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RESEARCH ARTICLE

Bio-pesticidal, Antimicrobial, and Anti-inflammatory Potentials of n-Hexane Fraction of *Zanthoxylum armatum* DC and Its Chemical Profiling

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Abstract:

Zanthoxylum armatum DC, commonly known as toothache tree, is utilized for treating inflamed gums. The plant's volatile constituent possesses a robust fragrance and contributes to its tangy taste. This study investigates the bioactivities, including bio-pesticidal, antimicrobial, and anti-inflammatory properties as well as the chemical profiling of the n-hexane fraction based on GC-MS analysis. The evaluated activities involve contact toxicity, microplate alamar blue assay, against three different insects, five bacteria, and seven fungi, and oxidative burst assay. The NIST library serves as a standard reference database for constituent identification. Remarkable insecticidal activities comparable to the standard drug permethrin were observed, particularly against *Rhyzopertha dominica* (100%), *Tribolium castaneum* (60%), and *Sitophilus oryzae* (50%). The fraction exhibited significant antifungal activity against *Fusarium lini* (85%) and notable inhibition against *B. subtillis* (67.27%) and *S. aureus* (65.25%). Potent anti-inflammatory effects were noted with an IC_{50} value of 11.2±1.9 µg/ml, equivalent to standard ibuprofen at various concentrations. GC-MS analysis identified twenty compounds, with major ones including trans-13-Octadecenoic acid (36.08%), Cis-9 hexadecenoic acid (18.66%), and 2-propenoic acid 3 phenyl methyl ester (11.08%). The diverse bioactivities observed may be attributed to the varied nature of compounds such as polyunsaturated fatty acids and their oxides. This research revealed the potential of *Z. armatum* as a potential bio-pesticide, anti-inflammatory, and antimicrobial agent.

Keywords: Anti-inflammatory, Bio-pesticide, GC-MS, Insecticidal, *Zanthoxylum armatum* DC

Introduction

Synthetic pesticides have adverse effects on all consumers in the food chain, impacting the entire ecosystem [1]. Pathogens such as insects, fungi, and bacteria contribute to significant losses in productivity and yield in agriculture. To combat these challenges, farmers often resort to the excessive use of chemical pesticides, resulting in acute pesticide poisoning, particularly in developing nations [2]. The concern for unintentional acute pesticide poisoning poses a major health challenge, with Southern Asia reporting the highest number of cases [3]. Embracing traditional practices, such as using specific plants for food storage by indigenous communities, presents an alternative approach to preserving food and managing pests. Natural resources like plants, animals, bacteria, and minerals can serve as bio-pesticide due to their insecticidal, antibacterial, or antifungal properties The use of various natural resources such as plants, animals, bacteria, and minerals can serve as bio-pesticides in controlling pests, insects, weeds, and various diseases due to their insecticidal, antibacterial, or antifungal properties, offering a more sustainable and eco-friendly means of pest control [2]. The adaptation of eco-friendly pesticides can have long-term positive impacts on overall plant and animal species with numerous modern medications being derived from conventional practices [4].

Zanthoxylum armatum, locally known as Timur in Nepal, is an economically valued medicinal plant belonging to the Rutaceae family. Widely distributed in Nepal ranging from 1000-2500m in uncluttered places. Besides this plant is also distributed in India, Bhutan, China, Taiwan, the Philippines, Malaysia, Pakistan, and Japan, at an altitudes ranging from 1000-1500m [5], [6].Renowned as a toothache tree, it contains various phytochemicals, including alkaloids, sterols, flavonoids, saponins, coumarins, glycosides, benzoids fatty acids, alkenes acids, and amino acids [7], [8]. Studies have demonstrated the antioxidant, anti-inflammatory, cytotoxic, and hepato-protective activities of phytochemicals found in different parts of the plant [9]. The alkaloids in *Z. armatum* exhibit diverse biological and pharmacological properties including larvicidal, antinociceptive, antioxidant, antibiotic, hepatoprotective, antiplasmodial, cytotoxic, antiproliferative, anthelminthic, antiviral, antifungal, and anti-inflammatory activities [10]. Locals use crude extracts for their purported anti-helminthic, stomachic, and carminative properties to treat bacterial infections [11]. Additionally, the essential oil from leaves and fruits, rich in fatty acids and esters, adds to the plant's economic value.

This study specifically focuses on investigating the bioactivities on the n-hexane fraction of *Zanthoxylum armatum* DC and its constituents through GC-MS. The synergistic effects of natural compounds may reveal various biological benefits and provide essential dietary supplements. The hydrophobic nature of hexane as a solvent makes it adept at extracting volatile compounds like fatty acids and their derivatives, underscoring the potential significance of the identified constituents.

Material and Methods

I) Collection of plant materials and partitioning

Matured fruit pericarp of *Zanthoxylum armatum* DC were collected in October 2018 from higher altitude (>2200 m above sea level) Pyuthan district of Nepal. Authentication of the herbarium of *Zanthoxylum armatum* was done at the Central Department of Botany, Tribhuvan University Central Herbarium, Kritipur. Specimen voucher no TUCH (201016) was deposited in the same department. The powdered form of the extract five kilograms was soaked in 80% EtOH/H2O, one kilogram in four litres. It was shacked manually for homogeneous distribution. This procedure was repeated thrice. The clear filtrate was taken and concentrated in rotatory evaporator IKA (Werke GmbH &Co. KG, Germany) at 40 ºC and dried in the freeze drier (HETOSICC, Heto Lab Equipment, Denmark) at 55 oC. The concentrated ethanolic extract (600 g) was then fractionated to obtain a lower polarity hexane fraction of 150 g.

II) Characterization of hexane fraction of *Zanthoxylum armatum*

The Agilent technologies 7000 GC/MS triple quadrupole acquisition method was used to characterize the hexane fraction (MS-7000, GC 7890A). The ZEBRONZB-5HT column was used at 400 ºC: 30m×320µm × 0.25 µm, In Front SS inlet He and Out Vacuum. The oven was equilibrated for 5 minutes before running for 72 minutes at 60ºC (8ºC/min to 240 ºC for 20 minutes and 15 ºC/min to 300 ºC for 5 minutes). The injected sample volume was 1.5 µL. The spectra were computer-matched using the NIST Mass Spectrometry Data Center and the WILEY 7.0 library, and the retention times of known species injected in the chromatographic column were used to identify the peaks.

III) Identification of Components

Each of the mass spectra in GC-MS was identified by comparing the mass spectral fragmentation patterns head to tail of each constituent with those in the National Institute of Standards and Technology's (NIST) Mass Spectral Libraries version 2.2 database [12].

IV) Microplate Alamar Blue Assay (MABA)

Microplate alamar blue assay test used five different bacteria: *Bacillus subtilis* ATCC23875, *Staphylococcus aureus* NCTC 6571, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, and *Salmonella typhi* ATCC 14028. MABA was performed on hexane fraction at the concentration of 3000 µg/ml whereas the concentration of standard ofloxacin used was 100 µg/ml.

V) Insecticidal activity by contact toxicity method

For insecticidal activity by contact toxicity method, three major insects were used namely *Tribolium castaneum, Sitophilus oryzae, Rhyzopertha dominica*. The hexane fraction used for the activity was 2038.20 µg/cm^2 whereas the standard Permethrin used was 239.5 µg/cm^2.

VI) Antifungal activities

In vitro antifungal bioassay was performed with a concentration of sample as 3000 µg/mL of DMSO with an incubation time of 7 days at a temperature of 27 ºC. Seven different fungi; *Trichophyton rubrum, Candida albicans, Aspergillus niger, Microsporumcanis, Fusarium lini, Candida glabarata and Aspergillus fumigatus* were used for the antifungal activities.

VII) Oxidative Burst Assay using Chemiluminescence Technique

The anti-inflammatory activity was determined using a luminol-enhanced chemiluminescence test, based on a predetermined protocol [13]. In brief, 25 µL of diluted whole blood HBSS++ (Hanks Balanced Salt Solution, containing calcium chloride and magnesium chloride) [Sigma, St. Louis, USA] was added to 25 µL of the hexane fraction in triplicate at three different concentrations (10, 50 and 250µg/ml) and incubated. HBSS++, cells were added to the control wells only. The experiment was carried out in a white half-area 96-well plate [Costar, NY, USA], which was incubated for 15 minutes at 37 ºC in a luminometer's thermostat chamber [Labsystems, Helsinki, Finland]. Following incubation, 25 µL of serum opsonized zymosan (SOZ) [Fluka, Buchs, Switzerland] and 25 µL of an intracellular reactive oxygen species detecting probe, luminol [Research Organics, Cleveland, OH, USA] were added into each well beside the blank wells containing HB++. Ibuprofen was used as a standard. A luminometer was used to calculate the amount of ROS in terms of relative light units (RLU).

Result and Discussion

The result section consists mainly of two parts chemical and biological.

A) Chemical part

This portion consists of the analysis of the GC-MS portion, the structure of major compounds, and the mass spectra of major compounds as follows.

I) GC-MS Analysis

Gas Chromatography coupled with mass spectrometry is a direct and quick analytical approach. It operates with high separation efficiency flexibility and selectivity with mass sensitivity in detection [14]. Identification of volatile matters generally includes long and short-chain polyunsaturated fatty acids, hydrocarbons, esters, alcohols, etc. The GC chromatograms of the hexane fraction indicating total ion concentration are shown in Fig1 and Fig. 2 below. Table 1 consists of the compounds identified from each constituent's mass spectral fragmentation pattern from head to tail. The sample mainly consists of hydrocarbons, polyunsaturated fatty acids, long and short-chain hydrocarbons, terpenoids, and esters. The aromatic flavor of the fruit was found to be a mixture of esters and various compounds.

SN	t_R(min.)	Compound	Compound nature	Peak area
1	5.96	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	2-Pinen-4-one	0.04
2	7.16	2Furanmethanol,5ethanyltetrahydro-α,α,5-trimethyl-cis-	*cis*-Linaool Oxide	0.16
3	7.49	*α*-methyl-α[4-methyl-3pentrnyl]oxiranemethanol		0.16
4	7.86	1,6-Octadien-3-ol,3,7,dimethyl-	*β*-Linalool	6.77
5	9.4	3-Cyclohexen-1-ol, 4-methyl-1-(methylethyl)-	*p*-Menth-1-en-4-ol	0.03
6	9.6	3,7-octadione-2,6-diol,2,6-dimethyl	1,5-Octadiene-3,7-diol,3,7-dimethyl-	1.72
7	13.51	2-propenoic acid,3 phenylmethylester	Methyl cinnamate	11.08
8	14.06	Bicyclo[7.2.0]undec-4-ene,4,11,11-trimethyl-8-methylene-[IR-(1R*,4Z,95*)]	1.4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene	0.12
9	14.89	2-Propenic acid, 3-phenyl	Cinnamic acid	0.22
10	16.9	Caryophyllene oxide	*β*-Caryophyllene oxide	0.61
11	21.85	Methylhexadec-9-enoate		5.58
12	22.2	Hexadecanoic acid, methylester	Palmitic acid, methyl ester	2.77
13	23.8	*Cis*-9 hexadecenoicacid	Palmitoleic acid	18.66
14	24.2	n-hexadecanoic acid	Palmitic acid	7.5
15	26	9-octadecenoicacid(Z)-methylester	Oleic acid, methyl ester	2.93
16	27.85-29.46	9,12-Octadecadienoic acid (Z, Z)-	Linoleic acid	1.78
17	29.36	Trans-13-Octadecenoic acid		36.08
18	29.63-36.25	Icosapent	*ω*-3 Marine Triglycerides	3.28
19	51.42	Isopulegol acetate	Isopulegyl acetate	0.37
20	57.67	*β*-Amyrin	Olean-12-en-3-ol, (3β)-	0.14

Table 1: Phytoconstituent identified in the hexane fraction of *Zanthoxylum armatum* DC.

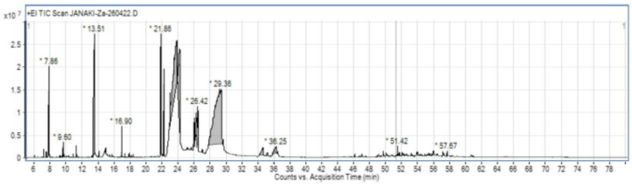


Fig 1 GC Chromatogram showing tR between 6.0 and 68 min

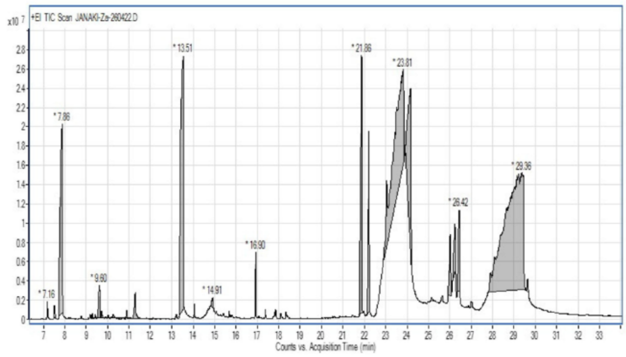
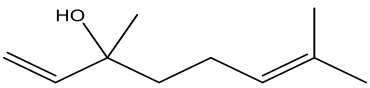


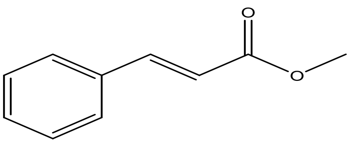
Fig 2 GC Chromatogram showing tR between 7.0 and 31 min

II) Structures of major compounds

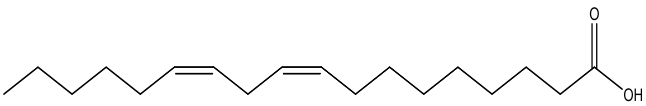
The structure of some compounds of hexane fraction *Zanthoxylum armatum* is presented below.



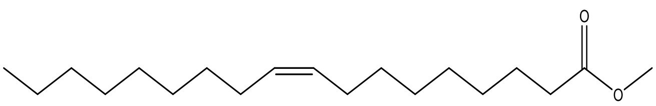
linalool



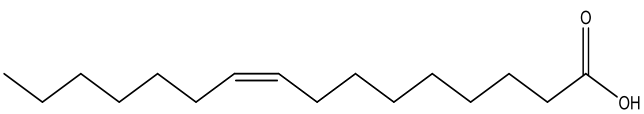
Cinnamic acid methyl ester



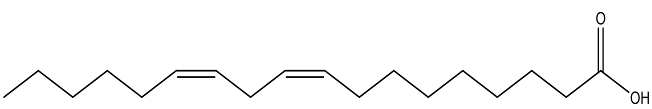
linoleic acid



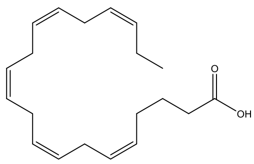
9-octadecenoic acid (Z) methyl ester



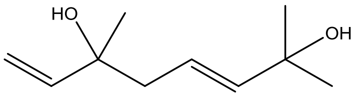
cis-9 hexadecenoic acid



cis-9-cis-12-octadecadienoic acid



Icosapent



3,7-octadiene-2,6-diol,2,6-dimethyl

III) Bioactive constituents

Among the twenty compounds identified from the hexane fraction, some compounds with remarkable activities reported are listed below. Compound linalool is one of the major constituents reported with diverse activities along with palmitic acid, cinnamic acid methyl ester, methyl oleate, trans-13 – octadecenoic acid, palmitoleic acid, and methyl palmitoleate. Studies show these compounds as anticancer, antibacterial, and anti-inflammatory along with diverse applications as mentioned in Table 2.

Table 2. Phytoconstituents with reported activities

Compound	**Activities**	**Citation**
Linalool	Anti-inflammatory, anticancer, anti hyperlipedemic, antimicrobial, antinoceptive, analgesic, anxiolytic, antidepressive, neuroprotective, anticonvulsant	[15] [16] [17]
Palmitic acid	Anti-inflammatory	[18]
Cinnamic acid methyl ester	Anticancer, Antibacterial, Anti-fungal, Neurological disorders	[19]
Methyl oleate	Antifungal	[20]
Trans-13-octadecenoic acid	Human metabolite	
Palmitoleic acid	Anti-inflammatory	[21]

IV) Major compounds of hexane fraction of *Zanthoxylum armatum* DC

Predominant compounds with area percentages above 10% are presented in Table 3 below with their name/nature, molecular weight, molecular formula, and NIST matching as observed from GC-MS.

Table 3: List of major compounds from hexane fraction of *Zanthoxylum armatum* DC.

Peak no	Retention time	Area %	Compound name/nature	MF/Mwt	NIST matching
7	13.51	11.08	2-propenoicacid,3 phenylmethylester(Methyl cinnamate)	$C_{10}H_{10}O_2$ 162	229225
13	23.81	18.66	Cis-9-hexadecenoicacid (Palmitoleic acid)	$C_{16}H_{30}O_2$ 254	333195
18	29.36	36.08	Trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$ 282	333615

NB: MF: Molecular Formula, Mwt: Molecular weight

B) Biological Activities

I) MABA Bioassay

The preliminary screening of antibacterial assay of hexane fraction revealed good percentage inhibition against *S. aureus* (65.25%) and *B. subtilis* (67.27%) only but no activity against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 10145, and *Salmonella typhi* ATCC 14028. The standard drug ofloxacin possesses 91.23%. The inhibition against particular bacteria could be related to the major constituents of hexane fraction possessing a hydrophobic nature which makes it easier during partition with the lipids in the cell membrane of bacteria thus making it more permeable by disrupting the cell membrane leading to changes in cytoplasm, and leakage of critical molecules eventually causing the death of the bacterial cells [23].

II) Insecticidal activity

The insecticidal activity was observed using the contact toxicity method in three types of insects namely *Tribolium castaneum, Sitophilus oryzae, Rhyzopertha dominica*. The hexane fraction used for the activity was 2038.20 μg/cm^2 whereas the standard insecticidal drug Permethrin used was 239.5 μg/cm^2. Insecticidal activity revealed hexane fractions as highly active. The percentage mortality of these insects observed was 100% against *Rhyzopertha dominica* and moderately active against *Tribolium castaneum* at 60% and *Sitophilus oryzae* at 50%. The hexane fraction of *Zanthoxylum armatum* was active against *Tribolium castaneum* and *Sitophilus oryzae*. The highest activity, comparable to the standard drug, was found to be against *Rhyzopertha dominica*. Fig. 3 illustrates the efficacy of hexane fraction equivalent to the standard drug in the case of *Rhyzopertha dominica* along with other insects used during insecticidal activity by contact toxicity method.

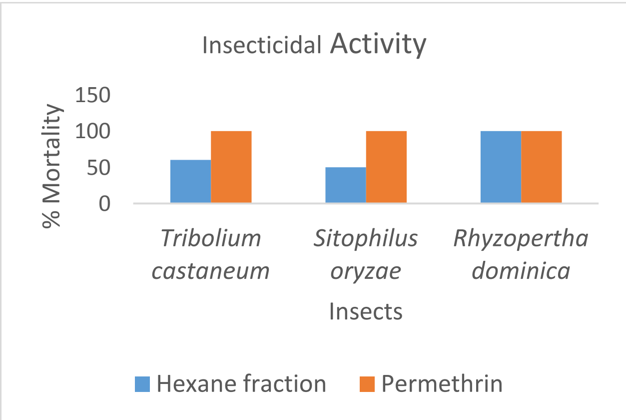


Fig: 3 Graphical representation of percentage mortality of various insects due to hexane fraction of *Z. armatum* and standard drug Permethrin

III) Antifungal activity

In vitro antifungal bioassay was performed with a concentration of sample as 3000 µg/ml of DMSO with an incubation time of 7 days and temperature of 27 ºC. Among seven different fungus; *Trichophyton rubrum, Candida albicans, Aspergillus niger, Microsporumcanis, Fusarium lini, Candida glabarata and Aspergillus fumigatus*. The hexane fraction was significant on only *Fusarium lini*. Hexane fraction of *Zanthoxylum armatum* showed highly significant activity against *F. lini* with linear growth of 15 mm and inhibition of 85%. Standard drug Miconazole possesses 73.25 µg/mL as its minimum inhibitory concentration. Particular antifungal properties of *F. lini* might be due to terpenes in this fraction [24].

IV) Anti-inflammatory activity

Inflammation is a non-specific immune response that occurs after physical injury with primary symptoms as changes in blood flow, cellular metabolism, and related. This disorder in some conditions leads to chronic inflammatory disease [25] amplifying stress in chronic condition cases disturbing the quality and productivity of life with huge financial loss [26]. For the anti-inflammatory activity determination, 1 mg of hexane fraction was used with the standard Ibuprofen. The percentage inhibition of Ibuprofen at 25 µg/mL was (73.2± 1.4) % with the inhibitory capacity IC_{50}=11.2±1.9µg/mL. Likewise, the hexane fraction at 10µg/ml concentration inhibited 79.4% revealing potency IC_{50} = 11.2±1.9µg/mL. The potency of this fraction against reactive oxygen species (ROS) might be due to the well-known anti-inflammatory compounds such as terpenes and sesquiterpenes. Studies show the use of terpenes as a skin penetration booster medium in the case of various inflammatory diseases probably supports the potency of hexane fraction [24]. Fig 4. Represents the anti-inflammatory activities of hexane fraction at various concentrations and standard drug ibuprofen used.

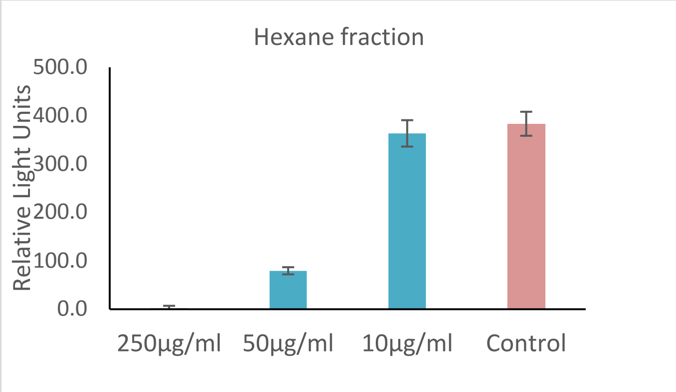


Fig: 4 ROS inhibition activity (IC_{50} value) of hexane fraction and standard drug ibuprofen

Discussions

This investigation of non-polar constituents through GC-MS revealed the polyunsaturated fatty acids and their oxygenated derivatives and terpenes as the majority. GC-MS quantitatively identified 20 phytoconstituents some of which are potent bioactive. The major bioactive compounds identified were 2-propenoic acid, and 3-phenylmethyl ester consisting of 11.08 percent of the area. Similarly, Cis-9-Hexadecenoic acid (18.66 %), and trans-13-octadecenoic acid (36.08 %) were found as major constituents. Studies show the essential oil from the fruit pericarp of *Z. armatum* possesses remarkable antibacterial activities with the majority of constituents as linalool, methyl trans-cinnamate, and limonene as revealed through GC-MS [27]. These degradable, environmentally friendly, non-toxic bioactive compounds are responsible for insecticidal and antimicrobial characteristics overall possessing the qualities of bio-pesticides [28]. Studies also suggest flavonoids isolated from the plants such as tambulin and prudomestin possess remarkable anti-inflammatory properties and hence are suitable drug candidates [29]. The present study revealed higher mortality of some insects by the n-hexane fraction of the plant may be due to the presence of the mono-terpenoids. The presence of various esters with a mixture of aromatic compounds is responsible for the strong odor of fruits. The potent bioactive properties of its volatile oil are also due to compounds like linalool, limonene, and lignin [27], [6]. Studies through GC-MS of hexane extract obtained through hot Soxhlet revealed 36 phytoconstituents in GC-MS with 2-hydroxy cyclopentadecanone as a major constituent [30]. Compounds like unsaturated fatty acids play an important role from an early stage of neurodevelopment [31], [32]. Polyunsaturated fatty acids due to their flexibility and conformational states are more significant than corresponding saturated fatty acids as the liver prefers to convert them to ketone bodies rather than to other atherogenic lipoproteins[33], [34]. Nutrition with polyunsaturated fatty acids has been employed to treat and fight against atherosclerosis as well as to inhibit thrombosis [35] thus dietary supplement of this highly valued *Zanthoxylum armatum* is quite relatable to the practical impacts. Together polyunsaturated fatty acids with hyperlipidemia and statin medication aid in lowering triglyceride levels [36]. Dietary saturated fatty acids influence inducing inflammation, in addition to their indirect role of raising low-density lipoprotein (LDL) cholesterol levels [37]. Such fatty acids affect the rate of LDL cholesterol synthesis and elimination besides preserving the steady state of LDL content of cholesterol in plasma. Hence dietary intake of polyunsaturated fatty acids obtained from plant *Zanthoxylum armatum* is of high importance in improving lipid profile as the length of the fatty acid chains of their cis/trans isomers also has drastic variation in cholesterol metabolism [38]. Potent insecticidal activity against *Rhyzopertha dominica* revealed the possible use of a hexane fraction of *Z. armatum* as a potential bio-pesticide.

Conclusions

The hexane fraction of *Zanthoxylum armatum* DC was found to be abundant in unsaturated fatty acids which may be beneficial for human health and can be developed as a biopesticide against *Rhyzopertha dominica*.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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