



Antifungal Activity of the Crude Extracts of the Neem Leaves (*Azadirachta indica*)

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Abstract: Our study investigated the antifungal properties of crude extracts obtained from *Azadirachta indica* (Neem) leaves against possible fungal pathogens, including *Aspergillus niger*, *Alternaria alternata*, and *Penicillium* sp. The study aimed to evaluate the effectiveness of methanolic extracts at different strengths (0.5%, 1%, 2%, 4%, and 8%) using the well diffusion method. The results demonstrated antifungal activity, with inhibition increasing as the extract concentration increased. Complete inhibition (100%) was observed at 4% and 8% strengths for all tested fungal infections. Phytochemical screening showed the presence of alkaloids, flavonoids, tannins, terpenoids, and phenolic chemicals, which are most likely responsible for the observed antifungal properties. Negative results were obtained for saponins, steroids, and anthraquinones, which may have been due to methodological or concentration-related factors. These results underscore Neem's potential as a natural antifungal agent and support its traditional medicinal uses. On the other hand, further research, including clinical trials, is recommended to confirm these results and explore practical uses in treating fungal infections.

Keywords: *Azadirachta indica*; Neem leaves; Antifungal activity; Methanolic extract; *Aspergillus niger*; *Alternaria alternata*; *Penicillium* spp.; Phytochemical screening.

1. Introduction

Medicinal plants have been an integral part of human health and wellness for thousands of years, forming the foundation of traditional medicine systems across cultures. These plants are defined as plants that contain bioactive compounds capable of preventing or managing diseases (Ekor, 2014). They have played a critical role in the development of pharmaceutical medicines and continue to provide valuable compounds for modern medicine.

The importance of medicinal plants lies in their ability to manage a wide variety of ailments, from minor infections to chronic diseases, often with fewer side effects compared to synthetic drugs (Petrovska, 2012). For example, medicines such as aspirin (derived from willow bark), morphine (from the opium poppy), and the anti-malarial drug artemisinin (from *Artemisia annua*) originated from plant-based sources. In resource-limited settings, medicinal plants often serve as the primary healthcare option, underscoring their accessibility and affordability (Fabricant & Farnsworth, 2001). Moreover, ongoing research on medicinal plants continues to yield promising discoveries, particularly in combating

antibiotic resistance and developing treatments for cancers and other autoimmune diseases.

The benefits of medicinal plants extend beyond their therapeutic applications. They contribute to biodiversity conservation, cultural heritage, and local economies. However, issues such as overharvesting, habitat destruction, and the lack of scientific validation for some classical remedies pose challenges to their sustainable application (Pan et al., 2014).

The increasing prevalence of multidrug-resistant microorganisms has necessitated research into novel antibacterial compounds from natural sources, especially plants. Plants have been a cornerstone of classical medicines for centuries, offering a rich repository of bioactive chemicals with diverse therapeutic properties. Among these, *Azadirachta indica* (Neem) stands out as a plant of significant medical value, with increasing use in Ayurvedic and traditional therapeutic systems for its antimicrobial, anti-inflammatory, and antifungal properties (Desalegn, 2014).

Neem, a member of the Meliaceae family, is renowned for its broad-spectrum biological efficiency, attributed to its complex phytochemical composition. Compounds

such as nimbin, azadirachtin, gedunin, and quercetin have been identified as key compounds responsible for its therapeutic effects. These compounds exhibit antimicrobial properties, making Neem a promising candidate for combating drug-resistant pathogens, including opportunistic fungal infections (Kirbag et al., 2009).

Opportunistic fungal infections can arise from organisms such as *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, posing a significant threat to immunocompromised persons, including those with HIV/AIDS, cancer patients undergoing chemotherapy, and organ transplant recipients. The rise of antifungal resistance to classical drugs such as fluconazole and amphotericin B has further exacerbated the need for alternative therapeutic medicines. Plant-derived compounds, with their diverse mechanisms of action and lower toxicity effects, offer viable solutions for this global health challenge (Derwich et al., 2010).

The essential oils and extracts obtained from medicinal plants have been extensively studied for their antimicrobial activities. These natural compounds are not only effective against pathogens but also serve as valuable resources for the development of new pharmaceuticals, nutraceuticals, and food compounds. The antifungal efficacy of Neem extracts, in particular, has been the subject of numerous studies, highlighting its potential as a natural antifungal agent (Tumwine, 2011).

The aim of our study was to evaluate the in vitro antifungal efficacy of crude extracts of *Azadirachta indica* leaves against some medically important opportunistic fungal infections.

The following specific objectives were achieved:

1. To collect and identify the medicinal plant *Azadirachta indica*.
2. To extract the bioactive compounds from *Azadirachta indica* leaves using ethanol and aqueous solvents.
3. To determine the minimum inhibitory concentrations (MIC) of the extracts against selected opportunistic fungal infections.

2. Materials and Methods

2.1 Material

2.1.1 Equipment and Apparatus

The table below illustrates the equipment and apparatus used in this study:

Apparatus	Manufactured company	Origin
Autoclave	Lab tech	Korea
Distiller	Gallen kamp	England
Haemocytometer	Marienfeld	Germany
Incubator	Shell lab	Holland
Laminar flow cabinet	Jeio-Tech	Korea
Light microscope	Olympus	Japan
Magnetic stirrer-Hot Plate	Gallenkamp	England
Millipor filter unit (4.5 µm)	Whatman	England
Oven	Memmert	Germany
pH-meter	Crison Instruments	Spain
Refrigerator	Concord	Lebanon
Micropipette	Eppendorf	Germany
Bunsen burner	AL-Murtajal	Iraq
Flasks	—	China
Petri dishes	—	China
Bunchner funnel	CNWTC	China
Soxhlet apparatus	Microlab scientific	China
Laboratory grinder	Justbuy	China

2.1.2 Chemicals

Materials Used in Scientific Research in Iraq (Al-Nahrain University).

Material	Manufacturing company	Origin
Neem leaves(<i>Azadirachta indica</i>)	Medicinal plants garden-Al-Nahrain university	Iraq
Ethanol	BDH/Scharlau/Himedia	Uk/spain/india
Methanol	BDH/sigma-aldrich/J.T.Baker	Uk/USA/Netherlands
Potato dextrose agar (PDA)	Himedia/Merck/oxid	India/Germany/UK
Sabouraud dextrose agar (SDA)	Himedia/Merck/oxid	India/Germany/UK

2.1.3 Qualitative Analysis of Phytochemicals in Neem Leaves (*Azadirachta indica*)

The leaves of *Azadirachta indica* (Neem) are rich in bioactive materials, including alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides, and phenolic compounds. These phytochemicals are responsible for the plant's medical properties, such as antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory effects.

Below is a summary of the qualitative analysis of phytochemicals commonly found in Neem leaves, the compounds detected, and references to studies.

2.1.3.1 Phytochemical Components and Compounds in Neem Leaves

2.1.3.1.1 Alkaloids

Qualitative Test:

Dragendorff's test:

The Dragendorff test is a qualitative analysis used to illustrate the presence of alkaloids in plant extracts, including Neem leaves. Below is the general procedure for performing the Dragendorff test on Neem leaf extracts, along with a reference formatted in APA style.

Procedure for the Dragendorff Test of Alkaloids in Neem Leaves

- **Preparation of Dragendorff Reagent:**

1. Mix 0.85 g of bismuth nitrate in 40 mL of distilled water and 10 mL of glacial acetic acid.
2. Separately, dissolve 8 g of potassium iodide in 20 mL of distilled water.
3. Combine the two solutions to form the Dragendorff reagent.

- **Testing for Alkaloids:**

1. Take 1 to 2 mL of the Neem leaf extract in a test tube.
2. Add a few drops of the Dragendorff reagent to the test tube.
3. Observe the formation of an orange or reddish-brown precipitate, which indicates the presence of alkaloids.

2.1.3.1.2 Flavonoids

Qualitative Test:

Shinoda test:

The Shinoda test is a qualitative test that illustrates the presence of flavonoids in plant extracts. Below is the step-by-step procedure for performing the Shinoda test on Neem leaves (*Azadirachta indica*).

Procedure

Performing the Shinoda Test:

1. Take 2 to 3 mL of the Neem leaf extract in a clean test tube.
2. Add a small piece of magnesium ribbon (or powdered magnesium) to the test tube.

3. Carefully add a few drops of concentrated hydrochloric acid (HCl) to the extract.
4. Observe the color change in the solution.

Ammonia test:

Performing the ammonia test:

1. Add one drop of extract on filter paper.
2. Expose to ammonia vapor.
3. Result: yellow spot on filter paper.

2.1.3.1.3 Tannins

Qualitative Test:

Ferric chloride test:

The Ferric Chloride Test is one of the most common qualitative tests used to detect the presence of tannins. Tannins are phenolic compounds, and they react with ferric ions (Fe^{3+}) to form colored complexes, usually blue, green, or black, depending on the type of tannin present.

Performing the Ferric chloride test:

1. Take 2 to 3 mL of the Neem leaf extract in a clean test tube.
2. Add 2 to 3 drops of 5% ferric chloride (FeCl_3) solution to the extract.
3. Shake the test tube gently and observe for any color change.

Lead acetate:

- Tannins form insoluble complexes with lead ions (Pb^{2+}), resulting in the observed precipitate. This reaction occurs due to the tannins' ability to chelate metal ions.

Performing the Lead Acetate Test:

1. Take 2 to 3 mL of the Neem leaf extract in a clean test tube.
2. Add 1 to 2 mL of 10% lead acetate solution to the test tube.
3. Shake the test tube gently and observe the reaction.

Potassium dichromate:

Tannins, being polyphenolic compounds, form insoluble complexes with potassium dichromate, leading to the formation of a precipitate.

Performing the Potassium Dichromate Test:

1. Take 2 to 3 mL of the Neem leaf extract in a clean test tube.
2. Add a few drops of 10% potassium dichromate solution to the test tube.
3. Shake the test tube gently and observe for the formation of a precipitate.

2.1.3.1.4 Saponins

Qualitative Test:**Foam test:**

Persistent froth formation after shaking indicates the presence of saponins.

Performing the Foam test:

1. Add 5 mL of distilled water to 0.5 mL of extract in a test tube, then boil the mixture for 5 minutes.
2. Add 3 drops of Na_2CO_3 .
3. Shake the test tube vigorously for 15 seconds.
4. Allow the test tube to stand for 10–15 minutes.

2.1.3.1.5 Terpenoids**Qualitative Test:****Salkowski test:**

Formation of a reddish-brown interface indicates terpenoids.

Performing the Salkowski test:

1. Take 2 mL of Neem leaves extract in a test tube.
2. Carefully add 2 mL of concentrated sulfuric acid along the sides of the test tube to form a separate layer.

2.1.3.1.6 Steroids**Qualitative Test:****Liebermann-Burchard test:**

A green color indicates steroids.

Performing the Liebermann-Burchard test:

1. Take 1 mL of extract in a clean dry test tube, then add 3 mL of acetic acid and mix them gently.
2. Slowly add 3–4 drops of concentrated sulfuric acid.

2.1.3.1.7 Phenolic Compounds**Qualitative Test:**

The lead acetate test can be used for detection of the presence of phenols in a sample. Phenols will react with lead acetate to form insoluble lead phenoxide; the formation of a white precipitate indicates the presence of phenol.

Performing the Phenol test:

Take two mL of Neem leaves extract in a test tube, then add 3 drops of 3% lead acetate solution.

2.1.3.1.8 Glycosides**Qualitative Test:****Keller-Killiani test:****Performing the Keller-Killiani test:**

1. Take 2 mL of the Neem leaves extract in a clean test tube.
2. Add 1 mL of glacial acetic acid containing one drop of 1% ferric chloride solution.

3. Slowly add 1 mL of concentrated sulfuric acid (H_2SO_4) along the side of the test tube to form a separate layer.

2.1.3.1.9 Anthraquinone**Borntrreger Test:**

This is a chemical test used to detect the presence of anthraquinone derivatives in plant extracts.

Performing the Borntrreger Test:

1. Transfer the Neem leaves extract into the separatory funnel.
 2. Add two mL of dilute hydrochloric acid (5%) to the funnel and shake gently. This helps to hydrolyze the anthraquinone glycosides into their aglycones.
 3. Add the chloroform to the separatory funnel and allow the funnel to settle and separate the layers. The chloroform layer (bottom) will contain the organic compounds, and the aqueous layer (top) contains the acid.
 4. Drain the lower chloroform layer into a clean beaker, leaving the aqueous acid layer in the separatory funnel.
- Reaction and Observation:**
5. To the aqueous layer in the separatory funnel, add 2 mL of 10% ammonia solution and shake gently.
 6. Allow the layers to separate.
 7. The upper ammoniacal layer should now be examined for any color changes.

2.1.4 Culture Media**2.1.4.1 Potato Dextrose Agar (PDA)**

Potato Dextrose Agar (PDA) is a commonly used medium that supports the growth of fungi, such as yeast and mold. It is widely used in microbiology for the isolation and cultivation of fungal species, especially in food, dairy, and environmental samples. PDA provides a nutrient-rich source due to its high carbohydrate content, which promotes the growth of fungal organisms.

This medium is particularly useful in mycological studies for the differentiation and enumeration of yeasts and molds. The inclusion of potato extract and dextrose supplies essential nutrients and energy sources, while agar acts as the solidifying agent. The pH of PDA can be adjusted to inhibit bacterial growth and increase fungal development.

Composition of PDA (per liter):

- Agar – 15.0 g/L
- Potassium Chloride – 0.02 g/L
- Dextrose – 20.0 g/L
- Starch – 3.5 g/L

- Peptones – 0.42 g/L
- Final pH – 5.6 ± 0.2 at 25°C

The medium was prepared according to the manufacturing instructions by dissolving 39.1 g of the dehydrated medium in 1000 mL of distilled water. The solution was heated with stirring and boiled for 1 minute to ensure complete dissolution. It was then distributed and sterilized by autoclaving at 121°C , 15 Psi for 15 minutes. If necessary, the pH was adjusted to 3.5 ± 0.1 before pouring the plates by adding 1 mL of sterile 10% lactic acid to 140 mL of sterile medium, which had been cooled to $45\text{--}50^{\circ}\text{C}$.

The plates were stored at $10\text{--}25^{\circ}\text{C}$ in a dark, dry place. The medium was inspected for changes in color or lump formation before use.

2.1.4.2 Sabouraud Dextrose Agar (SDA)

Sabouraud Dextrose Agar (SDA) is a selective medium primarily used for the isolation and cultivation of fungi, including yeasts and molds. It was originally formulated by Raymond Sabouraud and is commonly used in clinical and research laboratories for fungal identification and testing. The high dextrose concentration and slightly acidic pH of the medium favor fungal growth while inhibiting the growth of most bacteria.

SDA is especially useful in the detection of dermatophytes, *Candida* species, and other fungal pathogens. It can also be supplemented with antibiotics to further suppress bacterial contamination. The presence of peptone provides essential nutrients, while agar acts as the solidifying agent.

Composition of SDA (per liter):

- Peptone: 10.0 g/L
- Dextrose: 37.7 g/L
- Agar: 14.4 g/L
- Final pH: 5.6 ± 0.2 at 25°C

Preparation:

Sabouraud Dextrose Agar was prepared according to the manufacturer's instructions by dissolving 62.1 g of the powdered medium in 1000 mL of distilled water. The mixture was heated and stirred until completely dissolved. The medium was then sterilized by autoclaving at 121°C for 15 minutes and poured into sterile Petri dishes. The prepared plates were stored at $10\text{--}25^{\circ}\text{C}$ to maintain their stability until use.

2.2 Methods

2.2.1 Collection of the Plant Material

Leaves were collected in October 2024 from the medicinal plants garden of Al-Nahrain University in Iraq. The plants were identified as *Azadirachta indica* (Neem)

Table (3_1): Chemical tests on Neem leave extract

based on the morphological characteristics of the genus and species in the College of Sciences, Department of Biology.

2.2.2 Preparation of the Crude Aqueous-Methanolic Extract from Leaves

The plant leaves were thoroughly washed and cleaned before being dried at room temperature for about 15–20 days. The dried leaves were then ground using an herbal grinder to obtain a fine powder. Prior to extraction, the powder was stored in airtight polyethylene bags at room temperature. A total of 100 grams of the dried powder underwent extraction using 80% methanol with a Soxhlet apparatus. The crude extract was concentrated using a rotary evaporator under reduced pressure and then allowed to evaporate in an oven at 45°C .

The dried extracts were dissolved in about 20 mL of methanol, resulting in a stock solution with a concentration of 100%. The crude methanolic extracts were then filtered using a $0.45\text{ }\mu\text{m}$ Millipore filter unit and stored in a refrigerator at 4°C until further use.

2.2.3 Fungal Strains Used in This Study

All fungal strains used in this study were obtained from the Medical Garden of the College of Pharmacy / Department of Pharmacognosy. The tested microorganisms included:

► Three types of fungi:

Aspergillus niger, *Alternaria alternata*, *Penicillium* sp.

2.2.4 Determination of Minimum Inhibitory Concentration (MIC)

The crude methanolic aqueous extract prepared from the leaves was studied for its antimicrobial activity in vitro. The antimicrobial activity was evaluated using the serial dilution method.

Sterile Petri dishes were used and labeled with the names of the fungi. Serial dilutions were prepared from the stock solution of the crude extract to obtain the following concentrations: 0.5%, 1%, 2%, 4%, 8%.

2.2.5 Antifungal Screening

Potato Dextrose Agar (PDA) plates were inoculated with a 6 mm disk of the test fungus, placed using a sterile inoculation needle. The wells were filled with (200, 400, 800, 1000, 1600) μL of the leaf extract, and the plates were incubated at 28°C . The diameter of the inhibition zone was the basis for checking antifungal activity after (7) days of incubation. Results were observed on the basis of absence or presence of zone of inhibition and were measured. A zone reader was used to measure zone of inhibition in mm.

3. Results

3.1 Chemical Tests on Neem Leave Extract

Phytochemical class	Test	Result
Alkaloid	Dragendorff's test	+
Flavonoids	Shinoda test	+
Flavonoids	Ammonia test	+
Tannins	Ferric chloride test	+
Tannins	Lead acetate	+
Tannins	Potassium dichromate	+
Saponins	Foam test	-
Terpenoids	Salkowski test	+
Steroid	Liebermann-Burchard test	-
Phenolic Compounds	Lead acetate	+
Glycosides	Keller-Killiani test	+
Anthraquinone	Borntrager test	-

3.2 Antifungal Activity of Crude Extract of *A. indica* Leaves

Different concentrations (0.5, 1, 2, 4, 8)% of crude methanolic extract of *Azadirachta indica* leaves were evaluated by the well diffusion method against the pathogenic fungi *Penicillium* sp., *Alternaria alternata*, *Aspergillus niger*, and other fungal isolates. Results clearly show that the crude methanolic extract of *A. indica* leaves exhibited antifungal activity in varying degrees against all tested fungal strains. It was observed that the methanolic leaf extract of *A. indica* remained

active and showed an inhibition effect up to a dilution of (10 mg/mL) for all fungal isolates tested.

3.2.1 *Aspergillus niger*

The results in the table (3_1) demonstrated that the methanolic extract of *Azadirachta indica* leaves exhibited significant antifungal activity against *Aspergillus niger*, with inhibition increasing as the extract concentration increased. At lower concentrations (0.5% and 1%), partial inhibition of fungal growth was observed, while at higher concentrations (2% and above), the inhibition was more pronounced, reaching 100% at 4% and 8%.

Table (3-2): Antifungal activity of methanolic extract of *A. indica* leaves against *Aspergillus niger*

Microorganism	Extract concentration %	Colony diameter (Cm)	Inhibition rate %
<i>Aspergillus niger</i>	Control	4.5	0
	0.5	3.3	26.70
	1	2.5	44.44
	2	1.2	73.33
	4	0	100
	8	0	100

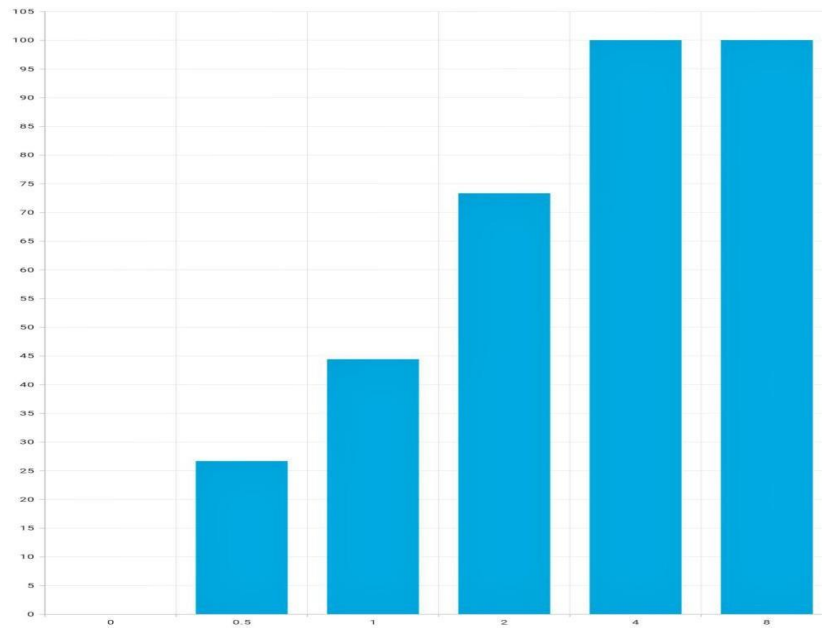


Fig. (3-1): Antifungal activity of methanolic extract of *A. indica* leaves against *Aspergillus niger*

3.2.2 *Alternaria alternata*

The results in the table (3_2) demonstrated that the methanolic extract of *Azadirachta indica* leaves exhibited significant antifungal activity against *Alternaria alternata*, with inhibition increasing as the extract concentration increased. At lower concentrations

(0.5% and 1%), partial inhibition of fungal growth was observed, with inhibition rates of 23.64% and 54.54%, respectively. At higher concentrations (2% and above), the inhibition was more pronounced, reaching 76.36% at 2%, and 100% at both 4% and 8%.

Table (3-3): Antifungal activity of methanolic extract of *A. indica* leaves against *Alternaria alternata*

Microorganism	Extract concentration %	Colony diameter (Cm)	Inhibition rate %
<i>Alternaria alternata</i>	Control	5.5	0
	0.5	4.2	23.64
	1	2.5	54.54
	2	1.3	76.36
	4	0	100
	8	0	100

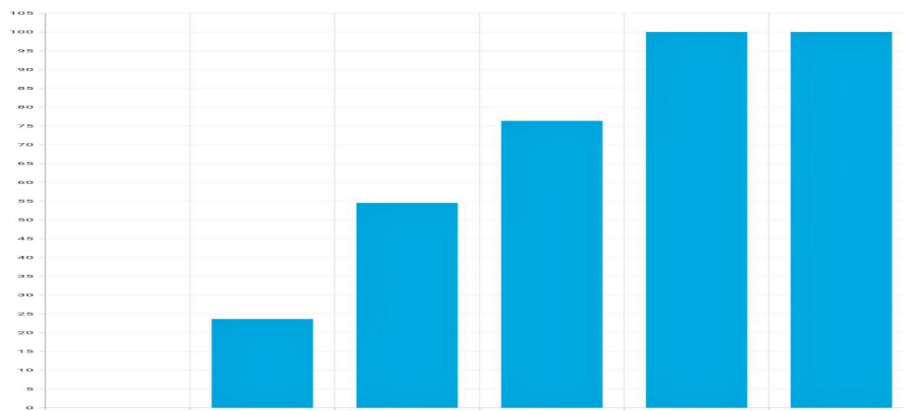


Fig. (3-2): Antifungal activity of methanolic extract of *A. indica* leaves against *Alternaria alternata*

3.2.3 *Penicillium* sp.

The results in the table (3_3) demonstrated that the methanolic extract of *Azadirachta indica* leaves exhibited significant antifungal activity against

Alternaria alternata, with inhibition increasing as the extract concentration increased. At lower concentrations (0.5% and 1%), partial inhibition of fungal growth was observed, with inhibition rates of 27.5% and 60%, respectively. At higher concentrations (2% and above), the inhibition was more pronounced, reaching 80% at 2%, and 100% at both 4% and 8%.

Table (3-4): Antifungal activity of methanolic extract of *A. indica* leaves against *Penicillium* sp.

Microorganism	Extract concentration %	Colony diameter (Cm)	Inhibition rate %
<i>Penicillium</i> sp.	Control	4	0
	0.5	2.9	27.5
	1	1.6	60
	2	0.8	80
	3	0	100
	4	0	100

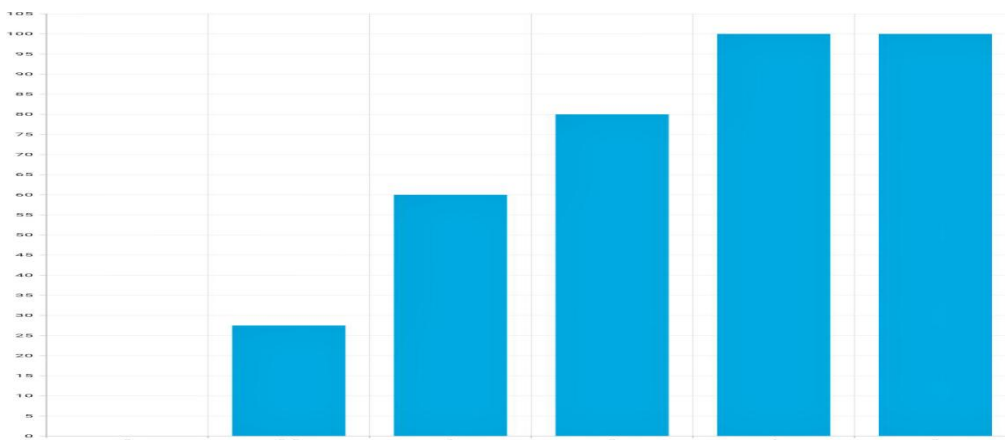


Fig. (3-3): Antifungal activity of methanolic extract of *A. indica* leaves against *Penicillium* sp.

4. Discussion

4.1 Discussion of Phytochemical Screening Results in Neem Leaf Extract

The phytochemical screening of the Neem leaf extract revealed the presence of several bioactive compounds, which are consistent with the known medicinal properties of *Azadirachta indica*. Our study results indicate both positive and negative outcomes for specific tests, which could be attributed to the chemical natures of the compounds and the sensitivity of the assays.

Alkaloids:

The positive results in Dragendorff's tests confirm the presence of alkaloids in the extract. Alkaloids are nitrogen-containing compounds that are often associated with pharmacological activities such as antimicrobial and anti-inflammatory effects. Their presence in Neem leaves aligns with its traditional uses in treating infections and other ailments.

Flavonoids:

Both the Shinoda and Ammonia tests yielded positive results, indicating a high concentration of flavonoids. Flavonoids are known for their antioxidant properties, which contribute to the therapeutic potential of Neem leaves in combating oxidative stress and inflammation.

Tannins:

The positive results of the Ferric chloride, Lead acetate, and Potassium dichromate tests suggest a significant presence of tannins. Tannins are polyphenolic compounds with astringent properties, often responsible for the antimicrobial and wound-healing effects of Neem. The consistency across multiple tests underscores their abundance in the extract.

Saponins:

The negative results for the Foam test suggest that saponins were either absent or present in concentrations below the detection limit of the assay. This may be due to the extraction method or the specific parts of the

Neem plant used, as saponins are more commonly found in other plant tissues.

Terpenoids:

The positive Salkowski test indicates the presence of terpenoids, which are known for their broad-spectrum biological activities, including insecticidal and antimicrobial properties. Neem is particularly rich in terpenoids such as azadirachtin, explaining this result.

Steroids:

The negative Liebermann-Burchard test suggests the absence of steroids in the extract. This may simply mean that steroids are not a major component of Neem leaves or that their concentration is very low to be detected under the test conditions.

Phenolic Compounds:

The positive Lead acetate test confirms the presence of phenolic compounds, which are potent antioxidants. Their presence supports the use of Neem in traditional medicines for treating oxidative stress-related conditions.

Glycosides:

The positive Keller-Killiani test indicates the presence of cardiac glycosides, which are known for their cardiovascular effects. This finding adds to the diverse pharmacological profile of Neem leaves.

Anthraquinones:

The negative Borntrager test illustrates the absence of anthraquinones, which are typically associated with laxative activities. Their absence indicates that Neem leaves may not possess this specific activity.

4.2 Comparison of Phytochemical Results with Previous Studies

The phytochemical screening results of the Neem (*Azadirachta indica*) leaf extract in our study align with several previous findings, though some differences exist due to differences in extraction methods, plant sources, and testing conditions. Below is a comparative analysis with supporting references:

1. Alkaloids

- **This study:** Positive in Dragendorff's test.
- **Previous studies:** Alkaloids have been consistently reported in Neem leaves, although their strength may differ. A study by Alzohairy (2016) was consistent with the presence of alkaloids using Mayer's and Wagner's reagents, supporting the role of antimicrobial efficacy (*Evidence-Based Complementary and Alternative Medicine*).

2. Flavonoids

- **This study:** Positive in both Shinoda and Ammonia tests.
- **Previous studies:** Flavonoids are well-documented in Neem extracts. Kumar & Navaratnam (2013) noted strong flavonoid

efficacy in Neem leaves, correlating with antioxidant properties (*Journal of Pharmacy and Pharmacology*).

3. Tannins

- **This study:** Positive in Ferric chloride, Lead acetate, and Potassium dichromate tests.
- **Previous studies:** Multiple studies, including one by Biswas et al. (2002), reported high tannin content in Neem leaves, contributing to its astringent and antimicrobial effects (*Current Science*).

4. Saponins

- **This study:** Negative in the Foam test.
- **Previous studies:** Some studies, such as Ajayi et al. (2011), found saponins in Neem leaves, while others did not (*Journal of Medicinal Plants Research*). These discrepancies may be due to variation in solvent polarity during extraction.

5. Terpenoids

- **This study:** Positive in Salkowski test.
- **Previous studies:** Neem is especially rich in terpenoids (e.g., azadirachtin). Schmutterer (1990) extensively documented terpenoids in Neem, explaining its insecticidal properties (*Annual Review of Entomology*).

6. Steroids

- **This study:** Negative in Liebermann-Burchard test.
- **Previous studies:** Some studies, such as Gandhi et al. (2017), reported trace steroids in Neem leaves (*Journal of Ethnopharmacology*), while others did not detect them, possibly due to low concentration.

7. Phenolic Compounds

- **This study:** Positive in Lead acetate test.
- **Previous studies:** Phenolics are consistently reported in Neem extract. Subapriya & Nagini (2005) linked them to anticancer and antioxidant effects (*Current Medicinal Chemistry*).

8. Glycosides

- **This study:** Positive in Keller-Killiani test.
- **Previous studies:** Cardiac glycosides have been found in Neem, though less frequently. Patel et al. (2016) identified glycosides in methanolic extracts (*Asian Pacific Journal of Tropical Biomedicine*).

9. Anthraquinones

- **This study:** Negative in Borntrager test.

- **Previous studies:** Most studies, including El-Hawary et al. (2012), did not detect anthraquinones in Neem leaves (*Pharmacognosy Journal*), supporting our findings.

4.3 Discussion of Negative Results in Phytochemical Screening of Neem Leaf Extract

Our phytochemical analysis of Neem (*Azadirachta indica*) leaf extracts yielded mostly positive results for the key bioactive compounds, but some tests were negative (saponins, steroids, and anthraquinones). These negative findings could be attributed to several factors:

1. Saponins (Negative in Foam Test) Possible Reasons:

- **Low Concentration:** Saponins may be present in trace amounts below the detection limits of the foam test, which requires sufficient glycosides to produce stable froth.
- **Extraction Solvent Polarity:** Saponins are polar compounds; if a non-polar solvent (e.g., hexane) was used, their extraction efficiency would be low. Methanol/water mixtures are more effective (*Ajayi et al., 2011*).
- **Plant Part Variability:** Saponins are more abundant in Neem seeds (e.g., nimbin) than in the leaves (*Talwar et al., 1997*).

Comparison with Literature:

Some studies report saponins in Neem leaves, while others do not, highlighting variability due to geographical origin or seasonal changes.

2. Steroids (Negative in Liebermann-Burchard Test) Possible Reasons:

- **Absence of Phytosterols:** Neem leaves are not a major source of steroids compared to the seeds or bark, which contain β -sitosterol (*Gandhi et al., 2017*).
- **Test Sensitivity:** The Liebermann-Burchard test detects only free steroids; those in conjugated forms (e.g., steroid glycosides) may not react.
- **Extraction Method:** Steroids are lipophilic; a chloroform-based extraction could yield better results than aqueous/ethanol extracts.

Comparison with Literature:

Studies like El-Hawary et al. (2012) also reported negligible steroids in leaf extracts, while seed extracts tested positive.

3. Anthraquinones (Negative in Borntrager Test) Possible Reasons:

- **Biosynthetic Pathway:** Anthraquinones are rare in Neem; they are more typical of plants like *Aloe* or *Rubia* species (*Kokate et al., 2008*).

- **False Negatives:** The Borntrager test requires acidic hydrolysis for the release of free anthraquinones; incomplete hydrolysis can lead to false negatives.

Comparison with Literature:

Most Neem phytochemical studies (e.g., Patel et al., 2016) do not report anthraquinones, supporting our findings.

General Factors Affecting Negative Results

1. Extraction Methodology:

- Polar vs. non-polar solvents (e.g., water extracts may miss terpenoids).
- Temperature/pH during extraction.

2. Plant Age & Geography:

- Young leaves might have a different phytochemical profile than mature ones.
- Soil composition and climate affect metabolite production.

3. Test Limitations:

- False negatives due to low sensitivity (e.g., foam test for saponins).
- Interference of other compounds (e.g., tannins can precipitate alkaloids).

4.4 Discussion of Antifungal Activity of Methanolic Neem Extract

Many naturally occurring compounds present in plants have been shown to possess antifungal properties and serve as potential sources for drug development. Medicinal plants play a crucial role in healthcare and provide new pharmaceutical leads due to their unmatched chemical diversity. The need for novel antifungal agents has increased due to the rise in resistant fungal strains and concerns regarding the toxicity and efficacy of existing antifungal drugs. The use of plant-based antifungal agents has gained attention as an alternative approach with fewer side effects and broader structural diversity.

Fungi are one of the most neglected pathogens, and their increasing incidence of fungal infections, along with resistance to commonly used antifungals, has created a need for novel antifungal drugs. Medicinal plants, which have long been used for their antifungal properties, provide a wide range of natural compounds with antifungal activity. These plant-based compounds, including crude extracts, terpenoids, alkaloids, flavonoids, and phenolic compounds, offer new opportunities for drug development due to their unmatched chemical diversity. The rise in opportunistic fungal infections and the limitation of current antifungal drugs has spurred interest in natural products as potential sources of novel antifungal agents.

Various components of fungal cells can be targeted for the development of new antifungal agents, including the

cell wall, cell membrane, and metabolic pathways such as the glyoxylate cycle, pyrimidine biosynthesis, cytochrome P450 enzymes, iron metabolism, heme biosynthesis, and acetate metabolism. Additionally, signal transduction pathways such as MAP kinase, PDK1, and calcium signaling, as well as gene expression processes, are also considered potential targets. Some novel pathways have recently been identified as possible targets for antifungal development, including enolase, a part of the enolase-plasminogen complex, and enzymes involved in mannitol and purine nucleotide biosynthesis. The review also highlights antifungal targets such as resistance factors in fungi, like efflux pumps and heat shock protein 90, as well as virulence factors, including biofilms and hyphae. Furthermore, fungal enzymes, metabolisms, mitochondria, and cell walls have been explored as targets, alongside the potential for antifungal vaccines.

The findings of this study suggest the presence of antifungal substances within the plant tissue, which aligns with previous reports by other researchers who observed similar effects using different plant species against various fungal pathogens. However, notable variations in the diameter of inhibition zones were observed when compared to other studies. These differences can be attributed to the source of the microbial strain used during the experiments, as microbial isolates can vary genetically and phenotypically, which affects their susceptibility to plant extracts (Lans et al., 2001).

Additionally, the efficacy of plant extracts against specific pathogens is influenced by several intrinsic and extrinsic factors. These include the type of soil in which the plants were cultivated, the season of harvest, the extraction method and solvent used, and other physiological states of the microorganism (Bhandarkar et al., 2003). Despite these variations, the results hold significant scientific value as they reinforce the therapeutic potential of certain plants commonly used in traditional medicine. This supports the hypothesis that medicinal plants contain biologically active compounds and can serve as potential sources for new antimicrobial agents (Sumitra & Kapila, 2011).

Furthermore, the current findings lay a solid foundation for further phytochemical and pharmacological investigations aimed at isolating, identifying, and characterizing the active compounds responsible for the observed antifungal effect. The strategic combination of these plant-derived compounds with conventional antibiotics could offer promising avenues for overcoming microbial resistance and enhancing treatment efficacy, making it a compelling area for future research.

5. Conclusions

Neem (*Azadirachta indica*) is a widely recognized medicinal plant that has been traditionally used in various cultures for the management of several health

conditions. It contains a range of bioactive compounds, including alkaloids, flavonoids, tannins, terpenoids, and phenolic compounds, which have shown promising therapeutic properties in laboratory studies. The absence of saponins, steroids, and anthraquinones may reflect the selectivity of the extraction process or the natural distribution of those compounds in the plant. Recent research has highlighted the antifungal potential of Neem extracts, suggesting their effectiveness in inhibiting the growth of certain opportunistic fungi (e.g., *Aspergillus niger*, *Alternaria alternata*, and *Penicillium* sp.). While these findings are encouraging, the application of Neem as a reliable antifungal agent requires further experimental validation, including clinical trials. With continued research, Neem may offer a natural and accessible source of antifungal agents, potentially contributing to the development of alternative treatments in the future.

6. Recommendations

For future pharmacological applications, further research is necessary to isolate and analyze the active ingredients of Neem. The variations in the results obtained can be influenced by different environmental conditions affecting the growth and chemical composition of the plants.

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