

Full Length Research Paper

# Antibacterial and anti-inflammatory effects of honeybee (*Apis mellifera*) venom against acne-inducing bacteria

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Acne vulgaris is a chronic inflammatory disorder of the sebaceous follicles. *Propionibacterium acnes* plays a critical role in the development of these inflammatory lesions. The present study was conducted to evaluate the antimicrobial property of honeybee (*Apis mellifera* L.) venom (BV) against the etiologic agents of acne vulgaris. Incubation of the skin bacteria *P. acnes*, clindamycin-resistant *P. acnes*, *Staphylococcus epidermidis* or *Streptococcus pyogenes* with BV yielded the minimal inhibitory concentration (MIC). Production of inflammatory cytokines (interleukin-8 (IL-8) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )) were examined in THP-1 cells. BV MIC values were 0.086, 0.067, 0.104 and 0.121  $\mu\text{g/ml}$  against *P. acnes*, clindamycin-resistant *P. acnes*, *S. epidermidis*, and *S. pyogenes*, respectively. In time-kill studies, BV was bacteriostatic in action. In addition, BV exhibited low cytotoxicity at 10  $\mu\text{g/ml}$  in human epidermal keratinocytes and monocytes. In addition, BV reduced the *P. acnes*-induced secretion of IL-8 and TNF- $\alpha$  in THP-1 cells, an indication of its anti-inflammatory effects. Based on these results, BV has effective antimicrobial and anti-inflammatory activity against *P. acnes*, and we suggest that BV is an alternative treatment for antibiotic therapy of acne vulgaris.

**Key words:** Bee venom, acne, *Propionibacterium acnes*, IL-8, TNF- $\alpha$ .

## INTRODUCTION

Acne vulgaris is the most common skin disease that affects areas containing the largest oil glands, including the face, back, and trunk (Van De Kerkhof, 2006). Normal skin commensals including *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Streptococcus pyogenes* and *Staphylococcus aureus*, proliferate rapidly during puberty and are often involved in the development of acne (Chomnawang et al., 2005; Nakatani et al., 2009). *P. acnes* is a Gram-positive anaerobic bacterium that mostly resides in the pilosebaceous follicles of the skin. Although *P. acnes* is a member of the normal skin commensal, bacterial flora, it plays a critical role in the development of inflammatory acne when it becomes overgrown and colonizes the pilosebaceous unit. On the other hand, aerobic organisms such as *S. epidermidis*, *S. pyogenes*

and *S. aureus* usually cause superficial infections within the sebaceous unit (Leyden et al., 1999; Bojar and Holland, 2004). It has also been widely accepted that inflammatory acne induced by host immune reactions to *acnes* releases chemoactive factors that attract immune system cells such as neutrophils, monocytes, and lymphocytes (Burkhart et al., 1999). Previous studies have found that *P. acnes* stimulates the production of proinflammatory cytokines such as interleukins-1, -8, -12 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Nagy et al., 2006). As therapeutic agents for acne, antibiotics are typically employed to inhibit inflammation or kill bacteria (Odou, 2007; Nakatani et al., 2009). Tetracycline, erythromycin, roxithromycin, clindamycin, benzoyl peroxide, and azelaic acid are some examples of these antibiotics. However, antibiotic resistance has been increasing in prevalence within the dermatologic setting (Swanson, 2003). To overcome the problem of antibiotic resistance, natural products have been extensively studied as alternative treatments for diseases.

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In addition to the venoms from snakes and scorpions, honeybee (*Apis mellifera*) venom (BV) is also known to have a natural antimicrobial effect (Fennell et al., 1968). The polypeptide melittin is the main component of honeybee venom. Melittin has a moderate antibacterial and antifungal activity against many kinds of organisms. Using bee venom to treat acne caused by infection is effective, if not, more effective, than known antibacterial drugs and it has no side effects at all (Kwon et al., 2002; Perumal et al., 2006). It is also quite inexpensive. Bee venom therapy is considered a psychoneurological approach for autoimmune and nervous system diseases (Somerfield et al., 1984; Kwon et al., 2001). In this study, we have demonstrated the potential of BV as an alternate to antibacterial therapy in acne treatment. BV was examined for antimicrobial and anti-inflammatory activity against skin bacteria such as *P. acnes* and clindamycin-resistant *P. acnes*.

## MATERIALS AND METHODS

### Collection of BV

Experimental colonies of honeybees (*A. mellifera*, L) were maintained at the National Academy of Agricultural Science, Korea. BV was collected with a Bee Venom Collector (ChungJin Biotech, Korea). The collected BV was diluted in cold water and then centrifuged at 10,000 g for 5 min at 4°C to discard residues from the supernatant. BV was lyophilized by a freeze dryer and stored in a refrigerator for later use.

### Preparation of bacteria

*P. acnes* (ATCC 6919), *S. epidermidis* (ATCC 12228) and *S. pyogenes* (ATCC 12353) were obtained from Korean Culture Center of Microorganisms, Seoul, Korea. Antibiotic-resistant *P. acnes* (CCARM 9010) were obtained from the Culture Collection of Antimicrobial Resistant Microbes, Seoul, Korea. *P. acnes* were cultured at 37°C on Reinforced Clostridium Medium (BD, MD, USA) under anaerobic conditions before the assay. *S. pyogenes* and antibiotic-resistant *P. acnes* were cultured on tryptic soy broth (BD, MD, USA) with 5% (v/v) defibrinated sheep blood at 37°C. Antibiotic-resistant *P. acnes* was cultured under anaerobic conditions. *S. epidermidis* was cultured at 37°C for 24 h in nutrient broth (BD, MD, USA).

### Determination of minimal inhibitory concentration (MIC)

The MIC of BV was determined by the broth antimicrodilution method in 96 - well microtiter plates (Cos et al., 2006). BV was dissolved in distilled water and then filtered by membrane filter (0.2 µm pore size, Millipore, MA, USA). Two fold serial dilutions of BV were prepared in appropriate broth media. *P. acnes* or clindamycin-resistant *P. acnes* ( $1 \times 10^6$  CFU/ml) were incubated in the two fold serial dilutions of BV in appropriate broth media under anaerobic conditions for 72 h. *S. pyogenes* or *S. epidermidis* ( $1 \times 10^6$  CFU/ml) were incubated with the same concentrations of BV in appropriate broth media at 37°C for 48 h. The MIC was defined as the lowest concentration of BV that inhibited all visible growth of the test organism (optically clear). No trailing was observed.

### Time - kill assays

Time-kill assays were performed using previously described standard CLSI methods (Clinical and Laboratory Standards Institute, 2008). Bacterial suspensions of *P. acnes*, clindamycin-resistant *P. acnes*, *S. pyogenes* or *S. epidermidis* diluted with appropriate broth media to  $1 \times 10^8$  CFU/ml were pre-incubated at 37°C. These samples were then co-incubated with BV adjusted to 1.0% in appropriate broth media to give a final concentration of two times the MIC and ten times the MIC. Aliquots of 100 µl of the culture before (0 h, positive control) and after (1, 3, 6, 12 and 24 h) the addition of BV were used to estimate CFU on appropriate agar plates with adequate dilution using buffered saline supplemented with 0.01% gelatin. Three plates were used for one sample and the estimation of CFU was repeated separately.

### Cytotoxicity of BV

Cytotoxicity of BV was examined using human epidermal keratinocyte (HEK) and human monocytic THP-1 cells (Korean cell line bank, Seoul, Korea). HEKs from neonatal foreskin were purchased from Modern Cell and Tissue Technologies (Seoul, Korea) and cultured in keratinocyte growth media supplemented with bovine pituitary extract, human recombinant epidermal growth factor, insulin, hydrocortisone, gentamicin and amphotericin-B (Cambryx, MD, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. THP-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, NY, USA) and penicillin-streptomycin in the same conditions as the HEK cells. HEK or THP-1 cells were seeded on 96-well plates and BV treatment at various concentrations began 24 or 48 h after seeding. HEK or THP-1 cells were then incubated with BV for 24 or 72 h. After incubation, cell viability of HEK or THP-1 cells was determined by MTT assay. The absorbance was measured at 570 nm with a spectrophotometer.

### Measurement of cytokine production

THP-1 cells ( $1 \times 10^6$  CFU/ml) in serum-free medium were stimulated with 100 µg/ml (wet weight) of heat-killed *P. acnes*, alone or in combination with the indicated concentrations of BV, and were incubated for 48h (Cos et al., 2006). The culture supernatants were then harvested. The concentrations of TNF-α and IL-8 in the culture supernatant were measured using ELISA (Enzyme-linked Immunosorbent Assay) kits (R and D Systems, MN, USA)

### Statistical analyses

All experiments were performed in triplicate. All data are presented as mean ± s.e., and statistical significance was determined via Duncan's t-tests with  $p < 0.05$  considered to be significant. (SAS enterprise guide 3.0).

## RESULTS

### Antimicrobial effects of BV against skin bacteria

BV was tested for antibacterial activity against *P. acnes*, clindamycin-resistant *P. acnes*, *S. epidermidis* and *S. pyogenes*, which are involved in formation of acne. The BV investigated in this study was found to possess

**Table 1.** MIC and EC<sub>50</sub> of honeybee venom on treatment of *P. acnes*, antibiotic-resistant *P. acnes*, *S. epidermidis* and *S. pyrogenes*.

Bacteria	MIC (µg/ml)	EC <sub>50</sub> (µg/ml)
<i>P.acnes</i>	0.086	0.097
clindamycin-resistant <i>P. acnes</i>	0.067	0.086
<i>S.epidermidis</i>	0.104	0.156
<i>S. pyrogenes</i>	0.121	0.2

marked antibacterial activity against *P. acnes* (MIC, 0.086), clindamycin-resistant *P. acnes* (MIC, 0.067), *S. epidermidis* (MIC, 0.104) and *S. pyrogenes* (MIC, 0.121). The half maximal effective concentration of BV against clindamycin-resistant *P. acnes* was the lowest among the bacteria tested, suggesting that *P. acnes* and clindamycin-resistant *P. acnes* more sensitive to BV than *S. epidermidis* and *S. pyrogenes* (Table 1).

The antibacterial effects of BV were also analyzed in time - kill assays. Figure 1a shows the bactericidal effect associated with BV against *P. acnes*. The CFU value was rapidly reduced below detectable levels after co-incubation with BV for 4 h at ten times the MIC and after 8 h at two times the MIC. The antibacterial effects of BV showed the same pattern against clindamycin-resistant *P. acnes* (Figure 1b). The CFU value of *S. pyrogenes* was reduced below the detection limit after co-incubation with BV for 14 h at ten times the MIC and after 22 h at 2 times the MIC (Figure 1c). At a BV concentration of 10 times the MIC for *S. epidermidis*, the CFU value was reduced below detectable levels after a 16 h co-incubation (Figure 1d). Although the CFU value continued to decrease gradually at two times the MIC, the CFU did not decrease beyond the detection limit after 24 h. Thus, this study demonstrates that BV exhibits both bactericidal and bacteriostatic effects towards *P. acnes*, clindamycin-resistant *P. acnes*, *S. epidermidis*, and *S. pyrogenes* depending on the concentration. When low concentrations (two times the MIC) of BV were administered in time-kill assays, the CFU were rapidly reduced, and this effect was maintained over time.

### Effects of BV on *P. acnes*-induced inflammation

In order to use BV as a therapeutic agent for acne, BV should not induce cytotoxicity in human skin cells. We examined the cytotoxic effects of BV on human epidermal keratinocyte (HEK) and human monocytic THP-1 cultured cells via the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

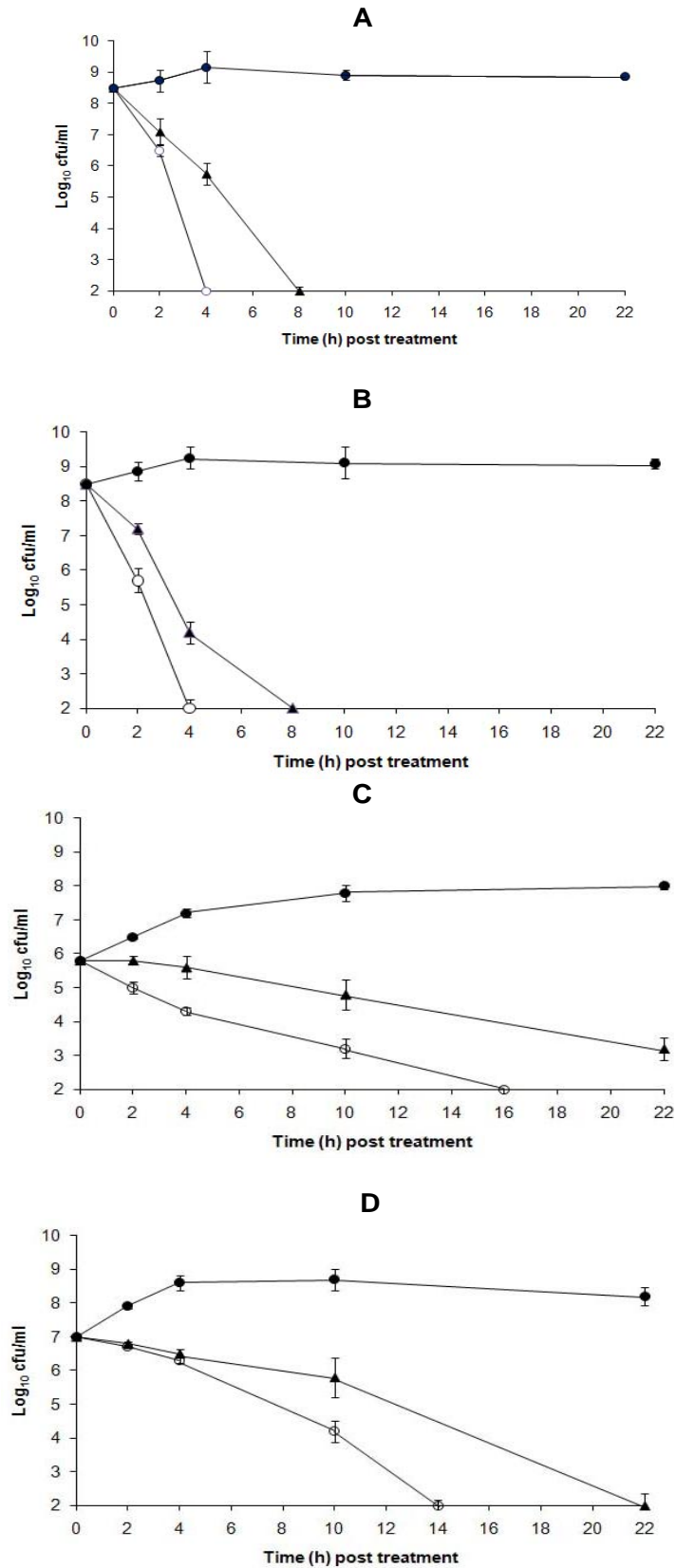
To determine whether BV induced cytotoxicity, we performed MTT assays in THP-cells. BV had low cytotoxic effects at 10 µg/ml (Figure 2a). As shown in Figure 2b, over 70% of cells were viable at 10 µg/ml of BV. We found that BV did not affect cell viability at the

concentrations tested. BV was examined for biological action against inflammatory acne induced by *P. acnes* in terms of the inhibition cytokine secretion. To determine the effect of BV on the production of proinflammatory cytokines such as TNF-α and IL-8, THP-1 cells were treated with various concentrations of BV in the presence of heat - killed *P. acnes* (100 µg/ml; wet weight) for 48 h, and cytokine concentration was measured in the culture media. As shown Figure 3, cells treated with heat-killed *P. acnes* showed an increase of TNF-α and IL-8 secretion. However, cells treated with broth heat-killed *P. acnes* and BV showed significantly reduced production of proinflammatory cytokines (Figure 3a, b).

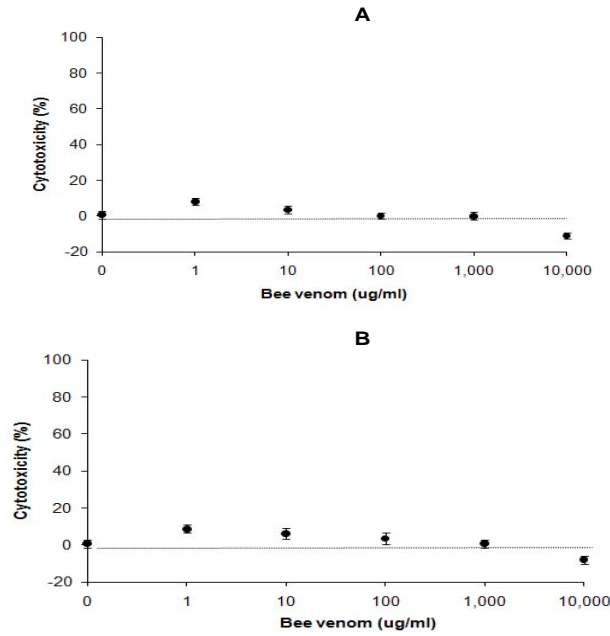
### DISCUSSION

In the present study, we demonstrated that BV exhibits potent antibacterial activity against the growth of skin bacteria such as *P. acnes*, *S. epidermidis* and *S. pyrogenes*. A previous study reported that BV has antibacterial activity against *S. aureus*, which plays an important role in the pathogenesis of inflamed lesions in the case of acne vulgaris (Nakatuji et al., 2009). Moreover, significant antibiotic resistance and multiple drug resistance have been identified in *P. acnes* strains from acne patients after long-term antibiotic treatment (Eady et al., 2003). Biofilm formation by *P. acnes* increases its resistance to antimicrobial agents (Coenye et al., 2007). These problems of resistance may cause the failure of antibiotic treatment for acne. BV, however, was shown to exert an inhibitory effect on the growth of clindamycin-resistant *P. acnes*. Thus, BV may have potential for use as an effective antibacterial treatment for antibiotic-refractory acne.

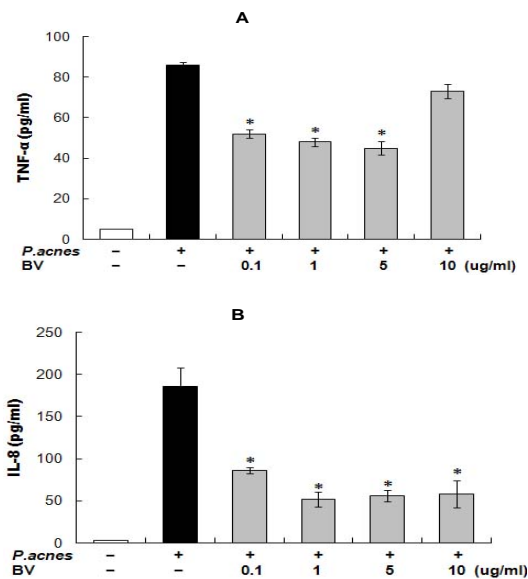
The main components of the pilosebaceous unit of the skin, such as keratinocytes and sebocytes, can be activated by *P. acnes*, leading to the production of proinflammatory cytokines (Nagy et al., 2006). It has been reported that a secreted peptidoglycan of *P. acnes* stimulates the production of proinflammatory cytokines, such as IL-1, IL-8, and TNF-α, by human monocytic cell lines and freshly isolated peripheral blood mononuclear cells from acne patients (Chen et al., 2002). In a recently published study, heat-killed *P. acnes* stimulated THP-1 cells to produce IL-8 and TNF-α (Jain, 2003; Kim et al.,



**Figure 1.** Time-kill curves for honeybee venom against *P. acnes* (a), clindamycin-resistant *P. acnes* (b), *S. epidermidis* (c), and *S. pyrogenes* (d). Control (●), 2 × MIC (▲), 10 × MIC (○).



**Figure 2.** Cytotoxicity of honeybee venom on human epidermal keratinocyte (HEK, A) and human monocytic (THP-1, B) cells. Cells were cultured with the indicated concentrations of BV in media at 37°C for 24 or 48 h. The viability of cells was determined by the MTT assay. The absorbance was measured at 570 nm using a spectrophotometer. Data represent mean ± s.e. of three individual experiments.



**Figure 3.** Honeybee venom BV inhibits *P. acnes*-induced secretion of pro-inflammatory cytokines. BV treatment demonstrates a dose-dependent effect on *P. acnes*-induced TNF-α (A) or IL-8 (B) release. THP-1 cells were stimulated with or without heat-killed *P. acnes*, and then the supernatants were harvested. The secreted TNF-α and IL-8 in the culture supernatant were measured. Data are expressed as mean ± s.e.

2008). Therefore, to determine the anti-inflammatory effects of BV, we measured IL-8 and TNF- $\alpha$  concentration in THP-1 cells by ELISA. Our data showed that production of IL-8 and TNF- $\alpha$  in THP-1 cells induced by *P. acnes* was reduced by treatment with BV.

In conclusion, this study indicates that BV has potential as an anti-acne agent and may be useful in the pharmaceutical and cosmetic industries. However, although we identified antimicrobial and anti-inflammatory effects of BV against acne-inducing bacteria, we did not determine its mechanisms of action. In particular, the possible inhibition of proinflammatory cytokine secretion remains to be evaluated in further studies.

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## REFERENCES

- Bojar RA, Holland KT (2004). Acne and *Propionibacterium acnes*. Clin. Dermatol. 22: 375-379.
- Burkhart CG, Burkhart CN, Lehmann PF (1999). Acne: a review of immunologic and microbiologic factors. P. Ostgrad. Med. J. 75: 328-331.
- Chen Q, Koga T, Uchi H, Hara H, Terao H, Moroi Y, Urabe K, Furue M (2002). *Propionibacterium acnes*-induced IL-8 production may be mediated by NF-kappaB activation in human monocytes. J. Dermatol. Sci. 29: 97-103.
- Chomnawang MT, Surassmo S, Nukoolkarn VS, Gritsanapan W (2005). Antimicrobial effects of Thai medicinal plants against acne-inducing bacteria. J. Ethnopharmacol. 101: 330-303.
- Clinical and Laboratory Standards Institute (2008). Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing, 18<sup>th</sup> informational supplement, Wayne, PA.
- Coenye T, Peeters E, Nelis HJ (2007). Biofilm formation by *Propionibacterium acnes* is associated with increased resistance to antimicrobial agents and increased production of putative virulence factors. Res. Microbiol. 158: 386-392.
- Cos A, Vlietinck AJ, Berghe DV, Maes L (2006). Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. J. Ethnopharmacol. 106: 290-302.
- Eady EA, Gloor M, Leyden JJ (2003). *Propionibacterium acnes* resistance: a worldwide problem. Dermatol. 206: 54-56.
- Fennell JF, Shipman WH, Cole LJ (1968). Antibacterial action of melittin, a polypeptide from bee venom. Proc. Soc. Exp. Biol. Med. 127: 707-710.
- Jain A (2003). Basal E. Inhibition of *Propionibacterium acnes*-induced mediators of inflammation by Indian herbs. Phytomedicine 10: 34-38.
- Kim SS, Baik JS, Oh TH, Yoon WJ, Lee NH, Hyun CG (2008). Biological activities of Korean Citrus obovoides and Citrus natsudaidai essential oils against acne-inducing bacteria. Biosci. Biotechnol. Biochem. 72: 2507-2513.
- Kwon YB, Lee HJ, Han HJ, Mar WC, Kang SK, Yoon OB, Beitz AJ, Lee JH (2002). The water-soluble fraction of bee venom produces antinociceptive and anti-inflammatory effects on rheumatoid arthritis in rats. Life Sci. 71: 191-204.
- Kwon YB, Lee JD, Lee HJ, Han HJ, Mar WC, Kang SK, Beitz AJ, Lee JH (2001). Bee venom injection into an acupuncture point reduces arthritis associated edema and nociceptive responses. Pain 90: 271-280.
- Leyden JJ, Del Rosso JQ, Webster GF (2009). Clinical considerations in the treatment of acne vulgaris and other inflammatory skin disorders: a status report. Dermatol. Clin. 27: 1-15.
- Nagy I, Pivarcsi A, Kis K, Koreck A, Bodai L, McDowell A, Seltmann H, Patrick S, Zouboulis CC, Kemény L (2006). *Propionibacterium acnes* and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. Microbes Infect. 8: 2195-2205.
- Nakatuji T, Kao MC, Fang JY, Zouboulis CC, Zhang L, Gallo RL, Huang CM (2009). Antimicrobial Property of Lauric Acid Against *Propionibacterium Acnes*: Its Therapeutic Potential for Inflammatory Acne Vulgaris. J. Invest. Dermatol. < <http://www.nature.com/jid/journal/vaop/ncurrent/abs/jid200993a.html>.
- Odou MF, Muller C, Calvet L, Dubreuil L (2007). In vitro activity against anaerobes of retapamulin, a new topical antibiotic for treatment of skin infections. J. Antimicrob. Chemother. 59: 646-651.
- Perumal SR, Pachiappan A, Gopalakrishnakone P, Thwin MM, Hian YE, Chow VT, Bow H, Weng JT (2006). In vitro antimicrobial activity of natural toxins and animal venoms tested against *Burkholderia pseudomallei*. BMC Infect. Dis. 6: 100.
- Somerfield SD, Stach JL, Mraz C, Gervais F, Skamene E (1984). Bee venom inhibits superoxide production by human neutrophils. Inflammation 8: 385-391
- Swanson JK (2003). Antibiotic resistance of *Propionibacterium acnes* in acne vulgaris. Dermatol. Nurs. 15: 359-362
- Van De Kerkhof PCM, Kleinpenning MM, De Jong EM, Gerritsen MJ, Van Doorne-Greebe RJ, Alkemade HAC (2006). Current and future treatment options for acne. J. Dermatolog. Trea. 17: 198-204.