Antioxidant and HPLC analysis of an Indian Medicinal Herb: Paederia foetida L. (Prasarini)

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ABSTRACT

Background: Paederia foetida (Prasarini) is a traditionally used herb majorly against diarrhoea and dysentery. It is well known for its antimicrobial and anti-inflammatory properties. They are also used for increasing male testosterone level.

Aim: This study was conducted to evaluate the antioxidant properties of the medicinal herb P. foetida. Further, High performance liquid chromatography (HPLC) of P. foetida extracts were done in order to determine the presence of phenolics and flavonoid compounds which essential for the antioxidant property of the plant.

Methods: The leaf extracts were prepared using 4 solvents, methanol, acetone, ethyl acetate and water. The antioxidant property was evaluated by DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS method using Ascorbic acid as standard. In HPLC, Caffeic acid, Ferrulic acid, Galic acid, p-Coumaric acid, Quercetin, Myricetin, Kaemperol and Isorhamnetin were used for the analysis. The standards were dissolved in HPLC grade methanol. The dilutions of the standards were done from the range of 20μg/ml to 100μg/ml followed by making the standard curve.

Results: It was concluded from the DPPH assay that the ethyl acetate extract of P. foetida leaves had the highest free radical scavenging activity than other extracts, however, from the ABTS assay it was concluded that the acetone extract of P. foetida had the highest free radical scavenging activity. From the HPLC analysis it was confirmed that the ethyl acetate extract and the highest number of phenolic compounds, however they had moderate number of flavonoid compounds.

Conclusion: The antioxidant property of P. foetida leaves is very high as they are rich in phenolic compounds and can be extracted using ethyl acetate and acetone.

Keywords: Paederia foetida; Antioxidant assay; DPPH; ABTS; HPLC; Phenolic compounds
INTRODUCTION

Antioxidants prevent the slow damage to cells caused by the release of free radicals or free-radical scavengers or reactive oxygen species (ROS), which are unstable molecules produced in the body in reaction to defective metabolism, environmental and other related stress on the body. Antioxidants are both natural and artificial. Several plant-based products are well known antioxidant drugs (Wilson et al., 2017). Human body also produces few antioxidants, known as endogenous antioxidants. Antioxidants from external sources are called exogenous. If the body is unable to process and remove such free radicals efficiently, it results in oxidative stress and lead to defective metabolism and diseases (Ramana et al., 2018). The number of free radicals in the body escalates because of internal factors, such as inflammation, or external factors like, environmental pollution, ultraviolet rays, indifferent food habits and tobacco use (Lobo et al., 2010). Oxidative stress may lead to cardiac defects, cancer, arthritis, strokes, respiratory problems and immunodeficiency or suppressed condition, emphysema, Parkinson’s disease and other inflammatory or ischemic diseases (Pizzino et al., 2017). Therefore, antioxidants become much more important as they are required to neutralize free radicals and to keep the body healthy.

Medicinal plants since ancient times were known to be the potent source of antioxidants as they contain a mixture of different secondary metabolites and phytochemicals, that may act individually or in synergy as therapeutic agent against various health ailments (Xu et al., 2017). In last 4 decades, research on antioxidants and medicinal plants has significantly increased and the popularity of natural antioxidant products has escalated, particularly in the urban society. A single medicinal plant may have variety of phytochemicals, which may have several pharmacological properties (Kasote et al., 2015; Machado et al., 2018). Unfortunately, several herbal medicines which are in use today have not gone under proper scientific evaluation and some of them have life threatening side/toxic effects. Therefore, careful scientific and clinical studies of the herbal medicine for the safety and toxicity has become essential (Asif, 2012). With high prevalence of medicinal plants in India and their potential as a source of antioxidants need to be judiciously investigated (Sen and Chakraborty, 2016; Kumar et al., 2016).

*Paederia foetida* is an Indian edible herb that has traditionally been used for a various health ailment, particularly against diarrhoea and dysentery and in male infertility problems. It has both antioxidant and anti-inflammatory properties, like most medicinal plants (Wang et al., 2014). Some scientific reports also validate in erogogenic and aphrodisiac properties. The medicinal properties are majorly due to presence of phytochemicals present in this herb, which acts in combinations or in solitary mode against various human ailments. Hence, investigating the major bioactive chemicals of the plant has become essential (Wang et al., 2014; Kumar et al., 2014). No report of toxicity has been reported for this plant, however, very few studies has been conducted in this aspect (Chanda et al., 2015). This study was conducted to evaluate the antioxidant properties of the medicinal herb *P. foetida*. Further, High performance liquid chromatography (HPLC) of *P. foetida* extracts were done in order to determine the presence of phenolics and flavonoid compounds which are essential for the antioxidant property of the plant.

MATERIALS AND METHODS

Processing of plant material

Fresh young leaves of *P. foetida* were collected from the Chandaka forest region of Bhubaneswar, Odisha in September 2018 and it was initially identified by its morphological features. The fresh leaves were washed properly with pyrogen free water and were shed dried and powdered in a mixer grinder. Further the powdered leaves were stored in airtight polybags for future use.

Extraction

A total of 15-gram of powdered leaf samples was taken and extraction was carried out with the help of a Soxhlet apparatus by using different solvent on their polarity basis. Different solvents used for extraction process were ethyl acetate, acetone, methanol and water respectively. After the extraction, solvent evaporation was done by using a rotary evaporator, where the solvent and extract got separated resulting in the yield of pure extract. Further the final crude extracts were stored in a glass airtight container and were kept at 4°C until further use.
In vitro Antioxidant Assay
The in vitro antioxidant properties of the 4 solvent leaf extracts from P. foetida were determined by using 2 methods: (a) The DPPH method; (b) The ABTS method.

The DPPH method
One ml of DPPH solution in methanol (0.1 mMol/L-1) was mixed with 3.0 ml of leaf extract in several concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml) and the mixture was incubated for 30 min at room temperature in dark and the absorbance was recorded at 517 nm using a UV - Visible spectrophotometer. Ascorbic acid was used as standard to plot the standard curve. The antioxidant potentiality of these extracts was recorded as IC50. The IC50 value is the concentration (in µg/ml) of extract that inhibits the formation of DPPH radicals by 50% (Kedare and Singh, 2011).

ABTS + - Radical scavenging assay
ABTS + was prepared by mixing 1:1 ratio of ABTS and potassium per sulphate and kept in dark for 12 hours to generate the free radicals. Then its absorbance was adjusted up to 0.700 ± 0.025 at 734 nm before use. 0.5 ml of sample of different concentration was added with 2.5 ml of ABTS was prepared. Then control is prepared without sample 0.5 ml of methanol with 2.5 ml of ABTS. According to the sample type, blank is prepared. The absorbance was measured at 734 nm. The scavenging activity of the extract based on the % of ABTS radical scavenged (1%) using equation,

\[
\text{DPPH scavenging/ABTS scavenging} = \frac{A_{\text{control}} - A_{\text{Sample}}}{A_{\text{control}}} \times 100
\]

Acontrol - Absorbance of the control sample.
A sample - Absorbance in the presence of the extracts.

Ascorbic acid was taken as the standard antioxidant compound. A standard curve was plotted using different concentrations of ascorbic acid and IC50 value were determined for both the test samples as well as standard sample and the IC50 value of test samples were compared (Rajurkar and Hande, 2011).

HPLC ANALYSIS

Standards used: Caffeic acid (CA), Ferrulic acid (FA), Galic acid (GA), p-Coumaric acid (p-CA), Quercetin, Myricetin, Kaemperol and Isorhamnetin were used for the analysis. The standards were dissolved in HPLC grade methanol. The dilutions of the standards were done from the range of 20µg/ml to 100µg/ml followed by making standard curve.

Samples: 5mg/ml stock solutions were prepared for ethyl acetate, acetone and methanol leaf extract of P. foetida with HPLC grade methanol. But the stock solution of water extract was prepared with mili Q water. All the stock solutions were filter sterilised properly with 0.2 mm filter.

Apparatus: Reverse phase HPLC apparatus consisting of 2 pumping systems, injector, C18 column and UV visible detector of Shimadzu JAPAN was used. The data were collected and analysed by using “LC solution” software.

Method: Quantitative analysis of phenolic compounds was carried out with GA, FA, Caffeic acid and p-CA as the standards. The mobile phase was consisted of Solvent A (1% ortho-phosphoric acid in mili Q water) and Solvent B (1% ortho-phosphoric acid in acetonitrile). The ratio of solvent was 63:37. That means solvent A consisting of 63% where solvent B is 37%. The flow rate of the mobile phase was 0.5 ml/min and the injection volume 20µl. The peaks were monitored at 280nm for GA, 324nm for CA and FA, 310nm for p-CA. Quantitative analysis of flavonols was carried out by using Quercetin, Myricetin, Kaemferol and Isorhamnetin as standards. The mobile phase was methanol, acetonitrile and water (40:15:45). The flow rate of the mobile phase was kept at 0.5ml/min and the injection volume were 20µl. The peaks were monitored at 368nm for all (El-Nabi et al, 2018).

RESULTS

DPPH assay
Leaf extracts of P. foetida exhibited a concentration response relationship in DPPH scavenging. The increase in concentration was synchronous with the increase in the scavenging capacity. Lower the IC50 value greater of its scavenging activity. As the positive control, Ascorbic acid had the highest scavenging activity with IC50 value 6.758 µg/ml. Comparing IC50 values of all the extracts, it was recorded, that the acetone extract had more scavenging activity than rest three extracts. The IC50 value of acetone extract was 6.964 ± 16. Further, the ethyl acetate, methanol and aqueous extracts had the scavenging activity of IC50 value 27.363 ± 3.093363, 13.336 ± 0.456353 and 16.308 ± 0.636684 respectively. From the above readings and the graph, it was concluded that the ethyl acetate extract of P. foetida leaves had the highest scavenging activity than others (Table 1, Figure 1).
Table 1. Antioxidant activity of P. foetida leaf extracts in DPPH method

<table>
<thead>
<tr>
<th>Extracts/Standard</th>
<th>IC50 value in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>27.363 ± 3.093363</td>
</tr>
<tr>
<td>Acetone</td>
<td>6.964 ± 1.634197</td>
</tr>
<tr>
<td>Methanol</td>
<td>13.336 ± 0.456353</td>
</tr>
<tr>
<td>Aqueous</td>
<td>16.308 ± 0.636684</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>2.52 ± 0.81</td>
</tr>
</tbody>
</table>

Figure 1: IC50 value of different extracts of P. foetida leaf extracts by DPPH method

Table 2. Antioxidant activity of P. foetida leaf extracts in ABTS method

<table>
<thead>
<tr>
<th>Extracts/Standard</th>
<th>IC50 value in µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate</td>
<td>118.4562 ± 3.410</td>
</tr>
<tr>
<td>Acetone</td>
<td>28.55942 ± 0.367</td>
</tr>
<tr>
<td>Methanol</td>
<td>38.66273 ± 0.106</td>
</tr>
<tr>
<td>Aqueous</td>
<td>78.04537 ± 3.096</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>24.52042 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 2. IC50 values of different extracts of P. foetida leaf extracts by ABTS method
Determinant of ABTS assay

P. foetida leaf extracts exhibited a concentration response relationship in ABTS radical scavenging. An increasing in concentration is synchronous with an increasing in scavenging capacity. Lower the IC$_{50}$ value greater of its scavenging activity. As the positive control, Ascorbic acid exhibited high scavenging activity with IC$_{50}$ value 24.52042µg/ml. Comparing IC$_{50}$ value of all the leaf extracts of P. foetida, acetone extract recorded the highest scavenging activity than other extracts with an IC$_{50}$ value of 28.55942 ± 0.367. It was followed by methanol and aqueous extracts have the scavenging activity of IC$_{50}$ value 38.66273 ± 0.106 and 78.04537 ± 3.096 respectively. Ethyl acetate extract had and IC$_{50}$ value of 118.4562 ± 3.410. Hence, it was concluded that the acetone extract of P. foetida had the highest scavenging activity than others (Table 2, Figure 2).

HPLC ANALYSIS

HPLC method was developed for the analysis of GA, ferrulic acid, caffeic acid and p-coumaric acid in the leaf extracts of P. foetida. The polyphenolic content list of P. foetida leaves are recorded in table 3. The quantification of polyphenolic compounds by HPLC exhibited that ethyl acetate extract had GA (316.83 mg/gm), FA (37.459mg/gm), CA (111.225mg/gm) and p-CA (31.529mg/gm) by comparing the chromatogram of the reference standards. Retention time (RT) of the ethyl acetate extract 7.441, 10.185, 8.288, 9.818 min corresponded well with the standard chromatogram peaks of GA, FA, CA and p-CA at 7.652, 10.169, 8.062 and 9.827 min respectively. From the results it was concluded that ethyl acetate extract of P. foetida leaves has a high concentration of phenolic compounds (Table 3).

Similarly, the quantification of polyphenolic compounds by HPLC exhibited that acetone extract of P. foetida leaves had GA (766.73mg/gm), FA (27.00mg/gm), CA (85.842mg/gm) and p-CA (55.27mg/gm) by comparing the chromatogram of the reference standards. RT of the acetone extract of P. foetida leaves at 7.442, 10.152, 8.275 and 9.808 min corresponded well with the standard chromatogram peaks of GA, FA, CA and p-CA at 7.652, 10.169, 8.062 and 9.827 min respectively. From the results it was concluded that acetone extract of P. foetida leaves has a high concentration of phenolic compounds (Table 3).

Likewise, the quantification of polyphenolic compounds by HPLC exhibited that methanol extract of P. foetida leaves had GA (785.964mg/gm), FA (0), CA (113.94mg/gm) and p-CA (65.12mg/gm) by comparing the chromatogram of the reference standards. RT time of the acetone extract of P. foetida leaves at 7.435, 10.968, 8.279, 9.840 min corresponded well with the standard chromatogram peaks of GA, FA, CA and p-CA at 7.652, 10.169, 8.062 and 9.827 min, respectively. From the results it has been shown that methanol extract of P. foetida leaves had a rich content of GA, FA, p-CA and comparatively less amount of FA (Table 3).

Further, the quantification of polyphenolic compounds by HPLC exhibited that aqueous extract of P. foetida leaves had GA (121.22mg/gm), FA (0), CA (8.56mg/gm) and p-CA (0) by comparing the chromatogram of the reference standards. RT time of the aqueous extract of P. foetida leaves at 7.024, 8.261, 9.831min corresponded well with the standard chromatogram peaks of GA, FA, CA and p-CA at 7.652, 8.062 and 9.827 min respectively. From the results it has been shown that aqueous extract of P. foetida leaves had a rich amount of GA, caffeic acid, however, p-CA and FA is absent in case of aqueous extract. Phenolic compounds are a category of phytonutrients with strong antioxidant properties (Table 3).

### Table 3. HPLC analysis for phenolic content

<table>
<thead>
<tr>
<th>Standards</th>
<th>Std. Equation</th>
<th>Ethyl Acetate extract</th>
<th>Acetone extract</th>
<th>Methanol extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>$Y= 41355X+10355$</td>
<td>316.837</td>
<td>766.732</td>
<td>785.964</td>
<td>121.225</td>
</tr>
<tr>
<td>Ferrulic acid</td>
<td>$Y=1402X-21127$</td>
<td>37.459</td>
<td>27.009</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>$Y= 12936X-36524$</td>
<td>111.225</td>
<td>85.842</td>
<td>113.942</td>
<td>8.560</td>
</tr>
<tr>
<td>P coumaric acid</td>
<td>$Y= 17581X+26496$</td>
<td>31.523</td>
<td>55.271</td>
<td>65.123</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4. HPLC analysis of flavonoid content

<table>
<thead>
<tr>
<th>Standards</th>
<th>Standard Equation</th>
<th>Ethyl acetate extract</th>
<th>Acetone extract</th>
<th>Methanol extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>Y=68662X-35060</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Myricetin</td>
<td>Y= 89948X+39064</td>
<td>0</td>
<td>2.806855072</td>
<td>0</td>
<td>0.247843198</td>
</tr>
<tr>
<td>Kaemferol</td>
<td>Y= 10861X-55166</td>
<td>0</td>
<td>7.080287266</td>
<td>5.975876991</td>
<td>5.69349047</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>Y= 15595X+48534</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

For flavonoids
HPLC method was developed for the analysis of Quercetin, Myricetin, Kaemferol and Isorhamnetin in the extracts of P. foetida leaves. The flavonoids profile of P. foetida leaves are shown in the table 4. The quantification of flavonoid compounds by HPLC exhibited that ethyl acetate extract of P. foetida leaves did not had Quercetin, Myricetin, Kaemferol and Isorhamnetin by comparing the chromatogram of the reference standards. In the acetone extract, Myricetin (2.806mg/gm) and Kaemferol (7.0802mg/gm) were present. RT of the acetone extract at 11.283 and 26.345 min corresponded well with the standard chromatogram peaks of myricetin and kaemferol at 11.626 and 26.787 min respectively. In the methanol extract, Kaemferol (5.97mg/gm) was present. RT time of the methanol extract at 26.635min corresponded well with the standard chromatogram peaks of kaemferol 26.787 min. Others are absent. In the aqueous extract, Myricetin (0.2478mg/gm) and Kaemferol (5.693mg/gm) were present. RT time of the aqueous extract of P. foetida leaves at 11.603 and 26.836 min corresponded well with the standard chromatogram peaks of myricetin and kaemferol at 11.626 and 26.787 min respectively. Quercetin and Isorhamnetin were absent in the aqueous extracts (Table 4).

DISCUSSION
The present investigation study discusses about the antioxidant potential effect of the 4-leaf extract of P. foetida. In general, phenolic compounds and flavonoids exhibits a wide range of medicinal activities in vivo. Previous studies suggest that P. foetida extracts can prevent the oxidation of β-carotene and linoleic acid and exhibit antioxidant property when tested in ABTS free radical scavenging assay system (Osman et al., 2009; Upadhyaya, 2013). In this study, we concluded that P. foetida leaf extract also have strong antioxidant activity in DPPH radical scavenging assay. DPPH antioxidant assay is primarily depending upon the presence of antioxidants, a stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) will decolorize. The DPPH radical has free electron, which is responsible for the absorbance at 515-517 nm and develops a visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance (Sahoo and Bhatnagar, 2016). P. foetida exhibited scavenging activity of DPPH radicals. This activity was increased with the increasing concentration of sample extract.

Phyto-polyphenols are secondary metabolites/ their derivatives/ isomers of flavones, isoflavones, flavonols, catechins and phenolic acids. More than 9000 structural versions of polyphenols and 3,000 flavonoids are distributed as unique elements of plant life. Considering, their structure, their solubility varies in polar to non-polar solvents. Most of the phenolic extracts are soluble in organic polar solvents, e.g. methanol, ethanol and acetone. A correct mixture of polar and non-polar solvents results in higher extraction of these phytophenols (Ojha et al., 2018). Absence of phenolic constituents can be attributed the physical properties of a specific solvent. The polarity of solvents and physical/ chemical houses of active components play an essential role on number of phenolic compounds at some point of extraction. Phenolic and flavonoid components like quercetin, sebiferine and litseferine are extracted with water in 80% polar solvents. So, increased numbers of peaks in natural solvent systems are due to presence of these additives (Wang et al., 2014). Phenolic components as glycosides/ aglycones has lower RF values (zero. 00- 0. 25) and oligo-hydroxylated and methylated additives has more RF values (0, 50, 75). The versions in quantity of peaks of phenolics between solvents can be because of their prevalence with appreciate to RF values. Quercetin-3-glucosides, quercetin-three-arabinosides, quercetin-3-rhamnoglucosides and kaempferol.
glucoside has 0.33, 0.35, 0.29 and zero. 54 Rf values respectively (Wang et al., 2014; Kumar et al., 2014).

**CONCLUSION**

From the study, it was concluded that the antioxidant property of *P. foetida* leaves is very high as they are rich in phenolic compounds and can be extracted using ethyl acetate and acetone. The results of this study justify the use of this plant as food product and in the pharmaceutical industry. It also validates the ethnomedicinal values of this plant.

**Conflict of Interest:** The authors declare no conflict of interest.

**REFERENCES:**


