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Azadirachta indica leaf and bark methanolic and aqueous extracts: phytochemical analysis and antibacterial activity against *Escherichia coli*

Rachna Bharti & Rimjhim Sheel*

Department of Botany, Ganga Devi Mahila College, Patliputra University, Patna, Bihar, India

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Abstract- Neem, or *Azadirachta indica*, is a tropical evergreen tree that grows quickly and is mostly found in America, Africa, and India. It is known in Sanskrit as 'arishtha,' meaning 'perfect, complete, imperishable, and healer of diseases.' India has supported neem tree scientific study in an attempt to boost business interests and revive traditional customs. Among the phytochemicals identified in the *A. indica* leaf and bark extracts employed in this study were flavonoids, phenolics, tannins, saponins, alkaloids, and steroids. Phenolics and tannins were present in differing amounts in the methanolic and aqueous extracts. The bark and leaves of *A. indica* exhibited antibacterial activity against *E. coli* in both methanolic and aqueous preparations. The aqueous extract had better antibacterial activity than the methanolic extract. The antibacterial activity of *A. indica* extracts was concentration-dependent, as evidenced by the various zones of inhibition with concentration variations.

Keywords: *Azadirachta indica*, *Escherichia coli*, antibacterial, phytochemical

INTRODUCTION

Azadirachta indica, commonly known as neem, is a fast-growing tropical evergreen tree primarily found in India, Africa, and America. In Sanskrit, it is called 'arishtha,' which translates to 'perfect, complete, imperishable, and reliever of sicknesses.' India has promoted scientific research on the neem tree as part of its efforts to revitalize traditional practices while enhancing commercial interests.[1]

In India, neem is referred to by various names, such as the "Divine Tree," "Wonder Tree," "Heal All," "Materia Medica," "Free Tree of India," "Nature's Drugstore," "Village Pharmacy," and "Panacea for All Diseases".[2-4] For centuries, Indians have utilized neem in various forms, including using neem leaf juice for skin disorders, drinking neem tea as a tonic, cleaning teeth with neem twigs, and using neem leaves to repel pests. Its benefits are documented in ancient texts like the 'Charak Samhita' and 'Susruta Samhita.'

The *Azadirachta indica* tree exhibits numerous properties, including antimicrobial, antimalarial, insecticidal, antiviral, anti-inflammatory, analgesic, hypoglycemic, anti-ulcer, antipyretic, anticarcinogenic, hepatoprotective, antioxidant, antifertility, anxiolytic, molluscicidal, acaricidal, and antifilarial effects.[5-7] In recent years, the benefits of neem have gained recognition worldwide, particularly for its medicinal and insecticidal properties.[8]

Almost all parts of the neem tree serve various purposes in agriculture. Since these trees flourish in both urban and rural settings, communities can enhance their economic conditions by producing products from neem

*Corresponding author :
Phone : 9334331800
E-mail : rimjhimsheelppu@gmail.com

seeds and leaves in a short time. The leaves of neem trees, in particular, are widely used across various agricultural industries.[9] Neem also contains bioactive components with considerable health benefits.[10,11] One of the most abundant compounds in neem leaves is azadirachtin, which has been shown to be safe for humans.[12] While numerous studies have summarized the therapeutic value of various components of *A. indica*, this particular study focused on the effects of total phenolic and flavonoid contents, as well as the antioxidant potential of *A. indica* seeds collected from rural and suburban areas of Patna in the Gangetic plains, Northern India.

MATERIALS & METHODS

Collection of Plant Materials

In January 2025, *A. indica* leaves and bark were gathered at the Ganga Devi Mahila Mahavidyalaya. To get rid of any dirt, the plant material was first soaked in regular water for two to five minutes. It was then rinsed twice with deionised water. Following washing, the material was allowed to dry at room temperature in the dark while being closely monitored to avoid contamination. In the end, an electric grinder was used to grind it. After that, the powdered material was kept for future research in sealed vials.

Preparation of extract

In a conical flask, roughly 10 grammes of powdered *A. indica* leaves and bark were individually steeped in 100 millilitres of distilled water and methanol. After that, the mixes were shaken at 100 rpm for 72 hours at 25ºC in an orbital shaking incubator. The mixtures were then passed through Whatman No. 1 filter paper, and the filtrates were centrifuged for 10 minutes at 3000 rpm. For a later activity analysis, the supernatant was gathered and kept in a refrigerator at 4°C.

Chemicals

Gallic acid, quercetin, ascorbic acid, sodium carbonate, methanol, aluminium chloride, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azino-bis 3-ethylbenzo thiazoline-6-sulphonic acid (ABTS), 2,4,6-tripyridyl-S-triazine (TPTZ), Folin-Ciocalteu Phenol Reagent, and all other chemicals used in this study were purchased from Merck Pvt. Ltd. in India. Every chemical used in the examination was of analytical quality.

Phytochemical screening

The bark and leaves of *A. indica* were subjected to phytochemical screening using the procedures described by Ayoola *et al.* (2008)[13]. Terpenoids, saponins, flavonoids, tannins, and phenols were screened for in the extracts from various plant components.

Test for terpenoids (Salkowski test)

Two millilitres of chloroform were added to 0.5 grammes of each plant component extract. After that, three millilitres of sulphuric acid concentrate were carefully added to create a layer. Terpenoids were present because of the interface's reddish-brown colouring.

Test for flavonoids

Five millilitres of diluted ammonia and one millilitre of concentrated sulphuric acid were added to 0.5 millilitres of filtrate of each plant component extract. The solution's yellow hue, which goes away after it stands, indicated the presence of flavonoids.

Test for saponins

Five millilitres of distilled water were added to 0.5 grammes of each plant extract in test tubes. After giving the mixture a good shake, a stable, long-lasting froth was looked for. After adding three to four drops of olive oil to the foam and shaking it violently once more, the production of emulsions was monitored.

Test for tannins

In each test tube, roughly 0.5 g of each plant extract was cooked in 10 ml of water before being filtered. After adding a few drops of 0.1% ferric chloride, the presence of tannins was determined by looking for the creation of a brownish-green or blue-black colour.

Test for phenols (ferric chloride test)

Two millilitres of distilled water were used to dissolve small amounts of alcoholic and aqueous extracts, and a few drops of a 10% aqueous ferric chloride solution were then added. The presence of phenols was indicated by the production of a blue or green colour.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The techniques described by Ebrahimzadeh *et al.* (2008)[14] were used to determine the flavonoid content of *A. indica's* leaves and bark. This procedure involved mixing 1.5 ml of methanol with 0.5 ml of each extract, then adding 0.1 ml of 10% aluminium chloride and 0.1 ml of 1M potassium acetate. The mixture was then supplemented with 2.8 millilitres of distilled water. After that, this mixture was incubated for half an hour at room temperature. A spectrophotometer was used to test the resultant solution's absorbance at 415 nm. Using quercetin

as a reference, the total flavonoid content was reported in mg/g as quercetin equivalent. Every determination was carried out in duplicate.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The technique outlined by Kim *et al.* (2003)[15] was used to assess the total phenolic content of *A. indica's* leaves and bark. This procedure involved mixing 4.5 ml of distilled water with 0.5 ml of the extract, then adding 0.5 ml of Folin-Ciocalteu reagent. The mixture was thoroughly mixed and then left to stand for five minutes at room temperature. The mixture was then mixed with 2 millilitres of distilled water and 5 millilitres of 7% sodium carbonate. After that, the solution was incubated at 23°C for 90 minutes. A spectrophotometer was used to measure the absorbance at a wavelength of 750 nm. As a standard, the total phenolic content was stated in milligrammes per gramme of gallic acid equivalent. Three separate measurements of the total phenolic content were made.

DETERMINATION OF 1, 1 - DIPHENYL - 2 - PICRILHYDRAZYL RADICAL SCAVENGIN ACTIVITY

The Von Gadow *et al.* (1997)[16] method was used to evaluate the DPPH radical scavenging activity of *A. indica's* leaves and bark. 50 µl samples of each plant extract were put in a different cuvette for this technique, and then 2 ml of a 6×10^{-5} M methanolic solution of DPPH radical was added. At 517 nm, the absorbance was measured right away. Following a 16-minute incubation period, each sample's absorbance was tested again. The standard was an ascorbic acid methanolic solution at a concentration of 1 µl/mg. Every determination was made three times. The colour reduction % was used to represent the DPPH radical scavenging activity. The Yen and Duh (1994)[17] formula was used to determine the sample extracts' % inhibition of the DPPH radical. At time zero, the control measurement was made to reflect the situation prior to the plant extracts' antioxidants starting to work.

% inhibition = {(*AC* (0) - *AA* (*t*)))/*AC* (0)} × 100

Where,

***AC* (0)** = Absorbance of control at time, $t = 0$ min

AA (*t*) = Absorbance of antioxidant at time, $t = 16$ min.

ANTIMICROBIAL ACTIVITY TEST

Test organisms

The antimicrobial activity was assessed using a bacterial cultures: *E. coli* (MTCC40) from The Microbial Type Culture Collection and Gene Bank (MTCC), a national facility founded in 1986 and supported by the Government of India's Department of Biotechnology (DBT) and Council of Scientific and Industrial Research (CSIR).

Preparation of Bacterial Isolates

According to Beyene and Tsegaye (2011)[18], the bacteria were kept at 37°C in a controlled acidity environment while being fed the right amount of food to encourage their growth.

Inoculum Preparation

Saline & 0.5 McFarland turbidity principles were utilised to compare turbidity because this study employed the direct state suspension method. Saline was used to balance the turbidity until it matched the 0.5 McFarland turbidity measurements. The suspension and the 0.5 McFarland turbidity gauges were held in front of a light source against a white foundation with distinct dark lines to complete this.[19]

Inoculation

For *E. coli*, Muller-Hinton agar plates were used. The agar surface was carefully checked for excessive wetness prior to inoculation, and the plates were checked for excessive dryness (a wrinkled surface indicates excessive dryness). The suspension of microscopic organisms was put onto a sterilised cotton swab. By inserting the swab into the suspension tube for the microscopic organisms, the abundance inoculum was released. Before using the test plant removes, the media was inoculated by swabbing the agar surfaces in two directions at a 90-degree edge to each surface and the third line at a 45-degree edge. This was done for a total of 20 minutes to promote assimilation of the abundant inoculum.[20]

Disc Diffusion Method

The bactericidal qualities were tested using the disc diffusion method. The extract was applied to the disc, allowed to permeate the disc, and then set on the bacterial agar medium.[21] To compare the results with those of experimental ethanol and water extracts, Amikacin 5 µg standard discs were used as the positive control against *E. coli.*

Antimicrobial Activity Determination

The aqueous and methanolic extracts of *A. indica* were evaluated on *E. coli* culture strains on agar plates at various concentrations (10 mg/mL, 20 mg/mL, and 30 mg/mL). Zones of inhibition during incubation demonstrated

the studied pathogenic organisms' susceptibility to aqueous and methanolic extracts. Additionally, the Vernier scale was used to measure the zone of inhibition's (ZOI) diameter in millimetres.[22] To reduce error, the zone of inhibition was measured three times for each extract concentration, and the mean was noted. After that, the statistical analysis was carried out. The agar-disc diffusion method was used to calculate the crude extract's Minimum Inhibitory Concentration (MIC). According to Margaret *et al.* (2020)[23], MIC is the lowest concentration at which a distinct zone of microbial growth inhibition will be visible.

RESULTS

Phytochemical screening

Several phytochemicals were found in both methanol and aqueous extracts after preliminary phytochemical screening, as indicated in Table 1. Similar phytochemicals, such as alkaloids, glycosides, carbohydrates, phenols, flavonoids, steroids, proteins, and amino acids, were present in both extracts.

Total phenolic content

The Folin-Ciocalteu reagent was used to calculate the total phenol (TPC) content in terms of gallic acid equivalent (standard curve equation: y=0.0035x + 0.0989 R2 = 0.9732). The aqueous extract of leaves had a total phenolic content of 144.28 ± 2.5 mg GAE/g, which is significantly higher than the methanol extract, which was found to be 86.45 ± 2.21 mg GAE/g. In contrast, the aqueous extract of bark had a total phenolic content of 89.05 ± 1.42 mg GAE/g, which is significantly higher than the methanol extract, which was found to be 66.2 ± 2.06 mg GAE/g.

Antioxidant activity

As the concentration rose, so did the extracts' and standard ascorbic acid's capacity to scavenge free radicals. The aqueous and methanol extracts of *A. indica* leaves showed the highest % inhibition of DPPH free radical at 517 nm, with the aqueous extract showing 82.35 ± 2.46 µg/ml and the methanol extract showing 81.60 ± 1.8 µg/ml. *A. indica* bark aqueous and methanol extracts showed the highest % suppression of DPPH free radicals at 67.5 ± 1.7 µg/ml and 88.20 ± 1.6 µg/ml, respectively.

Determination of total flavonoid content

The techniques outlined by Ebrahimzadeh *et al.* (2008)[14] were used to ascertain the content of total flavonoids. The aqueous extract of leaves had a total flavonoid concentration of 143.28 ± 2.7 mg QE/g, which is much greater than the methanol extract of leaves, which had a content of 90.45 ± 1.88 mg QE/g of sample. The amount of total flavonoids in the bark's aqueous extract was 95.67 ± 1.96 mg QE/g, while the amount in the methanol extract was 71.12 ± 2.mg QE/g of the sample. The standard unit of measurement for the total flavonoid content was mg QE/g of quercetin equivalent.

Table 1: Qualitative chemical profiling analyses of *A. indica*

Sl. No.	Name of the phytochemical	Methanol extract		Aqueous extract	
	A. indica	LEAVES	BARK	LEAVES	BARK
1.	Alkaloids	Present	Present	Present	Present
2.	Flavonoids	Present	Present	Present	Present
3.	Phenolic compounds	Present	Present	Present	Present
4.	Saponins	Present	Present	Present	Present
5.	Tannins	Present	Present	Present	Present
6.	Terpenoids	Present	Present	Present	Present

Table 2: Yield and assays of total phenol, total flavonoid, and DPPH radical scavenging activity of *A. indica* extract

Solvent	Plant Parts	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)	DPPH radical scavenging activity IC_{50} (µg/mL)
Aqueous	Leaves	144.28 ± 2.5	143.28 ± 2.7	82.35 ± 2.46
	Bark	89.05 ± 1.42	95.67 ± 1.96	67.5 ± 1.7
Methanol	Leaves	86.45 ± 2.21	90.45 ± 1.88	81.60 ± 1.8
	Bark	66.2 ± 2.06	71.12 ± 2.4	88.20 ± 1.6

Values represent mean ± SD of triplicate, *Significant at P<0.05.
Extracts of *A. indica's* leaves and bark.
DPPH=1,1-diphenyl-2-picrylhydrazyl; SD=Standard deviation

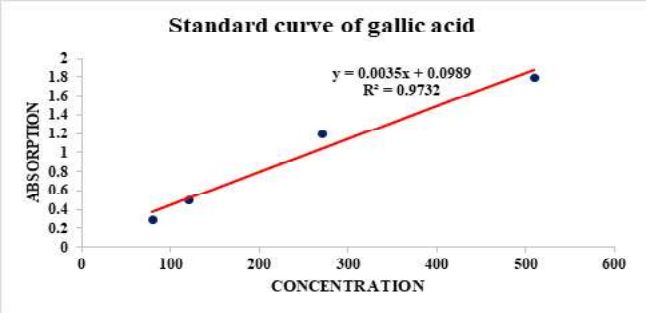

Fig. 1: Standard curve of gallic acid in mg GAE/g for total phenol content estimation

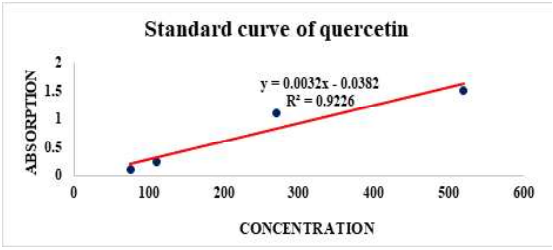

Fig. 2 : Standard curve of quercetin in mg QE/g total flavonoid estimation

Antibacterial Activity of Aqueous and Methanolic leaf and bark extracts of *A. indica* against *E. coli*

The methanolic bark extract of *A. indica* produced a zone of inhibition of 8.42 ± 0.26 mm against *E. coli*, while the aqueous bark extract at 30 mg/ml produced the highest zone of inhibition (7.88 ± 1.3 mm) compared to the methanolic bark extract (8.42 ± 0.26 mm). The mean zone of inhibition for amikacin (control) was 14.24 ± 0.32 mm. The aqueous bark extract's zone of inhibition against *E. coli* was 10 mg/ml (2.8 ± 2.14 mm), while the methanolic bark extract's was 20 mg/ml (5.9 ± 0.42 mm).

Table 4. Mean zone of inhibition of aqueous and methanolic bark extracts against *E. coli.*

Concentration of extract	Aqueous extract Mean zone of inhibition (mm)	Methanolic extract Mean zone of inhibition (mm)	Amikacin (control) Mean zone of inhibition (mm)
10 mg/mL	2.8 ± 2.14	0	14.24 ± 0.32
20 mg/mL	5.2 ± 1.4	5.9 ± 0.42	
30 mg/mL	7.88 ± 1.3	8.42 ± 0.26	

*- MZI of aqueous and methanolic bark extracts against *E. coli*.

In comparison to the methanolic leaf extract (8.34 ± 0.48 mm), the aqueous bark extract at 30 mg/ml provided the maximum zone of inhibition (7.23 ± 0.27 mm) against *E. coli*, whereas the methanolic leaf extract produced a zone of inhibition of 8.34 ± 0.48 mm. Amikacin (control) had an average zone of inhibition of 14.24 ± 0.32 mm. The methanolic leaf extract had a zone of inhibition of 20 mg/ml (5.72 ± 0.32 mm) against *E. coli*, but the aqueous leaf extract had a MIC of 10 mg/ml (2.5 ± 2.02 mm).

Table 5. Mean zone of inhibition of aqueous and methanolic leaf extracts against *E. coli.*

Concentration of extract	Aqueous extract Mean zone of inhibition (mm)	Methanolic extract Mean zone of inhibition (mm)	Amikacin (control) mean zone of inhibition (mm)
10 mg/mL	2.5 ± 2.02	0	14.24 ± 0.32
20 mg/mL	4.9 ± 0.18	5.72 ± 0.32	
30 mg/mL	7.23 ± 0.27	8.34 ± 0.48	

*- MZI of aqueous and methanolic leaf extracts against *E. coli*.

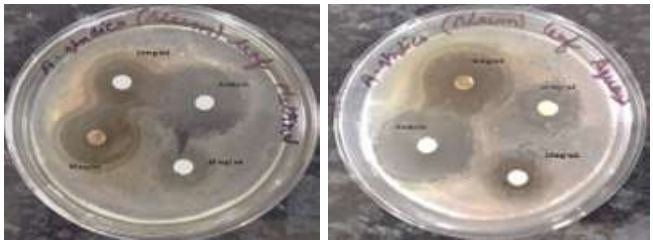

Fig 3: Zone of inhibition of aqueous and methanolic leaf extracts against *E. coli.*

Fig 4: Zone of inhibition of aqueous and methanolic bark extracts against *E. coli.*

DISCUSSION

Chemical compounds that have a specific physiological effect on the human body are responsible for the secondary metabolites' therapeutic qualities. Alkaloids, glycosides, steroids, flavonoids, fatty oils, resins, mucilage, tannins, gums, and calcium and phosphorus for bodybuilding, cell growth, and replacement are some examples of these chemicals.[24]

Flavonoids, phenolics, tannins, saponins, alkaloids, and steroids were among the phytochemicals found in the *A. indica* leaf and bark extracts used in this investigation. The aqueous and methanolic extracts showed varying degrees of phenolics and tannins. The findings of this study are consistent with those of Sahrawat *et al.* (2018)[25], who found that the methanolic extract of *A. indica* grown in India included phenolics and tannins, whereas the aqueous extract contained saponins. Gupta *et al.*, (2013)[26] reported the presence of terpenoids in both the aqueous and methanolic extract, in contrast to this work where they were lacking in both.

Similar to findings by Susmitha *et al.* (2013)[27], who also discovered steroids and tannins in the methanolic extract, steroids were only found in the *A. indica* methanolic extract. Variations in the solvents' polarity are the cause of the observed variation in phytochemical presence. Additionally, research conducted in other regions of the world may reflect the presence or absence of phytochemicals described in this study due to the geographical and environmental conditions that the plant is exposed to.

Amikacin 5 µg, the positive control, provided an average zone of inhibition of 14 mm with p-value = 0.025, whereas the highest concentration of 30 mg/ml of both aqueous and methanolic leaf extracts demonstrated an average zone of inhibition of 7.23 ± 0.27 mm and 8.34 ± 0.48 mm, respectively. Since Amikacin had 100% of its

active component and was in its purest antibacterial form, there was a statistically significant difference between the three outcomes. The highest concentration of 30 mg/ml of both aqueous and methanolic bark extracts showed an average zone of inhibition of 7.88 ± 1.3 mm and 8.42 ± 0.26 mm, respectively, while amikacin 5 µg, the positive control, produced an average zone of inhibition of 14 mm with p-value = 0.025. The methanolic and aqueous extracts did not differ statistically at 30 mg/ml. The aqueous extract performed statistically considerably better than the methanolic extract at different doses (p-value = 0.004).

The methanolic extract's activity against *E. coli* was 8 mm, whereas the aqueous extract exhibited no activity, according to Susmitha *et al.* (2013).[27] However, as solvents with varying polarity were utilised, the observed variation in inhibitory zones may be the result of variations in the phytochemical content in the extracts. As this study showed, different polarities had an impact on the kinds of phytochemicals that were extracted and, consequently, the plant extract's medicinal properties. Furthermore, variations in harvesting seasons, rainfall patterns, and geographic locations may be the cause of observed discrepancies among the published research. The methanolic extract of *A. indica* had a minimum inhibitory concentration (MIC) of 20 mg/mL, whereas the aqueous extract had a MIC of 10 mg/mL. The MIC for *A. indica* methanolic extract against *E. coli* was found to be 83.3 ± 29.0 mg/ml in a study by Nigussie *et al.* (2021).[28]

Shu'aibu *et al.*, (2015)[29] investigation, however, found that the petroleum ether extract of *A. indica* exhibited a minimum inhibitory concentration (MIC) of 100 µg/mL against *E. coli*, although the methanolic extracts showed no action at that concentration. This indicates that the polarity of the extraction solvent has a major impact on *A. indica* activity.

CONCLUSION

The methanolic and aqueous extracts of leaf and bark of *A. indica* showed antibacterial activity against *E. coli*. Compared to the methanolic extract, the aqueous extract exhibited superior antibacterial activity. As seen by the different zones of inhibition with concentration changes, the antibacterial activity of *A. indica* extracts was concentration-dependent.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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