subtilis, S. aureus, S. boydii, S. flexneri, P. aeruginosa and S. typhi.* The essential oil displayed high to low activity in killing the *S. boydi* (56%), *E. coli* (44%), *S. flexneri* (36%), *S. aureus* (25%), *B. subtilis* (17%) and *P. aeruginosa* (10%) but no activity was seen against the *S. typhi* (Table II).

B. asiatica essential oil displayed significant activity in killing *A. flavus* with 77% zone of inhibition (Table III). Optimum activity was observed against *M. canis* (66%), *F. solani* (60%) *and T. longifusus* (59%) while no activity were reported against *C. glaberata* and *C. albicans.*

B. asiatica essential oil revealed to be much potent towards AChE and BChE by inhibiting AChE and BChE in a dose dependent manner (Table IV). The IC_{50} values of essential oil was determined to be 5.2 µM and 27.9 against AChE and BChE respectively.

Discussion

Our results showed that both monoterpenes and sesquiterpenes were detected in the leaves of essential oil. They included four monoterpenes hydrocarbons, four oxygenated monoterpenes, one hydrocarbon sesquiterpenes and five oxygenated sesquiterpenes. The major constituent being found was 1,8-cineole (38.1%) while β-sinensal, 1,10-seco-1-hydroxycalamenen-10-one and α-phellandrene were found to be 11.8%, 10.2%, 5.8% respectively. α-Pinene and α-thujene were present in 5.0% and 3.4%. While furan, 2-(1-pentenyl)-(E), terpinen-4-ol, bicyclo[5.1.0]octane, 8-(1-methylethylidene) and eugenol were found in traces. Because of the high content of monoterpenes, especially, 1,8-cineole, the oil may be classified as a medicinal type (Oyedeji et al., 1992). Other sesquiterpenes like

Table I

GC/MS analysis of essential oil from leaves of *Buddleja asiatica* essential oil

Sl. No	R.I	Compound identified	M. F	% Abundance	*m/z* (%)
1	3.1	*a –Pinene*	$C_{10}H_{16}$	4.9	136, 121, 105, 93, 77, 65, 53, 41
2	3.8	*a-Phellandrene*	$C_{10}H_{16}$	5.8	136, 121, 107, 93, 77, 67, 55, 43
3	4.4	*a –Thujene*	$C_{10}H_{16}$	3.4	136, 121, 105, 93, 77, 65, 51, 41
4	5.3	*1,8-cineole*	$C_{10}H_{18}O$	38.1	154, 139, 107, 96, 81, 71, 68, 59, 43
5	6.3	*Furan, 2-(1-pentenyl)- (E)*	$C_9H_{12}O$	1.8	136, 121, 107, 93, 79, 65, 43
6	9.0	*Terpinen-4-ol*	$C_{10}H_{18}O$	1.9	154, 136, 111, 93, 86, 77, 71, 55, 43
7	9.6	*Bicyclo[5.1.0]octane, 8-(1-methylethylidene)*	$C_{11}H_{18}$	1.0	150, 139, 135, 121, 107, 96, 93, 81, 71, 67, 59, 55, 43
8	15.0	*Eugenol*	$C_{10}H_{12}O_2$	1.5	164, 149, 137, 121, 103, 91, 77, 55
9	17.0	*Kushimone*	$C_{14}H_{20}O$	1.0	204, 189, 161, 147, 133, 121, 105, 91, 79, 67, 55
10	17.6	*a –Cubebene*	$C_{15}H_{23}$	1.4	204, 189, 174, 161, 147, 133, 119, 105, 91, 81, 67, 55
11	18.7	*12-Nor-preziza-7(15)-en-2-one*	$C_{14}H_{20}O$	1.8	204, 189, 173, 161, 136, 121, 107, 93, 81, 67, 55, 43
12	22.0	*1,10-seco-1-hydroxycalamenen-10-one*	$C_{14}H_{20}O_2$	10.2	220, 202, 187, 177, 159, 147, 133, 119, 107, 95, 81, 69
13	22.0	*Unidentified alcohol*	$C_{16}H_{14}O$	4.0	222, 204, 189, 175, 161, 147, 133, 121, 107, 95, 81, 69
14	23.4	*Isospathulenol*	$C_{15}H_{24}O$	0.9	220, 205, 187, 177, 162, 147, 134, 119, 105, 93, 79, 69
15	23.8	*Unknowen Diphenyl, di-ketone*	$C_{15}H_{10}O_2$	0.9	222, 204, 189, 177, 161, 147, 133, 121, 109, 95, 79, 69
16	26.0	*β-Sinensal*	$C_{15}H_{22}O$	11.8	218, 203, 189, 175, 161, 147, 133, 121, 107, 91, 79, 69
17	44.2	*Unidentified*	$C_{18}H_{17}O_2N$	9.4	279, 248, 167, 149, 131, 113, 104, 97, 83, 71, 57, 43

Table II

Antibacterial activities of *B. asiatica* essential oil

Bacteria	Imepinem (Zone of inhibition)	Essential oil	
		Zone of inhibition	% Inhibition
E .coli	34	15	44
B. subtilis	30	5	17
S. aureus	28	7	25
S. boydii	25	14	66
S. flexneri	31	11	36
P. aeruginosa	30	3	10
S. typhi	29	-	-

The plates were inculcated at a concentration of mg/mL of DMSO

isospathulenol, kushimone, 12-nor-preziza-7(15)-en-2-one, α-cubebene, isospathulenol and germacrene-B were found in traces. Three of the constituents remain unidentified even from their molecular formulas due to the need of more spectroscopic evidences. In our continued search for biologically active constituents, we are interested to evaluate the AChE and BChE inhibiting potential of essential oils from herbal medicinal plants. Currently, AChE and BChE represent the most attractive target for drug design and discovery of mechanism-based inhibitors for the treatment of Alzheimer's disease (Zhang, 2004). The percentage inhibition against AChE and BChE of *B. asiatica* essential oil was first determined at 0.1 mM and further subsequently assayed for IC_{50} determination. Due to phytochemical deviations between essential oil components, many essential oils differ in their anticholinesterase activities. Since essential oils were shown to be complex mixtures of a number of components with a high percentage of monoterpenes,especially the oxygenated monoterpenes, hence it is not conclusive to determine which single component is responsible for most of the biological activities. Several other studies have been carried out on the anticholinesterase activity of essential oils but the lack of sufficient knowledge has prompted us to work on this basis. In a recent study, essential oil of *F. vulgare* was tested for their AChE inhibitory activities (Mata et al., 2007) which revealed to be reasonably potent.

Table IV

AChE and BChE inhibitory activities of *B. asiatica*

	AChE ± SEM[a]	BChE ± SEM[a]
Essential oil	5.2 ± 0.06	27.9 ± 0.03
Allanzanthane [b]	6.9 ± 0.05	25.0 ± 0.06
Galanthamine [b]	7.9 ± 0.01	16.3 ± 0.01

a = Standard error of mean of five assays; b = Positive control used in the assays; Data shown are values from triplicate experiments

As compared to the AChE and BChE inhibitory effects of the single compounds of the essential oils, it may be deduced that the activity may not be due to a single compound. Similarly the essential oils of *Melissa officinalis* and *Mentha suaveolens* were reported to have strong AChE inhibitory activity. The anticholinesterase activity of several *Mentha* essential oils of Japanese origin were carried out (Miyazawa et al., 1998) in which, sesquiterpenes alcohols were the main components of these. *Mentha* oil showed the most potent inhibition. But none of them showed a stronger inhibitory activity than the essential oils. Although this was a report on a species different from ours, the difference in the activity may doubtlessly depend on chemical compositions.

Conclusion

The promising antimicrobial and anti-cholinesterase activities of the essential oil from *B. asiatica* are due to the presence of several minor multifunctional components, oxygenated mono and sesquiterpenes, specially 1,8-cineole and *a*-pinene.

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Table III

Antifungal activities of *B. asiatica* essential oil

Fungi	Linear growth (mm) Control	*B. asiatica* essential oil		Standard drugs	
		Linear growth (mm)	%Inhibition	Name	MIC (µL/mL)
A. flavus	100	21	79	Amphotericin B	30
M .canis	100	70	66	Miconazole	105
T. longifusus	100	50	59	Miconazole	88
F. solani	100	40	60	Miconazole	94
C. glaberata	100	100	-	Miconazole	103
C. albicans	100	100	-	Miconazole	20

The plates were inculcated at a concentration of mg/ml of DMSO

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Author Info
Farman Ali Khan (Principal contact)
e-mail: alinuml@gmail.com Phone: 00923339724044