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Screening of Crude Extracts of *Holarrhena antidysenterica* Wall. Against Clinically Important Pathogenic Strains

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ABSTRACT

The crude ethanol, chloroform and petroleum ether extracts of *Holarrhena antidysenterica* (stem bark, leaf and inflorescence) were tested against 18 clinically isolated strains including identified strains using the agar-well diffusion method. Of all the extracts the activity was more pronounced against Gram-negative bacteria than against Gram-positive ones. Amongst the evaluated extracts, the ethanolic stem bark extract showed the strongest antibacterial effect. The inhibitory effect of the extracts was compared with standard antibiotic Ciprofloxacin. Our results offer a scientific basis for the traditional use of *H. antidysenterica* in the treatment of infectious diseases.

Keywords: antibacterial, clinical isolates, plant extracts

INTRODUCTION

Since ancient times, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made a large contribution to human health and well-being. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants which have folklore reputation in a more intensified manner (Roja and Rao 2000; Awadh Ali *et al.* 2001; Nitta *et al.* 2002). Over the past few years, many efforts have been made to discover new antimicrobial compounds from various kinds of natural sources such as microorganisms, animals and plants. In this regard several Indian medicinal plants have been evaluated, a fair number of which possess potential antimicrobial activity (Ahmad *et al.* 1998) and few natural products have been approved as new antibacterial drugs (Kameshwara Rao 2000; Subramani and Goraya 2003). However, the increased prevalence of antibiotic resistant bacteria due to the extensive use of antibiotics may render the current antimicrobial agents insufficient to control some bacterial diseases and hence research for identifying novel substances that are active against human pathogens is an urgent need (Shahidi and Karimi 2004).

Plants are known to produce some chemicals that are naturally toxic to bacteria (Basile *et al.* 1999). Plant-based natural constituents can be derived from any part of the plant (Cragg and Newman 2001). The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Wink 1999).

The present study was undertaken to investigate the antibacterial activity of *Holarrhena antidysenterica* (Apocynaceae) against some enteric pathogens. The crude extracts of stem bark, leaf and inflorescence were tested for their potential antibacterial property. The selection of this plant for evaluation was based on its traditional use. *H. antidysenterica* has amoebicidal, antidysenteric, anthelmintic, antiperiodic, febrifugic and diuretic activities (Bhattacharjee 2000; Prajapati *et al.* 2004). The plant also finds folkloric usage in the treatment of bronchitis, hematuria, spermatorrhoea, epilepsy, asthma, piles, leprosy and eczema (Guha Bakshi 2001).

MATERIALS AND METHODS

Plant materials

Fresh materials of plants in the flowering stage above 20 cm diameter at breast height were collected locally from Bhadra Wild Life Sanctuary, Karnataka (Southern India) in May 2006. The taxonomic identification of the plant was confirmed by Dr. Y. L. Ramachandra, Department of Biotechnology, Kuvempu University, Shankaraghatta (Voucher specimen number YLR204).

Extraction

Freshly collected whole stem bark, leaf and inflorescence of *H. antidysenterica* were shade-dried and then powdered using a mechanical grinder. Ten grams of each pulverized plant part were separately soaked in 100 ml of petroleum ether, chloroform and ethanol (LR grade, Merck, India) and kept on a rotary shaker for 24 h. Each extract was filtered under vacuum through a Whatman No. 1 filter paper and the process repeated until all soluble compounds had been extracted. Extraction was considered to be complete when the filtrate had a faint colour. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). A portion of the residue was used for the antibacterial assay.

Bacterial culture

The bacterial strains used in this study were clinical isolates from different infection status of patients presenting symptoms of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*-associated diseases. The isolates were identified by a standard method (Cowan and Steel 1993). The standard strains used were *Pseudomonas aeruginosa* (ATCC-20852), *Klebsiella pneumoniae* (MTCC-618) and *Staphylococcus aureus* (ATCC-29737). The organisms were maintained on nutrient agar slope at 4°C and sub-cultured into nutrient broth by a picking-off technique (Aneja 2003) for 24 hrs before use.

Bacterial susceptibility testing

In vitro antibacterial activity of the crude extracts was studied against Gram-negative and Gram-positive bacteria by the agar

Received: 4 March, 2008. Accepted: 9 June, 2008.

Original Research Paper

well diffusion method (Nair *et al.* 2005). Nutrient agar (Hi Media, India) was used as the bacteriological medium. The extracts were dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 100 mg/ml. Pure DMSO was taken as the negative control and 50 mg/ml Ciprofloxacin as the positive control.

100 µl of inoculum was aseptically introduced on to the surface of sterile agar plates and sterilized cotton swabs were used for even distribution of the inoculum. Wells were prepared in the agar plates using a sterile cork borer of 6.0 mm diameter. 100 µl of test and control compound was introduced in the well. The same procedure was used for all the strains. The plates were incubated aerobically at 35°C and examined after 24 hours (Collins *et al.* 1989; Ali-Stayeh *et al.* 1998). The diameter of the zone of inhibition produced by each agent were measured with a ruler and compared with those produced by the commercial antibiotic Ciprofloxacin.

Statistical treatments

The results of the experiment are expressed as mean ± SE of three replicates in each test. The data were evaluated by one-way Analysis of Variance (ANOVA) followed by Tukey's multiple pairwise comparison tests to assess the statistical significance. $P \leq 0.05$ was considered as statistically significant, using software ezANOVA ver. 0.98. The data are presented in **Tables 1, 2** and **3**.

RESULTS AND DISCUSSION

The antibacterial activity of the crude extracts of *H. antidysenterica* was determined against 18 strains which include Gram-negative and Gram-positive bacteria. The plant extracts differ significantly in their activity. The antibacterial activity was observed to be in dependent on solvent i.e. the ethanol extracts showed more significant activity (8.73 ± 0.40 to 24.07 ± 0.06) than chloroform (8.53 ± 0.29 to

Table 1 Antibacterial activity of stem bark extracts against clinically important bacterial strains.

Bacterial strains tested and source	Stem bark			Ciprofloxacin
	PE	CE	EE	
Pa1 Urine	21.53 ± 0.14	23.00 ± 0.25	23.80 ± 0.32	24.53 ± 0.17
Pa2 Ear swab	22.13 ± 0.35	23.27 ± 0.39	23.67 ± 0.36	24.27 ± 0.30
Pa3 Pus	21.60 ± 0.15	23.07 ± 0.08	23.73 ± 0.37	23.60 ± 0.18
Pa4 Stool	21.80 ± 0.17	23.27 ± 0.20	24.07 ± 0.06	24.73 ± 0.24
Pa5 Sputum	21.67 ± 0.18	23.33 ± 0.20	23.53 ± 0.19	23.00 ± 0.18
Pa6 ATCC 20852	16.93 ± 0.33	18.07 ± 0.38	20.33 ± 0.18	22.80 ± 0.18
F-value	59.1	48.4	22.7	12.0
Kp1 Urine	22.00 ± 0.14	22.27 ± 0.28	22.40 ± 0.29	22.53 ± 0.13
Kp2 Urine	22.40 ± 0.09	23.13 ± 0.21	23.33 ± 0.12	22.73 ± 0.20
Kp3 Urine	22.33 ± 0.36	22.53 ± 0.25	22.67 ± 0.26	24.00 ± 0.22
Kp4 Urine	22.00 ± 0.44	22.80 ± 0.12	22.87 ± 0.17	22.93 ± 0.73
Kp5 Urine	22.27 ± 0.26	22.87 ± 0.44	23.27 ± 0.16	23.27 ± 0.55
Kp6 MTCC 618	17.33 ± 0.74	18.40 ± 0.42	19.40 ± 0.38	22.60 ± 1.13
F-value	20.5	28.0	29.7	0.68
Sa1 Wound swab	18.00 ± 0.63	19.33 ± 0.21	20.87 ± 0.30	20.53 ± 0.60
Sa2 Mucus	18.80 ± 0.10	19.80 ± 0.14	21.33 ± 0.42	21.67 ± 0.15
Sa3 Hospital effluent	18.40 ± 0.61	19.53 ± 0.01	20.33 ± 0.22	20.33 ± 0.27
Sa4 Pus	18.40 ± 0.64	20.20 ± 0.14	21.47 ± 0.33	21.87 ± 0.28
Sa5 Pimples	18.20 ± 0.14	19.60 ± 0.40	20.73 ± 0.30	21.73 ± 0.84
Sa6 ATCC 29737	12.87 ± 0.49	13.47 ± 0.07	13.87 ± 0.28	19.67 ± 0.13
F-value	17.6	134	71.3	3.33

The values are the mean of three experiments ± S.E. the F-value is significantly different at the 0.05% probability level

Abbreviations: Pa, *Pseudomonas aeruginosa*; Kp, *Klebsiella pneumoniae*; Sa, *Staphylococcus aureus*; PE, petroleum ether extract; CE, chloroform extract; EE, ethanol extract.

Table 2 Antibacterial activity of leaf extracts against clinically important bacterial strains.

Bacterial strains tested and source	Leaves			Ciprofloxacin
	PE	CE	EE	
Pa1 Urine	13.00 ± 0.50	16.33 ± 0.56	19.00 ± 0.39	24.53 ± 0.17
Pa2 Ear swab	13.00 ± 0.10	16.00 ± 0.56	17.33 ± 0.76	24.27 ± 0.30
Pa3 Pus	12.33 ± 0.40	15.33 ± 0.17	18.73 ± 0.19	23.60 ± 0.18
Pa4 Stool	12.80 ± 0.27	15.67 ± 0.33	17.00 ± 0.44	24.73 ± 0.24
Pa5 Sputum	13.00 ± 0.45	15.73 ± 0.62	16.67 ± 0.63	23.00 ± 0.18
Pa6 ATCC 20852	11.27 ± 0.34	11.93 ± 0.16	13.67 ± 0.44	22.80 ± 0.18
F-value	2.90	11.1	11.8	12.0
Kp1 Urine	12.73 ± 0.25	17.00 ± 0.22	17.67 ± 0.69	22.53 ± 0.13
Kp2 Urine	12.77 ± 0.30	17.33 ± 0.29	18.67 ± 0.69	22.73 ± 0.20
Kp3 Urine	12.20 ± 0.15	16.33 ± 0.29	16.67 ± 0.84	24.00 ± 0.22
Kp4 Urine	11.93 ± 0.13	16.67 ± 0.11	19.33 ± 0.69	22.93 ± 0.73
Kp5 Urine	12.27 ± 0.26	17.67 ± 0.68	19.00 ± 0.51	23.27 ± 0.55
Kp6 MTCC 618	10.87 ± 0.53	13.67 ± 0.48	14.67 ± 0.51	22.60 ± 1.13
F-value	4.5	11.2	5.83	0.68
Sa1 Wound swab	11.87 ± 0.52	13.67 ± 0.43	16.00 ± 0.27	20.53 ± 0.60
Sa2 Mucus	11.53 ± 0.20	13.33 ± 0.44	15.00 ± 0.36	21.67 ± 0.15
Sa3 Hospital effluent	11.73 ± 0.44	13.53 ± 0.28	14.00 ± 0.27	20.33 ± 0.27
Sa4 Pus	11.47 ± 0.16	14.00 ± 0.12	15.33 ± 0.40	21.87 ± 0.28
Sa5 Pimples	12.00 ± 0.04	13.33 ± 0.56	13.73 ± 0.10	21.73 ± 0.84
Sa6 ATCC 29737	9.67 ± 0.40	10.60 ± 0.23	11.33 ± 0.32	19.67 ± 0.13
F-value	5.36	9.10	25.0	3.33

The values are the mean of three experiments ± S.E. the F-value is significantly different at the 0.05% probability level

Abbreviations: Pa, *Pseudomonas aeruginosa*; Kp, *Klebsiella pneumoniae*; Sa, *Staphylococcus aureus*; PE, petroleum ether extract; CE, chloroform extract; EE, ethanol extract.

Table 3 Antibacterial activity of inflorescence extract against clinically important bacterial strains.

Bacterial strains tested and source	Inflorescence			Ciprofloxacin
	PE	CE	EE	
Pa1 Urine	9.00 ± 0.52	12.00 ± 0.42	15.33 ± 0.52	24.53 ± 0.17
Pa2 Ear swab	8.67 ± 0.63	12.33 ± 0.19	14.00 ± 1.19	24.27 ± 0.30
Pa3 Pus	9.33 ± 0.71	11.67 ± 0.52	14.00 ± 0.15	23.60 ± 0.18
Pa4 Stool	9.33 ± 0.39	11.67 ± 0.24	13.33 ± 0.73	24.73 ± 0.24
Pa5 Sputum	9.87 ± 0.18	12.33 ± 0.82	14.67 ± 0.58	23.00 ± 0.18
Pa6 ATCC 20852	5.53 ± 0.24	9.40 ± 0.42	11.47 ± 0.17	22.80 ± 0.18
F-value	8.67	4.38	3.39	12.0
Kp1 Urine	10.33 ±0.98	11.07 ± 0.50	13.67 ± 0.39	22.53 ± 0.13
Kp2 Urine	9.67 ± 0.42	11.33 ± 0.26	13.00 ± 0.40	22.73 ± 0.20
Kp3 Urine	11.00 ± 0.92	11.67 ± 0.33	13.33 ± 0.82	24.00 ± 0.22
Kp4 Urine	10.33 ± 0.42	10.67 ± 0.59	12.67 ± 0.53	22.93 ± 0.73
Kp5 Urine	10.00 ± 0.92	11.00 ± 0.68	12.33 ± 0.37	23.27 ± 0.55
Kp6 MTCC 618	7.67 ± 0.25	9.07 ± 0.07	9.87 ± 0.29	22.60 ± 1.13
F-value	2.16	3.34	6.27	0.68
Sa1 Wound swab	7.33 ± 0.16	9.67 ± 0.34	10.67 ± 0.68	20.53 ± 0.60
Sa2 Mucus	7.67 ± 0.25	9.67 ± 0.66	12.67 ± 0.68	21.67 ± 0.15
Sa3 Hospital effluent	7.67 ± 0.33	10.67 ± 0.34	12.67 ± 0.58	20.33 ± 0.27
Sa4 Pus	8.00 ± 0.18	10.33 ± 0.32	12.00 ± 0.66	21.87 ± 0.28
Sa5 Pimples	8.33 ± 0.16	10.00 ± 0.58	12.33 ± 0.53	21.73 ± 0.84
Sa6 ATCC 29737	4.87 ± 0.22	8.53 ± 0.29	8.73 ± 0.40	19.67 ± 0.13
F-value	25.5	2.28	5.59	3.33

The values are the mean of three experiments ± S.E. the F-value is significantly different at the 0.05% probability level

Abbreviations: Pa, *Pseudomonas aeruginosa*; Kp, *Klebsiella pneumoniae*; Sa, *Staphylococcus aureus*; PE, petroleum ether extract; CE, chloroform extract; EE, ethanol extract.

23.33 ± 0.20) and petroleum ether (4.87 ± 0.22 to 22.40 ± 0.09) extracts. Among all, the ethanol extract of stem bark was most active against the Gram-negative bacteria *Pseudomonas aeruginosa* from stool sample in comparison to all the bacteria tested at the same concentration.

These observations may be attributed to two reasons; firstly, due to the nature of biologically active components (alkaloids, flavonoids, sterols, quinine, tannins etc.) which might be enhanced in the presence of ethanol (Kabir *et al.* 2005). It has been documented that alkaloids, flavonoids and tannins are plants metabolites well known for their antimicrobial activity (Tschesche 1971). Secondly, the stronger extraction capacity of ethanol could have produced a greater number of active constituents responsible for antibacterial activity.

Gram-negative bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were more susceptible to the plant extracts than Gram-positive bacterium *Staphylococcus aureus.* This observation contradicts the earlier reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria (Vlietinck *et al.* 1995; Rabe and van Staden 1997).

This could be attributed to the fact that the cell wall in Gram-positive bacteria has a single layer, whereas the Gram-negative cell wall is a multi-layered structure (Yao and Moellering 1995; Ozcelik 1998), acting as a barrier to many environmental substances, including antibiotics (Tortora *et al.* 2001).

However, our results reveal that the crude ethanol extracts contain certain constituents like flavonoids which are known to be synthesized by plants in response to microbial infection. Hence, it is apparent that they have been found to be effective antimicrobial substances against a wide range of microorganisms. Their activity is probably due to their ability to react with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan 2002).

Ciprofloxacin, which was used as a positive experimental control against all bacterial strains assayed, produced a zone of inhibition of 19.67 ± 0.13 to 24.73 ± 0.24 while no inhibitory effect could be observed for DMSO used as negative control.

Recent studies have focused on the antibacterial effect of *H. antidysenterica* on several pathogenic as well as antibiotic resistant strains (Kavitha *et al.* 2003; Aqil *et al.* 2005, 2006). However, the significance of the present work lies in the fact that geographical variations play a crucial role in the production of secondary metabolites (Srivastava *et al.* 2002).

The present results offer a scientific basis for the therapeutic potency of *H. antidysenterica* used in traditional medicine. However, the activity level of the extracts may be more accurately evaluated in terms of MIC values as the zone of inhibition might be influenced by solubility and diffusion rate of the phytocompounds. In addition, *in vivo* studies are necessary to determine the toxicity of the active constituents, their side effects, circulating levels, pharmacokinetic properties and diffusion in different body sites. The antimicrobial activities could be enhanced if the active components are purified and adequate dosage determined for proper administration. This may go a long way in curbing administration of inappropriate concentration, a common practice among many traditional medical practitioners in India.

ACKNOWLEDGEMENTS

We acknowledge Mr. K. P. Prasanna for his assistance and encouragement.

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