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ORIGINAL ARTICLE

Phenolic Compounds of *Heliotropium europaeum* and their Biological Activities

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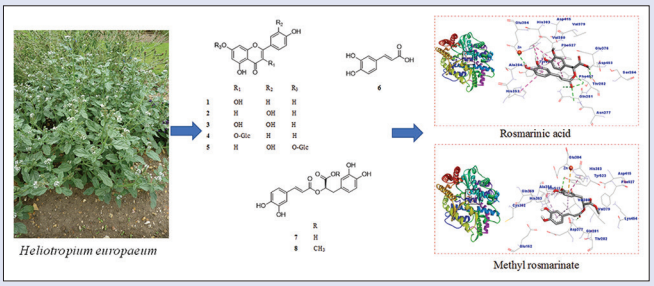
ABSTRACT

Background: *Heliotropium europaeum* L. is known to contain pyrrolizidine alkaloids and possesses a wide variety of biological activities. **Objective:** The objective of the study was to discover antioxidant phytochemical compounds in *H. europaeum* and assess their *in vitro* lipoxygenase (LOX)- and angiotensin-converting enzyme (ACE) inhibitory activities. **Materials and Methods:** *H. europaeum* herbs were extracted exhaustively by maceration using 90% aqueous ethanol. Solvent fractionation with *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol was performed with the dried extract until complete exhaustion. Two antioxidant active fractions (CH_2Cl_2 and EtOAc) were combined and subjected to medium pressure liquid chromatography followed by reversed-phase high-performance liquid chromatography. Quantitative evaluation in terms of the antioxidant activities of the isolated compounds was performed using the 2,2-diphenyl-1-picrylhydrazyl assay. The potential limiting effects on the action of ACE and soybean LOX were assessed quantitatively. **Results:** Isolates of eight newly identified phenolic compounds from *H. europaeum* exhibited antioxidant and ACE- and LOX-inhibitory activities. The isolated compounds were identified as kaempferol (1), luteolin (2), quercetin (3), kaempferol-3-O-glucoside (4), and luteolin-7-O-glucoside (5), in addition to caffeic acid (6), rosmarinic acid (7), and methyl rosmarinate (8). Among the isolated compounds, quercetin possessed the most potent antioxidant activity (IC_{50} = 8.1 μM) and ACE-inhibitory activity (IC_{50} = 17.5 μM), whereas rosmarinic acid and its methyl ester showed the strongest LOX-inhibitory activity (IC_{50s} = 4.2 μM and 3.6 μM, respectively). **Conclusion:** Our study is the first report on the phenolic constituents of the *H. europaeum* herbal extract and their biological activities. A total of eight phenolics were identified in this extract and isolated for the first time. Our results on *H. europaeum* extract constituents provide some scientific evidence on the beneficial effects of its traditional uses.

Key words: Angiotensin-converting enzyme inhibition, antioxidant, Boraginaceae, *Heliotropium europaeum,* lipoxygenase inhibition, rosmarinic acid

SUMMARY

- A total of eight phenolic compounds were identified from the herbal extract of *Heliotropium europaeum* for the first time
- Quercetin possessed the most potent antioxidant and angiotensin-converting enzyme (ACE)-inhibitory activities
- Rosmarinic acid and its methyl ester showed the strongest lipoxygenase (LOX)-inhibitory activity. The flavonoid glycosides exhibited weak ACE- and LOX-inhibitory activities.



Abbreviations used: LOX: Lipoxygenase; ACE: Angiotensin-converting enzyme; MPLC: Medium pressure liquid chromatography; DPPH: 2,2-diphenyl-1-picrylhydrazyl; CVD: Cardiovascular disease; ROS: Reactive oxygen species; HRESIMS: High-resolution electrospray ionization mass spectrometry; NMR: Nuclear magnetic resonance; TLC: Thin layer chromatography; RP: Reversed phase; His: Histidine; Glu: Glutamic acid; Asp: Aspartic acid; Lys: Lysine; Tyr: Tyrosine; Val: Valine; Thr: Threonine; Ser: Serine; Phe: Phenylalanine; Ala: Alanine; SD: Standard deviations; Arg: Arginine; Gln: Glutamine; Trp: Tryptophan; SAR: Structural activity relationship.

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INTRODUCTION

Cardiovascular disease (CVD) accounts for majority of the death cases reported in modern society.[1] Hypertension, the most critical risk factor of CVD renal disorders, is called the silent killer affecting one-quarter of the adult population globally and one-third of people residing in the Western countries.[2,3] Hypertension is still not adequately controlled and approximately 60% of the hypertension cases are either undetected or inadequately treated.[4] Furthermore, drug therapy for hypertension management also relies on lifestyle modifications.[5] The

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angiotensin-converting enzyme (ACE) inhibitors are first-line drugs in hypertension treatment.

Maintenance of the arterial blood pressure involves a crucial role of the renin–angiotensin–aldosterone system. Of the various components that play a central role in the system, ACE acts to convert angiotensin I into the active vasoconstrictor angiotensin II and also degrades bradykinin, a potent vasodilator. This helps to regulate the arterial blood pressure and electrolyte balance.[6-9] ACE inhibitors are typically used to control hypertension and to treat general cardiovascular and renal diseases, such as acute myocardial infarction, congestive heart failure, asymptomatic left ventricular dysfunction, and renal dysfunction.[10-13]

Many chemically synthesized ACE inhibitors, such as captopril and enalapril that helps to control hypertension, have several undesirable side effects, including angioneurotic edema, renal impairment, loss of taste, and cough.[14,15] Thus, the search for natural ACE inhibitors is critical to discover safe and economical alternatives to synthetic drugs.

A large number of reactive oxygen species (ROS) are produced in the case of CVD diseases and hypertension. Studies have indicated an antithetical relationship prevails between antioxidant intake and mortality due to cardiovascular disorder.[16-18] Therefore, expanding the search for natural therapeutics with antioxidants could identify drugs that render protection to the body from the free radicals and subsequently the development of many persistent cardiovascular disorders.[19,20]

The genus *Heliotropium* (Boraginaceae) comprises 250 species, which are found in warm regions. *Heliotropium europaeum* L., also known as the common heliotrope, European heliotrope, barooga weed, and caterpillar weed, is a herbaceous annual plant, native to Europe, Asia, North Africa, Australia, and Northern America.[21] *H. europaeum* possesses a wide variety of biological activities, including antibacterial, antifungal, antitumor, anti-inflammatory, insecticidal, antispasmodic, cholagogue, emmenagogue, antipyretic, and anthelmintic effects and is used externally to treat warts and to promote wound healing.[21-25] *H. europaeum* is known to contain pyrrolizidine alkaloids, such as heliotrine, heleurine, supinine, europine, and lasiocarpine, which are linked to the reported hepatotoxic, teratogenic, pulmotoxic, and mutagenic effects.[25,26] Many reported cases of *H. europaeum* poisoning in sheep have resulted from ingesting food contaminated with seeds of this species.[27-29] In India, Hong Kong, and South America, there have been some reports of human poisoning by *H. europaeum* due to the consumption of contaminated herbal teas with its seed hepatotoxic pyrrolizidine alkaloids.[29-33]

Phytochemical analysis of *H. europaeum* indicated the presence of alkaloids, terpenoids, steroids, saponins, flavonoids, phenols, and tannins.[21,26] Most of the previous reports were focused on the alkaloid content and the associated toxic effects, while other active metabolites and their beneficial biological activities were mostly neglected.

In continued search for drug leads from natural sources, this study extensively investigated dried *H. europaeum* herb to characterize its phenolic compounds. To our knowledge, this study is the first detailed analysis of the phenolic constituents of *H. europaeum* and their potential pharmacological activities. The aim of the current study is to evaluate the antioxidant and ACE- and lipoxygenase (LOX)-inhibitory actions of the phenolic metabolites obtained from the herbal extracts of *H. europaeum*.

MATERIALS AND METHODS

General

Ultraviolet (UV) spectral analyses were carried out using Varian Cary® 50 UV-Vis Spectrophotometer. High-resolution electrospray ionization mass spectrometry analysis was performed using a Bruker micrOTOF mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DRX600 spectrometer (Bruker GmbH, Rheinstetten, Germany). Silica gel was utilized for performing column chromatography (Merck 60 Å, 70–230 mesh ASTM, Darmstadt, Germany). Purification was carried out using flash medium-pressure liquid chromatography (22 mm, i.d. × 30 cm, Kusano Scientific Co., Tokyo, Japan). Silica gel 60 F_{254} (0.25 mm, Merck, Darmstadt, Germany) and RP-18 F_{254S} plates (0.25 mm, Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). Reversed-phase high-performance liquid chromatography (HPLC) was conducted using a Phenomenex Luna C_{18} column (Luna C_{18}, 5 µm, 250 mm × 10 mm) on a Shimadzu HPLC-LC-20 AD binary gradient pump (Tokyo, Japan) combined with a SPD-M20A detector.

Plant materials

H. europaeum herbs were collected on March 2016 from the garden of medicinal plant, Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The identity and authenticity of the plants were confirmed by an adept taxonomist at the Herbarium Unit and the voucher specimen (SY 017/2016) has been kept at the herbarium of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Extraction and isolation

Air-dried powdered plant material weighing 450 g was extracted three times by maceration using 2 L of 90% aqueous ethanol. Dry extract of 67 g (14.9%) was obtained from the alcoholic extract through filtration and concentration techniques performed under conditions of reduced pressure at 40°C. The dry material was suspended in 750 ml of distilled water and thereafter solvent fractionation was performed with *n*-hexane, followed by dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and finally, *n*-butanol until complete exhaustion. The process yielded the *n*-hexane fraction (HEPM1; 21.2 g), CH_2Cl_2 fraction (HEPM2; 13.6 g), EtOAc fraction (HEPM3; 17.2 g), and *n*-butanol fraction (HEPM4; 6.1 g).

The CH_2Cl_2 (HEPM2) and EtOAc (HEPM3) fractions were combined due to their similarities on TLC and were chromatographed using flash medium-pressure chromatography on a normal silica gel column and $CHCl_3$–MeOH mixtures for separation according to the increasing polarity. TLC silica gel was used to keep a check on the 25 mL fractions that were collected using different solvent systems of $CHCl_3$–MeOH (90:10) and $CHCl_3$–MeOH–H_2O (80:20:2) and (70:30:3), and 0.2% 2,2-diphenyl-1-picrylhydrazyl (DPPH) in MeOH and/or 15% v/v H_2SO_4 in ethanol were used as spraying agents. Thereafter, the fractions were pooled to obtain nine main fractions (A–I) and were separated by reversed-phase HPLC on a Phenomenex Luna C_{18} column (250 mm × 10 mm, 5 µm) using a 5%–100% CH_3CN–H_2O gradient over 40 min, yielding the following compounds: rosmarinic acid (7) and methyl rosmarinate (8), 16.1 mg and 7.9 mg yellow amorphous powders, respectively, from fraction C; kaempferol (1), luteolin (2), and quercetin (3), 10.2, 12.5, and 9.8 mg yellow amorphous powders, respectively, from fraction E; caffeic acid (6) 11.3 mg yellow amorphous powder from fraction G; and kaempferol-3-O-glucoside (4) and luteolin-7-O-glucoside (5), 12.7 and 8.5 mg yellow amorphous powders, respectively, from fraction H.

2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay

Rapid TLC screening method was used to qualitatively assess the radical-scavenging activities of the isolated compounds against 0.2% DPPH* (Sigma-Aldrich Chemical Co., St. Louis, USA) in MeOH. Quantitative determination with ascorbic acid as a positive control was carried out according to previously described methods.[34,35]

Angiotensin-converting enzyme inhibition assay

The ACE-inhibitory activity was determined spectrophotometrically using rabbit lung ACE (EC 3.4.15.1) (Sigma-Aldrich, Munich, Germany) and substrate (Hippuryl-histidyl-leucine [Bz-Gly-His-Leu]) (Sigma-Aldrich, Munich, Germany) adopting a previously described procedure of Cushman and Cheung method.[36] All the experiments were performed thrice. ACE inhibitor captopril (Sigma-Aldrich, Munich, Germany) was used a positive control. Data were expressed as mean ± standard deviation; negative control reactions were performed without the test material and the resulting values were used to calculate the ACE-inhibitory activity expressed as percentage inhibition.

Percentage inhibition = $([Abs_{control} - Abs_{Samples}]/Abs_{Control}) \times 100$

Lipoxygenase inhibition assay

The LOX-inhibitory activity of isolated phenolics was assessed spectrophotometrically using the soybean LOX (EC 1.13.11.12) type 1-B (Sigma-Aldrich, Munich, Germany) with linoleic acid (Sigma-Aldrich, Munich, Germany) as substrate adopting the method of Tappel.[37] The reaction was performed using SpectraMax Plus 384 (Molecular Devices, USA). EZ-Fit Enzyme kinetics program was utilized to calculate the IC_{50} value (Perrella Scientific Inc., Amherst, USA). All the reactions were carried out in triplicate. The formulae used to calculate the percentage (%) of LOX inhibition was $([C - S]/C) \times 100$, where C and S are the LOX activity without and with the test compound, respectively.[37]

Molecular docking

AutoDock4.2 was used to perform molecular docking between ligands (Kaempferol, Luteolin, Quercetin, Caffeic acid, Rosmarinic acid, and Methyl rosmarinate) and protein (ACE) as described earlier.[38,39] Briefly, the structures of ligands were downloaded from the PubChem database and were prepared for docking by adding Gasteiger partial charges, assigning rotatable bonds, and merging non-polar hydrogen atoms. The energy of each ligand was minimized using Universal Force Field with the help of Open Babel. The three-dimensional coordinate of ACE was retrieved from the RCSB database (Pdb Id: 1O8A, 2.0 Å resolution).[40] The structure of ACE was optimized for docking by adding essential hydrogen atoms, Kollman united atom type charges, and solvation parameters. A grid of 63 × 73 × 67 Å dimensions with 0.375 Å spacing was created using AutoGrid. Molecular docking was performed using Lamarckian Genetic Algorithm and Solis and Wets local search methods. A total of 2,500,000 energy calculations were computed for each run and a total of ten runs were performed for each ligand. All the other parameters were set to default values. Further, the binding affinity (K_b) was computed from binding energy using the following relation.[41]

$$\Delta G = -RT \ln K_b$$

Where R and T are the universal gas constant and temperature, respectively.

RESULTS

The phytochemical examination of the herbal extract of *H. europaeum* guided by the results of the DPPH antioxidant assay facilitated separation and identification of eight phenolics (1–8), including five flavonoids, kaempferol (1),[42] luteolin (2),[42,43] quercetin (3),[43] kaempferol-3-O-glucoside (4),[44] and luteolin-7-O-glucoside (5),[45] in addition to caffeic acid (6),[46] rosmarinic acid (7),[47] and methyl rosmarinate (8) [Figure 1].[47] Light was shed on their structures through extensive NMR analyses, precise mass measurement, and by collating with the published data.

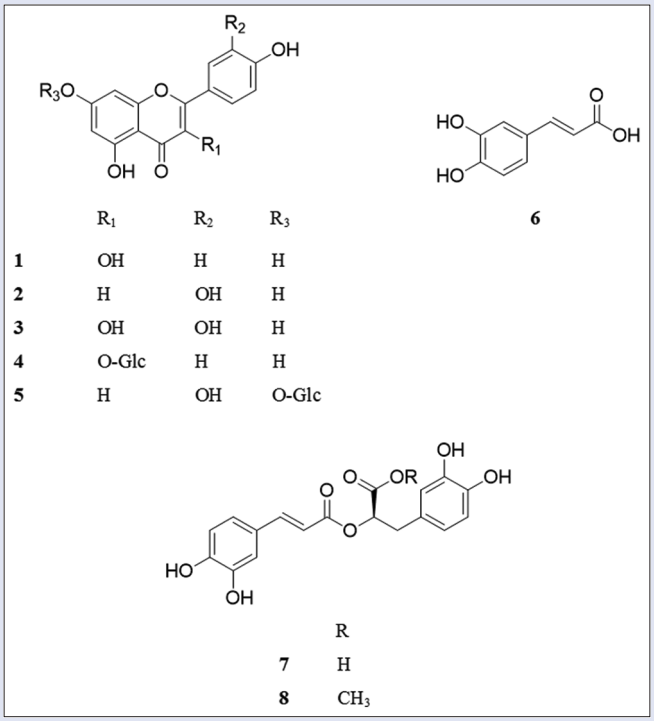

Figure 1: Chemical structures of isolated compounds (1 – 8)

Quantitative assessment of the antioxidant activity of the isolated phenolics using the DPPH assay [Table 1] revealed that compounds kaempferol, luteolin, and quercetin (1–3) and luteolin-7-O-glucoside, caffeic acid, rosmarinic acid, and methyl rosmarinate (5–8) showed significant free radical scavenging activity. Quercetin (3) with an IC_{50} of 8.1 µM was identified to be more formidable than the positive control, ascorbic acid.

The results of the ACE-inhibitory activity test were expressed in percentage inhibition of ACE. Interestingly, all compounds possessed ACE-inhibitory activity in a concentration-dependent manner [Table 1]. Among all isolated compounds, quercetin (3) with an IC_{50} of 17.5 µM exhibited the highest ACE-inhibitory effect.

Furthermore, the anti-inflammatory activities of the phenolic isolates were assessed against the soybean LOX (EC 1.13.11.12), as summarized in Table 1. The findings revealed that both the free flavonoids (1–3) as well as the caffeic and rosmarinic acid derivatives (6–8) possess strong LOX-inhibitory activity with IC_{50} values within the range of 3.6–31.2 µM. The strongest activity was observed for rosmarinic acid (7) and its derivative methyl rosmarinate (8) with IC_{50} values of 4.2 µM and 3.6 µM, respectively.

In this study, molecular docking of the isolated phenolics kaempferol (1), luteolin (2), quercetin (3), caffeic acid (6), rosmarinic acid (7), and methyl rosmarinate (8) with ACE was performed to gain an insight into their mechanism of ACE inhibition. The data from docking experiments suggested that all the selected compounds were bound to the active site of ACE and hence acted as a potential inhibitor of ACE activity [Figures 2-7 and Tables 2, 3]. According to the molecular docking, kaempferol interacted with ACE by forming one Pi-Donor hydrogen bond with His383, four conventional hydrogen bonds (Glu376, Asp415, Lys454, and Tyr523), two Pi–Pi stacked hydrophobic interactions (His383 and Tyr523), one Pi–Pi T-shaped hydrophobic interaction (His383), and one Pi-alkyl hydrophobic interaction (Val379). Other amino acid residues such as Thr282, Ser284,

His353, Asp453, His513, and Phe527 also interact with ACE and stabilize the ACE-kaempferol complex [Figure 2]. The kaempferol-ACE complex was stabilized by −8.6 kcal/mol of binding energy, which corresponded to a binding affinity of 2.03×10^6/M [Table 2]. Similarly, luteolin formed one carbon–hydrogen bond with Ser355, three conventional hydrogen bonds (Glu143, His513, and Tyr523), two Pi–Pi T-shaped hydrophobic interactions (His387 and Phe512), and one Pi-alkyl hydrophobic interaction (Val518) with ACE [Table 2]. Other amino acid residues involved in stabilizing the luteolin-ACE complex were Asn70, His353, Ala356, Lys368, His383, Phe391, Ser516, Val518, and Arg522 [Figure 3]. The binding energy and the corresponding binding affinity of luteolin and ACE complex were −8.6 kcal/mol and 2.03×10^6, respectively [Table 2]. Furthermore, quercetin formed four hydrogen bonds (Thr282, His353, and Lys454; Lys454 formed two hydrogen bonds), one Pi–Pi stacked hydrophobic interaction (Tyr523), one Pi–Pi T-shaped hydrophobic interaction (His383), and one Pi-alkyl hydrophobic interaction (Val379) with ACE. Other amino acid residues involved in stabilizing the quercetin-ACE complex were Gln281, Ser284, Glu376, Asp415, Asp453, His513, Tyr520, and Phe527 [Figure 4]. The binding energy and the corresponding binding affinity of quercetin and ACE complex were −9.0 kcal/mol and 3.99×10^6, respectively [Table 2].

Likewise, caffeic acid interacted with ACE by forming five hydrogen bonds (His353, Ala354, Asp415, His513, and Tyr523), one electrostatic interaction (Asp415), one Pi–Pi stacked hydrophobic interaction (His383), and one Pi-alkyl hydrophobic interaction (Val380). Other amino acid residues such as Val379, Glu384, Lys454, and Phe527 also interact with ACE and stabilize the ACE-caffeic acid complex [Figure 5]. The caffeic acid-ACE complex was stabilized by −6.5 kcal/mol of binding energy, which corresponded to a binding affinity of 5.85×10^4/M [Table 3]. Moreover, rosmarinic acid interacted with ACE by forming one Pi-donor hydrogen bond (His383), six conventional hydrogen bonds (Asn277, Gln281, Thr282, Glu376, and Glu384; Thr282 formed two hydrogen bonds), and one Pi-sigma hydrophobic interaction (Val380), one Pi–Pi stacked hydrophobic interaction (His353), four Pi–Pi T-shaped hydrophobic interaction (His383, Phe457, and Phe527; His383 formed two Pi-Pi T-shaped hydrophobic interactions), and one Pi-alkyl hydrophobic interaction (Ala354). Other amino acid residue stabilizing rosmarinic acid-ACE complex were Ser284, Val379, Asp415, Asp453, and

Table 1: Biological activities of isolated compounds (1-8)

Compound	IC_{50} (μM)		
	DPPH	ACE inhibition	LOX inhibition
1	29.4±1.64	26.9±1.48	31.2±1.39
2	16.6±0.97	22.6±0.81	4.8±0.37
3	8.1±0.43	17.5±0.95	5.1±0.29
4	>100	>100	>100
5	17.2±1.06	>100	>100
6	12.8±0.75	52.7±2.36	12.7±0.54
7	14.4±0.92	24.3±1.14	4.2±0.26
8	12.5±0.58	26.1±1.52	3.6±0.32
Captopril	-	0.02±0.003	-
Ascorbic acid	12.7±0.66	-	-
Baicalein	-	-	18.4±0.42

DPPH: 2,2-diphenyl-1-picrylhydrazyl; ACE: Angiotensin-converting enzyme; LOX: Lipoxygenase

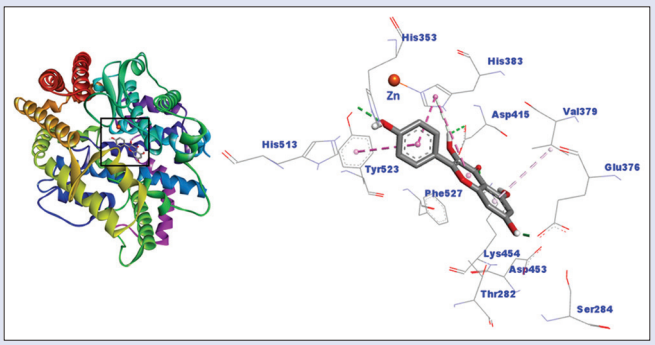

Figure 2: Molecular docking and interaction pattern of kaempferol with angiotensin-converting enzyme

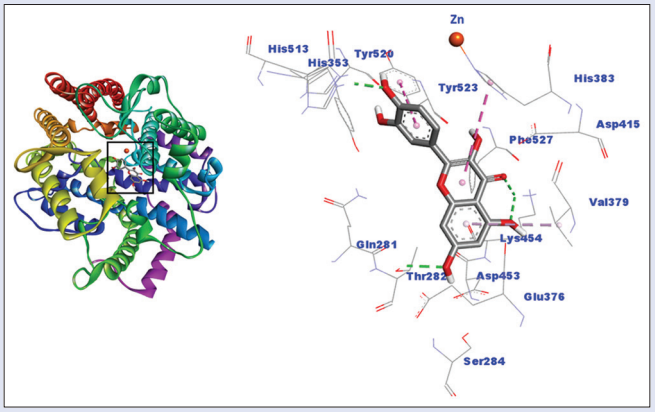

Figure 4: Molecular docking and interaction pattern of quercetin with angiotensin-converting enzyme

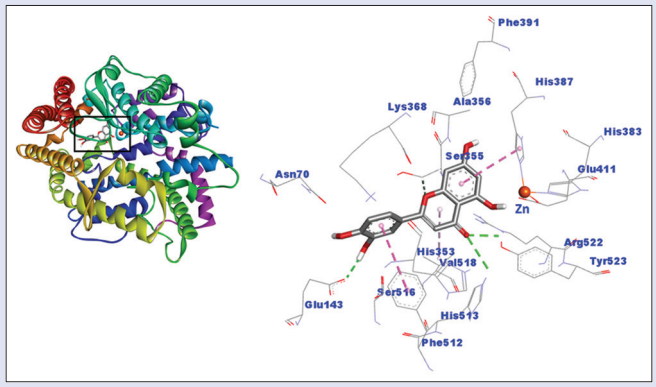

Figure 3: Molecular docking and interaction pattern of luteolin with angiotensin-converting enzyme

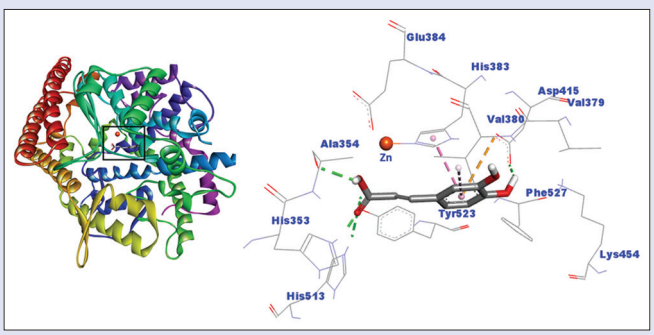

Figure 5: Molecular docking and interaction pattern of caffeic acid with angiotensin-converting enzyme

Table 2: Molecular docking parameters for the interaction between flavonoids (1-3) and angiotensin-converting enzyme

Name of bond	Nature of interaction	Distance (Å)	Binding energy (kcal/mol^{1})	Binding affinity (M^{-1})
		Kaempferol		
Lys454:HZ2 - UNK: O	Hydrogen bond	2.5535	−8.6	2.03×10^{6}
Tyr523:HH - UNK: O	Hydrogen bond	2.7849		
UNK: H - Asp415:OD1	Hydrogen bond	3.0613		
UNK: H - Glu376:OE2	Hydrogen bond	1.9514		
UNK: H - His383	Hydrogen bond (Pi-donor)	2.7188		
His383 - UNK	Hydrophobic (Pi-Pi stacked)	4.6688		
Tyr523 - UNK	Hydrophobic (Pi-Pi stacked)	4.9869		
His383 - UNK	Hydrophobic (Pi-Pi T-shaped)	5.9714		
UNK - Val379	Hydrophobic (Pi-alkyl)	5.3863		
		Luteolin		
His513:HE2 - UNK: O	Hydrogen bond	3.0181	−8.6	2.03×10^{6}
Tyr523:HH - UNK: O	Hydrogen bond	2.4821		
UNK: H - Glu143:OE1	Hydrogen bond	2.2949		
Ser355:CB - UNK: O	Carbon hydrogen bond	3.3532		
His387 - UNK	Hydrophobic (Pi-Pi T-shaped)	4.8305		
Phe512 - UNK	Hydrophobic (Pi-Pi T-shaped)	4.9122		
UNK - Val518	Hydrophobic (Pi-alkyl)	4.8308		
		Quercetin		
Thr282:HG1 - UNK: O	Hydrogen bond	2.5144	−9.0	3.99×10^{6}
His353:HE2 - UNK: O	Hydrogen bond	2.3339		
Lys454:HZ2 - UNK: O	Hydrogen bond	2.5455		
Lys454:HZ2 - UNK: O	Hydrogen bond	2.6263		
Tyr523 - UNK	Hydrophobic (Pi-Pi stacked)	4.8911		
His383 - UNK	Hydrophobic (Pi-Pi T-shaped)	5.9863		
UNK - Val379	Hydrophobic (Pi-alkyl)	5.3861		

His: Histidine; Glu: Glutamic acid; Lys: Lysine; Tyr: Tyrosine; Thr: Threonine; Ser: Serine; Ala: Alanine

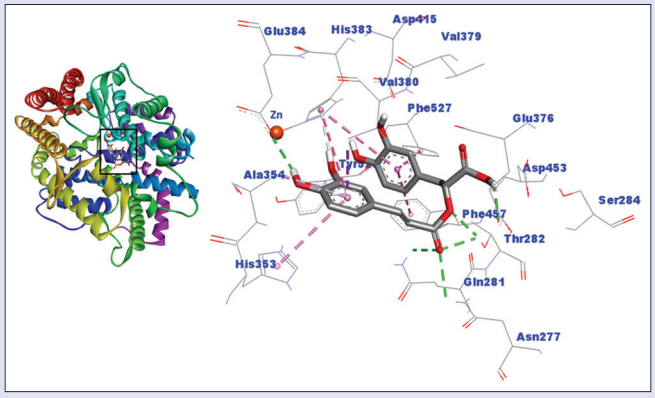

Figure 6: Molecular docking and interaction pattern of rosmarinic acid with angiotensin-converting enzyme

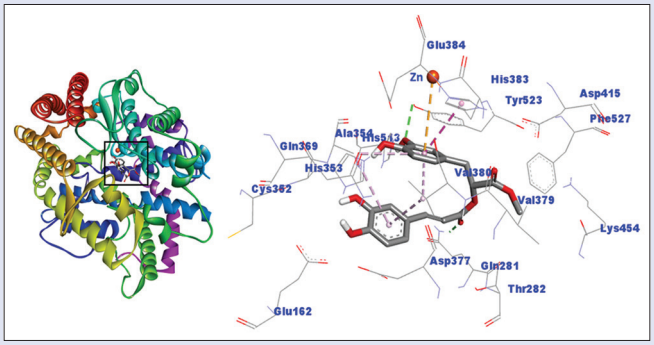

Figure 7: Molecular docking and interaction pattern of methyl rosmarinate with angiotensin-converting enzyme

Tyr523 [Figure 6]. The binding energy of rosmarinic acid for ACE was −8.5 kcal/mol which corresponded to a binding affinity of 1.72×10^{6}/M [Table 3]. Finally, methyl rosmarinate formed two hydrogen bonds (Gln281 and Tyr523), one electrostatic interactions (Zn^{2+}), one Pi–Pi stacked hydrophobic interaction (His383), and three Pi-alkyl hydrophobic interactions (Ala354 and Val380; Ala354 formed two Pi-alkyl hydrophobic interactions) with ACE. Other amino acid residues involved in stabilizing the quercetin-ACE complex were Glu162, Thr282, Cys352, His353, Gln369, Asp377, Val379, Glu384, Asp415, Lys454, His513, and Phe527 [Figure 7]. The binding energy and the corresponding binding affinity of methyl rosmarinate and ACE complex were −8.2 kcal/mol and 1.03×10^{6}, respectively [Table 3].

DISCUSSION

In this study, eight phenolic compounds isolated for the first time were identified in the herbal extract of *H. europaeum*. They include five flavonoids in addition to caffeic acid (6) and rosmarinic acid as well as its methyl derivative (7 and 8). The first report that examines the main phenolic constituents in *H. europaeum* herbal extracts and assesses their biological activities are hereby presented.

The isolated phenolics possessed strong radical-scavenging activity. The antioxidant activity depended on the presence of the (*ortho* 3',4'-dihydroxy) (catechol) structure as found in compounds 2, 3, and 5–8. This could explain the weak scavenging activity of kaempferol-3-O-glucopyranoside (4) (IC_{50} >100) which lacks the catechol substructure in ring B.[34,35,48] The (catechol) structure in the

Table 3: Molecular docking parameters for the interaction between phenolic compounds (6-8) and angiotensin-converting enzyme

Name of bond	Nature of interaction	Distance (Å)	Binding energy (kcal/mol)	Binding affinity (M^{-1})
Caffeic acid				
His353:HE2 - UNK: O	Hydrogen bond	1.9185	−6.5	5.85×104
His513:HE2 - UNK: O	Hydrogen bond	2.1624		
Tyr523:HH - UNK: O	Hydrogen bond	2.5417		
UNK: H - Ala354:O	Hydrogen bond	2.2084		
UNK: H - Asp415:OD2	Hydrogen bond	2.3926		
Asp415:OD1 - UNK	Electrostatic (Pi-anion)	4.5331		
His383 - UNK	Hydrophobic (Pi-Pi stacked)	3.8087		
UNK - Val380	Hydrophobic (Pi-alkyl)	5.3546		
Rosmarinic acid				
ASN277:HD21 - UNK: O	Hydrogen bond	2.7453	−8.5	1.72×106
Gln281:HE22 - UNK: O	Hydrogen bond	2.5575		
Thr282:HG1 - UNK: O	Hydrogen bond	2.3318		
Thr282:HG1 - UNK: O	Hydrogen bond	2.3844		
UNK: H - Glu384:OE2	Hydrogen bond	2.4915		
UNK: H - Glu376:OE2	Hydrogen bond	2.2486		
UNK: H - His383	Hydrogen bond (Pi-donor)	2.6755		
Val380:CG1 - UNK	Hydrophobic (Pi-sigma)	3.9371		
His353 - UNK	Hydrophobic (Pi-Pi stacked)	5.1363		
His383 - UNK	Hydrophobic (Pi-Pi T-shaped)	5.9371		
His383 - UNK	Hydrophobic (Pi-Pi T-shaped)	5.7716		
Phe457 - UNK	Hydrophobic (Pi-Pi T-shaped)	5.0333		
Phe527 - UNK	Hydrophobic (Pi-Pi T-shaped)	4.8377		
UNK - A: Ala354	Hydrophobic (Pi-alkyl)	4.8407		
Methyl rosmarinate				
Gln281:HE22 - UNK: O	Hydrogen bond	2.0298	−8.2	1.03×106
Tyr523:HH - UNK: O	Hydrogen bond	2.7644		
ZN701:ZN - UNK	Electrostatic (Pi-cation)	4.7172		
His383 - UNK	Hydrophobic (Pi-Pi stacked)	4.0978		
UNK - Ala354	Hydrophobic (Pi-alkyl)	5.0918		
UNK - Val380	Hydrophobic (Pi-alkyl)	4.2872		
UNK - Ala354	Hydrophobic (Pi-alkyl)	5.4509		
UNK - Val380	Hydrophobic (Pi-alkyl)	5.1749		

His: Histidine; Glu: Glutamic acid; Asp: Aspartic acid; Tyr: Tyrosine; Val: Valine; Thr: Threonine; Phe: Phenylalanine; Ala: Alanine; Gln: Glutamine

B-ring bestows a high stability to the flavonoid phenoxyl radicals through hydrogen bonding and/or via expanding electron delocalization.[49,50] Furthermore, the presence of the *α,β*-unsaturated carbonyl moiety and *α*-hydroxyketone groups at ring C potentiated the antioxidant activity of flavonoids as observed with compound (3) through their chelating properties and enhancement of the radical stabilization via electron delocalization over all three-ring systems after the initial oxidation steps.[34,49-51] The glycosylation of the free hydroxyl group at C-3 (*α*-hydroxyketone) position reduces the antioxidant activity as observed with the compound (6).[34,49-51]

For ACE-inhibitory activity, the isolated phenolic compounds were tested and quercetin exhibited the most potent ACE inhibitors (IC_{50} 17.5 μM). In fact, the renin-angiotensin system acts as a crucial component in the process of controlling ion-water balance, blood pressure regulation, and pathophysiology of hypertension and renal failure. Renin acts through converting angiotensinogen to angiotensin I, which is converted subsequently to angiotensin II by ACE. The production of aldosterone from the adrenal cortex is triggered by the resulting angiotensin II. Aldosterone, in turn, raises the blood pressure via retention of sodium. Thus, ACE inhibition is considered an effective way of managing renal dysfunction and hypertension. Novel natural ACE inhibitors such as flavonoids or phenolic compounds are promising alternatives to the synthetic ACE inhibitors, such as enalapril or captopril, which cause many side effects during treatment of heart failure and essential hypertension in humans. In recent times, ACE has emerged as a critical target in the development of inhibitors/drugs to treat hypertension, heart failure, myocardial infarction, and diabetic nephropathy.

An insight into the mode of binding, and hence, inactivating ACE was gained by performing molecular docking of the isolated compounds with ACE. The active site of ACE comprises three parts: a carboxylate group crucial for enzymatic activity, a pocket that accommodates a hydrophobic side chain of *C*-terminal amino acid residues, and a zinc ion. Interaction between the zinc ion and the carbonyl of the penultimate peptide bond of the substrate (e.g., hippuryl-histidyl-leucine) results in its polarization and thereby poses for an electrophilic attack. The amino acid residues lining the active site of ACE comprises Tyr224, His353, Ala354, His383, Glu384, His387, Glu411, Lys511, His513, Tyr520, Arg522, and Tyr523.[40] ACE has a characteristic HEXXH zinc-binding motif where His383 and His387 are directly coordinated with the zinc. In addition, Glu411 provides addition coordination to zinc through acetate ion from the solvent medium. The hydrolysis of substrate by ACE is initiated by two chloride ions; first, Cl^- ion is bound to Arg186, Trp485, Arg489, and a water molecule, while the second Cl^- ion is bound to Tyr224, Arg522, and a water molecule.[52] Moreover, the X-ray crystal structure of ACE bound with a potential inhibitor (lisinopril) shows that several amino acid residues such as Glu162, Glu384, Lys511, Val518, and Tyr520 contribute significantly in the recognition and binding of the inhibitor.[40]

ACE is a Zn-containing metalloenzyme, in which Zn ion at the active site is essential for ACE catalysis. Thus, the interaction of any ligand with Zn or the residues of ACE interacting with Zn ion is considered a significant parameter for the inhibition of ACE activity.

The results of this study indicate that all the isolated compounds interacted with Zn ion or at least one amino acid residue of the HEXXH zinc-binding motif. Kaempferol interacted with the key residues of ACE such as His383 through electrostatic and hydrophobic interactions, His513 through hydrogen bonding, and Tyr523 via hydrophobic interaction. Likewise, luteolin interacted with some key residues of ACE through hydrogen bonding (His387, Glu411, and Tyr523) and hydrophobic interactions (His387 and Val518). Similarly, quercetin interacted with some key residues of ACE through hydrogen bonding (Tyr520 and Tyr523) and hydrophobic interactions (His383 and Tyr523). Caffeic acid was also bound to the active site of ACE and inhibited it by interacting with the key active site and catalytic residues such as His353, Ala354, Glu384, His513, and Tyr523 through hydrogen bonding, and His383 via electrostatic and hydrophobic interactions. Moreover, rosmarinic acid also interacted with key residues of ACE through hydrogen bonding (Ala354 and Glu384), carbon–hydrogen bonding (His383), and hydrophobic interactions (His353, Ala354 and His383). Further, methyl rosmarinate was also bound to the active site of ACE and inhibited it by binding with its key catalytic residues through hydrogen bonding (His353 and His383), carbon–hydrogen bonding (His513), and electrostatic (His383) and hydrophobic interactions (His383). The binding energy of all the isolated compounds was in the range of −6.5-9.0 kcal/mol, which corresponded to a binding affinity of 5.85×10^4- to 3.99×10^6/M. The results suggested that these compounds have a high probability to bind to the active site of ACE and thus act as a potential inhibitor of ACE activity. Moreover, the ACE-compound complex was stable in nature, thereby implying a low dissociation rate. The previous report by Al Shukor *et al*. also suggested that natural compounds such as gallic acid, protocatechuic acid, and caffeic acid act as an inhibitor of ACE by interacting with Zn ion through their carboxylate groups, while syringic acid interacts with Zn ion of ACE through its hydroxyl group. On the other hand, some natural compounds such as rutin, resveratrol, catechol, and pyrogallol inhibit ACE activity through an interaction with amino acid residues of the active site.[53]

Many studies reported that phenolic compounds including flavonoids exert the ACE-inhibitory activity via the zinc chelate complex within the ACE active center.[54-57] The presence of an ortho-dihydroxy group, the catechol group in the flavonoids, is essential for zinc chelation and ACE-inhibitory activity.[58] The reported structural activity relationship of flavonoids revealed that the 3',4'-catechol B-ring pattern was accompanied by the conjugated 4-oxo and α,β-unsaturated (2-3) bond, as found in luteolin (2) and quercetin (3) which are essential for the activity.[58] Compounds (6–8) exhibit similarities with the flavonoids in terms of the presence of ortho-dihydroxy and α,β-unsaturated ester groups. The α,β-unsaturated esters are electrophiles and can bind to the enzyme with its carbonyl group that is coordinated to the zinc ion, and then, a Michael-type addition reaction may occur on the α,β-unsaturated acid or ester by the catalytically active carboxylate in ACE. The reaction results in irreversible inhibition of the enzyme.[58]

The anti-inflammatory activity tests revealed that the free flavonoids (1–3) as well as the caffeic acid and rosmarinic acid with its derivative (6–8) were potent LOX inhibitors. The LOX-inhibitory effect was decreased by adding a sugar group to the core structure. The introduction of the sugar group increased the hydrophilicity, leading to a low affinity toward the lipophilic active site of LOX.[34,59,60] These activities may contribute to the anti-inflammatory effects of *H. europaeum* and support its use in treating many inflammatory conditions.

It should be noted that methyl rosmarinate had slightly better antioxidant and anti-inflammatory activities than rosmarinic acid. This indicated that the methyl esterification might be favorable for improving both the activities. These findings are corroborated by many previous studies.[61-63] Furthermore, several studies have reported that the esterification of rosmarinic acid suppresses its ionization at the physiological pH *in vivo*, which enhances its cellular uptake and intracellular biological activity.[61] The present findings verified a good correlation of intrinsic antioxidant activity between cellular and non-cellular assays and suggested that further studies on methyl rosmarinate, rather than rosmarinic acid, may generate promising results.

CONCLUSION

Eight phenolic compounds were identified in the herbal extract of *H. europaeum* and isolated for the first time. They include five flavonoids in addition to caffeic acid, rosmarinic acid as well as its methyl derivative. The isolated phenolics possessed strong antioxidant, ACE-inhibitory, and anti-inflammatory activities. Quercetin possessed the most potent antioxidant and ACE-inhibitory activities, whereas rosmarinic acid and its methyl ester showed the strongest LOX-inhibitory activity.

For the ACE-inhibitory activity, the flavonoids and phenolic acids bind at the active site and interact with some key residues of ACE with high affinity. The ACE inhibitory activity of the isolated compounds was in the following order: 3 > 2 > 7 > 8 > 1 > 6. This order can be explained by understanding that the ortho-dihydroxy and α,β-unsaturated oxo groups that are essential for activity. Thus, these compounds hold the promise to be used directly or developed into more potent ACE inhibitors. Our results on *H. europaeum* extract constituents provided some scientific evidence on the beneficial effects of its traditional uses and could be considered as a promising drug candidate.

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Conflicts of interest

There are no conflicts of interest.

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