In vitro evaluation of antibacterial, anticollagenase, and antioxidant activities of hop components addressing acne vulgaris

Naoto Yamaguchi, Keiko-Satoh Yamaguchi and Mitsunori Ono*

Betal, LLC, 5185 MacArthur Blvd, NW, Suite 300, Washington, DC 20016, USA

*Tel (U.S.): 339-223-3008; email: mono411@rcn.com

Abstract

Seven naturally derived components from hop plant (*Humulus lupulus L.)* extracts were tested for evaluation of biological activities affecting acne vulgaris. Five strains, *Propionibacterium acnes, Staphylococcus epidermidis, Staphylococcus aureus, Kocuria rhizophila and, Staphylococcus pyogenes,* were selected as the main acne-causing bacteria. Hop extracts xanthohumol and the lupulones showed strong inhibitory activities against all of the strains. Although hydrogenated derivatives did not show the same level of activity, naturally occurring xanthohumol, humulones, and lupulones all showed moderate to strong anticollagenase inhibitory activities. Antioxidant capacity was also evaluated with seven different methods based on different reactive oxygen species. Xanthohumol showed the highest activity in total oxygen radical absorbance capacity as well as singlet oxygen absorbance capacity.

Keywords: *Humulus lupulus*, Acne-causing bacteria, Oxygen radical absorbance, Singlet oxygen absorbance, Matrix metalloproteinases, Xanthohumol

Introduction

Acne vulgaris is one of the most common skin diseases affecting children and adolescents. The pathogenesis of acne is multifactorial, with primary accompanying features being increased sebum production during early puberty; the proliferation of bacteria such as *P. acnes*, *S. epidermidis, S. aureus, K. rhisophila and S. pyogenes* causing primary or secondary skin infections; and abnormal follicular keratinization and inflammation. *P. acnes* and *S. epidermidis* are pus-forming organisms that trigger inflammation in acne. *S. pyogenes, K. rhizophila and S. aureus* are often isolated from patients with clinical symptoms of acne.

Acne skin care preparations containing antibiotics, which are useful for treatment of mild to moderate inflammatory acne, partially exert their beneficial effects by decreasing the follicular population of *P. acnes* (Gollnick et al., 1998; Del Rosso, 2007). However, the widespread use of antibiotics in dermatological treatments has led to the development of drug-resistant *P. acnes* strains (Esperson et al., 1998). Benzoyl peroxide (BP) is widely used as a topical treatment for acne because of its antibacterial activity. However, it has been reported that BP generates free radicals in the skin; its effect is similar to that of unprotected exposure to the sun (Kennedy et al., 1995). In 1995 the U.S. Federal Drug Administration changed BP from a Category I (safe) to a Category III (safety is uncertain) ingredient based on information that raised a safety concern regarding BP as a tumor promoter in mice (Kraus et al., 1995).

Topical retinoids are important tools in the management of acne because they act against comedones and microcomedones, and have direct anti-inflammatory effects. The retinoids approved for acne treatment include tretinoin (all-trans-retinoic acid) and isotretinoin (13-cis retinoic acid), as well as the synthetic third-generation polyaromatic retinoids adapalene and tazarotene. Retinoids in fact account for roughly half of the U.S.

prescription acne medicine market. Although the minimal systemic availability of topical retinoid creams has been confirmed (Buchan, 1993), teratogenicity seems to be the most worrisome effect of the retinoids since it was first observed in rat experiments (Cohlan, 1954).

To overcome the potential risk of adverse effects and antibiotic resistance from prescription medications, traditional herbal medicines have been extensively studied as alternative treatments for many diseases. In this same vein, the potential use of herbal medicines as a basis for new skin-care cosmetics has been emphasized recently. However, most of the natural herbal components (isolated from several different plant species) have antimicrobial activity considerably less potent than that of synthetic drugs (Viyoch et al., 2006).

Dried hop flowers (hops) have attracted a great deal of attention as a source of small molecules such as humulones **1**, lupulones **2**, isohumulones **3** and xanthohumol **7** (Fig. 1) with potential for beneficial effects on health. It is known that these naturally occurring molecules possess antibacterial, antioxidant, anti-inflammatory, and anticancer activities (Schmalreck et al., 1975; Haas and Barsoumian, 1994; Gerhauser, 2005; Zanoli et al., 2008). Although possible applications of hop components to skin disorders have been proposed in several patents (Tripp et al., 2007), *in vitro* biological activities against acne vulgaris are yet to be reported. The aim of the present work was to explore the effects of hop components on the main factors involved in the pathogenesis of acne vulgaris.

Materials and Methods

Hop components

With reference to Fig. 1, three compounds, humulones **1**, lupulones **2** and xanthohumol **7** are naturally occurring hop components. Isohumulones **3**

are isomerized molecules that are converted from humulones **1**. Other molecules, tetrahydroisohumulone **4,** reduced isohumulone **5,** and hexahydroisohumulone **6**, are hydrogenated derivatives of isohumulones **3** with improved stability. The humulones-rich fraction (containing 34% of humulones **1**; calcd for $C_{21}H_{30}O_6$) was produced by fractionation of a CO_2 extract (obtained from John I Haas Inc., Yakima, WA) followed by purification through a column packed with Amberlite 20 FPX66 (food grade), with alkaline water used as an eluent aqueous at pH 10 (Yamaguchi and Ono, 2008). The lupulones-rich fraction (containing 10% of lupulones **2**; calcd for $C_{26}H_{38}O_4$) was produced by fractionation of the CO_2 extract, followed by purification through active charcoal treatment in an aqueous solution at pH 10 (Yamaguchi and Ono, 2007a).

Isohumulones **3** (calcd for $C_{21}H_{30}O_5$, 10% aqueous solution) converted by treatment of humulones **1** at 70°C under alkaline conditions, were also obtained from John I Haas Inc. Reduced isohumolones **5** (calcd for $C_{21}H_{32}O_5$), synthesized by reduction of isohumulones **3** with sodium borohydride ($NaBH_4$) in water at pH 10, were likewise obtained from John I Haas Inc. Similarly obtained were tetrahydro- and hexahydroisohumulones **4** (calcd for $C_{21}H_{32}O_5$) and -**6** (calcd for $C_{21}H_{34}O_5$), prepared by hydrogenation of isohumulones **3,** and reduced isohumulones **5**, respectively, in the presence of 5% palladium on charcoal (Pd/C) in water at pH 10.

A xanthohumol-rich fraction (containing 50% of xanthohumol **7**, calcd for $C_{21}H_{22}O_5$) was prepared by extraction of spent hops (obtained from Nateco2, Wolnzach, Germany) with acetone, and subsequent purification by a pH-adjusted salting-out method employing aqueous ethanol in the presence of NaCl (Yamaguchi and Ono, 2007b).

Antimicrobial assays

Antimicrobial susceptibility testing was performed using a broth dilution method. Gentamicin or ampicillin was used as a control to verify the methodology. A stock solution (20 mg/mL) of the test substance (or vehicle control) was prepared in dimethyl sulfoxide. Serial dilution was done in microwell plates. Reinforced Clostridial Medium was used for *P. acnes* (ATCC 6919). Three hundreds μL of the test substance was added to the test tube containing *P. acnes* (10^5 CFU/mL) in 2.7 mL of cultures grown under anaerobic conditions. Mueller-Hinston Broth suitable for culturing *S. epidermidis* (ATCC 12228*), K. rhizophila (formerly M. luteus*) (ATCC 9341), and *S. aureus* (ATCC 6538P), and Brain Heart Infusion Broth suitable for culturing *S. pyogenes* (ATCC 14289) were used. 100 μL of the test substance was added to the test tube containing the other microorganisms (10^5 CFU/mL) in 0.9 mL of cultures grown under controlled conditions. After 2 days for *P. acnes* and 1 day for the others, growth of the culture was examined and scored positive for inhibition of growth, or negative for no effect upon growth. Samples from those tubes that scored positive was plated onto an agar plate and incubated under controlled conditions. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that resulted in no visible growth after 2 days for *P. acnes* or 1 day for the others. The minimum bactericidal concentration (MBC) was defined as the lowest concentration at which the microorganisms failed to grow in each medium and on each agar plate.

Antioxidant assays

Both hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) assays were carried out at Brunswick Laboratory (Wareham, MA) based on the modified $ORAC_{fl}$ method reported by Ou et al. (2001, 2002a). Trolox, a water-soluble vitamin E analog, was used as the calibration standard. The data are expressed as μmole of Trolox equivalents per gram (μmol TE/g). The acceptable precision of the ORAC assay is a 15% relative standard deviation. Caffeic acid was used as the calibration standard. Hydroxyl radical ORAC (HORAC) is expressed as μmole caffeic acid equivalent per

gram (μmol CAE/g). Trolox was used as the calibration standard. The peroxyl nitrite ORAC (NORAC) result is expressed as μmol TE/g. Alpha-tocopherol (vitamin E) was used as the calibration standard, and the singlet oxygen absorbance capacity (SOAC) result is expressed as μmole alpha-tocopherol equivalent (μmol VtE) per gram (Aubry et al., 1982).

The abbreviation for the 1,1-diphenyl-2-picrylhydrazyl radical is DPPH. Radical-scavenging activity was measured by the change in absorbance at 517 nm with the DPPH result expressed as μmol TE/g. Similarly, FRAP is an abbreviation for the ferric reducing antioxidative power method, which utilizes chemical conversion of the yellow Fe^{3+}- 2,4,6-tripyridyl s-triazine (TPTZ) complex to the blue Fe^{2+}-TPTZ complex by electron donation under acidic conditions (Okada and Okada, 1998). The FRAP result is expressed as μmol TE/g (Ou et al., 2002b). Superoxide dismutase (SOD) was used as a calibration standard; the SOD result is expressed as kilo unit SOD equivalent (kunitSODeq) per gram.

Anticollagenase assays

Human recombinant matrix metalloproteinase-1 (MMP-1) pro-enzyme, expressed in mammalian cells, and human nutrophil MMP-8 pro-enzyme were activated with 4-aminophenylmercuric acetate for 60 minutes at 37°C, respectively (Knight et al., 1992). Hop components and vehicle were preincubated with 5 nM MMP-1 and 6 nM MMP-8 active enzymes in a modified MOPS buffer, pH 7.2, for 60 minutes at 37°C. The reaction was initiated by addition of 4 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg for another 120-minute incubation period. The determination of the amount of Mca-Pro-Leu-Gly formed was read spectrofluorimetrically at 340 nm/400 nm. Tissue inhibitors of metalloproteinase TIMP-1 and TIMP-2 were used as positive controls (Olson et al., 1997).

Results and Discussion

As shown in table 1, we have evaluated, with broth dilution methods, the antibacterial effects of these seven hops components against five different strains of bacteria involved in primary or secondary skin and soft tissue infections. The lowest MIC values against *P. acnes* and *S. pyogens* were observed for lupulones **2,** the values having reached 0.1 and 0.3 μg/mL, respectively (Table 1). The lowest MIC values against *S. epidermidis, K. rhizophila* and *S. aureus* were observed for lupulones **2** and xanthohumol **7,** the value having reached 1 μg/mL. It is also important that all the strains are sensitive not only to naturally occurring hop components **1, 2,** and **7**, but also the chemically modified ones, **3, 4** and **5**. Such strong inhibitory activities of lupulones **2** and xanthohumol **7** against acne-related bacteria have not been reported among natural products derived from edible plants. The low MIC values are comparable to the most commonly prescribed antibiotics for topical acne treatment (e.g., clindamycin and erythromycin). It is noted that lupulones **2** and xanthohumol **7** exhibited bactericidal activity against *P. acnes* and the MBC values were 0.3 and 3.0 μg/mL, respectively (Table 1). Recently, however, the development of resistance to hop constituents of certain lactobacilli from beer (containing mostly isohumulones **3**) has been observed (Suzuki et al., 2006). This finding suggests that the potential large-scale use of hop components may bring the possibility of hop-resistant acne bacteria development.

Results of activity against interstitial collagenase (MMP-1) and nutrophil collagenase (MMP-8) are listed in Table 2. Xanthohumol **7** showed the highest activity against both collagenases; the IC_{50} values were 20.5 and 16.8 μg/mL, respectively , whereas humulones **1** and lupulones **2** showed only weak activity with 27% and 28% inhibition for MMP-1, and 61% and 62% for MMP-8, respectively. Chemically modified derivatives **3**, **4**, **5** and **6** did not show activity up to a concentration of 100 μg/mL.

Extracellular proteases, in particular MMPs, have been implicated in a number of dermatological conditions; for example, in chronological aging,

inflammatory matrix remodeling, and hyperproliferative skin disorders (Choi et al., 2008). These processes involve the increased breakdown of various components of the extracellular matrix in the skin, notably collagen, elastin, and fibronectin. Enhanced expression of the collagenases MMP-1 and -8 has been described as playing a central role in connective type-1 collagen breakdown in the skin (Brenneisen et al., 2002). The transcription factors nuclear factor-B (NFkappaB) and activator protein-1 (AP-1) are activated in acne lesions with consequent elevated expression of their target gene products, inflammatory cytokines and matrix-degrading metalloproteinases, respectively. These elevated gene products are molecular mediators of inflammation and collagen degradation in acne lesions *in vivo*. Recently, this new knowledge has enabled a rational strategy for the development of drugs that can target the inflammation and matrix remodeling that occurs in severe acne (Kang et al., 2005).

It is well documented (Shi et al., 2007; Rahimi et al., 2005) that inflammation caused by oxidative damage has been implicated in not only skin disorders but also various systemic chronic diseases such as cancer, Alzheimer's disease, rheumatoid arthritis, cardiovascular disease, cataracts, and other ageing processes. Reactive oxygen species are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS in various active forms can damage tissues. The radical scavenging activity of hop components **1** and **2** has been evaluated previously using a conventional DPPH assay, employing one of the stable nitrogen-centered free radicals. These components have promise for their antioxidant potential (Tagashira et al., 1995). Xanthohumol was also shown to scavenge hydroxyl and peroxyl radicals, and superoxide anion radicals, in an ORAC assay (Gerhauser, 2005; Vogel et al., 2008).

A standardized ORAC value shows the scavenging capacity of antioxidants against the peroxyl radicals, which are among the most common ROS found in the body. Thus the ORAC method has become a widely used

method for assessing antioxidant capacity in biological samples and foods. However, because of its inability to measure both hydrophilic and lipophilic antioxidants, the method has its limitations.

An $ORAC_{fl}$ method for lipophilic antioxidants was further developed and validated using fluorescein as the fluorescent probe (Prior et al., 2003). This method has the advantage that similar assay conditions and standards can be used for both hydrophilic and lipophilic antioxidant components, such that the two values can be added together to obtain a total antioxidant capacity.

Table 3 shows six assays for the antioxidant capacity of hop components against various ROS formed in human skin (Bickers and Athar, 2006). In the present work, the ORAC value is expressed as the sum of values of both the water-soluble and fat-soluble parts. We used green tea catechins (Polyphenon 60) as the control with the highest ORAC value among edible plants, and also used vitamin C and vitamin E as controls for water- and fat-soluble molecules, respectively.

The results are shown in Table 4. The higher the total ORAC score, the higher the antioxidant capacity. The total ORAC value of xanthohumol **7** was comparable to that of Polyphenon 60, and much higher than that of vitamins C and E. In addition xanthohumol **7** shows equal activity in both fat-soluble and water-soluble antioxidant capacity. These data raise the possibility that xanthohumol **7** may serve as a well-balanced antioxidant similar to the combination of vitamins C and E. Taken altogether, the antioxidant properties of xanthohumol **7** deserve attention.

Table 5 shows the singlet oxygen-quenching activity of hop components. Alpha-tocopherol (vitamin E) was used as the calibration standard, and the SOAC result is expressed as μmol VtE/g. The higher the SOAC score, the higher the singlet oxygen-quenching capacity. It is noted that the

xanthohumol SOAC value was about eight to fifteen times higher than that of vitamin E or Polyphenon 60. Singlet oxygen has been postulated to be a highly reactive and toxic intermediate against skin. It is known that the acne-causing bacterium *P. acnes* naturally produces high amounts of intracellular porphyrins, mostly coproporphyrin (Ashkenazi et al., 2003). Singlet oxygen can be generated on the skin surface from *P. acnes* porphyrins under sunlight and induce serious skin damage (Arakane et al., 1996). (Various skin disorders progress through a singlet oxygen-dependent mechanism, including acne, atopic dermatitis, and skin aging.)

Squalene is also a component of acne sebum. Both porphyrins and squalene are directly exposed to the external environment and play a key role in skin physiology. Recently it was demonstrated that squalene peroxidation during solar exposure is mainly caused by singlet oxygen and by free radical attack, suggesting that sun skin-care cosmetics should make use not only of free radical scavengers but also of singlet oxygen quenchers (Auffray, 2007). Xanthohumol's singlet oxygen quenching activity might be widely used to develop new therapeutic medications for immune and inflammatory diseases (Moan et al., 2006).

Recently many other scientific papers have addressed the relationship between lifestyle diseases and chemical attacks by singlet oxygen and free radicals (Rao and Rao, 2007). We can see that xanthohumol must be ranked among the best naturally occurring antioxidants in terms of singlet oxygen-quenching activity. Our results suggest that its singlet oxygen-quenching activity comes close to that of β-carotene, a precursor of retinoids (Sies and Krinsky, 1995).

The radar chart in Fig. 2 exhibits how the hop components have ranked in our six antioxidant assays. The SOAC scores are not included in this figure; ORAC values used are the sum of lipophilic and hydrophilic results. For each assay category, scaling was made logarithmic so that the plot on the

chart resembles the associated rating. The chart graphically shows areas of relative strength and relative weakness, as well as depicting the general overall antioxidative capacity. The larger the spatial area, the higher the overall antioxidative capacity. We note that xanthohumol **7** (green) shows the highest quenching activity against peroxyl radical (ORAC-total), superoxide (SOD), ferric ion (FRAP) and hydroxyl radical (HORAC). Humulones **1** (yellow) show the strongest quenching activity against the nitrite radical (NORAC) and DPPH (data not shown).

On the other hand, chemically reduced derivatives **3**, **4** and **5** show poor antioxidant capacity in comparison to natural compounds. As active ingredients with anti-inflammatory activity due to their improved chemical stability and ease of handling, chemically reduced hop derivatives **4**, **5** and **6** are used for relief of arthritis symptoms. However, it must be kept in mind that the chemical modifications significantly impair one of the most important biological properties essential for anti-inflammatory efficacy, antioxidant capacity (Altindag et al., 2007).

In this study, we have employed biological assays related to the three main factors responsible for the pathogenesis of acne. First is bacterial proliferation. Second is increased sebum production caused by ROS, particularly singlet oxygen. Third is excessive matrix remodeling through type-1 collagen breakdown by MMPs.

Over-the-counter and other nonprescription medications can often be more effective if they are addressing more than one of the above factors when treating acne. As a result, dermatologists have generally concluded that taking a more comprehensive approach gives better results. Thus the recommendations given to patients frequently suggest using more than one agent at a time. Unfortunately, mixing medications does not always work because of unwanted interactions that lead to a decrease or loss of efficacy.

Results reported here confirm that xanthohumol **7** and lupulones **2** demonstrate multiple activities against the main biological events responsible for the pathogenesis of acne. It is clear that the anti-inflammatory activity of naturally occurring hop components inhibits the proinflammatory mediators (e.g., COX-2, PGE2, NO, NFkappaB, IL-1β and TNF-α) responsible for skin inflammation (Zhao et al., 2003; Hougee et al., 2006; Lee et al., 2007; Monteiro et al., 2008). Thus, the unique multifunctional activities of lupulones **2** and xanthohumol **7** among hop components, could prove to be of value for future human clinical studies in comparison with commercial combinations of synthetic drugs. On the other hand, it has been long known that fresh and dried hops occasionally induce contact dermatitis in hop workers (Cookson and Lawton, 1953; Raith and Jäger, 1984; Spiewak and Dutkiewicz, 2002). Since the allergens have not been specified yet, a careful skin test of 2 and 7 must be carried out prior to their topical use.

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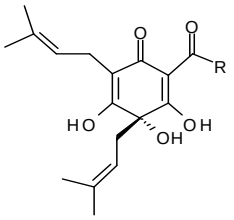
Figure 1. Structures of hop components (**1–7**)



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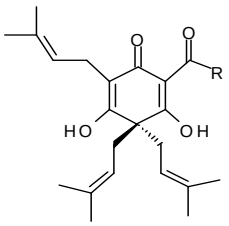
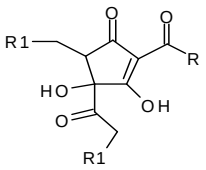
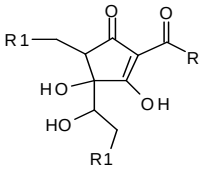
- CH(CH3)CH2CH3

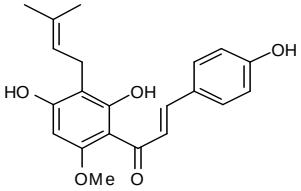


1: Humulones

2: Lupulones







3: Isohumulones
R1 = - CH=C(CH3)2

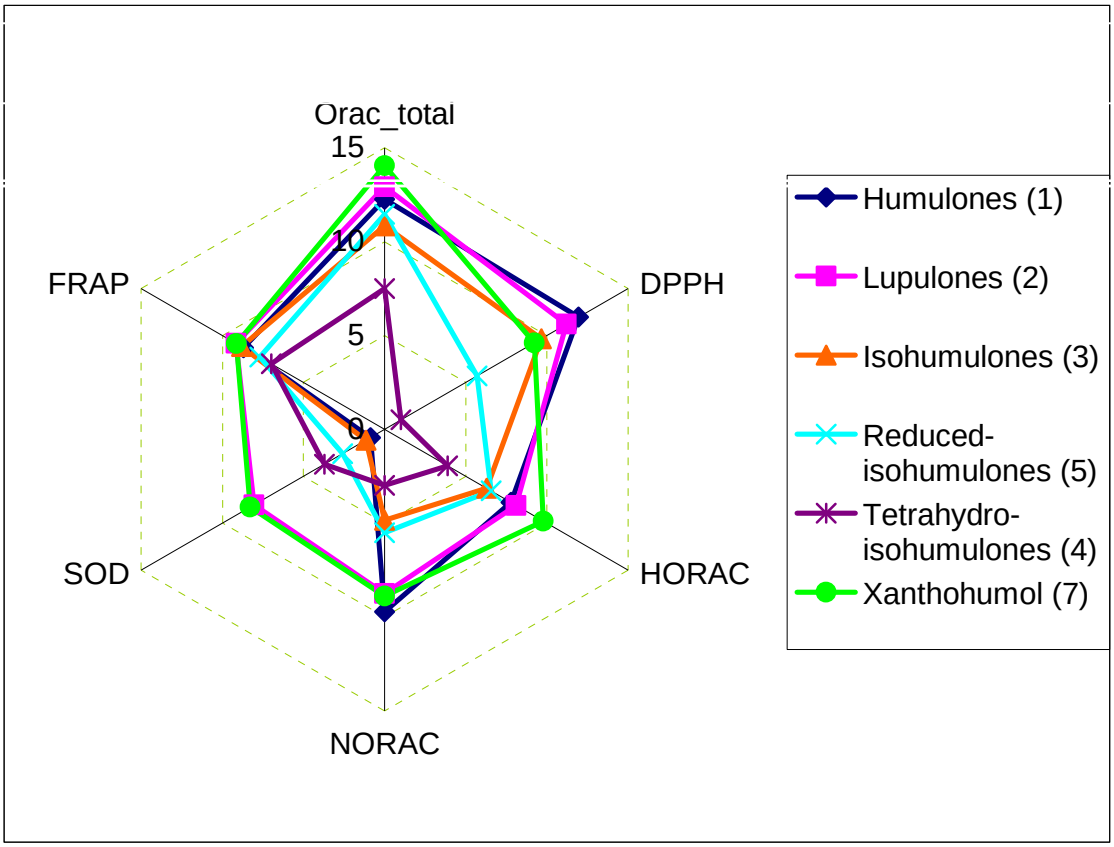
4: Tetrahydroisohumulones
R1 = -CH2-CH(CH3)2

5: Reduced isohumulones
R1 = - CH=C(CH3)2

6: Hexahydroisohumulones
R1 = -CH2-CH(CH3)2

7: Xanthohumol

Figure 2. Antioxidant activities of hop components in six assay categories



LEGENDS

Figure 1. Structures of hop components (1–7)

Table 1. Antibacterial activities (MIC[a] and MBC[b]) of hop components against the most common bacteria causing primary or secondary skin or soft tissue infections

Table 2. Anticollagenase activities[a] of hops against MMP-1 and MMP-8 involved in acne pathogenesis

Table 3. Reactive oxygen species (ROS) related to inflammation

Table 4. Oxygen radical absorbance capacity (ORAC) of hop component

Table 5. Singlet oxygen absorbance capacity (SOAC) of hop components

Figure 2. Antioxidant activities of hop components in six assay categories

Table 1. Antibacterial activities (MIC[a] and MBC[b]) of hop components against the most common bacteria causing primary or secondary skin or soft tissue infections

Conpound	MIC (μg/mL) [MBC (μg/mL)]				
	P. acnes	S. aureus	S. epidermidis	S. pyogenes	K. rhizophila
Humulones (1)	10 [30]	10 [100]	10 [100]	3 [100]	30 [100]
Lupulones (2)	0.1 [0.3]	1 [100]	10 [10]	0.3 [30]	1 [10]
Isohumulones (3)	30 [>100]	10 [>100]	30 [>100]	10 [>100]	100 [100]
Reduced isohumolones (5)	10 [>100]	3 [>100]	10 [>100]	10 [>100]	ND [ND]
Tetrahydro-isohumulones (4)	3 [10]	3 [>100]	10 [100]	10 [100]	ND [ND]
Hexahydro isohumulones (6)	3 [100]	10 [>100]	10 [100]	3 [>100]	ND [ND]
Xanthohumol (7)	3 [3]	1 [100]	3 [10]	1 [3]	1 [10]

ND: Not Determined

[a]MIC indicates minimal 100% inhibitory concentration.

[b]MBC indicates the lowest concentration required to kill an organism.

Table 2. Anticollagenase activities[a] of hops against MMP-1 and MMP-8 involved in acne pathogenesis

Compound	Concentration (μg/mL)	Inhibitory ratio (%)	
		MMP-1	MMP-8
Humulones (1)	100	62	62
	30	11	29
Lupulones (2)	100	27	28
	30	6	12
Isohumulones (3)	100	0	0
Reduced isohumulones (5)	100	0	0
Tetrahydroisohumulones (4)	100	0	0
Hexahydroisohumulones (6)	100	0	0
Xanthohumol (7)	100	91	99
	30	65	70
	10	25	31

[a] Data represent the mean of three independent measurements

Table 3. Reactive oxygen species (ROS) related to inflammation

	ROS	Assay
1	Peroxyl nitrite	NORAC
2	Hydroxyl radical	HORAC
3	Peroxyl radical, fat-soluble	ORAC-L
4	Peroxyl radical, water-soluble	ORAC-H
5	Superoxide	SOD
6	Heavy metal cation Fe^{3+}	FRAP
7	Singlet oxygen $^1O_2$.	SOAC

ORAC-H: $ORAC_{hydro}$, water-soluble antioxidant capacity
ORAC-L: $ORAC_{lipo}$, lipid soluble antioxidant capacity

Table 4. Oxygen radical absorbance capacity (ORAC) of hop components

Conpound	ORAC-H (Trolox equivalent)	ORAC-L (Trolox equivalent)	ORAC-Total (Trolox equivalent)
Humulones (1)	0.62	0.60	1.2
Lupulones (2)	0.84	1.1	1.9
Isohumulones (3)	0.41	0.054	0.46
Reduced isohumolones (5)	0.64	0.062	0.65
Tetrahydro-isohumulones (4)	0.034	0.011	0.045
Xanthohumol (7)	2.1	2.1	4.2
Vitamin C	1.4	-	1.4
Vitamin E	-	0.75	0.75
Polyphenon 60	4.2	0.006	4.2

ORAC-Total: $ORAC_{lipo} + ORAC_{hydro}$

- No activity detected

Table 5. Singlet oxygen absorbance capacity (SOAC) of hop components

Conpound	SOAC (Vitamin E equivalent)
Humulones (1)	0.75
Lupulones (2)	0.80
Isohumulones (3)	0.40
Reduced isohumolones (5)	0.55
Tetrahydro-isohumulones (4)	0.59
Xanthohumol (7)	14.1
Vitamin E	1.0
Polyphenon 60	1.8