



***In vitro* evaluation of anti-microbial spectrum of *Acacia nilotica* leaves and bark extracts against pathogens causing otitis infection**

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Abstract: The antimicrobial activity of *Acacia nilotica* leaves and bark extracts were assayed against the six ear pathogens causing otitis infection namely, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter* sp. and *Candida albicans* using agar well diffusion method. Organic leaves and bark extracts displayed antimicrobial activity against all the tested ear pathogens, while aqueous leaves extract showed activity against three tested bacterial ear pathogens. Aqueous bark extracts were unable to exhibit any antimicrobial activity. The highest antimicrobial activity of *A. nilotica* leaves and bark extracts were found against *S. aureus* with zone of inhibition of 23.6 mm and 20.6mm in acetonic extract with minimum inhibitory concentration value of 6.25mg/ml and 12.5 mg/ml. The MBC values ranged between 6.25mg/ml and 505mg/ml for the different bacterial ear pathogens while showed the common MFC value of 50 mg/ml against *C. albicans*. These results indicate that acetonic leaves extract of *A. nilotica* could be used as a source of antimicrobial agents to treat otitis infections.

Keywords: Otitis infection, antimicrobial activity, *Acacia nilotica*, Organic and aqueous extracts

Received: 15 January 2014 / Accepted: 01 February 2014 / Published Online: 30 March 2014

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An otitis infection is an inflammation of the epithelial lining of the external auditory canal and auricle. Otitis infection is mainly caused by bacterial (*Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Proteus mirabilis*) and fungal pathogens (*Aspergillus niger*, *A. flavus*, *A. fumigatus*, *A. luchuensis*, *A. terreus*, *Penicillium* sp. and *Candida albicans*) (Hawke et al. 1984; Clark et al. 1997; Aneja et al. 2010).

Infectious diseases show an important health problem and represent one of the main causes of morbidity and mortality worldwide, due to the indiscriminate use of antibiotics and incidence of multiple antibiotic resistances in human pathogens. Due to report of increasing developments of drug resistance in human pathogen, it is necessary to search for new agents that are better and without side effect for treating infectious diseases especially in developing

countries (Marjorie, 1999; Kahkashan et al. 2012). A wide variety of plant/natural products are used in the treatment of different ailments. In traditional medicine diverse infectious diseases have been treated with herbal products. Approximately the 80% of the world population have used herbal products to satisfy their primary health care (Shubhi et al. 2010). It has been scientifically demonstrated that plants contain secondary metabolites (alkaloids, volatile essential oils, phenols and phenolic glycosides, resins, oleosins, steroids, tannins and terpenes etc) to which biological properties are attributed, and from these properties, drugs have been developed to cure diseases. Due to this, there is a constant need to find and develop new compounds with antimicrobial potential, and to continue the search of medicinal plants with new mechanisms of action to treat infectious diseases (Egwaikhide et al. 2009). *Acacia nilotica* L. commonly known as Babul, Kikar and Gum Arabic tree is a

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member of the family *Mimosaceae*. It is a medium size thorny tree growing up to a height of 5-20m and mainly found in the drier parts of India. It has yellow mimosa like flowers and long grey pods constricted between seeds. The leaves are fine and densely hairy with 3-6 pairs of pinnate consisting of 10-20 pairs of leaflets that are narrow with parallel margins. The bark has a tinge of orange and/or green (young tree), but older trees have dark, rough bark and tend to lose their thorns (Beniwal et al. 1992; Mann et al. 2003; Khan et al. 2009). *Acacia nilotica* are used in folk medicine by people in rural areas as a remedy for curing different disorders. The bark of plant is used extensively for colds, bronchitis, diarrhea, leprosy, bleeding piles and leucoderma. Pods and tender leaves are given to treat diarrhea and also considered in folk medicine to treat diabetes mellitus. The plant has been shown to exhibit antibacterial, antiinflammatory, vasoconstrictor actions, antihypertensive, antispasmodic activities, inhibitory effect against hepatitis virus, cytotoxic activity and antioxidant activity (Gilani et al. 1999; Del, 2009; Malviya et al. 2011).

Various parts of this plant are known to be important source of secondary metabolites as alkaloids, cyanogenic glycosides, fluoroacetate, gums, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), hydrolyzable tannins, flavonoids and condensed tannins (Seigler, 2003). Therefore, the present study was designed to evaluate the antibacterial and antifungal potential of leaves and bark extracts of this plant against the locally isolated microorganism from the patients having ear infection and its comparison with locally available ear drops.

MATERIAL AND METHODOLOGY

Plant collection

The bark and leaves of *Acacia nilotica* were collected from the trees alongside the roads of University of Kurukshetra, Haryana. The taxonomic identity of the plant was confirmed by Dr. BD Vashishta, plant taxonomist, Chairman of Botany Department, Kurukshetra University, Kurukshetra.

Extraction of plant material

The samples were carefully washed under running tap water followed by sterile distilled water and air dried at room temperature (35-40°C) for 4-5 days, homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. Four different solvents, namely ethanol, methanol, acetone and aqueous (hot and cold), were used for extraction. Homogenized bark and leaves, 10 g each, were separately soaked in conical flasks each containing 100 ml of acetone, ethanol and methanol (95%) and sterile distilled water. Also, an equal amount (i.e. 10 g) of homogenized bark and leaves was immersed separately in 100 ml of hot sterile distilled water in conical flasks and allowed to stand for 30 minutes in a water bath (at 100°C) with occasional shaking, followed by keeping all the flasks on rotary

shaker at 200 rpm for 24 hours. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rota evaporator. The dried extract thus obtained was sterilized by overnight UV-irradiation, checked for sterility on nutrient agar plates and stored at 4°C in labelled sterile bottles until further use (Aneja and Sharma, 2010).

Test microorganisms

Five bacteria, namely *Staphylococcus aureus* (HM626197)*, *Acinetobacter* sp. (HM626198), *Proteus mirabilis* (HM626199), *Escherchia coli* (HM626200), *Pseudomonas aeruginosa* (HM626201) and one yeast, *Candida albicans*, were isolated from patients with an ear infection from the local ENT clinics in Kurukshetra (Aneja et al. 2010). Bacterial strains were identified on the basis of Gram staining, biochemical and molecular characteristics (16S rRNA sequencing) (Lawongsa et al. 2008) and on the basis of staining, morphological and cultural characteristics for the yeast (Aneja, 2003; Cappuccino and Sherman, 2008). The bacterial isolates were subcultured on nutrient agar and *C.albicans* on malt yeast agar and incubated aerobically at 37°C. The media were procured from Hi Media Laboratory Pvt. Ltd., Bombay, India.

*(Nucleotide sequence of all the five bacteria have been submitted to GenBank database which provided the GenBank accession number, HM626197-HM626201)

Ear drops

Three commonly prescribed ear drops by otolaryngologists, two allopathic ciplox (antibacterial), candid (antifungal) and a herbal ear drop Kan pip (antimicrobial), were procured from the local market in Kurukshetra.

Screening for antimicrobial activity

The acetone, methanol, ethanol and hot and cold aqueous *Acacia nilotica* bark and leaves extracts were used for evaluation of antimicrobial activity by the agar well diffusion method. In this method, a pure isolate of each microbe was grown on agar plates at 37°C for 24 hours. One plate of each microorganism was taken and a minimum of four colonies were transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted to be equal to that of 10⁶ cfu/mL (standardized by 0.5McFarland standard) and used as the inoculum for performing an agar well diffusion assay. One hundred microliter (100 µL) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8 mm wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with molten agar medium. The dried extracts were reconstituted in dimethylsulphoxide (DMSO) for the bioassay analysis. A 100 µL volume of each extract was propelled directly into the wells (in triplicate) of the inoculated agar plates for each test organism. The

plates were allowed to stand for 1 hour at room temperature (40°C) for diffusion of the extract into agar and incubated at 37 °C for 24 hours. Sterile DMSO served as the negative control and ciplox (for bacteria), candid (for fungi) and Kan pip (antimicrobial) ear drops served as the positive controls. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone was greater than 8 mm. The experiments were performed in triplicate and the mean values of the diameter of inhibition zones and \pm standard deviations were calculated (Aneja and Sharma, 2010).

Determination of minimum inhibitory concentration (MIC)

MIC for each test organism was determined by the macrodilution broth method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100 mg/mL) in DMSO, followed by dilution in Mueller-Hinton broth (bacteria) and Malt yeast broth (yeast) to achieve a decreasing concentration range of 50 mg/mL to 0.39 mg/mL. Each dilution was seeded with 100 μ L of the standardized microbial inoculum (1.5×10^6 cfu/mL). The inoculated culture tubes were incubated at 37°C for 24 hours. A set of tubes containing only broth was kept as control. Afterwards, incubation tubes were examined for changes in turbidity as an indicator of growth. The lowest concentration that did not permit any visible growth was considered as MIC (Ncube et al. 2008; Das et al. 2010).

Determination of minimum bactericidal concentration (MBC)

MBC is the lowest concentration of antimicrobial agent that will not allow the growth of an organism after sub-culturing on antibiotic free media. MBC was determined by sub-culturing the preparations that did not show any bacterial growth in the MIC determination.

A 100 μ L aliquot from the selected tube (showing MIC) was spread over the Mueller-Hinton agar plate and incubated at 37°C for 24 hours and examined for bacterial growth. The MBC, lowest concentration of the plant extract giving 99.9% reduction of the bacterial growth of various plants parts against the bacterial pathogens, was recorded (Ncube et al. 2008).

Determination of minimum fungicidal concentration (MFC)

A loopful of culture from each set of tubes that did not show any visible growth of the yeast in MIC determination was subcultured on to fresh plates of MEA and incubated at 37°C for 24-48 hours. Minimum fungicidal concentration for each plant extracts against the tested yeast was recorded as the lowest concentration that did not yield any fungal growth on the solid medium (Mann, 2008; Doughari and Obidah, 2008).

RESULTS AND DISCUSSION

Antimicrobial activity (assessed in terms of inhibition zone) of *A. nilotica* leaves and bark extracts, tested against selected microorganisms were recorded in Table 1. The antimicrobial activity of *A. nilotica* leaves and bark extracts on the agar plates varied in different solvents. Positive controls produced significantly sized inhibition zones against the tested bacteria and the yeast. Negative control produced no observable inhibitory effect against any of the tested microorganism. In this study, all the organic solvent extracts of both leaves and bark possessed antimicrobial activity against all six tested ear pathogens while only aqueous leaves extracts exhibit activity against *S. aureus*, *P. mirabilis* and *Acinetobacter* sp.

Of the tested organic leaves extract of *A. nilotica*, acetonic extract was found most active against all the five tested bacterial pathogens with the zone of inhibition

Table 1: Antibacterial and antiyeast activity of *Acacia nilotica* leaves and bark extracts on ear pathogens

Solvent extracts (mg/ml)	Diameter of inhibition zones (mm)									
	Sa		Pm		Pa		Ec		As	
	Le	Be	Le	Be	Le	Be	Le	Be	Le	Be
Acetone	23.6 \pm 1.15 ^b	20.6 \pm 0.57	21.6 \pm 1.15	19.6 \pm 1.52	15.3 \pm 1.52	17.6 \pm 0.57	18.3 \pm 1.15	17.6 \pm 1.52	18.3 \pm 0.57	16.3 \pm 1.52
Ethanol	21.3 \pm 1.52	18.6 \pm 1.57	18.3 \pm 1.52	17.3 \pm 1.52	14.6 \pm 1.15	16.3 \pm 1.52	16.6 \pm 0.57	14.6 \pm 1.52	17.6 \pm 1.15	14.6 \pm 0.57
Methanol	22.6 \pm 1.15	19.3 \pm 1.52	20.3 \pm 0.57	18.6 \pm 1.52	15.3 \pm 1.15	16.3 \pm 1.15	17.6 \pm 1.15	15.3 \pm 0.57	18.6 \pm 1.52	15.6 \pm 1.52
Hot aq	19.3 \pm 1.52	-	16.3 \pm 1.15	-	-	-	-	-	17.3 \pm 0.57	-
Cold aq	17.3 \pm 1.52	-	14.6 \pm 1.15	-	-	-	-	-	15.6 \pm 1.52	-
Ciplox ED	56.3 \pm 0.57 ^b		46.3 \pm 1.52		34.0 \pm 1.0		36.3 \pm 0.57		32.6 \pm 0.57	
Kan pip ED	26.3 \pm 1.52		20.3 \pm 0.57		18.3 \pm 1.52		23.6 \pm 1.15		21.6 \pm 1.52	
Candid ED	nt		nt		nt		nt		nt	

Sa- *Staphylococcus aureus*, Pm- *Proteus mirabilis*, Pa- *Pseudomonas aeruginosa*, Ec- *Escherchia coli*, As - *Acinetobacter* sp., Ca- *Candida albicans* - No act extract, Be- Bark extract, nt – Not tested, ^a Values, including diameter of the well (8mm), are means of three replicates, ^b \pm Standard deviation, ED- ear drop

ranging between 23.6 mm and 15.3 mm (Fig. 1) followed by methanolic (ranging between 22.6 mm and 15.3 mm) and ethanolic (ranging between 21.3 mm and 14.6 mm) extract. The acetonic leaves extract was found most effective against *S. aureus* (23.6mm), followed by *P. mirabilis* (21.6mm), *E.coli* & *Acinetobacter* sp. (18.3 mm), *C. albicans* (17.6mm) and *P. aeruginosa* (15.3mm). Aqueous leaves extracts (hot and cold) displayed bioactivity against three pathogens only, *S. aureus*, *P. mirabilis* and *Acinetobacter* sp. with zone of inhibition ranging between 19.3mm and 14.6mm (Fig. 2).

However, in the case of bark, acetonic extract was again the best extract with zone of inhibition ranging between 20.6 mm and 16.3 mm followed by methanolic (19.3 mm and 15.3 mm) and ethanolic (18.6 mm and 14.3 mm) extract. Acetonic leaves and bark extract was found to be best against *S. aureus* (20.6mm). Aqueous bark extracts, both hot and cold totally lacked antibacterial activity. All the six organic extracts of leaves and bark possessed bioactivity against *C.albicans* that ranged between 17.6 mm and 14.6 mm. The acetonic extracts were found more active than the methanolic and ethanolic extracts. The aqueous extracts completely lacked antiyeast activity. Bark extracts were found slightly less inhibitory to the tested pathogens as compared to the leaves extracts. A perusal of the tables 1 reveals that antibacterial and antiyeast activity shown by organic leaves extract was almost equal to the Kan pip ear drop but lower than the ciplox ear drop.

The MIC and MBC value ranged between 6.25mg/ml and 50mg/ml for different bacterial ear pathogens and showed MFC value of 50mg/ml against *C.albicans*. The result revealed that the MBC and MFC values were either equal or twofold higher than the MIC values against the corresponding pathogens. Of all the leaves and bark extracts in different solvent tested, the acetonic extract was the best solvent where the lowest MIC of 6.25 mg/ml and 12.5 mg/ml was found against *S.aureus* and MBC value of 6.25 mg/ml and 25 mg/ml against *S.aureus*. Therefore, *S. aureus* was found the most susceptible bacterium and *Pseudomonas aeruginosa* the least susceptible bacterium with MIC and MBC value of 50mg/ml. In case of yeast, MIC value ranged between 25mg/ml and 50mg/ml and showed a common MFC value of 50mg/ml in all the organic solvents (Table 2 and 3).

DISCUSSION

Medicinal plant based antimicrobials represent a vast untapped source of pharmaceuticals and further exploration of plant antimicrobials need to occur for treatment of infectious diseases. The antimicrobial potential of this plant extracted in different solvents (e.g. petroleum ether, ethanol, benzene, chloroform, aqueous, ethyl acetate, methanol, ethanol and chloroform) has been evaluated against different bacterial

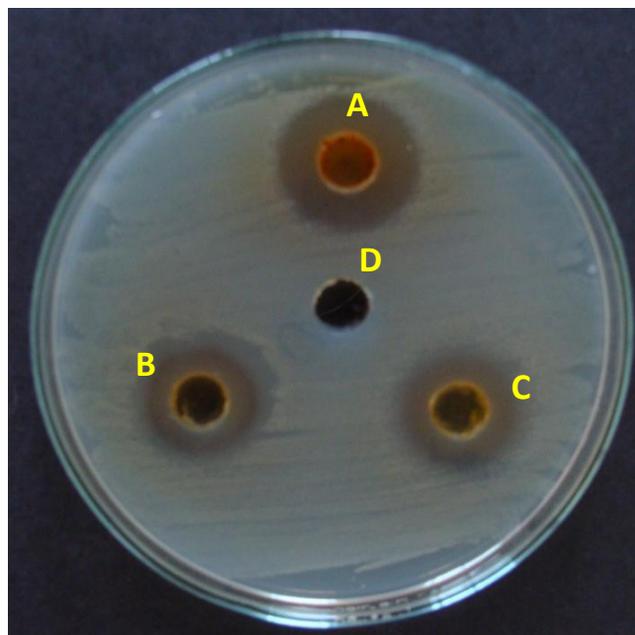


Figure 1 Zone of antibacterial inhibition of *A. nilotica* leaves organic extracts against *S. aureus* by acetonic (A), methanolic (B), ethanolic extract (C), and negative control DMSO (D)

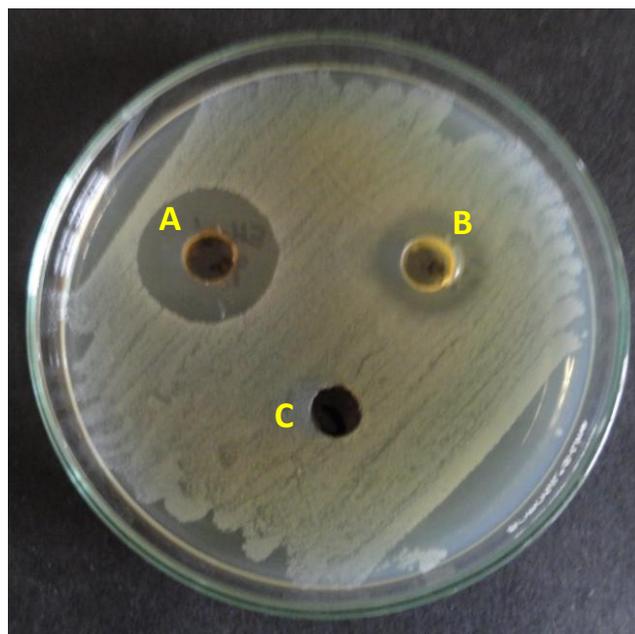


Figure 2 Zone of antibacterial inhibition of *A. nilotica* leaves aqueous extracts against *S. aureus* by hot aqueous (A), cold aqueous extract (B), and negative control DMSO (C)

and fungal human pathogens and variable activities of different plant parts including leaves, seeds, stem bark, roots in different solvents has been reported (Malviya et al. 2011; Choudhari, 2011; Deshpande, 2013)

Table 2 MIC of *Acacia nilotica* leaves and bark extracts against bacterial and candidal ear pathogens

Solvent extracts	MIC (mg/ml)											
	Sa		Pm		Pa		Ec		As		Ca	
	Le	Be	Le	Be	Le	Be	Le	Be	Le	Be	Le	Be
Acetone	6.25	12.5	12.5	25	50	50	25	25	25	50	25	25
Ethanol	12.5	25	25	25	50	50	50	50	25	50	50	50
Methanol	6.25	25	12.5	25	50	50	25	50	25	50	25	50
Hot aq	25	nt	50	nt	nt	nt	nt	nt	25	nt	nt	nt
Cold aq	25	nt	50	nt	nt	nt	nt	nt	50	nt	nt	nt

Sa- *Staphylococcus aureus*, Pm- *Proteus mirabilis*, Pa- *Pseudomonas aeruginosa*, Ec- *Escherchia coli*, As - *Acinetobacter sp.*, Ca- *Candida albicans*, nt = not tested, aq- aqueous , Le- Leaves extract, Be- Bark extract

2010).

In our study, the organic leaves and bark extracts of *Acacia nilotica* were found to be the most active in inhibiting the growth of all the five tested bacterial and fungal ear pathogens compared to aqueous extracts. Our work is supported by earlier studies on an alcoholic extract that exhibited greater activity than the aqueous extracts against bacteria (Phadke and Kulkarni, 1989; Ahmad et al. 1998). Among the tested bacterial ear pathogens, Gram-positive bacterial strains have been found to be more susceptible than Gram-negative bacterial strains. This may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas, the gram-negative cell wall is a multilayered structure bounded by an outer cell membrane (Yao and Moellering, 1995).

A majority of the described antimicrobial effects of *A. nilotica* extracts have been attributed to their secondary metabolites. In our study leaf extract showed better antimicrobial activity compare to bark extract might be due to the presence of saponins, saponin glycosides, volatile oil, hydrolysable tannin, triterpenoid, tannins, flavonoids, phenol and alkaloids. Leaves also contain apigenin, 6-8-bis-D-glucoside and rutin (Chaubal and Tambe, 2006; Wisdom and Shittu,

Of the three organic extracts of this plant screened, the acetonc extract has been found to be more active and have a better antibacterial activity than the corresponding ethanolic and methanolic extracts. Our results confirm the finding of Nair et al. 2005, Cowan, 1999 and Eloff (1998) who rated acetone as the best solvent. The results of present study support the valuable use of *A. nilotica* in traditional medicines for treatment of different infections.

Conclusion

From the present screening, it could be concluded that the leaves of *A. nilotica* is more potent antimicrobial agent than the bark and could be compared to the known herbal drugs. Further it is recommended that research should be carried out to investigate the bioactive component of this plant and *in vivo* studies to determine its toxicity and their pharmacokinetics properties.

Acknowledgements

We would like to thank Dr. BD. Vashishta, Department of Botany, Kurukshetra University, Kurukshetra, for rendering help in confirmation of the identification of the plant.

Table 3 Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of *Acacia nilotica* leaves and bark extracts

Solvent extracts	MBC (mg/ml)										MFC(mg/ml)	
	Sa		Pm		Pa		Ec		As		Ca	
	Le	Be	Le	Be	Le	Be	Le	Be	Le	Be	Le	Be
Acetone	6.25	25	25	25	50	50	25	50	25	50	50	50
Ethanol	12.5	50	25	50	50	50	50	50	50	50	50	50
Methanol	12.5	25	12.5	50	50	50	50	50	50	50	50	50
Hot aq	25	nt	50	nt	nt	nt	nt	nt	50	nt	nt	nt
Cold aq	50	nt	50	nt	nt	nt	nt	nt	50	nt	nt	nt

Sa- *Staphylococcus aureus*, Pm- *Proteus mirabilis*, Pa- *Pseudomonas aeruginosa*, Ec- *Escherchia coli*, As - *Acinetobacter sp.*, Ca- *Candida albicans*, nt = not tested, aq- aqueous , Le- Leaves extract, Be- Bark extract

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