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EXTRACTION, ANTIOXIDANT AND *IN-VITRO* AND *IN-VIVO* ANTI-ACNE POTENTIAL OF *SYMPLOCOS RACEMOSA* EXTRACT

Ankita Shrivastava*, Mr. Salaj Khare, Dr. Deepak Kumar Basediya, Sandra Gautam, Dr. B. K. Dubey

TIT Pharmacy Education & Research, Bhopal (M.P.)

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Abstract

Medicinal plants play an important role in the development of potent therapeutic agents. Plant based drugs provide outstanding contribution to modern therapeutics as a source of many valuable secondary metabolites which serves as plant defence mechanisms against predator such as microorganism, insects and herbivores which have been proved to be potentially active compounds. There is a tremendous increase in search of antimicrobial plant extracts due to the fact that the resistance offered against antibiotic by the microorganism, in short the effective life span of any antibiotic is limited. Acne is a common but serious skin disease, which affects approximately 80%adolescents and young adults in 11-30 age groups. 42.5% of men and 50.9% of women continue to suffer from this disease into their twenties. Acne vulgaris (acne) is a cutaneous pleomorphic disorder of the pilosebaceous unit involving abnormalities in sebum production and is characterized by both inflammatory (papules, pustules and nodules) and non-inflammatory (comedones, open and closed) lesions. Propionibacterium acnes are common pus-forming microbes responsible for the development of various forms of acne. In the present study anti-acne activity of the hydroalcoholic extracts of Leaves of Symplocos racemosa evaluated. In- vitro anti acne of Clindamycin and hydroalcoholic extract of Symplocos racemosa against Propionibacterium acne was performed using well diffusion method. The presence of various phytoconstituents including the leaves extract in Symplocos racemosa i.e. flavanoids, alkaloids showed significant anti-acne properties. In conclusion, the presented data indicate that the administration of hydroalcoholic extract of Symplocos racemosa have potent anti-acne activity.

Keywords: *Propionibacterium acnes, Hibiscus Rosa-Sinesis Linn, Embelia Ribes, Allium Cepa Carbopol 940, Anti-acne activity, Well diffusion method, Clindamycin*

Corresponding author:
Ankita Shrivastava,
TIT Pharmacy Education & Research, Bhopal (M.P.)
shrivstavaankita83@gmail.com

QR code

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INTRODUCTION:

Acne is considered as one of the most widespread skin diseases[1]. When extreme disfiguration occurs, it results in the development of severe consequences among the young people and may result in depression and suicide. *Acne vulgaris* is the second uppermost reason of suicide among skin diseases. When a person suffering from acne is compared with an individual who is not suffering from acne than it is found that the former has higher level of anxiety, more socio inhibition and has more aggressiveness. Acne is an exclusive disease associated with skin occurs when sebaceous glands (SGs) attain special conditions. This disease occurs in both male and female; there is no preference among them, but the course is more severe in males[2].

Acne is a chronic inflammatory disease of the pilosebaceous follicle that affects about 85% of adolescents. It is estimated that the prevalence of the disease is about 1-12 % in the adult males and 12-17% in adult females. It is more frequent and severe in males, but more persistent in women. The disease has four main causes: sebaceous hyperplasia and hyperseborrhoea; hyperkeratinization and consequent keratinocyte accession; colonization of *Propionibacterium acnes* (*P. acnes*) and respective Staphylococcus albus and inflammation and immune response. The production of sebum by sebocytes is stimulated by androgens, such as testosterone, which in turn stimulate the production of sebocytes. The type I 5α - reductase, present in sebocytes, converts androgens into a more active molecule, 5α - dihydrotestosterone (5α -DHT), which stimulates production and differentiation of sebocytes with subsequent rupture and release sebum, called hyperseborrhoea. The defects in the differentiation of keratinocytes and scaling result in increasing its stickiness, are the cause of clogging of the follicle, which prevents the flow of sebum and leads to the formation of the blackhead[3].

The blackhead is the primary lesion of acne, characterized by the appearance of a slight bulge on the skin intact and can be classified into closed when the follicle blocks the drain hole and the sebum tends to rupture or open when it is distended and is commonly dubbed the “black spot”. The black color is due to the oxidation of lipids contained in the blackhead, the accumulation of melanin and the cells. The accumulation of sebum in the hair follicle promotes proliferation of *P. acnes*, an anaerobic bacterium and normal resident skin. This bacterium produces the lipase responsible for the metabolism of lipids in sebum free fatty acids, which have pro - inflammatory properties. At this stage, acne is considered non-inflammatory. Substances with proinflammatory properties, together with hyperproliferative keratinocytes may leak into the dermis culminating in a cascade of immune events in the inflammatory response.

The disruption occurs because of the enzymes produced by *P. acnes*, which will increase the permeability of the wall and alter the follicular epithelium. Furthermore, these bacteria release chemotactic factors that attract neutrophils to the blackhead, causing inflammatory lesions characteristic of inflammatory acne. Neutrophils can be added to the surface and cause pustules, characterized by the appearance of inflammation and pus. When inflammation is deep, papules are formed, pustules similar to, but with erythema and edema. If there is extensive inflammatory infiltration, the nodules are formed, hot and soft texture and which can be hemorrhagic or suppurative, giving rise to the cysts. All these lesions can converge in a single injury, the scar, an indoor irregular depression of atrophic skin and telangiectasia, resulting from the destruction of the pilosebaceous follicles by inflammatory reaction.

Acne vulgaris is a chronic inflammatory disorder in adolescents consists of the pilosebaceous follicles, characterized by comedones, papules, pustules, cysts, nodules and often scars, mainly of face, neck, back and trunk. The microorganisms involved include *Propionibacterium acnes* and *Staphylococcus epidermidis*. *Propionibacterium acnes* have been described as an obligate anaerobic organism. It is implicated in the development of inflammatory acne by its capability to activate complements and by its ability to metabolize sebaceous triglycerides into fatty acids, which chemotactically attract neutrophils. On the contrary, *Staphylococcus epidermidis*, an aerobic organism, usually involves in superficial infections within the sebaceous unit. These factors provide a potential target for treatment. *Propionibacterium acnes* and *Staphylococcus epidermidis* are the target sites of antiacne drugs. Long term use of antibiotics against acne is outdated because of exacerbated antibiotic resistance.

Symplocos racemosa Roxb. belongs to a unigeneric family Symplocaceae, known as lodhra in Sanskrit; is a small evergreen tree, found throughout the tropical and sub-tropical countries. Ethnobotanical literature indicates use of *S. racemosa* in treatment of eye disease, skin diseases, ear diseases, liver and bowel complaints, tumors, uterine disorders, spongy and bleeding gums, asthma, fever, snake-bite, gonorrhea and arthritis.

To minimize the side effect of topical antibiotics and problem of antibiotic resistance, present study aim to study *In-vitro* and *In-vivo* anti acne potential of herbal plant extract of *Symplocos racemosa*.

MATERIAL AND METHODS:

Material

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals and solvent used in this study were of analytical grade. The pathogenic microbes used in the current study are obtained from Microbial Culture collection, National Centre Forcell Science, Pune, Maharashtra, India.

Methods

Extraction procedure

Following procedure was adopted for the preparation of extract from the shade dried and powdered herbs [4-5]:

Defatting of plant material

Leaves of *Symplocos racemosa* was shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by soxhlet extraction. The extraction was continued till the defatting of the material had taken place.

Extraction by maceration process

56.4 gm of dried powdered leaves of *Symplocos racemosa* has been extracted with hydroalcoholic solvent (ethanol : water, 80:20 v/v) using soxhlet extraction process for 24-48 hrs, filtered and dried using vacuum evaporator at 40°C.

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

Percentage yield = Weight of Extract/ Weight of powder drug Taken x 100

Phytochemical Screening

Phytochemical screening: Phytochemical examinations were carried out for all the extracts as per the standard methods.

1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

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

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

4. Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

5. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

6. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

7. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

8. Detection of proteins

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

9. Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes[6-8].

Total Phenolic content estimation

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method[9].

Preparation of Standard: 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 5-25µg/ml was prepared in methanol.

Preparation of Extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation of phenol.

Procedure: 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Total flavonoids content estimation

Determination of total flavonoids content was based on aluminium chloride method[10].

Preparation of standard: 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.

Preparation of extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Antioxidant activity of hydroalcoholic extract of *Symplocos racemosa* using DPPH method

DPPH scavenging activity was measured by the spectrophotometer with slightly modification[11]. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10- 100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm.

The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

In vitro* antiacne activity of hydroalcoholic leaves extract of *Symplocos racemosa

This agar medium was dissolved in distilled water and boiled in conical flask of sufficient capacity. Dry ingredients are transferred to flask containing required quantity of distilled water and heat to dissolve the medium completely. The flask containing medium was cotton plugged and was placed in autoclave for sterilization at 15 lbs /inch^2 (121^{o}C) for 15 minutes. After sterilization, the media in flask was immediately poured (20 ml/ plate) into sterile petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37^{o}C overnight to check the sterility of plates. The plates were dried at 50^{o}C for 30 minutes before use.

The well diffusion method was used to determine the antiacne activity of the hydroalcoholic extract prepared from the leaves of *Symplocos racemosa* using standard procedure[55.] There were 3 concentrations used which are 25, 50 and 100 mg/ml for extracted phytochemicals in antibiogram studies. It's essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted over night broth cultures should never be used as an inoculum. The plates were incubated at 37^{o}C for 24 hr. and then examined for clear zones of inhibition

around the wells impregnated with particular concentration of drug.

***In vivo* anti acne activity**

Animals

Wistar rats (180-220g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55–65%). Rats received standard rodent chow and water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. All the experiments were carried in a noise-free room between 08.00 to 15.00 h. Separate group (n=6) of rats was used for each set of experiments. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute toxicity studies

Acute oral toxicity was conducted according to the method of Organisation for Economic Co-operation and Development (OECD)[12]. Animals were kept fasting providing only water, leaves hydroalcoholic extract of *Symplocos racemosa* (250, 500, 1000, 2000mg/kg/day) was administered orally for 4 days of five groups of rats (n=6) and the animals were kept under observation for mortality as well as any behavioral changes for evaluation of a possible anti-acne activity.

Induction of acne by *Propionibacterium acnes*

The acne like inflammatory model was produced in the ears of rats by subcutaneous injection of 140 µg of heat-killed bacteria (65°C for 30 min) [13].

Experimental designs

Group –I: control (acne induced)
Group -II: Leaves hydroalcoholic extract of *Symplocos racemosa* (100mg/kg, p.o.)
Group –III: Leaves hydroalcoholic extract of *Symplocos racemosa* (200mg/kg, p.o.)
Group –IV: Clindamycin (200mg/kg, p.o.)
Animals were divided into four groups of 6 animals each. The group I received subcutaneous injection of 140µg of heat-killed bacteria. The groups II, III and IV received 100 mg/kg and 200 mg/kg of leaves hydroalcoholic extract of *Symplocos racemosa* and Clindamycin (200 mg/kg p.o.), respectively.

Measurement of ear thickness

Ear thickness was measured as an index of inflammatory strength and acne. Thickness was measured by using a vernier calliper. Thickness was measured once every two day until the 10th day.

Statistical analysis

All statistical analysis is expressed as mean ± standard error of the mean (SEM). Data were analyzed by one way ANOVA, where applicable $p<0.05$ was considered statistically significant, compared with vehicle followed by Dunnett's test.

RESULTS AND DISCUSSION:

Percentage yield of hydroalcoholic extract of *Symplocos racemosa* exhibited comparable yield 5.85% respectively. Preliminary phytochemical analysis generally helps identify and classify the plant extracts' bioactive constituents. For extracts of all samples, a small portion of the dried extracts of plant leaves underwent phytochemical screening using Kokate (1994) methods for chemical testing of alkaloids, glycosides, flavonoids, saponins, phenolics, proteins and amino acids, tannins separately.

The content of total phenolic compounds (TPC) content was expressed as mg/100mg of gallic acid equivalent of dry extract sample using the equation obtained from the calibration curve: Y = 0.042X-0.002, R^2= 0.999, where X is the gallic acid equivalent (GAE) and Y is the absorbance.

The content of total flavonoid compounds (TFC) content was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: Y = 0.06X+0.019, R^2= 0.999, where X is the quercetin equivalent (QE) and Y is the absorbance. The total phenolic content in hydroalcoholic extract was found 0.586 mg/100mg and total flavonoid content was found 0.769 mg/100mg respectively in hydroalcoholic extract of *Symplocos racemosa.*

In- vitro anti acne of Clindamycin and hydroalcoholic extract of *Symplocos racemosa* against *Propionibacterium acne* was performed using well diffusion method. The results of in vitro anti acne activity showed the zone of inhibition against clindamycin 18±0.57, 15±0.47 and 11±0.94nm at the concentration of 30 µg/ml, 20µg/ml and 10 µg/ml respectively. The zone of inhibition of Hydroalcoholic extract of leaves was found to be 13±0.86, 10±0.5 and 8±0.74nm at the concentration of 30µg/ml, 20µg/ml and 10 µg/ml respectively.

Acne vulgaris is a chronic inflammatory disease results in the formation of inflamed and/or noninflamed eruptions *Propionibacterium acnes* are the anaerobes, in the skin which grow in the sebaceous region. Various antibiotics like tetracycline, Clindamycin, and erythromycin etc and

other drugs like benzoyl peroxide are used for acne treatment. The various drawbacks of synthetic drugs are different side effects and resistant developed towards these drugs. Herbal therapy is required to overcome the above drawbacks and treat the acne. So in the present study leaves hydroalcoholic extract of *Symplocos racemosa* was selected for the anti-acne activity.

Table 1: % Yield of leaves of *Symplocos racemosa*

S. No.	Extract	% Yield (w/w)
1.	Hydroalcoholic	5.85%

Table 2: Phytochemical screening of extract of *Symplocos racemosa*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Mayer's Test:	-ve
	Wagner's Test:	-ve
	Dragendroff's Test:	-ve
	Hager's Test:	+ve
2.	**Glycosides**	
	Legal's test	+ve
3.	**Flavonoids**	
	Lead acetate	+ve
	Alkaline Reagent Test:	-ve
4.	**Phenolics**	
	Ferric Chloride Test	+ve
5.	**Proteins**	
	Xanthoproteic test	+ve
6.	**Carbohydrates**	
	Molisch's Test:	-ve
	Benedict's Test:	-ve
	Fehling's Test:	+ve
7.	**Saponins**	
	Froth Test:	+ve
	Foam Test:	+ve
8.	**Diterpins**	
	Copper acetate test	-ve
9.	**Tannins**	
	Gelatin Test:	+ve

Table 3: Total phenolic and total flavonoid content of *Symplocos racemosa*

S. No.	Total Phenol content	Total flavonoid content
1.	0.586 mg/100mg	0.769 mg/100mg

Table 4: % Inhibition of ascorbic acid and Hydroalcoholic extract of *S. racemosa* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	10	38.65	16.85
2	20	52.36	21.52
3	40	65.87	36.85
4	60	75.45	50.63
5	80	80.39	53.21
6	100	89.65	59.52
IC 50		**19.27**	**72.47**

Table 5: Antiacne activity of standard drug and hydroalcoholic extract against *Propionibacterium acnes*

S. No.	Drug	Zone of Inhibition (nm)		
		30 µg/ml	20 µg/ml	10 µg/ml
1	Clindamycin	18±0.57	15±0.47	11±0.94
		100 mg/ml	50 mg/ml	25mg/ml
2	Hydroalcoholic extract	13±0.86	10±0.5	8±0.74

Table 6: Effect of Clindamycin (standard) and hydroalcoholic extract of *Symplocos racemosa* on acne induced by *Propionibacterium acnes* in rats

Treatment	Dose	Mean thickness ±SEM				
		Day2	Day4	Day6	Day8	Day10
Control	140 µg	1.45± 0.15	1.35 ± 0.15	1.28± 0.20	1.25± 0.20	1.24± 0.15
S. racemosa extract	100mg/kg p.o.	1.43±0.35*	0.35±0.32*	0.22±0.35*	0.22±0.40*	0.22±0.35*
S. racemosa extract	200mg/kg p.o.	1.05±0.25**	0.26±0.40**	0.20±0.40**	0.18±0.40**	0.18±0.40**
Clindamycin	200 mg/kg p.o.	0.95±0.30**	0.18±0.30***	0.10±0.30***	0.09±0.30***	0.09±0.30***

Values are expressed as the mean ± SEM of six observations. *** $P<0.001$ vs. control treatment (One-way ANOVA followed by Dunnett's test)

CONCLUSION:

The acne like inflammatory activity was carried out by measuring the ear thickness. Hydroalcoholic extract of *Symplocos racemosa* showed significant reduction in the ear thickness. It seems that the increased ear thickness and inflammation caused due to various biochemicals, viz. various kinins, histamine and 5-HT is significantly reduced. The presence of various phytoconstituents including the leaves extract in *Symplocos racemosa* i.e. flavanoids, alkaloids showed significant anti-acne properties. In conclusion, the presented data indicate that the administration of hydroalcoholic extract of *Symplocos racemosa* have potent anti-acne activity.

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