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Sensitivity of *Propionibacterium acnes* towards Commercial Anti-Acne Formulations

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Propionibacterium acnes are aerotolerant anaerobic, gram-positive bacilli that form part of normal flora. They produce several pro-inflammatory substances that can trigger an immune response in the host by an influx of inflammatory leukocytes into the strands, causing inflammatory lesions that

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leave behind scars. Repeated isolation of Propionibacterium acnes may reduce efficacy among the resistant types, clearly explaining Acne lesions' importance. The Counter acne therapies are often the first treatment choice 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. The microscopic observation and biochemical studies conform to the presence of anti-acne activity. A sensitivity test was performed on all the isolates of *Propionibacterium acnes* by well diffusion technique. The selected over-the-counter anti-acne gel formulations failed to produce any inhibition zone.

Keywords: Acne vulgaris; propionibacterium; facial acne lesions; sensitivity test.

1. 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 [1]. Human skin is one of the biggest organs present across the body, which consists of various tiny microorganisms, including *Malassezia, Propionibacterium, Corynebacterium, Streptococcus,* and *Staphylococcus*. The most common human skin microbiota is a gram-positive anaerobic bacteria called *Propionibacterium acnes* (*P. acnes*) which colonizes the pilosebaceous. The uniqueness of these propionibacterium is that they can maintain skin through environmental niches occupied by various pathogenic microbes. They produce bacteriocins, short-chain fatty acids, thiopeptides, and a few other molecules that are capable of inhibiting other organisms [2]. *P. acnes* and *P. granulosum* are commonly found in sebaceous gland-rich areas of skin while *P. acnes* can also be seen in other parts of the body such as the gastrointestinal system, prostate, and mouth surface [3-9]. The propionibacteria provide support and maintain the microbial balance in the skin but they may also cause diseases under improper conditions [10,11]. The disease in some cases may leave permanent scars on the skin diminishing which causes negative effects on psychological and social well-being in young adolescents such as discomfort, emotional stress [12] anxiety, and embarrassment [13]. 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 [14-22]. The pathogenesis of acne is multifactorial and the four main pathological factors involved include sebum production, epidermal hyper-proliferation, irregular follicular desquamation, and bacterial proliferation and inflammation [23,24]. 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 [25]. 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 [26-28]. Few studies suggest that non-antibiotic agents are used to treating mild to moderate acne, which can be used as monotherapy or in combination with antibiotics to enhance the efficacy of treatment and reduce antibiotic resistance in *P. acnes*. Combined agents are found to be more effective, due to the synergistic effect [29-33]; these combinations show antibacterial resistance in *P. acnes* and are much more effective in combination when they are used individually [34,35,36-38]. Combination exerts bactericidal effects which are capable of decreasing *P. acnes* counts [39]. Prolonged usage of antibiotics, especially by topical application, results in the development of *P. acnes* resistant strains [40,41]. 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 [42-47]. Similarly in the present study, we tried to examine the efficacy of Commercial Anti-Acne formulations against *propionibacterium acnes.*

2. MATERIALS AND METHODS

2.1 Isolation of *P. acnes* Aerobically

P. acnes was isolated from acne lesions. Three samples were randomly collected from patients

18 - 21 years old. The samples were collected using a sterile Himedia swab 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 smeared with sterile swabs and stored in a test tube containing 10 ml of nutrient broth (NB) and was incubated for 4 days in Anaero Gas Pak. The incubated samples were later streaked on nutrient agar and incubated at 37^{o}C for 4 days. The obtained colony morphology was observed and stained using gram staining [48].

2.2 Staining and Bacterial Observation

Gram stain was performed as described previously with slight modifications. A loop full of the samples was smeared on clean glass slides, air-dried, and heat-fixed. Crystal violet was added to the samples and incubated for 5 min at room temperature. After incubation, the glass slides were gently rinsed under tap water in order to remove excess crystal violet. Gram iodine was then added and kept for 2 min before washing with tap water. The grams decolorizer was added in order to remove excess crystal violet stain for about 30s and quickly rinsed under tap water. A drop of Safranin stain was added and kept for 1 min, followed by dehydration using 70% ethanol and coverslip were placed [49,50].

2.3 Isolation and Purification of *P. acnes* Colony

In order to isolate the *P. acnes* bacteria from a cluster of bacteria, 1 ml of culture nutrient broth was spread on nutrient agar plates. The collected samples were serially diluted, 1 ml of 5-fold serial diluted samples were spread on nutrient agar plates. The culture plates were incubated at 37^{0}C for about 24 hours. The obtained colonies were further counted, characterized, and recorded. The obtained colonies were purified by repeated subculturing using the 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 tests.

2.4 Biochemical Characterization of *P. acnes*

2.4.1 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 bubbles indicates the presence of catalase and no air bubble indicates the absence of catalase [49].

2.4.2 Indole test

Indole test is used to determine the presence of *P. acnes*. The test organism was cultured on Tryptone broth media in a bijou bottle and incubated at 37℃ for four days. To the media 0.5 ml Kovac's reagent was added and gently shaken until the obtained colored ring was observed [150.

2.4.3 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℃ for four days. To the culture tube 2 to 3 drops of nitrite reagent A and B were added and the reaction culture was observed [51].

2.4.4 Sugar fermentation test

Two purple base broth was prepared with an inverted Durham's tube 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℃ for about 24 hours and a yellow color confirms the positive results of sugar fermentation test [51].

2.4.5 Hemolytic test

To 1.25 ml of 5% defibrinated sheep blood was added on Blood Agar Base (Fluka Analytical) and were incubated at 37°C, 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℃ for four days [51,52].

2.4.6 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℃ for 24 hours. To the plate an iodine solution was added to check the starch utilization [51].

2.4.7 Methyl red test

The test organisms were culture in MR broth and incubate at 37℃ for about 48 hours. After incubation 1 ml of broth was transferred into two test tubes, where one of the tubes is used as control. To these tubes 2 to 3 drops of methyl red were 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 [50].

2.4.8 Antimicrobial activity by well diffusion method

50 µl 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, 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 [53,54,52]. 200 µl of Clindamycin phosphate Cleargel and Biotique bio chlorophyll and anti-acne gels were added to the plate wells and labeled A, B, C, and D respectively. The Ampicillin was used as positive control and distilled water was used as negative control and the plates were then incubated at 37℃ for 24 hours [52,55].

3. RESULTS AND DISCUSSION

3.1 Isolation and Culture of *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; turbidity was also found at the bottom of the tube which confirms the presence of *P. acnes.*

3.2 Gram Staining

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

3.3 Serial Dilution

In order to obtained pure culture of *Propionibacterium acnes*, serial dilution was carried out using the spread plate method. The obtained colonies were further characterized and confirms the presence of *P.acnes*. In the present

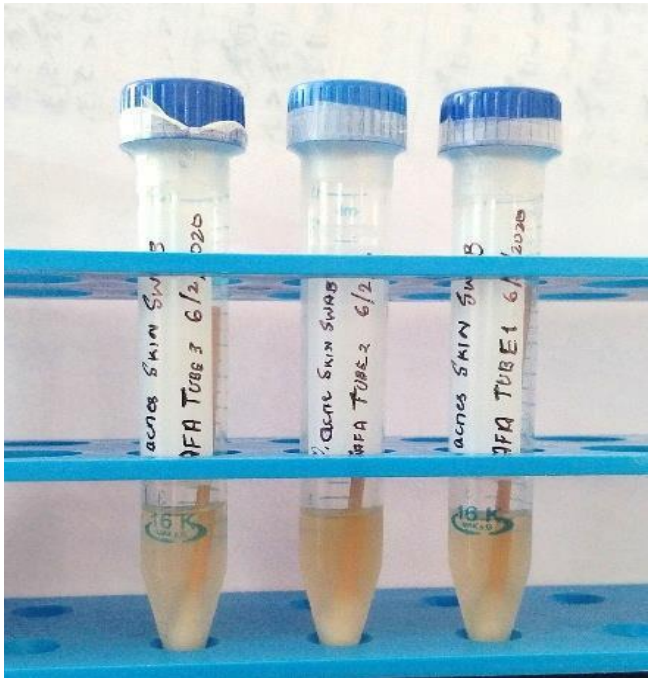

Fig. 1. Formation of biofilms confirms the presence of *P. acnes* after postincubation

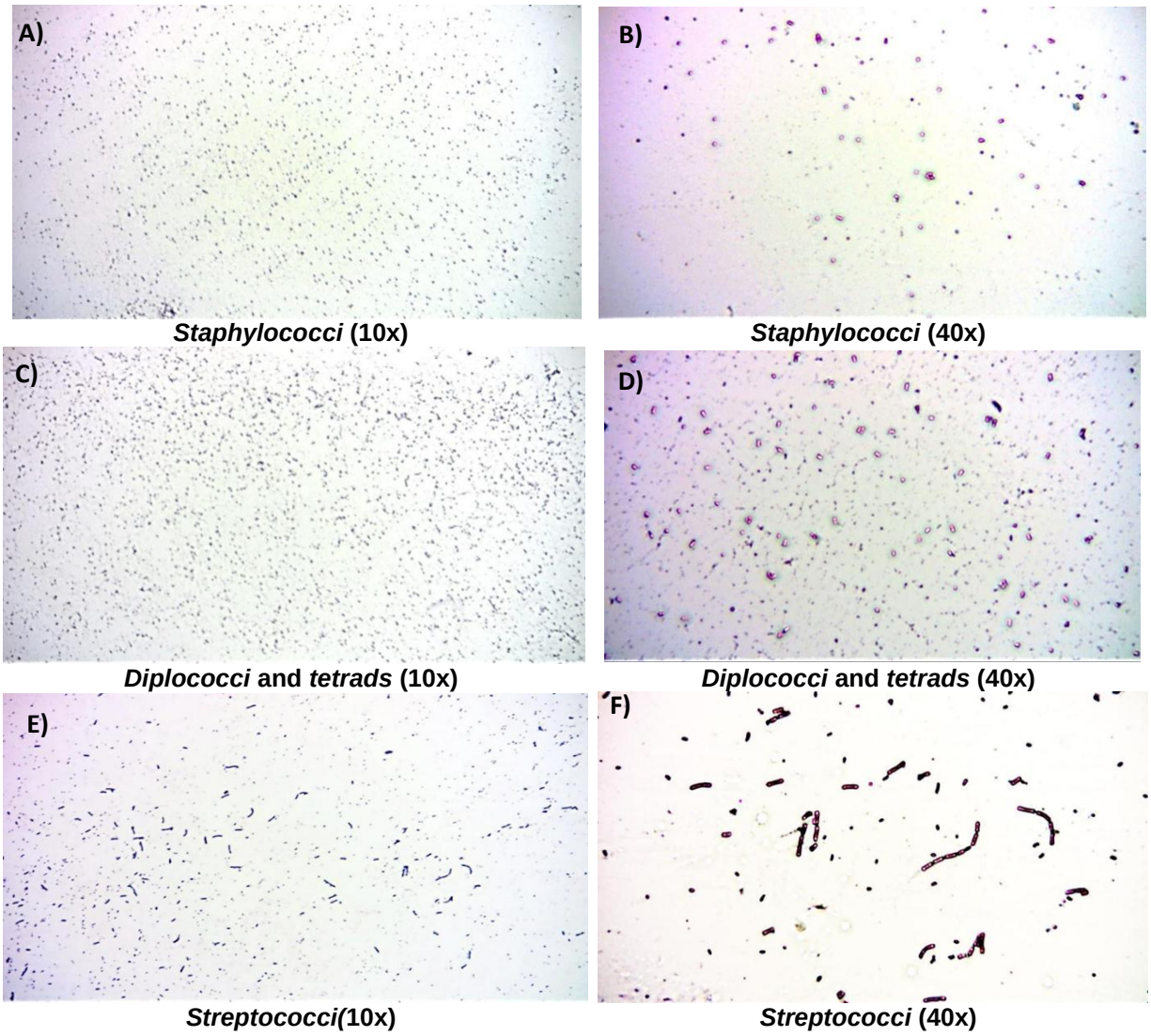

Fig. 2. Obtained gram-positive bacteria were stained namely *staphylococci*, *diplococci, tetrads,* and *streptococci*. Figure A,C,E were observed under the magnification of 10x and figure B,D,F were observed under the magnification of 40x

study the obtained bacterial colonies were subcultured 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}.

3.4 Culture Isolation and Purification of *P. acnes*

The serially diluted samples were further inoculated on nutrient agar plates using the streak plate method. The dilution was repeated several times 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.

3.5 Microscopic Observation of *P. acnes* Using Gram Staining

The colonies stained by Gram staining were observed as Gram-positive bacilli (Fig. 4).

3.6 Biochemical Characterization for *P. acnes*

The obtained bacterial colonies were further characterized and conforms the presence of *P. acnes* by biochemical analysis as described (Table 2).

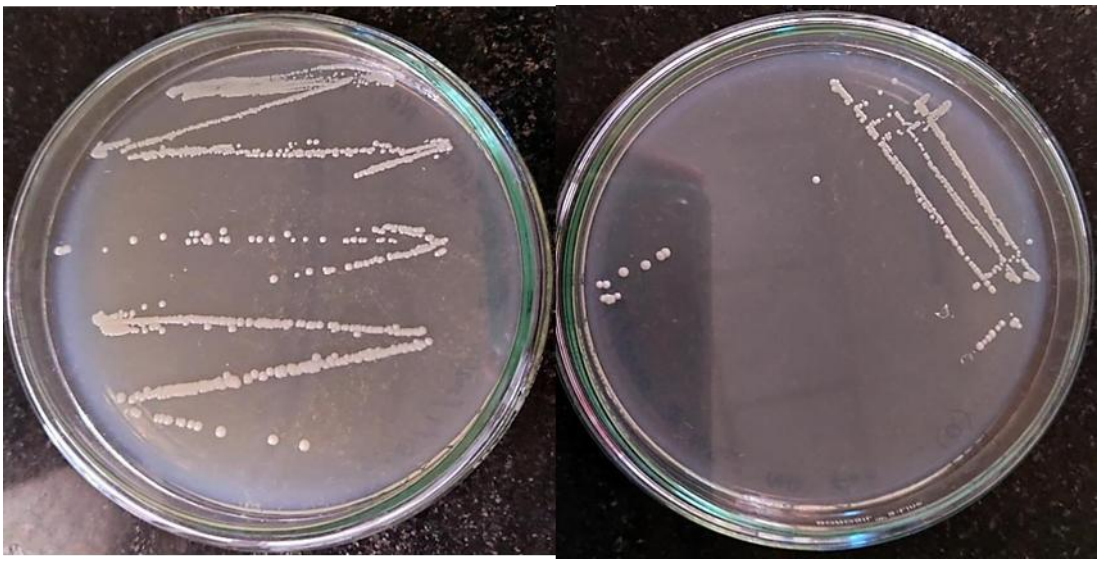

Fig. 3. Sticking plate of *P. acnes* pure culture on brain heart infusion agar plate

Table 1. Characteristics of the purified culture of *P. acnes* colonies

Form	Elevation	Margin	Color
Circular	Convex	entire	white

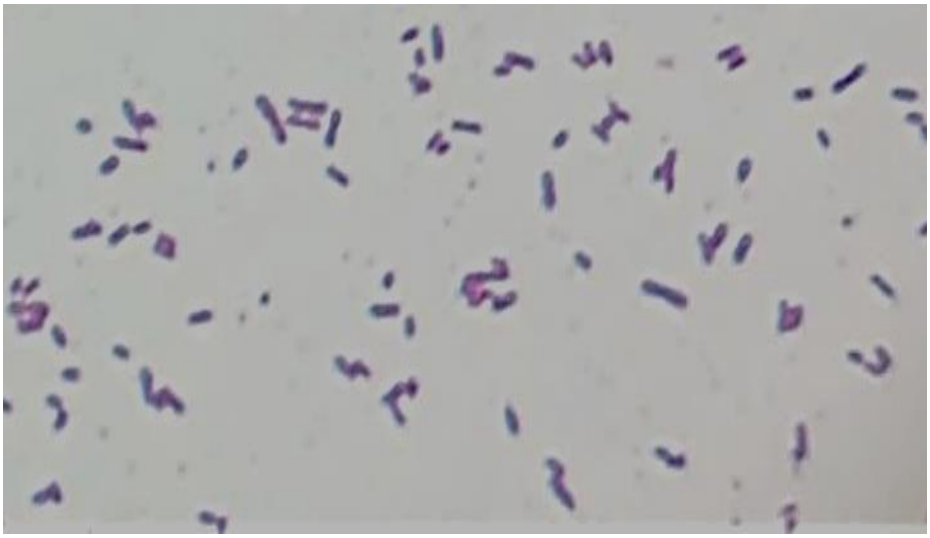

Fig. 4. Gram-positive *P. acnes* were observed under the magnification of 40x

Table 2. 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	+

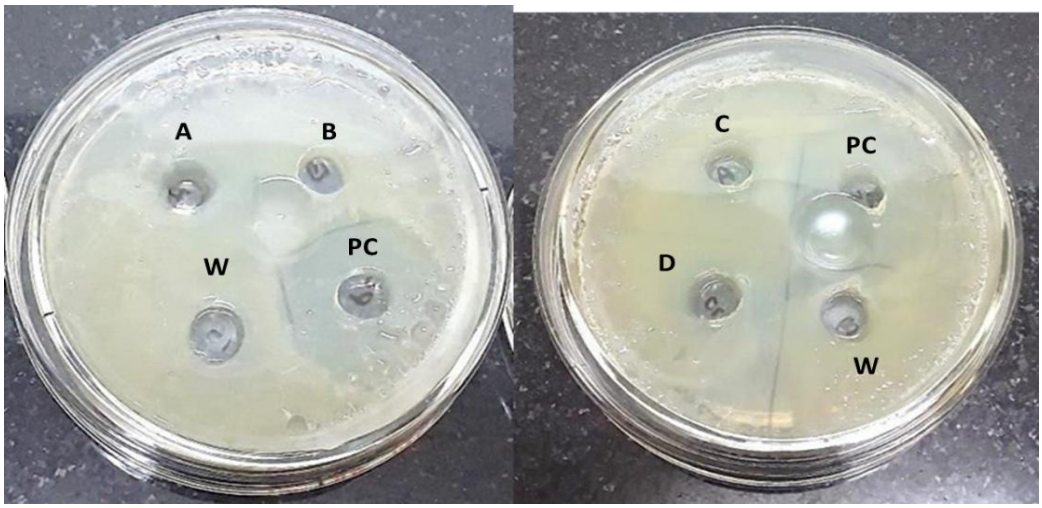

Fig. 5. Antimicrobial activity of *P. acnes* against the selected drugs, were A and B are Clindamycin phosphate Cleargel, C and D are Biotique bio chlorophyll, ampicillin was used as positive control (PC) and water (W) was used as negative control

3.7 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. Two selected anti-acne gels, namely 'Clindamycin phosphate Cleargel' and 'Biotique bio chlorophyll anti-acne gel' (A,B,C and D) failed to produce any inhibition zones. The zone of inhibition was only found in a positive control well (PC) that is ampicillin with an inhibition about 30 mm or 3 cm.

4. 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 rod shape of bacteria 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 even though they have an antibiotic property they fail to inhibit the clinically isolated samples dew to their bacterial resistance property. 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*, whereas a positive control ampicillin shows a good zone of inhibition. 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.

DISCLAIMER

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.

CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline Patient's consent and ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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