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EVALUATION OF PHYTOCHEMICALS, ANTIBACTERIAL, ANTIOXIDANT ACTIVITY, TOTAL FLAVONOID, AND PHENOLIC CONTENT OF ZANTHOXYLUM ARMATUM FOUND IN PALPA DISTRICT OF NEPAL

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ABSTRACT

Zanthoxylum armatumis an important medicinal plant. This study was to analyze phytochemicals, antibacterial, antioxidant activity, TPC, and TFC quantitatively in wild fruit extract. The wild fruits of the plant were collected from the Palpa district. Fruits were shaded dried and ground and extracted with methanolic solvent by cold percolation method. The extract was then examined for qualitative and quantitative analysis. Preliminary phytochemical screening revealed the presence of flavonoids, Polyphenol, Alkaloids, steroids, Tannins, Terpenoids, Coumarins, Quinones, Glycosides, Saponins, etc. The antimicrobial activity was analyzed by the Agar Well Diffusion method, antioxidant activity was evaluated by DPPH radical scavenging assay, total phenolic content was determined by using Folin-Ciocalteu method, and flavonoid content was determined by using aluminium chloride colorimetric method. The DPPH radical scavenging activity in terms of IC_{50} value of the wild fruit sample was 51.596µg/ml. The total phenolic content was found 244.46±0.03 (mg of GAE/g),total TFC content was found 131.66±0.06(mg of QE/ g).Upon antibacterial test, the extract was more sensitive to gram-positive bacteria S. aureus (ZOI= 8 mm) but remains insensitive to E. coli.

KEYWORDS: *Z. Armatum, Rutaceae Family, Phenolic, Flavonoid, Antibacterial Analysis.*

INTRODUCTION

Nepal is a beautiful landlocked country in South Asia, with a total area of 147, 181 km^2.Its altitude ranges from 70m to 8848 m, and the highest point in the world. Nepal occupies only 0.03 percent of the global surface area, is enriched by several climatic conditions, and geographical variations, and an immense variety of medicinal plants have contributed to about 10% medicinal plants of the expected 7000 species of flowering plants(Muhammad et al., 2011).In this research, the wild fruit of Zanthoxylum armatum was collected from the Palpa district from1 August to 20 August. It is a district in Lumbini Province Nepal, where the altitude ranges from 250m to 2000 m from sea levels. Z. armatumis an important medicinal plant commonly known as winged prickly ash in English and Timur in Nepali. It is a small xerophytic shrub, growing to 3.5 meters

in height, that belongs to the Rutaceae family. It grows throughout Nepal between 1000-2400m altitude. It is endemic from Pakistan across to South Asia up to Korea and Japan. The fruits and seeds of the plant are used as a spice; as folk medicine, essential oil production, and as an ornamental garden plant. This is a highly pungent, sharp-tasting dried berry with a "biting taste and anesthetic feeling on the tongue. Eight species of Zanthoxylum have been reported from Nepal till now: Z. acanthopodium DC., Zanthoxylum armatum DC., Z. floribunda Wall., Z. nepalense Babu, Z. nitidum (Roxb.) DC., Z. oxyphyllum Edgew., Z. simularis Hance and Z. tomentellum Hook. f. (Rajbhandari et al., 2015). Among these species Zanthoxylum armatum is the most common and one of the 30 medicinal plants of the country, which has been prioritized by the government of Nepal for economic development with a high emphasis on cultivation and agro-technology development (DPR, 2006).Thus different parts of the plants have been used in several indigenous medicinal practices as carminative, antipyretic, appetizer, stomachic, toothache, dyspepsia.

Different phytochemical constituents (alkaloids, flavonoids, lignins, coumarins, phenols, terpenoids) have been found in this plant. Its trade value is also very high with its manifold applications in Ayurveda, allopathy, general pharmacy, and other industries there is a huge demand for Z. armatum in the both domestic and international markets due to which the market price has been escalating in the last two decades (Hertog & Wiersum, 2000).Acetone fraction of methanolic extract, from the Z. aramatum fruit, has strong antibacterial activity against the gram-positive bacteria Staphylococcus aureus and gram-negative bacteria Escherichia coli.(Tiwari et al. 2020).The antifungal activity of essential oil of methanol extract from Z. armatum leaves was tested against fungi, A. brassicae, A. alternate , and C. lunata. The methanol extract gave significant results for antifungal activity. It could be due to the presence of phenolic and flavonoid compounds (Tiwari et al. 2020).The oil of the plant also showed antifungal properties against 24 different fungi. It also exhibited repellent and larvicidal activity against three mosquito vectors, also recommended for treating ailments like rheumatism, bronchitis, indigestion, asthma, varicose veins, toothaches, and cardiovascular, respiratory, and gastrointestinal disorders. And cholera. Powdered fruit, mixed with Menthaspp and table salt is eaten with a boiled egg for a chest infection and other digestive problems (Islam et al., 2009).

Indigenously Z. armatumis mainly used in chronic problems that is skin disease, rheumatism, toothache, gum bleeding, etc. It has anticancer antibacterial, antiviral, antifungal, anti-inflammatory, antioxidants activity etc. It have various phytochemicals such as lignins, alkaloids, monoterpenoid, polyphenolic and flavonoid groups, etc. Where mainly the phenolic and flavonoids give antioxidant activity and anticancer activity. It is widely used in Ayurveda and allopathy. Oxidative stress inside the body produce chronic diseases such as diabetes, heart disease, and cancer (Rezaeizadeh et al., 2011). In oxidative stress, the balance between the formation of reactive oxygen species (ROS) and the amount of antioxidants in the body is destroyed which causes damage to cell components

such as proteins, lipids, and nucleic acid and eventually leads to cell death (Nazıroğlu et al., n.d.). ROS and reactive nitrogen species (RNS) are the main sources of free radicals which lead to serious disorders such as Alzheimer's disease, Parkinson's disease, and Strokes (Willcox et al., 2004). Overproduction of these two radicals contributes to the pathogenesis of inflammatory diseases(medicine & 1989, n.d.). Antioxidants are compounds that hinder oxidative processes and

thereby delay or prevent oxidative stress (Shyur et al., 2005). Increasing the intake of antioxidants can prevent diseases and lower health problems. In the present context, widely used synthetic oxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylated hydroxyquinone (TBHQ), and gallic acid ester etc. These possess certain side effects and toxic effects. Natural products, mainly obtained from dietary sources provide a large number of antioxidants that decreases oxidative injury (Elmastaş et al., 2006). Plants contain many phytochemicals that are useful sources of natural antioxidants, such as phenolic diterpenes, flavonoids, tannins, and phenolic acids(Lee et al., n.d.). Polyphenols, especially flavonoids are strong antioxidant in plant extracts(Zoology & 2010, 2010). Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen and various free radicals.

Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen and various free radicals. By Kharel et.al., among 9 different medicinal plants in the Kavre district of Nepal, the extract of *S. pinnata* showed the highest percentage of radical scavenging activity up to 87.94±1.88 with 50% inhibitory concentration (IC_{50}) 17.51±1.27 μg/mL (KHAREL et al., 2020). The presence of phenolic compounds suggests that antioxidant activity may be due to ferulic acid content (Khare et al., 2012).According to Nooreen et al., 2017, from Z. armatum fruits four chemically distinct compounds were isolated namely Tambulin (6), Prudomestin (7), Ombuin (8), and 3, 4, 5, 3′, 4′, 5′-hexahydroxydiphenyl ether (9) have been isolated and quantified from the fruits, further studied the antiproliferative, antimicrobial and antioxidant efficacy. The Tambulin revealed significant antiproliferative activity (IC50 37.96 ± 0.36 to 48.7 ± 0.21 μg/mL) against breast, liver, colon, and skin cancer cells. Hexane and ethyl acetate fraction exhibited moderate antibacterial efficacy (MIC: 250–1000 μg/mL) against selected pathogenic microbes while Ombuin displayed a broad-spectrum antibacterial effect. The high TPC (5.27 ± 0.06 to 46.12 ± 0.40 mgGAE/g, highTFC (6.05 ± 0.24 to 14.46 ± 0.73 mgQE/g and ferric reducing power (42.35 ± 0.85 to 62.52 ± 0.66 mg/g of ferrous sulfate equivalents) and high percent free radical scavenging activity (59.56 ± 0.38 to 64.85 ± 1.78) were estimated and gave more positive attribute to its traditional usage(Nooreen et al., 2017).The different plant parts like leaves, fruits, stems, bark, seeds, and roots show antioxidative, antitumor, anti-inflammatory, analgesic, antimicrobial and insecticidal/larvicidal activity (Brijwal et al., 2013).

(Phuyal et al., 2020b)Natural antioxidants present in several medicinal plants are responsible for inhibiting the harmful effects of oxidative stress. These plants contain polyphenols and flavonoids that act as free radical scavengers and reduce oxidative stress and may be an alternative remedy to cure various harmful human diseases. In a study of methanolic extracts of fruits, seeds, and bark of Zanthoxylum armatum collected from wild and cultivated populations in Nepal, the highest TPC value was 226.3 ± 1.14mg GAE/g in wild fruits, and the lowest was 137.72± 4.21mg GAE/g in cultivated seeds. Similarly, the highest TFC value was 135.17± 2.02mg QE/g in cultivated fruits, and the lowest was 76.58 ± 4.18mg QE/g in cultivated seeds. The extracts showed variable antioxidant properties. The fruits exhibited excellent antioxidant properties with IC50 values of 40.62 μg/mL and 45.62 μg/mL for cultivated and wild fruits, respectively. Similarly, the IC50 values of the bark were 63.39 μg/mL and 67.82 μg/mL, respectively, for cultivated and wild samples. And the least antioxidant capacity was shown by the seeds extract with IC50 values of 86.75 μg/mL and 94.49 μg/mL for wild and cultivated seeds, respectively. The IC50 value of the standard ascorbic acid was 36.22 μg/mL.

Different extracts of Z. armatum contain a considerable amount ofphenols and flavonoids, including antioxidant properties, suggesting the potential use of this speciesin pharmacy and phytotherapy as a source of natural antioxidants (Phuyal et al., 2020b, 2020a)- Crude methanol extracts of fruits, seeds, and bark of Zanthoxylum armatum were investigated in vitro for antimicrobial activities against 9 different bacterial strains using the agar well diffusion method, and the MBC values were determined. Only 5 bacteria, i.e., Bacillus subtilis, Enterococcus faecalis, MRSA, Staphylococcus aureus, and Staphylococcus epidermidis exhibited antibacterial properties against the different extracts. The fruit and seed extracts showed activities against 5 bacteria. Staphylococcus aureus was found to be more susceptible for all the extracts compared to other strains. The maximum ZOI of20.72mm was produced by fruits (wild) and 18.10mm (cultivated) against Staphylococcus aureus. And highest ZOI value of 50 mg/mL for fruits and seeds extracts against S. epidermidis. The fruits, seeds, and bark extracts of Z. armatum exhibited remarkable antibacterial properties. It suggests the potential use of this plant for treating different bacterial diseases such as skin infection, urinary tract infection, dental problems, diarrhea, and dysentery (Phuyal et al., 2020a)**.**

SPECIFIC OBJECTIVES

The specific objectives of this research study are:

- To perform photochemical screening, of the methanolic extract of the wild fruits of the plant.
- To find the antioxidant activity, total phenolic, and total flavonoid content of methanolic extract of wild fruit of Zanthoxylum armatum.
- To perform antimicrobial activity test.

MATERIAL AND METHOD

Equipment Used

The types of equipment used in this research work were beakers, conical flasks, test tubes, reagent bottles, bio safety cabinet, vials, burettes, Digital balance(GT 210), Cuvettes (Quartz), Incubator and autoclave(S.N Scientific Instrument(p)Ltd Delhi), Graduated pipettes, Laminar air flow (Indosati scientific lab equipment), Mechanical grinder, pipettes, micropipettes, thermometer, condensers, round bottom flasks, Rotary evaporator, water bath, UV-visible spectrophotometer (Aczel 2306, Product ID:201605028).

Chemicals:

All chemicals used are of analytical grades. Methanol, Quercetin, Galic acid (Loba Chemie Pvt Ltd.), 2,2-diphenyl-1-PycrylHydrazyl,Folin-Ciocalteu reagent, DMSO (Qualigenes), Mueller-Hinton Agar (Himedia), NH_4SCN, $FeSO_4$, NH_4OH, Distilled water, $NaNO_2$, $AlCl_3$, Aluminium chloride, ascorbic acid (LOBA Chemie Pvt. Ltd), hydrochloric acid, Fehling's solution, alpha-Naphthol, $FeCl_3$, $NaHCO_3$, $Bi(NO_3)_3$, KI, $HgCl_2$, Picric acid, disodium hydrogen phosphate, sodium carbonate, sodium chloride, sodium hydroxide, sodium nitroprusside, etc. Different reagentsviz: Meyer's reagent, Dragendorff's reagent, Molisch's reagent, etc were prepared in laboratory reagent grade

Collection and Identification of Plant Sample

The fresh wild fruits of Zanthoxylum armatum were collected from August 1 to 20, 2021 fromthe Palpa district of Nepal. The collected fruits were identified by Prof. Dr. Ananta Gopal Singh Head of the Department of Botany Tribhuvan University, Butwal Multiple Campus, Butwal.

Drying and extract Preparation of Extract

The collected fresh fruits were washed with tap water to remove the contaminants. Then the fruits were shade dried. The shade dried fruits were grounded into powder form in an electric grinder and the powdered sample were collected in clean plastic bag and were stored in cool and dry place, the powdered fruits extract was extracted by cold percolation method using methanol as a solvent. 84 g of powdered samples were weighed out by digital balance and kept in clean and dry conical flask. 250 ml methanols were added to flask sealed tightly and extraction was done repeatedly for 72 hrs with frequent shaking and filtered after complete maceration. The filtrate was concentrated with the help of a rotary evaporator. The solid methanolic extract of plants was obtained and stored at 4ºC in a refrigerator until analyses.

Phytochemical analysis

The method used for phytochemical screening was based on the protocol put forward by Ciulei I et, al. (2013). Phytochemical screening helps to identify secondary metabolites (bioactive compounds) present in plants. The analysis was done by the color reaction using different specific reagents. The qualitative results are expressed as (+) for the presence and (−) for the absence of phytochemicals.

1. **Test for Alkaloids-**About 500 mg extract was dissolved in 3 mL of 2 % (v/v) HCl. The solution was equally divided into two test tubes. It gave positive Meyer's Test and Dragendorff's Test. Presence of alkaloids was confirmed.
2. **Test for Terpenoids-**To about 200 mg extract, 2 mL of chloroform ($CHCl_3$) and then 3 mL concentrated sulphuric acid (H_2SO_4) were added carefully. The formation of reddish-brown coloration at the interface indicated the presence of terpenoids.
3. **Test for Coumarins-**To about 1 mL of extract, 1 mL of 10 % sodium hydroxide (NaOH) solution was added. The formation of yellow color indicated the presence of coumarins.
4. **Test for Flavonoids/Shinoda's Test-**About 200 mg extract was dissolved in 2 mL methanol. To this solution, a small piece of magnesium and 4-5 drops of concentrated hydrochloric acid (HCl) were added. The formation of orange color indicates the presence of flavonoids.
5. **Test for Quinones-**To with about 2 mL extract, 1 mL freshly prepared ferrous sulfate ($FeSO_4$) solution, and a few crystals of ammonium thiocyanate (NH_4SCN) were added and the solution was treated with conc. sulphuric acid (H_2SO_4) drop by drop. The appearance of persistent deep red coloration indicates the presence of quinones.
6. **Test for Polyphenols/ $FeCl_3$ Test**

   To about 1 mL extract, 1 mL distilled water was added followed by the addition of a few drops of 10 % (w/v) ferric chloride ($FeCl_3$) solution. The appearance of greenish-blue

coloration indicates the presence of polyphenols.

7. Test for Glycosides

About 500 mg extract was dissolved in 2 mL methanol and divided into two parts and the following tests were performed.

8. Molisch's Test

I) The first part was treated with 5 mL of Molisch's reagent and conc. H_2SO_4 was added drop by drop from the side of the test tube without disturbing the solution. The appearance of a violet ring at the junction of two liquids which on shaking turns the solution into a violet color indicates the presence of glycosides.

ii) To the second part 2 mL of 25 % (v/v) NH_4OH solution was added and shaken vigorously. The appearance of the cherry red color indicated the presence of glycosides.

9. Test for Reducing Sugars

To about 1 mL extract, 1mL distilled water was added followed by the addition of 1 mL Fehling's reagent (1, 1 mixture of Fehling's solution A and B). Then the mixture was warmed over a water bath for 30 minutes. The appearance of a brick-red precipitate indicated the presence of reducing sugars.

10. Test for Saponins-About 500 mg extract was treated with hot water followed by shaking for 30 seconds. The formation of thick forth indicates the presence of saponins. 10)Test for Tannins

11. Test for Tannins-About 200 mg extract was boiled adding 10 mL distilled water. The mixture was cooled and filtered and a few drops of $FeCl_3$ solution were added to the filtrate. The appearance of a blue-green or black precipitate indicated the presence of tannin (Salkowaski test).

12. Carbohydrate (Molisch's Test) About 2 mg of extracts were dissolved individually in 5 ml distilled water and filtered. Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicated the presence of carbohydrates

13. Test for Proteins (Xanthoproteic test): In 2 ml of extract, 2 ml of conc. HNO_3 was added formation of orange-yellow color indicated the presence of protein.

TABLE 1: PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT OF PLANT SAMPLES

S.N	Test	Result
1.	Alkaloids	+++
2.	Terpenoids	+
3.	Coumarins	+++
4.	Flavonoids	++
5.	Quinones	+++
6.	Polyphenols	++

7.	Glycosides	+
8.	Reducing sugar	-
9.	Saponins	+++
10.	Tannins	++
11.	Carbohydrates	+
12.	Proteins	+
13.	Steroids	+

Where, +++: Significantly present, ++: Moderately present, +: Weakly present -: Absent

Determination of Antioxidant Activity

To determine antioxidant activity, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the method of Jamuna et al. (2012). 2 ml of different concentrations (31.25 µg/ml to 500 µg/ml) of extract solution of plant sample was mixed with 2 ml of DPPH solution (60 µM). The mixture was allowed to stand in dark conditions for 30 minutes for complete reaction. Finally, the absorbance of each plant sample was measured at 517 nm by using a UV spectrophotometer. The radical scavenging activity of each sample was calculated by using the following formula:

Radical scavenging (%) = $[(A_0 - A_1 / A_0) \times 100\%]$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract. Control is the test solution without a sample. Ascorbic acid was taken as standard. A similar procedure was followed with an ascorbic acid solution of concentration (31.25 µg/ml to 500 µg/ml). The antioxidant activity of fruit sample was expressed in terms of IC_{50} (concentration required to inhibit DPPH radical formation by 50%). The 50% inhibitory concentration (IC50) value was indicated as an effective concentration of the sample that was required to scavenge 50% of the DPPH free radicals. IC50 values were calculated using the inhibition curve by plotting extract concentration versus the corresponding scavenging effect.

Determination of total Phenolic Content

The total phenolic content of the extract was determined by using the Folin-Ciocalteu method using gallic acid as standard based on the oxidation-reduction reaction. The total phenolic content determination was performed with the help of the standard procedure given by Kim et al., n.d. with few modifications (Shackelford et al., 2009). 1 ml of crude extract of different concentrations (31.25, 61.5, 125, 250 and 500 µg/ml) was separately mixed with 5 ml 10% of Folin–Ciocalteu reagent. After standing for 5 min, 4 ml of 7% (w/v) sodium carbonate was mixed and shaken separately. The mixture was incubated for 40 min, and absorbance was measured at 760 nm. All experiment was carried out in triplicate for different concentration of samples.. The calibration curve was prepared using gallic acid as the standard of different concentrations (31.25, 61.5, 125, 250 and 500 µg/ml) in methanol. The total phenolic content was calculated from the calibration curve and the results were expressed as mg of gallic acid equivalent (GAE) per grm dry weight of extract.

Weight of extract using the formula, TPC= C×V/M where C=conc of Galic acid obtained from the calibration curve in mg/ml, V= (R2) value and regression equation Volume of extract in ml,

M=weight of plant extract in mg. the linear correlation coefficient were obtained from the gallic acid calibration curve. The regression equation was used to calculate the concentration of each extract. Thus with the calculated value of the concentration of each extract, the TPC was calculated.

Determination of total Flavonoid Content

Aluminium chloride colorimetric method was used for the determination of flavonoid content with slight modification. 1 ml of extract of each different concentrations (31.25, 61.5, 125, 250, and 500 µg/ml) was mixed with 4 ml of distilled water, and readily added 0.3 ml of 5% sodium nitrite separately. After 5 minutes, added 0.3 ml 10% aluminium chloride and allowed to stand for 6 minutes. Then, added 2 ml of 1 M sodium hydroxide and finally added 2.4 ml of distilled water so that vol. was made to 10 ml, it was shaken well and absorbance was measured at 510 nm using the UV spectrophotometer. The calibration curve was prepared using quercetin as the standard of different concentrations (31.25, 61.5, 125, 250, and 500 µg/ml). Total flavonoid content was calculated from the calibration curve and results were expressed as mg of quercetin equivalent (QE) per gram dry extract weight of the extract.

Statistical Analysis

All the experiments were performed in triplicate. Results were expressed as mean ± Standard deviation (SD). The IC_{50} values and all the statistical analysis were carried out through Microsoft Excel 2016.

The linear correlation coefficient (R^2) value was calculated from the data recorded as a mean of triplicate absorbance for different concentration of Gallic acid and Quercetin. The regression equation is given as $Y = mx + c$ …… (4)

Where, Y = Absorbance of extract, m = Slope from the std. calibration curve, x = Concentration of extract, c = Intercept

The TPC and TFC value of extract in different concentration was calculated using this regression equation.

Antimicrobial Activity Test

Agar well diffusion method was used in the study of screening and the evaluation of the antibacterial activity of the crude plant extracts the diameter of zone of inhibition (ZOI) produced by the plant extracts on particular bacteria was measured for the estimation of their antimicrobial activity**.** In biological screening, the effect of the crude plant extract at a fixed dose level in species of the organism was studied. In this work, the antibacterial assay was performed. Inhibition of the bacteria was tested by agar well diffusion method and measured in the form of the zone of inhibition (ZOI). The antimicrobial assay was performed at, Crimson College of Technology (CCT), BMLT Department Butwal Rupandehi.

Collection of antibiotics and Test organisms

Commercially available Gentamyc in (as gram-positive bacteria) was used as positive control and Ciprofloxacin (as a gram-negative standard) was used. Gram-positive bacteria were: *Staphylococcus aureus* and Gram-negative bacteria was: *Escherichia coli.* DMSO was used as a negative control. All the strains were obtained from Crimson College of technology, BMLT

Department, Rupandehi, Nepal.

Preparation of Plant Extract (Working Solution)

100 mg/mL of working solution was made by transferring 0.01 g of the crude extract to a sterile vial aseptically containing 1 ml of DMSO solvent and dissolved thoroughly in DMSO to prepare stock solution so each 10ul of the sample contained 1 mg of plant extract. After making up a stock solution, the test tubes were capped, sealed, and stored in the refrigerator (2-8 ºC) until use.

Collection of Antibiotics and Test organisms

Commercially available Gentamycin (as a gram-positive bacteria) was used as positive control and Ciprofloxacin (as a gram-negative standard) was used. Gram-positive bacteria were: *Staphylococcus aureus and* Gram-negative bacteria were: *Escherichia coli. DMSO was used as a negative control.* All the strains were obtained from Crimson College of technology, BMLT Department, Rupandehi, Nepal.

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Preparation of Culture Medium-The media used in the study were prepared according to the manufacturer's recommendation. The detailed procedure is given below:

Mueller Hinton Agar (MHA) Media Preparation

38 g of MHA was placed in 1000 ml of the conical flask and 1000 ml distilled water was added. In the conical flask, the media dissolved completely and closed with a cotton plug and aluminium foil. Then the conical flask was sterilized in an autoclave at 121°C for 15 minutes at 15 Ibs pressure for sterilization. The hot conical flask media was allowed to cool to 40- 50°C in sterilized laminar airflow. This media was poured into each petri plate and dropped to set.

Preparation of nutrient Agar Media

It was prepared by adding distilled water to13 g of nutrient agar in a conical flask and made final vol.1000 ml(13g/L) and boiled with continuously shaking . Then it was sterilized by autoclaving at 15 Ibs pressure (121^0C) for 15 minutes. The sterilized media was allowed to cool about 50 ºC. They were distributed in the sterilized petri-plates of 90 mm diameter in the ratio of 25 ml per plate aseptically and labeled properly. Plates were then left as such for solidification.

Inoculation of Bacteria into the Media, Screening and Evaluation of Antimicrobial Activity:

Already prepared Sterile Mueller-Hinton Agar (MHA) plates were dried to remove the excess moisture from the surface of the media. The sterile cotton swab was dipped into the standard inoculums and the excess of the inoculums was removed by pressing and rotating against the upper inside wall of the tube above the liquid level and then swabbed carefully all over the plates This process was repeated two more times to maintain the uniform distribution of microorganism. The plate was rotated at an angle of 60° after each swabbing. Finally, the swab was passed around the edges of the agar surface. The inoculated plates were left to dry for a day in laminar

air flow. The wells were made in the incubated media plates with the help of sterile cork borer of the diameter of 6mm and labeled properly. Then, 20 µl of the working solution of the plant extracts was loaded into the respective wells with the help of micropipette. DMSO was used as negative control and Gentamycin was used as a positive control in the separate well for the antibacterial activity. The plates were then left for half an hour with the lid closed so that extracts diffuse into the media. The plates were incubated overnight at 37°C.The plates were then observed for the zone of inhibition (ZOI) produced by the antibacterial activity of plant extracts and the inhibition zones were measured by the use of a scale.

Measurement of ZOI

The zone of inhibition was determined. After 24 hours of incubation, the culture media was taken out from the incubator, the inhibited area (ZOI) by extract and antibiotics were measured in mm, with the help by ruler.

RESULT AND DISCUSSION

Extraction Yield Value;

The percentage yield of methanolic extract of wild fruit of *zanthoxylum aramatum* was found to be 12.65 % (w/w).

Phytochemical Screening

Phytochemical screening revealed the presence of flavonoids, Polyphenol, Alkaloids, steroids, Tannins, Terpenoids, Coumarins, Quinones, Glycosides, Saponins, Proteins, Phenolic compounds, etc. These compounds are responsible for several pharmacological activities.

Determination of Antioxidant Activity

Variation of DPPH Radical Scavenging Activity

The percentage of DPPH radical scavenging activity of samples at different concentrations in methanol is shown in figure 1.The DPPH radical scavenging activity in terms of IC_{50} value of wild fruit sample was51.596µg/ml. DPPH radical scavenging activity regarding ascorbic acid standardIC_{50} value was61.474µg/ml. This assay is a simple and widely used and most acceptable technique to evaluate the antioxidant potency of plant extracts. The antioxidants are the chemical compounds of the plant which are capable of enacting the visually noticeable quenching of the stable purple-colored DPPH radical to the yellow-

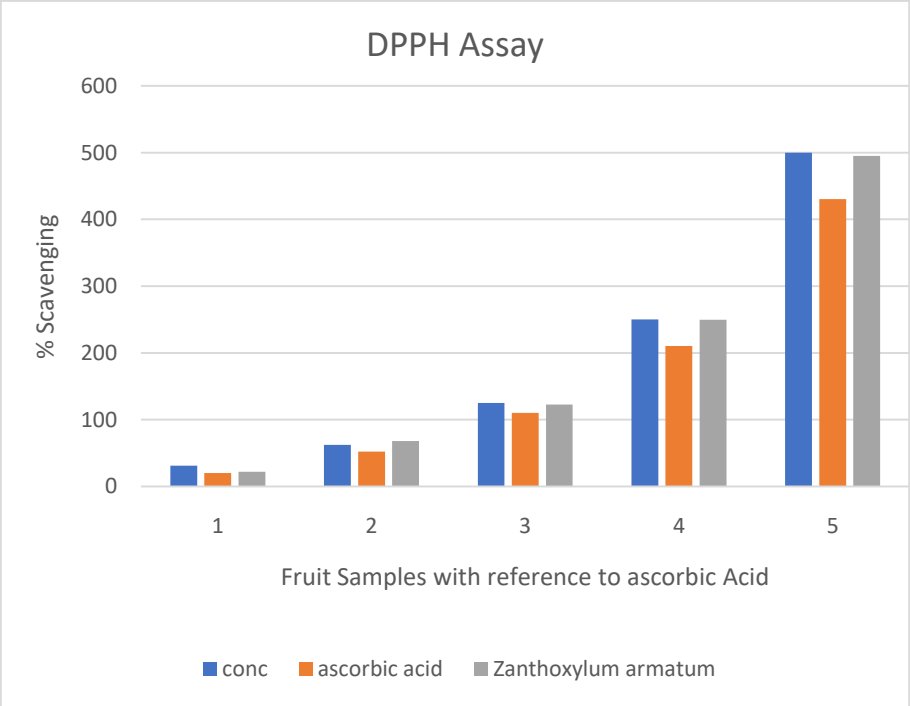

Fig. 1: Percentage Scavenging of DPPH free radicals by methanolic extract of fruit with reference to ascorbic acid, results expressed as the mean ± standard deviation (n=3) at a concentration of 31.25 to 500ppm.

Determination of Total Phenolic Content (TPC)

The total phenolic content of methanolic extracts was estimated by Folin- Ciocalteu's method using gallic acid as standard. Gallic acid solution of concentration (31.25-500 µg/ml) confirmed Beer's Law at 760 nm with a regression coefficient (R^2) = 0.9983 (Figure 2). The highest total phenolic content was found in wild fruit extract 244.46±0.03 mg GAE/g dry extract weight.

Calibration curve for standard Gallic Acid

TABLE 2: ABSORBANCE OF GALLIC ACID AT DIFFERENT CONCENTRATION

Concentration of Gallic acid (PPM)	Absorbance(mean)
500	2.343
250	1.131
125	0.579
62.5	0.294
31.25	0.253

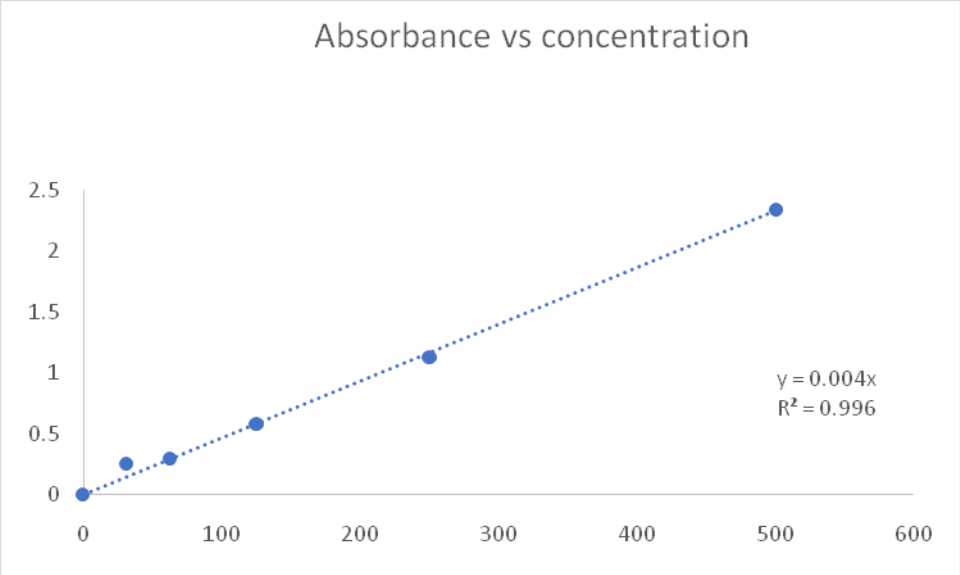

Figure2: Calibration curve for standard Gallic acid

TABLE 3: TOTAL PHENOLIC CONTENT (TPC) OF EXTRACT

Concentration of extract (ppm)	Absorbance(mean)	Total Phenolic Content (TPC) mgGAE/gm
500	1.149±0.02	244.46±0.03
250	1.124±0.01	239.14±1.43
125	0.738±0.04	157.021±4.33
62.5	0.411±0.023	87.44±7.35
31.25	0.395±0.012	84.042±5.34

4.5 Determination of Total Flavonoid Content (TFC)

The total flavonoid content for methanolic extracts was measured with the aluminium chloride colorimetric assay using quercet in as standard. The quercet in solution of concentration (31.25-500 µg/ml) confirmed to Beer's Law at 510 nm with a regression co- efficient (R^2) = 0.9989 (Figure 2). Total flavonoid content was found 131.66±0.06 mg QE/g

TABLE 4: ABSORBANCE OF QUERCET IN AT DIFFERENT CONCENTRATION

Concentration of Standard Quercetin (ppm)	Absorbance (mean)
500	0.305
250	0.156
125	0.085
62.5	0.046
31.25	0.023

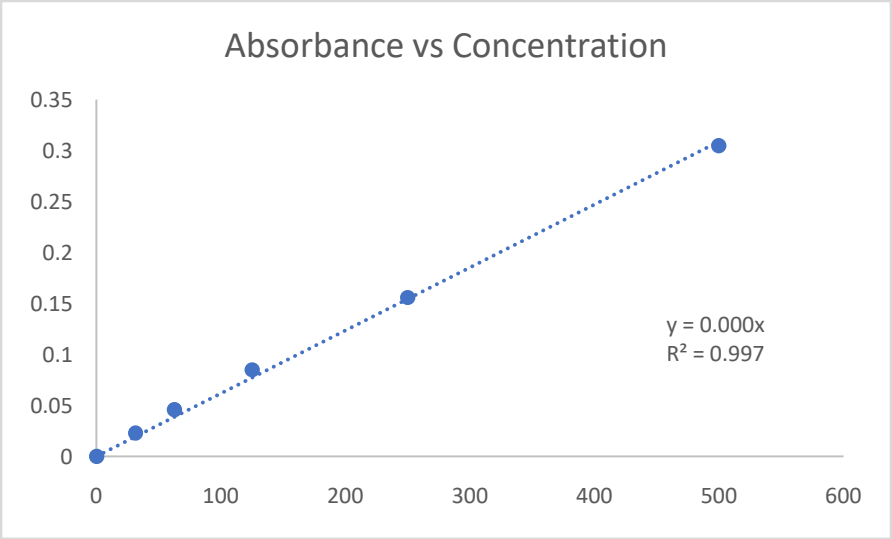

Figure 3: Calibration curve for standard Quercet in

TABLE 5: TOTAL FLAVONOID CONTENT (TFC) OF EXTRACT

Concentration of extract (PPM)	Absorbance(mean)	Total Flavonoid Content (TFC) mgQE/gm
500	0.079±0.001	131.66±0.06
250	0.074±0.012	123.33±0.44
125	0.071±0.003	118.33±0.54
62.5	0.050±0.02	83.33±1.03
31.25	0.043±0.01	71.66±3.12

Result of Antibacterial Test

The methanolic extract of fruit was screened for its antibacterial activity against two bacterial strains.Its antibacterial potency was confirmed by the absence or presence of an inhibition zone all over the disc loaded with the extract, Gentamic in Ciprofloxacin, and DMSO as reference.

The result showed that extract was more sensitive to gram- positive bacteria as compared to gram- negative bacteria. The methanolic fruit extract was reported to be most significant against *S. aureus* (ZOI= 8 mm). However, the extract remains insensitive against *E. coli.*

Figure 4: Antibacterial activity of methanolic fruit extract of Z. aramatum

TABLE 6: ANTIBACTERIAL ACTIVITY OF METHANOLIC FRUIT EXTRACT OF Z. ARAMATUM

Sample	Zone of Inhibition(mm)	
	E. coli	***S.auerus***
Methanolic Extract of fruit	-	8 mm
Gentamicin(+ve control)	15 mm	25 mm
Ciprofloxacin	10 mm	15 mm
DMSO(-ve control)	-	-

Note: (-) Indicate inactive in the evaluated concentration.

The calculation for Total Phenolic Content (TPC) in different concentration of plant extract.

TABLE 7: TOTAL PHENOLIC CONTENT FOUND IN DIFFERENT CONCENTRATION OF THE EXTRACT

Conc^{n} of Extract(ppm)	Weight of dry extract mg/ml	Absorbance(y)	GAEconc µg/ml (mean x)	GAE conc^{n} mg/ml (mean C)	TPC(mean) = $\frac{CXV}{M}$ (mg of GAE/gm)
500	0.0005	1.149±0.02	244.46	0.24446	244.46±0.03
250	0.000250	1.124±0.01	239.14	0.23914	239.14±1.43

125	0.000125	0.738±0.04	157.021	0.15702	157.02±4.33
62.5	0.000062	0.411±0.023	87.44	0.08744	87.44±7.35
31.25	0.000031	0.395±0.012	84.042	0.084042	84.042±5.34

The calculation for Total Flavonoids Content (TFC) in different concentration of extract

TABLE 8: TOTAL FLAVONOID CONTENT FOUND IN DIFFERENT CONCENTRATION OF THE EXTRACT

Conc^n of Extract (ppm)	Weight of dry extract mg/ml	Absorbance (mean) (y)	QEconc^n µg/ml (mean x)	QEmg/ml (meanC)	TFC(mean) = (mg of QE/gm)
500	0.0005	0.079±0.001	131.66	0.13166	131.66±0.06
250	0.000250	0.074±0.012	123.33	0.1233	123.33±0.44
125	0.000125	0.071±0.003	118.33	0.1183	118.33±0.54
62.5	0.0000625	0.050±0.02	83.33	0.08333	83.33±1.34
31.25	0.00003125	0.043±0.01	71.66	0.07166	71.66±3.12

CONCLUSIONS

The tudy shows the methanolic extract of wild fruit Z.armatum possesses potent anti-oxidant and antibacterial activity.The methanolic fruit extract was significantagainst *S. aureus*.The DPPH radical scavenging activities and subsequently the IC50 values of methanolic extracts of the plants showed a high degree of antioxidant property.The greater antioxidant property might be due to the presence of bio-active constituents such as polyphenol,flavonoids, etc. The TPC and TFC were also found inthe highest amount inwild fruit extract.Hence, this plant could be a potent source of natural drugs. Further extensive phytochemical and pharmacological investigation must be done.

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