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Evaluation of traditionally claimed *Salmalia malabarica* (DC) Schot & Endlicher for anti - acne activity: An *in-vitro* and *in-vivo* approach

Shailju G Gurunani and Ravindra V Karadi

Abstract
In Indian tradition, aerial parts of *Salmalia malabarica* (Bombacaceae) has been used in the treatment of various skin troubles, especially paste of thorns work out on A*cne vulgaris*. The objective of the present study is to evaluate leaves, bark and thorns of *S. malabarica* for anti-acne activity in animal model. Phytochemical screening revealed the presence of flavonoids, steroids, tannins in the plant extract. Anti-acne properties of all extracts were assayed using Clindamycin as standard drug. The drug was studied against *P. acnes* by *in-vitro* anti-bacterial studies. The MIC's for this assay were determined by a broth dilution method. The Sprague-Dawley male rats (180-220 g) were used for *in-vivo* studies which were divided into six groups of six animals each. Acne (granulomatous inflammation) was induced by injecting heat killed *Propionibacterium acnes* strain in normal saline to the sub cutaneous regions in ear of each rat regardless of weight. Then, the ethanolic extract of leaves (AEL), bark (AEB) and thorns (AET) with the dose 200mg/kg b.w., p.o. each, were administered to the three different groups of animals for 35 days and thickness of the ear was measured daily. On the day 10th, ear was assessed for histopathology parameters with respect to infiltration of neutrophils and lymphocytes. The AET exhibited noteworthy activity in all the parameters of the study. The results of *in-vitro* and *in-vivo* studies strongly indicate that the AET has potent anti-acne property than AEL and AEB in comparison to the standard drug Clindamycin. These findings divulge that the present study corroborate the ethnomedicinal use of *S. malabarica* in the treatment of acnes.

Keywords: *Salmalia malabarica*, *Bombax ceiba* Linn., *Propionibacterium acnes*, Anti-acne activity, granulomatous inflammation, anti- bacterial

1. Introduction
Thousands of phytochemicals with inhibitory effects on micro-organisms have been found to be active *in vitro* but have not been tested *in vivo* and therefore activity cannot be claimed but one must take into consideration that many, if not all, of these plants have been used for centuries by various cultures in the treatment of disease [1].

Acne vulgaris is a chronic inflammatory disease of the pilosebaceous apparatus, the lesions occurring on face, neck & back. The inflamed glands form small pink papules, surrounded by comedomes form pustules or cysts, caused by stress, hereditary factors, hormones, drugs and bacteria i.e. *Propionibacterium acnes* [2]. *P. acnes* is the bacterial species which performs abnormal bacterial function by producing biologically active substances which activate the complement system and stimulate the release of hydrolyses from neutrophils [3]. Inflammatory lesions have traditionally been attributed to the accumulation of neutrophils within microcomedomes or comedomes with subsequent rupture of the follicle [4, 5].

Medical researchers are working on new drugs to treat acne, particularly antibiotics to replace some of those in current use. As with many other types of bacterial infections, the bacteria that are associated with acne are becoming resistant to treatment with certain antibiotics. Research is also being conducted by industry on the potential side effects of isotretinoin and the long-term use of medicines used for treating acne. Scientists are working on other means of treating acne. For example, researchers are studying the biology of sebaceous cells and testing a laser in laboratory animals to treat acne by disrupting sebaceous glands by inducing acnes. Depending upon the degree of involvement, treatment of acne varies from topical application to systemic therapy with antibiotics, many remedies have been employed to treat acne from long period. Most of the remedies were taken from plants and proved to be useful, though the rationale behind their use is not scientifically established except for a few plants [6, 7]. This has given rise to stimulate the search for investigating the plant Silk Cotton tree known as *Salmalia malabarica* (DC) Schott & Endlicher synonym *Bombax ceiba* Linn. belonging to the family Bombacaceae.

In the Ayurvedic system of medicine, the aerial parts of this plant also known as 'semul' are used in treatment of various skin troubles and especially paste of thorns (prickles) works out on acne vulgaris [8-11]. Also some observations in view concept of doctorine of similarity, states that, the shape of the plant part resembles the human organ for which it has a medicinal value for example *Centella asiatica,* a brain tonic and it resembles structure of brain [12]. Thus, in present study thorns of *Salmalia malabarica (DC) Schott & Endl.* has been evaluated in comparison with bark and leaves, for anti-acne activity by screening the extracts against the causative organism i.e. *Propionibacterium acnes.*

Salmalia malabarica is an important medicinal plant of tropical and sub- tropical India. It is a tall, lofty, deciduous, cotton tree with straight buttressed trunk, covered with stout hard sharp conical prickles at base [13-15]. Leaves contain mangiferin and shamimin, [16] stem bark contains lupeol and β- sitosterol. [17] Root bark contains phenolic components, [18], triterpene, sterol, [19-21] lactone, [22, 23] and sesquiterpenoids. [22]. Leaves are anti-inflammatory and are good for strangury, skin eruptions and in anemia. Roots are consumed as vegetable; it is sweet, cooling, stimulant, tonic, demulcent, antidysenteric, aphrodisiac, astringent and also used for gonorrhea. Bark is mucilaginous, demulcent, styptic, tonic and emetic. The aqueous extract of bark with curd arrests blood dysentery, and is used for fomenting and healing wounds. Its paste when applied externally is good for skin eruptions, snake bite and in acne and pimples. [8, 9]. Seeds are evaluated for anti-microbial and anti-inflammatory activities [24]. A paste made out of thorns is good for restoring skin colour especially on the face and ground spike- paste is recommended for acne [10, 11]. However, literature review revealed that *Salmalia malabarica* (DC) Schott & Endl. has not been scientifically investigated for anti-acne activity. Hence, the aerial parts such as bark, leaves and thorns were selected for the investigation.

Materials and Methods

Plant Material

The aerial parts such as leaves, bark and thorns of *Salmalia malabarica* Schott & Endl. were collected from the local areas of Belgaum (15.85^{0}N 74.55^{0}E) Karnataka state of India and were authenticated by Dr. G.R. Hegde, Professor and Head, PG Department of Botany, Karnataka University, Dharwad, Karnataka. The voucher specimen no. DU /BD/2005-279 is available in the herbarium file of the University Department for the future reference.

Preparation of extract by Successive Solvent Extraction [25, 26]

The shade dried parts were reduced to coarse powder in a mechanical grinder and passed through a sieve no.40 to obtain powder of desired particle size. The powdered material was subjected to successive solvent extraction in soxhlet assembly with the solvents in increasing polarity such as petroleum ether, benzene, chloroform, ethanol and chloroform water I.P. The extracts were filtered and concentrated by distillating off the solvent and then evaporated to dryness by rota-evaporator. The extract thus obtained was weighed and percentage was calculated in terms of air-dried weight of plant material.

Selection of extract for the pharmacological activity

The preliminary phytochemical investigation, anti-oxidant activity & *in-vitro* anti- bacterial assay for all the extracts of three parts were performed which reveals that the ethanolic extract shows better results. On the basis of which it was decided to select ethanolic extract of leaves (AEL), ethanolic extract of bark (AEB), and ethanolic extract of thorns (AET) for *in-vivo* anti-acne activity.

AEL, AEB, and AET were taken in the concentration of 1mg/ml (1000 micrograms /ml) in distilled water and used for antibacterial assay. The extracts were suspended in distilled water using 2% Tween 80 as suspending agent for toxicity and *in- vivo* anti-acne study.

Standard drug and Chemicals

Clindamycin HCl capsules USP, 150 mg was used as a standard drug. The dose was selected on the basis of adult human effective dose. Tween ® 80 (Sigma Ultra Product Number P 8074), Thioglycollate broth (Sigma Aldrich), Phosphate Buffer Solution (Loba), Formalin Solution, hematoxylin-eosin dye, diphenyl xylene. The solvent and other chemicals of analytical grade were used.

Bacterium and medium

The bacterium *Propionibacterium acnes* (MTCC 3297) used for experiment were procured from Microbial type culture collection and gene bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The thioglycollate broth was used for the antibacterial assay. 3% Thioglycollate broth medium (TBM) containing various concenration of extract and standard was used to estimate MIC against *Propionibacterium acnes*. Although only one strain of *P. acnes* was tested, a compound active against one strain is expected to retain a similar order of activity against a variety of strains of this species. On the other hand, the possibility of resistance among strains is always present. With these considerations in mind, the experiments were carried out.

Experimental Animals [27, 28]

Sprague- Dawley male rats (180-220gm) were selected for acute toxicity studies and *in-vivo* anti-acne activity. They were procured from NIMHANS, Bangalore, Karnataka, India. The animals were acclimatized to standard laboratory conditions of temp ($25^0 \pm 2\ ^0$C) and maintained on 12:12 h light: dark cycle. The animals were provided with regular rat chow (Lipton India Ltd., Mumbai) and distilled water *ad libitum.* The pharmacological study was undertaken after the prior approval from CPCSEA/IAEC (registration no. : 221/ CPCSEA). The animal care and experimental protocols were in accordance with its guidelines.

Acute toxicity study [28, 29]

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD) revised draft guidelines 423 B ("Up and Down" method) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Based on the cut-off value of the median lethal dose (LD 50), the therapeutically effective dose was derived.

Experimental groups

The rats were divided into following 6 groups of 6 animals each:

Group I - given distilled water.

Group II - induced by heat killed *P. acnes* in PBS and given distilled water.

Group III - induced by heat killed *P. acnes* in PBS and treated with Standard Clindamycin 200 mg/kg b.w., p.o.

Group IV - induced by heat killed *P. acnes* in PBS and treated with AEL 200 mg/kg b.w.,p.o
Group V - induced by heat killed *P. acnes* in PBS and treated with AEB 200 mg/kg b.w.,p.o
Group VI - induced by heat killed *P. acnes* in PBS and treated with AET 200 mg/kg b.w.,p.o

Pharmacological screening methods

***In-vitro* anti-bacterial model**

The MIC's for this assay were determined by a broth dilution method [30]. The serial two-fold dilutions of the test compounds were made in PBS. This test broth was then inoculated with 10µl of each solution, which was added to 1ml of thioglycollate broth. It was then inoculated with 3 day old culture of *Propionibacterium acnes*. After 2 days of incubation at 37 ^{0}C, the growth of *P. acnes* was examined on the culture plates. The lowest concentration of the test compound, which inhibited the growth, was reported as MIC. The assay was repeated twice with two replicates per assay.

In-vivo anti- acne model [31-35] :

Based on the pilot screening the following protocol was carried out. In pilot screening, 6 rats were taken under study which showed that the granulomatous inflammation remain constant from day 6th day to 10th day. Thus, depending on the protocol given, the animals were divided into 6 groups containing 6 in each and kept in metabolic cages. All animals had free access to regular rat show and drinking water *ad libitum* during the study.

Induction of acne by Propionibacterium acnes

The acne like inflammatory model was produced in the ears of male Sprague Dawly rats (180-220g) by subcutaneous injection of 0.14 mg, heat-killed bacteria.

Measurement of ear thickness

Ear thickness was measured as an index of inflammatory strength and acne. Thickness was measured by using a vernier calipers (Esel International, Ambala). Thickness was measured once every day for the first week of induction, then every other day until 35th day. The % inhibition of *P. acnes* induced granulomatous inflammation by the test or standard drug was calculated by using following formula;

[(1-Vt)/Vc] x 100

Where; Vc: Mean thickness increased (mm) in Control group,
Vt: Mean thickness increased (mm) in Treated or Standard group

Statistical analysis

Results are expressed as Mean ± S.E.M. The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The results were considered statistically significant when ***P<0.001.

Histopathology

On the 10th day after the induction of acne, 3 animals from each group were sacrificed and ears were excised and fixed in 10% formalin (pH 7.2) and then embedded in paraffin and thick sections were taken to stain using hematoxylin-eosin dye and mounted in diphenyl xylene [7]. The changes were observed and rest of the three animals in each group were observed till 35th day.

Results

No adverse effect or mortality was observed upto 2000 mg/kg. Hence, 1/10th of LD_{50} dose of AEL, AEB, AET was taken as therapeutic dose i.e. 200 mg/kg body weight was selected as a test dose. The anti-bacterial assay against *P. acnes* was performed for the standard, AEL, AEB, AET. The table no. 1 reveals the results of antibacterial assay showing Minimal Inhibitory Concentration (MIC). It was found that AEB and AET has lowest MIC i.e. the lowest concentration that can inhibit the growth of the microorganisms. Thus, the MIC for Clindamycin was found to be 1mg/ml, for AEL it was 500µg/ml where as AEB and AET showed the lowest MIC of 250µg/ml.

Table 1: Antibacterial assay showing MIC

S. No.	Concentration	Clindamycin	AEL	AEB	AET
1.	1000µg/ml	N G+	NG	NG	NG
2.	500µg/ml	G+	NG+	NG	NG
3.	250µg/ml	G+	G+	NG+	NG+
4.	125µg/ml	G+	G+	G+	G+

G+ = Growth NG= No Growth,

The bacteria when injected in ear, the granulomatous inflammation at the site was observed and once a day the thickness was measured using vernier calipers. The maximum inflammation on ear at the induced site was on 4th day in all the groups. In test group, on 5th day there was an sudden decrease in inflammation which was constant till the end of the study, but in control group constant thickness was observed till 10th day and at around 20th day the inflammation reduced and came to normal. The observations were made till 35th day where the thickness was found to be normal. Table no.2 shows the mean thickness of ear increased in each group separately from day 1st to 35th with respect to that of day 0. Table No.3 shows percentage inhibition in which 5th day to 10th day shows maximum inhibition as compared to day 1st to 4th. The comparative results are shown in graph no.1.

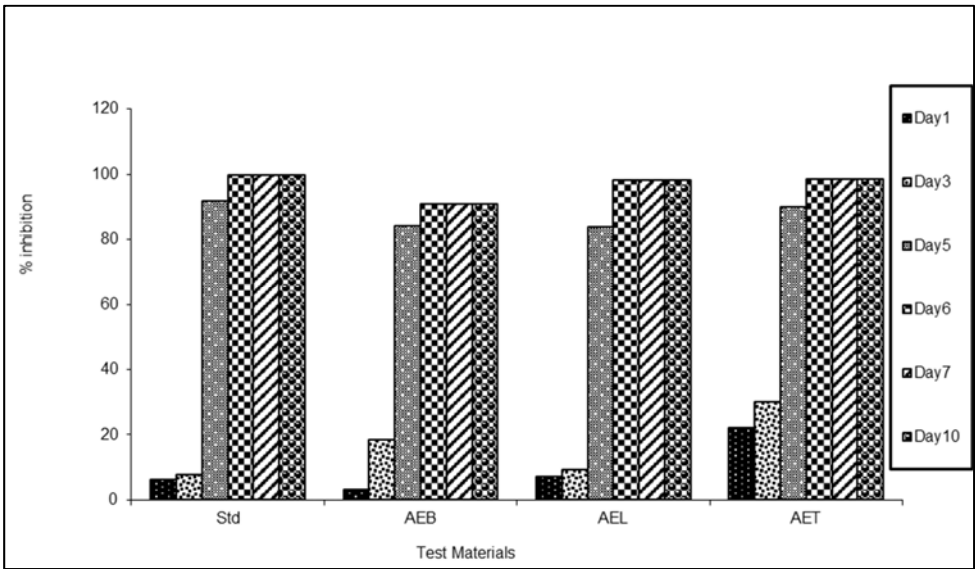
Table 2: Effect of Clindamycin (standard), AEL, AEB, and AET of *Salmalia malabarica* on *P. acnes* induced granulomatous inflammation in terms of Mean thickness increased (mm)

SR. NO	GROUP	Mean thickness increased (mm) ± S.D								
		Day1	Day3	Day5	Day6	Day7	Day10	Day15	Day 20	Day25-35
1	CONTROL	1.438 ± 0.017	1.377 ±0.014	1.270 ±0.021	1.268 ±0.0215	1.268 ±0.021	1.268 ±0.021	1.268 ±0.021	0.1280 ±0.001	0.1280 ±0.001
2	CLIND AMYCIN	1.350 ±0.0054**	1.270 ±0.0031**	0.1040 ±0.0040**	0.0020 ±0.0020**	0.0020 ±0.002	0.0020 ±0.002	0.0020 ±0.002	0.0020 ±0.002	0.0020 ±0.002
3	AEL	1.406 ±0.0024	1.342 ±0.018	0.2080 ±0.019**	0.1180 ±0.015**	0.1180 ±0.015	0.1180 ±0.015	0.1180 ±0.015	0.1180 ±0.015	0.1180 ±0.015
4	AEB	1.392 ±0.0040	1.308 ±0.0047**	0.2033 ±0.0049**	0.1167 ±0.011**	0.1167 ±0.011	0.1167 ±0.011	0.1167 ±0.011	0.1167 ±0.011	0.1167 ±0.011
5	AET	1.342 ±0.0016**	1.262 ±0.0016**	0.1383 ±0.0016**	0.01833 ±0.003**	0.01833 ±0.003	0.01833 ±0.003	0.01833 ±0.003	0.01833 ±0.003	0.01833 ±0.003

The average thickness of ear of an normal rat was 0.5 mm. the above data shows difference in the thickness of ear on day 0 and day 1 to day 35
***P<0.001, **P<0.01, *P<0.05

Table 3: % inhibition of *P. acnes* induced granulomatous inflammation treated with Clindamycin and AEL, AEB, AET of *Salmalia malabarica.*

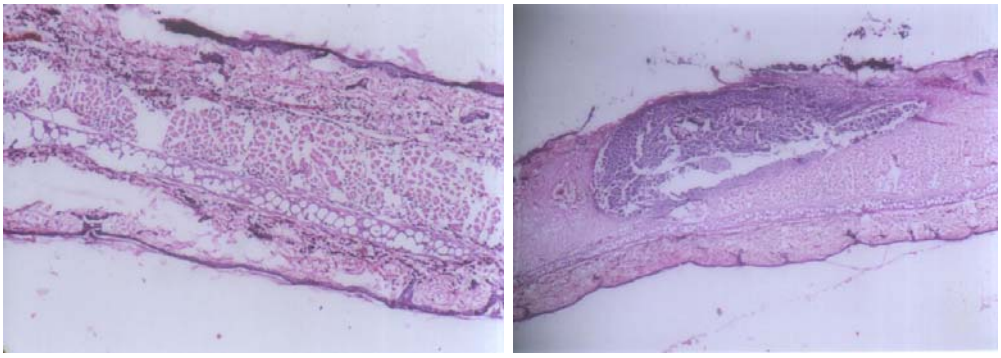
Test Material	Percentage inhibition (%)					
	Day1	Day3	Day5	Day6	Day7	Day10
Clindamycin	6.12	7.7	91.94	99.85	99.85	99.85
AEB	3.2	18.4	84.3	90.8	90.8	90.8
AEL	7.3	9.3	84	98.2	98.2	98.2
AET	22.0	30	90	98.6	98.6	98.6



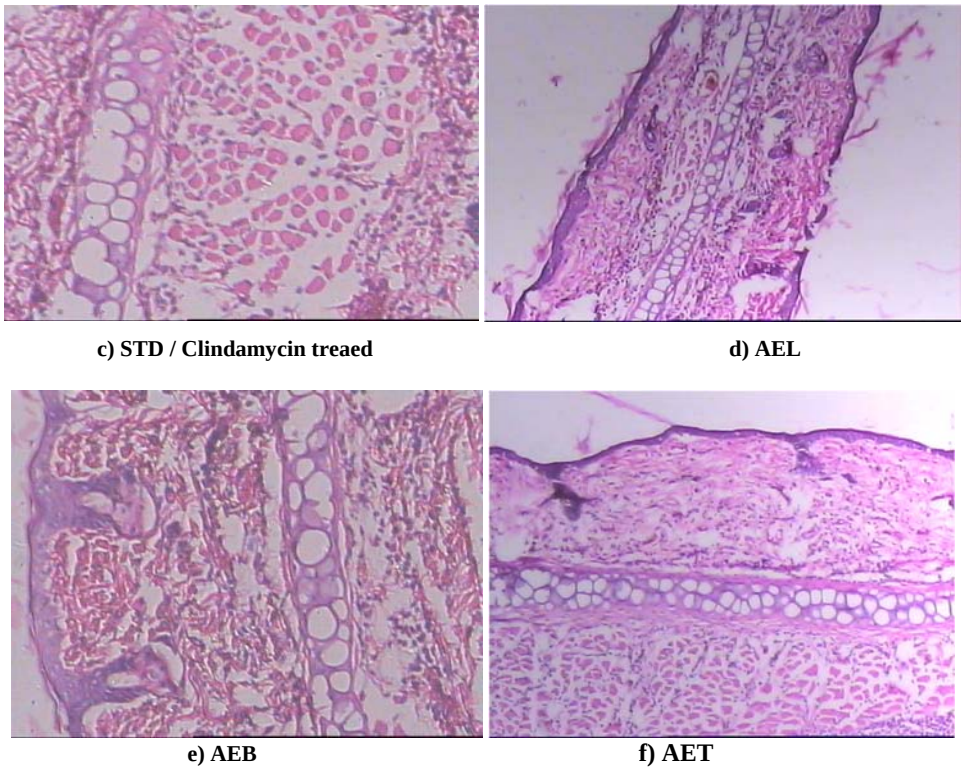
Graph 1: Histogram showing the effect of test materials on% inhibition of inflammation in *P. acnes* induced acnes in rats ear

As per the preliminary studies carried out in animals, the thickness of the ear (inflammation) was observed on 10th day. The thickness was found to be constant between 6th and 10th day. Hence, histopathology of the ear was assessed on 10th day.

In histopathology study, it was found that, there was an accumulation of neutrophils on inflammatory lesions site with subsequent rupture of the follicle and formation of a pustule in the dermis. And the transmigration of lymphocytes into the wall of the follicle associated with increasing spongiosis of the follicular epithelium. During 24-72 hours, the accumulation of neutrophils within the follicle led to its distension and subsequent rupture. There was a localized loss of the granular layer in the region of the eventual rupture. This shows the difference in normal and acne-induced ear section, the figure no. 1 a and b. clarifies this. The figure no. 1.b. shows the accumulation and rupture along with infiltered inflammatory cells surrounding the injection site of *P. acnes* at an H&E stained frozen section of the *P.acnes* injected ear but not in normal ear figure no. 1.a. The figure no. 1.c shows section of ear treated by the standard drug. And the figure no. 1.d,1.e, 1.f are the sections of the group treated with AEL, AEB, AET respectively which are also similar to that of standard. The histopathology study supports the results shown in Table No.1, 2 and 3.



a) Normal group **b) Acne induced group**



c) STD / Clindamycin treaed **d) AEL**

e) AEB **f) AET**

Fig 1: Histopathology of ear a) Normal group b) Acne induced group c) STD / Clindamycin treaed group d) AEL e) AEB f) AET

Discussion

Propionibacterium acnes is the gram-positive bacterial species, non-spore forming, anaerobic to aero tolerant diphtheroid bacillus that produces propionic acid, as its name suggests, it also has the ability to produce catalase along with indole, and/or nitrate, and acetic acids as major end products of fermentation. It leads to severe infection of skin commonly called as acne vulgaris, also leads to corneal ulcers, heart valves and prosthetic devices. *P. acnes* is also of little interest to neurosurgeons as cases of serious intracranial infection due to this organism are also reported. A heart disease known as *P. acnes* endocarditis has been discovered in a prosthetic valve infected with *P. acnes* [36]. *P.granulosum* is found in the same areas but at numbers about one hundredth of those of *P. acnes*. Both *P. acnes* and *P. granulosum* may be isolated from the gastrointestinal tract. *P. avidum* is found in the axilla, rather than on exposed areas, and increases in numbers at puberty. *P. propionicus* has been implicated as a less common causative agent of a disease process similar to actinomycosis. Thus, the germ theory of this century enabled the eradication of most microbial infections through the use of antibiotics and anti-viral drugs. Thus, the rapid and accurate analysis of *P. acnes*, an invading organism, is needed.

In current study, we examined the anti-bacterial and anti – inflammatory properties of ethanolic extract of leaves, bark and thorns of the plant *S. malabarica* to evaluate its anti-acne activity in the *P. acnes* induced animal model. The results demonstrated that administration of ethanolic extract of thorns was capable of inhibiting the growth of bacteria *P. acnes* at MIC 250 µg/ml which was lower than Std, AEL, AEB. Also, AET showed strongest anti-acne activity against *P. acnes* in animal model.

This may be due to the presence of antioxidants and polyphenols in the plant extracts, which can be beneficial in the reduction of inflammatory response in acne vulgaris. The treatment modalities of acne are usually aimed at decreasing the *P. acnes* population, producing anti-inflammatory effect and decreasing the sebaceous gland activity[11].

Although only one strain of *P. acnes* was tested, a compound active against one strain is expected to retain a similar order of activity against a variety of strains of this species. On the other hand, the possibility of resistance among strains is always present. With these considerations in mind, the plant can be tested for other diseases or disorders caused by *Propionibacterium* strains. The animal model given here can also be used further for other anti-acne agents. However, further studies are needed to comment more in this respect.

Conclusion

Our findings demonstrated the favorable anti-bacterial and anti-inflammatory activities supporting the anti-acne activity. The in-vivo and in-vitro study affirmed the claim done by traditional medicine practitioner that thorns of *S. malabarica* could treat acne, reduces inflammation and that it possesses anti-bacterial property. Nevertheless this plant contains too many compounds and possibly several other properties. Therefore, it is necessary to further investigate the chemical components of this plant to clearly elucidate its mechanism of action.

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