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# Antimicrobial potential of the extracts of the twigs of *Azadirachta indica* (Neem): an *in vitro* study

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**Abstract**
**Objective:** Various medicinal plants have been used since ancient times in daily life to treat diseases all over the world. Almost every part of the *Azadirachta indica* A. Juss (syn. Melia azadirachta) tree commonly known as neem has been used for years to treat a number of human diseases. As neem is known to possess antimicrobial activity against a number of organisms, the present study was carried out to reveal the antibacterial effect on common oral cariogenic and periodontal pathogens and antifungal activity against *Candida albicans.*
**Methods:** The twigs of *Azadirachta indica* were collected and different solvents were used for the preparation of the extract namely petroleum ether, dichloromethane, ethyl acetate, methanol and aqueous solvent. The antibacterial potential was evaluated using the agar well diffusion assay. Sodium hypochlorite and dimethyl sulfoxide were used as positive and negative controls, respectively.
**Results:** Methanol extract of neem exhibited the highest antimicrobial activity at 500 mg/ml concentration, whereas the aqueous extract did not demonstrate any antibacterial and antifungal activities at any concentration tested.
**Conclusion:** Evidence of antimicrobial activities of neem twig extract against cariogenic and periodontal pathogens indicates the presence of bioactive compounds which need to be isolated and identified for the incorporation in the modern oral health care system.

**Keywords:** Antibacterial, Antimicrobial, *Azadirachta indica*, cariogenic, Neem, periodontal.

## 1. Introduction
Plants have been a source of herbal remedies throughout the history of mankind. Various medicinal plants have been used for years in daily life to treat diseases all over the world. [1, 2] According to reports of the World Health Organization, 80% of the world's population relies mainly on traditional therapies which involve the use of plant [3]. Medicinal plants are a rich source of novel drugs that form the ingredients in traditional systems of medicine, modern medicine, food supplements, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs [4]. In recent years, multiple drug resistance in both human and plant pathogens has developed due to indiscriminate use of synthetic drugs. Therefore, the search for novel bioactive compounds from medicinal plants has gained immense importance as the plant based drugs are biodegradable, safe and have fewer side effects [5, 6].
*Azadirachta indica* (*A. indica*) A. Juss (syn. Melia azadirachta) belonging to the Meliaceae family commonly known as neem is one of the most versatile medicinal plants having a wide spectrum of biological activity [7, 8, 9]. *A. indica* has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a part of modern medicine. *A. indica* contains a vast range of biologically active compounds that are chemically diverse and structurally complex. More than 140 compounds have been isolated from different parts of it [7, 10, 11]. The Chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids and ketones [12].
Almost every part of the tree including *A. indica* tree, leaves, flowers, seeds, fruits, roots and bark have been used traditionally to treat various diseases [7, 10, 11, 13]. *A. indica* known to have anti-inflammatory, antibacterial, antimalarial, antiulcer, antiparasitic, antifungal, antiprotozoal, and antiviral properties [7, 11, 13]. It is also been studied for antidiabetic, [14] antifertility [15] and

antioxidant activity [16, 17]. The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children [13]. Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial action against Gram negative and Gram positive microorganisms, including *M. Tuberculosis* and *Streptomycin* resistant strains [9]. Various antimicrobial activities of different parts of *A. indica* have been studied previously [18-21]. In this study, antimicrobial activities of *A. indica* have been studied against isolated cariogenic and periodontal pathogens.

## 2. Materials and methods

### 2.1 Preparation of plant extract

**a) Preparation of petroleum ether extract**

The twigs of *A. indica* were collected locally and authenticated by a botanist. It was washed with distilled water to remove dirt, cut into smaller pieces, and air dried for 21 days and pulverized to a coarse powder. 100 grams of the plant powder were soaked in 1000 ml of petroleum ether and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and the filtrate was concentrated to dryness at 45 $^0$C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

**b) Preparation of dichloromethane extract**

Residue obtained after extraction with petroleum ether was further soaked in dichloromethane and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45 $^0$C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

**c) Preparation of ethyl acetate extract**

Residue obtained after extraction with dichloromethane was further soaked in ethyl acetate and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45 $^0$C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

**d) Preparation of Methanol extract**

Residue obtained after extraction with ethyl acetate was further soaked in methanol and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45 $^0$C on a rotary evaporator. The extract used was refrigerated to be used as such for further studies. The residual precipitate was extracted further as described below.

**e) Preparation of Aqueous extract**

Residue obtained after extraction with methanol was further soaked in distilled water and stirred at room temperature for 24 hours. The mixture was filtered through Whatman filter paper and filtrate was concentrated to dryness at 45 $^0$C on a rotary evaporator. The extract prepared was refrigerated to be used as such for further studies.

### 2.2 Collection of test organisms

Test organisms were collected from carious cavities of affected teeth by scraping soft caries using excavators and from periodontal pockets using paper points. After collection the paper points were dropped into 20 ml of brain heart infusion broth (BHI broth) which was used as transport media.

The inoculated Sheep blood agar plates were incubated at 37 $^0$C for 48 hrs aerobically and anaerobically (media streaked in duplicate – one for aerobic and the other for anaerobic culture). The different types of colonies were picked up, isolated and subcultured onto sheep blood agar plates for further identification. Colonies of different test organisms were identified by colony morphology, gram staining, catalase test, pigment production, aerotolerance and sugar fermentation tests. The organisms isolated from the samples included *Streptococcus mutans* (*S. mutans*), *Streptococcus salivarius* (*S. salivarius*), *Streptococcus mitis* (*S. mitis*), *Lactobacillus* species, *Prevotella intermedia* (*P. intermedia*), and *Candida albicans* (*C. albicans*). These organisms were preserved for studies by repeated subculturing on blood agar slants and maintained in a deep freezer at -80 $^0$C. For antibacterial activity studies, fresh subcultures were done in BHI broth and used as inocula.

### 2.3 Determination of antimicrobial activity

The different extracts of *A. indica* were dissolved in dimethyl sulfoxide (DMSO), in the concentration of 5G/10 ml and then filtered using whatman filter paper no.1. The dissolved extract was then diluted using DMSO to obtain a concentration of 500 mg/ml, 250 mg/ml and 125 mg/ml.

The susceptibility of the test bacteria to plant extracts was determined using an agar well diffusion assay on 5% sheep blood agar plates. Fresh 24 hour old broth cultures of bacteria was adjusted to 0.5 McFarland turbidity (1-2 x $10^6$CFU mL) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 min following which 5 wells (6 mm holes) were cut into the agar using sterile steel borer.

Individual wells were filled with plant extracts (50 µL). Three wells were filled with the different concentrations of extract 500 mg/ml, 250 mg/ml and 125 mg/ml. 2.5% Sodium hypochlorite and DMSO were pipetted into the other two wells and used as positive and negative/solvent control respectively. The plates were incubated at 37 ºC for 48 hours period. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was measured and recorded. Each concentration included triplicates and the results are average of three independent experiments.

### 2.4 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) of the different extracts of *A. indica* was determined by broth dilution methods. Sterile Brain Heart Infusion broth, 1 ml was taken in test tubes to which 10 microlitres of the fresh bacterial inoculums were added. Then the extract was added in the concentrations of 125, 60.5, 30.25, 15.13, 7.56 mg/ml to each tube. Tube containing only bacterial inoculums served as growth control and sterile BHI broth served as negative control. The tubes were incubated at 37 degree centigrade for 24 hours. The tubes were checked for turbidity and the lowest dilution showing turbidity was taken as MIC. Subcultures were done on Blood agar from each of the tubes and the plates incubated for 24 hours at 37 $^0$C. The lowest dilution that did not grow any colony was taken as MBC.

## 3. Results

All test strains of bacteria and *Candida albicans* were found to be sensitive to sodium hypochlorite which acted as positive control. DMSO was used as the negative control which did not show any zone of inhibition against test organisms. From the result obtained, it has been observed that *A. indica* have varying inhibition potential on the test organisms. All the 5 different extracts of *A. indica* at a concentration of 125 mg/ml did not show zone of inhibition against any of tested bacteria and fungi. The methanolic extract of neem at 250 mg/ml concentration showed an inhibition zone of 10 mm for Lactobacilli. The neem at 250 mg/ml concentration also showed an inhibition zone of 12 mm for Prevotella species in methanol extract. Different extracts of *A. indica* did not show any inhibitory activity against *S. mutans*, *S. salivarius*, *S. mitis*, and *C. albicans* at 250 mg/ml concentration. The results of antimicrobial activity of *A. indica* at 500 mg/ml concentration are shown in table-1.

**Table 1:** Showing the zone of inhibition in mm for *Azadirachta indica* extract at 500 mg/ml concentration

| Extracts of *Azadirachta indica* | *S. mutans* | *S. mitis* | *S. salivarius* | *Lactobacillus* species | *P. intermedia* | *C. albicans* |
|---|---|---|---|---|---|---|
| **Petroleum ether** | 12 | 10 | 8 | 14 | 14 | 8 |
| **Dichloro methane** | 6 | 6 | 6 | 6 | 10 | 6 |
| **Ethyl acetate** | 10 | 10 | 6 | 10 | 12 | 6 |
| **Methanol** | 20 | 16 | 10 | 22 | 20 | 10 |
| **Aqueous** | 6 | 6 | 6 | 6 | 6 | 6 |
| **Positive control (Sodium hypochlorite)** | 16 | 18 | 12 | 20 | 16 | 10 |

The petroleum ether and methanol extracts of *A. indica* showed significant antimicrobial activity against all the test organisms. The bacteria *S. mutans*, *S. mitis*, Lactobacilli and *P. intermedia* were found to be sensitive against the ethyl acetate extract of *A. indica*. The dichloromethane extract significant zone of inhibition only against *P. intermedia*. The aqueous extracts of *A. indica* did not show any significant antimicrobial activity.

MIC was carried out for methanolic extract of *A. indica* as maximum inhibitory effect was exhibited by this extract. The results are shown in the table-2.

**Table 2:** Showing MIC and MBC value of methanolic extract of *Azadirachta indica*.

| *Azadirachta indica* | MIC (mg/ml) | MBC (mg/ml) |
|---|---|---|
| *S. mutans* | 60.5 | 125 |
| *S. mitis* | 60.5 | 125 |
| *Lactobacillus* species | 60.5 | 125 |
| *P. intermedia* | 60.5 | 125 |
| *S. salivarius* | >125 | >125 |
| *Candida albicans* | >125 | >125 |

All the concentrations tested showed growth in MIC tubes of *S. mutans*, *S. salivarius* and *C. albicans*. Hence the MIC and MBC values for these organisms was more than the highest concentration tested (125 mg / ml).

## 4. Discussion

Dental caries and periodontal diseases are two most common oral health problems. Dental caries is caused by acidogenic and aciduric Gram-positive bacteria, primarily the streptococci mutans, lactobacilli and actinomycetes [22]. The periodontal diseases have been linked to anaerobic Gram-negative bacteria such as Porphyromonas gingivalis, Actinobacillus species, Prevotella species and Fusobacterium species [23]. *Candida albicans* is the most important causative organism of oral candidiasis which is a common oral fungal infection [24]. Since various parts of *A. indica* are known to possess antimicrobial properties, we tried to explore the antibacterial and antifungal activities of neem twig against selected oral cariogenic and periodontal pathogens.

Variable results have been observed in different studies of antimicrobial activities of neem against bacterial and fungal organisms. Aqueous extract of *A. indica* did not show any significant activity against the isolates obtained from the oral cavity namely *Staphylococcus auricularis*, *Micrococcus* species, *Acinetobacter lwoffii* and *C. albicans.* [25] Aqueous extract was found to be less effective in antimicrobial activity in comparison to ethanol extract in a study conducted on bark and leave extracts of neem [26]. In a study which evaluated the antimicrobial activity of leaf extracts of *A. indica*, ethanolic and dichloromethane extracts were found to be more effective among the different extracts used [27]. In our study, methanol extract was found to be most effective followed by petroleum ether and ethyl acetate. Extract of dichloromethane exhibited effective zone of inhibition only against prevotella species and aqueous extract failed to demonstrate effective zone of inhibition against any tested organisms. Similar findings have been observed in other study, where methanol extract of *A. indica* leaves was reported to have highest antibacterial activity compared to chloroform extract which exhibited moderate to good antibacterial activity [21]. Thus, inhibitory activities of plant extracts may be both organism and solvent dependent as it has been observed in other study [27].

Different parts of *A. indica* have been reported to exhibit varying degrees of antimicrobial activities against bacterial and fungal species. When antibacterial activities of leaf and bark of *A. indica* were compared, bark was found to be more effective antibacterial agent against tested organisms [27]. In a study, aqueous extracts of *A. indica* leaf was found to exhibit strongest antimicrobial activity compared with the bark and seed of *A. indica.* The authors have attributed this difference in antimicrobial activity to variation in the distribution of phytochemical compounds in different parts of *A. indica*. [28] The strong antimicrobial properties of *A. indica* leaves have been related to the presence of high concentrations of azadirachtins, quercetin and ß- sitosterol in it [11]. In this study, the antimicrobial properties of twigs of *A. indica* were studied. Similar studies involving the different parts of *A. indica* can highlight the antimicrobial properties of this medicinal plant against common oral cariogenic and periodontal pathogens.

It has been demonstrated that *A. indica* stick extract can inhibit the growth of *S. mutans*, *S. mitis*, *S. salivarius* and S. sanguis which are important in the development of dental caries [29]. The ability of some streptococci to colonize the tooth surface

may be declined by *A. indica* stick extract. [30] In the present study, for methanolic extract, the highest zone of inhibition was observed in *Lactobacillus* species (22 mm), followed by prevotella and streptococcus mutan (20 mm) at the concentration of 500 mg/ml. *Streptococcus mitis* showed a zone of inhibition of 16 mm followed by *streptococcus salivarius* and *Candida albicans* which exhibited similar zone of inhibition (10 mm).

High antimycotic activity with extracts of different parts of neem has been reported [31]. The extracts of neem leaf, neem oil, and seed kernels were found to be effective against certain fungal organisms including Trichosporon, Microsporum, Trichophyton, Epidermophyton, Geotricum and Candida [32]. In our study, extracts of methanol and petroleum ether exhibited zone of inhibition (10 mm and 8 mm respectively) at concentration of 500 mg/ml. The leaf, bark and seed extracts of *A. indica* have shown antifungal activity at higher concentration in one of the study [28].

The MIC and MBC value for *S. mutans*, *S. mitis*, *Lactobacillus* species and *P. intermedia* was found to be 60.5 mg/ml and 125 mg/ml respectively. In one of the study conducted on the antimicrobial effect of *A. indica* leaf extract, the MIC value of both the aqueous and alcoholic extract for *Streptococcus mutans* was found to be 7.5% [33]. This variation can be attributed to the use of standard strains and also the difference in solvent and the part of *A. indica* used.

As twigs of *A. indica* is used in the oral hygiene practices and it is found to have antibacterial activity against the common oral cariogenic and periodontal pathogens, further studies should be carried out to isolate and purify bioactive compounds responsible for these antimicrobial activities.

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