Quantitative analysis of isolated phenolic fractions and evaluations of antioxidant potential of selected berries

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Abstract

The present study aimed at determining the antioxidant action of a five species of wild Himalayan berries, determination of the total flavonoid content and fractionation of the phenolic components from the extract. Successive solvent extraction in hexane, petroleum ether, chloroform, methanol and water were carried out by hot continuous extraction method. 25 extracts were obtained and screened for phytochemical class present in them. The total flavonoid content of the extracts was determined and the antioxidant action was assayed using ABTS radical scavenging assay. The extracts were fractionated to obtain the phenolic isolates; the yield and TFC of the isolates was studied. The extractibles were lowest in the hexane and aqueous extracts in all plants except *Rubus niveus.* High amounts of flavonoids in 23 of the 25 extracts and phenolics in 20 extracts. The highest flavonoid content was found in VMM (7.490 QE mg/g) followed by VMW (5.068 QE mg/g) while the least flavonoids were present in RNC (0.482 QE mg/g). All the extracts except WBP, WBM, GBH, GBC, REH, REC, and RNC exhibited more than 50% inhibition of the ABTS radical cation suggesting significant antioxidant activity. Fractions eluted with methanol-chloroform (10-90 & 20-80) contained the phenolics with highest yield of phenolic content in REP 10 (93.42% phenolic content).

Keywords: Antioxidant, Phenolic, ABTS radical, Ascorbic acid, berries
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Introduction

The recommendations in the dietary guidelines suggest high consumption of fruits and vegetables.[1] Fruits such as berries are found to be high in phytochemicals and nutrients that would be helpful in strengthening the immune system and preventing several physiological disorders.[2]Berries are a rich source of a wide variety of non-nutritive, nutritive, and bioactive compounds such as flavonoids, phenolics, anthocyanins, phenolic acids, stilbenes, and tannins, as well as nutritive compounds such as sugars, essential oils, carotenoids, vitamins, and minerals.[3,4]These colourful fruits can be consumed fresh or can also be frozen or processed to derive several products. Additionally, there has been a growing trend in the use of berry extracts as ingredients in functional foods and dietary supplements, which may be combined with other colourful

fruits, vegetables, and herbal extracts. Many laboratory and animal studies have shown that berries have anticancer, antioxidant, and antiproliferative properties.[5-7]

Free radical production and lipid peroxidation are involved in the pathogenesis of some chronic diseases, including atherosclerosis, cardiac and cerebral ischemia, neurodegenerative disorders, carcinogenesis, diabetes, and rheumatic disorders.[8]The compounds derived from secondary metabolism, specifically phenolic compounds, play a fundamental role against oxidative stress.[8]In the recent years, there has been an increased interest in phenolic compounds derived for their possible health benefits. These compounds are known to act as antioxidants not only for their ability to donate hydrogen or electrons but also because they are stable radical intermediates.[9]

This study aimed to determine the antioxidant action of a few wild berries, determination of the total flavonoid content and fractionation of the phenolic components from the extract.

Material and methods

Selection of the plants

Five wild berries found in the Himalayan region of India were selected. The species used for the study included the berries of *Euterpe oleraceae* (Acai) (EO), *Vaccinium myrtillu (*wild blue) (VM), *Phyllanthus embilica* (gooseberry) (PE), *Rubus ellipticus* (Himalayan Raspberry) (RE), *Rubus niveus* (Mysore raspberry) (RN).

The berries powder purchase from Herbalveda.

Extraction of phytoconstituents

The collected berries were washed with water followed by surface sterilized using 0.1 % mercuric chloride and finally washed under running tap water. The clean plant material was shade dried for 2 to 4 weeks, prevented from direct sunlight. The dried plant material were finely ground and stored in airtight container. The dried powder (5 g) was defatted hexane, followed by petroleum ether and the defatted material was successively extracted using chloroform, methanol and water utilizing hot continuous extraction method. The extracts were dried by solvent evaporation using rotary vacuum evaporator, weighed and stored in sterile containers. All the extracts were diluted to 20 ml in their respective solvents wherever needed.[10,11]

Phytochemical screening of the extracts

Alkaloids

A small amount of the extracts was dissolved in 2 N HCl by aid of slight heat.The solution was filtered and the filtrate was divided into 3 equal portions. One portion as treated with a few drops of Mayer's reagent; the second portion was treated with equal amount of Dragondroff's reagent and the last portion was treated with equal amount of Wagner's reagent. Occurrence of creamish, orange and brown precipitates respectively, indicates the presence of respective alkaloids.[12]

Flavonoids

The presence of flavonoids was estimated by Shinoda test. The extracts were treated with a few drops of concentrated HCl and magnesium ribbon was added to it. The appearance of pink or tomato red colour within a few minutes indicates the presence of flavonoids.[13]

Tannins

The extracts were treated with alcoholic $FeCl_3$ reagent. Occurrence of a blue color in the test solution indicates the presence of tannins.[14]

Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The powdered extracts were 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 $H_2SO_4$was added. Appearance of greenish blue color within a few minutes was taken as the indication of the presence of cardiac glycosides.[15]

Steroids

Liebermann-Burchard reaction was used to assess the presence of steroids. A chloroform solution of the extractwas treated with acetic anhydride and a few drops of concentrated

H_2SO_4wereflown down the sides of the test tube. A blue green ring indicates the presence of terpenoids.[13]

Saponins

Froth test was performed to observe saponins. The extracts were vigorously shaken with distilled water and allowed to stand for 10 minutes. Development of a froth of more than 1.5 cm height indicates the presence of saponins.[16]

Carbohydrates (Molisch's Test)

To the extracts, 1ml of the Molisch's reagent was added followed by flowing down of conc. H_2SO_4 along the walls of the test tube. Formation of a brown ring at the interface of two liquids was indication for carbohydrates.

Reducing Sugars (Fehling's Test)

To the extracts was added 1ml of the Fehling's solution (A and B) and the mixture was boiled on the water bath. The solution was observed for the color change.

Glucose (Benedict's test)

Few drops of Benedict solution was added in the plant extracts. The occurrence of brick rea color indicates glucose in the solution.

Total Flavonoid content (TFC)

The TFC of extracts was determined with the aid of aluminium chloride method using quercetin as the standard. To 1.5 mL of the extract solution, 0.5 mL of 1.2% aluminium chloride solution and 0.5 mL of 1M potassium acetate solution were added. The solution was allowed to stand for 60 minutes and the absorbance was measured using UV visible spectrophotometer at 420 nm.[17]

Antioxidant activity

The antioxidant activity was assayed using 2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical assay.[18,19] Briefly 7mM ABTS in water and 2.45 mM potassium persulphate were mixed in equal ratio and incubated in dark for 24 h. The solvent blend was diluted to obtain absorbance of ~0.7 at 734 nm. 5µl of extract and 3.395 mL of the diluted reagent were added and incubated in dark for 10 min. Absorbance were taken at 734 nm. Control was prepared by taking absorbance of ABTS and methanol. Standard curve of Ascorbic acid was prepared simultaneously at concentration levels of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 µg/ml. The ABTS radical scavenging percent was calculated using the formula

$$ABTSscavenging(\%) = \frac{(Ab - Aa)}{Ab} x100$$

Where,

Ab=Absorbance of control

Aa= ABTS+ sample/standard

CEAC (Vitamin C Equivalents Antioxidant Capacity) of all the extracts were estimated using the standard curve of $ABTS^+$ scavenging (%) of ascorbic acid standard solutions.

Extraction of Phenolics from crude extracts

A method of liquid liquid extraction was adapted and the extracts were acidified to pH 2.0 by adding 2M HCl solution. Briefly, 20 ml acidified extract was extracted thrice with 3 mL portions of n-hexane and the aqueous layer was separated. The aqueous layer was further extracted with seven portions of 1 mL ethyl acetate to collect the phenolic compounds. The ethyl acetate extracts were pooled and dried over anhydrous sodium sulphate and evaporated at 75°C to obtain the dried phenolic extract and weighed. The phenolic extractswere dissolved in equimolar mixture of chloroform &methanol and an equal weight of silica gel was added to the mixture. The solvent was evaporated in the fume hood, and the mixture was loaded on a silica gel column packed in chloroform. The column was eluted with chloroform, and then the polarity was gradually increased using methanol. Fractions of similar compositions were collected together to give a total of ten fractionated groups [A (I)–A (X)].

Group A (I) - 100% chloroform.
Group A (II) - 10% methanol: 90% chloroform.
Group A (III) - 20% methanol: 80% chloroform.
Group A(IV) - 30% methanol: 70 %chloroform.
Group A(V) - 40% methanol: 60 % chloroform.
Group A(VI) - 50% methanol: 50% chloroform.
Group A(VII) - 60% methanol: 40% chloroform.
Group A(VIII) - 70% methanol: 30% chloroform.

Group A(IX) - 80% methanol: 20% chloroform.
Group A(X) - 90% methanol: 10% chloroform.
Initial fraction of chloroform was discarded. TPC of all fractions were estimated and only the fraction of having maximum TPC was taken for further purification. Only the fractions of II & III were having the good phenolic content of which fraction II exhibited maximum phenolics and was subjected to further analysis.

Results and Discussion

Extraction yield

The extractibles were lowest in the hexane and aqueous extracts in all plants except *Rubus niveus* where the maximum extraction yield was obtained in aqueous (RNW) and hexane (RNH) extracts (Figure 1).

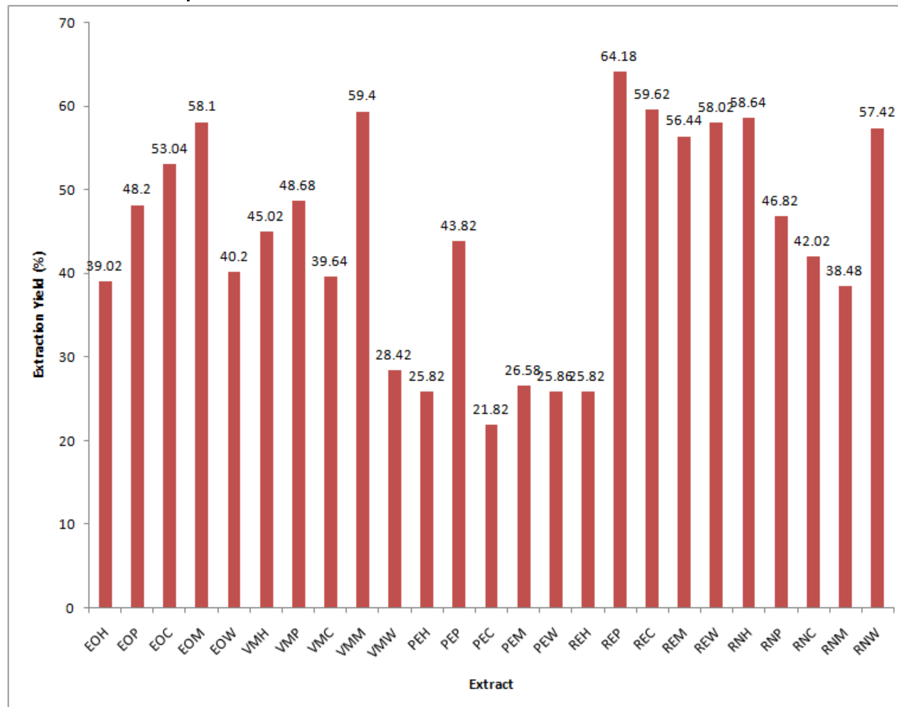

Figure 1 Extraction yield of various plant extracts

Phytochemical screening

The various extracts were screened for assessing the phytoconstituent class to reveal the presence of high amounts of flavonoids in 23 of the 25 extracts and phenolics in 20 extracts. Steroids were found only in 7 extracts while glycosides in 8, tannins and alkaloids in 13, carbohydrates in 18 and saponins in 10 (Table 1).

Table 1 Results of phytochemical screening of crude extracts

Class of Secondary Metabolite	Found in Extract
Alkaloids	EOH, EOP, VMM, VMW, PEC, PEM, REH, REP, REC, REM, REW, RNM, RNW
Glyosides	VMM, VMW, PEH, PEC, PEW, REH, REM, REW

Flavonoid	EOH, EOP, EOM, EOW, VMH, VMP, VMC, VMM, VMW, PEH, PEP, PEC, PEM, REH, REP, REC, REM, REW, RNH, RNP, RNC, RNM, RNW
Phenolics	EOH, EOP, EOM, EOW, VMH, VMP, VMC, VMM, VMW, PEH, PEM, REP, REC, REM, REW, RNH, RNP, RNC, RNM, RNW
Tannins	EOM, EOW, VMM, VMW, PEH, PEP, PEM, PEW, REM, REW, RNC, RNM, RNW
Carbohydrate	EOH, EOP, EOC, EOM, EOW, VMH, VMP, VMC, VMM, VMW, PEH, PEM, PEW, RNH, RNP, RNC, RNM, RNW
Sapnonins	VMC, VMM, VMW, PEH, PEP, PEC, REM, REW, RNM, RNW
Steroids	VMC, VMM, VMW, PEP, PEC,RNM, RNW

TFC estimation of crude extracts

The total flavonoid content was determined as quercetin equivalent per gram of the extract. The absorbance of solution at 420 nm was recorded and the concentration of quercetin was calculated in each extract using the calibration curve for quercetin (Figure 2).

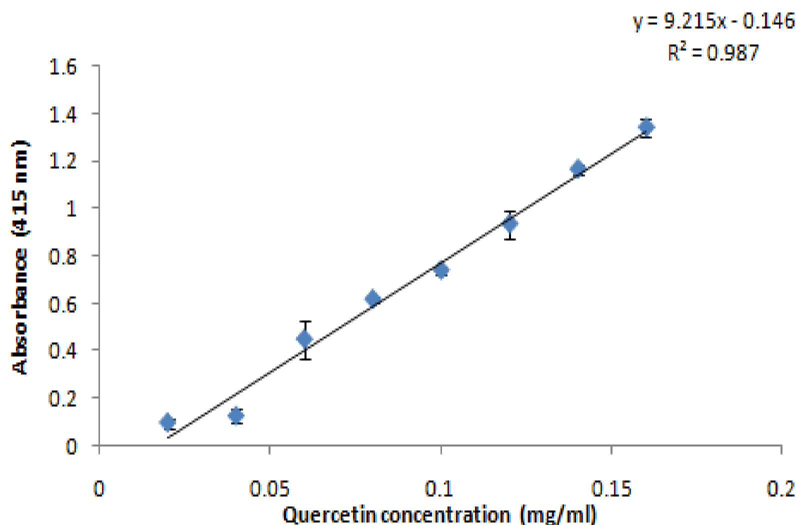

Figure 2 Calibration curve of quercetin standard at 420 nm

Table 2 TFC of crude extracts

Sample	**QE(mg/ml)**	**TFC (mg/ gm)**
EOH	0.026	0.633
EOP	0.025	0.591
EOM	0.202	4.860
EOW	0.156	3.740
VMH	0.125	3.011
VMP	0.035	0.828
VMC	0.031	0.742
VMM	0.312	7.490
VMW	0.211	5.068
PEH	0.046	1.112
PEP	0.032	0.763
PEC	0.039	0.945
PEM	0.103	2.469
REH	0.038	0.919
REP	0.068	1.636
REC	0.058	1.401
REM	0.101	2.433
REW	0.056	1.354
RNH	0.024	0.570
RNP	0.020	0.487
RNC	0.020	0.481
RNM	0.106	2.552
RNW	0.117	2.823

As shown in Figure 3, the highest flavonoid content was found in VMM (7.490 QE mg/g) followed by VMW (5.068 QE mg/g) while the least flavonoids were present in RNC (0.482 QE mg/g).

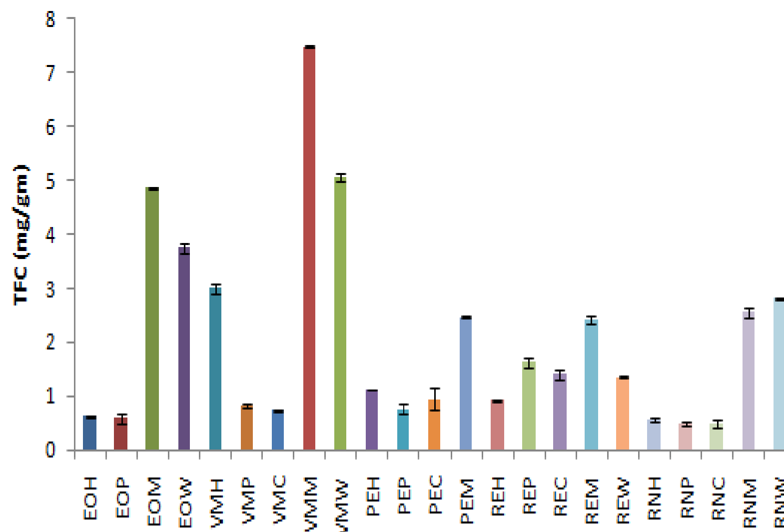

Figure 3 Comparative representation of TFC of various extracts

Antioxidant Activity

Antioxidant capacity is an overall ability of organisms or food to catch free radicals and prevent their harmful effect. Antioxidative effect includes protection of cells and cellular structures against harmful effect of free radicals, especially oxygen and nitrogen.[20] Antioxidative systems include antioxidative enzymes, that is, superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase, and nonenzymatic substrates, such as glutathione, uric acid, lipoic acid, bilirubin, coenzyme Q, vitamin C (L-ascorbic acid), vitamin A (retinol), vitamin E (tocopherol), flavonoids, carotenoids, theine compounds.[21]

The antioxidant activity of all the 25 crude extracts was determined using the 2,2' -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{•+}$) radical cation assay and determination of the CEAC thereafter. The basis of the ABTS assay is the interaction between an antioxidant and the pre-generated $ABTS^{•+}$ radical cation having an absorption maximum of 734 nm. $ABTS^{-}$ scavenging can be easily quantitatively detected due to the bleaching of absorption spectrum.

All the extracts except WBP, WBM, GBH, GBC, REH, REC, and RNC exhibited more than 50% inhibition of the ABTS radical cation suggesting significant antioxidant activity (Table 3, Figure 4). While the highest inhibition of ABTS inhibition was exhibited by EOC (97.76 ± 0.248), the lowest was presented by GBC (4.56 ± 0.759). The calibration curve using ascorbic acid as the sample for ABTS assay had linearity equation Abs = 22.82 (conc) – 3.315 with an R^2 value of 0.929 and was used for determining the CEAC of the extracts. The highest CEAC was 2.30 ± 0.005 (EOC) and the lowest was 0.17 ± 0.017 (GBC) (Figure 5).

CEAC (Vitamin C Equivalents Antioxidant Capacity) or ascorbic acid content of all the extract will be estimated by standard curve of $ABTS^{+}$ scavenging effect (%) linear curve equation.

Table 3 ABTS inhibition and CEAC of extracts

Extract	(% inhibition of ABTS radical)*	CEAC(mg/ml)*
EOH	81.84 ± 1.084	1.94 ± 0.024
EOP	77.94 ± 0.379	1.85 ± 0.008
EOC	97.76 ± 0.248	2.30 ± 0.005

EOM	82.66 ± 0.626	1.96 ± 0.014
EOW	77.77 ± 1.370	1.85 ± 0.031
WBH	68.73 ± 1.035	1.64 ± 0.023
WBP	28.68 ± 1.618	0.73 ± 0.036
WBC	41.87 ± 1.175	1.03 ± 0.026
WBM	51.82 ± 1.005	1.25 ± 0.022
WBW	81.67 ± 0.941	1.93 ± 0.021
GBH	30.34 ± 1.741	0.76 ± 0.039
GBP	68.82 ± 1.175	1.64 ± 0.026
GBC	4.56 ± 0.759	0.17 ± 0.017
GBM	96.10 ± 0.799	2.26 ± 0.018
GBW	94.19 ± 1.227	2.22 ± 0.028
REH	19.65 ± 1.316	0.52 ± 0.030
REP	22.88 ± 1.741	0.59 ± 0.039
REC	19.15 ± 2.372	0.51 ± 0.054
REM	52.37 ± 0.311	1.27 ± 0.007
REW	55.55 ± 1.276	1.34 ± 0.029
RNH	44.27 ± 0.995	1.086 ± 0.022
RNP	46.84 ± 1.252	1.14 ± 0.028
RNC	22.55 ± 2.311	0.59 ± 0.052
RNM	56.30 ± 1.227	1.36 ± 0.028
RNW	77.03 ± 0.287	1.83 ± 0.006

*Mean ± standard deviation, n = 3

Abbreviation: EA: *Euterpe oleraceae*(Acai), VM: *Vaccinium myrtillu(*wild blue), PE: *Phyllanthus embilica*(gooseberry), RE: *Rubus ellipticus*, RN:*Rubus niveus*

H: Hexane extract, P: Patroleum ether extract, C: Chloroform extract, M: Methanolic extract, W: Water extract

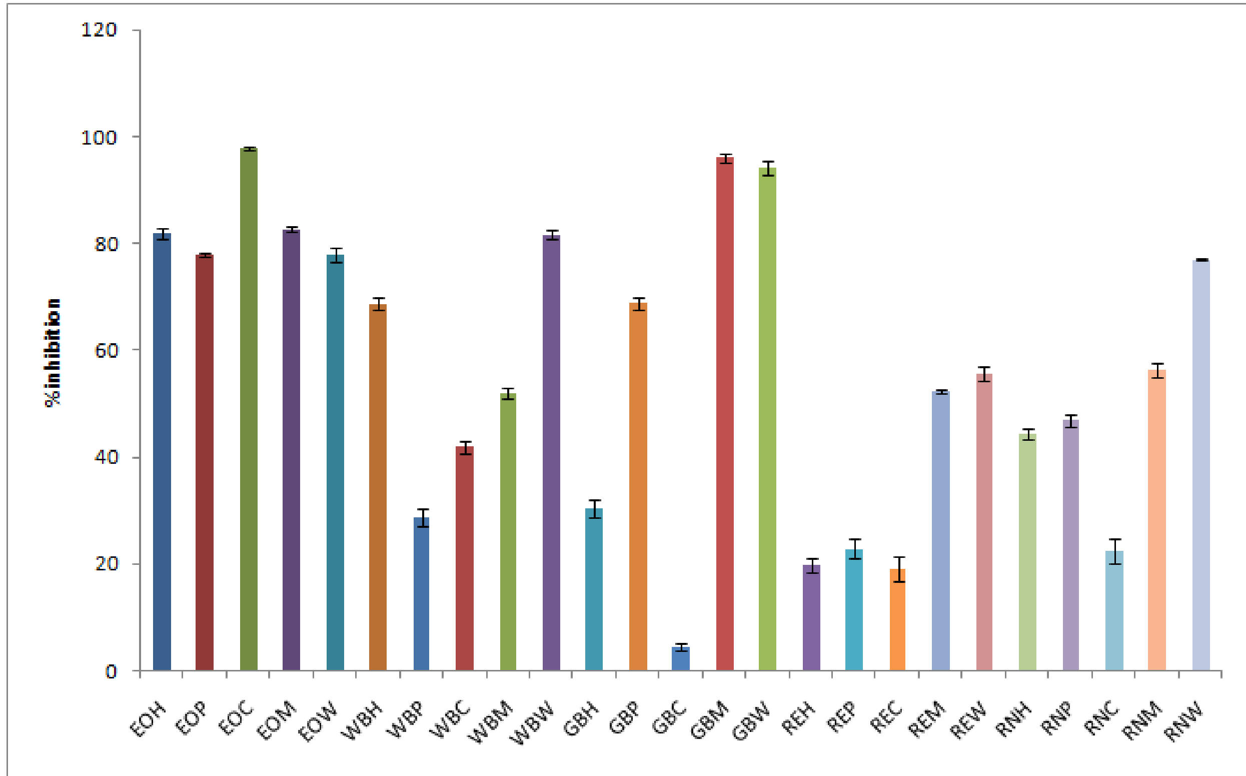

Figure 4 Percent inhibition of ABTS radical by extracts

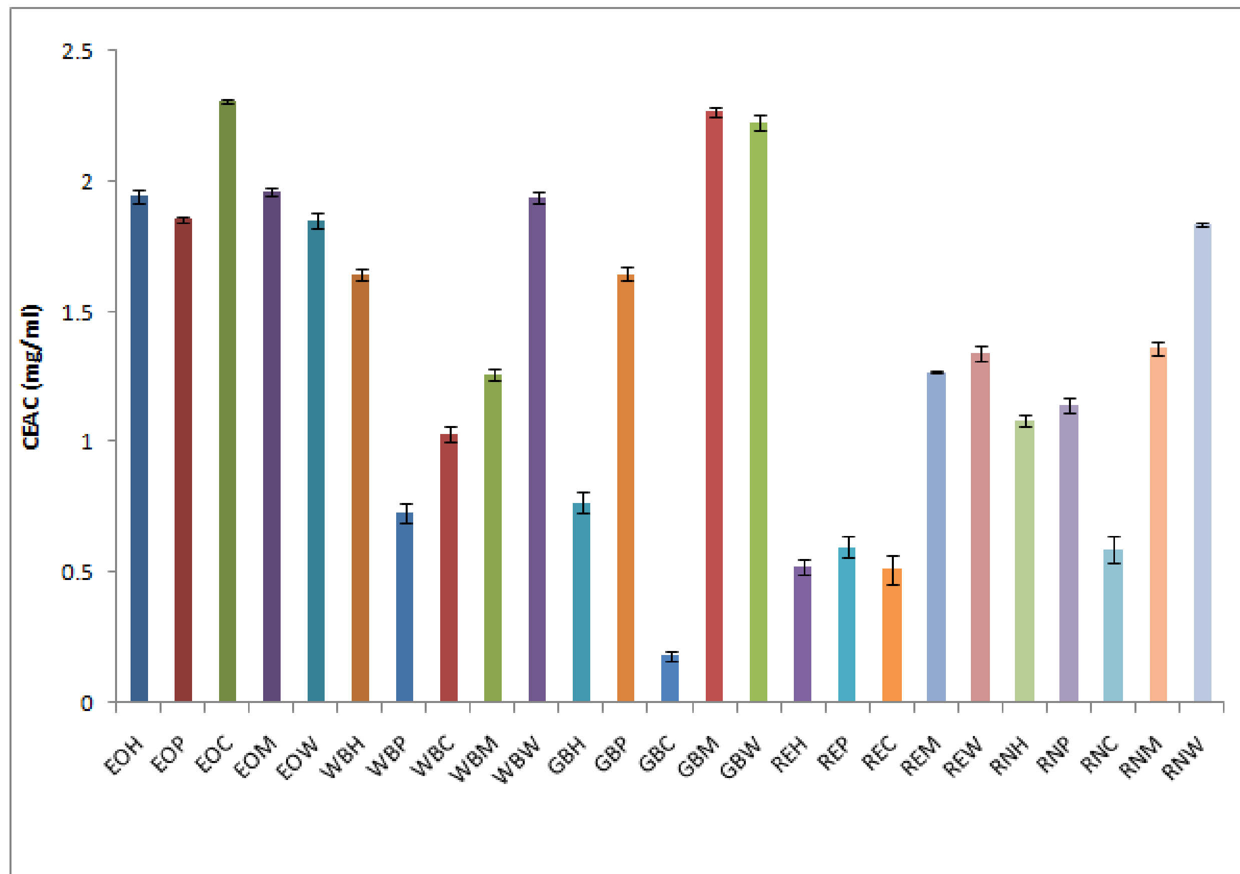

Figure 5 CEAC (mg/ml) calculated for all extracts

Extraction of phenolics

The phenolics were extracted out from each extract using the method reported in the previous section the crude phenolic weight was determined. The crude phenolics were further fractionated using column chromatography and of all the **X** fractions only the fractions **II** and **III** containing 10 and 20% methanol in chloroform were found to contain the phenolics in them. The percent yield of TFC of these fractions was determined with respect to the dry weight of the fraction obtained (Figure 6).

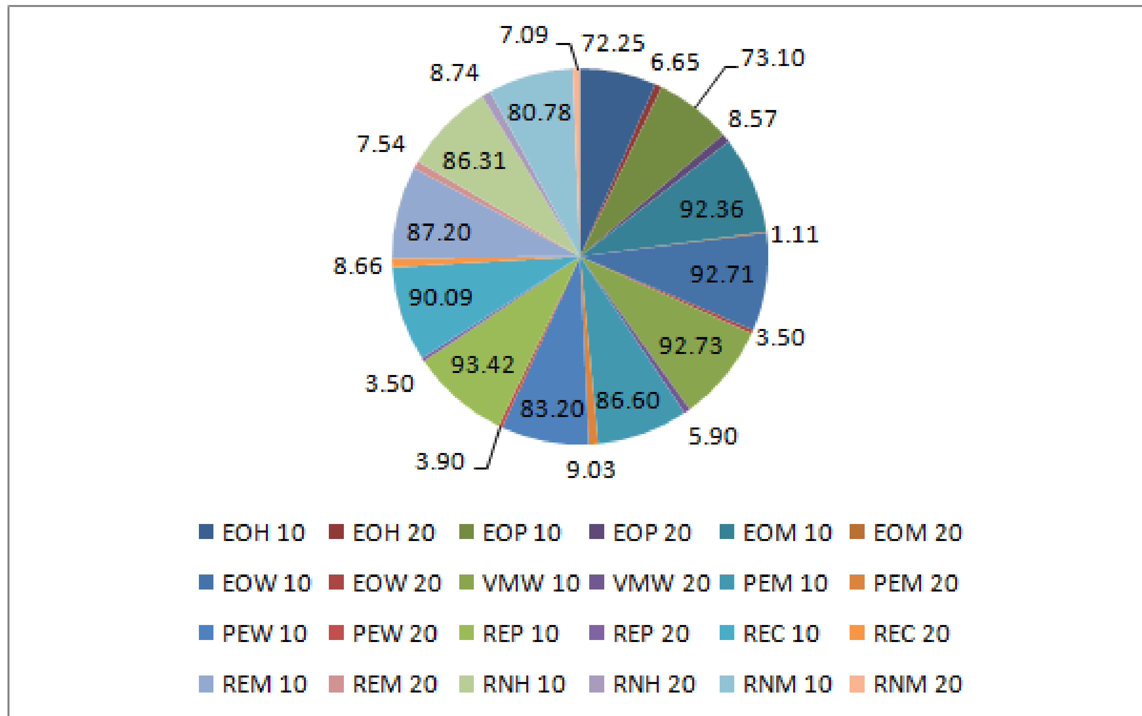

Figure 6 Percent yield of phenolic fractions of the extracts

The results of fraction revealed highest yield of phenolic content in REP 10 (93.42% phenolic content) suggesting highly non-polar constitutents in the fraction and the lowest in the fraction EOM 20 (1.11%).

Conclusion

The obtained results revealed that all the tested species of wild Himalayan berries exhibited the ability to scavenge ABTS radical. Furthermore the antioxidant potential of the methanolic and aqueous extracts of the berries was the most significant. It could be concluded from the study that the wild Himalayan berries could be a potential source of rich antioxidant molecules and may be helpful in obtaining new lead molecules for treating several conditions.

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