PHYTOCHEMICAL INVESTIGATION AND IN VITRO ANTIOXIDANT ACTIVITY OF SOME MEDICINALY IMPORTANT PLANTS OF UTTARAKHAND

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ABSTRACT
Antioxidants are the substances, compounds or nutrients in our foods which can prevent or slow oxidative damage to our bodies. These agents are able to remove the deleterious effects of free radicals within our body. Solvent extraction methods are widely used for extracting antioxidant in foods and other sources. In this study, Microwave Solvent Extraction (MSE) methods were utilized to determine the content of antioxidants in Ricinus communis (root), Aloe vera (leaves), Crateva nurvula (bark), Swertia chirayta (whole plant) and Bacopa monnieri (whole plant) extracts. MSE was performed at 80°C employing four solvents, methanol, aqueous, hexane and petroleum ether. The in vitro antioxidant activity was determined by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay and Superoxide Anion Radical Scavenging activity. The yield of antioxidants extracted using the MSE was achieved using methanol as the solvent, followed by aqueous extracts in all the plants in comparison to Ricinus communis. Antioxidant activities in hexane and petroleum ether extracts of Ricinus communis were efficient in comparison to that of methanol and aqueous extracts of other plants. The antioxidant capacities were found to be correlated with the Total Phenolic Content (TPC). The phytochemical active constituents in the potent extracts were determined by conventional methods. These studies thus lead to the isolation and characterization of some active components responsible for antioxidant activity.

Key words: Microwave Solvent Extraction (MSE), Antioxidant activity, DPPH assay, Superoxide Anion Radical Scavenging activity, Total Phenolic Content (TPC).

INTRODUCTION
India is one of the nations blessed with a rich heritage of traditional medical systems and rich biodiversity to complement the herbal needs of the treatment administered by these traditional medical systems. The recognized Indian Systems of Medicine are Ayurveda, Siddha and Unani, which use herbs and minerals in the formulations. Health advantages of diets rich in antioxidant plant compounds include lowering the risk of cardiovascular disease, certain cancers and the natural degeneration of the body associated with the aging process. Free radicals are unstable molecules formed when the body uses oxygen for energy. The instability of these molecules can damage tissues, alter DNA and change cell structure. Ultimately, free radicals start a chain reaction resulting in the reproduction of even more free radicals. Antioxidants can stabilize the free radicals, thus can lead to protection of the body by any of the serious disorder that can be caused by the attack of free radicals. The role antioxidants have in free radical stabilization involves the antioxidants donating one of their own electrons to the free radical. This electron donation is done without the antioxidant becoming unstable or damaging to the body. This remarkable action stabilizes the free radicals as quickly as they are produced in the human body. Recently, natural plants have received much attention as sources of biological active substances including antioxidants. Numerous studies have been carried out on some plants, vegetables and fruits because they are rich sources of antioxidants, such as vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds and flavanoids¹ which prevent free radical damage, reducing risk of chronic diseases. Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular disease². The search for newer natural antioxidants, especially of plant origin has ever since increased. The in vitro antioxidant activities in plants, citrus fruits, apple
varieties of Kashmir (J&K) were investigated. In this study, the microwave solvent extraction methods were investigated for extraction capabilities from different plants. The results can determine the natural antioxidants available in the plant parts. Also, the Microwave Solvent Extraction (MSE) method will determine the efficient solvent for extracting antioxidants and poly-phenolic compounds. This study may provide insight for future extraction solvents and natural potent antioxidants which can be used as dietary supplements.

MATERIALS AND METHODS
Collection of material
The plants materials were authenticated by Botanical Survey of India (BSI), Dehradun (U.K), India. The plant material was collected from the specific plant. The plants materials were dried under shade and were ground to form the fine powder.

Microwave Solvent Extraction (MSE)
Twenty grams of each of the plant material was weighed separately using a clean aluminum container and transferred into each of the Teflon vessels of the Ethos E Microwave Extraction System (Milestone, Inc. Monroe, CT). The Teflon vessels were covered with polymer material that can resist high inside pressure generated when extraction temperatures are higher than the used solvent’s boiling point. Specific solvent (40 ml) and a magnetic stirring rod were added in each vessel for each of the plant material. The vessels were sealed and properly placed inside the Microwave Extraction System. Then, the Microwave Extraction System was programmed to increase to the extraction temperature with a maximum energy level of 800 watts and held at that temperature for 20 minutes with an energy level of 500 watts. Extraction temperature 80°C was applied to perform the microwave-assisted solvent extraction, respectively. After a twenty-minute cool down period, the vessels were unsealed and transferred to each corresponding centrifuge tube. These tubes were centrifuged at 2000 rpm for 15 minutes to separate the supernatant and residue. The solvent supernatant was transferred to a clean test tube that had been previously weighed. The residues were mixed with 20 ml of same solvent again and vortexed. The solvent supernatant was separated by the centrifugation and combined with the previous one. The supernatant was then placed in a vacuum centrifuge evaporator to remove the solvent. The dried extract in the test tube was weighed to measure the extraction yield of the samples. The samples were stored in a -20°C freezer prior to testing.

Estimation of Total Phenolic Content (TPC)
The Total Phenolic Content of each fraction obtained of different extracts was determined and the phenolic content was expressed as μg/g Gallic acid equivalents. In brief a 100 μl aliquot of the sample was added to 2 ml of 0.2 % (w/v) Na₂CO₃ solution. After 2 minutes of incubation, 100 μl of 500 ml/l Folin-Ciocalteu reagent added and the mixture was then allowed to stand for 30 minutes at 25°C. The absorbance was measured at 750 nm using a UV-VIS Systronics spectrophotometer. The blank consist of all reagents and solvents but no sample. The total phenolic content was determined using the standard Gallic acid calibration curve.

Determination of Antioxidant Activity by DPPH Radical Scavenging Method
The extract solution for the DPPH test was prepared by re-dissolving 0.2 g of each dried extract in 10 ml methanol. Two ml of the DPPH solution was mixed with 40 μl of the plant solvent extract, the solution were transferred to a cuvette separately. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible Systronics spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. The reaction mixture without any sample was used as negative control. IC₅₀ is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Determination of Superoxide Anion Radical Scavenging Activity
Superoxide Anion Radical scavenging Activity was measured with some modifications. The various fractions of plants extracts were mixed with 3 ml of reaction buffer solution (pH 7.4) containing 1.3 μM riboflavin, 0.02 M methionine and 5.1 μM NBT. The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance was measured at 560 nm using Systronics UV-VIS spectrophotometer. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. The reaction mixture without any sample was used as negative control.
Phytochemical screening of plant extracts
Phytochemical screening of plants extracts was performed according to the conventional methods\(^9,10\).

**Test for Alkaloids**
0.5 g of extract was dissolved in 5 ml of 1% HCl and was kept in boiling water bath. About 1 ml of filtrate was treated with drops of Mayer’s reagent. Turbidity or precipitate observed was taken as indication for the presence of alkaloids.

**Test for Saponin**
0.5 g/ml of each sample was stirred with water in a test-tube. Frothing persist on warming was taken as evidence for the presence of saponin.

**Test for Tannins**
0.5 ml of each sample was mixed with 10 ml of boiling water and was filtered. Few ml of 6% FeCl\(_3\) was added to the filtrate. Appearance of deep green color confirmed the presence of tannins.

**Test for Flavanoids**
0.2 ml of the extract was dissolved in CH\(_3\)OH was heated and a chip of Mg metal was added to mixture followed by the addition of few drop of HCl. The appearance of reddish-orange color indicates the presence of flavanoids.

**Test for Steroids**
About 0.5 ml of the extract was dissolved in 3 ml of CHCl\(_3\) and was filtered. To the filtrate conc. H\(_2\)SO\(_4\) was added which formed a lower layer. A reddish brown color was taken as positive for the presence of steroid ring.

**Test for Cardiac Glycosides**
About 0.5 ml of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of 1% FeCl\(_3\) along with conc. H\(_2\)SO\(_4\). A brown ring obtained at the interphase indicated the presence of deoxy sugar which is the characteristic of cardiac glycoside. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout the layer.

**RESULTS**

**Antioxidant activity of various fractions of plants extracts**
The *in vitro* antioxidant activities of different fractions of plant extracts as extracted by MSE were determined and the results were analyzed. It was observed from the results that antioxidant activities of the plants were directly proportional to Total Phenolic Content (TPC). The results of DPPH assay and Superoxide Anion Radical Scavenging Activity were found to be in correlation with the Total Phenolic Content (TPC). All data are expressed as mean of the three readings for each of the extract. Differences between groups were considered significant at \(p<0.05\).

**Determination of Total Phenolic Content (TPC)**
Total phenolic content of each fraction obtained was expressed as \(\mu\)g Gallic acid equivalent. The experiments were performed in triplicates and mean values of TPC of each of the fraction of the plant extract were determined.

The order of total phenolic content obtained by different conventional solvent extractions from low to high was hexane, petroleum ether, water and methanol in all the plants in comparison to *Ricinus communis*. The values of TPC in *Bacopa monnieri* (whole plant) were found to be 59.1, 88.0, 107.5 and 241.3 \(\mu\)g Gallic acid equivalent/g of the plant material. The values of TPC in *Swertia chirayta* (whole plant) were found to be 56.1, 84.0, 100.5 and 237.2 \(\mu\)g Gallic acid equivalent /g of the plant material. The values of TPC in *Aloe vera* (leaves) were found to be 53.0, 82.0, 89.5 and 226.0 \(\mu\)g Gallic acid equivalent/g of the plant material. The values of TPC in *Crataeva nurvula* (bark) were found to be 50.1, 81.0, 87.5 and 220.6 \(\mu\)g Gallic acid equivalent /g of the plant material.

The values of TPC in *Ricinus communis* (root) were found to be highest in hexane extract (265.0 \(\mu\)g Gallic acid equivalent/g of the plant material) followed by petroleum ether extract (235.8 \(\mu\)g Gallic acid equivalent/g of the plant material). The values of TPC were found to be lowest in aqueous and methanolic extracts (156.7 and 137.8 \(\mu\)g Gallic acid equivalent/g of the plant material).

These results indicate that maximum TPC values in solvent extracts of different plants in comparison to *Ricinus communis* follow the order Methanol> Water> Petroleum ether > Hexane. In *Ricinus communis*, maximum TPC values in solvent extracts follows the order Hexane > Petroleum ether > Aqueous > Methanol, as shown in Table 1.
Determination of Antioxidant Activity by DPPH Radical Scavenging Method

DPPH Radical scavenging activity was determined of different solvent fractions of the plant parts used for the study. The experiments were performed in triplicates and mean values of IC$_{50}$ of each of the solvent fraction of the plant extract were determined. The values of antioxidant activity determination by DPPH Radical Scavenging method follow the same order as that of TPC. Among the different total solvent extracts of the plants (in comparison to *Ricinus communis*), the methanolic fraction showed maximum DPPH radical scavenging activity. Hexane extract fraction in *Ricinus communis* possessed maximum antioxidant activity in comparison to other fractions. The four extracts of *Ricinus communis* (root) tested for antioxidant activity using DPPH radical scavenging method were determined. The hexane and petroleum ether successive extracts showed the maximum antioxidant activity with IC$_{50}$ values of 47.15 µg/ml and 39.20 µg/ml respectively. The aqueous and methanol extracts also showed antioxidant activity with IC$_{50}$ values of 75.10 and 72.57 µg/ml.

The four extracts of *Swertia chirayta* (whole plant) tested for antioxidant activity using DPPH radical scavenging method were determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC$_{50}$ values of 36.15 µg/ml and 32.10 µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC$_{50}$ values of 45.10 and 42.57 µg/ml.

The four extracts of *Crataeva nurvula* (bark) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC$_{50}$ values of 33.10 µg/ml and 26.30 µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC$_{50}$ values of 43.10 and 36.27 µg/ml.

The four extracts of *Aloe vera* (leaves) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC$_{50}$ values of 25.00 µg/ml and 22.14 µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC$_{50}$ values of 37.10 and 31.07 µg/ml. The four extracts of *Bacopa monnieri* (whole plant) tested for antioxidant activity using DPPH radical scavenging was determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with IC$_{50}$ values of 46.00 µg/ml and 43.10 µg/ml, respectively. The petroleum ether and hexane extracts also showed antioxidant activity with IC$_{50}$ values of 52.18 and 50.07 µg/ml. The known antioxidant ascorbic acid exhibited IC$_{50}$ value of 78.17 µg/ml. The results are recorded in Table 2.

Determination of Antioxidant activity by Superoxide Anion Radical Scavenging Method

Superoxide anion radical scavenging was determined of each of the fractions of the plant extracts. The experiments were performed in triplicates and mean values of antioxidant activity of each of the fraction of the plant extract were determined. The values of antioxidant activity determination by Superoxide Anion Radical Scavenging method follow the same order as that of DPPH assay and TPC. Among the different total solvent extracts of the plants (in comparison to *Ricinus communis*), the methanolic fraction showed maximum Superoxide Anion radical scavenging activity. Hexane extract fraction in *Ricinus communis* (root) possessed maximum antioxidant activity in comparison to other fractions.

The four extracts of *Ricinus communis* (root) tested for antioxidant activity using Superoxide Anion radical scavenging method, the hexane and petroleum ether successive extracts showed the maximum antioxidant activity with 85% and 83.5% inhibition of Superoxide. The aqueous and methanol extracts also showed antioxidant activity with 75% and 72% inhibition.

The four extracts of *Swertia chirayta* (whole plant) tested for antioxidant activity using Superoxide Anion radical scavenging method were determined, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 87.62% and 84.34% inhibition respectively. The petroleum ether and hexane extracts also showed antioxidant activity with 71.13% and 68.56% inhibition. Among the four extracts of *Crataeva nurvula* (bark) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 72.3% and 68.67% inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 73.5% and 72.14% inhibition.

Among the four extracts of *Aloe vera* (leaves) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum antioxidant activity with 75.5% and 72.23% inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 74.3% and 73.10% inhibition. Among the four extracts of *Bacopa monnieri* (whole plant) tested for antioxidant activity using Superoxide Anion radical scavenging method, the methanol and aqueous successive extracts showed the maximum
antioxidant activity with 65.68% and 62.34% inhibition. The petroleum ether and hexane extracts also showed antioxidant activity with 56.67% and 54.18% inhibition. The known antioxidant ascorbic acid exhibited 87.8% inhibition as shown in Table 3.

**Phytochemical investigation of active constituents present in potent extracts of plants**

The portions of the dried extracts obtained were subjected to phytochemical screening using conventional methods to test for alkaloids, tannins, flavanoids, steroids, saponin and cardiac glycosides. Steroids were predominant in *Riccinus communis* (root). Alkaloids and Saponin were present in all the plants parts while Tannins were absent in all the plants. Flavanoids content was found to be present in *Riccinus communis* (root) and *Bacopa monnieri* (whole plant). Glycosides were present in *Bacopa monnieri* (whole plant) and *Aloe vera* (leaves) as shown in Table 4.

**DISCUSSION**

The present study illustrates the use of MSE as an effective technique for extraction of antioxidants. Various phytochemicals and secondary metabolites are responsible for pharmacological activity. The role of polyphenols in determination of antioxidant activity and free radical activity has been known from various plants. There are many reports on the ambiguous or even adverse relationships between polyphenols as a whole and antioxidant activity.

**CONCLUSION**

The studies shown that all the fractions of plant extracts contain antioxidant activity. The Total Phenolic Content (TPC) of each of the fractions of the plant extracts was determined. The *in vitro* antioxidant activities were determined by DPPH Radical Scavenging Activity and Superoxide Anion Radical Scavenging Activity. The antioxidant activities determined were correlated with the TPC values. The crude methanolic extracts (in all plants studied) and hexane extract (in *Riccinus communis*) can contain a large number of lipophillic and hydrophilic antioxidants. These studies thus lead to the formulation of some antioxidants. Further studies are needed for isolation and characterization of the active principle(s) in these plants extracts which are responsible for antioxidant activity.

**ACKNOWLEDGEMENT**

The authors are thankful to Dr. G.S. Rajwar, Professor, Department of Botany, Govt. P.G. College, Rishikesh, Uttarakhand, India for identification of plant parts along with further consultation by BSI, Dehradun (U.K), India.

**REFERENCES**


Table 1: Determination of Total Phenolic Content (TPC)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant</th>
<th>Part used</th>
<th>Hexane</th>
<th>Petroleum ether</th>
<th>Water</th>
<th>Methanol</th>
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</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ricinus communis</td>
<td>root</td>
<td>265.0</td>
<td>235.8</td>
<td>156.7</td>
<td>137.8</td>
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<tr>
<td>2.</td>
<td>Bacopa monnieri</td>
<td>whole plant</td>
<td>59.1</td>
<td>88.0</td>
<td>107.5</td>
<td>241.3</td>
</tr>
<tr>
<td>3.</td>
<td>Swertia chirayta</td>
<td>whole plant</td>
<td>56.1</td>
<td>84.0</td>
<td>100.5</td>
<td>237.2</td>
</tr>
<tr>
<td>4.</td>
<td>Aloe vera</td>
<td>leaves</td>
<td>53.0</td>
<td>82.0</td>
<td>89.5</td>
<td>226.0</td>
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<tr>
<td>5.</td>
<td>Crateva nurvula</td>
<td>bark</td>
<td>50.1</td>
<td>81.0</td>
<td>87.5</td>
<td>220.6</td>
</tr>
</tbody>
</table>

*The results are the average of three determinations for each of the extracts.

Table 2: Determination of Antioxidant activity by DPPH Radical Scavenging assay

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant</th>
<th>Part used</th>
<th>DPPH Radical Scavenging Method (IC50); µg/ml</th>
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<tr>
<td></td>
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<tr>
<td>1.</td>
<td>Ricinus communis</td>
<td>root</td>
<td>47.15</td>
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<td>2.</td>
<td>Bacopa monnieri</td>
<td>whole plant</td>
<td>50.07</td>
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<td>3.</td>
<td>Swertia chirayta</td>
<td>whole plant</td>
<td>42.57</td>
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<tr>
<td>4.</td>
<td>Aloe vera</td>
<td>leaves</td>
<td>31.07</td>
</tr>
<tr>
<td>5.</td>
<td>Crateva nurvula</td>
<td>bark</td>
<td>36.27</td>
</tr>
</tbody>
</table>

Ascorbic acid = 78.17 µg/ml

*The results are the average of three determinations for each of the extracts.

Table 3: Determination of Antioxidant activity by Superoxide Anion Radical Scavenging method

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant</th>
<th>Part used</th>
<th>Superoxide Anion Radical Scavenging Method (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Hexane</td>
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<tr>
<td>1.</td>
<td>Ricinus communis</td>
<td>root</td>
<td>85.00</td>
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<td>2.</td>
<td>Bacopa monnieri</td>
<td>whole plant</td>
<td>54.18</td>
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<td>3.</td>
<td>Swertia chirayta</td>
<td>whole plant</td>
<td>68.56</td>
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<td>4.</td>
<td>Aloe vera</td>
<td>leaves</td>
<td>73.10</td>
</tr>
<tr>
<td>5.</td>
<td>Crateva nurvula</td>
<td>bark</td>
<td>72.14</td>
</tr>
</tbody>
</table>

Ascorbic acid = 87.80%

*The results are the average of three determinations for each of the extracts.
Table 4: Phytochemical investigation of active constituents in potent extracts of the plant parts

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Plant</th>
<th>Extract used</th>
<th>Phytochemical constituents</th>
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<td>Alkaloids</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>2.</td>
<td><em>Bacopa monnieri</em></td>
<td>Methanolic</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td><em>Swertia chirayta</em></td>
<td>Methanolic</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td><em>Aloe vera</em></td>
<td>Methanolic</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td><em>Crateva nurvula</em></td>
<td>Methanolic</td>
<td>+</td>
</tr>
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+, present; -, absent; ++, prominent

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