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Hops (*Humulus lupulus* L.) Strobile Extract and Its Major Components Show Strong Antibacterial Activity against Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) can cause severe infections leading to sepsis and death. Antibiotic resistance is a growing burden in clinical settings, and new treatment strategies are required against MRSA infections and related health complications. Hops strobiles rich in bioactive compounds may provide an alternative solution to address the antibiotic resistance. Hops (*Humulus lupulus* L.) strobiles were extracted in five different ethanol concentrations; 10%, 30%, 50%, 70%, 95%, to determine optimal extraction of antibacterial compounds. The extracts and three major components of the strobiles; alpha acids, beta acids, and xanthohumol were assessed for antibacterial efficacy using standard well diffusion assay and micro-broth dilution method. The strobiles extracts showed the level of inhibition increasing with the higher ethanol concentration, 95% being the most effective. Minimum bactericidal concentrations (MBCs) ranged from 0.39 % (50%) to 0.01% (95%). Among the components tested, alpha and beta acids had the highest inhibitory action with MBCs 0.05% and 0.006% respectively. Time-kill studies conducted with 95% ethanol extract of hops showed that the bacterial growth reduced by more than 5 logs after 10 h, indicating strong bactericidal activity of the hops extract. To achieve optimal antimicrobial efficacy, extraction of hops with 95% ethanol is recommended.

Strong bactericidal activity of hops extracts and the components suggests the potential of hops strobiles for control of MRSA infections.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of healthcare-related infections in the United States and is associated with significant mortality and morbidity (CDC, 2013; Klein et al., 2007). Epidemiological studies suggest that community-associated MRSA infections are rapidly spreading into the healthcare system with increased total number of infections and are currently the dominant cause of skin and soft tissue infections (Kumar et al., 2015). A recent WHO report on surveillance data of drug resistance states that antibiotic resistance, including MRSA, is now a major threat to public health in all regions of the world (WHO, 2014). As pointed out by Moellering (2012), the efforts taken to control MRSA infection for almost 50 years have yielded only partial success, and the organism shows a remarkable ability to survive and continues to be a challenging pathogen. Novel approaches and novel antibacterial agents are therefore needed not only for MRSA infection but also for the treatment of other bacterial pathogens that are resistant to a number of antibiotics.

Plant-derived antimicrobial substances, which are widely used in traditional medicine around the globe, are considered as alternative strategies to control infectious diseases (Savoia, 2012). Whole plant extracts or isolated plant compounds have been

used for hundreds of years as natural medicines to combat diseases caused by bacterial, viral, and fungal pathogens (Hammer *et al.*, 1999). Plant secondary metabolites often have been shown to possess such antibacterial properties, and most of the bioactive compounds present in plants confer antimicrobial activity by damaging bacterial cell membranes and inhibition of proton motive force (Cowan, 1999; Cushnie and Lamb, 2005). Among the plants with antibacterial activity, hops (*Humulus lupulus* L.) has been gaining attention for its strong antibacterial properties. The female inflorescences (strobiles) of hops are well-known as a bittering agent in the brewing industry. Hops strobiles have been used in beer production to enhance the taste of beer as well as to increase its shelf life, which is mainly due to the fact that hops contain antibacterial compounds.

In addition to being used in brewing, hops have been used in herbal medicine for centuries to treat various ailments such as indigestion and bacterial infections. In North American and European folk medicine, hops were frequently mentioned as an infusion or a fomentation to relieve painful skin swelling, suppuration, skin sores, and injuries (Wichtl, 2004). Numerous studies have shown that its extracts are active against a wide range of microorganisms (Ohsugi *et al.*, 1997; Yamaguchi *et al.*, 2009). The major bio-active compounds present in hops extracts are alpha acids (humulone and its analogues), beta acids (lupulone and its corresponding isomers) and xanthohumol. In addition to the major bioactives, hops extracts contain over 100 different compounds, both volatile and non-volatile (Rój *et al.*, 2015), which may participate in inhibitory activity directly or synergistically enhancing the action of major compounds.

Ethanol is one of the most common organic solvents used in herbal medicine to extract plant compounds, and the bioactivity of plant components may depend on the ethanol concentration used in the extraction process (Green, 2000). Although a great amount of research has been performed to determine the antibacterial activity of medicinal plants, optimum extraction of antimicrobial compounds has not been well established for medicinal plants. The authors have previously shown that 90% ethanolic extracts of

several herbs including hops strobiles, all of which are used in herbal medicine for infectious diseases, possess strong antibacterial activity against several Gram-positive bacteria including a MRSA strain (Wendakoon *et al.*, 2012). Another study conducted by the authors (Gagnon *et al.*, 2014) has confirmed that the active components (alpha acids, beta acids, and xanthohumol) of hops can be extracted in optimum level only at a specific solvent concentration. In that study, hops strobiles extracted in different ethanol levels were subjected to HPLC analysis to assess the amount of each component present in different concentrations of ethanol, and the results showed that 95% ethanol is the ideal menstruum to extract maximum level of hops bioactive components. Further, when preparing hops strobiles extracts, a minimum of 70% ethanol is necessary not only to extract higher levels of the three bioactive constituents, but also to retain these constituents over a two-year period (Gagnon *et al.* 2014). Therefore, ethanol concentration is a critical factor to be considered when extracting hops to obtain bioactive components and maintain the components at the same level.

The aim of this study, therefore, is to investigate and compare the efficacy of hops extracted in different ethanol concentrations on the inhibition of MRSA. This comparative study on the antimicrobial properties of hops extracted in different levels of ethanol would reveal the optimum solvent concentration that may impart the strongest antimicrobial action on MRSA. In addition, three isolated compounds (alpha acids, beta acids, and xanthohumol) from hops strobiles were also studied for their antibacterial activity against Methicillin-resistant *Staphylococcus aureus*.

MATERIALS AND METHODS

Plant materials. Whole dried strobiles of hops grown in the Yakima Valley, Washington State, were obtained from HopSteiner, a division of S.S. Steiner, New York, NY. The strobiles were of the Super Galena variety. The strobiles were dried and stored under frozen conditions in warehouses in Yakima, WA, and then shipped to Herbs, Etc. Inc., where they were stored in a freezer at -15°C for about two

months. Identity of the material was confirmed by the author (DG) using macroscopic and organoleptic methods (Wichtl, 2004).

Cryogenic grinding and cold-process percolation extraction with ethanol. The strobiles were cryogenically ground to prevent the breakdown of heat-sensitive constituents, using a hammer mill (Fitzpatrick Manufacturing, Sterling Heights, MI) cooled by injecting liquid nitrogen into the grinding chamber. A sample of the ground material was set aside and kept frozen for future reference. The bulk of the ground material was used to produce ethanolic extracts. Ethanol (95% USP grade ethyl alcohol, Pharmco-Aaper, Shelbyville, KY) at different concentrations (10%, 30%, 50%, 70%) were prepared with water, and the solvent percentages were verified using a hydrometer and a thermometer. Dried hops strobiles were extracted in the different ethanol concentrations including 95%. On the same day that the strobiles were powdered using the cryogenic grinding method, a cold-process percolation extraction was carried out to extract the strobiles. A 1:5 herb-to-solvent ratio was used to extract the ground strobiles (Green 2000). The finished extracts were filtered to remove sediments and stored in amber-colored glass bottles. All extracted samples were kept at room temperature in a dark closet.

Hops Components. Alpha acids (40%) and beta acids (42%), both in propylene glycol, and xanthohumol powder (71.8%) were obtained from HopSteiner, a division of S.S. Steiner, New York, NY and stored at 4°C. Alpha acids and beta acids were further diluted appropriately in propylene glycol for the experiments. Xanthohumol was dissolved in 95% ethanol in order to obtain a 30% solution.

Bacterial cultures. *Staphylococcus aureus* (ATCC 25923), Methicillin-resistant *Staphylococcus aureus* (MRSA); (ATCC 43300) and *E. coli* (ATCC 25922) were used in the study. Stock cultures were maintained in tryptic soy broth (TSB) supplemented with 20% glycerol at -70°C. For use in the experiments, working cultures were grown in Mueller-Hinton broth for 20-22h at 37°C.

Antibacterial activity of the ethanol extracts and components of hops. The ethanolic extracts of hops strobiles were tested without further dilution.

The individual compounds (alpha acids, beta acids and xanthohumol) were tested after diluting at appropriate concentrations. The antibacterial activity was evaluated using well diffusion assay and broth dilution assay. All experiments were performed in duplicate and replicated at least three times with the same extracts using new bacterial cultures in each experiment. One-way ANOVA was used to determine whether there was any significant difference among the extracts.

(a) Well diffusion assay. The well diffusion assay was carried out using Mueller-Hinton agar plates according to the method described in NCCLS manual (NCCLS, 2003). Culture media and antibiotics [Ampicillin (10 µg) and Oxacillin (1 µg)] discs were purchased from BD, NJ, USA. For each experiment, appropriate dilutions of the bacterial cultures were prepared in phosphate buffered saline (PBS). Diluted bacterial cultures adjusted to a 0.5 McFarland turbidity ($1-2 \times 10^8$ CFU mL⁻¹) were spread over the entire surface of the agar plates using a sterile cotton swab. After allowing the plates to dry for about 10 min, wells (6mm holes) were cut in agar using sterile plastic straws. The plant extracts (150µL) were placed in each well. For sample controls, the wells were filled with appropriate concentrations of ethanol. Ampicillin (10µg) and Oxacillin (1 µg) were used as positive controls. Three replicates were performed for each of the extract. The plates were incubated at 37°C for 22-24h period. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was measured and recorded.

(b) Broth micro dilution method. Antimicrobial efficacy of the extracts and the components were also tested using the broth dilution technique and the minimum bactericidal concentration (MBC) was calculated. The test was performed in sterile 96-well micro titer plates (flat bottom). Each well was filled with a 100 µL aliquot of Mueller Hinton broth (MHB). The first column of wells received a 100 µL of the test compound and serial 2-fold dilutions were made to produce further dilutions. The bacterial culture was diluted in PBS and each well was inoculated with 50 µL of inoculum (final 10^6 CFU mL⁻¹) and incubated at 37°C.

Following overnight incubation, a small volume of the culture from each well was streaked on Tryptic Soy agar plates and the plates were incubated at 37°C for an overnight period. The lowest dilution that yielded complete inhibition of growth was taken as the minimal bactericidal concentration. Controls included: (i) uninoculated media without test compound; (ii) inoculated media without test compound to evaluate the microbial growth; (iii) inoculated media without test compound but containing corresponding amount of ethanol.

Bactericidal activity of hops extract. The time-kill test procedure was performed with the 95% hops extract according to the NCCLS guidelines. The bactericidal activity was determined in 100 mL of Mueller-Hinton broth containing two levels (0.1% and 0.2%) of the 95% ethanol extract with a starting inoculum of $\sim 10^6$ CFU mL⁻¹ of *S. aureus* (MRSA) culture. The inoculum was added to each flask, mixed well and incubated at 37°C. Aliquots were taken out at specific time intervals for 25 h and the surviving bacteria were enumerated by plating 100µL of serial dilution of the cultures. The control culture, without the test compound was also enumerated for 25h. Bactericidal effect was defined as $\geq 3 \log_{10}$ CFU mL⁻¹ decrease in comparison with the level for the initial inoculum after 24 h of incubation. The entire experiment was repeated and average values were expressed.

RESULTS AND DISCUSSION

In the present study, air-dried hops strobiles extracted in different ethanol concentrations were evaluated for their ability to inhibit the growth of Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and for determination of the ideal ethanol concentration to be used in the extraction process for optimal antibacterial activity. In addition, antibacterial efficacy of the isolated constituents (alpha acids, beta acids, and xanthohumol) of hops strobiles were also assessed. Both well diffusion assay and broth dilution method revealed the strong antibacterial activity of hops extracts as well as of the individual compounds against MRSA.

Of the five ethanol extracts tested using well diffusion assay, 95% extract showed the highest

antibacterial activity against the antibiotic-resistant bacterium (Table 1 & Figure 1). The results clearly showed that the bioactivity is dependent on the solvent concentration used in the extraction process, and the level of inhibition was directly proportional to the ethanol concentration. Since hops bioactives can be extracted at maximum level with 95% ethanol (Gagnon, 2014), it is likely that 95% extract containing all the three major components may have participated in the inhibition. The solvent, ethanol, used as the negative control did not show any inhibition. Ampicillin and Oxacillin used as positive controls confirmed the resistance of the microorganism to the antibiotics. The results were also compared with *E. coli* (ATCC 25922) and the extracts did not inhibit the growth of *E. coli*. It is not surprising that *E. coli* was not inhibited by the hops extracts since it is known that Gram-negative organisms are less susceptible to plant antibacterials. (Zaika, 1988; Cowan, 1999).

When the individual hops components were tested in well diffusion assay (Table 1), the growth of this methicillin-resistant strain was easily inhibited by the constituents. All the three constituents showed a strong inhibition, in particular, beta acids having the strongest activity. Hops strobiles extracted in 95% ethanol were shown to contain 2.12% alpha acids, 1.44% beta acids and .09% xanthohumol (Gagnon, 2014). Therefore, original hops components, alpha acids and beta acids used in this study were diluted to obtain similar concentrations for the well assay experiment. The concentrations of alpha acids and beta acids used in the well assay were 2% and 1.5% respectively. Numerous studies have shown that it is the hops bitter acids that account for its antibacterial action (Simpson and Smith, 1992) and other bioactive properties (Van Cleemput et al., 2009). Antibacterial action of xanthohumol at the concentration used was slightly lower compared to that of hops acids. Xanthohumol has been shown to inhibit the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus mutans* (Gerhauser, 2005) and may play a synergistic inhibitory role along with the acids. The synergy of these compounds in the whole hops extract may have contributed to its strong antibacterial activity. In addition to the three major compounds,

hops have been shown to contain a wide range of other compounds, some of which may also act synergistically along with the acids (Rój et al., 2015; Van Cleemput et al., 2009).

Table 1. Antibacterial activity of hops ethanolic extracts and its components against MRSA.

Hops extracts in ethanol and hops constituents	Inhibitory zone (mm) for <i>S. aureus</i> ATCC 43300	Inhibitory zone (mm) for <i>E. coli</i> 25922
Extracts		
10%	-	-
30%	8 (0.83) *	-
50%	24 (0.87) *	-
70%	29 (0.82) *	9 (0.94)
95%	34 (1.0) *	11(0.82)
Constituents		
Alpha acids	31 (1.22) **	-
Beta acids	48 (0.70) **	-
Xanthohumul	19 (0.71) **	-
Controls		
Ampicillin	10 (1.17)	19 (0.8)
Oxacillin	14 (0.98)	-

Mean diameters (mm) of inhibitory zones are given with standard deviation in parenthesis. The experiments were repeated at least three times in duplicates. */** denotes significant difference (P<0.05).

Table 2. Minimum bactericidal concentrations of hops extracts and its components against MRSA.

Extract/Compound	MBC % (vol/vol)
10%	>50
30%	>50
50%	0.39
70%	0.10
95%	0.01
Alpha acids	0.01
Beta acids	0.006
Xanthohumul	0.78

Average value for 3 determinations.

In the next step, we determined the minimum bactericidal concentrations (MBCs) for each extract and for each component. It was interesting to note that both ethanol extracts above 50% and the isolated components showed a stronger bactericidal activity towards MRSA (Table 2). MBCs ranged from 0.39% for 50% ethanol to 0.01% for 95% ethanol indicating strong bactericidal action. Alpha acids had the similar MBC as with 95% ethanol extract (0.01%) and beta acids was the most potent compound with 0.006% MBC, suggesting its potential applications in clinical or pharmaceutical preparations. The time kill assay conducted to evaluate the pattern of growth inhibition of MRSA in the presence of 95% ethanolic extract (Figure 2) showed a strong inhibition of the pathogen after being exposed to 0.1% and 0.2% of the extract. Compared to the control, a complete growth

inhibition occurred when the extract was added to the medium demonstrating a rapid concentration-dependent bactericidal activity of hops extract. This is a very important finding that a nearly 5-log reduction in growth occurred within 10-12 h in the presence of the 95% hops extract.

In addition to the essential role of hops in inhibiting beer spoilage bacteria, its ability to overcome pathogenic bacterial growth has been presented in a number of studies (Simpson and Smith, 1992; Bhattacharya et al., 2003). Hops have been frequently used in traditional European folk medicine for treating skin disorders (Van Hellemont, 1986). The present study clearly shows that there is a strong positive correlation between the ethanol level used for extraction and the antibacterial activity of hops. Additionally, the finding that each of the single isolated alpha and beta acids constituent is also very effective in killing MRSA seems promising as these results may inspire the designing of new antibacterial medicine for eradication of MRSA.

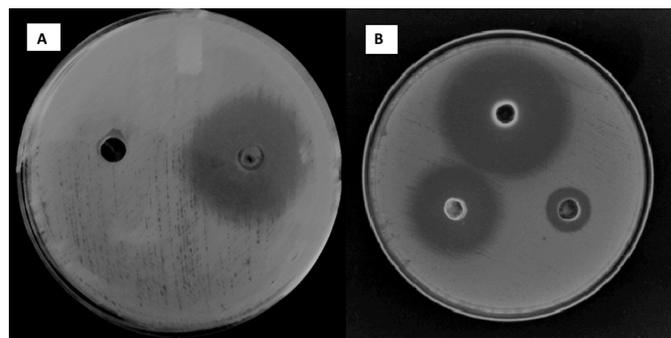


Figure 1. Antibacterial activity of (A) 95% ethanol extract; left - 95% ethanol only, right - hops ethanol extract, (B) Hops components; Top: Beta acid; Lower left: Alpha acid; Lower right: Xanthohumul.

It is known that antimicrobial resistance may develop as a result of serial passages of a microorganism through sub-lethal concentration of an antimicrobial agent (Andersson and Hughes, 2014). However, a study done by Bhattacharya et al. (2003) revealed that bacteria did not develop resistance to the isolated hops constituent beta acids even after 10 passages in a sub-inhibitory concentration. This is a valuable finding to support the idea of developing antibacterial medicines from hops constituents. Bacterial resistance to antibiotics is among the most

challenging problems in clinical medicine and health care settings, and there has been a remarkable increase of drug resistant bacteria including Methicillin-resistant *Staphylococcus aureus* (MRSA). This study reveals the strong antibacterial activity of major components and ethanol extracts of hops strobiles against MRSA with a potential for development of therapeutic products for drug resistant *S. aureus* infections.

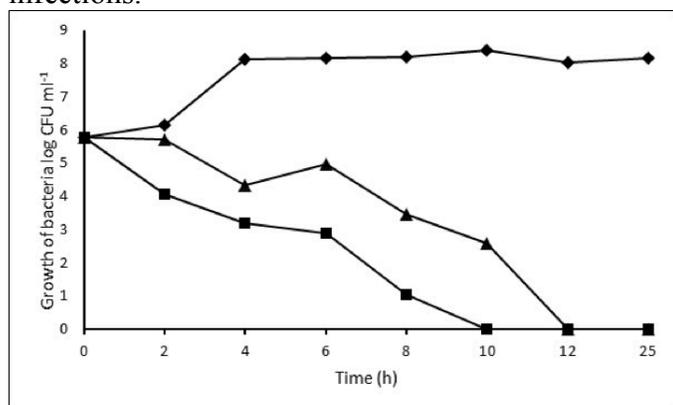


Figure 2. Bactericidal activity of 95% ethanolic hops extract against Methicillin-resistant *S. aureus* (ATCC 43300). Growth was monitored for 24h in the presence of 0.2% of hops extract (■), 0.1% of hops extract (▲), and in the absence of the extract (◆) in Mueller-Hinton broth

CONCLUSION

The aim of this research was to evaluate the antibacterial action of hops strobiles extracted in different ethanol concentrations on MRSA in order to determine the ideal concentration of ethanol for optimal extraction of antibacterial compounds. We conclude that 50% or greater ethanol extracts as well as two individual components of hops (alpha acids and beta acids) can inhibit the growth of MRSA very effectively. Furthermore, solvent concentration used in the extraction process has a significant influence on the antibacterial activity of hops, and use of 95% ethanol optimizes the extraction of antibacterial components from hops strobiles. The results of this study strongly support the likelihood that hops ethanolic extracts and/or the two major isolated components can successfully be used as an antibacterial agent against MRSA infection and warrant further research on developing hops derived medicines or pharmaceuticals, especially for skin infections. In addition, it may be useful to separate

other bioactive compounds from the hop extracts and test the synergistic action of different combinations. Hops strobile ethanolic extracts rich in naturally occurring bioactive compounds with antibacterial properties may provide an alternative solution to treat MRSA infections. There is a distinct possibility that either a single component or a mixture of hops compounds could become a new class of antibacterial for MRSA infections.

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