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Research Article

In Vitro Anti Acne Activity of Methanolic Extract of Dried Fruit of *Embelia ribes*

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ABSTRACT
In current investigation, an attempt has been taken to explore the in vitro antiacne activity of methanolic extract of dried fruit of *Embelia ribes*. The minimum inhibitory concentration value of the *Embelia ribes* fruits extract against test *S.epidermidis, Propionibacterium acne and Malassezia furfur* was found to be 500 µg/ml ,600µg/ml and 400µg/ml respectively. It clearly indicated that methanolic extract of dried fruit of *Embelia ribes* is promising anti-acne agent against the test microorganisms.

Keywords: Acne, Anti Acne Activity, *Embelia ribes*, Embelin.

INTRODUCTION

Medication and cosmetic measures to overcome skin problems continue to be a foremost research and development initiates by pharmaceutical and personal care industries. Plant based medicines have entered the growing 'cosmeceuticals' market for combating various skin problems[1].It is attracting renewed attention from both practical and scientific view even though the mode of action of phytoconstituents from herbal origin is more complex than mechanisms of one bioactive factor. Ancient records show that the varieties of herbal approaches are proven to be effective for primary health care and treatment of various diseases[2].

Skin is most important and sensitive part of the human body. The external environmental exposure leads to many kinds of skin problems and disorders like acne, sunburn and pigmentation[1]. Acne is superficial skin disorder encountered in the age group from 15 to 25 years owing to increased production of sebum followed by the attack of *Propionibacterium acnes* (*P.acne*)[3]. It usually begins at puberty and worsens during adolescent age, around 12 to13 years in females and 14 to 16 years in males. Statistic study shows that globally around 85% of young adults aged from 12 to 25, 8% of adults aged from 25 to 34, and 3% of adults aged 35 to 44 years experienced certain degree of acne during their lifetime. At age of 20 years both men and women suffers from acne. Recent research shows that, around 30% of women within their fertile period faced persistent acne[4]. One population study in Germany shows that 64% of population aged from 20 to 29 and 43% of aged from 30 to 39 had visible acne. Another study of more than 2000 adults found that 3% of men and 5% of women had mild acne at the age of 40 to 49 years. In USA, 61.9% of population aged 18 years and older were seen in clinics for acne vulgaris[5]

Natural alternatives are blooming as they are being explored for healing multiple factors related with acne[6]. Topical approach is useful in treatment of acne whereas it can also be effectively used for deramatophytosis, candidiasis, tineanigra and fungal keratitis[7]. Natural products research plays an important role in the identification of bioactive lead molecule for the management of acne[4]. The plants producing antioxidant, antimicrobial, anticomedogenic activity and, in certain cases hormone balancing properties can be beneficial as acne involve production of free radicals in inflammatory conditions, microorganism invasion and hormone imbalance. But still there is need for comprehensive studies on medicinal plants for preliminary stages of acne and other skin diseases[8]. Embelia ribes fruits is used as an anthelmintic, diuretic, carminative, contraceptive, anti-bacterial, anti-inflammatory astringent, antioxidant, anticancer agents and seed possessed antibiotic and antitubercular properties. In this context, Methanolic Extract of dried fruit of *Embelia ribes* has been screened for the aforesaid anti-acne activity.

MATERIALS AND METHODS

Plant material

Procurement and authentication of plant material

Dried fruits of *Embelia ribes* were procured from local commercial suppliers of Jalandhar. Authentication of

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Embelia ribes was done at Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar and the voucher specimens have been deposited at the school of pharmaceutical sciences, Lovely professional University.

Pulverization of plant material

The crude plant material (dried fruits) was pulverized in coarse powder form for the purpose of extraction.

Extraction

Method: The coarsely powdered dried fruit was extracted using Soxhlet apparatus. The Solvent used for extraction was methanol[11].

Phytochemical screening of extract

Methanol extract of dried fruits of *E.ribes* were subjected to various chemical tests to detect the chemical constituents which are present in them[12-14].

Test for Carbohydrates

Extracts were dissolved individually in 5 ml distilled water and were filtered. The filtrates were then used to test for the presence of carbohydrates.

Molisch's test

2ml of the extract was taken to which 1ml of 1-naphthol solution and concentrated sulphuric acid was added from the side of the test tube. At the junction, appearance of Purple to reddish violet colour confirmed carbohydrate presence.

Fehling's test

1ml of the extract was taken to which equal quantities of Fehling's solution A and B were added. The solution was heated which led formation of a brick red precipitate at bottom. This confirms the presence of sugars.

Benedict's test

5ml of Benedict's reagent was taken to which 1 ml of extract solution was added. The mixture was boiled for 2 minutes and then was cooled.There was formation of brick red precipitates at bottom which confirmed the presence of reducing sugars in the extract.

Test for Proteins

Biuret's test

1% copper sulphate solution was slowly added to 1 ml of 40% sodium hydroxide solution until there was appearance of blue colour. To above mixture, 1 ml of extract was added. Protein presence was confirmed as there was formation of pinkish violet colour.

Ninhydrin test

0.2% Ninhydrin reagent was freshly prepared using 0.1% solution in n-Butanol, which was added to slowly to test tube containing few drops of extract solution.Formation of blue colour confirms the presence of proteins, peptides and amino acids.

Test for Steroids

Salkowski test

Solution was made by dissolving extract in chloroform to which equal quantity of sulphuric acid was added. There was formation of bluish red colour in the ether layer and green fluorescence in the sulphuric acid layers which confirms the presence of steroids.

Libermann-Burchard's test

Acetic anhydride was added to extract and solution was cooled after boiling. Then, conc. Sulphuric acid was added through the side of the test tube which led to formation of brown ring at the junction of two layers. The upper acidic layer was green in colour which confirms the presence of steroids. Also, there was formation of deep red colour which confirms the presence of triterpenoid in the extract solution.

Test for Glycosides

General test

Test A

200 mg of drug was extracted with 5 ml of dilute sulphuric acid by warming on a water bath which was filtered. Then acid extract was neutralized with 5% solution of sodium hydroxide. 0.1 ml of Fehling's solution A and B was added until it becomes alkaline which can be tested with pH paper. Then the solution was heated on water bath for 2 minutes. The quantity of red precipitate formed was noted and at end the comparison was made with quantity obtained in Test B.

Test B

200 mg of the drug was extracted using 5 ml of water. After boiling the solution add same amount of water as it was added in Test A. Then add 0.1ml Fehling's solution A and B until the solution become alkaline, which can be tested using the pH paper. The quantity of the red precipitates was noted and was compared with the amount obtained in Test A. If the amount of precipitates in Test A is more than Test B then there are chances of presence of glycosides. Test B shows the quantity of free reducing sugars which may be present in crude drug whereas Test A shows both sugars which are based on acid hydrolysis of crude drug.

Test for Flavonoids

Alkaline reagent test

Solution of sodium hydroxide was added to extract which led to formation of intense yellow colour. The appearance of yellow colour indicates the presence of flavonoids, but it is not permanent as it fades away turning to colourless solution upon addition of dilute acid.

Lead acetate test

Few drops of lead acetate solution was added to extract. This led to formation of yellow precipitates at bottom of solution showing the presence of flavonoids.

Zinc-HCl reduction test

Concentrated hydrochloric acid and pinch of Zinc dust was added to alcoholic solution of extract. After few minutes there was formation of magenta colour which shows the presence of flavonoids.

Test for Alkaloids

Extract was dissolved in dilute hydrochloric acid. The solution was filtered which was used to test for the presence of alkaloids.

Dragendorff's test

1 ml Dragendorff's reagent solution, Potassium bismuth iodide solution, was added to 1 mL of extract. Appearance of Orange or red precipitates at the bottom of solution indicates the presence of alkaloids.

Mayer's test

1 ml of Mayer's reagent, Potassium mercuric iodide solution, was added to 1 mL of extract. Appearance of

Table 1: Phytochemical screening of methanol extract of *E.ribes.*

Sr.NO	Phytoconstituents	Test	Methanolic extract of *E.ribes*
1.	Carbohydrates	Molish's test	-
		Fehling's test	+
		Benedict's test	-
2.	Protein and amino acids	Biuret test	-
		Ninhydrin test	-
3.	Steroids	Salkowaski test	+
		Libermann-Buchard's	+
4.	Glycoside	General test	-
5.	Flavonoids	Alkaline test	+
		Lead acetate test	+
		Zinc-HCL reduction test	+
6.	Alkaloids	Dragendroff's test	+
		Mayer's test	+
		Hager's test	+
		Wager's test	+
7.	Tannin	Gelatin test	+
		Ferric chloride test	+
8.	Saponins	Froth test	-
9.	Napthoquinones	Dam-Karrer test	-
10.	Resin	Acetone-water test	+

Positive = + (Present); Negative = - (Absent)

Table 2: Antimicrobial screening of plant extracts against *S. epidermidis* MTCC 3382 using disc diffusion method.

	Different concentration	Zone of Inhibition (Mean±SD)
Plant extract	100mg/ml	7.88 ±0.02
	200mg/ml	10.92±0.02
	300mg/ml	10.93±0.05

Zone of inhibition of plant extracts (mm) against *S. epidermidis* in triplicate (Mean ± SEM)
Zone of inhibition of Clindamycin is 20mm

Table 3: Antimicrobial screening of plant extracts against *P. acnes* MTCC 1951 using disc diffusion method.

	Different concentration	Zone of Inhibition (Mean±SD)
Plant extract	100mg/ml	6.08±0.05
	200mg/ml	10.21±0.02
	300mg/ml	10.40±0.03

Zone of inhibition of plant extracts (mm) against *P. acnes* in triplicate (Mean ± SEM)
Zone of inhibition of Clindamycin is 20 mm

whitish yellow to cream coloured precipitates at the bottom of solution indicates the presence of alkaloids.

Hager's test

3 mL of Hager's reagent which is saturated aqueous solution of picric acid, was added to 1 mL of extract solution. Formation of yellow coloured precipitates at the bottom of solution indicates the presence of alkaloids.

Wagner's test

2 ml of Wagner's reagent which is iodine in potassium iodide, was added to 1 mL of extract solution. Appearance of reddish brown precipitates in the solution indicates the presence of alkaloids.

Test for Tannins

Gelatin test

1 % gelatin solution which contains 10 % sodium chloride was added to extract solution. Appearance of white precipitates shows the presence of tannins

Ferric chloride test

Ferric chloride solution was added to 1 mL of extract. The appearance of a dark blue or greenish black colour precipitates indicates the presence of tannins.

Test for Saponins

Froth test

20 ml of distilled water was added to test extract, after shaking the extract solution for 15 minutes there was formation of foam of height of 1cm which confirmed the presence of saponins.

Test for Naphthoquinones

Dam-Karrer test

10% of KOH was added to the chloroform extract of plant which led to formation of blue colour. The appearance of blue colour indicates the presence of naphthoquinones.

Test for Resins

Acetone-water test

Extract was treated with water and acetone after stirring, the appearance of turbidity confirmed the presence of resins.

Determination of in-vitro anti-acne activity

Microorganisms and culture media

Collection of Bacterial strains

Aerobic bacteria

S. epidermidis (MTCC 3382)

Anaerobic bacteria

P.acnes (MTCC 1951) were obtained from the Microbial

Table 4: Antifungal screening of plant extracts against *M. furfur* MTCC 1765 using disc diffusion method.

	Different concentration	Zone of Inhibition (Mean±SD)
Plant extract	100mg/ml	8.68±0.05
	200mg/ml	12.33±0.03
	300mg/ml	14.67±0.01

Zone of inhibition of plant extracts (mm) against *M.furfur* in triplicate (Mean ± SEM)

Zone of inhibition of Fluconazole is 26mm

The MIC value of the *E.ribes* fruits extract against test *S.epidermidis, P.acne* and *M.furfur* was found to be 500 µg/ml ,600µg/ml and 400µg/ml respectively.

Type Culture Collection Centre from Institute of Microbial Technology in Chandigarh.

Growth conditions and culture medium

The Freeze and dried microorganism was activated by suspending bacteria in 0.9% sodium chloride which was kept at 37±1ºC for half an hour. The suspension of *S. epidermidis* was cultured in sterile Mueller Hinton (MH) agar medium and incubated for 24 hours at 37^0C in aerobic conditions. The suspension of *P. acnes* was cultured in Nutrient agar and incubated anaerobically at 37^0C for 48 hours[17-18].

Collection of Fungal strain

Malassezia furfur (MTCC 1765) was obtained from the Microbial Type Culture Collection

Centre from Institute of Microbial Technology in Chandigarh.

Growth conditions and inoculum preparation

Freeze dried fungal strain was activated by suspending in sterilized double distilled water. The strain was grown on potato dextrose agar (PDA) following incubation at 30^0C in aerobic conditions during 2-7 days[19-20] .

Antimicrobial activity of plant extract

Anti-microbial activity of plant extract was tested using agar disc diffusion method. In order to evaluate anti-microbial activity of plant extract, *P. acnes*, *S. epidermidis* and *M. furfur* were incubated in Nutrient agar, MH agar and PDA media respectively. Uniform sized wells were made with sterile borer on agar plates and were impregnated with plant extract of various concentrations. Antimicrobial activity was calculated by measuring the diameter of the growth inhibition zone (mm). For each isolated bacteria, three plates were prepared of given plant extract and control. Incubation was done for 24 hours. Three wells were made in each plate for comparative study[21-22].

Similarly anti-fungal activity of plant extract against *M. furfur* formerly called *Pityrosporum ovale* was tested using agar disk diffusion method. The agar plates were impregnated with plant extract of various concentrations and incubation was done for 2-7 days[22]

Antibacterial screening by disc diffusion method

Bacterial suspensions were uniformly spread on each agar plates. Three uniform sized wells were made with sterile borer on agar plates that had been seeded with the organism to be tested and in each well 50µl of plant extract of various concentrations were added. Plates were then incubated at 37^0C for 48 hours under anaerobic conditions. *S. epidermidis* was also incubated in MH agar for 24 hours under aerobic conditions. Controls were also prepared and incubated under same condition. The anti-microbial agent clindamycin with concentration of 15µg per disc, was used as a positive control and methanol which was used as solvent for dilutions served as negative control. Zone of inhibition in mm was measured to determine anti-microbial activity of plant extracts[18].

Antifungal activity by disc diffusion method

Fungal suspensions were uniformly spread on PDA plates. Three uniform sized wells were made with sterile borer on agar plates that had been seeded with the organism to be tested and in each well 50µl of plant extracts of various concentrations were added. Plates were then incubated at 30^0c for 2-7 days under aerobic conditions. Control was prepared and incubated at same condition. The anti-fungal agent fluconazole (10mg/ml) served as a positive control in the assay. The plates were sealed and kept in incubator for 2-7 days. Zone of inhibition in mm was measured to determine anti-fungal activity of plant extract[19].

Determination of MIC of plant extract

Collection and preservation of culture.

Determination of MIC of plant extract.

"The MIC is defined as the lowest concentration of the extract at which the bacterium does not demonstrate visible growth"[18].

Protocol for evaluation of MIC by broth dilution method

Evaluation of MIC was done by addition of different concentrations of plant extract in previous cultured bacterium and fungal strain test tubes and incubated at 37^0c and 30^0c for specified period of time and observed for any microbial growth in form of turbidity. The test procedure was carried out by preparing test samples containing different concentrations of 50,100, 200, 300, 400, 500, 600, 700, 800, 900,1000 µg/ml among which the lowest concentration of extract was determined at which bacteria showed no visible growth[3].

RESULT AND DISCUSSION

The yield of methanolic extract (colour: Reddish brown; Odour: Characteristics) was found to be 9.2%. The phytochemical screening of methanol extract of *E.ribes* showed presence of alkaloids, flavanoids, tannin, carbohydrates, resin and steroids (Table 1). These are secondary plant metabolites which are known to be possess various pharmacological effects.

In vitro antimicrobial screening using clindamycin as a positive control clearly indicates that methanol extract of *E. ribes* show promising antimicrobial activity against the test micro-organisms i.e. *P. acnes* and *S. epidermidis*. It was observed that the methanol extract of plant extract showed significant antimicrobial activity against test organisms. Zone of inhibition (in mm) was measured to determine the antimicrobial and antifungal activity of the plant extract (Table 2, 3, 4).

CONCLUSION

As *E.ribes* extract show prominent result against *P. acnes, M. furfur (yeast),* and *S. epidermidis*, so *E.ribes* extract could be a good source for the anti-acne medicine.

CONFLICT OF INTEREST

Nil

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