Hops (*Humulus lupulus*) and Methicillin-Resistant *Staphylococcus aureus* (MRSA): A Tale of Discovery

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Staph infection sparks curiosity

- In 1997, I contracted a head-to-toe *Staph* infection
- MD diagnosed infection and prescribed antibiotics
- Leaving for Nepal one week later
- Concerned about effect of antibiotics on GI system
- Unwilling to take them
- Decided on a combination of Usnea (*Usnea barbata*) lichen and Hops (*Humulus lupulus*) strobile extracts externally and internally
- Infection gone within a week
- Traveled without any GI incidents

Skin infections and MRSA

- Herbalists frequently encounter skin infections in their practice
- Skin infections getting more challenging because of ever-increasing microbial multi-drug resistance
- Community-associated MRSA infections now the dominant cause of skin and soft tissue infections
- CA-MRSA infections represent the majority of patients with skin and soft tissue infections being treated at emergency departments
- MRSA-associated hospitalization have increased from 8% in 1993, to 44% in 1998, and to 65% in 2005

Evaluation of Selected Medicinal Plants Extracted in Different Ethanol Concentrations for Antibacterial Activity Against Human Pathogens

Chitra Wendakoon, PhD
Peter Calderon, PhD
Daniel Gagnon, MS

Study published in *Journal of Medicinally Active Plants*, 2012. 1(2): 60-68.

Initial *in vitro* study

- In 2007, Herbs, Etc. sponsored a study at New Mexico State University of eight herbs: boldo, buchu, *Echinacea angustifolia*, hops, licorice, Oregon grape, usnea, and yerba mansa

Aim of the study

- Perform a comparative study to evaluate the relative effectiveness of the antimicrobial properties of eight medicinal herb extracts

Study design

- Extracts were prepared with 3 different ethanol concentrations (50%, 70% and 90%)
- Screened for antibacterial activity against four Gram-positive and four Gram-negative pathogens
- Used standard well assay and micro-broth dilution method
- Determined minimum bactericidal concentrations (MBCs) for each extract

Plants and Bacterial Strains Used in the Study

Table 1. Plants used in the study

Common name	Latin binomial	Family	Plant part
Boldo	*Peumus boldus*	Monimiaceae	Leaf (dry)
Buchu	*Agathosma betulina*	Rutaceae	Leaf (dry)
Echinacea angustifolia	*Echinacea angustifolia*	Asteraceae	Root (dry)
Hops	*Humulus lupulus*	Cannabaceae	Strobile (dry)
Licorice	*Glycyrrhiza glabra*	Fabaceae	Root (dry)
Oregon grape	*Mahonia aquifolium*	Berberidaceae	Root (dry)
Usnea	*Usnea barbata*	Usneaceae	Lichen (dry)
Yerba mansa	*Anemopsis californica*	Saururaceae	Root (dry)

Table 2. Bacterial strains used in the study

Gram-positive:	Gram-negative:
Staphylococcus aureus (ATCC 25923)	*Escherichia coli* (ATCC 25922)
Staphylococcus aureus COL (MRSA)*	*Pseudomonas aeruginosa* (ATCC 27853)
Staphylococcus epidermidis (ATCC 12228)	*Salmonella enteritidis* (ATCC 13076)
Streptococcus pyogenes (ATCC 19615)	*Klebsiella pneumoniae* (ATCC 13883)

*The MRSA culture was kindly provided by Dr. J. Gustafson, Department Biology, New Mexico State University

Antibacterial activity of ethanol extracts of 8 plants on Gram-positive bacteria

Plant	Ethanol	*Staphylococcus aureus*	*Staphylococcus aureus* (MRSA)	*Staphylococcus epidermidis*	*Streptococcus pyogenes*
	Inhibitory zone diameter* (%)	(mm)	(mm)	(mm)	(mm)
Boldo	50	18	22	22	20
	70	19	23	23	22
	90	17	20	23	21
Buchu	50	14	21	N	18
	70	12	14	11	20
	90	10	9	10	18
Echinacea angustifolia	50	N	N	N	12
	70	N	N	N	10
	90	N	N	N	11
Hops	50	32	31	35	24
	70	32	31	34	24
	90	36	37	34	25
Licorice	50	18	21	19	20
	70	18	21	19	25
	90	20	18	18	25
Oregon grape	50	13	15	20	28
	70	13	16	19	28
	90	12	12	17	25
Usnea	50	N	N	11	16
	70	N	N	18	13
	90	N	16	32	16
Yerba mansa	50	14	18	19	19
	70	15	19	19	20
	90	14	15	17	20
Ampicillin	(10ug)	34	12	25	35
Ethanol	50	N	N	N	N
	70	N	N	N	N
	90	N	N	N	N

*Mean value for 5 samples

Analysis

- Boldo, hops, licorice, and yerba mansa possess strong inhibitory activities on all four Gram-positive organisms tested at all levels of ethanol
- No inhibitory action detected against the four Gram-negative bacteria tested in this study
- Antibacterial activity of the other plant extracts varied depending on the ethanol level (Table 3)
- Buchu at 50% ethanol, Oregon grape at 70 % ethanol were more inhibitory towards MRSA than either at 90% ethanol level
- Hops showed the strongest antibacterial activity and, further, its activity was highest at 90% ethanol level
- The results from the broth dilution study were in agreement with the well assay, confirming that the extracts of boldo, hops, licorice, and yerba mansa are potentially effective antibacterial agents

Conclusions

- Thousands of scientific papers describe the antimicrobial activities of plant extracts
- However, no systematic studies have been conducted on the effects of ethanol concentration on the antimicrobial activity of specific herbs
- Due to the complex nature of the phytochemicals present in herbs, extraction solvent system should be taken into consideration
- This study provides data on the importance of various ethanol concentration on the antimicrobial activity of herbs
- It also demonstrated that the ethanol extracts of Hops offered significant potential for the development of novel antibacterial therapies
- More studies on the antimicrobial activity of Hops against specific bacteria are needed

Second study

The potential antibacterial properties of Hops (*Humulus lupulus*) extracts against methicillin-resistant *Staphylococcus aureus* (MRSA)

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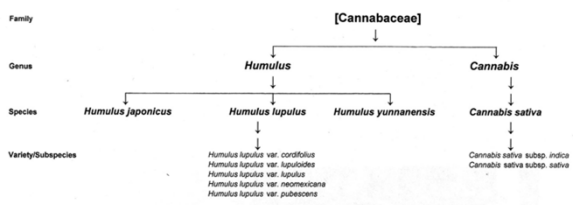
Jeremy Leker, BS, HopSteiner

Study published in *European Journal of Medicinal Plants,* 2014. 4(11): 1302-1312.

Appendix I: Taxonomy of Cannabaceae Family



USDA ARS (United States Department of Agriculture), Agricultural Research Service, Beltsville Area. Germplasm Resources Information Network (GRIN). Available at: http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?19415. [Accessed 1/14/2009] and http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?8862. [Accessed 1/12/2009].

Hops strobiles in September (a.k.a. cones)

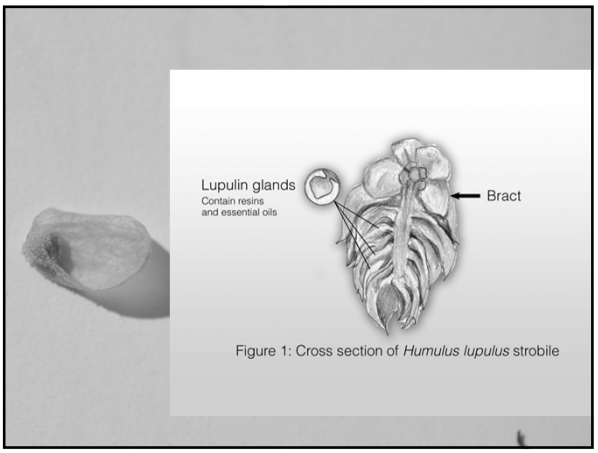


Figure 1: Cross section of *Humulus lupulus* strobile

Primary aim of the study

- Investigate the effectiveness of the *in vitro* antibacterial activity of five Hops (*Humulus lupulus)* strobile ethanolic extracts containing 10%, 30%, 50%, 70%, and 95% ethanol and three *H. lupulus* isolated constituents, alpha acids (α-acids), beta-acids (β-acids), and xanthohumol against methicillin-resistant *Staphylococcus aureus* (MRSA)

Secondary aims of the study

- Chemically analyze whole and ground strobiles as well as the extracts by quantifying the amounts of three constituents: α-acids, β-acids, and xanthohumol immediately after making the extracts as well as one year later and two years later
- Compare and contrast the constituents in the five ethanolic extracts and the three isolated constituents as to their relative MRSA antimicrobial activity

Results of HPLC analysis:

Chemical characterization of *H. lupulus* extracts

- Performed a HPLC analysis of five *H. lupulus* ethanolic extracts, as well as whole and ground strobiles after one and two year storage
- Evaluated the amount of constituents loss during cryogenic grinding process of whole *H. lupulus*
- Calculated the difference in amount of constituents immediately after extraction, as well as after one year and two years of storage

HPLC analysis of *Humulus lupulus* ethanolic extracts and strobiles at the beginning of the experiment

Concentrations of compounds g/100 ml for ethanolic extracts and percentage of *H. lupulus* whole and ground strobiles

Compounds	10% Ethanol	30% Ethanol	Menstruum 50% Ethanol	70% Ethanol	95% Ethanol	Whole Hops Strobiles	Ground Hops Strobiles
Alpha Acids	0.040	0.060	0.630	2.010	2.120	11.400	10.400
Beta Acids	0.015	0.017	0.053	1.050	1.440	7.480	6.940
Xanthohumol	0.002	0.002	0.026	0.078	0.090	0.459	0.445

HPLC analysis was performed March 9, 2010

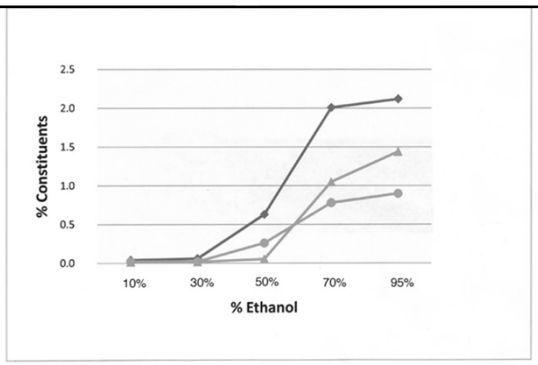


Figure 4: Concentration of *H. lupulus* Selected Constituents in Ethanolic Extracts

Alpha Acids
Beta Acids
Xanthohumol X10

HPLC analysis of *Humulus lupulus* ethanolic extracts and strobiles stored for one year

Concentrations are expressed in g/100 ml for ethanolic extracts and as a percentage of *Humulus lupulus* for whole and ground strobiles

Compounds	10% Ethanol	30% Ethanol	Menstruum 50% Ethanol	70% Ethanol	95% Ethanol	Whole Hops Strobiles	Ground Hops Strobiles
Alpha Acids	0.003	0.016	0.520	1.810	2.030	9.90	9.66
Beta Acids	0.0004	0.0005	0.036	1.000	1.370	5.64	6.03
Xanthohumol	0.00009	0.0004	0.020	0.080	0.081	0.43	0.43

These *Humulus lupulus* ethanolic extracts were made in January of 2010 and were stored at room temperature in a dark closet for one year.
Both whole and ground *Humulus lupulus* strobiles were stored in a freezer kept at -15°C for one year.
HPLC analysis was performed February 3, 2011

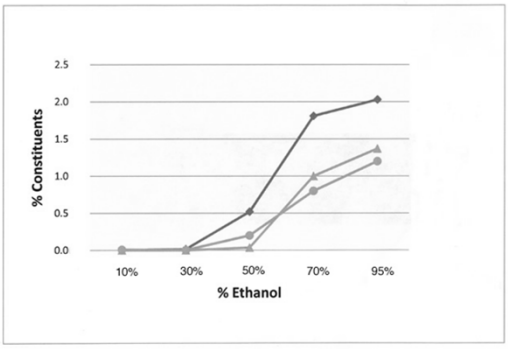


Figure 5: Concentration of *Humulus lupulus* Selected Constituents in Ethanolic Extracts Stored One Year

Alpha Acids
Beta Acids
Xanthohumol X10

HPLC analysis of *Humulus lupulus* ethanolic extracts after one year and two years of storage at room temperature (21°C)

Ethanol (%)	Constituents (g/100ml) Alpha acids Y1	Alpha acids Y2	Beta acids Y1	Beta acids Y2	Xanthohumol Y1	Xanthohumol Y2
10	0.003	0.0008	0.0004	*N.D.	0.00009	*N.D.
30	0.016	0.0068	0.0005	0.0001	0.0004	0.0003
50	0.520	0.404	0.036	0.030	0.020	0.0096
70	1.810	1.500	1.000	0.892	0.070	0.043
95	2.030	1.660	1.370	1.170	0.081	0.059

N.D.: Not Detectable

Percent of constituents from *Humulus lupulus* ethanol extracts **remaining** when stored for one or two years

One years old extracts (2011)

Ethanol concentration (%)	Alpha acids	Beta acids	Xanthohumol
10%	7.5	2.7	4.5
30%	26.7	2.9	20.0
50%	82.5	67.9	76.9
70%	90.1	95.2	102.5
95%	95.8	95.1	90.0
Whole Strobiles	86.8	75.4	95.6
Ground Strobiles	92.9	86.9	95.6

Two years old extracts (2012)

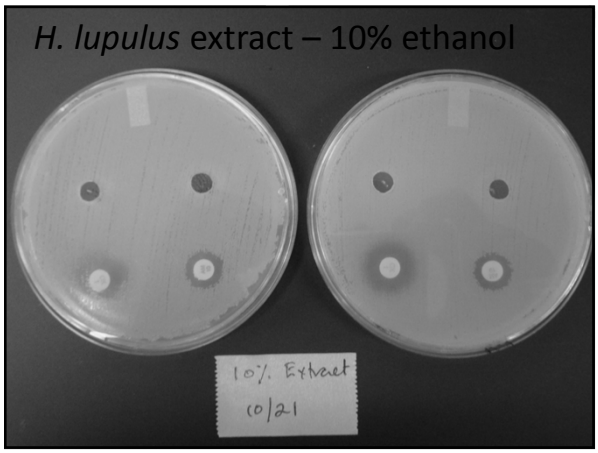
Ethanol Concentration	Alpha acids	Beta acids	Xanthohumol
10%	0.2	0.0	0.0
30%	11.3	0.6	15.0
50%	64.1	56.6	36.9
70%	74.6	85.0	55.1
95%	78.3	81.3	65.6
Whole Strobiles	102.4	90.1	95.6
Ground strobiles	69.7	70.0	72.9

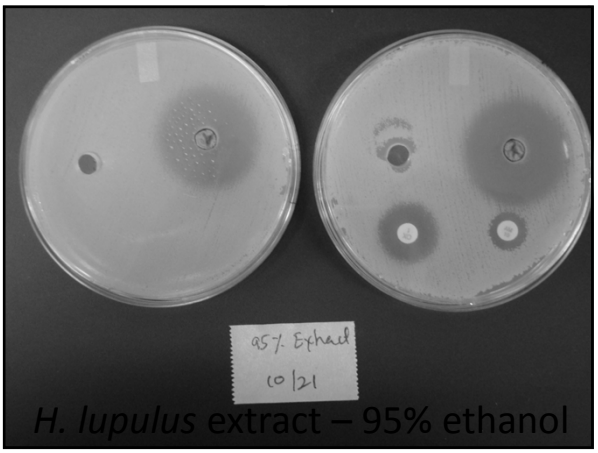
Concentrations are expressed in g/100 ml for ethanolic extracts

Antibacterial activity of *H. lupulus* strobile extracts against methicillin-resistant *S. aureus* (MRSA)

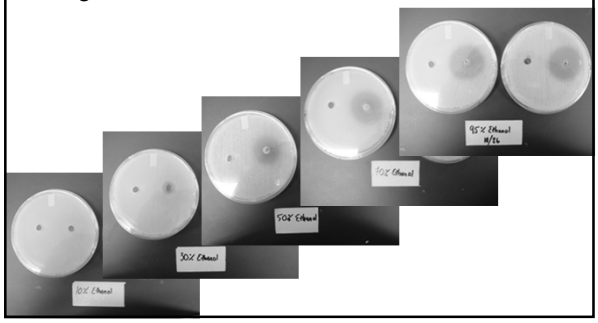
Ethanol concentration (%)	Zone Diameter (mm)* *S. aureus* (MRSA)	*E. coli*
10	N	N
30	8	N
50	24	N
70	30	8
95	34	12
Ampicillin (10µg)	10	1
Oxacillin (1µg)	14	N
Ethanol (10, 30, 50, 70, 95%)	N	N

N= no inhibitory zones were observed





Antibacterial activity of *H. lupulus* ethanolic extracts against methicillin-resistant *S. aureus* ATCC 43300



Antibacterial activity of diluted *H. lupulus* isolated constituents against methicillin-resistant *S. aureus* (MRSA)

Compound	Zone Diameter (mm)						
	Replicate						
Isolated Constituents	1	2	3	Average	Ethanol	Ampicillin	Oxacillin
α-acid	32	30	31	31	-	10.5	16
β-acid	49	50	50	50	-	11	-
Xanthohumol	15	15	15	15	-	11	-
Propylene glycol	-	-	-	-			

All assays were done in duplicate
α-acids was diluted to 1.95g/100ml in a base of propylene glycol. Β-acids was diluted to 1.44g/100ml in a base of propylene glycol.

Minimum bactericidal concentrations (MBC) of *Humulus lupulus* ethanolic extracts

Extract	Extract concentration (%) in microtiter well										Control	
	Well 1 (1:2)	Well 2 (1:4)	Well 3 (1:8)	Well 4 (1:16)	Well 5 (1:32)	Well 6 (1:64)	Well 7 (1:128)	Well 8 (1:256)	Well 9 (1:512)	Well 10 (1:1,024)	Positive	Negative
	50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	S. aureus	Broth
10%	+	+	+	+	+	+	+	+	+	+	+	-
30%	+	+	+	+	+	+	+	+	+	+	+	-
α-acids β-acids xanthohumol								2.46µg/ml 0.20µg/ml 0.10µg/ml				
50%	-	-	-	-	-	-	-	-	+	+	+	-
α-acids β-acids xanthohumol										<1.96µg/ml <1.00µg/ml <0.08µg/ml		
70%	-	-	-	-	-	-	-	-	-	-	+	-
α-acids β-acids xanthohumol										<2.07µg/ml <1.40µg/ml <0.09µg/ml		
95%	-	-	-	-	-	-	-	-	-	-	+	-

All experiments were done in duplicate
The 10% *H. lupulus* ethanolic extracts contained 40µg of α-acids, 15µg of β-acids and 2µg of xanthohumol.
The 30% *H. lupulus* ethanolic extracts contained 60µg of α-acids, 17µg of β-acids and 2µg of xanthohumol.
The 50% *H. lupulus* ethanolic extracts contained 630µg of α-acids, 53µg of β-acids and 26µg of xanthohumol.
The 70% *H. lupulus* ethanolic extracts contained 2,010µg of α-acids, 1,060µg of β-acids and 78µg of xanthohumol.
The 95% *H. lupulus* ethanolic extracts contained 2,120µg of α-acids, 1,440µg of β-acids and 90µg of xanthohumol.

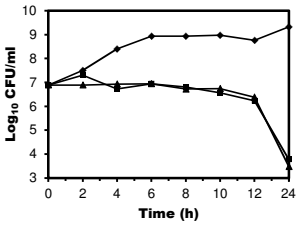
Minimum bactericidal concentrations (MBC) of diluted *H. lupulus* isolated constituents

Extract	Extract concentration (%) in microtiter well																Control	
	1 (1:2)	2 (1:4)	3 (1:8)	4 (1:16)	5 (1:32)	6 (1:64)	7 (1:128)	8 (1:256)	9 (1:512)	10 (1:1024)	11 (1:2048)	12 (1:4,096)	13 (1:8,192)	14 (1:16,384)	15 (32,768)		Positive	negative
	50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	0.05%	0.026%	0.013%	0.006%	0.003%		S. aureus	Broth
1,946µg α-acid	-	-	-	-	-	-	-	-	-	-	-	-	0.24µg -	+	+	+	+	-
1,440µg β-acid	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09 µg -	+	+	+	-
60µg Xanthohumol	-	-	-	-	-	-	0.7µg -	+	+	+	+	+	+	+	+	+	+	-
Propylene Glycol	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

The first 100µl of α-acid rich extract added to well #1 contained 1,946µg of α-acids.
The first 100µl of β-acid rich extract added to well #1 contained 1,440µg of β-acids.
The first 100µl of xanthohumol-rich extract added to well #1 contained 60µg of xanthohumol.

Bactericidal activity of *H. lupulus* extract (95% ethanol) against methicillin-resistant *S. aureus* (MRSA)

Growth was monitored for 24h in the presence of 100µl of the extract (■), 200µl of the extract (▲) and in the absence of the extract (♦).



Discussion

- Plant metabolites that display super potent biological activity are relatively rare
- Very large amounts of active constituents, essential oils, and other compounds are needed to act effectively as antibiotics
- The minimum inhibitory concentration for most herbs are in the range of 100 to 1,000µg/ml of active constituents or higher
- However, the *Humulus lupulus*' constituent beta-acids was active in the 0.09µg/ml range
- *In vitro* Hops (*Humulus lupulus)* is active against MRSA and is comparable to the few antibiotics that are still active against this bacteria

Discussion

- Lab research is often disconnected from clinical application
- Lessons learned from the study are applicable to herbalists
  - Keep *H. lupulus* strobiles frozen until used
  - Minimize the loss of *H. lupulus* active constituents during the grinding process
  - Use a high ethanol content menstruum to extract ground *H. lupulus* because:
    - High ethanol extracts yield more active constituents
    - High ethanol extracts preserves a larger amount of active constituents over a longer period of time
  - High ethanol *Humulus lupulus* extracts are effective against methicillin-resistant *Staphylococcus aureus (MRSA)*

Clinical notes: MRSA skin infection

- 24 years old man, mechanic by trade, diagnosed with MRSA skin infection under his nail bed
- Second MRSA infection affecting a finger in six years
- Doctors cut the nail bed to drain the infection
- First infection took two rounds of antibiotics to stop it. This time, on third round of antibiotic
- MD told him he would need to cut his finger at the first joint if unable to stop the infection – kept getting worse
- Applied Hops (*Humulus lupulus*) 95% ethanol extract compresses on the infection
- Within two weeks the infection was gone
- Six years have now elapsed since that incident and he has remained free from MRSA infections

Conclusions

- Much work needed to be done to elucidate the secrets of the antibacterial properties of Hops (*Humulus lupulus*)
- The research presented here points toward the usefulness of the strobile against methicillin-resistant *S. aureus* (MRSA)
- Initial clinical results suggest that Hops may be useful against MRSA
- Keep Hops strobiles frozen until use
- High ethanol level essential to make a strong and effective Hops extract
- High ethanol level is essential to safeguard the potency of Hops extracts over time

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