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SECONDARY METABOLITES EXTRACTION USING POTENT BIOLOGICALS & PHYTOCHEMICAL SCREENING

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Antioxidants present in the diet may have a significant effect on the prophylaxis and progression of various diseases associated with oxidative stress like cancer diabetes, cardiovascular and neurodegenerative. Berries and fungal species contain a range of secondary metabolites with antioxidant properties, including phenolic compounds. The objective of this study is to extract secondary metabolites from biologicals (wild berries and fungal species from the Himalaya region) and to conduct the preliminary phytochemical screening, total flavonoid and phenolic contents assays of various solvent extracts.

In this study, wild species were collected from the Himalayan region and extracted with different solvents by Soxhlet extraction. Phytochemical screening was carried out using standard procedures, total flavonoid content was measured by the aluminum chloride colorimetric assay and total phenolic content was estimated spectrophotometrically by the Folin-Ciocalteau method. Preliminary phytochemical screening reveals that except a few all extracts show the presence of alkaloids, tannin, carbohydrate, glycosides phenol, and flavonoid. Only Water, methanolic, and Chloroform extract of *Rubus indicus* (RIW, RIM, RIC) and water, methanolic extract of *Solanum nigrum* (SNW and SNM) contains steroids. Saponins are found only in extracts of *Solanum nigrum* and *Morchella esculenta.* Water extract of *Rubus armeniacus*, *Solanum nigrum*, *Morchella esculenta* and methanolic extract of *Rubus indicus*, *Lycium barbarum* (RAW, SNW, MEW, RIM, and LBM) shows higher content of flavonoid and phenol.

Methanolic extract of *Lycium barbarum* and *Rubas armeniacus* has the richest content of phenolics and flavonoids i.e. (102.16 mg GAE/g and 2.95 mg QE/g) respectively, on the contrary, hexane extract of *Morchella esculenta* and chloroform extract of *Lycium arbarum* was the least i.e. (6.64 mg GAE/g and 0.59 mg QE/g). All the extracts were not significantly different from one another ($p > 0.05$). It can be hypothesized that the high

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contents of phenolic compounds of these sample extracts indicated that these compounds contribute to the antioxidant activity and can be regarded as promising plant species for natural sources of radical scavenging activity with potential value for the treatment of many life-threatening diseases.

Keywords: Secondary metabolite; extraction; antioxidants; phytochemical screening.

1. INTRODUCTION

Plants have been a rich source of affordable natural compounds, explicitly the secondary metabolites that possess sufficient structural complexity so that their synthesis is difficult or at this time not yet accomplished and exhibit a broad spectrum of bioactivities including antitumor activity [1,2] Secondary metabolites are mostly small organic molecules, produced by an organism, that aren't essential for its growth, development, and reproduction. They can be classified based on the pathway by which they are synthesized. Additionally, an easy classification includes three main groups: terpenoids, phenolics, and extremely diverse alkaloids [3,4,5].

Phenolic compounds are secondary metabolites possessing an aromatic ring with one or more hydroxyl groups, and their structures can be a simple phenolic molecule or a complex polymer. They exhibited a wide range of physiological properties, such as anti-allergenic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective, and vasodilator effects. The main characteristic of this group of compounds has been associated with its antioxidant activity because they act as reducing agents, radical scavengers, singlet oxygen quenchers, or metal ion chelators [6,7].

This property is related to their capacity to act as antioxidants; they can scavenge free radical and reactive oxygen species. The process of oxidation is essential for living organisms; it is necessary for the production of energy. However, the generation of free radicals has been implicated in several human diseases [8,9 10,11].

In recent years consumption of berries and fruit increased. Research suggests that this increased intake of fruits and berries could also be related to a reduced incidence of disorders induced by reactive oxygen species (ROS), including cardiovascular disorders, cancer, and inflammatory processes [12]. Berries and their products (i.e., berry juice and jam) are very often recognized as “superfoods.” Berries belong to several families, although the two key examples are the Rosaceae, including black chokeberry (*Aronia melanocarpa*), strawberry (*Fragaria ananassa*), red raspberry (*Rubus ideaus*), black raspberry (*Rubus occidentalis*), blackberry (*Rubus fruticosus*), and cloudberry (*Rubus chamaemorus*), and the Ericaceae, including cranberry (*Vaccinium macrocarpon*), bilberry (*Vaccinium myritillus*), lowbush blueberry (*Vaccinium angustifolium*), highbush blueberry (*Vaccinium corymbosum*). Examples of berries from other families include blackcurrants (*Ribes nigrum*, family: Grossulariaceae), sea buckthorn (*Elaeagnus rhamnoides* (L.); family: Elaeagnaceae), and grapes (*Vitis vinifera*, family: Vitaceae) [13]."

They possess high concentrations of phenolic compounds, which have been found in vitro and in vivo studies to possess a range of biological activities, including anticancer and antiplatelet activities, as well as antioxidant properties [14]. Biological activities of berry phenolics are hooked into a variety of things including the class of phenolics, their concentration, the type of berry, and even the form consumed, be it fresh berries, juice, wine, jam, oil, or medicinal products.

A huge variety of phenolic compounds are produced by these plants, with 1000s recognized throughout the plant kingdom. They can be found in various parts of the plant, but particularly the fruits, leaves, and seeds, where they are typically involved in the defense against ultraviolet radiation and pathogens [15].

Recent advancements in biotechnology, microbiology, and progress in genetic research have made it easy for researchers to explore, detect and identify primary and secondary metabolites which have high antioxidant capacity, anticancer, anti-inflammatory, and antibacterial characteristics from cheap and easily available non-traditional sources such as fungi. Fungi are a distinct group of organisms that include species with large and visible fruiting bodies (macrofungi). The best-known samples of macrofungi are mushrooms.

Secondary metabolites derived from fauna, flora, and mycobiota are very important natural resources used in the construction of many medicines. These emerging bioactive compounds are used as medicines for several years and today they still are reserves of potential drugs [16]. The pharmacological evaluation of fungi from these bioactive compound sources is increasing day by day. In past, people consumed mushrooms as nutrients, especially during the season. In addition to their nutritive properties, mushrooms are considered significant medical sources thanks to

the biologically active compounds they contain everywhere. Those fungi are important biological resources in terms of secondary metabolites they produce in their bodies.

These secondary metabolites are chemically quite diverse and have an honest range of biological activity sought in traditional medicines [17]. Their ingredients include several secondary metabolites like phenolic compounds, polyketides, terpenes, and steroids [18].

There is a direct correlation between antioxidant activities and phenolic compounds in mushrooms. Mushrooms contain phenolic acids as phenolic compounds. The antioxidant capacities of phenolic acids are thanks to their phenolic hydrogen content. Especially natural mushrooms contain several phenolic acids. These include acid, caffeic acid, quercetin, hesperidin, and cinnamic acid.

Mushrooms have several pharmacological effects including the stimulation of the system, anti-carcinogen, anti-inflammatory, antimicrobial, anti-allergic, antioxidant, DNA protective properties thanks to their phenolic acid content [19,20].

A detailed review of literature in recent years revealed the role of phenolic compounds in berries as antioxidants protecting against the most common diseases related to oxidative stress however, the phenolic compound content of berries and berry products is not always well described, and further studies are required. Numerous clinical trials are confirming the applicability of mushrooms and their extracts as components of many therapeutics. But the complex modes of action and molecular targets, as well as exact structures of the active molecules from these mushrooms, still have to be studied in more detail. So, in the present study, the secondary metabolite is extracted from the potent biological to explore future advancement to cure many life-threatening diseases.

2. MATERIALS AND METHODS

2.1 Preparation of Plant Extract

The collected fruits/seeds were washed with running tap water surface sterilized with 0.1% mercuric chloride and allowed to air dry in shade for two to four weeks. After drying, the fruits/seed were ground finely and stored in an airtight container. The air-dried powder (5 g) was successively extracted by the Hot-extraction method i.e., Soxhlet extraction with solvents of different polarity i.e., hexane, petroleum ether, chloroform, methanol, and water. 450 ml of hexane solvent was used for extraction and time duration was maintained for 6-8 hrs. (4-6 cycles per hour i.e., 24-48 cycles). The hexane solvent containing extract was dried, collected in a sterile container, and stored in an airtight container. Similarly, the different extract was prepared in different solvents. All the dried extracts were weighed. All the extracts were diluted to 20 ml in their respective solvents. For each extract, the yield was expressed in percentage by dividing the quantity of dry mass obtained after extraction by the dry weight of the powder used before extraction the extraction yield percent was determined by the following formula:

$$\textit{Extraction yield \%} = (W_o/W_t)*100$$

W_o = *weight of initial fruit/seed sample*
W_t = *weight of dried extract after Soxhlet extraction*

2.2 Preliminary Phytochemical Screening

The phytochemical screening of the extracts was conducted using standard procedures. The following qualitative tests were carried out:

Test for Alkaloids: The extract of each fruit is evaporated to dryness in a boiling water bath. The residue is dissolved in 2 N HCl. The mixture is filtered and the filtrate is divided into 3 equal portions. One portion is treated with a few drops of Mayer's reagent; one portion is treated with an equal amount of Dragondroff's reagent and the other portion is treated with an equal amount of Wagner's reagent. The creamish precipitate, orange precipitate, and brown precipitate indicate the presence of respective alkaloids [21].

Test for Flavonoids: The presence of flavonoids is estimated by the Shinoda test. The extract of each fruit is treated with a few drops of concentrated HCl and magnesium ribbon. The appearance of pink or tomato red color within a few minutes indicates the presence of flavonoids [22].

Test for Tannins: The extract of each fruit is treated with an alcoholic $FeCl_3$ reagent. The blue color indicates the presence of tannins [23].

Test for Cardiac glycosides: Keller-kiliani test is performed to assess the presence of cardiac glycosides. The crude dry powder of each fruit is treated with 1 ml of $FeCl_3$ reagent (mixture of 1 volume of 5% $FeCl_3$ solution and 99 volumes of glacial acetic acid). To this solution, a few drops of concentrated H_2SO_4 are added. The appearance of greenish-blue color within a few minutes indicates the presence of cardiac glycosides [24].

Test for Steroids: Liebermann-Burchard reaction is performed to assess the presence of steroids. A chloroform solution of the crude dry powder of each fruit is treated with acetic anhydride and a few drops of concentrated H_2SO_4 are added down the sides of the test tube. A blue-green ring indicates the presence of terpenoids.

Test for **Saponins:** The presence of saponins is determined by the Frothing test. The crude dry powder of each fruit is vigorously shaken with distilled water and is allowed to stand for 10 minutes and classified for saponin content as follows: no froth indicates the absence of saponins and stable froth more than 1.5 cm indicates the presence of saponins [25].

Test for Carbohydrates (Molisch's Test): To the extract, 1ml of the Molisch's reagent was added then along the walls of the test tube carefully conc H2SO4 was added. The formation of a brown ring at the junction of two liquids was observed.

Test for Reducing Sugars (Fehling's Test): The extract was taken in a test tube, and 1ml of Fehling's solution (A and B) was added and boiled on the water bath. The solution was observed for the color change reaction.

Test for Glucose (Benedict's test): Few drops of Benedict solution were added to the plant extract if it shows the brick read color Confirm the presence of glucose.

Total phenol determination: Total phenolic content of the extracts was determined by the Folin Ciocalteu reagent method [26]. Fruit extract (1 ml) mixed with Ciocalteu reagent (0.1 ml, 1 N), and allowed to stand for 15 min. Then 5 ml of saturated Na_2CO_3 was added. The mixture was allowed to stand for 30 min at room temperature and the total phenol was determined spectrophotometrically at 760 nm. Gallic acid is used as a standard. Total phenol value was expressed in terms of gallic acid equivalent (mg g^{-1} of the extracted compound).

Flavonoid determination: Flavonoid was estimated by the protocol of Chang et al., 2002. 1ml of fruit extract mixed with 1ml of methanol, 0.5 ml aluminium chloride (1.2 *%)* and 0.5 ml potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature; then the absorbance was measured at 415 nm. Quercetin is used as a standard. Flavonoid content was expressed in terms of quercetin equivalent (mg g-1 of the extracted compound) [27].

3. RESULTS AND DISCUSSION

Percentage yield of extracts: Initial weight of the plant sample was 5 gm. The percentage yield for the various solvent extracts is shown in Table 1.

Table 1. Percentage yield of various solvent extracts

Sample	Initial weight	Final dry weight	Extraction yield (%)
RAH	5	1.124	22.48
RAP	5	2.091	41.82
RAC	5	2.004	40.08
RAM	5	1.982	39.64
RAW	5	1.743	34.86
SNH	5	1.2	24
SNP	5	2.32	46.4
SNC	5	2.41	48.2
SNM	5	2.49	49.8
SNW	5	2.89	57.8
MEH	5	2.91	58.2
MEP	5	2.81	56.2
MEC	5	1.209	24.18
MEM	5	2.078	41.56
MEW	5	2.79	55.8
RIH	5	2.81	56.2
RIP	5	2.704	54.08
RIC	5	2.12	42.4
RIM	5	2.43	48.6
RIW	5	2.94	58.8
LBH	5	3.8	76
LBP	5	2.4	48
LBC	5	2.8	56
LBM	5	3.2	64
LBW	5	2.1	42

(RA: Rubus armeniacus (Himalayan blackberry), SN: Solanum nigrum (Makoy), ME: Morchella esculenta, RI: Rubus indices (Indian Raspberry) LB: Lycium barbarum(Gojiberry) H: Hexane extract, P: Petroleum ether extract, C: Chloroform extract, M: Methanolic extract, W: Water extract)}.

As shown in the table LBH extract has the highest yield followed by LBM, RIW, MEH, and SNW

Preliminary phytochemical screening: Phytochemical analysis of plant samples shows in Table 2. Except for a few extracts that show the presence of alkaloids, tannin, carbohydrate, glycosides phenol, and flavonoid. Only RIW, RIC, RIM, SNW, and SNM extracts contain steroids. Saponins are found only in extracts of *Solanum nigrum* and *Morchella esculenta.* RAW, SNW, MEW, RIM, and LBM show higher content of flavonoid and phenol.

Table 2. Preliminary phytochemical screening of various solvent extracts

Sample	Alkaloid			Flavonoid	Phenol	Glycosides	Tannins	Carbohydrate			Saponins	Steroids
	Mayer's test	Dragendorff's test	Wagner test					Molisch	Fehling's	Benedicts		
RAH	-	-	-	+	+	-	+	++	+	-	-	-
RAP	-	-	-	+	+	-	+	++	+	-	-	-
RAC	+	+	+	++	+	+	+	++	+	-	-	-
RAM	++	+	+	+++	++	+	++	+++	++	-	-	-
RAW	++	++	+	+++	+++	+	++	+++	++	-	-	-
SNH	-	-	-	-	-	-	-	+	-	-	+	-
SNP	-	-	-	-	-	-	-	+	-	-	+	-
SNC	+	-	+	+	-	-	-	+	-	-	+	-
SNM	++	++	++	++	++	-	-	++	++	+	++	++
SNW	++	++	++	+++	+++	-	-	++	++	+	++	++
MEH	+	+	+	-	+	+	+	+	+	+	+	-
MEP	+	+	+	-	+	+	+	+	+	+	+	-
MEC	+	+	++	+	+	+	+	+	+	+	+	-
MEM	++	++	++	++	++	+	+	++	++	+	++	-
MEW	++	++	++	++	++	++	+	++	++	++	++	-
RIH	+	+	+	-	+	++	+	+	-	+	-	-
RIP	+	+	+	-	-	-	-	+	-	+	-	-
RIC	+	+	+	+	+	-	+	+	-	+	-	+
RIM	+++	+++	+++	+++	+++	++	++	++	++	++	-	++
RIW	+	++	+	-	+	+	-	++	++	++	-	++
LBH	+	-	-	-	+	-	+	-	-	-	-	-
LBP	-	-	-	-	-	-	-	-	-	-	-	-
LBC	+	++	-	+	++	-	+	-	-	-	-	-
LBM	++	++	+	++	+++	-	++	-	-	-	-	-
LBW	++	+	+	-	+++	-	+	-	-	-	+	-

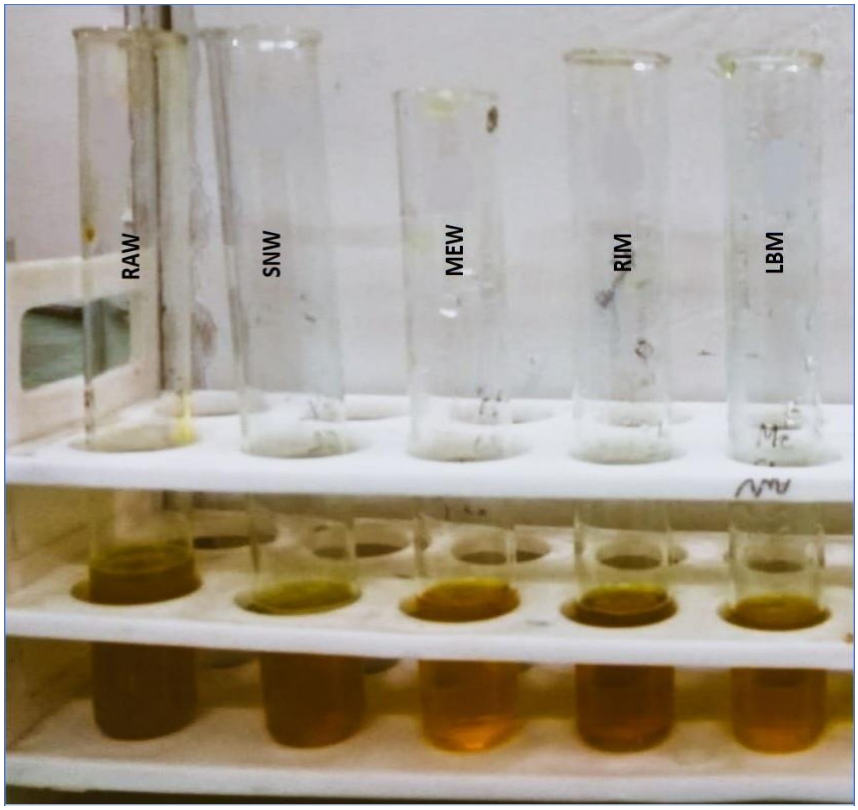

Fig. 1. Test tubes of samples with higher Flavonoid content

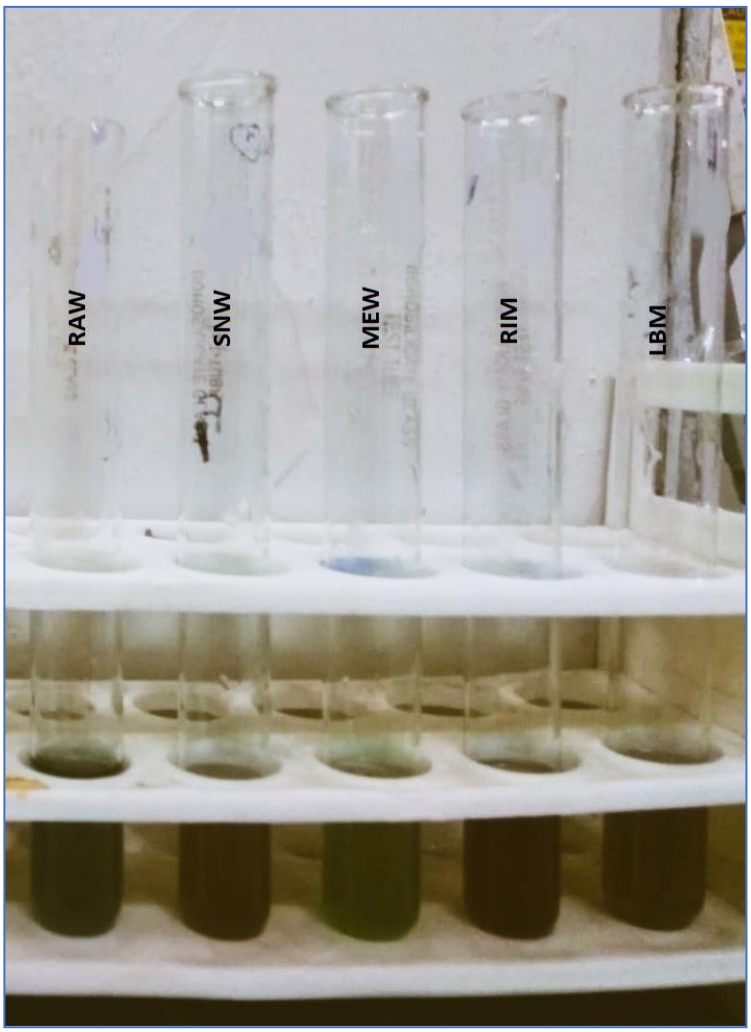

Fig. 2. Test tubes of samples with a higher phenol content

(RA: Rubus armeniacus (Himalayan blackberry), SN: Solanum nigrum (Makoy), ME: Morchella esculenta, RI: Rubus indices (Indian Raspberry), LB: Lyciiim barbarum(Gojiberry) # H: Hexane extract, P: Petroleum ether extract, C: Chloroform extract, M: Methanolic extract, W: Water extract}

3.1 Total Phenolic Content (TPC)

The TPC of the extracts was determined by extrapolation from the calibration curve (Y = 11.86x - 0.075; R^2 = 0.9826) prepared from the gallic acid concentrations (Fig. 1) and expressed in mg of gallic acid equivalence (GAE) per gram. The number of phenolic compounds in the various extracts was obtained from the regression equation and the values were expressed in gallic acid equivalence (Fig. 2). There was no significant difference ($p > 0.05$) in all the different extracts. The TPC of different extracts is shown in Table 3.

3.2 Total Flavonoid Content (TFC)

The TFC content of the 1 extracts was also determined by extrapolation from the calibration curve (Y = 9.2159x - 0.1466; R^2 = 0.9877) prepared from the quercetin concentrations (Fig. 3) and expressed in mg of quercetin equivalence (QE) per gram. The amounts of flavonoid compounds in the various extracts were obtained from the regression equation and the values were expressed in quercetin equivalence (Fig. 4). The values from all the extracts were not significantly different ($p > 0.05$). The TFC of different extracts is shown in Table 4.

Table 3. TPC of various solvent **extract**

Sample	TPC (mg/gm)
RAW	21.28
RAH	7.42
RAP	6.75
RAC	7.62
RAM	10.46
SNW	16.42
SNM	11.3
MEH	6.64
MEC	7.42
MEM	8.47
MEW	8.57
MEP	7.66
RIM	11.5
RIH	7.02
LBW	95.08
LBM	102.16

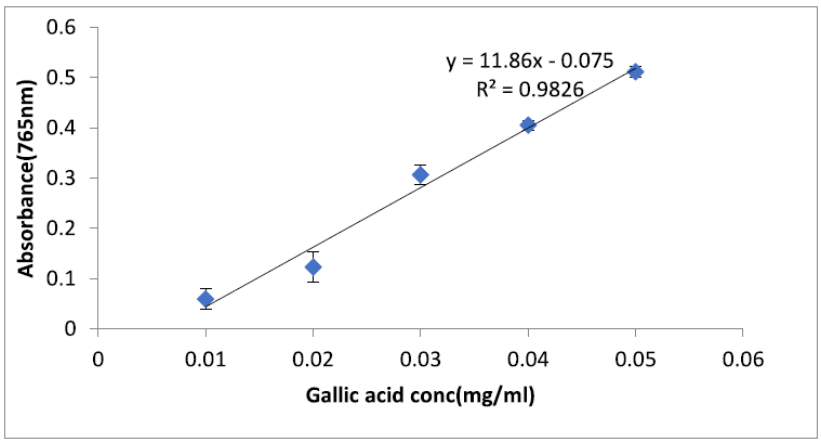

Fig. 3. Gallic acid calibration curve

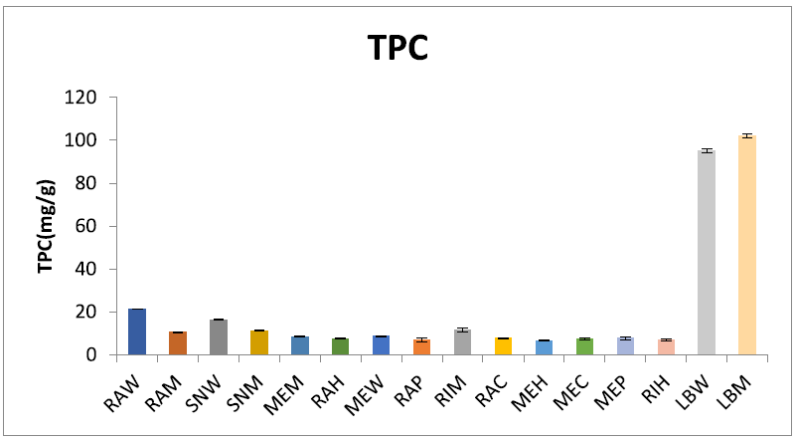

Fig. 4. Total phenolic content of various solvent extracts

Table 4. TFC of various solvent extract

Sample	TFC (mg/gm)
RAH	0.65
RAP	0.92
RAC	1.44
RAM	2.95
RAW	2.55
SNC	0.95
SNM	1.21
SNW	2.93
MEC	0.72
MEM	1.52
MEW	1.47
RIC	0.64
RIM	2.68
LBC	0.59
LBM	1.27

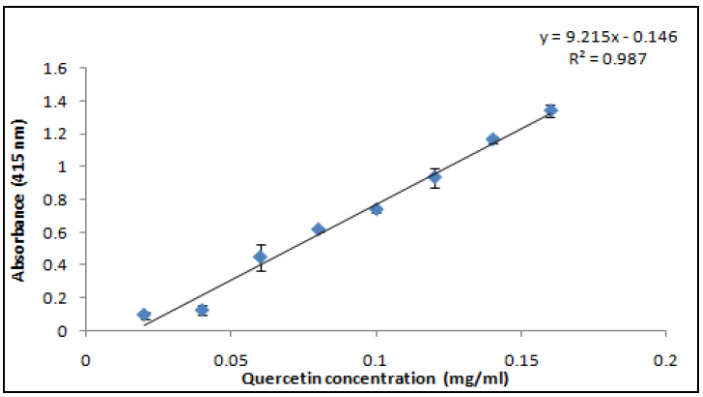

Fig. 4. Quercetin calibration curve

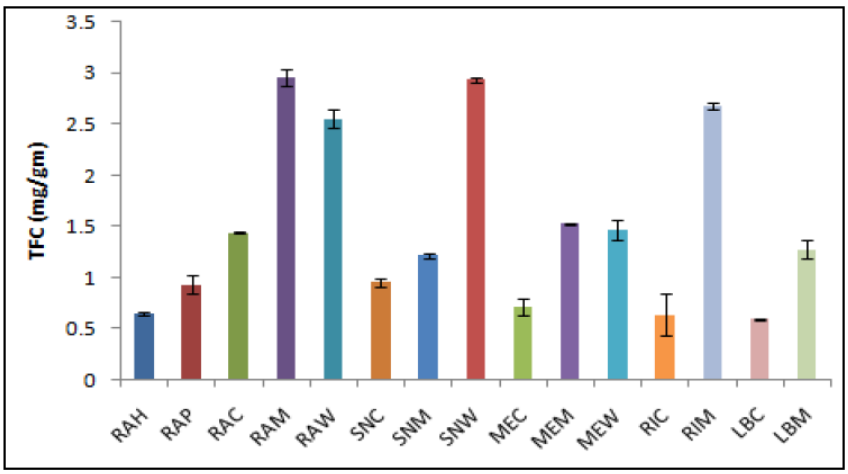

Fig. 5. Total flavonoid content of various solvent extracts

Preliminary phytochemical screening reveals that expect few all extracts show the presence of alkaloids, tannin, carbohydrate, glycosides phenol, and flavonoid. Only RIW, RIC, RIM, SNW, and SNM extracts contain steroids. Saponins are found only in extracts of *Solanum nigrum* and *Morchella esculenta.* RAW, SNW, MEW, RIM, and LBM show higher content of flavonoid and phenol.

The total phenolic content (TPC) of all extracts was determined using the Folin - Ciocalteau method. This method allows the estimation of all the flavonoids, anthocyanins, and non-flavonoid phenolic compounds of all the phenolics available in the samples. The results for total phenolic content in the studied extracts are presented in Table 3. The results show that LBM has the richest source of phenolics, total phenolic content is 102.16 mg GAE/g while on the contrary, MEH possesses the lowest amount of phenolics i.e., 6.64mg GAE/g. The total phenolic contents did not vary significantly ($p > 0.05$) in different solvent extracts. So found that yield of total phenolic was dependent on the method and choice of solvent. The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvent used in the extraction process. Furthermore, solvent polarity will play a key role in increasing phenolic solubility. Thus, it's difficult to supply a typical extraction procedure suitable for phenol extraction of all the plant samples.

As the results indicated, it's apparent that the recovery of phenolic compounds was hooked into the solvents used and their polarity. This may be attributed to the fact that a wide range of phenols can dissolve in aqueous methanol mixtures. On the opposite hand, methanol may be a good solvent system for the extraction of polar antioxidants. There is a robust link between phenolic content and radical scavenging activity [28-31] The results obtained in the present study revealed that the level of phenolic compounds in all the (especially water and methanolic) extracts of all the samples was considerable and falls within the values of commonly consumed green vegetables and fruit as compared to extracts of other solvents. Hence consumption of those plants will enhance the health benefits by absorbing and neutralizing free radicals, quenching singlet oxygen, or decomposing peroxides.

The total flavonoid content (TFC) of different extracts was determined by the $AlCl_3$ method. Determining the total flavonoids by using $AlCl_3$ is based upon the formation of a stable complex between $AlCl_3$, keto, and hydroxyl groups of flavones and flavonoids. The results for total flavonoid content in the studied extracts are presented in Table 4. The results show that RAM has the richest source of flavonoids, i.e., the total content is 2.95 mg QE/g while on the contrary, LBC possesses the lowest number of flavonoids i.e., 0.59 mg QE/g. Flavonoids display an important role in scavenging the free radicals and these are the phytoconstituents that ought to be focused on for investigation of the many biological activities. Phytochemicals especially polyphenols constitute a serious group of compounds that act as primary antioxidants [32].

4. CONCLUSION

It can be postulated that the contents of phenolic and flavonoid compounds of various sample extracts (especially water and methanolic extracts) contribute to the radical scavenging activity and can be regarded as promising plant species for natural sources of antioxidants with potential value for the treatment of many life-threatening diseases. The process of extraction and identification of active principles responsible for the free radical scavenging property of different extracts of plant sample through bioactivity guided fraction is under progress to understand the possible mechanism of action of these compounds. Utilization of these plants will be of advantage to mankind and an increase in their consumption will help in the prevention of chronic lifestyle diseases like cancer, cardiovascular and diabetes.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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