Original Research Article

Stability of Active Constituents of Hops (*Humulus lupulus*) Strobiles and their Ethanolic Extracts during Storage

ABSTRACT

Aims: The purpose of this study was to evaluate the stability of three major active constituents in dried hops (*Humulus lupulus*) strobiles and their ethanolic extracts during storage.

Methodology: A comparative study of the levels of alpha acids, beta acids, and xanthohumol of *H. lupulus* strobiles during storage was carried out. Dried whole strobiles and cryogenically ground dried strobiles stored at -15°C as well as ethanol extracts of the strobiles prepared using different ethanol concentrations (10%, 30%, 50%, 70%, and 95%) and stored at room temperature, were analyzed by HPLC to quantify each constituent. The hops samples were analyzed immediately after preparation, and then one year and two years later to determine the concentrations of the constituents.

Results: HPLC analysis of *H. lupulus* dried and ground samples indicated a gradual decrease in the components over a two year period. The ethanol content of the extract was a strong determinant to predict the constituent levels found in the extract; the higher the ethanol level, the higher the initial and subsequent constituent levels. The 10% and 30% ethanol extracts had very low amounts of constituents initially and were practically devoid of constituents at the end of two years. The 50% ethanol extract contained low beta acid levels as well as higher alpha acid and xanthohumol levels initially, but lost substantial amounts over time. The 70% and 95% ethanol extracts showed high levels of all three constituents, while the 95% *H. lupulus* ethanol extract contained the highest levels of constituent both initially and at the end of the two-year testing period.

Conclusion: These results suggest that both whole and ground hops lose active components over storage time. A minimum amount of 70% ethanol is necessary to extract high levels of all three bioactive constituents and to retain them over a two-year period.

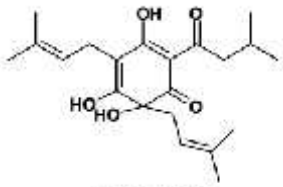
Keywords: [Hops, ethanol extracts, alpha acids, beta acids, xanthohumol, storage stability]

1. INTRODUCTION

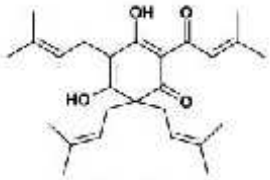
Humulus lupulus L., commonly known as hops, belongs to the Hemp [Cannabaceae] family [1] and has been cultivated throughout the temperate regions of the world primarily for the brewing industry [2]. The strobiles (also known as cones) produced by female plants of hops are the desired parts for the brewing industry. For this reason, only the female plants are cultivated, and the strobiles are harvested during late summer for further processing [3]. The brewing industry generally grows *H. lupulus* from “root” cuttings and not from seeds since this cultivation method maintains a genetically consistent product. It also aids in controlling the aroma characteristics and the amount of active constituents found in the strobiles [4]. However, the overall strobile chemical composition still depends on a multitude of factors

including variety, growing region, growing conditions, harvesting time, as well as drying and storage conditions [5, 6].

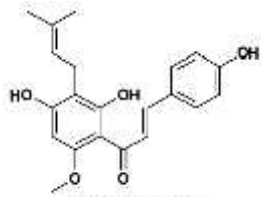
Oleoresin glands present in the strobiles produce a resinous yellowish/reddish powder called lupulin [7]. Numerous compounds present in lupulin are of economical interest. The volatile oils and bitter acids are the most significant classes of compounds in terms of economic value. Two of the major constituents found in lupulin resin are alpha acids (humulones) and beta acids (lupulones) [8], which are phloroglucinol derivatives. Xanthohumol, a bioactive flavonoid, has also been isolated from the hops resin [9].



Humulone



Lupulone



Xanthohumol

The stability of the constituents of the strobile pellets or extracts used in the brewing industry has been of great importance [10]. Moreover, the stability of alpha acids in *H. lupulus* during storage of strobiles has been recognized as a critical issue since alpha acids provide most of the bitterness in beer [11]. When the hop cones are harvested, the moisture content is around 75-80% and, in order to prevent deterioration, reduction of moisture content is necessary before storage or processing. Skinner et al. [12] demonstrated that the constituents' rate of deterioration was related to the storage temperature; that every 15°C rise in the storage temperature doubled the deterioration rate. In order to prevent the loss of hops' active constituents, it is important to store the strobiles at a low temperature; preferably below 0° C. Weber et al. [13] studied the effects of post-harvest handling on the quality and storage stability of strobiles. Their study showed that decreasing the kilning temperature, using a lower compression force during bundling of strobiles, and wrapping the strobiles in burlap instead of plastic were instrumental in producing a superior product. These results concluded that, of the three factors tested (temperature, compression of the strobiles, and the material used to wrap the strobiles), elevated temperature had the most negative influence.

Clinical herbalists frequently use hops to treat a variety of ailments [14]. In England, *H. lupulus* strobiles have been recommended for their skin anti-infective properties for hundreds of years [15] and the strobiles have been used as a wash for impetigo, boils, and abscesses [16]. In traditional European folk medicine, *Humulus lupulus* was frequently mentioned as an infusion or a fomentation to treat skin sores, cuts, and injuries [17, 18, 19]. More recently, Bartram [20] suggested that the antimicrobial properties of hops could be used to treat skin infections. At present, however, *H. lupulus* is mainly used in modern phytotherapy for its nervous system sedative effects, to stimulate gastric secretions, and to improve digestive function [21]. Additionally, *H. lupulus*' phyto-estrogenic properties [22] and antiviral activities [23] have recently been investigated and ascribed to its constituents.

It is generally acknowledged that clinical results can only be achieved if the herbs' active constituents are present in sufficient quantity to reach therapeutic levels [24]. This important issue, although of considerable concern to practitioners, has not been properly addressed by researchers [25]. Additionally, research suggests that the ethanol percentage used to extract herbs has a significant impact on the amount of active constituents found in the final extract [26]. In some instances, a high percentage of ethanol yields higher levels of active

constituents [27] while, in other instances, a low ethanol level actually yields higher levels of active constituent [28]. The present study focuses on the amounts of bioactive constituents present in dried *H. lupulus* strobiles and extracts made with varying ethanol concentration, and the storage stability of these constituents over time.

2. MATERIAL AND METHODS

2.1 Plant materials

Humulus lupulus L. plant materials used in this study consisted of whole dried strobiles grown in the Yakima Valley, Washington State, USA. Strobiles of the Super Galena variety were obtained from HopSteiner, a division of S.S. Steiner, New York, NY. The strobiles were collected in the autumn of 2009, dried, and stored in warehouses under frozen conditions until shipped to Herbs, Etc., Inc. where they were stored in a freezer maintained at -15°C. Identity of the material was confirmed by the first author using macroscopic and organoleptic methods. A voucher specimen of whole strobiles was stored in a freezer maintained at -15°C.

2.2 Solvents

Five concentrations of ethanolic extracts (10%, 30%, 50%, 70% and 95%) were prepared using 95% USP grade ethyl alcohol (Pharmco-Aaper, Shelbyville, KY) and water. The ethanol concentrations were verified using a hydrometer. A sample of each ethanol concentration was set aside in an amber-colored glass bottle as reference material and stored at room temperature in a dark closet for solvent control studies.

2.3 Preparation of hops strobile extracts

2.3.1 Cryogenic grinding

In order to prevent the loss of heat-sensitive constituents, the strobiles were cryogenically ground using a hammer mill (Fitzpatrick Manufacturing, Sterling Heights, MI) cooled by the injection of USP-grade liquid nitrogen into the grinding chamber. Samples of ground strobiles were then stored at -15°C.

2.3.2 Cold-process percolation extraction

On the same day that the *H. lupulus* strobiles were powdered using the above-described cryogenic grinding method, a cold-process percolation extraction method using a 1:5 herb-to-solvent (ethanol) ratio was used to extract the ground strobiles. The finished products were filtered to remove the sediments present in the liquid extracts and stored in amber-colored glass bottles. Three lots of samples were set aside, a) one lot for immediate analytical purposes, b) the second lot as reserve samples for quality assurance purposes and c) an additional lot for analytical purposes at the end of one year and two year storage. All the ethanol extract samples were kept at room temperature in a dark closet.

2.4 Chemical analysis

Chemical analysis of the whole and ground strobiles stored at -15°C as well as the five ethanolic extracts prepared from the same strobile lot and stored at room temperature was performed by S.S. Steiner, in Yakima, WA. The amount of the three bioactive constituents [alpha acids (humulones), beta acids (lupulones), and xanthohumol] in each sample was

quantified using an HPLC method. The samples were analyzed again one year and two years later to determine the changes in constituents during storage.

2.4.1 Standards and Sample Preparation for HPLC analysis

Working standard solutions of alpha acids and beta acids were prepared by bath sonication of 0.5 g (measured to 0.1 mg) of the international calibration extract (ICE-3) obtained from American Society of Brewing Chemists (ASBC, St. Paul, MN). The ICE-3 extract was dissolved in 50 ml of methanol and diluted (1:10) in acidic methanol (0.5 ml of 85% phosphoric acid in 1 liter of methanol). The standard solution of xanthohumol was prepared by dissolving approximately 20 mg of xanthohumol (in-house standard) in 100 ml of acidic methanol. Samples were diluted (1:20) with acidic methanol prior to analysis.

2.4.2 HPLC Analysis

Quantification of the three constituents, alpha acid, beta acid, and xanthohumol, were carried out by the methods described by the European Brewing Congress, method EBC 7.7, and the American Society of Brewing Chemists, method ASBC HOPS-14, using a Shimadzu HPLC system equipped with diode array detector. The mobile phase was composed of 72.5% methanol, 26.5% water, 0.85% phosphoric acid, and 0.075 mM Sodium EDTA. A C-18 column, Kinetex 2.6 um, 4.6 x 50 mm (Phenomenex) was used to separate the compounds. The flow rate was adjusted to 1.3 ml/minute at 40°C, and 10 µl of samples and calibration solutions were injected into the column. The detector wavelength was set at the absorbance of 270 nm for alpha acids and beta acids, and at 367 nm for xanthohumol.

3. RESULTS AND DISCUSSION

3.1 HPLC analysis of whole and ground *H. lupulus* strobiles before and after storage

Figures 1 and 2 show the HPLC chromatograms of the standard compounds and the 95% ethanol extract. The total alpha acids (cohumulone plus n+adhumulone), total beta acids (colupulone plus n+adlupulone) and Xanthohumol levels were determined using the ICE-3 standard.

Initial analysis: The HPLC results revealed that the whole dried *H. lupulus* strobiles initially contained 11.4g of alpha acids, 7.4g of beta acids, and 0.46g of xanthohumol per 100 grams of strobiles (Table 1). Immediately after the grinding process, the cryogenically-ground strobiles from the same lot were shown to contain lesser amounts of the three constituents. Even though the strobiles were ground using ultra-cold cryogenic technology, they were shown to have lost 8.8% of their alpha acids and 7.2% of their beta acids (Table 2). With a net 2.2% loss, xanthohumol showed the smallest constituents loss of all.

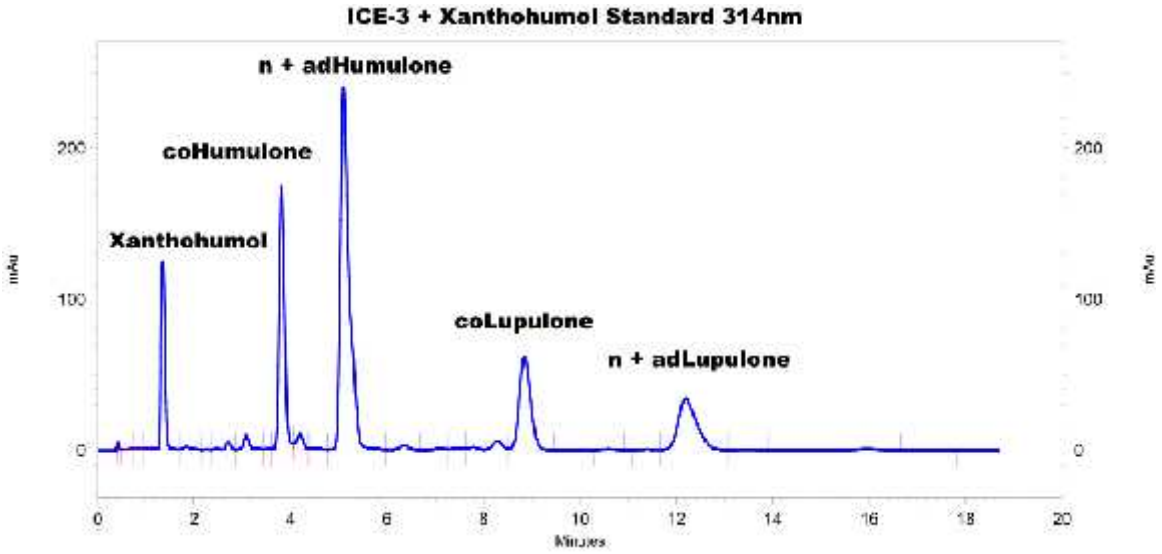


Figure 1. HPLC chromatogram of ICE-3 standards showing Humulones (alpha acids) lupulones (beta acids) and Xanthohumol

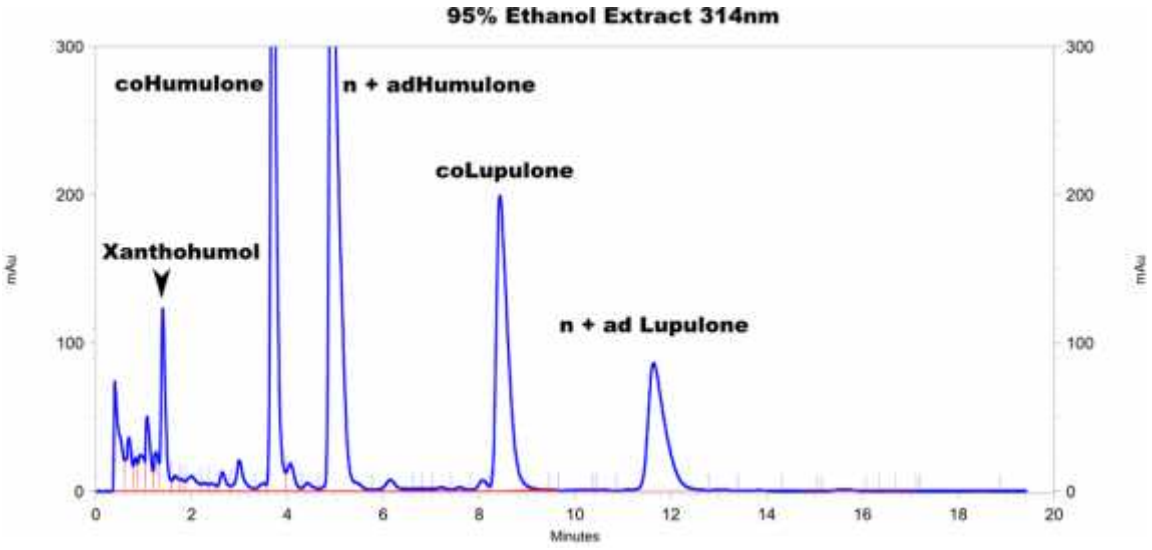


Figure 2. HPLC chromatogram of the 95% ethanol extract

Table 1. HPLC analysis of dried whole and cryogenically-ground *Humulus lupulus* strobiles (stored at -15°C) immediately after grinding, one year, and two years after the grinding process

Constituents (%)	Storage Time					
	Whole Strobiles			Ground Strobiles		
	Initial	Year 1	Year 2	Initial	Year 1	Year 2
Alpha acid	11.40	9.90	11.67	10.40	9.66	7.98
Beta Acid	7.48	5.64	6.78	6.94	6.03	5.34
Xanthohumol	0.46	0.43	0.43	0.45	0.43	0.35

One year later. The total amount of alpha acids found in whole strobiles was slightly higher than the amount found in ground strobiles (9.9g/100g vs. 9.7g/100g) after one year of storage, while the amount of beta acids was higher in ground strobiles than in whole strobiles (6 g/100g vs. 5.6 g/100g). The amount of xanthohumol found in both whole and ground strobiles was exactly the same (0.43 g/100g) after one year of storage (Table 1).

When comparing the percentage loss of whole and ground strobiles stored at -15°C after one year (Table 1), the approximate amount of alpha acids (13% vs. 7%), beta acids (25% vs. 13%) and xanthohumol (7% vs. 5%) lost in the first year of storage was greater in the whole strobile sample than in the ground sample (Table 2)

Table 2. Percentage loss of constituents in whole and ground strobiles (stored at -15°C) immediately after grinding (initial), after one year and two years of storage

Constituents (%)	Percentage Loss					
	Whole Strobiles			Ground Strobiles		
	Initial	Year 1	Year 2	Initial	Year 1	Year 2
Alpha acid	0	13.2	0	8.8	7.1	23.3
Beta Acid	0	24.5	9.4	7.2	13.1	23.0
Xanthohumol	0	6.5	6.5	3.0	4.4	22.2

Two years later. After two years of storage at -15°C, whole hops strobiles were found to contain higher levels of alpha acids and beta acids. The anomaly of higher levels of

constituents in whole hops after two years of storage may stem from the fact that each individual strobile is highly variable in its active constituent levels, while ground strobiles are a mixture of many ground and homogenized strobiles. The ground strobiles showed an almost 23% decrease in alpha acids and beta acids as well as a 22% decrease in xanthohumol levels compared to the initial levels (Table 2). The ground strobiles powder tended to show more uniformity in their levels of active constituents.

3.2 HPLC analysis of ethanol extracts of *H. lupulus* strobiles immediately after extraction

Initial analysis: The results from the chemical analysis of *H. lupulus* ethanolic strobile extracts showed that alpha acids (2,120mg/100ml), beta acids (1,440mg/100ml) and xanthohumol (90mg/100ml) were highest in the 95% ethanolic extract (Table 3). There was a striking difference between the amount of active constituents reported in the 10% ethanolic extract and the 95% ethanolic extract. The 10% ethanolic extract had 53 times less alpha acids, 96 times less beta acids, and 45 times less xanthohumol than the 95% ethanolic extract.

Table 3. Changes in the amounts of bioactive constituents of *Humulus lupulus* strobiles ethanol extracts (stored at room temperature) immediately after extraction, one year, and two years after storage.

	Constituents (mg/100ml)								
	Alpha acid			Beta acid			Xanthohumol		
Ethanol %	Initial	Year 1	Year 2	Initial	Year 1	Year 2	Initial	Year 1	Year 2
10	40	3	0.8	15	0.4	ND	2.0	0.09	ND
30	60	16	6.8	17	0.5	0.1	2.0	0.4	0.3
50	630	520	404	53	36	30	26	18	9.6
70	2,010	1,810	1,500	1,050	1,000	892	78	70	43
95	2,120	2,030	1,660	1,440	1,370	1,170	90	97	59

These results clearly demonstrate that, as the amount of ethanol in the menstruum used to extract the strobiles increased, the level of active constituents extracted also increased. The biggest increase in alpha acids level occurred when the ethanol percentage increased from 30% to 50%; the alpha acids amount increased from 60 to 630mg/100ml, a ten-fold increase. A similar increase in xanthohumol levels was observed when the ethanol percentage increased from 30% to 50%; the amount of xanthohumol increased from 2 to 26mg/100ml, a thirteen-fold increase. Conversely, the biggest increase in the percentage of beta acids occurred when the ethanol content increased from 50% to 70%; at that level, the amounts of beta acids increased from 53 to 1,050mg/100ml, a twenty-fold increase of beta acids. At an ethanol concentration of 70%, or greater, a substantial increase in the amount of all three constituents present in the extract was noted (Table 3).

3.3 HPLC analysis of *Humulus lupulus* ethanol extracts after one year and two years in storage

HPLC analysis of the five ethanol extracts stored at room temperature for 2 years showed that the different ethanol levels used to extract the strobiles greatly influenced the stability of each constituent over time (Table 3).

One year later: The 10% *H. lupulus* ethanol extract, which began with 40mg/100ml alpha acids, was found to contain only 3mg/100ml of the constituent one year later indicating a loss of 92.5% of alpha acids. In comparison, not only did the 95% ethanol extract start with a much higher level of alpha acids (2,120mg/100ml), but one year later, the amount of alpha acids remained at 2,030mg/100ml, representing a relatively small loss of 4.25%.

Overall, beta acids levels showed a greater loss over a one year period when compared to alpha acids. The 10% *H. lupulus* ethanolic extract initially contained 15mg/100ml of beta acids but, one year later, only 0.4mg/100ml of beta acids remained. This represents a loss of 97.5% beta acids in one year. Conversely, the 95% ethanolic extract started with a beta acids level of 1,440g/100ml and one year later was still found to contain 1,370mg/100ml of beta acids. The 95% ethanol extract had lost only 5% of its beta acids, while the 10% ethanol extract had lost most of its beta acids.

Xanthohumol analysis results showed a smaller loss when compared to alpha acids and beta acids constituents. The 10% ethanol extract initially contained 2mg/100ml of xanthohumol. One year later, HPLC analysis revealed that it contained 0.09mg/100ml, a loss of nearly 96%. However, the 95% ethanol extract started with a xanthohumol level of 90mg/100ml and ended with a level of 81mg/100ml indicating a loss of only 10%.

Two years later. The 10% ethanol extract showed 0.8mg/l00ml of alpha acids, while the 95% ethanol extract showed an alpha acid level of 1,660mg/100ml(Table 3). The 10% ethanol extract lost 98% of its alpha acids during the 24 month period, while the 95% ethanol extract lost only 22% of its alpha acids. The HPLC analysis revealed that, the lower the ethanol level used to make the *H. lupulus* ethanolic extract, the greater the loss of alpha acids over time. As the ethanol content used to extract the strobiles increased, higher levels of alpha acids were also retained over time. The higher ethanol content seemed to act as a preservative of alpha acids.

After a two year storage period, the beta acids were not detectable in the 10% ethanol extract, while the 95% ethanol extract still contained 1,170mg/ml or 81% of its beta acids. In effect, the 10% ethanol extract lost 100% of its beta acids, while the 95% ethanol extract lost only 19% of its beta acids. At the end of two years, analysis of xanthohumol levels revealed that there were no detectable levels of this constituent in the 10% ethanol extract, while the 95% ethanol extract had levels of 59mg/ml or 52% of the initial levels of xanthohumol.

When lower levels of ethanol were used to extract *H. lupulus* strobiles, concomitant lower levels of active constituents were extracted. Conversely, the higher the ethanol levels used to extract the strobiles, the less active constituents were lost over a one year or two year storage period. The 95% *H. lupulus* ethanolic extract lost less than 5% of both alpha acids and beta acids over a one year period. However, the 10% ethanolic extract lost more than 92% and 97% respectively of the same two compounds. Over a two-year storage period, the 95% ethanolic extract lost 25% and 15% of its alpha acids and beta acids while the 10% ethanolic extract lost over 99% and 100% of these two constituents. A summary of the percentage of active constituents remaining in the ethanolic extracts over a one and two year period is shown in Table 4.

When the stability of the three major active constituents is compared over time, it was observed that there was a strong correlation between the amount of ethanol used to extract the strobiles and the amount of active constituents retained in the extract after a storage

period of one and two years. Similar results were obtained in previous research for other herbs including hops [26]. One observation is that the higher the level of ethanol used to extract *H. lupulus* strobiles, the better the stability of the constituents in the ethanol extract stored at room temperature over time. The two extracts that contained the most constituents were the 70% and 95% ethanol extract; they both retained approximately three-quarter of their alpha acids and beta acids over a two year period. The extract that contained 50% ethanol lost substantial levels of constituents while the two extracts with 10% and 30% ethanol lost virtually all of their constituents. These last two extracts are of particular concern in that they did not contain significant levels of constituents to start with and, then, proceeded to lose all of them over the two year storage period. The present study showed for the first time that ethanol concentration is a very important factor to be considered in hops extraction process and storage.

Table 4. Percentage of constituents remaining in *Humulus lupulus* ethanolic extracts after one year and two years of storage at room temperature (21°C).

	Constituents (%)					
	Alpha acid		Beta acid		Xanthohumol	
Ethanol (%)	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
10	7.5	0.2	2.7	0	4.5	0
30	26.7	11.3	2.9	0.6	20	15
50	82.5	64.1	67.9	56.6	76	36.9
70	90.1	74.6	95.2	85.0	103	55.1
95	95.8	78.3	95.1	81.3	90	65.6

4. CONCLUSION

Whole dried *Humulus lupulus* strobiles contained the constituents, alpha acids, beta acids and xanthohumol at 11.4%, 7.5%, and 4.6% levels respectively. After grinding the strobiles under cryogenic conditions, the levels of alpha and beta acids were reduced by nearly 10%. During the storage for two years at -15°C, the levels were further reduced. This study also revealed that *Humulus lupulus* strobiles extracted with an ethanol level of 70% or higher yields more active constituents than extracts made with lower concentrations of ethanol. Further, ethanol levels of 70% or higher helps to preserve the active constituents found in *H. lupulus* extracts for a longer period of time (at least over a two-year period) than extracts made with lower ethanol levels. The data obtained in this study would be helpful to clinical herbalists, as well as the dietary supplements or pharmaceutical industry, in developing nutraceutical and pharmaceutical products using *H. lupulus* extracts for human ailments. It is recommended that clinical herbalists use at least 70% *H. lupulus* ethanolic extracts in their clinical practice. Thus, they will gain an additional assurance that the *H. lupulus* ethanolic extract dispensed to their clients contains the level of constituents necessary to achieve the clinical results desired.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTION

Daniel Gagnon designed the study, wrote the protocol, supervised the preparation of the *H. lupulus* samples, and helped in revising the manuscript. Chitra Wendakoon provided advice throughout the project, and prepared the final manuscript. Bob Smith and Jeremy Leker performed the HPLC analysis of the *H. lupulus* samples. All authors read and approved the final manuscript.

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