Comparative Studies on Antioxidant Activity, Total Phenol Content and High Performance Thin Layer Chromatography Analysis of Seabuckthorn (Hippophae rhamnoides L) Leaves

Amrit Kumar Singh1*, Prakash Deep1, Suchita Dubey1, Dharam Paul Attrey2, Tanveer Naved3

1Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow Campus, Uttar Pradesh, India, 2Amity Institute of Seabuckthorn Research, Amity University Uttar Pradesh, Noida Campus, Uttar Pradesh, India, 3Amity Institute of Pharmacy, Amity University Uttar Pradesh, Noida Campus, Uttar Pradesh, India

ABSTRACT

Background: Seabuckthorn (SBT) is a high altitude medicinal plant with vast history of use in traditional medicinal systems such as Tibetan and Chinese systems. SBT leaves have shown range of pharmacological properties suggesting their importance to be used for product development. Objective: The aim of this study was to compare 75% ethanolic extracts of male and female SBT leaves on the basis of antioxidant activity, total phenol content and high performance thin layer chromatography (HPTLC) estimation of β-sitosterol and ursolic acid. It also involved comparison of total phenol contents of successive soxhlet extracts (pet ether, chloroform, ethyl acetate, ethanol, and aqueous) of above leaves. Materials and Methods: Antioxidant activities and total phenol contents of the extracts were evaluated by using 1,1-diphenyl-2-picryl-hydrazyl free radical scavenging assay and Folin–Ciocalteu reagent based assay, respectively. Results: Male leaf extract was found to show significantly higher antioxidant activity and total phenol content than that of female leaves. Furthermore, the successive extracts of male leaves showed higher phenol contents than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts. In HPTLC estimation, concentration of β-sitosterol in female leaf extract was observed to be less than that of male leaf extract. However, ursolic acid concentration was found to be almost same in both the type of leaf extracts. Conclusion: The results suggest the need for developing standard quality control profile of SBT leaves, especially for product development.

Keywords: Antioxidant activity, 75% ethanolic extract, high performance thin layer chromatography, seabuckthorn, total phenol content

INTRODUCTION

Free radical formation inside the body is a natural and unavoidable phenomenon. Free radicals cause damage to various biomolecules such as lipid, protein, and DNA of the living systems.1 There are two interdependent systems in the body, oxidants and antioxidants. Increase in the amount of free radicals shifts the balance toward oxidants resulting in oxidative stress.2 Plant derived antioxidants have the potential to reduce the incidence of oxidative stress by shifting the balance towards antioxidants.3 There are reports of increased interest on plant based antioxidants.4,5

Hippophae rhamnoides L., commonly known as seabuckthorn (SBT); Family: Elaeagnaceae, growing in North-West Himalayas at high altitude (7,000-15,000 feet), is a dwarf to tall (3-15 feet), branched, and Thorny nitrogen fixing deciduous shrub, native to Europe and Asia.6 SBT is a good source of a large number of nutrients and phytochemicals, especially phenolic compounds.6-9 Guliyev et al. (2004) have reported that the phenolic acids found in the leaves of SBT include gallic, protocatechuic, p-coumaric, ferulic, p-hydroxybenzoic and ellagic acids.10 Suryakumar et al. (2011) have reported high contents of ursolic acid in SBT.11

SBT leaf extracts have been reported to possess many medicinal properties, including antioxidant.6,12-15 Medicinal effects of SBT are due to the presence of high antioxidant...
There may be some difference between antioxidant properties and phytochemical contents of male and female SBT leaves. Furthermore, no such studies have been found comparing male and female SBT leaves on the basis of these parameters. In view of the above, the present study was undertaken to compare 75% ethanolic extracts of male and female SBT leaves on the basis of antioxidant activities, total phenol contents and high performance thin layer chromatography (HPTLC) estimation of \( \beta \)-sitosterol and ursolic acid contents.

**MATERIALS AND METHODS**

**Plant material**

Leaves of SBT were collected from Leh (Ladakh), India, and authenticated by National Institute of Science Communication And Information Resources, New Delhi, India.

**Chemicals**

Gallic acid, 2,2-di phenyl-1-picryl hydrazyl (DPPH), Folin–Ciocalteu reagent (FCR) were purchased from sigma. All other reagents were of analytical grade and were procured from Ranbaxy Fine Chemicals Ltd., Punjab, Fischer Inorganics and Aromatics Ltd., Madras, NICE Chemicals Ltd., Cochin and Central Drug House Pvt., Ltd., New Delhi.

**Preparation of extracts**

**75% ethanolic extract**

Powdered SBT leaves were extracted with 75% ethanol by cold percolation method.\(^6\) The powdered leaves were soaked in above solvent (1:8 w/v) at room temperature. After 24 h, the supernatant was decanted and the residue was re-soaked in fresh solvent. This process was repeated 5 times for complete extraction. After completion of the extraction process, the supernatants were pooled and filtered through 250 mesh nylon cloth. This filtrate was dried under reduced pressure until a solid mass was obtained. Same procedure was repeated for, both male and female leaves. The above extracts were used for estimation of total phenol contents, antioxidant activities and for HPTLC estimation of \( \beta \)-sitosterol and ursolic acid.

**Successive extracts**

Powdered SBT leaves were extracted, one by one, with petroleum ether (50-60°C), chloroform, ethyl acetate, ethanol and distilled water, by soxhlet extraction process for 4 h. After completion of extraction process, solvents were removed by distillation and concentrated under reduced pressure till a solid mass was obtained. Same procedure was repeated for, both male and female leaves. The above extracts were used for estimation of total phenol contents.

**Total phenol content**

Total phenol content was estimated in above prepared SBT extracts by FCR based assay.\(^{16}\) To the aliquot (50 \( \mu \)l) taken from stock solution (1 mg/ml) of the extract, 3.5 ml distilled water and 250 \( \mu \)l of FCR was added, the mixture was kept at room temperature for 1-8 min and 750 \( \mu \)l of 20% sodium carbonate solution was added. Mixture was kept at room temperature for 2 h and absorbance of the color developed was recorded at 765 nm with the help of a ultraviolet (UV)-visible spectrophotometer against blank. Total phenolic content was determined using gallic acid standard curve (\( R^2 = 0.986 \)) and expressed in mg/g as gallic acid equivalents. Same procedure was repeated for estimation of total phenolic content in all the extracts.

**Antioxidant activity**

DPPH based assay was used for determination of antioxidant activities of above prepared extracts.\(^{17}\) A 0.1 mm solution of DPPH was prepared by using methanol. A volume of 2 ml of this solution was added to 2 ml of solution of extract and the mixture was kept in dark for 20 min. After 20 min. absorbance of the color developed was recorded at 517 nm with the help of a UV-visible spectrophotometer against blank. Control was prepared by adding 2 ml of the DPPH solution to 2 ml methanol. Same procedure was repeated for estimation of antioxidant activity in all the above extracts.

IC50 values were calculated using the formula:

\[
\text{Percentage Scavenging} = \left( \text{Absorbance [Control-Test]} / \text{Absorbance of Control} \right) \times 100
\]

**HPTLC analysis**

This was performed by using 2 different reference standards, namely \( \beta \)-sitosterol and ursolic acid. A stock solution was prepared for each reference standard i.e. \( \beta \)-sitosterol and ursolic acid (1 mg/ml) and sample that is., 75% ethanolic extract of SBT leaves, both male and female (40 mg/ml). Firs, the TLC plates (precoated silica gel 60-F254) were kept at 110°C for 1 h for activation, followed by the test sample and reference solutions (5 \( \mu l \) each with band length of 8 mm) were applied on the TLC plates through
and female SBT leaves may lead to significant variation in amounts of pharmacological effects precipitated by these extracts. Hence, one should be careful while collecting the SBT leaves especially for use in product development because variation in amount of pharmacological effects of the extract may result in variation in medicinal effects of the finished products also, which may cause batch to batch consistency problems. These observations suggest the need of quality control of SBT leaves.

Table 2 represents total phenol content of successive extracts of male and female SBT leaves. It revealed that, even in successive extracts, total phenol contents of male leaf extracts were more than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts. Both the leaves were found to show maximum content of total phenols in aqueous extracts, which indicate that large quantity of phenolic compounds, present in both male and female SBT leaves, are hydrophilic (polar) in nature. However, they appear to be present in more quantity in male leaves since total phenol content in aqueous extract of male leaves was slightly higher than that of female leaves (Table 2).

In case of ethyl acetate and ethanol extracts also, total phenol contents of male leaves was much higher than that of female SBT leaves, which suggests that phenolic compounds with hydrophobic (non-polar) nature are present in significantly lower amounts in female SBT leaves than in male leaves (Table 2). These findings support the lower content of total phenols in 75% Ethanolic extract of female leaves (Table 1) since ethanol is a non-polar solvent.

HPTLC analysis

It involved simultaneous estimation of β-sitosterol and ursolic acid in the 75% Ethanolic extracts of both, male and female SBT leaves.

Table 3 represents the results of HPTLC analysis. It stated that concentration of ursolic acid was almost same in case of both the type of leaf extracts but concentration of β-sitosterol was higher in male leaf extract than that of female.

### Table 1: Total phenol content and IC₅₀ values of 75% ethanolic extracts

<table>
<thead>
<tr>
<th>75% ethanolic extracts</th>
<th>Total phenol content (GAE mg/g)</th>
<th>IC₅₀ For antioxidant activity (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male leaf</td>
<td>398.86±2.20*</td>
<td>5.99±0.25*</td>
</tr>
<tr>
<td>Female leaf</td>
<td>134.95±2.72*</td>
<td>30.39±0.23*</td>
</tr>
</tbody>
</table>

*Significant difference (P<0.01). GAE: Gallic acid equivalent

<table>
<thead>
<tr>
<th>Successive extracts</th>
<th>Total phenol content (GAE mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male leaf</td>
<td></td>
</tr>
<tr>
<td>Female leaf</td>
<td></td>
</tr>
<tr>
<td>Pet ether</td>
<td>63.45±2.10*</td>
</tr>
<tr>
<td>Chloroform</td>
<td>52.17±1.73*</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>146.29±3.12*</td>
</tr>
<tr>
<td>Ethanol</td>
<td>432.19±2.56*</td>
</tr>
<tr>
<td>Aqueous</td>
<td>599.67±3.60*</td>
</tr>
</tbody>
</table>

*Significant difference; **Non significant difference. GAE: Gallic acid equivalent, SBT: Seabuckthorn

**RESULTS AND DISCUSSION**

Total phenol content and antioxidant activity

Table 1 represents the total phenol content and IC₅₀ values (antioxidant activities) of 75% ethanolic extracts of male and female SBT leaves. This table showed that total phenol content of 75% ethanolic male leaf extract was much higher than that of female leaf extract. Also, the IC₅₀ value of male leaf extract was significantly lower than that of female leaf extract, which indicate that antioxidant activity of male leaf extract is significantly higher than that of female leaf extract.

On comparing the total phenol contents and antioxidant activities of 75% ethanolic extracts of both, male and female SBT leaves, an increase in antioxidant activity with increase in total phenol content was observed. This observation indicates that phenolic compounds may contribute toward antioxidant activity observed (Table 1). Further, it also supports the work of other researchers that phenolic compounds are known to have various properties including antioxidant property.5,18,19 Also, the beneficial effects of natural phenolic compounds of plant origin, on coronary heart diseases and cancers have been reported to be mainly due to their antioxidant activity.5 Considering these points, the significant difference in total phenol contents of 75% ethanolic extracts of male and female SBT leaves may lead to significant variation in amounts of pharmacological effects precipitated by these extracts. Hence, one should be careful while collecting the SBT leaves especially for use in product development because variation in amount of pharmacological effects of the extract may result in variation in medicinal effects of the finished products also, which may cause batch to batch consistency problems. These observations suggest the need of quality control of SBT leaves.

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HPTLC analysis

It involved simultaneous estimation of β-sitosterol and ursolic acid in the 75% ethanolic extracts of both, male and female SBT leaves.

Table 3 represents the results of HPTLC analysis. It stated that concentration of ursolic acid was almost same in case of both the type of leaf extracts but concentration of β-sitosterol was higher in male leaf extract than that of female.
Above results can be summarized as follows.

a. 75% ethanolic male leaf extract was observed to have significantly higher total phenol content and antioxidant activity (DPPH scavenging activity) than that of female leaf extract.

b. Increased antioxidant activity of 75% ethanolic male leaf extract may be due to the presence of higher amount of total phenol content. However, it needs to be investigated further.

c. Even successive extracts of male leaves was observed to show higher quantities of total phenols than that of female leaves. However, it was not significant in case of pet ether and chloroform extracts.

d. Concentration of β-sitosterol in female leaf extract was observed to be less than that of male leaf extract. However, ursolic acid concentration was found to be almost same in both the type of leaf extracts.

**CONCLUSION**

The results of the present investigation demonstrate some important differences between 75% ethanolic extracts of male and female SBT leaves with respect to total phenol content, antioxidant activity and HPTLC estimation of β-sitosterol and ursolic acid contents. Results also indicate the need for quality control of SBT leaves, especially for product development.

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