

**IN VITRO: EVALUATION OF ANTIBACTERIAL ACTIVITY OF  
AZADIRACHTA INDICA A. JUSS. FRUIT EXTRACTS AGAINST  
GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA**

**Manoj Kumar\***

Department of Botany, Govt. College, Kosli- 123302, Haryana, India.

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**\*Corresponding Author**

**Manoj Kumar**

Department of Botany, Govt.  
College, Kosli- 123302,  
Haryana, India.

**ABSTRACT**

Antibacterial activity of *Azadirachta indica* A. Juss. fruit extracts in different solvents (methanol, ethanol and aqueous) was carried out against seven different bacterial strains, including four gram-positive (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus* and *Streptococcus* sp.) and three gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacterial strains by using the agar well diffusion assay (AWDA) method. The obtained results indicated that the methanol extract gave the highest yield percentage as compared to aqueous as well as ethanol extracts.

The patterns of inhibition varied with the solvent as well as the tested bacterial strains. The results showed that *B. subtilis* among the gram-positive and *E. coli* among the gram-negative were highly susceptible as compared to other tested bacterial strains. It has been showed that the methanol extract had widest range of inhibitory activity as compared to the ethanol and aqueous extracts. The minimum inhibitory concentrations (MIC) as well as minimum bactericidal concentration (MBC) of the extracts were determined for the various tested bacterial strains which ranged between 12.5 to 100 mg/ml. The remarkable antibacterial activity of the fruit extracts against tested gram-positive and gram-negative bacteria suggests that the fruit extracts of *A. indica* plant could be a possible source of novel broad spectrum drug for the treatment of various infectious diseases.

**KEYWORDS:** Antibacterial activity; *Azadirachta indica*; Solvent extracts; Zone of Inhibition.

## INTRODUCTION

Antibiotic resistance has become a serious and widespread problem in developing countries, both in hospitals and the community, causing high mortality in every year.<sup>[1]</sup> The development of antibiotic resistant bacteria stems from a number of factors which include the prevalent and sometimes inappropriate use of antibiotics, extensive use of these agents as growth enhancers in animal feed, and increased trans boundary passage of antibiotic-resistant bacteria.<sup>[2-4]</sup> The problem of antibiotic resistance in humans and animals will continue for a long time.<sup>[5]</sup> Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased. Antibiotic resistance results in reduced efficacy of antibacterial drugs, making the treatment of patients difficult, costly, or even impossible. The impact on particularly vulnerable patients is most obvious, resulting in prolonged illness and increased mortality.<sup>[6]</sup> Such a fact is cause for concern, because of the number of patients in hospitals who have suppressed immunity, and due to new bacterial strains, which are multi-drug-resistant. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. Consequently, new infections can occur in hospitals resulting in high mortality and the problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain.<sup>[7]</sup> Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, to develop the new drugs, either synthetic or natural.<sup>[8]</sup> The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient. For a long period of time, plants have been a valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. Plants have continued to be a valuable source of natural products for maintaining human health.<sup>[9-10]</sup> According to World Health Organization (WHO) medicinal plants would be the best source to obtain a variety of drugs. WHO estimated that 80% of the populations rely on traditional medicines, mostly plant drugs, for their primary health care needs in developing countries.<sup>[11]</sup> Globally, about 85% of the traditional medicines used for primary healthcare are derived from the plants. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plants is found in “Rigveda”, which is said to have been written between 4500-1600 B.C. and is supposed to be the oldest repository of human knowledge.<sup>[12-13]</sup> Plants have an amazing ability to produce a wide variety of secondary metabolites, acts as antimicrobial agents.<sup>[4,8,14]</sup>

*Azadirachta indica* A. Juss. belongs to the family Meliaceae, commonly known as neem. Neem tree has adaptability to a wide range of climatic, topographic and edaphic factors. It thrives well in dry, stony shallow soils and even on soils having hard calcareous or clay pan, at a shallow depth. Neem tree requires little water and plenty of sunlight.<sup>[15]</sup> Neem is used in traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries.<sup>[16]</sup> Neem is the most versatile, multifarious trees of tropics, with immense potential. It possesses maximum useful non-wood products (leaves, bark, flowers, fruits, seed, gum, oil and neem cake) than any other tree species. Various parts of the neem tree have been used as traditional Ayurvedic medicine in India. Seed oil, bark and leaf extracts have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, and constipation and also as a general health promoter. Seed oil of neem plant finds use to control various skin infections. Bark, leaf, root, flower and fruit together cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and phthisis.<sup>[15]</sup>

Different parts of *A. indica* (leaf, bark and seed) have been shown to exhibit wide pharmacological activities including; antioxidant, antimalarial, antimutagenic, anticarcinogenic, antiinflammatory, antihyperglycaemic, antiulcer and antidiabetic properties. The biological activities are attributed to the presence of many bioactive compounds in different parts.<sup>[17-18]</sup>



**Figure. 1: Photo of *Azadirachta indica* A. Juss. Plant.**

Hence, the present study was initiated to evaluate the antibacterial activity of methanol, ethanol and aqueous fruit extracts of *A. indica* against seven different bacterial strains, comprising four gram-positive and three gram-negative bacterial strains.

## MATERIALS AND METHODS

### Sources of bacterial strains

Bacterial strains, including both gram-positive and gram-negative obtained from M.D. University, Rohtak, Haryana and Microbial Type Culture Collection and Gene Bank Institute of Microbial Technology, Chandigarh. The bacterial strains include *Bacillus subtilis*, *Micrococcus luteus* (MTCC106), *Staphylococcus aureus* (MTCC6908), *Streptococcus* sp. (MTCC9724), *Escherichia coli* DH5 $\alpha$ , *Pseudomonas aeruginosa* (MTCC4673) and *Salmonella typhimurium* (MTCC3224) have been selected for the present study.

### Culture of bacterial strains

The bacterial strains were propagated in the nutrient broth medium (5g/l peptone, 3g/l beef extract, 5g/l NaCl and pH 7.0) incubated for 18hr at 37°C temperature. Slants were prepared from the separated colonies of bacteria, stored at 4°C temperature and sub-cultured in a nutrient broth medium before testing the antibacterial activity. The chemicals were purchased from Hi-media, Mumbai, India.

### Preparation of plant material

The collected fresh fruits were thoroughly washed under tap water, dried in the shade for one month and then ground into coarse powdered with the help of mortar and pestle. These powders were stored in airtight brown bottles at 4°C until needed for future use.

### Extraction of plant material (Maceration)

The shade dried 100 gm coarse powdered of fruits of *A. indica* plant was immersed in 200 ml of different solvents (methanol, ethanol and aqueous) contained in 500 ml sterile conical flasks and covered with cotton wool separately. It was placed aside with intermittent shaking for one week. They were first filtered with double layered muslin cloth and then through Whatman No. 1 filter paper, and the marc was discarded. The filtrate was subjected to evaporation by treating at 40°C in an oven to obtain a dried extract. The dried extract was stored at 4°C until used for further study.<sup>[19-20]</sup>

**Yield percentage of solvents extracts**

After the drying, yield of each extraction was measured separately and the extraction efficiency was quantified by determining the weight each of the extracts and the yield percentage was calculated as dry weight/dry material weight  $\times 100$ .<sup>[21]</sup>

**Antibacterial activity by agar well diffusion assay (AWDA) method**

The antibacterial activity of crude solvents (methanol, ethanol and aqueous) fruit extracts of *A. indica* against gram-positive as well as gram-negative bacterial strains were evaluated by agar well diffusion assay (AWDA) method.<sup>[21-22]</sup> The diameters of the inhibition zones were measured in millimeters (mm). For this, a well (6 mm diameter) was made with the help of a borer in cooled nutrient agar plate, overlaid with soft agar (5 ml), seeded with a target strain ( $\sim 1.0 \times 10^6$  cfu/ml). Aliquots of the test compound (100  $\mu$ l) were introduced into the well and the plates were incubated for overnight at 37°C. For each bacterial strain, the dissolving solvent 10% DMSO and streptomycin (50  $\mu$ g/ml) were used as negative and positive controls respectively. To test the antibacterial activity of all extracts were dissolved in 10% DMSO solvent to make a final concentration 200 mg/ml.

**Determination of minimum inhibitory concentration (MIC)**

The MIC is the concentration giving the least inhibitory activity and below which there is no further inhibition were determined by using the Broth dilution method.<sup>[23]</sup> Briefly, 1.0 ml of the reconstituted extract solution at a concentration of 200 mg/ml was added to another test tube containing 1.0 ml of sterile broth so as to obtain a concentration of 100 mg/ml. 1.0 ml of this dilution was transferred to another test tube till the 7<sup>th</sup> test tube was reached. The 8<sup>th</sup> test tube did not contain any extract, but a solution of pure solvent and served as negative control. Then 1.0 ml of 18 hr grown cultures of each of bacterial strains, adjusted at  $\sim 1.0 \times 10^6$  cfu/ml was put into each tube and thoroughly mixed by vortex mixer. The tubes were incubated at 37°C for 18 hr and observed the growth in the form of turbidity. The test tube with the lowest dilution with no detectable growth by visual inspection considered the MIC's value.

**Determination of minimum bactericidal concentration (MBC)**

The MBC values were determined by removing 100  $\mu$ l of bacterial suspension from the MIC positive tube as well as one above and one below the same tube, spread on nutrient agar plates and incubated at 37°C for 18 hr. After incubation, the plates were examined for colony growth and MBC's were recorded.<sup>[24-25]</sup>



### Statistical analysis

The experiments were carried out in three independent sets, each consisting of three replicates. Values shown here represent mean  $\pm$  standard error of the mean (SEM).

### RESULTS

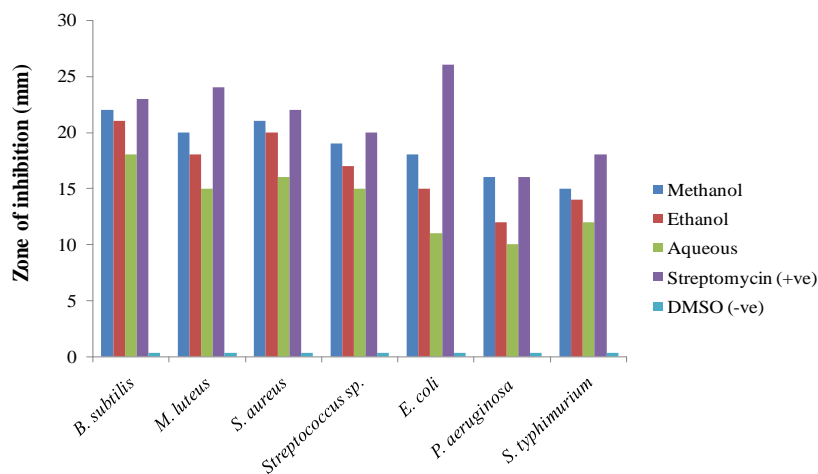
After complete drying, the yield percentage (gms) of *A. indica*. plant fruit extracts with the various solvents (methanol, ethanol and aqueous) was measured separately and quantified the efficiency of extraction. The results of the present study, methanol extraction gave the highest yield percentage (10.25%) followed by aqueous (9.43%) and ethanol (8.68%) are illustrated in Table 1.

**Table. 1: Yield percentage of *A. indica*. plant fruit extracts in different solvents.**

Solvent	Yield percentage of extracts (gms)		
	Weight of dry powder	Weight of dry extracts	Yield percentage
Methanol	100	10.25	10.25
Ethanol	100	08.68	08.68
Aqueous	100	09.43	09.43

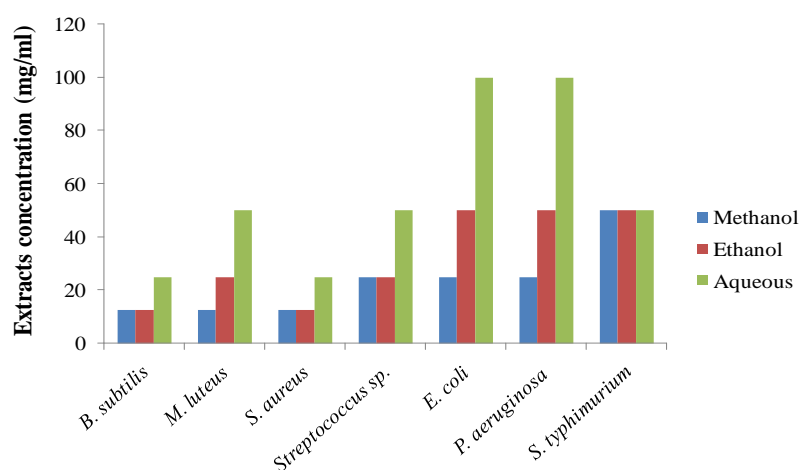
The obtained results showed that the tested different bacterial strains responded differently to the different solvents extracts (methanol, ethanol and aqueous). However, different solvents extracts exhibited the inhibitory activity against all the tested seven bacterial strains, consisting both the gram-positive as well as gram-negative as shown in Figure 1. The maximum zone of inhibition was recorded for the methanol extract against *B. subtilis* (22), *M. luteus* (20), *S. aureus* (21), *Streptococcus* sp. (19), *E. coli* (18), *P. aeruginosa* (16) and *S. typhimurium* (15). The ethanol extract showed against *B. subtilis* (21), *M. luteus* (18), *S. aureus* (20), *Streptococcus* sp. (17), *E. coli* (15), *P. aeruginosa* (12) and *S. typhimurium* (14). Similarly, aqueous extract produced inhibitory zone towards *B. subtilis* (18), *M. luteus* (15), *S. aureus* (16), *Streptococcus* sp. (15), *E. coli* (11), *P. aeruginosa* (10) and *S. typhimurium* (12).

However, streptomycin used as a positive control exhibited higher inhibition as compared to the used different solvent extracts with the zones against *B. subtilis* (23), *M. luteus* (24), *S. aureus* (22), *Streptococcus* sp. (20), *E. coli* (26), *P. aeruginosa* (16), and *S. typhimurium* (18), while DMSO doesn't shows any activity.



**Figure. 2: Antibacterial activity of *A. indica* fruit extracts.**

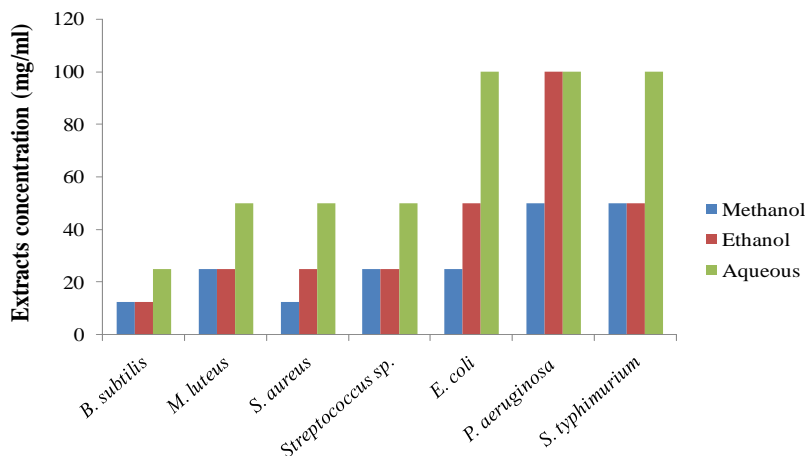
The minimum inhibitory concentration (MIC) for the methanol, ethanol and aqueous fruit extracts are shown in Figure 2. In the present study, methanol extract exhibited 12.5mg/ml against *B. subtilis*, *M. luteus* and *S. aureus*; 25mg/ml against *Streptococcus sp.*, *E. coli* and *P. aeruginosa*; 50mg/ml against *S. typhimurium*. Samples of ethanol extract possessed 12.5mg/ml against *B. subtilis* and *S. aureus*; 25mg/ml against *M. luteus* and *Streptococcus sp.*; 50mg/ml against *E. coli*, *P. aeruginosa* and *S. typhimurium*. Similarly, aqueous extract showed 25mg/ml against *B. subtilis* and *S. aureus*; 50mg/ml against *M. luteus*, *Streptococcus sp.* and *S. typhimurium*; 100mg/ml against *E. coli* and *P. aeruginosa*.



**Figure. 2: MIC (mg/ml) values of *A. indica* fruit extracts.**

The results of MBC values of methanol, ethanol and aqueous fruit extracts are shown in Figure 3. Methanol extract exhibited 12.5mg/ml against *B. subtilis* and *S. aureus*; 25mg/ml against *M. luteus*, *Streptococcus sp.* and *E. coli*; 50mg/ml against *P. aeruginosa* and *S.*

*typhimurium*. Ethanol sample possessed 12.5mg/ml against *B. subtilis*; 25mg/ml against *M. luteus*, *S. aureus* and *Streptococcus* sp.; 50mg/ml against *E. coli* and *S. typhimurium*; 100mg/ml against *P. aeruginosa*. Similarly, aqueous extract exhibited 25mg/ml against *B. subtilis*; 50mg/ml against *M. luteus*, *S. aureus* and *Streptococcus* sp.; 100mg/ml against *E. coli*, *S. typhimurium* and *P. aeruginosa*.



**Figure. 3: MBC (mg/ml) values of *A. indica* fruit extracts.**

## DISCUSSION

Herbal medicines are a valuable and readily available resources for primary health care as well as complementary health care system, undoubtedly the plant kingdom still holds numerous plants, possessing the substances of medicinal value that have yet to be discovered, though large number of plants are constantly being screened for their antimicrobial effect, these plant may prove to be a rich source of compounds with possible antimicrobial activities.<sup>[26]</sup> In the recent years there is resurgence of scientific interest to use the medicinal plants for newer pharmaco-therapeutics. Thus the need of cost effective and safe phytochemicals with therapeutic potential is urged as alternative sources that can control the microbial infections.<sup>[27]</sup>

Many studies have been undertaken with the aim of determining the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections as possible alternatives to the synthetic chemical drugs to which many infectious microorganisms have become resistant.<sup>[28-30]</sup> The ethnomedicinal value of *A. indica* has been reported by many literatures, but there is little scientific proof for further using this plant commercially or in a more effective form. For this, the yield of extraction was calculated because the crude plant extracts are generally a mixture of active and non-active



compounds. A number of medicinal plants described in Ayurveda still need to be testified, according to the modern parameters to ensure their activity and efficacy. Drugs used in Ayurveda are mostly prepared by extraction with water. Therefore healers may not be able to extract all the active compound(s).<sup>[31]</sup>

The yield percentage of medicinal plant extracts which contain the bioactive metabolites vary considerably with plant species and the method or solvent used for extraction. Also, factors like age of the plant and the polarity of the solvent used may have affected the yield percentage.<sup>[20,32]</sup> In the present study, methanol solvent extract gave the highest yield of extraction followed by aqueous and ethanol, and the used various solvent extracts exhibited inhibitory activity against all the tested seven different bacterial strains comprising both gram-positive as well as gram-negative with varying degrees. Among the different solvents used, methanol extract was found to be most effective in comparison to other solvents. Similar findings have been observed in other study, where methanol extract of *A. indica* was reported to have highest antibacterial activity compared to other extract which exhibited moderate to good antibacterial activity.<sup>[33]</sup> Abalaka, et al.<sup>[34]</sup> reported that the organism's *P. aeruginosa* showed the highest susceptibility followed by *S. aureus*, *Klebsiella ozanae* and *E. coli* of on treating with the *A. indica* plant solvent extract. However, variable results have been observed in different studies, aqueous extract of *A. indica* did not show any significant activity against the isolates obtained from the oral cavity namely *S. auricularis*, *Micrococcus* species, *Acinetobacter lwoffii* and *Candida albicans*.<sup>[35]</sup> Rathod, et al.<sup>[36]</sup> reported that the aqueous extract proved less effective in antimicrobial activity as compared to ethanol extract. In one of the study ethanol extract were produced more antimicrobial activity among the different extracts of *A. indica* and reported the inhibitory activities of extracts may be dependent on both the tested organism as well as the solvent used for the extraction.<sup>[37]</sup> The differences in the antimicrobial activity produced by the extracts can be due to the variation in the distribution of various phytochemical compounds in the different parts of *A. indica* plant.<sup>[38]</sup>

In the present study, samples of methanol, ethanol and aqueous extracts produced the MIC as well as MBC values a range between 12.5 to 100 mg/ml against the tested different bacterial strains. However, in one of the study, *A. indica* extracts produced the MIC and MBC 5 mg/ml and 50 mg/ml respectively for *P. aeruginosa*, *Kl. ozanae*, *S. aureus* and *E. coli*.<sup>[34]</sup> The antibacterial effect of methanol and aqueous extracts of the stem bark of *A. indica* was

determined by using MIC, MBC and Kill-time of extracts as indices against clinical bacterial isolates such as *E. coli*, *Salmonella* spp and *S. aureus* were used as the test organisms.<sup>[39]</sup>

## CONCLUSION

Evaluation of the medicinal plants is not only to find out the scientific rationale for their usage, but also to contribute in the global scientific efforts toward exploring new antibiotics and antimicrobial drugs to eradicate the growing phenomenon of multi-drug resistant microorganisms. In the present investigation, *A. indica* plant fruit extracts with various solvents possesses significant inhibitory activity against tested gram-positive (*B. subtilis*, *M. luteus*, *S. aureus* and *Streptococcus* sp.) as well as gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*) and the results in agreement to a certain degree with the traditional uses of this plant. The methanol and ethanol extracts possessed strong antibacterial activity as compared to the aqueous extract which showed the compounds extracted in the alcoholic solutions were more effective than aqueous extraction. The present study indicated that the *A. indica* fruit extracts are effective antibacterial agents that can be used in the folk medicine and will be a good source to treat and control various diseases. Newer antimicrobials from the plant fruit extracts could also be of commercial interest to pharmaceutical companies and research institutes in designing and developing new drugs.

## COMPETING INTERESTS

The author declared that he has no competing interests.

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