IJPT
1735-2657/12/112-68-72
IRANIAN JOURNAL OF PHARMACOLOGY & THERAPEUTICS

IJPT 11: 68-72, 2012

ORIGINAL ARTICLE

Major Constituents, Antioxidant and Antibacterial Activities of *Zanthoxylum armatum* DC. Essential Oil

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Received January 12, 2012; Revised May 3, 2012; Accepted June 27, 2012

This paper is available online at *http://ijpt.iums.ac.ir*

ABSTRACT

Chemical compositions, antioxidant and antibacterial activities of *Zanthoxylum armatum* essential oil was analyzed. A total of 3 samples (500 g/each) were collected at 6:00 am; 12:00 noon; and 6:00 pm in the same day. The essential oil was extracted by hydro distillation in Clevenger apparatus and their chemical compositions were determined by the GC-MS system. The eleven most abundant ingredients were bornyl acetate (16.61-22.66%), cymene (8.25-12.50%), α-copaene (7.54-7.59%), γ-terpinene (5.33-7.66%), camphene (4.32-4.66%), limonene (2.66-4.68%), linalool (3.28-3.58%), β-ocimene (3.24-3.36%), *trans*-caryophyllene (2.54-3.46%), α-terpinolene (2.32-3.36%) and germacrene (2.02-2.85%). Antioxidant activity was examined by 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. Ascorbic acid was used as standard. Essential oil exhibited significant antioxidant activity. The IC_{50} of the oil was found 27.0± 0.1 µg/ml and that of ascorbic acid 15.0± 0.5 µg/ml. Essential oil of *Z. armatum* also exhibited moderate antibacterial activity. The results showed that the gram-positive bacteria are more sensitivity to the essential oil than gram negative bacteria.

Keywords: *Zanthoxylum armatum, Essential oil, antioxidant, anti-bacterial, GC-MS, DPPH, Ciprofloxacin*

Zanthoxylum are deciduous aromatic shrubs and trees (family Rutaceae) comprises 250 species native to warm temperate and subtropical region of the world. Traditionally, leaves and fruits are used for mouth fresh and tooth care while bark is used for intoxicating the fishes [1] and leaves, fruits and barks are also used as spice [2]. Plants essential oils, commonly used as fragrances and flavoring agents for foods and beverages, are also recommended as an alternative source for constituting numerous bioactive phytochemicals that can be potentially used for insect control [3]. The main components of *Zanthoxylum* oil are oleic acid, palmitic acid, linoleic acid methyl ester, limonene and linalool [4]. β-Caryophyllene, α- and β-farnesene, β-bisabolol, γ-cadinene, nerolidol, 2-undecanone and dodecanal were identified from the essential oil of *Z. gillettii* [5]. Chemical constituents of *Z. bungeanum* and *Z. piperitum* essential oils have been determined [6,7]. Altitudinal variations of linalool and limonene in leaves of *Z. alatum* have also been determined by Gupta et al. [8].

Medicinal plants are good source of remedies for human health problems due to the presence of bioactive compounds. Recently wide ranges of medicinal plants have been screened for antioxidant and antimicrobial activities [9-11]. These medicinal plants are also used in different countries as a source of many potent and powerful drugs. Antimicrobial property of essential oils of medicinal plants have been reviewed [12]. *Zanthoxylum tingoassuiba* and *Z. hyemale* essential oils have also been reported for their antimicrobial activity [13,14] and *Z. leprieurii* and *Z. xanthoxyloides* for their antioxidant activity [15]. In view of strong antioxidant and antimicrobial properties of essential oils, the present study was designed to determine major constituents, antioxidant and antibacterial activities of *Zanthoxylum armatum* essential oil.

Table 1. Chemical composition of essential oil of *Z. armatum*

Constituents	Composition (%)			
	6 am	12 noon	6 pm	Mean ± SD
Camphene	4.59	4.66	4.32	4.52 ± 0.14
γ-Terpinene	5.38	5.33	7.66	6.12 ± 1.08
Cymene	8.25	8.35	12.50	9.70 ± 1.98
Limonene	3.26	2.66	4.68	3.53 ± 0.84
β-Ocimene	3.24	3.36	3.36	3.32 ± 0.56
α-Terpinolene	2.32	2.32	3.36	2.66 ± 0.49
Linalool	3.28	3.28	3.58	3.38 ± 0.14
Bornyl Acetate	17.82	16.61	22.66	19.03 ± 2.61
Trans-Caryophyllene	3.46	2.59	2.54	2.86 ± 0.42
Germacrene	2.02	2.53	2.85	2.46 ± 0.34
α-Copaene	7.54	7.54	7.59	7.55 ± 0.02

MATERIALS AND METHODS

Plant material and extraction of essential oil

Leaves of *Z. armatum* were collected from Mandal forest of Uttarakhand, India. A total of 3 samples (500 g/each) were collected at 6:00 am; 12:00 noon; and 6:00 pm in a same day. The plant was authenticated through existing literature (herbarium No. GUH 3802). The fresh leaves of *Z. armatum* were cut into small pieces and subjected to hydro-distillation in a Clevenger apparatus (5 h). The pale yellow colored essential oils were collected, dried over anhydrous sodium sulphate and stored in a sealed glass vials at low temperature (0-4°C) prior to analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

Qualitative and quantitative analysis of extracted essential oils of *Z. armatum* were performed on Perkin-Elmer make Clarus-500 GC equipped with Perkin-Elmer-Clarus-500 MS and capillary column (60m×0.25mm, film thickness 0.25 µm). Injector and detector temperatures were 210°C and 280°C, respectively, while the helium was used as carrier gas. Oven temperature was held for 5 minutes at 50°C with 5 min solvent delay, then programmed at 3°C/min up to 220°C/min, and then maintained isothermally at 220°C for 20 min. GC-MS was operated in EI mode at 70 eV.

Antioxidant activity

DPPH radical scavenging activity of *Z. armatum* oil isolated from the sample collected at 6 pm (high yield) was determined according to the Zhishen et al. [16] and Brand-Williams [17] with slight modifications. The working solutions (1, 5, 10, 20, 50, 70, 100 µg/ml) of the *Z. armatum* oil were prepared in methanol. Ascorbic acid was used as standard in 1-100 µg/ml. Briefly, 1 ml of DPPH (1, 1-diphenyl-2-picrylhydrazyl) solution (0.1 mM in methanol) was mixed with 3 ml of oil samples and standard solutions separately. The mixture was shaken and kept for 30 minutes at room temperature. The decrease of solution absorbance due to proton donating activity of components of oil was determined at 517 nm using Elico SL-159 UV-Vis spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. DPPH (3 ml of 0.1 mM) and methanol (1 ml) was used as blank. The DPPH radical scavenging activity was calculated using the following formula:

DPPH Radical Scavenging Activity (% inhibition) = $[(A_0–A_1/A_0) \times 100]$, where A_0 is the absorbance of the blank, and A_1 is the absorbance of oil mixed with DPPH. IC_{50} values were measured from % inhibition versus concentration graph.

Antibacterial activity

Antibacterial activity of *Z. armatum* oil isolated from the sample collected at 6 pm (high yield) was determined by well diffusion method described according to Cutler and Wilson [18] and Gupta et al. [19] with slight modifications. Bacterial cultures of *Streptococcus faecalis, Staphylococcus aureus, Proteus vulgaris* and *Klebsiella pneumoniae* were obtained from Department of Microbiology, HNB Garhwal University and used for antibacterial test organism. The bacteria were maintained on nutrient broth at 37°C. The gram positive (*Streptococcus faecalis* and *Staphylococcus aureus*) and gram negative (*Proteus vulgaris* and *Klebsiella pneumoniae*) bacteria were precultured in nutrient broth overnight in incubator. The stock culture suspensions were diluted with sterile Saline water. The Petri dishes were flooded with Mueller Hinton Agar and after solidification of agar 0.1 ml of diluted inoculums were spread over Mueller Hinton Agar in the dishes using sterile L spreader to achieve confluent growth. Six millimeter diameter wells were cut from the agar using a sterile cork borer, and 10 µl of the essential oil was delivered into the wells. Ciprofloxacin (5 µg/well) was used as standard. The antibacterial assay plates were incubated at 37°C for 24 h. The diameters of the zone of inhibition (ZoI) were measured in mm.

Table 2. Comparative accounts of major constituents (%) of *Zanthoxylum* oil

Species	Plant part	γ-Terpinene	Limonene	β-Ocimene	α-Terpinolene	Linalool	Bornyl Acetate	Trans-Caryophyllene	Germa-crene
Z. armatum	Seeds[21]	-	19.8	-	-	57.00	-	-	-
	Leaves[8]	-	1.59-2.76	-	-	34.06-35.57	-	-	-
Z. bungeanum	Fruits[22]	7.30	-	1.10	-	3.70	0.30	0.60	-
	Fruits[38]	-	12.00	-	-	13.00	-	-	-
	Fruits[28]	-	21.00	-	-		-	-	-
Z. acanthopodium	Leaves[23]	0.40	14.80-18.30	-	0.30	0.80	0.10	2.2-2.6	0.6
Z. rhoifolium	Leaves[39]	-	-	-	-		-	34.00	-
	Fruits[39]	-	-	-	-	15.00	-	-	-
Z. limonella	Fruits[29]	-	31.09	-	-		-	-	-
	Fruits[30]	6.60	12.90	-	-		-	-	-
	Leaves[30]	-	33.10	6.20	-	23.30	-	-	-
Z. schinifolium	Fruits[38]	-	14.00	-	-	29.00	-	-	-
Zanthoxylum species	Leaves[27]	-	12.00	-	-	13.00	-	-	-

RESULTS AND DISCUSSION

Chemical composition of essential oil

The hydro-distillation of *Z. armatum* yielded pale yellow colored oil (yielded~0.088%-0.176%, w/v). The individual constituents separated by gas chromatography were identified by comparing their MS with those of standard NIST (National Institute of Standards and Technology, U.S. Department of Commerce) and Wiley (John Wiley & Sons Ltd) libraries. Upon GC-MS analysis, the hydro-distilled oils were found to contain forty two constituents eluted between 18 and 65 min. Among detected constituents eleven are found to be major constituents, which are mainly comprised of mono and sesquiterpenoids (Table 1). Comparative accounts of major constituents of *Zanthoxylum* oils are summarized in Table 2. The lowest yield (0.088%) was recorded for the sample harvested at 12:00 noon, and the highest (0.176%) was obtained at 6.00 pm. Thus it can be perceived that to obtain the highest essential oils yield, the collection must be comprehended near about 6.00 pm. Variation in the aerial part and seed essential oil yield of the genus *Zanthoxylum* were observed 0.12-0.42% [20] and 1.3-1.36%, respectively. In many other reports [21,22] 0.019% and 0.017% dry weight essential oil was also recorded in *Z. acanthopodium* leaves collected in summer and winter, respectively [23]. Quantitative differences can be observed, when a comparison is made between the different daytime collections.

The major constituents were found in the range 2.02-22.66%. Among these, bornyl acetate was the major component (16.61-22.66%) followed by cymene (8.25-12.50%), α-copaene (7.54-7.59%), γ-terpinene (5.33-7.66%), limonene (2.66-4.68%), camphene (4.32-4.66%), linalool (3.28-3.58%), β-ocimene (3.24-3.36%), *trans*-caryophyllene (2.54-3.46%), α-terpinolene (2.32-3.36%) and germacrene (2.02-2.85%). Bornyl acetate was identified as the main constituent, which was found with the concentration of 22.66% at 6:00 pm, 17.82% at 6:00 am and 16.61% at 12 noon. And germacrene was present at the lowest concentration of 2.02% at 6:00 am, 2.53% at 12 noon and 2.85% at 6.00 pm. The mean values of % composition of three collections are presented in Table 1. Bisht and Chanotiya [24] analyzed the essential oil of *Z. armatum* leaf by capillary gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) and found 2-undecanone as main component followed by 2-tridecanone. Although linalool and limonene were also reported as dominant content in several other studies on *Z. armatum* oil [21,25]. There are number of reports [26,27] on the essential oil analysis of *Zanthoxylum.* One study showed that linalyl acetate, linalool and limonene were major components in the essential oil of *Zanthoxylum* [26]. GC-FID and GC-MS analysis of the essential oil of *Z. schinifolium* and *Z. bungeanum* showed that limonene was the main component, followed by 4-terpineol, γ-terpinene, α-terpineol acetate, β-pinene, α-terpineol and β-linalool [28]. Itthipanichpong et al. [29] had also reported limonene, terpin-4-ol and sabinene as major components of essential oil of *Z. limonella* Alston. Another study showed that major components in the fruit oil were linalool, limonene, aterpineot, α-pinene, γ-terpinene, *cis*-β-ocimene, terpinen-4-ol and isomenthone, while in leaf oil, the predominant was limonene, followed by geraniol, carvone, 7-hydroxy-3,7dimethyloctanal, geranial and nerol [30]. Our results

Table 3. Antibacterial activity of essential oil of *Z. armatum*

Test organism	Zone of inhibition (mm)	
	Z. armatum oil (10 mg/well)	Ciprofloxacin (5 µg/well)
Streptococcus faecalis	23	38
Staphylococcus aureus	21	33
Proteus vulgaris	10	29
Klebsiella pneumoniae	12	26

are different from theirs. The difference in chemical composition may be attributed to a different geographical environment, growth and physiological development of the plant.

Antioxidant activity

Decrease in the absorbance of DPPH in the presence of antioxidants correlates with the free radical scavenging potential of the antioxidant. The scavenging activity might be due to the presence of different compounds. *Z. armatum* oil showed significant antioxidant activity with IC_{50} value of 27.0± 0.1 µg/ml while IC_{50} value for ascorbic acid was 15.0± 0.5 µg/ml. The results indicate that the antioxidant activity of the oil of *Z. armatum* is lower than that of ascorbic acid. The antioxidant effectiveness of the essential oil is probably due to a relatively high content of bornyl acetate, cymene, α-copaene, γ-terpinene, camphene, β-ocimene and linalool. *Z. leprieurii* and *Z. xanthoxyloides* are also exhibited antioxidant activity [15].

Antibacterial activity

The essential oil of *Z. armatum* exhibited moderate antibacterial activity against tested bacterial organisms as compared to the standard ciprofloxacin. The results were summarized in Table 3. Zone of inhibition (mm) are average of triplicate experiments. The highest zone of inhibition was observed against *Streptococcus faecalis* (23 mm) and lowest against *Proteus vulgaris* (10 mm). The study revealed that essential oil of *Z. armatum* is very effective against gram positive bacteria (*Streptococcus faecalis* and *Staphylococcus aureus*) and moderately effective against gram negative bacteria (*Klebsiella pneumoniae* and *Proteus vulgaris*) which may be because of their impenetrable wall. Rui-Xue Zhu et al. [31] reported that gram positive bacteria, *S. aureus* was the most sensitive (20 mm) while the gram negative bacteria, *E. coli* was the most resistant (5.4 mm) to the *Zanthoxylum bungeanum* oil (9 mg/ well). The fruits extract of *Z. armatum* has been tested for their antibacterial activity against *S. aureus*, *E. coli, Pseudomonas aeruginosa* and *Shigella boydii*. And observed strong antibacterial activity against gram positive and gram negative bacteria [32]. The essential oils of *Z. xanthoxyloides, Z. leprieurii*, *Z. chalybeum* and *Z. usambarense* have also been reported for their antibacterial activity against *E. coli*, *S. aureus* [33], *B. subtilis*, *Micrococcus luteus* and *S. aureus* [34]. The results of the present study are similar to previous reports, in which gram positive bacteria showed more sensitivity to the essential oil than gram negative bacteria [31,35]. The antibacterial activity of *Z. armatum* essential oil could be associated with its main constituents such as bornyl acetate, cymene, γ-terpinene, limonene, linalool, caryophyllene, α-terpinolene, α-terpineol and β-pinene, some of which are well known to possess significant antibacterial activity [36-39].

CONCLUSION

The concentration of plant active substance may vary according to collection period (daytime). GC-MS analysis of essential oil of *Z. armatum* leaf showed high concentration of bornyl acetate followed by cymene, α-copaene, γ-terpinene, camphene, limonene, linalool, β-ocimene, *trans*-caryophyllene, α-terpinolene and germacrene. The IC_{50} of the extracted oil was found to be 27.0 ± 0.1 µg/ml and that of ascorbic acid 15.0 ± 0.5 µg/ml. This investigation reveals that essential oil of *Z. armatum* is an excellent antioxidant and also exhibited moderate antibacterial activity. Its activities were quite comparable with the standards. Antioxidant and antibacterial activities of *Z. armatum* essential oil may be due to the presence of terpenoids. The study also reveals that the consumption of *Zanthoxylum* would exert several beneficial effects by virtue of their antioxidant and antibacterial activities.

ACKNOWLEDGEMENT

The authors are thankful to Prof. MSM Rawat, Dean School of Sciences, HNB Garhwal University, Uttarakhand, India for providing GC-MS facility and Dr. Sanjay, Department of Microbiology for providing bacterial strain.

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