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Synergistic Antibacterial Effects of Geranium Essential Oil against Skin Pathogenic Antibiotic Resistant Acne Related Bacteria

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Abstract

Acne is a chronic and sometimes severe skin disorder affecting an estimated 85% of adolescents and 50% of adults older than age 20 years. Bacterial pathogens are associated with acne and hence antibiotic therapy some time lead to development of antibiotic resistant bacteria. Four factors including Androgen-mediated stimulation of sebaceous gland activity, follicular hyper keratinization, colonization of the bacterium *P. acnes*, and inflammation, play important roles in the pathogenesis of acne. In addition to *P. acnes*, as the main causative bacteria, *S. epidermidis* is also present in acne lesions. Therefore, alternative acne control therapies with minimal side effects are in demand. Given the trend of increased multidrug resistance, there is an urgent need to discover and development of promising new antimicrobials. In the present study, the antimicrobial activity of six essential oils (EO) namely, lavender, eucalyptus, lemon, rosemary, tea tree, and

geranium oils were investigated against *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* by broth micro dilution method and synergistic effects in combination with antibiotics and tropical forms of Vitamin-A. From the results of preliminary investigation, among the six essential oils tested, geranium oil was found to be better antimicrobial in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against skin pathogens tested in the present study. The results of the present study confirmed that geranium oil exhibited significant MIC/MBC values ranging from, 128-512 µg/mL, 512-2,048 µg/mL against *P. acnes* reference and clinical isolate strains tested several other Gram-positive skin pathogens such as *S. aureus* and *S. epidermidis* were found to be susceptible for geranium oil treatment MIC in range of 512-2,048 µg/mL. Also the MIC of both retinol and all trans-retinol were ranging from 64-1,024 µg/mL. The analysis of fractional inhibitory concentration (FIC) indices clearly showed a synergistic antibacterial effect between geranium EO and commercial antibiotics, also between tropical forms of Vitamin-A and geranium EO. Thus, the median of ∑FIC values against *P. acnes* were ranged from 0.09-0.565 and 0.26-0.56 in combination with commercial antibiotics and Vitamin-A. The results of the present study suggested that geranium oil can be a possible natural antimicrobial candidate to control skin related pathogens.

1. Introduction

The gram-positive anaerobic bacteria *Propionibacterium acnes* of family *Propionibacterium spp* are the most causative organisms for Acne vulgaris, which is one of the most common chronic skin disease affecting an estimated 85% of adolescents and 50% of adults older than age of 20 years (Fledman et al., 2004). Acne vulgairs mainly affects hair follicles and oil glands found in the face, back, chest and upper arms (Leyden, 1997) due to factors including follicular keratinization, bacterial colonization, and metabolizing sebaceous triglycerides into fatty acids inside sebaceous glands which leads to the increase of sebum production in epidermis of skin and results in comedo (Chomnawang et al., 2005). The other skin pathogenic bacteria involved in acne vulgaris is *Staphylococcus epidermidis* and least involvement of *Staphylococcus aureus*.

From many years antibiotics are used in the treatment of acne vulgaris but sometimes it leads to the skin irritation, dryness, redness also in case of oral intake of antibiotics can cause gastro-intestinal problems (Hamdy et al., 2017) and also increased resistance to developed existing anti-microbial compounds and high cost of treatment (Hamburger and Hostettmann, 1991) suggests an immediate development of natural low cost alternative therapeutic agents with strong antibacterial activity and lesser side effects.

In general plant materials are used in health and skin care from olden days as folk medicines. In current study the commonly stated Geranium essential oil of *Pelargonium* genus of flowering plant of more than 200 species is used as antibacterial agent against skin pathogenic bacteria *Propionibacterium acnes*. Rose-scented geranium is semi herbal ornamental plant native to South Africa, Egypt, India, France and China in producing its essential oil (Ravindra and Kulkarni 2015). The aromatic essential oil of geranium was produced by hydro distillation (Gulati, 1960; Douglas, 1969). The geranium oil consists of mono and sesqui-terpenes of low molecular aroma compounds with citronellal, geraniol, linalool and their esters as major components (Charlwood and Charlwood 1991). The geranium oil is mainly used in aromatic perfumes, diffusers, cosmetic industries and a few applications in health industries (Misra and Srivastava 2010) and (Rao et al., 2002). Although it has promising phytochemical compounds with rich anti-inflammatory properties the use of geranium oil against food borne and skin pathogenic bacteria are very less reported. In the present study the novel In-vitro antibacterial activity of geranium oil against skin pathogenic bacteria

P. acnes was evaluated with high synergistic effect with commercial antibiotics and tropical Vitamin-A forms retinol and al-trans retinal which has highest demand in the treatment of acne vulgaris.

2. Materials and Methods

2.1 Materials

The essential oils used in this study are Geranium essential oil, Tea tree, Lavender, Lemon, Rosemary and Eucalyptus. Antibiotics (erythromycin and tetracycline) and Vitamin-A were purchased from Sigma-Aldrich Chemicals Co. (St. Louis. MO, USA). And all the chemicals used this study are of analytical grade and commercially available.

2.2 Bacterial strains and culture conditions

The bacterial strains used were obtained from Korean Collection of Type Culture (KCTC, Daejeon, Korea). P. acnes KCTC 3314, S. aureus KCTC 1927, S. epidermidis KCTC 1370 and P. aeruginosa KCTC 1637. Five of P. acnes clinical strains were kindly provided by the member of national biobank of Korea, Gyeongsang National University Hospital (Jinju, Korea).

P. acnes stains was cultured anaerobically in Brain Heart Infusion Broth (Difco, Detroit, MI, USA) supplemented with 1% glucose and incubated at 37 ℃ for 48-72 h in CO_2 Incubator with presence of 10% CO_2 – humidified atmosphere (NAPCO 5400; General Laboratory supply, Pasadena, TX, The other strains of *Staphylococcus* genera was cultured in Tryptic soy broth at 37 ℃ for 24 h (TSB, Difco).

Table 1. List of essential oils used in this study

Essential oils	Solubility
Lavender	DMSO
Lemon	
Geranium	
Tea Tree	
Rosemary	

Table 2. List of antibiotics and Vitamin-A used in this study

Antibiotics & chemicals	Company	Solubility	Application
Benzyl Peroxide	SIGMA ALDRICH	Acetone, H_2O	Treatment of *P. acnes* infection
Tetracycline		H_2O	Antibacterial
Erythromycin		95% EtOH	
Retinal		95% EtOH, DMSO	Vitamin-A
All-trans Retinal		95% EtOH, DMSO	

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Table 3. List of bacterial strains used in this study

Strains	Medium	Culture conditions	Source
Staphylococcus aureus KCTC 1927	Tryptic soy broth	37 ℃, 24h under aerobic condition	Korean collection for type cultures (KCTC; Daejeon, Korea)
Staphylococcus epidermidis KCTC 1917	Tryptic soy broth		
Pseudomonas aeruginosa KCTC 1637	Tryptic soy broth		
Escherichia coli KCTC 1682	Tryptic soy broth		
Propionibacterium acnes KCTC 3314	Brain heart infusion broth supplemented with 1% glucose	37 ℃, 72h under anaerobic condition	Gyeongsang National Hospital (Jinju, Korea)
P. acnes isolate 2874			
P. acnes isolate 2875			
P. acnes isolate 2876			
P. acnes isolate 2877			
P. acnes isolate 2878			

2.3 A quantitative antibacterial assay against acne-related bacterial cells

Minimum inhibitory concentration (MIC) is the method of evaluating the antimicrobial activity quantitatively. It is defined as the lowest concentration of antimicrobial agents which will inhibit the visible growth of a microorganism after 20-24 h of incubation (Grierson and Afolayan, 1999). Minimum inhibitory concentration test can be used to determine best fit for a particular application from a list of various antibacterial agents. The experiment procedures were followed by the guideline of Clinical and Laboratory Standards Institute (CLSI, 2012). Minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by sub culturing to agar plates that do not contain the test agent. MBC was defined as the highest dilution showing ≥99.9% kill after 24 h of incubation (Saginur et al., 2006).

The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death.

2.4 Morphology by Scanning Electron Microscopy (SEM)

The morphological changes of *P. acnes* cells was monitored by Scanning Electron Microscopy (SEM) Vega Ⅱ LSU (Tescan Orsay Holding; Libušina, Ceska) by the treatment of geranium EO. Briefly, the cells were directly fixed through the addition of glutaraldehyde (2.5% in the final concentration) and formaldehyde (2% in the final concentration) and incubated at 4 °C overnight. Then, the cells were collected by filtering with a 0.45 mm Nylon filter (Nalgene, New York, USA) under vacuum. The filter, which contained the cells, was cut into 0.5 × 0.5 mm squares and placed in clean ampoules. For biofilm cells, a nylon filter was cut into 0.5 × 0.5 mm square and placed in 96-well plates with 300 mL of cells with an initial turbidity of 0.05 at 600 nm. Cells and the nylon filter were incubated together at 37 °C for 24 h without shaking. After fixation with glutaraldehyde and formaldehyde, *P. acnes* cells were washed three times with a 0.2 M sodium phosphate buffer (pH 7.2) for 20 min each time at 4 °C and stored in the same buffer at 4 °C overnight. Then, the samples were washed twice with a 0.2 M sodium phosphate buffer for 20 min before they were post fixed for 90 min with an Osmium solution [containing 1.5 mL of a sodium phosphate buffer (0.2 M),

3 mL of 2% OsO4 and 3 mL deionized water]. The cells were washed again four times with 1.5 mL of a sodium phosphate buffer (0.2 M) for 20 min. In the next step, cells were dehydrated through successive 20 min incubations in 50%, 70%, 80%, 90% and 95% ethanol, and then two successive 20 min incubations in 100% ethanol. After the dehydration process, the cells were incubated twice in isoamyl acetate for 20 min. These filter pieces, which contained the cells, were dried using a freeze dryer. Then, the dried filters were affixed to SEM stubs, and coated with white gold for 200 s using an Ion-sputter (E-1030, Hitachi, Japan). The specimens were examined using SEM S-4100 (Hitachi, Japan) at a voltage of 15 kV and magnifications ranging from X 2000 to X 5000.

2.5 Antibiotic susceptibility test (AST)

Antibiotic susceptibility test (AST) is used to determine whether an organism is susceptible or resistant to an antimicrobial agent (Jenkins and Schuetz, 2012). The antibiotic resistance of test strains was confirmed against few types of commercial antibiotics by MIC assay. An antibiotic was serially diluted and the bacterial growth was visually checked.

2.6 Determination of fractional inhibitory concentration (FIC)

Fractional inhibitory concentration (FIC) assay is widely used to evaluate the *in vitro* synergy for multiple agents (Odds, 2003). This method analyze the interaction between antimicrobial agents by exposing bacteria to various concentrations of the antibacterial drugs (Hsieh et al., 1993). In this study, the synergy between geranium EO, antibiotics (benzyl peroxide, tetracycline, and erythromycin) and different forms of Vitamin-A (al-trans retinol, retinol) against *P. acnes*. FIC index was calculated by using the following formula:

$$\sum FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B,$$

Where MIC_A and MIC_B are the MICs of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination respectively. The synergistic effect was evaluated as a fractional inhibitory concentration (FIC) index. The interaction was defined as synergistic if the FIC index was <1.0, additive if the FIC index was 1.0, sub additive if the FIC index was between 1.0 to <2.0, indifferent if the FIC index was 2, and antagonistic if the FIC index >2. Synergy was further sub-classified as marked (FIC index, <0.50) and weak (FIC index, between 0.50 to <1.0).

2.7 GC–MS analyses of geranium EO

GC analyses were performed using a Hewlett-Packard (HP, Palo Alto, CA, USA) gas chromatograph equipped with a flame ionization detector and HP5-MS capillary column (30 m × 0.32 mm, 0.25 μm film thickness). The oven temperature was programmed isothermally for 8 min at 45°C and then 45–240°C at 2°C/min for 15 min. Injector and detector temperatures were 250 and 280°C, respectively. Carrier gas was nitrogen at a flow rate of 1.2 mL/min in split mode 1:70 with an injection volume of 1 μL. The composition of RGEO was computed by the normalization method from the GC peak areas.

GC–MS analyses were performed using a Hewlett-Packard GC system interfaced with a mass spectrometer equipped with an HP5-MS capillary column (30 m × 0.32 mm, 0.25 μm film thickness). For GC–MS detection, electron ionization with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1.2 mL/min with an injection volume of 1 μL. Injector and detector temperatures were set at 250 and 280°C, respectively.

Identification of the components of rose geranium volatile oil were made by matching their recorded mass spectra with the mass spectra data bank (Wiley 7N and NIST 2002 libraries) and by comparing their retention indices (RIs) relative to a series of hydrocarbons (C7–C28) with literature values.

2.8 Investigation of anti-inflammatory activity

Human HaCaT cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco BRL, Germany) supplemented with 1%antibiotic-antimycotic solution (Gibco BRL) and 10% fetal bovine serum at 37℃ and in a 5% CO_2 atmosphere. For experiments, the cells were harvested by trypsin-EDTA (Gibco BRL) treatment and seeded in 96-well microplates (Greiner, Germany) at a density of 40,000 cells/cm^2.

2.8.1 Cytotoxicity assessment by MTT assay

The cytotoxicity was carried out by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma-Aldrich Chemicals Co.) assay for investigating changes in mitochondrial/non-mitochondrial dehydrogenase activity. In brief, HaCaT cells were seeded on 96-well plates and cultured at a density of 1X 10^5 cells/mL. The plate was incubated overnight and treated with 100μL of DMEM medium containing different concentrations of geranium EO (50, 100, 200, 300 μg/mL). After 24 h of incubation, MTT solution (1 mg/mL) was added to each well and the plate was incubated for another 4 h at 37℃. The blue formazan salt was dissolved in DMSO and then optical density was measured at 540 nm with a GENios microplate

reader (Tecan Austria GmbH, Salzburg, Austria). The optical density of formazan formed by untreated cells were taken as 100% viability.

2.8.2 Measurement of nitric oxide production

Nitric oxide (NO) levels in the HaCaT cell culture supernatants were determined by measuring nitrate by Griess reaction (Sun et al., 2003). The HaCaT cells were cultured in a 96-well plate with 1X 10^5 cells/well and allowed to adhere for 2 h at 37℃. The cells were then incubated for 24h with presence of sample geranium EO. As a parameter of NO synthesis, the nitrate concentration was measured by Griess reaction using the supernatant of the HaCaT cells as described. Briefly, 100 µg/mL of cell culture supernatant was reacted with 100 µL of Griess reagent (0.1% naphtilethylenediamine di-hydrochloride and 1% sulfanilamide in 5% H_3PO_4). The absorption of the mixture was measured with an Infinite F200 pro-microplate reader (TECAN, Mannedrof, Switzerland) at 540 nm.

2.9 Data interpretation

For the data interpretation of MIC values, Geometric mean values of MIC,

MBC, and FIC values are represented. The geometric mean (G-mean) values which are transformed data derived by using logarithms to generate a normal distribution was used (Bland and Altman 1996). G-mean values were compared using logarithms to generate a normal distribution was used. G-mean values were compared with two tailed t-tests using graph pad quick calc.

3. Results and Discussion

3.1 Determination of MIC and MBC values of essential oils

The antibacterial activity of essential oils was evaluated by MIC assay (Table 4). The MIC values of essential oils were in range of 256 to 5,120 μg/mL against acne related bacteria tested in this study. Interestingly geranium EO showed strong antibacterial activity against *P. acnes* with a MIC value of 128-512 μg/mL. However the other EO's showed very less antibacterial activity compared to the geranium EO. Other cutaneous acne related bacteria tested in this study are *S. aureus* KCTC 1927, *S. epidermidis* KCTC 1917, *P. aeruginosa* KCTC 1637, *Escherichia coli* KCTC 1682 are also showed less antibacterial activity in the treatment with lavender, lemon, tea tree, rosemary EO's but in the presence of geranium EO the MIC values are in satisfactory range of 256 to 1,024 μg/mL by proving that the geranium EO can be a potential antibacterial agent for the treatment of acne vulgaris.

In order to evaluate the bactericidal effect of essential oils MBC assay was performed. The MBC values of essential oils were ranged in 256-16,384 μg/mL against the acme related bacteria causing acne vulgaris. The MBC

results as seen in table 5 the geranium EO showed the MBC values of ranging of 256 to 2,048 μg/mL for the acne related bacteria.

According to previous studies, the number of commercial essential oils recommended for acne treatment, less than half of the commercial essential oils have actually focused on *S. epidermidis* and *P. acnes (*Halls 2011; Lawless 1995 and Vuuren 2017). The use of an essential oil in combination resulted in a decrease in inflammation, reduction of the odor, and improved healing rates (Jones et al., 2004). Considering these reports proving that geranium EO is suitable in treatment of acne vulgaris which exhibiting MIC values of 256 to 1,024 μg/mL. Furthermore, geranium EO possess specific and strong antibacterial activity against *P. acnes*. In addition these results indicate that geranium EO will be a potential candidate to develop an alternative therapeutic agent for the treatment of *P. acnes* infection.

Table 4. Minimum inhibitory concentrations (MIC) of the essential oils against skin pathogenic bacteria

Strains	MIC (μg/mL)				
	Lavender EO	Lemon EO	Geranium EO	Tea Tree EO	Rosemary EO
S. aureus KCTC 1927	4,096	4,096	512	5,120	4,096
S. epidermidis KCTC 1917	4,096	4,096	256	4,096	2,048
P. aeruginosa KCTC 1637	2,048	8,192	512	5,120	2,048
E. coli KCTC 1682	8,192	8,192	1,024	5,120	4,096
P. acnes KCTC 3314	2,048	4,096	256	5,120	4,096
P. acnes isolate 2874	2,048	4,096	256	2,048	4,096
P. acnes isolate 2875	4,096	2,048	512	2,048	2,048

P. acnes isolate 2876	2,096	8,192	128	4,096	5,120
P. acnes isolate 2877	5,120	4,096	512	5,120	2,048
P. acnes isolate 2878	4,096	4096	512	2,048	4,098

Table 5. Minimum bactericidal concentrations (MBC) of the essential oils against skin pathogenic bacteria

Strains	MBC (μg/mL)				
	Lavender EO	Lemon EO	Geranium EO	Tea Tree EO	Rosemary EO
S. aureus KCTC 1927	8,192	5,120	2,048	8,192	8,192
S. epidermidis KCTC 1917	8,192	5,120	1,024	5,120	4,096
P. aeruginosa KCTC 1637	4,096	16,384	1,024	8,192	4,096
E. coli KCTC 1682	16,384	16,384	1,024	8,192	8,192
P. acnes KCTC 3314	4,096	5,120	256	8,192	8,192
P. acnes isolate 2874	4,096	4,096	1,024	4,096	5,120
P. acnes isolate 2875	5,120	4,096	1,024	4,096	4,096
P. acnes isolate 2876	4,096	10,240	512	6,144	8,192
P. acnes isolate 2877	8,192	8,192	1,024	8,192	4,096
P. acnes isolate 2878	5,120	8,192	1,024	4,096	5,120

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3.2 Effect of geranium EO on morphology of *P. acnes* cells

As mentioned above the geranium EO with a concentration of 256 μg/mL exhibited higher antibacterial activity against *P. acnes* cells. To investigate the antibacterial effect of geranium EO on *P. acnes* cells were monitored by a change of cell morphology by using the SEM analysis. Considering the MIC values of geranium EO, the *P. acnes* cells were treated with different concentrations of geranium EO (0, 64, 128, 256 and 512 μg/mL) and the cells were incubated at 37℃ under anaerobic conditions for 48 h. As seen in Fig 1 the SEM micro images shows the distorted and decreased cells by the treatment of geranium EO at 256 μg/mL. In detail the untreated *P. acne* cells shows normal cell surface fig d. In the treatment of 64 μg/mL the *P. acne* cells looked more shrunken and visibly small as seen in fig b. As followed by the treatment at 128 μg/mL of geranium EO the *P. acne* cells are visibly small and also the number of bacteria has been decreased as seen in Fig.1c as compared to the control. Whereas the treated *P. acne* cells with 256 μg/mL of geranium EO were completely invisible in the SEM micro images proving that the geranium EO can be a suitable agent for treating the acne vulgaris skin disease using a minimal amount of 256 ppm. Since geranium EO has antibacterial and antiseptic properties, when applied topically, it may

be helpful for preventing the bacteria on to skin from entering the pores. As previously reported by other research gropus, some of the essential oils are capable of treating chronical acne infections, based on this geranium EO is highly potent and effective in the treatment of *P. acnes* infection, suggesting that the antibacterial activity of geranium EO against *P. acnes* is different from other essentials oils by acting on the viability of *P. acne* cells (Orafidiya et al., 2002).

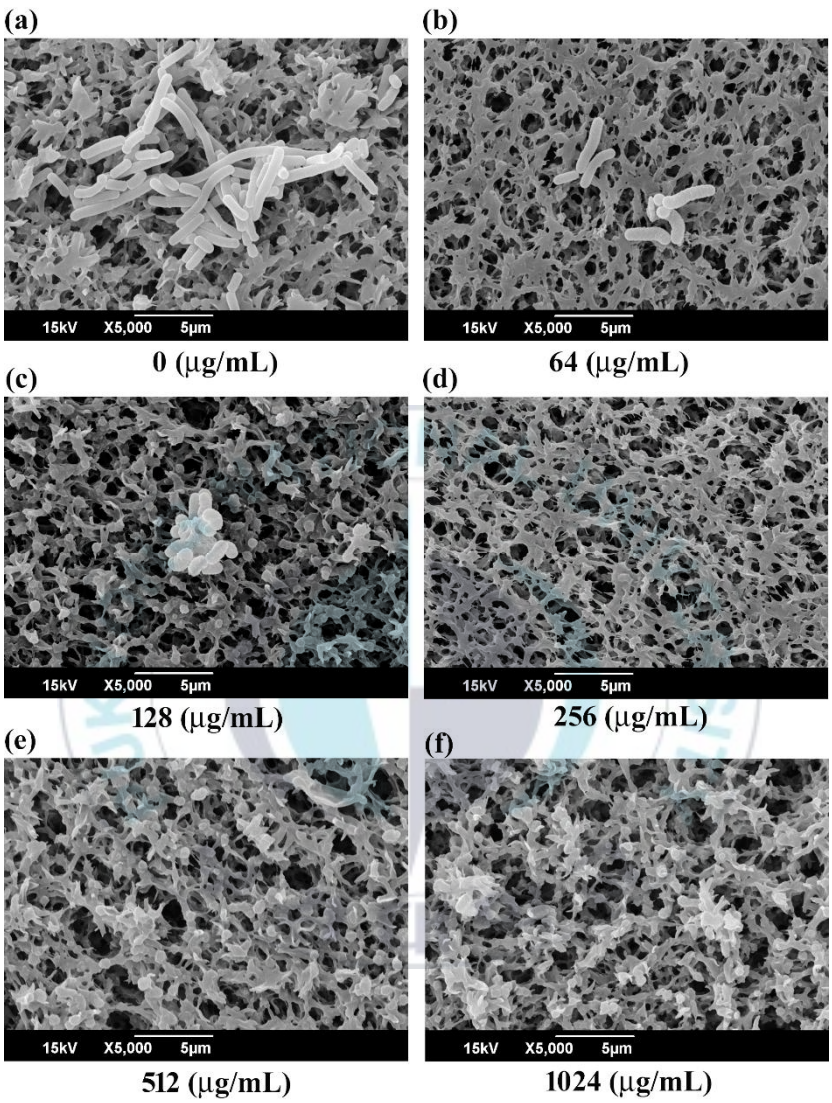

Figure 1. SEM profiles of the antibacterial effect of geranium EO against *P. acnes* (a) control, ×5,000 magnification (b) treated with geranium EO concentration of 64 μg/mL (c) treated with geranium EO concentration of 128 μg/mL (d) treated with geranium EO concentration of 256 μg/mL (e)

treated with geranium EO concentration of 512 μg/mL (f) treated with geranium EO concentration of 128 μg/mL.

3.3 Antibiotic and Vitamin-A resistance of *P. acnes*

The performance of antibiotic susceptibility testing by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antibiotic agents, or to detect resistance in individual bacterial isolates. One of the earliest antimicrobial susceptibility testing methods was the macro broth or tube-dilution method based on the analysis of MIC breakpoint (Soussy et al., 1995). The MIC break point values of erythromycin, tetracycline and benzyl peroxide were 1-4 μg/mL and 4-8 μg/mL, respectively. In the current study *P. acnes* and their isolates were tested for the antibiotic susceptibility, for erythromycin the *P. acnes* were higher than the breakpoint values suggesting the resistance of the strains to the tested antibiotic.

However the MIC of tetracycline to the *P. acnes* cells were in range of general Soussy's MIC breakpoint indicating susceptibility to the antibiotic. The MIC of *P. acnes* standard strain against tetracycline is 8 μg/mL, which is within the range of MIC breakpoint, also the *P. acnes* isolates was found to be resistant to tetracycline. However the *P. acnes* strains tested in this

study is suggested to be sensitive to the benzyl peroxide based on the analysis of MIC values obtained.

In addition to the antibiotics Vitamin-A was also tested against *P. acnes* strains to check whether it is susceptibility as this study is a trail to test retinal and Al-trans retinal as they are both an antioxidant and a cell-communicator to produce new skin cells and less irritable than the other forms of Vitamin-A. In the present study retinol and all trans-retinal are used in antibacterial activity against skin pathogenic *P. acnes* strains and also synergy with Geranium essential oil. The MIC values of *P. acnes* strains against retinal and all-trans retinal is in range of 32-1,024 µg/mL and 128-256 µg/mL as shown in table 6.

Also these antibiotic resistant profiles against skin microbial pathogens are similar to the previous reports (Lee et al., 2014; Kim et al., 2016b). In addition, the profiles of MICs of antibiotics were similar with the previous results (Lee et al., 2004; Kim et al., 2016a; Kim et al., 2016b). The results obtained in this study indicated that some commercial antibiotics are no longer useful for treating bacterial infections related with pathogens of human skin.

Furthermore as shown in table 6, several cutaneous pathogens including P. acnes isolates exhibited multi-antibiotic resistance. It has been reported the multiple antibiotic resistances in skin microbial strains isolated from acne

patients (Eady et al., 1994; Eady et al., 1996; Lee et al., 2014; Kim et al., 2016a; Kim et al., 2016b). Thus it urgently needs to develop some alternative therapeutic agents for replacing antibiotics that has lost their efficiency.

Table 6. Minimum inhibitory concentration (MIC) of antibiotics and Vitamin-A against *P. acnes* strains

Strain	MIC (μg/mL)				
	Erythromycin	Benzyl peroxide	Tetracycline	Retinal	Al-trans Retinal
Break point	1-4	-	4-8	128	-
P. acnes KCTC 3314	16	32	16	32	256
P. acnes isolate 2874	32	32	8	64	256
P. acnes isolate 2875	32	128	4	1,024	128
P. acnes isolate 2876	16	32	16	1,024	256
P. acnes isolate 2877	32	64	8	128	256
P. acnes isolate 2878	16	64	8	128	128

3.4 Synergistic antibacterial effect between geranium EO, antibiotics and Vitamin-A against acne-related bacteria

Due to emergence of multidrug resistant bacteria, the need for new antibiotics or therapeutic agents is increased (Eom et al., 2011; Kim et al., 2014). It has been demonstrated that one of the more effective strategies in developing new drugs or alternative therapies is the restoration of antibiotic activity in combination with antibacterial materials derived from natural products and traditional medicines against drug-resistant bacteria (Eom et al., 2016a; Eom et al., 2016b; Nshmiyumukiza et al., 2015; Eom et al., 2015b; Eom et al., 2012; Gislene et al., 2000). Based on these reports, an interaction between geranium EO with commercial antibiotics and Vitamin-A were estimated by the checkerboard method as stated above and the results were presented in Table 6. Among of essential oils used in this study geranium EO was chosen for further study since geranium EO exhibited the highest antibacterial activity against acne-related bacteria.

As shown in Table 7, the MICs of tetracycline, erythromycin and benzyl peroxide against antibiotic resistant *P. acnes* strains were ranged from 8 μg/mL to 128 μg/mL. However, the MICs against the *P. acnes* strains were

dramatically reduced in combination with geranium EO. The MICs of tetracycline against *P. acnes* KCTC 3314 and isolates strains were fairly reduced up to 1 μg/mL when applied in combination with 256 μg/mL of geranium EO. Comparing the MICs of the tetracycline alone (16 μg/mL to 32 μg/mL), the MICs has decreased 2- to 3-fold in the combination of tetracycline-geranium EO. In addition, the MICs of benzyl peroxide against the *P. acnes* strains were also dramatically decreased 2- to 4-folds in the combination with geranium EO, as resulting the median FIC indices were from 0.078 to 0.562. Thus, these results indicated that the combination of geranium EO with commercial antibiotics used in acne infection resulted in a synergistic effect against the antibiotic resistant *P. acnes* strains.

Vitamin-A forms retinal and al-trans retinal are used in this study to check the synergistic effect in combination with geranium EO as of date this work is not performed. Retinal and al-trans retinal showed a strong minimal MIC values against *P. acnes* as shown in table 8. The MICs of retinal and al-trans retinal against *P. acnes* KCTC 3314 and isolates strains were fairly reduced up to 1 μg/mL when applied in combination with 256 μg/mL of geranium EO as shown in table 8. Comparing the MICs of the retinal and al-trans retinal alone (128 μg/mL to 256 μg/mL), the MICs has decreased 4- to 5-fold in the combination of retinal, al-trans retinal and geranium EO separately, as resulting the median FIC indices were from 0.312 to 1.5. Thus, these results

indicated that the combination of geranium EO with Vitamin-A forms used in this study resulted in a synergistic effect against the antibiotic resistant *P. acnes* strains.

The median ΣFIC indices were in the ranges of 0.078 to 0.563. Also, it has been previously reported that phlorotannins of *Eisenia bicyclis* exhibited a synergistic antibacterial effect with the FIC indices ranging from 0.502 to 1.000 against antibiotic resistant *P. acnes* strains in combination with the antibiotics used in this study (Lee et al., 2014c). Kim et al. (2016b) reported that a synergy effect between an edible brown alga (*Sargassum serratifolium*) extract and the antibiotics with the median ∑FIC indices from 0.270 to 0.550 against *P. acnes* strains. Compared with these results, it was clear that geranium EO showed comparatively strong synergistic antibacterial effect against antibiotic resistant acne-related bacteria in combination with the antibiotics.

This finding showed that the geranium EO has synergistic antibacterial effects in combination with tetracycline, benzyl peroxide and Vitamin-A against acne-related bacteria. Furthermore, these results strongly suggested that geranium EO would restore the antibacterial activity of old commercial antibiotics, which were lost its antibacterial activity against some antibiotic resistant bacteria.

Thus, the geranium essential oil might have potential for use as an adjunct in the treatment of the antibiotic-resistant bacteria as solely or in combination with Vitamin-A forms. However, some issues still remain to be examined in future studies.

Table 7. FIC of antibiotics, treatment in combination with geranium essential oil

Strains	Test compound	MIC (μg/mL)	$\sum FIC_{max}$[a)]	$\sum FIC_{min}$[b)]	Median $\sum FIC$[c)]	Minimum Concentration for observing synergy
P. acnes **KCTC 3314**	Geranium EO	256	0.1875	0.062	0.078	0.25
	Benzyl Peroxide	32				2
	Geranium EO	256	0.5625	0.125	0.125	2
	Tetracycline	16				1
P. acnes isolate **2874**	Geranium EO	256	0.2813	0.125	0.265	0.25
	Benzyl Peroxide	32				1
	Geranium EO	256	0.531	0.187	0.312	0.1

	Tetracycline	8				1
P. acnes isolate **2875**	Geranium EO	512	1.003	0.187	0.562	1
	Benzyl Peroxide	128				1
	Geranium EO	512	0.503	0.156	0.281	0.1
	Tetracycline	4				0.5

Table 7. Continues

Strains	Test compound	MIC (μg/mL)	$\sum FIC_{max}$[a)]	$\sum FIC_{min}$[b)]	Median $\sum FIC$[c)]	Minimum Concentration for observing synergy
P. acnes isolate **2876**	Geranium EO	128	1.007	0.25	0.375	2
	Benzyl Peroxide	32				0.5
	Geranium EO	128	0.312	0.093	0.148	0.2
	Tetracycline	16				0.5
P. acnes isolate **2877**	Geranium EO	512	1.03	0.25	0.531	2
	Benzyl Peroxide	64				1
	Geranium EO	512	1.031	0.125	0.187	0.1
	Tetracycline	8				2

	Geranium EO	512	1.06	0.32	0.45	2
P .acnes	Benzyl Peroxide	64				0.5
isolate **2878**	Geranium EO	512	1.03	0.14	0.18	0.1
	Tetracycline	8				1

[a)] $\sum FIC_{max}$ is maximum FIC, [b)]$\sum FIC_{min}$ is minimum FIC, [c)]$\sum FIC_{med}$ is median of FIC. The FIC index indicated synergistic effect: < 0.5, marked synergy; 0.5 to < 1.0, weak synergy; 1.0, additive; > 1.0 to < 2.0, sub additive; 2.0, indifferent; > 2.0, antagonistic

Table 8. FIC of Vitamin-A, treatment in combination with geranium essential oil against *P. acnes* strains

Strains	Test compound	MIC (μg/mL)	$\sum FIC_{max}$ [a)]	$\sum FIC_{min}$ [b)]	Median $\sum FIC$ [c)]	Minimum Concentration for observing synergy
P .acnes KCTC **3314**	Geranium EO	256	0.565	0.1875	0.565	0.5
	Retinal	32				1
	Geranium EO	256	1.03	0.312	0.625	0.1
	All-trans retinal	256				4
P. acnes isolate **2874**	Geranium EO	256	0.562	0.25	0.281	0.2
	Retinal	64				1
	Geranium EO	256	1.5	0.53	1.5	1
	All-trans retinal	256				4

P. acnes isolate **2875**	Geranium EO	512	-	-	-	
	Retinal	1024				
	Geranium EO	512	1.5	0.375	1.5	0.2
	All-trans retinal	128				2

Table 8. Continues

Strains	Test compound	MIC (μg/mL)	$\sum FIC_{max}$[a)]	$\sum FIC_{min}$[b)]	Median $\sum FIC$[c)]	Minimum Concentration for observing synergy
P. acnes isolate **2876**	Geranium EO	128	-	-	-	
	Retinal	1024				
	Geranium EO	128	1.125	0.125	0.187	0.05
	All-trans retinal	256				4
	Geranium EO	512	0.562	0.069	0.36	0.2

P. acnes isolate **2877**	Retinal	128	1.03	0.25	0.53	2
	Geranium EO	512				0.2
	All-trans retinal	256				4
P. acnes isolate **2878**	Geranium EO	512	0.375	0.25	0.312	0.2
	Retinal	128				2
	Geranium EO	512	0.562	0.187	0.5625	0.2
	All-trans retinal	128				2

[a)]$\sum FIC_{max}$ is maximum FIC, [b)]$\sum FIC_{min}$ is minimum FIC, [c)]$\sum FIC_{med}$ is median of FIC. The FIC index indicated synergistic effect: < 0.5, marked synergy; 0.5 to < 1.0, weak synergy; 1.0, additive; > 1.0 to < 2.0, sub additive; 2.0, indifferent; > 2.0, antagonistic

3.5 Chemical composition of geranium EO

The EO was obtained by steam distillation in a stainless steel alembic from fresh aerial part of geranium. The geranium oil obtained is a yellowish-green liquid. It has a strong lemon-rose odor. The EO was obtained with a yield of 0.15% (v/w). In a recent study (Mosta et al., 2006) it has been reported that a higher yield is obtained during spring/summer (0.1%) than during autumn/winter, with an average yield of 0.06%.

The volatile oil was analyzed by GC-MS. Qualitative and quantitative studies of the oil volatile profiles are listed in order of their retention indices. In total, 20 compounds representing 83.5% of the EO were identified. Citronellal (29.1%), geraniol (12.6%), citronellyl formate (8.1%), geranyl tiglate (7.1%), and linalool (4.5%) were the major compounds in the oil, with minor quantities of geranyl butyrate (2.0%) and geranyl acetate (1.6%). Other constituents were found in smaller amounts (<2%). The rose geranium oil consisted mainly of oxygenated monoterpenes (76.9%) and oxygenated sesquiterpenes (3.3%).

The data presented here are consistent with previous reports (Bouaziz et al., 2012), which demonstrated that rose geranium oils are characterized by citronellol (22.0–32.9%) as the most important component. However, our results diverge from those published by other studies Juliani HR, Koroch A

et al., 2006 Generally, the observed differences in chemical composition of rose geranium oils, when compared with those reported in previous studies could be due to a number of factors, including differences in climatic conditions and geographical locations, season at the time of collection, and fertilization (Kaul et al., 2002, Rahman et al., 2013). Previous reports revealed that although the chemical composition of the geranium EO differed owing to the geographical origin, compounds such as alcohols, ketones, esters, and mainly aldehydes have consistently been recorded (Balchin 2002, Rao, Kaul et al., 2002).

3.6 Cytotoxicity analysis by MTT assay

Our MTT test results showed that geranium EO decreased the cell viability significantly to less than 70% from the 250 µg/mL concentration, showing that, after its metabolization by phase I and phase II hepatic enzymes, its cytotoxicity is increased from lower concentrations. Several studies assign potent cytotoxic effect of monoterpenes, including geranium, on human HaCaT cells (Bakkali et al., 2008; Vieira et al., 2011; Crespo et al., 2013; Sobral et al., 2014; Lee et al., 2016). Considering that HaCaT is a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin,[1] widely used in scientific research. In this study geranium EO was used from 100 µg/mL onwards, might, therefore, be justified, once the same cytotoxicity pattern was not obtained when used normal cells. Literature data demonstrate that geranium EO specifically suppresses the cell growth (Cho et al., 2016).

Therefore, to evaluate anti-inflammatory effect of geranium EO on HaCaT cells depending on the geranium EO density by MTT assay. Generally, (Garcia et al., 2014) over 80 % of cell viability is considered as non-cytotoxicity within 60-80% weak, 40-60% is moderate and below 40% is strongly cytotoxic. Thus no cytotoxicity of geranium EO was observed at

200 μg/mL. Based on this results it was evaluated an anti-inflammatory effect of geranium EO at the concentration of 200 μg/mL by determining an inhibitory effect of NO, as an inflammatory marker.

It was previously reported that *P. acnes* can trigger inflammation that leads to the symptoms associated with some common skin disorders, such as folliculitis and acne vulgaris (Kim et al., 2002). Grange et al., 2009 also reported that inducible NO production by *P. acnes* cells inoculation in HaCaT cell. Therefore in this study it was examined whether the inoculation of *P. acnes* was capable of inducing NO production in HaCaT cells.

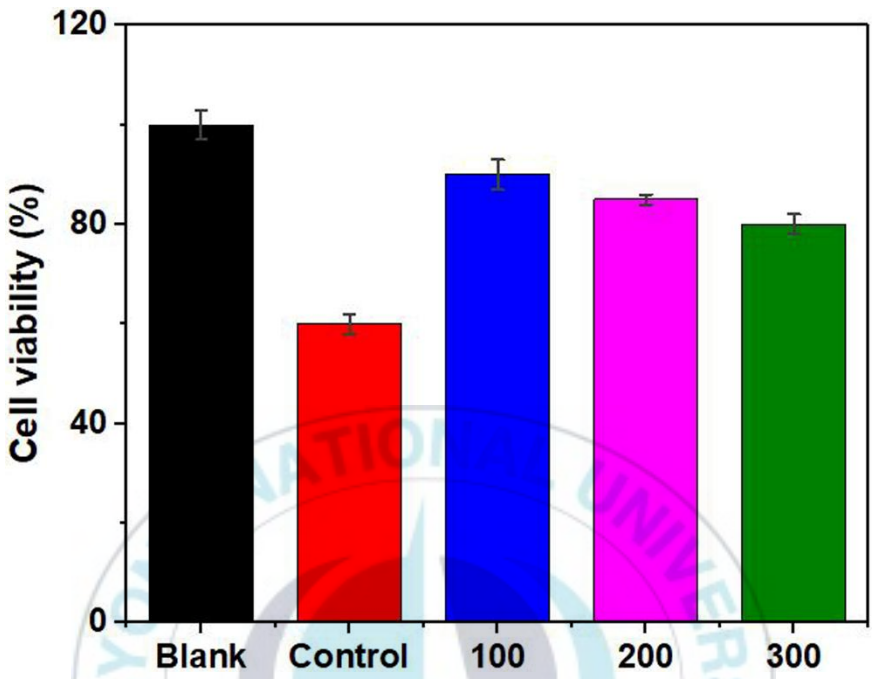

Fig 2. Effect of geranium EO (μg/mL) on cell viability in HaCaT cells.

3.6.1 Inhibitory effect of geranium EO on *P. acnes* induced NO production in HaCaT cells

P. acnes increased the production of $O_2^{\bullet -}$, NO and H_2O_2 by the immortalized keratinocyte cell line HaCaT in a dose-dependent manner. We then evaluated the ability to reduce NO production in the presence of geranium EO which is exhibiting highest antibacterial activity against *P. acnes* cells.

In order to progress further study on inhibitory effect of the geranium EO on NO production was monitored in *P. acnes* treated HaCaT cells in the presence of various concentrations of geranium EO, as shown in fig 3, the geranium EO with concentration of 200 μg/mL inhibited NO production in a concentration dependent manner. Describing more in detail, the NO production was reduced up to 65% exhibiting no cytotoxicity compared to the control. The inhibitory effect was almost similar with the recent report obtained by *E. bicyclis* against *P. acnes* induced inflammation in HaCaT cells. This result indicates that the geranium EO possessed considerable anti-inflammatory effect in *P. acnes*-induced HaCaT cells. These findings also suggest that the inhibition of NO production by the geranium EO might suppress *P. acnes* cells.

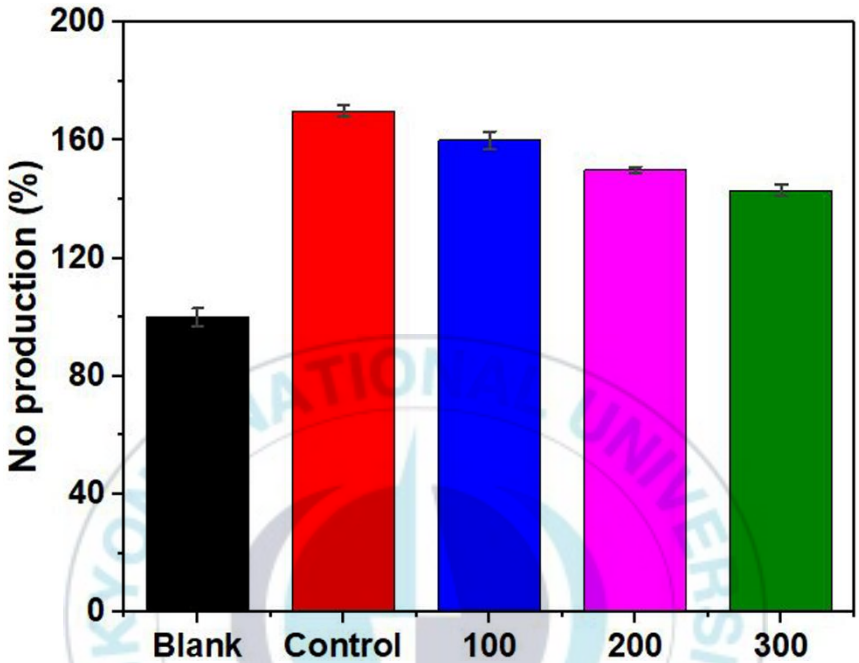

Fig 3. Effect of geranium EO (μg/mL) on nitric oxide (NO) production in HaCaT cells.

4. Conclusions

Acne is a chronic and sometimes severe skin disorder affecting an estimated

85% of adolescents and 50% of adults older than age 20 years. Bacterial pathogens are associated with acne and hence antibiotic therapy some time lead to development of antibiotic resistant bacteria. In this study, we evaluated the antibacterial activity of essential oils against acne-related bacteria such as *P. acnes, S. epidermidis* and *S. aureus*. Among the 5 EO's used in this study geranium EO showed a higher antibacterial activity of against acne related bacteria. The FIC of antibiotics showed a marked synergy with combination of geranium EO. Due to the side effects of antibiotics it is always necessary to opt for natural products as shown in the results Vitamin-A retinal and all-trans retinal showed a strong synergy with the combination of geranium EO.

In general plant materials are used in health and skin care from olden days as folk medicines. In current study the commonly stated Geranium essential oil of *Pelargonium* genus of flowering plant of more than 200 species is used as antibacterial agent against skin pathogenic bacteria *P. acnes.* Rose-

scented geranium is semi herbal ornamental plant native to South Africa, Egypt, India, France and China in producing its essential oil. The aromatic essential oil of geranium was produced by hydro distillation .The geranium oil consists of mono and sesqui-terpenes of low molecular aroma compounds with citronellol, geraniol, linalool and their esters as major components. The geranium oil is mainly used in aromatic perfumes, diffusers, cosmetic industries and a few applications in health industries. Although it has promising phytochemical compounds with rich anti-inflammatory properties the use of geranium oil against food borne and skin pathogenic bacteria are very less reported. In the present study the novel In-vitro antibacterial activity of geranium oil against skin pathogenic bacteria *P.acnes* was evaluated with high synergistic effect with commercial antibiotics and tropical Vitamin-A retinal and al-trans retinal which has highest demand in the treatment of acne vulgaris.

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