



ISSN: 2455-0485

Biological activities and Phytochemical analysis of *Zanthoxylum armatum* DC. leaves and bark extracts collected from Kumaun region, Uttarakhand, India

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ABSTRACT

The methanolic and chloroform extracts of leaves and bark of *Zanthoxylum armatum* DC. were evaluated for their phytochemical analysis and biological activities. In phytochemical analysis, fargesin was identified as major constituent in leaves methanolic and leaves chloroform extracts. t-butylamine and benzoxazole, 2-(isobutylamino) were identified as the major constituents in bark methanolic and bark chloroform extracts respectively. Both the extracts exhibited moderate antioxidant activity with IC₅₀ values ranging from 19.42±0.07 to 78.01±0.31 µg. These extracts also possess moderate anti-inflammatory activity with IB₅₀ values ranging from 28.53 ± 0.06 to 89.80 ± 0.05 µg. Moderate anti-bacterial activity against *E. coli* and *S. aureus* has also been observed in both the extracts. The total flavonoids, orthodihydric phenols and phenolic contents were also quantified in the extracts. Based on these observations, it can be concluded that *Zanthoxylum armatum* DC. may be used as herbal antioxidant, food preservative, natural anti-inflammatory drug and natural bactericidal, besides generation of data base for judicious exploitation in future.

KEYWORDS: *Zanthoxylum armatum*, antioxidant activity, anti-inflammatory activity, anti-bacterial activity, total phenolics, total flavonoids, orthodihydric phenols

Received: August 10, 2019
Accepted: December 12, 2019
Published: January 12, 2020

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INTRODUCTION

From the beginning of human civilization, natural products, including terrestrial plants, marine organisms, animal products, and products produced by micro-organism have been used in traditional medicines. The use of plants based natural products as medicines could be traced as far back as the beginning of human civilization and dominated the human pharmacopoeia for thousands of years [1]. Bioactive compounds isolated from raw plant material have proven to be valuable sources of metabolites which can hardly be obtained from other sources [2]. The most bioactive constituents are mainly derived from plants includes tannin, alkaloids, saponins, terpenoids, flavonoids and phenolic compounds, which form the backbone of the drugs.

Rutaceae is one of the major Angiosperm families with 153 genera and 1975 species distributed worldwide and major

species rich genera are *Zanthoxylum* L., *Melicope* Forst. & Forst.f., *Boronia* Sm., *Glycosmis* Corr. Serr., *Haplophyllum* A.Juss., *Vepris* Com. ex A.Juss., *Zieria* Sm., *Agathosma* Willd., *Citrus* L., *Ruta* L. and *Murraya* Koenig. The members are trees, shrubs, lianas, or rarely herbs, often with spines or prickle and with secretory glands containing ethereal oils in many tissues and appearing as pellucid-punctate glands in the leaves and pericarp [3]. The genus *Zanthoxylum* L. with 225 tropical species is a rich source of various phytochemicals such as amides, alkaloids, flavanoides, lignans, sterols and terpenes, etc. Many species of genus *Zanthoxylum* (*Z. acanthopodium* DC., *Z. americanum* Mill., *Z. armatum* DC., *Z. bungeanum* Maxim., *Z. beecheyanum* K.Koch, *Z. capense* (Thunb.) Harv., *Z. caribaeum* Lam., *Z. clava-herculis* L., *Z. flavum* Vahl, *Z. gillettii* (de Wild) Waterm., *Z. piperitum* (L.) DC., *Z. rhetsa* (Roxb.) DC., *Z. simulans* Hance, *Z. xanthoxyloides* (Lam.) Zepernick & Tilmer etc.) are of economic importance as source of edible fruits, essential oils, wood, ornamentals, raw materials

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for industries, medicinal plants and culinary applications. Almost all the species of genus *Zanthoxylum* have great ability to produce tires which could be used as encapsulates in the pharmaceutical industry, diluents and emulsifying agents [4,5,6,7,8]. *Zanthoxylum armatum* DC., commonly called as 'Prickly ash' or 'Timur' or 'Kababe Tejal', is a shrub or small tree which predominately grows in well drained alluvial, black soil and have a strong aroma. The plants are armed scandent or erect, 6 m tall or more, with dense foliage [9]. In India, it is found in the warmer valleys of the Himalaya from Jammu and Kashmir to Assam and Khasi (1,000 to 2,100 m), in the Eastern Ghats in Orissa and Andhra Pradesh (1,200 m) and the lesser Himalayan regions in the northeastern part of India for example, Naga Hills, Meghalaya, Mizoram, and Manipur [10,11,12,13]. Essential oils and different extracts of aqueous ethanol, dichloromethane, acetone, methanol, petroleum ether have been reported to shows many biological activities viz; larvicidal, antiviral, antifungal, keratolytic, anti-protozoan, pesticidal/insecticidal, hepatoprotective, antibacterial, antihelminthic and allelopathic [14,15]. The fruits and seeds are extensively used as tonic in fever, dyspepsia and cholera, eliminate pain, used to treat heart diseases, piles, diseases of mouth, teeth and throat disorder and the bark is used for intoxicating fishes [16]. We already have reported the chemical composition and biological activities of seeds, bark and leaves essential oil of *Zanthoxylum armatum* DC. collected from different altitudes of Kumaun region, Uttarakhand [17,18,19]. The present study assesses the chemical composition and biological activities of methanolic and chloroform extracts of *Zanthoxylum armatum* DC. leaves and bark collected from Kumaun region of Uttarakhand (India).

MATERIALS AND METHODS

Collection of Plant Material

The plants material was collected from Aadi Kailash region (Bhimtal) (1370 m elevation), Nainital, Uttarakhand in the month of July, 2017. The plant material was properly identified by one of the author and Plant Taxonomist (DSR) and the identity was further confirmed by comparing the specimens with authentically identified specimens at the herbarium of Botanical Survey of India, Dehradun, India (BSD). The voucher specimen (GBPUH-917/28.5.2018) was deposited to the Herbarium of Department of Biological Sciences, C.B.S.&H., G.B.P.U.A. & T. Pantnagar for future reference.

Preparation of Extracts

The bark and fresh leaves of *Z. armatum* were finely chopped, and shade dried and grinded. The resultant powdered material (250g) was subjected for extraction by cold percolation method in organic solvents with varying polarity. The solvents from extracts were evaporated by using rotatory vacuum evaporator and the final yield of the extracts was weighed. The yields in percentage (w/w) obtained were 2.4% leaves methanol extract, 4.8% bark methanol extract, 7.2% leaves chloroform extract and 4.4% bark chloroform extract.

GC-MS Analysis

GC/MS analysis of the different extracts was performed using a GC MS-QP 2010. The GC capillary column DB-5 (30 m × 0.25 mm i.d.; 0.25 μm film thickness; J&W Scientific, Agilent, Santa Clara, CA, USA) was used. Helium was used as a carrier gas with a flow rate of 1.21 mL/min, at a pressure of 73.3 kPa. The extracts were injected at temperature: 260 °C with oven temperature programme as: Initial temperature 60°C, RAMP 3°C/min upto 210°C (isotherm for 2 min), then RAMP 6°C/min upto 280°C (isotherm for 2 min), finally hold for 11 min. The compounds were identified with the help of NIST-MS, FFNSC Wiley Library, and comparing the data with literature reports and retention indices (RI) [20].

Antioxidant Activity

DPPH (1, 1-diphenyl -2-picryl -hydrazyl) radical scavenging activity

This activity was evaluated according to the developed protocols with slight modifications [21,22,23]. The tested extract samples (50-250 μg/mL) were taken and mixed with 5 mL of a 0.004% methanolic solution of freshly prepared DPPH. The O.D. (optical density/optical absorbance) was measured by using UV-visible spectrophotometer (Thermo Scientific EVOLUTION-201 series) at 517 nm. All the observations were recorded in triplicate with reference to the standard antioxidants catechin and BHT. Inhibition of free radical in percent (IC%) was calculated by using the equation: $IC\% = (A_0 - A_t)/A_0 \times 100$ where, A_0 = absorbance value of control sample, A_t = absorbance value of test sample, IC = inhibitory concentration. Percent inhibition was plotted against concentrations in graph. The standard curve was drawn using standard antioxidant (BHT and catechin) to calculate the IC₅₀ values for different extracts and standard.

Reducing power

The reducing power of extracts was evaluated by the method developed earlier and is being practiced [24]. Various amount of extracts (50-250 μg/mL) were mixed with 2.5 mL of phosphate buffer (pH= 6.6, 200 mM,) and 2.5 mL of 1% potassium ferricyanide, K₃[Fe(CN)₆]. After 20 minute incubation at 50±1°C, 2.5 mL of trichloroacetic acid was added to the mixtures, followed by centrifugation at 650 rpm for 10 min. The supernatant (1 mL) was mixed with 5mL distilled water followed by 1 mL of 0.1% ferric chloride. The absorbance of the resultant solution was measured spectrophotometrically at 700 nm. All the readings were recorded in triplicate. Ascorbic acid was used as standard. The percent reducing power of samples was calculated using the formula: Reducing power % = $(A_0 - A_t)/A_0 \times 100$ where, A_0 = absorbance value of control sample, A_t = absorbance value of test sample. Percent inhibition was plotted against concentrations in the graph. The standard curve was drawn using standard antioxidant (BHT) to evaluate the RP₅₀ values for standard and different extracts.

Metal chelating activity

The chelation of Fe+2 was evaluated using the method developed earlier [24]. 0.1 mL of 2mM FeCl₂.4H₂O, 0.2mL of 5mM ferrozine and 4.7 mL of methanol was added to various concentrations of test sample (50-250 µg/mL). The solutions were mixed thoroughly and incubated for 10 min. At 562 nm, the absorbance of test sample was measured in a UV spectrophotometer (Thermo Scientific EVOLUTION 201 series). All the readings were recorded in triplicate; EDTA (0.01 mM) was used as the standard. The metal-chelating activity of tested samples, expressed as percentage was calculated by using the formula: $IC\% = (A_0 - A_t)/A_0 \times 100$ where, A_0 = absorbance value of control sample, A_t = absorbance value of test sample, IC = inhibitory concentration. The percent of chelating ability was plotted against concentrations in graph. The standard curve was drawn using standard antioxidant (EDTA) to calculate the IC₅₀ values for standard and different extracts.

Estimation of Phenols

The phenolic assay of both the extracts of *Z. armatum* was calculated quantitatively by spectrophotometer in terms of total phenols, flavonoids, and orthodihydroxy phenols. The concentrations of these samples were measured with the help of working calibration curve by the relation among concentration and absorbance of the sample.

Total phenolic assay

The total phenols were determined by the Folin-Ciocalteu reagent (FCR) method [25]. In brief, 1 mL of the sample extract was poured into a test tube and mixed with 1 mL of 80% methanol and 8 mL of distilled water. In each sample 0.5 mL of 1 N FCR was added and mixed. After 5 min., 1 mL of saturated Na₂CO₃ was added to the reaction mixture and allowed to stand for 60 min. The absorbance of test sample was observed at 650 nm. The standard curve was drawn using various concentrations of gallic acid and results were expressed as mg of gallic acid per gram of sample in dried weight.

Estimation of flavanols

Aluminum chloride colorimetric assay [26] was applied for the estimation of flavanols. 10 mg of extract were dissolved in 10 mL of 80% methanol to prepare stock solution. In a test tube, 0.1 mL of stock solution was mixed with 1.25 mL water and 0.75 mL of 5% NaNO₂. The mixture was incubated for 5 min. 0.15 mL of 10% AlCl₃ was added to the mixtures after incubation. After 6 min, 275 µL of distilled water and 0.5 mL of 1 N NaOH were added, after thoroughly mixing of the solution. At 510 nm, the intensity of pink colour was obtained. The standard curve was established using different concentrations of catechin and the concentrations were evaluated with the help of calibration curve and expressed in mg/100gm of dry material [27].

Estimation of orthodihydric phenols (OHP)

10 mg of extract was added in 10 mL of 80% methanol to prepare stock solution. 0.1 mL of the extract solution was poured in a test tube and mixed with 0.4 mL of water and 1 mL of 0.05N HCl, 1 mL of Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate made up to 100 ml with distilled water), 10 mL of water and 2 mL of 1 N NaOH. The resultant solutions were thoroughly mixed (appearance of pink colour) and at 515 nm absorbance was measured. The standard curve was prepared with the help of working standard catechol solution at different concentrations. The concentration was calculated and expressed in mg per 100gm of material [28].

In-vitro Anti-inflammatory Activity

The *in-vitro* anti-inflammatory activity of extracts was evaluated by using inhibition of albumin denaturation technique, by the standard protocols [29,30,31]. The reaction mixture (5 mL) was comprised of 0.2 mL of egg albumin, 2.8 mL of phosphate buffer solution (pH= 6.4) and 2 mL of varying amount of extracts (25 µg/mL, 50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL, 250 µg/mL and 500 µg/mL). Double distilled water was used as control. At 37±2°C, the mixtures were incubated in a BOD incubator (for 15 min and then heated for 5 min at 70°C in water bath). Subsequent to cooling, the absorbance was observed at 660nm. All the readings were observed in triplicate, diclofenac sodium was used as the standard. The percentage inhibition of protein denaturation was calculated using the formula: $IB\% = (A_0 - A_t)/A_0 \times 100$ where, A_0 = absorbance value of control sample, A_t = absorbance value of test sample, IB = inhibitory concentration. The extract/drug concentration for 50% inhibition (IB₅₀) was determined by plotting percentage inhibition with respect to control against treatment concentration.

Antibacterial Activity

The antibacterial activity was determined by using Agar well diffusion method [32,33]. It was expressed as the mean of zone of inhibition (ZOI) diameters (mm) produced by the various extracts. For screening plates were prepared by using nutrient agar. The inoculums (50µL) of different bacterial strains were spread evenly on respective plates with sterile spreader. A borer (8mm diameter) was used to cut well. 20µL of different concentrations of the extracts were poured in each well and incubated at 24 hrs at 37±2°C. The diameter of ZOI was measured and the mean was recorded. The experiment was performed in triplicate.

Statistical Analysis

The data were analyzed by using Analysis of Variance (ANOVA) using STPR. All the values were taken in triplicate. IC₅₀ was determined by linear regression analysis using MS Excel 2007.

RESULTS AND DISCUSSION

Phytochemical Studies

In ZNLME (*Zanthoxylum armatum* leaves methanolic extract), 78 constituents were identified which contributed 79.4% of the total extract. Fargsin (21.9%) was detected as the major constituent. The other identified constituents were (+)-eudesmin (15.4%), (+)-sesamin (6.9%), linolenic acid (3.6%), methanol (trimethylsilyl), acetate (3.3%), methylvanillin (2.9%), 5,8,8-trimethyl-3-oxatricyclo [5.1.0.0~2,4~] octane (2.8%) and marmesin (1.9%). Sixty two constituents were identified in ZNLCIE (*Zanthoxylum armatum* leaves chloroform extract), which contributed 82.2% of the total extract. Similar to ZNLME, ZNLCIE (*Zanthoxylum armatum* leaves chloroform extract) was also dominated by fargsin (18.9%). The other identified constituents were γ -sitosterol (12.8%), *cis*-5,8,11-eicosatrienoic acid, trimethylsilyl ester (6.4%), *D*-(+)-sesamin (6.2%), palmitic acid, trimethylsilyl ester (6.1%), stigmast-5-ene, 3- β - (trimethylsilyloxy)-(24*S*)- (4.2%), linoleic acid, TMS (3.5%) and phytol, TMS derivative (2.8%). ZNBME (*Zanthoxylum armatum* bark methanolic extract) revealed the presence of 56 constituents contributing 92.2% of the total extract. In identified compounds *t*-butylamine (23.1%) was the major constituent. The other

constituents were 1-[(trimethylsilyl) oxy] propan-2-ol (7.5%), propylene glycol, 2-TMS derivative (5.8%), glycerol, 1-*tert*-butyl 3-trimethylsilyl ether (5.5%), doxepin (4.9%), acetin, bis-1,3-trimethylsilyl ether (3.8%), laudanosine (3.7%) and 2-methyl-1,2-butanediol 2-TMS (3.1%). Similarly in ZNBCIE (*Zanthoxylum armatum* bark chloroform extract), 82 constituents were identified which contributed 93.0% of the total extract. Among all the constituents benzoxazole, 2-(isobutylamino) (42.7%) was the major constituent. The other identified constituents were (*Z,Z*)-6,9-*cis*-3,4-epoxy-nonadecadiene (22.0%), (+)-eudesmin (5.4%), thujaplicatin, tri-*O*-methyl (4.1%), (+)-sesamin (3.7%), [(2*E*)-3,7-dimethyl-2,6-octadienyl] benzene (3.5%) and 1,3,14,16-nonadecatetraene (3.1%).

The literature search reveals no report on GC/MS analysis of chloroform and methanolic extracts of *Z. armatum* leaves and bark (ZNLCIE, ZNLME, ZNBME and ZNBCIE) hence is being reported first time. The major fargsin detected in leaves has also been reported in the leaves essential oil of *Z. acanthopodium* from north-eastern region of India [34]. Hence, the present analysis reveals the first report on it. The study on comparative chemical composition among ZNLME, ZNBME, ZNLCIE and ZNBCIE has been represented in Table 1 and structures of major chemical constituents in extracts of *Z. armatum* has been illustrated in Figure 1.

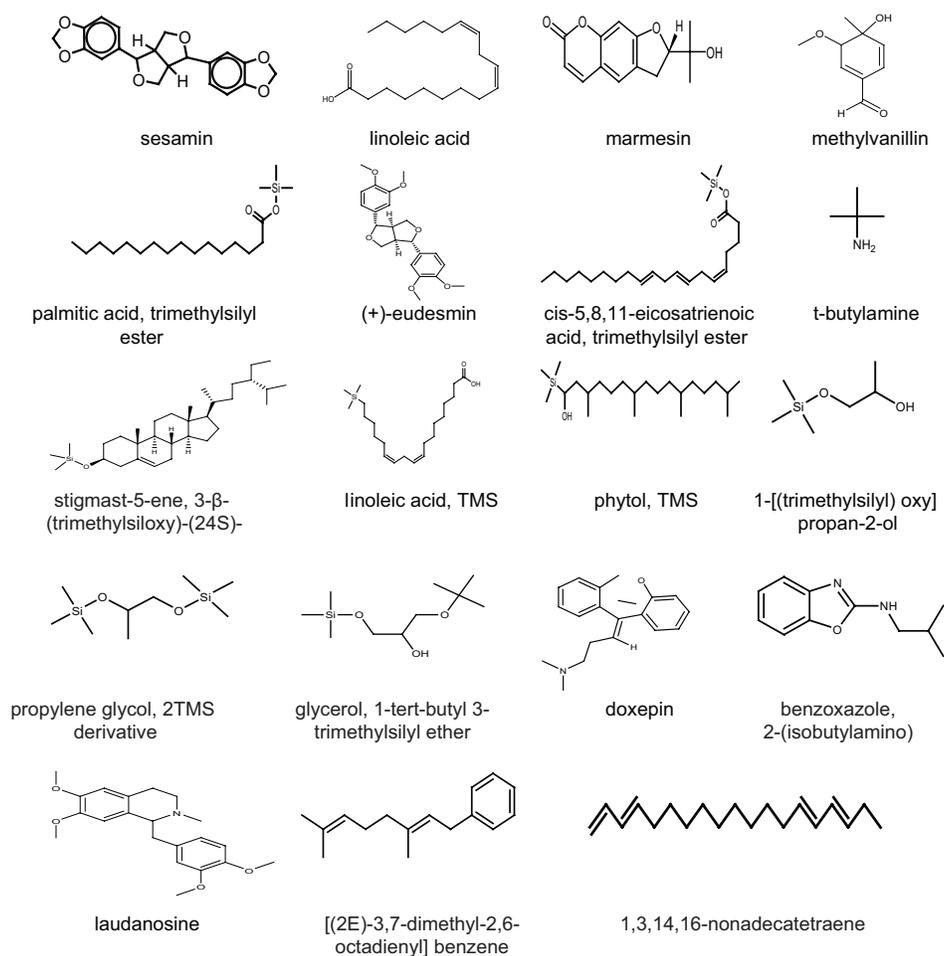


Figure 1: Structures of major chemical constituents in extracts of *Z. armatum*

Table 1: Comparative study of GC-MS analysis of *Z. armatum* leaves and bark methanolic and chloroform extract

S.N.	Constituents	K.I.	% Contribution			
			Leaves		Bark	
			ZNLCIE	ZNLME	ZNBCIE	ZNBME
1.	methanol, (trimethylsilyl)-, acetate	695	-	3.3	-	-
2.	trimethylsilyl methacrylate	762	0.9	-	-	-
3.	1-[(trimethylsilyloxy) propan-2-ol	766	-	-	-	7.5
4.	propylene glycol, 2TMS derivative	824	-	-	-	5.8
5.	trimethylsilyl 2,2-dimethyl- 3,6,9,12-tetraoxa-2-silatetradecan-14-oate	851	-	-	-	0.6
6.	L-(+)-lactic acid	857	-	-	-	0.9
7.	1,3-diisopropoxy-1,3 dimethyl-1,3-disilacyclo-butane	860	-	t	-	0.5
8.	caproic acid, trimethylsilyl ester	993	-	-	-	0.6
9.	2-furancarboxylic acid, trimethylsilyl ester	1018	-	-	-	1.9
10.	isovaleric acid, 2-hydroxy, di-TMS	1050	t	-	-	0.7
11.	2-hydroxyisocaproic acid, trimethylsilyl ester	1091	-	-	-	2.4
12.	glycerol, 1-tert-butyl 3-trimethylsilyl ether	1108	-	-	-	5.5
13.	1,2,3-butanetriol, tris-o- (trimethylsilyl)	1143	-	-	-	0.9
14.	α -(trimethylsilyloxy) styrene	1144	-	-	-	1.1
15.	butane, 1,2,3-tris (trimethylsilyloxy)	1147	-	-	-	1.1
16.	butanenitrile, 4,4-diethoxy	1149	-	0.6	-	-
17.	4-ethyl-2-methoxyanisole	1271	1.3	-	-	-
18.	syringol	1279	-	t	t	0.5
19.	ethanesulfonic acid, 2-[(trimethylsilyl oxy)-, trimethylsilyl ester	1295	-	-	-	0.6
20.	5-trimethylsilyloxymethyl-2-trimethylsilylfuroate	1392	-	-	-	0.7
21.	2-deoxy ribose O,O',O''-tris (trimethylsilyl)	1431	-	-	-	1.2
22.	benzoxazole, 2-(isobutyl-amino)	1578	-	-	42.7	-
23.	[(2E)-3,7-dimethyl-2,6-octadienyl] benzene	1658	-	-	3.5	-
24.	bis (trimethylsilyl) derivative of mephenesin	1686	-	-	-	1.4
25.	4- β -H,5- α -eremophil-1(10)-ene, 11 (trimethyl-siloxy)	1706	-	t	-	0.9
26.	isopsoralen	1711	-	0.5	-	-
27.	5-allyl-1-methoxy-2,3-dihydroxybenzene, di(trimethylsilyl) ether	1767	-	-	-	0.6
28.	2-propanone,1-hydroxy -3-(4-hydroxy-3-methoxy-phenyl)	1781	-	-	-	1.1
29.	1-(4-methoxyphenyl)-1,5-pentanediol	1785	-	-	-	0.5
30.	2,2-dimethyl-5- [2- (2-trimethylsilyl-ethoxy-methoxy)-propyl]-[1,3] dioxolane-4-carboxaldehyde	1816	-	-	-	1.6
31.	1,3,14,16-nonadecatetraene	1924	-	-	3.1	-
32.	palmitic acid	1968	-	-	0.5	t
33.	palmitic acid, trimethylsilyl ester	1987	6.1	t	-	-
34.	phytol	2045	-	0.5	-	-
35.	phytol, TMS derivative	2086	2.8	1.0	-	-
36.	9,12-octadecenoic acid, methyl ester	2093	-	0.9	-	-
37.	linolenic acid, methyl ester	2101	-	2.9	-	-
38.	2-carboxymethyl-3-n-hexylmaleic acid anhydride	2110	-	-	-	0.6
39.	linoleic acid	2183	3.5	-	-	-
40.	stearic acid, trimethylsilyl ester	2186	1.3	-	-	-
41.	linolenic acid	2191	-	3.6	-	-
42.	doxepin	2254	-	-	-	4.9
43.	cis-5,8,11-eicosatrienoic acid, trimethylsilyl ester	2409	6.4	-	-	-
44.	eudesmin	2731	12.8	15.4	5.4	-
45.	squalene	2914	1.6	-	-	-
46.	yangambin	3243	-	-	2.4	-
47.	thiazolidin-4-one, 3-[2-(3,4-dimethoxyphenyl) ethyl]-2-(2,6-dimethylphenylimino)	3304	-	-	0.7	-
48.	4-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-purin-7-ylmethyl)-dibenzo-18-crown-6	4774	-	-	-	0.5
49.	5-(2-hydroxyethylamino)-9-methyl-N,N'-diphenyl-6,7,8,9-tetrahydro-6,8a-ethano-8ah-carbazole-7,8,10,11-tetracarboxylic 7,8:10,11-diimide	4869	-	-	-	3.2
50.	neophytadiene	-	1.3	-	-	-
51.	2-methyl-1,2-butanediol 2 TMS	-	-	-	-	3.1
52.	cyclopenten, 3,3-dimethyl-4-methylen-1,2-bis (trimethyl silyloxymethyl)	-	-	-	-	0.7
53.	benzeneacetic acid, 4-trimethylsilyloxy, trimethylsilyl ester	-	-	-	-	1.2
54.	9-cis-octadecenoic acid	-	-	2.0	-	-
55.	sesamin	-	6.2	6.9	3.7	-
56.	thujaplicatin, tri-O-methyl	-	t	0.6	4.1	-
57.	5,8,8-trimethyl-3-oxatricyclo [5.1.0.0~2,4~] octane	-	-	2.8	-	-
58.	(Z,Z)-6,9-cis-3,4-epoxy-nonadecadiene	-	-	-	22.0	-
59.	marmesin	-	0.7	1.9	-	-
60.	cholest-5-en-3-yl	-	-	-	-	0.5
61.	retinylidene malodinitrile	-	-	-	1.0	-
62.	5-(propenyl-2)-1,3,7-nonatriene	-	-	-	1.0	-

(Contd...)

Table 1: (Continued)

S.N.	Constituents	K.I.	% Contribution			
			Leaves		Bark	
			ZNLCIE	ZNLME	ZNBCIE	ZNBME
63.	acetamide, N-(2-piperidin-4-ylethyl)	-	-	-	-	0.7
64.	N-[(dimethylammonio) methyl] trifluoromethane-sulfoSnamidate	-	-	-	-	0.9
65.	laudanoline	-	-	-	-	3.7
66.	(E)-gingerenone A	-	-	-	-	0.6
67.	t-butylamine	-	-	-	-	23.1
68.	oxetane	-	-	-	-	1.4
69.	tricyclo [4.3.1.1 2,5] undecan-10-ol	-	0.8	-	-	-
70.	hydroquinone, trimethyl	-	-	1.3	-	-
71.	methylvanillin	-	-	2.9	-	-
72.	1,4-benzenediol, 2-(1,1,3,3-tetramethylbutyl)	-	0.5	-	-	-
73.	bis (2-ethylhexy) phthalate	-	0.6	-	-	-
74.	fargisin	-	18.9	21.9	-	-
75.	N-(2-adamantan-1-yl-ethyl)-N-[1-(2-methoxy-phenyl)-2,5-dioxo-pyrrolidin- 3-yl]-acetamide	-	-	0.5	-	-
76.	γ -sitosterol	-	1.4	-	-	-
77.	stigmast-5-ene, 3- β - (trimethylsiloxy)	-	4.2	-	-	-
78.	(13R)-8 β ,13:13, 20-diepoxy-5 β , 9 β , 10 α -14,15-dinorlabdan	-	1.0	-	-	-
79.	stigmast-4-en-3-one	-	1.1	-	-	-
	TOTAL		82.2	79.4	93.0	92.2

(where t = trace compounds which is less than 0.5), ZNLME = *Zanthoxylum armatum* leaf methanolic extract, ZNBME = *Zanthoxylum armatum* bark methanolic extract, ZNLCIE = *Zanthoxylum armatum* leaf chloroform extract and ZNBCIE = *Zanthoxylum armatum* bark chloroform extract

Biochemical Assay

Total phenols

The total phenolics content in different extracts of *Zanthoxylum armatum* DC. were observed in the order of: ZNLME (31.23 \pm 0.03 mg/g GAE) > ZNBME (27.58 \pm 0.05 mg/g GAE) > ZNBCIE (25.02 \pm 0.04 mg/g GAE) > ZNLCIE (17.74 \pm 0.02 mg/g GAE). The variation in total phenolic content in different extracts might be because of different solubility of phenolics in organic solvents with varying polarity. Among different plant extracts from different parts of plant, it has been observed that ZNLME contained maximum total phenolic content (31.23 \pm 0.03 mg/g GAE) while ZNLCIE showed minimum among the extracts (17.74 \pm 0.02 mg/g GAE) (Table 2).

Total flavonoids

Flavonoids are one of the most important groups of bioactive secondary metabolites in plants and are known for their health promoting properties due to protective effects against cancer, cardiovascular disease and other diseases [35]. In the present study, the total flavonoids content in different extracts were observed in the order of: ZNLCIE (77.18 \pm 0.06 mg/g CNE) > ZNBME (68.04 \pm 0.06 mg/g CNE) > ZNBCIE (56.72 \pm 0.03 mg/g CNE) > ZNLME (47.63 \pm 0.14 mg/g CNE) (Table 2).

Orthodihydric phenol

The orthodihydric phenol content in different extracts were found in the order: ZNLME (26.69 \pm 0.05 mg/g CLE) > ZNBME (16.56 \pm 0.02 mg/g CLE) > ZNBCIE (8.48 \pm 0.03 mg/g CLE) > ZNLCIE (3.34 \pm 0.01 mg/g CLE). The ZNLME showed the highest total orthodihydric phenol content

(26.69 \pm 0.05 mg/g CLE), while ZNLCIE showed the minimum (3.34 \pm 0.01 mg/g CLE).

The total phenolics and flavonoids in bark ethanolic extracts from Pakistan have also been reported [36]. The results revealed the presence of total phenolics (11.66 \pm 0.33 mg/g) and flavonoids (13.68 \pm 0.66 mg/g) in leaves and total phenolics (16.48 \pm 1.33 mg/g) and flavonoids (18.33 \pm 1.22 mg/g) in bark. Our study revealed high phenolic and flavonoids content in leaves methanolic extract (31.23 \pm 0.03 mg/g GAE and 47.63 \pm 0.14 mg/g GAE respectively) and bark methanolic extract (27.58 \pm 0.05 mg/g GAE and 68.04 \pm 0.06 mg/g GAE respectively) compared to Barkatullah et al., 2017 [36] (Table 2). This discrepancy in results might be due to diverse natural habitats of sampled plants.

Antioxidant Activity

DPPH radical scavenging activity

All the extracts exhibited good radical scavenging activity as a function of their concentrations. ZNLME (IC₅₀ = 50.87 μ g) showed maximum antioxidant property while ZNLCIE (IC₅₀ = 78.01 μ g) exhibits minimum antioxidant activity, compared to the standard antioxidant. The decreasing order of radical scavenging activity in terms of their IC₅₀ values among extracts were observed in the order of ZNLME (IC₅₀ = 50.87 μ g) > ZNBCIE (IC₅₀ = 59.16 μ g) > ZNBME (IC₅₀ = 63.47 μ g) > ZNLCIE (IC₅₀ = 78.01 μ g). (Table 3)

Reducing power

The extract ZNLME (RP₅₀ = 28.93 μ g/mL) showed maximum reducing power and ZNLCIE (RP₅₀ = 62.87 μ g) showed minimum reducing power. The decreasing reducing power in

Table 2: Total phenolics, flavonoid and orthodihydric phenols content of leaves and bark extractsof *Z. armatum*

S.N.	Sample name	Phenolic content		
		Total phenolic content (mg/g GAE)	Total flavonoid content (mg/g CNE)	Orthodihydric content (mg/g CLE)
1.	ZNLCIE	17.74 ± 0.02	77.18 ± 0.06	3.34 ± 0.01
2.	ZNLME	31.23 ± 0.03	47.63 ± 0.14	26.69 ± 0.05
3.	ZNBCIE	25.02 ± 0.04	56.72 ± 0.03	8.48 ± 0.03
4.	ZNBME	27.58 ± 0.05	68.04 ± 0.06	16.56 ± 0.02

ZNLCIE= *Z. armatum* leaves chloroform extract; ZNLME= *Z. armatum* leaves methanol extract; ZNBCIE= *Z. armatum* bark chloroform extract; ZNBME= *Z. armatum* bark methanol extract

Table 3: Antioxidant activity of *Z. armatum* leaves and bark extracts

S.N.	Sample name	Mean values (μg) \pm SD		
		DPPH activity (IC_{50})	Reducing power activity (RP_{50})	Metal chelating activity (IC_{50})
1.	ZNLCIE	78.01 ± 0.31 ^a	62.87 ± 0.33 ^a	39.02 ± 0.69 ^a
2.	ZNLME	50.87 ± 0.14 ^d	28.93 ± 0.46 ^d	19.42 ± 0.07 ^d
3.	ZNBCIE	59.16 ± 0.57 ^c	43.5 ± 0.11 ^b	28.81 ± 0.52 ^b
4.	ZNBME	63.47 ± 0.78 ^b	34.69 ± 0.13 ^c	24.21 ± 0.12 ^c
5.	BHT (Standard)	10.95 ± 0.13 ^f	-	-
6.	Catechin (Standard)	17.55 ± 0.44 ^e	-	-
7.	Ascorbic acid (Standard)	-	23.03 ± 0.31 ^e	-
8.	EDTA (Standard)	-	-	14.08 ± 0.11 ^e

Values are mean \pm standard deviation, within a column, mean values followed by the same letters are not significantly different according to Tukey's test ($p < 0.05$). ZNLCIE= *Z. armatum* leaves chloroform extract; ZNLME= *Z. armatum* leaves methanol extract; ZNBCIE= *Z. armatum* bark chloroform extract; ZNBME= *Z. armatum* bark methanol extract

different extract was in the order of: ZNLME ($\text{RP}_{50} = 28.93 \mu\text{g}$) > ZNBME ($\text{RP}_{50} = 34.69 \mu\text{g}$) > ZNBCIE ($\text{RP}_{50} = 43.5 \mu\text{g}$) > ZNLCIE ($\text{RP}_{50} = 62.87 \mu\text{g}$), compared to the standard antioxidants. The dose dependant reducing power in extract was observed along with the values of standard antioxidant. (Table 3)

Metal chelating activity

ZNLME ($\text{IC}_{50} = 19.42 \mu\text{g/mL}$) showed highest chelating power and ZNLCIE ($\text{IC}_{50} = 39.02 \mu\text{g}$) possessed least chelating power. The decreasing chelating power in different extract was in order ZNLME ($\text{IC}_{50} = 19.42 \mu\text{g}$) > ZNBME ($\text{IC}_{50} = 24.21 \mu\text{g}$) > ZNBCIE ($\text{IC}_{50} = 28.81 \mu\text{g}$) > ZNLCIE ($\text{IC}_{50} = 39.02 \mu\text{g}$), compared to the standard antioxidants. The dose dependant chelating power in extracts was observed along with the values of standard antioxidant. (Table 3)

Kanwal et al., 2015 [37] has reported the moderate antioxidant activity of *Z. armatum* leaves methanolic extract by DPPH and reducing power assay. Present study exhibited good antioxidant assay in all the extracts. Among all the extracts ZNLME exhibited maximum antioxidant activity while the

minimum was found in ZNLCIE. The phenolics are found to be related and reported to possess the antioxidant activity [37]. In present study, a good quantitative amount of phenolics, flavonoids and orthodihydric phenols have been observed. Hence, the good antioxidant activity of extract is because of the presence of phenols. Further, it has been observed that the phenolic content in the extract exhibited positive correlation with antioxidant activity and negative with IC_{50} values. The antioxidants are used to prevent the oxidative deterioration of foods and food products and can be used as food preservation for long-life of the food material. The synthetic antioxidants possess deleterious effects. Hence, in present scenario, the herbal antioxidants are in demands. Based on these facts, it can be concluded that the medicinal herb *Z. armatum* can be used as an herbal source of natural antioxidants and phytochemical source of nutraceuticals.

In-vitro Anti-inflammatory Activity

The *in-vitro* anti-inflammatory activity of extracts was performed by inhibition of egg albumin denaturation method as described in materials and methods section. Denaturation of proteins is well documented and is caused by inflammation process, mostly in conditions like arthritis. In protein denaturation mechanism, due to external stress, influence of chemical reactions results in distortion of protein's tertiary and secondary structure and leads to denaturation of proteins [38]. As the part of study on mechanism of anti-inflammation activity, capability of plant extracts were studied. It was observed that the extracts ZNLCIE, ZNLME, ZNBCIE and ZNBME inhibited the heat induced albumin denaturation in a dose dependent manner as monitored spectrophotometrically.

The extracts, ZNLME ($\text{IB}_{50} = 28.53 \mu\text{g}$) possessed maximum anti-inflammatory activity and ZNLCIE ($\text{IB}_{50} = 89.80 \mu\text{g}$) showed minimum anti-inflammatory activity. The decreasing inhibition of protein denaturation in different extract was in the order of: ZNLME ($\text{IB}_{50} = 28.53 \mu\text{g}$) > ZNBME ($\text{IB}_{50} = 42.22 \mu\text{g}$) > ZNBCIE ($\text{IB}_{50} = 55.42 \mu\text{g}$) > ZNLCIE ($\text{IB}_{50} = 89.80 \mu\text{g}$), compared to standard anti-inflammatory drug. The dose dependant anti-inflammatory activity in extracts were observed along with the values of standard anti-inflammatory drug diclofenac sodium (Table 4) and IB_{50} values of individual extract has been represented in Figure. 2.

The *in-vivo* anti-inflammatory activity of ethyl acetate extract from stems and roots of *Z. armatum* has been reported. The study revealed that the constituents like eudesmin, pinoresinol, sesamin and yangambin were responsible for *in-vivo* anti-inflammatory activity [39]. In present study most of the extracts exhibit moderate anti-inflammatory activity. The compounds eudesmin, sesamin and yagambin are also present in our extracts. Hence, it can be inferred that the anti-inflammatory activity is possibly due to the presence of these compounds or synergetic effect of co-occurrence of major, minor or trace constituents. Presence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols has also been reported to posses anti-inflammatory

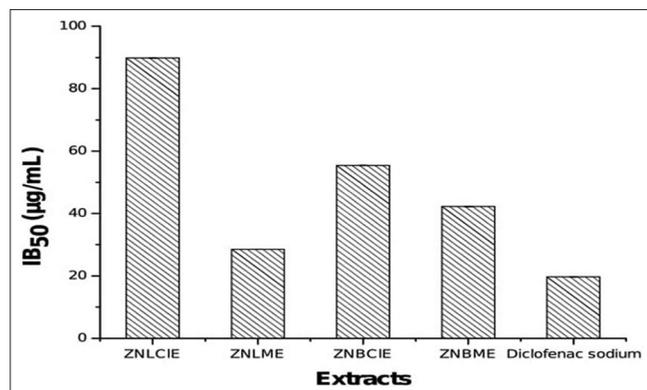


Figure 2: IB₅₀ values of different extracts of *Z. armatum*

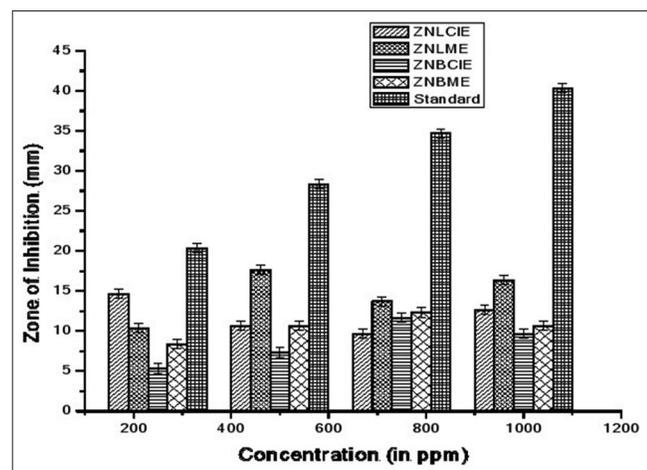


Figure 3: Screening of antibacterial activity of different extracts of *Z. armatum* against *E. coli*

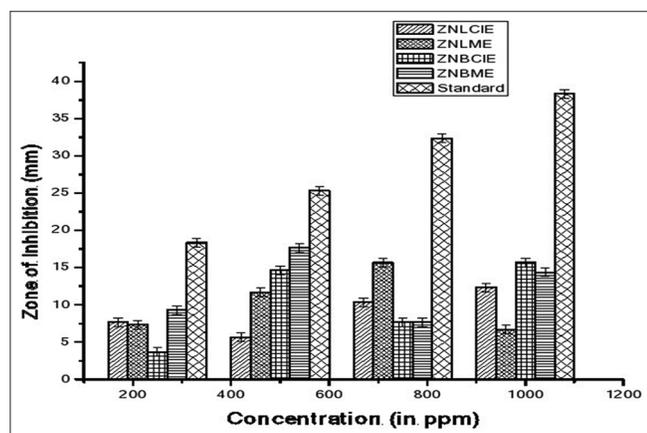


Figure 4: Screening of antibacterial activity of different extracts of *Z. armatum* against *S. aureus*

activity [40]. It has been reported that the total phenolics and antioxidants possess anti-inflammatory activity [41,42]. There is a positive correlation between antioxidant activity and total phenolics in present study. Hence, the constituents responsible for antioxidant activity may also be responsible for anti-inflammatory activity, i.e. there is positive co-relation between anti-inflammatory activity and phenolics/antioxidants.

Table 4: Anti-inflammatory activity of *Z. armatum* leaves and bark extracts

S.N.	Sample name	Mean IB ₅₀ values (µg) ± SD
1.	ZNLCIE	89.80 ± 0.05 ^a
2.	ZNLME	28.53 ± 0.06 ^d
3.	ZNBCIE	55.42 ± 0.11 ^b
4.	ZNBME	42.22 ± 0.17 ^c
5.	Diclofenac Sodium (Standard)	19.63 ± 0.06 ^e

Values are mean ± standard deviation, within a column, mean values followed by the same letters are not significantly different according to Tukey's test ($p < 0.05$)

Table 5: Antibacterial activity of *Z. armatum* leaves and bark extracts against *Escherichia coli* and *Staphylococcus aureus*

S.N.	Sample Name	Concentration (in ppm)	Mean R ₁ ± SD (<i>Escherichia coli</i>)	Mean R ₂ ± SD (<i>Staphylococcus aureus</i>)
1.	ZNLCIE	250	14.67 ± 0.58 ^g	7.67 ± 0.58 ^m
		500	10.67 ± 0.58 ^l	5.67 ± 0.58 ^p
		750	9.67 ± 0.58 ⁿ	10.33 ± 0.58 ^k
		1000	12.67 ± 0.58 ⁱ	12.33 ± 0.58 ⁱ
2.	ZNLME	250	10.33 ± 0.58 ^m	7.33 ± 0.58 ⁿ
		500	17.67 ± 0.58 ^e	11.67 ± 0.58 ^j
		750	13.67 ± 0.58 ^h	15.67 ± 0.58 ^f
		1000	16.33 ± 0.58 ^f	6.67 ± 0.58 ^o
3.	ZNBCIE	250	5.33 ± 0.58 ^q	3.67 ± 0.58 ^a
		500	7.33 ± 0.58 ^p	14.67 ± 0.58 ^g
		750	11.67 ± 0.58 ^k	7.67 ± 0.58 ^m
		1000	9.67 ± 0.58 ⁿ	15.67 ± 0.58 ^f
4.	ZNBME	250	8.33 ± 0.58 ^o	9.33 ± 0.58 ^l
		500	10.67 ± 0.58 ^l	17.67 ± 0.58 ^e
		750	12.33 ± 0.58 ^j	7.67 ± 0.58 ^m
		1000	10.67 ± 0.58 ⁱ	14.33 ± 0.58 ^h
5.	Gentamicin sulphate (Standard)	250	20.33 ± 0.58 ^d	18.33 ± 0.58 ^d
		500	28.33 ± 0.58 ^c	25.33 ± 0.58 ^c
		750	34.67 ± 0.58 ^b	32.33 ± 0.58 ^b
		1000	40.33 ± 0.58 ^a	38.33 ± 0.58 ^a

Values are mean ± standard deviation, within a column, mean values followed by the same letters are not significantly different according to Tukey's test ($p < 0.05$). ZNLCIE = *Z. armatum* leaves chloroform extract; ZNLME = *Z. armatum* leaves methanol extract; ZNBCIE = *Z. armatum* bark chloroform extract; ZNBME = *Z. armatum* bark methanol extract

Antibacterial Activity

The antibacterial efficiency of the ZNLCIE, ZNLME, ZNBCIE and ZNBME has been presented in Table 5. The extracts were tested for antibacterial activity against gram-positive and gram-negative bacteria and were found to be effective against all the tested bacterial strains as compared to the antibiotic gentamicin sulphate, taken as standard. It was observed that ZNLME showed maximum ZOI (zone of inhibition) of 17.67 mm at 500 ppm against *E. coli* while, ZNBME exhibits maximum ZOI of 17.67 mm at 500 ppm against *S. aureus*. The minimum ZOI was observed in ZNBCIE (5.33 mm), at 250 ppm against *E. coli* while, in case of *S. aureus* ZNBCIE showed minimum ZOI (3.67 mm) (Table 5). The screening of antibacterial activity of different extracts of *Z. armatum* against *E. coli* has been represented in Figure 3 while the screening of antibacterial activity of different extracts of *Z. armatum* against *S. aureus* has been represented in Figure 4.

A comparative antibacterial study against *S. aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Enterococcus faecalis* on hot water and ethanolic extract of *Z. armatum* has been reported from Pakistan [43]. It has been reported that the ethanolic extract was more effective than hot water extract [43]. From India, Srivastava et al., 2013 [44] reported the antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa* in *Z. armatum* chloroform, methanol and acetone bark extracts collected from lesser and higher Himalaya (altitude 700- 2000 m). The acetone and methanol extracts of bark were found to be more effective for *S. aureus* and chloroform extract for *P. vulgaris*.

CONCLUSIONS

The above results show that the *Z. armatum* is a good source of major compounds like eudesmin, sesamin, methyl-vanillin, linolenic acid, fargsin, γ - sitosterol, doxepin, besides other major and minor constituents. These compounds find their wide applications in perfumery, preservation, pharmacological activities and starting material for the synthesis of novel molecules. The compounds like doxepin and (Z, Z) -6, 9- cis-3, 4-epoxy-nonadecadiene has also been reported to possess anti-depressant activity and sex pheromone, respectively. The extracts have also been found to possess moderate antioxidant, anti-inflammatory and antibacterial activity. The total flavonoids, orthodihydric phenols and phenolic contents were also quantified in methanolic and chloroform extract of leaves and bark. In present scenario, food and pharmaceutical industries are in search of environmentally benign novel lead molecules from herbal origin. The present study concludes that the entire plant of *Zanthoxylum armatum* might be used as a good source of herbal antioxidants, food preservative, natural anti-inflammatory drug and natural anti-bacterial agent after proper clinical trials. The present study contributes for preparation of database on this species so that it can be exploited judiciously and scientifically.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

The authors are thankful to Advanced Instrumentation Research Facility, JNU, New Delhi for GC/MS. Thanks are due to Dr. Rishendra Kumar, Department of Biotechnology, Bhimtal Campus, Kumaun University, Nainital for antibacterial activity. The Department of Chemistry, GBPUAT, Pantnagar is thankfully acknowledged for providing laboratory facilities to carry out this work.

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