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Phytochemical, Cytotoxic and Immunomodulatory Analysis of an Indian Blackberry *Rubus fruticosus*

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Abstract: The present study was carried out for cytotoxic potential, immunomodulatory response, antioxidant potential and phytochemical analysis of *Rubus fruticosus* (leaf and fruits). *In vitro* cytotoxicity of methanolic extract of fruit possess higher activity than the leaf part against lung (A549) cancer cell line with 72 % growth inhibition at 100 µg/mL concentration. Similarly, when administered orally at 100 mg/kg p.o., fruit (crude extract) showed an increase of 130 % activity in delayed type hypersensitivity (DTH) response and 79 % activity in humoral antibody response in immune suppressed Balb/c mice. On fractionation of the methanolic fruit extract, the acetone fraction of fruit showed remarkable antioxidant potential. Phytochemical analysis of acetone fraction showed the presence of higher content of phenols (290.81 mg GAEs/g dry weight) and flavonoids (50 ± 0.12 mg QEs/g dry wt). Further, HPLC analysis confirmed the presence of cyanidin and ellagic acid in acetone fraction. The above results showed that the fruits of *Rubus fruticosus* as a very good source of herbal preparation with its significant antioxidant potential, immunomodulation and cytotoxic effect, that can be further exploited in food and pharmaceutical industry.

Key words: cytotoxic, ellagic acid, free radical scavenging, immunomodulatory, phytochemicals.

Introduction

Reactive Oxygen Species (ROS) are formed in body during cellular metabolism at moderate levels. It plays a significant role in redox signaling [28]. ROS is also essential in apoptosis, gene expression and ion transportation. Excessive accumulation of ROS causes oxidative stress which can lead to several diseases such as inflammatory diseases, cardiovascular diseases, cancer, diabetes, cataract, autism, Alzheimer's disease, aging etc. Antioxidants react with ROS, destroy them and can, thus, decrease the oxidative damage [22].

Many ready-to-eat food products contain ingredients that have unsaturated fatty acids. Under oxidative stress, the fatty acids get oxidized and form new products. These break down products cause change in colour, flavor, off-odour and loss of nutrient content of the food. This can be avoided by adding antioxidants. Both natural and synthetic antioxidants can be used. Antioxidants act as chain breakers by interfering with the free radicals produced in chain reactions. The efficiency of aromatic antioxidants is directly proportional to number of hydroxyl groups present on aromatic ring(s). Synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxyl toluene (BHT) and propyl gallate and natural antioxidants such as ascorbic acid (AA) and α-tocopherol have only one aromatic ring. Natural antioxidants such as flavonoids and anthocyanins have more than one aromatic ring, thus making them more effective [5]. Foods that have natural ingredients are preferred by consumers over synthetic ones. This has forced the food industry world-wide to meet the demand of the consumers [3]. Berry phenolics are known to possess strong antioxidant activity [31].

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Rubus fruticosus (blackberry) belongs to family Rosaceae. It has good medicinal and nutritional value. Fruit, leaves and stem of the plant have good potential against common pathogens [29]. Leaves are used in diarrhoea, cough and fever. They are also noted for their use as diuretic and carminative [17]. Decoction of root bark is used in treating diarrhea [18]. Cyanidin-3-glucoside, a natural product present in blackberries, possesses chemo-preventive and chemotherapeutic activities in experimental models [8]. Ripened fruit is consumed to control stomachache and to enhance digestion [32]. Ripened fruit when taken in combination with leaves of *Achyranthes aspera* is used in treating eye diseases [31]. Besides fruits are eaten and used in making jellies and jams [17]. The objective of this study was to explore the cytotoxicity, immuno-modulatory and antioxidant potential of *R. fruticosus*, identify its active fractions from which potential antioxidant compound could be further isolated that could be used in food and pharmacy.

Materials and methods

Collection of plant material

Plant material (fruits and leaves of *Rubus fruticosus*), uniform in shape and without disease and mechanical injury was hand-picked in the month of August, 2012 from the hilly regions (at an approx altitude of 5000 m above sea level) of Bhadarwah, Jammu and Kashmir, India. It was identified by an expert taxonomist in the Department of Botany, University of Jammu and a sample was kept in the herbarium of the department.

Preparation of extracts

The plant material (fruit and leaves of *Rubus fruticosus*) was dried and then grounded into fine powder. 50 g of fruit powder and 25 g of leaf powder was extracted with 250 mL and 125 mL of methanolic at 40°C by continuous stirring for 6 h. Supernatant was collected and extraction was repeated thrice from the residue; extracts were combined, filtered and evaporated [13]. The percentage yield of extract (dry weight of the extract obtained/total plant material taken) was 14.04 % in fruit and 6.23 % in leaf.

Fractionation of extract

The methanolic extract of fruit was sequentially fractionated by using different solvents of wide range polarity *viz*. chloroform, ethyl acetate, acetone and methanol (Fig 1). All the fractions

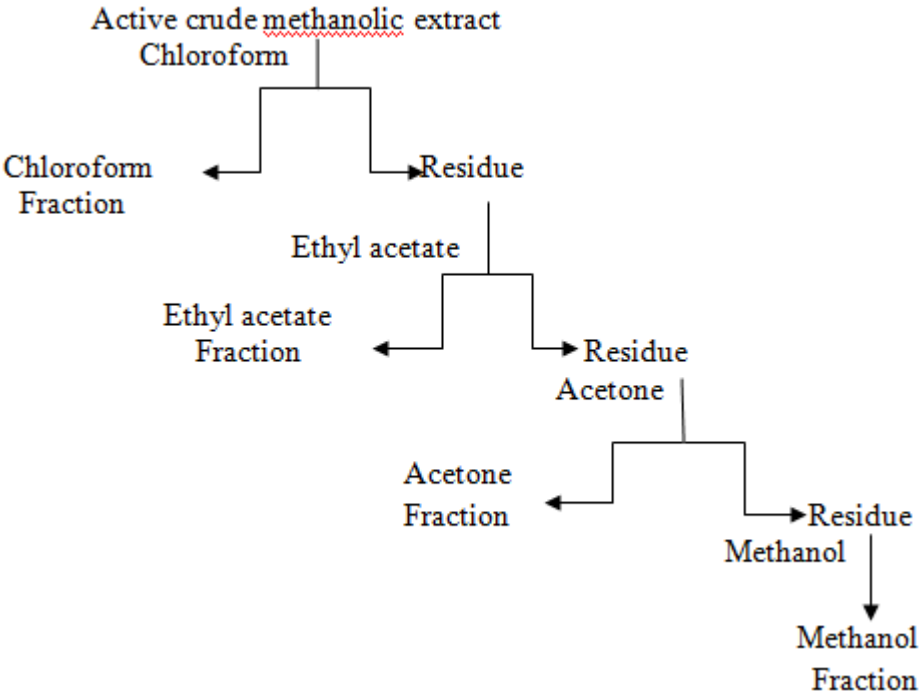

Fig 1. Flowchart of sequential partitioning of active crude methanolic extract of fruits of *R. fruticosus* to obtain fractions

obtained were dried by evaporation and then dissolved in dimethyl sulfoxide (10 mg/mL). The stock thus obtained was diluted in methanol to obtain various final working concentrations.

In vitro Anti-proliferative activity

Crude methanolic extracts of fruit and leaf were evaluated for anti-proliferative activity against four human cancer cell lines *i.e.,* lung (A549), breast (MCF-7), colon (COLO-205) and pancreatic (MIAPACA) by SRB assay in 96 well microplates [34]. In each well, cell suspension (100 µL) containing 10^5 to 2 x10^5 cells/ml and 100 µL of test sample were added. This was incubated in 5 % CO_2 at 37 °C for 72 h. In brief, after 48 h incubation of cell lines, the medium was discarded and cells were fixed with 100 µL ice-cold 40 % trichloroacetic acid (TCA, Aldrich Chemical). The cells were then incubated at 4°C for 1 h and plates were washed five times with cold water. Excess water was drained off and the plates were left to dry, 50 µL of SRB stain (10 mg w/v in 1 % acetic acid) was added in all the wells for 30 min and subsequently washed with 1 % acetic acid to remove unbound stain, 10 mM tris base (pH: 10.5) was used to solubilize the dye. The plates were shaken vigorously, and the absorbance was measured at 540 nm using an ELISA reader (model 450, BIO-RAD, USA). Untreated cells were used as negative control.

Immunomodulatory activity

Delayed Type Hypersensitivity Response (DTH)

SRBC induced DTH response in mice was assessed by the previously described method [10]. Mice were immunized by injecting 20 µL of $5_ß10^9$ SRBC/ml subcutaneously into the right hind foot pad. After seven days, the thickness of the left hind foot was measured with a spheromicrometer (0.01 mm pitch) and was taken as a control. The mice were then challenged by injecting the same amount of SRBC intradermally into the left hind foot pad. The foot thickness was measured again at 0, 4 and 24 h after challenge.

Humoral antibody response (Hab)

Groups of six mice each were immunized by injecting 0.2 mL of $5_ß10^9$ SRBC/mL intra-peritoneally (i.p.) on day 0 and challenged seven days later by injecting an equal volume of SRBC i.p. Blood samples were collected on day +7 (before challenge) for primary antibody titre and on day +14 (7 days after challenge) for secondary antibody titre. Haemagglutination antibody titres were determined following the microtitration technique [23]. The value of the highest serum dilution causing visible haemagglutination was taken as a titre. BSA-saline alone served as a control.

In vitro free radical scavenging activities

DPPH radical scavenging assay

Free radical scavenging activity was assessed according to a previously described method with slight modifications [1]. 1 mL of methanolic solution of the DPPH radical (0.5 mM) was added to different dilutions of the test sample (10, 20, 40, 80, 100 µg/ml). Then 2 mL of 0.1 M sodium acetate buffer (pH 5.5) was added. The mixtures were incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a spectrophotometer. Methanol was used as a negative control whereas BHA, BHT and ascorbic acid were used as standard antioxidants. The radical scavenging activity (RSA) was calculated according to the equation:

$$\% \text{ RSA} = [(A_0 - A_s) / A_0] \times 100$$

where, A_0 is the absorbance of the control and A_s is the absorbance of test compound.

Chelating power assay

The chelating power on ferrous ions was determined by the previously described method with slight modifications [9]. The test sample (100 µg/ml) was raised to 3 mL with methanol. 20 µL of 2 mM $FeCl_2$ was added. Then 40 µL of 5 mM ferrozine was added to the mixture. This mixture was then incubated at room temperature for 10 min before and the absorbance was measured at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the equation: Percentage inhibition = ([absorbance of control – absorbance of test sample]/ absorbance of control) × 100.

Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay was done by the method of Li *et al.*,[20]. The FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2-pyridyl)-s-triazine solution in 40 mM HCl and 20 mM $FeCl_3$ solution in proportion of 10:1:1 (v/v), respectively. Extract/fraction (50 µL) was added to 1.5 mL of the FRAP reagent. The absorbance was measured at 593 nm after 4 min. The standard curve was constructed using iron (II) sulphate (100-2000 µM), and the results were expressed as *i*mol Fe (II)/g dry weight of the plant material.

Reducing power assay

The reducing power of the samples was assessed according to the previously described method with slight modifications [25]. Different dilutions of extracts/fractions (10, 20, 40, 80, 100 µg/ml) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6). 2.5 mL of 1% potassium ferricyanide ($K_3Fe[CN]^6$) was added and the mixture was incubated at 50°C for 20 min. After incubation, trichloroacetic acid was added to the mixture. The mixture was then centrifuged at 1036 × *g* for 10 min. The upper layer of the solution (2.5 mL) was taken and mixed with 2.5 mL of distilled water. To this, 2.5 mL of 0.1% ferric chloride solution was added and the absorbance was noted at 700 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract/fraction concentration.

Phytochemical analysis

Total phenolic content

Total phenolic content was evaluated by Folin-Ciocalteu method [6]. Extracts/fractions solution was mixed with 0.5 mL of 1 N Folin-Ciocalteu reagent. The mixture was kept for 5 min. Then 1 mL of 20% Na_2CO_3 was added. 10 min of incubation at room temperature was given and the absorbance was measured at 750 nm using a spectrophotometer. Phenolic compounds concentration was evaluated according to the equation from the standard gallic acid graph:

Absorbance = 0.037 gallic acid (µg) - 0.047 (R^2 = 0.998)

Total Flavonoids

The flavonoids present in the extracts/fractions were determined by previously described method [18]. Plant extracts/fractions were diluted with distilled water to a volume of 1.25 mL and 75 µL of a 5 % $NaNO_2$ solution. After 5min, 150 µL of 10 % $AlCl_3 \cdot H_2O$ solution were added. After 6 min, 500 µL of 1 M NaOH and 275 µL of distilled water was added. The solution was mixed and the absorbance was observed at 510 nm. The concentration of flavonoid compounds were evaluated by the equation derived from the standard quercetin graph:

Absorbance = 0.001 quercetin (μg) + 0.002 (R^2 = 0.998)

HPLC Analysis

HPLC was done on a Shimatzu HPLC equipped with reverse phase column coupled with PDA detector. Sample was injected (2 µL) with varying concentration using gradient of 3.5% phosphoric acid and acetonitrile with a flow rate 0.75 mL/min.

Statistical analysis

For all the experiments, three samples were analysed and all the assays were carried out in triplicate. The results were expressed as mean values with standard deviation (SD).

Results

Fractionation of extract

Percent extraction yield of fractions obtained from fruit extract is shown in Table 1. Maximum yield was obtained in the methanolic fraction (24.26 %).

Antiproliferative activity

Crude methanolic extract of fruit showed potential cytotoxic activity at 100 µg/ml concentration with a growth inhibition of 72 % (A549) < 63 % (PC-3) < 56 % (MCF-7) < 17 % (NCI-H322) whereas in leaf extract it was 67 % (A549) < 65 % (PC-3) < 54 % (MCF-7) < 30 % (NCI-H322) respectively.

Immunomodulatory activity

Delayed type hypersensitivity response

In delayed type hypersensitivity (DTH)

Table 1. Relative proportion of solvent fractions obtained during fractionation of crude methanol extract (fruit) of *Rubus*

Solvent fractions	Fruit fractions (%)
Chloroform	20.7 ± 0.04
Ethyl acetate	1.78 ± 0.10
Acetone	12.03 ± 0.12
Methanol	24.26 ± 0.03

response, crude extracts of fruit and leaf, when administered orally at 100 mg/kg p.o., showed an increase of 130 % and 123 % activity respectively. Levamisole was used as standard which showed an increase of 140 % activity.

Humoral antibody response

Fruit extract showed 79 % activity whereas leaf extract showed 65 % activity in humoral antibody response in immune suppressed Balb/c mice.

DPPH radical scavenging assay

The use of DPPH free radical provides an easy and rapid way to find the antiradical activities of antioxidants [4]. In the present study, DPPH radical scavenging activity of crude extract of fruit was more than the leaf extract as shown in Table 2. The IC_{50} value of crude extracts of fruit and leaf was 30.74 ± 0.03 µg/mL and 56.23 ± 0.06 µg/mL respectively. Among the fractions, acetone fraction exhibited highest DPPH radical scavenging activity with an IC_{50} value of 30.36 ± 0.05 µg/mL followed by methanolic fraction (IC_{50}= 39.96 ± 0.12 µg/mL). Chloroform fraction showed negligible activity. BHA, BHT and Ascorbic acid (positive controls), well known antioxidants, showed IC_{50} value of 17.93 ± 0.12 µg/mL, 21.36 ± 0.04 µg/mL and 48.16 ± 0.03 µg/mL respectively.

Chelation power assay

Ferrous ions, among other transition metal ions, are considered as strong pro oxidants. They help in controlling the lipid peroxidation reactions [36]. In this assay, the metal binding capacity was determined by assessing the ability of antioxidants to compete with ferrozine to complex with ferrous ions in solution [22]. At 100 µg/mL, crude extract of fruit showed higher chelating activity (28.22 %) than the leaf extract (24.54 %). Among the fractions (fruit) **(Figure 2),** only methanolic fraction showed chelating activity (43.74 % at 100 µg/mL).

Ferric reducing antioxidant potential (FRAP) assay

FRAP is an electron transfer type assay based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in presence of TPTZ (2,4,6-tripyridyl-s-triazine)

Table 2. DPPH radical scavenging activity of crude extracts and fractions of *Rubus fruticosus*

Extract/Fractions[A]	IC_{50} (µg/mL)	
	Fruit	Leaf
Crude extract	30.74 ± 0.03	56.23 ± 0.06
Chloroform fraction	na	-
Ethyl acetate fraction	293.19 ± 0.03	-
Acetone fraction	30.36 ± 0.05	-
Methanol fraction	39.96 ± 0.12	-

Values are expressed as mean ± standard deviation of 3 parallel measurements
na = not active

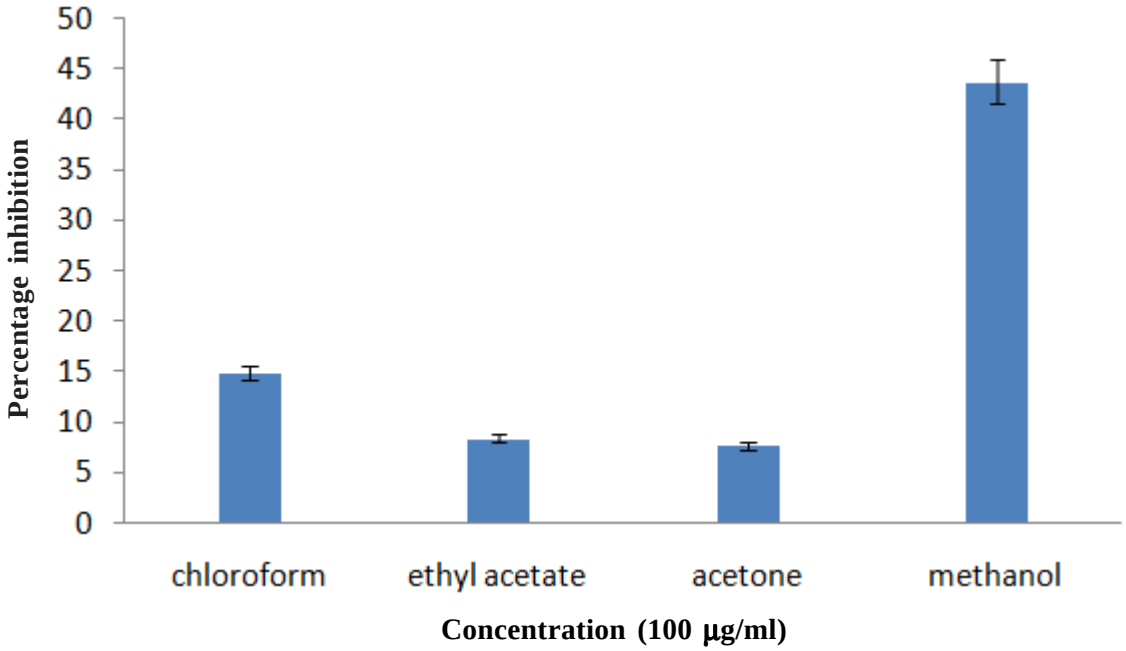

Figure 2. Chelating activity of *Rubus fruticosus* (fruit) fractions

forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm [14]. In the present study, the FRAP values were divided into different groups on the basis of previous classification [35]. As shown in Table 3, both fruit and leaf showed extremely high antioxidant activities (fruit = 955.4± 0.03 µM Fe(II)/g dry weight of the plant material, leaf = 935.4 ± 0.12 µM Fe(II)/g dry weight). On fractionation, acetone (2423.4 ± 0.03 µM Fe(II)/g dry weight) and methanolic (2405.4 ± 0.02 µM Fe(II)/g dry weight) fractions of fruit exhibited even higher antioxidant activity to reduce Fe^{3+} ions.

Reducing power assay

In this assay, the presence of reductants (antioxidants) in test samples results in the reduction of Fe^{3+}/ferricyanide complex into its ferrous form. Fe^{2+} is thus monitored by measuring the formation of Prussian blue colour at 700 nm [7]. The reducing power of the crude extracts/fractions was assessed by using potassium ferricyanide reduction method (Table 4). Reducing power (EC_{50}) of crude extract of fruit and leaf was 437 ± 0.01 µg/mL and 505 ± 0.03 µg/mL respectively. Among the fractions (fruit), moderate reducing power potential was observed in acetone and methanolic

Table 3. Ferric reducing antioxidant power (FRAP) of crude extracts and fractions of Rubus fruticosus

Extract/Fractions [A]	(µM Fe(II)/g dry wt. of plant material)	
	Fruit	Leaf
Crude extract	955.4 ± 0.03	935.4 ± 0.12
Chloroform fraction	187.4 ± 0.07	-
Ethyl acetate fraction	421.4 ± 0.04	-
Acetone fraction	2423.4 ± 0.03	-
Methanol fraction	2405.4 ± 0.02	-

[A]Values are expressed as mean ± standard deviation of 3 parallel measurements

Table 4. Reducing power of crude extracts and fractions of *Rubus fruticosus*

Extract/Fractions[A]	EC_{50} (µg/mL)	
	Fruit	Leaf
Crude extract	437 ± 0.01	505 ± 0.03
Chloroform fraction	na	-
Ethyl acetate fraction	na	-
Acetone fraction	162.33 ± 0.12	-
Methanol fraction	220 ± 0.03	-

[A]Values are expressed as mean ± standard deviation of 3 parallel measurements
na = not active

fraction (EC_{50} of 162.33 ± 0.12 µg/mL and 220 ± 0.03 µg/mL) respectively. EC_{50} of ascorbic acid (standard) was 103.2 µg/mL.

Total phenols and flavonoids

Total phenols and flavonoids of crude extracts/ fractions of fruit and leaf parts of *Rubus fruticosus* is shown in Table 5. Crude methanolic extract of fruit possessed more phenols whereas the leaf part possessed more flavonoids. Acetone fraction of fruit possessed the highest content of phenolic compounds (290.81 ± 0.03 mg GAEs/g dry wt.). Methanolic fraction also possessed significant level of total phenolics (276.75 ± 0.17 mg GAEs/g dry wt.)

Amount of flavonoids in fruit was comparatively low as compared to total phenols. Higher flavonoid content was observed in the crude extract of leaf part (280 ± 0.04 mg QEs/g dry wt.). Flavonoid content in crude methanolic extract of fruit was (80 ± 0.09 mg QEs/g dry wt.).

HPLC analysis

Acetone fraction of methanolic extract of fruit showed the presence of cyanidin predominantly. Significant amount of ellagic acid was also present on comparison with reference ellagic acid as shown in Figure 3.

Discussion

Rubus fruticosus is a well known household remedy used by indigenous communities due to its nutritional and medicinal uses. Fruit, leaves and stem of the plant have good potential against common pathogens [29]. Leaves are noted for their use as diuretic and carminative [17]. Decoction of root bark is used in treating diarrhea [18]. Cyanidin-3-glucoside, a natural product present in blackberries, possesses chemopreventive and chemotherapeutic activities in experimental models [8]. Ripened fruit is consumed to control stomachache and to enhance digestion [32]. Ripened fruit when taken in combination with leaves of *Achyranthes*

Table 5. Total phenol and flavonoid content in extracts and fractions of *Rubus fruticosus*

Extract/Fractions[A]	Total phenolic content (mg GAEs/g dry wt.)		Totalflavonoid content (mg QEs/g dry wt.)	
	Fruit	Leaf	Fruit	Leaf
Crude extract	162.16 ± 0.03	177.02 ± 0.01	80 ± 0.09	280 ± 0.04
Chloroform fraction	33.51 ± 0.12	-	130 ± 0.03	-
Ethyl acetate fraction	55.40 ± 0.06	-	90 ± 0.07	-
Acetone fraction	290.81 ± 0.03	-	70 ± 0.12	-
Methanol fraction	276.75 ± 0.17	-	80 ± 0.04	-

Values are expressed as mean ± standard deviation of 3 parallel measurements

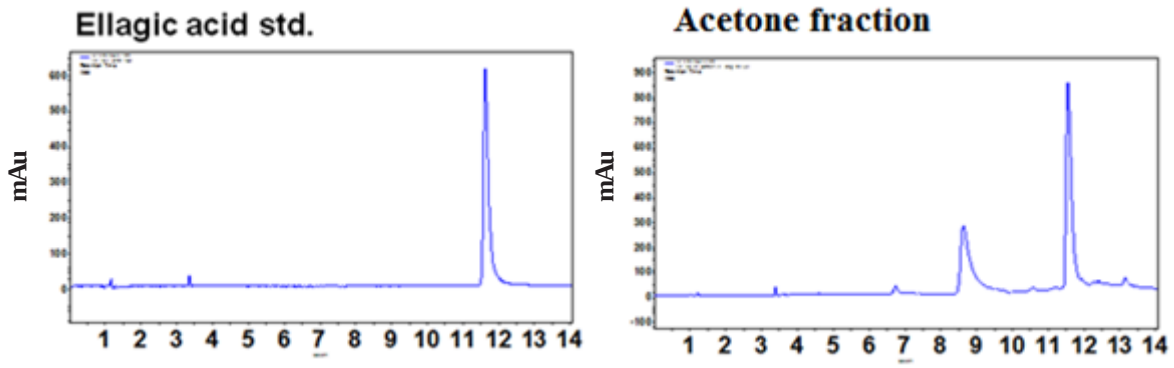

Figure 3. HPLC chromatograms of ellagic acid stdandard and acetone fraction from fruits of *Rubus fruticosus*

aspera, used in treating eye diseases [31]. Besides, fruits are eaten and used in making jellies and jams [17]. The present study results, indicate that fruit of *Rubus fruticosus* possessed significant anti-proliferative activity against lung (A549) cancer cell line (72 %) growth inhibition.. Crude extract of fruit showed maximum abrogation on humoral as well as cell-mediated immune response in immune suppressed Balb/c mice whereas leaf extract showed moderate stimulation in comparison to levamisole (standard) which shows the immunomodulatory activity of fruit of *Rubus fruticosus*. Thus, bioactive molecules from the fruit could be used as therapeutics.

In terms of free radical scavenging capacity, fruit of the plant exhibited better antioxidant potential as compared to the leaf. The DPPH radical scavenging activity (IC_{50}) of crude extract of fruit and leaf was 30.74 ± 0.03 µg/mL and 56.23 ± 0.06 µg/mL respectively. Fruit showed more chelating activity, FRAP and reducing activity than the leaf. Crude extract of fruit on evaluation, possessed more phenolic content whereas the leaf part possessed more flavonoids. A high positive correlation has been established between total phenols and antioxidant capacity [29]. Assessment of Amazonian plant species (leaves, bark, stems, fruits, and seeds) used in folk medicine for their polyphenolic contents and antioxidant activities revealed a strong correlation between total phenolics and antioxidant activity [33].

On fractionation of crude methanolic extract of fruit, acetone fraction showed the highest antioxidant potential as compared to other fractions. Higher DPPH free radical antioxidant activity of acetone fraction can be attributed to its higher content of phenols. In previous studies also, *Rubus fruticosus* has shown good DPPH radical scavenging activity (EC_{50} value of 6.4 mg FW) [2]. However, the chelating activity of acetone fraction was lower as compared to the methanolic fraction. Earlier studies suggest that chelating activity of extracts may not be associated with phenolic content [8]. Higher FRAP values of acetone fraction suggest the presence of antioxidants that can reduce ferric ions. A linear correlation between total phenolic content and FRAP has previously been established [19]. Acetone fraction also showed a moderate reducing activity (162.33 µg/mL) which may be associated with their higher content of phenolics [26]. HPLC analysis of acetone fraction confirmed the presence of cyanidin and ellagic acid. Cyanidin-3-glucoside, present in blackberry, is known to possess strong antioxidant, chemo-preventive and chemotherapeutic activities [8]. Ellagic acid has been reported to possess high DPPH radical scavenging activity, lipid peroxidation inhibition and anticancer activities[11, 15,24].

On the basis of these results, it can be concluded that fruit of *Rubus fruticosus* is a potent anti-proliferative, immunostimulant and a good source of natural antioxidants. The antioxidant activity in fruit could be attributed to its high phenolic content. This is the first study demonstrating the comparative antioxidant activity of fruit and leaf by using crude methanolic extracts and then identifying the active fraction from which potential antioxidant compound could be isolated for further use in food and pharmaceutical industry. The acetone fraction of fruit had appreciable phenolic

content and a remarkable antioxidant potential. These phenols can contribute to potential health benefits by exhibiting not only antioxidant activity but anti-inflammatory, antiproliferative and antimicrobial activities as well. Furthermore, the presence of ellagic acid makes the fruit an excellent source of dietary ellagitannins.

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