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PHYTOCHEMICAL STUDY, ANTIBACTERIAL, ANTIOXIDANT AND ANTIINFLAMMATORY ACTIVITY OF FRUITS OF ZANTHOXYLUM ARMATUM

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ABSTRACT

This study examines the phytochemicals of Zanthoxylum armatum, a medicinal plant known for its antibacterial, antioxidant, and anti-inflammatory properties. The plant, also known as Timur in Nepal, is used in indigenous Asian practices. Its therapeutic efficacy is attributed to its use in fruits, leaves, stems, bark, and roots. The study utilized solvents like chloroform, ethanol, and methanol for extracting plant fruit, followed by hydro distillation for essential oils. The phytochemicals were analyzed qualitatively,with antioxidant properties assessed using DPPH free radical scavenging, antibacterial activities determined using disc diffusion, and anti-inflammatory activity determined using human RBSs membrane stabilization. The study reveals the presence of alkaloids, steroids, flavonoids, terpenoids, tannins, and saponins in methanolic extracts, while carbohydrates are absent, and these compounds are also present in ethanolic extracts. The different solvent extracts showed ZOI for all the tested bacteriaat the range of 6-11 mm. The antibacterial potency of the extracts was found to be concentration-dependent. The bacterial activity of essential oil against S. typhi and S. aureus was found to be more sensitive than Azithromycin and Ciprofloxacin. The bacterial activity of methanolic extracts of Z. armatum against KLE was found to be more sensitive than Azithromycin and Ciprofloxacin. The chloroform extract of Z. armatum exhibited low antioxidant activity, while the ethanolic

and methanolic extracts exhibited high antioxidant activity and good anti-inflammatory potency, respectively. Hence, essential phytochemicals with antibacterial, antioxidant, and anti-inflammatory qualitieswere discovered in the study, laying the groundwork for additional investigation and the creationof new drug entities.

KEYWORDS: *Zanthoxylum armatum,* Phytochemical study, Antibacterial activity, Anti-oxidant, Anti-inflammatory activity.

1. INTRODUCTION

Herbal medicine, a form of medicine derived from plants, utilizes their medicinal properties to treat various ailments.

Plants have been used for medicinal purposes since ancient times, with ancient Chinese and Egyptian papyrus writings mentioning their use as early as 3,000 BC. Indigenous cultures like African and Native American used herbs in healing rituals, while traditional medical systems likeAyurveda and Traditional Chinese Medicine utilized herbal therapies.[1]

Herbal medicine is described as a method that uses herbs, herbal materials, preparations, and final products that include active substances derived from plants or other plant materials by the World Health Organization (WHO).[2]

With its interdisciplinary medicinal and therapeutic value, Zanthoxylum armatum plays a significant role in this area.

1.1.Plant Profile[3,4]

- Kingdom: Plantae
- Phylum: Streptophyta
- Class: Equisetopsida
- Subclass: Magnoliidae
- Order: Sapindales
- Family: *Rutaceae*
- Genus: *Zanthoxylum*
- Species: armatum
- Common name: Timur in Nepal, Rattan pepper in English

Zanthoxylum armatum, a prickly shrub in the Rutaceae family, is referred to as Timur in Nepal. This plant is indigenous to parts of Southeast Asia, including Korea and Japan, and stretches fromPakistan. It usually reaches a height of around 3.5 meters, and its characteristic thorny branchesand fragrant qualities define it.[3]

1.2.Features of Zanthoxylum armatum[3,5]

- Leaves: opposite, lanceolate to obovate, with branchlets bearing spines.
- Flowers: Usually flowering from March to May, these green to yellow blooms are found inthick panicles.
- Fruits: Reddish-purple follicles with a diameter of around 4-5 mm that hold 4-5 mm black seeds.

  Zanthoxylum armatum is valued not only for its culinary uses but also for its medicinal properties, making it a versatile plant in various cultural and traditional contexts. The plant is known for its aromatic berries, which are sometimes used in traditional Ayurvedic and Chinese medicine practices. It's important to handle the plant with care due to its thorny branches.[3]

1.3.Traditional Uses of Zanthoxylum armatum

A wide range of conditions have been treated using zanthoxylum armatum, including.

- Management of Fever: Because of its actions on inflammatory mediators, it is also used as an antipyretic, which helps to reduce fever.[6]
- Inflammation and Pain: The plant is historically used to treat inflammation and pain, and studies have shown that its extracts have strong analgesic and anti-inflammatory properties.[6,10]
- Oral Health: Dental conditions like toothaches and bleeding gums have been treated withthe herb.[8]
- Digestive Disorders: Its carminative qualities are well-known for helping with dyspepsia and other digestive problems.[6]
- Treatment of Respiratory Conditions: Conventional uses involve the management of asthmaand other respiratory disorders.[9]

1.4. Pharmacological activities of Zanthoxylum armatum[9,11]

Zanthoxylum armatum contains a variety of bioactive substances, including flavonoids, essential oils, and alkaloids, which are responsible for some of its therapeutic qualities,

according to phytochemical studies. Important pharmacological tasks consist of.

- **Antioxidant Properties**: The plant has strong antioxidant activity that helps lessen damage caused by oxidative stress.
- **Antibacterial Effects:** Extracts are efficient against a variety of diseases due to their documented antibacterial characteristics.
- **Hepatoprotective Activity**: Research suggests that Zanthoxylum armatum may shield the liver from toxicity-related liver damage.
- **Antifungal and Antiparasitic Activities**: The plant's antifungal and antiparasitic propertieshave been demonstrated, confirming its use in traditional medicine as a treatment for diseasescaused by specific parasites and fungi.

1.5. Chemical constituents of Zanthoxylum armatum[12,13]

- **Alkaloids:** Known for their biological properties, alkaloids are abundant in the plant. Prominent alkaloids consist of: Magnoflorine, Xanthoplanine, Dictamnine, and Fragarine
- **Essential Oils**: Several important components may be found in the essential oil that is derived from the leaves and fruits. Fruit essential oils contain linalool (73.57%), D-limonene(10.7%), and eucalyptol (29.65%) in their essential oils.
- **Phenolic Compounds:** These substances, which include flavonoids and several phenolic acids, are linked to antioxidant qualities.
- **Other constituents**: α-copaene, γ-terpinene, camphene, limonene, β-ocimene, trans-caryophyllene, α-terpinolene, and germacrene are some of the other components.
- **Additional chemicals**: The plant also has carbonyl and resinous chemicals, which might beresponsible for some of its taste and therapeutic properties.

1.6.Statement of Problem

Antibiotic resistance, oxidative stress-related diseases, and inflammatory disorders are posing significant public health challenges worldwide. In 2019, antimicrobial resistance caused at least 1.27 million deaths and 2.8 million infections in the US, resulting in over 35,000 deaths. Antibiotics are effective in killing microorganisms but can cause side effects like bloating and abdominal pain.

Traditional medicine has been valuable in discovering new bioactive compounds to address these health issues. Zanthoxylum Armatum, also known as Toothache tree or Timur, is an indigenous plant with medicinal properties.[13,14]

This research aims to investigate the antibacterial, antioxidant, and anti-inflammatory activities of Zanthoxylum Armatum fruit, potentially leading to the development of new natural remedies or drug leads for various health conditions.

1.7.Rationale of the Study

Nepal, a country with diverse natural resources and biodiversity, has a significant impact on phytochemicals. The number and percentage of chemicals in plants vary greatly depending on location, altitude, storage conditions, and growth period. This is true for plants of varying ages and storage conditions.

Modern research should focus on the chemical content of plants, their proportion, and the best cultivation methods to maximize yield. Zanthoxylum armatum, a plant with various chemical compounds, is used to scavenge free radicals and has potential applications as an antioxidant, anti-inflammatory, antimicrobial, and antioxidant.

2. MATERIALS AND METHOD

2.1.Plant Materials

The fruits of *Zanthoxylum armatum* was collected from the local market of Lalitpur, Nepal. Herbarium of both the plant and seeds were prepared and taken to the National Department of Herbarium for Identification in Godawari, Lalitpur, Nepal.

2.2.Extraction Process[11]

The fruits of Zanthoxylum armatum were cleaned and shade-dried for 1-2 weeks, then pulverized into coarse powdered form.

The powdered fruit materials were extracted using gradient extraction methods, including chloroform, ethanol, and methanol. The powdered samples were loaded into a soxhlet apparatus and extracted using the Hot Soxhlet Extraction method.

The apparatus was run for 17 hours until the crude extracts appeared in a siphon. The dried extracts were packed and kept in a refrigerator at 2-8°C for further examination after the solvents were evaporated in a hot water bath at about 80-90°C.

2.3.Isolation of essential oil

Using Clevenger equipment, freshly powdered fruit samples were hydro-distillated for four to five hours. After obtaining the essential oil, it was promptly sealed and stored at 4°C in a

glass vial for further examination.

2.4. Phytochemical studies[15]

The tests below are carried out to screen for phytochemicals.

a. Test for alkaloids

A few milligrams of residue from each extract were extracted and filtered separately in 5ml of 1.5% v/v hydrochloric acid. The alkaloids were then tested using the filtrates.

- **Mayer's Test:** 1 ml of Mayer's reagent (potassium mercuric iodide) was added to 2 ml of filtrate. The presence of alkaloid is indicated by the production of a yellow-colored precipitate.
- **Wagner's Test:** A small amount of Wagner's reagent (potassium iodide) was added to 2ml of filtrate. The emergence of a brownish-yellow precipitate signifies the existence of alkaloids.
- **Hager's Test**: A few drops of Hager's reagent (saturated picric acid solution) are added to 2 ml of filtrate. The presence of alkaloids is indicated by the production of a yellow precipitate.

b. Detection of flavonoids

- **Shinoda Test**: A few drops of concentrated hydrochloric acid (HCL) and 0.5 grams of magnesium turnings were added to the extract from the test tube's side until the formation of a pink appearance which suggested the presence of flavonoids.
- **Alkaline Reagent Test**: 2 ml of each extract was mixed with a few drops of sodium hydroxide solution. The presence of flavonoids is indicated by the production of a bright yellow hue that becomes colourless when a few drops of diluted acetic acid are added.

c. Test for Steroids

- **Salkowski's Test**: A few mg of each extract's residue was added to 2 ml of chloroform, and then 2 ml of concentrated sulfuric acid was added from the test tube's side. A few minutes were spent shaking the test tube. The presence of sterols was shown by the chloroform layer becoming red.

d. Detection of Tannins

The test residue of each extract was taken separately in water, warmed and filtered. Testswere carried out with the filtrate using the following reagents.

- **Ferric Chloride Test:** 90% alcohol was mixed with 5% w/v ferric chloride. A tiny bit of

the filtrate mentioned above was mixed with a few drops of this solution. Tannins are present if a vivid green or blue hue is achieved.

- **Gelatin Test:** A 1% w/v solution of gelatin in water was made, and 10% of the solution included sodium chloride. The filtrate was mixed with a small amount of this solution. Tannins are present if a white precipitate forms.

e. Detection of Carbohydrate

- **Molisch's Test:** 2 drops of Molisch's reagent were added to a test tube containing 0.5 ml of water and a few milligrams of the test residue. 1 ml of concentrated sulfuric acid was added to this solution from the side of the angled test tube, forming a layer beneath the aqueous solution without interacting with it. The presence of carbohydrates is indicated by the appearance of a reddish-brown ring at the intersection of the two layers.
- **Fehling's Test:** Just before usage, equal quantities of Fehling's solutions A and B were combined. A small amount of Fehling's solution was added to a small amount of the test residue that had been dissolved in water. This mixture was then warmed until a red precipitate of cuprous oxide was obtained which indicates reducing sugars are present.

f. Detection of Saponins

- **Foam Test:** A small amount of sodium bicarbonate and water were mixed with a few milligrams of the test residue in a test tube, and the mixture was agitated violently. Saponins are present, and it is a stable, distinctive foam that resembles a honeycomb.

g. Detection of Terpenoids

5 ml of each extract was combined with 2 ml of chloroform and 3 ml of concentrated sulfuric acid, then slowly added to create a layer. There is a terpenoid present because a layer of reddish-brown hue has formed at the contact.

2.5. Antimicrobial Activity[11]

Preparation of test sample: The Methanolic and Ethanolic extracts were used as the test sample. The antimicrobial activity was screened by using the disc diffusion method.

Bacterial Strains: For the antibacterial assay, gram-positive (Staphylococcus aureus) and gram-negative (Escherichia coli, Salmonella typhi, and Klebsiella pneumoniae) bacteria were used. Throughout the investigation, the bacterial cultures were kept in nutritional broth at 4°C, and thisbroth served as the stock solution.

Preparation of bacterial suspension: Ethanol and methanol extracts were soaked on WhatmanNo. 1 filter paper to create the sterile disk. Muller Hinton agar was used to disperse the bacterialstrains S. aureus, S. typhi, E. coli, and KLE. The bacterial lawn was covered with the extract- loaded discs, which were then incubated for 24 hours at 37°C. Within 24 hours, ZOI was measured using a scale.

2.6. Antioxidant activity

a. DPPH Radical Scavenging Activity[16]

Preparation of DPPH solution: 3.94 mg of DPPH were measured in an analytical balance and dissolved in methanol to create 0.1mM DPPH. The final volume was created with methanol up to 100 ml.

Preparation of test and standard solution: 100 mg of each of the following were dissolved in 100 mL of ethanol to create a stock solution of the fruit extract in methanol, ethanol, chloroform, and standard ascorbic acid. All of the seed extracts were produced from their corresponding stock solutions at different concentrations of 10 mcg/ml, 20 mcg/ml, 40 mcg/ml, 80 mcg/ml, and 100 mcg/ml, along with standard ascorbic acid.

DPPH radical scavenging assay: In a test tube, 3 ml of DPPH and 1 ml of the test or standard of the appropriate concentrations were combined, and the mixture was incubated in the dark for about 30 minutes. A UV-visible spectrophotometer was used to measure the absorbance(abs) ofeach test sample and the standard amounts at 517 nm.

Next, the DPPH Radical Scavenging activity was computed using the following formula,

$$DPPH\ Radical\ Scavenging\ Assay = \frac{\text{abs of control} - \text{abs of test or standard}}{\text{abs of control}} \times 100$$

Where Methanol was taken as a blank and
(3 ml DPPH + 1ml methanol) was taken as a control

2.7.Anti-inflammatory activity

a. HRBC membrane stabilization method[17]

Preparation of HRBC suspension: Before the experiment, volunteers in good health who had not taken any NSAIDS for around two weeks had their fresh human blood drawn. Then, it was combined with an equivalent volume of Alsever's solution, which contained 100 milliliters of distilled water, 0.8% sodium citrate, 0.05% citric acid, and 2% dextrose.

Following that, the mixture was centrifuged for around ten minutes at 3000 rpm. The cells were packed and repeatedly rinsed with 0.85% isosaline (0.85g of NaCl in 100 mL water, autoclaved for 15 minutes at 121°C, and chilled to room temperature). A 10% suspension was then prepared.

Preparation of test and standard solution: A stock solution containing fruit extract in methanol, ethanol, chloroform, and standard aceclofenac was created by dissolving 100 mg of each substance in a small amount of methanol. Distilled water was then added to bring the volume up to 100 mL. All fruit extracts were prepared from their corresponding stock solutions at different concentrations of 100 mcg/ml, 300 mcg/ml, and 500 mcg/ml along with standard ascorbic acid.

HRBC membrane stabilization assay: After mixing 1 mL of the sample, 1 mL of phosphate buffer, 2 mL of 0.36% hyposaline (0.5 mL of dissolved NaCl in 100 mL of water, autoclaved for 15 minutes at 121°C, and then cooled to room temperature), and 0.5 mL of HRBC suspension, the mixture was incubated for 30 minutes at 37°C before being centrifuged at 3000 rpm. Using a spectrophotometer set at 560 nm, the amount of haemoglobin in the supernatant solution was calculated. The following formula was used to get the protection percentage.

$$\%\ of\ Protection\ = \frac{\text{OD of test solution}}{OD\ of\ control} \times 100$$

Test Control: 1 mL of sample + 1 mL phosphate buffer + 2 mL DW (instead of hyposaline toproduce 100% hemolysis) + 0.5% of HRBC suspension.

3. RESULTS AND DISCUSSION

3.1. Extractive value

The study analyzed the phytochemical screening, antibacterial, antioxidant, and anti-inflammatory properties of Zanthoxylum armatum fruits extracted from different solvents, Methanol, Ethanol, and Chloroform. The extracts' weights were 3.71 gm, 5.05 gm, and 4.13 gm, respectively.

Table 1: Extractive Yield of fruit extract in different solvents.

S.N	Solvent	Sample Weight (gm)	Weight of extract (gm)	% Yield
1	Methanol	35	3.71	10.6
2	Ethanol	35	5.05	14.42
3	Chloroform	35	4.13	11.8

3.2.Phytochemical Screening

The extracts of Methanol, Ethanol, and Chloroform underwent phytochemical screening to identify secondary metabolites such as alkaloids, steroids, flavonoids, terpenoids, tanins, carbohydrates, and saponins.

3.3.Antibacterial activity

The antibacterial activity of *Zanthoxylum armatum* fruit extracts and its oil were done bydisc diffusion method.

3.4. Antioxidant activity

The anti-oxidant property of *Zanthoxylum armatum* fruit exract was observed by DPPH free radical scavenging activity. The methanolic, ethanolic and chloroform extracts of *Zanthoxylum armatum* at various concentrations such as 10, 20, 40, 80, 100 mcg/ml was estimated with Ascorbic acid as standard.

3.5. Anti-inflammatory activity

The anti-inflammatory activity of *Zanthoxylum armatum* fruit extracts was observed invitro using HRBC membrane stabilization method. The methanolic, ethanolic, chloroform extracts of *Zanthoxylum armatum* at various concentrations such as 100, 300, 500 mcg/ml was estimated with Aceclofenac as standard.

Table 2: Phytochemical screening of fruit extract of Zanthoxylum armatum.

S.N	Test	Methanol	Ethanol	Chloroform
1	Alkaloid Test **a.** Mayor's test **b.** Wagner's test **c.** Hager's test	 + + +	 + + +	 + + +
2	Test for steroids a. Salkowski test	 +	 +	 -
3	Test for flavonoid **a.** Shinoda test **b.** Alkaline test	 + +	 - -	 - -
4	Test for terpenoid	+	+	+

5	Test for saponin a. Foam test	+	+	+
6	Test for tanins a. Ferric chloride test	+	-	-
7	Test for carbohydrate **a.** Molish test **b.** Fehling test	- -	- -	+ +

(+): Positive(-): Negative

Table 3: Antibacterial activity of methanolic, ethanolic extracts and oil in E.coli, S.typhi, S.aureus and Kle.

Bacteria	Concentration(mg)	Zone of Inhibition(mm)		
		Methanol	Ethanol	Oil
E.coli	100	9	8	11
S.typhi	100	7	6	16
S.aureus	100	9	10	14
Kle	100	11	9	9

Table 4: Antibacterial activity of Azithromycin and Ciprofloxacin as positive control in different bacteria.

Name of Bacteria	Zone of Inhibition(mm)	
	Azithromycin (30 mcg)	Ciprofloxacin (5 mcg)
E.coli	18	25
S.typhi	10	23
S.aureus	17	12
Kle	5	6

Table 5: Antibacterial activity of Methanol and ethanol as negative control in different bacteria.

Name of Bacteria	Concentration(mg/ml)	Zone of Inhibition(mm)	
		Methanol	Ethanol
E.coli	100	5	5
S.typhi	100	5	11
S.aureus	100	5	12
Kle	100	5	13

Table 6: DPPH Free radical scavenging assay.

SN	Concentration (ppm)		Inhibition %		
		Methanol	Ethanol	Chloroform	Ascorbic acid
1	10	33.14	35.15	17.86	42.36
2	20	37.75	40.34	19.16	58.06
3	40	46.25	48.55	23.19	60.66
4	80	64.26	64.84	30.40	88.04
5	100	73.91	71.75	35.01	98.41
	IC50 (mcg)	47.63	44.74	180.79	16.73

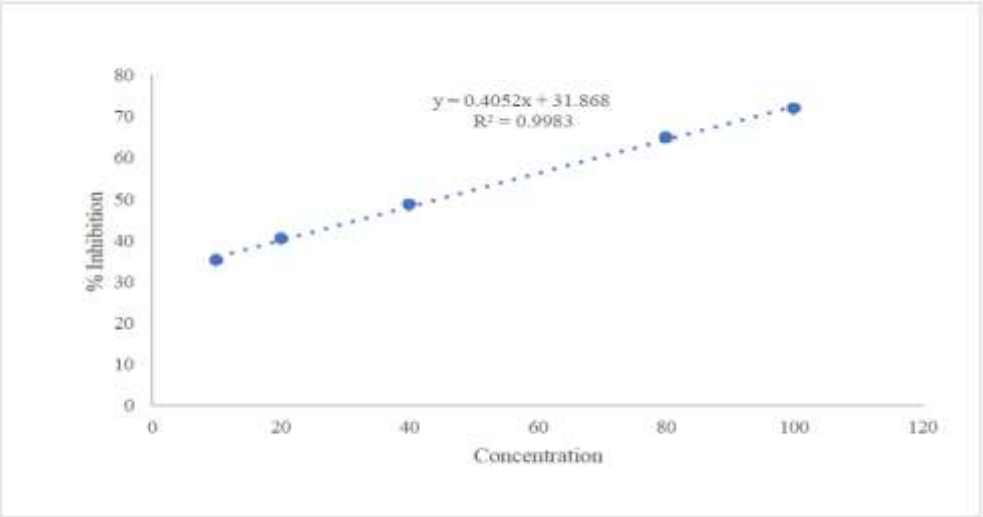

Figure 1: Correlation curve of scavenging activity(%) of Ethanol via DPPH.

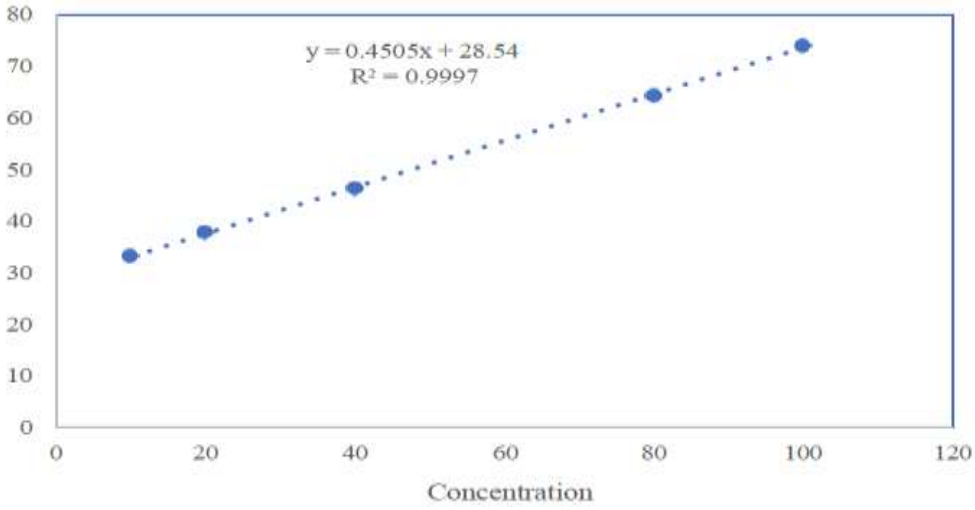

Figure 2: Correlation curve of scavenging activity(%) of Methanol via DPPH.

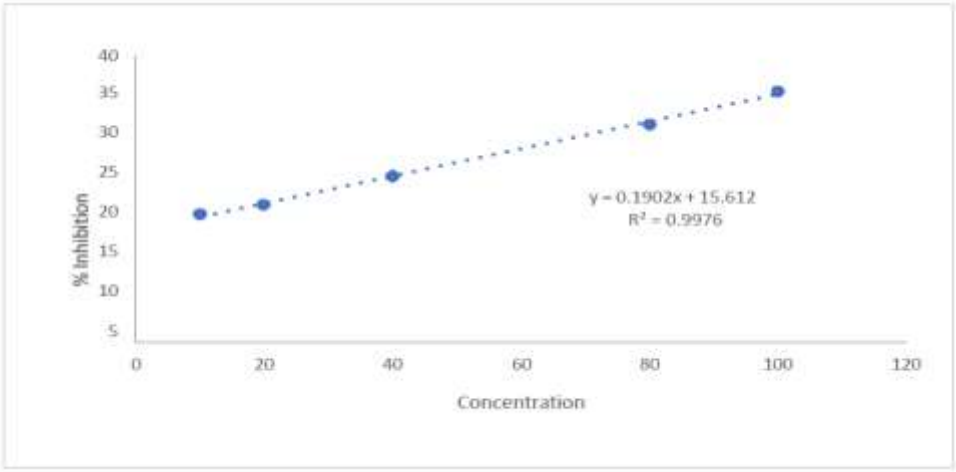

Figure 3: Correlation curve of scavenging activity(%) of Chloroform via DPPH.

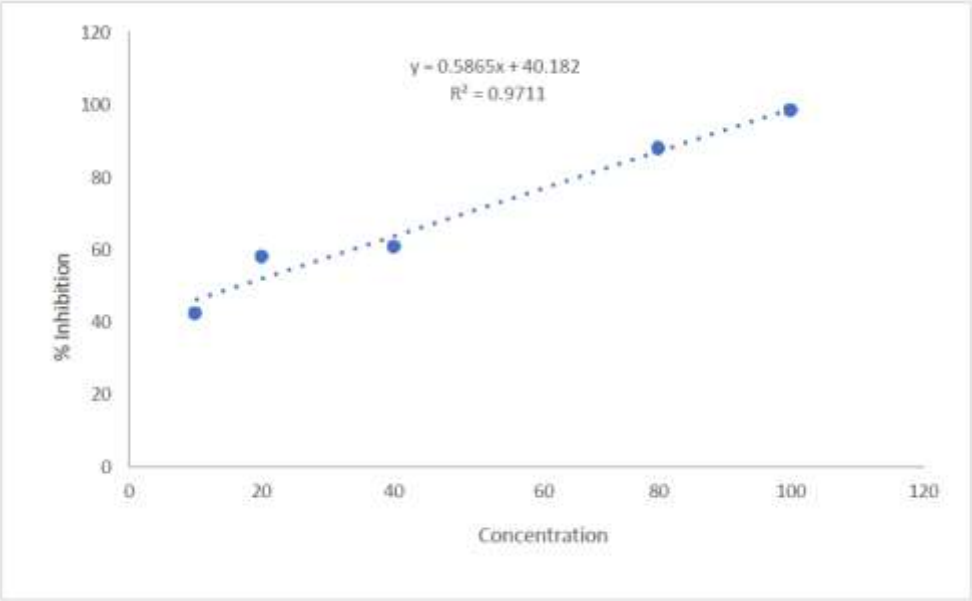

Figure 4: Correlation curve of scavenging activity(%) of Standard Ascorbic acid via DPPH.

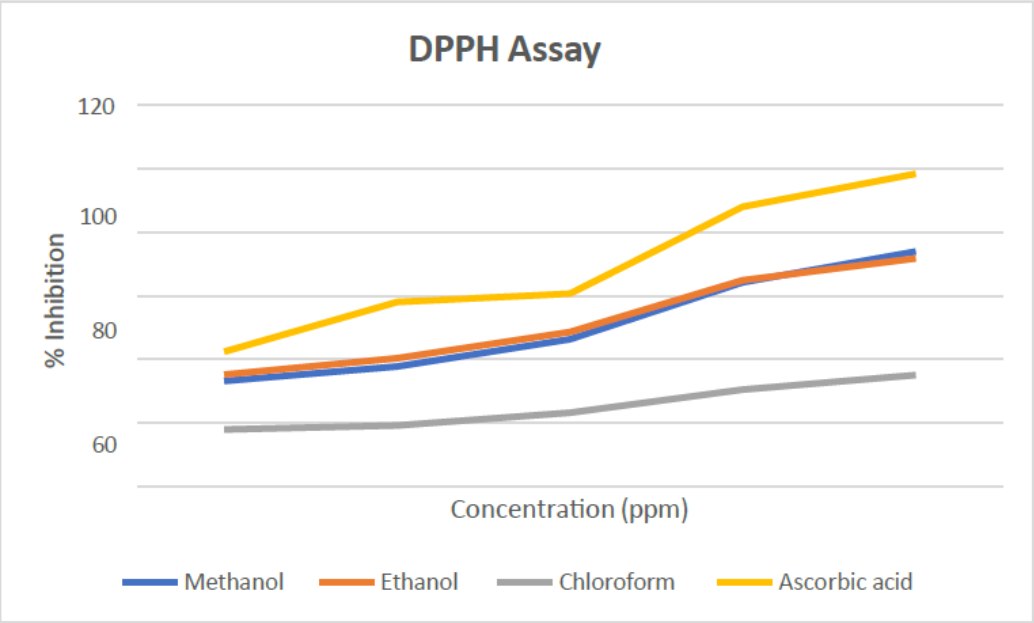

Figure 5: Inhibition % of methanol, ethanol and chloroform extract with reference to standard (ascorbic acid).

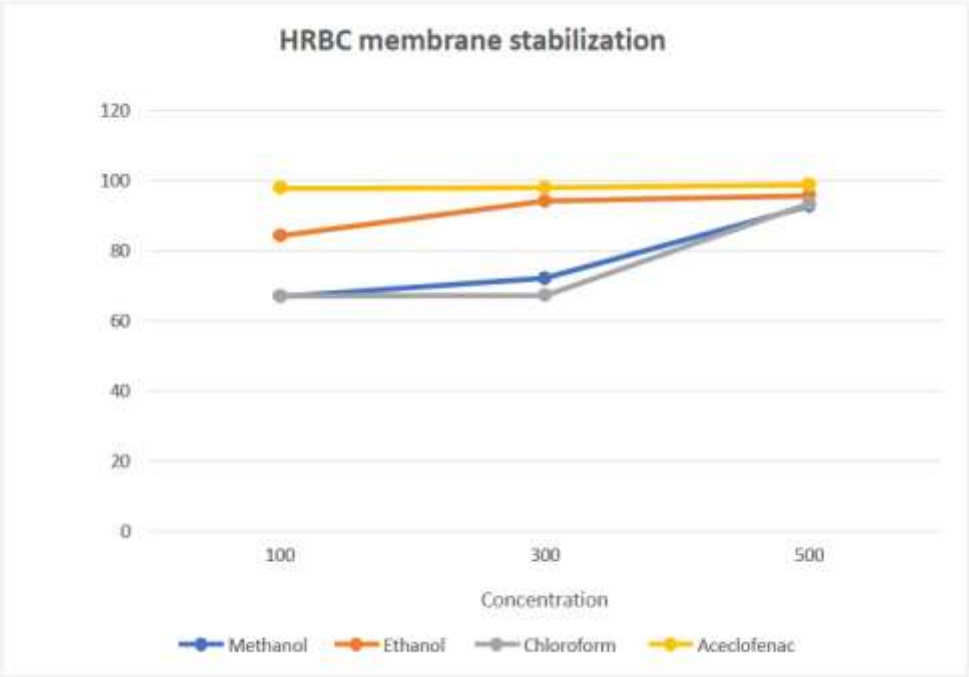

Figure 6: Protection percentage of methanol, ethanol, and chloroform extract with reference to standard(aceclofenac).

3.6. DISCUSSION

Proximate analysis: The percentage yield value of the methanol, ethanol, and chloroform extracts from Zanthoxylum armatum fruits was determined to be 10.6, 14.42, and 11.8%, respectively. The percentage yield value of the methanol extract produced is higher than the 9.1% achieved by Karmakar et al. usingchloroform, and it is comparable to the result obtained by Karmakar et al. using methanol.[18] The yield value gained as a percentage is likewise higher than the value obtained with ethanol (6.67%) by Nitin Verma et al.[19]

Phytochemical Screening

From the study conducted by Joshi et al., the methanolic extract of *Z.armatum* fruits confirms the presence of various secondary metabolites like alkaloids, flavonoids, saponins and tannins.[11] Similarly in our study, *Z.armatum* fruits that were extracted from methanol showed the presenceof alkaloids, saponins, flavonoids, terpenoids, steroids and tannins.

Antibacterial activity

The antibacterial efficacy of Zanthoxylm armatum fruits was examined by the utilization of several solvent extracts, including methanol and ethanol, as well as its essential oil. All the extracts and essential oils against specific gram +ve (S. aureus) and gram -ve (Kle, E. coli, and S. typhi) bacteria showed different degrees of Zone of Inhibition.

The ethanolic extract of Zanthoxylum armatum was shown to have a ZOI of 7 mm against Staphylococcus aureus in research by Bishnu Joshi et al.[20]

In contrast, our investigation revealed a much higher ZOI of 10 mm against S. aureus.

Antioxidant activity

According to the Phuyal et al. study, the DPPH test revealed that the standard ascorbic acid's IC50 value was 36.22 mcg/ml. The cultivated and wild fruits showed IC50 values of 40.62 µg/mLand 45.62 µg/mL, respectively, indicating their exceptional antioxidant activities. They said that the high concentration of total phenolic and total flavonoids found in Z.armatum fruits accountsfor their strong antioxidant activity.[21]

Anti-inflammatory activity

According to Chaitanya et al.'s study, membrane hemolysis reduced and membrane stabilization/protection rose with increasing concentration. This led to the conclusion that the extract's anti-inflammatory properties depended on concentration.[22]

Our results also support the findings of the aforementioned study, which showed that membrane stability and protection increased while membrane hemolysis decreased with concentration. At 500 ppm, the ethanolic extract of Z. armatum fruit had the highest percentage of protection (95.67%), followed by the chloroform extract (93.41%). At 500 ppm, the ethanolic extract demonstrated a significantly lower percentage of protection—92.80%. In our investigation, the typical Aceclofenac had a protective value of 98.82% at 500 ppm.

4. CONCLUSION

The study reveals the presence of alkaloids, steroids, flavonoids, terpenoids, tanins, and saponinsin methanolic extracts, while carbohydrates are absent. The antibacterial potency of the extracts was found to be concentration-dependent, with the essential oil of Z. armatum being more sensitive than other solvent extracts. Methanol was found to be more sensitive against Klebsiellaand S. aureus.

The chloroform extract of Z.armatum showed low antioxidant activity, while the ethanolic and methanolic extracts showed high potency. The anti-inflammatory activity of the extract was concentration-dependent, with an increase in concentration.

The results suggest the potential of plants as a source of bioactive natural products and drugs to reduce multidrug resistance, inflammation, and synthetic antioxidants. The study suggests the potential of plants as a potential source of bioactive natural products to combat multidrug resistance, inflammation, and synthetic antioxidants.

5. LIMITATIONS

i. The research ignored the possible advantages of utilizing the plant's bark, seeds, and leavesin favor of concentrating just on the fruits of the plant.
ii. Only a small number of characteristics were included in the proximate analysis, which mayhave left out other crucial elements.
iii. The study only took into account four different kinds of bacteria: E. Coli, Salmonella typhi, S. aureus, and Kle. It did not assess the possible impacts on other strains of bacteria.
iv. Because antibacterial activity was only evaluated once, it was not possible to determine theplant's relative effectiveness over time or against other microorganisms.
v. No comparison of the plant's antibacterial activity with other plants or variants from thesame plant grown in different places or climates was made in this study.

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