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Phytochemical Analysis, Antibacterial Activity, and GC-MS Characterization of Methanolic Extract of *Azadirachta indica* from Nuwakot, Nepal

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Abstract

Azadirachta indica, known as *Neem*, is renowned for its diverse pharmacological properties. The present work was carried out to examine the bio-efficacy of the leaves of *Azadirachta indica* by examining phytochemical screening, GC-MS analysis, and antibacterial properties. Phytochemical screening of the extract revealed the presence of bioactive secondary metabolites like alkaloids, carbohydrates, flavonoids, tannins, steroids, and phenolic compounds. The GC-MS analysis revealed the presence of 30 compounds, among which 3- Oxatricyclo[20.8.0.0(7,16)triaconta-1(22),7(16),9,13,23,29-hexene, Phytol, 9,12,15-Octadecatrien- 1-ol, (Z, Z, Z), 3E,5E,7E-6-methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-one, n- Hexadecanoic acid, *dl*-alpha,-Tocopherol, and Hexanedioic acid were found as major compounds. The antibacterial activity of the *Azadirachta indica* was evaluated against the Gram-positive bacterium *Bacillus subtilis* and the Gram-negative bacterium *Enterobacter aerogenes* using the filter disc diffusion technique on the Muller Hilton Agar plate (MHA). A significant zone of inhibition of 7 mm and 11 mm was observed, indicating the extract's efficacy against both Gram-positive and Gram-negative bacteria. These findings underscore the potential of *Azadirachta indica* from *Nuwakot* district Nepal, as a source of natural bioactive compounds.

Keywords: *Azadirachta indica, pharmacology, phytochemical screening, GC-MS, antibacterial*

1. Introduction

The *Azadirachta indica (neem)* tree, scientifically known as *Azadirachta indica*, gets its name from the Sanskrit word 'nimba' which implies "throw away" because of its bitter taste, making it unpleasant to eat directly. However, the word "Nimba" also connects to another Sanskrit word, "Aristha", which means reliever of sickness (Janika Sitasiwi et al., 2018). This shows its importance even in the Veidic system of medicine(Devi & Sharma, 2023).

Azadirachta indica is an important member of the family Meliaceae, which contains alkaloids, flavonoids, polyphenols, and triterpenoids as the major bioactive compounds that play an important role in disease management through the modulation of variousgenetic pathways and other activities. *Neem* is an exotic plant that is believed to have been introduced to Nepal by traders and farmers bringing the seeds from India after discovering their multiple benefits and by other agents of seed propagation. However, due to a lack of knowledge, its use has been limited to shade, toothbrush sticks, medicinal herbs, pest repellents, fodder, firewood, timber, and animal bedding materials. Studies conducted previously using GC-MS showed *Azadirachta indica* to contain nimbin, nimbolide, nimbidin, and limolides as major bioactive compounds (Alzohairy, 2016; Mondall et al., 2009).

All these bioactive compounds have been proven promising for leprosy, intestinal worms, eye disorders, a bloody nose, loss of appetite, stomach upsets, skin ulcers, diseases of the heart and blood vessels (cardiovascular disease), fever, diabetes, gum disease (gingivitis), and liver problems (Meena et al., 2014). Also, their leaf causes abortions. Every part of the tree is bitter and contains compounds with proven antiviral, antiretroviral, anti-inflammatory, antiulcer, antifungal, antibacterial, anti-plasmodial, antiseptic, antipyretic, and anti-diabetic properties (Dubey & Kashyap, 2014). The present study focuses on the phytochemical analysis, GC-MS analysis, and antibacterial activity study of the methanol extract of the *Azadirachta indica (Neem)* plant from the Nuwakot district of Nepal.

As far as the author's present knowledge is concerned, no study on the bio-efficacy of this plant from the Nuwakot district has been carried out. Although *Azardirachta indica* has been extensively studied for its medicinal properties in different regions, this research specifically investigates the chemical framework and pharmacological potential of the species from Nuwakot. It provides a perception of the regional differences in bioavailable potents and opens up possibilities for drug development, reinforce the understanding of plant-based medicines, especially from under-researched geographical areas.

2. Materials and methods

2.1 Collection of plant materials

The leaves of *Azadirachta indica* were collected from Bidur, Nuwakot district, latitude $27^0$52'48.89''N and longitude 85 ^{0}7'56.56''E, in October 2019. The collected plant material was identified as *Azadirachta indica* by the National Herbarium and Plant Laboratories (NHPL); Godavari, Ministry of Forests and Environment, Department of Plant Resources, Nepal.

2.2 Preparation of plant extract

The collected sample was washed and rinsed with normal running tap water multiple times, followed by sterile double-distilled water. The sample was allowed to air dry for three weeks in a shady place before being ground into a coarse powder using a mechanical grinder and then stored in a polyethylene bag for future use. For the extraction, 50 g of coarse powder was suspended in 300 mL of methanol in a Soxhlet apparatus at 40 °C for about 5 hours. After this, the extract was transferred to a beaker, and the solvent was evaporated using a water bath at 40 °C. Once fully dried, the required extract was obtained for subsequent analysis.

2.3 Phytochemical screening

The phytochemical screening of *Azadirachta indica* was performed using the standard method mentioned in the literature (Kancherla et al., 2019). By using specific reagents, the main group of compounds present in the leaf of *Azadirachta indica* were analyzed.

2.3.1 Tests for alkaloids

To the extract, dilute hydrochloric acid was added, shaken well, and filtered. With the filtrate, the following tests were performed.

Mayer's reagent test: To 3 mL of filtrate, a few drops of Mayer's reagent were added along the sides of the tube. The formation of a creamy precipitate indicates the presence of alkaloids.

Wagner's test: To 2 mL of filtrate, a few drops of Wagner's reagent were added to a test tube. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

2.3.2 Tests for carbohydrates

Molisch test: 2 mL of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 mL of concentrated sulphuric acid was added carefully along the sides of the test tube. The formation of a violet ring at the junction indicates the presence of carbohydrates.

2.3.3 Tests for reducing sugars

Fehling's test: 1 mL of aqueous extract, 1 mL of Fehling's A, and 1 mL of Fehling's B solutions were added to a test tube and heated in a water bath for 10 minutes. The formation of a red precipitate indicates the presence of reducing sugar.

Benedict's test: Equal volumes of Benedict's reagent and extract were mixed in a test tube and heated in a water bath for 5-10 minutes. The solution appears green, yellow, or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

2.3.4 Tests for flavonoids

Alkaline reagent test: The extract was treated with a few drops of sodium hydroxide solution separately in a test tube. The formation of an intense yellow color, which becomes colorless with the addition of a few drops of dilute acid, indicates the presence of flavonoids.

Lead acetate test: The extract with a few drops of lead acetate solution. The formation of a yellow precipitate indicates the presence of flavonoids.

2.3.5 Tests for glycosides

Borntrager's test: To 3 mL of test solution, dilute sulphuric acid was added, boiled for 5 minutes, and filtered. To the cold filtrate, an equal volume of benzene or chloroform was added and it was shaken well. The organic solvent layer was separated and ammonia was added to it. The formation of pink to red color in the ammonical layer indicates the presence of anthraquinone glycosides.

2.3.6 Tests for tannin and phenolic compounds

Ferric chloride test: A small amount of extract was dissolved in distilled water. To this solution, 2 mL of 5% ferric chloride solution was added. The formation of blue, green, or violet color indicates the presence of phenolic compounds.

2.3.7 Lead acetate test

A small amount of extract was dissolved in distilled water. To this solution, a few drops of lead acetate solution were added. The formation of a white precipitate indicates the presence of phenolic compounds.

2.3.8 Test for saponin

Froth test: The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of a layer of foam indicates the presence of saponin.

2.3.9 Test for steroids

Salkowski's test: The extract was treated with chloroform and filtered. The filtrate was added with a few drops of concentrated sulphuric acid, shaken, and allowed to stand. If the lower layer turns red, sterol is present.

2.4 GC-MS analysis

The GC MS-QP 2010 Ultra SHIMADZU instrument at the National Forensic Science Laboratory, Dhangadhi, Nepal was used for GC-MS analysis. The methanolic extract of *Azadirachta indica* was injected into the GC inlet, maintaining a column flow rate of 0.95 mL/min and a purge flow of 3 mL/min. The ion source temperature was set at 200 °C, with the interface temperature at 280 °C. Mass spectra were scanned at a speed of 1000, covering a range from m/z 30 to 500. The chemical constituents present were identified by comparing them with the NIST Library.

2.5 Antibacterial activity

The antibacterial activity of *Azadirachta indica* leaves was evaluated using the filter disc diffusion method on a Muller Hinton Agar plate (MHA). To make the MHA plate, 0.5 g of media were dissolved in 250 mL of distilled water, brought to a boil, and then autoclaved for 15 minutes at 121 degrees Celsius and 15 lb of pressure. After that, the medium was chilled to around 50 °C,poured into 20 mL plates, and allowed to solidify. To remove any surface moisture, the sterilized MHA plates were dried. After dipping sterile cotton swabs into the *Bacillus subtilis* and *Enterobacter aerogenes* produced inoculums, surplus inoculum was removed by pushing and spinning the swabs against the tube's upper sidewall above the liquid level. Next, the inoculums were dispersed equally throughout the plates. MHA plates with the corresponding test strains were covered with a sterilized Whatman filter paper disc (0.6 mm) impregnated with 10 µL of the test solution; methanol was used as a control test. On the same plate, the separate activities of the solvent and extract were tested at the same time on the different filter paper discs. The plates were then left for half an hour with the lid closed so that the extract diffused through the whole medium. These plates were incubated for 24 hours at 37 °C and a zone of inhibition was observed.

3. Results and discussion

3.1 Phytochemical analysis

To observe the presence of various secondary metabolites in the methanol extract, phytochemical screening was performed using standard phytochemical screening methods. The results from the phytochemical screening have been displayed in Table 1.

Table 1: Phytochemical screening of the Leaves of *Azadirachta indica*

S. N.	Phytochemicals	Methods/ tests	Result
1	Alkaloids	Mayer's Reagent test	+
		Wagner'S test	+
2	Carbohydrates	Molisch Test	+
3	Reducing Sugars	Fehling'S test	+
		Benedicts test	+
4	Flavonoids	Alkaline Reagent Test	+
		Lead Acetate Test	+
5	Glycosides	Bontrager's Test	+
6	Tannins and Phenolic compounds	Ferric Chloride test	+
7	Saponin	Froth test	+
8	Steroids	Salkowski''s test	+

The phytochemical screening revealed the presence of all the bioactive secondary metabolites like alkaloids, phenols and tannins, flavonoids, steroids, glycosides, terpenoids, and saponins. These results vouch that the leaf of *Azadirachta indica* is a potential source of bioactive compounds, which can be applied as natural drugs.

3.2 GC-MS analysis

The methanol extract of *Azadirachta indica* was subjected to GC-MS analysis. The chromatogram of the methanolic extract showed different peaks at different retention times. The list of the compounds with retention time and area % have been shown in Table 2. The chromatogram of the same has been displayed in Figure 1.

Table 2: Chemical constituents of methanolic leaf extract as obtained from GC-MS analysis

Peak	Retention Time	Area %	Compound
1.	20.803	17.46	3-Oxatricyclo[20.8.0.0(7,16)triaconta-1(22),7(16),9,13,23,29-hexene
2.	10.164	16.17	Phytol
3.	10.476	15.99	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)
4.	18.864	8.39	3E,5E,7E-6-methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-one
5.	9.299	6.45	n-Hexadecanoic acid
6.	17.901	4.98	dl- alpha-Tocopherol
7.	6.710	4.36	Hexanedioic acid
8.	24.355	3.89	Lycoxanthin
9.	19.761	2.94	Resorcinol,2-p-mentha-1,8,-dien-3-yl-5-pentyl

10.	8.318	2.69	Neophytadiene
11.	19.446	2.62	Stigmasterol
12.	6.937	2.31	Cyclooctasiloxane, hexadecamethyl-
13.	22.583	2.13	2H-1-Benzopyran-5-ol, 2- methyl-2-(4-methyl
14.	20.125	1.43	gamma,-Sitosterol
15.	11.025	0.90	Heptasiloxane,hexadecamethly-
16.	8.483	0.80	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
17.	13.876	0.74	1,2-Benzenedicarboxylic acid, dioctyl ester
18.	8.617	0.73	Neophytadiene
19.	15.518	0.58	Squalene
20.	14.790	0.51	Octocrylene
21.	8.083	0.50	Cyclohexasiloxane,dodecamethyl-
22.	18.058	0.49	Heptasiloxane, Hexadecamethyl-
23.	5.432	0.47	Garama-Elemene
24.	15.108	0.43	1,3-Benzenedicarboxylic acid bis(2-ethylhex
25.	7.992	0.42	1-Heptadecene
26.	8.931	0.40	Hexadecanoic acid, methyl ester
27.	9.616	0.39	3,9-Dioxa-2,10-disilaundecane,2,2,10,10,ter
28.	17.256	0.37	Nanocosane
29	5.633	0.22	Cycloheptasiloxane, tetradecamethyl
30	15.327	0.21	Cyclononasiloxane, octadecamethyl

GC-MS analysis revealed the presence of 30 different peaks with retention times ranging from 5.432 to 24.355 sec. These peaks correspond to bioactive substances, and their identification was achieved by comparing their mass spectrum fragmentation patterns with those of known substances listed in the NIST collection. Seven predominant peaks were obtained, occupying 74.42% of the total area, which were identified as 3-Oxatricyclo [20.8.0.0(7,16)triaconta-1(22),7(16),9,13,23,29- hexene, Phytol, 9,12,15-Octadecatrien-1-ol, (Z, Z, Z), 3E,5E,7E-6-methyl-8-(2,6,6-trimethyl-1- cyclohexenyl)-3,5,7-octatrien-2-one, n-Hexadecanoic acid, dl-alpha,(-)-Tocopherol, an Hexanedioic acid with 17.46%, 16.17%, 15.99%, 8.39%, 6.45%, 4.98%, and 4.96% area. The compound phytol is known for its anti-inflammatory, antinociceptive, antioxidant, and anti-allergic activities (Ryu et al., 2011; Santos et al., 2013). Recent research highlights its remarkable immunostimulant properties, surpassing those of many commercial adjuvants in promoting long-term memory and enhancing both innate and acquired immunity (Chowdhury & Ghosh, 2012). Additionally, 9, 12, 15-Octadecatrien-1-ol, (Z, Z, Z) (Linolenyl alcohol) functions as an effective antibacterial agent (Osman et al., 2023) . The n-hexadecanoic acid (palmitic acid) exhibits notable antibacterial and cholesterol-lowering effects (Casillas-Vargas et al., 2021). The compounds Methyl-8-(2,6,6- trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-one and 3-Oxatricyclo [20.8.0.0(7,16)] triaconta- 1(22),7(16),9,13,23,29-hexene are distinct from those identified in previous studies by Balasubramanian et al. (Sathyamurthy, 2019). It may be due to the different origins of the

plants in these studies. The secondary metabolites modify their constituents according to their environment, altitude, and region to adapt to their surroundings. These results reveal a great potential of this plant as a source of several drugs.

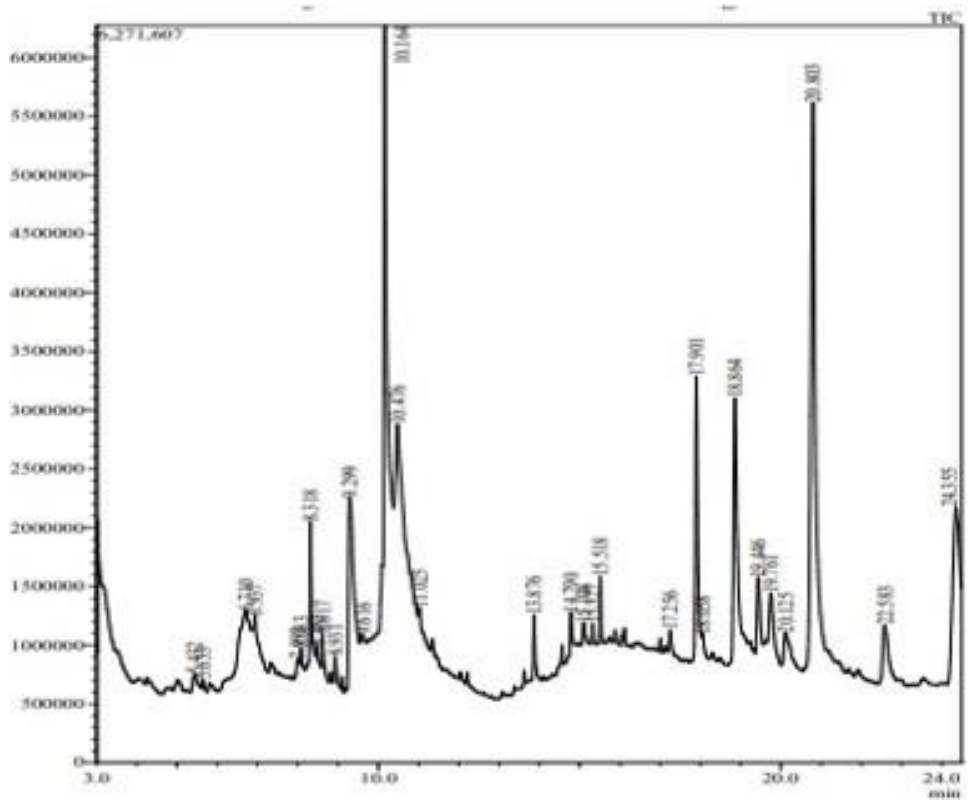

Fig. 1. Chromatogram of methanol extract *of Azadirachta indica*

3.3 Antibacterial activity

The antibacterial activity analysis of the *Azadirachta indica* methanolic extract was conducted to assess the antibacterial activity against Gram-positive bacteria *Bacillus subtilis* and Gram-negative bacteria *Enterobacter aerogenes*. The antibacterial activity was analyzed by measuring the zone of inhibition with kanamycin as a positive control. The results of the same has been shown in Figure 2 and Table 3.

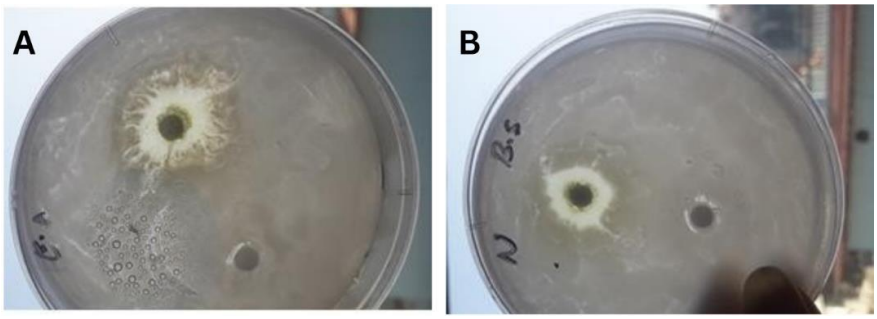

Fig. 2. Antibacterial activity of the methanolic extract in *(A) Enterobacter aerogenes, (B) Bacillus subtilis*

Compared to the *Bacillus subtilis* whose zone of inhibition was 7 mm, Enterobacter aerogenes showed a greater zone of inhibition of 11 mm, which showed the extract's special antibacterial activity against Gram-negative bacteria.

Table 3: Antibacterial activity of methanolic extract of *Azadirachta indica.*

S. N	Tested bacteria	Zone of inhibition
1	*Enterobacter aerogenes*	11 mm
2	*Bacillus subtilis*	7 mm

4. Conclusion

The present study explored the phytochemical profile, GC-MS analysis, and antibacterial properties of *Azadirachta indica*. The methanolic extract of *Azadirachta indica* leaves was found to contain alkaloids, carbohydrates, flavonoids, tannins, steroids, and phenolic compounds, all contributing to its bio-efficacy. The GC-MS analysis identified 30 compounds, with seven compounds accounting for 74.42% of the total composition. These key compounds include 3- Oxatricyclo[20.8.0.0(7,16)triaconta-1(22),7(16),9,13,23,29-hexene, Phytol, 9,12,15- Octadecatrien-1-ol, (Z,Z,Z), 3E,5E,7E-6-methyl-8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7- octatrien-2-one, n-Hexadecanoic acid, dl-alpha-Tocopherol, and Hexanedioic acid. Furthermore, the methanolic extract demonstrated effectiveness against both Gram-positive *Bacillus subtilis* and Gram-negative bacteria *Enterobacter aerogenes*. However, the extract was found to be more effective against Gram-negative bacterium *Enterobacter aerogenes*.

These results unveil the potential of *Azadirachta indica* from Nuwakot district, Nepal as a source of medically important compounds. The plant can be used as a source of several drugs.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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