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Evaluation of Antibacterial Activity of *Euryale ferox* Salisb., a Threatened Aquatic Plant of Kashmir Himalaya

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ABSTRACT

The antibacterial activity of methanolic extract of seeds and leaves of *Euryale ferox* was tested against nine clinically isolated bacterial strains (*Staphylococus aureus*, *Escherichia coli*, *Pseudomonas aureoginosa*, *Citrobacter freundi*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Salmonella typhi and Salmonella typhimurium*) and subsequently was also tested for minimum inhibitory concentration (MIC) values which ranged from 0.25 to 500 mg/l against six ATCC bacterial strains using the micro broth dilution method. The broad spectrum activity displayed by the seed and leaf extracts appears to provide a scientific basis for the use of *E. ferox* in kidney problems and urinary tract infections in ethnomedicines.

Keywords: inhibition zones, methanol, MIC, Nymphaceae, seed extract
Abbreviations: CFU, colony forming unit; **GNB**, Gram-negative bacilli; **GPC**, Gram-positive cocci; **IZD**, inhibition zone diameter; **MIC**, minimum inhibitory concentration

INTRODUCTION

Euryale ferox, commonly known as makhana, is an annual aquatic medicinal plant that belongs to the family Nymphaceae. It is widely distributed throughout Russia, Korea, China, Japan, Bangladesh and India (Wu and Raven 1994). It grows wild in Kashmir and is a non-endemic threatened aquatic species (Khan 2000; Dar *et al*. 2002; Khan *et al.* 2004).The plant produces starchy seeds and is edible. Traditionally the plant has been used throughout the world to cure many diseases including chronic diarrhhoea, kidney problem, leucorrehea and spleen hypofunction (Brown 1995). The plant as a whole is used as an analgesic, aphrodisiac, astringent, deobstruent, oxytonic and tonic in China (Duke 1985). Leaves are used in difficult parturition (Shankar 2010). The plant is internally taken to treat vaginal discharge, impotency, premature and involuntary ejaculation and nocturnal emission (Brown 1995). In China this plant is cultivated for its stem, rhizomes and seeds (Sturtevant 1972). In the Indian traditional system of medicines the dry seeds of the plant are being used as an immunostimulant for mothers after childbirth and invalids with a relatively poor immune status (Puri *et al*. 2000). Various aspects of significant antioxidant activity have been evaluated in total extracts and fractions derived from *E. ferox.* The plant has also been shown to enhance the activities of Superoxide dismutase, Catalase and Glutathione peroxidase in V79-4 cells (Lee *et al.* 2002). Recent studies on antioxidant activities of *E. ferox* and its glycoside composition *in vitro* reveal that the plant extracts have potent scavenging activity against reactive oxygen species suggesting that seed of this plant have cardioprotective properties which may link with the ability of makhana to induce TRP32 and TrX-1 protein and to scavenge ROS (Samarajit *et al*. 2006; Verma *et al*. 2010).

In view of its traditional use, we decided to determine the antibacterial activity of *E. ferox* extracts for the development of possible new antimicrobial compounds.

MATERIALS AND METHODS

Plant material

E. ferox was collected as a whole plant from Manasbal Lake of Kashmir Himalaya, J&K, India in September 2008. Sampling was carried out immediately after seed formation and plants were collected manually at the age of 7 months in bulk from the Lake. After that four parts i.e., leaves, petioles, rhizomes and seeds were separated and dried separately. The plant was identified by the HOD, Kashmir University Herbarium (KASH), Centre of Plant Taxonomy, Department of Botany, University of Kashmir, Srinagar. A sample was deposited in the herbarium as voucher no. 1015.

Preparation of plant extracts (hot process)

Dried powder (50 g) from the seeds, leaves, petioles and rhizomes of *E. ferox* were extracted successively with petroleum ether, chloroform and methanol in a Soxhlet extractor. The solvents from all 12 extracts were concentrated under vacuum at 40–50°C using a rotary flash evaporator (Heidolph, Germany) and crude extracts were air dried at room temperature in a steady air current. The weight of the solid residue was recorded and assumed as the yield of crude extract. The dried extracts were then stored in air-tight jars at 4°C for microbial analysis.

Microbial strains

All bacterial strains used in this study were clinical strains, namely *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Salmonella typimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Citrobacter fruendii*, *Proteus vulgaris* and were obtained from the Bacteriology Laboratory, Department of Microbiology, SKIMS, Soura, Srinagar, J&K.

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Research Note

Antibacterial susceptibility tests

The disc diffusion method (Bauer *et al.* 1966) was used to determine the antibacterial activity of all 12 extracts which was determined in a mixed culture of Gram-positive cocci (GPC) and Gram-negative bacilli (GNB). The extracts which exhibited inhibitory activity were then tested against clinically isolated strains using Muller Hinton agar (MHA; Hi-Media). The MHA plates were prepared by pouring 15 ml of MHA into sterile Petri dishes. The plates were allowed to solidify for 15 min and 0.1 ml (0.5 MacFarland standard) inoculum suspension was swabbed uniformly and the plates were allowed to dry for 5-10 min. The concentrations of extracts (160 and 320 µg/ml/disc) were loaded onto 6 mm sterile discs (Hi Media). The loaded discs were placed on the surface of the medium and the compound was allowed to diffuse for 5 min and the plates were incubated. Inhibition zones formed around the discs were measured with a transparent ruler (in mm). Antibiotic discs and solvents were taken as positive and negative controls, respectively.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the extracts was performed by using a broth micro dilution method on 96 micro-well plates based on recommendations of the National Committee for Clinical Laboratory Standards (NCCLS 2000). Stored bacteria were resituated, identified and incubated in 5 ml of Muller Hinton broth (HMB) in an incubator at 37°C for 24 hrs to a density equal to that of No.1 McFarland standard. The bacterial suspension was further diluted with a broth to give a final inoculum of 10^6 CFU/ml. To evaluate bacterial sensitivity, serial two-fold dilutions of 500, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/l were prepared with HMB (Phillips *et al*. 1990). Each micro-well of the 96 micro-well plates contained 50 µl diluted agents and 50 µl bacterial strain suspension. A final test volume of 100 µl was dispensed into each well. Both methanol and gentamicin (Hi Media) were kept in one set of wells to act as negative and positive control, respectively. After 24 hrs incubation of the micro-well plate in an incubator at 37°C, bacterial growth was observed by visual inspection. At the same time, from every micro-well, 50 µl of treated suspension was removed and was inoculated on new MHA plates; bacterial colonies were count after incubation for 48 hrs. The initial concentration of no bacterial growth on the plates was considered as the individual agent minimum inhibitory concentration (MIC) in the micro dilution corresponding to individual strains.

RESULTS AND DISCUSSION

The present investigation of antimicrobial activity of *E. ferox against* a mixed culture of GPC and GNB reveals that the methanolic extracts of seeds and leaves possessed antibacterial activity (**Table 1, Fig. 1A**). The inhibition zone diameters (IZD) obtained through the disc diffusion assay of methanolic extracts of seeds and leaves of *E. ferox* against clinically isolated bacterial strains is shown in **Table 2**. The MIC values of the methanolic extract of seed and leaf extracts of *E. ferox* with gentamicin and methanol as positive and negative control, respectively against six standard strains are shown in **Table 2**. Among the strains, *K. pneumoniae* and *C. fruendii* were resistant towards the methanolic extract of both seeds and leaves (**Figs. 1B, 1C**). *P. aeruginosa* showed the maximum IZD (**Fig. 1D**) followed by *S. typhi* (**Fig. 1E**), *E. coli* (**Fig. 1F**) and *S. flexnerii* (**Fig. 1G**). *S. aureus* and *S. typhimurium* (**Figs. 1H, 1I**) were sensitive towards only the methanolic extract of seeds whereas *P. vulgaris* (**Fig. 1J**) was sensitive to higher concentrations of seed and leaf extracts. The antimicrobial activity of crude extracts from numerous plants has been evaluated by the agar disc dif-fusion assay (Guven *et al*. 2005) and MIC was determined, based on which anti-infective lead molecules were isolated or used as a therapeutic agent (Sawer *et al.* 2005). The IZD of some Gram-negative strains like *P. aeruginosa* was greater than Gram-positive strains (Venkata and Venkata 2008), as determined for fruit extracts

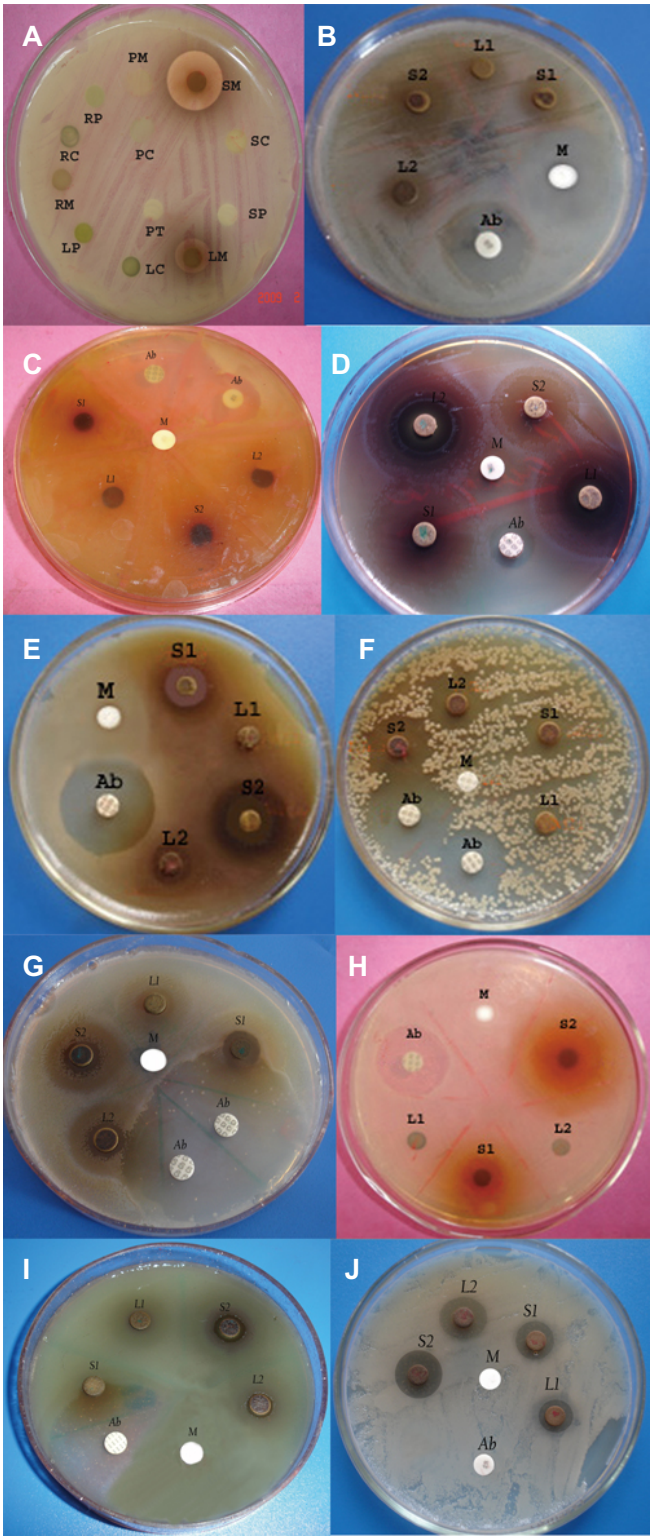

Fig. 1 (**A**) Gram-positive cocii and Gram-negative bacillus. (**B**) *Klebsiella pneumoniae*; (**C**) *Citrobacter fruendi*; (**D**) *Pseudomonas aeruginosa*; (**E**) *Salmonella typhii*; (**F**) *Escherichia coli*; (**G**) *Shigella flexneri*; (**H**) *Staphylococcus aureus*; (**I**) *Salmonella typhimurium*; (**J**) *Proteus vulgaris.* M = methanol: SI = 160 µg/ml, S2 = 320 µg/ml = seed methanol extract. L1 = 160 µg/ml, L2 = 320 µg/ml = leaf methanol extract, Ab = antibiotic; GNB = Gram-negative bacilli, GPC = Gram-positive cocci; Pt = petroleum ether; C = choloroform; Sm = seed methanol; SC = seed chloroform; SP = seed pet ether; LM = leaf methanol; LC = leaf chloroform; LP = leaf pet ether; PM = petiole methanol; PC = seed chloroform; PP = seed pet ether; RM = leaf methanol; RC = leaf chloroform; RP = leaf pet ether

of two *Syzygium* spp. The higher the extract concentration, the greater the inhibitory effect, supporting similar studies such as that by Boakye-Yiadom (1977), who discovered antimicrobial properties of some West African medicinal

Table 1 Antimicrobial activity of *Euryale ferox* extracts against mixed culture of GPC and GNB bacteria.

Bacterial strains	Inhibition zone diameter (mm)											
	Rhizome			Petiole			Leaf			Seed		
	Pt	C	M	Pt	C	M	Pt	C	M	Pt	C	M
Mixed culture of GPC and GNB	NA	NA	NA	NA	NA	NA	NA	IA	I A	NA	IA	IA

GPC = Gram-positive cocci, GNB = Gram-negative bacilli, Pt = petroleum ether extract, C = chloroform extract, M = methanol extract, IA = inhibitory activity; NA = no activity.

Table 2 Anti bacterial activity of methanolic extracts of seeds and leaves of *E. ferox* against clinically isolated bacterial strains.

Bacterial strains	Inhibition zone diameter (mm)				Antibiotics
	Methanolic seed extract		Methanolic leaf extract		
	160 µg/ml	320 µg/ml	160 µg/ml	320 µg/ml	
Staphylococcus aureus	12	23	NA	NA	Erythromycin (25)
Pseudomonasaeruginosa a	18	28	16	22	Amikacin (15)
Shigella flexneri	8	12	10	15	Ciprofloxacin (29)
Klebsiella pneumoniae	NA	NA	NA	NA	Gentamycin (25)
Salmonella typhi	14	19	8	12	Imipenin (28)
Salmonella typhimurium	0	15	0	10	Gatifloxacin (20)
Proteus vulgaris	NA	19	NA	20	Cefixime (0)
Citrobacter fruendii	NA	NA	NA	NA	Vancomycin (22)
Escherchia coli	12	15	8	12	Ceftazidime (30)

Results are mean of three readings; NA = no activity.

plants. Among the solvents, methanol extracts showed a higher activity than other solvents (petroleum ether and chloroform). Pavithra *et al*. (2010) also found that the methanolic extracts of *M. cerviana* exhibited significant antibacterial activity against Gram-positive and -negative strains with minimum bactericidal concentration (MBC) ranging from 1.5 to 100 mg/ml. This may be due to the higher polarity and maximum number of compounds extracted in methanol, which has also proved to be the most effective solvent for extracting a broad spectrum of antimicrobial compounds from plants (Vlachos *et al*. 1996).

From our study, the methanolic seed extract exhibited more inhibitory activity than the leaf extract against all tested bacterial strains and hence showed bactericidal activity against standard strains with *S. aureus* ATCC 25923 having a low MIC value (64 mg/l) and *S. flexneri* the highest (500 mg/l). The methanolic leaf extract of *E. ferox* showed bactericidal activity against some standard strains i.e., with an MIC value of 128 mg/l against *S. aureus* ATCC 25923, 256 mg/l against *Pseudomonas aeruginosa* ATCC 27853 and no activity against a standard *P. vulgaris* strain. Among the bacterial strains tested *S. aureus* and *P. aeruginosa* showed higher activity towards both seed and leaf extracts and some tested strains like *Acintobacter* sp., *K. pneumoniae* were least effective since they are naturally resistant to antibacterial agents (Walker and Edward 1999). The results of MIC revealed a decreasing trend in activity as the concentrations of extracts decreased, which implies that the extracts were more active at higher concentrations. The active results of most of strains tested were in the range of 256-500 mg/l and our findings are in agreement with the results of Idu *et al*. (2007) for the methanolic extracts of *Senna allata* flowers. The methanolic seed extract exhibited more inhibitory activity than the leaf extract against all tested bacterial strains and hence showed bactericidal activity against standard strains with *S. aureus* ATCC 25923 having a low MIC value of 64 mg/l and *S. flexneri* having the highest MIC value of 500 mg/l. while *S. aureus* and *E. coli* were inhibited by both seed and leaf extracts. Similarly, Pongpaichit *et al.* (2005), for the crude methanolic extract of *Acorus calamus* seed and leaf extracts and Panda *et al*. (2009), for the methanolic leaf and bark extracts of *Vitex negundo*, reported significant antibacterial activity against *E. coli* and *S. aureus*, respectively. However, results against *P. auruginosa* were contradictory to that observed by Pongpaichit *et al.* (2005). The antibacterial activity of *E. ferox* may be due to the presence of some antimicrobial compounds like cerebrosides and glycosterols (Zhao *et al*. 1989, 1994; Lin *et al*. 2003; Li and Xu 2007; Row *et al*. 2007).

Table 3 Minimum inhibitory concentration (MIC) of methanolic extracts of seed and leaf extracts of *E. ferox* against Standard strains (mg/l).

Standard bacterial strains	MIC (mg/l)		
	Seed extract	Leaf extract	Gentamycin 10 mg/l
Staphylococcus aureus ATCC 25923	64	128	32
Escherichia coli ATCC 25922	128	256	128
Pseudomonas aeruginosa ATCC 27853	64	256	16
Shigella flexneri ATCC 12022	ND	500	ND
*Proteus vulgaris**	500	ND	256
*Salmonella typhi**	256	500	ND

The seed and leaf extracts exhibited marked inhibitory action against *S. typhi*, a virulent strain that causes typhoid (Prescott 2005). With the increase in resistance to antityphoid drugs, medicinal plants have gained popularity among both urban and rural dwellers in the treatment of the ailment. Similarly, *Seena siamae* leaf extracts have been shown to have antibacterial activity against *S. typhi* and *S. typhimurium* (Doughari and Okafor 2008).

K. pneumoniae was not inhibited by any of the extracts tested and this may be due to the fact that it produces a capsule (Sule and Aghabiaka 2008). Methanolic extracts exhibited higher activity, as also concluded by Babayi *et al.* (2004) for *Eucalyptus camaldulensis* and *Terminalia catappa*. The crude methanolic extracts of *E. ferox* extract exhibited highest activity against *P. aeruginosa* and *E. coli* and similar activity has been reported from other medicinal plants, namely *Grewia erythraea*, *Hymenocrater sessilifolius*, *Vincetoxicum stocksii*, *Zygophyllum fabago* and *Arcenthobium Oxycedri* (Zaidi *et al*. 2005; Mudassir *et al*. 2006).

Among the strains, *P. aureoginosa*, *S. aureus* and *E. coli* exhibited higher zones of inhibition against both methanolic extracts. The antibacterial activity of seed extracts was also reported by Murtaza *et al.* (1994) while the antibacterial activity of the leaf extract may be due to the presence of alkaloids and flavonoids as reported by Ahmad *et al.* (2001) in the leaves of *Adhantoda vasica* Nees. However, the antibacterial activity may be due to the presence of one or more phytocompounds present in plants as most of them are known to have antimicrobial activity (Bruenton 1995). The MIC results depicted that gentamicin exhibited higher activity than the methanolic extracts of both plant parts of *E. ferox* (**Table 3**). This could be attributed to the fact that, unlike conventional antibiotics and other pharmaceutical products which are usually prepared from synthetic materials by means of reproducible manufacturing techniques and proce-

dures, herbal medical products are prepared from materials of plant origin which may be subjected to contamination and deterioration (Babu *et al*. 2002). The literature reveals that no prior work has been done on evaluating the antimicrobial activity of *E. ferox* and it is now expected that screening of this plant for antibacterial activity against a wide variety of test organisms will be helpful in obtaining a broad spectrum herbal formulation as well as new antimicrobial substances for the treatment of diseases like kidney problems and urinary tract infections.

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