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EVALUATION OF ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF *ZANTHOXYLUM ALATUM* FRUIT AND LEAVES EXTRACTS AGAINST SELECTED PATHOGENIC BACTERIA

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ABSTRACT

Zanthoxylum alatum (*Z. alatum*), an important medicinal plant is used for various ailments including chest infection, cough, cholera, fever, stomach disorders, gas problems, indigestion, piles, toothache, gum problems, dyspepsia and stomachic worldwide. Keeping in view the medicinal potential of this plant, fruit and leaves methanolic (MeOH) extracts were prepared, evaluated for antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and for antibacterial potential by well diffusion and macrodilution methods *in-vitro*. Our results confirmed that *Z. alatum* fruit and leaves extracts have significant antioxidant activity with IC_{50} values 0.28 ± 0.07 mg/ml and 0.34 ± 0.05 mg/ml, respectively. The inhibitory trend at highest tested concentration (120 mg/ml) at 24 hr incubation in well diffusion method was recorded as 0.39, 0.30, 0.28, 0.27 and 0.18 cm against *S. pyogenes, B. cereus, E. coli, S. aureus and S. enterica* for leaf extract, respectively. In case of fruit extract inhibitory trend at highest tested concentration was observed as 0.33, 0.32, 0.31, 0.30 and 0.28 cm against *S. aureus, S. enterica, S. pyogenes, B. cereus* and *E. coli*, respectively. The fruit extract showed higher zones of inhibition than leaves extracts against all the test bacteria except *S. pyogenes.* Moreover, highest zones of inhibition were observed at lowest incubation (24 hr) and lowest zones were observed at highest incubation period (72 hr) for all tested concentrations. Macrodilution method showed antibacterial susceptibility in liquid medium with different levels of IC_{50} values ranging from 1.6±0.13 mg/ml to 10.3±0.7 5mg/ml. Interestingly, none of the tested bacteria showed resistance against any of the test extract in well diffusion or macrodilution method expressing the *Z. alatum* as potent candidates to kill bacteria in semisolid or in liquid medium to fulfill medical needs in future.

Keywords: bacteria, macrodilution method, *Zanthoxylum alatum,* zones of inhibition

INTRODUCTION

Bacteria cause infections which are responsible for heavy losses of human as well as animal lives and resources. Antibiotics have shown significant role in controlling infections. However, time proved that the bacterial control by antibiotics is temporary, and we expect to face perhaps never-ending struggle to cope because of the high risk of resistance development by bacteria. Recent studies reported that, the development of *Escherichia coli* resistance to commonly available drugs such as ciprofloxacin, ampicillin, cephalosporins, trimethoprim, sulfamethoxazole (Rodríguez-Bano *et al.,* 2004), *Staphylococcus aureus* resistance to penicillin, ampicillin (Yılmaz *et al.* 2017), *S. enterica* resistance to cephalosporin, macrolides, tetracycline, aminoglycosides, ketolides ampicillin (Afolami and Onifade, 2018), *S. pyogenes* resistance to most macrolides, lincosamide floroquinolone macrolide, streptogramin antibiotics (Ali *et al.,* 2001) and *Bacillus cereus* resistance to cephalosporin, penicillin. Therefore, search for new effective alternatives to combat microbial infections is always desirable.

The plant domain has a major contributions in health management that was remarkable worldwide even when no synthetic medicines or surgical progress evolved (Sher *et al.,* 2011). Our study plant *Zanthoxylum armatum* (*Z. armatum*), commonly known as Indian prickly shrub, while locally it is called as Nepal pepper

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or Tejphal in Hindi language. In Sanskri, Nepali and Chinese language it is known as Tejowati, Timur and Szechuan pepper, respectively (Singh *et al.,* 2011). *Z. armatum,* a thorny tree or shrub up to 5m tall, is distributed worldwide. Varieties of this precious tree are found in countries like Pakistan, Jammu and Kashmir, Bangladesh, Nepal, India, China, Taiwan, Vietnam, Japan, Korea, Indonesia, Bhutan, Laos, Myanmar and Thailand (Batool *et al.,* 2010). Phytochemical analysis of seed, leaves, fruit, root and barks of *Z. armatum* reveals the presence of medicinally important chemicals including terpenoids, alkaloids, sterols, steroids, lignins, flavonoids, coumarins, amides, carbonyl compounds, aromatic compounds and other aliphatic compounds (Singh *et al.,* 2011).

This plant is considered as a spiritually benefited and used in the religious festivals in different parts of the subcontinent. In Unani or herbal medicine system, its fruits, seeds and bark are used extensively for the preparation of medicines against different diseases. The fruit and bark have many therapeutic uses such as cough, asthma, colic, convulsion, general debility, cephalalgia, heart debility, dyspepsia and fever. Besides this, the different parts are being utilized for the treatment of helminthiasis, eye and ear diseases, skin disease, splenopathy strangury, goitre, difficult micturition, odentalgia, pharyngopathy, diabetes, leprosy, stomach disorders, tumors, paralysis, leucoderma, ulcers, diarrhoea, hepatopathy, flatulence, otopathy, and wounds.

The seeds of *Z. armatum* are very effective to strengthen the liver, in cholera, intestinal worms, remove bad breath from mouth, insanity and diseases related to brain (Barua *et al.,* 2018). Moreover, the studies proved that the seeds have some important biological activities such as analgesic, antiproliferative, antifungal, antioxidant, larvicidal, anthelminthic, anti-inflammatory, antibiotic, hepatoprotective, antiplasmodial, antiviral, antinociceptive, anti-tumor and cytotoxic (Barua *et al.,* 2018).

Keeping in view the medicinal potential of *Z. alatum* plant, fruit and leave MeOH extracts were prepared, their antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and, bacterial inhibition in concentration and incubation dependent manner by well diffusion (semi solid medium) method against selected bacteria was evaluated. To recapitulate inhibitory effect of test extracts in liquid medium, macrodilution method was applied aiming to find the possibility of test extracts to inactivate bacteria to meet the medical needs in future.

MATERIALS AND METHODS

Selected plant and preparation of extracts

Z. alatum leaves and fruits were collected from Nakyal, AJK, Pakistan. Selected plant parts were washed with tap water and air dried under shade for 2 weeks. Then, ground to fine powder and stored in airtight glass bottles. The powdered leaf and fruit samples were dipped in 1mg /10ml of methanol in separate flasks for 7 days with daily 5 min shaking. After filtration, filtrate was evaporated by rotary evaporator, air dried and saved as extract stock for further use according to experimental design.

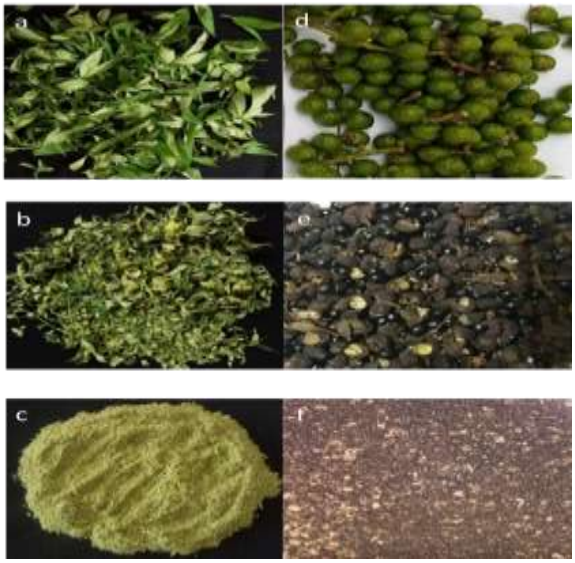

Figure 1. *Zanthoxylum alatum* leaves and fruit samples. a-c) *Z. alatum* leaves a) fresh, b) dry and c) grinded and, d-f) *Z. alatum* fruit d) fresh, e) dry and f) grinded form is shown

Chemicals

During this study 1, 1-di-phenyl-2-picrylhydrazyl (Sigma), ascorbic acid, analytical methanol (Sigma), Nutrient broth (Merck, Germany) and Nutrient agar (OXOID CM003, UK) were used.

Bacterial strains and culture conditions

The bacterial strains *Bacillus cereus* (*B. cereus*) ATCC 10876, *Staphylococcus aureus* (*S. aureus*) ATCC 2592, *Streptococcus pyogenes* (*S. pyogenes*) ATCC 12384, *Salmonella enterica* (*S. enterica*) and *Escherichia coli* (*E. coli*) ATCC 8739 were used in present study. Bacteria were streaked on agar plates, incubated at 37°C for 17 hrs then plates were stored at 4°C or used to prepare liquid culture in nutrient broth.

Antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to check antioxidant activity of our plant extracts. The stock solution was prepared by dissolving DPPH in methanol. Then, DPPH working solution OD= 0.98 ± 0.02 was obtained at 490 nm wavelength. DPPH working solution was mixed with test extract and read the sample at 490 nm after 10min incubation to find IC_{50} for antioxidants activity.

Evaluation of antibacterial susceptibility by well diffusion method

Well diffusion method was used to calculate the effects of prepared extracts on selected bacteria as described previously (Khurshid *et al.,* 2019). Bacteria were spread homogeneously on agar plates and 5 wells (6 mm in diameter) on each plate were made by sterilized cork borer. To find bacterial susceptibility, 50 µl of 120 mg/ml, 60 mg/ml, 30 mg/ml, 15 mg/ml and 0 mg/ml (control) of test extract was loaded in each well. Plates were incubated at 37°C, and inhibitory zones around the wells were measured in cm after 24 hrs, 48 hrs and 72 hrs.

Testing bacterial inhibitory concentration (IC_{50}) by broth dilution method

Macrobroth dilution method was used to determine bacterial inhibitory concentration of test extracts. Serial dilutions of test extract were prepared and added in sample tubes having bacteria and, control tubes having broth only (without bacteria). The total volume as 1 ml per tube was adjusted with broth. Then, samples were incubated at 37°C for 24 hrs and read in plate reader (BioTek Lx800) at 470 nm wave length. Later, IC_{50} in mg/ml was evaluated and compared with control to find bacterial susceptibility against test extract in liquid medium.

Statistical analysis

All experiments were performed twice in triplets. Significant difference was calculated by Student's t-test using lowest zone of inhibition (except zero) against each tested zone of inhibition ($p^a < 0.05$; $p^b < 0.005$, $p^c < 0.0001$) (Khurshid *et al*., 2019).

RESULTS AND DISCUSSION

In present study, *Z. alatum* fruit and leaves MeOH extracts were prepared aiming to evaluate their antibacterial potential in concentration dependent and incubation dependent manners against *B. cereus, E. coli, S. enterica, S. aureus* and *S. pyogenes*. Later, antioxidant activity was evaluated.

Antioxidant activity of test extracts

To find antioxidant activity of test extracts, DPPH assay was performed. The extract concentration required to achieve 50% inhibition was obtained and expressed as inhibitory concentration (IC_{50}). The IC_{50} values recorded for *Z. alatum* fruit and leaves were 0.28 mg/ml and 0.34 mg/ml, respectively (Table 1). The antioxidant activity of fruit is shown more than leaves, but still less than ascorbic acid (a reference). The MeOH fruit extract of *Z. alatum* showed DPPH free radical scavenging activity so its extract can be used as a good source of antioxidant. Strong antioxidant properties of *Z. alatum* are associated with high contents of phenols and flavonoids (Sharififar *et al*., 2009; Tosun *et al*., 2009).

Table 1. Evaluation of antioxidant activity of *Z. alatum* fruit and leaves MeOH extracts

***Z. alatum* extract type**	**DPPH inhibition IC_{50} ± SEM (mg/ml)**
Fruit	0.28 ± 0.07
Leaves	0.34 ± 0.05
Ascorbic acid	0.1 ± 0.02

Antibacterial activity of leaf and fruit extracts by well diffusion method

Bacterial zones of inhibition as the measure of antibacterial potential of MeOH leaf extract at concentrations i.e. 120 mg/ml, 60 mg/ml, 30 mg/ml and 15 mg/ml were measured as 0.30 cm, 0.20 cm, 0.13 cm, 0.07 cm against *B. cereus,* 0.27 cm, 0.20 cm, 0.10 cm, 0.06 cm against *S. aureus,* 0.18 cm, 0.03 cm, 0.029 cm, 0.003 cm against *S. enterica*, 0.28 cm, 0.16 cm, 0.09 cm, 0.03 cm against *E. coli* and, 0.39 cm, 0.28 cm, 0.23 cm, 0.18 cm against *S. pyogenes*, respectively at 24 hrs of incubation (Table 2). Moreover, zones of inhibition by MeOH fruit extract at 120 mg/ml, 60 mg/ml, 30 mg/ml and 15 mg/ml concentrations were measured as 0.30 cm, 0.23 cm, 0.15 cm, 0.09 cm against *B. cereus*, 0.33 cm, 0.28 cm, 0.20 cm, 0.12 cm against *S. aureus,* 0.32 cm, 0.23 cm, 0.13 cm, 0.07 cm against *S. enterica,* 0.28 cm, 0.16 cm, 0.09 cm, 0.03 cm against *E. coli* and, 0.31 cm, 0.23 cm, 0.17 cm, 0.09 cm against *S. pyogenes*, respectively at 24 hrs of incubation (Table 2).

Table 2. Evaluation of bacterial activity of *Z. alatum* MeOH fruit and leaves extracts by well diffusion method. Student's t-test was applied to check significant difference using lowest zone of inhibition (except zero) vs each tested zone of inhibition, where $p^a < 0.05$, $p^b < 0.005$ and $p^c < 0.0001$

Z. alatum **MeOH extract type**	**Concentration (mg/ml)**				**Concentration (mg/ml)**				**Concentration (mg/ml)**				**Test bacteria**
	15	**30**	**60**	**120**	**15**	**30**	**60**	**120**	**15**	**30**	**60**	**120**	
	Zone of inhibition (cm) at 24 hrs incubation				**Zone of inhibition (cm) at 48 hrs incubation**				**Zone of inhibition (cm) at 72 hrs incubation**				
Fruit	0.09± 0.01	0.15± 0.01	0.23± 0.02	0.30± 0.01	0.07± 0.01	0.14± 0.02	0.22± 0.02	0.29± 0.02	0.06± 0.01	0.13± 0.02	0.22± 0.02	0.29± 0.02	*B. cereus*
Leaves	0.07± 0.02	0.13± 0.02	0.2± 0.02	0.30± 0.02	0.06± 0.01	0.13± 0.02	0.19± 0.02	0.29± 0.02	0.06± 0.01	0.12± 0.02	0.18± 0.02	0.28± 0.02	
Fruit	0.12± 0.01	0.20± 0.01	0.28± 0.01	0.33± 0.02	0.11± 0.01	0.19± 0.01	0.27± 0.01	0.32± 0.02	0.11± 0.01	0.18± 0.01	0.27± 0.01	0.32± 0.03	*S. aureus*
Leaves	0.06± 0.009	0.10± 0.01	0.20± 0.02	0.27± 0.02	0.05± 0.009	0.09± 0.01	0.19± 0.02	0.26± 0.02	0.04± 0.008	0.08± 0.02	0.17± 0.02	0.25± 0.02	
Fruit	0.07± 0.02	0.13± 0.02	0.23± 0.02	0.32± 0.02	0.06± 0.02	0.12± 0.01	0.23± 0.02	0.32± 0.02	0.06± 0.02	0.12± 0.01	0.24± 0.06	0.31± 0.02	*S. enterica*
Leaves	0.003± 0.002	0.029± 0.006	0.03± 0.004	0.18± 0.03	0.002± 0.002	0.029± 0.006	0.03± 0.004	0.17± 0.03	0.002± 0.002	0.026± 0.006	0.027± 0.005	0.16± 0.03	
Fruit	0.03± 0.01	0.09± 0.02	0.18± 0.01	0.28± 0.02	0.02± 0.01	0.08± 0.02	0.17± 0.01	0.27± 0.03	0.02± 0.01	0.08± 0.01	0.17± 0.01	0.27± 0.02	*E. coli*
Leaves	0.03± 0.01	0.09± 0.01	0.16± 0.01	0.28± 0.03	0.026± 0.01	0.08± 0.01	0.15± 0.02	0.27± 0.03	0.02± 0.01	0.07± 0.01	0.14± 0.02	0.25± 0.03	
Fruit	0.09± 0.02	0.17± 0.02	0.23± 0.02	0.31± 0.02	0.14± 0.02	0.27± 0.03	0.35± 0.01	0.46± 0.02	0.14± 0.02	0.26± 0.02	0.36± 0.02	0.45± 0.02	*S. pyogenes*
Leaves	0.18± 0.02	0.23± 0.02	0.28± 0.02	0.39± 0.03	0.17± 0.02	0.23± 0.02	0.29± 0.02	0.42± 0.03	0.17± 0.02	0.24± 0.02	0.29± 0.02	0.42± 0.04	

Table 3. Evaluation of *Z. alatum* fruit and leaves MeOH extracts bacterial inhibitory concentration (IC50)

Z. alatum **extract type**	**Bacterial inhibition IC_{50} ± SEM (mg/ml)**				
	B. cereus	***S. aureus***	***S. enterica***	***E. coli***	***S. pyogenes***
Fruit	2.2±0.06	4.8±0.13	4±0.4	4.4±0.09	2.5±0.02
Leaves	10.3±0.75	1.6±0.13	1.7±0.07	2.8±0.25	1.95±0.05

Thus, susceptibility trend at highest tested concentration (120 mg/ml) at 24 hr incubation was as *S. pyogenes* > *B. cereus* > *E. coli* > *S. aureus* > *S. enterica* for leaf extract and *S. aureus* > *S. enterica* > *S. pyogenes* > *B. cereus* > *E. coli* for fruit extract, respectively. Moreover, fruit extract showed higher zones of inhibition than leaves extracts against all test bacteria except *S. pyogenes*. Many studies have shown biological activities of *Z. alatum* including anti-inflammatory, analgesic, antinociceptive, antioxidant, antibiotic, hepatoprotective, antiplasmodial, larvicidal, cytotoxic, antiproliferative, anthelminthic, antiviral and antifungal (Barua *et al.,* 2018). Srivastava *et al.* (2013) evaluated antimicrobial efficacy of *Zanthoxylum* bark extracts prepared with acetone, chloroform and methanol by using diffusion method *S. aureus*, *E. coli*, *P. vulgaris* and *P. aeruginosa*. The results the research work confirmed that methanol and acetone extract of bark are more efficient as there was more inhibition of bacterial zones was observed as compare to chloroform when tested against *S. aureus*. Furthermore, it was also observed that chloroform extract has highest inhibition potential against *P. vulgaris* as compare to others (Srivastava *et al., 2013*). Concurrently, in the present study, we evaluated antibacterial activity of leaf and fruit MeOH extracts of *Z. alatum*. None of the tested bacteria showed any resistance. Interestingly, highest zones of inhibition were observed at highest concentration and lowest incubation period in order of 24 hrs > 48 hrs > 72 hrs for both fruit and leave extracts which is in accordance to our previous findings (Rafiq *et al.,* 2020).

Testing bacterial inhibitory concentration (IC_{50}) by broth dilution method

To evaluate bacterial susceptibility of test extracts in suspension state, calorimetric method was used (Rafiq *et al.,* 2020). The IC_{50} value for MeOH fruit extract was calculated as 2.2±0.06 mg/ml, 4.8±0.13 mg/ml, 4±0.4 mg/ml, 4.4±0.09 mg/ml and 2.5±0.02 mg/ml against *B. cereus, S. aureus, S. enterica, E. coli* and *S. pyogenes,* respectively (Table 3). Moreover, IC_{50} value for MeOH leave extract was obtained as 10.3±0.75 mg/ml, 1.6±0.13 mg/ml, 1.7±0.07 mg/ml, 2.8±0.25 mg/ml and 1.95±0.05 mg/ml against *B. cereus, S. aureus, S. enterica, E. coli* and *S. pyogenes,* respectively (Table 3). The trend in IC_{50} for MeOH fruit extract was *B. cereus* > *S. pyogenes* > *S. enterica* > *E. coli* > *S. aureus*. However, the IC_{50} trend for MeOH leaves extract was as *S. aureus* > *S. enterica* > *S. pyogenes* > *E. coli* > *B. cereus*. According to Guleria *et al.* (2013) essential oil and methanolic extracts from leaves of *Z. alatum* showed similar

inhibitory effect against crop infecting fungi namely *Alternaria alternate* with IC_{50} values 1623 ± 41.5 μg/ml and 1071 ± 26.2 μg/ml and *Curvularia lunata* with IC_{50} values 1322 ± 24.9 μg/ml and 948 ± 21.8 μg/ml, respectively (Guleria *et al.,* 2013).

Moreover, Guleria *et al.* (2013) reported antibacterial activity of the essential oil of *Z. alatum* against *B. subtilis, M. luteus, S. aureus, E. coli* and *P. aeruginosa* with minimum inhibitory concentration (MIC) as 500 μg/ml, 62.5 μg/ml, 125.5 μg/ml, 250 μg/ml and 1000 μg/ml, respectively. Recent reports expressed significant resistance at an alarming rate of medically important bacteria such as *B. cereus, S. pyogenes, S. aureus, S. enterica* and *E. coli* against commonly used antibiotics (Ali *et al.,* 2001; Rodríguez-Bano *et al.,* 2004; Bottone, 2010; Preethi *et al.,* 2010; Mahalingam *et al.,* 2011; Yılmaz *et al.,* 2017; Afolami and Onifade, 2018). However, in our study, no bacteria showed resistance against *Z. alatum* fruit or leaves MeOH extract in well diffusion or macrodilution method expressing the *Z. alatum* as a potent candidate to kill bacteria in semisolid or in liquid medium to fulfill medical needs in future.

CONCLUSION

In the present study *Z. alatum* fruit and leaves MeOH extracts were prepared. Both extracts exhibited good antioxidant activity. Moreover, both test extracts showed successful bacterial inhibition in concentration and incubation dependent manner in well diffusion (semi solid medium) method against all tested bacteria i.e. *B. cereus*, *E. coli, S. enterica, S. aureus* and *S. pyogenes.* Moreover, macrodilution method recapitulate inhibitory effect of test extracts in liquid medium presenting the *Z. alatum* fruit and leaves extracts as potent candidates to treat tested bacteria in future.

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AUTHOR'S CONTRIBUTION

A. Sharif: Conducted the experiments
H. Javed: Collection and data punching
A. Ali: Designed and supervised the experiments
I. Ahmed: Prepared initial draft
F. N. Khoso: Statistical analysis and proof reading

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