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### Comparative analysis of phytochemical, antibacterial and antioxidant activity of different extracts of *azadirachtaindica* leaves

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#### ABSTRACT

##### Introduction

The present study was designed to compare phytochemicals, antimicrobial and antioxidant activity of different extracts of leaves of *Azadirachtaindica* from Himachal Pradesh. In this study, different extracts of *Azadirachtaindica* leaves are screened for the occurrence of bioactive compounds.

##### Objectives

Analysis of Phytochemicals in various extracts of *Azadirachtaindica* leaves.

Comparative evaluation of antibacterial and antioxidant activity of various extracts of *Azadirachtaindica* leaves against *E. coli* and *S. aureus*, using DPPH radical scavenging assay.

##### Material & Methods

The disc diffusion method was used to detect antibacterial activity of different extracts of *Azadirachtaindica* leaves against gram positive and gram negative bacteria such as *S. aureus*, and *E. coli* respectively. The methanolic extract showed the more antibacterial and antioxidant activity as compared to that of other extracts. DPPH free radical scavenging assay was performed to check antioxidant potential of *A. indica*. Phytochemical screening of different extracts showed the presence of flavonoids, tannins, phenols, alkaloid, protein/amino acid, carbohydrates and steroids in different extracts of the *A. indica* leaves.

##### Results

The findings suggested that phytochemicals present in *A. indica* leaves are potentially beneficial as therapeutic and antioxidative agents in pharmaceuticals, medicinal and food industries.

**Keywords:** *Azadirachtaindica*, methanol extract, Chloroform extract, Ethyl acetate extract, Aqueous extract, Antimicrobial activity, *S. aureus*, *E. coli*, DPPH.

## INTRODUCTION

Azadirachtaindica (Neem) is a fast growing evergreen popular tree of meliaceae family found in most tropical countries like India, Africa, America [1]. It has been used in Ayurvedic medicine for more than 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. The importance of the neem tree has been recognized by the US National Academy of Science, which publish a report in 1992 entitled “Neem- a tree for solving global problems” [3]. It possesses maximum useful non-wood products (leaves, fruits, bark, flowers, seed, gum, oil, and neem cake) than any otherspecies. It is known to have Antiallergenic, antidermatic, antifungal, antiinflammatory, antipyorrhoeic, antiscabic, cardiac, diuretic, insecticidal, larvicidal, nematocidal, spermicidal and other biological activities. Quercetin and  $\beta$ -sitosterol were first polyphenolic flavonoids purified from fresh leaves of neem and were known to have antifungal and antibacterial activities. Numerous biological and pharmacological activities have been reported including antibacterial [4], antifungal [Kher and Chaurasia 1997] and anti-inflammatory. Earlier investigators have confirmed their role as anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, antigastric ulcer, antifungal, antibacterial, and antitumour activities [5].

Azadirachtaindica is considered harmless to human, animal, birds, beneficial insects and earthworms and has been approved by the US Environment Protection Agency for use on food crops [6]. Biological active compound isolated from different parts of the plant include: azadirachtin, meliacin, gedunin, salanin, nimbin, valassin and many other derivatives of these principles. 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. Cyclic trisulphide and tetrasulphide isolated from the steam distillate of fresh, matured neem leaves have antifungal activity against trichophytonmentagrophytes.

It has been accepted that drugs based on allopathy are expensive and also exhibit toxic effect on normal tissues and on various biological

activities. It is largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants [7]. *Azadirachtaindicahas* complex of various constituents including nimbin, nimbidin, nimbolide, and limonoids and such types of ingredients play role in diseases management through modulation of various genetic pathway and other activities. Quercetin and  $\beta$ -sitosterol were first polyphenolic flavonoids purified from fresh leaves of neem and were known to have antifungal and antibacterial activities [8]. Numerous biological and pharmacological activities have been reported including antibacterial [4], antifungal [9] and anti-inflammatory. Leaf and bark extracts of *A. indicahave* been studied for their antioxidant activity and results of the study clearly indicated that all the tested leaf and bark extracts/fractions of neem grown in the foothills have significant antioxidant properties [10].

A study confirmed that ethanolic fraction of neem leaf (EFNL) treatment effectively up regulated the pro-apoptotic genes and proteins including p53, Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter protein (Bad) caspases, phosphatase and tensin homolog gene (pTEN), and c-Jun N-terminal kinase (JNK) [11].

A methanol extract of the leaves exerts antipyretic effect in male rabbits [12]. The plant also possesses analgesic activity mediated through opioid receptors in laboratory animals.

The aqueous extract of leaf also possesses potent immune stimulant activity as evidenced by both humoral and cell-mediated responses [13]. Leaf extract at 100 mg/kg after three weeks of oral administration causes higher IgM and IgG levels along with increased titer of anti albumin antibody [14].

Aqueous extract of neem leaves significantly decreases blood sugar level and prevents adrenaline as well as glucose-induced hyperglycaemia [15]. The aqueous leaf extract when orally fed, also produces hypoglycaemia in normal rats and decreased blood glucose levels in experimentally-induced diabetes in rats [16]. Neem leaf aqueous extract produces antiulcer effect in rats exposed to restraint – cold stressor ethanol orally by preventing mucus depletion and mast cell degranulation [17].

Oral administration of aqueous extract of Neem leaf shows anti-fertility effect in mice [18].

Neemseed and leaf extracts are effective against malarial parasites [19]

In the present work, we have tried to analyse various extracts from *azadirachtaindica* leaves for its phytochemical, antibacterial and antioxidant activity. The aim of phytochemical screening is to confirm the presence of various constituents for assessing their biological activity or medicinal use. Comparative evaluation of antibacterial activity of various extracts of *Azadirachtaindicaleaves* against *E.coli* and *S.aureus*. Comparative evaluation of antioxidant activity in various extracts of *Azadirachtaindicaleaves* using DPPH radical scavenging assay.

## MATERIALS AND METHODS

### Materials

*Azadirachtaindica* leaves, HgCl<sub>2</sub>, methanol, ethyl acetate, chloroform, DMSO, Bacterial strain (*Staphylococcus aureus*, *Eschericia. Coli*), Ampicillin, Nutrient agar, Fehling reagent, millon reagent

### Sample Collection

*A. indica* leaves were collected from Bilaspur district of Himachal Pradesh in the month of January 2016.

### Washing and Drying

Leaves were washed thoroughly with distilled water and surface sterilized with 0.1% HgCl<sub>2</sub> and then air dried for 4-5 days.

### Grinding and Preparation of extracts

*A. indicaleaves* were ground into fine powder using mortar and pestle. To 10 g of *A.indicaleaves* powder were soaked in 100 ml methanol, ethyl acetate and chloroform separately and kept on shaker for 3-4 days. The extracts were then filtered through whatman filter paper and filtrate were collected in a china dish. The filtrates were dried by evaporates and dissolved in DMSO (100 mg/ml).

### Preparation of the aqueous extract

The aqueous extract was prepared according to Sripanidkulchai et al., (2000), with the slight changes. 5g of *A. indicapowder* was boiled in 80 ml distilled water for 2-3 hours. Then solution was filtered through a whatman filter paper and filtrate

was collected in a Petri dish. The filtrate was evaporated by heating over the hot plate at temperature 60° C for 2-3 hours. The weight of the crude extract was recorded. The crude extract dissolved in the DMSO at concentration of 100 mg/ml.

### Bacterial strains and Antibiotic used

Two bacterial strains were used in this study- one gram positive (*Staphylococcus aureus*) and one gram negative (*Eschericia. coli*). Both the strains were obtained from Yeast Biology Lab, Shoolini University, Solan. Pure cultures were maintained on nutrient agar plates and stored at 4°C. Ampicillin (100µg/ml) was used in this study.

### Preparation of inoculum

Bacterial suspensions were prepared from overnight cultures by the direct colony method. Colonies were taken directly from the plate and suspended into 1 ml of sterile 0.85% saline. The turbidity of initial suspension was adjusted comparing with 0.5 McFarland Standard which is equivalent to 10<sup>8</sup> colony forming units (CFU)/ml. Bacterial lawn was spread on nutrient agar plates using a sterile cotton swab.

### Antimicrobial susceptibility test

The qualitative antimicrobial activity of different extracts of *A. indica leaves* were performed by using well diffusion method (Felten et al., 2002). 50 µl of plant extracts (50µg) were poured into 6mm well. 10 µl ampicillin of concentration 100 µg/ml was used as a positive control and DMSO (solvent) was taken as a negative control. The plates were kept in the incubator at 37°C aerobically for 18 hours. The inhibition zones obtained around the disc were measured using HI Antibiotic Zone Scale-C.

## PHYTOCHEMICAL TESTS (QUALITATIVE ANALYSIS)

### Detection of phenolics (Ferric Chloride Test)

Ferric chloride is acidic in nature; it produces H<sub>3</sub>O<sup>+</sup> ions when dissolved in water. Compounds with a phenol group will form a blue, violet, purple, green, or red-brown colour upon addition of aqueous ferric chloride. Extracts were 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 bluish black colour indicates the presence of phenols.

#### Detection of flavonoids (Lead acetate Test)

The conjugation in flavonoid compounds produces a yellow colour, while the extended conjugation in the resultant anthocyanidin shifts the colour further out to the red region of the visible spectrum. The dramatic change in colour makes this a simple visual test for the presence of flavones. Extracts were treated with few drops of 10% lead acetate solution. Formation of yellow colour precipitate indicated the presence of flavonoids.

#### Detection of carbohydrates (Fehling's test)

Fehling's solution contains blue alkaline cupric hydroxide solution, which upon heating with plant extract gets reduced to yellow or red cuprous oxide. Extracts were treated with 2-3 drops of Fehling reagent and heated to 10 minutes. Appearance of red colour precipitate indicated the presence of reducing sugars.

#### Detection of alkaloids (Mayer's Test)

Mercury salt reacts with the lone pairs of nitrogen that are present, then the typical off-white precipitate falls out. An uncharged mercury salt is, thus obtained. 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 coloured precipitate indicated the presence of alkaloids.

#### Detection of Proteins (Millon test) and amino acids (xanthoprotein test)

The reaction is due to the presence of the hydroxyphenyl group,  $C_6H_5OH$  in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colours or yellow precipitates which react with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol. 20  $\mu$ l of millon reagent was added to the test tube containing 1ml of extract and then

heated in water bath for 10 minutes. The samples were cooled and 10  $\mu$ l of 1% sodium nitrite solution. Red colour indicated the presence of proteins.

Xanthoprotein test gives positive results with proteins, peptones, peptides, amino acids and other primary amines, including ammonia. Proline and hydroxyproline give yellow colour with ninhydrin, while other acids give blue to purple colour. 4-5 drops of 0.1% ninhydrin solution were added to 1ml of extract. Purple colour indicated the presence of amino acids.

#### Detection of triterpenoids (Salkowski's Test)

Salkowski's test detect presence of cholesterol. Presence of double bond in one cholesterol ring is responsible for its ability to form coloured product in the presence of concentrated inorganic acid and it should yield a layering of colour. Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicated the presence of triterpenes.

#### ANTIOXIDANT ACTIVITY (DPPH RADICAL SCAVENGING ASSAY)

The DPPH radical scavenging assay of various extracts of *A. indicawas* conducted (Shahet *al.*, 2013). In this method, 900  $\mu$ l of 0.1 mM DPPH solution (prepared in ethanol) was mixed with 100  $\mu$ l of methanolic, ethyl acetate, chloroform and aqueous extracts of *A.indicaw* ranging from 2.5-20  $\mu$ g/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 standard. DPPH radical scavenging activity was calculated from the following equation-  
DPPH radical scavenging activity (%) =  $1 - A_s/A_c \times 100$   
Where  $A_c$ -Absorbance of control,  $A_s$ -Absorbance of test sample.

#### RESULTS

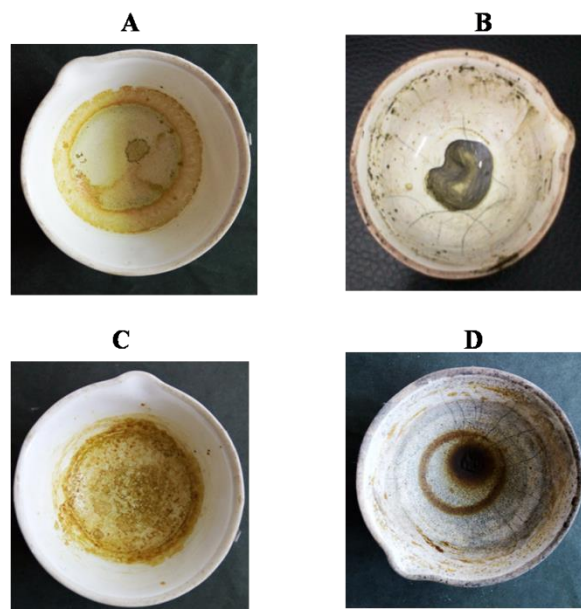
##### Extract yield

The yield obtained of the different extracts was shown in.

**Table -1: Percentage Yield of different extracts of *A. indica* leaves. Maximum yield was obtained with ethyl acetate followed by methanol and water.**

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

The result showed that the yield of ethyl acetate extract was higher than other extracts. Fig. 1



**Extracts of *A. indica* leaves: A. Methanol extract; B. Chloroform extract; C. Ethyl acetate extract; D. Aqueous extract.**

It may be due to the different polarity of the solvents used.

#### **Analysis of Phytochemicals in various extracts of *Azadirachta indica* leaves**

The screening of phytochemicals was done in all the four extracts by performing various tests as listed in materials and method section.

#### **Test for phenolics**

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

#### **Tests for flavonoids**

In the lead acetate test formation of yellow precipitate revealed that flavonoids are present in all the extracts (fig. 4.3). Control contains lead acetate. The yellow precipitate was detected in all the extracts.

#### **Test for carbohydrates**

The Fehling's test for carbohydrates revealed the presence of reducing sugars in aqueous, methanol and ethyl acetate extracts, whereas chloroform extract showed absence of carbohydrate. Formation of green or red colour indicated the presence of reducing sugar (Fig. 4.3).



### Test for alkaloids

The Mayer's test for alkaloids revealed that aqueous, methanol, chloroform, ethyl acetate and crude methanol show presence of alkaloids (Fig. 4.4). In this (A) is control which contains only reagent. In all extracts, yellow colour precipitate or turbidity was observed. Formation of yellow colour indicated presence of alkaloids.

### Ninhydrin test

Formation of yellow colour in the ethyl acetate extract revealed the presence of amino acids. The control in this contains the conc. HNO<sub>3</sub>. The yellow colour was absent in the aqueous, methanol and chloroform extract, showing the absence of amino acids.

### Tests for triterpenoids

In salkowski's test, formation of reddish brown colour revealed that triterpenoids were present in all the extracts. Reddish brown colour was formed in the aqueous, methanol, chloroform and ethyl acetate extract of *A. indica* leaves.

The result showed that various phytochemicals were present in the aqueous, methanol, chloroform and ethyl acetate extract. Alkaloids, flavonoids, 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 colour as compared to other extracts.

### Comparative evaluation of antibacterial activity of various extracts of *azadirachta 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, chloroform extracts showed no zone of inhibition. The ampicillin (+ve control) showed the inhibition zone of 30 mm. DMSO (-ve control) showed no zone against the *S. aureus*. In case of *E. coli* bacteria, zone of inhibition of 18.5 mm was observed for methanol extract, 10 mm for ethyl acetate extract and of 9 mm for aqueous extract. Chloroform extract showed no zone of inhibition. The ampicillin (+ve control) showed the inhibition zone of 10 mm. DMSO (negative control) showed no zone of inhibition.

### Comparative antibacterial activity of different extracts of *A. Indica* leaves

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.

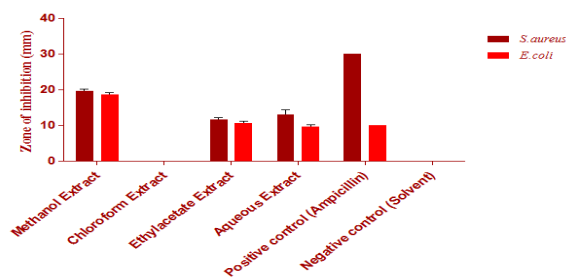


Fig-2 Comparative antibacterial activity of different extracts of *A. indica* leaves

### In vitro antioxidant activity

*A. indica* leaves extract exhibited good antioxidant activities in comparison to ascorbic acid. IC<sub>50</sub> value (half maximal inhibitory

concentration) indicates how much of a particular drug or other substance is required to inhibit a given biological process.

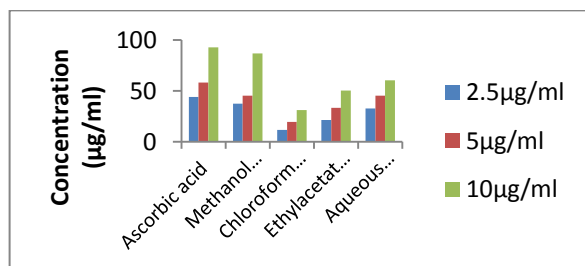
### DPPH radical scavenging activity

In DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity the results showed that percentage inhibition increased with increasing concentration from 2.5µg/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, ethylacetate and aqueous extracts were determined spectrophotometrically at

517nm. 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 test sample.

IC<sub>50</sub> value is a measure of the effectiveness of substance in inhibiting a specific biological or biochemical function. The IC<sub>50</sub> is the concentration of an inhibitor where the response (or binding) is reduced by half. The IC<sub>50</sub> 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 significant scavenging effect on the DPPH radical which was generally significantly increasing with the increase in the concentration (Fig-3)



**Fig-3 DPPH radical scavenging activity of methanol, chloroform, ethyl acetate and aqueous extracts of *A. indica* leaves.**

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.

### DISCUSSION

Many of the existing synthetic drugs cause various side effects. Hence, natural plant based drug compounds could be useful in meeting this demand for newer drugs with minimal side effects. *A. indica* leaves possessed good antibacterial activity confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care. In the present work, we elucidated that different extracts of *A. indica* have antimicrobial activity and contains 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, saponins, steroids, phenols, flavonoids and tannins. Preliminary phytochemical screening of methanolic, chloroform, ethyl acetate and aqueous extracts of 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 extra ordinary array of pharmacological activities. 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 flavonoids content indicates the probability of significant antioxidant potential of the *A. indicaleaves* [20]. Here in the present study, found flavonoids in all different extracts of *A. indica*.

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* leaves extract had a characteristic effect on dermatophytes especially for lower polar extracts over high polar ones. [21] Showed that *A. indicaleaves* extract was found to have interesting inhibitory action on a wider spectrum of microorganisms including *C. albicans*, *C. tropicalis*, *Neisseria gonorrhoea*, multi drug resistant *S. aureus*, *E. coli* and *Herpes simplex* [22]. Showed the fungicidal and bactericidal properties of extracts from leave. 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 of 13 mm for aqueous extract ,chloroform extracts showed no zone of inhibition against *S.aureus*. 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 of 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*. also showed antioxidant activity of methanolic extract of leaves of *A. indica*[23]

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## CONCLUSION

In the present project entitled “**Comparative analysis of Phytochemicals, antibacterial and antioxidant activity of different extracts of *Azadirachtaindicaleaves***” we investigated various phytochemicals in different extracts of leaves. The result suggested that alkaloids, flavonoids, 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 *Staphylococcus aureus* and *Escherichia coli* were used against the different extracts of *Azadirachtaindica* leaves *i.e.* methanol, chloroform, ethyl acetate and aqueous. The investigation revealed that, methanol showed the maximum antimicrobial activity against *Staphylococcus aureus* followed by *E.coli*. Chloroform extract showed no antimicrobial activity against any bacterial strains. Therefore the results suggested that bioactive compounds found in leaves of *Azadirachtaindica*, contribute to its pharmacological activities.

The DPPH free radical scavenging activity was performed to study the antioxidant potentials of *A. indica* leaves. Methanolic extract of *A. indica* showed more antioxidant activity as compared to chloroform, ethylacetate and aqueous extracts. The result suggested the antimicrobial and antioxidant potential of *A. indicaleaves*.



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