Screened Phytochemicals of A. esculentus Leaves and their Therapeutic Role as an Antioxidant

Tiwarl Ayushi, Dubey Prachee, Gupta S K, Watal Geeta*

Department of Chemistry, University of Allahabad, Allahabad - 211 002, UP, India.

Available Online: 10th September, 2016

ABSTRACT
Medicinal plants are valuable natural source for the development of potentially safe drugs. The biological activities associated with these plants are due to the presence of certain phytochemicals which act either individually or synergistically. Hence, the present study deals with phytochemical screening and assessment of antioxidant activity of aqueous extract of Abelmoschus esculentus leaves using different assays viz. total phenolics, total flavonoids, total flavonols, reducing power, free radical scavenging capacity for 1.1-diphenyl-2-picrylhydrazyl (DPPH), NO and superoxide anion radicals. The phytochemical analysis of the extract revealed the presence of alkaloids, coumarins, flavonoids, glycosides, saponins, steroids, tannins and terpenoids. The total phenolics, flavonoids and flavonols present in the Abelmoschus esculentus leaf extract were 9.61 mg of Gallic Acid Equivalent (GAE)/g, 9.25 mg of Quercetin Equivalent (QE) /g and 6.12 mg of QE/g of dry extract, respectively. Reducing power of the extract was found to be concentration-dependent as it increase with increase in concentration and it was maximum at the highest evaluated concentration of 80 µg/ml. Significant antioxidant efficacy of A. esculentus leaves was further confirmed by IC50 values of DPPH, NO and superoxide anion radical scavenging assays as it was found to be 53.96, 59.15 and 52.50 µg/ml respectively. Since, the results were almost at par with, Ascorbic acid taken as reference therefore, the aqueous extract of A. esculentus leaves, having therapeutically important phytochemicals, could be developed not only as an antiaging agent but also as an agent for managing oxidative stress due to diabetic complications.

Keywords: Abelmoschus esculentus, Phytochemical, antioxidant, radical scavenging activity.

INTRODUCTION
Reactive oxygen species (ROS) are continuously produced in the system being essential for various biological processes but their high levels, due to an imbalance between formation and neutralization of these ROS, causes oxidative stress leading to cell damage. Antioxidant agents protect the cell against such damages caused by ROS/free radicals. These antioxidants may be either natural or synthetic. Medicinal plants play vital role in the discovery and development of natural antioxidants with improved efficacy and lower toxicity over synthetic agents. Thus, the Natural antioxidant are always found cost effective and safe. Various medicinal plants as Natural antioxidant have already been reported in ethnomedical literature. Phytochemicals which are secondary metabolites of plants viz tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and phenols are responsible for their bioactivities such as antimicrobial, anti diabetic and antioxidant etc. Abelmoschus esculentus is a flowering plant commonly known as ‘Bhindi’ in Hindi, belongs to the family Malvaceae, valued for its edible fruits and therapeutic importance. Hence, the present study deals with the phytochemical screening of Abelmoschus esculentus leaves and assessment of their antioxidant potential since its seeds have already been reported for its antioxidant effect. Whereas, its leaves are yet to be explored for assessment of their antioxidant efficacy and therefore it is of our choice for the present study.

MATERIALS AND METHODS
Plant material
Fresh leaves of Abelmoschus esculentus (500g) were collected from the local area of Allahabad-U.P. (India) and authenticated by Professor Satya Narayan, Taxonomist Department of Botany, University of Allahabad, India. Collected leaves of A. esculentus were washed with distilled water and dried completely under shade. The shade dried leaves (150g) were mechanically crushed, powdered and preserved for further use.

Chemicals
1,1-Diphenyl-2-picrylhydrazyl (DPPH), Trichloroacetic acid (TCA), Nitro blue Tetrizolium (NBT), and Quercetin were purchased from Alfa Aesar Pvt. Ltd., Nicotinamide adenine dinucleotide (NADH), Phenazonium Methosulphate (PMS), Folin-Cioalcalteu reagent were purchased from Merck India Pvt Ltd. Sodium phosphate dibasic (Na2HPO4), Sodium phosphate monobasic (NaH2PO4) were purchased from Hi Media Laboratories and Potassium ferricyanide, Ascorbic acid, Sodium acetate, AlCl3, FeCl3 were purchased from Sisco Research Laboratories Pvt. Ltd.

*Author for Correspondence
India. All other chemicals used including the solvents were of analytical grade.

**Preparation of Extract**

The dried leaves (150g) were extracted with distilled water (500ml) repeatedly till there was no coloration. All fractions were collected, filtered and concentrated on rotary evaporator at 40°C - 50°C under reduced pressure to get a semisolid material which was then lyophilized the yield dark brown powder (~13.5% w/w).

**Chemical test for Screening of Phytoconstituents**

Screening for the phytoconstituents of aqueous extract of *A. esculentus* leaves was carried out using standard methods as given below:

**Antioxidant Assays - in vitro**

Antioxidant study of *aqueous extract of Abelmoschus esculentus* leaves was carried out using various in vitro assays viz estimation of total phenolics, flavonoids and flavanols in addition to reducing power, and free radical scavenging capacity for DPPH, NO and superoxide anion radicals. All the assays were carried out in triplicate and their average values were taken into consideration.

**Estimation of Total Phenolics**

Total phenolic content was estimated spectrophotometrically using Folin–Ciocalteu reagent. Different concentrations of the Extract and Gallic acid, taken as standard, ranging from 25 to 400µg/mL, were prepared in 60:40 acidified methanol/water (0.3% HCl). 2.0 mL of Na2CO3 (2%) was added to 100 µl of each test solution. After 2 minutes of incubation, 2.5 mL of Folin-Ciocalteu reagent (diluted with water 1:1 v/v) was added and allowed to stand at room temperature for 30 minutes. Absorbance was measured at 750 nm. Gallic acid was used for standard curve. The total phenolic content of the sample was estimated by comparing with the standard calibration curve and was expressed as mg/g of Gallic Acid Equivalent (GAE).

**Estimation of Total Flavonoids**

Total flavonoid content was estimated using the method of Ordon.et.al. Different concentration of the Extract and Quercetin as standard ranging from 5 to 25µg/mL were prepared. 0.5 µl of ethanolic AlCl3 (2%) was added to 0.5 µL of each test solution. Appearance of yellow colour indicated the presence of flavonoids after 1 hour of incubation at room temperature. The absorbance was measured at 420 nm. Quercetin was used for standard curve. The total flavonoid content of the sample was estimated by comparing with the standard calibration curve and was expressed as mg/g Quercetin Equivalent (QE).

**Estimation of Total Flavanols**

Total flavanol content was estimated by the method of Oyaizu. Different concentration of the Extract and Quercetin as standard ranging from 5µg/mL to 25µg/mL, were prepared. 0.5 µl of 2% AlCl3 in 95% ethanol was added to 0.5 µl of each test solution. After 2.5 hours of incubation at 20°C the absorbance was measured at 440 nm. Quercetin was used for standard curve. The total flavanol content of the sample was estimated by comparing with standard calibration curve and was expressed as mg/g Quercetin Equivalent (QE).

**Determination of Reducing Power**

Reducing power was estimated by the method of Oyaizu. An aliquot of 1.0 ml extract at various concentrations, ranging from 10 to 1000 µg/mL, was mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min followed by addition of 2.5 ml of trichloroacetic acid (10%) and then centrifuged at 3000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 0.5 ml of FeCl3 and then absorbance was measured at 700 nm. Ascorbic Acid was used as standard.

**Free radical Scavenging Assays**

These assays were carried out for DPPH, Nitric oxide and Superoxide anion radicals and the ability to scavenge these radicals was calculated in terms of % inhibition using the following formula:

\[
\text{Inhibition (%) = } \left( \frac{A_0 - A_t}{A_0} \right) \times 100
\]

where \(A_0\) is the absorbance of the control, and \(A_t\) is the absorbance of the sample. The lower the value of absorbance of the sample, the higher the radical scavenging ability will be.

**DPPH Radicals**

Ability of the Extract to scavenge the stable DPPH radical was evaluated by using the method of Mensor. For this assay 0.3 mM methanolic solution of DPPH was prepared, and 1 mL of this solution was added to 1 mL of different concentrations ranging from 20-1000 µg/mL of the Extract as well as Ascorbic acid as standard. The mixture was then shaken vigorously and allowed to stand for 30 min at room temperature in dark and finally absorbance of each sample was measured at 517 nm.

**Nitric Oxide Radicals**

Scavenging of Nitrosyl radical was determined by incubating 0.5ml of 5 mM SNP in 10mM PBS (Phosphate Buffered Saline), with different concentrations ranging from 20-1000 µg/ml of the Extract as well as Ascorbic acid as standard. After 150 min, the incubation solution was mixed with 500µl of Griess reagent and then the absorbance was measured at 540 nm, once, pink colour appeared.

**Superoxide Anion Radicals**

Superoxide anion radicals were generated by the PMS/NADH system according to the method of Kakkar et al. The reaction mixture was composed of 1 ml of NBT (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH (468µM in 100 mM phosphate buffer, pH 7.4) and 100 µl of varied concentrations ranging from 20-1000 µg/ml of the Extract as well as ascorbic acid as standard. The reaction was started by addition of 100 µl of PMS (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. After 5 min incubation at 25°C, absorbance was measured at 560 nm against an appropriate blank to determine the quantity of formazan generated.

**RESULTS AND DISCUSSION**

**Screened Phytoconstituents and their Bioactivities**

Table 1. shows the correlation between phytochemicals and bioactivity of *aqueous extract of Abelmoschus esculentus* leaves. The data reveals the significant
<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>Colour Tests</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td><em>(Hager’s Test)</em>&lt;br&gt;<em>(Wagner’s test)</em>&lt;br&gt;2 ml extract + few drops of Hager’s reagent + 2 drops HCl (1.5%) + 3 drops Wagner’s reagent</td>
<td>Yellow precipitate&lt;br&gt;Brown precipitate</td>
</tr>
<tr>
<td></td>
<td><em>(Mayer’s test)</em>&lt;br&gt;<em>(Borntrager’s test)</em>&lt;br&gt;2 ml extract (EtOH) + few drops Mayer’s reagent + 3 ml Benzene + 5 ml NH₃ (10%)</td>
<td>Yellow precipitate&lt;br&gt;Pink, Violet or Red colour</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>2 ml extract + 2 ml HCl (2N) + NH₃</td>
<td>Pinkish Red to Bluish Violet colour</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td><em>(Molisch’s test)</em>&lt;br&gt;2 ml extract (EtOH) + 10 ml H₂O + 2 drops ethanolic α-naphthol (20%) + 2 ml conc. H₂SO₄</td>
<td>Reddish Violet ring at the junction</td>
</tr>
<tr>
<td></td>
<td><em>(Fehling’s test)</em>&lt;br&gt;2 ml extract + 1 ml of Fehling’s solution A and B + heat</td>
<td>Red precipitate&lt;br&gt;Yellow colour</td>
</tr>
<tr>
<td>Coumarins</td>
<td>2 ml extract + 3 ml NaOH (10%)</td>
<td>Red colour</td>
</tr>
<tr>
<td>Emodins</td>
<td>2 ml extract + 2 ml NH₄OH + 3 ml Benzene</td>
<td>Yellow precipitate</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>1 ml extract + 1 ml Pb(OAc)₂ (10%)</td>
<td>Violet to Blue to Green colour</td>
</tr>
<tr>
<td>Glycosides</td>
<td><em>(Liebmann’s test)</em>&lt;br&gt;2 ml extract + 2 ml CHCl₃ + 2 ml CH₃COOH</td>
<td>Reddish Brown ring at the junction</td>
</tr>
<tr>
<td></td>
<td><em>(Salkowski’s test)</em>&lt;br&gt;2 ml extract + 2 ml CHCl₃ + 2 ml conc. H₂SO₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Fehling’s test)</em>&lt;br&gt;5 ml extract + 5 ml Isoamyl alcohol</td>
<td>Organic layer turns into Red colour</td>
</tr>
<tr>
<td>Leucoanthocyanins</td>
<td><em>(Precipitate test)</em>&lt;br&gt;2 ml extract + 2 ml HCl (1%) + boil</td>
<td>Red precipitate</td>
</tr>
<tr>
<td>Proteins</td>
<td><em>(Xanthoproteic test)</em>&lt;br&gt;1 ml extract + 1 ml conc. H₂SO₄</td>
<td>White precipitate to Yellow on heating</td>
</tr>
<tr>
<td></td>
<td><em>(Biuret’s test)</em>&lt;br&gt;1 ml extract + 5-6 drops w/v NaOH + 2 drops CuSO₄ (30% w/v)</td>
<td>Violet Red colour</td>
</tr>
<tr>
<td>Saponins</td>
<td><em>(Foam Test)</em>&lt;br&gt;5 ml extract + 5 ml H₂O + heat</td>
<td>Froth appearance</td>
</tr>
<tr>
<td>Steroids</td>
<td><em>(Emulsion test)</em>&lt;br&gt;5 ml extract + Olive oil (few drops)</td>
<td>Emulsion formation</td>
</tr>
<tr>
<td></td>
<td><em>(Salkowski’s Test)</em>&lt;br&gt;2 ml extract + 2 ml CHCl₃ + 2 ml conc. H₂SO₄</td>
<td>Reddish Brown colour at interface</td>
</tr>
<tr>
<td>Tannins</td>
<td><em>(Braymer’s Test)</em>&lt;br&gt;2 ml extract + 2 ml H₂O + few drops of FeCl₃ (5%)</td>
<td>Green colour</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>2 ml extract + EtOH+2ml CHCl₃ Δ (2 mint.) + 3 drops conc. H₂SO₄</td>
<td>Deep red colour</td>
</tr>
</tbody>
</table>

The presence of alkaloids, carbohydrate, coumarins, flavonoids, terpenoids and tannins in comparison to saponins, steroid and glycosides which were present in lesser extent. Whereas anthocyanins, anthraquinones, emodins, leucoanthocyanins, phlobatannins and proteins were totally absent. Since all these secondary metabolites are associated with various biological activities therefore the antimicrobial activity of *A. esculentus* leaves must be due to the presence of alkaloids as they are ranked the most efficient therapeutically significant plant substance used as a basic medicinal agent for its bactericidal effect. The presence of coumarins can be accounted for antiproliferative activity of *A. esculentus* leaves on the basis of its anti-inflammatory and antimicrobial activities, whereas the presence of carbohydrate, coumarins and glycosides are beneficial for the action of immune system by increasing the strength of body and hence these are valuable as dietary supplements. The other important bioactivities of *A. esculentus* leaves viz. antioxidant and anticancer are solely due to the presence of flavonoids, as a potent water soluble antioxidant and free radical scavenger, which prevent oxidative cell damage and also have strong anticancer activity. It manages diabetes induced oxidative stress as well. Since, terpenoids are well known for their number of bioactivities therefore its presence in *A. esculentus* leaves must be responsible for their antioxidant, hypoglycemic, antimicrobial and anticancer activities. Tannins are polyphenolic compounds and their derivatives are also considered as primary antioxidants or free radical scavengers. The presence of Saponins due to their general characteristics of cholesterol binding property and presence of steroids due to their cholesterol reducing property collectively enhance the therapeutic efficacy of the aqueous extract of *Abelmoschus esculentus* leaves.

**Antioxidant Assays - in vitro**

Table 2, shows the results of quantitative estimation of total phenolics, flavonoids and flavanols. Standard curve equation of Gallic acid was calculated with $y = 0.0032x + 0.111$, $R^2 = 0.941$ for total phenolics. Whereas, standard curve equation of Quercetin was calculated with $y =$
concentrations of aqueous extract of *Abelmoschus esculentus* leaves and of Ascorbic acid taken as standard. The reducing power of both, the sample and the standard were found to be concentration-dependent as the absorbance recorded has increased with increase in concentration. Moreover, the reducing power of the *A. esculentus* was found to be significantly greater than that of the standard, at the highest evaluated concentration of 800 μg/ml, confirming thereby the antioxidant potential of *A. esculentus* leaves. The highly significant reducing power of *A. esculentus* leaves have which is even greater than that of the standard, Ascorbic acid must be phenolic contents mediated. Thus, the reducing capacity of the extract is related to the presence of electron-donating reductants which react with free radicals and convert them to stable products resulting into terminate radical chain reaction.

**Assessment of Free radical Scavenging Activity**

**DPPH radical scavenging activity**

Fig.2, exhibits the results of DPPH radical scavenging activity of both, the Extract and the Standard, Ascorbic acid, at varied range of concentration. Results depict that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 80μg/ml with inhibition of 81.73% in case of Extract and 96.47% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaf extract. The IC₅₀ values of the Extract and the Standard were found to be 53.96 and 32.25 μg/ml respectively, which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent.

**NO radical Scavenging Activity**

Fig.3, exhibits the results of NO radical scavenging activity of both, the Extract and the Standard, Ascorbic acid, at varied range of concentration. Results depict that the scavenging ability in both the cases was concentration dependent and was found to be maximum at a concentration of 80μg/ml with inhibition of 77.59% in case of Extract and 85.99% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaves extract. The IC₅₀ values of the Extract and the Standard were found to be 59.15 and 50.19 μg/ml which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent. With the help of this data, selected medicinal plant could be further explored for its therapeutic efficacy as an antioxidant.

**Superoxide anion radical scavenging activity**

Fig.4, exhibits the results of Superoxide anion radical scavenging activity of both, the Extract and the Standard at varied range of concentration. Results depicts that the scavenging ability in both the cases was concentration

---

**Table 1: Screened Phytoconstituents and their Bioactivities**

<table>
<thead>
<tr>
<th>Phytoconstituents</th>
<th>A. esculentus leaf</th>
<th>Bioactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids++</td>
<td>Antimicrobial²⁵</td>
<td></td>
</tr>
<tr>
<td>Carbohydrate++</td>
<td>Dietary supplement²⁷</td>
<td></td>
</tr>
<tr>
<td>Coumarins++</td>
<td>Antiproliferative activity²⁶</td>
<td></td>
</tr>
<tr>
<td>Flavonoids++</td>
<td>Anticancer³⁰, Antioxidant²⁸, ²⁹</td>
<td></td>
</tr>
<tr>
<td>Glycosides+</td>
<td>Dietary supplement²⁷</td>
<td></td>
</tr>
<tr>
<td>Saponins+</td>
<td>Cholesterol binding property³⁹</td>
<td></td>
</tr>
<tr>
<td>Steroids+</td>
<td>Cholesterol reducing property⁴⁰</td>
<td></td>
</tr>
<tr>
<td>Tannins++</td>
<td>Free radical scavenger⁸</td>
<td></td>
</tr>
<tr>
<td>Terpenoids++</td>
<td>Hypoglycemic³¹, ³²</td>
<td></td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Emodins</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Leucoanthocyanins</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Flabobatanins</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

0.049x-0.325, R² = 0. 896 for total flavanoids and with y = 0.032x+ 0.002, R² = 0.998 for total flavanols. Since, Antioxidant efficacy is directly proportional to the quantity of total phenolic content due to the ability of their hydroxyl groups to scavenge free radicals, therefore the total estimated phenolic content of 9.65mg/g GAE must be contributing towards the significant antioxidant potential of the Extract⁴¹. Flavonoids are important secondary metabolite of plants and play vital role in modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. Since, pharmacological effect of flavonoids is associated with their antioxidant activities⁴² therefore the total estimated flavonoid content of 9.25 mg/g QE must be responsible for the significant antioxidant efficacy of the Extract. Flavonol is another important phytoconstituent which helps in treating cardiovascular diseases caused by oxidative stress. Hence, estimated flavonol content of 6.12mg/g QE must be attributing to the antioxidant profile of *A. esculentus* leaves⁴³. Thus, the extent of significant presence of total phenolics, flavanoids and flavanols may attribute to the antioxidant and free radical scavenging potential of the aqueous extract *A. esculentus* leaves.

**Assessment of Reducing Power**

Fig.1, depicts the results of Reducing Power of varied

---

**Table 2: Quantitative Estimation of Total Phenolics, Flavonoids and Favonols of aqueous extract of *Abelmoschus esculentus* leaves.**

<table>
<thead>
<tr>
<th>Dry extract</th>
<th>Total Phenolic (mg/g GAE)</th>
<th>Total Flavonoid (mg/g QE)</th>
<th>Total Flavonol (mg/g QE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abelmoschus esculentus</em></td>
<td>9.65±0.08</td>
<td>9.25±0.05</td>
<td>6.12±0.14</td>
</tr>
</tbody>
</table>
Figure 1: Reducing Power of *A. esculentus* leaves and Ascorbic acid, the standard.

Figure 2: DPPH radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.

Figure 3: NO radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.

Figure 4: Superoxide anion radical Scavenging Activity of *A. esculentus* leaves and Ascorbic acid.
dependent and was found to be maximum at a concentration of 80µg/ml with inhibition of 74.69% in case of Extract and 87.4% in case of Standard, validating thereby the significant antioxidant potential of *A. esculentus* leaves extract. The IC₅₀ values of the Extract and the Standard were found to be 52.50 µg/ml and 42.26µg/ml respectively, which further confirmed that *A. esculentus* leaves could be developed as an effective antioxidant agent.

**CONCLUSION**

Conclusively it could be stated that *A. esculentus* leaves can be explored further in order to develop a novel antioxidant agent with high therapeutic efficacy due to high content of their polyphenolics including terpenoids in addition to flavanoids. The highly significant antioxidant efficacy of *A. esculentus* leaves was also evident from its high free radical scavenging potential and low IC₅₀ values in addition to high reducing power in comparison to the standard drug, ascorbic acid, taken as reference.

**ACKNOWLEDGEMENT**

The first author, Ayushi Tiwari is thankful to University Grants Commission (UGC), NewDelhi, India for providing financial assistance.

**CONFLICT OF INTEREST**

All authors declare no conflict of interest.

**REFERENCES**