EVALUATION OF ANTIOXIDANT AND TOTAL FLAVANOID CONTENT OF MIRABILIS JALAPA LINN USING IN VITRO MODELS

Subin Mary Zachariah*, 1, 3 N.A. Aleykutty2, B. Jaykar3, Vidya Viswanad1, Halima O.A1
1Amrita School of Pharmacy, Ponekkara, Kochi-41, Kerala India
2Pushpagiri College of Pharmacy Tiruvalla, Kerala, India
3Vinayaka Missions College of Pharmacy, Salem, Tamil Nadu, India

Article Received on: 21/01/12 Revised on: 05/03/12 Approved for publication: 18/03/12

*Email:subinzac@gmail.com

ABSTRACT
The herbal drugs form the backbone of all traditional system of medicine around the world due to their endless therapeutic activity. Mirabilis jalapa Linn has been hailed as a cure for many common pathological conditions affecting the human body. It has folkloric use as antiinflammatory, antiinflammatory agent, laxative, and a host of other uses. The present study was conducted to evaluate the plants potential as an antioxidant lead by using various in vitro models like FRAP (Ferric reducing ability of plasma) DCF(AAPH), superoxide anion scavenging activity, nitric oxide scavenging activity and by the estimation of total flavanoid content. The plant exhibited significant inhibition of the superoxide anion and the nitric oxide radicals even at low concentration. The total flavanoid content of the extract was found to be 4.41 ± 0.02 mg /gram. The findings indicate that the methanolic extract of Mirabilis jalapa has potential antioxidant activity which is comparable to the standards. The results concluded that the extract have a potential source of antioxidants of natural origin.

KEY WORDS: Mirabilis jalapa Linn, Antioxidant, FRAP, Superoxide anion scavenging activity, nitric oxide scavenging activity, flavanoid content

INTRODUCTION
There has always been a continuing emphasis on the herbal medicines as a potential pipeline for novel bioactive molecules that encompass a varied field of application from cancer treatment and Alzheimer’s to autoimmune diseases. Mirabilis jalapa Linn of family Nyctaginaceae has been called by various vernacular names around the world like 'Four o’ clock' in English, Gulambasa” in Ayurveda, and ‘Gulabbas’ in Hindi. Mirabilis jalapa has been extensively used in almost all folklore remedies around the world for treating a variety of conditions. It has been reported that indigenous Mexican population uses various decoctions and preparations of Mirabilis jalapa for muscular pain, diarrhoea, dysentery, and abdominal colic. The plant has been extensively studied for a variety of bioactive principles and screened for different pharmacological activities. The ethanolic extract of the leaves and the stem was found to have potent antinociceptive activity in experimental mice. The plant has also proved to possess antibacterial, antiviral, and antioxidant activity. Furthermore studies have also tried to elucidate the role played by adrenergic and serotonergic receptors in the inhibitory effect of the flower extracts of Mirabilis jalapa on smooth muscle contractility. Studies on isolated jejunum muscles indicated that methanolic flower extracts possessed potent contractile activity. Efforts towards the identification and isolation of active principles from the plant has resulted in the isolation of eleven compounds including gingerlycolipid, 4'-hydroxy-2, 3-dihydroflavone, astragaloside VI etc. Moreover numerous components like β-sitosterol, stigmasterol, ursolic acid, oleanolic acid, brassicasterol, and Mirabilis antiviral protein, rotenoids (mirabijalone A-D, boerainones C and F) have been successfully isolated and characterised. The flowers possess antispasmodic activity and investigation of the mechanism of action indicated a role of serotoninerger receptor. The aqueous extract of the leaves possess potential anti inflammatory activity. Mirabilis jalapa has also been evaluated for its anti histaminic activity and it has been found that in concordance with the folkloric use of the plant for allergy and asthma it has significant inhibitive action on the release of histamine and subsequent typical allergic responses. It has also been evaluated for the antihelmentic activity using in-vitro models and was found to possess vermicidal activity. The present study aims to evaluate the methanolic extracts of the aerial parts of the Mirabilis jalapa Linn for potential antioxidant activity using conventional in vitro models like the Superoxide anion scavenging assay, FRAP, DCF (AAPH), Nitric oxide radical scavenging assay and the total flavanoid content estimation.

MATERIALS AND METHODS
Collection and identification of plant material
The fresh plants of Mirabilis jalapa Linn were collected in the months of July-August from the local areas of Kochi and authenticated by the authority of the botany department, S.H. College, Kochi. The aerial parts were washed with water, shade dried powdered in a mechanical grinder and kept in air tight container till use.

Preparation of the plant extract
The extraction of the aerial parts of Mirabilis jalapa were carried out by known standard procedures. The plant materials were dried in shade and powdered in a mechanical grinder. The powder (100gm) of the aerial parts each were initially de-fatted with petroleum ether (60-80°C), followed by 500ml methanol by Soxhlet extraction method for 72 hrs separately. Solvent elimination under reduced pressure afforded the petroleum ether and methanol extract of which methanol extract was further used for antioxidant assay methods. The extract was dried in a vacuum desiccator to obtain constant weight. The phychochemical screening was carried out as described by Norman. The methanolic extract of the aerial parts yielded a dark brown residue (2.5 %). The extracts were then kept in sterile bottles, under refrigerated conditions, until further use. The dry weight of the plant extracts were obtained by the solvent evaporation and used to determine the concentration in mg/ml. The extract was used directly for the superoxide anion scavenging assay, FRAP, DCF (AAPH) assay nitric oxide radical scavenging assay and the total flavanoid content estimation.
The plant extract (50 mg) were dissolved separately in methanol to obtain lower dilutions. The solution was used as the control after the incubation of alkaloid. Preparation of test and standard solutions

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction.

Phytochemical evaluation

The dried methanolic extract was used to analyze qualitatively various phytoconstituents such as alkaloids, glycosides, steroids, saponins, phenolic compounds, tannins, flavanoids, carbohydrates and proteins using standard procedures.

**Estimation of flavonoid content using Swain and Hillis method (1959)**

Preparation of test and standard solutions

The plant extract (50 mg) were dissolved separately in 50 ml of methanol. These solutions were serially diluted with methanol to obtain lower dilutions. Floroglucinol (50 mg) was dissolved in 50 ml of distilled water. It was serially diluted with water to obtain lower dilutions.

**Protocol for total Flavonoid content**

0.2 ml of the extract was taken in a test tube and the final volume was made up to 2 ml with distilled water. To this 4 ml of vanillin reagent was added rapidly. Exactly after 15 min absorbance was recorded at 500 nm against blank. The unknown was read from a standard curve prepared using different concentration of phlobaginic. In the phytochemical identification, the aqueous extract of Mirabilis jalapa with 5% ferric chloride solution gave deep blue colour and with lead acetate solution gave white precipitate indicated the presence of tannin and phenolic compounds. The extract with 5ml 95% ethanol, few drops of concentrated HCl and 0.5 g magnesium turnings gave pink colour indicated the presence of flavanoids. The extract with dragendoff reagent gave reddish brown precipitate showed the presence of alkaloid.

**Superoxide anion scavenging activity**

The scavenging activity of extract towards superoxide anion radicals was measured by the standard method. About 1 ml of nitro blue tetrazolium solution (156μM in 100 mM phosphate buffer, pH 7.4), 1 ml nicotine amide adenine dinucleotide solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different concentrations (10, 20, 30, 40, 50μg) of extract and standard in solvent were mixed. The reaction was initiated by adding 100 μl of phenazine methosulfate (PMS) solution (60 μM) in 100 mM phosphate buffer, (pH 7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance at 560 nm was measured against reagent blank in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation.

**Percentage inhibition = [1-(Abs sample/Abs control)]**

Where, Abs control was the absorbance of the control (without extract) at 560 nm; Abs sample was the absorbance in the presence of the extract at 560 nm. The experiment was repeated in triplicate.

**Nitric oxide Radical Scavenging Activity**

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. 1 ml of sodium nitroprusside (10 mM) in phosphate buffered saline (0.2 M, pH 7.4) was mixed with 100ml sample solution of various concentrations (10, 20, 30, 40, 50μg) and incubated at room temperature for 150 min. The same reaction mixture without the sample was used as the control after the incubation period; 0.5 ml of Griess reagent (1% sulfuric acid, 2% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore (pink colour) formed was read at 546 nm.

**Ferric reducing ability of plasma (FRAP) expressed as a function of time**

Ferric reducing ability of plasma is one of the method which directly analyses total antioxidant. FRAP assay is based on the ability of antioxidants to reduce Fe³⁺ to Fe²⁺ in the presence of 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe²⁺-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH dependent (optimum pH 3.6). FRAP agent was prepared by mixing acetate buffer (500 mM/l) with tryipyridyltrazaine (TPTZ) (10 mM/l) and 2.5 ml of ferric chloride (20 mM/l) solution. The reaction mixture contained freshly prepared FRAP reagent warmed to 37 °C, added to test along with water. Absorbance of this solution was taken at 593 nm, just after 4 min from the time of addition of FRAP reagent. An increase in absorbance indicated enhanced reducing potential of plasma. Quantitative calculation for each sample was done using an equation obtained from the standard curve of Fe⁺⁺-TPTZ. About 1 ml sample was added to 2.5 ml of concentrated HCl and 0.5 g magnesium turnings gave pink colour indicated the presence of flavonoids. The extract with dragendoff reagent gave reddish brown precipitate showed the presence of alkaloid.

**Total flavonoid content**

The flavonoid content was found to be 4.41 ± 0.02 mg/gram of dried extract equivalent to phloroglycinol (Figure1). The total flavonoid content shows good linear relation in both standard as well as sample extract.
Superoxide anion scavenging activity
Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species. The superoxide radical is produced in vivo and can result in the formation of H₂O₂ via dismutation reaction. Moreover, the conversion of superoxide and H₂O₂ into more reactive species, e.g., the hydroxyl radical, has been thought to be one of the unfavorable effects caused by superoxide radicals. The results are represented in Table 1 and Figure 2. The IC₅₀ value of melanocholic extract was found to be 18.49 µg/ml.

Nitric oxide radical scavenging activity
Mirabilis jalapa extract also caused a moderate dose-dependent inhibition of nitric oxide. The results are indicated in Table 2 and Figure 3. The IC₅₀ value of melanocholic extract was found to be 17.28 µg/ml.

Ferric Reducing ability of the plasma (FRAP) expressed as a function of time
The results of the FRAP assay are reported in Table 3 and Figure 4. The IC₅₀ value was found to be 40.03 µg/ml. FRAP assay is based on the ability of antioxidants to reduce Fe³⁺ to Fe²⁺ in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe²⁺-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). Linearity of FRAP (dose–response line) for standard solutions is shown in Figure 5.

DCF (AAPH) assay
The azo scavenging activity of melanocholic extracts were examined and it showed a dose dependent activity with concentration. But the antioxidant activity was comparatively less than the standard quercetin. The results of the assay are represented in Table 4 and figure 6. The IC₅₀ value of melanocholic extracts was found to be 1752 µg/ml.

CONCLUSION
The in-vitro antioxidant activity was evaluated by nitric oxide and superoxide, FRAP and DCF/AAPH assay. In nitric oxide method, the melanocholic extract effectively reduced the generation of nitric oxide from sodium nitroprusside. The percentage scavenging activity increased with increasing concentration of the extract. The cytotoxic and antioxidant properties, of flavonoids are considered in an evaluation of their biological and nutritional values of these compounds. The results of the present study shows that the melanocholic extract of the aerial parts of Mirabilis jalapa Linn possess antioxidant activity through, nitric oxide, superoxide radical scavenging assay, FRAP assay and DCF/AAPH assay. The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging. The preliminary phytochemical investigation indicated the presence of flavonoids, tannins and phenolics in the plant which are proved to be well known natural antioxidants. The separation and identification of flavonoids present in the plant can help researchers find new molecules which can be used as natural antioxidants.

REFERENCES
TABLE 1. FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS BY SUPEROXIDE ANION SCAVENGING ASSAY

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Percentage inhibition of the standard</th>
<th>Percentage inhibition of the methanol extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>19.01±0.019</td>
<td>12.58±0.299</td>
</tr>
<tr>
<td>20</td>
<td>43.24±0.204</td>
<td>38.18±0.312</td>
</tr>
<tr>
<td>30</td>
<td>76.09±0.098</td>
<td>74.20±0.324</td>
</tr>
<tr>
<td>40</td>
<td>85.41±0.259</td>
<td>82.16±0.120</td>
</tr>
<tr>
<td>50</td>
<td>86.44±0.722</td>
<td>84.27±0.025</td>
</tr>
<tr>
<td>EC50</td>
<td>23.20µg/ml</td>
<td>18.49µg/ml</td>
</tr>
</tbody>
</table>

TABLE 2. FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS BY NITRIC OXIDE ANION RADICAL SCAVENGING ASSAY

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Percentage inhibition of the standard</th>
<th>Percentage inhibition of the methanol extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>19.01±0.019</td>
<td>16.07±0.037</td>
</tr>
<tr>
<td>20</td>
<td>43.24±0.204</td>
<td>45.42±0.223</td>
</tr>
<tr>
<td>30</td>
<td>76.09±0.098</td>
<td>65.29±0.146</td>
</tr>
<tr>
<td>40</td>
<td>85.41±0.259</td>
<td>70.24±0.102</td>
</tr>
<tr>
<td>50</td>
<td>86.44±0.722</td>
<td>81.28±0.038</td>
</tr>
<tr>
<td>EC50</td>
<td>23.20µg/ml</td>
<td>17.28µg/ml</td>
</tr>
</tbody>
</table>

TABLE 3. FERRIC REDUCING ABILITY OF THE PLASMA (FRAP) EXPRESSED AS A FUNCTION OF TIME

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance in nm Methanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.3075667 ± 0.001283657</td>
</tr>
<tr>
<td>20</td>
<td>0.4836 ± 0.001299997</td>
</tr>
<tr>
<td>30</td>
<td>0.7358667 ± 0.001560269</td>
</tr>
<tr>
<td>40</td>
<td>1.143333 ± 0.001614869</td>
</tr>
<tr>
<td>50</td>
<td>1.313533 ± 0.001398025</td>
</tr>
<tr>
<td>EC50</td>
<td>40.03 µg/ml</td>
</tr>
</tbody>
</table>

TABLE 4: FREE RADICAL SCAVENGING ACTIVITY OF METHANOLIC EXTRACTS BY DCF/AAPH ASSAY

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Quercetin±SD</th>
<th>Concentration (µg/ml)</th>
<th>Methanolic extract±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>11.66 ± 0.13</td>
<td>100</td>
<td>29.57 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>17.95 ± 0.08</td>
<td>200</td>
<td>32.00 ± 0.09</td>
</tr>
<tr>
<td>30</td>
<td>49.41 ± 0.11</td>
<td>300</td>
<td>34.24 ± 0.08</td>
</tr>
<tr>
<td>40</td>
<td>53.57 ± 0.24</td>
<td>400</td>
<td>36.34 ± 0.04</td>
</tr>
<tr>
<td>50</td>
<td>65.54 ± 0.07</td>
<td>500</td>
<td>38.48 ± 0.05</td>
</tr>
<tr>
<td>IC-50</td>
<td>31.47 µg/ml</td>
<td>1752 µg/ml</td>
<td></td>
</tr>
</tbody>
</table>

Values are Mean ± SD (n=3) SD-Standard deviation

Figure 1. Quantitative estimation of flavanoids
Figure 2. Super oxide anion radical scavenging assay

Figure 3. Nitric oxide anion radical scavenging assay

Figure 4. Ferric reducibility assay of plasma (FRAP) expressed as a function of time

Figure 5. Linearity of FRAP assay method
Figure 6. DCF/AAPH

Source of support: Nil, Conflict of interest: None Declared