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Research Article

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 *Riccinus communis* (root), *Aloe vera* (leaves), *Crateva nurvula* (bark), *Swertia chirayta* (whole plant) and *Bacopa monnieri* (whole plant) extracts. MSE was performed at 80ºC 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 *Riccinus communis*. Antioxidant activities in hexane and petroleum ether extracts of *Riccinus 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 flavanoids[1] which prevent free radical damage, reducing risk of chronic diseases. Thus, the consumption of dietary antioxidants from these sources is beneficial in preventing cardiovascular disease[2]. 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[3-5]. 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^{o}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^{o}C freezer prior to testing.

Estimation of Total Phenolic Content (TPC)

The Total Phenolic Content of each fraction obtained of different extracts was determined[6] 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) Na_2CO_3 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^{0}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[7] 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. IC_{50} 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[8] 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.

The Superoxide anion radical scavenging activity (%) was calculated as :

$$\frac{Ao - As}{Ao} \times 100$$

Where, Ao = absorbance of positive control
As = absorbance of sample

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_3OH 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_2SO_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_2SO_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 µg/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 *Riccinus communis*. The values of TPC in *Bacopa monnieri* (whole plant) were found to be 59.1, 88.0, 107.5 and 241.3 µg 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 µg 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 µg Gallic acid equivalent/g of the plant material. The values of TPC in *Crateva nurvula* (bark) were found to be 50.1, 81.0, 87.5 and 220.6µg Gallic acid equivalent /g of the plant material.

The values of TPC in *Riccinus communis* (root) were found to be highest in hexane extract (265.0 µg Gallic acid equivalent/g of the plant material) followed by petroleum ether extract (235.8 µg 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 µg Gallic acid equivalent/g of the plant material).

These results indicate that maximum TPC values in solvent extracts of different plants in comparison to *Riccinus communis* follow the order Methanol> Water> Petroleum ether > Hexane. In *Riccinus 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 *Riccinus communis*), the methanolic fraction showed maximum DPPH radical scavenging activity. Hexane extract fraction in *Riccinus communis* possessed maximum antioxidant activity in comparison to other fractions. The four extracts of *Riccinus 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 47.15µg/ml and 39.20µg/ml respectively. The aqueous and methanol extracts also showed antioxidant activity with IC_{50} values of 75.10 and 72.57µg/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 32.10µg/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 *Crateva 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 26.30µg/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 *Riccinus communis*), the methanolic fraction showed maximum Superoxide Anion radical scavenging activity. Hexane extract fraction in *Riccinus communis* (root) possessed maximum antioxidant activity in comparison to other fractions.

The four extracts of *Riccinus 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 *Crateva 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[11]. There are many reports on the ambiguous or even adverse relationships between polyphenols as a whole and antioxidant activity[12, 13].

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.

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Table 1: Determination of Total Phenolic Content (TPC)

S. No.	Plant	Part used	Total Phenolic Content (TPC); µg/g Gallic acid equivalent			
			Hexane	Petroleum ether	Water	Methanol
1.	*Riccinus communis*	root	265.0	235.8	156.7	137.8
2.	*Bacopa monnieri*	whole plant	59.1	88.0	107.5	241.3
3.	*Swertia chirayta*	whole plant	56.1	84.0	100.5	237.2
4.	*Aloe vera*	leaves	53.0	82.0	89.5	226.0
5.	*Crateva nurvula*	bark	50.1	81.0	87.5	220.6

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

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

S. No.	Plant	Part used	DPPH Radical Scavenging Method (IC50); µg/ ml			
			Hexane	Petroleum ether	Water	Methanol
1.	*Riccinus communis*	root	47.15	39.20	75.10	72.57
2.	*Bacopa monnieri*	whole plant	50.07	52.18	43.10	46.00
3.	*Swertia chirayta*	whole plant	42.57	45.10	32.10	36.15
4.	*Aloe vera*	leaves	31.07	37.10	22.14	25.00
5.	*Crateva nurvula*	bark	36.27	43.10	26.30	33.10

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

S. No.	Plant	Part used	Superoxide Anion Radical Scavenging Method (%)			
			Hexane	Petroleum ether	Water	Methanol
1.	*Riccinus communis*	root	85.00	83.50	75.00	72.00
2.	*Bacopa monnieri*	whole plant	54.18	56.67	62.34	65.68
3.	*Swertia chirayta*	whole plant	68.56	71.13	84.34	87.62
4.	*Aloe vera*	leaves	73.10	74.30	72.30	75.50
5.	*Crateva nurvula*	bark	72.14	73.50	68.67	72.30

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

S. No.	Plant	Extract used	Phytochemical constituents					
			Alkaloids	**Flavanoids**	**Tannins**	**Steroids**	**Saponin**	**Glycosides**
1.	*Riccinus communis*	Hexane	+	+	-	++	+	-
2.	*Bacopa monnieri*	Methanolic	+	+	-	+	+	+
3.	*Swertia chirayta*	Methanolic	+	-	-	+	+	-
4.	*Aloe vera*	Methanolic	+	-	-	+	+	+
5.	*Crateva nurvula*	Methanolic	+	-	-	+	+	-

+, present; -, absent; ++, prominent

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