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High Throughput Antibacterial Screening of Plant Extracts by Resazurin Redox with Special Reference to Medicinal Plants of Western Ghats

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Abstract: A new high throughput antibacterial method employing the dye resazurin as an indicator of bacterial growth was developed to evaluate drug susceptibility by combined measurements of microtitre-plates, colorimetric and haemocytometric assays. The bacterial strains *E. coli, B. subtilis, P. aeruginosa* and *S. aureus* (multi-drug resistant strain) were used to evaluate the preliminary screening of plant extracts, minimum inhibition concentration (MIC) and minimum bacterial concentration (MBC) with streptomycin and tetracycline as reference antibiotics. The antibacterial activity of selected medicinal plant extracts were carried out against all the four bacteria by the same method and the values compared the value with reference antibiotics. The present study resulted in a rapid, reliable, simple and inexpensive method suitable for testing the susceptibility of bacterial strains to screen the medicinal plant extracts; its employment in evaluating new antibacterial molecules from the plant source is also suggested.

Key words: Antibacterial activity · Resazurin indicator · Medicinal plants · Western Ghats · India

INTRODUCTION

With the increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotics. Most of the antibiotics available today come from natural origin, especially from various microbial or plant sources. Higher plants also produce compounds to protect themselves from microbial attacks. For screening antimicrobial properties of plant extracts used agar plate techniques world wide.

The present investigation introduced an *in vitro* antibacterial assay that is simple, rapid, efficient, reliable, sensitive and cost-effective. However, most often the small quantities of the extracts or isolated fractions can be a limiting factor in any viable screening programme. The conventional methods, such as diffusion method, may be time consuming and require significant quantities of the materials. The aim of the present study is employing a high-throughput antibacterial screening programme for medicinal plants of Western Ghats. Already Drummond and Waigh [1] explained the microtitre-plate assay using resazurin for screening of natural compounds for microbial susceptibility tests. This method used an indicator, resazurin, which allows the detection microbial growth in extremely small volumes of solution in microtite-plates without the use of spectrophotometer. However, it was soon realized that this published method incorporated changes in the concentration of the both the test material as well as the bacterial suspension and also extended the spectrophotmetric and haemocytometric methods to determination of minimum inhibition concentration and minimum bacterial concentration.

Resazurin is a blue coloured redox dye commonly used as an indicator of chemical cytotoxicity in cultured cells [2]. The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and finally colourless dihydroresorufin. Resorufin produced as a result of resazurin bioreduction is measured colorimetrically. Resazurin is non-toxic to cells and stable in culture medium, allowing continuous measurement of cell proliferation *in vitro* [3,4]. When we testing the method the toxic result of drugs on bacterial cells that impairs cell viability and proliferation also affect the capacity of cultures to reduce resazurin and the rate of dye reduction is directly proportional to the number of viable cells present [5]. Therefore, as a direct measure of the metabolic competence of cell cultures, resazurin reduction may provide a convenient index of cell proliferation. The present study employed the resazurin dye reduction for an indicator of bacterial growth against

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the selected plant extracts of medicinal plants from Western Ghats, which is reported first time to describe a new approach for high throughput antibacterial screening progrmme.

MATERIALS AND METHODS

Plant Materials: Plant material were collected from the Palni hills of Western Ghats during the year 2008 by different field trips and the voucher specimens were deposited in the herbarium, department of Botany, The Madura College, Madurai. The botanical identity of the plant specimens were initially identified with local floras [6,7] and finally confirmed with the comparison of authentic specimens in the Madras Herbarium, Botanical Survey of India, Southern Circle, Coimbatore, India. The plant materials were shade tried, powdered and the powders were subjected separately to the extraction.

Extraction: 10 g of dried powder was added to distilled water and macerated well with help of pistle and motar. It was then filtered through two-layered muslin cloth and centrifuged at 5000 g for 10 min. The supernatant was collected. This procedure was repeated twice. Final volume of the supernatant was concentrated under the room temperature and percentage of extracts yield was calculated for each plant materials. Then the extracts were again dissolved in 100 ml of distilled water which is served as stock solution and it was autoclaved at 121°C and 15 lbs pressure and stored at 20±2°C.

Bacterial Strains and Culture Conditions: Bacterial strains procured from IMTECH, Chandigarh and cultured on Mueller-Hinton agar media with regular interval for subculture and stored in 20°±2°C. Stock cultures containing 1 x 10^7 cfu x ml (0.5 MacFarland) of each bacterial strains were saved frozen at -20°C, thawed when required to perform the test and grown for 2 days in complete nutrient agar broth. The culture obtained were vortexed, large agglomerates allowed to sediment completely and the supernatant further diluted 1:5 in complete minimal broth.

Titres were determined by viable counting on haemocytometer under microscope, giving 1 x 10^3 per ml. These strain dilutions were used as inoculum in both microtitre assay and colorimetric assy.

Resazurin: Resazurin was obtained form Sigma chemicals and prepared as 10g /l sterile water stock solution, saved frozen at -20°C, thawed and diluted 1:10 in sterile water when required.

Titreplate Resazurin Assay: The titreplate resazurin assay was performed in 96-well plates. Two-fold dilutions of each antibiotics and plant extracts were prepared in the test wells in complete nutrient broth, the final antibiotic concentrations being streptomycin 0.06 mg/l and tetracycline 0.12 mg/l. Twenty microlitres of each bacterial suspension was added to 180 µl of antibiotics and plant extracts containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. The plates were sealed and incubated for 12 hr at 37°C. After each incubation time, 5µl of resazurin solution were added per well, colouring them blue. Plates were incubated at 37°C for additional 5 hr. After every one hour incubation time intervals plates were read for colour change from blue to pink and pink to colourless in live-bacterial strains containing wells. Extracts that showed preliminary microtitre-plate assay were reveled the fast decolouration of resazurin which extracts does not have possessed antibacterial potential. The bioactivity of the extracts were screened by which are all the extracts inhibit the dye reduction.

Colorimetric Resazurin Assay: Inocula were prepared by various dilutions of (1 x 10^{-1}-1 x 10^{-7}) growing bacterial strains in Mueller-Hinton broth in a 10 ml test tubes. The tubes were sealed and incubated under 37°C for 24h. After the incubation, test tubes were added various concentrations of the plant extracts prepared in the same broth ranges between 0.1 mg/l to 10 mg/l. Positive controls were prepared with only 9 ml of broth containing 1 ml of 0.1% resazurin solution without plant extracts and antibiotics. Antibiotic control tubes were also maintained aliquots of antibiotic solutions with respective bacterial strains in serial concentrations. In each test tubes added 1 ml of 0.1% resazurin solution and the tubes were further incubated at 37°C for 5 h. After the incubation, 1 ml of solution were taken out form each test tube and read the absorbancy (OD) at 590nm in a spectrophotometer for every one hour up to 5^{th} hour. The minimum inhibition concentration (MIC) was defined as the lowest concentration of the extract that prevent colour change in the test tubes as OD is very close to positive control tube.

Haemocytometric Measurement: From the each test tubes of the above MIC assay, 1 µl of inoculum subjected to haemocytometric assay for bacterial cell count in RBC counterparts. The result indicate the number of viable and dead cells in each concentration of the tested plant extracts and also minimum bacterial concentration (MBC) was inferred based on the highest percentage of dead cell counts in lowest concentration of the plant extracts.

RESULTS

The antibacterial screening of 61 medicinal plant species extract was assayed *in vitro* by microtitre-plate resazurin reduction method against 4 bacterial strains. Table 1 summarizes the microbial growth inhibition of aquous extracts of different parts of selected medicinal plants with the presence of indicator dye resazurin. The data indicates that about 30 plant extracts showed their colour retaining property against any one bacterial strain from their well. If the colour changed in the well is inferred that the metabolic activity of viable bacteria released CO_2 and O_2 which will be reduced the dye becomes pink and fluorescent when reduced to resorufin by oxidoreductase acticity. Resorufin is further reduced to hydroresorufin as colourless. Due to inhibition of bacterial activity by potential plant drug that the dye colour is remain unchanged in the well. From the microtitre-plate assay easily screen the plant extracts which are having antimicrobial potential within short time. In this study, the aquous extracts of *Celastrus paniculata, Gualtheria fragrantissima* and *Vanasushava pedata* showed the fast colour changes in their well against all four bacterial strains (Table 1). The extracts of *Achillia millifolium, Lobelia nicotianifolia,*

Table 1: Antibacterial screening of medicinal plant extracts from Western Ghats by microtitreplate resazurin reduction assay

		OD at 590nm			
Plant species	Plant part extracted	*Staphylococcus aureus*	*Pseudomonos aeruginosa*	*Bascillus subtilis*	*Escherischia coli*
Acalypha fruticosa	Whole plant	--	--	--	--
Achillea millifolium	Whole plant	+	++	+	--
Allophylus concanicus	Stem bark	--	--	--	--
Anogeissus latifolia	Leaves	--	--	--	--
Aristolochia tagala	Leaves	--	--	--	--
Atalantia monophylla	Leaves	--	--	--	--
Balanophora fungosa	Whole plant	--	--	--	--
Berberis tinctoria	Stem bark	+	+	--	--
Bridelia crenulata	Stem bark	--	--	--	--
Buchanania axillaries	Stem bark	--	--	--	--
Bupleurum distichophyllum	Root	++	++	--	--
Cadaba fruticosa	Leaves	--	--	--	--
Canavalia mollis	Seeds	--	--	--	--
Cayratia pedata	Leaves	--	--	---	--
Celastrus paniculata	Seeds	++++	+++	+++	+++
Clematis gouriana	Leaves	--	--	--	--
Clerodendrum viscosum	Leaves	--	+	--	--
Combretum albidum	Flowers	--	--	--	--
Cynanchum tunicatum	Leaves	--	--	--	--
Disporum leschenaultianum	Whole plants	--	--	--	--
Elaeocarpus munronii	Stem bark	--	--	--	--
Eranthemum capense	Leaves	--	--	--	--
Erolaena hookeriana	Stem bark	--	--	--	--
Gaultheria fragrantissima	Leaves	++	++	+	++
Glochidion velutinum	Stem bark	+	+	+	--
Hedychium coronarium	Rhizome	+	--	+	+
Hedyotis swertioides	Whole plant	+	+	+	--
Holarrhena pubescens	Stem bark	--	--	--	--
Hypericum mysurense	Aerial parts	+	--	++	--
Ipomoea eriocarpa	Leaves	++	+	--	+
Justicia betonica	Leaves	--	--	--	--
Lannea coromandelica	Stem bark	--	--	--	--
Lobelia nictonianifolia	Leaves	++	++++	+	--
Melia dubia	Stem bark	--	--	--	--
Meliosma pinnata	Stem bark	--	--	--	--
Michelia nilagirica	Stem bark	+	++	++	-
Mukia madraspatana	Leaves	--	--	--	--
Naringi crenulata	Leaves	--	--	--	--

Table 1: Continued

Notopegia colebrookiana	Stem bark	--	--	--	--
Olea dioica	Leaves	--	--	--	--
Persea macrantha	Stem bark	++	+++	++	--
Phoebe paniculata	Stem bark	++	+	+	+
Pilea microphylla	Whole plant	+	+	++	--
Piper wightii	Fruits	+++	++	--	--
Pittosporum nepaulense	Stem bark	--	--	--	--
Rheum rhaponticum	Leaves	--	--	+	--
Rhus mysorensis	Leaves	--	--	--	--
Schefflera racemosa	Stem bark	--	--	+	--
Scutellaria colebrookiana	Whole plant	+	--	--	--
Sesamum radiatum	Leaves	+	+	+	--
Sida acuta	Whole plant	--	--	--	--
Sonchus wightianus	Leaves	--	--	--	--
Swertia angustifolia	Whole plant	++++	+++	+	--
Symphorema involucratum	Leaves	--	--	--	--
Symplocos cochinchinensis	Stem bark	+++	++	--	--
Syzygium lanceolatum	Leaves	+	+	+	--
Tephrosia maxima	Whole plant	--	--	--	--
Tragia involucrata	Leaves	--	--	--	--
Trochodesma zeylanicum	Whole plant	--	--	--	--
Vanasushava pedata	Leaves	+++	+	+	+
Vitex altissima	Leaves	--	--	--	--
Zizyphus xylopyrus	Seeds	--	---	--	--
Streptomycin		++++	++++	++++	++++
Tetracyclin		++++	++++	++++	+++

+ indicates positive antibacterial activity; -- indicates no antibacterial activity.

Table 2: Minimal inhibitory concentration (MIC) and minimal bacterial concentration (MBC) obtained for aqueous extracts of medicinal plants of Western Ghats (Results obtained after 5 hours of inoculation of sample extracts and dye)

		Staphyococcus aureus		*Bascillus subtilis*		*Pseuomonas aeruginosa*		*Escherichia coli*	
Plant species	Plant part extracted	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC	MBC
Achillea millifolium	Whole plant	150	200	180	= 200	250	190	= 250	200
Allophylus concanicus	Stem bark	170	>200	120	180	220	>200	190	= 200
Berberis tinctoria	Stem bark	100	200	170	250	230	= 200	200	= 200
Bupleurum distichophyllum	Root	190	= 200	230	> 250	100	160	140	230
Canavalia mollis	Seeds	200	= 250	190	> 250	180	240	150	200
Celastrus paniculata	Seeds	10.5	10.0	10.5	10.0	16.5	20	25	40
Clerodendrum viscosum	Leaves	200	250	190	= 250	230	> 250	100	210
Cynanchum tunicatum	Leaves	> 200	> 250	180	250	190	> 200	250	> 250
Disporum leschenaultianum	Whole plants	120	240	160	200	160	> 200	190	> 250
Elaeocarpus munronii	Stem bark	200	> 250	150	200	200	> 250	200	= 200
Gaultheria fragrantissima	Leaves	110	290	100	190	130	200	200	> 200
Glochidion velutinum	Stem bark	200	> 250	190	250	110	200	150	250
Hedychium coronarium	Rhizome	100	230	80	140	100	150	200	200
Hedyotis swertioides	Whole plant	110	200	200	> 250	140	230	200	= 200
Hypericum mysurense	Aerial parts	110	190	200	250	50	150	40	200
Ipomoea eriocarpa	Leaves	200	250	190	> 250	180	= 250	200	= 200
Lobelia nictonianifolia	Leaves	8.0	100	19	60	100	120	100	150
Michelia nilagirica	Stem bark	110	230	180	200	150	> 250	200	> 250
Persea macrantha	Stem bark	100	200	210	250	200	250	150	230
Phoebe paniculata	Stem bark	190	> 250	120	= 200	130	240	100	> 250
Pilea microphylla	Whole plant	200	250	140	= 200	200	250	150	250
Piper wightii	Fruits	80	100	100	130	140	200	130	210
Rheum rhaponticum	Leaves	140	200	190	240	230	> 250	200	= 200
Schefflera racemosa	Stem bark	90	100	20	80	130	240	100	200
Sesamum radiatum	Leaves	190	200	180	> 250	140	230	100	= 200
Sonchus wightianus	Leaves	200	200	110	> 250	150	240	200	250
Swertia angustifolia	Whole plant	90	18	190	200	140	230	180	220
Symplocos cochinchinensis	Stem bark	100	200	200	> 250	140	130	190	= 200
Syzygium lanceolatum	Leaves	90	200	120	200	180	200	120	190
Vanasushava pedata	Leaves	110	200	210	200	200	230	230	> 250
Streptomycine		0.5	1	32	30	8.0	20	0.5	16
Tetracyclin		0.2	10	5.0	20	0.15	25	0.25	25

Michelia nilagirica, Persia macrantha and *Swertia angustifolia* possessed dye colour retaining against at least three bacterial strains in their respective wells. The plant species of *Bupleurum distichophyllum, Hypericum mysurense, Piper wightii* and *Symplocos cochinchinensis* extracts are showed considerable activity against at least bacterial strains. The dye reduction activity of the plant extracts have compared with standard antibiotics and some of the plant extracts results was comparable (Table 1). About 30 plant extracts were selected for further study based on the performance of titre-plate assay.

The resazurin reduction test cane be used for colorimetric determination of minimum inhibitory concentration (MIC) of the plant extracts on par with earlier method [8]. After 5 hours of inoculation of sample extracts in different concentrations with marker dye solution were taken the absorbancy of the cultured broth. The colour changes in the tubes can be markedly visible and also obtained MIC (maximum absorbancy) for potential antibacterial extracts showed the values close to the antibiotic control tubes (Table 2). The MIC values of *Celastrus paniculatus* and *Lobelia nicotianifolia* showed the range of concentration 10-25 µg/ml which is more or less equal to reference antibiotic concentrations. These two medicinal plants species inferred that they are having more potential antibiotic properties against selected bacteria. The moderate bacterial susceptibility and MIC was obtained from the plant extracts of *Schefflera racemosa, Swertia angustifolia* and *Syzygium lanceolatum*. Remaining plant extracts showed that minimum susceptibility against the selected bacterial strains which all extracts possess the antibacterial property above 150 µg/ml concentrations.

DISCUSSION

The minimum bacterial concentrations (MBC) were performed by the dead cell count from each MIC cultured broths in the haemocytometer (Table 2). MBC results are also supported the potential medicinal extracts possessed high dead cell count in respective MIC tubes. Cell counting with a haemocytometer is the most direct way of quantifying cell numbers. Resazurin is not able to penetrate viable cells because of the membrane potential. However, dead cells lose their membrane integrity and are stained slightly bluish pink. This method is inexpensive and easy to perform, bur unsuitable for all type of bacterial cells [9].

The study evaluated the recently described resazurin dye reduction method for rapid screening of plant extracts for its antibacterial potential. Results were obtained in a short period of time and with very good sensitivity. Our results confirm the recent reports of the performance of resazurin reduction assay in other settings [10]. The cost and time of the test will very less when compared with conventional agar plate assay. However, one important concern of this type of the test performed in microtitre-plate with liquid medium relates to the biosafety requirements. This can be overcome by performing the test in individual closed tubes; however, the test is recommended for reference laboratories that already have the necessary biosafety facilities.

CONCLUSION

The study describes the application of resazurin reduction test for high throughput antibacterial screening of plant extracts. MIC and MBC results obtained with colorimetric and haemocytometric assays were comparable with conventional agar plate dilution method.

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