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Evaluation of the biological efficacy of neem oil against some pathogens

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ABSTRACT

Background: The neem tree (Azadirachta indica) is an evergreen species native to regions such as India and Pakistan and is also cultivated in parts of the Americas. Products derived from neem have numerous applications, particularly in skin, hair, and nail care, as well as in food preservation, due to their ability to inhibit the growth of various microorganisms.

Objective: Three organic solvents, hexane, ethanol, and petroleum ether (80-100°C(, wereused to extract neem oil from dried, ground seeds using the Soxhlet extraction method. High-performance liquid chromatography (HPLC) was employed to determine the fatty acid composition of the extracted oil. The antibacterial activity of neem seed oil was evaluated against four bacterial strains (*Staphylococcus aureus*, *Bacillus* spp., *Pseudomonas aeruginosa*, and *Escherichia coli*) and one yeast (*Candida* spp.) at three different concentrations.

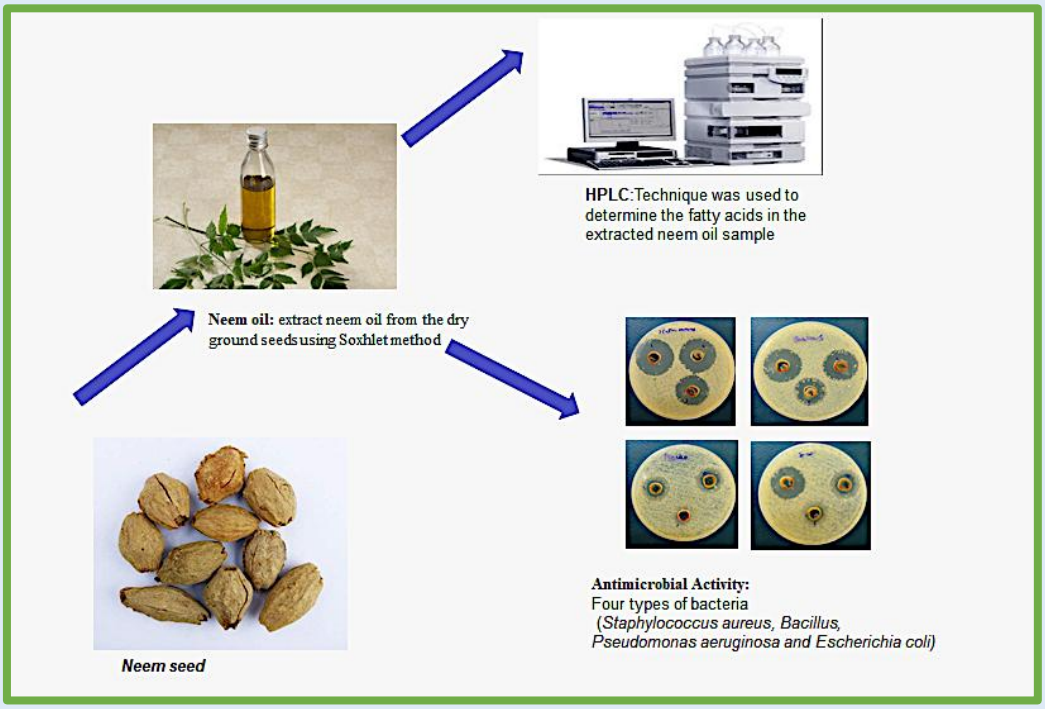
Results: The extraction yields were 44.89% using petroleum ether, 42.63% using hexane, and 39.17% using ethanol. Analysis revealed that long-chain fatty acids accounted for 19.77% of the neem seed oil composition, including palmitoleic acid (14.23%), oleic acid (2.49%), palmitic acid (1.50%), stearic acid (0.90%), linoleic acid (1.50%), and linolenic acid (0.05%). Neem seed oil exhibited strong antibacterial activity. *Staphylococcus aureus* showed inhibition zones of 36, 30, and 28 mm at concentrations of 1000, 500, and 250 µg/mL, respectively. *Bacillus* spp. demonstrated inhibition zones of 28 , 26 , and 24 mm at the same concentrations. *Escherichia coli* exhibited inhibition zones of 26 , 22

, and 10 mm, respectively. Lower inhibitory effects were observed against *Pseudomonas aeruginosa*, with inhibition zones of 18 , 19 , and 3 mm.

Novelty: Neem seed oil represents an effective, sustainable, and environmentally friendly alternative to synthetic antimicrobial agents.

Conclusion: Neem seeds are a valuable source of oil rich in long-chain fatty acids with significant therapeutic potential. The oil demonstrated notable antibacterial and antifungal activity, emphasizing its potential importance for medical and pharmaceutical applications**.**

Key words: Neem tree, Oil extraction, Fatty acid and Antimicrobial, antifungal activity, long-chain fatty acids.



Graphical Abstract: Investigation of the biological effectiveness of neem seed oil against various pathogenic microorganisms.

INTRODUCTION

Azadirachta indica A. Juss (neem tree) is an evergreen tropical tree belonging to the family Meliaceae. It is widely distributed in tropical and subtropical regions, particularly in India, which is considered one of the most important countries for neem cultivation. The tree is also found in other regions, including Pakistan and parts of the Americas. Neem trees produce green fruits that typically turn yellow during the month of July [1]. Archaeological evidence indicates that cosmetics, perfumes, and skin and hair care products were widely used in ancient civilizations, such as those of Greece and Egypt. These products were commonly extracted from plants native to those regions, including lily, mint, rosemary, rose, aloe vera, sesame oil, olive oil, almond oil, and neem oil [2]. Various methods have been

employed to extract neem oil from its seeds, including mechanical pressing, solvent extraction using different organic solvents, and modern techniques such as steam pressure extraction and supercritical fluid extraction [3-4]. Neem seed oil is widely available in commercial markets and stores specializing in natural plant products. Traditional knowledge and cultural practices in many countries highlight the significant benefits associated with its use [5-6]. Numerous studies have demonstrated neem seed oil's ability to inhibit the activity of a wide range of bacterial and fungal species [2,8]. The oil has been used to treat nail infections, with excellent results attributed to its antibacterial and anti-inflammatory properties; additionally, its long-chain aliphatic carboxylic acids, including oleic, linoleic, and palmitic acids, play roles in energy storage and hormone production [9]. Azadirachtin is one of the most important bioactive compounds found in the leaves, seeds, and oil of the neem tree. This compound exhibits strong antibacterial activity by interfering with bacterial cell division and inhibiting essential metabolic processes, thereby disrupting pathogen growth and reproduction [10-11]. Azadirachtin also inhibits chitin synthesis, leading to cell lysis and microbial death. Other compounds present in neem oil, such as nimbin and nimbidin, have also been reported to exhibit antiviral and antifungal activities [12-13]. In addition, neem oil has been used in food preservation, where it has shown significant antimicrobial activity against foodborne pathogens and food spoilage fungi, even at low concentrations [14-15]. The aim of this study was to extract neem oil from Azadirachta indica seeds using different organic solvents, to identify its fixed fatty acid composition, and to evaluate its effectiveness against selected bacterial and yeast strains.

MATERIALS AND METHODS

Materials: All materials used in the research are provided by specialized international companies and are of high purity and suitable for laboratory use.

Sample preparation: Dried neem seeds were transported to the laboratory, washed with distilled water, and dried using blotting paper. The seeds were then oven-dried at 55°C until a constant weight was achieved. The dried seeds were ground into a fine powder using a laboratory grinder and prepared for extraction.

Neem Oil Extraction: Neem seed oil was extracted using a multi-unit Soxhlet apparatus at the College of Agriculture, University of Anbar. Twenty grams of dried seed powder were placed into the extraction thimbles of three separate units. Each unit employed a different solvent: hexane, ethanol, or petroleum ether (80-100°C). The solvents were heated under reflux, and the extraction process was carried out for 6 hours. After extraction, the solvent was recovered using a rotary evaporator (ISOLAB Laborgerate GmbH). The extracted oil, along with any residual solvent, was placed in a drying oven to ensure complete solvent removal. The oil yield was calculated using the following equation [16]:

$$\text{Oil yield \%} = \frac{M2-M1}{M1} \times 100$$

Estimation of Fatty Acids: High-performance liquid chromatography (HPLC) (YL9100 Series QC Analyzer) was used to determine the fatty acid composition of the extracted neem oil sample. Separation was performed using a C18 column (4.6×250 mm, 5µm) with UV detection at 210 nm. The flow rate was set at 0.8 mL/min, the injection volume was 20 µL, the column temperature was maintained at 30°C, and the mobile phase consisted of water containing 0.1% H3PO4.

For sample preparation, 1 g of neem oil was mixed with 3 mL of hexane and vortexed for 3 minutes. The mixture was then centrifuged for 10 minutes to separate the precipitate from the filtrate. The filtrate layer was dried using liquid nitrogen, redissolved in methanol, and injected into the HPLC system. Fatty

acids were identified by comparing the retention times and peak areas of the sample with those of standard fatty acids. The concentration of each fatty acid was calculated using the following equation [17]: Fatty acid concentration = $\frac{A\ sample}{A\ standard}$ × C Standard

While: A sample is the peak area of the sample, A standard is the peak area of the standard, and C Standard is the concentration of the standard.

Antimicrobial Activity: The antimicrobial activity of neem seed oil was evaluated against four bacterial strains, *Staphylococcus aureus*, *Bacillus*, *Pseudomonas aeruginosa,* and *Escherichia coli,* in addition to *Candida* as a type of yeast. The agar well diffusion method was used to measure inhibition zones, as previously described [18]. Three concentrations of neem oil (250, 500, and 1000 µg/mL) were tested. All tools and culture media were sterilized using an autoclave (Hirayama HV-85) prior to use. Each bacterial isolate was cultured in nutrient broth and incubated at 37°C for 18-24 hours (Gallenkamp IEF097). Subsequently, 0.1 mL of each bacterial suspension was spread evenly onto nutrient agar plates and incubated at 37°C for 24 hours. A single colony from each culture was transferred into a test tube containing 5 mL of sterile normal saline to obtain a bacterial suspension with turbidity comparable to the McFarland standard (approximately 1.5 x 10^8 CFU/mL). Using a sterile cotton swab, the bacterial suspension was uniformly spread onto Mueller-Hinton agar plates and allowed to stand for 10 minutes. Wells with a diameter of 5 mm were punched into the agar (three wells per plate). The agar plugs were removed, and 50µL of neem seed oil solution was added to each well using a micropipette. The central wall served as a control. The plates were incubated at 37°C for 18 hours, after which the diameters of the inhibition zones were measured and recorded [19].

RESULTS AND DISCUSSION

Three different organic solvents, hexane, ethanol, and petroleum ether, were used to extract neem seed oil. The seed-to-solvent ratio was 1:6 for all three solvents, as shown in Table l. The extraction times were 4 hours for hexane and petroleum ether and 6 hours for ethanol. The Soxhlet extraction temperatures were 70°C for hexane and ethanol, and 90°C for petroleum ether. Petroleum ether yielded the highest oil extraction (44.89%), followed by hexane (42.63%), while ethanol gave the lowest yield (39.17%). These results indicate that the optimal extraction time for the highest oil yield was 4 hours when using petroleum ether. These findings are consistent with previous studies that retained organic solvents for oil extraction [20]. Other research has shown that neem seeds predominantly contain fixed fatty acids rather than volatile ones, with a reported yield of 34.85% when polar solvents were used [21].

Table 1. Neem seeds oil extraction and extraction conditions.

Solvent	Seed/solvent Ratio(g/ml)	Time extraction (hour)	Temperature of Soxhlet °C	Oil yield $\%_{wt/wt}$
hexane	1:6	4	70	42.63
ethanol	1:6	6	70	39.17
petroleum ether (80-100°C)	1:6	4	90	44.89

Estimation of Fatty Acids*:* High-performance liquid chromatography (HPLC) was used to determine the percentage of fixed fatty acids in neem seed oil. Six long-chain fatty acids were identified: palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids (Table 2). The total percentage of these fatty acids in neem seed oil was 19.77%, with palmitoleic acid accounting for 14.23%, oleic acid 2.49%, palmitic acid 1.5%, stearic acid 0.9%, and linoleic and linolenic acids 0.6% and 0.05%, respectively. Figure 1 illustrates that

palmitoleic acid accounted for 71.99% of the total long-chain fatty acids. These results are consistent with previous studies using GC/MS analysis of neem seed oil. The presence of fixed fatty acids contributes to the diverse applications and therapeutic benefits of neem oil, including the treatment of various skin and dermatological conditions, and its use in food preservation [22]. Although vegetable oils can be extracted from multiple plant parts, seed-derived oils are particularly versatile due to their high triglyceride content, which serves as an energy reserve and makes them suitable for both body care and food applications [23].

Table 2: Estimation of fatty acid by HPLC technique.

No	Fatty Acid	Area of standard	Rt.Time (min)	Area of sample	Concentration of fatty acid mg/kg	Concentration of fatty acid %	Fatty acid percentage %
1	Palmitic	483.86	7.032	7245041.9	14973.43	1.50	7.574
2	Palmitoleic	77.26	13.256	10994287	142302.45	14.23	71.985
3	Stearic	376.201	14.884	3384376.4	8996.192	0.90	4.551
4	Oleic	333.737	15.924	8325023.7	24944.863	2.49	12.619
5	Linoleic	149.809	20.036	894001.49	5967.609	0.60	3.019
6	Linolenic	776.08	21.66	388021.45	499.976	0.05	0.253
Total					197684.51	19.77	100

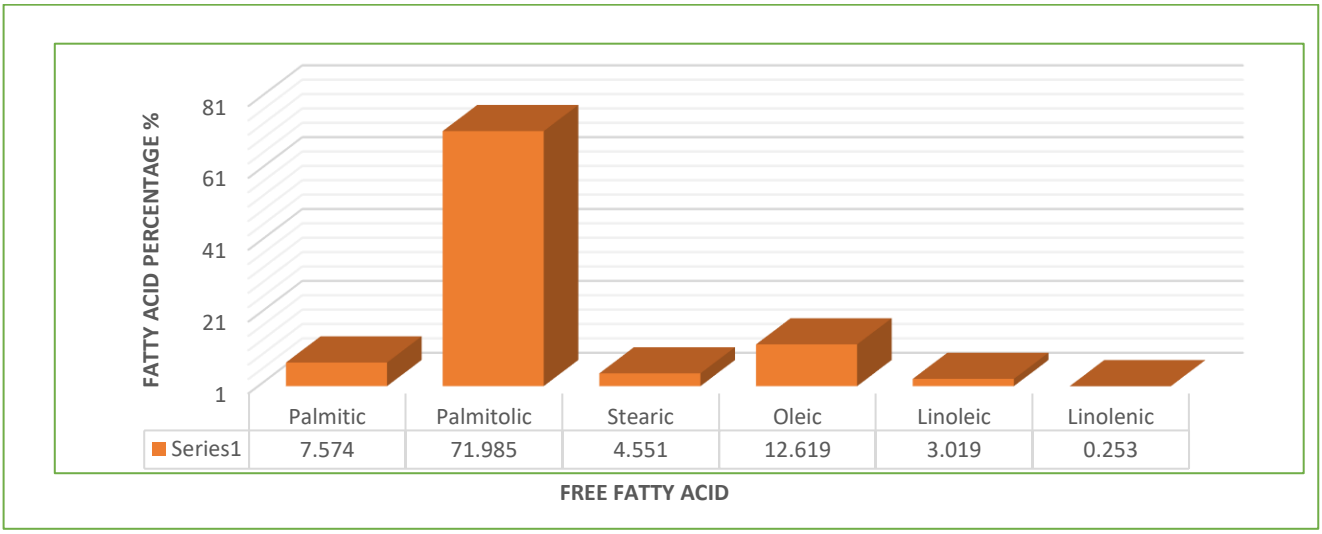
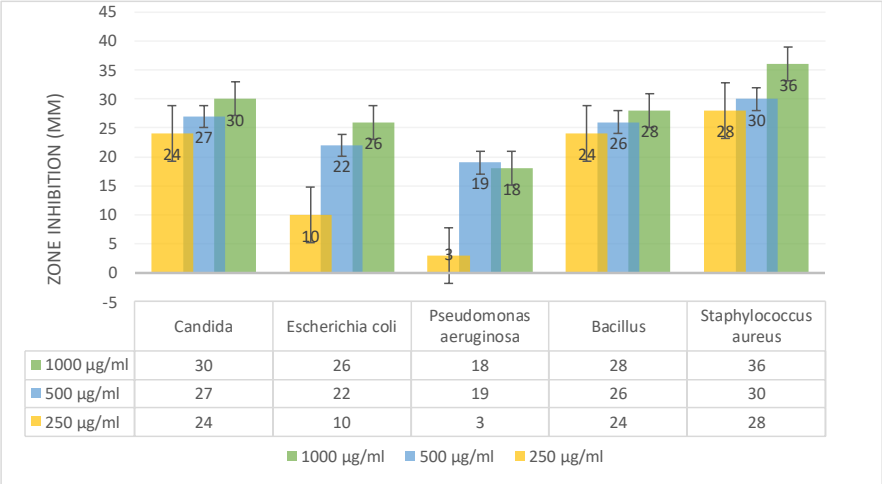


Figure 1: Fatty acids fixed of neem seeds

Antimicrobial Activity: Neem seed oil demonstrated significant antibacterial and antifungal activity, inhibiting four bacterial strains, *Staphylococcus aureus*, *Bacillus* spp., *Pseudomonas aeruginosa*, and *Escherichia coli*, as well as one yeast strain (*Candida* spp.). Three concentrations of neem seed oil (250, 500, and 1000 µg/mL) were tested, as shown in Figures 2 and 3. *Staphylococcus aureus* exhibited the highest inhibition, with zones of 36, 30, and 28 mm at 1000, 500, and 250 µg/mL, respectively. *Bacillus* spp. showed inhibition zones of , 26, and 24 mm at the same concentrations. *Escherichia coli* displayed zones of 26, 22, and 10 mm, while *Pseudomonas aeruginosa* showed lower inhibition, with zones of 18, 19, and 3 mm, respectively. Notably, the inhibitory effect decreased significantly at 250 µg/mL for *E. coli* and *Pseudomonas*. Neem seed oil also inhibited *Candida* spp., producing inhibition zones of 30, 27, and 24 mm at 1000, 500, and 250 µg/mL, respectively (Figure 3). These results are consistent with

previous studies reporting that neem seed oil exhibits activity against both Gram-positive and Gram-negative bacteria [22-24].



	Candida	Escherichia coli	Pseudomonas aeruginosa	Bacillus	Staphylococcus aureus
1000 µg/ml	30	26	18	28	36
500 µg/ml	27	22	19	26	30
250 µg/ml	24	10	3	24	28

1000 µg/ml 500 µg/ml 250 µg/ml

Figure 2. Inhibitory ability of neem seed oil against various types of microbes.

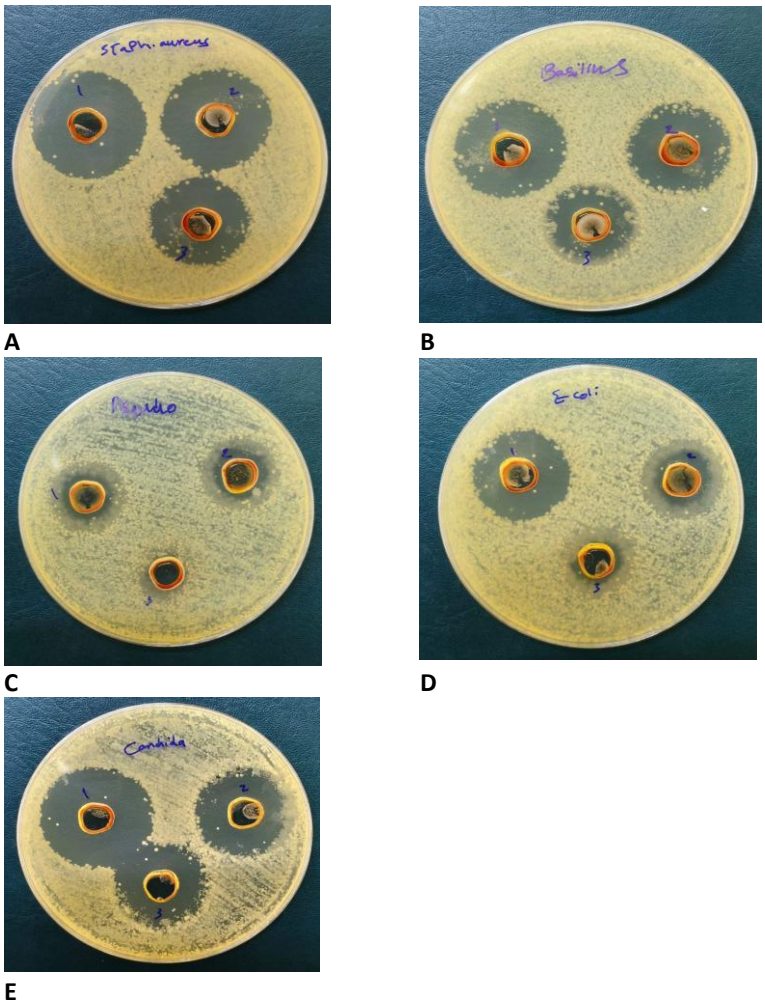


Figure 3: **A**-Zone inhibition of *Staphylococcus aureus* , **B-** *Bacillus,* **C-** *Pseudomonas* **D-** *E. coli* , **E-** *Candida spp* ,.against three concentrations of seed neem oil *(*250 µg/mL, 500 µg/mL, and 1000 µg/mL). **B**-Zone inhibition of *Bacillus* spp. against three concentrations of seed neem oil *(*250 µg/mL, 500 µg/mL, and 1000 µg/mL). **C**-Zone inhibition of *Pseudomonas aeruginosa* against three concentrations of seed neem oil *(*250 µg/mL, 500 µg/mL, and 1000 µg/mL). **D**-Zone inhibition of *Escherichia coli.* against three concentrations of seed neem oil *(*250 µg/mL, 500 µg/mL, and 1000 µg/mL). **E**-Zone inhibition of *Candida spp.* against three concentrations of seed neem oil *(*250 µg/mL, 500 µg/mL, and 1000 µg/mL).

Previous research has demonstrated the antibacterial activity of neem fixed oil against Gram-positive bacteria, including *Bacillus cereus, Lactobacillus acidophilus, Streptococcus luteum, Staphylococcus aureus,* and *Streptococcus pneumoniae,* as well as Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. Neem oil effectively inhibited *Lactobacillus acidophilus*, with a low minimum bactericidal concentration (MCC) of 0.125 µmol/mL. Our study corresponds to Step 6 of the Functional Food Center's (FFC) 17-step framework, which focuses on establishing relevant biomarkers; the primary aim of our experiment was to identify and validate biochemical markers associated with the observed functional effects [7, 25].

Conclusions: Neem tree seeds are a valuable source of high-quality oil, rich in long-chain fatty acids that provide numerous therapeutic benefits. Neem seed oil exhibits strong antibacterial and antifungal activity, demonstrating effectiveness against a wide range of bacteria, molds, and yeasts. Its composition and bioactivity make it a promising natural product for medical, pharmaceutical, and food preservation applications.

Authors' Contribution: Aseel Abdulsattar Gburi: Formal analysis and Methodology; Validation and Writing original draft. Ayyad Wajeh Raof Al-Shahwany, Project administration; Funding acquisition. Sara Thamer Hadi: Data duration; Formal analysis and Methodology.

Competing Interests: The authors declared no conflict of interest.

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