DOI:10.7598/cst2018.1513
ISSN:2278-3458

Chemical Science Transactions
2018, **7(3),** 499-505

RESEARCH ARTICLE

Essential Oil Composition and Antibacterial Activity of *Agrimonia Pilosa* Ledeb (Rosaceae)

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Received 21 February 2018 / Accepted 12 March 2018

Abstract: Essential oil composition of the aerial parts of *Agrimonia pilosa* Ledeb growing wild in the central Himalayan region of Uttarakhand, India was analyzed by capillary gas chromatography (GC-FID) and gas chromatography–mass spectrometry (GC–MS). A total of 15 constituents were identified, representing 95.29% of the oil composition. The oil consisted mainly of oxygenated monoterpenes (75.74%) followed by monoterpene hydrocarbons (18.68%). Major constituents identified were methyl myrtenate (71.39%), limonene (11.86 %), α-thujone (5.66%) and myrtenyl acetate (3.14%). The essential oil showed a broad spectrum of antibacterial activity against both the human and plant pathogenic bacteria. Oil showed the highest activity against human pathogenic bacteria *K. pneumonia* (7.33 mm, MIC 175 µL/mL) and *P. aeruginosa* (7.00 mm, MIC 175 µL/mL). Plant pathogenic bacteria *R. solanacearum* (6.70 mm, MIC 175 µL/mL) was found less resistant against the oil. The results showed that oil containing methyl myrtenate a major constituent has potential for treatment of infections caused by these pathogenic bacterial strains.

Keywords: Rosaceae, *Agrimonia pilosa* Ledeb, Essential oil, Antibacterial activity, Methyl myrtenate.

Introduction

The genus *Agrimonia*, family Rosaceae having pinnate leaves and yellow flowers followed by bristly fruits is found chiefly in north temperate regions. The species of *Agrimonia* belonging to the Rosaceae, are listed in the oriental medicine as an astringent hemostatic to treat bleeding, curing disorders related to the liver and bile, gastrointestinal and respiratory tract in traditional Austrian medicine[1]. Many pharmacological studies have reported that *Agrimonia pilosa* Ledeb showed broad biological properties, such as antioxidant activity, anti-viral effect and acetylcholinesterase inhibitory effect[2-4]. *A. pilosa* contains abundant flavonoids *viz*. catechin, hyperoside, quercitrin, quercetin and rutin. These compounds are demonstrated to be the major bioactive constituents having anti-inflammatory, anti-tumor, anti-virus, anti-bacteria and anti-oxidation functions[5]. Over the last two decades several

research groups have reported the isolation of various biologically active compounds from this plant[6-9]. The major components in the essential oil of *A. Aitchisonii* were reported to be methyl myrtenate, limonene, linalool, myrtenyl acetate , linalyl acetate and zingiberene[10,11]. Essential oil from wild growing leaf and flower oil of *Agrimonia eupatoria* has been reported to possess β-caryophyllene, caryophyllene oxide, α-humulene and *E*-β-farnesene[12]. *A.pilosa* collected from three different locations of China contained hexadecanoic acid (11.83– 27.34%) as the main compound[13].

To the best of our knowledge, there is no report on the essential oil composition and antibacterial activity of *A. Pilosa* from Uttrakhand, India. Therefore, this study was aimed to determine the antibacterial activity of *A. Pilosa* essential oil.

Experimental

The plant material was collected in the month of September (flowering stage) from Binsor forest (Almora District), Uttarakhand, India, at an altitude of 2700 meters. A voucher (specimen No. 116125) has been deposited at the Botanical Survey of India (BSI), Dehradun, India and Phytochemistry laboratory, Department of Chemistry, Almora, Kumaun University.

Chemicals and reagents

All chemicals and reagents used were of analytical grade. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) were obtained from Hi-Media, India.

Extraction of the essential oil

Fresh aerial parts (~4 kg) were subjected to steam distillation. The distillate obtained after steam distillation of fresh plant material was treated with *n*-hexane for the extraction of organic constituents. The distillate was further shaken with dichloromethane to ensure complete extraction of constituents. The *n*-hexane and dichloromethane extracts were combined and dried over anhydrous Na_2SO_4. Solvent was distilled off in a rotary vacuum evaporator (Perfit-RV 1240, Buchi type) to get residual oil which was stored at ~4 ºC.

GC-FID analysis

A gas chromatographic analysis of essential oil was performed on a Shimadzu GC-2010 Ulta gas chromatograph equipped with flame ionization detector and a Rtx-5MS fused silica capillary column (25 m × 0.25 mm, 0.25 µm film thickness). The injector and detector temperature were maintained at 260 and 270 ºC, respectively. Helium at a flow rate of 1.21 mL/min and 69.0 kPa inlet pressure was employed as the carrier gas. The oven temperature was programmed at 50 ^{o}C (first 2 min), then increased at the rate: 3 ^{o}C/min to 280 ^{o}C, after which it was maintained at 280 ^{o}C for 18 minutes. The sample (1.0 µL) was injected with 10:1 spilt ratio.

GC-MS analysis

GC-MS was carried out on Shimadzu GC-MS-QP2010 Ultra using identical oven temperature programming with following conditions: Rtx-5MS capillary column (25 m × 0.25 mm, film thickness 0.25 µm); carrier gas: He (flow rate was 1.21 mL/min), injection in Split mode (10:1); injector temperature: 260 ^{o}C. The MS was used in the electron impact (EI) conditions (70 eV), ion 230 ^{o}C, mass scan mode: 2.41 scan/second, mass range: 40-650 *m/z*; a 5% solution of oil in hexane (1.0 µL) was injected.

The identification of individual compound was carried out by the calculation of Retention Indices (RI) using homologous C_8-C_{34} (Supplier- Restek's ISO 9001:2008) *n*-alkane series and a comparison with available mass spectral data (NIST 11, Wiley 8 and FFNSC 2) and confirmed by comparison of their retention indices with those of reported in the literature[14]. For quantification purposes, relative area percentages obtained by FID were used without the use of correction factors.

Antimicrobial screening of essential oils

Pathogenic bacterial strains

The *in vitro* antibacterial activity was evaluated against five human pathogenic bacterial strains *Bacillus subtilis* (MTCC No. 441), *Escherichia coli* (MTCC No. 443), *Klebsiella pneumoniae* (MTCC No. 3384), *Pseudomonas aeruginosa* (MTCC No. 424), *Salmonella typhimurium* (MTCC No. 3224) and six plant pathogenic bacterial strains *Agrobacterium tumefaciens* (MTCC No. 609), *Erwinia crysanthemi* (KUMSCC 328), *Ralstonia solanacearum* (BI0012), *Xanthomonas campestris* (BB0006), *X. oryzae* (BH0007) and *X. phaseoli* (KUMSCC 327)]. Some of the test strains were purchased from Indian Type Culture Collection (ITCC), ICAR, New Delhi and some provided by the Department of Biotechnology, Bhimtal, Kumaun University, which were procured from the Institute of Microbial Technology, Chandigarh. Indian Type Culture Collection (ITCC) and Microbial Technology Culture Collection (MTCC) numbers represent the standard strain numbers assigned to these microorganisms. The cultures of bacteria were maintained throughout the experiment at 4°C on their appropriate nutrient agar and used as stock cultures.

Antimicrobial activity by disc-diffusion method

Evaluation of antimicrobial activity of essential oil samples was done by disc-diffusion method described by Clinical and Laboratory Standards Institute[15]. The samples were dissolved in dimethyl sulphoxide (DMSO) to prepare desired concentrations. Inoculums of the microbial strains (1×10^6 CFU/mL) were plated using sterile swabs into petri dishes (90 mm) with 20 mL of Nutrient Agar, and then discs of Whatman paper-42 were soaked in sample solution (15 µL/mL) and placed onto inoculated petri dishes. Standard antibiotic streptomycin (15 mg/mL) was used as a positive control and DMSO as negative control. The petri dishes were pre-incubated for 3 h at room temperature, allowing the complete diffusion of the samples and then, incubated at 37±1 ºC for 24 h[16]. Finally the zones of inhibition were measured.

Antimicrobial activity by broth dilution method

The evaluation of MICs was done using the agar dilution method with slight modifications described by the National Committee for Clinical Laboratory Standards[17]. Equal volumes of each microbial strain culture, containing approximately 1×10^6 CFU/ml, were applied onto MHB supplemented with the essential oil at concentration ranging from 25-250 µl/ml in tubes. These cultures were then incubated at 37 ºC for 24 hrs and then the cultures were finally inoculated on nutrient agar media to determine the growth of bacteria. Controls of bacteria without the oil were also applied. The concentration at which no visible growth was observed is considered as MICs.

Statistical analysis

Mean value ± SD was determined by using XLSTAT 14 statistical computer software package.

Results and Discussion

The essential oil (yield 0.1%; v/w) obtained from aerial parts of *A. pilosa* was analyzed by using GC-FID and GC-MS. A total of 15 constituents, representing 95.29% of the total oil have been identified. The retention index of volatile compounds (RI^a and RI^b) and their percentage are summarized in Table 1. Essential oil showed the dominant presence of oxygenated monoterpenes (75.74%) followed by monoterpene hydrocarbons (18.68%). The sesquiterpene hydrocarbons accounted only for 0.87%. The essential oil has been characterized by a high amount of methyl myrtenate (71.39%). The other major components are limonene (11.86%), α-thujone (5.66%) and myrtenyl acetate (3.14%). Previous report of *A. Pilosa* collected from different regions of China was characterised by the presence of hexadecanoic acid (11.83– 27.34%) as main constituent[13] while in present study, oil was rich in methyl myrtenate (71.39%) with absence of hexadecanoic acid. Chemical variation of essential oil may be attributed due to influences of environmental, genetic and ecological factors such as light and temperature[18-24].

Table 1. Chemical composition of essential oil from *Agrimonia pilosa*

S. No.	Compound	RI^a	RI^b	% Composition	Method of identification
1.	*α*-Thujone	932	930	5.66	c
2.	Camphene	957	954	0.18	c
3.	*β*- Pinene	979	979	0.83	c
4.	Myrcene	991	990	0.15	c
5.	Limonene	1031	1029	11.86	c
6.	Linalool	1101	1096	0.27	c
7.	*α*-Campholenal	1128	1126	0.13	c
8.	*trans* -Verbenol	1147	1144	0.12	c
9.	Myrtenal	1199	1195	0.26	c
10.	Linalool acetate	1259	1257	0.25	c
11.	Methyl myrtenate	1300	1294	71.39	c
12.	Myrtenyl acetate	1329	1326	3.14	c
13.	Methyl perrilate	1396	1393	0.18	c
14.	*α*-Zingiberene	1493	1493	0.72	c
15.	*E,E*, *α*-Farnesene	1508	1505	0.15	c
	Total identified			95.29%	
	Monoterpene hydrocarbones			18.68%	
	Oxygenated monoterpenes			75.74%	
	Sesquiterpene hydrocarbones			0.87%	
	Total unidentified			4.71%	
	Oil yield (%, v/w)			0.11	

[a]Retention index (RI) calculated relative to homologous series of n-alkanes (C_8-C_{24}) on Rtx-5 non-polar fused silica capillary column; [b]Retention index (RI) Adams; [c]MS, NIST and WILEY libraries spectra and the literature

Essential oil of *A. pilosa* was tested against five human and six plant pathogenic bacteria. Results presented in Table 2 showed that oil exhibited different antibacterial activity against both the tested bacterial strains. The oil showed the maximum activity against human bacterial strain *K. pneumonia* (7.33 mm, MIC 175 µL/mL) followed by *P. aeruginosa* (7.00 mm, MIC 175 µL/mL) and *S. typhimurium* (6.66 mm, MIC 200 µL/mL) while Modest activity was observed against *E. coli* (5.00 mm, MIC 200 µL/mL). The order of resistivity of human pathogenic bacteria against oil was found *E. coli*> *B. subtilis*> *S. typhimurium*> *P. aeruginosa*> *K. pneumonia.* The oil demonstrated significant activity against plant pathogenic bacteria. The result showed highest antibacterial activity against *R. solanacearum* (6.75 mm, MIC 175 µL/mL). The *A. pilosa* was found to be less active to the control of *X. phaseoli* (5.50 mm) and *X. oryzae* (5.00 mm) at 250 µL/mL MIC value. The order of resistivity of plant pathogenic bacteria against oil was found *X. oryzae*> *X. phaseoli*> *X. campestris*> *A. tumefaciens*> *E. crysanthemi* > *R. solanacearum.* Based on the zone of inhibition and minimum inhibitory concentrations (MIC) values *K. pneumonia* and *R. solanacearum* were the most sensitive strains tested to the oil. While *E. coli* and *X. oryzae* were the most resistant towards the oil. As compared to the standard antibiotic (streptomycin) oil showed significant activity against all the tested bacteria. The activity of the oil may be due to the presence of higher percentage of methyl myrtenate a oxygenated monoterpene and the synergetic effect of other major and minor compounds present in the oil.

Table 2. Antibacterial activity of the *Agrimonia pilosa* essential oil by disc-diffusion method

Bacterial strains	*Agrimonia pilosa*		Reference antibiotic	
	ZOI (mean ± SD)[a]	MIC (µL/mL)	ZOI (mean ± SD)[b]	MIC (mg/mL)
Human Pathogenic				
B. subtilis	6.33±0.57	200	26.33±0.57	100
E. coli	5.00±0.33	200	31.66±0.66	50
K. pneumoniae	7.33±0.57	175	30.33±0.57	50
P. aeruginosa	7.00±0.15	175	27.33±1.00	75
S. typhimurium	6.66±0.57	200	24.66±0.33	100
Plant pathogenic				
A. tumefaciens	6.00±0.57	200	33.00±1.00	50
E. crysanthemi	6.30±0.33	200	25.66±0.57	50
R. solanacearum	6.70±0.55	175	25.66±0.57	100
X. campestris	6.00±0.57	225	23.66±0.52	100
X. oryzae	5.00±1.00	250	24.33±0.52	100
X. phaseoli	5.50±0.50	250	32.66±1.00	50

[a] *Inhibition zone diameter includes Whatman paper-42 (3 mm) at15 µL/mL,* [b] *Inhibition zone diameter includes Whatman paper-42 (3 mm) at15 mg/mL*

Conclusion

The essential oil composition and antibacterial activity of *A. pilosa* is being reported for the first time from Uttarakhand Himalayan region. The essential oil of aerial parts of *A. pilosa* containing methyl myrtenate as major constituents showed significant antibacterial activity against both the human and plant pathogenic bacteria. All the bacterial strains showed the sensitivity towards the essential oil, thus indicated the importance of this plant as natural agents for the treatment of infectious diseases caused by respective bacteria.

Acknowledgement

The authors are thankful to Department of Chemistry, Kumaun University Campus Almora for providing laboratory facilities and Department of Botany, Kumaun University Campus Almora for bioactivity.

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