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Development and Evaluation of Anti-Acne Products from *Terminalia arjuna* Bark

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Abstract : *Propionibacterium acnes* and *Staphylococcus epidermidis* have been recognized as pus-forming bacteria triggering an inflammation in acne. The present study was conducted to evaluate antimicrobial activities of *Terminalia arjuna* bark against these etiologic agents of acne vulgaris. Topical formulations (cream) have been developed containing flavonoid (FF-I to III) and tannin fraction (TF-I to III) of *Terminalia arjuna* bark at different concentrations i.e. 0.5%, 1% and 2% (w/w). These topical formulations were tested for pH, viscosity, spreadability, stability, drug contents uniformity and *in vitro* diffusion. The drug content uniformity of creams were found within the range of 97.31% to 98.80% (FF-I to III) and 96.84% to 98.66% (TF-I to III) respectively. The formulations of FF -III and TF –III showed maximum drug release of 83% and 78% over a period of 8h. All the formulations were evaluated for its acute skin irritancy activity in Swiss Albino rats. These formulations did not produce any skin irritation for about a week when applied over the skin. Comparative studies showed that the viscosity of the formulations increases, spreadability decreases and vice versa. From the stability studies, creams showed no changes in pH, viscosity, spreadability and drug contents, after keeping at different temperatures for 90 days.

In vitro antibacterial activity was performed against *Propionibacterium acnes* (P. acnes) and *Staphylococcus epidermidis*, a causative organism for Acne vulgar for the developed formulations using agar well diffusion method. The measured zones of inhibitions of the formulations were compared with standard marketed topical herbal preparation for acne. Results of the investigation showed that formulation FF-III (cream containing 2% flavonoid fraction) has greater antibacterial activity against *Propionibacterium acnes* (zones of inhibition >17 mm) and *Staphylococcus epidermidis* (zones of inhibition >20 mm) than other formulations and which is comparable to that of standard marketed topical herbal preparation. Therefore, this plant would possibly for an alternative treatment for acne.

Key words: *P. acnes, Terminalia arjuna,* Cream, Flavonoid fraction, Tannin fraction.

Introduction

Acne vulgaris is a most common skin disorder of Pilosebaceous unit, that affects areas containing the largest oil glands, including the face, back, and trunk [1]. It is generally characterized by formation of seborrhea, comedone, inflammatory lesions and presence of bacteria *Propionibacterium acnes, Staphylococcus epidermidis* and *Malassezia furfur* in the follicular canal and sebum production [2]. *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 [3]. These factors provide a potential target for treatment. *Propionibacterium acnes* and *Staphylococcus epidermidis* are the target sites of antiacne drugs [4,5].

Long term use of antibiotics against acne is outdated because of exacerbated antibiotic resistance [6,7]. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotics, how the antibacterial is used, host characteristics, and environmental factors. To overcome the problem of

antibiotic resistance, medicinal plants have been extensively studied as alternative treatments for diseases. In the present study, the bark of the plant *Terminalia arjuna* belonging to the family Combretaceae, which have been traditionally used as astringent**,** wound healing, cardiac stimulant, heamoptysis, lithontriptic and also useful in bilious infections, diarrhoea and in acne [8] was selected for the proposed study. The use of flavonoids and tannins in the treatment of diseases is much older than the science of chemistry. Flavonoids exhibit several biological effects such as anti oxidant, anti cancer, anti-inflammatory, anti ulcer, hepatoprotective, and anti microbial [9]. Tannins exhibit cytotoxic, anti-tumour, astringent, anti-hemorrhagic, anti-oxidant, anti-bacterial [10] etc. From folklore claiming and literature review, bark of *Terminalia arjuna* was found to contain flavonoids, tannins, triterpenoid and glycosides [11].

In the present study, 3 topical formulations (Cream) have been developed containing flavonoid fraction and 3 topical formulations containing tannin fraction of *Terminalia arjuna* bark. The developed formulations were examined for antimicrobial activities against microorganism frequently involved in acne inflammation, *P. acnes* and *S. epidermidis.*

Materials and Methods

Plant material

The Plant specimen for the proposed study was purchased from commercial source in Hyderabad, Andhra Pradesh and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Center, (PARC) Tambaram, Chennai. The specimens were deposited at Department of Pharmacognosy (No. 390), School of pharmaceutical sciences, Vels University, Tamilnadu, India.

Isolation of Flavonoid fraction

The stem bark of *Terminalia arjuna* (W$A) was dried, coarsely powdered and extracted using a soxhlet apparatus with methanol (18 h) [12]. The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator). The concentrated extract was again exhaustively defatted by refluxing with n-hexane and benzene (15 h twice). The two fractions were negative for polyphenols. Then the defatted bulk residue was successively extracted by refluxing with ethyl acetate (15 h twice). Total flavonoids [13] were spectrophotometrically estimated in the ethyl acetate fraction which was found to contain the bulk of flavonoids (total flavonoids content 410.57 ± 16.23 mg QE/g dry weight) and this fraction was evaporated in a rotary evaporator under reduced pressure, freeze-dried and used for the study.

Isolation of Tannin rich fraction

The stem bark of *Terminalia arjuna* (W$A) was dried and coarsely powdered. About 100 gms of powder was defatted with petroleum ether. The defatted bark powder was extracted with acetone (70 % v/v) by cold maceration method and this fraction was evaporated in a rotary evaporator under reduced pressure, freeze-dried and used for the study. Total tannins [14] were spectrophotometrically estimated in the acetone fraction which was found to contain the bulk of tannins (total tannins content 320.14 ± 12.47 mg QE/g dry weight).

Thin layer chromatography

A number of developing solvent systems were tried for fractions showing presences of flavonoids, but the satisfactory resolution was obtained in the solvent systems Toluene: Ethyl acetate: Formic acid (6:5:1) and the plates were dried and detected by spraying with NaOH solution, a reagent specific for flavonoids. For tannin fraction, the solvent 5% acetic acid was used. After development, the plates were air dried and detected with 5% anisaldehyde sulphuric acid [15].

Microorganisms and media

The test organisms used in this study were as followed: *Propionibacterium acnes* (MTCC 1951) and *Staphylococcus epidermidis* (MTCC 931). These bacteria were obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. All media were purchased from Himedia.

Determination of minimum inhibitory concentrations

The minimal inhibitory concentration (MIC) values were determined by broth dilution assay [16]. The cultures were prepared at 24 h and 48 h broth cultures of *Staphylococcus epidermidis* and *Propionibacterium acnes*, respectively. The MIC was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. Six sterile test tubes with 9 ml sterile nutrient broth were taken. 1ml of different concentration of drug solution was added and 0.1ml inoculum was also added to the test tube aseptically and media blank with the nutrient broth and the drug solution was also prepared. A positive control, containing media with 0.1ml inoculum was maintained to indicate the growth promotion capacity of the media. Test samples of *Staphylococcus epidermidis* were incubated at 37°C for 24 hours and those of *Propionibacterium acnes* were incubated under anaerobic condition in an anaerobic jar (Hi-Media) with gas pack for 48h.

MBC was determined by subculturing the samples on to sterile nutrient agar plates, from the three test tubes which had shown no growth during determination of MIC. The plates were incubated following the procedure as described in MIC determination. The minimum bactericidal concentration values were interpreted as the highest dilution (lowest concentration) of the sample, which showed no growth on the agar plates.

Development of Formulations

Six batches of the cream were prepared and used in the study. Batches FF-I to FF-III contained varying concentration of the flavonoid fraction (0.5 %, 1.0 % and 2% respectively) Batches TF-I to TF-III contained varying concentration of the tannin fraction (0.5 %, 1.0 % and 2% respectively). The oily phase that consisted of paraffin oil (16 %) and surfactant ABIL- M 90 (3.5 %) was heated up to 75 $\pm 1^{O}$C. The aqueous phase consisting of water (quantity sufficient to make 100 %) and flavonoid rich fraction (0.5 %, 1.0 % and 2%) was heated to 75 $\pm 1^{O}$C. After that, aqueous phase was added to the oil phase drop by drop using a stirring speed of 2000 rpm by the mechanical mixer for about 15 minutes until complete aqueous phase was added. Mean while few drops of lemon oil were added during this stirring time to give good fragrance to the formulation. After the complete addition of the aqueous phase, the speed of the mixer was reduced to 1000 rpm for homogenization, for a period of 5 minutes, and then the speed of the mixer was further reduced to 500 rpm for 5 minutes for complete homogenization; until the cream cooled to room temperature. A similar procedure was followed for developing a cream containing tannin rich fractions (0.5 %, 1.0 % and 2%) of *Terminalia arjuna* bark.

Evaluation

Drug content

Each formulation (1gm) was accurately weighed and transferred to 100 ml volumetric flask to which about 70 ml of methanol was added. After shaking, the volume was made up to 100 ml with methanol. The content was filtered through a suitable filter paper. 1ml filtrate was taken and suitable diluted and the drug content (extract) was estimated by using UV/Visible spectrophotometer, (SL-159-Shimadzu-1700, SI-164 Double Beam) at 271nm. Results given in **Table. 3** are the average of triplicate values. Drug contents values are expressed as Mean ± Standard deviation.

Drug release

The *in vitro* diffusion studies of the creams were performed by using dialysis membrane (Sigma Inc. MO, USA; dry, unwashed, pre-cut and open ended; fiat width: 35 mm; inflated diameter, 21mm; Length: 30mm). The membrane soaked in phosphate buffer pH 7.4 for 6-8 h was clamped carefully to one end of the hollow glass tube of dialysis cell (2.3 cm diameter, 4.16 cm2 area). 100 ml of phosphate buffer was taken in a beaker, which was used as receptor compartment for the study. 1gm of each formulation were spreaded uniformly on the membrane. The donor compartment was kept in contact with the receptor compartment and the temperature was maintained at 37 ± 0.5^{0}C. The solutions on the receptor side were stirred by externally driven Teflon-coated magnetic bars. At pre-determined time intervals, 5 ml of solution from the receptor compartment was pipetted out and immediately replaced with 5 ml fresh phosphate buffer solution. The drug concentration of the receptor fluid was determined spectrophotometrically at 271nm against appropriate blank [17]. The amount of drug permeation of all the formulations were calculated. This experiment was carried out in triplicate and the results were extrapolated in the Fig. 2.

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Physical evaluations

Preliminary evaluation of formulations was carried out as follows:-

pH [18]

The pH of various formulations was determined by using Digital pH meter (Digital pH meter 335, Systronics, Noroda, Ahmedabad). One gram of cream was dissolved in 100 ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were depicted in Table. 3.

Viscosity [19]

The measurement of viscosity of prepared creams was carried out with Brookfield Viscometer (model LV-DV-II, Helipath-spindle type S-96). The values of each formulation were depicted in Table. 3.

Spreadability [20]

Spreadability denotes the extent of area to which the gel readily spreads on application to skin or the affected part. The bioavailability efficiency of a cream also depends on its spreading value. The Spreadability was expressed in terms of time in seconds taken by two slides to slip off from the cream, placed in between the slides, under certain load. Lesser the time taken for separation of the two slides, better the Spreadability. Two sets of glass slides of standard dimensions were taken. The cream formulation was placed over one of the slides. The other slide was placed on the top of the cream, such that the cream was sandwiched between the two slides in an area occupied by a distance of 6.0 cm along the slide. 100

gm weight was placed upon the upper slides so that the cream between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of cream adhering to the slides was scrapped off. The two slides in position were fixed to a stand without slightest disturbance and in such a way that only the upper slide to slip off freely by the force of weight tied to it. A 20 gm weight was tied to the upper slide carefully. The time taken for the upper slide to travel the distance of 6.0 cm and separated away from the lower slide under the influence of the weight was noted. The experiment was repeated by three times and the mean time taken for calculation.

Spreadability was calculated by using the following formula:

$$S = m \times \frac{l}{t}$$

Where,

S - Spreadability
m - Weight tied to the upper slide (20gm)
l - Length of the glass (6 cm)
t - Time taken in seconds.

Acute skin irritation study [21]

The primary skin irritation test was performed on albino rats and weighing about 150-200 gm. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing seven animals. Two batches of each were used for control and test. Dorsal hairs at the back of the rats were clipped off one day prior to the commencement of the study. Animals showing normal skin texture were housed individually in cages with copography meshes to avoid contact with the bedding. 50 mg of the each formulation of different concentrations were applied over one square centimeter area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for seven days for any signs of oedema and erythema.

Stability studies [22]

The stability studies were carried out in all formulations at different temperature conditions (4^o, 25^o and 37^oC) for 3 months. All the evaluation parameters i.e. pH, viscosity, spreadability, drug contents, consistency and phase separation studied at different time intervals i.e. 15^{th} , 30^{th} , 60^{th} and 90^{th} days.

Sample Preparations

Solutions of creams were prepared using 100 mg of cream in 10 ml of dimethyl sulfoxide (DMSO). Similarly, Solution of marketed formulation clindamycin phosphate cream [Clincitop cream, universal twin labs] was prepared. Clindamycin (10 µg/ml) was used as a positive control and DMSO as a negative control.

Antimicrobial testing using agar well diffusion method

The antibacterial activity of different formulations was determined by modified agar well diffusion method [23]. *Propionibacterium* acnes was incubated in brain heart infusion medium (BHI) with 1% glucose for 48 h under anaerobic conditions and adjusted to yield approximately 1.0×10^8 CFU/ml. Aliquots of molten BHI with glucose agar were used as the agar base. A prepared inoculum was added to the molten agar, mixed, poured over the surface of the agar base and left to solidity. A sterile 8 mm borer was used to cut wells of equidistance in each of plates; 0.5 ml of solutions of marketed formulations and standard cream containing clindamycin (10 µg/ml) were intoduced in to the wells at randomly. The plates were then incubated at 37°C for 48 h under anaerobic conditions in an anaerobic jar (Hi-Media) with gas pack and indicator strip and the jar was kept in an incubator for 48 h at 37 ± 1°C. Gas packs containing citric acid, sodium carbonate and sodium borohydride were used to maintain and check the anaerobiosis, where citric acid releases carbon dioxide and sodium borohydride releases hydrogen when they come in contact with oxygen. An indicator strip of methylene blue, when introduced into the jar, changes in colour from white to blue in the absence of anaerobiosis. *Staphylococcus epidermidis* was incubated in tryptic soy broth (TSB) for 24 h at 37°C and adjusted to yield approximately 1.0×10^8 CFU/ml. The procedures were the same as mentioned above except the plates were incubated at 37°C for 24 h under aerobic conditions The antibacterial activity was evaluated by measuring the diameter of zones of inhibition (in mm). The experiments were performed in three separate experiments. (Table. 4).

Table. 1: Thin layer chromatography of flavonoid fraction and tannin fraction of *Terminalia arjuna* bark

Test extract	Solvent system	Number of spots	R_f values	Detecting agent
Ethyl acetate Fraction	Toluene : Ethyl acetate : Formic acid (6:5:1)	5	0.87 0.76 0.62 0.38 0.17	NaOH
Acetone extract	5 % acetic acid	6	0.87 0.77 0.65 0.64 0.59 0.17	5 % Anisaldehyde sulphuric acid

Table. 2: The MIC and MBC values of flavonoid and tannin fraction against *Propionibacterium acnes* and *Staphylococcus epidermidis*

Drug	*Propionibacterium acnes*		*Staphylococcus epidermidis*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Flavonoid fraction	0.315	0.625	0.186	0.312
Tannin fraction	0.425	1.25	0.156	0.420

Table. 3: Physicochemical evaluations of different formulation of creams

Formulations	Drug Content (%)	pH	Viscosity (cps)	Spreadability (gm-cm/sec)
FF-I	97.31±0.32	6.36	267500	2.935
FF-II	98.28±0.56	6.18	378660	3.690
FF-III	98.80±0.47	5.80	535480	5.192
TF-I	96.84±0.22	6.90	267700	2.736
TF-II	97.46±0.43	6. 24	330166	4.057
TF-III	98.66±0.27	6.06	534333	4.970

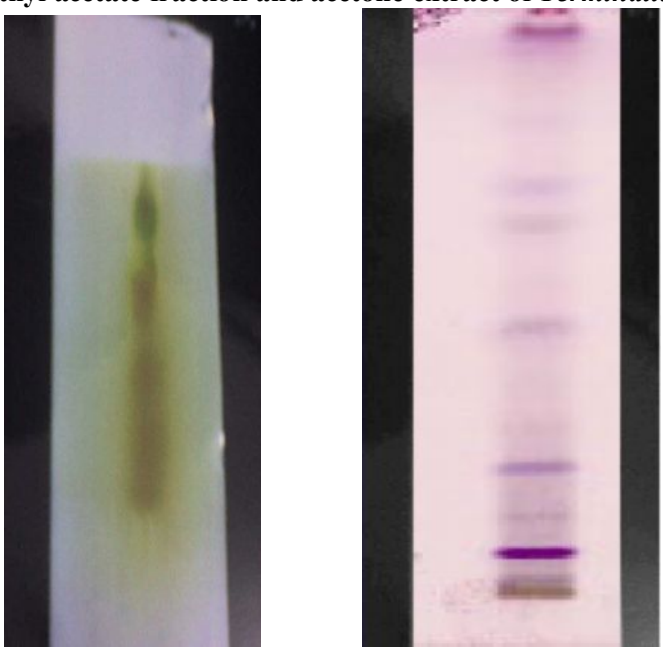
FF-I to FF – III – Cream containing flavonoid fraction (0.5 %, 1.0 % and 2% respectively)
TF-I to TF – III – Cream containing tannin fraction (0.5 %, 1.0 % and 2% respectively)

Table. 4: Antibacterial activity of various formulation against *Propionibacterium acnes* and *Staphylococcus epidermidis*

Formulation	Diameter of Zone of Inhibition (mm)	
	Propionibacterium acnes	*Staphylococcus epidermidis*
FF-I	12.70±0.47	13.50±0.41
FF-II	15.30±0.47	17.30±0.82
FF-III	17.90±1.8	20.50±1.20
TF-I	10.80±0.63	13.60±0.82
TF-II	14.45±0.40	16.80±0.47
TF-III	15.60±0.31	19.50±0.80
Marketed herbal preparation	17.15±0.31	20.15±2.40
Clindamycin (10 µg/ml)	18.75±0.69	22±1.30
DMSO	-	-

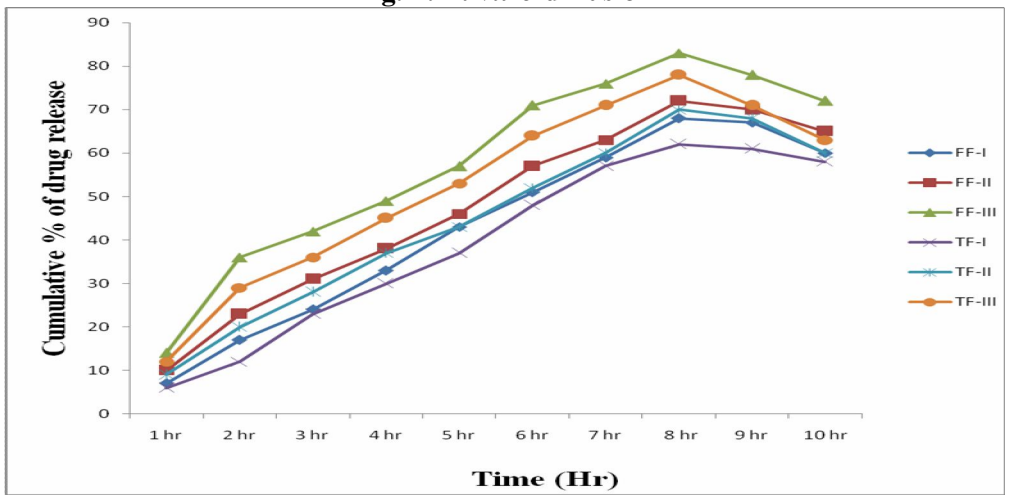
FF-I to FF – III – Cream containing flavonoid fraction (0.5 %, 1.0 % and 2% respectively)
TF-I to TF – III – Cream containing tannin fraction (0.5 %, 1.0 % and 2% respectively)
Standard - Cream containing clindamycin (10 µg/ml) **,Mean ±S.D (n = 4)**

Fig. 1: TLC of ethyl acetate fraction and acetone extract of *Terminalia arjuna* (W&A)



Ethyl acetate fraction **Acetone extract**

Fig. 2: *In-vitro* diffusion



Results

In the present study, flavonoid and tannin fraction were isolated from *Terminalia arjuna* bark and their percentage yield was 14.3 and 22.3% w/w respectively. Preliminary phytochemical screening of ethylacetate fraction showed presence of flavonoids and acetone extract showed presence of tannins. TLC findings were in agreement with the data of qualitative chemical tests. Ethyl acetate fraction (flavonoid fraction) showed well marked 5 spots and acetone extract (tannin fraction) showed well marked 6 spots, shown in Fig. 1 and Table. 1. Topical formulations (cream) have been developed containing flavonoid (FF-I to III) and tannin fraction (TF-I to III) and the developed formulation were examined for antimicrobial activity against *Propionibacterium acnes* and *Staphylococcus epidermidis*. MIC and MBC values of flavonoid and tannin fraction against *P. acnes* and *S. epidermidis* were determined by broth dilution assay. MIC values of flavonoid fraction were 0.315 and 0.186 mg/ml against *P. acnes* and *S. epidermidis* respectively, whereas MIC values of tannin fraction were 0.425 and 0.156 mg/ml, against *P. acnes* astringent**,** wound healing, cardiac stimulant, heamoptysis, lithontriptic and also useful in bilious infections, diarrhoea and in acne respectively. MBC values of flavonoid fraction were 0.625 and 0.312 mg/ml against *P. acnes* and *S. epidermidis* respectively, whereas MBC values of tannin fraction were 1.25 and 0.420 mg/ml, against *P. acnes* and *S. epidermidis* respectively (Table. 1).

The various physicochemical parameters utilized to evaluate the prepared cream formulations are shown in Table. 2. *In vitro* release of cream prepared from flavonoid fraction (FF-I to III) and tannin fraction (TF-I to III) of *Terminalia arjuna* bark were found to be slow and extended over the longer period of time. All the formulations showed only slight difference in release profile at the particular time period. The FF-III and TF-III showed maximum release of 83% and 78% over a period of 8 h. The pH of the formulations from FF-I to FF-III and TF-I to TF-III were in between 6.36 to 5.80 and 6.90 to 6.06 respectively, which lie in the normal pH range of the human skin. The drug content uniformity of the formulations were found within the range of 97.31% to 98.80% (FF-I to III) and 96.84% to 98.66% (TF-I to III) respectively. All the formulations did not produce any skin irritation, i.e., erythema and edema for about a week when applied over the skin. The rheological behaviors of the different formulations of cream were studied with Rotational Brookfield Viscometer. The results indicated that the torque and shear stress increases where as viscosity decreases. A comparative study of viscosity and spreadability showed that the viscosity of the formulations increases, spreadability decreases and vice versa. From the stability studies, creams at the concentrations of 0.5 %, 1.0 % and 2%, showed no changes in pH, viscosity, spreadability, extrudability, drug contents, consistency and phase separation after keeping at different temperatures for 90 days.

The results of this investigation showed that all developed formulations had inhibitory effect on the *P. acnes* and *S. epidermidis*. Formulation FF-III has higher activity than that of other developed formulations. The activity of the developed formulation FF-III has been comparable to that of marketed preparation. Standard Clindamycin was more than that of all developed formulations, marketed herbal anti-acne preparation. DMSO did not have activity. The diameter of zones of inhibitions is given in Table-3.

Discussion

Acne vulgaris is an extremely common skin disorder that affects virtually all individuals at least once during life. The incidence of acne peaks at teenage, but substantial numbers of men and women between 20-40 years of age are also affected by the disorder. Acne can have important negative psychosocial consequences for the affected individual, including diminished self-esteem, social withdrawal due to embarrassment and depression. Herbal medication are considered safer than allopathic medicines as allopathic medicines are associated with side effects such as like contact allergy, local irritation, scaling, photosensitivity, itching, pruritus, redness, skin peeling, xerosis of the skin etc [24]. The present research work deals with formulation and evaluation of herbal anti- acne creams containing flavonoid fraction and tannin fraction from *Terminalia arjuna* bark. The plant has been reported in the literature as wound healing and in acne.

The developed formulations were evaluated for their in vitro antibacterial activity against *P. acnes* and *S. epidermidis*. The Zones of inhibitions for the antibacterial activity of the formulations were compared with the standard clindamycin and herbal marketed preparation for acne vulgaris. Formulation FF-III and TT-III has shown comparable zones of inhibitions to that of the marketed preparation. Formulation FF-III exhibited more zone of inhibition than TT-III. *Terminalia arjuna* bark with active constituent flavonoids and tannins may be responsible for the significant antibacterial activity. The antimicrobial activity of phenolics (tannins, flavonoids) and saponins has been established in some plants [25, 26]. Flavonoids were becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity [27]. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. The mechanism of action of flavonoids and tannins is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds [28]. These results suggested that the preparations incorporating flavonoids and tannins of the *Terminalia arjuna* bark could be used as an alternative treatment for acne.

Conclusion

Natural remedies are more acceptable in the belief that they are safer with fewer side effects than the synthetic ones. So, herbal anti-acne cream which is non-toxic, safe, effective and improves patient compliance by the utilization of herbal extracts would be highly acceptable.

Therefore, the active component flavonoids and tannins of the *Terminalia arjuna* bark could be of interest for further development as anti-acne products, however further clinical research will be necessary.

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