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Research Article

Evaluation Of Antioxidant And Antibacterial Activity Of *Zanthoxylum Armatum*: A Research

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ABSTRACT

The genus Zanthoxylum of the family Rutaceae is economically important due to its nutritional, industrial and therapeutic values. Z. armatum is a short tree or a shrub that grows under dry conditions. The current examination manages the full scale concentrates just as investigations of assessment of pharmacognostical, phytochemical, antioxidants and antibacterial movement of Zanthoxylum armatum leaves some particular characters were noticed while contemplating their cross over areas. Physiochemical and primer phytochemical investigations of the leaves of plant Zanthoxylum armatum were additionally done. The antioxidants assay performed by various methods like DPPH, nitric oxide, hydrogen peroxide. The extract of the plant show various effects as antioxidants on various concentrations and showed graphically. The antibacterial examinations affirmed that the ethanolic remove was very compelling for Staphylococcus aureus, Bacillus subtilis, E. coli and Pseudomonas aeruginosa at 25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 500 µg/ml and tetracycline (100 µg/ml) individually. The current investigation may be helpful to enhance data with respect to its distinguishing proof boundaries expected essentially in the method of adequacy of home grown medications in the current situation lacking administrative laws to control nature of natural medications and furthermore to discover the antioxidants and antibacterial action.

INTRODUCTION

Medicinal property of plants are comes in highlight during ancient period. During Ancient times for rescue of diseases, people depend on nature. At that time they are not aware about medicinal nature of plants. Hence we can say that healing with medicinal plants as old as mankind itself. Human his research for medicine from plants dates from the far past. Human struggles for many years to learned about therapeutic nature of many plants. Contemporary science has acknowledged their active action, and it has included in modern pharmacotherapy, known by

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Ancient Civilization and used throughout millennia. Medicine from plants can derived from different plant parts i.e. leaves, roots, bark , fruits, seeds, flower. The different plant parts contain different-different ingredients within a plant. Hence, one part of the plant may be toxic while another part of the same plant harmless. Plants are an important source of medicine and plays important role in world health. Many countries in the world that is two third of the world population depends on herbal medicine for primary health care. In India and other developing countries herbal or medicinal plants use to cure the therapeutic curative diseases. India is mainly the largest country all over the world which has highest growth of herbal plants which may have medicinal properties and also used for preparation of herbal drugs. Around 20,000 of herbal plants may exist in India which may have registered in Indian system of Ayurveda. Approx7500 plants are scientifically proved as medicinal drugs [1]. In India around 2500 herbal plants which used for the different formulation of herbal products. Lots of 7800 manufacturing company practitioners worked on herbal plants by using traditional system of India. India has been proved its therapies by scientific researches. Herbal medicines may have the dietary supplements which may have used as diet food for the good health. There are many ingredients included in dietary supplements such as minerals, vitamins, herbs, amino acids and other supplements, antioxidants, macronutrients, tonics and some of herbal preparation also involves such as Muslipak, Chyawanprash, Ashwagandha etc. They administered as oral pills, capsules, tablets, syrups etc. Some of botanicals found in any forms such as dried food, liquid or solid extracts, tablets, capsules etc. Functional foods of herbal remedies may have flour, oil, probiotics food etc. which may reduce the risk factors of chronic diseases [2, 3]. Zanthoxylum armatum is also known as winged prickly ash or timru. It belongs to the family Rutaceae. It is an aromatic, deciduous, spiny and shrubs. Timru is an evergreen, small tree and height 6m. Leaves are around 20 cm long, aromatic and compound. Fruits are reddish in colour and around 5 mm in diameter [4]. Flowers are green to yellowish colour. Seeds are shiny and bitter. Fruits, seeds and bark and are used as cure of dyspepsia and fever. Fruits and seeds are useful for dental trouble thus many kind of dental paste and powder are made from it. It has many antiseptic properties. Mainly found in Himalayas Warmer valley 1000 to 2100 m from the sea level and moves from Jammu Kashmir to Assam. Synonyms of Z. armatum are Z. planispinum, Timru, Timber, Toothache tree, winged prickly ash. Found in India, Meghalaya, Mizoram and Manipur [5]. There are 11 species and genus of the Z.armatum that mainly found as medicinal plants, Z. budrunga, Z. oxyphyllum, Z. ovalifolium, Z. acanthopodium, Z. planispinum, Z. armatum, Z. nitidium, Z. rhesta, Z. simulans, Z. avicennae and Z. limonella. Out of these, 4 species are Z. armatum DC, Z. acanthopodium DC, Z. oxyphyllum Edgew, and Z. budrunga are present in India [4]. These species which may use as medicinal plants have more effectiveness against the diseases and more curable without having any side effects [6]. Z.armatum are used as a medicine from ancient time for cure of various diseases such as toothache and problems related to tooth, asthma, used for gum bleeding, fever, dyspepsia and tonics etc. [7, 8]. The fruit part of the plant may use to purify the water. The wood of this pant may be very heavier and strong then it is used for walking sticks. Z.armatum also gives and showed work against antioxidants [9] antinociceptive, antifungal, [10] anti-inflammatory, hepatoprotective, [11] pesticides, anthelmintic, ant proliferative [12] etc. The phytochemical investigation shows the presence of volatile oil and resins.1-alphaphellandrene, linalool, carbonyl compound, methyl n-nonyl ketone. Linalyl

acetate, sesquiterpene, hydrocarbon, tricosaine, dictamine, fragarine, magnoflorine, skimmianine, xanthoplanine [13, 14]. Essential oils known as fennel, citronella, geranium, lavender and rosemary are used to be fragrance and in food and beverages [15, 16].

Fig: 1 Plant of Zanthoxylum Armatum

METERIAL AND METHODS:

Collection and Authentication:

The plant of Zanthoxylum armatum was collected from local village of Chail Chowk, District Mandi, Himachal Pradesh. Selected plant may mostly cultivate in the forest region of Mandi district place named as Kamrughati jyuni valley. This experimental work was carried out at Abhilashi University Mandi in Pharmacognosy department. Leaves of the plant were collected from the forest region in the month of August and September 2020. After collection of the plant has been washed and dried thoroughly, it has been dried under the shade which takes around a month. When plant became dry than it was crushed into mortar pestle and converted into powder form.

Macroscopical evaluation:

Organoleptic evaluation means the study of drugs using organs of senses. It refers to the methods of analysis like colour, odour, taste, size, shape and special features, such as touch, texture, etc. Obviously, the initial sight of the plant or extract is so specific that it tends to identify itself. If this is not enough, perhaps the plant or extract has a characteristic odour or taste. The study of form of a crude drug is morphology while description of the form is morphography. It influences on the colour, odour, taste, texture, size of the crude drug which depends on the sense organs also called sensory characters [17].

Study of Macroscopically Characters:

Optical microscopy:

Free-hand sections of the lamina of the leaf of Zanthoxylum armatum were cleared in warm chloral-hydrate solution in a flask over a boiling water bath. The cleared specimens were mounted and photomicrographs of the epidermal features were captured from the slides using an Olympus light microscope fitted with camera using AM cap software. The transverse sections (TS) of the lamina and the midrib of the fresh leaf were also cleared, mounted, observed and their features photographed [17].

Powder microscopy:

Little quantity of leaf powder of Zanthoxylum armatum occupy on microscope slide and small amount of phloroglucinal and hydrochloric acid then a small drop of glycerol to the slide and observe under the microscope with 45X magnification, and the powder characters are observed like xylem, phloem, calcium oxalate crystals and starch grains by adding a drop of iodine the starch grains observed in blue colour [18].

Ash values:

The determination of ash is useful for detecting low grade products, exhausted drugs, and excess of sandy or earthy matter. The residue remaining after incineration of the crude drug is designated as ash. The residue obtained usually represents the inorganic salts naturally occurring in the drug and adhering to it. It varies with in definite limits according to the soils. Ash values are helpful in determining the quality and purity of a crude drug, especially in the powdered form. The objective of ashing vegetable drugs is to remove all traces of organic matter, which may otherwise interfere in an analytical determination. On incineration, crude drugs normally leave an ash usually consisting of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. The total ash of a crude drug reflects the care taken in its preparation. A higher limit of acid insoluble ash is imposed, especially in cases where silica may be present or when the calcium oxalate content of the drug is very high. The ash remaining after complete ignition of the medicinal plant materials is determined by three different methods known as Total ash, Acid-insoluble ash and water-soluble ash [18].

Total ash:

Total ash is useful in detecting the crude drugs that are mixed with various mineral substances like sand, soil, calcium oxalate, chalk powder or other drugs with different inorganic contents to improve their appearance, as is done with nutmegs and ginger. About 2 g of the powdered drug was weighed accurately and spreaded as a fine layer at the bottom in a tared silica crucible. The crucible was incinerated at a temperature to 500-600°C until it is white, indicating the absence of Carbon. The crucible was cooled and weighed. The entire procedure was repeated till a constant weight was observed. The percentage of the total ash was calculated with reference to the weight of the air-dried drug using following formula [18].

% Total Ash=(Weight of total ash)/(Weight of crude plant material taken)×100

Acid-insoluble ash:

Acid insoluble ash means the ash insoluble in dilute hydrochloric acid. The majority of crude drugs contain calcium oxalate, and the quantity of calcium oxalate varies very frequently. The ash obtained in the total ash was boiled with 25 ml of hydrochloric acid for 5 minutes. The insoluble ash was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred to a tared silica crucible together with ash less filter paper and ignited at a temperature not exceeding 600˚C, cooled and weighed. The procedure was repeated till a constant weight was observed. This measures the amount of silica present, especially as sand and siliceous earth. The percentage of acid insoluble ash was calculated with reference to the air-dried drug using following formula [18].

% Acid insoluble ash=(Weight of the acid insoluble ash)/(Weight of crude plant material taken)×100

Water-soluble ash:

The water-soluble ash is used to detect the presence of material exhausted by water. Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. The ash obtained as described in the total ash, was boiled with 25 ml of hot water for 5 minutes and filtered on an ash less filter paper, washed with hot water. The insoluble ash was transferred to a tarred silica crucible and ignited at 600°C. The procedure was repeated to get a constant weight. The weight of the insoluble matter was subtracted from weight of the total ash. The difference in weight was considered for water-soluble ash. The percentage of the water-soluble ash was calculated with reference to the air dried drug using the following formula [18].

% Water soluble ash=(Weight of the total ash-Weight of the water insoluble ash)/(Weight of crude plant material)×100

Sulphated ash:

Sulphated ash is done by addition of sulphuric acid in order to get sulphate salts, and the percentage ash is calculated with reference to the air -dried drug. The silica crucible was heated to redness for 10 minutes. Allowed to cool in a desiccator and silica crucible was heated to redness for 10 minutes, allowed to cool in a desiccator and weighed. 1 to 2 g of the test drug substance accurately weighed in to the crucible was ignited gently at first, until the substance is thoroughly charred. The residue was cooled and moistened with 1 ml of sulphuric acid, heated gently until white fumes are no longer evolved and then ignited at 800 ± 25°C until all the black particles disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool and then a few drops of sulphuric acid was added and ignited as before. It was allowed to cool and then weighed to give the Sulphated ash content. The % water soluble ash is determined by the following equation [18].

% Sulphated as=(Weight of the Crucible with ash-Weight of the Crucible)/(Weight of Plant material)×100

Moisture content determination:

The moisture content of a drug will be responsible for decomposition of crude drugs either producing chemical change or microbial growth. So, the moisture content of a drug should be determined and controlled. The moisture content is determined by heating a drug at 105°C in an oven to a constant weight. Weigh 2 gm. of the crude drug and place in the china dish and dried in the oven at 105°C for 5 hrs. and weigh the drug continuously. This procedure was repeated till a constant weight was obtained. The moisture content of the sample was calculated as percentage with reference to the shade-dried material [18].

Extraction with different solvents:

A Soxhlet extractor has three main sections: a percolator (boiler and reflux) which circulates the solvent, a thimble (usually made of thick filter paper) which retains the solid to be extracted, and a siphon mechanism, which periodically empties the thimble. Mechanism: The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is emptied by the siphon. The solvent is returned to the distillation flask. The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent.

Process:

The powdered plant samples (50 g/250 mL) were extracted successively with petroleum ether, chloroform, methanol and water using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract the polar and non-polar compounds. For each solvent extraction, the powdered pack material was air dried and then used. The solvents of the respective extracts were reduced under room temperature and stored at 4 °C for further use. The dried plant extracts were then re-dissolved in their respective solvent and to get the solution of 10 mg/10 mL for each extract which was subjected to analysis of in vitro antioxidant activities and antibacterial activity [19].

Figure 3: Assembly of Soxhlet extraction apparatus

Table 1: Extraction of plant parts of Zanthoxylum armatum in different solvents

S. No.	Code	Parts of plant	Solvent for Extraction	Amount of plant material (gm.)	Volume of solvent
1	ZAP	Leaves	Petroleum ether	50	250
2	ZAC	Leaves	Chloroform	50	250
3	ZAM	Leaves	Methanol	50	250
4	ZAW	Leaves	Water	50	250
5	ZAE	Leaves	Ethyl acetate	50	250

In-Vitro characterization of leaf Extract:

Extractive values:

The term "extraction" is used, pharmaceutically to indicate "The process of separating the medicinally active portion of plant or animal tissues from the inactive or inert components by using selective solvent in standard extraction procedure". The products, thus obtained, are relatively impure liquids, semisolids or powders intended for oral or external use. The mode of extraction selected greatly depends on the texture and water content of the plant material and in the type of substance to be isolated.

Petroleum Ether soluble extractive value:

The powdered plant samples (50 g/250 mL) were extracted with petroleum ether using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract non-polar compounds. The solvent with extracted material filtered through Whatman filter paper no. (41) to attain a clear filtrate. The filtrate was reduced, evaporated and dried under reduced pressure using rotatory vacuum evaporator. The extract was stored in a small bottle in fridge at 5°C. The percentage yield of petroleum ether extract was calculated with using the following formula [19].

% Extractive value=(Weight of dried extract)/(Weighed of plant material)×100

Ethyl acetate soluble extractive value:

The powdered plant samples (50 g/250 mL) were extracted with Ethyl acetate using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract non-polar compounds. The solvent with extracted material filtered through Whatman filter paper no. (41) to attain a clear filtrate. The filtrate was reduced, evaporated and dried under reduced pressure using rotatory vacuum evaporator. The extract was stored in a small bottle in fridge at 5°C. The percentage yield of chloroform extract was calculated with using the following formula [19].

% Extractive value=(Weight of dried extract)/(Weight of plant material)×100

Chloroform soluble extractive value:

The powdered plant samples (50 g/250 mL) were extracted with chloroform using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract non-polar compounds. The solvent with extracted material filtered through Whatman filter paper no. (41) to attain a clear filtrate. The filtrate was reduced, evaporated and dried under reduced pressure using rotatory vacuum evaporator. The extract was stored in a small bottle in fridge at 5°C. The percentage yield of chloroform extract was calculated with using the following formula [19].

% Extractive value=(Weight of dried extract)/(Weight of plant material)×100

Alcohol soluble extractive value:

The powdered plant samples (50 g/250 mL) were extracted with methanol using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract non-polar and polar compounds. The solvent with extracted material filtered through Whatman filter paper no (41) to attain a clear filtrate. The filtrate was reduced, evaporated and dried under reduced pressure using rotatory vacuum evaporator. The extract was stored in a small bottle in fridge at 5°C. The percentage yield of methanol extract was calculated with using the following formula [19].

% Extractive value=(Weight of dried extract)/(Weighed of plant material)×100

Water soluble extractive value:

The powdered plant samples (50 g/250 mL) were extracted with water using Soxhlet apparatus at 55-85 °C for 8-10 h in order to extract polar compounds. The solvent with extracted material filtered through Whatman filter paper no. (41) to attain a clear filtrate. The filtrate was reduced, evaporated and dried under reduced pressure using rotatory vacuum evaporator. The extract was stored in a small bottle in fridge at 5°C. The percentage yield of water extract was calculated with using the following formula [19].

% Extractive value=(Weight of dried extract)/(Weighed of plant material)×100

Preliminary phytochemical analysis:

The extract was tested for the presence of bioactive compounds by using following standard methods [20].

Detection of Carbohydrates:

100 mg of extract was dissolved in 10 ml of water and filtered. The filtrate prepared was used to test the presence of proteins and amino acids.

a) Molisch's Test

To the 1 ml of filtrate add 2 drops of Molisch's reagent in a test tube and add 2 ml of concentrated sulphuric acid carefully along the sides of the test tube. Formation of violet colour at the interface of two liquids indicates the presence of carbohydrates.

b) Fehling's Test

To the 1 ml of filtrate add 4 ml of Fehling's reagent (2 ml Fehling A and 2 ml Fehling B solutions) in a test tube and heated for about 10 minutes in a water bath. Formation of red precipitate indicates the presence of reducing sugar.

c) Barfoed's Test

1 ml of Barfoed's reagent is heated with 5 drops of filtrate in a test tube on water bath. Formation of a brick-red precipitate within 5 minutes indicates the presence of mono saccharides. Disaccharides generally don't give any reaction even for ten minutes.

Detection of Proteins and Amino acid:

100 mg of extracts were dissolved in water (10 ml) and then it was filtered. The filtrate was used to test the presence of proteins and amino acids.

a) Millon's Test

2 ml of filtrate was treated with 2 ml of Millon's reagent in a test tube and it was heated in a water bath for about 5 min, cooled and few drops of $NaNO_2$ solution were added to the test tube. Formation of white precipitate and it turns to red upon heating indicates the presence of proteins and amino acids.

b) Ninhydrin Test

To the 2 ml of filtrate add 2-3 drops of Ninhydrin reagent in a Test tube and boiled for about 2 min.

Formation of deep blue colour indicates the presence of amino acids.

Detection of Glycosides:

0.5 g of extract was hydrolysed with 20 ml of dilute 0.1 N HCL and then filtered. The filtrate obtained was used to test the presence of glycosides.

a) Borntrager Test

To the 1ml of filtrate add 2 ml of 1% ferric chloride solution in a test tube and heated for about 10 min in boiling water bath. The mixture was cooled and was shaken with equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Appearance of pink colour in the ammonical layer indicates the presence of glycosides.

Detection of Flavonoids:

Shinoda Test To the extract (100 mg) in a test tube adds few fragments of magnesium metal. To the test tube add 3 to 4 drops of conc. HCL. Formation of magenta colour or light pink colour indicates the presence of flavonoids.

Detection of Alkaloids:

0.5 g. of extract was taken and it was dissolved in 10 ml of dilute 0.1 N HCL and then filtered. The filtrate was used to test the presence of alkaloids.

a) Dragendorff's Test

To the 2 ml of filtrate, Dragendorff's reagent (2-3 drops) was added. Appearance of reddish brown coloured precipitate indicates the presence of alkaloids.

b) Mayer's Test

To the 2 ml of filtrate, 2-3 drops of Mayer's reagent were added, this leads to formation of cream coloured precipitate indicates the presence of alkaloids.

c) Wagner's Test

To the 1 ml of the extract, add 2 ml of Wagner's reagent. Appearance of reddish brown precipitate indicates the presence of alkaloids.

Detection of Phenolic Compounds:

100 mg of extract mixed with 1 ml of water and then it was boiled and filtered. The filtrate was used for the following test.

a) Ferric Chloride Test

Take 2 ml of filtrate in a test tube to that add 2 ml of ferric chloride solution (1%). Formation of bluish to black colour indicates the presence of phenolic nucleus.

b) Lead Acetate Test

To the 2 ml of filtrate in a test tube add 2 to 3 drops of lead acetate solution. Appearance of yellowish precipitate indicates the presence of tannins.

c) Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

Antioxidant activity:

Determination of DPPH Free Radical Scavenging Activity

Principle:

This method is based on an antioxidant compounds' hydrogen donating or radical scavenging ability to reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to 2,2-diphenyl-1-picrylhydrazine resulting in a pale-yellow solution. The decrease in the absorbance as the colour of the solution fades (from deep violet to light yellow) is monitored at 517 nm.

Procedure:

The plant extract was prepared at various concentrations ranging from 100µg/ml to 700µg/ml in methanol. The reaction mixture consisted of 1 mL of sample, 3 mL of methanol and 0.5 mL of 1 mm methanolic solution of DPPH. The reaction mixture was then vortexes and left to stand for 5 minutes. The absorbance of the resulting solution was measured at 517 nm. A mixture of methanol and DPPH solution served as a blank while a reaction mixture of methanol, DPPH, and standard (vitamin C) served as the positive control. All tests were run in triplicates. The percentage radical scavenging activity was

calculated according to the following formula [21].

% DPPH Radical Scavenging activity = (A0-A1)/A1×100

Where, control A0 = Absorbance of the control, A1 =Absorbance of extract. The percentage radical scavenging activity was then plotted against various concentrations and the IC50 (half maximal inhibitory concentration) was determined graphically

Determination of Nitric Oxide Radical Scavenging Activity

Principle:

This assay is based on the theory that sodium nitroprusside (SNP) spontaneously generates nitric oxide which interacts with molecular oxygen to form nitrite ions that may be estimated using Griess reagent. Scavengers of nitric oxide in the extract compete with molecular oxygen resulting in reduced production of nitrite ions.

Procedure:

Nitric oxide radical scavenging activity of methanolic extract of leaf of Zanthoxylum armatum was determined. The reaction mixture constituting a solution of SNP (5 mole/l) in phosphate-buffered saline pH 7.4 and different concentrations of the extract ranging from 100µg/ml to 700µg/ml, prepared in methanol, was incubated for 30 minutes at 25C. After incubation, an aliquot of the incubated solution (1.5 mL) was diluted with 1.5 mL of Griess reagent (0.1% N-1-naphthyl ethylene diamine di-hydrochloride [NED], 1% sulphanilamide, and 2% phosphoric acid). Quercetin was used as a standard drug. Diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED generated a pink chromosphere, whose absorbance was measured spectrophotometrically at 546 nm against a blank. The blank contained all the reactants except the extract. All the tests were performed in triplicate. The percentage of radical scavenging activity was computed using the formula below [21].

% Nitric oxide radical scavenging activity = (A0-A1)/A1×100

Where, A0= control reaction absorbance (blank) and A1=extract or quercetin absorbance.

Determination of Hydrogen Peroxide Radical Scavenging Activity

Principle:

This method is based on the decrease in absorbance of H2O2 following reduction of H2O2 by the antioxidant compound.

Procedure:

A solution of 40 mm hydrogen peroxide H2O2was prepared in phosphate buffer Ph. 7.4. The plant extract (at different concentrations of 100µg/ml to 700µg/ml) was added to hydro6gen peroxide solution, incubated for 10 minutes, and absorbance measured at 230 nm against a blank solution containing phosphate buffer without the hydrogen peroxide. Ascorbic acid was used as a positive control. All tests were run in triplicate and hydrogen peroxide radical scavenging activity was calculated using the following formula [21].

% Hydrogen peroxide radical scavenging activity = (A0-A1)/A1×100

Where, A0 = control absorbance (blank) and A1 ¼ extract or ascorbic acid absorbance.

Antibacterial Activity:

In-vitro antibacterial evaluation:

2 gram negative Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853) and 2 gram positive Staphylococcus aureus (ATCC: 25923), and Bacillus subtilis (ATCC: 6633) were used to check the effectiveness of methanolic extract of the Zanthoxylum armatum medicinal plants. These bacterial strains were procured from the IMTECH.

Preparation of Inoculums:

The inoculum of each bacterium was developed by growing the organism overnight in Mueller Hinton medium at 37°C and then subcultures in Mueller

Hinton agar at 37°C overnight. Four or five colonies of the bacteria were selected using a sterile inoculating loop and suspended in 2 ml of sterile saline. The turbidity of the bacterial suspensions was adjusted to the 0.5 McFarland standards by diluting with sterile saline. Sterile swabs were dipped into the inoculum tubes. Mueller Hinton agar plates were inoculated with bacteria by streaking the swabs [22].

Preparation of Stock Solution and Dilutions:

The plant extract was stored in the refrigerator at 4°C until required for use. Stock solution (1000mg/ml) of methanolic plant extract of leaves was prepared by solubilizing of 10gm of plant extract powder initially with 10 ml of the 1% DMSO. Further filtration for each stock solution by using Millipore unit and Whatman filter paper no. (1). the serially dilutions were prepared for stock solution with sterile distilled water to give seven dilutions 500, 300, 200, 100, 50, 25, 10 µg/ml.

Antibacterial activity of plants extract:

The antimicrobial susceptibility was initially assayed by the agar disk diffusion method. Base plates were prepared by pouring 15 ml Mueller Hinton (MH) agar (Bio lab, UK) into sterile Petri dishes (14 cm diameter) and allowed to set. The sterilized standard discs (6.4 mm) were impregnated with three seven different concentration 500, 300, 200, 100, 50, 25, 10 µg/ml of methanolic extract of the Zanthoxylum armatum and Tetracycline as positive control and then incubated at 55°C for 1 h. All discs were utilized to assess the antibacterial activity against four different bacterial strains on Mueller Hinton agar plates. The impregnated discs were placed on the Mueller Hinton agar plates were inoculated with bacteria. Petri dishes were incubated for 24 h at 37°C. Finally, antibacterial capacity of the methanolic extract of the Zanthoxylum armatum was measured by the zone of inhibition around discs as bacterial growth inhibition, measured in mm using a Venire calliper. The disc diffusion test was performed in triplicate [22].

Minimum Inhibitory Concentration (MIC):

MIC was the lowest concentration of the methanolic extract of with no visible bacterial growth. Plant extracts that gave a positive result for the disk diffusion assay were used to determine MIC using the microplate dilution method. Serial dilutions of the plant extracts were prepared in the water from the stock solution, yielding five serial dilutions of the original extract. Inoculum of organism was prepared in Mueller-Hinton broth, and the turbidity was adjusted to approximately 0.5 McFarland turbidity standard to prepare 1 × 108 bacterial/ml. 150 µl of plant extract was added to each well of the 96-well microplate. 50 µl of bacterial suspension was added to each well. Tetracycline drug was used as the positive control. Microliters plates were incubated at 35 ± 2°C for 24 hr. The lowest concentration that inhibits the growth of bacteria were noted and considered as the MIC value for each of the bacteria strain [22].

RESULTS AND DISCUSSION:

Collection of Plant material:

The plant of Zanthoxylum armatum was collected in village of Chail Chowk, District Mandi, Himachal Pradesh.

Assessment of quality of plant materials:

Macroscopic evaluation:

Macroscopic evaluation was conducted to evaluate the morphological parameters of the leaves of Zanthoxylum armatum. The morphological parameters include size, colour, shape, texture, fracture, taste and odour as displayed in Table 2.

Table 2: Macroscopic evaluation parameters of Zanthoxylum armatum leaves

Sr. No	Parameters	Observations
1.	Colour	Dark green upper surface, light green lower surface
2.	Size	Lanceolate, acuminate, inparipinnate
3.	Shape	Small leaves 2.3-3 cm, large leaves 12-14 cm
4.	Taste	Spice
5.	Odour	Aromatic, spicy
6	Fracutre	Brittle when dry

Figure 3: Leaves of the Zanthoxylum armatum

Microscopically Characters:

T.S of leaf lamina of Z. armatum showed upper epidermis followed by palisade mesophyll, spongy mesophylls and then lower epidermis. Collateral Vascular bundle was also present (Figure 4) Upper epidermis was non stomatiferous and covered with thin cuticle, composed of rectangular shaped compactly arranged cells. The spongy mesophyll cells (some of which were idioblast i.e., containing Cu-oxalate crystals) were rounded to somewhat elongated in shape and were arranged loosely with large intercellular spaces. Adaxial surface of Z. armatum leaf midrib was planoconvex while the adaxial surface was semicircular in appearance. There was completeabsence of any kind of appendage on it. T.S of midrib showed upper epidermis, hypodermis, vascular bundle, cortex and lower epidermis. Upper epidermal was a single layered with oval to rectangular shaped cells. Phloem was characterized by rounded cells with almost the same dimensions as that of xylum. Vascular bundle was surrounded by iodioblast cells having calcium oxalate crystals which were either Present solitary or either aggregated in clusters. The lower epidermis of the midrib region was composed of rectangular cells.

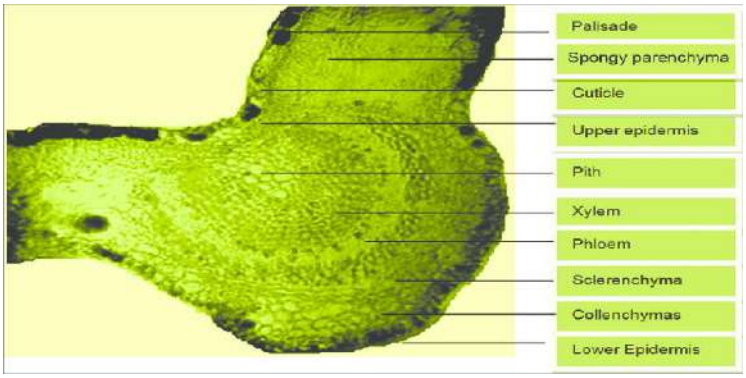

Figure 4: T. S. of Zanthoxylum armatum leaf.

Moisture content determination:

Moisture is one of the major factors responsible for the deterioration of drugs and herbal formulations. The moisture promotes the degradation processes caused by enzymes, development of microorganisms, oxidation and hydrolysis reactions. The moisture content was dried leaves of the Zanthoxylum armatum was shown in table 3.

Table 3: % Moisture content of leaves of the Zanthoxylum armatum

Plant material	% Moisture content
Leaves	0.53± 0.16

This study recorded moisture range of 0.3-0.7% which is deemed to be good as water content in vegetable drugs should not be greater than 14%.

Ash values:

Ash is an inorganic substance left over from the combustion of an organic material. Ignition process that is all organic substances will burn into black charcoal, with continuous heating, all organic substances (charcoal) will be burned out and ash will be obtained in the form of the remaining substances consisting of inorganic substances in the form of metal oxides. The growth process of a plant in nature requires nutrients, including those from minerals and other organic compounds. Total ash values for the leaves of the Zanthoxylum armatum were observed 2.60±0.28% (table 4) lied within the standard range of % moisture content recommended by the Indian pharmacopoeia. A high ash value is indicative of contamination, substitution or adulteration by minerals. Furthermore, the value of water-soluble ash, a part of the total ash content, got easily soluble in the water for the leaves of the Zanthoxylum armatum was observed to be 1.47±0.50%. Similarly, the value of acid insoluble ash and sulphated ash value was found to be 0.97±0.49% and 1.14±0.23 respectively. Value of all parameters for ash value of was found to be in the range of pharmacopoeia (Figure 6).

Figure 5: Ash value determination of crude drug

Table 4: Total Ash value, Acid-insoluble ash, Water-soluble ash, Sulphated ash of leaves of the Zanthoxylum armatum

S. No.	Plant material	Total Ash value (%w/w)	Acid-insoluble ash (%w/w)	Water-soluble ash (%w/w)	Sulphated ash (%w/w)
1	Leaves	2.60±0.28	0.97±0.49	1.47±0.50	1.14±0.23

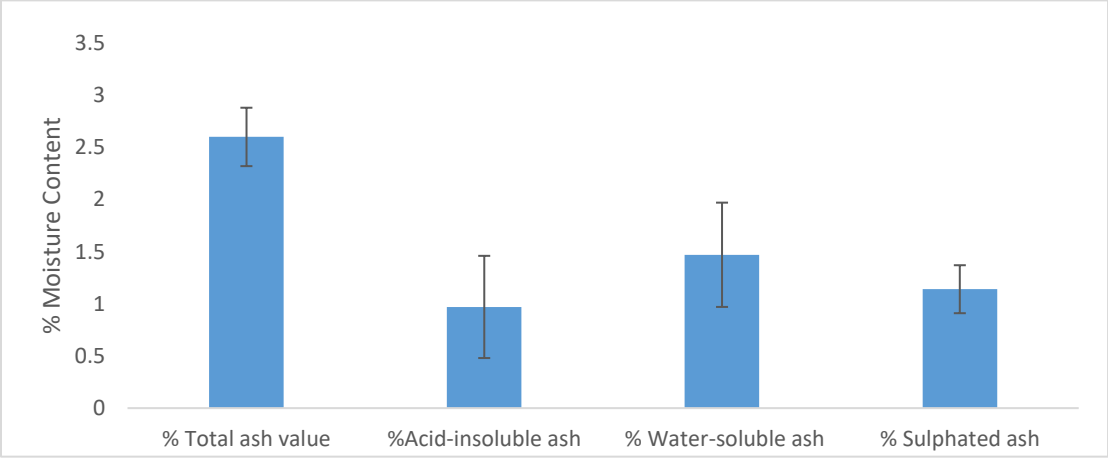

Figure 6: Bar graph of the total Ash value, Acid-insoluble ash, Water-soluble ash, Sulphated ash of leaf of the Zanthoxylum armatum

Extraction with different solvents using Soxhlet process:

The dried leaves of Zanthoxylum armatum was reduced to fine powder (40 size mesh) and around 50 gm. of crude dried powder was subjected to successive extraction with different solvent based on the polarity. The dried extract was first subject to the non-polar solvent petroleum ether the remove the fatty Material and further the plant residue material in each step material solvent was subjected to the extraction with solvent of increasing polarity and finally the dried material was subjected to the extract with water to extract the water soluble phytoconstituent. The effective extraction process yielded the liquid solution was further concentrated and drying using rota evaporator. The residue obtained after evaporation was weighed and percentage yield was calculated. The above residue was subjected to successive washed with petroleum ether, Ethyl acetate, Chloroform, Methanol and water. Each time before extracting with next solvent the extracted material (Residue) was dried at room temperature.

In-vitro characterization of extract of Zanthoxylum armatum leaves:

Physical observation of Extract of leaf of Zanthoxylum armatum:

All prepared extract demonstrated the light greenish brown colour on visually observed as shown in table 5.

Table 5: Physical appearance of all prepared extract

S. No.	Code	Physical appearance
1	ZAP	Light greenish brown colour
2	ZAE	Light greenish brown colour
3	ZAC	Light greenish brown colour
4	ZAM	Light greenish brown colour
5	ZAW	Light greenish brown colour

Extractive value:

It is employed for material to which as yet no suitable chemical or biological assays exist. The extracts obtained by exhausting crude drugs with different solvents are approximate measures of their chemical constituents. Extractive values determine amount of active constituents present in given plant material in given solvent. Extracts were prepared with various solvents by Soxhlet methods as described. Percentage of dry extract was calculated in terms of air-dried crude drug powder. Various solvents are used according to the type of the constituents to be analysed. Water soluble extractive is used for crude drugs containing water-soluble constituents like glycosides, tannins, mucilage etc. alcohol- soluble extractive is used for crude drugs containing tannins, glycosides, resins, etc. and ether-soluble extractives are used for drugs containing volatile constituents and fats. The extract value of all prepared extract of leaves of Zanthoxylum armatum has been shown in table 6.

Table 6: Extractive value of different extract of leaves of Zanthoxylum armatum

S. No.	Code	% Extractive Value
1	ZAP	13.81 ± 0.25
2	ZAE	16.70 ± 0.89
3	ZAC	20.60 ±0.69
4	ZAM	36.32 ± 0.86
5	ZAW	14.18 ± 0.99

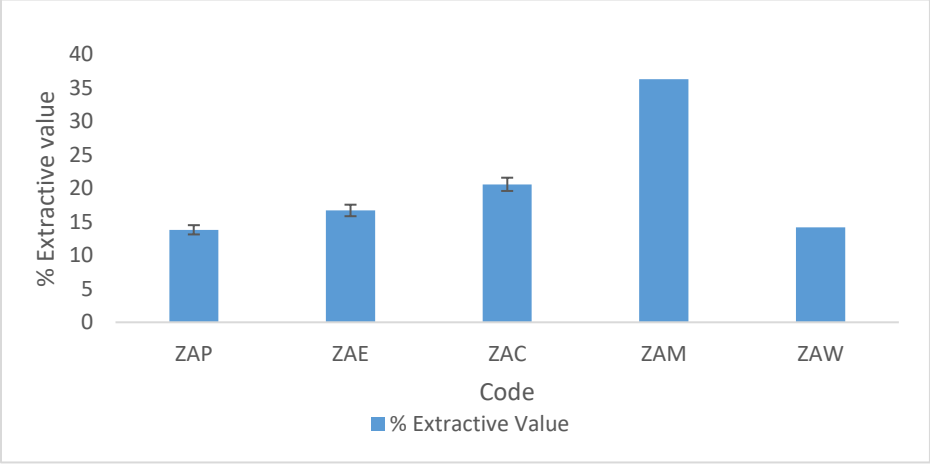

Figure 7: % Extractive value of different extract of leaves of Zanthoxylum armatum

The extractive value of leaf extracts of Zanthoxylum armatum in different solvent was found to be in a range of 13-36%. The maximum extractive yield of leaf extract of Zanthoxylum armatum has been found to be higher with the methanol 36.32 ± 0.86% and least with the petroleum ether solvent respectively indicated in table 6 and figure 7.

Preliminary phytochemical analysis:

Qualitative analysis of leaves, Z. armatum showed the presence of various phytochemicals. More than one test was employed in the case of alkaloids, flavonoids, tannins, saponins and steroids. Alkaloids contents are detected in leaves. Analysis showed the presence of flavonoids, saponins, phenolic compound and terpenoids in all five extract including petroleum ether extract, ethyl acetate extract, methanolic extract, aqueous extract and chloroform extract with their respective tests.

Table 7: Phytochemical test different leaves extract of Zanthoxylum armatum

Phytochemical Tests	Methanolic Extract	Aqueous Extract	Chloroform Extract	Ethyl acetate extract	Petroleum ether Extract
Carbohydrates	Molisch's test	+	+	-	-
	Barfoed's Test	+	+	-	-
	Fehling's test	+	+	+	+
Proteins and Amino acids	Millon's Test	-	+	-	-
	Ninhydrin Test	-	+	-	-
Phytosterols	Libermaan - Burchard test	+	+	+	
Glycosides	Brontranger's Test	+	+	+	+
Flavonoids	Shinoda test	+	-	+	+
Alkaloids	Wagner's	+	-	+	+
	Mayer's	+	-	+	+
	Draggendrof's test	+	+	+	+
Phenolic Compounds	Ferric chloride test	+	+	+	-
	Alkaline reagent test	+	+	+	-
	Lead Acetate test	+	+	+	+
Tannins	Ferric chloride test	+	+	+	-
Saponin	Foam test	+	+	+	+
Triterpenoids	Salkowasky test	+	+	+	+
	Hishron test	+	+	+	+
Gum and Mucilage	Alcohol test	+	+	-	-

Optimisation of Extract:

Based on above In vitro characterization parameters including % extract value the methanolic of the leaves was selected for further In vitro antibacterial and antioxidant activity.

In vitro antioxidant activity of methanolic extract of leaves of Zanthoxylum armatum:

There is no universal method by which antioxidant activity can be measured quantitatively and precisely. Thus, in this study we have opted to evaluate the antioxidant activities in vitro via the three most commonly used tests and to combine the results of these complementary tests in order to have an indication on the antioxidant capacities of methanolic leaf extract of Zanthoxylum armatum.

Measurement of antioxidant activity by DPPH test:

The chemical compound 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is one of the first free radicals used to study antiradical activity. It has an unpaired electron on an atom of the nitrogen bridge; this relocation is responsible for the violet colour characteristic of the DPPH radical. The measure of the efficacy of an antioxidant is done by measuring the decrease in violet coloration that is due to the recombination of DPPH radicals measurable at 517 nm. The trapping of radicals by the antioxidant (hydrophilic or lipophilic molecules) is attributed principally to the liberation of the electron which is unpaired. In this study, we realized the DPPH trapping test in order to measure the antiradical activity of Methanolic leaf extract of Zanthoxylum armatum extracts and we expressed our results in IC50 (µg/mL). We noted that the percentage of inhibition increases as the concentration of the extracts is increased. The comparison of the % inhibition of methanolic extracts 84.04± 0.63% and that of reference antioxidants ascorbic acid 0.40 ± 0.35% showed that Methanolic leaf extract of Zanthoxylum armatum extracts has a good anti-free radical activity. The Value of IC50 for the methanolic extract of the leaves of the Zanthoxylum armatum was found to 301±0.12, (Table 8)

Table 8: Percentage inhibition of the Extract in DPPH assay

Concentration (µg/ml)	% Inhibition
Control	0.40±0.35
100	15.35±0.463
200	35.67±0.606
300	52.53±0.763
400	62.12±0.606
500	73.64±0.303
600	84.04±0.631
700	83.48±0.214

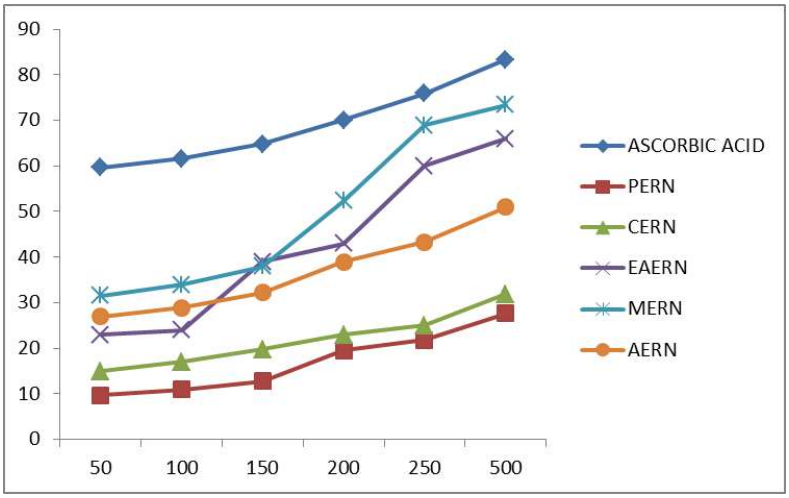

Figure 7: Bar graph of the Percentage inhibition of the Extract in DPPH assay

Hydrogen peroxide (H2O2) scavenging activity:

H2O2 is not a free radical, but it has the capacity to generate in the presence of transition metals (iron and copper) highly reactive radicals such as hydroxyl-radical OH−. It can be formed by the dis-stimulation of O2− by the superoxide dismutase, or produced by the bivalent reduction of oxygen through a high number of dehydrogenase. The percentage of trapping of hydrogen peroxide by methanolic extract of the leaves of the Zanthoxylum armatum extracts is illustrated in Table 9. The extracts at the concentration of (600 µg/ml) have the capacity to trap the radical H2O2 with an advantage for methanolic extract 77.85 ± 1.308% which has a higher activity than the positive control (ascorbic acid) 1.04 ± 1.248%. The value of IC50 was found to be 312 ± 0.99%.

Table 9: Percentage inhibition of the Extract in Hydrogen peroxide (H2O2) scavenging activity

Concentration (µg/ml)	% Inhibition
Control	1.04±1.248
100	12.46±0.692
200	30.68±0.720
300	48.44±0.34
400	55.71±0.69
500	70.13±0.87
600	77.85±1.03
700	76.99±0.24

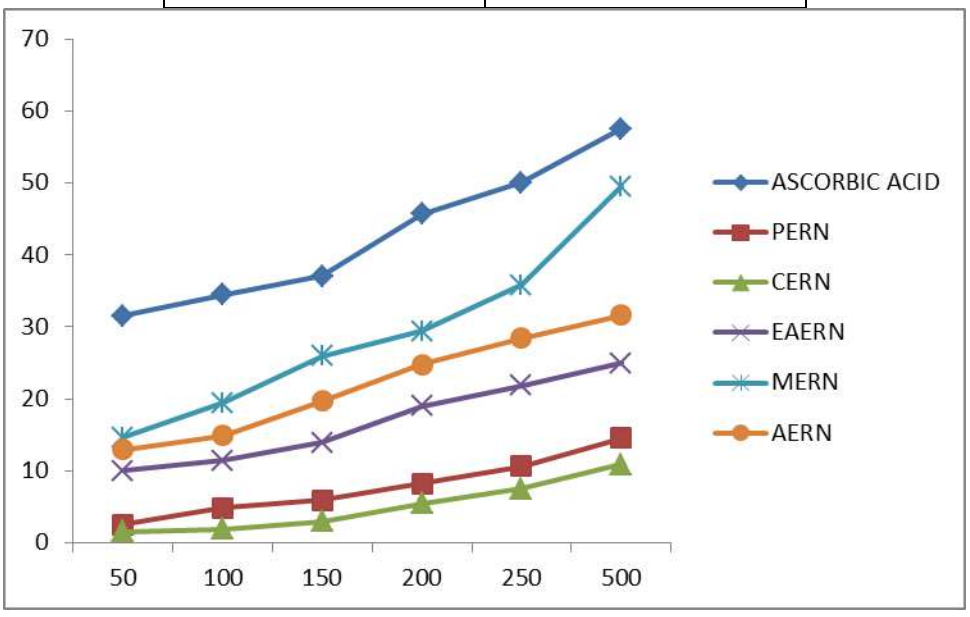

Figure 8: Bar graph of the Percentage inhibition of the Extract in Hydrogen peroxide (H2O2) scavenging activity

Nitric Oxide Scavenging Activity:

The study showed that the methanolic leaf extract of Zanthoxylum armatum caused a concentration-dependent increase in nitric oxide radical scavenging activity (Figure 10).

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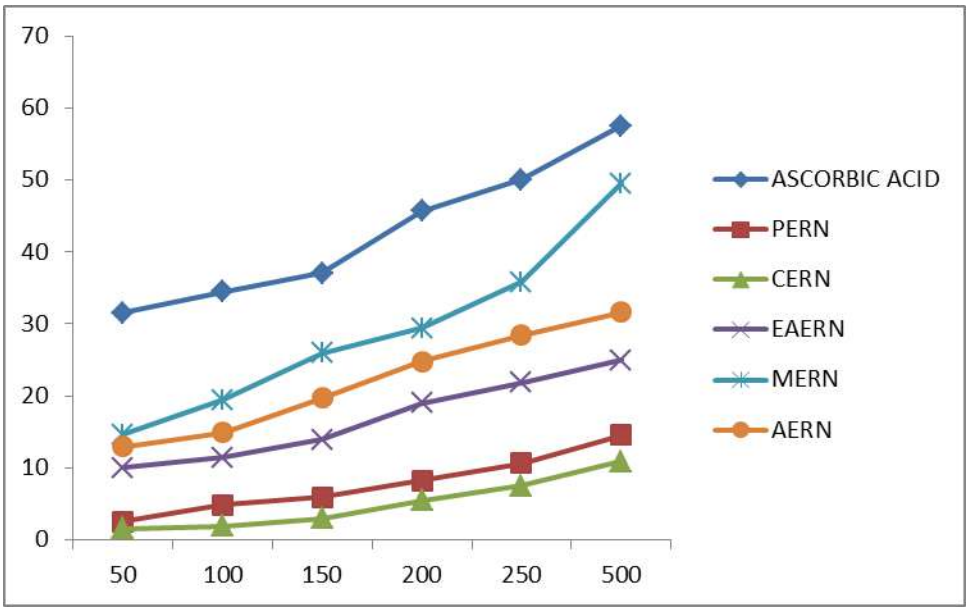

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Nitric Oxide Scavenging Activity:

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Table 10: Percentage inhibition of the Extract in Nitric Oxide Scavenging Activity

Concentration (µg/ml)	% Inhibition
Control	0.89±0.88
100	11.70±0.67
200	30.52±0.68
300	49.48±0.92
400	60.59±0.25
500	72.15±0.93
600	75.56±0.44
700	73.78±0.40

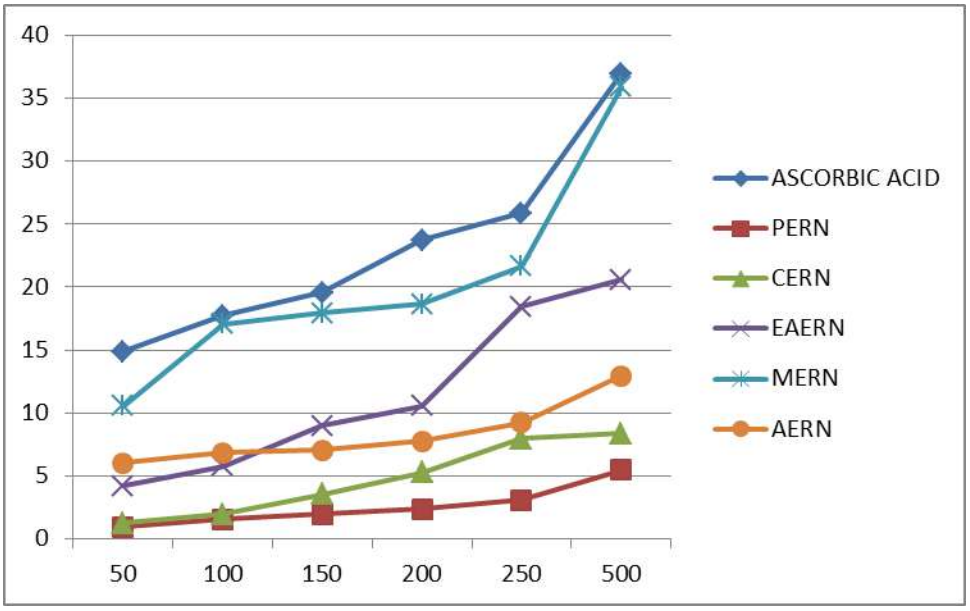

Figure 9: Bar graph of the Percentage inhibition of the Extract in Nitric Oxide Scavenging Activity

We noted that the percentage of inhibition increases as the concentration of the extracts is increased. The comparison of the % inhibition of methanolic extracts 75.56±0.44% and that of reference compound control 0.89±0.88% showed that Methanolic leaf extract of Zanthoxylum armatum extracts has a good anti-free radical activity. The figure demonstrated that the IC50 value of methanolic extract of the leaves of Zanthoxylum armatum was observed to be 318±0.56.

Antibacterial activity of Methanolic extract of leaves of Zanthoxylum armatum:

The methanolic extract of leaves of Zanthoxylum armatum was tested for their putative antibacterial activity against 4 different kinds of bacteria. The Zone of inhibition value extract against each bacteria was presented in Table 11. The results showed that the extract had certain antibacterial activity on all of the tested bacteria including both Gram-positive and Gram-negative bacteria. A narrow spectrum antibiotic (tetracycline) was used as positive controls to determine the antagonistic spectrum of the extract. The results also indicated that the methanolic extract of leaves of Zanthoxylum armatum had antibacterial function like tetracycline. The zone of inhibition value of the methanolic extract of leaves of Zanthoxylum armatum against four different bacteria Varied from to 18 mm (Table 11). The largest zone of inhibition value was obtained for E. coli, followed by the Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis. We could see that the methanolic extract had a better antibacterial activity at 500 (µg/ml) on these bacteria because the zone of inhibition value was comparable with control (12-17mm).

Table 12: Zone of inhibition of the methanolic extract of leaves of Zanthoxylum armatum

Concentration (µg/ml)	Zone of Inhibition (mm)			
	E. Coli	P. Aeruginosa	S. Aureus	B. Subtilis
Tetracycline (100 µg/ml)	19.33±1.52	17±1	19.33±1.55	16±2.0
500	17.33±0.57	15.33±1.14	17±1.73	12.66±1.89
300	11.3±1.15	12.66±1.52	13.66±0.58	7.33±1.02
200	9±1	8±1	11±1	5.66±1.48

100	4.33±1.16	4.66±2	7±1.9	1.33±1.51
50	1.33±1.56	1.33±1.32	3.33±1.51	0±0
25	0.66±0.58	0.33±1.50	1.66±1.38	0±0
10	0±0	0±0	0±0	0±0
300	11.3±1.15	12.66±1.52	13.66±0.58	7.33±1.02

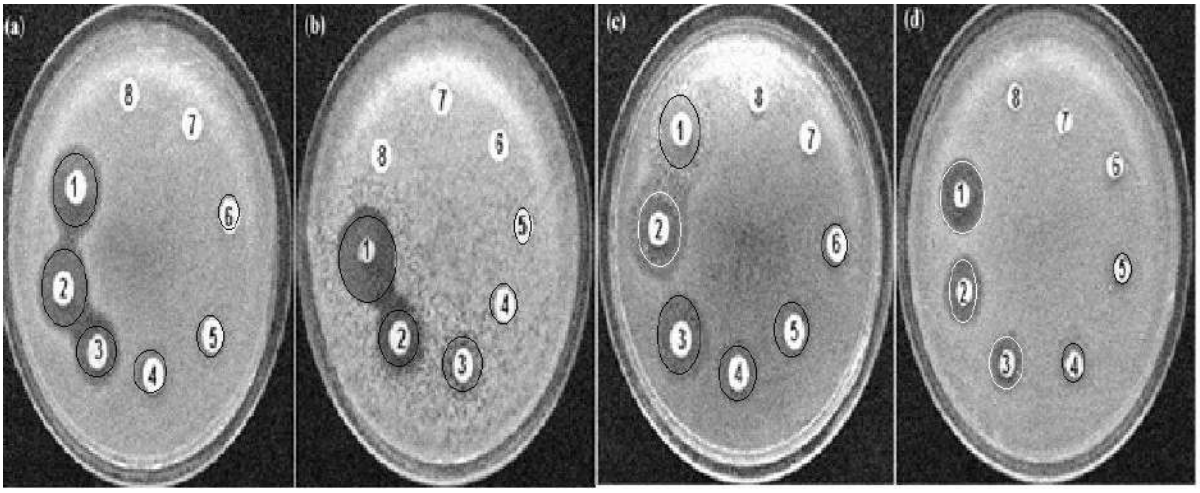

Figure 11: Images of Zones of inhibition of Methanolic extract of leaves against the pathogenic strains E. coli (a), P. aeruginosa (b), S. aureus (c) and Bacillus subtilis (d) in antimicrobial susceptibility disk diffusion method.

1-Tetracycline, 2-500 µg/ml, 3-300 µg/ml, 4-200 µg/ml, 5-100 µg/ml, 6-50 µg/ml, 7-25 µg/ml, 8-10 µg/ml

MIC value:

MIC was the minimum essential oil concentration that can prevent the bacteria from obvious growth. The MIC value of extract against different bacteria was determined by measuring the zone of inhibition against two gram positive bacteria and two gram negative bacteria. The MIC value of the methanolic extract of leaves of Zanthoxylum armatum against different gram positive and gram negative bacteria was presented in the Table 12. The results showed on increasing the concentration of the extract the area of the zone of inhibition was increase as displayed that the methanolic extract had certain antibacterial activity on all of the tested bacteria, according to the report above, the study about the methanolic extract of leaves has important implications for the traditional medicine.

Table 13: MIC value of thee methanolic extract of leaves at different concentration against four different bacteria.

Parameter	Bacteria			
	E coli	P. Aeruginosa	S. Aureus	Bacillus Subtalis
MIC value	19.34±1.32	32.67±1.15	17.11±0.67	75.36±0.54

CONCLUSION:

The present, study deals with the evaluation of phytochemical screening, in vitro antioxidant, antibacterial activities on Zanthoxylum armatum leaves. T.S of leaf lamina of Z. armatum showed upper epidermis followed by palisade mesophyll, spongy mesophyle and then lower epidermis. The spongy mesophyll cells (some of which were idioblast i.e., containing Cu-oxalate crystals) were rounded to somewhat elongated in shape and were

arranged loosely with large intercellular spaces. Phloem was characterized by rounded cells with almost the same dimensions as that of xylum. Percentage moisture range leaves of 0.3-0.7% which is deemed to be good as water content in vegetable drugs should not be greater than 14%.

Total ash values for the leaves of the Zanthoxylum armatum were observed 2.60±0.28%, acid insoluble ash and sulphated ash value was found to be 0.97±0.49% and 1.14±0.23 respectively. dried leaves of Zanthoxylum armatum was reduced to fine powder (40 size mesh) and around 50 gm. of crude dried powder was subjected to successive extraction with different solvent petroleum ether, Ethyl acetate, Chloroform, Methanol and water based on the polarity using Soxhlet apparatus. The extractive value of leaf extracts of Zanthoxylum armatum in different solvent was found to be in a range of 13-36%. The maximum extractive yield of leaf extract of Zanthoxylum armatum has been found to be higher with the methanol 36.32 ± 0.86% and least with the petroleum ether solvent.

Preliminary phytochemical Analysis showed the presence of flavonoids, saponins, phenolic compound and terpenoids in all five extract including petroleum ether extract, ethyl acetate extract, methanolic extract, aqueous extract and chrloroform extract with their respective tests In DPPH antioxidant assay, The comparison of the % inhibition of methanolic extracts 84.04± 0.63% and that of reference antioxidants ascorbic acid 0.40 ± 0.35% showed that Methanolic leaf extract of Zanthoxylum armatum extracts has a good anti-free radical activity. The Value of IC50 for the methanolic extract of the leaves of the Zanthoxylum armatum was found to 301±0.12, In Hydrogen peroxide (H2O2) scavenging activity, the extracts at the concentration of (600 µg/ml) have the capacity to trap the radical H2O2 with an advantage for methanolic extract 77.85 ± 1.308% which has a higher activity than the positive control (ascorbic acid) 1.04 ± 1.248%. The value of IC50 was found to be 312 ± 0.99%. Nitric Oxide Scavenging Activity, the comparison of the % inhibition of methanolic extracts 75.56±0.44% and that of reference compound control 0.89±0.88% showed that Methanolic leaf extract of Zanthoxylum armatum extracts has a good anti-free radical activity. The figure demonstrated that the IC50 value of methanolic extract of the leaves of Zanthoxylum armatum was observed to be 318±0.56. In antibacterial activity, the zone of inhibition value of the methanolic extract of leaves of Zanthoxylum armatum against four different bacteria varied from to 18 mm. The largest zone of inhibition value was obtained for E. coli, followed by the Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis. We could see that the methanolic extract had a better antibacterial activity at 500 (µg/ml) on these bacteria because the zone of inhibition value was comparable with control (12-17mm). The MIC value of methanolic extract of leaves of the Zanthoxylum armatum was found to be 19.34±1.32, 32.67±1.15, and 17.11±0.67, 75.36±0.54, against E coli, P. Aeruginosa, S. Aureus, and Bacillus Subtalis.

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