PHYTOCHEMICAL SCREENING, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF MELIA AZEDARACH LEAVES IN METHANOL SOLVENT

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ABSTRACT

Natural products continue to play an important role in the discovery and development of new pharmaceuticals. According to WHO, about 80% of the population in the developing countries use traditional medicine in the treatment of various ailments. The present study was conducted to evaluate the physicochemical and preliminary phytochemical studies, antimicrobial and antioxidant efficiency of Melia azedarach L. a medicinal plant (leaf extracts) using methanol as a solvent. Phytochemical screening revealed that methanolic leaf extract, contained, steroids, alkaloids, phenol, flavonoids, polyphenols and glycosides. Antimicrobial efficiency of Melia azedarach L. a medicinal plants (leaf extracts) were tested against six human pathogens: Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae and Proteus vulgaris. The diameter of zone of inhibition (ZOI) was measured and it was found that M. azedarach showed maximum zone of inhibition against gram +ve S. aureus and minimum zone of inhibition against gram –ve E. coli. Also the zone of inhibition was increased on increasing concentration. Extracts were also subjected to total phenolic content, total flavonoid content, total proanthocyanidins content and antioxidant activity using DPPH assay. 500µg methanolic extract showed the inhibition activity of 65.4%. Results observed in the present study may be indicative of the alcoholic extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat infections, which justify the ethnic uses of M. azedarach against various infectious diseases.

KEYWORDS: Melia azedarach, antimicrobial, antioxidant, phenols.
1. INTRODUCTION

Medicinal plants constitute the major constituents of most indigenous medicines and also a large number of Western medical preparations contain one or more ingredients of plant origin.\textsuperscript{[12]} According to WHO, about 80% of the population in the developing countries use traditional medicine in the treatment of various ailments.\textsuperscript{[13]} Therefore, plant species used by different ethnic groups should be investigated in order to tap the incredible bio resources for sustainable harvesting of novel bioactive phyto-pharmaceuticals.\textsuperscript{[14]}

\textit{Melia azedarach}, is among one of these important medicinal plants. Being the native of South Asian region including Iran, India, and south of China it is a very important plant in traditional medicine in Nepal due to its ethnomedicinal properties. The generic name is derived from the Greek word “Melia” means “the ash” and the specific name comes from the Persian “azzadirackt” i.e. the noble tree. \textit{M. azedarach} is around 14-16 meter-tall deciduous tree with smooth, greenish-brown bark during young age and with age turning grey and fissured. The main habitat of the plant is subtropical climatic zone including Bangladesh, India, Laos, Indonesia, Nepal and Malaysia and exotic are Argentina, Australia, USA, Brazil, Iran etc.\textsuperscript{[1]}

The plant consists of various biologically active compounds i.e. terpenoids, flavonoids, steroids, alkaloids, saponins, tannins, anthraquinones.\textsuperscript{[1, 3]} Similarly various phytochemical studies have shown the presence of vallinic acids, glycosides, phenol, annamic acids, gums, resins, glycosides, meliotannic acid and margosine.\textsuperscript{[5, 6]} The oil is the most active medicinal product of this plant. It is used as antiseptic for sores and ulcers that show no tendency to heal. It is also used for rheumatism and skin diseases such as ringworm and scabies. Internally, the oil is useful in malaria fever and leprosy. Powdered dust of fruit, crude extract from wood and bark and oil is insecticidal as well as antibacterial. Alcoholic extract of leaf is antihelminthic and mild analgesic whereas alcoholic extract of stem and bark show anticancerous, antispasmodic and antiviral property.\textsuperscript{[19]} Roots showed the presence of terpenoids and limonoids like 6-Acetoxy-7a-hydroxy-3-oxo 14beta, 15beta-epoxymeliac-15-diene, 6-Aceto-3beta-hydroxy-7-oxo 14beta, Azecline-1, Azecline-2, Azecline-3 and Azecline-4.\textsuperscript{[6]}

This plant is considered as resolvent, deobstrucent and alexipharmic. The leaves, fruits, roots and barks are used for curing various types of skin diseases like eczema, ulcerative, wounds, syphilitic ulcers, leprosy, scrofula etc. in form of oil, lotion and ointment. Systematically it is
used as an emetic, cathartic, antihelminthic, antipyretic, expectorant and diuretic.¹² ⁴ ⁸ ¹¹ Commercially oil of *M. azedarach* is used in soap and cosmetics.¹¹ The present study was conducted to evaluate the physicochemical and preliminary phytochemical studies, Antimicrobial and antioxidant efficiency of *Melia azedarach* L. a medicinal plants (leaf extracts) using Methanol as a solvent.

2. METHODS AND METHODOLOGIES

2.1 Specimen collection

The fresh leaves of *Melia azedarach* were collected from Sunsari district, Nepal in the month of July 2014. The plant was identified with the available literature and authenticated by botanist Rita Chhetri (1982) of National Herbarium Department, Godawori, Lalitpur, Nepal. The voucher specimens have been deposited at Universal Science College, Nepal. The healthy leaves of *M. azedarach* were taken, washed with distilled water and shade dried for 4 weeks.

2.2 Preparation of extract

Dried leaves of plant were grinded in a sterile blender to fine powder; thirty gram of powder was soaked in 300 ml of methanol as a solvent in conical flask and stored at room temperature for 72 hours. The extract was filtered through Whatman No.1 filter paper and was evaporated to dryness using rotary evaporator at reduced pressure. This was followed by the dilution of the crude extract (concentrated using a rotary evaporator) with mother solvent to produce a stock solution of 100mg/ml; from which a series of dilutions were made to obtain solutions of 10, 20, 30, 40, and 50 μg/μl concentrations.

2.3. Preliminary phytochemical screening

2.3.1 Test of alkaloids

In 1ml of sample extract, a few drops of Dragendorff’s reagent were added. Appearance of orange red colour indicated the presence of the alkaloids.¹⁵

2.3.2 Test of glycosides

To 0.5 g of extract diluted in 5 ml of water, 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface was observed which indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout the layer.¹⁷
2.3.3 Test of phenols and tannins
Each Extract was diluted with water and 3-4 drop of 10% ferric chloride solution was added. Appearance of the blue-green or black colour indicated the presence of phenol and tannins.\textsuperscript{[15]}

2.3.4 Test for steroids
Each extract was treated with acetic anhydride (2.5 ml) and chloroform (2.5 ml). Then concentrated solution of sulphuric acid was added slowly and green bluish color was observed for steroids.\textsuperscript{[17]}

2.3.5 Test of flavonoids
10% of dilute ammonia (5ml) was added to different extract of plants. Concentrated sulphuric acid (1ml) was added. A yellow coloration that disappears on standing indicates the presence of flavonoids.\textsuperscript{[17]}

2.3.5 Test for volatile oil
1ml extract of plant was taken and concentrated to get residue and mixing with 0.5ml methanol. This solution was shaken vigorously and filtered, few drops of filtrate is pour into filter paper with capillary tube and observed for persistent yellow spot on filter paper.\textsuperscript{[17]}

2.3.6 Test for carotenoid
Extract was treated with 1ml conc. sulfuric acid to examine the presence of carotenoids. Development of orange-yellow color confirms the presence of carotenoids.\textsuperscript{[17]}

2.3.7 Test for fatty acids
2ml of extract was concentrated and spotted on filter paper. It was then observed for yellow spot persistent even after the evaporation of solvents.\textsuperscript{[17]}

2.3.7 Test for polyphenol
1ml of extract of plant was mixed with 1ml water and 3 drops of 1 % (w/v) ferric chloride solution was added. Presence of polyphenol was confirmed by appearance of greenish blue colour.\textsuperscript{[17]}

2.3.8 Test for Anthocyanosides
2ml acidic layer of the different plant extract was treated with sodium carbonates until it becomes basic. Appearance of green color indicated the presence of anthocyanosides.\textsuperscript{[17]}
2.3.8 Test for saponins

2ml of extract was taken and treated with hot water and vigorously shaken for 30 sec. Thick forth was formed which confirmed the presence of saponins.\textsuperscript{[17]}

2.4 Quantitative phytochemical screening

2.4.1 Determination of total phenolics

The total amount of phenolic content of plant extract was determined by Folin Ciocalteu method. The standard solutions were prepared by taking 1, 2, 3, 4, 5μl sample from the stock of 100mg/ml and maintained final volume of 1ml. To this 1ml standard solution, 1ml Folin Ciocalteu’s reagent, previously diluted (1:4) was added. To the mixture, 4ml of sodium carbonate (75g/L) and 10 ml of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of Gallic acid. The total phenolic content was expressed as Gallic acid equivalents (GAE).\textsuperscript{[17]}

2.4.2 Determination of total proanthocyanidins

The presence of proanthocyanidins content was determined according to the procedure reported by Kikuzaki & Nakatani. \textsuperscript{[15]} In this method again 1, 2, 3, 4, 5μl samples were taken from original stock solution of 100mg/ml and final volume was maintained 0.5ml. This 0.5ml sample was mixed with 3ml of 4% vanillin methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500nm. A standard curve was obtained using various concentrations of rutin. The total proanthocyanidins content were expressed as rutin equivalents.

2.4.3 Determination of total flavonols

The total flavonols in the plant extract were estimated using the method of Kumaran et., al. \textsuperscript{[16]} In this method again 1, 2, 3, 4, 5μl samples were taken from stock of 100mg/ml and final volume of 2ml was maintained. To this, 2ml of 2% AlCl3 ethanol and 3.0 mL (50 g/L) sodium acetate solutions were added to 1ml standard solution of different concentration. The total flavonoids content were expressed as rutin equivalents.

2.5 Antimicrobial screening of the plants

The antimicrobial activity of plant extract was evaluated by well diffusion methods given by Perez et., al. 1990. \textsuperscript{[18]} The microbial strains of six bacterial samples which were used for the
test were obtained from National Public Health laboratory, Teku Kathmandu. The six different strains employed were *Staphylococcus. aureus* (ATCC 25923), *Proteus vulgaris* (ATCC 15028), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhi* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853). They were taken and cultured on broth containing nutrient agar. The sterile nutrient agar plates of Muller-Hinton agar were prepared. A McFarland 0.5 standard was prepared and the bacterial suspension was compared to the 0.5 McFarland standards to adjust the turbidity of the inoculums for the susceptibility test. The inoculums of bacteria were transferred into Petri-plates containing solidified agar using sterile cotton swab. The swab was used to spread the bacteria on the media in a confluent lawn. Wells were prepared by punching the agar plate already inoculated with a pure culture of the test organism with the help of sterile glass pipe. Well were made at the equidistance on the Petri plate. Then 40, 50, 60, 70μl of samples were added in wells from the stock solution of 10mg/ml. Also mother solvent was kept as a control. The plates were then incubated for 24 hour at 37°C and zone of inhibitions were measured.

2.6 Evaluation of antioxidant assay

2.6.1 DPPH (1, 1-diphenyl-2-picryl hydrazyl) radical scavenging activity

1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity was measured according to the method of Ilhami et.al 2005 [18]. Extract solutions were prepared by dissolving of different dry extract in methanol to produce a solution of 10mg/ml. 600μM DPPH was dissolved in 300 ml methanol and used as stock solution. The plant extract in methanol at various concentrations (1, 2, 3, 4, and 5mg) whose final volume was maintained 1ml and were mixed with an aliquot of 2ml of 600μM DPPH solution in methanol and incubated at 25°C for 30 min. Absorbance of the test mixture was read at 517nm using a spectrophotometer against a DPPH control containing only 1 ml of methanol in place of the extract. All experiments were performed thrice and the results were averaged. Ascorbic acid was used as a standard. Percent inhibition was calculated using the following expression

\[
\text{Inhibition} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100
\]

Where, \(A_{\text{control}}\) and \(A_{\text{sample}}\) stand for absorbance of the control and absorbance of tested extract solution respectively.
3. RESULTS

3.1 Preliminary phytochemical screening

The preliminary phytochemical screening of the methanolic extract of *M. azedarach* leaves showed the presence of various secondary metabolites. Alkaloids, phenol, tannin, flavonoids, saponins were the most prominent and result of phytochemical test has been summarized in (Table 1). Flavonoids, alkaloids, and phenolic compounds are a major group of compounds that act as primary antioxidants or free radical scavenger. Subsequently it may be used for the preparation of drug in a systematic way which may lead to the cure of many ailments in the future. So due to the presence of such secondary metabolites *M. azedarach* may have higher medicinal value.

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volatile oil</td>
<td>++</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+++</td>
</tr>
<tr>
<td>Fatty acid</td>
<td>+</td>
</tr>
<tr>
<td>Polyphenol</td>
<td>+</td>
</tr>
<tr>
<td>Antracenosides</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>++</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>+++</td>
</tr>
<tr>
<td>Phenol &amp; tannin</td>
<td>+++</td>
</tr>
</tbody>
</table>

+++ Maximum presence of the compound, ++ moderate, + least presence

3.2 Total amount of plant phenol

The phenolic content of the extracts in terms of Gallic acid equivalent (the standard curve equation: y =0.357x +0.103 Figure no1) is shown in Table no 2. Table shows the contents of total phenols that were measured by Folin-Ciocalteu reagent in terms of Gallic acid equivalent. Phenol was found to be increased with increase in concentration. as shown in figure 2. The results of this study show that the alcoholic extract of plant can be used as easily accessible source of natural antioxidants. The content of phenol expressed as GAE, in *M. azedarach* is 92±5mg/g extract.
3.3 Total amount of plant flavonoids
The content of flavonoids compounds (mg/ml) in plant extract, was determined from regression equation of calibration curve \( y = 0.249x - 0.096, \ R^2 = 0.973 \) Figure 3) and expressed in rutin equivalents. Table 2 shows the concentration of flavonoids in sample. Flavonoid was found to be increased with increase in concentration as shown in figure 4. Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics .Our present investigation depicts high content of flavonoids in the plant extract as compared to other phenolic compound.
Figure 3: Standard calibration curve for the quantification of total flavonoids content.

Figure 4: Flavonoid content of methanolic extract of *M. azedarach*

Figure 5: Standard calibration curve for the quantification of Total proanthocyanidins content.
3.4 Total amount of plant proanthocyanidins
The content of proanthocyanidins compounds (mg/ml) in plant extract, determined from regression equation of calibration curve (y = 0.046x – 0.037, R² = 0.994 Figure 5) and expressed in rutin equivalents. Table 2 shows the concentration of proanthocyanidins. Total proanthocyanidins play a major role in controlling antioxidants and act as antimicrobial agents.

\[
y = 0.046x - 0.037, \quad R^2 = 0.994
\]

![Figure 6: Proanthocyanidin content of methanolic extract of M. azedarach](image)

Table 2: Concentration of phenols, flavonoids and proanthocyanidins in mg/g gallic acid, rutin equivalent

<table>
<thead>
<tr>
<th>Extract</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenols</td>
<td>92±5</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>286±10</td>
</tr>
<tr>
<td>Proanthocyanidins</td>
<td>330±15</td>
</tr>
</tbody>
</table>

3.5 Antibacterial Activity
The result for antibacterial activity of *M. azedarach* is shown in Table 3. The antibacterial potential may be due to various phytochemicals like phenols and flavonols. *M. azedarach* was most effective against *S. aureus* and *Pseudomonas aeruginosa* whereas it was less effective against *E. coli* and *K. pneumonia* and was moderately effective against *S. typhi* and *Proteus vulgaris*. The diameter of zone of inhibition (ZOI) was measured and it was found that *M. azedarach* showed maximum zone of inhibition against Gram +ve *S. aureus* and minimum zone of inhibition against Gram –ve *E. coli*. Also the zone of inhibition was
increased on increasing concentration with maximum inhibition of 18mm on *S. aureus* when 700µg extract was used.

### Table 3: Result of antibacterial activity of *M. azedarach* plant extracts

<table>
<thead>
<tr>
<th>Amount of sample(µg)</th>
<th>E. coli (ATCC 25922)</th>
<th>K. pneumonia (ATCC 700603)</th>
<th>S. typhi (ATCC 14028)</th>
<th>Pseudomonas aeruginosa (ATCC 27853)</th>
<th>Proteus vulgaris (ATCC 14028)</th>
<th>S. aureus (ATCC 25923)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>6</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>500</td>
<td>7</td>
<td>9</td>
<td>11</td>
<td>9</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>600</td>
<td>7</td>
<td>8</td>
<td>11</td>
<td>12</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>700</td>
<td>10</td>
<td>10</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>18</td>
</tr>
</tbody>
</table>

**3.6 DPPH Radical Scavenging Activity**

The DPPH radical scavenging activity of *M. azedarach* is shown in Figure 7. Antioxidant studies of the plant extract, the extent of DPPH radical scavenging at different concentrations 100-500µg/ml of *M. azedarach* extracts was measured, with ascorbic acid as the standard. The inhibition activity of the plant extract was comparatively lower than the ascorbic acid. The result revealed that 5mg plant extract showed the highest inhibition activity 65.4% followed by decreasing inhibition activity in lower concentrations with lowest inhibition of 45.04% when 1mg was used.

![Figure 7: Antioxidant activity by DPPH Assay](image)

**4. DISCUSSION**

Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism. Phytochemicals such as phenol and flavonoids are well known for their antioxidant potential. Presence of such compounds in extracts act as reducing agents and antioxidants by the hydrogen-donating property of their hydroxyl groups. These compounds have significant therapeutic application...
against human pathogens including bacteria, fungi or virus. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity, antioxidant potential as well as for the discovery of new antimicrobial compounds. Therefore, medicinal plants are finding their way into pharmaceuticals, nutraceuticals and food supplements.

In the present investigation, methanolic extracts of *M. azedarach* was evaluated for total phenol, flavonoid, proanthocyanidins, exploration of antimicrobial activity against certain Gram negative and Gram positive bacteria, and assesement of antioxidant potential was done. Results observed in the present study may be indicative of the alcoholic extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat infections, which justify the ethnic uses of *M. azedarach* against various infectious diseases.

The antioxidant activity has a positive correlation with phenolic content of extracts of the plant. This confirms the assertion that phenolic content of plants contribute directly to their antioxidant properties. The DPPH scavenging capacity of the plant extracts may therefore be related to the phenolic compounds present. The values recorded for extract of the plant, even though lower than the standard antioxidant (ascorbic acid), showed that *M. azedarach* is a relatively good source of antioxidant activity.

The alcoholic extracts of *M. azerarach* showed significant antimicrobial activity against multi-drug resistant clinically isolated microorganisms. Though, the mechanism of the action of these plant constituents is not yet fully known. This observation clearly indicates that the existence of residues in the extracts which have higher both bactericidal and bacteristatic abilities.

In conclusion, of the present investigation *Melia azedarach* contain potential antimicrobial and antioxidant components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases.

5. CONCLUSION

From this study it has been clear that crude extract of *Melia azedarach* contains some medicinal active components. The phytochemical compounds contained in the different extract shows positive effect or relatively high inhibitory power on both gram positive and negative bacteria. The *Melia azedarach* leaves therefore contain anti-bacterial agents and nutrients. The result of the present study suggests that the *Melia azedarach* plant can be used as a source of antioxidant and antimicrobial for pharmacological preparations.
6. ACKNOWLEDGEMENTS

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7. REFERENCES


