Original Research Article

SENSITIVITY OF *PROPIONIBACTERIUM ACNES* TOWARDS COMMERCIAL ANTI-ACNE FORMULATIONS

Abstract

Propionibacterium acnes is a gram-positive anaerobe found prevalently in the sebum-rich follicles of the skin. They produce several pro-inflammatory substances that can trigger an immune response in the host by an influx of inflammatory leukocytes into the follicles, causing inflammatory lesions that leave behind scars. Repeated isolation of *Propionibacterium acnes* may reduce efficacy among the resistant types which clearly explains the importance of Acne lesions. The Counter acne therapies are often the first choice of treatment due to the convenience of cost and time over clinical appointments. However, not all of the commercially available anti-acne formulations are supported by clinical studies. The present study was conducted to test the efficacy of selected commercial anti-acne gel formulations. Microscopic observation and biochemical tests were performed and confirmation results were obtained. A sensitivity test was performed on all the isolates of Propionibacterium *acnes* by well diffusion technique, in which the selected over counter anti-acne gel formulations failed to produce any inhibition zone.

Keywords: *Acne Vulgaris, Propionibacterium, facial acne lesions, Sensitivity test*

Introduction

Acne vulgaris is a common skin disorder of the pilosebaceous unit, with the severity

ranging from mild to chronic. The condition can most commonly be seen in 80% of adolescents and young adults (Khorvash*et al.,* 2012). Human skin in one of the biggest organs present across the body, which consist of various tiny microorganism that are associated with skin, includes *Malassezia, Propionibacterium, Corynebacterium, Streptococcus* and *Staphylococcus*. Out of which a gram-positive anaerobic bacteria called *Propionibacterium acnes* a common human skin microbiota and also controls the pilosebaceous. The uniqueness of this propioni bacteria is they are capable in maintaining skin through environmental niches which occupied by various pathogenic microbes. They produce bacteriocins, short chain fatty acids, thiopeptides and few other molecules which have a capability of inhibiting various organisms (Pochi*et al.,* 1988*). P. acnes* and *P. granulosum* are commonly found in sebaceous gland-rich areas of skin while *P. acnes* can also see in other parts of the body such as gastrointestinal system, prostate and also found on the surface of mouth.The propioni bacteria provide a support and maintain the microbial balance in skin but they are not so beneficial which may cause diseases with improper set of conditions. The disorder in some cases may leaves permanent scars on the skin diminishing which causes psychological and social well-being leads to negative effects in young adolescents such as discomfort, emotional stress (Fabbrocini*et al.,* 2010) anxiety, and embarrassment (Purvis *et al.,* 2006). In acne-prone skin, hyper-proliferation of the keratinocytes occurs and the abnormally desquamated corneocytes accumulate in the sebaceous follicle along with other lipids and debris, which blocks the follicle, and hence a non-inflammatory micro papule is formed. The pathogenesis of acne is multifactorial and the four main pathological factors involved are increased which includes sebum production, epidermal hyper-proliferation, irregular follicular desquamation, and bacterial proliferation and inflammation (Dessinioti*et al.,* 2010; Roselin*et al.,* 2010).The microflora present in a normal sebaceous follicle is qualitatively similar to that found in papules which includes three coexisting groups of bacteria namely coagulase-negative staphylococci,

anaerobic diphtheroids, and lipophilic yeasts.The main goal of acne treatment is to control existing acne lesions, permanent scarring, limit the duration of the disorder, and minimize morbidity. A combination treatment that targets more than one of the mechanisms of acne pathogenesis is often successful. Few studies suggest the non-antibiotic agents are used for the treatment of mild to moderate acne which can be used as monotherapy or in combination with antibiotics to enhance the efficacy of treatment and also reduce the development of antibiotic resistance in *P. acnes*. Combined agents are found to be more effective, due to the synergistic effect; these combinations show antibacterial resistance in *P. acnes* and are much more effective in combination when they are used individually. Combination exerts bactericidal effectswhich are capable in decrease in *P. acnes* counts (Alexeyev*et al.,* 2012). Prolonged use of antibiotics, topical application in particular results in the development of *P. acnes* resistant strains (Achermann*et al.,* 2014; Ramasamy *et al.,* 2019). Among various antibiotics over the counter (OTC) anti-acne formulations consist of antibiotics and non-antibiotics either as monotherapy or most often in combination, designed to target at least one of the pathogenic pathways that are reported to be involved in the development of acne. Similarly in the present study we tried to check the efficacy of Commercial Anti-Acne formulations against *propionibacterium acnes.*

Materials and Methods

Isolation of *P. acnes* aerobically

The *P. acnes* was isolated from acne lesions. Three samples were randomly collected from a volunteer between the age group 18- 21 years. The samples were collected using a sterile Himedia swabs and were stored in a brain heart infusion broth (BHI) and Nutrient broth (NB). 1 cm2 area from the facial skin from three volunteers was smear with sterile swabs and were stored in test tube containing 10ml of nutrient broth (NB) and was incubated from 4days in Anaero Gas Pak. The incubated samples were later

streaked on nutrient agar and incubated at 37°C for 4 days. The obtained colony morphology was observed and stained using gram staining. (Hug *et al.,* 1999).

Staining and bacterial observation

Gram stain was performed as described previously with slight modifications, From the collected samples, a loop full of the samples was smeared on clean glass slides, air-dried and heat-fixed. Crystal violet was added on to the samples and incubate for 5min at room temperature. After incubation the glass slides were gently rinsed under tap water in order to remove excess of crystal violet. Further Gram iodine were added and kept it for 2 min and washed with tap water. The grams decolorizer was added in order to remove excess of crystal violet stain for about 30s and quickly rinsed under tap water. The drop of Safranin stain was added and kept it for a 1 minute and followed by dehydration using 70% ethanol and coverslip were placed. (Bisen*et al.,* 2014; Abiola *et al.,* 2016).

Isolation and purification of *P.acnes*colony

In order to isolate the *P.acnes* bacteria from a cluster of bacteria's, 1ml of culture nutrient broth were spread on nutrient agar plates. The collected samples were serially diluted, 1ml of 5-fold serial diluted samples were spread on nutrient agar plates. The culture plates were incubated at 37^0C whereas mFC agar were incubated at 45^0C for about 24 hours. The obtained colonies were further counted, characterized and recorded.Colonies were purified by twice subculturing using the streaking plate method. The obtained colonies were further purified by repeated subculturing using streak plate technique. The cultures were subjected to gram staining and were identified as gram positive *P.acnes*. Further the isolated bacteria were subjected to biochemical identification test.

Biochemical characterization of *P.acnes*

Catalase test

A loop of the colony was smeared on a clean glass slide and a few drops of 3%

hydrogen peroxide were added. The production of air bubble indicates the present of catalase and no air bubble indicates the absence of catalase. (Bisen*et al.,* 2014).

Indole test

Indole test is used to determine the presence of *P.acnes*. The test organism was cultures on Tryptone broth media cultured in bijou bottle and incubated at 37°C for four days. To the media 0.5 ml Kovac's reagent were added and gently shake and the obtained colored ring was observed (Abiola *et al.,* 2016).

Nitrate test

Nitrate broth is prepared and inverted Durham's tube is added into the medium without any appearance of air bubbles, and then a loop of the colony was inoculated into the medium and incubated at 37°C for four days. To the culture tube add 2 to 3 drops of nitrite reagent A and B and the reaction culture was observed (Moss *et al.,* 1967).

Sugar fermentation test

Purple base broth was prepared and added to two test tubes with an inverted Durham's tube added without the appearance of air bubbles, one of the tubes is marked as control. A loop of the colony was inoculated into the medium and incubated at 37°C for about 24 hours and the obtained yellow color conforms the positive results of sugar fermentation test (Moss *et al.,* 1967).

Hemolytic test

To 1.25 ml of 5% defibrinated sheep blood was added and mixed, the prepared medium was poured into a Petri plate and allowed to solidify, after which the culture was inoculated on the medium by spread plate technique and kept for incubation at 37°C for four days (Moss *et al.,* 1967; Bakht *et al.,* 2011)

Gelatin hydrolysis

From the culture test bacterial plates, a loop of colony was stabbed into the gelatin media using a streaked as a single line and incubated at 37°C for 24 hours. To the plate a

iodine solution were added to check the starch utilization (Moss et al., 1967).

Methyl red test

The test organisms were culture of MR broth and incubate at 37°C for about 48 hours. After incubation 1ml of broth was transfer into two test tubes, where one of the tubes is used as control. To these tubes 2 to 3 drops of methyl red was added, the formation of red color indicates the presence of positive methyl red test whereas yellow color indicates the negative results of methyl red test. (Abiola *et al.,* 2016)

Antimicrobial activity by well diffusion method

50µl of bacterial samples were pipetted onto two solidified brain heart infusion agar plates and spread evenly on the surface using a glass rod until completely absorbed by the media. The two agar plates were then labeled as plate 1 and 2, each of which was divided and marked as four quadrants namely A, B, positive control (PC), and anti-acne gel (Cl). Four wells were then made in the four quadrants of each plate using a cork borer (Valgas*et al.,* 2007; Magaldi*et al.,* 2004; Bakht *et al.,* 2011). Ampicillin was used as the positive control and therefore 200µl was pipetted into the (PC) labeled well of the two plates. 200µl of Distilled water used for the preparation of stock solution was poured into well (A) as a negative control in both the plates. 200µl of anti-acne gels from both the stock solutions C1 and C2 was added to well (Cl) and well (B) of plate 1, and stock solutions B1 and B2 were pipetted into the wells marked as (Cl) and B of plate 2 in the respective order. The plates were then kept for incubation at 37°C for 24 hours (Bakht *et al.,* 2011; Holder *et al.,* 1994).

Results and Discussion

Isolation and cultureof*P.acnes*

*Propionibacterium acnes*was collected from a surface swab of facial acne skin lesions and suspended in a nutrient broth; post aerobic incubation growth was seen by the appearance of biofilms and also the turbidity was found at the bottom of tube

which conforms the presence of *P. acnes.*

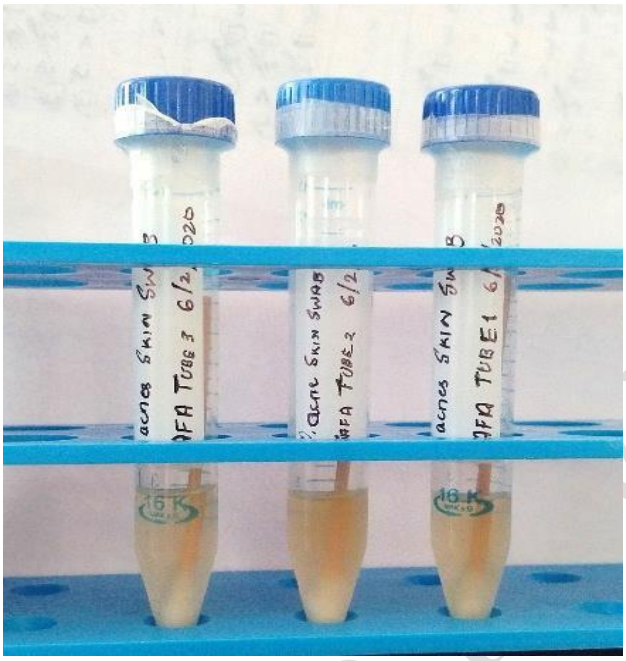
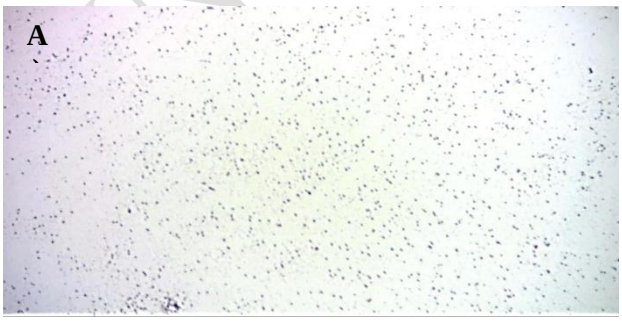
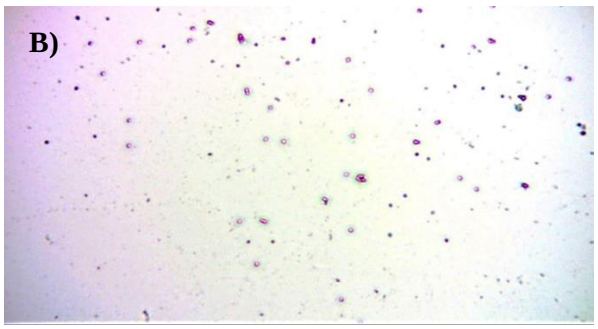


Figure 1: Formationofbiofilms conforms the presence of *P. acnes* after postincubation

Gram staining

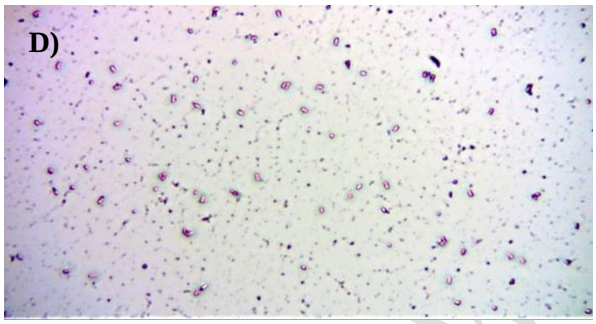
The obtained isolates were further examined using gram staining. Through this staining technique it conforms that the isolate consists of numerous gram-positive bacteria. The isolated Gram-positive bacteria were stained namely *staphylococci*, *diplococci, tetrads* and *streptococci* were conformed under the magnification of 10x and 40x



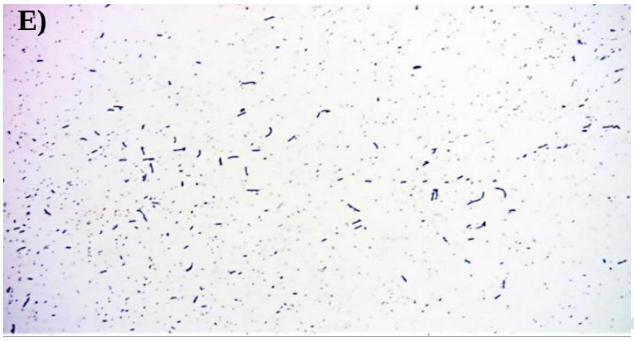


***Staphylococci* (10x)**

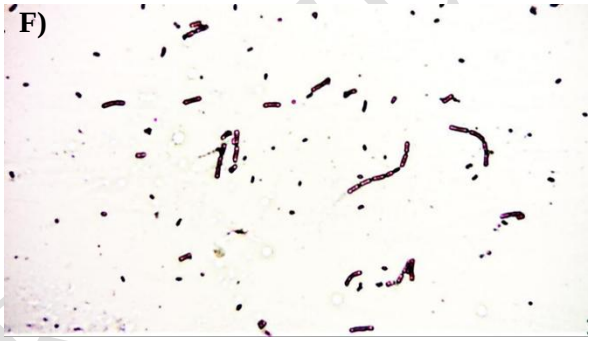
***Staphylococci* (40x)**



***Diplococci* and *tetrads* (10x)**



***Diplococci* and *tetrads* (40x)**



***Streptococci*(10x)**

***Streptococci* (40x)**

Figure2: Obtained gram-positive bacteria were stained namely *staphylococci*, *diplococci, tetrads* and *streptococci*where, figure A,C,E were observed under the magnification of 10x and figure B,D,F were observed under the magnification of 40x

Serial dilution

In order to obtained pure culture of *Propionibacterium acnes* serial dilution was carried out using spread plate method and the obtained colonies were further characterized and conforms the presence of *P.acnes*. In the present study the obtained bacterial colonies were subculture and serially diluted in order to obtained a pure culture from the bulk samples. The samples were serially diluted ranging from 10^{-1} to 10^{-5} and were showedin table 1

Table 1: Serial diluted bacterialcolonycharacteristics

Dilution factor	Total No. of colonies	Colony forming unit (CFU)	Colony number	Form	Elevation	Margin	Color
10-1	638	638×10^{-1}	1	Irregular	Crateriform	Filamentous	Pale White
10-2	5	5×10^{-2}	1	Circular	Convex	Entire	Yellow
10-3	27	27×10^{-3}	1	Irregular	Convex	Filamentous	Pale White
10-4	0	0×10^{-4}	-	-	-	-	-
10-5	3	3×10^{-5}	1	Irregular	Crateriform	Filamentous	Pale White

Culture isolation and Pure of *P. acnes*

The serially diluted samples were further inoculated on nutrient agar plates using streak plate method. The dilution was repeated for several time and the pure culture colony of *P. acnes* was further conformed using gram staining and biochemical characterization. The morphology, elevation, margin and color conform the presence of

P. acnes bacteria.

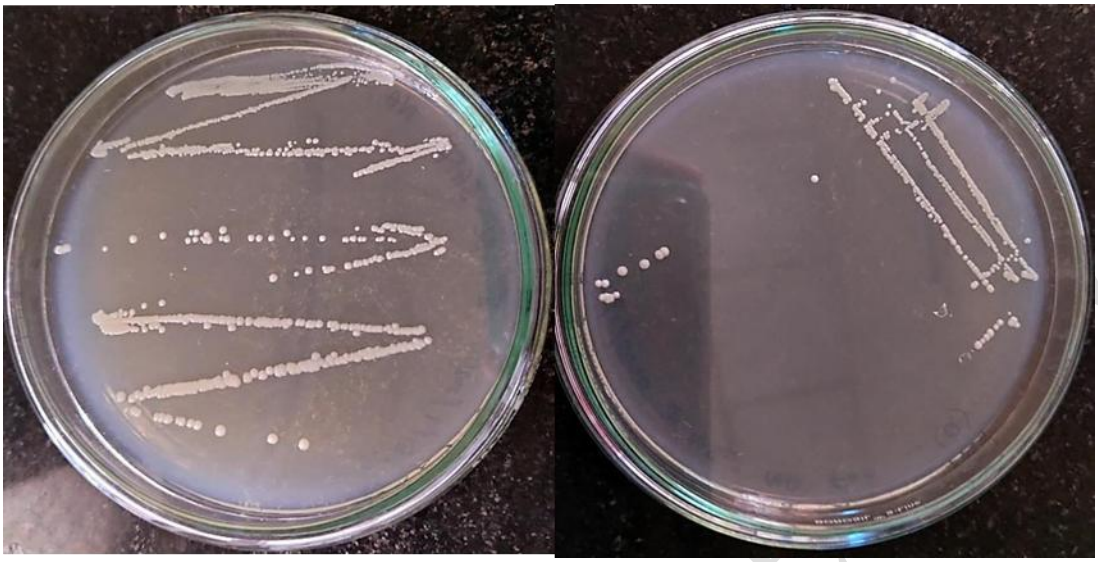


Figure 3: Sticking plate of *P. acnes* pure culture on brain heart infusion agar plate

Table2: Isolated pure culture of P. acnes colony characters

Form	**Elevation**	**Margin**	**Color**
Circular	Convex	entire	white

Microscopicobservation of *P.acnes* using Gramstaining

Thecoloniesstainedby Gramstainingwere observedasGrampositive bacilli

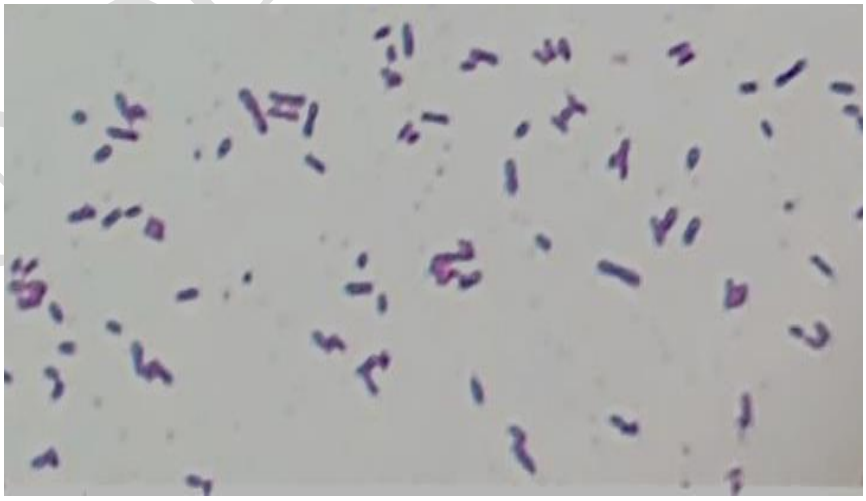


Figure4: Grampositive*P. acnes*were observed under the magnification of 40x

Biochemical characterization for *P.acnes*

The obtained bacterial colonies were further characterized and conforms the presence of *P.acnes* by biochemical analysis as described below.

Table 3: Biochemical characterization of *P. acnes*

No. of tests	**Biochemical test**	***P. acnes* result**
1	Catalase test	+
2	Indole test	+
3	Nitrate reduction test	+
4	Sugar fermentation test	+
5	Hemolytic test	+
6	Gelatin hydrolysation test	+
7	Methyl red test	+

Antimicrobial activity by well diffusion method

The culture was further confirmed positive with biochemical tests characteristic of *Propionibacterium acnes*. The sensitivity of the isolated Propionibacterium acnes to commercial anti-acne gels was tested by a well diffusion method, the two selected anti-acne gels, namely 'Clindamycin phosphate Cleargel' and 'Biotique bio chlorophyll anti-acne gel' failed to produce any inhibition zones. The inhibitory zone of size 30mm (3cm) was seen only around well (PC) in both the platesandno inhibition zonewas seen aroundwell A, Bor Cl in eitherof theplates.

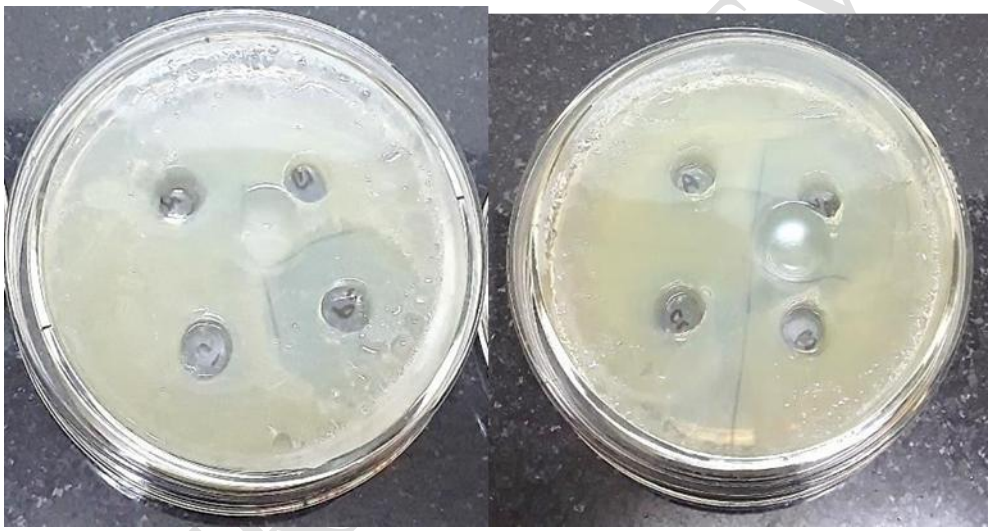


Figure 4: Antimicrobial activity of *P. acnes* against the selected drugs

Conclusion

The limited presence of clinically supported over-the-counter topical anti-acne treatments makes it difficult for the consumer to find an effective treatment from a wide range of products. These treatments are mainly designed to target the reduction in bacterial colonization of the skin to reduce inflammation induced by the organism. The most probable organism among the skin commensal that can proliferate in the anaerobic condition of the plugged follicle is *Propionibacterium acnes*, making it the most

efficient target of topical anti-acne treatments. Antibiotics like macrolides, tetracyclines, and antimicrobial non-antibiotic agents like benzoyl peroxide and zinc that can inhibit *Propionibacterium acnes* are most commonly used.

The colonies conform the shape of cocci by staining and microscopy observation. *Propionibacterium acnes* was then isolated anaerobically by taking a facial skin swab of acne lesion, in a brain heart infusion broth using an Anaerobic Gas Pak jar. After incubation for 4 days, a sample from the broth was stained and observed microscopically as Gram-positive bacilli. The culture was further confirmed positive with biochemical tests characteristic of *Propionibacterium acnes*. The sensitivity of the isolated Propionibacterium acnes to commercial anti-acne gels was tested by a well diffusion method, the two selected anti-acne gels, namely 'Clindamycin phosphate Cleargel' and 'Biotique bio chlorophyll anti-acne gel' failed to produce any inhibition zones.It was concluded that Over the Counter 'Clindamycin phosphate Cleargel' and 'Biotique bio chlorophyll anti-acne gel' was unable to inhibit the growth of isolated *Propionibacterium acnes*. Further susceptibility tests of *Propionibacterium acnes* towards other Over Counter anti-acne gels containing different macrolides and tetracyclines as monotherapy or in combination with non-antibiotic agents like benzoyl peroxide will be carried out in the future.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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