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Full Length Research Paper

In vitro antibacterial activity of plant extracts against Gram positive and Gram negative pathogenic bacteria

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Medicinal plant extracts prepared with selected various solvents from ten species, *Murraya koenigii, Syzygium aromaticum*, *Piper nigrum*, *Ocimum tenuiforum*, *Laurus nobilis*, *Cinnamomum zeylanicum, Phyllanthus niruri*, *Cuminum cyminum, Trilobatum* sp. and *Hibiscus rosasinensis* were screened for antibacterial activity against bacterial pathogens by using disc diffusion method. The antibacterial activities of the extracts (methanol and aqueous) were gave maximum inhibition zone when compare with other solvent extracts. Plant extracts showed strong antibacterial action against pathogens, among the plant extracts the methanol extracts of *Ocimum tenuiforum* and *Syzygium aromaticum* against *B. subtilis* and *Cuminum cyminum* against *E. coli* while aqueous extracts of *Piper nigrum* gave maximum inhibition against *Proteus* sp. However no antibacterial activities noted in methanol extracts of *Hibiscus rosasinensis* against *E. coli* and *P. aeruginosa*.

Key words: Medicinal plants, antibacterial, methanol, aqueous, inhibition.

INTRODUCTION

Medicinal plants is very important one in human health, it will act as an antibactericide activity against the bacterial pathogens, this is followed from ancient times (Zaika, 1988). Plant extracts have numerous health-related effects such as antibacterial, antimutagenic, anticarcinogenic, antithrombotic and vasodilatory activities (Bidlack et al., 2000). *Allium vineale*, *Chaerophyllum macropodum* and *Prangos ferulacea,* have been used for cheese production and it is traditionally believed by local people that these plants have antibacterial activity (Durmaz et al., 2006). Organic solvents such as ethanol, acetone, and methanol are often used to extract bioactive compounds (Eloff, 1998)They also studied Four solvent extracts (methanol, ethanol, *n*-hexane and water) of the plants were investigated against gram positive and gram negative bacteria by using disc diffusion method. However, Wendakoon et al. (2012) showed strong antibacterial action against gram-positive bacteria and no inhibitory activity against gram-negative bacteria was observed. Nascimento et al. (2010) observed the antibacterial activity of various plants extracts *Achillea millifolium*, *Caryophyllus aromaticus*, *Melissa offficinalis*, *Ocimun basilucum*, *Psidium guajava*, *Punica granatum*, *Rosmarinus officinalis*, *Salvia officinalis*, *Syzygyum joabolanum* and *Thymus vulgaris*.

In this study, we investigated the antibacterial activity of methanol, aqueous, ethanol, acetone and petroleum ether extracts of *Murraya koenigii, Syzygium aromaticum*, *Piper nigrum*, *Ocimum tenuiforum*, *Laurus nobilis*, *Cinnamomum zeylanicum, Phyllanthus niruri*, *Cuminum cyminum, Trilobatum* sp. and *Hibiscus rosasinensis* against Gram positive and Gram negative bacteria. To our knowledge, antibacterial activities of these plants against pathogenic bacteria.

MATERIALS AND METHODS

Selection of medicinal plants for the study

Five medicinal plants viz. *Ocimun tenuiflorum, Murryaya*

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koennigii, Hibiscus rosasinesis, Phyllanthus niruri, Trilobatum leaves and five plant materials viz *Piper nigrum, Laurus nobilis, Syzygium aromaticum*, *Cuminum cyminum* and *Cinnamom zeylanicum* were selected based on ethanomedical importance. Healthy, disease free leaves of selected plants were collected in around Sivakasi, Virudhunagar District, Tamil Nadu (India) were used for the preparation of aqueous and solvent extracts.

Test strains

Authentic pure cultures of human pathogenic bacteria of Gram-positive (*Staphylococcus aureus*) and five negative (*Escherichia coli, Klebsiella pneumoniae, Proteus sp., Pseudomonas aeruginosa,* and *Bacillus subtilus*) bacterial stains were used in the study.

The organisms were sub-cultured on Mueller Hinton Agar medium, incubated at 37°C for 24 h and stored at 4°C in the refrigeration to maintain stock culture. The gram positive and gram negative were pre-cultured in nutrient broth overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometer.

Preparation of plant extracts

The plant materials were shade dried and ground to powder form using the grinder mixer. Exposure to direct sunlight was avoided to prevent the loss of active components.

Preparation of plant materials aqueous extract

Fifty grams of selected fresh leaf materials was macerated with 50 ml of sterile distilled water in a grinding machine for about 10 to 15 min. The macerate was first filtered through double layer muslin cloth then centrifuged at 3500 rpm for 30 min. The supernatant was filtered through. Whatman No. 1 filters paper and sterilized at 120°C for 30 min. The extracts were preserved aseptically at 5°C for further use.

Preparation of plant materials solvent extract

Fifty gram of dried plant materials were powdered and soaked separately for 48 h in 50% (v/v) methanol, ethanol, acetone and petroleum ether. The soaked material was agitated at regular time intervals. After 48 h the soaked material was filtered using muslin cloth. Then the filtrate were again filtered using Whatman filter paper No. 1 on separate filtration setups. The final filtrates were collected in wide mouthed evaporating bowls and dried under room temperature. The dried extracts were weighed to calculate the extractability percentage. The extracts were stored at 40°C until further use.

Antibacterial activity assay

Antibacterial activity of aqueous and solvent extracts of all the selected plant extracts was determined by the cup diffusion method on nutrient agar medium. Both the aqueous and solvent extracts of plants were screened for the antibacterial assay.

Aqueous extract

The organism to be tested was inoculated into sterile nutrient agar. After incubation period of 24 h at 37°C, a loop of inoculum was transferred into 5 ml of nutrient broth and incubated for 2 h at 37°C which served as fresh suspension inoculum. Five wells (5 mm diameter) were made in sterile nutrient agar plate using cork borer (one in the centre and four wells at the corner) and inoculum containing 10^6 CFU/ml of test bacteria were spread on solid plates with the help of sterile swab moistened with the bacterial suspension. Then 50 µl of aqueous extract of all the leaves were placed in the wells made in inoculated plates. The treatment also includes 50 µl of sterilized distilled water as control. All the plates were incubated for 24 h at 37°C and zone of inhibition if any around the well were measured in millimetre (mm). For each treatment six replicates were maintained.

Solvent extract

One gram of all the selected plant leaf extract was dissolved in 9 ml of each solvent of methanol, acetone and petroleum ether for aqueous, alcoholic, acetone and petroleum ether. The sterile nutrient agar medium in petridishes was uniformly smeared with test culture. Well (5 mm) were made in each petridish to which 50 µl of solvent extracts dissolved in each solvents were added. For each treatment six replicates were maintained.

***In vitro* antibacterial assay**

3.8 g of Mueller Hinton agar powder was dissolved in 100 ml distilled water and autoclaved at 121°C for 15 min. About 25 ml molten agar was poured into each petridish (9 cm in diameter) and allowed to set. Sterile filter paper disks were prepared and sterilized in a Pasteur-oven,

(at 170~ for 2 h). The plant extracts was pipette into the centre of each disk to achieve the desired potency. Disks were air-dried in a contamination free environment. The antibacterial activities of isolates to the different extracts were tested using the disk-agar method standardized by the National Committee for Clinical Laboratory Standards. In brief each extract was pipette onto a sterile paper disc (Hi Media Laboratories Ltd., Mumbai, India) that had been suspended on top of a dissecting needle. The volume added was chosen because it represents the approximate volumetric capacity of each disc. The solvent was allowed to evaporate and the discs (upto 9 plate-1) were then placed onto the surface of petridishes that had previously been surface-inoculated with individual test strains at 106 dilution. Plates were then incubated at 37°C for 24 h. Solvent control discs were prepared in the same manner and were never observed to inhibit bacterial growth. Antibacterial activity was evaluated by measuring inhibition zone diameters. After incubation, the diameter of clear zone surrounding the disc was measured to the nearest mm. An extract was considered active if one or more replicate extracts produced a zone of inhibition >1 mm beyond the edge of the disc. The area of this zone of inhibition was calculated for each replicate assay of discs treated with extracts.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration of plant extracts were found using broth dilution technique. Seven test tubes containing 1 ml of sterile Sabouraud's Dextrose broth were prepared. For assaying plant extract, the starting concentration kept at 8mg/ml in the first tube containing 1 ml of sterile Sabouraud's dextrose broth. The plant extract were serially diluted at the concentration 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/ml. To each of this test tube, 0.1 ml of 6 hr culture of test strains was added. The tubes were incubated at 30 °C for 24 to 48 h. The test tubes were examined for visible turbidity. 1ml of the above mentioned tube was transferred to a microfuge and centrifuged at 5,000 rpm for 4 min. The supernatant was completely removed using micropipette and the pellet was suspended in 0.1 ml sterile distilled water. The resulting bacterial suspension was serially diluted and plated on Sabouraud's dextrose agar plates. The end point of complete inhibition was defined as the minimum inhibition concentration of the test compound in the original tube which fails to yield discernible growth when sub cultured. Results were recorded as presence or absence of zone of inhibition (Lennette et al., 1985). The inhibitory zone around test paper disks indicated absence of bacterial growth and it was reported as positive (growth inhibition observed) and absence of zone as negative. The test was repeated once again to insure reliability of the results.

RESULT AND DISCUSSION

The present study was designed to obtain preliminary information on the antibacterial activity of ten plants. The disc diffusion method was preferred to be used in this study. Among the extractions assayed, the methanol extracts of *Ocimum tenuiforum*, *Hibiscus rosasinensis*, *Trilobatum* sp., *Phyllanthus niruri*, *Murraya koenigii*, *Piper nigrum*, *Syzygium aromaticum*, *Laurus nobilis*, *Cuminum cyminum* and *Cinnamomum zeylanicum* gave the maximum inhibition of all the pathogens followed aqueous extract. Eloff (1998) reported that methanol was the most effective solvent for plant extraction than ethanol, *n*-hexane and water. The methanol extracts of *A. vineale* showed the higher antibacterial activity as compared with *C. macropodum* and *P. ferulacea* (Durmaz et al., 2006). The methanol extracts of *Murraya koenigii* have less activity against *P. aeruginosa* and *E. coli,* whereas aqueous extract gave maximum zone of inhibition in the same pathogen but Vlientinck *et al*., (1995) reported that water extracts of plants do not have much activity against bacteria. The bioactivity of plant extracts depends on the water and ethanol concentration used in the extraction process (Ganora, 2008). However the no inhibition zone noticed against *S. aureus* in both solvent. *Syzygium aromaticum* and *Hibiscus rosasinensis* extracts have no clear zone in *P. aerugenosa* and *E. coli*, *B. subtilis*. Our results indicated that methanol and aqueous extraction is a good method to extract antibacterial compounds found in these species. Ethanol and petroleum ether extracts of *Murraya koenigii* gave clear zone of *E. coli* and *S. aureus* remaining extracts were fail produce the clear zone against these pathogens. Wendakoon et al. (2012) reported that ethanol extracts active against gram positive and gram negative bacteria. Solvents (negative control) used for extraction showed no activity against any bacteria tested. The inhibition zones varied depending depends on the type of extract, plant species and bacterial species. In general, methanol extracts of the three species were found to be more effective than other extracts. The largest diameter of inhibition zone was observed from methanol extracts of *Ocimum tenuiforum*, *Cuminum cyminum* and *Piper nigrum* against *B. subtilis*, *E. coli* and *Proteus* sp. However, the clear zone of water extracts results on par with methanol extracts activity against the bacteria tested. Among the plant species tested, *Ocimum tenuiforum* exhibited higher antibacterial activity compared with *Cuminum cyminum* and *Piper nigrum*. *Ocimum sanctum* (Mishra and Mishra, 2011), *Cuminum cyminum* (Iacobellis et al., 2005) and *Piper nigrum*

Table 1. Antibacterial activities of medicinal plants against gram negative and gram positive bacteria

S/No	Organisms	Methanol	Aqueous	Ethanol	Acetone	Petroleum ether
Murraya koenigii	*S. aureus*	-	-	2	-	5
	K. pneu	9	7	4	3	2
	E. coli	-	-	2	-	4
	P. aeru	6	8.5	3.5	2.5	1
	B. subti	15	12	8	5	2
	Proteus sp	8	5	3	2	1
Syzygium aromaticum	*S. aureus*	14	13	2	-	4
	K. pneu	-	2	4	3	1
	E. coli	15	14	13	8	6
	P. aeru	-	2	2	2	1
	B. subti	19	18	16	15	9
	Proteus sp	9	8	6	5	4
Piper nigrum	*S. aureus*	12	9	11	8	6
	K. pneu	14	13	12	6	4
	E. coli	11	10	10	7	5
	P. aeru	10	9	9	7	5
	B. subti	14	13	10	8	6
	Proteus sp	13	14	12	8	5
Ocimum tenuiforum	*S. aureus*	15.5	14.5	13.0	7.0	5.0
	K. pneu	13.0	12.0	11.0	10.0	6.0
	E. coli	21.0	18.0	16.0	10.0	8.0
	P. aeru	11.0	9.0	8.0	6.0	5.0
	B. subti	24.0	22.0	18.0	16.0	15.0
	Proteus sp	8.0	7.0	6.0	4.0	2.0
Laurus nobilis	*S. aureus*	3.5.0	3.0	2.8	2.0	1.8
	K. pneu	3.3.0	3.0	2.8	2.0	1.8
	E. coli	4.2.0	4.0	3.8	3.0	2.8
	P. aeru	2.0	1.8	1.6	1.5	1.0
	B. subti	2.8	2.0	1.8	1.6	1.4
	Proteus sp	1.4	1.0	0.8	0.5	0.2
Cinnamomum zeylanicum	*S. aureus*	2.8	2.0	1.8	1.5	1.2
	K. pneu	2.5	2.4	2.2	1.8	1.5
	E. coli	3.2	3.0	2.8	2.5	2.0
	P. aeru	2.3	2.2	2.0	1.8	1.0
	B. subti	4.8	4.0	3.8	3.0	2.8
	Proteus sp	1.8	1.5	1.0	0.8	0.5
Phyllanthus niruri	*S. aureus*	4.0	3.8	3.0	2.8	2.0
	K. pneu	3.0	2.8	2.5	2.0	1.8
	E. coli	3.5	3.3	3.2	2.8	2.0
	P. aeru	3.4	3.2	3.0	2.0	1.8
	B. subti	5.5	5.0	4.5	4.0	3.8
	Proteus sp	2.5	2.2	2.0	1.8	1.0

Table 1 Contd.

Cuminum cyminum	*S. aureus*	14.0	12.0	10.0	8.0	5.0
	K. pneu	16.0	15.0	12.0	10.0	8.0
	E. coli	20.0	18.0	14.0	10.0	8.0
	P. aeru	17.0	15.0	12.0	10.0	8.0
	B. subti	16.0	15.0	13.0	12.0	10
	Proteus sp	11.0	8.0	6.0	4.0	3.0
Trilobatum sp.	*S. aureus*	6.5	6.0	5.5	5.0	4.0
	K. pneu	4.0	3.8	3.0	2.8	2.0
	E. coli	6.2	5.0	4.8	4.6	4.0
	P.aeru	4.8	4.0	3.8	3.0	2.8
	B.subti	4.4	4.2	4.0	3.2	3.0
	Proteus sp	1.2	1.0	0.8	0.6	0.4
Hibiscus rosasinesis	*S. aureus*	9.0	7.5	6.5	5.5	6.5
	K. pneu	8.0	6.8	6.0	5.8	5.0
	E. coli	-	0.2	0.8	-	0.5
	P. aeru	9.0	8.8	8.0	-	6.0
	B. subti	-	0.5	0.8	1.0	1.2
	Proteus sp	6.5	6.0	5.8	5.0	4.5

(Karsha and Lakshmi, 2010) were reported equally effective against the gram negative and gram positive bacteria and. In conclusion, methanol and aqueous extracts of studied plants showed antibacterial activity. Among the plants tested, *Ocimum tenuiforum* showed higher antibacterial activity against tested bacteria. The results were noted in Table 1. This gives an indication of the presence of promising antibacterial compounds. In future phytochemical studies are needed to elucidate the components responsible for antibacterial activity of these extracts against bacteria.

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