Estimation of Total Flavonoids and Antioxidant Activity of Spilanthes acmella Leaves

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Abstract

Flavonoids are a group of polyphenolic compounds, which are widely distributed throughout the plant kingdom. Flavonoids of different classes exhibited various pharmacological and biological activities. The present study was undertaken to investigate the total flavonoids and antioxidant effect of Spilanthes acmella leaves. The ethanol extract of Spilanthes acmella leaves were prepared and performed its phytochemical screening. The total flavonoids and polyphenol were investigated to quantify the presence of polyphenol compounds. The 2,2-diphenyl-1-picryl-hydrazil stable radical (DPPH) and Superoxide scavenging radical were used to determine extract antioxidant activity. The concentrations of flavonoids polyphenol in ethanol extract of Spilanthes acmella were 72.14 QE mg/gm and 84.52 GAE mg/gm, respectively. The extract exhibited the strongest antioxidant activity, with the lowest IC50 value for DPPH and Superoxide scavenging. The IC50 value for DPPH and Superoxide scavenging were 134.11 µg/ml and 104.51 µg/ml, respectively. The strongest antioxidant activity of ethanol extract could be due to the presence of flavonoids and phenols.

Keywords:
Spilanthes acmella, Total flavonoids, Total polyphenol, Antioxidant activity

1 Introduction

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrene structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for the variety of pharmacological activities. The flavonoids are mostly used for the health benefits due to antioxidant properties. The hydroxyl group present in the flavonoids regulates the antioxidant activity by scavenging free radicals and/or by chelating metal ions. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules. The various studies reported protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases.

Spilanthes acmella L. belongs to the genus Spilanthes, family Asteraceae (Compositae). It is an herb found all around the world and widely distributed throughout the tropics and subtropics. It is native to the tropics of Brazil, and is grown as an ornamental (and as a medicinal) in various parts of the world. Commonly it is known as Toothache plant or Paracress or Eyeball plant. The name Eyeball plant should be obvious to anyone who is familiar with the plant’s flowers, which are yellow and gradually turn to dark red in the center. The active constituent spilanthol chiefly present in leaves and flower heads, and produce analgesic activity used to numb toothache. The whole plants can be used in the treatment of dysentery and rheumatism. A decoction of the plant can be taken internally as a diuretic and able to resolve stones in the bladder, while a decoction of the roots can be used as a purgative. It is also used as a defensive medicine for scurvy and stimulates digestion. Besides these medicinal uses, the flower heads have been used as a spice for appetizers by the Japanese.

The various extracts and active metabolites namely spilanthol (N-isobutyl-2E, 6Z, 8E-decatrienamide), butylated hydroxytoluene and fatty acids (n-Hexadecanoic acid and tetradecanoic acid), sesquiterpenoids, polygodial and eudesmanolide II from various parts of this plant possess useful pharmacological activities. The researchers documented...
numerous pharmacological actions due to its flavonoids property namely antifungal, antipyretic, local anaesthetic, bioinsecticide, anticonvulsant, antioxidant, aphrodisiac, analgesic, pancreatic lipase inhibitor, antimicrobial, antinociception, diuretic, vasorelaxant, anti-human immunodeficiency virus, toothache relieve and anti-inflammatory effects. Hence, the study was planned to estimate the total flavonoids and antioxidant activity of Spilanthes acmella leaves extract.

2 Materials and Methods

2.1 Plant collection

The fresh leaves of Spilanthes acmella were collected from the Green House Pipani, Barkhera Nursery, Bhopal, Madhya Pradesh, India during month of May- June, 2016. Collected material was washed thoroughly in running tap water, rinsed in distilled water and shade dried in open air and grinded into powder for further use.

2.2 Preparation of the crude extracts

The powder of the leaves of Spilanthes acmella, was packed in the Soxhlet apparatus and extracted with ethanol, until the completion of the extraction. The extract was filtered while hot, and the resultant extract was distilled in vacuum under reduced pressure in order to remove the solvent completely, and later dried in a desiccator. After that ethanol extract of leaves was kept in air tight container for further study.

2.3 Preliminary Phytochemical analysis

The extracts were analyzed by the following procedures. To test for the presence of the alkaloids, saponins, tannins, Terpenoids, flavonoids, glycosides, volatile oils and reducing sugars.

2.3.1 Test for alkaloids

(a) Dragendorff’s test: To 1 ml of the extract, add 1 ml of dragendorff’s reagent (Potassium Bismuth iodide solution). An orange-red precipitate indicates the presence of alkaloids.

(b) Mayer’s test: To 1 ml of the extract, add 1 ml of mayer’s reagent (Potassium mercuric iodide solution). Whitish yellow or cream coloured precipitate indicates the presence of alkaloids.

(c) Hager’s test: To 1 ml of the extract, add 3 ml of Hager’s reagent (Saturated aqueous solution of picric acid), yellow coloured precipitate indicates the presence of alkaloids.

(d) Wagner’s test: To 1 ml of the extract, add 2 ml of wagner’s reagent (Iodine in Potassium iodide), Formation of reddish brown precipitate indicates the presence of alkaloids.

2.3.2 Test for saponins

Take small quantity of alcoholic and aqueous extract separately and add 20 ml of distilled water and shake in a graduated cylinder for 15 minutes lengthwise. A 1cm layer of foam indicates the presence of saponins.

2.3.3 Test for Glycosides

(a) Legal test: Dissolve the extract in pyridine and add sodium nitroprusside solution to make it alkaline. The formation of pink red to red colour shows the presence of glycosides.

(b) Baijet test: To 1ml of the test extract, add 1ml of sodium picrate solution and the yellow to orange colour reveals the presence of glycosides.

(c) Keller-Killiani test: 1gm of powdered drug is extracted with 10ml of 70% alcohol for 2 minutes, filtered, add to the filtrate, 10ml of water and 0.5ml of strong solution of lead acetate and filtered and the filtrate is shaken with 5ml of chloroform. The chloroform layer is separated in a porcelain dish and removes the solvent by gentle evaporation. Dissolve the cooled residue in 3ml of glacial acetic acid containing 2 drops of 5% ferric chloride solution. Carefully transfer this solution to the surface of 2ml of concentrated sulphuric acid. A reddish brown layer forms at the junction of the two liquids and the upper layer slowly becomes bluish green, darkening with standing.

(d) Borntrager’s test: Add a few ml of dilute Sulphuric acid to 1ml of the extract solution. Boil, filter and extract the filtrate with chloroform. The chloroform layer was treated with 1ml of ammonia. The formation of red colour of the ammonical layer shows the presence of anthraquinone glycosides.

2.3.4 Test for carbohydrates and sugars

(a) Molisch’s test: To 2 ml of the extract, add 1ml of α-napthol solution, add concentrated sulphuric acid through the side of the test tube. Purple or reddish violet colour at the junction of the two liquids reveals the presence of Carbohydrates.

(b) Fehling’s test: To 1 ml of the extract, add equal quantities of Fehling solution A and B. upon heating formation of a brick red precipitate indicates the presence of sugars

(c) Benedict’s test: To 5 ml of Benedict’s reagent, add 1ml of extract solution and boil for 2 minutes and cool. Formation of red precipitate shows the presence of sugars.
2.3.5 Test for tannins and phenolic compounds

(a) Take the little quantity of test solution and mixed with basic lead acetate solution. Formation of white precipitates indicates the presence of tannins.

(b) To 1 ml of the extract, add ferric chloride solution, formation of a dark blue or greenish black colour product shows the presence of tannins.

(c) The little quantity of test extract is treated with Potassium ferric cyanide and ammonia solution. A deep red colour indicates the presence of flavonoids.

2.3.6 Test for flavonoids

(a) The drug in alcoholic and aqueous solution with few ml of ammonia is seen in U.V. and visible light, formation of fluorescence indicates the presence of flavonoids.

(b) Little quantity of extract is treated with amyyl alcohol, sodium acetate and ferric chloride. A yellow colour solution formed, disappears on addition of an acid indicates the presence of flavonoids.

(c) Shinoda’s test: The alcoholic extract of powder treated with magnesium foil and concentrated HCl give intense cherry colour in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

(d) The extract is treated with sodium hydroxide, formation of yellow or orange colour indicates the presence of flavones.

(e) The extract is treated with concentrated H₂SO₄, formation of yellow or orange colour indicates flavonols.

2.3.7 Test for steroids

(a) Libermann-Burchard test: 1 gm of the test substance was dissolved in a few drops of chloroform, 3 ml of acetic anhydride, 3 ml of glacial acetic acid were added, warmed and cooled under the tap and drops of concentrated sulphuric acid were added along the sides of the test tube. Appearance of bluish-green colour shows the presence of sterols.

(b) Salkowski test: Dissolve the extract in chloroform and add equal volume of conc. H₂SO₄. Formation of bluish red to cherry colour in chloroform layer and green fluorescence in the acid layer represents the steroidal components in the tested extract.

2.3.8 Test for triterpenoids

Noller’s test: Dissolve two or three granules or tin metal in 2 ml thionyl chloride solution. Then add 1 ml of the extract into test tube and warm, the formation of pink colour indicates the presence of triterpenoids^{11-13}.

2.4 In vitro antioxidant activity

2.4.1 Total flavonol content

Flavones and flavonols contents were analyzed by the colorimetric method. 9.8 ml of the prepared extract was mixed with a 10% solution of aluminum chloride (200 µl). After 30 min, absorption was measured at a 425 nm wavelength. The amount was calculated using quercetin calibration curve. The results were expressed as the quercetin equivalent (QE) mg per 100 ml of the sample.

2.4.2 Total polyphenol content

Total polyphenol content was determined using colorimetric method. 2.0 ml of the prepared extract was oxidized using Folin-Ciocalteu reagent (400 µl), and sodium carbonate solution (75 g/l) was then added to the reaction mixture to reach a 10.0 ml volume. After 2 h, the suspension was centrifuged for 10 min at 5000 rpm, and absorption was measured at a 760 nm wavelength. The amount was calculated using the gallic acid calibration curve. The results were expressed as gallic acid equivalent (GAE) mg per 100 ml of the sample.

2.4.3 Hydrogen-donating activity

This assay was used in many studies for testing antioxidant activity. 2,2-diphenyl-1-picryl-hydrazil stable radical (DPPH) evidently offers a convenient and accurate method for titrating the oxidizable groups of natural and synthetic antioxidants. This assay was based on the reduction of a methanolic solution of the colored free radical DPPH by free radical scavenger. The degradation of DPPH was evaluated by comparison with a control sample without hydrogen-donating compounds. The decrease in absorbance of DPPH at its absorbance maximum of 517 nm was proportional to the concentration of free radical scavenger added to DPPH reagent solution. Lower absorbance of reaction mixture indicated higher antioxidant activity.

In this study, methanolic solution of DPPH (100 mM, 2.95 ml), 0.05 ml of extracts dissolved in methanol was added at different concentrations (50-250 μg/ml). Reaction mixture was shaken and after 30 min at room temperature, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity (% AA). Ascorbic acid was used as standard. The degree of discoloration indicates the scavenging efficacy of the extract, was calculated by the following equation^{13-15}.

\[
\% \text{ AA} = 100 - \left( \frac{[\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}] \times 100}{\text{Abs}_{\text{DPPH}}} \right)
\]

2.4.4 Superoxide scavenging activity

Superoxide scavenging (SOD) was carried out by using alkaline Dimethyl sulfoxide (DMSO). Solid potassium superoxide was allowed to stand in contact with dry DMSO for at least 24 h and the solution was filtered immediately before use. Filtrate (200 ml) was added to 2.8 ml of an aqueous solution containing nitrobutetrazolium (56 mM), EDTA (10 mM) and potassium phosphate buffer (10 mM, pH 7.4). Sample extract (1 ml) at various concentrations (50-250 μg/ml) in water was added and the absorbance was recorded at 560 nm against a control in

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which pure DMSO has been added instead of alkaline DMSO.  

3 Results

3.1 Phytochemical screening of extract

The phytochemical screening of Spilanthes acmella leaves powder of ethanol extracts demonstrated the presence of alkaloids, polyphenol, tannins, flavonoids and glycoside (Table 1). The presence of these phytochemical components may be responsible for the various pharmacological activity of the plant leaves extract.

Table 1: Phytochemical analysis of ethanol extracts of Spilanthes acmella leaves

<table>
<thead>
<tr>
<th>Plant constituents</th>
<th>Ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>+</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenes</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
</tr>
</tbody>
</table>

-ive: absence of plant constituents, +ive: presence of plant constituents

3.2 In vitro antioxidant activity

3.2.1 Total flavonol content of extract

The concentration of flavonoids in ethanol extract of Spilanthes acmella was determined spectrophotometrically using aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalents. The content of flavonoids identified in the tested extracts is shown in table 2. The concentrations of flavonoids in ethanol extract of Spilanthes acmella was 72.14 QE mg/gm.

3.2.2 Total polyphenol content of extract

The ethanol extract of Spilanthes acmella was evaluated for investigation of the total phenolic content concentrations in extracts. The total phenolic content of ethanol extract of Spilanthes acmella was found to be 84.52 GAE mg/gm (Table 2).

3.2.3 Hydrogen-donating activity of extract

2,2-Diphenyl-1-picrylhydrazyl radical is a stable organic free radical with an absorption band at 517 nm. It loses this absorption when it accepts an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. As shown in table 3, ethanol extract of Spilanthes acmella strongly scavenged DPPH radical with the IC_{50} being 134.11 µg/ml (Fig. 1). The scavenging was found to dose dependent.

Table 2: Determination of total flavonol and polyphenol content of Spilanthes acmella leaves

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total flavonol content (QE mg/gm)</th>
<th>Total polyphenol content (GAE mg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol extract</td>
<td>72.14±0.32</td>
<td>84.52±0.74</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of triplicate determinations

Table 3: Free radical scavenging capacity of ethanol extracts of Spilanthes acmella leaves

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>DPPH Scavenging %</th>
<th>Ethanol Extract</th>
<th>Ascorbic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>24.19±0.25</td>
<td>94.13±0.53</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>42.85±0.94</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>53.71±0.57</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>69.43±0.41</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>82.63±0.62</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>IC_{50}</td>
<td>134.11</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SEM of six determinations

Fig 1: IC_{50} values of ethanol extract of Spilanthes acmella leaves by DPPH scavenging capacity

3.2.4 Superoxide scavenging activity of extract

As it is a reactive oxygen species, superoxide has some damaging properties that can be imposed to the cells and DNA and subsequently invites various diseases. Thus, a proposal has been established to gauge the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. Table 3 demonstrated the changes in the activity of SOD upon treatment with the extracts. The ethanol extract...
Table 4: Superoxide scavenging capacity of ethanol extracts of Spilanthes acmella leaves

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Superoxide scavenging%</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol Extract</td>
<td>Ascobic Acid</td>
</tr>
<tr>
<td>50</td>
<td>34.28±0.58</td>
<td>88.31±0.68</td>
</tr>
<tr>
<td>100</td>
<td>49.17±0.61</td>
<td>-</td>
</tr>
<tr>
<td>150</td>
<td>61.74±0.82</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>76.12±0.35</td>
<td>-</td>
</tr>
<tr>
<td>250</td>
<td>96.34±0.42</td>
<td>-</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>104.51</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of six determinations

Fig 2: IC<sub>50</sub> values of ethanol extract of Spilanthes acmella leaves by superoxide scavenging capacity

4 Discussions

Preliminary phytochemical screening showed the presence of alkaloids, polyphenol, tannins, flavonoids and glycoside in ethanol extract of Spilanthes acmella leaves. It was supported by earlier reports for the presences of phenols and flavonoids in the Spilanthes acmella leaves. Flavonoids and phenols exhibit a wide range of biological activities, one of which is they have the properties of antioxidant activity. Being plant secondary metabolites, the phenolics or polyphenols are very important judging from the virtue of their antioxidant activities by chelating redox-active metal ions, inactivating lipid free radical chains, and avoiding the hydroperoxide conversions into reactive oxyradicals.

The ethanol extract of Spilanthes acmella leaves illustrated the highest total flavonol and polyphenol content. The rich-flavonoid plants could manifest themselves as good sources of antioxidants that would assist in the enhancement of the overall antioxidant capacity of an organism and protection against lipid peroxidation. The total flavonoid content results were entirely synchronous with those of the total phenolic. It was successfully shown that samples with high level of phenolic content also contain flavonoids in great amount. The rich-flavonoid plants could be a good antioxidant source that would help increase the overall antioxidant capacity of an organism and guard it against lipid peroxidation<sup>16</sup>.

The plant extract was able to reduce the stable free radical of DPPH to the yellow coloured diphenylpicrylhydrazine. This evidences that the Spilanthes acmella extract contains some active constituents that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical’s reactivity. DPPH radical scavenging method has been proven to be good because its results are not affected by substrate polarity. Scavenging ability of the Spilanthes acmella extract shows the potential decrease in the concentration of DPPH.

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals and also very harmful to cellular components. It has been reported that flavonoids are found to be most effective antioxidants mainly because they can easily scavenge superoxide anions<sup>17</sup>. The results suggest that radical scavenging effect of extract is significant.

5 Conclusion

The findings of study indicates that Spilanthes acmella leaves extract contains large amounts of phenolic and flavonoids compounds and exhibits high antioxidant and free radical scavenging activities. The in vitro assays of antioxidant exhibit Spilanthes acmella extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Further studies are in progress for the isolation of active constituents responsible for antioxidant activity.

6 Conflicts of Interests

We have not declared any conflict of interest.

7 Author's contributions

NGN carried out the complete experimental work. The entire work was carried out under the Supervision of MS.

8 References


