



# Microbes and Infectious Diseases

Journal homepage: <https://mid.journals.ekb.eg/>

## Original article

### Evaluation of *in vitro* antimicrobial effects of Azadirachta indica (Neem) leaves extracts against selected pathogens

Waleed Omer Fasher Bory<sup>1</sup>, Mohammed Aldai Hammad<sup>2</sup>, Alkhair Abd Almahmoud Idris<sup>\*3</sup>

1- Sudan International University-Sudan.

2- Dar ALaloum College-Sudan.

3- Ahfad University for Women, Sudan.

#### ARTICLE INFO

##### Article history:

Received 6 December 2021

Received in revised form 20 December 2021

Accepted 22 December 2021

##### Keywords:

Azadirachta indica

Neem extracts

*Staphylococcus aureus*

Methicillin resistant *Staphylococcus aureus*

##### List of Abbreviations

ATCC: American Type Culture Collection

MDR: Multi-drug resistant

MHA: Mueller Hinton agar

MRSA: Methicillin resistant

*Staphylococcus aureus*

WHO: World Health Organization

#### ABSTRACT

**Background:** Azadirachta indica has been used against a few disease in traditional medicine and have long history of use, and their use is worldwide in both developing and devolved countries, also have multiple health benefits. **Objectives:** This study aimed to evaluate the antibacterial activity of Neem leaves extract against standard bacterial strain and only one drug resistant strain of important human pathogenic bacteria by using *in vitro* methods. **Methods:** This study aimed to evaluate the antimicrobial effects of Azadirachta indica (Neem) leaves extracts against selected pathogens. The present study was carried out to screen and evaluate antimicrobial activity of leaves of Azadirhnta indica (Neem)methanol and petroleum ether extracts. Azadirchta indica extracts were tested against standard strains of *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *K. pneumonia* ATCC 700603, and *S. typhimurium* ATCC 14028. The effeminacy of the extracts was studied and determined by applying different concentration of extracts on the selected bacterial strains and synthetic antimicrobial discs were used as positive control by using disc diffusion method. **Results:** With the statistical test analysis the comparison done showed that, no significant different between the two extracts used, and *Staphylococcus aureus* and *Methicillin resistant staphylococcus aureus* were the only bacteria susceptibly affected by this Neem extracts used. **Conclusions:** The result obtained indicate the leaves extracts of Neem plant have enormous antimicrobial effect, and may be exploited for the treatment of various infectious diseases.

#### Introduction

Antibiotics provide the basis for the fungal and bacterial infections therapy. The discovery of antibiotics and making use of them as chemotherapeutic agents has made the medical fraternity to believe that they will eradicate various infectious diseases. However, indiscriminate use of antibiotics in human and veterinary healthcare systems has lead to the emergence of multi-drug resistant (MDR) strains of different groups of

microorganisms [1,2]. The emergence and dissemination of MDR bacteria has made chemically synthesized antibiotics ineffective for the treatment of infectious diseases caused by such bacteria [3]. These circumstances have propelled the researchers and scientists to explore new antimicrobial substances from various sources such as medicinal plants [4]. There are many studies that have described different type of plants such as herbs, shrubs and trees with the aim of knowing their

DOI: 10.21608/MID.2021.109886.1213

\* Corresponding author: Alkhair Abd Almahmoud Idris

E-mail address: alkhair20@hotmail.com

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phytoconstituents and using them for the treatment of various diseases as possible alternatives to the synthetic drugs [5]. The screening of plants for medicinal purposes represents a serious effort to discover newer, safer, and possibly more effective drugs with the potential of fighting pathogenic bacteria and fungi [6]. The green medicines are widely believed as safe and dependable in contrast with expensive synthetic drugs that have undesirable side effects along with beneficial effects [7]. The plants have been in use in traditional medicine worldwide since long time but are still understudied, particularly in clinical microbiology [8]. In past few decades, the curiosity to evaluate plants possessing antimicrobial, anti-inflammatory activity for various diseases has grown many folds and a large number of biologically active compounds has been characterized [9].

*Azadirachta indica* (*A. indica*), (Neem, Indian Lilac or margoes tree) is a native tree of India, is widely grown and cultivated throughout the country especially in semi-arid condition. It is a tropical evergreen plant with a wide adaptability. Earliest report on the isolation of first Neem component, nimben, was obtained in 1942 [10] and since then extensive research has been carried out to study the various product of Neem tree [11]. Presently more than 135 compound have been isolated from different parts of Neem, which have tremendous biological activity as anti-arthritis, antipyretic, hypoglycemic, spermicidal, antifungal, antibacterial, diuretic, anti-malaria, anti-tumor, anti-inflammatory, immune-modulatory...etc [12]. Many studies have been carried out so far on the medicinal properties of *A. indica*. The inhibitory effect of the different phyto-constituents of Neem have been reported to exhibit antibacterial [13, 14] as well as antifungal activities [15, 16]. This study aimed to find out the potentiality of crude organic extracts of leaves of Neem (*A. indica*) to inhibit bacterial activities.

### Materials and Methods

This was laboratory In vitro based study conducted to evaluate the antimicrobial effects of *Azadirachta indica* (Neem) leaves extracts against selected pathogens.

### Preparation of extracts

Fresh leaves of *A. indica* were collected from Omdurman Hai El-Omda area. Leaves were washed gently under tap water and left to dry under shady place for 5 days and grinded. And then grinded leaves were prepared in two solvents which are

methanol and petroleum ether. For methanol extract a weight of 50 g of powdered material was soaked in 95% methanol for about 72 hours, with daily filtration and evaporation under reduced pressure to dryness using rotary evaporator apparatus, and then extract was exposed to air till complete dryness. And for petroleum ether extract a weight of 100 g of coarsely powdered material was soaked in 250 ml of petroleum ether and kept at 4 °C overnight. It was then centrifuged at 100,000 rpm for 20 minutes. Pellet was further dissolved in 250 ml of ethyl acetate in sterile conical flask and kept at freezing temperature over-night. Then solution was filtered and dried at room temperature to evaporate the organic solvent. The extraction was carried out in the Medicinal and Aromatic Plants and Medicine Research Institute in Khartoum.

### Microbial strains

Antimicrobial activities of extracts of *A. indica* leaves were evaluated against clinical isolate of Meticillin resistant *staphylococcus aureus* (MRSA). Standard strains include *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *K. pneumonia* ATCC 700603, and *S. typhimurium* ATCC 14028. All pathogens were obtained from National Health Laboratory in Khartoum.

### Preparation of culture media (according to manufacturer instruction)

Mueller Hinton agar (MHA) media was prepared by suspending 38 g in 1000 ml of distilled water. The media was sterilized by autoclaving at 121 °C for 15 min and poured into sterile petri plates at around 50 °C up to a uniform thickness of approximately 4 mm (+ or - 4 mm) and agar was allowed at ambient temperature before use.

### Preparation of different concentrations of the extracts

The solvent of 10% DMSO was used to prepare stock solutions of methanol and petroleum ether leaves extract with concentration of 400 mg/ml. and then concentrations of 75%, 50%, and 25% of each extract were also prepared by the same solvent.

### Antimicrobial assay

The antimicrobial assay of both extract of methanol and petroleum ether of Neem leaves were performed by agar well diffusion method. Plates were swabbed with cotton wool impregnated with the bacterial suspension containing 106 CFU/ML and allowed to dry. Five wells (6 mm diameter) were bored on the surface of the agar media on each plate. The 5 wells were filled with 100 µl solution of the extract at concentration 100%, 75%, 50%, and 25% respectively and the last well was filled with

(DMSO) solvent as negative control of each extract. The inoculated plates were kept in the refrigerator at 4 °C for 1 hour to allow the diffusion of the extract into the agar before growth of the organism commenced. A disk of appropriate antibiotics based on antibiogram of each bacterial strain were used as positive control (ampicillin, ciprofloxacin, gentamycin, and meropenem), the three antibiotic were used on a separate plate by using the conventional disc diffusion described by Kirby – Bauer.

All inoculated plates were incubated at 37 °C for 24 hour, and then the antibacterial activity was assessed based on measurement of the zone diameter of the inhibition formed around the well by using a ruler.

### Data analysis

The data collected were statistically analyzed to generate Pearson correlation coefficient (*p*-value) using Statistical Package for Social Sciences (IBM SPSS Statistics 21) to compare the values observed.

All information gathered via data master sheet then coded into variables involving T and one way ANOVA tests were used to present results, a *p* value of less than 0.05 was considered as statistically significant.

### Results

**Table 1.** Effect of different concentrations of Neem petroleum ether and methanol extract on the selected bacteria by measuring zone of inhibition in mm.

Neem extract with Concentration in %	Zone of inhibition of bacteria					
	<i>S.aureus</i>	<i>MRSA</i>	<i>E.coli</i>	<i>Klebsiella pneumonia</i>	<i>Salmonella typhi murium</i>	<i>Pseudomonas aeruginosa</i>
100	14	16	0	0	5	0
75	14	17	0	0	3	0
50	5	3	0	0	0	0
25	0	0	0	0	0	0
Neem methanol extract with concentration in %						
100	13	9	0	4	5	0
75	12	11	0	0	0	0
50	6	5	0	0	0	0
25	0	0	0	0	0	0

**Table 2.** The result of commercial antimicrobial disc measured by mm:

Antimicrobial discs used:	Zone of inhibition of bacteria					
	<i>S.aureus</i>	<i>MRSA</i>	<i>E.coli</i>	<i>Klebsiella Pneumonia</i>	<i>Salmonella typhi murium</i>	<i>Pseudomonas aeruginosa</i>
Ampicillin	0	0	18	13	15	0
Gentamicin	10	10	14	13	12	10
Ciprofloxacin	20	18	17	16	21	25
Meropenem	20	17	20	16	19	17

The effect of different concentrations of petroleum ether and methanol extracts of Neem leaves (*A.indica*) against bacteria was determined by disc diffusion method, and varying of results were obtained of our experiment according to the solvent of extract used and its concentration and the type of bacteria. Both extracts of Neem demonstrated antimicrobial activities against *staphylococcus aureus* and *Meticillin resistant staphylococcus aureus (MRSA)* but not from Gram negative bacteria (**Table 1**). The results of the commercial antimicrobial discs used as positive control are shown in **table (2)**

The study results showed there are insignificant statistical differences regarding the inhibition zone diameters of both methanol and petroleum ether extract of Neem leaves was detected. These data are shown in **table (3)**.

Also when compared the susceptibility of different species of pathogens of two extracts with different concentrations the results showed there are significant different (*p* <0.5) according to the type of pathogen in the concentration of extract used.

The antimicrobial effect of Neem extracts and commercial antimicrobial discs against the same selected pathogens when compared together showed that there is significant difference between them (*p* > 0.05).

**Table 3.** T-Test (comparison) between two extracts.

Group Statistics					
	Extract	N	Mean	Std. Deviation	Std. Error Mean
Zone	Methanol	24	2.71	4.379	.894
	Petroleum	24	3.33	5.700	1.164

## Discussion

World Health Organization (WHO) has recently reported that the world is coming into a post – antibiotic era and most of current antibiotics will become inefficient [17]. Appearance of antimicrobial reactance strains is a threat to the public health [18]. It imposes huge economic burden due to increasing morbidity and mortality [19,20]. On the other hand, most of the new antibiotics are expensive and have threatening side effects [21]. Research is under way to identify the effective and safe alternatives of current antibiotics from plant source [22]. The antimicrobial compound extracted from plants have great therapeutic potentials against microbes as they can help in aliment without undesirable side affect which are usually occur with synthetic antimicrobial agents [23].

*Azadirachta indica* leaves extracts used in this study have shown an antimicrobial effect as presented in different table used in the result interpretation. The effect was seen on *staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA), however, the international studies were able to produce larger zone of inhibition (18-19 mm) with the same or lower concentration this might be due to the known botanical fact that are grown in different locations may demonstrate different properties since they are grown on different soils and in varying climates and are exposed to different chemical and biological flora. While the other pathogens which are Gram negative bacteria were showed resistance to Neem extracts. This might be due to the fact that Gram negative bacteria change their cell wall slightly, so the Neem extracts cannot attach or they produce enzymes to disable the antibiotic.

This study is supported by the previous study results of **Kirtikar and Basu** [24], who reported similar findings, they revealed that the extract were the best term of effectiveness against *S.aureus*. **Hala and Alfadil** [25] reported that Neem leaves extract inhibited the growth of most gram positive and gram negative pathogens used in their experiment.

The result of present study may justify and support the use of extract of this plant in traditional medicine for the treatment of certain infections caused by *Staphylococcus aureus*.

**Vimala et al.** mentioned that *Neem (Azadirachta indica)* is a traditional and naturally available medicinal plant in India, South Africa, and Southeast Asia [26]

**Mahfuzul Hoque et al.** reported that chloroform extracts of neem inhibited the growth of *Listeria monocytogenes* while ethanolic extracts showed higher inhibition for *Staphylococcus aureus* [27].

**Ravva and Korn** reported that foodborne pathogen contamination from “ready to eat” produce is difficult to remove. Treating with *Neem* supplements could be an inexpensive way to prevent pre-harvest contamination via manure from nearby animal raising operations. Supplementation of *Neem* leaf and bark to manure resulted in elimination of pathogenic *EcO157* in less than 10 days [28].

An aqueous *Neem* bark preparation inhibited oral streptococci and induced a significant reduction in their surface adhesion [29]. Indeed, a significant decrease in plaque accumulation and bacterial counts were found following oral treatment with *Neem* extract [30]. However, to the best of our knowledge, no studies were performed on the possible beneficial effects of *Neem* extracts on the microbiota involved in periodontitis [31- 34]

In addition to their antimicrobial capacity, *Neem* extracts possess potent anti-inflammatory [35] and antioxidant properties, which may suppress oxidative stress that accompany periodontal pathologies [36, 37].

**Heyman et al.** concluded that *Neem* extracts possess prominent dose-dependent bacteriostatic activity against *P. gingivalis*. It possesses strong anti-oxidant activity, which is amplified following adherence to bacteria, to red blood cells and to the polycationic peptide lysozyme. The clinical relevance of these findings is based on the co-existence of bacteria, RBCs and

polycationic compounds in the oral cavity. Conceivably, Neem extract could adhere to these compounds, and provide a long lasting bacteriostatic as well as anti-oxidant activity at the site of inflammation [38].

All these previous reports supported findings of the current study.

### Conclusions

From this study, we conclude that Neem extract is effective against *S. aureus* and MRSA at 100% and 75% concentrations but not effective against Gram negative bacteria. These results provide a potential for development of this drug for treatment of MRSA infection. Further studies including greater range of organisms especially gram positive and drug resistant pathogens are recommended to study the role of Neem extract on different organisms. We also recommend isolating and separating the bioactive compound responsible for this antimicrobial activity using advanced scientific techniques.

**Ethics approval and consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

### Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

Authors declare that they have no competing interests.

**Funding:** Not applicable.

### Authors' contributions

WOFB and MAH conceived the design and carried out the experiments. AAI obtained, analyzed and interpreted the data. WOFB and MAH wrote and revised the manuscript. AAI provides financial support for all experiments. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

### Acknowledgements

Thanks for all participants involved in this research.

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