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Original Research

Bioactivity evaluation and HPLC UV-VIS based quantification of antioxidant secondary metabolites from extract and fractions of *Bistorta amplexicaulis* rhizome

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Abstract: *Bistorta amplexicaulis* is a popular medicinal plant and reported as rich source of antioxidant compounds. The present study was designed for antioxidant and anticancer potential of polarity based fractions of *B. amplexicaulis* and its correlation to the secondary metabolites quantified by HPLC-UV/VIS.Crude extract was prepared by maceration method and polarity based fractions were prepared by solvent-solvent extraction. Antioxidant and anticancer potential was investigated by using various physiological and non-physiological assays while secondary metabolites rutin, naringin and quercetin present in extract and fractions were quantified by using HPLC- UV/VIS. All extracts showed Antioxidant potential but highest activity was obtained with ethyl acetate fraction (DPPH IC_{50} 5.76±0.03 µg/ml, ABTS IC_{50} 0.74±0.1 µg/ml, Total Antioxidant Assay 72.55±0.098 Ascorbic acid equivalents, Super oxide radical scavenging assay IC_{50}6.86±0.1909 µg/ml, Hydroxyl radical scavenging assay IC_{50} 0.96±0.1690 µg/ml). The cytotoxicity of fractions against HepG2 cell lines showed lowest ell viability in n-hexane fraction (11%). The results revealed that ethyl acetate fraction of *B. amplexicaulis* can be a potential source of novel antioxidant compounds while n hexane fraction could provide anticancer compounds. A new method of simultaneous quantification of three flavonoids by using UV/VIS detector is reported in this study.

Key words: Antioxidant; Anticancer; *B. amplexicaulis*; HPLC- UV/VIS; Flavonoids.

Introduction

It is clearly understood that reactive oxygen species (ROS), play an important part in the progress of oxidative stress that can prompt variousailments including cardiovascular disease, diabetes, anemia, degenerative diseases, ischemia, malignancy and inflammation(1,2). The condition of High ROS production becomes lethal when antioxidant system of the cells is compromised (3, 4). In aerobic organisms an antioxidative system has been evolved to protect the cell against oxidative damage and cancer, this antioxidant system include antioxidant enzymes and plant secondary metabolites (3,5). In plants, secondary metabolites, which are biosynthesized from primary metabolites are found to have antioxidantand anticancer activities when tested invitro and in vivo (6, 7, 8, 9). The antioxidant potential of secondary metabolites is due to their redox properties(10, 11) which play a significant part in quenching singlet and triplet oxygen, scavenging of free radicals, and decomposition of peroxides. Polygonaceae family have many plantsthat have been traditional medicinal useas antioxidants (12, 13).

Genus Bistorta belongs to family Polygonaceae consists of 50 species (14). In Pakistan, genus Polygonum is distributed in temperate zone with nearly 7 species (Flora of Pakistan). The *B. amplexicaulis* (*Persicaria amplexicaulis*) commonly known as Atrosanguineum/Mountain fleece is widely distributed in North Pakistan, Azad Kashmir, and Galliat. The flowering season of this plant is from June to September. Conventionally, *B. amplexicaulis* rhizome tea is utilized to treat fever, flue, gastrointestinal disorders and joint pain. (15, 16, 17, 18). *B. amplexicaulis* have been used traditionally for the treatment of internal bleeding and hepatic ailments. There is no much work reported on its antioxidant activities, and the work presented in this paper covers the detailed free radical scavenging and anticancer evaluation of the crude extract and the polarity based fractions quantification of different phytochemicals from *B. amplexicaulis*.

Materials and Methods

Extraction and fractionation

Plants of *B. amplexicaulis* were collected in July 2015 from Miran Jani Tract, NathiaGali, District Abbottabad, and Pakistan. The herbarium Specimens were authenticated by examining the morphological and anatomical features in the Department of Botany, PMAS Arid Agriculture University Rawalpindi. The extraction was done by maceration method (18) and obtained material was referred to as crude methanolic extract that was placed at -4°C for storage.

Solvent-solvent extraction of crude extract

The methanolic extract 936g was suspended in 3 liters water and extracted with n-hexane in 1:1 ratio in a separating funnel; n-hexane layer was separated and filtered through filter paper to obtain the particle free extract. The residue aqueous layer was extracted twice with n hexane and n hexane soluble layer was separated, filtered and concentrated under vacuum to obtain n-hexane fraction (2g). The same procedure was followed for the other solvents, such as chloroform, ethyl acetate, and butanol to give chloroform (4g), ethyl acetate (200g) and butanol (400g) extracts, respectively.

Total phenolic contents and total flavonoid contents

Total phenolic contents (TPC) of *B. amplexicaulis* were evaluated by utilizing Folin–Ciocalteu reagent(19). Briefly 0.2ml of each fractions (1.0 mg/ml) was taken in Pyrex test tube and 1.5 ml of Folin–Ciocalteu reagent was added into it and incubated at room temperature for 5 mints. In the next step this solution was mixed with 1.5 ml of 6% Na_2CO_3 solution and left for 90 min at room temperature. Absorbance was measured at 725 nm.TPC were calculated as micro grams Gallic acid (GAE) equivalents present per mg of fraction.

Total flavonoid contents were estimated by a calorimetric assay by employing the method of(20, 21). 1.5ml of methanol was separately mixed with 0.5 ml of crude methanolic extract and its fractions (1mg/ml), and then 100 µl aluminum chloride (10%), 100 µl of 1M potassium acetate and 2.8ml of distilled water was added to premixed solution. The resultant solution was incubated for half hour. The absorbance was taken at 415nm TFCwere determined as mg Quercetin equivalents per gram of dried fraction (mg/g).

Ascorbic acid and vitamin E equivalents

Ascorbic acid equivalents were evaluated by following the modified method described by (22, 23). The solution of extract and fractions (200µl) was taken into Pyrex tube and mixed with 500µl solution of 0.2M phosphate buffer (pH 6.6) and 500µl solution of 1% ($K_3Fe(CN)_6$) potassium ferricyanide. The mixture was set at 50 centigrade for twenty minutes , a 500 µl solution of 10 percent trichloro acetic acid (TCA) was introduced to the mixture,1500 µl of this mixture was mixed with 1500 µl of deionized water along with a 100 µl solution of FeCl3 (0.1%), Absorbance of solution was taken at 700 nm through a spectrophotometer.

Vitamin E equivalents were determined by employing the method given by (24, 25). 0.1ml solution of sample was taken into Pyrex tubes along with 1.0 ml of the working solution; made up of phosphate buffer, 600mM sulfuric acid, 28 mM Na2MoO4 and 4 mM ammonium molybdate. The mixture was incubated for 90 min in a water bath at 95°C. The absorbance was taken by spectrophotometer at 765 nm.

Free radical scavenging activities

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was used to access the free radical scavenging activity by using the method of (21). Initially, 60 µM stock solution of DPPH was prepared through mixing 2.4 mg DPPHin 0.1L of methanol for the generation of free radicals. Then methanol was added to the diluted stock solution to attain an absorbance less than 1 at 517 nm. 2000 µl DPPH solution was added to 200 µl of each fraction at varying concentrations (1.25–12.5 µg/ml) and vortexed. Solution was left in dark for 30 minutes and after that absorbance was measured at 517 nm. The assay was performed in triplicates. The anti-radical potential of crude extract and fractions was evaluated by means of the following formula:

$$\text{Scavenging (\%)} = \frac{\text{absorbance of control} - \text{absorbanceof sample}}{\text{absorbance of control}} \times 100$$

IC_{50} values showed the 50% scavenging of DPPH radicals. Ascorbic acid was utilized as standard.

$ABTS^+$ radical scavenging activity

ABTS radical scavenging potential wasestimatedby standard procedure slight modifications (23). Master solution was made by mixing 7×10^{-3} M ABTS and 2.4×10^{-3}M potassium persulphate. It is then placed for overnight at room temperature for the generation of ABTS radical. After 16 hours ABTS stock solution was then diluted with 60% of methanol. Dilution should be done in a way that final absorbance of solution should be less than 1 at 734 on spectrophotometer. Every time it is mandatory to prepare fresh ABTS solution. 10µl of each fraction at varying concentrations (1.25–12.5 µg/ml) was added to 2000µl of the ABTS solution. Care should be taken to avoid exposure of ABTS solution to light because it can be decolorized when exposed to light. The absorbance was measured at 734 nm. This decrease is time dependent so absorbance was taken at 1 min and6 mints. Percent scavenging was calculatedthrough formula given below:

$$\text{Percentage Scavending (\%)} = \frac{\text{absorbance of control} - \text{absorbanceof sample}}{\text{absorbance of control}} \times 100$$

H_2O_2 scavenging activity

The hydrogen peroxide scavenging potential by various fractions was evaluatedthrough the method of (24, 25). 4mM H_2O_2 solution was made in phosphate buffer-saline at the pH 7.4 at room temperature. Concentration of hydrogen peroxide was found spectrophotometrically at 230nm absorption by means of molar extinction coefficient of $81m^{-1}cm^{-1}$ for H_2O_2. Extract and fractions were dissolved in DMSO and added to the H_2O_2 solution at final concentration of 0.5-500µg/ml at room temperature. After ten minutes incubation H_2O_2 absorbance was determined by spectrophotometer at 230 nm. Separate blank was prepared for each concentration containing extract and fractions at 0.5-500µg/ml in PBS without adding H_2O_2. The percentage scavenging was determinedthrough the formula given below:

$$\text{Percentage scavenging (\%)} = \frac{\text{absorbance of control} - \text{absorbanceof sample}}{\text{absorbance of control}} \times 100$$

Scavenging of hydroxyl radical scavenging activity

Scavenging ability of Hydroxyl radical by various fractions evaluatedthroughthe methodof (26). The reaction solution was made up of 100 µl solution of 10 mM ferric chloride, 250 µl solution of 10 mM 2-deoxyribose prepared in phosphate buffer saline; and 350µl of 1mM EDTA solution. 200µl of 10 mM H_2O_2 was added and reaction was started by addition of 50 µl of 1 mM ascorbate and left for 1 hr at room temperature. 0.5ml solution of 0.5% TBA and 0.5ml of 10% Trichloro acetic

acid was introduced in the solution and placed in boiling water for 15 min. Absorbance was recorded at 532 nm. Percentage scavenging of hydroxyl radical was determined as:

$$\text{Percentage scavenging (\%)} = \frac{\text{absorbance of control} - \text{absorbanceof sample}}{\text{absorbance of control}} \times 100$$

Superoxide radicals scavenging activity

The superoxide radical scavenging ability of crude extract and fractions of B. amplexicaulis was determined through riboflavin-light-NBT system(27). The reaction solution was made up of 1000µl of PBSat the pH of 7.6, 500 micro liter of 60 µM riboflavin, 0.25ml of 20×10^{-3} M phenazinemetho sulfate and 0.1ml of 0.5 $\times10^{-3}$M Nitro Blue Tetrazolium before mixing of 1000µl of all fractions. This reaction was triggered by enlightening the above solution utilizing a fluorescent light. After 20 min of lighting the absorbance was measuredon 560 nm. The percentage of superoxide anion scavenging was determined as:

$$\text{Percentage scavenging (\%)} = \frac{\text{absorbance of control} - \text{absorbanceof sample}}{\text{absorbance of control}} \times 100$$

***In vitro* lipid peroxidation assay**

The *in vitro* Lipid peroxidation assay was utilized to determine the antioxidant activity of *B. amplexicaulis* extracts. Formation of malondialdehyde (MDA) by using the thiobarbituric acid-reactive species (TBARs) was described by standard method (32). To determine the protective effect of the extracts on Hela cells, cells were first incubated with extracts at various concentrations for 48 hours. After the completion of incubation $FeSO_4$ was added and incubated for 1 hour, lysing buffer and SDS added and mixture was boiled with TBA solution. Absorbance was taken at 532 nm.

Anticancer activity

MTT Assay

HepG2 cells (100 µL/each well, 20000 cells/mL) were seeded in a 96-well culture plate and allowed to grow for 24 h in medium (RPMI 1640 medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin sulfate. The cells were treated with varying concentrations of fractions and incubated in a 5 % CO2 incubator for 72 hour using DMSO as negative control. At the end of incubation, 10 µl of a MTT stock solution (5 mg/ml) was added into each well. The plate was incubated at 37 °C for 4 hour, medium was removed and 100 µl of DMSO was added into each well, followed by thorough shaking. The absorbance of the formazan product was measured at 570 nm on Thermo Scientific Varioskan Flash Multimode Reader (28)

HPLC analysis of phytochemicals

A new method was designed for simultaneous quantification of three flavonoids by using UV/VIS detector. The chemicals used for the method development included the HPLC gradient water, from Sigma-Aldrich (99.8% pure), acetonitrile (98.9% pure), Quercitin , linear formula (LF) = C15H10O7, molecular weight (MW) = 302.2357 g /mol, 97% pure), rutin, linear formula (LF) = C27H30O16, molecular weight (MW) = 610.52 g /mol, 97% pure, naringin, linear formula (LF) = C27H32O14, molecular weight (MW) = 580.53 g /mol, 97% pure), that was purchased from sigma-Aldrich. All the glassware (Pyrex) was thoroughly washed with 5% HNO3, including subsequent washing with distilled water, and oven drying. The standards were prepared in the HPLC-grade acetonitrile (mmol/l). Serial dilutions of standards were carried out using the stock of 1mg/ml in strength for each standard. The stock standard was diluted up to ng/ml. each dilution was made fresh each day before initializing the calibration protocol. The standards were placed in a refrigerator to avoid sol-vent evaporation and contamination.

Standard solutions

Quercetin, rutin and naringinStock solutions (1000 µg/ml) were prepared in absolute methanol: suitable dilutions of the stock solutions were made to obtain working solutions from 0.05 µg/ml to 100 µg/ml of rutin, naringin and quercetin for the determination of LOD and LOQ for each standard

Analytical procedures

All the samples were filtered by mean of 0.02µl filters. A 20 µl injection of sample was used for manual injection into the HPLC Shimadzu equipped with the SPD-20 A UV-visible detector, the LC-20AT pump and the DGU-20A5 degasser. The analysis was done at C-18 column.

HPLC optimization for the quercetin, rutin and naringin analysis

The HPLC conditions were optimized for the three standards. The optimization was carried out by using a sequence of standard dilutions for many times, and the conditions were watched for three consecutive days to authenticate optimization. The optimum conditions for the all three standards were as follows: 20 µl injection, 167 bars pressure, a flow rate of 1 ml/minute, with a run time of 20 min. The mobile phase consisted of acetonitrile and the 0.05M buffer prepared in HPLC gradient Water. The column temperature was set at 30°C. The mobile phase was acetonitrile (A) and buffer (B).0.2 µmpore size hydrophilic polypropylene filter was used to filter all solvents. Filters were degassed in an ultrasonic bath before use. Separation was achieved by using a gradient program as follow: 0–6 min 30% A; 6–16 min 70% a linear; 16–20 min 70%. The wavelength was set according to; 0-4.5min 257nm, 4.6-6 min 280nm, 6.01-20min 368nm. Rutin peak was detected at retention time of 4.0min, naringin and Quercetin peaks were detected at 5.1 and 12.2min respectively.

Results

Extraction and fractionation

Total 936g of crude methanolic extract was subjected to solvent-solvent extraction. To obtain n hexane fraction (2g), chloroform fraction (3g), Ethyl acetate fraction (200g) andbutanolic fraction (400g).

Total phenolic contents, total flavonoids contents, ascorbic acid equivalents and vitamin E equivalents

Total phenolics (TPC), and total flavonoids (TFC) contents , ascorbic acid equivalents (AAE) and vita-

Table 1. Total phenolic contents (TPC) and total flavonoid contents (TFC) of *B. amplexicaulis* fractions.

Fraction	TPC	TFC	AA Eq.	Vit. E Eq.
	µg GAE/mg	µg QE/mg	µg AAE/mg	µg ViEE /mg
BANH	35.66±2.24^{c}	46.65±1.35^{a}	63±2.003^{c}	22.45±0.0^{d}
BABU	183±1.22^{c}	31.5±2.2^{b}	253.39±1.35^{c}	102.44±1.3^{b}
BACH	588.66±2.35^{b}	46.4±1.11^{a}	278.02±2.1^{b}	55.47±0.04^{c}
BAME	650.33±0.12^{b}	34.15±2.12^{b}	279.9±1.11^{b}	116.85±0.45^{b}
BAEA	808.66±2.12^{a}	32.1±2.98^{b}	350.98±2.76 ^{a}	149.22±0.06^{a}

Each value represents mean ±standard deviation of the same experiment performed in triplicates, LSD was performed with P value <0.05, BANH (*B. amplexicaulis* n-hexane fraction); BAEA (*B. amplexicaulis* ethyl acetate fraction); BAME (*B. amplexicaulis* methanol fraction); BABU (*B. amplexicaulis* butanol fraction); BACH (*B. amplexicaulis* chloroform fraction).

Table 2. Free radical scavenging activities of *B. amplexicaulis* fractions.

Fraction	Antioxidant Scavenging Assays IC_{50}(µg/ml) values					
	DPPH Assay	ABTS assay	H_2O_2 radical scavenging	Superoxide radical scavenging	Hydroxyl radical scavenging	Mean Values
BANH	131.71±2.2	27.42±0.9	24.18±0.23	11.96±2.60	78.08±3.35	54.67±2.31^{d}
BABU	7.09±0.02	1.52±0.12	12.845±0.72	79.23±2.13	16.80±2.04	23.49±1.45^{b}
BACH	19.735±0.31	2.49±0.16	39.13±0.35	77.27±1.80	288.95±2.35	85.515±5.66^{e}
BAME	5.311±0.02	0.74±0.08	39.7±0.35	68.99±5.11	80.23±2.22	38.994±1.28^{c}
BAEA	5.76±0.03	0.748±0.1	25.84±0.28	1.37±0.19	0.96±0.16	6.936±0.02^{a}
Standard	3.728±0.040	1.429±0.1	15.62±1.1313	26.14±2.22	0.66±0.12	9.5162±1.11^{a}
Assay mean	28.88±0.261^{b}	5.72±2.11^{a}	26.21±1.39^{b}	44.16±3.06^{c}	77.613±2.43^{d}	

BANH (*B. amplexicaulis* n-hexane fraction); BAEA (*B. amplexicaulis* ethyl acetate fraction); BAME (*B. amplexicaulis)* methanol fraction); BABU (*B. amplexicaulis* butanol fraction); BACH (*B. amplexicaulis* chloroform fraction); Each value represents mean ±standard deviation of same experiment performed in triplicate, LSD was performed with P value <0.05.

min E equivalents (ViEE)were significantly different (P<0.05) among various fractions of *B. amplexicaulis* (Table 1) TPC was used to detect the number of phenols and reducing potential of fractions. Phenols of different polarities were extracted by using different solvents ranged from 35.66 µg GAE/mg plant extract to 808.66 µg GAE/mg plant extract.

Total flavonoids content was used to quantify flavonoids in various fractions spectrophotometrically. . Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various other free radicals. Total flavonoids contents in different fractions ranged from 32 µg QE/mg plant extract to 46.65 32 µg QE/mg plant extract. Ascorbic acid equivalents and vitamin E equivalents are used to represent mg of ascorbic acid equivalents and vitamin E equivalents present per gram of plant extract respectively. It is used to represent antioxidant capacity of plant extracts. AAE in different fractions ranged from 60-350 µg AAE/mg plant extract while vitamin E equivalents were 22.45-149.2 µg ViEE/mg plant extract. Ethyl acetate fraction contain highest of TAA and ViEE.

Free radical scavenging assays

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was used to measure the antiradical potential of plant fractions. All fractions of *B. amplexicaulis* showed the concentration dependent decrease in absorbance related to increase in radical-scavenging activity (Table 2). These results showed that ethyl acetate extract showed the highest electron/hydrogen donating capacity. IC_{50} values ranged from 131.71±2.2 µg/ml for n hexane, 5.7675±0.03 µg/ml for ethyle acetate fraction, 19.735±0.31 µg/ml for chloroform fraction, 7.09±0.02 µg/ml for butanol fraction, and 5.311±0.002 µg/ml for methanolic fraction Ascorbic acid, Quercetin was tested as references. The IC50 values were 3.728±0.040 µg/ml for ascorbic acid and Quercetin showed 50% inhibition at 3.17±0.565 µg/ml. (Table 2).

ABTS free radical scavenging assay

The ABTS method is used to monitor the decay of the ABTS• radical-cationbased on absorbance.

The ABTS scavenging effect of *B. amplexicaulis* extract and fractions varied considerably (Table 2). Extract scavenge ABTS+ radicals in a concentration dependent manner. Highest scavenging activity was shown by ethyl acetate fraction with the IC_{50} value of 0.748±0.1 l and 0.64±0.01 µg/ml at 1 and 6 minutes respectively as compared with the IC_{50} value of ascorbic acid 1.429±0.1 µg/ml and 1.265±0.1µg/ml at 1 and 6 minutes respectively. The order of reactivity for all other fractions was methanolic>butanolic>chloform>n hexane. IC_{50} of other fractions are shown in the Table 2

Scavenging of hydrogen peroxide

The *B. amplexicaulis* extract and fractions showed H_2O_2 scavenging effect in a dose dependent manner (Table 2). The n-butanolic fraction has showed highest effect with IC_{50} value of 12.845±0.7283 µg/ml compared to control ascorbic acid 15.62±1.13µg/ml. The reactivity of other fractions in an order of n-hexane > Ethyl acetate >Chloroform >Methanolic fractions (Table 2).

Superoxide radical scavenging

The *B. amplexicaulis* SOD activities works in a dose-dependent manner and generation of superoxide radicals by NBT/riboflavin system, the superoxide radi-

cal is significantly decreased in higher doses as shown in (Table 2).

Among all fractions ethyl acetate fraction showed the highest scavenging potential with IC_{50} value of 6.86±0.1909 µg/ml as while the ascorbic acid's IC_{50} value of 130.72±2.2203 µg/ml. The order of scavenging of superoxide radical in other fractions was n hexane > methanol > chloroform > n-butanolic fractions.

Hydroxyl radical scavenging activity

Scavenging ability of the hydroxyl radical of crude extract and fractions of *B. amplexicaulis* was compared with the gallic acid standard as shown in Table 2. The lowest IC_{50} value is shown by ethyl acetate fraction 0.96±0.1690 µg/ml compared to gallic acid 0.66±0.1202. The order of reactivity of other fractions was butanolic fraction> n hexane fraction >methanolic fraction >chloroform fraction.

***In vitro* lipid peroxidation assay**

Lipid peroxidation assay on Hela cell lines showed concentration dependent inhibition of Malondialdehyde (MDA) formation. Table 3 showed the relative MDA formed in µM. Ethyl acetate fraction showed the highest activity as it inhibit the formation of MDA to the highest extent as shown in Table 3.

Anticancer activity (MTT assay)

Liver is the major place of metabolism for drugs and other xenobiotic. The Human HepG2 is a hepatic origin cancer cell which is widely used as model for biochemical and nutritional studies.

Preliminary screening of *B. amplexicaulis* extract and all of four fractions (n-hexane fraction, chloroform fraction, ethyl acetate fraction and butanolic fraction) for cytotoxicity against HepG 2 cancer cell lineat 200 µM concentration of each fraction. The order of cytotoxicity (% inhibition) of various fractions is given as; hexane fraction (89 %) > methanol fraction (78 %) > ethyl acetate fraction (73 %) > chloroform fraction (63 %) >butanol fraction (58 %) > ethanol fraction (54 %) (Fig. 1).

HPLC analysis of phytochemicals

The identification and quantification of quercitin, rutin and naringin (i.e., the therapeutic flavonoids) from

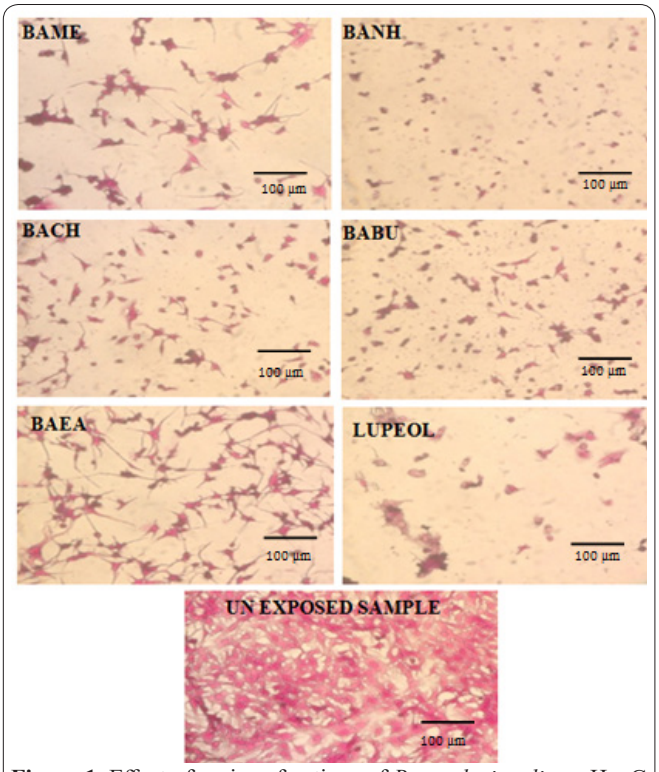

Figure 1. Effect of various fractions of *B. amplexicaulis* on HepG 2 cancer cells at 200 µM concentration. BAME (*B. amplexicaulis* methanol fraction), BANH (*B. amplexicaulis* n-hexane fraction); BAEA (*B. amplexicaulis* ethyl acetate fraction); BABU (*B. amplexicaulis*butanol fraction); BACH (*B. amplexicaulis* chloroform fraction).

Table 3. Lipid peroxidation assay on extracts and fractions of *Bistorta amplexicaulis*.

No	Sample (µM)	Antioxidant activity (Relative MDA in µM)
Unexposed sample		1.04±0.21
Ethanol		1.31±1.23
$FeSO_4$		3.75±1.1
$FeSO_4$+ Ethanol		4.98±0.2
BANH	100	4.39±0.5
	200	3.21±0.45
	300	3.02±1.02
BAEA	100	3.33±1.0
	200	2.98±0.021
	300	1.04±0.1
BACH	100	4.656±0.21
	200	4.44±0.6
	300	3.13±0.45
BAME	100	4.88±80.8
	200	4.34±1.2
	300	3.43±1.01
BABU	100	3.76±1.21
	200	3.02±0.23
	300	2.11±1.1

BANH (*B. amplexicaulis* n-hexane fraction); BAEA (*B. amplexicaulis* ethyl acetate fraction); BAME (*B. amplexicaulis)* methanol fraction); BABU (*B. amplexicaulis* butanol fraction); BACH (*B. amplexicaulis* chloroform fraction); Each value represents mean ±standard deviation of same experiment performed in triplicate, LSD was performed with P value <0.05.

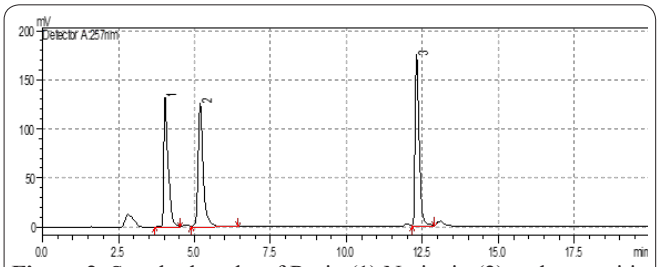

Figure 2. Standard peaks of Rutin (1) Naringin (2) and querecitin (3).

the plant extract in shown in (Figure 2). In order to develop a new method based on the UV-visible detector system to quantify the amount of flavonoids from the plant extract, we used concentrations of all three standards up to the nm/ml level, with an average elusion time less than 20 min.

Quantification of three flavonoids was done in the various fractions and results are shown in Table 4. A correlation among free radical scavenging assays and antioxidant phytochemicals was established and results are shown in the table 5.

Correlation among free radical scavenging assays and antioxidant phytochemical were calculated by using Pearson's correlation coefficient. DPPH and ABTS radical scavenging assay showed high correlation to TPC, TFC, AA Eq and vitamin E eq. Hydrogen peroxide radical scavenging assay showed high correlation with TPC, AA Eq, Rutin, quercetin and Naringin. Superoxide radical scavenging assay showed high correlation with quercetin and naringin. The OH scavenging assay showed high correlation with TFC, quercetin and naringin. So these compounds are the major contributor to antioxidant activity.

Methanolic crude extract and its polarity based four factions were used in the calculation of correlations

Discussion

The oxidative stress is the fundamental cause of many diseases including diabetes, hypertension, ulcer and cancer(1, 2). Medicinal plants could be used to tackle the oxidative stress and countering many other follow up diseases and disorders. Rhizome of *Bistorta amplexicaulis* has been traditionally used to treat various ailments (18). We hypothesis that the medicinal properties of this plant may be attributed to the phytochemicals and antioxidant potential of this plant. To identify phytochemicals and antioxidant properties,Polarity based fractions were prepared from the crude extract. The presence of different antioxidant compounds with different chemical nature and polarities could possibly be insolublein a specific solvent (10). Polar solvents are preferably used for extraction of phytochemicals

Table 4. Quantification of three antioxidant phytochemicals by HPLC.

Fraction	Rutin (µg/ml)	Naringin (µg/ml)	Quercetin (µg/ml)
BANH	0.80	N.D	0.99
BAEA	13.87	N.D	6.00
BABU	5.33	N.D	N.D
BAME	192.56	N.D	3.31
BACH	0.53	0.88	0.19
LOD	0.08	0.045	0.024

BANH (*B. amplexicaulis* n-hexane fraction); BAEA (*B. amplexicaulis* ethyl acetate fraction); BAME (*B. amplexicaulis* methanol fraction); BABU (*B. amplexicaulis* butanol fraction); BACH (*B. amplexicaulis* chloroform fraction); LOD (limit of detection).

from plant materials because they can easily disrupt cell wall of plant cells (29). Ethyl acetate fraction contains highest total phenols. Highest total flavonoids were present in chloroform fraction. Antioxidant compoundsofcrude extract and polarity based fractions of *Bistorta amplexicaulis* was extensively studied by using DPPH radical scavenging assay, ABTS radical scavenging assay, super oxide radical scavenging assay, hydroxyl radical scavenging assay and hydrogen peroxide assay, that confirm its ability to scavenge the excessive radicals of all major types. DPPH free radical scavenging assay and ABTS free radical scavenging assay was used as the basic assays because it isconsidered as the most accurate screening method used to evaluate the free radical scavenging potential of the plant (30). DPPH radical is a stable non physiologicalfree radical used to evaluate the anti-radical potential of various fractions. All fractions showed antioxidant potential butethyl acetate fraction provided maximum number of electron to the DPPH radical that is converted to a stable diamagnetic colorless molecule. The decrease in the absorption is stoichiometricaly related to the number of electrons captured (30,31). IC_{50} value of DPPH radical scavenging assay has a strong negative correlation with total phenolics, vitamin E and ascorbic acid equivalents with the R2 value of -0.709, -0.803 and -0.581. Where the negative sign indicates that with an increase in the phenolics, vitamin E equivalents and ascorbic acid equivalents, the corresponding IC_{50} value decrease that indicate the increase in antioxidant activity.

In published literature studies, the florescence detector is preferably used for the quantification of the flavonoids because of its high sensitivity, however, this is undermining the importance of the UV-visible detections for this purpose since the UV-visible detector also gives good results, but not as sensitive as the florescent detector. We have demonstrated that quantification of the quercitin, rutin and naringin using the UV-visible

Table 5. Correlations among free radical scavenging assays and antioxidant phytochemicals.

ASSAYS (IC_{50} Values)	Correlations						
	TPC	TFC	AA Eq.	Vit. E Eq.	Rutin	Quercetin	Naringin
DPPH Assay	-0.709	0.695	-0.581	-0.803	-0.323	-0.157	-0.144
ABTS Assay	-0.725	0.655	-0.604	-0.78	-0.311	-0.205	-0.196
Hydrogen peroxide radical scavenging Assay	0.589	0.363	0.671	-0.056	0.546	0.850	0.534
Superoxide radical scavenging Assay	-0.037	-0.06	-0.121	-0.051	0.282	0.525	0.435
Hydroxyl radical scavenging Assay	0.119	0.73	0.209	-0.539	-0.105	0.938	0.951

detector can also provide good results.

The results of this study suggest that ethyl acetate fraction of *Bistorta amplexicaulis* may serve as a source of natural antioxidants and n hexane fraction of *Bistorta amplexicaulis* contains natural anticancer compounds. This is the first report on the new method of simultaneous quantification of three flavonoids by using UV/VIS detector. This study provides an introduction to more comprehensive work on bioactive compounds present in *Bistorta amplexicaulis*.

Conflict of interest statement

We declare that we have no conflict of interest.

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