Plants of Acanthaceae family: Phenolic composition, enzyme inhibitory and antioxidant activities

Neha Sikri and Sunita Dalal

Abstract
The plants of Acanthaceae family traditionally been used as significant source of therapeutic preparation. For the treatment of various diseases like gastrointestinal tract & hepato-protective disorders, inflammatory diseases, fevers and snake poisoning as well. In the present study we have examined the antioxidant and urease inhibitory potential of aqueous and methanol extracts of leaves of two plants of Acanthaceae family viz. Andrographis paniculata and Justicia adhatoda. Different analysis revealed that the aqueous extract of Andrographis paniculata leaves not only exhibited significant antioxidant potential in different assays performed but also exhibited considerable urease inhibitory potential(45.32%) as compared to the standard inhibitor, thiourea exhibited percent inhibition 71.47%. Quantitative evaluation of phytochemicals also revealed that the aqueous extracts of Andrographis paniculata leaves possessed the high flavonoid and phenolic content (73.67 mgGAE/gm and 85.66 mgGAE/gm) respectively. This study validates that Andrographis paniculata leaves, part of various therapeutic preparations for many years, can be used for the treatment of gastrointestinal disorders, stomach related disorders and other urease related infections as well.

Keywords: Acanthaceae, urease inhibitory, thiourea, Andrographis paniculata

1. Introduction
Number of infections like gastro duodenal infections, hepatic coma, urothliasis, ammonia encephalopathy, gastric and peptic ulcers and many more are mainly associated with ureolytic activity of bacterial and fungal pathogens such as Helicobacter pylori, Clostridium perfringens, klebsiella pneumonia, proteus mirabilis and fungal species Cryptococcus neoformans (a basidiomyce) and Coccidioides posadasii (an ascomycete) [1-3]. Out of these, one of the significant bacterial pathogens, H. pylori is able to develop infection in stomach due to the hydrolysis of urea by enzyme urease that provide ammonia and carbon dioxide which enable H. pylori to raise pH of its environment. [2, 4, 5] it leads to its survivability in human stomach. H. pylori infection also leads to the gastric mucosal injury [6]. The urease activity associated with other pathogens leads to stone formation and other urinary tract infections. However eradication of these pathogens can be done by two or more antibiotics and a proton pump inhibitor [7]. To fight the infection the combination can be given with Bismuth salt (a metal) that have antiucrease properties [8-11]. But the treatment efficacy can be reduced by acquisition of resistance by the bacterial pathogen to antibiotics [12]. So there is a need for the designing of new therapy for eradication of infections caused by urease enzyme. Till now, different organic compounds, five and six member heterocyclic compounds, barbituric acid derivatives and also the organophosphorus compounds do act as urease inhibitors [13]. But features like low hydrolytic stability and toxicity, renders their use. So, the natural sources can act as better alternatives.

Since the ancient times, people of different cultures and regions are using the medicinal plants traditionally due to their therapeutic properties [14-16]. So, the medicinal plants can prove to be an alternative source for ingredients that can be used in the therapeutic preparations and this can also be termed as alternative medicine.

Various metabolic processes like respiration, cell mediated immune function and many more leads to the continuous production of ROS (reactive oxygen species) and free radicals. These reactive species are also generated by environmental pollutants, smoking and pesticides also [17]. These free radicals could not be able to cause the deleterious effects on the body due to the antioxidiant mechanisms present in the body in the balanced way [17]. But sometimes the imbalance generated in the cell system leads to the instability of the reactive species as they have lone pairs in their outer shell. In order to attain the stability they then attack the biological
molecules of the living cells such as lipids, proteins, DNA & RNA. This bimolecular degradation associated with various diseases like cardiovascular diseases, inflammatory diseases, liver diseases, cancer, AIDS, premature aging and neurodegenerative diseases due to a phenomenon lipid per oxidation [18, 19]. Generally, the antioxidants functions to deter the deleterious effects of the free radicals on human body [20]. Till date, synthetic antioxidants like BHT, BHQ are used as drugs but these possess the potential risk. So, there is need for some natural antioxidants with no side effects.

Acanthaceae, a family of dicotyledened flowering plants spread all over the world. Out of the 4300 species and 346 genera’s, With 600 species Justicia is the largest genus of the family [21]. ‘Acan’ word in the Acanthaceae means ‘it washes’ because leaves of the plants of this family contains oil and it is used in laundry as well [22]. The different plants of this family possesses compelling therapeutic uses due to the presence of bioactive compounds like alkaloids, terpenoids, phenols, cardiac glycosides and different proteins also. Different plant extracts contains chemicals like acetaldehyde, cyclopendate and many more so they are used in pharmaceutical and cosmetic industries as well [23]. Paste of the leaves of the plants like Justicia flava and Justicia betonica is used for treatment of snake bites, dry cough, ulcers, diarrhea and eye infections [24]. Acanthaceae family members like Hygrophila spinosa, Barleria prionitis and Adhatoda vasica are traditionally used as antipyretic, anti-asthmatic and in respiratory diseases as well. In the present study, leaf extracts of two plants of Acanthaceae family viz. Andrographis paniculata and Justicia adhatoda were evaluated to validate their use in therapeutic preparations. Out of these, Andrographis paniculata known as ‘King of Bitters’ use in folk medicine from ancient times. Traditionally, it is being used as antimicrobial, anti-diabetic, sex hormone modulatory and also as anti-insecticides [25, 26]. The other member used in the study was of Justicia species viz. Justicia adhatoda used for swellings, pneumonia malaria, cold and cough [27].

The purpose of this study is to evaluate the in vitro antioxidant and urease inhibitory activities of methanol and water extracts of Andrographis paniculata and Justicia adhatoda leaves. Antioxidant activity was analyzed by using DPPH, metal chelating, β-carotene bleaching assay and reducing power assays. Additionally, the relationship between the phytochemical composition and biological activity also discussed.

2. Materials and methods

2.1 Chemicals

Urease type 1X (specific activity: 50,000-100000units/g) from Canavalia ensiformis (L.) DC. (Fabaceae) commonly known as Jack-Bean was purchased from Sigma Aldrich (St. Louis, MO). 2, 2’ diphenylpicrylhydrazyl, Folin-ciocalteu’s reagent, ascorbic acid, trichloroacetic acid, and glacial acetic acid were purchased from Hi Media Pvt. Ltd. All other chemicals and reagents used in this study were of analytical grade.

2.2. Plant material and preparation of extract

Leaves of the selected flora were collected from surroundings of Kurukshetra University, Kurukshetra, Haryana, India (29° 57’ 31.353” N, 76° 48’52.128” E). The identifications and authentication of the specimens was done from the Department of Botany, Kurukshetra University, Kurukshetra. The collected leaves were washed under tap water followed by distilled water, shade dried for 7 days and then milled to a coarse powder by a mechanical grinder and stored till the further use.

The powders of dried leaves (10g) were separately soaked in distilled water and methanol (100ml) in a reagent bottle covered with a lid at 37 °C for 24h. The powder were packed into Soxhlet column for 48 h. Resulting extracts in different solvents were evaporated and concentrated to dryness using the rotatory evaporator at 50 °C. The yield of extraction of dried plant material was calculated. The extracts were stored at -4 °C.

2.3 Qualitative and quantitative phytochemical screening

- Qualitative phytochemical screening

Both extracts of the selected plants were evaluated for the screening of the various phytochemicals using standard phytochemical procedures [28].

- Estimation of total phenolic content

The content of total phenols was determined according to the Folin-Ciocalteu’s method [29]. Aliquots of 0.5mL of extracts were mixed with 2.5mL of 10-fold-diluted Folin-Ciocalteu reagent and 2mL of 7.5% sodium carbonate (Na2CO3). The mixture was allowed to stand for 90min at room temperature before the absorbance taken at 760nm spectrophotometrically. The final results were calibrated to deduct the contribution from ascorbic acid and expressed as Gallic acid equivalent (mg of GA/g of extract).

- Estimation of total Flavonoid content

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method by Quettier et al., 2000 [30]. A volume of 2.5mL of extract was transferred to a test tube, mixed with 0.15mL of 5% Sodium nitrite for 5min. Then, 0.15mL of 10% aluminium nitrate was added. After 6mins the reaction stopped by adding 1mL of 1M sodium hydroxide. The mixture was further diluted with distilled water up to 5mL. The absorbance of the mixture immediately measured at 510nm. The flavonoid content was calculated and expressed as rutin equivalent (mg of RU/g of extract).

- Estimation of total tannin content

Total Tannin content was measured by Folin-Ciocalteu’s method [31]. 7.5mL of distilled water was added in0.1ml of the extracts followed by addition of 0.5ml Folin-Ciocalteu reagent. 1ml of 35% Na2CO3 was added to the above mixture then it was diluted up to 10ml with distilled water. Mixture was kept at room temperature for 30 minutes and absorbance was taken at 725 nm. The final results were calibrated to deduct the contribution from ascorbic acid and expressed as gallic acid equivalent.

- Estimation of Ascorbic acid

Ascorbic acid content was determined by using method of Roe and Keuther, 1953 [32]. Briefly, ascorbic acid was oxidized by activated charcoals to yield dehydroascorbic acid, which further react with 2,4-dinitrophenylhydrazine to form osazone, a light-absorbing substance. The absorbance read at 490nm spectrophotometrically. Ascorbic acid was taken as standard and the levels of ascorbic acid in the sample expressed as mg ascorbate/ g sample.
2.4 In vitro antioxidant evaluation
- 2, 2-diphenyl-1-picyrylhydrazyl scavenging assay
The ability of the plant extract to scavenge DPPH free radicals was assessed by the method described by Mensor et al., 2001[33]. The L-ascorbic acid was used as a positive control. The reaction mixture was prepared containing 300 µl of extract of varying concentrations (1-100 µg/ml) and 2 ml of DPPH (0.1 mM in methanol). The reaction mixture was then placed in the cuvette holder of the spectrophotometer and the absorbance measure at 517 nm against the blank. The per cent DPPH decolorization of the sample was calculated by the equation: % inhibition = B0-Bt/B0×100, Where, B0 is the absorbance of negative control and Bt is the absorbance of reaction mixture.
- Chelating effects on ferrous ions
The measurement of meal chelating activity of the different extracts was estimated by the method of Dinis et al. (1994) [34]. The extracts (0.25 ml) were mixed with 1.75 ml of methanol and 0.25 ml of 250 mM FeCl3. This was followed by the addition of 0.25 ml of 2 mM ferrozine, and kept at room temperature for 10 min. before determining the absorbance of the mixture at 562 nm. The chelating effect (%) was calculated from the formula as given for DPPH scavenging assay.
- Ferric reducing antioxidant power assay (FRAP)
FRAP assay of the extracts was determined by a method based on the reduction of ferric-tripryidyltriazine complex to a blue coloured ferrous form described by Benzie and Strain et al., 1996 [35]. The FRAP reagent containing 2.5mL of 10mM 2,4,6-tripryidy-s-triazine solution in 40mM HCl and 2.5mL of 20mM FeCl3 and 25mL of 0.3M acetate buffer, pH 3.6, and prepared freshly and pre-warmed at 37 °C. Aliquots of 40µL of extracts mixed with 0.2mL of distilled water and 1.8mL of FRAP reagent. The absorbance of reaction mixture at 593nm was measured spectrophotometrically after incubation at 37 °C. In this assay, the final result expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1mM FeSO4.
- Coupled oxidation of β-carotene and linoleic acid
β-carotene linoleic acid assay of the listed plants was carried out as described by Miller, 1971 [36]. BHT was used for comparison. 2 mg of β-carotene dissolved in chloroform (10 ml) was pipette into a small, round-bottom flask. After the chloroform was removed at 40 °C under vacuum, 100 µl of linoleic acid, 400 mg of tween 20 and 100 ml of oxygenated water was added to the flask followed by vigorous mechanical stirring. 5 ml of the above prepared emulsion was transferred to a series of test tubes containing 200 µl of extracts of various concentrations. As soon as the emulsion was added to each tube, the zero time absorbance is measured at 470 nm using a spectrophotometer (T60 UV visible). After initial vortexing, the test tubes were incubated in a water bath at 50 °C for 120 min. The absorbance of each sample was measured at 470 nm. Blank, devoid of β-carotene was prepared for background subtraction. Antioxidant activity calculated using the following equation: 
\[ AA = 100 \times \left(\frac{A_{o} - A_{t}}{A_{o}}\right) \]
Where, A0 and A∞ are the absorbance at zero time of test and blank, while Ao and At are the absorbance at time t of test and blank respectively.

2.5 Urease inhibition studies
The enzyme activity and inhibition was measured through catalytic effects of urease on urea by measuring change in absorbance in the absence and in the presence of inhibitor at 640nm, using spectrophotometer (T60 UV visible). All phytochemicals were tested for urease inhibition activity at concentration of 1.0 mg/ml. and that exerted significant inhibition, and tested in a concentration range of 100 to 1000µg/ml. Thiourea as standard inhibitor. For urease inhibition assays after addition of 10 ml of phosphate buffer to accurate weight of enzyme, sonication was performed, followed by centrifugation and absorbance of upper solution at 280 nm. By using equation \[ I\% = \frac{1}{T} \times \left(\frac{C - B}{C} \times 100\right) \]
Where I (%) is the inhibition of the enzyme, T (test) is the absorbance of the tested sample in the presence of enzyme C (control) is the absorbance of the solvent in the presence of enzyme.

2.6 Statistical analysis
All the assays were done in triplicates to test the reproducibility of them. All results are presented as mean ± S.E. SPSS 15.0 (statistical software) was used for statistical analysis of results. The values of p<0.05 were considered statistically significant. Correlations among data obtained were calculated using Pearson’s coefficient (r).

3. Results and Discussion
3.1. Extraction Yield: Aqueous and methanol extracts were prepared of the selected medicinal herb to examine phytochemicals qualitatively and quantitatively, antioxidant activity, and urease inhibitory activity. The yield of extract obtained from 10g of dry plant material was measured for each extract by formula% yield = weight of extract obtained / weight of powder taken × 100 (Table 1).

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Aqueous extract</th>
<th>Methanol Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographis paniculata</td>
<td>9.76%</td>
<td>7.31%</td>
</tr>
<tr>
<td>Justicia adhatoda</td>
<td>8.79%</td>
<td>7.68%</td>
</tr>
</tbody>
</table>

3.2. Qualitative & quantitative phytochemical screening
- Qualitative Phytochemical screening
The studied plant extracts revealed the presence of various phytochemicals viz. phenols, flavonoids, saponins, alkaloids and tannins. Out of various phytochemicals analyzed, in
Andrographis paniculata leaf extracts flavonoids and phenols and in Justicia adhatoda tannins, alkaloids and flavonoids are present in significant quantity (Table 2).

### Table 2: Qualitative phytochemical screening of Aqueous and methanol extract

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Flavonoids</th>
<th>Phenols</th>
<th>Saponins</th>
<th>Alkaloids</th>
<th>Tannins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographis paniculata</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Justicia adhatoda</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>


- **Quantitative phytochemical estimation**
  
  Total phenolic content varies from 18.91±0.10mgGAE/gm in methanol extract of Justicia adhatoda to 87.66±0.34mg GAE/gm of extract in aqueous extract of Andrographis paniculata (Table 3). The Flavonoid content varies from 51.04±0.39 mgRU/gm in aqueous extract of Justicia adhatoda to 95.67±1.08mgRU/gm in aqueous extract of Andrographis paniculata. Current study evaluated that total tannin content varies from 20.04±0.98 mg GAE/gm in methanol extract of Justicia adhatoda to 45.70±1.75mg GAE/gm in aqueous extract of Andrographis paniculata. The ascorbic acid content varies from 12.54±1.02 mg GAE/gm in methanol extract of Justicia adhatoda to 47.98±1.42 mgGAE/gm in aqueous extract of Andrographis paniculata.

### Table 3: Quantitative estimation of phytochemicals of aqueous and methanol extract

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Phenolic (mg GAE/g)</th>
<th>Flavonoid (mg RU/g)</th>
<th>Tannin (mg GAE/g)</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographis paniculata</td>
<td>87.66±0.04</td>
<td>18.91±0.04</td>
<td>95.67±1.08</td>
<td>21.98±1.57</td>
</tr>
<tr>
<td>Justicia adhatoda</td>
<td>26.36±0.65</td>
<td>18.91±0.04</td>
<td>75.04±1.09</td>
<td>25.67±0.21</td>
</tr>
</tbody>
</table>

Aq: Aqueous extract, Met: Methanol extract.

3.3 Evaluation of Antioxidant activity

In the present study, a total of four methods including, DPPH free radical-scavenging activity, ferric reducing/antioxidant power (FRAP)- reducing assay, Chelating effect on ferrous ions and β-carotene bleaching assay were studied. In order to become stable diamagnetic molecules, they have the ability to release hydrogen radical or electron to DPPH and this phenomenon is called as antioxidant activity. So, the reason for the higher antioxidant activity of the above-mentioned extracts with DPPH might be the same. However, antioxidant activity of the extracts is also well explained by FRAP, chelating capacity and bleaching assay. Antioxidant activity of the compounds is summarized in figures1, 2, 3&4. However, Andrographis paniculata (aq) shows the maximum scavenging activity of 69.70±0.40% in case of DPPH scavenging assay. The antioxidant evaluation by metal chelating effect, antioxidant activity ranges from 32.14±0.09% in Andrographis paniculata (met) to 45.62±0.20% in Andrographis paniculata (aq). In case of FRAP assay, antioxidant activity ranges from 7.80±0.40 in Andrographis paniculata (met) to 17.44±0.26% in Justicia adhatoda (met). However, the maximum antioxidant activity by β-carotene bleaching assay by Justicia adhatoda (aq). DPPH, L-Ascorbic acid as standard: 78.26% Chelating effect on ferrous ions, EDTA as standard: 72.8% FRAP assay, Ferrous sulphate as standard.

β-carotene bleaching method, Butylatedhydroxytoluene (BHT) as standard: 85.24%.

**Fig 1:** Scavenging activity (%) of different plant extracts on DPPH radicals
**Fig 2:** Ferrous ion chelating activity (%) of various extracts

**Fig 3:** Ferric reducing antioxidant capacity (%) of various plant extracts

**Fig 4:** Antioxidant activity (%) of various plant extracts by β-carotene bleaching assay
3.4 Urease inhibition studies

As in the occurrence of various pathological conditions such as gastric and peptic ulcers, stomach cancers, urolithiasis and in many more, urease enzyme plays an important role. However, there are numbers of inhibitors synthetically prepared but due to their toxic and unstable nature; there is a need to discover natural and safe ones. In the present study, urease inhibitory potential of aqueous and methanol extract of two plants viz., Andrographis paniculata and Justicia adhatoda was studied (Table 4). The urease inhibitory potential varies from 19.21±0.09 to 45.23±0.02%.

Table 4: Urease inhibitory activity of tested plant extracts at a concentration 1mg/ml

<table>
<thead>
<tr>
<th>Plant species</th>
<th>% inhibition</th>
<th>IC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrographis paniculata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aq</td>
<td>32.23±0.02</td>
<td>1.02</td>
</tr>
<tr>
<td>Met</td>
<td>45.21±0.03</td>
<td>0.86</td>
</tr>
<tr>
<td>Justicia adhatoda</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aq</td>
<td>15.21±0.08</td>
<td>1.28</td>
</tr>
<tr>
<td>Met</td>
<td>21.24±0.34</td>
<td>1.64</td>
</tr>
</tbody>
</table>

Out of the two plants of Acanthaceae family studied, Andrographis paniculata posses high amount of flavonoids, phenolic content and other phytochemicals, good antioxidant potential as well as significant urease inhibition activity. There are many reports in the literature that show the strong correlation between flavonoids and antioxidant capacity exhibited by different medicinal plant extracts [38]. In the present study, Andrographis paniculata extracts exhibited high flavonoid content also possesses strong antioxidant capacity. Lalitha et al., [39] and Malahubban et al., [40] revealed that methanol extract of the Andrographis paniculata leaves possesses higher antioxidant capacity as compared to the aqueous extract which validates the present study. Literature revealed Andrographolide (C20H30O5), a diterpenoid lactone and the effective constituent of Andrographis paniculata act as competitive inhibitor of urease enzyme which can be correlated with significant urease inhibition activity of one of the extracts in our study [41].

4. Conclusion

The present study reveals a strong likelihood of correlation between flavonoid content and antioxidant potential. The study verifies availability of some phytochemicals that could be responsible for the significant urease inhibitory potential of the Andrographis paniculata. The work validates traditional application of Andrographis paniculata as antioxidative and anti-ure. Further research towards the identification of the compound responsible need to be carried out by bioassay guided studies.

5. Acknowledgements

The authors greatly acknowledge the Haryana State Council for Science and Technology (HSCST) for financial support for the project entitled “Urease Inhibitors from Medicinal plants of Haryana in context to agricultural soil protection and therapy against clinically significant virulence factor” (Endst. No. HSCST/R&D/2015/ 2383).

6. References

18. Nagmoti DM, Khatri DK, Juvekar PR, Juvekar AR.


