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Phytochemical investigation and antimicrobial anthelmintic activities of the leaves of *Rubus moluccanus* Linn

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Abstract
Objective: To evaluate the leaves of the endangered traditional Indian medicinal plant *Rubus moluccanus* for its pharmacologically active chemical constituents and assessment of its antimicrobial and anthelmintic activities.
Methods: Shade dried and pulverized leaves of the plant were macerated with different solvents to get the respective extracts that were used for chemical examination using simple test tube chemical reactions. The extract that shown to possess maximum chemical constituents was selected for Physicochemical and Pharmacological investigation. Antibacterial and antifungal activities were studied by agar disc diffusion method in comparison with standard drugs amoxicillin and ketaconazole respectively against antimicrobial sensitive and resistant, pathogenic strains of microorganisms say *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger* and *Candida albicans*. The anthelmintic activity was studied by using adult Indian earthworms *Pheretima postuma* using parameters like, time taken for paralysis and death of the worms and compared with standard albendazole drug.
Results: The ethanolic extract of the leaves of the plant was selected for Physicochemical and Pharmacological evaluation as it was found to possess maximum chemical constituents. The crude plant extract was known to illustrate the significant antimicrobial and anthelmintic activities in comparison with reference drugs.
Conclusion: More studies are recommended for isolation and chemical, pharmacological characterization of the chief chemical constituents responsible for the above discovered activities of the plant extract.

Keywords: *Rubus moluccanus,* leaves, ethanolic extract, antimicrobial and anthelmintic activity.

Introduction
Bacterial, fungal and helminthic infections of human beings, animals and plants emerging as a major threat to the productivity and food security of the world especially, the third world group of developing countries such as India due to poor management practices and lack of awareness. Conventional especially, the synthetic drugs become noneffective against several pathogenic microbes and helminths and these drugs also possess several unwanted adverse effects and drug interactions. Therefore, it is challenging for the scientific world to discover safe and effective drugs to deal with this threat. The nature provided us with enormous resources containing wide range of Pharmaceutical agents that are able to cure several ailments of all the types. Several natural products from plants and other natural sources were scientifically proven to be safe and effective antimicrobial and anthelmintic agents raised the hopes for the future scientific world to discover novel drugs [1-4]. The present study is an attempt in this regard to study an endangered Indian medicinal plant *Rubus moluccanus* of family Rosaceae leaves for its Phytochemical and Pharmacological screening to scientifically establish it as a lead for future discovery of safe and effective drugs.

Materials and Methods
Plant material: The leaves of *Rubus moluccanus* were collected from Nilgiri hills region of Tamilnadu state. The collected leaves were shade dried and subjected to pulverization and sieving to get powder of uniform size. The powdered plant material was extracted by using different menstruum say Formaldehyde, Ethyl alcohol, Acetic acid and Chloroform by maceration for seven days. Dried extracts were prepared by desiccation after evaporation of the solvents from filtered extracts in rotary evaporator. The plant material was authenticated by department of Botany, Osmania University, Hyderabad.

Test organisms: Bacterial cultures of *E-Coli*, *Staphylococcus aureus*, *Bacillus subtilis* and Fungal cultures of *Candida albicans* and *Aspergillus niger* were procured from Institute of Microbial technology, Chandigarh. The adult Indian earth worms-*Pheretima postuma* were collected from college premises. All the test organisms were stored under standard conditions till their use for evaluation.

Chemicals: All the chemical reagents and culture requirements were procured from authenticated sources in Hyderabad and stored under standard conditions in stores of the college till usage.

Preliminary Phytochemical investigation: The dried extracts were reconstituted in suitable solvent and are subjected to the following test tube reactions for different chemical compounds as per the procedure of CK. Kokate *et al* [5]:

A). Detection of Alkaloids

a). Dragandorff's test: To 1 ml of sample, two drops of Dragandorff's reagent (Potassium bismuth iodide solution) was added and observed for the formation of precipitate. Formation of prominent reddish-brown precipitate indicates positive test for the presence of alkaloids.

b). Mayer's test: 1 ml of sample was taken into a test tube and added two drops of Mayer's reagent (Potassium mercuric iodide solution) along the sides of the test tube and observed for white or creamy precipitate, which indicates the presence of alkaloids in the extract.

c). Wagner's test: 1 ml of sample was taken into a test tube, added two drops of Wagner's reagent (Iodine-Potassium iodide solution) along the sides of the test tube and observed for reddish brown precipitate, which indicates the presence of alkaloids in the extract.

d). Hager's test: To 1 ml of sample, two drops of Hager's reagent (Picric acid) was added and observed for prominent yellow precipitate, which indicates positive test for the presence of alkaloids

B). Detection of Carbohydrates

a). Molish's test: 1 ml of the test solution was taken and two drops of alcoholic solution of α– naphthol (Molish's reagent) was added. The mixture was shaken and 1 ml of conc. H_2SO_4 was added slowly from the sides of the test tube. The test tube was cooled in ice water and allowed to stand. Then the test tubes were observed for violet ring formation at the junction which indicates the presence of carbohydrates.

b). Fehling's test: 1 ml of test solution was boiled on a water bath with a mixture of 1 ml each of Fehling's solutions A and B and allowed to boil for 1min and observed for the formation of red precipitate, which indicates the presence of reducing sugar.

c) Benedict's test: To 0.5 ml of test solution, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes and observed for the formation of yellow, green or red colored precipitate, which indicates the presence of reducing sugar.

d). Barfoed's test: To 1 ml of test solution, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes and observed for the formation of red precipitate which indicates the presence of non-reducing sugar.

C). **Detection of Proteins and Amino acids**

a). Biuret test: To 3 ml of extract, two drops of 4% Na OH was added and treated with two drops of 1% $CuSO_4$ solution. Formation of pink color indicates the presence of proteins.

b) Ninhydrin test: To 3 ml of extract, three drops of 5% Ninhydrin reagent was added and heated in boiling water bath for 10 minutes. Formation of a characteristic purple color indicates the presence of amino acids.

D). Detection of Steroids and Terpenoids

a) Salkowski test: To the test solution, 2 ml of chloroform and 2 ml of concentrated sulphuric acid were added, shaken well and observed the coloration of chloroform and acid layers. Appearance of chloroform layer as red in color and acid layer as greenish yellow fluorescence indicates the presence of steroids.

b) Liebermann – Burchard's test: To the test solution, 2 ml of acetic anhydride, 2 ml of chloroform were added and heated to boiling and cooled. Then 1 ml of concentrated sulphuric acid was added along the sides of the test tube and observed for the formation of color at the junction. Formation of red, pink or violet color at the junction of the liquids indicates the presence of steroidal triterpenoids (Harborne 2005).

E). Detection of Phenolic compounds and Tannins

a) Ferric chloride test: The extracts were taken and added two drops of neutral 5% ferric chloride solution and observed for blue, green or violet color, which indicates the presence of phenolic compounds. Test solution treated with few drops of ferric chloride solution gives dark color.

b) Lead acetate test: The extract was taken and to this 3 ml of 10% lead acetate solution was added. Formation of bulky white precipitate indicates the presence of phenolic compounds.

c) Bromine water test: The extracts were taken and 1ml of bromine water was added and observed for the discoloration of bromine water. Discoloration of bromine water indicates the presence of phenolic compounds.

F). Detection of Glycosides

1). Tests for cardiac glycosides

a) Legal test: The test samples were taken and added few drops of pyridine and 1 drop of 2% sodium nitroprusside and a drop of 20% sodium hydroxide solution was added. Formation of deep red color indicates the presence of cardiac glycosides.

b) Keller - Killiani test: The test samples were taken and added 2 ml of glacial acetic acid and two drops of 5% ferric chloride solution and mixed. Then 1 ml of sulphuric acid was added. Reddish brown color appear at the junction of the two liquid layers and upper layer appear bluish green color indicates the presence of steroidal glycosides.

2). Test for anthraquinone glycosides

a) Borntrager's test: To 2 ml of the sample, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added. Formation

of pink color indicates the presence of anthraquinone glycosides.

3). Test for saponin glycosides

a) Foam test: Extracts were taken and 20 ml of distilled water was added and shaken for 15 min in a graduated cylinder. A layer of stable foam indicates the presence of saponin glycosides.

Evaluation of Antibacterial and Antifungal activities: It was done by agar disc diffusion method as described by of Omer Erturk [6]. With minor modifications. Sterile Mueller-Hinton agar plates were used for the screening of antibacterial and sterile Potato dextrose agar plates were used for the screening of antifungal activities. Preserved cultures of bacteria and fungi were inoculated under aseptic conditions of laminar airflow chamber into the respective petri plates by spread plate method and discs were made by using sterile borer of 10 mm diameter under aseptic conditions. 100 µl each of sterile Vehicle-2% DMSO (Di methyl sulphoxide), standard drug says, Amoxicillin (10 µg/ml) for antibacterial and ketaconazole (10 µg/ml) for antifungal activities and different concentrations of the plant extract says, 100, 200 and 300 10 µg/ml were added to each disc after filtration through sintered glass filters. The bacterial culture plates were incubated for 24 hours at 37℃ and fungal culture plates were incubated at 28℃ for 24 hours. Finally, the results were evaluated by comparing the zone of inhibition noted from test cultures with that obtained from standard drug cultures. Vehicle plates were used as control to test the inhibitory potential (or) sterility of vehicles used.

Assessment of anthelmintic activity: The method of Ghosh *et al*., [7] was followed for screening of anthelmintic activity with minor modifications. Anthelmintic activity was evaluated on adult *Pheretima posthuma*. Earthworms were divided into eight groups (5 each). The first group (I) serviced the standard drug albendazole at a dose level of 10mg/ml. Groups of (VI) to (IX) received different doses of extracts says 10 mg/ml, 15mg/ml, 20mg/ml, 25mg/ml, 30mg/ml, and 35mg/ml respectively. Observations were made for the time taken to cause paralysis and death of individual worm for two hours. Paralysis was confirmed when the worms did not revive even in normal saline water. Death was concluded when the warms lost their lost motility followed by fading away of their body color.

Results

Extraction: The percentage of the yield of different extracts is illustrated as follows in table 1:

Table 1: Yield of different extracts of the leaves of *Rubus moluccanus*:

S. No.	Menstrum	Percentage of yield (w/w)
1	Formaldehyde	1
2	Ethyl alcohol	4
3	Acetic acid	2
4	Chloroform	2

From the above table 1, it can be known that, maximum extraction of the leaves of the plant was taken place with Ethyl alcohol.

Preliminary Phytochemical examination: The results obtained for the phytochemical investigation of different extracts of the plant material can be illustrated in following table 2:

Table 2: Phytochemical investigation of the different extracts of the leaves of *Rubus moluccanus*:

S. No	Test	Formaldehyde Extract	Ethanol Extract	Acetic Acid extract	Chloroform Extract
1	Alkaloids	-	+	+	+
2	Carbohydrates	-	-	-	-
3	Proteins and Amino Acids	-	+	+	+
4	Steroids and Terpenoids	-	-	-	-
5	Phenolic Compounds	+	+	-	-
6	Tannins	+	+	-	-
7	Glycosides	-	+	+	+
8	Flavanoids	+	+	+	+
9	Saponins	-	+	-	-

From table.2, it can be known that, ethanolic extract of the plant leaves possess maximum biologically active chemical compounds. Based on the literature review and obtained results, the ethanolic extract was taken for Pharmacological investigation of antimicrobial and anthelmintic activities.

Screening of antibacterial activity: The antibacterial activity with respect to zone of inhibition of different concentrations of the plant extract and standard drug-Amoxicillin against different test organisms is represented in the following table.3:

Table 3: Antibacterial activity of the ethanolic extract of the leaves of *Rubus moluccanus*:

Test Organism / Sample (Conc. µg/ml)	Zone of inhibition (mm)			
	Amoxicillin (10)	EE (100)	EE (200)	EE (300)
1. *E. Coli*	85	60	68	75
2. *Bacillus subtilis*	80	55	64	70
3. *Staphylococcus aureus*	85	67	75	80

From tabe.3, it was found that, the ethanolic extract of the plant leaves shown to possess significant and dose dependent inhibitory activity against tested organisms and it is comparable with standard drug.

Evaluation of antifungal activity: The antifungal potential of the different concentrations of the plant extract against the selected fungal organisms in comparison with standard drug-Ketaconazole with respect to the zone of inhibition is

represented in the following table. 4:

Table 4: Antifungal activity of the ethanolic extract of the leaves of *Rubus moluccanus*:

Test Organism / Sample (Conc. µg/ml)	Zone of inhibition (mm)			
	Amoxicillin (10)	EE (100)	EE (200)	EE (300)
1. Candida albicans	36	19	25	31
2. Aspergillus niger	34	20	26	29

From the above table 4, it was come to know that, the plant extract possesses significant and dose dependent antifungal activity against the test organisms in comparison with the standard drug.

Screening of anthelmintic activity: The febrifugal and febricidal activity of the plant extract against adult Indian earth worms-*Pheretima postuma* in comparison with standard drug-Albendazole with respect to time taken for paralysis and death time is represented in the following table.5:

Table 5: Anthelmintic activity of the ethanolic extract (EE) of the leaves of *Rubus moluccanus*:

Groups	Treatment	Concentration Used(mg/ml)	Time Taken for Paralysis(min) (X=S.D)	Time Taken for Death(min) (X=S.D)
1	Vehicle Normal Saline	-	-	-
2	Standard (Albendazole)	10	20±0.23	40.23±0.4
3	EE 1	10	80.3±0.6	111.31±0.6
4	EE 2	15	71±0.4	103.2±5
6	EE 3	20	59±0.5	88±0.9
7	EE 4	25	40±0.7	60.9±1
8	EE 5	30	22±0.4	43±.6±0.5

All the values were expressed as mean ± SD

From table.5, it can be expressed that, the plant extract possesses significant and dose dependent anthelmintic activity against earth worms tested that is comparable with reference drug-Albendazole.

Discussion

Several natural products from Medicinal plants like extracts and pure phytopharmaceutical agents who were previously reported to contain the chemical constituents like, Phenolic compounds, Flavanoids, Tannins and Saponins etc., were also reported to possess significant antimicrobial and anthelmintic activities [8-10]. Berries of the plant *Rubus moluccanus* were reported to contain several biologically active chemicals and also reported to possess anti-microbial activities [11]. The ethanolic extract of the leaves of *Rubus moluccanus* taken in the current investigation was also found to possess the similar compounds and exhibit significant antimicrobial and anthelmintic activities against the tested organisms. Therefore, the Pharmacological potential of the plant extract may be due to the same chemical compounds. Probably, it was the first report in this regard.

Conclusion

The ethanolic extract of the leaves of the plant *Rubus moluccanus* was found to possess several pharmacologically active chemical constituents can be used for further scientific evaluation to discover novel drugs for various pathological conditions.

The ethanolic extract was also found to possess significant antibacterial, antifungal and anthelmintic activities against the test pathogenic organisms like, *E-Coli*, *Candida albicans* and earthworms that is comparable with standard drugs like, Amoxicillin, Ketaconazole and Albendazole.

However, it is highly recommended to further the research in this regard for the isolation and characterization both chemically and biologically to discover the safe and effective Pharmaceutical agents from this source of the nature.

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