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ANALYSIS OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF LEAF AND BARK EXTRACTS OF *ZANTHOXYLUM ARMATUM* DC.

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ABSTRACT

The present study was executed on *Zanthoxylum armatum* DC. a medicinal, aromatic, deciduous, erect, spiny tree/shrub, traditionally used by people for tooth cleaning, toothache, and mouth care of cattle. The antibacterial screening was done by using the Agar-well diffusion method against four human pathogenic bacteria viz. *Listeria monocytogenes*, *Salmonella typhi*, *Shigella dysenteriae*, *Escherichia coli*. Antibacterial results concluded that methanol extracts showed higher zones of inhibition against *Escherichia coli* i.e., 20.33 mm in the case of bark and 18.67 mm in the case of leaf, and acetone extracts showed higher zones of inhibition against *Salmonella typhi* (16.67 mm) in case of bark and against *Shigella dysenteriae* (16.67 mm) in case of a leaf. Antioxidant activity was examined by using DPPH free radical scavenging assay. Among leaf and bark, bark extracts showed higher antioxidant potential (IC_{50} values for acetone-88.16 µg/mL and methanol-76.37 µg/mL) as compared to leaf extracts ((IC_{50} values for acetone-143.46 µg/mL and methanol-98.86 µg/mL).
Keywords: Plant extracts, Agar-well diffusion, and DPPH

Introduction

Himachal Pradesh is a hilly state situated in the Trans and Northwest Himalayas (30°22′40″- 33°12′40″ N to 75°45′55″- 79°04′20″ E) and covers 55,673 km^2 area (Kumar, 2015). Out of a total 55,673 km^2 area 37948, km^2 area is legally classified as forest land, among this classified forest land, 15434 km^2 areas is under tree cover (actual forest area). Among the total plant species, there are about 1500 species of medicinal and aromatic plants found in Himachal Pradesh (Chauhan,1999).

The use of plant products has a long history, beginning with folk medicine and covering all aspects of traditional and allopathic medicine (Dubey *et al.,* 2011). Since ancient times, plants have been used by humans to treat common infectious diseases and still some of these traditional medicines are included as part of the habitual treatment of various diseases (Rios and Recio, 2005). Still, 75-80% of the world population of developing countries relies on herbal medicine, for their primary health care because of lesser side effects, better cultural acceptability, and better compatibility with the human body (Kamraj, 2000). The use of traditional medicine and medicinal plants as a normative basis for the maintenance of good health in most developing countries has been widely observed (UNESCO, 1996).

At present time microbial infections are one of the great challenges for human health. Microorganisms cause many diseases in humans e.g., *Staphylococcus* and *Streptococcus* species cause respiratory and skin infections, along with *Pseudomonas* and members of the Enterobacteriaceae causing gastrointestinal, and urogenital diseases and wound contamination (Neu, 1992). With the advancement in the field of Science and Technology, there is a remarkable boost in discoveries of many natural and synthetic drugs (Preethi *et al.,* 2010). But in recent years, human pathogenic bacteria have become drug-resistant and are commonly reported from all over the world (Piddock *et al.,*1989). Antioxidants are compounds that delay or inhibit the oxidation of lipids or other molecules and act as free radical scavengers, helping in minimizing the effect of oxidative stress in various diseases like cardiovascular diseases, Parkinson's disease, Alzheimer's disease, cancerogenesis, Neuro-degenerative, nephrotoxicity, diabetes and the aging (Chew *et al.,* 2011). Synthetic antioxidants such as butylated hydroxytoluene (BHT) are more effective, but these may have side effects and are responsible for liver damage and carcinogenesis (Barlow, 1990). Due to possible side effects and the toxic nature of synthetic antioxidants, there is an increase in attention toward natural antioxidants (Naimiki, 1990). Naturally, plants contain a big variety of compounds that possess antioxidant activity, like vitamin C, vitamin E, carotenes, xanthophyll, tannins, and phenols (Chanwitheesuk *et al.*, 2005).

Zanthoxylum armatum DC. belongs to the family Rutaceae. The genus *Zanthoxylum* includes about 200 species, it grows in the valleys of the sub-tropical Himalayas (Majid *et al*., 2004). The plant *Z. armatum* is an aromatic, deciduous, erect, spiny tree/shrub. The plant is mainly distinguished by thorns on the stem and foliage and ash-like leaves. The ripped fruits are usually reddish in colour of about 4-5 mm in size. Local people use the stem and bark of

this plant for tooth cleaning, and toothache. The dry leaf powder is used for mouth care of cattle. The leaves are also used by people for making chutney. The stem of *Z. armatum* is also used by local people as a pestle for crushing various items in mortar, because of its long life and the stem didn't get any fungal/ bacterial growth easily.

In India, different parts of the plant are used in Ayurvedic practices for the treatment of skin diseases, abdominal pain, anorexia, and ataxia (Chaudiere and Ferrari-Iliou 1999). The plant is used for the treatment of stomach and toothaches, intestinal worms, snake bites, rheumatism, scabies, fever, and cholera (Anonymous, 1976).

A lot of work has been done in the Microbiology Lab of the Department of Biosciences, Himachal Pradesh University Shimla on the antioxidant and antibacterial potentials of various medicinal plants (Prakash *et al.,* 2016; Rana *et al.,* 2017; Sagar *et al*., 2018; Bala *et al.,* 2019; Singh *et al.,* 2020; Prakash and Sagar 2021). The present investigations were undertaken on *Zanthoxylum armatum* DC. a medicinal plant of Mandi District of Himachal Pradesh, traditionally used by local people. The objectives of this work were to accumulate ethnobotanical knowledge and examined the antibacterial and antioxidant activities of the leaf and bark extracts of the selected plant.

Materials and Methods

Survey and collection

Galma village at an altitude of 1360 m in Mandi district of Himachal Pradesh was selected as the collection area of study material.

Procurement of bacteria

Different strains of bacteria (*Salmonella typhi, Escherichia coli, Shigella dysentriae,* and *Listeria monocytogenes*) were procured from the Department of Biotechnology, HPU Shimla for screening antibacterial properties of different plant extracts.

Revival of pathogen

The collected pathogens were revived in nutrient broth and stored in nutrient agar slants at 4°C.

Maintenance and preservation of pure cultures

Pure cultures of all the bacteria were maintained in nutrient medium broth and preserved in a refrigerator. Sub-culturing was done at regular intervals in order to maintain the cultures. Each bacterial species was transferred from the parent source to maintain and preserve the cultures.

Preparation of plant extracts

Extracts (acetone and methanol) of different parts of plants were prepared to check antibacterial and antioxidant activities. 5 gm dried plant material was taken in separate Erlenmeyer flasks to which 50 mL of required solvents i.e., methanol and acetone were added. The flasks were covered with aluminum foil and allowed to stand for 3-5 days for extraction. These extracts were filtered through Whatman filter paper no. 1 and allowed to evaporate at room temperature. The dry extracts were collected and weighed. Finally, a stock solution of conc. 50 mg/mL was prepared in Dimethyl sulfoxide (DMSO).

Screening of extracts for antibacterial activity

Agar-well Diffusion Method:

Different extracts (methanol and acetone) of medicinal plants were screened using the Agar-well diffusion method. Nutrient agar medium (Beef extract 1 g, Yeast extract 2 g, Sodium Chloride 1 g, Peptone 5 g, Agar 20 g, Distilled Water 1000 mL) was used throughout the investigation. The medium was autoclaved at 121.6°C for 30 minutes and poured into Petri plates. Bacteria were grown in nutrient broth for 24 hours. A 100 µL of bacterial suspension was spread on each nutrient agar plate. Agar wells of 8 mm diameter were prepared with the help of a sterilized stainless steel cork borer in each Petri plate. The wells in each plate were loaded with 25, 50, 75, and 100% concentrations of prepared plant extracts. A Petri plate contained Streptomycin, kept as a positive control. The plates were incubated at 37±2°C for 24 hours in the incubation chamber. The zone of growth inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the good diameter. The readings were taken in a perpendicular direction in all three replicates and the average values were tabulated (Hemashenpagam and Selvaraj, 2010).

Antioxidant activity

DPPH Radical Scavenging Activity Assay:

The free radical scavenging activity of plant extracts was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Blois (1958). Briefly, to 1 mL of different concentrations (20, 40, 60, 80, and 100 µg/ml) of plant or test extract, 1 ml of DPPH (0.1 mM in methanol) was added. A corresponding blank sample was prepared and ascorbic acid was used as a reference standard. The mixture of 1 mL DMSO and 1 mL DPPH solution (without plant extract) was used as a control. All the tests were carried out in triplicate and the decrease in absorbance was measured at 517 nm after 30 minutes in dark using a UV-VIS spectrophotometer. The percentage of inhibition was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Where, $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of the sample

Graphs were plotted against percentage inhibition v/s concentration of plant extracts and standard ascorbic acid in order to find out the values of slope and y-intercepts. IC_{50} value (the amount of antioxidant required to decrease the initial DPPH concentration by 50%) for each extract and ascorbic acid was evaluated using the following equation given below:

$$IC_{50} = \frac{50 - Y\text{-}Intercept}{Slope}$$

Results

Antibacterial activity

Acetone and methanol extracts of leaf and bark are used to test the antibacterial potential of plants against some human pathogenic bacteria (*Listeria monocytogenes*, *Salmonella typhi, Shigella dysenteriae, Escherichia coli*) and the results of the above assay are presented in Figs. 1-4.

Antibacterial activity of bark extracts:

Acetone bark extract showed maximum zones of inhibition i.e., 16 mm, 16.67 mm, 14.67 mm, and 15.67 mm at 100% concentration against bacteria *Listeria monocytogenes*, *Salmonella typhi, Shigella dysenteriae,* and *Escherichia coli,* respectively and minimum i.e., 11.67 mm, 13.33 mm, 12.33 mm, 12 mm, respectively at 25% concentration. Acetone bark extract was most effective against *S. typhi* with a maximum zone of inhibition i.e., 16.67 mm.

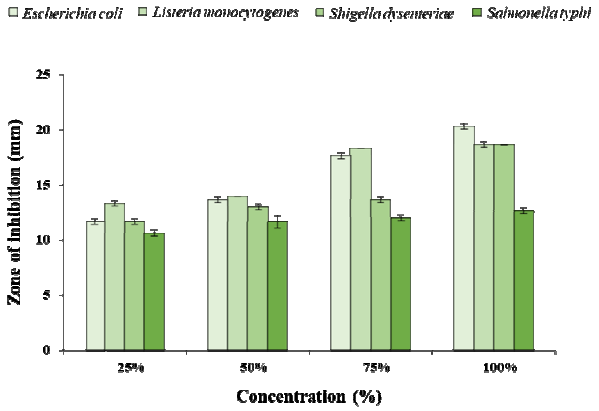

Fig. 1 : Antibacterial activity of methanol bark extract of *Z. armatum*

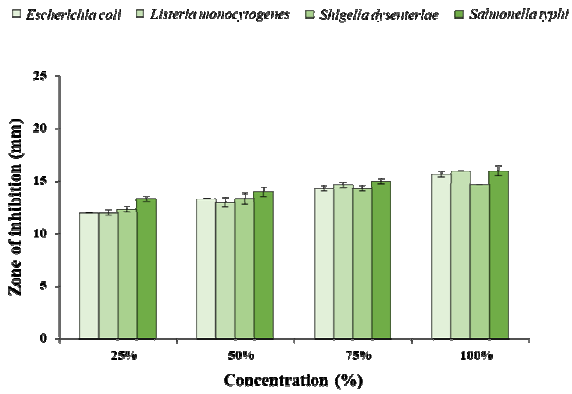

Fig. 2 : Antibacterial activity of acetone bark extract of *Z. armatum*

In methanol bark extract maximum zones of inhibition were i.e., 19 mm, 12.67 mm, 18.67 mm, and 20.33 mm at 100% concentration against bacteria *Listeria monocytogenes*, *Salmonella typhi, Shigella dysenteriae, Escherichia coli,* respectively and minimum 13.33 mm, 10.67 mm, 11.67 mm, 11.33 mm, respectively at 25% concentration. Methanol bark extract was most effective against *E. coli*, showing a maximum zone of inhibition of 20.33 mm.

Antibacterial activity of leaf extracts

In the case of acetone, leaf extracts maximum zones of inhibition were 16.33 mm, 15.67 mm, 16.67 mm, and 16 mm at 100% concentration against bacteria *Listeria monocytogenes*, *Salmonella typhi, Shigella dysenteriae,* and *Escherichia coli,* respectively and minimum 12.67 mm, 12.33 mm, 12.33 mm and 12 mm, respectively at 25% concentration. Acetone leaf extract was most effective against *S. dysenteriae*, with a maximum zone of inhibition of 16.67 mm.

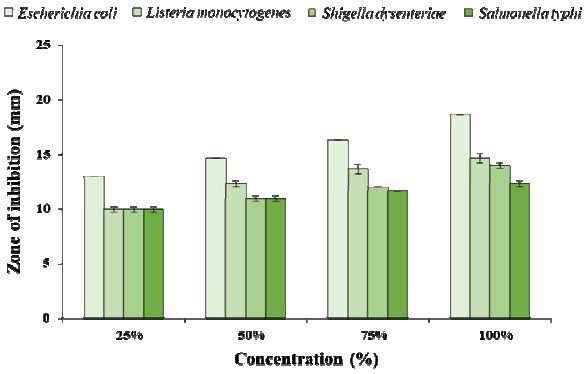

Fig. 3 : Antibacterial activity of methanol leaf extract of *Z. armatum*

In the case of methanol leaf extract the zones of inhibition were maximum i.e., 14.67 mm, 12.33 mm, 14 mm, and 18.67 mm at 100% concentration against bacteria *Listeria monocytogenes*, *Salmonella typhi, Shigella dysenteriae,* and *Escherichia coli,* respectively and minimum 16.33 mm, 15.67 mm, 16.67 mm and 16 mm, respectively at 25% concentration. Methanol leaf extract was most effective against *E. coli* with a maximum zone of inhibition of 18.67 mm.

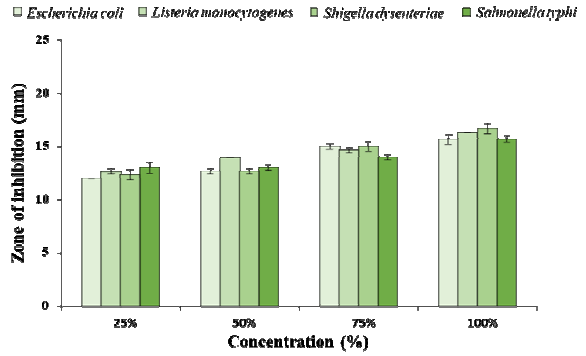

Fig. 4 : Antibacterial activity of acetone leaf extract of *Z. armatum*

Antioxidant activity

In this study acetone and methanol extracts of the bark and stem of *Z. armatum* have been used to determine the antioxidant potential of the plant. The antioxidant activity of extracts was evaluated by using DPPH free radical scavenging assay and results are shown in Table 1 and Figs. 6-7. Ascorbic acid was taken as standard which shows an IC_{50} value of 43.07 as shown in Fig.- 5.

Table 1 : IC_{50} values (µg/mL) of acetone and methanol extracts of Leaf and Bark of *Zanthoxylum armatum* DC.

Plant Part	IC_{50} value (µg/mL) of Acetone	IC_{50} value (µg/mL) of Methanol
Bark	88.16	76.37
Leaf	143.46	96.86

Free radical scavenging activity of bark extracts:

Results presented show that the scavenging activity of both methanol and acetone extracts of bark increase with the increase in the concentrations of the extracts. Methanol extract of the bark has the least IC_{50} value (76.37 µg/mL)

thus, exhibited higher antioxidant activity as compared to acetone extract (IC_{50} = 88.16 µg/mL).

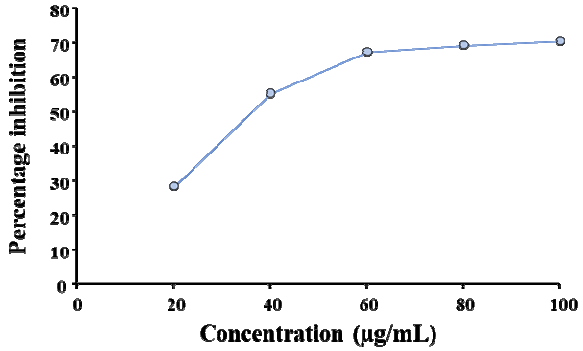

Fig. 5 : Free radical scavenging activity of Ascorbic acid

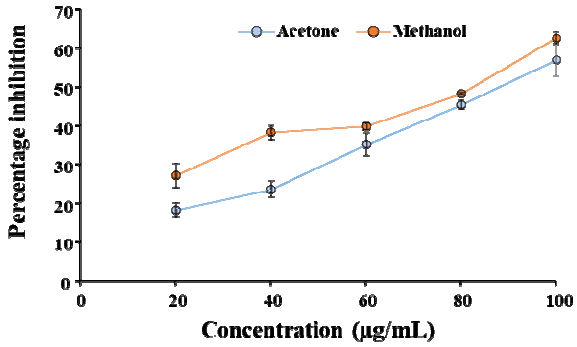

Fig. 6 : Free radical scavenging activity of acetone and methanol bark extracts of *Zanthoxylum armatum* DC.

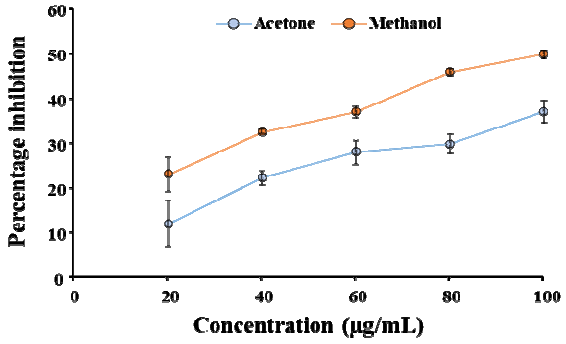

Fig. 7 : Free radical scavenging activity of acetone and methanol leaf extracts of *Zanthoxylum armatum* DC.

Free radical scavenging activity of leaf extracts

Results recorded show that the free radical scavenging activity of both methanol and acetone leaf extracts, increase with increase in the concentrations of the extracts and methanol leaf extract expressed the least IC_{50} (96.86 µg/mL) and higher antioxidant activity as compared to acetone extract (IC_{50} = 143.46 µg/mL).

Discussion

Antibacterial activity screening

From the study, we found that both the bark and stem of *Zanthoxylum armatum* DC. possessed significant antibacterial activity. Methanol extracts of both plant parts showed high antibacterial activity than that of acetone extracts. Methanol extracts showed higher zones of inhibition against *Escherichia coli,* 20.33 mm in the case of bark and 18.67 mm in the case of the leaf. Acetone extracts showed higher zones of inhibition against *Salmonella typhi* (16.67 mm) in the case of bark and against *Shigella dysenteriae* (16.67 mm) in the case of the leaf.

Srivastava *et al.* (2013) examined the antibacterial activity of bark extracts of *Z. armatum*. The antibacterial potential was examined against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris,* and *Pseudomonas aeruginosa* using the well diffusion method. The highest inhibition zone was observed in acetone extract against *S. aureus* (42.3 mm) followed by acetone extract against *E. coli* (31.7 mm) followed further by methanol extract against *S. aureus* (28.7 mm).

Antioxidant activity

In the present study, it was observed that both leaf and bark possessed considerable antioxidant potential. Methanol extract showed higher antioxidant activity than acetone extract. Among leaf and bark, bark extracts showed least IC_{50} value (acetone-88.16 µg/mL and methanol-76.37 µg/mL) as compared to leaf extracts (acetone-143.46 µg/mL and methanol-98.86 µg/mL).

Phuyal *et al.* (2020) studied the antioxidant activity of fruit, seed, and bark extracts of *Z. armatum* DC. From their study they evaluated, the IC_{50} value of the standard (ascorbic acid) as 36.22 µg/mL. Fruit extract showed the highest oxidant activity having an IC_{50} value of 45.62 µg/mL. Seeds showed an IC_{50} value of 86.75µg/mL and bark showed a moderate IC_{50} value of 67.82 µg/mL. Among fruit, bark, and seed, the fruit showed good antioxidant activity which lies near the standard.

Conclusion

From the present investigation, it has been concluded that both acetone and methanol extracts of bark possess significant antibacterial potential. Methanol extracts of both the plant parts exhibited considerable antioxidant and antibacterial activity. The leaf and bark extracts utilized for the study were in crude form, and may contain other compounds than specific secondary metabolites which are responsible for antibacterial and antioxidant potentials. For better results on *Zanthoxylum armatum* DC. further study on the identification and isolation of specific metabolites is required.

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