Research Article

Exploration of total flavonoids, phenolic contents, antioxidant and anti-bacterial activities of *Rubus fruticosus* L. as a new sources of drug

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Abstract
This study was carried out in order to screen new drug candidate for many of human diseases. The study was carried out for the exploration of total flavonoids, phenolic contents, antioxidant and anti-bacterial activities of *Rubus fruticosus* as a new source of drug. *Rubus fruticosus* L. was screened for the estimation of total Flavonoid and Phenolic contents and antioxidant and anti-bacterial activities. *R. fruticosus* exhibited 50.01 ± 0.03 mg/100 g of alkaloids in leaf, 21.1 ± 0.07 mg/100 g in stem and 18.65 ± 0.13 mg/100 g in root. Total phenolic content (TPC) ranged from 51.02 ± 0.12 to 42.01 ± 0.08. The maximum content found in stem was 71.31 ± 0.04 mg GAE/g. Ethanol extract (EtE) of leaf, stem and root showed that the flavonoid content (TFC) of *R. fruticosus* extracts varied from 94.23 ± 0.10 to 47.13 ± 0.05 mg Rutin/g. Ethanolic extract of stem showed maximum DPPH scavenging activity (27±0.11). Above results revealed that ethanolic extract is reliable for proton-donating capability and could work, as free radical inhibitors and utilizing the plant extracts in traditional medicines can aid to cure most persistent human diseases. Keeping in view its medicinal significance with regards to community it is best suited antibacterial and antioxidant plant in order to cure many bacterial and metabolic disease. It will be a new insight for drugs screening and discovery of new compounds in order to use it for the benefit of society.

Keywords: Antibacterial; Ethanolic extract; Ethnobotany; Medicinal plants

Introduction
Medicinal plants are important all over the world for the income of poor societies. Estimates suggest that worldwide medicinal plant business will spread up to US$ 5 trillion in 2050 [1]. Medicinal plants are not only used by human beings but also used for animals. Such ethno botanical knowledge was evaluated by studying animal behavior, generally with respect to sick animals [2]. The important goal of ethno medicinal studies is to discover drugs from local medicinal plants. These useful plant species can be focused for drug discovery [3, 4]. In Pakistan, generally 60% population of villages is getting health care through
Hakims who suggest herbal preparation [5]. Almost 70 to 80% of the world people use old-style medicine for illness treatment [6]. Azad Jammu and Kashmir (AJK) is in the form of long narrow strip lies in the North East of Pakistan, situated between 73-75 longitude and 33-36 latitude North [7]. There is no reliable data on distribution of medicinal plants in Bagh. As in AJK some initial studies have conducted by some researcher but still a lot to be explored about upper and lower lying flora of district Bagh. Bagh Valley, from the ethno botanical point of vision, is one of the smallest investigated area of the Azad Kashmir with exception of a few contributions made by [8, 9].

Phytochemicals are present in various plant parts. These bioactive constituents of plants are steroids, terpenoids carotenoids, flavonoids alkaloids, tannins and glycosides. These compounds have strong antimicrobial and antibacterial activities [10]. New interest in antioxidants due to their involvement in the health benefits has directed to the development of assays. Plants contain high concentrations of antioxidants. Living systems comprise some difficult enzymatic antioxidants like catalase glutathione, peroxidase, and superoxide dismutase that can block the initiation of OH and the free radical chain reaction [11]. The aim of present study was to investigate the phytochemical studies on selected medicinal plants.

**Materials and Methods**

During plant collection, six field trips were carried out in the month of spring and autumn season, from February 2016 to September 2018 and collected the information about ethno medicinal plants. Information was collected from various localities. Meetings, interviews, thoughts and discussion were arranged with rural people, colleagues, hakims, and old people. Information was also gathered from local inhabitants of study area. Local people particularly plant collector got valuable information about medicinal plants. These citations were confirmed through field visits.

**Preparation of plant extract of Rubus fruticosus**

Fresh plant material (leaves) were washed through water, being purified by distillation and was allowed to dry in air under shadow away from sunlight. Extracts were set by drenching powder of plant part e.g. leaf, stem and root in methanol alcohol. The deposits were taken out twice or repeatedly many times in the similar manner as had done for the first time. Blend was additionally made more concentrated and was moved in a rotatory evaporator at a fixed temperature of about 42°C. Semi-fluid concentrates were made dry in fume cupboard and was kept at -20°C for further utilization.

**Estimation of the alkaloid content**

Alkaloid contents were estimated through Harborne-1973 method. 500 milligram of the plant sample was weighed and was liquefied in 10 mL of 5% acetic acid in ethyl alcohol then the blend was set aside for 4 hours. After this, the blend was filtered through the filter paper. After filtration, the beaker containing filtrate was heated in another beaker containing hot water, up to 90°C till one fourth of sample remained. Then concentrated NH₄OH was poured drop wise till the formation of precipitates. The whole solution was left to clear up and the deposited solid form was collected, rinsed with lower concentrated NH₄OH and then was made refined through filter paper. The remainder on filter paper was alkaloid content, which was allowed to dry and then its weight was measured through an electronic balance.

**Determination of total phenolic content**

Folin-Ciocalteu reagent technique was utilized to estimate total Phenolic content [12]. In short, extracts (10, 100 and 1000 ppm) were blended with Folin-Ciocalteu reagent 0.7 mL and was diluted with distilled water for 10 times. The resultant blend was
left at room temperature and right after 511 minutes, sodium carbonate (0.75 mL of 6 %) was put into it. After 90 minutes of re time, in the end, its absorbance was recorded at wavelength of 725 nm through Spectrophotometer. The standard configuration (0-25 μg/mL) curve was plotted by utilizing Gallic acid as standard and aggregate Phenolic content were shown as mg Gallic acid equal in value per gram (GAE/g) dry weight of the extract.

**Determination of total flavonoid content**

Aluminum chloride colorimetric technique [12] was utilized to check aggregate with a few alterations to find out the total Flavonoid contents. In short, a sample of 0.5 mL of different extracts (10, 100 and 1000 ppm) was treated with methanol of 1.5 mL. Thereafter, aluminum chloride (0.1 mL, 10 %), potassium acetate (1 M, 0.1 mL) and dH2O (2.8 mL) were also added. These whole contents were mixed thoroughly and were placed at normal temperature for 30 minutes. With the help of spectrophotometer, the absorbance of the sample was noted at wavelength of 415 nm. The configuration bend (0-8 μg/mL) was contrived with quercetin. The aggregate Flavonoid were indicated as mg quercetin equal in value per gram (QE/g) dry weight of the extract.

**Determination of antioxidant assay**

The extracts were set up in diethyl sulfoxide (DMSO). Ascorbic acid was utilized as positive control. For a more responsible assumption, antioxidant activity was calculated with the help of DPPH and H2O2 assay.

**Diphenyl-1-Picryl-Hydrazyl Radical (DPPH)**

The DPPH radical is carbon based nitrogen radical and has a profound purple pigment. It is economically accessible and is not essentially required before test. In this test, the purple chromogen radical is made lesser by antioxidant compounds to the comparable light yellow hydrazine. The decreasing capacity of antioxidants towards DPPH can be analyzed by observing the absorbance decline at 515–528 nm until the point that the absorbance stays stable in carbon based (organic) media [13]. Previously revealed technique was taken after for DPPH test with a few adjustments. In short, 950 μL of DPPH solution, being prepared in methanol (3.2 mg/100mL) was blended with 50 μL of each extract in eppendorf tubes blended and kept dark place at temperature of 37 °C for about 1 hour. Alteration in absorbance was recorded at wave length of 517 nm with spectrophotometer. The half maximal inhibitory concentration (IC50) value was determined by table curve technique and %age inhibition was estimated by using the formula. The percentage radicals scavenging activity was calculated by using following formula:

$$S_e(\%) = \frac{A_o - A_i}{A_o} \times 100$$

Where

- S_e = Scavenging effect
- A_o = Absorbance of control
- A_i = Absorbance of sample

**Hydrogen peroxide (H2O2) scavenging assay**

Hydrogen peroxide radical searching action of the extracts was estimated through well recognized protocol with a few adjustments [14]. In short, a solution of H2O2 (2mM) was set up in phosphate buffer (50mM, pH 7.4) and 0.3 mL of new buffer was blended with 0.1 mL of different plant extracts individually. After that addition of H2O2 solution (0.6 mL) and brief vortexing through vortex mixer. The reaction blend was left for 10 min and absorbance was recorded at wavelength of 230 nm.

**Antibacterial activity of Rubus fruticusus**

Disc diffusion essay proposed by [15] was used to test the extracts of plants for their antimicrobial activities. All the tested bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*) were obtained
from laboratory of Bacteriology & Mycology, Department of Pathobiology, University of Poonch Rawalakot while the tested yeasts (Candida albicans and Sccharomyces cerevisiae) were grown in Laboratory of pathology, Department of Pathobiology, University of Poonch Rawalakot. Nutrient agar medium (28g dehydrated nutrient agar in 1000 ml distilled water, warmed and shake) was used for culturing bacterial species. The fungal species were cultured in Sabouraud dextrose agar (65 g Sabouraud dextrose agar in 1000 ml distilled water). 2.5g MacConkey in 1000ml distill water. Hot stirrer plate was used. Medias were shacked on a stirrer and pH was adjusted to 7.0. Both media were autoclaved for 15 min at 121 °C.

The microorganisms were suspended in 10 ml distilled water by dipping a loop of organism in sterilized labeled test tube. From test tube, 1 ml dilution was transported in the corresponding sterilized Petri plates. The dilution and medium were mix in Petri plates by gently shaking and kept at room temperature for solidification. Sterilized filter paper discs of 6 mm in diameter were dipped in 10 mg/ml methanolic extracts of Rubus fruticosus and placed on agar medium in Petri plates commercially available antibiotics (Trimethoprim and oxacillin were used to stop the growth of bacteria as positive control and water, chloroform, ethanol and methanol as negative control. The experiment was performed in aseptic environment. The plates containing the bacteria and yeasts culture were incubated at 37 °C for 24h and 25 °C for 72 h respectively. The zones of inhibition were measured in millimeter by using mean.

**Statistical analysis**
The recorded data was tested in Microsoft excel and Statistical software (8.1). Mean values were tested through least significance difference (LSD) test.

**Results**
In the present study ethno botanical survey of important wild medicinal plants of Bagh Azad Kashmir was carried out in which 117 plant species belonging to 61 families were collected and identified during our field trips. Total alkaloid content in ethanol extract of R. fruticus L. were shown in (Graph. 1).

**Morphology of Rubus fruticosus**
*Rubus fruticosus* L. is perennial, deciduous prickly, shrub with entangling stem growing up to 3 m at a fast rate. It grows in forests and shady edges. *Rubus fruticosus* L is often spiny and have an erect stem, which are rough or smooth with weak prickles, bear ovate, pinnate leaves with 3 to 7 leaflets, which are white and tomentose on the underside. The perennial root stock sends out biennial stems, which produce leaves in the first year and flowering side shoots in the second year. Racemes of five pedaled white flowers (4-7mm) occur in summer. The fruit is usually yellow and. The fruit is an aggregate of drupelets each containing a small seed. Genetically, *Rubus fruticosus* L is diploid specie, branched shrub, 5 to 6m tall. Leaves are broad ovate, variable in size, 2.6-3.7 mm long and 1.6-2.5 mm wide. Leaves are usually green. The plant is green in color with aromatic odor [16]. The quantitative phytochemical analysis of *Rubus fruticosus* L exhibited the presence of alkaloids, total Phenolic content and total Flavonoid in significant quantity. The in-vitro antioxidant activity of the specie clearly demonstrated prominent antioxidant properties in leaves stem and root parts.

**Estimation of Alkaloids**
Alkaloids were observed in the residual ethanol fraction of *Rubus fruticus* L as orange, red-orange, or brown-orange precipitates. The total alkaloid content of stem and leaves were measured at 345.65 ± 15.52 mg in 100 g of the dry sample (RSD = 4.4%). The alkaloid contents of ethanolic extract of Rubus fruticus L. showed that
leaf extract had maximum alkaloid content as compared to stem and root extract. Ethanolic extract of *Rubus fruticosus* exhibited 50.01 ± 0.03 mg/100 g of alkaloids in leaf, 21.1 ± 0.07 mg/100 g in stem and 18.65 ± 0.13 mg/100 g in root.

![Graph 1. Total alkaloid content in ethanol extract of *R. fruticosus* L.](image)

**Determination of Total Phenolic Content (TPC)**
A higher phenolic content was found in stem of species under study as compared to the other parts of the plants. The TPC obtained range from 51.02 ± 0.12 to 42.01 ± 0.08 in *Rubus fruticosus* L (Graph 2 & Table 1). Ethanol extract (EtE) showed the highest TPC, which was 51.02 ± 0.12 mg GAE/g in stem of *R. fruticosus* L. Leaves showed also high TPC 50.60 ± 0.03 mg GAE/g and root showed 42.01 ± 0.08 mg GAE/g however, the difference of TPC in stem and leaves of *R. fruticosus* L was statistically non-significant.

**Determination of Total Flavonoid Content**
The highest TFC were found in the stem 94.23 ± 0.10 mg Rutin/g of *R. fruit cosus* followed by leaf 61.31 ± 0.02 (Graph 3 & Table 2). The minimum TFC were found in roots 47.13 ± 0.05 mg Rutin/g.

**Determination of antioxidant assay**
**Diphenyl-1-picryl-hydraziyl radical (DPPH) assay**
Results showed antioxidant activity of extracts of plants in different solvents by DPPH scavenging activity. Results indicated that all the extracts potentially scavenged the free radicals. They were expressed as the half maximal (50%) inhibitory concentration (IC$_{50}$). The lowest IC$_{50}$ represent the highest antioxidant activity. The ability of extracts to inhibit the discoloration of DPPH from purple to yellow was spectrophotometrically measured. The results showed that the stem of *Rubus fruticosus* exhibited relatively high antioxidant activity (Graph. 4 & Table 2). The lowest antioxidant activity was found in root of *R. fruticosus*. This could be due to less Phenolic compound in extract. In *R. fruticosus* ethanolic extract of stem showed maximum DPPH scavenging activity 27±0.11 μg/mL followed by leaf 24±0.14 μg/mL and root 17±0.14 μg/mL.

**H2O2 Scavenging**
In *R.fruticusus* L on hydrogen peroxide showed in leaf has maximum scavenging ability as compared to stem and root extract.100ug/mL of ethanolic extract of *R. fruticosus* L exhibit 29.30±0.02 in leaf (Graph 5 & Table 1). In stem21.13ug/mL ±0.10.In root18.65ug/mL ±0.05 was.
In extractive values, leaves showed higher ethanol soluble extractive that is 62.52±1.47% respectively than water. Phytochemical studies show that leaves contain all the chemical compounds like alkaloid and flavonoid.

**Antibacterial activity**

The antibacterial activity of methanolic extract *R. fruticosus* L. (stem, root, leaves) was performed against *Staphylococcus aureus*, *Bacillus subtillus* and *E. coli*. Data of table 2 shows the MIC values of all three plant extracts. The diameter of zone of inhibition represents the strongest antibacterial activity. At concentration of 5mg/20 ml root extract showed MIC=10±0.10 for *E. coli*, MIC= 8±0.12 for *Staphylococcus aureus* and 8.0±0.17 for *Bacillus subtillus*. Stem extracts had given the activity of 9.2±0.18 against *E. coli* while, MIC values were 8.8±0.28 and 8±0.09 for both *Staphylococcus aureus* and *Bacillus subtillus*. By changing concentration from 5mg/10ml to 10mg/20ml MIC values were changed and were little higher.

![Graph 2. Total phenolic content of ethanol extract of *R. fruticosus* L.](image)

**Table 1. Antimicrobial activity of *R. fruticosus* L (root, stem and leaves) against gram positive and gram negative bacteria**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Plant part</th>
<th>Antimicrobial activity MIC(μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. aureus</em></td>
</tr>
<tr>
<td>5mg /20 ml</td>
<td>Root</td>
<td>8.0±0.17</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>8.8±0.28</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>8.2±0.12</td>
</tr>
<tr>
<td>10/mg/20ml</td>
<td>Root</td>
<td>13±0.12</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>10±0.11</td>
</tr>
<tr>
<td></td>
<td>Leaves</td>
<td>11±0.14</td>
</tr>
</tbody>
</table>
Graph 3. Total Flavonoid content of ethanol of *R. fruticosus* L.

Table 2. Phytochemical analysis of total alkaloid, phenolic, flavonoid and antioxidant activities of leaf, stem and root of *R. fruticosus*

<table>
<thead>
<tr>
<th>Plant</th>
<th>Rubus fruticosus</th>
<th>Alkaloid (mg GAE/g DW)</th>
<th>Total phenols (mg GAE/g DW)</th>
<th>Total Flavonoid (mg Rutin /g DW)</th>
<th>IC50 (DPPH) ug/mL</th>
<th>% age H₂O₂ Scavenging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>50.01+_0.3</td>
<td>50.40+_0.03</td>
<td>61.31+_0.02</td>
<td>24+_0.14</td>
<td>29.30+_0.02</td>
<td></td>
</tr>
<tr>
<td>Stem</td>
<td>21.1+_0.07</td>
<td>51.02+_0.12</td>
<td>94.23+_0.10</td>
<td>27+_0.11</td>
<td>21.13+_0.10</td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>18.65+_0.13</td>
<td>42.01+_0.08</td>
<td>47.13+_0.05</td>
<td>17+_0.14</td>
<td>18.65+_0.05</td>
<td></td>
</tr>
</tbody>
</table>

Graph 4. DPPH assay of ethanol extract of *R. fruticosus* L.
Discussion
Ethanolic extracts of leaf stem and roots of *Rubus fruticosus* L. was evaluated for the antioxidant potential of plant. This plant has high medicinal potential widely utilized to treat disorders like diarrhea, bleeding, released bowel, dysentery, whooping cough, sore throat, tonsils, diabetes mellitus, and hepatic disorders. The quantitative phytochemical analysis of *Rubus fruticosus* exhibited the presence of alkaloids, total phenolics, and total flavonoids in significant quantity. The in-vitro antioxidant activity of this specie clearly demonstrated antioxidant properties in leaves, stem and root parts. Ethanol extract had the highest TPC and TFC. Ethanol has been demonstrated as the most potent solvent to obtain benzenol (Phenol). Apart from that, ethyl alcohol is secure for human utilization [17]. The oxidation inhibitor action of benzenol (phenol) are due to their oxidation-reduction (redox) reaction that plays a vital job in compelling and counterbalancing high energy rich particles, extinguishing high energy form of oxygen (O_2) and ground state oxygen (3O_2) and a metal chelation potential. Flavonoids have the capability as radical scavenger due to molecular structure of flavonoids which contained hydroxyl group that could provide a proton (H+) to high energy particles like hydroxyl radical (•OH), hydroperoxide (O_2−) and peroxy (ROO•) and thus counter balancing these free radicals (Harborne and Williams, 2000). They are present in plants in large quantities in the form of their glycosides [18]. Plants are thought to be under oxidative stress of free radicals thus developed an efficient antioxidant system to cope with these stresses. These antioxidant systems are helpful in controlling lipid oxidation, free radicals, and breakdown of secondary products [19]. Many compounds in plants are antioxidants including phenolics, flavonoids and carotenoids [20].

The scavenging ability of ethanolic extract of *R. fruticosus* on hydrogen peroxide showed that leaf extract had maximum scavenging ability as compared to stem and root extract. 100 µg/mL of ethanolic extract of *R. fruticosus* exhibited 29.33 % activity in leaf, 21.13 % in stem and 18.65 % in root. The free radical rummaging impact of unrefined methanolic extract of *R. fruticusus* was determined using the DPPH method. DPPH is nitrogen focused free radical, possessing an odd electron that gives a solid assimilation at 517 nm, its pigment alters from purple to yellow when DPPH odd electron matched off within the sight of radical scrounger to frame...
the lessened DPPH-H [21]. The results revealed that ethanolic extract has high scavenging impact of DPPH radicals that has been increased with increase in the sample concentration from 50-250 ug/mL. [22] reported similar trend in DPPH assay. This may be to the presence of high flavonoid content which is required for high scavenging potential in plants [23]. Antioxidant are significantly involved in the defense mechanism of the plant and prevent the formation of reactive oxygen species and free radical within tissues [24]. Recent studies suggested their role in the repairing of DNA, proteins, and prevention from diseases. This has brought the attention of research to use various methods and extracts from different sources to estimate the oxidation inhibitor action of medicinal plants [25].

A higher content of phenolics was found in stem of specie under studied. Ethanol extract (EtE) showed the highest TPC in stem of R. fruticosus than in leaves and roots. However, the difference of TPC in stem and leaves of R.fruticosus was statistically nonsignificant. Ethanol extract (EtE) of leaf, stem and root showed that the flavonoid content (TFC) of R.fruticosus extracts varied from 94.23 ± 0.10 to 47.13 ± 0.05 mg Rutin/g in R.fruticosus. The highest TFC were found in the stem of R.fruticosus followed by leaves. The minimum TFC were found in roots. There is no universal method of estimation of antioxidant potential of medicinal plants [26, 27]. Those plants, which are widely used as food source, have high antioxidant contents. [28] have devised a number of standard methods and requirements for the antioxidant activities of food components.

There are many factors, which affect the outcomes of antioxidant results in different ways. These factors are pH and hydrogen bonding acceptance ability of the solvent, nature of solvent and antioxidant themselves becoming radical species that can change the nature of results [29]. Content of alkaloids in leaves of this medicinal plant were higher as compared to the other plant parts such as root and stem. This is because leaves are functionally most important plant parts and are eaten by most of the herbivores. High alkaloid content may prevent plants from herbivores. Methanolic extract of R. fruticosus L. (root, stem and leaves) showed zone of inhibition both at minimum and maximum dose level (5mg/20 ml and 10/mg/20ml) against E. coli, B. subtilis and S. aureus. Root extract of the plant had most potent effect compared to stem and leaves. E. coli was more sensitive to the root extract. The antibacterial action may be due to the presence of tannins [30]. Flavonoids and tannins are among the important constituents of R. fruticosus leaves [31].

Authors’ contributions
Conceived and designed the experiments: N Nazir & T Batool, Performed the experiments: N Nazir, Analyzed the data: SA Khan & N Nazir, Contributed materials/analysis/tools: N Nazir & SA Khan, Wrote the paper: SA Khan & N Nazir.

References
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