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Research Article

COMPARATIVE ANALYSIS OF PHYTOCHEMICAL, ANTIBACTERIAL, AND ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF *AZADIRACHTA INDICA* LEAVES

GAURAV SHARMA*, ANKITA THAKUR, SOHAN LAL, ROHIT KUMAR NADDA

Department of Biotechnology, Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan, Himachal Pradesh, India.
Email: dr.gorav143@gmail.com

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ABSTRACT

Objective: The objective of the present study was the analysis of phytochemicals in various extracts of *Azadirachta indica* leaves, comparative evaluation of antibacterial activity of the various extracts of *A. indica* leaves against *Escherichia coli* and *Staphylococcus aureus*, and comparative evaluation of antioxidant activity in various extracts of *A. indica* leaves using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.

Methods: Various extracts were prepared by crushing the samples. Antibacterial susceptibility test, various phytochemical tests for qualitative analysis, and DPPH radical scavenging assay for antioxidant activity were performed.

Results: The result suggested that alkaloids, flavonoids, and terpenoids were present in all the four extracts. Tannins were absent in the ethyl acetate extract, and phenols were only present in the ethyl acetate extract. Sterols and phlobatannins were absent in all the four extracts. Saponins were only present in the aqueous extract, and amino acids were only present in the ethyl acetate extract. The bacterial strains *S. aureus* and *E. coli* were used against the different extracts of *A. indica* leaves, i.e., methanol, chloroform, ethyl acetate, and aqueous.

Conclusion: The results suggested that bioactive compounds found in leaves of *A. indica* contribute to its pharmacological activities.

Keywords: Antioxidant activity, 1, 1-Diphenyl-2-picrylhydrazyl, Radical assay, Triterpenoids, Ninhydrin.

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INTRODUCTION

Azadirachta indica is a fast growing evergreen popular tree found commonly in India, Africa, and America [1]. It has been used in Ayurvedic medicine for >4000 years due to its medicinal properties. Neem is called "arista" in Sanskrit, a word that means "perfect," complete, and imperishable [2]. The tree is regarded as "village dispensary" in India. Each part of the neem tree has some medicinal property and is thus commercially exploitable. Several pharmacological activities and medicinal applications of various parts of neem are well known. Although large numbers of compounds have been isolated from various parts, a few have been studied for biological activity. Nimbidin, a major crude bitter principle extracted from the oil of seed kernels of *A. indica,* demonstrated several biological activities. Two polymers isolated from neem bark possess anti-complement activity among which the compound NB-II, a peptidoglycan of lower molecular weight, was found to be more potent. A significant antiulcer effect was observed with nimbidin in preventing acetylsalicylic acid, indomethacin, stress, or serotonin-induced gastric lesion as well as histamine or cysteamine-induced duodenal ulcers. Nimbidin can also suppress basal as well as histamine and carbachol-stimulated gastric acid output and may act as an antihistamine by blocking hydrogen receptors, thereby helping as an antiulcer agent. The spermicidal activity of nimbidin and nimbin was reported in rats and human as early as 1959. From this crude principle, some tetranortriterpenoids, including nimbin, nimbinin, nimbidinin, nimbolide, and nimbidic acid, have been isolated. Nimbidin and sodium nimbidate possess significant dose-dependent anti-inflammatory activity against carrageenin-induced acute paw edema in rats and formalin-induced arthritis [3]. Antipyretic activity has also been reported and confirmed in nimbidin. Oral administration of nimbidin demonstrated a significant hypoglycemic effect in fasting rabbits. Phytosterol fraction, an active ingredient isolated from the lipid part of neem fruits, exhibits antiulcer activity in stress-induced gastric lesions [4]. The present study was the analysis of phytochemicals in various extracts of *A. indica* leaves, comparative evaluation of antibacterial activity of various extracts of *A. indica* leaves against *Escherichia coli* and *Staphylococcus aureus*, and comparative evaluation of antioxidant activity in various extracts of *A. indica* leaves using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay.

Plant authentication

Plant authentication was done at Dr. Y.S. Parmar University of Horticulture and Forestry under the Herbarium No. - 1015.

MATERIALS AND METHODS

Materials

A. indica leaves were collected from Bilaspur district of Himachal Pradesh, India.

Preparation of the aqueous extract

The aqueous extract was prepared according to Sripanidkulchai *et al.,* with the slight changes. 5 g of *A. indica* powder was boiled in 80 ml distilled water for 2–3 h. The solution was filtered through a Whatman filter paper, and the filtrate was collected in a Petri dish. The filtrate was evaporated by heating over the hot plate at a temperature of 60°C for 2–3 h. The weight of the crude extract was recorded. The crude extract was dissolved in the dimethyl sulfoxide (DMSO) at the concentration of 100 mg/ml.

Bacterial strains and antibiotic used two bacterial strains were used in this study - one Gram-positive (*S. aureus*) and one Gram-negative (*E. coli*). Both the strains were obtained from Yeast Biology Lab, Shoolini University, Solan. Pure cultures were maintained on NA plates and stored at 4°C. Ampicillin (100 µg/ml) was used in this study.

Phytochemical tests (qualitative analysis)

Detection of phenolic (ferric chloride test) extracts was treated with 3–4 drops of 5% ferric chloride solution. The appearance of bluish

or greenish-black coloration indicates the presence of pyrogallol or catechol tannins. Formation of a bluish black color indicates the presence of phenols.

Detection of flavonoids (lead acetate test)

Extracts were treated with few drops of 10% lead acetate solution. Formation of yellow color precipitate indicated the presence of flavonoids.

Detection of carbohydrates (Fehling's test)

Extracts were treated with 2–3 drops of Fehling's reagent and heated to 10 min. The appearance of red color precipitate indicated the presence of reducing sugars.

Detection of alkaloids (Mayer's Test)

Extracts were dissolved individually in dilute hydrochloric acid and filtered. Filtrates were treated with 2–3 drops of Mayer's reagent (potassium mercuric iodide). Formation of a yellow-colored precipitate indicated the presence of alkaloids.

Detection of Proteins (Millon's test) and amino acids (xanthoproteic test)

For proteins

About 20 µl of million reagents were added to the test tube containing 1 ml of extract and then heated in the water bath for 10 min. The samples were cooled and 10 µl of 1% sodium nitrite solution was added to the samples.

For amino acids

To 1 ml of extract, added 4–5 drops of 0.1% ninhydrin solution. Purple color indicated the presence of amino acids.

Detection of triterpenoids (Salkowski's test)

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated sulfuric acid, shaken, and allowed to stand. The appearance of golden yellow color indicated the presence of triterpenes.

Antioxidant activity (DPPH radical scavenging assay)

The DPPH radical scavenging assay of various extracts of *A. indica* was conducted. In this method, 900 µl of 0.1 mM DPPH solution (prepared in ethanol) was mixed with 100 µl of methanolic, ethyl acetate, chloroform, and aqueous extracts of *A. indica* ranging from 2.5 µg/ml to 20 µg/ml. The reaction mixture was shaken and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm against the blank. Ascorbic acid was used as the standard. DPPH radical scavenging activity was calculated from the following equation:

DPPH radical scavenging activity (5) = $1-A_s/A_c \times 100$

Where A_c - Absorbance of control and A_s - Absorbance of the test sample.

RESULTS AND DISCUSSION

Extract yield

For aqueous extract preparation, 5 g of *A. indica* leaf powder was boiled in 80 ml distilled water for 2–3 h. The solution was filtered and the filtrate was evaporated by heating over the hot plate at a temperature of 60°C for 2–3 h. The weight was recorded. For methanol, chloroform, and ethyl acetate, 10 g of *A. indica* leaf powder was added into 150 ml solvents and kept at shaker for 3–4 days. The solutions were then filtered, and the filtrate was evaporated in the incubator at 37°C temperature for 24 h. The extracts were prepared, and their weight was recorded and stored at 4°C. The yield obtained of the different extracts is shown in Table 1. The result showed that the yield of ethyl acetate extract was higher than other extracts. It may be due to the different polarities of the solvents used (Fig. 1).

Table 1: Percentage yield of different extracts of *Azadirachta indica* leaves

S. No	Extracts	Weight of extracts (mg)	Percentage yield (%)
1	Aqueous	95	1.9
2	Methanol	200	2
3	Chloroform	60	0.6
4	Ethyl acetate	530	5.3

Maximum yield was obtained with ethyl acetate followed by methanol and water

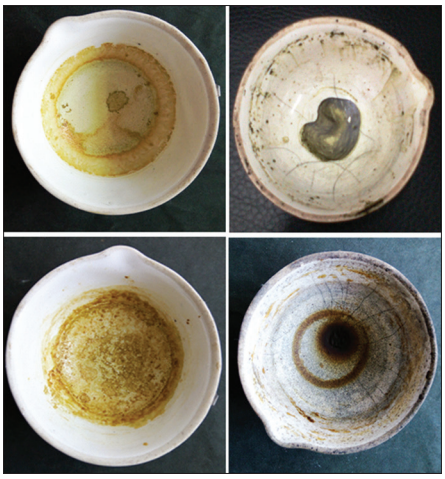

Fig. 1: Extracts of *Azadirachta indica* leaves: (a) Methanol extract, (b) chloroform extract, (c) ethyl acetate extract, (d) aqueous extract

Analysis of phytochemicals in various extracts of *A. indica* leaves

The screening of phytochemicals was done in all the four extracts by performing various tests.

Test for phenolics

The ferric chloride test formation of greenish black color revealed that phenolics were present in the methanol, ethyl acetate, and aqueous extracts. The chloroform extract did not reveal the presence of phenolics.

Tests for flavonoids

In the lead acetate test, formation of yellow precipitate revealed that flavonoids are present in all the extracts. The control contains lead acetate. The yellow color precipitation was detected in all the extracts.

Test for carbohydrates

Fehling's test for carbohydrates revealed the presence of reducing sugars in aqueous, methanol, and ethyl acetate extracts, whereas chloroform extract showed the absence of carbohydrate. Formation of green or red color indicated the presence of reducing sugar.

Test for alkaloids

Mayer's test for alkaloids revealed that aqueous, methanol, chloroform, ethyl acetate, and crude methanol shows the presence of alkaloids. Other than the aqueous, methanolic, chloroform and ethyl acetate there is also another solution (A) which is marked as Control and consists only reagent. In all extracts, yellow color precipitate or turbidity was observed. Formation of yellow color indicated the presence of alkaloids.

Ninhydrin test

Formation of yellow color in the ethyl acetate extract revealed the presence of amino acids. The control contains the concentrated HNO_3.

Table 2: Phytochemical constituents present in different extracts of *Azadirachta indica* leaves

Phytocompound	Test	Methanol extracts	Chloroform extracts	Ethyl acetate extracts	Aqueous extracts
Phenolic	$FeCl_3$ test	++	–	+	+
Flavonoids	Lead acetate test	+	+	++	+
Carbohydrate	Fehling's test	+	+	–	+
Proteins/amino acids	Ninhydrin test	–	–	–	+
	Millon's test	–	–	–	+
Alkaloids	Dragendorff's test	+	+	+	+
Terpenoids	Salkowski's test	+	+	+	+

Table 3: Comparative antibacterial activity of different extracts of the leaves of *Azadirachta indica*

Plant extract	Zone of inhibition (mm)	
	Staphylococcus aureus	***Escherichia coli***
Methanol extract	19.5±0.707107	18.5±0.707107
Chloroform extract	0	0
Ethyl acetate extract	11.5±0.707107	10.5±0.707107
Aqueous extract	13±1.414214	9.5±0.707107
Positive control (ampicillin)	30	10
Negative control (solvent)	0	0

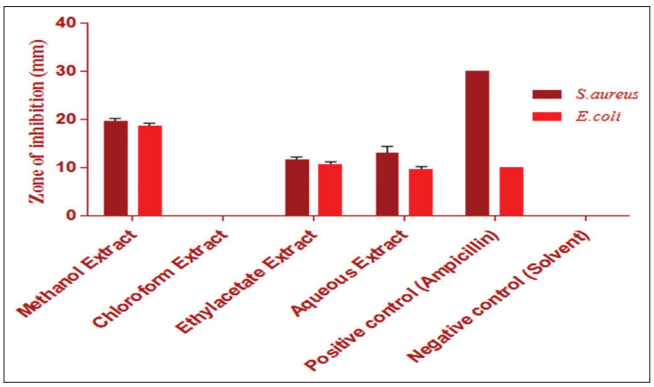

Fig. 2: The comparative antibacterial activity of different extracts of *Azadirachta indica* leaves

The yellow color was absent in the aqueous, methanol, and chloroform extract, showing the absence of amino acids.

Tests for triterpenoids

In Salkowski's test, the formation of reddish-brown color revealed that triterpenoids were present in all the extracts. It contains chloroform in Control-1 and concentrated H_2SO_4 in control-2. The reddish brown color was formed in the aqueous, methanol, chloroform and ethyl acetate extract of *A. indica* leaves (Table 2).

The result showed that various phytochemicals were present in the aqueous, methanol, chloroform, and ethyl acetate extract. Alkaloids, flavonoids, and terpenoids were present in all the four extracts. Tannins were absent in the ethyl acetate extract, and phenols were only present in the ethyl acetate extract. Sterols and phlobatannins were absent in all the four extracts. Saponins were only present in the aqueous extract, and amino acids were only present in the ethyl acetate extract. "++" indicates the more intense color as compared to other extracts.

Comparative evaluation of antibacterial activity of various extracts of *A. indica* leaves against *E. coli* and *S. aureus*

The antimicrobial activity of different extracts of *A. indica* was tested against the *S. aureus* and *E. coli* bacteria. The zone of inhibition against *S. aureus* was 19.5 mm for methanol extract, 11.5 mm for ethyl acetate extract, and of 13 mm for aqueous extract, and chloroform extracts showed no zone of inhibition. The ampicillin (positive control) showed the inhibition zone of 30 mm. DMSO (negative control) showed no zone against the *S. aureus.* In case *of E. coli* bacteria, a zone of inhibition of 18.5 mm was observed for methanol extract, 10 mm for ethyl acetate extract, and 9 mm for aqueous extract. Chloroform extract showed no zone of inhibition. The ampicillin (positive control) showed the inhibition zone of 10 mm. DMSO (negative control) showed no zone of inhibition (Fig. 2 and Table 3).

In the present study, we found that methanolic extract of *A. indica* leaves showed more antibacterial activity as compared to that of chloroform, ethyl acetate, and aqueous extracts. The increasing order of antibacterial activity against *S. aureus* is ethyl acetate<aqueous extract<methanolic extract, while in case of *E. coli,* aqueous extract<ethyl acetate<methanolic extract.

In vitro antioxidant activity

A. indica leaf extract exhibited good antioxidant activities in comparison to ascorbic acid. IC_{50} value (half maximal inhibitory concentration) indicates how much of a particular drug or other substance is required to inhibit a given biological process.

DPPH radical scavenging activity

In DPPH radical scavenging activity, the results showed that percentage inhibition increased with increasing concentration from 2.5 µg/ml to 10 µg/ml. DPPH antioxidant assay is based on the ability of a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron at 517 nm and is visible with deep purple color. When DPPH accepts an electron donated by an antioxidant compound, it is decolorized and can be quantitatively measured from the changes in the absorbance. Free radical scavenging effect of the corresponding different *A. indica* extracts of methanol, chloroform, and aqueous extracts was determined spectrophotometrically at 517 nm. Ascorbic acid was used as standard free radical scavenger reference compound.

DPPH radical scavenging activity (%) = $1-A_s/A_c \times 100$

Where A_c - Absorbance of control, A_s - Absorbance of the test sample.

IC_{50} value is a measure of the effectiveness of the substance in inhibiting a specific biological or biochemical function. The IC_{50} is the concentration of an inhibitor where the response (or binding) is reduced by half.

In the present study, the different extracts of *A. indica* had a significant scavenging effect on the DPPH radical which was generally significantly increased with the increase in the concentration. Methanolic extract of *A. indica* leaves showed comparable antioxidant activity with "ascorbic acid." In general, antioxidant activity of methanolic extracts was higher than that of chloroform, ethyl acetate, and aqueous extracts (Table 4).

CONCLUSION

In the present work, we elucidated that different extracts of *A. indica* have antimicrobial activity and contain various phytochemicals and antioxidant activity. The aim of phytochemical screening is to confirm the presence of various constituents for assessing their biological activity or medicinal use. The most important of these are alkaloids,

Table 4: The antioxidant activity of leaf extract of *Azadirachta indica* using DPPH assay

Antioxidant assay	Half-maximal inhibitory concentration (IC_{50}) (µg/ml)				
	Ascorbic acid	Methanolic extract	Chloroform extract	Ethyl acetate extract	Aqueous extract
DPPH	1.384	1.737	5.011	3.040	2.278

DPPH: 1, 1-diphenyl-2-picrylhydrazyl

saponins, steroids, phenols, flavonoids, and tannins. Preliminary phytochemical screening of methanolic, chloroform, ethyl acetate, and aqueous extracts of the leaves of *A. indica* showed the presence of phenols, tannins, flavonoids, carbohydrates, terpenoids, and alkaloids. Protein and amino acids were absent in all extracts of *A. indica.* Alkaloids are organic nitrogenous substances. These are alkaline in nature and exhibit an extraordinary array of pharmacological activities [5]. The flavonoids act as antioxidants which provide protection against free radicals that damage cells and tissues. Tannins promote healing of wounds. These are effective in diarrhea, colitis, and peptic ulcers. High flavonoid content indicates the probability of significant antioxidant potential of the *A. indica* leaves. In the present study, we found the presence of flavonoids in all different extracts of *A. indica* [6].

The antimicrobial activity of the aqueous, methanol, chloroform, and ethyl acetate extracts of *A. indica* was checked against the bacterial strains of *S. aureus* and *E. coli.* In an earlier study, Khan *et al.,* 1987, reported that *A. indica* leaf extract had a characteristic effect on dermatophytes, especially for lower polar extracts over high polar ones. Subapriya *et al.,* 2005, showed that *A. indica* leaf extract was found to have interesting inhibitory action on a wider spectrum of microorganisms including *Candida albicans*, *Candida tropicalis, Neisseria gonorrhea*, multidrug-resistant *S. aureus*, *E. coli,* and herpes simplex. Singh *et al.,* 1987, showed the fungicidal and bactericidal properties of extracts from leaves [7]. Methanolic extract of *A. indica* leaves showed more antibacterial activity as compared to that of the chloroform, ethyl acetate, and aqueous extracts. The zone of inhibition against *S. aureus* was 19.5 mm for methanol extract, 11.5 mm for ethyl acetate extract, and 13 mm for aqueous extract, and chloroform extracts showed no zone of inhibition against *S. aureus* [8], while in case of *E. coli* bacteria, the zone of inhibition of 18.5 mm was observed around the methanol extract, 10 mm around the ethyl acetate extract, and 9 mm around the aqueous extract. Chloroform extract showed no zone of inhibition. The increasing order of antibacterial activity against *S. aureus* - ethyl acetate<aqueous extract<methanolic extract while in case of *E. coli* - Aqueous extract<ethyl acetate<methanolic extract. Methanolic extracts showed more antioxidant activity as compared to other extracts of *A. indica.* Dekha *et al.,* 2013, also showed the antioxidant activity of methanolic extract of leaves of *A. indica.* All the plant extracts showed potent activities among the various tests. Oral care product can be developed if the active constituents responsible for the activities were analyzed [9]. The administration of the aquatic extracts of Aloe vera, Neem, and Moringa (separately/mix) played a therapeutic role against CCl4-induced liver damage by improving liver enzyme activities and antioxidant blood parameters [10]. Plant essential oils and extracts may have a role as pharmaceuticals and preservatives [11]. *A. indica* had shown almost equal antimicrobial activity, against all species of microbes which was taken in a study as compared to standard ofloxacin. These results indicate that the anti-bacterial and antifungal activity of these extracts might be due to the presence of phytochemicals, i.e., alkaloids, saponins, flavonoids, tannins, terpenoids, and amino acids [12].

AUTHORS' CONTRIBUTIONS

Gaurav Sharma (Research scholar, Shoolini University) (First and corresponding author) has designed and conducted the work keeping in view the medicinal importance of the *A. indica.* Other author Ankita Thakur has the analysis and framing work of the manuscript. Statistical analysis was performed by both the authors.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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