

EVALUATION OF ANTIBACTERIAL POTENTIAL OF PLANT EXTRACTS USING RESAZURIN BASED MICROTITER DILUTION ASSAY

ANJUM GAHLAUT AND ANIL K CHHILLAR*

*Centre for Biotechnology, Maharshi Dayanand University, Rohtak-124001, India. Email: anil.chhillar@gmail.com

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ABSTRACT

Objective: Antimicrobial potential of ten medicinal plants i.e. *Picrorhiza kurroa*, *Datura metel*, *Acacia catechu*, *Cissus quadrangularis*, *Cassia tora*, *Berberis aristata*, *Pongamia pinnata*, *Emblia officinalis*, *Saraca asoca* and *Tinospora cordifolia* was evaluated against six bacterial strains i.e. *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

Methods: Plant extracts were prepared by using Soxhlet extraction. Five extract from each plant were prepared using five solvents on the basis of increasing polarity. Minimum inhibitory concentration of extracts was determined by resazurin based microtiter dilution assay.

Results: Percent yields of petroleum ether and chloroform extracts of plant leaves was found to be in the range of 0.80 - 2.98%. Percent yields of acetone, methanol and water extract were found to ranging from 2.87- 5.77%. *S. asoca* extracts were found to be endowed with highest antimicrobial activity out of the ten plants used in the study. Leaves water extract of *S. asoca* showed lowest minimum inhibitory concentration (0.15 mg/mL) against *Pseudomonas aeruginosa*.

Conclusion: It was observed that leave water extracts of *S. asoca* could be potential reservoir of bioactive compounds. Post treatment analysis of proteome of test micro-organisms could explore potential anti-bacterial targets.

Keywords: Resazurin, Microtiter dilution assay.

INTRODUCTION

In the era of modernization and changed environmental conditions man frequently encounters pathogenic microorganisms causing infectious diseases. The indiscriminate use of commercially available antibiotics for the treatment of infectious diseases developed multiple drug resistance in the microorganisms, putting new challenge before the drug industries for identification of new efficient antimicrobial compounds. Herbal drugs therapy is regarded as an important alternate, leading the researchers to focus and evaluate the traditionally recommended medicinal plants for their efficacy in various disease conditions [1].

Medicinal plants are major part of new pharmaceuticals and health care products. Due to the availability of medicinal plants throughout the world, herbal drugs are being used by 75-80% of world

population, especially in developing countries. As reported by World Health Organization (WHO), traditional medicinal plants are the best reservoirs to develop newer pharmaceuticals [2]. Medicinal plants are renewable sources therefore farmers get encouraged to include them in traditional agriculture [3]. India has highly diverse vegetation and herbal plants which are rich source of bioactive compounds. To ensure the commercial medicinal potential of plants depicted in Ayurveda, the antimicrobial potential need to be evaluated against present day pathogens according to new parameters to ensure their efficacy and reliability. In the present study, ten plants were shortlisted on the basis of literature survey and their extracts were evaluated for antimicrobial potential against six bacterial strains (Table 1). Minimum inhibitory concentration (MIC) of plant extracts was determined using microbroth dilution assay.

Table 1: Traditional and Medicinal properties of Plants used in the study

S. No.	Botanical Name	Local name	Medicinal property	Reference
1.	<i>Picrorhiza kurroa</i>	Katuka, Kutki and Hellbore	Skin, urinary tract, diarrheal infections, gastrointestinal infections, antioxidant, antidiabetic, anti-allergic, anti-hyperglycaemic, hepatoprotective, immunostimulating, anti-cancer, and anti-inflammatory.	[5-7]
2.	<i>Datura metel</i>	Dhatura	Antibacterial, antioxidant, herbicidal, analgesic, anesthetic, antispasmodic, antitussive, bronchodilator and hallucinogenic.	[8-10]
3.	<i>Acacia catechu</i>	Black Cutch, Khair and Katha	Sore throats and diarrhoea, high blood pressure, dysentery, colitis, gastric problems, bronchial asthma, cough, leucorrhoea, immunomodulatory, antipyretic, antimycotic.	[11-14]
4.	<i>Cissus quadrangularis</i>	Hadjod, Asthisamdhani	Bone fracture healing activity, antiulcer, antiosteoporotic effect, antibacterial, antiprotozoal.	[15-19]
5.	<i>Cassia tora</i>	Chakunda	Hepatoprotective, antigenotoxic, hypotensive, antibacterial and antifungal.	[20-24]
6.	<i>Berberis aristata</i>	Indian barberry, Daru haldi, Chitra	Ear infection, antibacterial, Antifungal, anti-inflammatory, analgesic, antipyretic and anticancer.	[25-27]
7.	<i>Pongamia pinnata</i>	Karanja, Karanj	Anti-inflammatory, anti-plasmodial, anti-nonciceptive, anti-hyperglycaemics, anti-lipidoxidative, antidiarrhoeal, anti-ulcer, anti-hyperammonic, CNS depressant activity, antioxidant and antibacterial, antifungal	[28-30]
8.	<i>Emblia officinalis</i>	AmLa, Amalaka, Aavala, AmLaki and Indian gooseberry	Anti-viral, antibacterial, anti-cancer, anti-allergy, and anti-mutagenic.	[31]
9.	<i>Saraca asoca</i>	Ashok briksh, Ashoka	Antioxidant, antibacterial, anticancer, oxytocic and antilarval.	[32-37]
10.	<i>Tinospora cordifolia</i>	Gulanca, Giloy	Anti-HIV, anti-parkinson's disease, Anti-stress, anti-inflammatory, antibacterial.	[38-40]

MATERIALS AND METHODS

Plant material

Picrorhiza kurroa, *Datura metel*, *Acacia catechu*, *Cissus quadrangularis*, *Cassia tora*, *Berberis aristata*, *Pongamia pinnata*, *Embllica officinalis*, *Saraca asoca* and *Tinospora cordifolia* were selected for study (Table 1) and identified by Department of Botany, Maharshi Dayanand University, Rohtak (India). Cross authentication of selected plant was done with the help of flora of Haryana [4]. The herbarium specimens were preserved at Centre for Biotechnology, M D University, Rohtak.

Preparation of Crude Extract

Leaves of ten different medicinal plants were collected and air dried by keeping in shade for 3 weeks. Afterward, the plant materials were transferred to oven at 40°C for 20-24 hrs. The properly dried plant leaves were grinded to fine powder with the help of electronic grinder. Sixty gram leaves powder of each plant was extracted by Soxhlet's method. For crude extraction, five solvents (300 mL each) were used in ascending order of polarity i.e. petroleum ether, chloroform, acetone, methanol and water. The leaf powder extracts were filtered twice, firstly filtered under the vacuum through a double layer of Whatman filter paper (No. 3 and No. 1) and secondly through a single sheet of Whatman No. 1 filter paper under gravity. The clear supernatants were subjected to vacuum distillation at 30-35 °C using a Buichi Rotary Evaporator for removing the solvent. The remaining residues were stored in refrigerator till further use.

Micro-Organisms and reference strain

Six Microbial Type Culture Collection (MTCC) registered bacterial isolates were obtained from Institute of Microbial Technology, Chandigarh (Table 2) and used for the evaluation of antibacterial activity of crude plant extract.

Table 2: Six standard strains of bacteria given below were used in the study.

Name	Types	Strain type
<i>Escherichia coli</i>	Gram Negative	MTCC433
<i>Salmonella typhi</i>	Gram Negative	MTCC531
<i>Staphylococcus aureus</i>	Gram Positive	MTCC9011
<i>Bacillus subtilis</i>	Gram Positive	MTCC441
<i>Pseudomonas aeruginosa</i>	Gram Negative	MTCC7925
<i>Klebsiella pneumoniae</i>	Gram Negative	MTCC3384

Preparation of bacterial culture

Using aseptic techniques, six 100 mL bottles of Luria broth were inoculated with six bacterial strains (each bottle contain only one type of bacteria) and kept in the incubator overnight for 12-18 h at 35° C. After incubation, all bacterial cultures were centrifuged at 4000 rpm for 5 min. Supernatants were discarded and pellets were re-suspended in 20 mL of sterile normal saline and re-centrifuged at 4000 rpm for 5 min. The pellet was dissolved in 20 mL of sterile normal saline and was labelled as bacterial solution. Optical densities (OD) of the bacterial solutions were measured at 600 nm

and serial dilutions were made until the OD was in the range of 0.5-1.0. The viability graph was used to calculate the actual number of colony forming units. The dilution factor was calculated and the dilution was performed to obtain a final concentration of 5×10^6 CFU/mL [41].

Preparation of Resazurin Dye Solution (RDS)

Resazurin dye (300 mg) was dissolved in 40 mL sterile water. Vortex mixer was used to homogenize the solution. This solution was then referred as Resazurin dye solution. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth, particularly in various cytotoxicity assays [42]. It is purple non-fluorescent and non-toxic dye becomes pink and fluorescent when reduced to resorufin by oxidation reduction within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured). Resazurin reduction test has been used from decades to demonstrate bacterial and yeast contamination of milk [43].

Resazurin based Microtiter Dilution Assay (RMDA)

Under aseptic conditions, 96 well microtitre plates (Tarson) were used for Resazurin based Microtitre Dilution Assay. The first row of microtiter plate was filled with 100 µl of test materials in 10% (v/v) DMSO or sterile water. All the wells of microtitre plates were filled with 100 µl of nutrient broth. Two fold serial dilution (through out the column) was achieved by starting transferring 100 µl test material from first row to the subsequent wells in the next row of the same column and so that each well has 100 µl of test material in serially descending concentrations. 10 µl of resazurin solution as indicator was added in each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of 5×10^6 CFU/mL. To avoid the dehydration of bacterial culture, each plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. Each microtitre plate had a set of 3 controls: (a) a column with Streptomycin as positive control, (b) a column with all solutions with the exception of the test extract and (c) a column with all solutions except bacterial solution replaced by 10 µl of nutrient broth. The plates were incubated in temperature controlled incubator at 37° C for 24 h. The colour change in the well was then observed visually. Any colour change observed from purple to pink or colourless was taken as positive. The lowest concentration of plant leaf extract at which colour change occurred was recorded as the MIC value. All the experiments were performed in triplicates. The average values were calculated for the MIC of test material.

RESULT AND DISCUSSION

Percentage Yield

Different plant extracts were prepared from selected ten plants using five solvents of different polarity. Percent extract yield of these plants varies from 0.80 to 5.77% (Table 3). In most of the cases the amount of residue extracted with acetone, methanol and water is higher than other two solvents. Percent yields of petroleum ether and chloroform extracts of plant leaves was found to be in the range of 0.80 - 2.98%. Percent yields of acetone, methanol and water extract were found to ranging from 2.87- 5.77% (Table 3).

Table 3: Percent (%) yield of various plant extracts isolated by Soxhlet's method in serial solvents.

S. No.	Plant Name	Plants Part Used	Percent Yield of Plant Extracts In Serial Solvents				
			Petroleum Ether	Chloroform	Acetone	Methanol	Water
1	<i>D. metel</i>	Leaves	0.89	1.29	2.87	3.10	3.40
2	<i>C. quadrangularis</i>	Leaves	0.92	2.01	2.40	4.01	4.05
3	<i>P. kurroa</i>	Leaves	1.04	2.98	2.97	3.56	3.80
4	<i>A. catechu</i>	Leaves	0.94	2.97	3.12	3.15	4.17
5	<i>B. aristata</i>	Leaves	0.80	1.90	3.00	4.77	5.76
6	<i>T. cordifolia</i>	Leaves	1.33	2.67	3.09	3.02	4.99
7	<i>S. asoca</i>	Leaves	1.60	2.87	3.44	4.88	3.88
8	<i>E. officinalis</i>	Leaves	0.90	2.90	4.00	4.77	5.77
9	<i>P. pinnata</i>	Leaves	1.03	1.90	3.10	3.23	3.11
10	<i>C. tora</i>	Leaves	1.44	2.54	5.70	3.66	3.89

MIC of plant extracts using Resazurin Microtitration Dilution Assay (RMDA)

Fifty different crude extracts of ten medicinal plants were screened for their antibacterial potential. MIC values for different extracts obtained for the pathogenic bacteria species representing antibacterial activity (Table 4). Methanol extract of *C. quadrangularis* showed promising activity where as water extract of *S. asoca* showed maximum activity against *Pseudomonas aeruginosa* (MIC 0.15 mg/mL). Antimicrobial activity of bark of *S. asoca* was reported by Dabur et al [36]. It has been shown that antimicrobial activity of *S. asoca* bark is depends upon the

catechins present in the bark [37]. Less or negligible activity was observed in all the extracts of *Tinospora cordifolia*. Aqueous and methanol extract of other nine plants normally showed antimicrobial activity in a range of 5.0 to 0.62 mg/mL. Comparatively lower activities were observed in petroleum ether and chloroform extracts (MIC 5.0 to 1.25mg/mL). Streptomycin is used in this study as positive control shows MICs in the range of 0.15-1.25 mg/mL against the bacterial strains used in the study. After the overall observation of the MIC results it is concluded that the aqueous and methanol extracts from the studied plants showed broad range of activity and could be potential source of antimicrobial compounds.

Table 4: MIC of plant extracts against the pathogenic bacteria by Resazurin microtitre dilution assay.

Plant name	Solvent Fraction	MIC (mg/mL)					
		B s	S a	S t	E c	K p	P a
<i>P. kurroa</i>	Petroleum Ether	5.0	-	-	-	5.0	2.5
	Chloroform	-	2.5	-	5.0	1.25	-
	Acetone	2.5	5.0	2.5	-	-	-
	Methanol	1.25	1.25	-	0.62	5.0	2.5
	Water	-	-	-	1.25	1.25	1.25
<i>D. metel</i>	Petroleum Ether	5.0	-	-	-	1.5	1.25
	Chloroform	1.25	-	5.0	5.0	-	2.5
	Acetone	2.5	-	-	5.0	2.5	-
	Methanol	2.5	5.0	-	1.25	-	-
	Water	1.25	1.25	-	-	5.0	1.25
<i>A. catechu</i>	Petroleum Ether	2.5	-	-	-	-	5.0
	Chloroform	2.5	2.5	2.5	5.0	3.0	-
	Acetone	5.0	-	-	5.0	3.0	-
	Methanol	1.25	1.25	-	0.62	2.5	2.5
	Water	5.0	-	-	2.5	5.0	2.5
<i>C. quadrangularis</i>	Petroleum Ether	5.0	5.0	5.0	-	-	-
	Chloroform	-	-	2.5	-	-	-
	Acetone	-	5.0	5.0	-	1.25	2.5
	Methanol	0.62	0.62	1.25	-	-	2.5
	Water	-	2.5	-	-	1.25	-
<i>C. tora</i>	Petroleum Ether	5.0	-	-	-	5.0	-
	Chloroform	5.0	-	-	-	-	-
	Acetone	2.5	-	2.5	-	5.0	2.5
	Methanol	-	-	-	-	2.5	-
	Water	-	0.62	-	1.25	5.0	2.5
<i>B. aristata</i>	Petroleum Ether	-	-	5.0	-	-	-
	Chloroform	-	-	-	-	-	-
	Acetone	-	5.0	5.0	-	-	2.5
	Methanol	-	-	-	-	-	2.5
	Water	-	2.5	-	5.0	-	5.0
<i>P. pinnata</i>	Petroleum Ether	-	5.0	-	2.5	-	-
	Chloroform	5.2	5.0	-	5.0	-	5.0
	Acetone	-	5.0	-	2.5	2.5	5.0
	Methanol	2.5	5.0	-	5.0	-	5.0
	Water	-	5.0	2.5	-	-	-
<i>E. officinalis</i>	Petroleum Ether	-	-	-	-	-	-
	Chloroform	-	-	5.0	-	-	-
	Acetone	-	-	-	-	2.5	5.0
	Methanol	-	5.0	-	5.0	2.5	5.0
	Water	-	5.0	2.5	-	5.0	-
<i>S. asoca</i>	Petroleum Ether	-	-	-	-	5.0	2.5
	Chloroform	-	5.0	-	-	5.0	2.5
	Acetone	-	5.0	-	3.4	3.1	5.0
	Methanol	5.0	1.25	-	1.25	1.25	0.62
	Water	1.25	2.5	2.5	2.0	-	0.15
<i>T. cordifolia</i>	Petroleum Ether	-	-	-	-	-	-
	Chloroform	5.0	-	-	-	5.0	-
	Acetone	-	-	-	-	-	-
	Methanol	-	-	-	5.0	-	5.0
	Water	-	-	-	-	-	-

(B s= *Bacillus subtilis*); (S a= *Staphylococcus aureus*); (S t= *Salmonella typhi*); (E c= *Escherichia coli*); (K p= *Klebsiella pneumonia*); (P a= *Pseudomonas aeruginosa*) and (-) means no activity above 5 mg/mL.

CONCLUSION

The results of present study reveal that the antibacterial potential of medicinal plants varies with the species of the plants and solvents used for the extraction of phytoconstituents. The active extracts serve as reservoir of potential lead compounds. Further studies on *C. quadrangularis* and *S. asoca* may lead toward discovery of novel antimicrobial compounds. As on date new molecules are being identified and comprehensive Quantitative Structural Activity Relationship (QSAR) can be carried out in order to find an optimal combination of pharmacologically important substructure groups. Further structural correlation based studies lead the drug industries to develop compounds of more efficacy. Research may also be channelled towards proteome based studies of differential expressed microbial proteins (secretary & cellular) after treatment with bioactive compounds to identify the target proteins.

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