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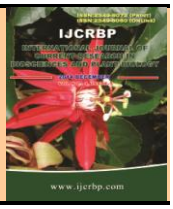

Original Research Article

Antibacterial Activity of *Morinda umbellata* L. (Rubiaceae) Leaves by Resazurin Redox Method

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Abstract

The present study investigated the antibacterial activity of leaf extracts of *Morinda umbellata* L. (Rubiaceae) against ten different bacterial species, *Bacillus megaterium, Bacillus subtilis, Escherichi coli, Klebsiella pneumoniae, Micrococcus luteus, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Staphylococcus aureus* and *Staphylococcus epidermidis* using resazurin microtiter plate assay and colorimetric resazurin assay. Out of five solvents used, ethanol and methanol extracts of *M. umbellata* leaves showed antibacterial activity against maximum number of bacterial species tested in the study followed by petroleum ether>ethyl acetate>acetone. The least Minimum Inhibitory Concentration (MIC) value of 12.0 µg/ml of methanolic leaf extract of *Morinda umbellata* was recorded against the bacterium, *Shigella flexneri*.

Keywords

Antibacterial activity
Eastern Ghats
Leaf extract
Medicinal plants
Morinda umbellata

Introduction

The genus *Morinda* L. is belonging to the family Rubiaceae comprising about 80 species distributed widely. Among the species of *Morinda*, *M. citrifolia* L. (Noni), is well known for its medicinal properties and has been extensively studied for various biological activities including antimicrobial activity (Jayaraman et al., 2008; Nayak et al., 2009; Kumar et al., 2010; West et al., 2012; Natheer et al., 2012; Candida et al., 2014) . Other species of *Morinda* are least studied for biological activities: *M. tinctoria* for antimicrobial activity (Deepti et al., 2012), *M. morindoides* for anti-*Vibrio cholarae* activity (Koffi et al., 2010) and *M. lucida* for antibacterial activity against *Escherichia coli* (Ogundare and Onifade, 2009).

M. umbellata L., a liana, is known for its traditional curative medicinal properties of stomach disorders and anti-diarrhoeal activity (Ismail and Sulthana, 2008; Nair et al., 2013). Though *M. umbellata* is known for its medicinal uses, scientific research on its biological activities is least studied. Hence in the present study, the leaf extracts of different solvents of *M. umbellata* has been subjected to antibacterial activity against ten bacterial species using resazurin dye reduction method.

Materials and methods

Plant collection and identification

The leaves of *Morinda umbellata* L. (Fig. 1) were collected from Sirumalai Hills (Eastern Ghats), Dindigul District, Tamil Nadu, India and the plant specimen was identified by Dr. S. Karuppusamy, Department of Botany, Madura College, Madurai, Tamil Nadu. The identification was confirmed with local floras. The leaves were collected and shade dried for a week and powdered using ball mill. A fine powder obtained was stored in air tight polythene bags and used for the preparation of extract.

Method of extraction

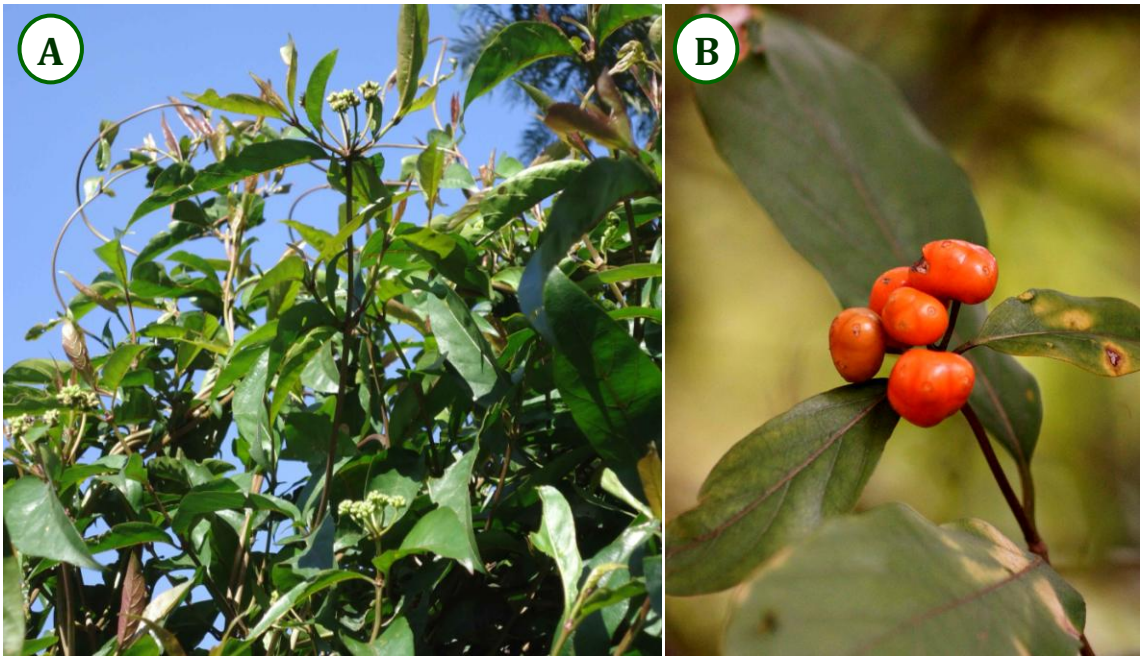
Five different solvents were used for extraction *viz.*, acetone, ethyl acetate, ethanol, methanol and petroleum ether. The powdered plant material (100g for each solvent separately) was taken in a thimble of Soxhlet apparatus and kept the thimble on round bottom flask, and 250ml of each solvent was added to round bottom flask then the condenser was fixed on upper part of the thimble, and finally heated to 50-80°C in a heating mantle. This was done individually for each solvent mentioned above. These steps were carried out for 24 h until the extract in the siphon tube became colourless. After separating the solvents and drying, the final crude extracts were collected and stored individually in air tight containers until use.

Antimicrobial activity

Ten bacterial strains listed below were procured from NCIM (National Centre for Industrial Microorganisms, Chandigarh, India), revived and sub-cultured for the present study. The bacterial species used were: *Bacillus megaterium* (NCIM-2187), *Bacillus subtilis* (NCIM-2063), *Escherichia coli* (NCIM-2065), *Klebsiella pneumoniae* (NCIM-2256), *Micrococcus luteus* (NCIM-2103), *Pseudomonas aeruginosa* (NCIM-2200), *Salmonella typhi* (NCIM-2501), *Shigella flexneri* (NCIM-2012), *Staphylococcus aureus* (NCIM-2079) and *Staphylococcus epidermidis* (NCIM-2493).

Bacterial strains procured were cultured on Mueller-Hinton agar media with regular interval for subculture and stored in 20±2°C. Stock cultures containing 1×10^7 cfu × ml (0.5 MacFarland) of each bacterial strain was 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 was further diluted to 1:5 in complete minimal broth. These strain dilutions were used as inoculum in both microtitre assay and colorimetric assay. Broad spectrum antibiotic, azithromycin was used for Gram positive bacterial strains and ciprofloxacin were used for Gram negative bacterial strains.

Fig. 1: *Morinda umbellata* L. (Rubiaceae) with flowers (A) and fruits (B).



Resazurin redox (dye reduction) method

The antibacterial activity of *Morinda umbellata* leaf extracts was determined by resazurin dye reduction method described by Kruppusamy and Rajasekaran (2009). The dye resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) was obtained from Sigma chemicals and prepared as 10g/l sterile water stock solution, stored in 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 of culture medium containing plant extracts in separate well plates for each bacterial strain. Control wells were prepared with culture medium and bacterial suspension only.

The plates were sealed and incubated for 12 h at 37°C. After incubation time, 5µl of resazurin solution were added per well, colouring them blue. Plates were incubated at 37°C for additional 5 h. After every one hour of incubation time interval, plates were read for colour change from blue to pink and pink to colour less in live-bacterial strains containing wells. The bioactivity of the extracts was assessed by the colour change.

Colorimetric resazurin assay

Inocula were prepared by various dilutions of (1×10^{-1} to 1×10^{-7}) growing bacterial strains in Mueller-Hinton broth in 10 ml test tubes. The tubes were sealed and incubated under 37°C for 24 h. 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 as aliquots of antibiotic solutions with respective bacterial strains in serial concentrations. In each test tube, 1 ml of 0.1% resazurin solution was added 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 590 nm in a spectrophotometer for every one hour up to 5 h.

The resazurin reduction test can be used for colorimetric determination of minimum inhibitory concentration (MIC) of the plant extracts on par with earlier methods. After 5 h of inoculation of 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 showing the values close to the antibiotic control wells.

Results and discussion

The antibacterial screening of different solvent extracts of *M. umbellata* leaves showed different degrees of activity against test bacteria (Table 1).

The acetone-leaf extract showed moderate activity only against *M. luteus* and low activity against *B. megaterium, B. subtilis, E. coli*, *P. aeruginosa* and *Staph. epidermidis*; *K. pneumoniae, Sal. typhi, Sh. flexneri* and *Staph. aureus* were resistant. Excepting *B. subtilis, K. pneumoniae* and *Sh. flexneri,* all the other bacteria were susceptible to the ethyl acetate-leaf extracts of *M. umbellata*. Petroleum ether leaf extracts showed moderate antibacterial activity all the bacteria tested except *K. pneumoniae, Mic. luteus* and *Sal. typhi*.

Ethanolic and methanolic extracts of *M. umbellata* leaves showed good antibacterial activity against most of the bacteria tested. The bacterial species that showed resistance against ethanolic extract was *Staph. aureus* and against methanolic extract was *B. megaterium* and *Staph. epidermidis*. The ethanolic extract showed strong antibacterial activity against *B. subtilis* and *P. aeruginosa*.

Similarly, methanolic extract showed strong activity against *K. pneumoniae* and *Sh. flexneri* (Table 1). All the solvent extracts of *M. umbellata* leaves showed antibacterial activity against one or the other bacteria among the bacteria, all extracts were subjected to study MIC values.

Table 1. Antibacterial screening of different solvent extracts of *Morinda umbellata* L. leaves (OD read at 590 nm).

Bacteria used	Leaf extract used					Standard Control
	Acetone	Ethanol	Ethyl acetate	Methanol	Petroleum-ether	
Bacillus megaterium (NCIM-2187)	+	+	+	-	++	+++
Bacillus subtilis (NCIM-2063)	+	+++	-	++	+	++++
Escherichia coli (NCIM-2065)	+	++	++	+	++	++++
Klebsiella pneumoniae (NCIM-2256)	-	+	-	+++	-	+++
Micrococcus luteus (NCIM-2103)	++	++	+	++	-	++++
Pseudomonas aeruginosa (NCIM-2200)	+	+++	+	++	++	++++
Salmonella typhi (NCIM-2501)	-	++	+	+	-	++++
Shigella flexneri (NCIM-2012)	-	+	-	+++	+	++++
Staphylococcus aureus (NCIM-2079)	-	-	++	+	++	+++
Staphylococcus epidermidis (NCIM-2493)	+	+	+	-	++	++++

The MIC values also vary greatly in coherence with the activity observed in screening test. The MIC values ranged from 12 µg/ml to above 250 µg/ml for different solvent extracts of *M. umbellata* leaves against test bacteria. The least MIC value of 90 µg/ml against *Mic. luteus* was recorded for acetone extract, followed by 100, 110, 140, 180 and 190 µg/ml against *Staph. epidermidis*, *B. megaterium*, *P. aeruginosa*, *E. coli* and *Sal. typhi* respectively. The least MIC for methanol, ethanol, petroleum ether and ethyl acetate extracts of *M. umbellata* leaves was 12, 20, 40 and 70 µg/ml respectively against *Sh. flexneri*, *B. subtilis*, *Staph. epidermidis* and *Staph. aureus* (Table 2).

Table 2. Minimal Inhibitory Concentration (MIC) obtained for various solvent extracts of *Morinda umbellata* L. (Results obtained after 5 h of inoculation of extracts and dye).

Bacteria used	Leaf extract used (µg/ml)					Standard Control (µg/ml)
	Acetone	Ethanol	Ethyl acetate	Methanol	Petroleum-ether	
Bacillus megaterium (NCIM-2187)	110	150	190	>200	85	0.4
Bacillus subtilis (NCIM-2063)	>250	20	>250	40	120	0.2
Escherichia coli (NCIM-2065)	180	80	140	115	70	1.5
Klebsiella pneumoniae (NCIM-2256)	>200	130	200	20	>250	10
Micrococcus luteus (NCIM-2103)	90	90	140	60	>200	0.2
Pseudomonas aeruginosa (NCIM-2200)	140	30	180	90	90	0.4
Salmonella typhi (NCIM-2501)	190	100	130	125	>200	0.1
Shigella flexneri (NCIM-2012)	230	175	>200	12	150	0.3
Staphylococcus aureus (NCIM-2079)	>250	>250	70	170	60	11
Staphylococcus epidermidis (NCIM-2493)	100	120	120	>200	40	0.2

The activity of *M. citrifolia* extracts is effective against many bacteria: *E. coli*, *Staph. aureus* (Sundar et al., 2011; West et al., 2012; Candida et al., 2014). The ethanolic extract of *M. citrifolia* fruit showed antimicrobial activity against *Staph. aureus* which was less resistant to ethanolic extract than *E. coli* (1 mg/ml and 10 mg/ml, respectively). Deepti et al. (2012) concluded that the methanolic extract of *M. tinctoria* is more effective against tested bacteria than ethyl acetate, chloroform and hexane extracts. In the current study also, methanolic extract of leaves of *M. umbellata* showed strong antibacterial activity with least MIC value. Variety of biological activities of ethanolic extracts of medicinal plants was reported to possess effective phytochemical principles with activity (Deepti et al., 2012; Anandkumar et al., 2014). However, many of these kinds of studies concerned with antimicrobial assays have adopted disc/well diffusion method.

The screening test of several plants collected from Western Ghats, India by Karuppusamy and Rajasekaran (2009) revealed the easy selection of effective medicinal plant/plant parts for antimicrobial activity using resazurin redox method, for example in their study: seeds of *Celestrus*

paniculata against *Staph. aureus*; leaves of *Lobelia nicotianaefolia* against *P. aeruginosa*. Their study also showed least MIC values of 10-25 µg/ml. In our study, the least MIC is very close to this value, i.e., 12-40 µg/ml. In the present study, comparably, methanol and ethanol extracts of *M. umbellata* leaves demonstrated good antimicrobial activity with least MIC values, with different degrees of variation. The resazurin redox method is time saving, effective and rapid for screening of plant extracts and for finding out MIC values.

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