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In-vitro Anthelmintic and Antimicrobial Activities of Stem Bark of *Eugenia jambolana* Lam.

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ABSTRACT

In order to scientifically appraise some of the folkloric uses of *Eugenia jambolana* Lam. (Myrtaceae), the present study was undertaken to examine the anthelmintic and antimicrobial properties of stem bark extracts. The anthelmintic activity of ethyl acetate and methanol extracts was tested on tape worm (*Moniezia expansa*) and hook worm (*Gaigeria pachyscelis*) at two concentrations, 100 and 200 mg/ml. Time taken for the inhibition of motility and the death were noted and compared with the standard drug, Piperazine citrate at 15 mg/ml. The plant extracts significantly paralysed the worms followed by death, which was comparable with that of standard. Further, these extracts along with petroleum ether extract were tested for their activity against 24 clinically isolated microbial strains, including identified strains, using agar well diffusion method. The methanol extracts showed highest activity followed by ethyl acetate, while the petroleum ether extract showed the lowest activity at 100 mg/ml. The inhibitory effect of extracts was compared with standard antibiotic Ciprofloxacin and antifungal miconazole. This study supports the folk claim.

Keywords: gastrointestinal, pathogen, plant extract, worm

INTRODUCTION

There are various infections with worms, most frequently in the gastrointestinal tract. Some of these conditions cause significant problems, most profusely diarrhea, anemia, liver diseases, lungs diseases, lethargy, dullness, loss of general body condition, loss of weight, pallor of visible mucous membrane, depression, protein-losing enteropathy leading to hypoproteinaemia, gastroenteritis, etc. (Adeolu *et al*. 2005). Most of these infections can be treated with anthelmintic medication. Presently in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases, but are also often with adulterations and side effects (Babayi1 *et al*. 2004).

Infectious diseases are the world's leading cause of premature deaths, killing almost 50 000 people every day. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Robin *et al*. 1998). The use of plant compounds to treat infections is an age-old practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases (Shibata *et al*. 2005; Gangoue-Pieboji *et al*. 2006). Interest in plants with antimicrobial properties has revived as a result of current problems associated with the use of antibiotics (Abu-Shanab *et al*. 2004; Shiota *et al*. 2004).

The present invention relates with the screening of *Eugenia jambolana* Lam., which has been found to have efficacy in reducing the above mentioned diseases. *E. jambolana* Lam. (Myrtaceae), commonly known as Jamun, Jam or Jambul in India is widely distributed through out India, Ceylon-Malaya and Australia. It has been valued in the Ayurveda and Unani systems of medication for possessing a variety of therapeutic properties. Most of the plant parts are used in traditional system of medicine in India.

Bark of the plant is digestive, anthelmintic, good against bronchitis, asthma, dysentery, ulcers and wound healing while the seeds are diuretic and stops urinary discharge, also used in the treatment of diabetes; fruit acts as liver tonic, enriches blood, teeth and gums (Prajapathi *et al*. 2003). Different parts were pharmacologically proved to possess hypoglycemic, antibacterial, anti-HIV activity, anti-diarrhea effects and anti-inflammatory activity (Teixeira *et al*. 2000; Muruganandan *et al*. 2001; Ravi *et al*. 2004). However, stem bark is the plant part most widely used in our study area by traditional practitioners. The vast ethnomedical applications to treat infectious diseases and several other ailments stimulated the investigation of the anthelmintic and antimicrobial activity of *E. jambolana* stem bark.

MATERIALS AND METHODS

Plant material

The bark of mature plants was collected from Sorab region of Shivamoga district, Karnataka, India during August to December (2008). The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number E/002/2008).

Preparation of plant extract

Freshly collected whole stem bark of *E. jambolana* (**Fig. 1**) was shade-dried and then powdered using a mechanical grinder. 100 g of pulverized plant part was extracted successively in 500 ml of petroleum ether, ethyl acetate and methanol (LR grade, Merck, India) using a Soxhlet apparatus. At the end of extraction, extracts were filtered under vacuum through Whatman No. 1 filter paper and the process repeated until all soluble compounds had been extracted. The filtrate obtained was concentrated *in vacuo* using a Rotavapor (Buchi Flawil, Switzerland). The extracts were stored at 4°C in an air-tight bottle until further use.

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Original Research Paper

Fig. 1 Tree (A) and stem bark (B) of *Eugenia jambolana*.

Phytochemical screening

The preliminary phytochemical analysis of petroleum ether, ethyl acetate, methanol extracts was carried out using the methods as described in Harborne (1984), Trease and Evans (1989), Kokate *et al*. (1998), and Khandelwal (2005).

Study of anthelmintic activity

The *in vitro* trial for anthelmintic activity of ethyl acetate and methanol extracts of *E. jambolana* was conducted on mature living tape worm (*Moniezia expansa*) and hook worm (*Gaigeria pachyscelis*) of sheep. The mature worms were collected from the freshly slaughtered sheep (*Ovis aries* L.) in the local butcher/slaughter house, Shivamogga and identified by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta. The worms were washed and finally suspended in 0.9 % phosphate buffer saline (PBS) (Zafar *et al*. 2006).

50 ml of the extract solution containing two different concentrations, each of ethyl acetate and methanol extract (100, 200 mg/ml in 1% Tween 80 in normal saline) were prepared and six worms were placed in it. Piperazine citrate (15 mg/ml; Glaxo Smithkline Pharmaceuticals (India) Ltd.) was used as reference standard while 1% Tween 80 in normal saline as control. The time taken to paralyze and kill individual worms was observed. Paralysis was noted when the worms became immobile even in the normal saline solution. Death was concluded when the worms lost their motility followed by fading body color.

Antimicrobial studies

The petroleum ether, ethyl acetate and methanol extracts were tested for antimicrobial activity by the agar well diffusion method (Nair *et al*. 2005).

Microorganisms used

The microbial strains used in this study were clinical isolates from different infection status of patients presenting symptoms of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Salmonella typhi*, *Salmonella paratyphi* and *Proteus vulgaris*, *Candida albicans*-associated diseases. The isolates were identified by a standard method (Cowan and Steel 1993). The standard strains used were *P. aeruginosa* ATCC-20852, *K. pneumoniae* MTCC-618, *S. aureus* ATCC- 29737, *S. typhi* MTCC- 3214, *S. paratyphi* MTCC- 735, *P. vulgaris* ATCC- 13315 and *C. albicans* ATCC-2091. The bacteria were maintained on agar slopes at 4°C and sub-cultured into nutrient broth by a picking-off technique (Aneja 2003) for 24 h before use.

Preparation of culture medium and inoculation

Nutrient agar (Hi Media, India) was used as the bacteriological medium. Yeast *C. albicans* strains were cultured in Potato Dextrose Agar (PDA) medium. Media were sterilized by autoclaving at 120°C for 20 min. Under aseptic conditions, in the laminar air flow 15 ml of culture medium was dispensed into presterilized Petri dishes to yield a uniform depth of 4 mm. After solidification of the medium, the microbial cultures were inoculated by spread plating technique.

Agar well diffusion

The extracts were dissolved in 10% DMSO to a final concentration of 100 mg/ml. Pure DMSO was taken as the negative control and 50 mg/ml Ciprofloxacin (fluoroquinolone antibiotic) and Miconazole as the positive control, both supplied by Cipla Ltd. Wells were prepared in the agar plates using a sterile cork borer of 6.0 mm diameter. 10 mg/100 µL of each extract were loaded in the corresponding wells. The plates were allowed to stand at room temperature for 1 h for extract to diffuse into the agar and then they were incubated at 37°C for 18 h. Subsequently, the plates were examined for microbial growth inhibition and the inhibition zone diameter (IZD) measured to the nearest mm.

Statistical analysis

The values of anthelmintic activity are presented as mean ± SEM for six observations. The results of the antimicrobial study are expressed as mean ± SEM of three replicates in each test. The data were evaluated by one-way Analysis of Variance (ANOVA) followed by Tukey's multiple pair-wise comparison tests to assess the statistical significance. The data were considered at $P < 0.001$.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical profile of various solvent extracts used in this study is presented in **Table 1**. The analysis revealed the presence of alkaloids, flavonoids, triterpenoids, sterols, tannins and glycosides. In particular, methanol extract showed positive for all the secondary metabolites tested ex-

Table 1 Phytochemical screening of extracts of *E. jambolana* stem bark.

Plant constituent	Extract		
	Petroleum ether	Ethyl acetate	Methanol
Alkaloids	-	-	+
Flavonoids	-	+	+
Triterpenoids	+	-	-
Sterols	+	+	+
Tannins	-	+	+
Saponins	-	-	-
Glycosides	-	+	+

Phytochemical test: - negative and + positive

cept for triterpenoids and saponins. Petroleum ether extract showed the presence of triterpenoids and sterols.

Anthelmintic activity

The extracts of *E. jambolana* stem bark exhibited anthelmintic activity in a dose-dependant manner giving shortest time of paralysis (P) and death (D) at 200 mg/ml. The worms incubated in the control medium showed physical activity for about 75-85 min. The ethyl acetate extract used in 100 and 200 mg/ml paralysed all the worms used between 30-40 and 20-26 min, respectively. Methanol extract at 100 and 200 mg/ml showed significant activity with paralysis of worms in less time (25-32 min and 15-23 min, respectively). Piperazine-treated worms paralysed between 12-16 min. The time taken for death of worms also varied in a similar manner. The plant extracts were more active against the tape worms tested. The data are depicted in **Tables 2** and **3**.

The predominant effect of Piperazine citrate on worm is to cause a flaccid paralysis those results in expulsion of the worm by peristalsis. Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyperpolarisation and reduced excitability that leads to muscle relaxation and flaccid paralysis (Martin 1985). Phytochemical analysis of the crude extracts revealed the presence of tannins among the other chemical constituents contained within them. Tannins were shown to produce anthelmintic activities (Niezen *et al*. 1995). Some synthetic phenolic anthelmintics e.g. niclosamide, oxyclozanide, bithionol, etc., are reported to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (Martin 1997). It is possible that tannins contained in the extracts of *E. jambolana* produced similar effects. Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal (Athnasiadou *et al*. 2001) or glycoprotein on the cuticle of the parasite (Thompson and Geary 1995) and may cause death. In the present study, the extracts not only demonstrated this property, they also caused death of the worms.

The traditional medicines hold a great promise as source of easily available effective anthelmintic agents to people, particularly in developing countries, including India. The origin of many effective drugs has been found in such medicinal practices and hence it is necessary to undertake studies pertaining to screening of the folklore medicinal plants for their proclaimed anthelmintic efficacy.

Antimicrobial studies

The extracts from stem bark of *E. jambolana* were effective in controlling the growth of clinical strains and also the standard strains tested (**Table 4**). The study revealed that the antimicrobial activity of methanol extract was highest with zone of inhibition in the range of 20.60 ± 0.31 to 13.43 ± 0.28. The lowest inhibition values were recorded by petroleum ether extract (17.80 ± 0.31 to 11.27 ± 0.71). The ethyl acetate fraction showed moderate activity. *P. aeruginosa* was the most susceptible and *S. aureus*, the least amongst all the bacterial strains investigated in the present work. Significant values were recorded against *C. albicans* strains (11.27 ± 0.71 to 14.93 ± 0.13), comparable with standard drug. Ciprofloxacin and miconazole, which were used as positive experimental controls against strains assayed, produced a zone of inhibition of 21.73 ± 0.55 to 29.43 ± 0.26 and 21.27 ± 0.65 to 23.97 ± 0.33, respectively while no inhibitory effect could be observed for DMSO used as negative control.

Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent (Shibata *et al*. 2005; Betoni *et al*. 2006). It has already been established that crude extracts of some medicinal plants and some pure compounds from such plants can potentiate the activity of antibiotics *in vitro* (Marquez *et al*. 2005; Smith *et al*. 2007). This search for antibiotic resistance modulators in plants represents a new dimension to addressing the problem of antibiotic resistance. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Lewis and Ausubel 2006). In the present work, the methanol extract was found to contain much of the bioactive constituents when compared to petroleum ether and ethyl acetate extracts. Our findings are in agreement with reports showing that polar extracts inhibited the growth of both Gram-positive and Gram-negative bacteria (Masika and Afolayan 2002; Karaman *et al*. 2003). The stronger extraction capacity of methanol could have produced a greater number of active constituents responsible for antimicrobial activity.

CONCLUSION

In conclusion, the traditional use of stem bark of *E. jambolana* Lam. as an anthelmintic and antimicrobial have been confirmed for the stem bark extracts displayed profound efficacy in the study. Further, it would be interesting to isolate the possible therapeutically useful compounds followed by *in vivo* assessments to determine the clinical

Table 2 Anthelmintic activity of *E. jambolana* stem bark extracts.

Worm	Time taken for paralysis					
	Control	EA		ME		Std
		100 mg/ml	200 mg/ml	100 mg/ml	200 mg/ml	15 mg/ml
Tape worm	80.67 ± 1.54	35.50 ± 1.18**	23.67 ± 0.67**	29.17 ± 0.75**	19.83 ± 0.95**	14.17 ± 0.60**
Hook worm	80.00 ± 1.29	32.17 ± 0.70**	23.50 ± 0.85**	27.67 ± 0.76**	18.17 ± 1.14**	13.17 ± 0.48**

The values are the mean ± SEM for six observations. *p< 0.001 vs. control (Tukey's pairwise comparison test); Abbreviations: ME, Methanol extract; EA, Ethyl acetate extract; Std, Piperazine citrate

Table 3 Anthelmintic activity of *E. jambolana* stem bark extracts

Worms	Time taken for death					
	Control	EA		ME		Std
		100 mg/ml	200 mg/ml	100 mg/ml	200 mg/ml	15 mg/ml
Tape worm	96.33 ± 3.11	47.33 ± 1.28**	37.17 ± 1.76**	40.67 ± 1.20	28.83 ± 1.22**	21.00 ± 1.21**
Hook worm	93.17 ± 2.57	44.33 ± 1.12**	34.00 ± 1.32**	34.67 ± 1.33**	26.50 ± 1.02**	19.17 ± 0.70**

The values are the mean ± SEM for six observations. *p< 0.001 vs. control (Tukey's pairwise comparison test); Abbreviations: ME, Methanol extract; EA, Ethyl acetate extract; Std, Piperazine citrate

Table 4 Antimicrobial activity of *E. jambolana* stem bark against clinically important microbial strains (inhibition zone in mm).

Microbial strains and source	Bark			Ciprofloxacin	Miconazole
	PE	EA	ME		
Pa 1 Urine	17.43 ± 0.58	18.33 ± 0.29	20.27 ± 0.25	28.33 ± 0.55	-
Pa 2 Pus	17.60 ± 0.50	18.40 ± 0.32	20.27 ± 0.20	28.00 ± 0.69	-
Pa 3 Stool	17.30 ± 0.21	18.40 ± 0.31	19.97 ± 0.19	29.43 ± 0.26	-
Pa 4 Sputum	17.57 ± 0.39	18.23 ± 0.15	20.23 ± 0.43	28.87 ± 0.44	-
Pa 5 ATCC-20852	17.80 ± 0.31	18.60 ± 0.31	20.60 ± 0.31	28.77 ± 0.64	-
Kp 1 Urine	12.93 ± 0.44	13.87 ± 0.29	16.53 ± 0.13	24.67 ± 0.35	-
Kp 2 Urine	13.10 ± 0.51	14.23 ± 0.30	16.13 ± 0.33	25.33 ± 0.57	-
Kp 3 Urine	12.90 ± 0.55	13.90 ± 0.52	16.43 ± 0.27	24.50 ± 0.35	-
Kp 4 Urine	13.20 ± 0.53	13.87 ± 0.44	16.20 ± 0.12	24.67 ± 0.24	-
Kp 5 MTCC-618	13.77 ± 0.58	14.60 ± 0.35	16.93 ± 0.61	25.40 ± 0.69	-
Sa 1 Wound swab	11.70 ± 0.32	12.33 ± 0.18	13.67 ± 0.48	21.73 ± 0.55	-
Sa 2 Mucus	11.87 ± 0.47	12.10 ± 0.06	13.43 ± 0.28	22.97 ± 1.02	-
Sa 3 Pus	12.13 ± 0.44	12.53 ± 0.29	13.47 ± 0.29	22.47 ± 1.33	-
Sa 4 Pimples	11.40 ± 0.69	12.40 ± 0.12	13.80 ± 0.23	23.30 ± 0.55	-
Sa 5 ATCC-29737	12.37 ± 0.52	12.83 ± 0.23	13.90 ± 0.17	24.20 ± 0.42	-
St 1 Blood	13.03 ± 0.58	15.13 ± 0.59	16.43 ± 0.39	23.43 ± 0.75	-
St 2 MTCC-3214	12.53 ± 0.35	17.10 ± 0.15	17.43 ± 0.32	24.63 ± 0.78	-
Sp 1 Blood	13.33 ± 0.70	14.00 ± 0.92	15.90 ± 0.40	24.33 ± 1.30	-
Sp 2 MTCC-735	13.83 ± 0.58	16.33 ± 0.29	16.93 ± 0.09	24.57 ± 0.90	-
P 1 Pus	12.23 ± 0.73	13.70 ± 0.25	15.13 ± 0.03	22.90 ± 0.61	-
P 2 ATCC-13315	12.70 ± 0.36	12.97 ± 0.45	15.43 ± 0.23	23.20 ± 0.75	-
Ca 1 Urine	11.37 ± 0.54	12.67 ± 0.24	14.93 ± 0.13	-	21.27 ± 0.65
Ca 2 Sputum	11.27 ± 0.71	12.93 ± 0.18	14.77 ± 0.43	-	22.63 ± 0.45
Ca 3 ATCC-2091	11.47 ± 0.66	13.13 ± 0.18	14.93 ± 0.57	-	23.97 ± 0.33

The values are the mean of three experiments ± S.E. Abbreviations: Pa, *Pseudomonas aeruginosa*; Kp, *Klebsiella pneumoniae*; Sa, *Staphylococcus aureus*; St, *Salmonella typhi*; Sp, *Salmonella paratyphi*; P, *Proteus* Spp.; ME, methanol extract; EA, ethyl acetate extract; PE, petroleum extract; -, not tested.

relevance of such compounds thus representing a potential area of future investigation.

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