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RESEARCH ARTICLE

BACTERICIDAL EFFECT OF ACACIA NILOTICA: IN VITRO ANTIBACTERIAL AND TIME KILL KINETIC STUDIES

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ABSTRACT

The study aimed at comparing antibacterial potential of 3 polar and 3 non-polar organic solvent extracts of *Acacia nilotica* leaves against 9 reference bacterial strains and time kill kinetic study of ethyl acetate extract against *S. typhi*. Antibacterial activity was assessed against 9 standard bacterial reference strains viz. *Shigella flexneri* ATCC 12022, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 259323, *Proteus mirabilis* ATCC 43071, *Salmonella typhi* ATCC 13311, *Serratia marcescens* ATCC 27137, *Klebsiella pneumonia* ATCC 700603, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 by Agar well diffusion method at a concentration of 50mg/ml. MIC was calculated by modified 96 well microtitre plate assay. Time kill kinetic study against *S. typhi* was performed at $\frac{1}{2}$ x MIC, MIC and 2 x MIC concentrations. Polar solvent extracts were more active against all test strains. Ethyl acetate extract was most active. Maximum activity was against *S. typhi* with a zone of inhibition of 25 mm. MIC values ranged from 0.39 mg/ml to 12.5 mg/ml for different test strains. Maximum reduction in CFU/ml was achieved at 8 hour of incubation at MIC and 2 x MIC concentrations. So we can conclude that *A. nilotica* leaves can be a potent source of new antibacterial agents for therapeutic applications.

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INTRODUCTION

Plants are the natural medicine gifted by nature to mankind. Medicinal plants contain a lot of active principles infused in different tissues, which are proposed to have a number of pharmacological effects (Banso, 2009). Undesirable side effects of common synthetic antibiotics and emergence of previously uncommon infections has augmented for search of new therapeutics from medicinal plants. Plants are a source of new prototype compounds as they host a great variety of secondary metabolites (Farombi, 2003; Okigbo et al., 2009). Most of the common human diseases are a result of bacterial infections (Djeussi et al., 2013). Bacteria are the cause of most dreadful human diseases like tuberculosis, typhoid, botulism, cholera etc. Use of medicinal plants as remedies for the treatment of bacterial infections depends on the indigenous knowledge about the plant. *Acacia nilotica* commonly known as babul or kikar is a member of family fabaceae and sub-family mimosoideae. *Acacia nilotica* is a single stemmed, dome shaped tree growing to 15-18 meters in height. Flowers are yellow globular heads (Brenan, 1983; Malviya et al., 2011).

Acacia nilotica is a weed with medicinal benefits. Stem exudates a reddish gum which is used in various food preparations (Kumar and Jain, 2013; Shetty and Pandey, 1983). Leaves are used as fodder for sheep and cattle. Leaf paste is used for treatment of headache (Bhalero and Kelkar, 2012). Leaves are used for treatment of diarrhea, dysentery (Mohanty et al., 1996), eczema and abscess (Siddiqui et al., 1989). Many compounds like androstene (Chaubal et al., 2003), D- pinitol (Chaubal et al., 2003) and ethyl gallate (Kalaivani et al., 2011) have been isolated from the plant leaves with various pharmacological effects.

These literature reports have shown the therapeutic potential of *Acacia nilotica* leaves. Extraction of desired compounds depends upon the choice of solvents used. Most common criteria for choosing a solvent are its polarity. Polar solvents extract out most of the polar molecules from plant material (Sujatha and Suresh, 2013). Present study was done to evaluate the comparative antibacterial potential of polar and non polar solvent leaf extracts of *Acacia nilotica* against various pathogenic reference ATCC strains. Time kill kinetic study was performed against *S. typhi*. This study is the first comprehensive attempt to assess the yield, antibacterial activity and minimum inhibitory concentration of different solvent extracts prepared from leaves of *Acacia nilotica*.

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MATERIALS AND METHODS

Plant Material

Fresh, mature and healthy leaves of *Acacia nilotica* were collected from Jhajjar district, Haryana, India in Oct. 2013. The plant material was identified and authenticated by comparing the herbarium specimen (MDU 2601) available in the Department of Genetics, M.D. University, Rohtak (India). Leaves were put in sterile plastic bags and brought to laboratory. Leaves were first washed with running tap water and then with distilled water. Properly washed leaves were dried in shade.

Preparation of Plant Extracts

Dried leaves were ground into coarse powder with the help of mixer grinder. Both polar and non-polar solvents were used for extract preparation. Polar solvents used in the study were methanol, acetone and ethyl acetate. Chloroform, benzene and petroleum ether were the non-polar solvents used. Extracts were prepared by cold percolation method in an incubator shaker at 120 rpm for 48-72 hrs. Extraction was carried out in 1:10 ratio. Extracts were filtered through Whatman filter paper No. 1. Filtrate was concentrated with help of rotary vacuum evaporator at 40 °C. Dried extracts were weighed and kept in freeze until further use.

Test Bacterial Strains

ATCC reference bacterial strains were used to screen the activity. 7 were gram negative bacterial strains viz. *E. coli* (ATCC 25922), *P. mirabilis* (ATCC 43071), *K. pneumonia* (ATCC 700603) *P. aeruginosa* (ATCC 27853), *S. flexneri* (ATCC 12022), *S. marcescens* (ATCC 27137), *S. typhi* (ATCC 13311). Two gram positive bacterial strains viz. *S. aureus* (ATCC 259323) and *E. faecalis* (ATCC 29212) were used.

Assay for Antibacterial Activity

Antibacterial activity was checked by modified Agar well diffusion method (Perez et al., 1990). Each experiment was performed in triplicates. Inoculums were prepared from overnight grown cultures of bacteria in peptone water. Turbidity was adjusted equivalent to 0.5 McFarland units (approximately 10^8 CFU/ml). 10 µg Streptomycin disc (HiMedia Laboratories Pvt. Ltd. India) was taken as positive control and DMSO as negative control. Inoculums (0.1 ml) were spread over fresh nutrient agar plates with a sterile spreader.

Wells of 6mm diameter were made with the help of a sterile cutter. Control disc was placed in centre. Stock solution of different extracts was prepared at a concentration of 50 mg/ml of DMSO. 10, 20, 30 and 40 µl of these prepared extracts were added to each well for all bacterial strains used. Plates were incubated for 24 hours at 37°C. Clear zone of inhibition around each well was measured with help of standard ruler HiAntibiotic ZoneScale™-C (HiMedia Laboratories Pvt. Ltd. India).

Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antibacterial compound or extract that will hinder the visible growth of bacteria after overnight incubation. The MIC values of leaf extracts were determined using micro broth dilution method in 96 multi-well microtitre plates developed by Sarkar et al. with slight modifications (Sarkar et al., 2007). 50 µl of nutrient broth and 50 µl of normal saline were added to each well of plate. A volume of 100 µl of test materials in DMSO (50 mg/ml) was added into the first row of the plate. Serial dilutions were performed using a multichannel pipette such that each well had total 100 µl of the test material in serially descending concentrations. 10 µl of resazurin indicator solution (prepared by dissolving 27 mg resazurin in 4 ml of sterile distilled water) was also added to each well. Finally 10 µl of bacterial suspension (5×10^6 CFU/ml) was added to each well. Each plate had a column with streptomycin as positive control and a column without streptomycin and plant extract as negative control. The plates were prepared in triplicate and placed in an incubator at 37°C for 24 hr. Any color change from purple to pink or to colorless indicates growth of microbes. The lowest concentration at which no color change observed was considered as the MIC value of extract.

Time Kill Assay

Time kill assays were performed to check rate of killing of *Salmonella typhi* by ethyl acetate leaf extract according to standard protocol (Jayaraman et al., 2010). Individual colonies of the microbe were isolated from overnight grown plates. These were suspended in sterile normal saline to approximate the density of 0.5 McFarland standards. This suspension was diluted 1:10 in nutrient broth. 100 µl of this prepared inoculum was added to 0.9 ml of nutrient broth. 1 ml samples of extract were prepared in concentration of $\frac{1}{2}$ x MIC, MIC and 2 x MIC. Above prepared inoculums were added to extracts and incubated at 35 °C for 24 hours. 2 tubes were taken as controls; one without extract and one without inoculums. From each tube 25 µl of sample was pipetted out at 0, 2, 4, 6, 8 and 24 hr and plated to count the CFU/ml. Experiments were performed in triplicates. The killing rate was determined by plotting viable colony counts (CFU/ml) against time.

Statistical Analyses

All experiments were performed in triplicates. Results are expressed as mean±SD (Standard Deviation). % Reduction (ASTM, 2008) in CFU was calculated as-

$$\% \text{ Reduction} = \frac{\text{Initial count} - \text{count at x interval}}{\text{Initial count}} \times 100$$

RESULTS

In present study different polar and non-polar solvents were used for preparation of leaf extracts of *Acacia nilotica*. Percentage yield of different extracts along with the solvent properties has been given in Table 1. Extract yield was more in polar solvents as compared to non-polar solvents.

All extracts showed activity against all the tested bacterial strains. Antibacterial activity of extracts at a concentration of 2 mg per well against 9 bacterial strains has been shown in Table 2. Antibacterial activity values ranged from 7.33 mm to 25 mm. Polar solvent extracts were very effective against all the tested bacterial strains whereas non-polar solvent extracts showed very little activity. Among the 3 polar solvent extracts, ethyl acetate extract was most effective followed by methanol and acetone. Maximum activity was shown by ethyl acetate extract against *S. typhi* with a zone of inhibition of 25 mm followed by 24.33 mm zone of inhibition for *K. pneumonia* in contrary to 18 mm zone of inhibition of Streptomycin against both of these bacterial strains. Methanol extract showed maximum 24 mm zone of inhibition against *S. marcescens* with respect to 23 mm of control.

In our study non-polar solvents extract activity was not very significant. Benzene extract was least active against most of the tested strains. Minimum 7.33 mm of inhibition zone was shown by petroleum ether extract against *P. mirabilis*. Petroleum ether and benzene extracts were comparatively more active than benzene extract. Extracts were equally active against both gram positive and gram negative strains used in the study. MIC values ranged from 0.39 mg/ml to 12.5 mg/ml for different extracts (Table 3). Extract showing maximum zone of inhibition showed minimum MIC value. Minimum MIC value of 0.39 mg/ml was shown by ethyl acetate and methanol extract against *S. typhi*, *P. aeruginosa*, *P. mirabilis* and *S. aureus*, *E. faecalis* respectively. Chloroform, benzene and petroleum ether extracts showed MIC values of either 6.25 mg/ml or 12.5 mg/ml against all test strains.

Table 1. Characteristics of different solvents and % yield of extracts in these solvents

Sr. No.	Solvent	Dielectric Constant	Boiling Point (°C)	Specific Gravity	% Yield
1	Methanol	33	64.7	0.791	12.96
2	Acetone	21	56.3	0.790	12.72
3	Ethyl acetate	6.02	77.1	0.895	12.90
4	Chloroform	4.81	61.2	1.498	1.34
5	Benzene	2.3	80.0	0.879	1.28
6	Petroleum ether	2.0	40-60	0.656	0.72

Table 2. Antibacterial activity of *Acacia nilotica* leaf extracts against reference bacterial strains

Bacterial strains	Zone of Inhibition (mm)						
	Methanol extract	Acetone extract	Ethyl Acetate extract	Chloroform extract	Benzene extract	Pet. Ether extract	Streptomycin (10µg)
<i>E. coli</i>	19.66±0.57	20.0±1.0	20.66±0.57	7.66±0.57	8.66±0.57	11.33±0.57	15.4±0.36
<i>S. aureus</i>	18.33±0.57	17.66±0.57	19.66±0.57	11.33±0.57	8.33±0.57	11.0±1.0	26.2±0.26
<i>S. typhi</i>	19.0±1.0	14.66±0.57	25.0±1.0	14.0±1.0	7.66±0.57	9.33±0.57	18.2±0.2
<i>S. marcescens</i>	24±1.0	19.33±0.57	22.66±0.57	10.66±0.57	7.66±1.15	8.66±0.57	23.1±0.17
<i>K. pneumonia</i>	17.33±0.57	16.33±0.57	24.33±0.57	8.66±0.57	8.66±0.57	15.66±0.57	18.16±0.28
<i>P. aeruginosa</i>	18.33±0.57	19.66±0.57	21.33±0.57	9.33±0.57	8.33±0.57	10.33±0.57	27.03±0.05
<i>P. mirabilis</i>	19.33±1.15	20.66±0.57	22.66±0.57	8.66±0.57	9.66±0.57	7.33±0.57	19.06±0.11
<i>E. faecalis</i>	17.66±0.57	17.33±0.57	18.0±1.0	11.33±0.57	8.33±0.57	8.0±1.0	23.1±0.17
<i>S. flexneri</i>	18.66±0.57	17.66±0.57	20.33±0.57	11.66±0.57	10.0±1.0	11.33±0.57	28.06±0.11

Table 3. Minimum Inhibitory Concentration (MIC in mg/ml) of all solvent extracts

Bacterial strains	Methanol extract	Acetone extract	Ethyl acetate extract	Chloro-form extract	Benzene extract	Pet. Ether extract
<i>E. coli</i>	1.56	1.56	0.78	12.5	6.25	6.25
<i>S. aureus</i>	0.39	3.12	1.56	6.25	12.5	6.25
<i>S. typhi</i>	0.78	3.12	0.39	6.25	6.25	6.25
<i>S. marcescens</i>	0.78	3.12	1.56	6.25	12.5	12.5
<i>K. pneumonia</i>	0.78	0.78	1.56	6.25	6.25	12.5
<i>P. aeruginosa</i>	0.78	0.78	0.39	12.5	6.25	6.25
<i>P. mirabilis</i>	1.56	0.78	0.39	12.5	12.5	12.5
<i>E. faecalis</i>	0.39	1.56	0.78	6.25	12.5	6.25
<i>S. flexneri</i>	1.56	1.56	0.78	6.25	12.5	6.25

Table 4. % reduction caused by Ethyl acetate extract against *S. typhi*

Time (hour)	% Reduction		
	½ x MIC	MIC	2 x MIC
0	N/A	N/A	N/A
2	20	32	48
4	52	76	84
6	64	92	95.2
8	80	99.9	99.9
24	97.2	99.9	99.9

Time kill kinetic study of ethyl acetate extract against *S. typhi* showed 99.9 % reduction at MIC and 2 x MIC concentration. Maximum reduction was achieved at 8 hour by both of these concentrations. At $\frac{1}{2}$ x MIC concentration extract was not effective in controlling all test microbes. 97.2 % reduction was achieved after 24 hours of incubation (Table 4). $5\log_{10}$ reduction was observed at MIC and 2 x MIC concentrations (Figure 1).

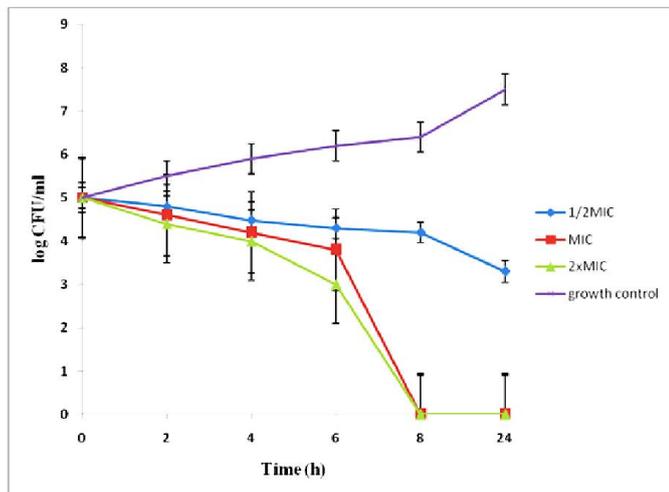


Figure 1. Time kill curve of ethyl acetate extract against *S. typhi*

DISCUSSION

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. In our study 3 polar and 3 non-polar solvents were used for the extraction process. Results indicate that polar solvents are more suited for extraction of phytochemicals to be used for antibacterial activity. Previous studies state that most of plant extracts are more active against gram positive bacteria than gram negative ones (Kollef et al., 2011). Gram negative bacteria are more difficult to control due to their double layered membrane (Beveridge, 1999). But in our study extracts were more effective against gram negative bacteria. Polar solvent extracts showed good activity against gram negative *E. coli*, *S. typhi* and *K. pneumonia*. Previous studies on *A. nilotica* also support the view that polar solvent extracts are more effective in controlling bacteria.

Ethanol extract of leaves showed good activity against *Campylobacter coli* isolated from goats (Solomon-Wisdom and Shittu, 2010). Ethanol, methanol and aqueous extracts showed good activity against *S. aureus* and *E. coli*. Maximum activity was shown by methanol extract of 29mm (Choudhary, 2011). Methanolic extracts showed activity against various hospital isolates (Kavitha et al., 2013). In our study methanol extract also showed good activity. Petroleum ether, benzene, chloroform, ethanol and methanol extracts showed activity against plant bacteria *Xanthomonas pathovars* (Raghvendra et al., 2013). Ethyl acetate as an extraction solvent has not been studied widely. Our results emphasize that ethyl acetate and methanol extracts are quite effective in controlling bacteria. Preliminary phytochemical studies state that *A. nilotica* is rich in alkaloids, flavonoids, tannins, saponins, steroids,

carbohydrates and glycosides (Choudhary, 2011; Solomon-Wisdom and Shittu, 2010). Polar solvent extracts out most of the polar biochemicals like phenols, polyphenols, flavonoids, tannins and alkaloids from plant tissues. All these phytochemicals are reported to have antimicrobial activity (Tiwari et al., 2011). Polyphenols and alkaloids are the major antimicrobial compounds present within plants (Vijyasanthi et al., 2012). Previous studies report that polyphenols and alkaloids are extracted in ethyl acetate and alcohols respectively (Singh et al., 2009; Rao, 2012).

Significant activity of methanol and ethyl acetate in this study may be contributed to extraction of alkaloids and polyphenols in these solvents. Non-polar solvents extract out less compounds than polar solvents as reported by previous studies. Phytochemical screening of petroleum ether extract of *A. nilotica* leaves showed presence of triterpenes only (Edriss et al., 2012). Terpenoids and flavonoids are extracted in chloroform extract (Tiwari et al., 2011). Both of these are antimicrobials. These contribute to little activity shown by these extracts. Higher activity of polar solvents may be due to synergistic effect of many phytochemicals. Time kill assays are performed to assess the pharmacodynamics of a particular drug or extract (Odenholt et al., 2001). It is used to assess both bacteriostatic and bactericidal activities of drugs (Bajak Souzian et al., 1997).

Present study shows the bactericidal effect of *A. nilotica* ethyl acetate leaf extract at MIC and 2 x MIC concentrations. At $\frac{1}{2}$ x MIC concentrations, extract showed bacteriostatic activity. In some cases it is more desirable to have drugs with bacteriostatic activity to control microbes (Pankey and Sabath, 2004). So we can propose that polar solvents i.e. methanol and ethyl acetate extracts of *Acacia nilotica* leaves are best suited for extraction process to be used in antibacterial assay. Also ethyl acetate leaf extract possess strong bactericidal effect at higher concentration and bacteriostatic at lower concentration. This study serves as an initial step for more comprehensive studies of compound isolation.

Conclusion

Acacia nilotica leaves are a rich source of various antibacterial compounds. Compound isolation studies on *Acacia nilotica* leaves can lead the research of new antibacterial agents with enhanced activity against gram negative bacteria. Good activity of leaf extract against *E. coli*, as shown in our study may be applied for treatment of various dermatological infections.

Conflict of Interest

All authors declare that they have no conflict of interest.

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