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Phytochemical And Biochemical Study Of The Hydroalcoholic Fruit Extract Of Zanthoxylum Armatum DC. For Its Anti-Oxidant And Anti-Inflammatory Potential

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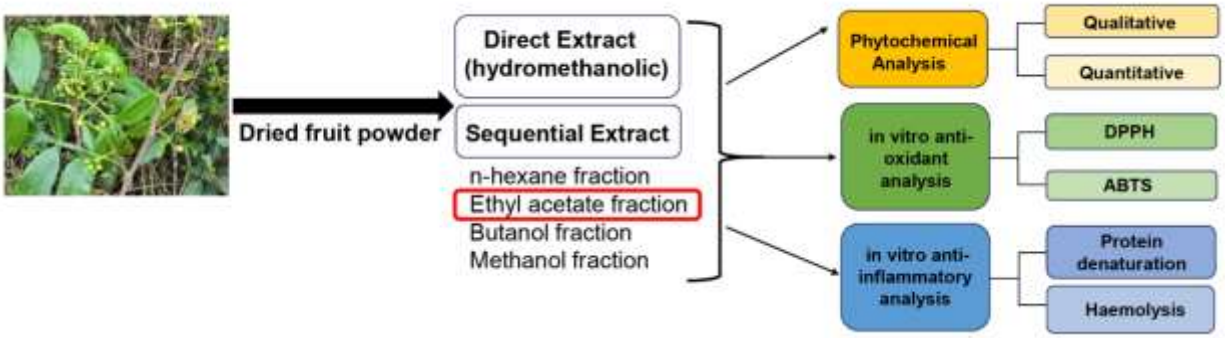
Abstract
Zanthoxylum armatum DC. is priced for both culinary as well as medicinal values. Fruits are used in local delicacies for its unique aroma with numbing and tingling sensation as well as in the treatment in oxidative as well as inflammatory problems by the indigenous people of South East Asian countries. This study aimed to examine the bioactive constituents of Zanthoxylum armatum DC. fruit through bioactivity-guided approach for its anti-oxidant and anti-inflammatry potential. Hydroalcoholic fruit extract was prepared from the mature fruits of Zanthoxylum armatum DC. The anti-oxidant activity was evaluated in vitro by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assays. Similarly, the anti-inflammatory activity was evaluated in vitro by heat induced haemolysis inhibition assay and albumin denaturation inhibition assay. The sequential ethyl acetate fraction of the hydroalcoholic fruit extract of Zanthoxylum armatum DC. showed anti-oxidant potential and anti-inflammatory potential. The results were in agreement with the ethnomedicinal use of Zanthoxylum armatum DC. as anti-oxidant and anti-inflammatory agent. This study further pave the way to further research exploring the mechanisms of action, safety profiles, and clinical efficacy is warranted.

Keywords: *Zanthoxylum armatum DC., Hydroalcoholic fruit extract, Sequential ethyl acetate fraction, Anti-oxidant, Anti-inflammatory.*

Graphical abstract:



1. INTRODUCTION

Studies on medicinal plants and its phyto-components are in ever increasing as it is one of the most popular method to explore useful natural components with rich anti-oxidant, anti-inflammatory, anti-cancer, etc. (1,2). Different medicinal properties of various plants were well known to man for centuries as man relied on nature for treatment of a variety of illnesses (1,3). Pharmacotherapies now recognize the huge potential of medicinal plants and its active phyto-components, for its fewer side effects, better efficacy, easy accessibility and cost effectiveness (4,5). In fact, almost two-thirds of all the approved drugs in the world are of herbal origin (6). According to WHO, in the developing countries, above 80% population use medicinal plants and its phyto-components in their primary healthcare needs which accounts to a huge number of around 21,000 plant species (2,5). And the interest of general people in developed countries is growing on natural care and natural therapies so as to gain a more balanced and more healthier lifestyle (7,8).

Naturally, our body metabolism generates reactive oxygen species (ROS) such as hydroxyl radicals, superoxide radicals, etc. and reactive nitrogen species (RNS) such as nitric oxide radicals, peroxynitrite radicals and S-nitrosothiols, during its cellular and extra cellular metabolic events and constitutes the metabolic stress (9). But as a part of our primary innate immune defence system, this oxidative stress along with the oxidative burst of macrophages and monocytes, are highly enhanced during harmful infection and in the state of inflammation (10,11,12). This overwhelmed free radicals can damage biomolecules such as DNA, proteins, lipids, etc., which in turn leads to carcinogenesis stimulation, aging of cells, cardiovascular diseases, inflammatory diseases, etc. (13).

Inflammation is a complex host body mechanism to maintain homeostasis in response to harmful perturbations (14). Primarily, associated with pain, heat, swelling, redness and may comprises events like increase permeability in vascular tissues, denaturation of protein and alteration of membrane (15). In acute inflammation, host body response to counter harmful perturbations mainly by macrophages and natural killer (NK) cells while in chronic inflammation, there is prolonged and over exaggerated response and thus typically involved in many diseases such as diabetes, rheumatoid arthritis, cancer and asthma (16,17,18). Such prolonged and exaggerated expression of pro-inflammatory mediators, cytokines, nitric oxide, prostaglandins, ROS and RNS are harmful to host body and their inflammatory reaction results in tissue damages by attacking macromolecules, lipid membrane peroxidation, increased vascular permeability, vasodialation and endothelial tissue damage which in turn took an important role in numerous inflammatory diseases (19). Both inflammation and oxidative stress are closely inter-related and inter-dependant of their pathophysiological nature. Either one of them may appear first or later, but when one of them appears, most likely the other one will appear and both of them take part in parthenogenesis of many diseases (20). So, phyto-components with anti-oxidant and anti-inflammatory properties may be ideal for the remedial treatment of many diseases.

Zanthoxylum armatum DC. is also known as Indian prickly ash tree or timur or toothache tree, is an important plant of citrus family Rutaceae, valued for both culinary spice as well as medicinal herb. It is aromatic, deciduous, spiny shrub which can height upto 6 metres. The genus has 250 species distributed throughout the glove and 11 species of *Zanthoxylum*, viz., *Z. budrunga, Z. oxyphyllum, Z. ovalifolium, Z. acanthopodium, Z. planispinum, Z. armatum, Z. nitidium, Z. rhesta, Z. simulans, Z. avicennae,* and *Z. limonella*, are significant medicinally (21). Fruit of *Z. armatum* has been being used as a medicine to treat a variety of illnesses such as (22,23). Pharmacological reports on antioxidant, antinociceptive, antifungal, anti-inflammtory, hepatoprotective, pesticidal, antihelminitic, antiproliferative, cytotoxic, antimicrobial, antiviral, insecticidal/larvicidal, etc. are evident (24,25,26). The plant reported to have a variety of phyto-components such as phenolics, flavonoids, alkaloids, terpinoids, sterols, etc. may be the reason behind such a varied bioactivity with potential mentions in numerous compositions in Ayurveda, allopathy, general pharmacy, and other fields (27).

For this study, mature fruits of *Zanthoxylum armatum* DC. growing in the foothill of Nongmaiching hill, Imphal East district of Manipur were collected for its phytochemical fingerprinting of the fruit through GC/MS and LC/MS and validates its potential use as an anti-oxidant and anti-inflammatory agent.

2. MATERIALS AND METHOD:

2.1. Sample collection, sample processing and authentication of the plant

Fruits of *Zanthoxylum armatum* DC. were collected during its mature stage (August, 2018) from Imphal East, Manipur, India (24°46'08.9"N 93°58'45.1"E). Fruits were washed for any dust and other surface contaminants properly in tap water and excess water was allowed to drained, dried in shade under room temperature for 5 days and grinded into fine power with the help of an electric grinder (Bajaj Pvt. Ltd.) and stored in 4°C in a fridge until further analysis. A specimen was submitted in the herbarium at Plant Systematics and Conservation Laboratory, Plant Bioresources Division, Institute of Bioresources and Sustainable Development (IBSD), Imphal (Voucher number IBSD/M-273). [Figure 1]

2.2. Preparation of fruit extract

1.21 kg of powdered *Zanthoxylum armatum* DC fruit was soaked in 6L of 70% Methanol for 6 days, filtered with Whatman filter paper I and the solvent was decanted completely from the filtrate by using a rotary evaporator (Buchi Rotavapor). Thus obtained hydromethanolic fruit extract of *Zanthoxylum armatum* DC. was assigned as ZF and kept at 4°C fridge until further analysis.

2.3. Fractionation of the fruit extract

The crude hydromethanolic fruit extract, (ZF) was given to primary fractionation by the method of liquid-liquid partition in a polarity gradient of organic solvents. This resulted in four fractions viz., hexane fraction (ZFH), ethylacetate fraction (ZFE), butanol fraction (ZFB) and methanol fraction (ZFM). All these four fractions were also concentrated to complete dry by using a rotary evaporator (Buchi Rotavapor) and stored in a 4°C fridge until further analysis.

2.4. Phytochemical profiling

2.4.1. Preliminary phytochemical profiling

The hydromethanolic fruit extract, (ZF) was analysed for different phytochemicals with standard phytochemical screening procedures. Qualitative test included- Dragendorff's test and Hager's test for alkaloids, - Shinoda test and Alkaline test for flavonoids, - Ferric chloride test and Lead acetate test for phenolics, - Molisch's test for carbohydrates, - Fehling's test for reducing sugars and - Iodine solution test for non-reducing sugars, - Biuret and ninhydrin test for proteins and amino acids, - Ferric chloride test for tannins, - Salkowski's test for steroids, - Concentrated sulphuric acid and Copper acetate test for terpinoids, - Keller-Kelliani test and 10% ammonia test for glycosides and - Foam test for saponin (28,29).

2.4.2. Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

Total phenolic content TPC of the hydromethanolic fruit extract, ZF and the four fractions, ZFH, ZFE, ZFB and ZFM were estimated using Folin-Ciocalteu method mentioned earlier (30,31). In short, 100µL test sample, 500µL 10% Folin-Ciocalteu reagent (v/v) and 400µL 7.5% Na_2CO_3 (w/v) were mixed and after 30 minutes, absorbance at 765nm was observed. Standard caliberation curve of gallic acid was prepared at concentrations 10 to 100µg/mL and comparing with it total phenolic contents of the hydromethanolic fruit extract and the four fractions were estimated.

Similarly, total flavonoid content (TFC) of the hydromethanolic fruit extract, ZF and the four four fractions, ZFH, ZFE, ZFB and ZFM were estimated using colorimetric aluminium chloride method as described earlier (32). In short, 1.5mL methanol, 0.5mL sample in 2.8mL of distilled water, 0.1mL 10% aluminum chloride and 0.1mL of 1M potassium acetate were mixed and after 30 minutes, absorbance at 415nm was observed. Standard caliberation curve of quercetin was prepared at concentrations 10 to100µg/mL and comparing with it total flavonoid contents of the hydromethanolic fruit extract and the four fractions were estimated.

2.4.4. Gas Chromatography Mass Spectrometry based phytochemical fingerprinting

Gas Chromatography Mass Spectrometry (GCMS) based phytochemical fingerprinting of the hydromethanolic fruit extract, ZF and its ethyl acetate fraction, ZFE were done at GCMS system Shimadzu GCMS QP-2010 Plus by comparing with relative retention indices of n-alkanes and mass spectra of available mass spectral libraries NIST14, WILEY8, FFNSC2, PubMed with 75% cut off similarity index at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi.

2.4.4. Liquid Chromatography Mass Spectrometry based phytochemical fingerprinting

Similarly, Liquid Chromatography Mass Spectrometry (LCMS) based phytochemical fingerprinting of the hydromethanolic fruit extract, ZF and its ethyl acetate fraction, ZFE were done at LCMS system Agilient 1260 Infinity-II system coupled with Agilient 6546 LC/Q-TOF Mass spec. by comparing with relative retention indices of n-alkanes and mass spectra of available mass spectral libraries NIST14, WILEY8, FFNSC2, PubMed with 90% cut off similarity index at Central Instrumentation Facility (CIF), Institute of Bioresources and Sustainable Development (IBSD), Imphal.

2.5. Evaluation of *in vitro* anti-oxidant activity

2.5.1. DPPH Free radical scavenging

Free radical scavenging activity of the hydromethanolic fruit extract and its four fractions were measured by 1,1-Diphenyl-2-picryhydrazil (DPPH, HiMedia) using the method of Sakthivel *et al.,* 2013 with slight modification (33). Briefly, 300µL of sample is mixed with 100µL of freshly prepared 0.1mM solution of DPPH in ethanol. This mixture is kept at room temperature for 30 minutes in dark condition and absorbance was measured at 517nm in Thermo Multiscan Spectrum. Ascorbic acid was used as positive control. Both control and samples were tested at different doses (10-100µg/mL).

2.5.2. ABTS Total free radical scavenging

Similarly, Total free radical scavenging activity of the hydromethanolic fruit extract and its four fractions were measured by using ABTS (Sigma) following the method of Hsu *et al.,* 2011 with slight modification (34). Briefly, $ABTS^{*+}$ solution was prepared by mixing 7mM ABTS and 2.45 mM $K_2S_2O_8$ in water and incubated for 16 hours at room temperature in dark condition. Just before use, this $ABTS^{*+}$ solution was diluted with ethanol to get an absorbance of 0.7±0.02 at 734nm. 200µL of this $ABTS^{*+}$ solution was mixed with 10µL of test samples and kept at room temperature for 10 minutes in dark condition. Absorbance was measured at 734nm in UV-Visible Thermo Multiscan Spectrum. Ascorbic acid was used as positive control. Both control and samples were tested at different doses (10-100µg/mL).

The percentage inhibition was calculated by using the formula

$$\% \text{ inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

A linear graph was plotted using % inhibition against concentration and IC_{50} was then calculated.

2.6. Evaluation of *in vitro* anti-inflammatory activity

2.6.1. Albumin denaturation inhibition assay

Heat induced albumin denaturation inhibition ability of the hydromethanolic fruit extract and its four fractions were estimated using the method by Sakat *et al.*, 2010 (30). In short, 500µL 1% bovine serum albumin aqueous solution (Sigma) and 500µL test sample were mixed and pH was kept at 6.8 using 1N HCl. After 20 minutes at 37°C and 20 minutes at 57°C incubation, absorbence was observed at 660nm. Water and diclofenac were taken as control and standard reference respectively. Both standard and samples were tested at different concentrations (5-100µg/mL).

2.6.2. Heat induced haemolysis inhibition assay

Similarly, heat induced haemolysis inhibition ability of the hydromethanolic fruit extract and its four fractions were also estimated following Gupta *et al.*, 2013 and Das et al., 2014 with some modification (35,36). In short, 500µL 10% RBC suspension and 500µL test sample were mixed and after 30 minutes incubation

at 56°C, cooled and centrifused at 2500rpm for 5 minutes. The supernatant absorbence was observed at 560nm. Normal saline and diclofenac were control and standard reference respectively and Both the standard and the test samples were tested at different concentrations (5-100µg/mL).

The percentage inhibition was calculated by using the formula

$$\% \text{ inhibition} = \frac{\text{Control Absorbance} - \text{Test Absorbance}}{\text{Control Absorbance}} \times 100$$

IC50 was calculated by plotting a linear graph with % inhibition against the concentration of the sample.

3. RESULTS

About 1.21 kg of powdered *Zanthoxylum armatum* DC. fruit was soaked in 6 litre of 70% Methanol and yielded 46 g of crude extract of fruit with a percentage yield of 3.8 %. Meanwhile, 40g of the crude methanolic fruit extract (ZF) yielded 10g Hexane Fraction (ZFH), 16g Ethyl Acetate Fraction (ZFE), 2g Butanol Fraction (ZFB) and 9g Methanol Fraction (ZFM) giving percentage yields of 25%, 40%, 5% and 22.5% resprctively.

3.1. Preliminary Phytochemical Profiling

Standard qualitative phytochemical screening of the extract are presented in Table 1. Hydromethanolic fruit extract of *Zanthoxylum armatum* DC. showed presence of various phytoconstituents like phenolic compoundss, flavonoids, alkaloids, diterpinoids, tannins and saponin glycosides. They may be responsible for its medicinal properties. Presence of carbohydrates, proteins and anthraquinone glycosides were not detected may be due to their limited solubility in high polar solvent like methanol.

Table 1. Preliminary phytochemical screening of hydromethanolic fruit extract of *Zanthoxylum armatum* DC.

Sl. No.	Tests conducted for phytochemical constituents	Hydromethanolic extract of *Z armatum* DC. fruit
1.	Alkaloids	
	Dragendorff's test	+
	Hager's test	+
2.	Flavonoids	
	Shinoda test	+
	Alkaline test	+
3.	Phenolics	
	Lead acetate test	+
	Ferric chloride test	+
4.	Carbohydrates	
	Molisch's test	-
	Reducing sugars	-
	Non-reducing sugars	-
5.	Proteins and amino acids	
	Biuret test	-
	Ninhydrin test	-
6.	Tannins	
	Ferric chloride test	-
7.	Steroids	
	Salkowski's Test	+
8.	Terpinoids	
	Triterpinoids	+

	Diterpinoides	+
9.	Glycosides	
	Cardiac glycosides	+
	Anthraquinone glycosides	-
	Saponin glycosides	+

'+'- presence, '-'- absence of tested phytochemicals.

3.2. Total phenolic and flavonoid content

Total phenolic content and total flavonoid content of the hydromethanolic fruit extract were found to be 114.5±1.56 µg Gallic acid equivalent and 134.19±0.71 µg Quercetin equivalent respectively. Among fractions, ethyl acetate fraction (ZFE) showed highest phenolic content 113.08±4.27 µg Gallic acid equivalent and highest flavonoid content 120.66±2.18 µg Quercetin equivalent followed by butanol fraction (ZFB) (phenolic content 87.66±1.01 µg Gallic acid equivalent and flavonoid content 85.90±1.22 µg Quercetin equivalent), methanol fraction (ZFM) (phenolic content 87.08±0.52 µg Gallic acid equivalent and flavonoid content 83.38±0.577 µg Quercetin equivalent) and hexane fraction (ZFH) (phenolic content 7.75±1.25 µg Gallic acid equivalent and flavonoid content 9.23±0.21 µg Quercetin equivalent). Standard calibration curves of gallic acid and quercetin are presented in [Figure 2].

Table 2. Quantification of total phenolic content and total flavonoid content of hydromethanolic fruit extract and its four fractions.

Sl. No.	Sample	Total phenolic content in µg/mL GAE* ± SD	Total flavonoid content in µg/mL QE** ± SD
1.	ZF	114.5±1.56	134.19±0.71
2.	ZFH	7.75±1.25	9.23±0.21
3.	ZFE	113.08±4.27	120.66±2.18
4.	ZFB	87.66±1.01	85.90±1.22
5.	ZFM	87.08±0.52	83.38±0.577

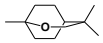
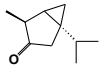
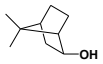
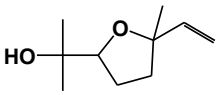
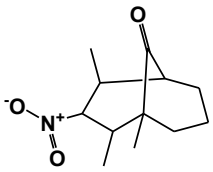
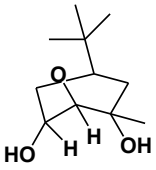
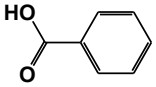
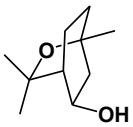
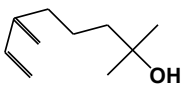
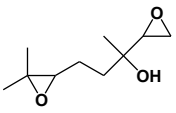
*expressed as GAE (Gallic acid equivalent) µg/mL, **expressed as QE (Quercetin equivalent) µg/mL, n= 3. ZF- hydromethanolic fruit extract of *Zanthoxylum armatum* DC. ZFH- hexane fraction of ZF, ZFE- ethyl acetate fraction of ZF, ZFB- butanol fraction of ZF and ZFM- methanol fraction of ZF, n= 3.

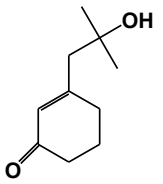
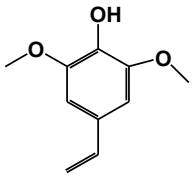
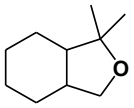
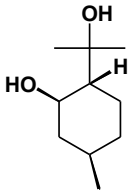
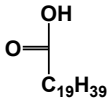
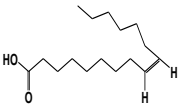
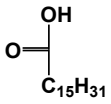
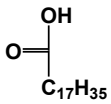
3.3. Gas Chromatography Mass Spectrometry based Phytochemical Fingerprinting

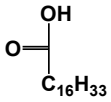
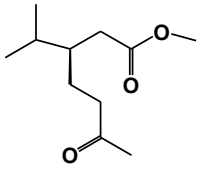
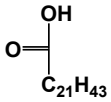
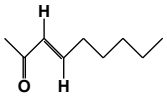
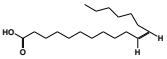
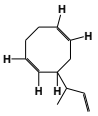
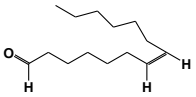
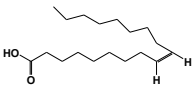
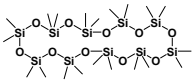
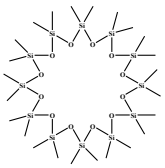
GCMS based phytochemical fingerprinting of hydromethanolic fruit extract and its ethyl acetate fraction of *Zanthoxylum armatum* DC. indicated the presence of 3-(2-Hydroxy-2-methyl-propyl)-cyclohex-2-enone, 4-Vinylsyringol, Eicosanoic acid, Hexadecanoic acid and Octadecanoic acid as major components common to both. In hydromethanolic fruit extract (ZF), a long chain carboxylic acid, Hexadecanoic acid has the highest concentration, a total of 26.72% of the total area percentage followed by another carboxylic acid, Palmitoleic acid with relative area percentage of 12.48%. Whereas, in ethyl acetate fraction of the hydromethanolic fruit extract, (ZFE), Tetracosamethyl-cyclododecasiloxane has the highest concentration with 25.22% relative area percentage followed by Hexadecanoic acid with relative area percentage of 12.77%. [Figure 3]

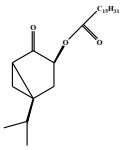
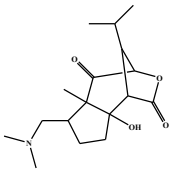
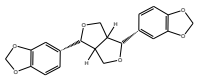
Table 3. GCMS based analysis of relative percentages of major chemical components found in hydromethanolic fruit extract of *Zanthoxylum armatum* DC. and its ethyl acetate fraction.

Compound	R.time in ZF	Area % in ZF	R.time in ZFE	Area % in ZFE	Formula	Mol. Mass	Structure

Eucalyptol	10.08	0.95	-	-	C10H18O	154.24	
Thujone	13.23	0.99	-	-	C10H16O	152.23	
2-Hydroxycineole	16.40	2.34	-	-	C10H18O2	170.24	
Borneol	21.84	1.67	-	-	C9H16O	140.22	
Linalyl oxide	-	-	22.20	0.5	C10H18O2	170.24	
1,2,4-trimethyl-3-nitro-Bicyclo[3.3.1]nonan-9-one	22.78	0.75	-	-	C12H19NO3	225.28	
Epomediol	-	-	22.79	0.50	C10H18O3	186.25	
Benzoic acid	23.10	0.74	-	-	C7H6O2	122.12	
3-Hydroxy-1,8-cineole	-	-	23.39	3.91	C10H18O2	170.28	
Myrcenol	23.49	1.38	-	-	C10H20O	156.26	
Epoxy-linalooloxide	-	-	23.62	1.06	C10H18O3	186.25	

3-(2-Hydroxy-2-methyl-propyl)-cyclohex-2-enone	25.70	4.29	26.93	7.00	C10H16O2	168.23	
4-Vinylsyringol	26.11	0.50	24.91	1.02	C10H12O3	180.20	
8-Oxabicyclo[4.3.0]nonane, 7,7-dimethyl-	26.43	2.44	-	-	C10H18O	154.25	
Menthoglycol	-	-	26.43	0.50	C10H20O2	172.26	
Eicosanoic acid	34.42	6.71	38.81	1.94	C20H40O2	312.5	
Palmitoleic acid	35.22	12.48	-	-	C16H30O2	254.41	
Hexadecanoic acid	35.60	26.72	33.75	12.77	C16H32O2	256	
Octadecanoic acid	36.04	7.48	37.51	4.76	C18H36O2	284	

Heptadecanoic acid	36.39	2.78	-	-	C17H34O2	270.5	
R-(+)-Methyl-3-isopropyl-6-oxoheptanoate	-	-	37.83	0.50	C11H20O3	200.27	
Docosanoic acid	37.86	3.85	-	-	C22H44O2	340.6	
3-Nonen-2-one	-	-	38.72	0.61	C9H16O	140.22	
Cis-vaccenic acid	38.97	1.61	-	-	C18H34O2	282.5	
1,5-Cyclooctadiene, 3-(1-methyl-2-propenyl)-	41.94	0.54	-	-	C12H18	162.27	
7-Tetradecenal, (Z)-	42.55	0.69	-	-	C14H26O	210.36	
Oleic Acid	42.67	0.70	-	-	C18H34O2	282.5	
Cyclodecasiloxane, eicosamethyl-	-	-	42.98	8.44	C20H60O10Si10	741.53	
Tetracosamethyl-cyclododecasiloxane	-	-	46.57	25.22	C24H72O12Si12	889.84	

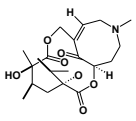
Sabinyl palmitate	47.39	0.61	-	-	C28H54O2	436.69	
Nobiline,6-hydroxy	53.26	1.38	-	-	C17H27NO4	309.4	
(+)-Sesamin	-	-	54.82	0.54	C20H18O6	354.35	

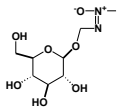
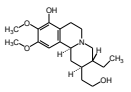
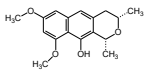
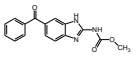
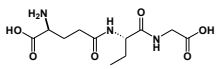
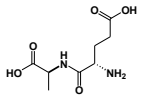
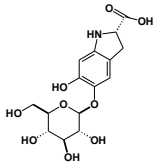
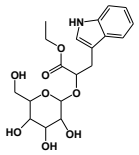
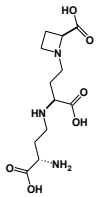
Minimum concentration of chemical components in Area% ≥ 0.5.
Total % of major chemical components in hydromethanolic fruit extract (ZF) =81.60
Total % of major chemical components in ethyl acetate fraction of fruit extract (ZFE) =69.27
Highest concentration of chemical component in hydromethanolic fruit extract (ZF) = Hexadecanoic acid (26.72%).
Highest concentration of chemical component in ethyl acetate fraction (ZFE) = Tetracosamethyl-cyclododecasiloxane (25.22%).

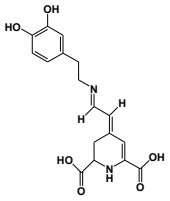
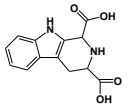
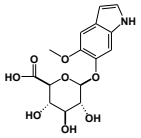
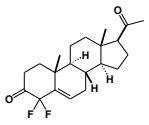
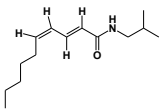
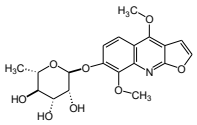
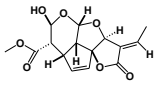
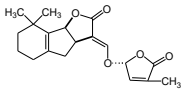
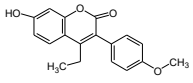
3.4. Liquid Chromatography Mass Spectrometry based Phytochemical Fingerprinting

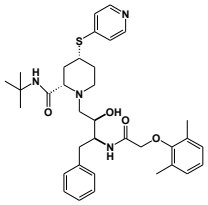
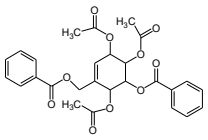
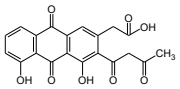
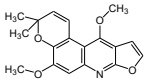
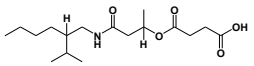
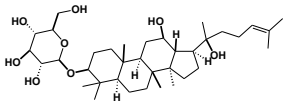
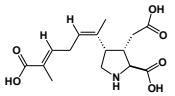
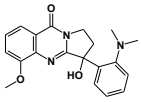
LCMS based phytochemical fingerprinting of hydromethanolic fruit extract of *Zanthoxylum armatum* DC. and its ethyl acetate fraction indicated the presence of Ophthalmic acid and Glycoperine common to both the extract and fraction. Ophthalmic acid has highest peak intensity in both hydromethanolic fruit extract (59908 units) and in its ethyl acetate fraction (161671 units). [Figure 4]

Table 4. LCMS based phytochemical analysis of chemical components found in hydromethanolic fruit extract of *Zanthoxylum armatum* DC. and its ethyl acetate fraction.

Compound	R.time in ZF	Peak intensity in ZF	R.time in ZFE	Peak intensity in ZFE	Formula	Mol. Mass	Structure
Petasitenine	0.78	8956	-	-	C19H27NO7	381.17	

Cycasin	2.84	26302	-	-	C8 H16 N2 O7	252.09	
Ankorine	3.76	20590	-	-	C19 H29 N O4	335.20	
Karwinaphthol B	4.34	53055	-	-	C17 H20 O4	288.13	
Mebendazole	4.35	11205	-	-	C16 H13 N3 O3	295.09	
Ophthalmic acid	4.42	59908	4.63	161671	C11 H19 N3 O6	289.12	
Glutamylalanine	-	-	4.82	37149	C8 H14 N2 O5	218.09	
cyclo-Dopa 5-O-glucoside	4.88	30350	-	-	C15 H19 N O9	357.10	
Ethyl 2-hydroxy-3-(3-indolyl)propanoate glucoside	-	-	5.10	20412	C19 H25 N O8	395.15	
Nicotianamine	-	-	6.47	48500	C12 H21 N3 O6	303.14	

Miraxanthin-V	-	-	7.22	47888	C17 H18 N2 O6	346.11	
1,2,3,4-Tetrahydro-b-carboline-1,3-dicarboxylic acid	-	-	7.35	47293	C13 H12 N2 O4	260.07	
6-Hydroxy-5-methoxyindole glucuronide	-	-	7.50	28364	C15 H17 N O8	339.09	
4,4-Difluoropregn-5-ene-3,20-dione	-	-	7.73	14563	C21 H28 F2 O2	350.20	
(E,E)-2,4-Decadienoic isobutylamide	-	-	10.79	26336	C14 H25 N O	223.19	
Glycoperine	11.13	13173	10.36	31625	C19 H21 N O8	391.12	
Allamandin	-	-	11.57	16121	C15 H16 O7	308.08	
5-Deoxystrigol	-	-	12.10	33654	C19 H22 O5	330.14	
4-Ethyl-7-hydroxy-3-(p-methoxyphenyl)coumarin	-	-	14.05	41693	C18 H16 O4	296.10	

BILA 2185BS	-	-	14.87	13221	C35 H46 N4 O4 S	618.32	
Piperenol A triacetate	15.31	26793	-	-	C27 H26 O10	510.15	
Aklanonic acid	15.32	35052	-	-	C21 H16 O8	396.08	
Acronidine	-	-	16.37	46764	C18 H17 N O4	311.11	
Butoctamide hydrogen succinate	-	-	16.77	47185	C16 H29 N O5	315.20	
(20R)-Ginsenoside Rh2	16.93	26423	-	-	C36 H62 O8	622.44	
Isodomoic acid A	17.78	23620	-	-	C15 H21 N O6	311.13	
Aniflorine	-	-	18.50	19314	C20 H21 N3 O3	351.15	

Minimum cut off similariry score of chemical components in the table =90%.
Ophthalmic acid has highest peak intensity in both hydromethanolic fruit extract (59908 units) and in its ethyl acetate fraction (161671 units).

3.5. *In vitro* anti oxidant activity

In vitro anti-oxidant potential of the hydromethanolic fruit extract and its four fractions are given in Table 5 & 6. The hydromethanolic fruit extract (ZF) showed anti-oxidant potential in both DPPH and ABTS free radical scavenging asssays with IC50 of 58.54 ± 0.08µg/mL ± SD and 73.28 ± 0.21 µg/mL ± SD respectively.

Standard anti-oxidant, ascorbic acid showed IC50 of 32.22 ± 0.14 µg/mL ± SD in DPPH assay and 49.30 ± 0.09 µg/mL ± SD in ABTS assay respectively.
Ethyl acetate fraction (ZFE) showed highest free radical scavenging potential in both DPPH assay and ABTS assay with IC50 of 56.09 ± 0.29 µg/mL ± SD in DPPH assay and 71.49 ± 0.41 µg/mL ± SD in ABTS assay followed by hexane fraction (ZFH) which showed IC50 of 121.86 ± 1.98 µg/mL ± SD in DPPH and 99.92 ± 2.07 µg/mL ± SD in ABTS. Methanol fraction (ZFM) showed highest IC50 of 224.13 ± 1.07 µg/mL ± SD in DPPH and 120.92 ± 4.77 µg/mL ± SD in ABTS and hence indicated least anti-oxidant potential followed by butanol fraction (ZFB) which showed IC50 of 171.90 ± 6.69 µg/mL ± SD in DPPH and 99.39 ± 0.19 µg/mL ± SD in ABTS. This indicates the comparatively low anti-oxidant potential of hexane fraction (ZFH), butanol fraction (ZFB) and Methanol fraction (ZFM). [Figure 5]

Table 5. Percentage radical scavenging activity of DPPH radical shown by the hydromethanolic fruit extract and its four fractions of *Zanthoxylum armatum* DC.

Parameters (µg/mL)	Percentage radical scavenging activity of DPPH radical					
	Abscorbic acid	ZF	ZFH	ZFE	ZFB	ZFM
100	86.72 ± 0.16	66.18 ± 0.16	39.13 ± 0.61	67.63 ± 0.33	15.16 ± 1.22	14.95 ± 0.61
80	86.43 ± 0.33	61.14 ± 0.33	36.45 ± 2.04	58.52 ± 0.60	8.81 ± 0.20	15.98 ± 0.00
60	86.62 ± 0.29	51.84 ± 0.16	29.09 ± 0.40	55.32 ± 0.44	1.22 ± 0.81	13.52 ± 0.40
50	86.53 ± 0.16	39.24 ± 0.29	18.44 ± 0.81	41.56 ± 1.81	0.40 ± 1.22	10.65 ± 0.40
40	54.74 ± 0.16	27.13 ± 1.17	13.11 ± 0.40	29.16 ± 1.02	0.00 ± 0.40	6.96 ± 0.81
20	42.53 ± 0.16	21.89 ± 0.16	11.27 ± 0.20	26.55 ± 0.73	6.14 ± 0.40	3.48 ± 0.20
10	23.83 ± 1.76	16.95 ± 0.33	6.96 ± 0.40	21.51 ± 0.29	7.17 ± 1.84	9.22 ± 0.61
IC50	32.22 ± 0.14	58.54 ± 0.08	118.47 ± 2.11	56.09 ± 0.29	171.90 ± 6.69	224.13 ± 1.07

All values are mean ± SD (n=3); ZF- *Zanthoxylum armatum* DC. hydromethanolic crude extracts of fruit; ZFH- hexane fraction of ZF; ZFE- ethyl acetate fraction of ZF; ZFB- butanol fraction of ZF; ZFM- methanol fraction of ZF. The standard anti-inflammatory drug diclofenac was used as positive control.

Table 6. Percentage scavenging activity of ABTS radical shown by the hydromethanolic fruit extract and its four fractions of *Zanthoxylum armatum* DC.

Parameters (µg/mL)	Percentage radical scavenging activity of ABTS radical					
	Abscorbic acid	ZF	ZFH	ZFE	ZFB	ZFM
100	95.21 ± 0.21	82.21 ± 0.07	49.28 ±0.57	89.07 ± 0.35	50.64 ± 0.21	37.64 ±1.21
80	94.85 ± 0.28	59.85 ± 0.14	35.07 ± 0.78	60.57 ± 0.57	29.42 ± 0.14	26.07 ±0.21

60	79.14 ± 0.14	30.5 ± 0.64	20.57 ± 0.14	35.71 ± 0.42	14.50 ± 0.07	16.35 ±1.92
50	51.78 ± 0.21	17.64 ± 0.35	7.64 ± 1.21	17.64 ± 0.92	5.35 ± 0.35	7.57 ± 0.14
40	26.00 ± 0.57	15.14 ± 0.14	1.14 ± 0.28	11.85 ± 1.28	2.92 ± 0.21	1.21 ± 1.21
20	13.50 ± 0.64	10.64 ± 0.35	-0.14 ± 1.71	6.57 ± 0.14	1.14 ± 0.85	-1.07 ±1.92
10	10.64 ± 0.5	9.71 ± 0.57	-3.71 ± 0.28	1.92 ± 0.21	0.92 ± 0.64	-2.92 ± 0.5
IC50	49.30 ± 0.09	73.28 ± 0.21	99.92 ± 2.07	71.49 ± 0.41	99.39 ± 0.12	120.92 ± 4.77
All values are mean ± SD (n=3); ZF- *Zanthoxylum armatum* DC. hydromethanolic crude extracts of fruit; ZFH- hexane fraction of ZF; ZFE- ethyl acetate fraction of ZF; ZFB- butanol fraction of ZF; ZFM- methanol fraction of ZF. The standard anti-inflammatory drug diclofenac was used as positive control.						

3.7. *In vitro* anti inflammatory activity

In vitro anti-inflammatory potential of the hydromethanolic fruit extract and its four fractions are shown in Table 7 & 8. The extract showed high anti-inflammatory potential both in heat induced haemolysis inhibition asssay and heat induced albumin denaturation inhibition assay with IC50 of 54.87 ± 0.00 µg/mL ± SD and 41.94 ± 0.48 µg/mL ± SD respectively. Standard anti-inflammatory drug, diclofenac showed IC50 of 58.86 ± 0.11 µg/mL ± SD in haemolysis assay and 47.90 ± 0.15 µg/mL ± SD in albumin assay respectively.

Ethyl acetate fraction (ZFE) showed highest anti-inflammatory potential in both heat induced haemolysis inhibition assay and heat induced albumin denaturation inhibition assay among four fractions with IC50 of 54.52 ± 0.12 µg/mL ± SD in haemolysis assay and 44.14 ± 0.24 µg/mL ± SD followed by hexane fraction (ZFH) which showed IC50 of 75.76 ± 0.57 µg/mL ± SD in haemolysis assay and 59.66 ± 0.24 µg/mL ± SD in albumin assay. Methanol fraction (ZFM) showed highest IC50 of 189.46 ± 3.58 µg/mL ± SD in haemolysis assay but in albumin assay, butanol fraction (ZFB) showed the highest IC50 of 90.72 ± 0.10 µg/mL ± SD. This indicates comparatively low anti-inflammatory potential of hexane fraction (ZFH), butanol fraction (ZLB) and Methanol fraction (ZLM). [Figure 5]

Table 7. Percent inhibition of RBC membrane lysis by the hydromethanolic fruit extract and its four fractions of *Zanthoxylum armatum* DC.

Parameters (µg/mL)	Percentage inhibition of RBC membrane lysis					
	Diclofenac	ZF	ZFH	ZFE	ZFB	ZFM
100	67.46 ± 0.41	62.19 ± 0.41	64.59 ± 0.41	55.97 ± 1.09	38.06 ± 1.09	28.94 ± 1.24
80	62.91 ± 0.41	62.67 ± 0.71	51.43 ± 0.41	53.82 ± 0.41	37.55 ± 0.00	29.42 ± 0.41
60	51.43 ± 0.41	55.02 ± 0.41	44.73 ± 0.71	51.43 ± 0.41	28.46 ± 1.49	26.07 ± 0.71
50	38.99 ± 1.89	45.21 ± 0.41	37.31 ± 0.41	48.80 ± 0.41	20.09 ± 0.82	17.70 ± 0.41

40	27.51 ± 0.71	33.02 ± 0.82	31.81 ± 2.15	33.96 ± 0.71	16.50 ± 1.09	16.98 ± 1.09
20	22.48 ± 0.71	28.94 ± 1.24	13.63 ± 0.41	26.79 ± 0.71	16.02 ± 0.71	16.02 ± 0.71
10	20.81 ± 1.09	24.40 ± 0.41	-702.41 ± 1.43	26.07 ± 0.72	15.54 ± 0.41	15.78 ± 0.41
IC50	58.86 ± 0.11	54.87 ± 0.01	75.76 ± 0.57	54.52 ± 0.12	130.00 ± 0.59	189.46 ± 3.58
All values are mean ± SD (n=3); ZF- *Zanthoxylum armatum* DC. hydromethanolic crude extracts of fruit; ZFH- hexane fraction of ZF; ZFE- ethyl acetate fraction of ZF; ZFB- butanol fraction of ZF; ZFM- methanol fraction of ZF. The standard anti-inflammatory drug diclofenac was used as positive control.						

Table 8. Percent inhibition of protein denaturation by the hydromethanolic fruit extract and its four fractions of *Zanthoxylum armatum* DC.

Parameters (µg/mL)	Percentage inhibition of protein denaturation					
	Diclofenac	ZF	ZFH	ZFE	ZFB	ZFM
100	69.98 ± 0.27	69.98 ± 0.27	49.44 ± 0.54	75.51 ± 0.27	53.08 ± 0.47	57.66 ± 0.72
80	69.03 ± 0.54	60.18 ± 0.94	48.97 ± 0.27	68.24 ± 0.47	46.44 ± 0.47	46.44 ± 0.47
60	61.13 ± 0.47	53.39 ± 0.54	48.34 ± 0.47	60.18 ± 0.47	44.86 ± 0.72	44.54 ± 0.47
50	51.50 ± 0.27	51.02 ± 0.27	47.39 ± 0.47	52.13 ± 0.47	44.54 ± 0.47	33.96 ± 0.72
40	44.39 ± 0.54	49.76 ± 0.00	41.70 ± 0.94	48.49 ± 0.27	44.23 ± 0.27	10.74 ± 0.27
20	41.54 ± 0.98	45.65 ± 0.54	33.96 ± 0.72	47.70 ± 0.72	43.44 ± 0.72	-0.78 ± 0.54
10	23.85 ± 0.98	38.70 ± 0.72	-4.10 ± 0.54	46.44 ± 0.47	44.23 ± 0.27	-4.10 ± 0.55
IC50	47.90 ± 0.15	41.94 ± 0.48	59.66 ± 0.24	44.14 ± 0.24	90.72 ± 0.10	86.32 ± 0.18
All values are mean ± SD (n=3); ZF- *Zanthoxylum armatum* DC. hydromethanolic crude extracts of stem; ZFH- hexane fraction of ZF; ZFE- ethyl acetate fraction of ZF; ZFB- butanol fraction of ZF; ZFM- methanol fraction of ZF. The standard anti-inflammatory drug diclofenac was used as positive control.						

4. DISCUSSION

In this study, phytochemical and pharmacological investigation of hydromethanolic fruit extract of *Zanthoxylum armatum* DC. and its four fractions are conducted. This study provides valuable insights into its potential medicinal properties and the findings are in line with several published reports, affirming the traditional use of fruit of this plant in herbal medicine. The preliminary phytochemical analysis of *Zanthoxylum armatum* DC. fruit extract revealed the presence of various important phytoconstituents such as alkaloids, flavonoids, phenolics, etc. which may be responsible for such varied pharmacological activities. In

fact, different parts of this plant has been reported to use in ethnopharmacology for many ailments throughout the glove such as against worm infestation, skin lotion, toothache and hence the name tootache tree. Powered dry fruit was used as tootpaste and tooth powder for its deodorising, santising and antiseptic nature. It showed bioactivity such as antioxidants, antinociceptives, antifungals, antinflammtory, hepatoprotective, pesticidal, antihelminitic, antiproliferative, cytotoxic, antimicrobial, antiviral, insecticidal/larvicidal, etc. (24,25,26). The fruit is rich in a variety of phytoingridients, phenolics, flavonoids, alkaloids, terpinoids, sterols, etc. with potential mentions in many formulations of Ayurveda, allopathy, general pharmacy, and other fields (27). In order to better understand the individual components responsible for various pharmacological effects and their underlying mechanisms of action, more attention should be placed on their screening, isolation, and characterisation. This is essential to forge the connection between the indigenous medicinal practices and contemporary scientific findings only then, the vast underlying potential of medicinal plants could be uncovered. Previously, *Z. armatum* DC. has been reported for its anticancer property and enhanced the therapeutic potential of cisplatin (37). Later, our earlier studies also isolated a phytoactive compound and identified as planispine A (a lignan) as the bioactive compound that enhanced the cisplatin induced apoptotic cell death (38) . It is consistant with our findings in this study as we also detected lignans in our GCMS and LCMS based metabolic profiling because lignan components are present, it is highly likely responsible for this plant's analgesic properties [24]. The results of this study are in agreement with anti-oxidant and anti-inflammatory potential of fruit which have been used by our forefathers. Overall, the comprehensive analysis of Zanthoxylum armatum DC. fruit extract gives the plant significant anti-oxidant and anti-inflammatory potential. These findings not only contribute to the understanding of the plant's therapeutic properties but also provide a basis for future research aimed at harnessing its medicinal benefits for the development of novel drugs and therapeutic interventions. Further studies exploring the mechanisms of action and safety profiles of the identified bioactive compounds are warranted to validate their therapeutic utility in clinical settings.

5. CONCLUSION

The findings of this study contribute to the growing evidence supporting the medicinal potential of fruit of Zanthoxylum armatum DC. The comprehensive phytochemical profiling and pharmacological evaluation provide a solid foundation for further research aimed at exploring its therapeutic applications. Further research exploring the bioactivity guided compound isolation, the mechanisms of action, safety profiles, and clinical efficacy of these compounds is warranted to fully harness their therapeutic benefits and translate them into clinical applications. The significance of this research lies in its contribution to expanding our understanding of the medicinal properties of the fruit of Zanthoxylum armatum DC., paving the way for their integration into pharmaceutical and biomedical practices for improved healthcare outcomes. The diverse phytochemical profile, coupled with demonstrated anti-inflammatory and antioxidant activities, positions Z. armatum DC. as a valuable natural resource with considerable therapeutic potential.

Credit and author contribution statement

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All authors have read and agreed to the published version of the manuscript.

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Declaration of copeting interest
All authors declare no conflict of interest.

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Figure legends

Figure 1: Collection, authentication and processing of *Zanthoxylum armatum* DC. (A) whole plant. (B) shade dried fruit. (C) a specimen submitted to the herbarium at Plant Systematics and Conservation Laboratory, Plant Bioresources Division, Institute of Bioresources and Sustainable Development (IBSD), Imphal.

Figure 2: (A) TPC- total phenolic content (in µg GAE/mg of DW) (B) TFC- total flavonoid content (in µg/mL of QE/mg of DW) of hydromethanolic fruit extract, ZF and its four fractions of *Zanthoxylum armatum* DC. ZFH- hexane fraction of ZF, ZFE- ethyl acetate fraction of ZF, ZFB- butanol fraction of ZF and ZFM- methanol fraction of ZF, n= 3. (C) Standard calibration curve of gallic acid. (D) Standard calibration curve of quercetin.

Figure 3: (A) GCMS chromatogram of hydromethanolic fruit extract (ZF) of *Zanthoxylum armatum* DC. (B) GCMS chromatogram of ethyl acetate fraction (ZFE) of hydromethanolic fruit extract of *Zanthoxylum armatum* DC.

Figure 4: (A) LCMS chromatogram of hydromethanolic fruit extract (ZF) of *Zanthoxylum armatum* DC. (B) LCMS chromatogram of ethyl acetate fraction (ZFE) of hydromethanolic fruit extract of *Zanthoxylum armatum* DC.

Figure 5: (A) DPPH radical scavenging activity, (B) ABTS total anti-oxidant activity, (C) Heat induced RBC membrane lysis inhibition activity (D) albumin anti-denaturation activity of hydromethanolic fruit extract and its four fractions of *Zanthoxylum armatum* DC. ZF= *Zanthoxylum armatum* DC. hydromethanolic crude extracts of fruit, ZFH- hexane fraction of ZF, ZFE- ethyl acetate fraction of ZF, ZFB- butanol fraction of ZF, ZFM- methanol fraction of ZF, n= 3.

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