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Original Research Article

LC-MS Based Phytochemical Perspective, ACE Inhibition Potential and Pharmacokinetics Study of *Humulus lupulus* Flower Extract

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ABSTRACT

The growing burden of cardiovascular diseases (CV) has been a major cause of concern for the world, which has further aggravated during the Covid-19 pandemic, due to post-SARS-CoV-2 mediated complications among recovered patients. The quest to explore new, and more efficacious, safe alternative therapeutic products for cardiovascular diseases and their associated symptoms have been the thrust of scientific importance. Angiotensin-converting enzyme (ACE) plays a pivotal role in hypertension, a disease condition associated with cardiovascular diseases. In the present study, *Humulus lupulus* was extracted in different solvents and quantified for total flavonoid, phenolic and ACE inhibition activity estimated using aluminium chloride, Folin Ciocalteu reagent, and Cushman and Cheung methods respectively. Further, liquid chromatography mass spectrometry (LC-MS) profiling of the phytocompound followed by Pharmacokinetics, drug-likeness behavior, and toxicity prediction using SWISS-ADME and Protox-II were analyzed. Results revealed the mixture to be rich in sphingolipids, polyphenolics, terpenes, flavonoids, and others. The study suggests the synergistic role of the compounds on ACE inhibition activity shown by the extract. However, the study needs to be further extended to screen the responsible compound for the inhibition activity and mechanism of action. The evaluation of these compounds for their pharmacokinetics properties opens up avenues to explore new molecules for the purpose of drug designing.

Keywords: *Humulus lupulus*, Angiotensin-converting enzyme, Phytocompounds, Pharmacokinetics, Toxicity

Introduction

The growing global disease burden of cardiovascular (CV) diseases had been a major cause of concern for the world, and it had further aggravated during the Covid-19 pandemic, due to post-SARS-CoV-2 mediated complications among recovered patients.[1,2,3,4] Limited but significant confirmatory studies exploring the possible association between CV diseases and Covid-19 revealed acute cardiac injury as one of the major complications manifested during cardiovascular disease.[5] Further studies had indicated severe clinical outcomes of SARS-CoV-2 infection with pre-existing CV diseases.[6,7] The existing studies and reports from World Health Organization (WHO) revealed an estimated 17.9 million global deaths due to CV diseases in 2019 accounting for 32% of the global death with around 85% of these deaths due to stroke and heart attack.[8] Angiotensin-converting enzyme (ACE) is a bivalent dipeptidyl carboxy metallopeptidase, a membrane enzyme in the epithelial, neuro epithelial, and endothelial cells. It is also present in soluble form in numerous body fluids and blood.[9] The enzyme ACE plays a significant role in fluid and electrolyte balancing and blood pressure regulation. The role of the enzyme has been explored in the development of the cardiovascular system and vascular remodelling. The enzyme is significant for catalyzing the hydrolysis of angiotensin I to angiotensin II, a well-known peptide.[9] It further deactivates the vasodepressor peptide bradykinin.[10]

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Also, ACE is well explored for its role in blocking angiotensin II and increasing the bradykinin level in the body that further maintains blood pressure, and associated cardiac cells (myocytes and smooth muscle cells) remodelling.[9] Different studies had shown an association between the SARS-CoV-2 virus and CV diseases, with ACE 2 being the receptor used by the virus to enter human tissues and cells.[5,11] The ACE 2 (Angiotensin converting enzyme-2) is reported to be the homolog of ACE (Angiotensin converting enzyme), with both being involved in the synthesis of biomolecules involved in Renin-Angiotensin System (RAS).[5,12,13] Existing studies have indicated that the increased activity of the ACE-2 linked receptor is triggered by ACE inhibitors.[13] The quest to explore ACE inhibitors further increased after the Covid-19 pandemic focusing on exploring efficacious ACE inhibitors with minimum side effects. The phytocompounds are safe and efficacious alternatives to their synthetic counterpart. The recent era has explored secondary metabolites as a protective dietary constituent and increasing shreds of evidence have suggested that prolonged consumption of these constituents can have beneficial effects on the regulation of cancers and other chronic diseases including diabetes and cardiovascular diseases.[14]
Polyphenols can be further divided into two categories namely flavonoids and non-flavonoids. The flavonoids fall under the category of aromatic compounds, comprising of 15 carbon which are arranged in two aromatic rings and are connected by a 3-carbon bridge.[14,15,16] Recent *in-vivo* studies have reported the therapeutic benefits of the consumption of phytocompounds[17]. These studies further validate their role in suppressing and improving the endothelium dysfunction associated with hypertension.[17] The therapeutic importance of polyphenol-rich natural sources has also been reported for improvement of the endothelial dysfunction in different types of cardiovascular diseases including (but not limited to) atherosclerosis and metabolic syndrome.[18] The studies on atherosclerosis-induced Golden Syrian hamsters further indicate the role of polyphenols

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(derived from grape) ingestion on the inhibition of fatty streak lesions in the aortic arch of the animal.[18] The plant is found to be a rich source of other phytocompounds like terpenes, chalcones, bitter acids, flavone glycosides, and catechins.[19] Terpenes have been well studied to have antimicrobial, anti-cancer, anti-inflammatory, anti-oxidant, anti-depressant, [20,21] and neuroprotective potential.[22] To the best of our knowledge, the ACE inhibition potential of the studied plant is not explored till date. This study sought to evaluate the ACE inhibition potential of the hop extract and its possible mechanism of inhibition.

Material and Methods

Plant material

The dried Hops (*Humulus lupulus*) powder was procured from Kshipra Biotech Pvt. Ltd., Madhya Pradesh, India. The solvent used for further extraction were hexane (99%), methanol (99%) and distilled water.

Extract preparation

For the study, Hop extract powder (10 g) was mixed with 100 mL of different solvents *viz.* methanol, hexane and distilled water. The choice of solvent was based on previous studies exploring phytocompounds yield in different solvents.[23] The solutions were incubated at 30 ℃ and 100 rpm for 48 h in a shaker incubator (Kuhner, Germany). Each sample was then centrifuged (REMI, India) for 30 minutes at 5,000 rpm at 4 - 6℃. The supernatants were collected and stored in glass bottles (Borosil, India) and stored at low temperatures for further analysis.

Quantitative Determination of Total Phenolic Content (TPC)

The TPC was estimated using Folin Ciocalteu reagent method.[24] Briefly, the 12.5 µL of each sample (in different solvents *viz.* methanol, hexane, distilled water) were mixed with 625 µL of Folin Ciocalteu's reagent adding 500 µL (7.5%, w/v) of sodium carbonate. The samples were incubated at room temperature for 3 hrs. Absorbance was recorded at 765 nm using Jenway 6850 UV/Vis (United Kingdom) spectrophotometer. The assay method involves blue color complex formation. Gallic acid was used as the standard for the estimation of TPC. The varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of gallic acid were used for quantification. The standard graph was plotted using gallic acid concentration *versus* absorbance (765 nm). The observations and results were expressed as mg gallic acid equivalent / mg hops extract. All the experiments were repeated in triplicates and average data were recorded.

Quantitative Determination of Total Flavonoid Content (TFC)

The TFC was estimated using the modified aluminium chloride method.[25] Briefly, 100 µL varying concentrations (0.2, 0.4, 0.6, 0.8, 1.0 mg/mL) of quercetin (standard) were prepared in water and used for quantification of flavonoid. Further, 30 µL sodium nitrite was added and the mixture was incubated for 5 minutes, followed by 30 µL aluminium chloride. Finally, 200 µL of sodium chloride was added. The volume was maintained at 1000 µL. The absorbance was measured using Thermo Scientific ELISA reader (United States) at 510 nm. Graph of the concentration of quercetin *versus* absorbance was plotted and used as a standard graph for the quantification of flavonoid concentration in unknown samples (extracts of the plant).

Angiotensin-converting enzyme (ACE) inhibitory activity

There are catalogues of methods for determination of ACE activity. The current study involves the use of method proposed by Cushman and Cheung (1971) with modifications suggested by Schnaith *et al*.[26,27] The HHL (Hippuryl-histidyl-leucine) is hydrolysed to HA (Hippuric acid) that was used to measure the ACE inhibitory action. The method analyzes the change in the catalytic efficiency of ACE on treatment with captopril and phytocompounds extract.[28]

The assay mixture containing the Incubation Buffer-1 was prepared for the enzymes by dissolving 2.91 g of boric acid (188 mmol/L,) and 25.63 g of potassium chloride (1.375 mol/L), dissolved in 200 mL of distilled water. pH was adjusted to 8.3 with 10 M potassium hydroxide (28.05 g in 50 mL water) and the final solution was made to 250 mL by adding distilled water. A separate 10 mL of this solution was used to dissolve the enzyme. For the preparation of Buffer-2, (188 mmol/L) boric acid, pH 8.3, (1.375 mol/L) potassium chloride was dissolved in 200 mL distilled water and the solutions (Incubation Buffer-1) were made exactly as discussed above, and instead of the enzyme, NaCl was added. The remaining 240 ml of the previously described Incubation Buffer-1 were dissolved in 4.2 g of 300 mM NaCl. The substrate solution (3 mM) was prepared by weighing 12.88 mg HHL which was further dissolved in 10 mL of Buffer-2. Varying concentrations of HHL (0.5, 1.0, 1.5, 2.0, 2.5,3 mM) were prepared. For inhibitor studies (10 µM) captopril was prepared in 50 mL Buffer-2. A dark-brown glass bottle was used to store 0.5 g of (136 mM) cyanuric chloride that had been dissolved in 20 mL of 1,4-dioxane. Enzyme stock (25 x 10^{-3} U/mL) of 10 µL was added with 40 µL HHL (3.0 mM) in 230 µL of Buffer-2 and incubated for different time intervals, *viz*.0 min, 5 min, 10 min, and 15 min. Then, a 40 µL cyanuric chloride was added. Absorbance was taken at 405 nm. The point beyond which no change in absorbance was observed was considered the optimum time. For inhibitory studies, 10 µL enzyme stock (25 x 10^{-3} U/mL) was incubated with 40 µL varying concentrations of HHL (1.0, 1.5, 2.0, 2.5, 3.0 mM) in 220 µL of Buffer-2, followed by 10 µL of inhibitors (10 µM captopril) and incubated for optimized time (5 min) followed by supplementation with 40 µL cyanuric chloride. The absorbance was measured at 405 nm. The same step was repeated with *Humulus lupulus* instead of an inhibitor. The samples were centrifuged at 3500 rpm at room temperature for 5 minutes and absorbance of the supernatant was measured at 405 nm (Jenway 6850 UV/Vis, United Kingdom). The activity of an enzyme corresponds to the amount of HHL degraded to Hippuric acid. The inhibition of ACE activity was estimated as % ACE inhibition (Equation1).

1 unit of the enzyme (25 x 10^{-3} U/mL) activity is the change of absorbance at 405 nm after 5 min incubation at room temperature (22℃ - 25℃).

$$ACE\ Inhibition\ (\%) = \frac{\text{Absorbance of Enzyme catalyzed Reaction} - \text{Reaction with inhibitor}}{\text{Absorbance of Enzyme catalyzed Reaction}} \times 100 \quad (1)$$

The enzyme inhibition (%) is the percentage of inhibition required to decrease the Hippuric acid (HA) and was calculated using the above equation.

ACE Inhibition Kinetics

In the current study, the Michaelis Menten's constant (K_m) of the catalytic reactions was compared with and without inhibitors. The constant (K_m) is significant in explaining the substrate affinity to the enzyme towards the substrate.[29] The prerequisite knowledge of enzyme catalysis is required for designing the inhibitors. The inhibitors can compete for the catalytic active site or interact with the alternative site to hinder enzyme catalysis. Previous studies had indicated that for allosteric enzymes, the inhibitions can be competitive, non-competitive or uncompetitive.[29] The Lineweaver Burk plot shows information pertaining to the kinetics of ACE inhibition. A calibration curve was plotted for the standard HA. The experiment was performed in duplicates. A graph between substrate concentration and standard inhibitor captopril was plotted to study the effect of inhibition as depicted in the graph (Figure 2B & 2C).

Qualitative determination of compounds in aqueous extract using Liquid chromatography mass spectrometry (LC-MS)

The LC-MS approaches have been used by researchers to explore phytocompounds profiling of plants. The setting of the Instrument (Dionex Ultimate 3000, Thermo Scientific) was as follows: Injection volume of sample; 15 µL**,** Column Used; Hypersil Gold C18 (2.1mm x 100mm, 3.0µm) Column temperature; 25 ℃**,** Flow rate; 0.350 mL/min (350 µL/min) with duration; 55 mins, buffers used Buffer A; 0.1% Formic Acid in Water and Buffer B; 0.1% Formic Acid in Acetonitrile.

The LC-MS settings of Instrument (Q Exactive, Thermo Scientific) have been done as follows, Scan type; Full MS Polarity; Positive (+),

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Negative (-), Scan Range; 120-800 m/z, Resolution; 70,000, AGC target; 1e6, Sheath Gas flow rate (arbitrary unit); 50, Aux Gas flow rate (arbitrary unit); 10, Sweep Gas flow rate; 1, Capillary voltage: (+) 3.5 kV, (-) 2.5 kV, Capillary Temperature; 325 °C, S-Lens RF Level; 55, Probe Heater Temp.; 350° C while MS2 settings were made as follows; Microscans 1, Resolution 35,000, AGC target 1e5, Maximum IT 50 ms, Loop count 5, MSX count 1, TopN 5, Isolation window 1.0 m/z, Isolation offset 0.3 m/z, Scan range 200 to 2000 m/z, (N) CE / stepped (N) CE; 15, 30, 45. The LC-MS data analysis was carried out using Thermo Fisher Scientific Compound Discoverer 3.3.

Statistical analysis

The experiments were performed in triplicates and the average values were used. Further standard deviation estimation was done to find variations in the phytocompound yield among different solvents ($p<0.05$) using R studio. The analysis of ACE inhibition activity was done using the average outcome of duplicate experiments and the standard deviations were plotted in the graphical data.

Pharmacokinetics study

Using the Swiss- ADME tool, the compounds were further evaluated for their Pharmacokinetics and drug-likeness behavior. Toxicity Prediction was carried out using Pro Tox-II, an online free tool available for predicting the toxicity of chemicals.

Results and Discussion

Extraction and Quantitative Determination of Phenolics and Flavonoids

Humulus lupulus has been reported to exhibit antiplatelet, antibacterial, antifungal, anti-collagenase, antioxidant, and anticancer activities.[30,31,32,33,34] In this current study, the phytocompounds were extracted from the dried plant material using three different solvents, differing in polarity *viz.* water, methanol, hexane and were further analyzed for phenolics and flavonoids. The TPC in the sample was calculated using linear correlation plot ($y = 0.144x$; $R^2 = 0.997$) (Figure1A) and TFC was further analyzed using the linear correlation ($y = 0.2031x$; $R^2 = 0.885$). The results are expressed as mg Quercetin equivalents / mg of hop extract (Figure1B). The results suggest that the TPC and TFC was highest in water with respect to other solvents (Table 1). The difference in solubility may be attributed to different chemical natures and solubility of phytocompounds present in the plant material. Similar results of high phenolic and flavonoid content have been observed with other phytocompound rich plant system.[35] The antimicrobial properties of the phenolic compounds have been well explored in plant–pathogen interactions and play a pivotal role in restricting the spread of a pathogen.[34] Different kinds of terpenoids, phenolics, flavonoids, and chalcones have been well-reported in the plant *Humulus lupulus*.[36]

ACE Inhibition activity

ACE enzyme is reported to be associated with hypertension.[9,10] The ACE enzyme converts angiotensin I to angiotensin II and modulate vasodilation.[9,10] Studies have shown *Momordica charantia*, *Angelica keiskei*, *Prunus domestica*, *Peperomia pellucida* and *Muntingia calabura* to possess ACE inhibitory activities.[37] Another study with *Syzygium polyanthum* have shown the ACE inhibition potential of aqueous extract exhibiting the ACE inhibitory activity of around 69.43 %.[38] ACE inhibition by reference standard and aqueous extract of the plant were analyzed by Lineweaver Burk's plot (Figure 2). Reciprocals of varying HHL concentrations on the x-axis were used as independent factors in the current study, and reciprocals of HA production were used as the dependent variable (y-axis), to create a linear regression. The (K_m) Michaelis Menten's constant which is a constant value that depicts the substrate specificity towards the enzyme was calculated to be 50 mM without any inhibitor with V_{max} maximum velocity of 20 mM/min. Further addition of inhibition decreases the maximum velocity and Michaelis Menten's constant **(**K_m). The V_{max} and the K_m with captopril as inhibitor were 1.92 mM/min and 19.95 mM. (Table 2) Further, extract inhibition was compared with captopril and results showed that the inhibition in ACE activity with extract was higher (47.97%) than captopril (33.78%). (Figure 3) Further, the result suggests phytocompound-rich aqueous extract from *Humulus lupulus* as a potential ACE inhibiting agent, for their suggestive role in alleviating cardiovascular diseases.

LC-MS profiling

The LC-MS profiling of the extract revealed the hop extract to be rich in sphingolipids, polyphenolics, terpenes, flavonoids, and others. The LC-MS was performed in both positive (Table 3) and negative (Table 4) modes as reported previously.[39] The LC-MS profiling of the extract run in positive mode revealed the extract to be rich in phytocompounds such as safingol, 2-amino-1,3,4-octadecanetriol, integracin B, (+)-absinthin, ziyuglycoside I, baicalin, bis(2-ethylhexyl) phthalate, skimmin, 1-aminocyclohexanecarboxylic acid, 7-methoxycoumarin-4-acetic acid, trimethadione, umbelliferone, lysolecithin, palmitoyl serinol, scoparone, kaempferol (Table 3). Other compounds like isocitric acid, apigenin 7-sulfate, oleanolic acid, (+)-[6]-gingerol, corchorifatty acid F, luteolin 7-sulfate, genistein, azelaic acid were identified in negative run (Table 4). Apart from this various other compound such as rutin, lupenone, coumarin, xanthine, quillaic acid, and others have also been found in the extract (Data not shown). The presence of polyphenols, particularly flavonoids, are frequent in ACE-inhibiting species, with quercetin being one particularly well-reported example.[40] The synergistic effect of these compounds on performing ACE inhibition activity cannot be ruled out. However, the role of such compounds on the inhibition activity of the Angiotensin-converting enzyme needs to be further explored.

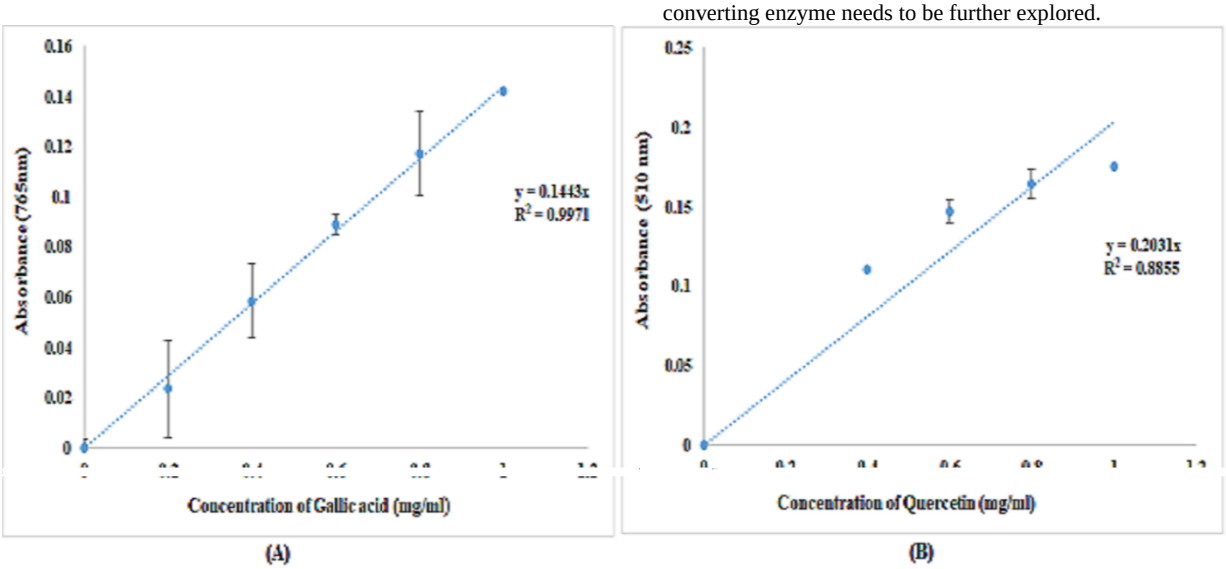

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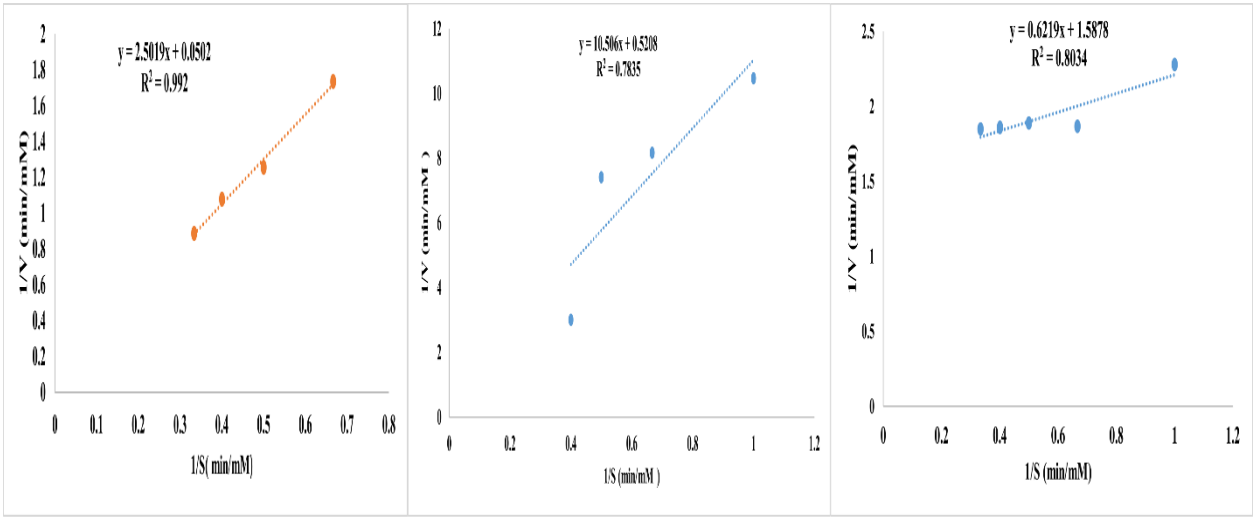
Figure 1: The standard plot for quantification of phytocompounds, *viz.* (A) Total Phenolic content; (B) Total Flavonoid content.

Table 1: Total Phenolic and Flavonoid contents in *Humulus lupulus* in different types of solvents

Hops extract in different types of solvent	Total Phenolic Content (mg gallic acid equivalent/mg of hop extract)	Total Flavonoid Content (mg Quercetin equivalent/mg of hop extract)
Hexane	0.009 ± 0.001	0.221 ± 0.001
Methanol	0.038 ± 0.000	1.885 ± 0.000
Water	0.083 ± 0.001	2.511 ± 0.036



(A) **(B)** **(C)**

Figure 2: The Lineweaver Burk's plot for analyzing the kinetic parameters of ACE catalytic activity with (A) HHL as substrate (B) catalysis of HHL with Captopril as inhibitor (C) catalysis of HHL substrate with aqueous plant extract.

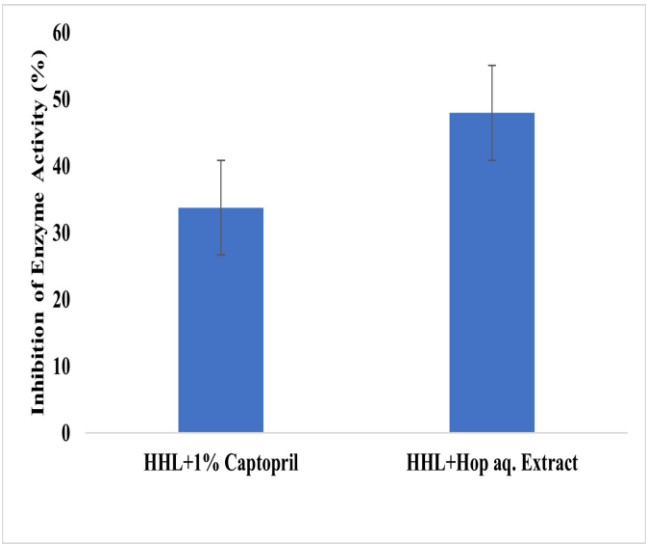


Figure 3: Percentage Inhibition of ACE Enzyme Activity

Table 2: Kinetic Parameters of ACE Inhibition Activity

Samples	$1/V_{max}$ (min/mM)	V_{max} (min/mM)	K_m (mM)
HHL	0.050	20	50
Captopril	0.520	1.92	19.95
Water	1.580	0.62	0.39

Pharmacokinetics study

The drug research and development heavily rely on the factors of chemical absorption, distribution, metabolism, excretion, and toxicity. In addition to demonstrating enough efficacy against the therapeutic target at a therapeutic dose, a high-quality drug candidate should also have appropriate ADMET characteristics.[41] The Swiss-ADME analysis revealed the majority of the compounds pass the Lipinski rule (Table 5). The compounds which have shown the potential to be used in drug-making were further screened for toxicity (Table 6). The analysis revealed that around 20 compounds were found to lie in the Toxicity class IV, suggesting the dose dependent harmful effects of some of these compounds ($300 < LD_{50} \leq 2000$).

The study is even more important amidst the global pandemic that the world is fighting, by suggesting an alternative plant extract with comparatively higher ACE inhibition than some of the other plants reported. The study will pave the way for further exploring the detailed mechanism of ACE inhibition, phytochemical profile and other biotherapeutic activities.

Conclusion

Current study is the first report on phytochemical associated ACE inhibitory property from the flower extract of *Humulus lupulus*. The aqueous extract of *Humulus lupulus* showed significantly higher inhibition than captopril. The diversity in the phytocompound available in the plant extract maybe pivotal and responsible for this property. However, the variations with other germplasms of *Humulus lupulus* cannot be ruled out. Also, the LC-MS profiling of the extract shows the synergistic role of these compounds responsible for ACE inhibition activity. Further ADMET analysis of these compounds paves the path to explore new molecules for drug designing. The study

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opens an avenue for exploring the potential of plants with a possible role in preventing cardiovascular diseases such as hypertension and other associated diseases.

Conflict of Interest

The authors declare no conflict of interest.

Table 3: Compounds in LC-MS (Positive mode) for aqueous plant extract

Compound	Formula	Molecular weight	m/z	Area	RT
Safingol	$C_{18}H_{39}NO_2$	301.29762	302.3049	2490302591.78086	12.024
2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$	317.29268	318.29996	2214830943.58038	9.641
Integracin B	$C_{35}H_{54}O_7$	586.38615	587.39343	479264625.327622	8.492
Diethylpyrocarbonate	$C_6H_{10}O_5$	162.05259	347.09433	379387318.306787	0.853
(+)-absinthin	$C_{30}H_{40}O_6$	496.28188	497.28915	324769236.004528	4.093
Ziyuglycoside I	$C_{41}H_{66}O_{13}$	766.44914	767.45642	312622270.173062	8.608
Baicalin	$C_{21}H_{18}O_{11}$	446.08468	447.09195	254243986.447954	1.311
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.27652	391.28378	247809496.816939	1.856
Skimmin	$C_{15}H_{16}O_8$	324.08409	325.09137	243361023.409473	1.051
1-Aminocyclohexanecarboxylic acid	$C_7H_{13}NO_2$	143.09444	144.10172	240253117.608486	0.854
7-Methoxycoumarin-4-acetic acid	$C_{12}H_{10}O_5$	234.0527	217.04945	236494752.766088	1.43
Trimethadione	$C_6H_9NO_3$	143.0583	127.03905	235960576.188552	1.003
Umbelliferone	$C_9H_6O_3$	162.03157	163.03885	229799994.850596	1.086
Lysolecithin	$C_{24}H_{50}NO_7P$	495.3322	496.33948	178922311.833466	15.387
Palmitoyl Serinol	$C_{19}H_{39}NO_3$	329.29259	330.29987	177614704.8178	12.384
Scoparone	$C_{11}H_{10}O_4$	206.05771	207.06499	174846110.08509	1.704
Kaempferol	$C_{15}H_{10}O_6$	286.04732	287.0546	156569469.345988	2.393

Table 4: Compounds in LC-MS (Negative mode) for aqueous plant extract

Compound	Formula	Molecular weight	m/z	Area	RT
Isocitric acid	$C_6H_8O_7$	192.02623	191.01895	672622179.399223	0.974
Apigenin 7-sulfate	$C_{15}H_{10}O_8S$	350.00957	349.00229	493844560.704445	2.227
Oleanolic acid	$C_{30}H_{48}O_3$	456.3603	455.35303	408603118.280022	17.552
(+)- [6]-Gingerol	$C_{17}H_{26}O_4$	294.18305	293.17578	292681908.776929	10.037
Corchorifatty acid F	$C_{18}H_{32}O_5$	328.22502	327.21774	196575657.330169	3.704
Luteolin 7-sulfate	$C_{15}H_{10}O_9S$	366.00465	364.99738	144461519.847906	1.7
Genistein	$C_{15}H_{10}O_5$	270.05287	269.04559	124081235.203285	3.783
Azelaic acid	$C_9H_{16}O_4$	188.10417	187.09689	97740853.9734879	1.634

Table 5: ADME analysis of LC-MS compounds

Groups	Compound Name	PubChem ID	GI absorption	BBB permeant	P-gp substrate	CYP 1A2 inhibitor	CYP2C19 inhibitor	CYP 2C9 inhibitor	CYP 2D6 inhibitor	CYP 3A4 inhibitor	Log K_p (skin permeation)	Lipinski Rule	Bioavailability Score
Sphingolipids	Safingol	3058739	High	Yes	Yes	No	No	No	Yes	No	-4.02 cm/s	Yes; 0 violation	0.55
Alcohol	2-Amino-1,3,4-octadecanetriol	248575	High	No	Yes	No	No	No	Yes	No	-4.94 cm/s	Yes; 0 violation	0.55
Benzoate ester	Integracin B	70678748	Low	No	No	No	No	No	No	No	-2.19 cm/s	No; 2 violations: MW>500,	0.17

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												MLOGP >4.15	
	Diethylpyrocarbonate	3051	High	Yes	No	No	No	No	No	No	-6.30 cm/s	Yes; 0 violation	0.55
Terpene	(+)-absinthin	442138	High	No	Yes	No	No	No	No	No	-7.74 cm/s	Yes; 0 violation	0.55
Glycosides	Ziyuglycoside I	71609288	Low	No	Yes	No	No	No	No	No	-9.13 cm/s	No; 3 violations: MW>500, NorO>10, NHorOH>5	0.17
	Baicalin	64982	Low	No	Yes	No	No	No	No	No	-8.23 cm/s	No; 2 violations: NorO>10, NHorOH>5	0.11
	Bis(2-ethylhexyl) phthalate	8343	High	No	Yes	No	No	Yes	No	Yes	-3.39 cm/s	Yes; 1 violation: MLOGP >4.15	0.55
	Skimmin	99693	High	No	No	No	No	No	No	No	-8.78 cm/s	Yes; 0 violation	0.55
	1-Aminocyclohexanecarboxylic acid	1366	High	No	No	No	No	No	No	No	-8.50 cm/s	Yes; 0 violation	0.55
	7-Methoxycoumarin-4-acetic acid	342221	High	No	No	No	No	No	No	No	-6.79 cm/s	Yes; 0 violation	0.56
	Trimethadione	5576	High	No	No	No	No	No	No	No	-6.96 cm/s	Yes; 0 violation	0.55
	Umbelliferone	5281426	High	Yes	No	Yes	No	No	No	No	-6.17 cm/s	Yes; 0 violation	0.55
	Lysolecithin	86554	Low	No	Yes	No	Yes	No	No	Yes	-5.32 cm/s	Yes; 0 violation	0.55
	Palmitoyl Serinol	9862307	High	Yes	Yes	No	No	No	Yes	No	-4.36 cm/s	Yes; 0 violation	0.55
	Scoparone	8417	High	Yes	No	Yes	No	No	No	No	-6.34 cm/s	Yes; 0 violation	0.55
	Kaempferol	5280863	High	No	No	Yes	No	No	Yes	Yes	-6.70 cm/s	Yes; 0 violation	0.55

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Isocitric acid	1198	Low	No	No	No	No	No	No	No	-8.75 cm/s	Yes; 0 violation	0.56
Apigenin 7-sulfate	14016776	Low	No	No	No	No	No	No	No	-6.71 cm/s	Yes; 0 violation	0.56
Oleanolic acid	10494	Low	No	No	No	No	No	No	No	-3.77 cm/s	Yes; 1 violation: MLOGP >4.15	0.85
(+)- [6]-Gingerol	1079	Low	No	No	No	No	No	No	No	-11.21 cm/s	Yes; 1 violation: NorO>10	0.55
Luteolin 7-sulfate	14016780	Low	No	No	No	No	No	No	No	-7.16 cm/s	Yes; 0 violation	0.11
Genistein	5280961	High	No	No	Yes	No	No	Yes	Yes	-6.05 cm/s	Yes; 0 violation	0.55
Azelaic acid	2266	High	Yes	No	No	No	No	No	No	-6.33 cm/s	Yes; 0 violation	0.85

Table 6: Toxicity Prediction of Compounds

Compound	Toxicity Class	LD_{50}	Carcinogenicity	Cytotoxicity
Safingol	4	1190 mg/kg	Inactive	Inactive
2-Amino-1,3,4-octadecanetriol	4	1190 mg/kg	Inactive	Inactive
Diethylpyrocarbonate	4	850 mg/kg	Inactive	Inactive
(+)-absinthin	4	1190 mg/kg	Inactive	Inactive
Bis(2-ethylhexyl) phthalate	4	1190 mg/kg	Inactive	Inactive
Skimmin	4	1190 mg/kg	Inactive	Inactive
1-Aminocyclohexanecarboxylic acid	4	1190 mg/kg	Inactive	Inactive
7-Methoxycoumarin-4-acetic acid	4	1190 mg/kg	Inactive	Inactive
Trimethadione	4	1190 mg/kg	Inactive	Inactive
Umbelliferone	4	1190 mg/kg	Inactive	Inactive
Lysolecithin	4	1190 mg/kg	Inactive	Inactive
Palmitoyl Serinol	4	1190 mg/kg	Inactive	Inactive
Scoparone	4	1190 mg/kg	Inactive	Inactive
Kaempferol	4	1190 mg/kg	Inactive	Inactive
Isocitric acid	4	1190 mg/kg	Inactive	Inactive
Apigenin 7-sulfate	4	1190 mg/kg	Inactive	Inactive
Oleanolic acid	4	1190 mg/kg	Inactive	Inactive
(+)- [6]-Gingerol	4	1190 mg/kg	Inactive	Inactive
Luteolin 7-sulfate	4	1190 mg/kg	Inactive	Inactive
Genistein	4	1190 mg/kg	Inactive	Inactive
Azelaic acid	4	900 mg/kg	Inactive	Inactive

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Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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