



**ESSENTIAL OIL CONSTITUENTS AND ANTIMICROBIAL ACTIVITY OF THE SEEDS
OF ZANTHOXYLUM ARMATUM DC. FROM CHAMOLI, UTTARAKHAND**

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ABSTRACT

Zanthoxylum armatum DC. (Rutaceae) is a thorny shrub or small tree found in south eathern Asia. Its seeds are used to treat arthritis, asthma, blood diseases, bronchitis, bruises, cancer, cholera, diabetes, depression, digestive impairment, dyspepsia, earache, fever, fibrositis, headache, heart diseases, microbial infections, piles, roundworms, skin diseases, swellings, toothache and worms. The present study was carried out to analyse an essential oil from the seeds of *Z. armatum* and to evaluate its antimicrobial activity. The seeds were hydrodistilled to get the essential oil which was analysed by GC and GC-MS techniques. The oil was evaluated for antimicrobial activity by disc diffusion method. The seed essential oil was characterized by high percentage of linalool (86.93%) followed by limonene (1.74 %), β -caryophyllene (1.53%) and β -phellandrene (1.32%). Oxygenated monoterpenes (91.4%) comprised major profile of chromatogram of the essential oil including eight monoterpenic alcohols (89.66%), two ketones (0.80%) and one oxide (0.94%) whereas seven monoterpenic hydrocarbons occurred up to 4.64%. There were only two sesquiterpene hydrocarbons (1.91%) identified as β -caryophyllene and alloaramadendrene. Three esters, viz., (E)-methyl cinnamate, ethyl 9-hexadecenoate and ethyl palmitate were positively identified. The chloroform, acetone and ethanolic extract and the essential oil of the seeds showed significant antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* when compared with standard antibiotics by using cup and plate method. The essential oil mainly contained a large number of monoterpenes and exhibited significant antimicrobial activity against pathogenic microorganisms.

KEYWORDS: *Zanthoxylum alatum*, seeds, essential oil, GC-MS analysis, antimicrobial activity.

INTRODUCTION

Zanthoxylum armatum DC., syn. *Z. alatum* Roxb., *Z. arenosum* Reeder et S.Y. Cheo, *Z. planispinum* Siebold et Zucc. (Rutaceae), known as Nepal pepper, tejbal, Nepali dhaniya, timura and winged prickly ash, is an aromatic shrub native to North America and north eastern India, distributed from Kashmir to Bhutan, Nepal, Myanmar, China, Japan, Korea, Thailand, Vietnam, Malaysia, Indonesia and Philippines. It is a small thorny tree or shrub, up to 6 m high, with dense foliage, branches having straight prickles; leaves trifoliate, imparipinnate, pungent, aromatic, the leaf-stalk winged, leaflets stalkless, elliptic - ovate, dentate, base oblique; flowers minute, yellow, in leaf axils; fruits 1-3 celled, small, pale red, subglobose; seeds round, shining black.^[1]

The seeds are regarded as analgesic, anthelmintic, anti-inflammatory, antiseptic, cardio-protective, carminative,

deodorant, disinfectant, pesticide, stomachic, tonic and vermifuge; used to treat for abdominal colic, abscesses, arthritis, asthma, blood diseases, bronchitis, bruises, cancer, cholera, diabetes, cough, depression, diarrhea, digestive impairment, dyspepsia, earache, fever, fibrositis, flatulence, gastritis, headache, heart diseases, hiccups, indigestion, microbial infections, piles, roundworms, skin diseases, swellings, throat disorders, toothache, varicose veins and worms. The stem bark is anthelmintic, carminative, fish poison and stomachic, taken to relieve asthma, cholera, cough, difficult breathing, fever, dyspepsia, gum bleeding, rheumatism, sores, throat complaints, toothache, ulcer of the tongue and wounds. The leaves are carminative and fish poison, useful to cure arthritis, cholera, cuts, eczema, indigestion, joint pain, leucoderma, skin diseases, stomachache, vitiation of blood and wounds. A root paste is applied to subside boils. A root decoction is drunk as an anthelmintic. The fruits are regarded as an

anthelmintic, antiseptic, antispasmodic, carminative, deodorant, disinfectant, fish poison, fungistatic, insect and leech repellent, stomachic and mouth-freshener, used to cure cholera, cold, cough, abdominal pains, diarrhoea, dysentery, fever, tonsillitis and limbs numbness, skin diseases and toothache. The twig is utilized to clean the teeth. The seed essential oil possesses deodorant and antiseptic properties and is used for soap and dental preparations.^[1-7]

The fruits possessed 2 α -methyl-2 β -ethylene-3 β -isopropyl-cyclohexan-1 β , 3 α -diol, phenol-O- β -D-arabinopyranosyl-4'--(tetramethyl)-hexadecan-1''-oate, phenyl derivatives, linoleyl-O- α -D-xylopyranoside, palmitic acid, tambulin, prudomestin, obmuin and hexahydroxydiphenyl ether.^[8,9] The roots furnished lignans, magnoflorine, xanthoplanine, skimmianine, dictamine and gamma-fagarine.^[10] The seeds gave a fixed oil, flavonoids tambulin and tambulol.^[11] The stem produced lignans and phenolic glycosides.^[10,12] The bark and branches yielded sesamin, fargesin, eudesmin, lignans, pulvatiide, dictamine, 8-hydroxydictamine, armatamide, asarinin, flavonoidal glycoside, α - and β -amyryns, lupeol, β -sitosterol glucoside, γ -fagarine, magnoflorine and xanthoplanine.^[13-17] Leaves contained methyl-n-nonylketone, linalyl acetate, lignans, sesquiterpene hydrocarbons and tricosane.^[10,18] The seeds produced 3-methoxy-11-hydroxy-6,8-dimethylcarboxylate biphenyl, 3,5,6,7-tetrahydroxy-3',4'-dimethoxyflavone-5- β -D-xylopyranoside, two anthraquinones, two 2-hydroxybenzoic acids, hydroxyalk-(4Z)-enoic acids and stigmasta-5-en-3 β -D-glucopyranoside.^[19,20] The essential oils of the fruits, pericarp and seeds were consisted of linalool, linalyl acetate, bornyl acetate, cymene, citral, geraniol, α -copaene, methyl cinnamate, limonene and sabinene, γ -terpinene, camphene, 1,8-cineole, undecan-2-one and β -ocimene.^[21-30] The leaf essential oil was composed mainly of 2-undecanone, linalool, 2-tridecanene and 1,8-cineole.^[23,31, 32]

The leaf, fruit and stem bark extracts exhibited anti-inflammatory antifungal, antiaflatoxin, antimicrobial, antioxidant, cytotoxic, hepatoprotective, phytotoxic, insecticidal and antileishmanial activities.^[8,33-47] The methanol extracts of the bark and leaf induced hypolipidemic and hypoglycemic effects in mice.^[48] The seeds displayed anthelmintic, anticholinergic, antihistaminic and antiserotonergic activities.^[49,50] The lignans from the stem bark showed antifeedant potential against *Tribolium castaneum*.^[17] The seed essential oil exhibited antioxidant, antiseptic, disinfectant, insecticidal and deodorant properties.^[26, 28, 51,52] An aqueous extract of the seeds elicited anthelmintic activity against *Haemonchus contortus* of small ruminants.^[53] The leaf essential oil exerted anti-inflammatory, analgesic, antiasthmatic, larvicidal, cytotoxic, antinociceptive, anticonvulsant and phytotoxic effects.^[26,32, 54-56] Toxicity of the oil constituents and related compounds to *Stomoxys calcitrans* was studied.^[57] An *n*-hexane

fraction of *Z. armatum* showed larvicidal activity against *Plutella xylostella*.^[30] The essential oil and plant extracts were tested against *Spodoptera litura*.^[58] Aqueous extracts of the dried fruits of *Z. armatum* induced cellular and nuclear damage coupled with inhibition of mitotic activity *in vivo*.^[59] The extracts and saponins from fruit, bark and leaves of *Z. armatum* had the potential to exert its cytotoxic effect on cancer cell lines isolated from human by a mechanism involving apoptosis.^[46] The stem bark extract showed cytotoxicity and antioxidant activities.^[60]

Keeping in view the high reputation and application of *Zanthoxylum armatum* in the indigenous medicinal systems, it has been aimed to carry out GC-MS analysis of the isolated essential oil from the seeds and screening of antimicrobial activity of the seed essential oil of this plant collected from Chamoli, Uttarakhand.

MATERIALS AND METHODS

Collection of plant material

The seeds of *Z. alatum* were collected from district Chamoli (Uttarakhand) and identified by Dr. M. P. Sharma, Professor and Taxonomist, Department of Botany, Jamia Hamdard, New Delhi. A voucher specimen is deposited in the herbarium of the Department of Pharmacognosy and Phytochemistry, Jamia Hamdard, New Delhi.

Isolation of the essential oil

The air dried powdered seeds (500 g) were hydrodistilled in a Clevenger type glass apparatus for 4 hr according to the method recommended in the British Pharmacopoeia, 1988. The volume of the oil was collected in the graduated tube. The collected pale yellow essential oil was dried over an anhydrous sodium sulphate and stored at 4 °C in the dark. The yield was of the essential oil was 2.8% based on dry weight of the sample. This oil was used for GC and GC-MS analysis and for evaluation of antimicrobial activity.

GC Analysis

The gas chromatographic analysis of the essential oil was carried out on a GC-2010 (Shimadzu) equipped with a flame ionization detector (FID) and ULBON HR-1 fused silica capillary column (60 m \times 0.25 mm \times 0.25 μ m). The injector and detector (FID) temperatures were maintained at 250 and 270 °C, respectively. The carrier gas used was nitrogen at a flow rate of 1.21 ml/min with column pressure of 155.1 kPa. The sample (0.2 μ l) was injected into the column with a split ratio of 80:1. Component separation was achieved following a linear temperature programmed from 60 to 230 °C at a rate of 3 °C/min and then held at 230 °C for 9 min, with a total run time of 55.14 min. Percentage of the constituents were calculated by electronic integration of FID peak areas. Injection volume for all samples was 0.1 μ l.

GC-MS Analysis

The GC-MS analysis was carried out on a GC-MS-QP 2010 Plus (Shimadzu) fitted with a Column AB-Innowax (60 m × 0.25 mm i.d., film thickness 0.25 µm), The carrier gas was nitrogen at a flow rate 1.21 ml/min. The oven column temperature was initially kept at 60 °C for 10 min and increased up to 230 °C at a rate of 4 °C/min, then held at 230 °C for 10 min and increased up to 260 °C at a rate of 1 °C/min and then held at 260 °C for 10 min. The split flow was 10 ml/min. The split ratio was 1:80. The injector temperature was 240 °C and detector temperature was 280 °C. Injection volume was 0.3 µl. The ionization energy (voltage) was 70 eV and mass scan range (*m/z*) was 40-850 amu. The percentage composition of the oil was calculated automatically from the FID peak area without any correction.

Identification of components

The individual compounds were identified by comparing their Kovat's indices (KI) of the peaks on Innowax fused silica capillary column with literature values, matching against the standard library spectra, built up using pure substances and components of known essential oils. Further identification was carried out by comparison of fragmentation pattern of the mass spectra obtained by GC-MS analysis with those stored in the spectrometer database of NBS 54 K L, WILEY 8 libraries and published literature.^[61,62] Relative amounts of identical components were based on peak areas obtained without FID response factor correction. The retention indices were calculated for all compounds using a homologous series of *n*-alkanes under the same operational conditions of analysis.

Preparation of extracts

The air-dried powdered seeds (50 g) were defatted with petroleum ether (b. p. 40 – 60 °C) in a Soxhlet apparatus. The defatted seeds (40 g) were extracted successively and exhaustively with chloroform, acetone and ethanol (95%) in the Soxhlet apparatus. All the extracts were concentrated under reduced pressure to get brown solid masses 2.6, 1.9 and 7.4 g, respectively. The ethanol extract was dissolved in sterile water, while chloroform and acetone extracts were dissolved in sterile water along Tween 80 (0.5 ml), previously tested for antimicrobial activity and found negative against all test microorganisms. The solutions were further diluted to get test solutions of required concentrations.

Antimicrobial activity

Microbial strains

Pure cultures of pathogenic bacterial species *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* were obtained from the Microbiology Department of Majeedia Hospital, Jamia Hamdard, New Delhi, India. All the bacterial cultures were maintained on nutrient agar medium and fungal culture were maintained on potato dextrose media at 4 °C.

Standard antimicrobial substance

Antimicrobial activity of the seed extracts was assayed by agar cup and plate method. Streptomycin sulphate and tetracycline hydrochloride were used as standard and nutrient agar was employed as medium.

Methods of preparation of test organisms

The antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth dilution method.^[63] The test organisms were maintained on slants of nutrient agar medium (for bacterial culture) potato dextrose agar medium (for fungal culture) and transferred to a fresh slant once in a month. The slants were incubated at 37 °C for bacterial culture for 24 hours. The amount of suspension to be added to each 100 ml agar or nutrient broth was determined by use of test plates or test broth. The test organisms were stored under refrigeration.

Determination of Zone of Inhibition (ZOI)

The antibacterial activity was assessed by agar well diffusion method. Muller Hinton agar medium was prepared by using 15 g agar dissolved in 1L distilled water. A previously liquefied and sterilized nutrient agar /potato dextrose agar medium (20 ml) was poured into petri-plates of 100 mm size (to make uniform thickness) and allowed to cool to 45°C to solidify. Microbial suspensions were spread over the solidified media. Holes were made in each plate with a stainless steel borer having 6 mm ID. Streptomycin sulphate and tetracycline solutions were used as standards. All dilutions were made in DMSO solvent. The freshly prepared inoculums were swabbed all over the surface of the MHA plate using sterile cotton swab. Wells of 8 mm diameter were made in the agar with a sterile cork borer. Hundred micro-liters of the working suspension/solution of different plant extracts were loaded in each well and same volume of extraction solvent for control was filled in the wells with the help of micropipette. Plates were left for some time till the extracts diffused in the medium with the lid closed and incubated at 37 °C. The zones of inhibition of microbial growths were measured after incubation for 24 hours. Each experiment was carried out in three replicates and the mean diameter of inhibition zone was recorded using a ruler and the results were studied.

RESULTS AND DISCUSSION

The chemical constituents of the seed essential oil were identified by analysis of GC and GC-MS and are tabulated in Table 1 with their Kovat's indices and respective percentage area. The essential oil was characterized by high percentage of linalool (86.93%) followed by limonene (1.74 %), β-caryophyllene (1.53%) and β-phellandrene (1.32%). Among 24 components comprising 99.36 % of the total oil, there were 19 monoterpenes (96.47) in all and two sesquiterpenes. Three esters, viz., (E)-methyl cinnamate (0.43%), ethyl 9-hexadecenoate (0.41 %) and ethyl palmitate (0.57 %) were positively identified. There were sixteen

monoterpenes occurring in trace amounts (0.15 – 0.94 %) and in addition of linalool, the predominant monoterpenes were limonene and β -phellandrene. Oxygenated monoterpenes (91.4%) comprised major profile of chromatogram of essential oil of *Z. armatum*, including eight monoterpene alcohols (89.66%), two ketones (0.80%) and one oxide (0.94%) whereas seven monoterpene hydrocarbons occurred up to 4.64 %. There were only two sesquiterpene hydrocarbons (1.91%) identified as β -caryophyllene (1.53 %) and alloaramadendrene (0.38 %).

The seeds essential oil from northern India was consisted mainly of linalool (71%), limonene (8.2%), β -phellandrene and (Z)-methyl cinnamate.^[25] The leaf essential oil of *Z. alatum* grown in north-western

Himalaya contained prominently linalool (30.58%), 2-decanone (20.85%), β -fenchol (9.43%), 2-tridecanone, β -phellandrene, sabinene and α -pinene.^[64] The major compounds found in the leaf essential oils from Kumaon were non-terpenic acyclic ketones, notably 2-undecanone and 2-tridecanone, in addition to 1,8-cineole, linalool, terpinen-4-ol, α -terpineol, *trans*-caryophyllene, α -humulene and germacrene.^[65] The seed essential oil from Balakot Mansehra (N.W.F.P., Pakistan) was consisted mainly of 3-borneol (9.57%), isobornyl acetate (9.57%), dihydrocarveol (8.81%), palmitic acid, 15-hexadecanolide, β -elemene, iso-thujanol and *trans*-caryophyllene; and no limonene was found in the essential oil.^[27]

Table 1: Chemical composition of the essential oil of the seeds of *Zanthoxylum alatum*.

S.No	Components	Kovats retention index	Percentage Area
1	α -Thujene	923	0.26
2	p-Myrcene	991	0.40
3	δ -Carene	1012	0.32
4	p-cymene	1027	0.45
5	Limonene	1029	1.74
6	β -Phellandrene	1031	1.32
7	1,8-Cineole	1039	0.27
8	<i>cis</i> -Ocimene	1043	0.15
9	(Z)- Linalool oxide	1074	0.94
10	Linalool	1098	86.93
11	α -Fenchol	1101	0.64
12	Camphor	1143	0.34
13	Terpinen-4-ol	1178	0.61
14	α -Terpineol	1185	0.29
15	(E)-Carveol	1217	0.26
16	Nerol	1228	0.35
17	Piperione	1232	0.28
18	Carvone	1243	0.18
19	Geraniol	1255	0.31
20	(E)-Methyl cinnamate	1379	0.43
21	β - Caryophyllene	1418	1.53
22	Alloaramadendrene	1461	0.38
23	Ethyl 9-hexadecenoate	1955	0.41
24	Ethyl palmitate	1983	0.57

The results of antimicrobial activity are summarized in Table 2. The table shows the diameter of zone of inhibition (mm) of test drugs and standard antibiotics against microorganisms. The plant extracts showed inhibitory action against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. Among all the pathogens, *B. subtilis* was inhibited by all type of the seed extracts. The acetone and ethanol (95%) extracts exhibited high antimicrobial activity against all the test

microorganisms. These extracts displayed significant inhibition of *S. aureus*, *B. subtilis*, and *E. coli* while chloroform extract showed activity only against *B. subtilis*. The degree of growth inhibition ranged from 12 to 15 mm against test microorganisms and was comparable with tetracycline hydrochloride and streptomycin sulphate as the standard antibiotics employed.

Table 2: Antimicrobial activity of the seeds extracts of *Zanthoxylum alatum*.

Microorganism	Diameter of zone of inhibition (mm)				
	Seed extracts			Standard antibiotics	
	Chloroform extract	Acetone extract	Ethanol extract	Streptomycin sulphate	Tetracycline
<i>Staphylococcus aureus</i>	--	12	13	14	25
<i>Bacillus Subtilis</i>	12	13	12	13	23
<i>Escherichia coli</i>	--	14	14	12	19

Conc: 1000 Mcg/ml (Tween 80 showed no activity against any of the organisms)

The agar cup and plate method results of the essential oil of *Z. alatum* are presented in Table 3. The oil showed the highest activity especially against *Bacillus subtilis* having 8.9 mm inhibition of zones at 0.1 % and the activity increased when the concentration of the oil was

1.0 %. *Escherichia coli* exhibited low inhibition of diameters of zones at all levels of the essential oil. The results were compared with the standard samples of chloramphenicol and tetracycline.

Table 3: Antimicrobial activity of the essential oil of the seeds of *Zanthoxylum alatum*.

Microorganism	Conc. of essential oil			Standard Chloramphenicol (0.1 mg/ml)	Standard Tetracycline (0.1 mg/ml)
	0.1 % v/v	0.5 % v/v	1.0 % v/v		
<i>Staphylococcus Aureus</i>	6.4	9.5	11.2	17.5	19.3
<i>Bacillus subtilis</i>	8.9	11.3	14.1	18.7	22.8
<i>Escherichia coli</i>	5.88.9	8.8	10.8	16.6	18.5

CONCLUSIONS

The seed essential oil of *Zanthoxylum alatum* was composed mainly of linalool (86.93%), limonene (1.74 %), β -caryophyllene (1.53%) and β -phellandrene (1.32%). Oxygenated monoterpenes (91.4%) comprised major profile of chromatogram of the essential oil including eight monoterpenic alcohols (89.66%), two ketones (0.80%) and one oxide (0.94%). The chloroform, acetone and ethanolic extracts and the essential oils of the seeds exhibited significant antimicrobial activity against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* when compared with standard antibiotics by using agar cup and plate method.

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