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Phytochemical and antimicrobial analysis of leaf samples of different Rubus species

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Abstract : Four blackberry cultivars (Rubus sp.) were evaluated for the presence of bioactive compounds, antibacterial and antifungal activities. The ash values (total ash (4.03-4.96% w/w), water soluble ash (0.96-2.51% w/w acid insoluble ash (0.25-0.61% w/w and sulphated ash (5.99-9.36% w/w)and percentage extractives of ethanol leaf extract of R. *ellipticus, R. niveus, R. racemosus* and *R. rugosus (*toluene - ethyl acetate (93:7) were analysed. Ehanol extracts of different Rubus Sp. Were evaluated for their phytochemical contents such as alkaloids, carbohydrate, steroid and sterols, glycosides, lactone ring in steroidal nucleus, saponin, flavonoids, tannin, triterpenoids and presence of protein. Carbohydrate, steroid and sterols, saponin, flavonoids, tannin and triterpenoids were present in all the Rubus Sp. Studied. TLC analysis was performed using two solvent systems. In the first solvent system, (toluene-ethyl acetate (93:7) the presence of four compounds with different Rf values were observed in all the test plants. In the second solvent system, chloroform- glacial acetic acid -methanol-water (60:32:12:8), three compounds were detected in R. *niveus, R. racemosus* and *R. rugosus* and two compounds were detected in *R. ellipticus. R.* For antibacterial assays, the highest activity was observed in *R. ellipticus* ethanol extract agains *S. aureus* (27.5 mm) and *S. epidermidis* (26.5 mm). The other extracts also showed considerable antibacterial activity. *R. racemosus* and *R. rugosus* extracts showed the highest antifungal activity of 21 mm and 22.5mm against *Candida krusei* respectively.
Keywords: Antibacterial activity, *Rubus* species, *Candida albicans*, *Pseudomonas aeruginosa*,pathogenic microorganism, phytochemical.

Introduction

Emerging microbial infections both by bacterial and fungal infections and microbial resistance to the available antibiotics play havoc around the globe and this has prompted researchers to investigate the antimicrobial activities of higher medicinal plants. The problem of antibiotic resistance is aggravated by the overuse of antibiotics both in humans/ animals and non-compliance of patients to the courses of treatment. Both the long-term exposure to low doses and the failure to finish a prescription encourage more resistant bacterial strains to thrive[1]. Despite the fact that there are several natural and synthetic products available to ameliorate fungal and bacterial maladies, it is recognized that both resistant fungi and bacteria are on the increase[2].The exploration of new antimicrobial compounds has led to collaborative interests in natural products chemistry, agriculture and medicine in fundamental research. Isolation and identification of compounds which have antibiotic effects without being cytotoxic to cells of higher plants and animals could be highly beneficial.

Medicinal plants are rich source of natural antioxidants[3]and have an appreciable role in the development of modern medicines as many diseases like cancer, hepatic diseases and arthritis have no complete cure in allopathy[4,5]. There is also increased use of plants extracts as cosmetics and pharmaceutical products, something which has generated a lot of interest in identification of active compounds[6].The renewed interest in the benefits of wild fruits and natural products has led to a substantial increase in the number of studies investigating active compounds in *Rubus* and their pharmacological effects. Berries contain a variety of phenolic compounds (phenolic acids, flavonoids, lignans and polymeric tannins) located in plant tissues, often in the surface layer of the plant or berry, which is in connection to their main natural function, to protect the plant against environmental stress and pathogens. The main phenolic compounds in raspberries are flavonoid anthocyanins (coloured substances), ellagic acid and ellagitannins, complex water- soluble phenolic polymers. From the phenolic acids, hydroxycinnamic or hydroxybenzoic acid derivatives are the most common in berries[7,8].

Rubus are are economically important for fruits crops and ecologically significant as invasive weeds. Rubus fruits are considered a healthy and nutritious food, containing phenolics, vitamin C, dietary fibre, α-tocopherol, tocotrienol, calcium, potassium, magnesium, carotenoids, and linoleic acid[7]. *Rubus* species are widely used as antibacterial, anti-inflammatory and pain relief drugs[9] because they are rich in carbohydrates, proteins, minerals, vitamins, superoxidase dismutase and phytochemicals[10]. The *Rosaceae* family is the 19th largest family of plantsrepresented by fruits as plums, cherries, damson plums, quinces, strawberries, pears and peaches. The genus *Rubus*, with almost 700 species, is the largest genus of this family[11].*Rubus* comprises 12 subgenera, with few domesticated species[12]. Members of this genus have been cultivated for centuries for their fruits and are consumed fresh or processed to make food products such as jam, wine, tea, ice cream, desserts, seedless jellies and bakery products. Extracted pigment from fruits is used as a natural colorant in baked products, jellies, chewing gums, fruit-wines and beverages[13]. Fruits and other parts of Rubus plants have had a significant effect on human health and nutrition in both ancient and modern times.

In the present study the ash values, phytochemical analysis; thin layer chromatography (TLC) analysis, antibacterial and antifungal assays were carried out to evaluate the phytochemial potential of four Rubus species namely, *Rubus ellipticus, Rubus niveus, Rubus racemosus* and *Rubus rugosus.*

Experimental

Plant material

The plant materials (leaf) of different species of Rubus, namely *Rubus ellipticus, Rubus niveus, Rubus racemosus* and *Rubus rugosus* were collected from Nilgiris, Ooty, Tamil Nadu, India. The plant species were identified by Dr. Shaji P.K., Scientist, Environmental Resources Research Centre (ERRC), P.B. No. 1230, P.O. Peroorkada, Thiruvananthapuram, Kerala state, India. The plant materials were initially cleaned, dried under shade and then pulverized to coarse powder in an electric grinder. The powder was then stored in airtight bottles for further studies.

Preparation of extracts

The dried leaf powder (30 g) was subsequently extracted with purified ethanol (250 ml) in a soxhlet apparatus for 72 hours and the final filtrate was concentrated using a rotary vacuum evaporator (IKA, RV 10 digital, Germany). The greenish brown sticky ethanol extracts obtained were collected, evaporated to dryness and stored at 4ºC for further studies. The ethanol extracts obtained were subjected to qualitative chemical test for the identification of presence of various chemical compounds

Physical evaluation

Ash value

The contents of total ash, acid insoluble ash and extractive values viz., alcohol and water soluble extractive values were determined following reported methods[14]. Determination of ash values are meant for detecting low-grade products, exhausted and sandy or earthy matter. It can also be utilized as a mean of detecting the chemical constituents by making use of water-soluble ash and acid insoluble ash.

Determination of total ash

Weighed 3 g of air dried leaf powder of four different species of *Rubus* sp. in a tarred silica crucible and incinerated at a temperature not exceeding 450^{o}C until free from carbon, cooled and weighed and then the percentage of total ash with reference to the air dried powdered drug was calculated.

Determination of water soluble ash

Second half of the ash obtained from total ash was boiled for 5 minutes with 25 ml of water. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited to constant weight at a low temperature. The weight of insoluble matter was subtracted from the weight of the ash. The difference in weights represents the water soluble ash. The percentage of water soluble ash with reference to the air dried drug was calculated.

Determination of acid insoluble ash

Half of the ash obtained from the above method was boiled for 5 minutes in 25ml of dilute HCl. The residue was collected in an ash less filter paper and washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Determination of sulphated ash

Moisten the weighed quantity (3gm) of the drug with concentrated sulphuric acid followed by gentle ignition and then repeating the moistening of charred drug with subsequent ignition at 800 ^{o}C. Ignition is repeated until a constant weight of ash is achieved.

Determination of extractive values

Alcohol soluble extractive value

Five grams of coarsely powdered air-dried drug was macerated with 100 ml of alcohol in a closed flask for 24 hour, shaking frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly taking precaution against loss of alcohol. 25 ml of the filtrate was evaporated to dryness in tared flat-bottomed shallow dish, dried at105^{o}C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried drug.

Water soluble extractive value

Five grams of coarsely powdered air-dried drug was macerated with 100 ml of chloroform water in a closed flask for 24 hours, shaking frequently for six hours and allowed to stand for eighteen hours. It was then filtered rapidly taking precautions against loss of chloroform water. 25 ml of the filtrate was evaporated to dryness in tared flat-bottomed dish dried at105^{o}C and weighed.

Phytochemical analysis

The phytochemical constituents present in different *Rubus* sp. were analysed using standard methods[15,16,17].

Detection of alkaloids

Wagner's Test: Extracts were dissolved in dilute hydrochloric acid and filtered. The filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of reddish brown precipitate indicates the presence of alkaloids.

Detection of Phenols

Crude extract was mixed with 2 ml of 2 % solution of $FeCl_3$. A blue green or black coloration indicates the presence of phenols.

Detection of Carbohydrates

Molisch's test was used to detect the presence of carbohydrate. The filtrate was treated with 2 drops of alcoholic α-naphthol solution. Formation of the violet ring at the junction indicates the presence of carbohydrates.

Test for sterols and steroids

Sterols and steroids were tested by the reaction of Lieberman. Ten ml of ethanol extract was evaporated and the residue was dissolved in 0.5 ml of hot acetic anhydride and added 0.5 ml of chloroform. The mixture was treated with the reagent of Lieberman Burchard. The appearance of blue-green ring at the interphase denotes a positive reaction.

Detection of glycosides

Extracts were hydrolyzed with dilute HCl and then subjected to test for glycosides and performed modified Borntrager's test for the detection of glycosides. Extracts were treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Test for intact Lactone ring in steroidal nucleus

Legal's test and Balget's test were done to test intact Lactone ring in steroidal nucleus.

Detection of Saponins

Foam test was performed to test the presence of saponins. To 2ml of the extract, 6 ml of water was added in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirms the presence of saponins.

Detection of Flavonoids

Alkaline reagent test was performed to test the presence of flavonoids. Crude extract was mixed with 2ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on addition of few drops of diluted acid which indicates the presence of flavonoids.

Detection of Tannins

Crude extract was mixed with 2 ml of 2% $FeCl_3$solution. A black coloration indicates the presence of tannins.

Detection of Triterpenoids

Liebermann Burchard's test was performed to detect triterpenoids. The extract was treated with chloroform and filtered. A few drops of acetic anhydride were added to the filtrate, boiled, cooled and concentrated sulphuric acid was added later on. Formation of deep red colour indicates the presence of triterpenoids.

Detection of Proteins

Ninhydrin test was employed to detect the presence of proteins. Crude extract was boiled with 2ml of 0.2% solution of ninhydrin, the appearance of violet colour suggest the presence of amino acids and proteins.

Thinlayer chromatography (TLC)

TLC profile of the extract was carried out on silica gel plates (Merck 60, F_{254}) using two solvent systems. They are toluene - ethyl acetate (93:7) and chloroform- glacial acetic acid –methanol – water (60:32:12:8).10 µl (25 mg/ml) of sample was applied at the center of the plate about 1 cm from the bottom. They were allowed to develop in a TLC chamber at 25°C which was already saturated with standardized mobile

phase (toluene: ethyl acetate and chloroform- glacial acetic acid –methanol – water) in an ascending manner. The spots developed in the toluene - ethyl acetate solvent system was identified by spraying freshly prepared reagent vanillin-sulphuric acid and spots developed in the chloroform - glacial acetic acid - methanol- water was sprayed with anesaldehyde – sulphuric acid reagent. The Rf values were calculated using the following formula.

$$\mathrm{Rf}value = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Antimicrobial analysis

Preparation of plant extracts

Approximately 25 g leaf powder of four *Rubus* sp. was subjected cold extraction using ethanol (250 ml). The final filtrate of each extract was concentrated using a rotary vacuum evaporator (IKA RV-10 Digital, Germany) were collected, evaporated to dryness and stored at 4 ^{0}C for further studies.

Pathogenic microbial strains

The following bacterial and fungal strains were used in this study. The bacterial strains include *Staphylococcus aureus* (NCIM 2492), *Staphylococcus epidermidis* (NCIM 2493), *Pseudomonas aeruginosa* (NCIM 2053) and *E. coli* (NCIM 2439) and the fungal strains such as *Aspergillus flavus* (NCIM 535), *Candida albicans* (NCIM 27), *Candida krusei* (NCIM 3129) and *Trichoderma lignorum* (NCIM 1195) were used in this study. The bacterial and fungal strains were sub cultured frequently in nutrient and Sabourauds dextrose agar slants respectively and are stored at 4ºC for further studies.

Microbial inoculum preparation

The bacterial strains were inoculated in nutrient agar medium and incubated at 37 ^{o}C for 24 hours and the slants were stored in refrigerator for further studies.

Antimicrobial assay

The antibacterial and antifungal sensitivity assays were carried out by disc diffusion method[18] and ethanol extract of the plant leaves were tested against the four bacterial and fungal strains. The bacterial and fungal cultures were evenly spread over Mueller Hinton agar and potato dextrose agar plates respectively using a sterile cotton swab. Then, sterile filter paper discs (6 mm diameter) impregnated with plant extract was placed over the respective agar plates. The bacterial plates were incubated at 37^{o}C for 24 hours and the fungal plates were incubated at 27 ^{o}C for 48 hours and the inhibition zones were measured after incubation. Ampicillin (100µg/ml) was used as a positive control and DMSO was used as the negative control for bacteria and Clotrimazole (10µg/ml) was used as a positive control for fungi and DMSO as the negative control. All tests were done in triplicate and the mean values were presented.

Results and Discussion

The total ash value of *Rubus* species varied from 4.03 % to 4.96 % and the highest percentage of ash was found in *R. racemosus* with 4.96 %. The highest water soluble ash was present in *R. racemosus* with 2.51 % and the lowest percentage was found in *R. rugosus* (0.96 %). In the case of acid-insoluble ash *R. racemosus* showed the lowest value with 0.25 % and the highest was found in *R. rugosus* (0.61). The highest sulphated ash content with 9.36 % was found in *R. rugosus* and the lowest sulphated ash content was found in *R. ellipticus* (5.99 %). The different ash contents of other *Rubus* sp. are given in table 1. The ash value represents the inorganic salts present in the drug[19].

Table 1. Ash values of different *Rubus* sp.

Plant species	Total ash (% w/w)	Water soluble ash (% w/w)	Acid-insoluble ash (% w/w)	Sulphated ash (% w/w)
R. ellipticus	4.03	1.74	0.51	5.99
R. niveus	4.58	2.06	0.27	8.08
R. racemosus	4.96	2.51	0.25	8.04
R. rugosus	4.85	0.96	0.61	9.36

The water soluble extractives observed were in the range of 18.42 % to 26.99 % and the ethanol soluble extractive found in *R. ellipticus* ranged from 8.13 % to 12.47 % from different *Rubus* species. In water soluble extract the highest percentage extractive was 26.99 % and the lowest was observed in *R. niveus* (18.42 %). It was observed that the ethanol soluble extractive was low when compared to water soluble extract (table 2).

Table 2. Percentage extractive of different *Rubus* sp.

Plants species	Water soluble extractive (% w/w)	Ethanol soluble Extractive (% w/w)
R. ellipticus	26.99	11.94
R. niveus	18.42	8.13
R. racemosus	25.81	12.19
R. rugosus	20.16	12.48

The phytochemical studies revealed the presence of phenols, carbohydrate, saponins, flavanoids, tannins and triterpenoids were present in all the four *Rubus* sp. (Table 3). Apart from this, alkaloids, Lactone ring in steroidal nucleus and proteins were absent in all the four *Rubus* sp. studied. Phytochemicals present in above *Rubus sp.* can be broadly classified into two categories: phenolics and terpenoids. Phenolics including flavonoids, tannins and lignan, are the primary group of phytochemicals which characterized by a hydroxyl group attached to anaromatic ring[21].

Various types of phenolics occur extensively in plants and are found in all plant organs. Flavonoids are medicinally used to reduce blood cholesterol and as anti-allergy, anti-inflammatory, anti-microbial and anti-cancer drugs. Most phenolics exhibit powerful antioxidant capability; such as polyphenolic catechins[22]. The largest category of phytochemicals is terpenoids and are widely distributed in plants and also classified into numerous subclasses based on the number of isoprene units: monoterpenoids have two isoprene units, sesquiterpenoids three, diterpenoids four and triterpenes with six isopreneunits. Different plant cells synthesize terpenoids for a variety of purposes; for example, leaf derived terpenoids deter herbivores and flower derived terpenoids attract pollinators[22].

Table 3. Data showing qualitative phytochemical analysis

Sl. No.	Tests for	Method	*R. ellipticus*	*R. niveus*	*R. racemosus*	*R. rugosus*
1	Alkaloids	Wagner's test	-	-	-	-
2	Phenol	Ferric Chloride test	+	+	+	+
3	Carbohydrate	Molisch's test	+	+	+	+
4	Steroid and sterols	Liebermann reaction	+	+	+	+
5	Glycosides	Keller-Killiani test	-	-	-	-
6	Lactone ring in steroidal	Legal's test and Balget's	-	-	-	-

	nucleus	test				
7	Saponin	Foam test	+	+	+	+
8	Flavonoids	Alkaline reagent test	+	+	+	+
9	Tannins	Ferric Chloride test	+	+	+	+
10	Triterpenoids	Salkowski's test	+	+	+	+
11	Protein	Ninhydrin test	-	-	-	-

Presence (+) or absence (-) of phytochemicals

Table 4. TLC of ethanol extract of different *Rubus* sp.

Plant species	**Solvent system: toluene – ethyl acetate (93:7) and spraying reagent: vanillin-sulphuric acid**	
	R_f value	**Colour of spot**
R. ellipticus	0.268	Yellowish green
	0.355	Violet
	0.439	Yellowish green
	0.848	Violet
R. niveus	0.275	Yellowish green
	0.369	Violet
	0.489	Yellowish green
	0.891	Violet
R. racemosus	0.289	Yellowish green
	0.398	Violet
	0.507	Yellowish green
	0.913	Violet
R. rugosus	0.289	Yellowish green
	0.398	Violet
	0.507	Yellowish green
	0.906	Violet

The TLC analysis of ethanol extract in solvent system toluene - ethyl acetate (93:7) and spraying reagent: vanillin-sulphuric acid, revealed the presence of four compounds in all the *Rubus* sp. studied with varying RF values (table 4). Similar R_f values were found in *R. racemosus* and *R. rugosus* species and this indicated the presence of similar compounds in them. As in the case of *R. ellipticus* and *R. niveus* the R_f values observed were almost similar and this indicated the presence of similar compounds.

Table 5. Data showing TLC of ethanol extract of various *Rubus* sp.

Plant extract	**Solvent system: chloroform, glacial acetic acid, methanol and water (60:32:12:8) and spraying reagent: anisaldehyde-sulphuric acid**	
	R_f value	**Colour of spot**
R. ellipticus	0.503	Yellow
	0.553	Brown
R. niveus	0.489	Yellow
	0.565	Brown
	0.782	Brown
R. racemosus	0.463	Brown
	0.531	Yellow
	0.809	Brown

R. rugosus	0.429	Brown
	0.503	Yellow
	0.768	Brown

In solvent system: chloroform, glacial acetic acid, methanol and water, similar R_f values of 0.503 were observed in *R. ellipticus* and *R. rugosus*. This indicated the presence of same compound in both the plants (table 5). In the case of *R. ellipticus* only two spots with R_f values 0.503 and 0.553 were observed and in the other three test plants three compounds each with varying R_f values observed (table 5).

Table 6. Antibacterial activity of ethanol extract (40 mg/ml) of different *Rubus* species

Bacteria	**Zone of inhibition in mm**					
	R. ellipticus	***R. niveus***	***R. racemosus***	***R. rugosus***	**Ampicillin (100 µg/ml)**	**DMSO**
S. aureus	27.5	26.5	23	24.5	26	-
S. epidermidis	26.5	19	23.5	27	18.7	-
P. aeruginosa	14.5	24.5	24.5	24	25.3	-
E. coli	13.5	23.5	25	24.5	28.3	-

The four different *Rubus* sp. tested for antibacterial activity showed promising results in ethanol extracts. *R. ellipticus*, showed the highest zone of inhibition of 27.5 mm against *S. aureus*, whereas the control drug ampicillin produced a zone of inhibition of 26 mm. *R. niveus*, *R. racemosus* and *R. rugosus* showed an inhibition zone of 26.5, 23 and 24.5 mm respectively against *S. aureus*. *R. ellipticus* showed a zone of inhibition of 26.5mm against *S. epidermidis* and *R. rugosus* showed 27 mm respectively and the control drug exhibited only 18.7 mm against *S. epidermidis*. *R. racemosus* showed highest activity against *P. aeruginosa* with 24.5 mm and *R. rugosus* showed highest activity of 27 mm against *S. epidermidis* and control drug ampicillin showed only 18.7 mm. The inhibition zone observed against other pathogenic microorganisms is given in table 6. The antibacterial activity exhibited by different *Rubus* sp. is mainly because of the presence of phytochemicals present in them. The phytochemical constituents present in berry fruits reported to have a wide range of biological effects, including antioxidant, anti-carcinogenic, anti-inflammatory, anti-neurodegenerative, antiviral, and antibacterial activities which are attributed to the phenolic compounds, such as flavonoids, phenolic acids and tannins[23].

Table 7. Antifungal activities of ethanol extract (40 mg/ml)

Fungi	**Zone of inhibition in mm**					
	R. ellipticus	***R. niveus***	***R. racemosus***	***R. rugosus***	**Clotrimazole (10 µg/ml)**	**DMSO**
A. flavus	-	-	-	-	25	-
C. albicans	13	17.5	19	16.5	35	-
C. krusei	15	14.5	21	22.5	33	-
T. lignorum	13.5	16.5	17.5	15.5	29	-

The test plants R. *ellipticus*, *R. niveus*, *R. racemosus* and *R. rugosus* showed no antifungal activity against *A. flavus*. The highest zone of inhibition of 22.5 mm was observed in the ethanol extract of *R. rugosus* and the control drug; clotrimazole (10 µg/ml) produced a zone of inhibition of 33 mm. The antifungal activity of other *Rubus* spp. against the test fungal pathogens is given in table 7.

Ethanol root extract of *R. ellipticus* at concentration 500µg/ml and 1000µg/ml showed significant antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *E. coli* but there was no significant antifungal activity against *Aspergillus niger*, *Saccharomyces cerevisiae*, *Candida albicans* and *Rhizopus nigricans*[24]. Phenolic compounds present in berries selectively inhibited the growth of human gastrointestinal pathogens such as *Salmonella typhimurium* and *Staphylococcus aureus*[25]. The genus *Rubus* (Rosaceae) is

employed to treat diabetes and some species reported to produce compounds that exert hypoglycemic, anti-bacterial, anti-allergic and anti-asthmatic activities[26].

Conclusion

In the present study, the ethanol extract contained important phytochemicals such as phenols, carbohydrate, saponins, flavanoids, tannins and triterpenoids in *R. ellipticus, R. niveus, R. racemosus* and *R. rugosus*. The TLC analysis also revealed the presence of different compounds. Ethanol extracts of the four *Rubus* sp. showed promising antibacterial and anti-fungal properties against the bacterial and fungal pathogens tested. This is mainly due to the presence of phytochemicals present in the test plants. Further screening is to be conducted against more bacterial and fungal pathogens and to isolate and elucidate the active compoundspresent in them.

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