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Mechanism & inhibition kinetics of bioassay-guided fractions of Indian medicinal plants and foods as ACE inhibitors

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ABSTRACT

Hypertension is a becoming a major threat to the world. Angiotensin converting enzyme (ACE) is a key part in the renin angiotensin aldosterone system (RAAS) which control blood pressure. Over expression of RAAS is related with vascular hypertension, ACE inhibition has turned into a noteworthy target for controlling hypertension. In the search of lead molecules from plant origin as a substitute for toxic synthetic drugs, 25 Indian medicinal plants and foods were screened for their ACE inhibitory activity. IC_{50} (50% inhibition of ACE) values of hydroalcoholic crude extracts and fraction were determined by a colorimetric method. Active fractions were further screened to determine the enzyme kinetics, mode, specificity and mechanism of inhibition. Standardization was done by determining total phenolics and flavonoids as gallic acid and quercetin equivalents/mg of extract respectively. Among 25 crude extracts, *Cynara scolymus* extract showed the best activity, IC_{50} value 356.62 μg/mL. ACE inhibition resulting from protein precipitation was highest in *Coscinium fenestratum*. Lineweaver-Burk plots revealed a competitive mode of inhibition for *Punica granatum* ethyl acetate fraction. Fractions of *Cassia occidentalis*, *Cynara scolymus* and *Embelia ribes* were found to be non-specific inhibitors of ACE. *Embelia ribes, Cassia occidentalis* and *Coscinium fenestratum* fractions inhibited the ACE by Zn^{2+} ion chelation. Research revealed the potential of tested plants fractions as ACE inhibitors along with their inhibition kinetics and mechanism of inhibition. These active plant fractions might find importance in the development of potential antihypertensive agents after further investigations using preclinical and clinical trials.

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1. Introduction

Hypertension is a major risk factor for many cardiovascular diseases such as arteriosclerosis, congestive heart failure, coronary heart disease, end-stage renal diseases, myocardial infarction, and stroke.[1] In the year 2000, about 26.4% of the world's population suffered hypertension, and it is increasing at an alarming rate with a prediction of 60% in 2025.[2] Blood pressure is regulated by different biochemical pathways, one of the major components of blood pressure regulation physiology is the renin-angiotensin system (RAS). Angiotensin I (an inactive form of decapeptide) is produced by catalytic cleavage by renin from angiotensinogen; Angiotensin I is further cleaved to angiotensin II(octapeptide) by angiotensin I converting enzyme (ACE), angiotensin II is a potent vasoconstrictor and an inhibitor of the bradykinin, which is vasodilator.[3] Inhibition of RAS is the mechanism of ACE inhibitors and they are the most commonly used classes of drugs for controlling elevated blood pressure (BP). ACE inhibitor drugs prevent the formation of angiotensin-II which is responsible for constriction of blood vessels and thereby lowers the blood pressure. By controlling hypertension through ACE inhibitors, cardiovascular diseases such as congestive heart failure,[4] myocardial infarction,[5] and diabetic

Abbreviations: ACE, Angiotensin I Converting Enzyme; BAPNA, a-N-benzoyl-DL-arginine-Pnitroanilide HCl; BP, blood pressure; BSA, bovine serum albumin; BSC, benzene sulphonyl chloride; CH_2Cl_2, dichloromethane; DMSO, dimethyl sulphoxide; EtOAc, Ethyl acetate; EtOH, ethanol; GAEs, gallic acid equivalents; HA, hippuric acid; HCl, Hydrochloric acid; HHL, hippury-l-histidyl-l-leucine; IC_{50}, half maximal inhibitory concentration; K_m, Michaelis-Menten constant; M, Molar; MeOH, methanol; mg, milligram; mL, milli litre; Mm, Milli mole; Mu, Milli units; n-BuOH, n-butanol; ng, nano gram; QEs, quercetin equivalents; RAS, renin-angiotensin system; TCA, Trichloroacetic acid; TFA, trifluoroacetic acid; UV, ultra violet; V_{max}, Maximum velocity; Zn^{2+}, Zinc ion; $ZnCl_2$, Zinc chloride.

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nephropathy[6] has been successfully controlled. Specific inhibitors of ACE have been shown to be useful as antihypertensive drugs. ACE inhibitors (viz., captopril, enalapril, fosinopril and ramipril) currently available in the market for clinical use, exert the antihypertensive effect by competitively binding to the active site of ACE.[7] Use of ACE inhibitor is associated with many adverse effects including bronchospasm and cough. ACE inhibitors are contraindicated in pregnancy.[8,9] Foods and plants are the perfect sources for exploring novel lead molecules for ACE inhibition. India being rich in biological diversity often been referred to as the "Medicinal Garden of the world". India is sitting on gold mine of well recorded and traditionally well-practiced knowledge of herbal medicine.[10] These herbs are used for variety of diseases including hypertension and many of them are unexplored for its phytopharmacological uses. Plant extracts and molecules isolated from them have previously shown inhibitory effects on ACE.[11,12] In the search for lead molecules for hypertension from plant origin, in this research article we have selected 25 plants for screening ACE inhibitory activity on the basis of their traditional use as cardiotonics, diuretics or modern research exploring cardiovascular potential.[11] The article aims toward identifying the potential plants and it fractions as ACE inhibitors, investigating the kinetics of ACE inhibition, thereby revealing the pharmacological mechanism of these fractions.

2. Material and methods

2.1. Enzymes and chemical reagents

ACE from rabbit lung (9015-82-1), chymotrypsin (9004-07-3), trypsin (9002-07-7), bovine serum albumin (BSA)(9048-46-8), hippury-l-histidyl-l-leucine (HHL)(207386-83-2), hippuric acid (HA)(495-69-2), Folin-Ciocalteu's phenol reagent (47641-100 ML-F), Trichloroacetic acid (TCA)(76-03-9), Gallic acid (149-91-7), Quercetin (117-39-5), Aluminium chloride (7446-70-0), trifluoroacetic acid (TFA)(76-05-1), Pyridine (110-86-1), benzene sulphonyl chloride (BSC)(98-09-9), BAPNA (a-N-benzoyl-DL-arginine-Pnitroanilide HCl) (911-77-3), dimethyl sulphoxide (DMSO)(67-68-5), Zinc chloride ($ZnCl_2$)(7646-85-7) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl acetate (EA), n-hexane, n-butanol (n-BuOH), methanol(MeOH), ethanol (EtOH) and dichloromethane (CH_2Cl_2) were purchased from S.D. Fine Chemical Ltd., Mumbai, India. All other chemicals used were of analytical grade.

2.2. Plant materials

Plant materials were collected from Whitestone Healthcare Pvt. Ltd. Bhopal in central India within the duration of 12 months during 2011–12. All Voucher specimens NIPPHYTO01-NIPPHYTO25 were authenticated by Dr. Brijesh Sahoo of Botanical Survey of India, Govt. of India, Ministry of Environment and Forests, India and Dr. Bhasker Punjani, Professor in P.G., Botany Department, Science college, Talod, Gujarat. All the voucher specimens (NIPPHYTO01-NIPPHYTO25) are deposited at the Herbarium of Institute of Pharmacy Nirma University of Science and Technology Ahmedabad for further reference.

2.3. Extraction and fractionation

Plant materials were dried under shade for a week, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. Extraction was carried out using analytical grade solvents obtained from S.D. Fine Chemical Ltd., Mumbai, India, for which 1 kg powder of each plant material was transferred into a container and hydroalcoholic solvent was added, until the coarse particles of the plant material was completely soaked. The container was gently shaken for 36 h at room temperature with intermittent shaking. The extract obtained was filtered using Whatman filter paper and the residue was again extracted with a fresh solvent for another 12 h. The extract was filtered and both the filtrates were pooled together. The solvent was removed using Buchi type rotary evaporator and the extract was subjected to freeze drying in a lyophilizer till dry powder was obtained for further use. As the plants and their parts were selected based on their traditional uses and modern research, fractionation was done only for those plants whose extract were found to be active, using a different fractionation approach for each plant material to obtain the bioactive enriched fraction. In general, all fractions were obtained by suspending a fixed quantity of crude extract in water forming the slurry which was successively partitioned with solvents of increasing polarity between water and n-hexane or Petroleum ether, followed by chloroform or dichloromethane, ethyl acetate, and n-butanol. The solutions were completely evaporated to give the respective fractions.[13]

2.4. Quantitative determination of total phenolic and flavonoids contents

Total phenolic and flavonoids contents were determined for the extracts which were found to be active. Determination of total phenolics compounds was performed on hydroalcoholic extracts by the Folin-Ciocalteu method. Each sample was mixed with 1 mL Folin-Ciocalteu reagent and 0.8 mL of 7.5% Na_2CO_3. This procedure involves the reduction of Folin-Ciocalteu reagent by phenolic compounds present in the extract, with concomitant formation of a blue complex determined at 765 nm by UV–visible spectrophotometer (UV-1700 Shimadzu, Japan) after 90 min at room temperature. Gallic acid was used for constructing the standard curve (Fig. 1) and the mean of three readings was used to determine the total phenolic content expressed as μg of gallic acid equivalents/mg of extract (GAEs).[14]

Determination of total flavonoids was done by the aluminum chloride colorimetric method. 0.6 mL of 2% aluminum chloride solution was mixed with 0.6 mL diluted standard quercetin solutions or extracts. The solution was incubated for 60 min at room temperature after proper mixing. The volume of 6% aluminium chloride was substituted by the same volume of distilled water in the blank. Reaction mixtures absorbance was measured against blank at 420 nm wavelength with a UV–visible spectrophotometer (UV-1700 Shimadzu, Japan). Concentration was determined by comparison with the standard calibration curve (2–200 μg/mL) of

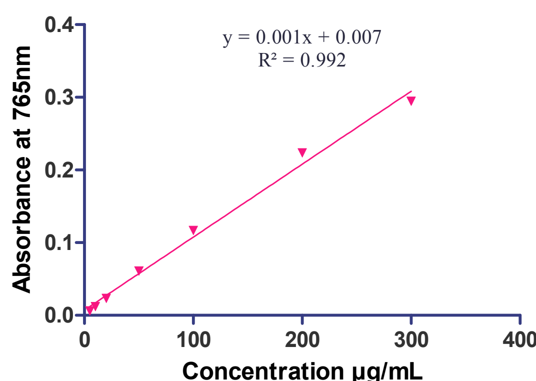

Fig. 1. Gallic acid standard curve.

quercetin. The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($Y = 0.0001x + 0.001$, $R^2 = 0.994$) (Fig. 2), and expressed as μg quercetin equivalents/mg of extract (QEs). All the quantifications were carried out in triplicate.[15]

2.5. Angiotensin I-converting-enzyme (ACE) inhibitory activity

There are several methods to determine the ACE inhibitory activity which can be used to determine the ACE activity. However, the method utilized here is a spectrophotometric method based on the commonly used method introduced by Cushman and Cheung (1971).[16] This method is simple, sensitive, and rapid, requiring no solvent extraction and can, therefore, be used for high-throughput screening of ACE inhibitors. ACE inhibitory activity was assayed by measuring the release of HA (Hippuric acid) from the substrate HHL.

Various types of substrates and methods used to analyze ACE inhibition activity are following: Cushman and Cheung Method using a substrate hippuryl-histidyl-leucine (HHL), Holmquist method using a substrate furanacryloyl-tripeptide, Elbl and Wagner method using a substrate benzoil-[l-14C] glicyl-L-histidine-L-leucine, Carmel and Yaron method using a substrate o-amino-benzoylglycyl-p-nitrophenylalanilproline, and Lam method using 3-hydroxybutyrylglycyl-glycyl-glycine as substrate. Methods to measure the results of enzymatic reactions are spectrophotometric, fluorometric, high-performance liquid chromatography, electrophoresis, and radiochemistry. A Radiometric assay using the labeled angiotensin I substrate, wherein the release of radioactive histidine-leucine which serves as an enzymatic activity index.[17] Carmel and Yaron (1977–1978) developed a measurement method of the ACE inhibitory activity using an o-amino-benzoylglycine-p-nitrophenylalanylproline as a substrate and then hydrolyzed into o-aminobenzoylglicyl.[18] Holmquist et al. in 1979 developed a method using FAPGG as a substrate. This method is based on the absorption spectrum blue shift that occurred on the substrate hydrolysis produces dipeptide and furanacryloyl-blocked amino acid.[19] Many of the methods described above require expensive instrumentation and a large volume of organic solvents,[20] so utilized a simple, sensitive, and rapid, requiring no solvent extraction method as described by Jimsheena and Gowda, 2009.[21]

The assay mixture contained 125 μL of a 0.05 M sodium borate buffer (pH 8.2), containing 0.3 M NaCl, 50 μL of 5 mM HHL and 25 μL of ACE (2.5 Milli units (mU)), which was pre-incubated with different sample concentrations of the plant inhibitor. The reaction was stopped after incubation at 37 °C for 30 min by the addition of 0.2 mL of 1 M HCl. Pyridine (0.4 mL) was added followed by 0.2 mL of BSC (the order of addition of reagents is critical) The solution was slowly mixed using a vortex mixer and cooled on ice. The yellow colour developed was measured at 410 nm using UV–visible spectrophotometer (UV-1700 Shimadzu, Japan). One unit of ACE activity is defined as the amount of enzyme, which releases 1 μmol of HA per min at 37 °C (Degree Celsius) and pH 8.2.[21]

The degree of ACE inhibition (%) was calculated with the following equation

$$\text{ACE inhibition (\%)} = \frac{A1 - A2}{A1 - A3} \times 100$$

Where.

A1 is absorbance of the ACE solution without an inhibitor (Plant extract and fractions)
A2 is absorbance of the tested sample of extract and fractions
A3 is absorbance of HHL solution (a buffer was added instead of the ACE solution and sample)

The IC_{50} value is defined as the concentration of inhibitor required to decrease the HA peak area by 50% (indicating 50% inhibition of ACE), and was calculated using a non-linear regression from a plot of activity versus inhibitory concentration of at least five separate concentrations. To obtain the standard deviation during the assay, each assay was performed in triplicate.

2.6. Kinetics of ACE inhibition

Study of enzyme kinetics is an important tool to investigate the chemical mechanism of catalysis. Kinetic studies provide information on substrate and product affinity to the enzyme. Knowledge of the dynamic properties of enzyme catalysis is a prerequisite for the design of inhibitors (drugs) directed against ACE.

Kinetic parameters were evaluated by the Lineweaver–Burk's method. The reaction conditions were the same as ACE inhibitory activity assay as described above. The enzyme activity was measured at various substrate (HHL) concentrations (0.5, 1, 2, 3.5, and 6 mM) in the presence of the inhibitor at three different concentrations (0, 100 μg/mL and 200 μg/mL) which was used to construct the Lineweaver-Burk plot. Classification of inhibition type as competitive, non-competitive or uncompetitive is done on the basis of these plots. In this plot, a linear regression was made using the reciprocals of various HHL concentrations as the independent variable (X-axis) and the reciprocals of HA formation rates as dependent variable (Y-axis). The rate of formation of HA was determined in the presence of different concentrations of the plant inhibitor. The Lineweaver-Burk plot provides information about $1/V_{max}$ (Y axis-intercept of the linear regression) and $-1/K_m$ (X axis-intercept) for the ACE inhibitory kinetics. A competitive inhibitor will have the same Y axis-intercept ($1/V_{max}$) in the presence of inhibitor (at 100 μg/mL and 200 μg/mL) as in the absence of inhibitor (since V_{max} is unaffected). A calibration curve for standard HA was constructed for reference. All experiments were conducted in triplicate.[22,23]

2.7. Assessment of mechanism and specificity of the ACE inhibition

Plants extracts and fractions can conjugate with protein and thus has the ability to non-specifically inhibit enzyme action, taking into consideration ACE inhibition with increasing concentrations of BSA (0, 25, 50, 125, 200 and 250 μg/mL) was accessed. The specificity of the inhibition of ACE was accessed by investigating the effect extract on the activity of chymotrypsin and trypsin as described by Liu et al. (2003).[24]

Briefly, in the test sample, 6 μL of plant extract in 100% DMSO

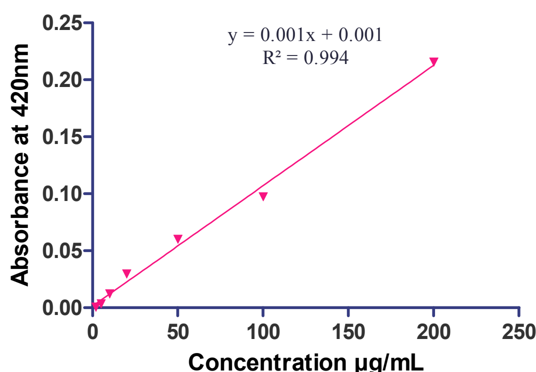

Fig. 2. Quercetin standard curve.

was mixed with 0.5 mL 20 μg/mL chymotrypsin under 0.1 M phosphate buffer (pH 8.0) and incubated at 37 °C for 5 min. 1000 μl of 2% casein in 0.1 M phosphate buffer (pH 8.0) was then added and incubated for a further 30 min at which time 2 mL of 10% trichloroacetate was added to quench the reaction. After 1 h, Absorbance of the supernatant was measured at 280 nm after the mixture was centrifuged for protein sedimentation. No plant fractions were added to the DMSO in the control group, and in the blank group, no enzyme was added.

Assay for trypsin inhibition was similar to that for chymotrypsin. In the test sample, 6 μL of plant extract or fractions solution was mixed with 0.5 mL (40 μg/mL) trypsin solution and incubated at 37 °C for 5 min 1 mL of BAPNA solution (25 mg BAPNA in 0.5 mL DMSO made up to 50 mL in 0.1 M Tris-HCl buffer) was added and incubated at 37 °C for a further 20 min. To quench the reaction, 0.5 mL of 10% acetic acid was added and the absorbency was measured at 410 nm.[24]

The Chymotrypsin/trypsin inhibitory activity is expressed as

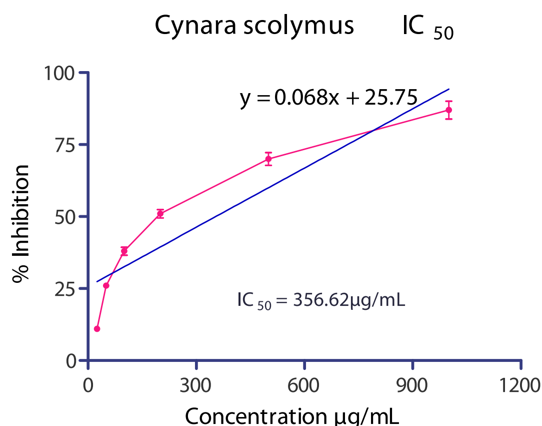

Fig. 3. IC_{50} of *Cynara scolymus* extract.

$$\text{Residual}\frac{\text{Chymotrypsin}}{\text{trypsin}}\text{activity }(\%) = \left(\frac{\text{absorbency of experimental group} - \text{absorbency of experimental group blank}}{\text{absorbency of control group} - \text{absorbency of control group blank}}\right) \times 100$$

2.8. The effect of $ZnCl_2$ on the inhibitory activity of ACE

As ACE is a Zn^{2+} containing enzyme, 1.5 mM $ZnCl_2$ was added to the reaction mixture according to the method of Liu et al. (2003) to assess whether the inhibitory action of the plant fractions resulted from the chelation of the Zn^{2+} ions. The experimental approaches including the supplementation of the ACE activity test system with or without $ZnCl_2$ were performed.

2.9. Statistical analysis

Each test was conducted in triplicate or otherwise mentioned, all data are presented as the mean (±SD) for the number of determinations shown in the tables and figures.

3. Results

3.1. Total phenolic and flavonoids contents

Phenolic and flavonoids content of tested plants extracts and active fractions are presented in Table 6. Total flavonoids ranged from 92.75 to 4.77(μg QE/mg dry wt). Total phenolic content varied from 94.42 to 3.27 (μg GAE/mg dry wt).

3.2. ACE inhibitory activity

Initially hydroalcoholic extracts of 25 plants were investigated for their IC_{50} value. Among 25 extracts studied, the best activity was found in *Cynara scolymus,* IC_{50} value 356.62 μg/mL(Fig. 3), activity decreases in following order *Cynara scolymus > Embelia ribes > Crataegus oxyacantha > Coscinium fenestratum > Stevia rebaudiana > Euphorbia prostrate > Piper betle > Punica granatum > Kalanchoe pinnata > Oryza sativa > Morus nigra > Polyalthia longifolia > Feronia limonia > Mucuna pruriens > Psoralea corylifolia > Cassia occidentalis > Asparagus racemosus > Cyperus rotundus > Carissa carandas > Elaeocarpus ganitrus > Bombax ceiba > Chenopodium album > Nepeta hindostana > Abutilon indicum > Nyctanthes* arbortristis. Least activity was found in *Nyctanthes arbortristis* IC_{50} value of 4478.01 μg/mL. IC_{50} values of all the plants are shown in Table 1.

17 out of 25 plants, whose IC_{50} value was less than 1 mg/mL were selected for fractionation. Based on the extensive literature survey of each plant, fractionation was done very carefully resulting 68 fractions from 17 plants. Out of 68 fractions screened, 11 fractions were found to be very active with the IC_{50} value less than 200 μg/mL. These active fractions were used for further study of enzyme kinetics (Table 2). Captopril, employed as positive control in the assays, presented $IC_{50} = 1.33 \pm 0.03$ ng/mL.

Results indicate that out of various solvents selected for fractionation, ethyl acetate fraction was found to be most potent for 7 fractions of plants, in which highest activity was found in ethyl acetate fraction of *Crataegus oxyacantha* with IC_{50} value of 50.91 μg/mL. Activity goes on decreasing with the following order of ethyl acetate extract of *Crataegus oxyacantha > Cynara scolymus > Mucuna pruriens > Morus nigra > Cassia occidentalis > Punica granatum > Euphorbia prostrata.* Butanol fraction of these plants *Coscinium fenestratum, Cynara scolymus, Punica granatum* was found to be active. Water and Hexane fraction of the plants were found to be least active except for *Stevia rebaudiana* water fraction(IC_{50} value of 137.23 μg/mL), Petroleum ether fraction of *Embelia ribes* was found to be most active (IC_{50} value of 39.32 μg/mL) among all fractions.

3.3. Kinetics of the ACE inhibition

The enzyme kinetics of the ACE in the presence of the inhibitor (eleven fractions) was determined from the Lineweaver-Burk plots. Values of K_m (mM) and V_{max} (μM/min) were calculated by fitting the slope of linear regression in Michaelis-Menten formula (Fig. 4). All the fractions exhibited a non-competitive mode of inhibition except *Punica granatum* Ethyl acetate fraction which exhibited a competitive mode of inhibition.

3.4. Mechanism of the ACE inhibition

The ACE inhibitory activity of six fractions was significantly

Table 1
IC_{50} Value of Extracts and fractions.

S.no	Family	Scientific name	Part	V·S	Use	Crude Extract Solvent ratio % Yield w/w (IC_{50} μg/mL)	Fraction 1% Yield w/w (IC_{50} μg/mL)	Fraction 2% Yield w/w (IC_{50} μg/mL)	Fraction 3% Yield w/w (IC_{50} μg/mL)	Fraction 4% Yield w/w (IC_{50} μg/mL)
1	Malvaceae	*Abutilon indicum* L.	Whole plant	NIPPYHTO01	Diuretic, Anti-hypertensive	Ethanol-Water (70–30) 16.43% 3159.46 ± 195.28	–	–	–	–
2	Liliaceae	*Asparagus racemosus* L.	Roots	NIPPYHTO02	Diuretic, Cardioprotective	Ethanol-Water (80–20) 18.56% 1065.2 ± 153.46	Chloroform 2.09 NA	**Ethyl acetate** **0.83** **417.26 ± 27.27**	**Butanol** **14.2** **323.66 ± 17.41**	Water 82.88 6019.23 ± 326.33
3	Bombacaceae	*Bombax ceiba* L.	Fruits	NIPPYHTO03	Diuretic	Ethanol-Water (80–20) 16.87% 2096.20 ± 167.02	–	–	–	–
4	Caesalpiniaceae	*Cassia occidentalis* L.	Leaves	NIPPYHTO04	Diuretic, High Blood Pressure	Ethanol-Water (50-50) 25.9% 1035.46 ± 175.61	Hexane 30.40 NA	**Ethyl acetate** **14.51** **199.48 ± 14.48**	Butanol 21.19 976.03 ± 28.37	Water 33.84 1879.25 ± 132.03
5	Chenopodiaceae	*Chenopodium album* L.	Arial Parts	NIPPYHTO05	Diuretic, Cardiotonic	Ethanol-Water (60–40) 17.49% 2486.54 ± 313.82	–	–	–	–
6	Menispermaceae	*Coscinium fenestratum* Gaertn.	Stem	NIPPYHTO06	Diuretic, Hypotensive	Ethanol-Water (80–20) 15.96% 837.96 ± 61.30	Hexane 12.8 NA	Chloroform 23.43 1523.76 ± 104.44	**Butanol** **16.30** **150.36 ± 13.8**	Water 47.40 1103.26 ± 41.01
7	Apocynaceae	*Carissa carandas* L.	Fruits	NIPPYHTO07	Diuretic	Methanol-Water (90:10) 31.13% 1635.23 ± 93.08	–	–	–	–
8	Rosaceae	*Crataegus oxyacantha* L.	Berries	NIPPYHTO08	Diuretic, Cardiotonic, Hypotensive	Ethanol-Water (70–30) 18.51% 796.25 ± 70.54	Chloroform 24.58 3265.23 ± 173.70	**Ethyl acetate** **15.12** **50.91 ± 3.52**	**Butanol** **23.33** **369.15 ± 26.78**	Water 36.33 1598.58 ± 104.32
9	Asteraceae	*Cynara scolymus* L.	Aerial Parts	NIPPYHTO09	Diuretic, Cardiotonic	Ethanol-Water (70–30) 25.67% 356.62 ± 22.15	Chloroform 23.67 453.16 ± 27.94	**Ethyl acetate** **09.23** **63.36 ± 3.62**	**Butanol** **17.37** **116.99 ± 7.21**	Water 49.7 3952.23 ± 260.56
10	Cyperaceae	*Cyperus rotundus* L.	Tubers	NIPPYHTO10	Diuretic	Methanol-Water (70:30) 9.17% 1416.39 ± 156.68	–	–	–	–
11	Elaeocarpaceae	*Elaeocarpus ganitrus* Roxb.	Seeds	NIPPYHTO11	Antihypertensive	Ethanol-Water (70–30) 6.53% 1869.39 ± 144.86	–	–	–	–
12	Myrsinaceae	*Embelia ribes* Burm.	Fruits	NIPPYHTO12	Diuretic, Cardioprotective	Ethanol-Water (90–10) 12.6% 761.53 ± 36.01	**Petroleum ether** **41.5** **39.32 ± 8.94**	Chloroform 13.2 NA	Butanol 16.96 1165.74 ± 63.32	Water 28.30 NA
13	Euphorbiaceae	*Euphorbia prostrata* Ait.	Whole Plant	NIPPYHTO13	Diuretic	Methanol-Water(80–20) 6.1% 863.23 ± 95.54	Dichloromethane 2.9 NA	**Ethyl acetate** **10.1** **405.46 ± 69.28**	Butanol 30.3 611.75 ± 58.89	Water 56.6 963.19 ± 61.94
14	Rutaceae	*Feronia limonia* L.	Fruits	NIPPYHTO14	Diuretic, Cardiotonic	Methanol-Water (80:20) 25.6% 981.29 ± 148.38	Petroleum ether 46.70 498.45 ± 31.14	**Ethyl acetate** **14.17** **315.17 ± 15.54**	Butanol 10.38 1633.43 ± 104.18	Water 28.72 3578.03 ± 214.47
15	Crassulaceae	*Kalanchoe pinnata* Lam.	Aerial Parts	NIPPYHTO15	Antihypertensive, Diuretic	Methanol-Water (80:20) 16.51% 916.49 ± 92.19	Hexane 6.35 NA	Chloroform 29.67 365.97 ± 5.70	Butanol 19.98 1830.19 ± 160.11	Water 43.97 230.41 ± 19.40
16	Moraceae	*Morus nigra* L.	Fruits	NIPPYHTO16	Diuretic, Hypotensive	Ethanol-Water (80:20) 28.08% 961.15 ± 69.90	Hexane 6.45 NA	Ethyl acetate 11.46 **197.15 ± 8.94**	Butanol 21.52 363.22 ± 5.40	Water 60.53 3956.95 ± 236.07
17	Fabaceae	*Mucuna pruriens* L.	Seeds	NIPPYHTO17	Diuretic, Hypotensive	Ethanol-Water (80:20) 39.46% 1003.15 ± 33.19	Hexane 31.39 NA	**Ethyl acetate** **7.54** **156.45 ± 6.75**	Butanol 6.53 1101.56 ± 30.36	Water 54.26 770.56 ± 61.28
18	Lamiaceae	*Nepeta hindostana* Roth	Aerial Parts	NIPPYHTO18	Diuretic, Cardiotonic	Ethanol-Water (80:20) 28.48% 2561.32 ± 186.86	–	–	–	–

(*continued on next page*)

Table 1 (*continued*)

S.no	Family	Scientific name	Part	V·S	Use	Crude Extract Solvent ratio % Yield w/w (IC_{50} μg/mL)	Fraction 1% Yield w/w (IC_{50} μg/mL)	Fraction 2% Yield w/w (IC_{50} μg/mL)	Fraction 3% Yield w/w (IC_{50} μg/mL)	Fraction 4% Yield w/w (IC_{50} μg/mL)
19	Oleaceae	*Nyctanthes arbortristis* L.	Flowers	NIPPYHTO19	Diuretic	Ethanol-Water (80:20) 17.23% 4478.01 ± 473.53	–	–	–	–
20	Poaceae	*Oryza sativa* L.	Bran	NIPPYHTO20	Diuretic, Antihypertensive	Methanol-Water (80:20) 18.15% 956.43 ± 151.78	Hexane 28.27 430.98 ± 26.64	Chloroform 1.02 NA	**Ethyl acetate** **2.54** **207.15 ± 11.71**	Water 67.20 NA
21	Piperaceae	*Piper betle* L.	Leaves	NIPPYHTO21	Diuretic, Antihypertensive	Ethanol-Water (80:20) 09.51% 889.01 ± 113.39	Hexane 32.10 NA	Chloroform 12.30 130.35 ± 8.94	Butanol 8.40 1135.88 ± 64.48	Water 44.20 870.56 ± 61.28
22	Annoaceae	*Polyalthia longifolia* Sonn.	Leaves	NIPPYHTO22	Diuretic, Hypotensive	Ethanol-Water (70:30) 14.63% 965.49 ± 57.4	Hexane 40.23 2863.56 ± 57.31	Chloroform 5.56 NA	**Butanol** **23.63** **401.56 ± 12.52**	**Water** **29.80** **293.01 ± 9.32**
23	Fabaceae	*Psoralea corylifolia* L.	Fruit	NIPPYHTO23	Diuretic, Vasodilator	Ethanol-Water (90–10) 22.5% 1013.98 ± 61.16	Hexane 13.56 NA	Ethyl acetate 30.61 **211.15 ± 8.94**	Butanol 18.14 363.22 ± 5.40	Water 20.78 2530.55 ± 137.35
24	Punicaceae	*Punica granatum* L.	Flowers	NIPPYHTO24	Diuretic, Cardiotonic	Ethanol-Water (80–20) 27.3% 905.94 ± 40.51	Hexane 11.2 NA	**Ethyl acetate** **15.7** **201.15 ± 5.72**	**Butanol** **27.8** **189.56 ± 3.76**	Water 45.3 1516.55 ± 12.45
25	Asteraceae	*Stevia rebaudiana* Bert.	Leaves	NIPPYHTO25	Diuretic, High Blood Pressure	Ethanol-Water (70–30) 10.1% 854.23 ± 54.09	Hexane 6.24 NA	DCM 8.8 993.16 ± 17.72	Butanol 70.32 677.12 ± 9.96	Water 10.72 **137.23 ± 9.40**

NA: Not active upto 3000 μg concentration.
Positive Control Captopril is 1.33 ± 0.03 ng/mL.

affected by the addition of different concentrations of BSA in the following order *Coscinium fenestratum* > *Mucuna pruriens* > *Cassia occidentalis* > *Crataegus oxyacantha* > *Morus nigra* > *Punica granatum* (Table 3). The ACE inhibition in the four plant fractions, viz *Cynara scolymus* Ethyl acetate fraction, *Embelia ribes* Butanol fraction, *Piper betle* Chloroform fraction and *Stevia rebaudiana* did not significantly reduce by addition of increasing concentration of BSA (Table 3).

3.5. Assessment of specificity of the ACE inhibition

3.5.1. Inhibition of chymotrypsin and trypsin enzymes

Plant fraction of *Cassia occidentalis* Ethyl acetate fraction, *Cynara scolymus* Ethyl acetate fraction and *Embelia ribes* Butanol fraction were able to non-specifically inhibit chymotrypsin and trypsin enzymes, whereas other fractions were not able to significantly inhibit these enzyme (Table 4).

3.5.2. Zinc ion chelation

The addition of 1.5 mM $ZnCl_2$ to the assay of the fraction induced ACE inhibitory action showed that *Embelia ribes, Cassia occidentalis, Coscinium fenestratum,* fractions reduced the inhibition of ACE activity by 58.39% (53.98%–22.46%), 38.91% (46.59%–28.46%) and 35.71% (51.3%–32.98%), respectively. The inhibition of ACE activity of the other fractions was insignificantly changed by the addition of $ZnCl_2$ (Table 5).

4. Discussion

Therapeutic potential of ACE inhibitors in the treatment of hypertension and management of cardiovascular diseases is well established.[25,26] Antihypertensive activity of some potential Indian medicinal plants and foods were evaluated by the inhibition of the angiotensin converting enzyme (ACE), using a colorimetric assay. In this article we have screened 25 extracts and 68 fractions for their IC_{50} value, which was a tedious job, so we utilized a simple, sensitive, and rapid method, which requires no solvent extraction and can therefore be utilized for high-throughput screening of ACE inhibitors.[21] Pharmacologically ACE removes histidyl-leucine from angiotensin I to form the physiologically active octapeptide, angiotensin II, a potent vasoconstrictor that inactivates the vasodilating nonapeptide, bradykinin. Medicinal plants were selected on the basis of their traditional usage as antihypertensive, cardiotonics and diuretics to obtain the active fractions for ACE inhibition, using hydro-alcoholic extraction. As far as possible, the traditionally used part of the plant was employed for the ACE assay.

Previous report suggest that fractions with a concentration of 330 μg/mL with inhibition rates between 50% and 100% deserve further investigation, aiming at the isolation of the active compound(s).[27] From the preliminary calculation of IC_{50} values, 11 out of the 68 fractions were whose IC_{50} value was found to be less than 200 μg/mL was used for further study of enzyme kinetics study (Table 2). Captopril, employed as a positive control in the assays, presented IC_{50} = 1.33 ng/mL. The limit of 200 μg/mL is a hypothetical value which is difficult to justify since crude extracts and fractions are complex mixtures of compounds.

4.1. Enzyme kinetics

Various kinetic parameters obtained from the Lineweaver-Burk plots are shown in Table 2. K_m of Angiotensin-converting enzyme varies according to the substrate, as a result, it is known as an unspecific enzyme. V_{max} is defined as the rate at which a substrate will be converted to a product once bound to the enzyme. K_m reflects how effectively the enzyme binds to the substrate, affinity is

Table 2
Kinetic parameters of the ACE inhibitor activity of most active fractions from various plants.

S.no	Fraction	Concentration (μg/mL)	K_m (mM)	V_{max} (μM/min)	Inhibition type
1	*Cassia occidentalis* Ethyl acetate fraction	200	3.540	0.086	Non-Competitive
2	*Coscinium fenestratum* Butanol fraction	200	4.065	0.088	Non-Competitive
3	*Crataegus oxyacantha* Ethyl acetate fraction	200	4.620	0.098	Non-Competitive
4	*Cynara scolymus* Ethyl acetate fraction	200	6.323	0.063	Non-Competitive
5	*Cynara scolymus* Butanol fraction	200	3.198	0.043	Non-Competitive
6	*Embelia ribes* Butanol fraction	200	6.449	0.049	Non-Competitive
7	*Morus nigra* Ethyl acetate fraction	200	3.025	0.046	Non-Competitive
8	*Mucuna pruriens* Ethyl acetate fraction	200	3.226	0.073	Non-Competitive
9	*Piper betle* Chloroform fraction	200	2.236	0.053	Non-Competitive
10	*Punica granatum* Ethyl acetate fraction	200	9.950	0.015	Competitive
11	*Stevia rebaudiana* Water fraction	200	2.495	0.064	Non-Competitive

reflected by these two parameters. When the enzyme binds strongly to its substrate, K_m value will be smaller, which means that lower concentration of substrate is needed to attain V_{max}. The lowest K_m is usually seen with the 'natural' substrates like angiotensin I and/or bradykinin for which the K_m value is 16–90 mM and 0.18–1.0 mM respectively.[28–30] Our result in Table 2 indicates that lowest K_m value is for *Piper betle* and highest is for *Punica granatum.* The maximum rate of substrate hydrolysis (V_{max}) and the apparent Michaelis constant (K_m) were determined to characterize the kind of inhibition exerted by fractions.[31] Table 2 shows the parameter V_{max} is significantly different in all the fractions which suggest a mixed inhibition. The Lineweaver-Burk plots Fig. 4 provides information about $-1/K_m$ (x-axis intercept) and $1/V_{max}$ (y-axis intercept of the linear regression) for the ACE inhibitory kinetics. In the presence of competitive inhibitor at different concentration (at 100 μg/mL and 200 μg/mL), the regression lines will have the same y-axis intercept ($1/V_{max}$) and it is same in the absence of inhibitor (since V_{max} is unaffected). Fig. 4(j) shows a competitive mode of inhibition for *Punica granatum* Ethyl acetate fraction. Three lines of the Lineweaver–Burk plot of *Punica granatum* representing 0, 100 μg/mL and 200 μg/mL of inhibitor concentration with different slopes shared the same y-intercept. This type of inhibition is competitive where inhibitor increases K_m and V_{max} remains unaffected, this type of inhibition suggest that fraction as well as the substrate interact with key residues in the active site of ACE, and fraction can compete with the substrate for access to the binding site. A competitive inhibitor can firmly bind to the active site and block access to other substrates.[32] In other words, competitive inhibitors might be able to enter the ACE protein and compete with HHL to interact with the active sites. Focusing on the K_m value, a competitive inhibitor increases the apparent K_m for a given substrate meaning that in the presence of a competitive inhibitor more substrate is needed to achieve half V_{max}. Fig. 4(a–d) shows Lineweaver-Burk plots for fractions of *Cassia occidentalis*, *Coscinium fenestratum*, *Crataegus oxyacantha*, *Cynara scolymus* EA, these fractions exhibited mixed inhibition mode, and the plots cross to the left of the 1/V axis but above the 1/S axis which indicates that these fractions showed a competitive-non-competitive inhibition mode with respect to the substrate (HHL). This mixed-type inhibition suggests that the fractions affected the affinity of the enzyme for the HHL substrate but did not bind at the active site.[33]

Fig. 4(e–i and k) shows Lineweaver-Burk plots for fractions of *Cynara scolymus* (BtOH), *Embelia ribes, Morus nigra, Mucuna pruriens, Piper betle, Stevia rebaudiana.* The ACE inhibition pattern of the fractions was analysed by the Lineweaver–Burk plot and was found to be non-competitive. These plots suggest that fractions might be able to enter the catalytic site, but could not bind to the catalytic site of ACE, and that it could not be hydrolyzed by ACE. Increasing angiotensin I to maintain angiotensin II levels would not overcome this mode of inhibition of the ACE. Fraction molecules can combine with an enzyme molecule to produce a dead-end complex by binding to different sites, from the substrate, regardless of whether a substrate molecule is bound or not.

To elucidate the mechanism of ACE inhibition through protein precipitation BSA (bovine serum albumin) was used. When the inhibition of ACE is due to protein precipitation addition of BSA in the test system will result in the decrease of the ACE activity, due to the reason that some of the fraction molecules will also inhibit the BSA, resulting the reduced activity of ACE. ACE inhibition resulting from enzymatic protein precipitation was observed in following fractions with decreasing order *Coscinium fenestratum* > *Mucuna pruriens* > *Cassia occidentalis* > *Crataegus oxyacantha* > *Morus nigra* > *Punica granatum.* Data in the (Table 3) indicates that inhibition of ACE due to above plants in a part may be in part due to precipitation of ACE. To elucidate the specificity of the fraction toward ACE, chymotrypsin and trypsin enzyme were used. Our result in Table 4 indicates that *Cassia occidentalis* ethyl acetate fraction, *Cynara scolymus* ethyl acetate fraction and *Embelia ribes* butanol fraction are non-specific inhibitors of ACE. These fractions were found to have reduced the activity of chymotrypsin and trypsin enzymes, thus, these fractions are non-specific inhibitors of ACE activity. Fractions of plants *Crataegus oxyacantha, Stevia rebaudiana, Piper betle Linn, Coscinium fenestratum, Mucuna pruriens, Punica granatum, Morus nigra* were not able to significantly inhibit the chymotrypsin and trypsin enzymes suggesting these fractions to be specific inhibitors of ACE.[24] ACE is a zinc metallopeptidase, its catalytic active site has zinc ion, which is essential for enzyme activity. This means that non-specific metal chelators may have apparent ACE inhibitor action. The active site of ACE is composed of three parts; a carboxylate binding functionality such as the guanidinium group of Arg, a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues, and a zinc ion.

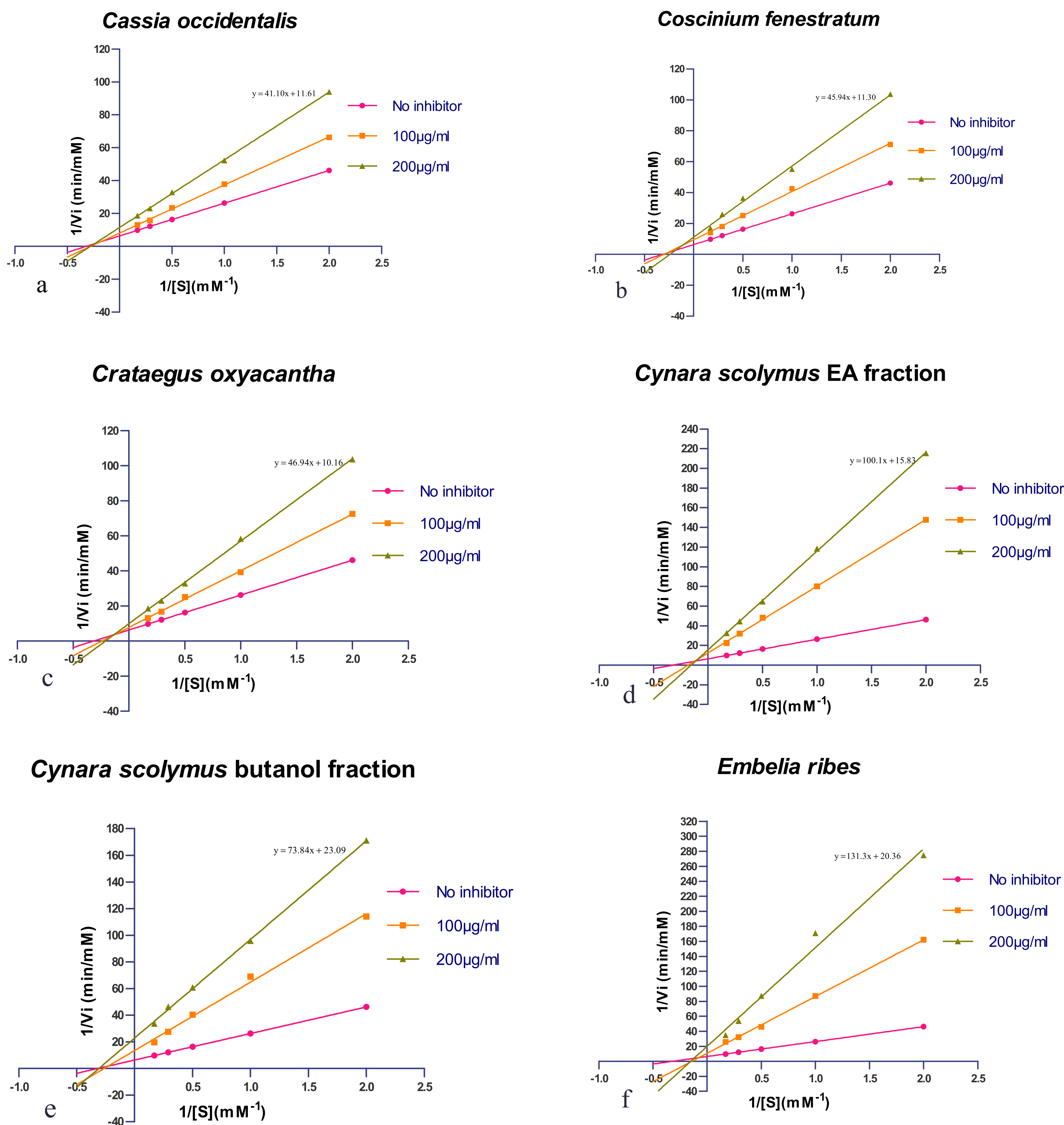

Fig. 4. Lineweaver–Burk plots derived from the inhibition of ACE by the active extract and fractions. 1/[S] and 1/Vi represent the reciprocal substrate (HHL) concentration and HA formation rate, respectively.

Supplementation of $ZnCl_2$ in the ACE activity test system is designed to reduce enzyme inhibition resulting from fraction-induced Zn^{2+} ion chelation. Our result in Table 5 indicates that out of 11 fraction only *Embelia ribes*, *Cassia occidentalis*, and *Coscinium fenestratum* fractions inhibited the ACE by Zn^{2+} ion chelation, $ZnCl_2$ decreases the inhibitory activity in these fractions suggesting that plant fractions apart from chelating the Zn^{2+} ion of ACE, they were involved in the chelation of Zn^{2+} ion released from $ZnCl_2$. Its shows that the chelation of Zn^{2+} may at least in part to be responsible for the ACE inhibitory activity of the fractions. Presence of flavonoids and other polyphenols is already reported in *Embelia ribes*,[34] *Cassia occidentalis*[35] and *Coscinium fenestratum*[36] and these molecules chelate complexes with the zinc atom within the active centre of zinc-dependent metallopeptidases.[37] Results from the previous study also suggest formation of hydrogen bridges between the inhibitor and amino acids near at the active site.[38] But

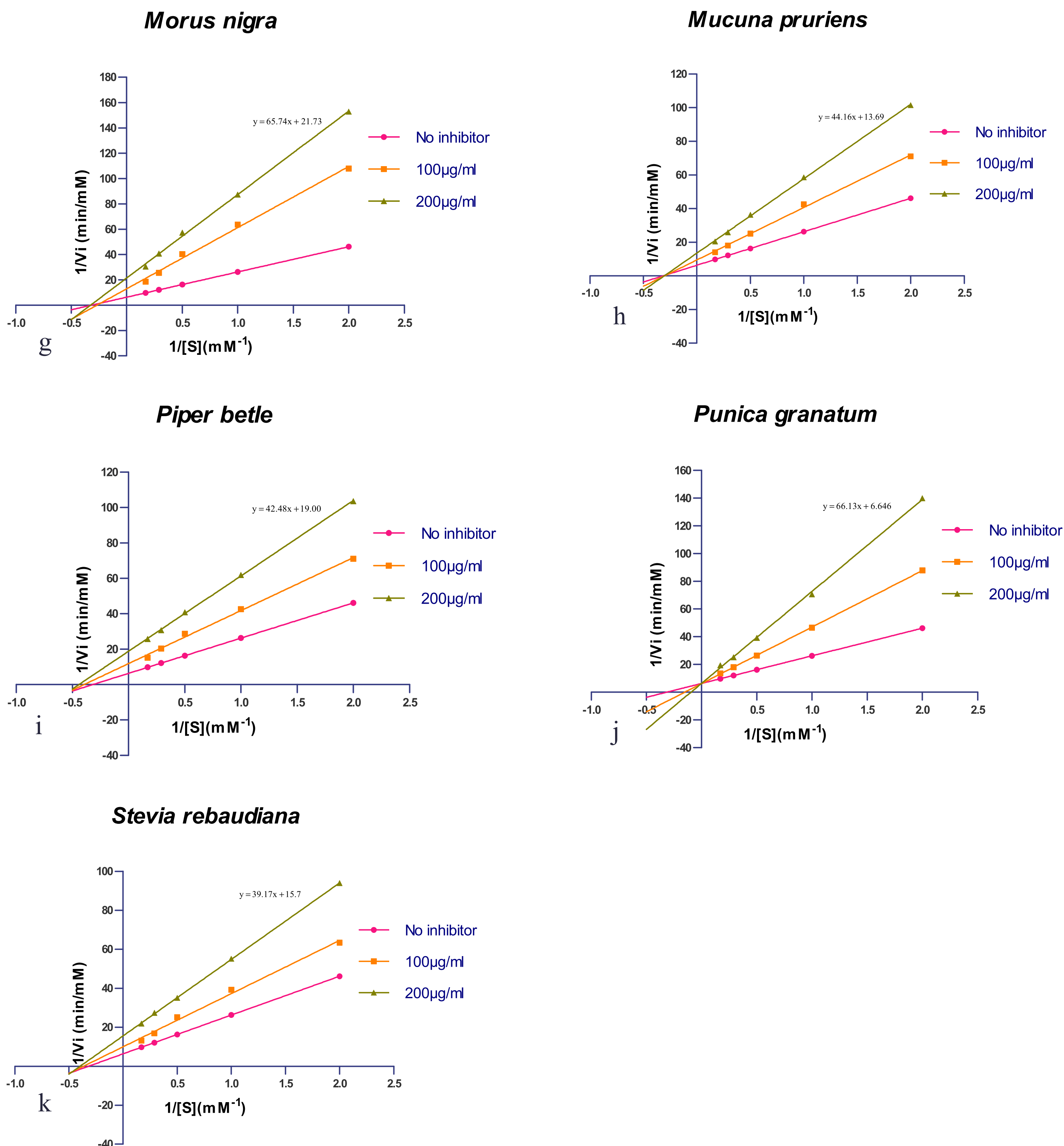

Fig. 4. (*continued*).

the exact molecules responsible for the ACE inhibition by Zn^{2+} ion chelation from these fractions require further study, which going in our laboratory.

Supplementation of $ZnCl_2$ to the fractions of plant *Stevia rebaudiana*, *Piper betle*, *Punica granatum*, *Crataegus oxyacantha*, *Morus nigra*, *Mucuna pruriens*, *Cynara scolymus* did not significantly reduce the ACE inhibition suggesting a different mechanism of enzyme inhibition apart from Zn^{2+} ion chelation.

Our result for the estimation of phenolic and flavonoid content indicates the presence of these compound in the extracts. Previous research shows that plants flavonoids are a wonderful source of ACE inhibitor.[39–41] Similarly, plant phenolics are the good source of ACE inhibitors.[42] Presence of high phenolic and flavonoid content may responsible for the bioactivity of these crude hydroalcoholic extracts. Best IC_{50} value of *Cynara scolymus* may be directly correlated to the very high content of flavonoid and phenolic compounds.

Out of 68 fractions screened, 11 fractions were found to be very

Table 3
Comparison of inhibitory effects of various plant fractions on angiotensin converting enzyme in the presence or absence of bovine serum albumin (BSA:25 μg/mL).

Plant Fraction	Concentration (μg/mL)	Inhibition of activity (%) in the absence or presence of BSA		
		BSA Absent	BSA Present	% Decrease in activity
Cassia occidentalis Ethyl acetate fraction	200	46.59 ± 2.17	13.25 ± 0.33	71.56
Coscinium fenestratum Butanol fraction	200	51.30 ± 1.49	6.93 ± 0.23	86.49
Crataegus oxyacantha Ethyl acetate fraction	50	47.63 ± 1.32	18.26 ± 0.33	61.66
Cynara scolymus Ethyl acetate fraction	50	40.41 ± 3.52	39.71 ± 1.58	1.73
Embelia ribes Butanol fraction	50	53.98 ± 3.20	50.19 ± 2.48	7.02
Morus nigra Ethyl acetate fraction	200	43.86 ± 1.94	20.01 ± 0.50	54.38
Mucuna pruriens Ethyl acetate fraction	200	48.62 ± 1.70	10.23 ± 0.57	78.96
Piper betle Chloroform fraction	200	54.38 ± 3.07	48.76 ± 3.57	10.33
Punica granatum Ethyl acetate fraction	200	45.91 ± 2.48	23.77 ± 0.99	7.02
Stevia rebaudiana Water fraction	200	52.64 ± 3.48	49.96 ± 2.96	5.09

Values are given as mean ± SD (n = 3).

Table 4
Inhibitory effects of various fractions on the activity of angiotensin converting enzyme, trypsin and chymotrypsin enzyme.

Plant Fraction	Concentration (μg/mL)	Inhibitory Activity (% inhibition)		
		ACE	Trypsin	Chymotrypsin
Cassia occidentalis Ethyl acetate fraction	200	46.59 ± 2.17	31.60 ± 1.52	37.94 ± 1.89
Coscinium fenestratum Butanol fraction	200	51.30 ± 1.49	7.23 ± 0.23	4.19 ± 0.14
Crataegus oxyacantha Ethyl acetate fraction	50	47.63 ± 1.32	11.56 ± 0.57	8.69 ± 0.61
Cynara scolymus Ethyl acetate fraction	50	40.41 ± 3.52	41.01 ± 2.93	24.58 ± 1.94
Embelia ribes Butanol fraction	50	53.98 ± 3.20	37.88 ± 1.65	26.13 ± 1.35
Morus nigra Ethyl acetate fraction	200	43.86 ± 1.94	2.93 ± 0.19	5.16 ± 0.35
Mucuna pruriens Ethyl acetate fraction	200	48.62 ± 0.98	5.98 ± 0.26	7.13 ± 0.52
Piper betle Linn Chloroform fraction	200	54.38 ± 3.07	7.56 ± 0.47	4.98 ± 0.21
Punica granatum Ethyl acetate fraction	200	45.91 ± 2.48	3.46 ± 0.28	4.16 ± 0.19
Stevia rebaudiana Water fraction	200	52.64 ± 3.48	8.43 ± 0.68	11.09 ± 0.92

Values are given as mean ± SD (n = 3).

active with the IC_{50} value less than 200 μg/mL. Most active was petroleum ether fraction of *Embelia ribes* (IC_{50} 39.32 ± 8.94), chemical profile of n-hexane fraction revealed embelin as the active compound.[43] *Crataegus oxyacantha* ethyl acetate fraction (IC_{50} 50.91 ± 3.52) was also very active with the highest content of the total phenolic compound (209.16 ± 7.48 μg/mg) of the fraction. Moreover several phenolic compounds like hyperoside, isoquercitrin, chlorogenic acid and epicatechin were reported in the extract of *Crataegus oxyacantha*.[44] Ethyl acetate fraction *Cassia occidentalis*, *Mucuna pruriens, Punica granatum* and *Morus nigra*, chloroform fraction of *Piper betle*, ethyl acetate and butanol fraction of *Cynara scolymus*, water fraction of *Stevia rebaudiana* were found to very active with high phenolic and flavonoidal content. Previous reports suggest that ethyl acetate fraction *Cassia occidentalis*, *Mucuna pruriens, Punica granatum* and *Morus nigra* contain anthraquinones,[35] L-dopa,[45] ellagitannins[46] and chlorogenic acid[47] respectively. Chloroform fraction of *Piper betle* reported hydroxychavicol as the bioactive constituent.[48] Chemical profile of butanol fraction of *Cynara scolymus* was reported to contain chlorogenic acid, cynarin, cynaroside, apigenin-7-rutinoside.[49] Water extract of *Stevia rebaudiana* contain phenolic compound pyrogallol.[50] Compounds reported in these plants may responsible for the bioactivity of these fractions but the exact molecules responsible for the ACE inhibition requires isolation of compound by various chromatographic techniques along with the animal studies which going in our laboratory.

5. Conclusion

Our study revealed utilization of Indian medicinal plants as a potential source of angiotensin-converting enzyme inhibitors. Among the assayed plants, the fraction of many plants produced

Table 5
Effects of $ZnCl_2$ on the inhibitory activity of tested extracts on angiotensin converting enzyme.

Plant Fraction	Amount added (μg/mL)	Inhibition of activity (%) in the presence or absence of $ZnCl_2$		
		None	$ZnCl_2$ (1.5 mM)	% Decrease in activity
Cassia occidentalis Ethyl acetate fraction	200	46.59 ± 2.17	28.46 ± 2.13	38.91
Coscinium fenestratum Butanol fraction	200	51.30 ± 1.49	32.98 ± 2.36	35.71
Crataegus oxyacantha Ethyl acetate fraction	50	47.63 ± 1.32	45.37 ± 3.19	4.74
Cynara scolymus Ethyl acetate fraction	50	40.41 ± 3.52	39.64 ± 1.45	1.91
Embelia ribes Butanol fraction	50	53.98 ± 3.2	22.46 ± 1.75	58.39
Morus nigra Ethyl acetate fraction	200	43.86 ± 1.94	42.13 ± 1.85	3.94
Mucuna pruriens Ethyl acetate fraction	200	48.62 ± 1.70	47.23 ± 3.57	2.86
Piper betle Chloroform fraction	200	54.38 ± 3.07	49.91 ± 3.26	8.22
Punica granatum Ethyl acetate fraction	200	45.91 ± 2.48	42.88 ± 1.78	6.60
Stevia rebaudiana Water fraction	200	52.64 ± 3.48	46.51 ± 3.91	11.65

Values are given as mean ± SD (n = 3).

Table 6
Total Phenolic & flavonoids contents of various plants.

Plant Hydroalcoholic Extract	Total phenolic content (μg(±)-gallic acid/mg of plant extract or fraction)*	Total flavonoid content (μg (±)-Quercetin/mg of plant extract or fraction)*
Asparagus racemosus	73.26 ± 5.65	47.26 ± 6.01
Cassia occidentalis	20.79 ± 1.75	58.16 ± 4.31
Coscinium fenestratum	17.96 ± 0.88	11.65 ± 0.66
Crataegus oxyacantha	19.32 ± 0.85	15.13 ± 0.21
Cynara scolymus	50.96 ± 2.37	92.75 ± 5.53
Embelia ribes	13.16 ± 0.21	8.17 ± 0.19
Euphorbia prostrata	8.4 ± 0.26	5.29 ± 0.16
Feronia limonia	34.76 ± 1.84	32.98 ± 2.06
Kalanchoe pinnata	3.27 ± 0.10	5.09 ± 0.24
Morus nigra	20.19 ± 0.57	11.78 ± 0.57
Mucuna pruriens	38.66 ± 1.77	4.77 ± 0.28
Oryza sativa	06.43 ± 0.16	9.69 ± 0.33
Piper betle	94.42 ± 3.79	40.98 ± 1.80
Polyalthia longifolia	80.76 ± 6.27	74.57 ± 3.48
Psoralea corylifolia	39.41 ± 2.01	14.85 ± 0.68
Punica granatum	27.23 ± 1.58	18.46 ± 0.36
Stevia rebaudiana	77.43 ± 3.26	52.36 ± 2.01
Cassia occidentalis Ethyl acetate fraction	109.38 ± 3.57	26.84 ± 1.14
Coscinium fenestratum Butanol fraction	14.23 ± 0.64	16.09 ± 0.31
Crataegus oxyacantha Ethyl acetate fraction	209.16 ± 7.48	19.18 ± 0.71
Cynara scolymus Ethyl acetate fraction	61.43 ± 2.06	109.68 ± 3.27
Cynara scolymus Butanol fraction	39.47 ± 1.44	72.98 ± 1.07
Embelia ribes Butanol fraction	7.84 ± 0.68	5.06 ± 0.23
Morus nigra Ethyl acetate fraction	18.42 ± 0.99	4.93 ± 0.31
Mucuna pruriens Ethyl acetate fraction	46.56 ± 0.57	6.19 ± 0.36
Piper betle Chloroform fraction	51.23 ± 1.77	18.63 ± 0.68
Punica granatum Ethyl acetate fraction	17.60 ± 0.48	24.12 ± 1.35
Stevia rebaudiana Water fraction	32.6 ± 1.44	41.72 ± 1.66

Each value represents a mean ± SD (n = 3).

significant ACE inhibition. Enzyme kinetics and mechanism of inhibition was determined by various parameters. The ACE inhibitory activity of the tested plants fractions might find importance in the development of potential antihypertensive agents after further investigations of each plant for active compound isolation by various chromatographic techniques and evaluating the activity of extract, fraction and isolated molecule using preclinical animal and human clinical trials.

Conflicts of interest

The authors declare that they have no conflict of interests.

For inhibition of ACE by inhibitors Km is defined as Kmapp since it is affected by the factor $(1 + [I])/Ki$ (Segel, 1975).

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References

1. Vasan RS, Larson MG, Leip EP, et al. Impact of high-normal blood pressure on the risk of cardiovascular disease. *N Engl J Med*. 2001;345:1291–1297.
2. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005: 365217–365223.
3. Weir MR, Dzau VJ. The renin-angiotensin-aldosterone system: a specific target for hypertension management. *Am J Hypertens*. 1999;12(S9), 205S–13S.
4. Carson PE. Rationale for the use of combination angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker therapy in heart failure. *Am Heart J*. 2000;140:361–366.
5. Shlipak MG, Browner WS, Noguchi H, Massie B, Frances CD, McClellan M. Comparison of the effects of angiotensin converting–enzyme inhibitors and beta blockers on survival in elderly patients with reduced left ventricular function after myocardial infarction. *Am J Med*. 2001;110:425–433.
6. Hebert LA, Falkenhain ME, Nahman Jr NS, Cosio FG, O'Dorisio TM. Combination ACE inhibitor and angiotensin II receptor antagonist therapy in diabetic nephropathy. *Am J Nephrol*. 1999;19:1–6.
7. Hemels ME, Bennett HA, Bonari L, Han D, Traverso ML, Einarson TR. HOPE study impact on ACE inhibitors use. *Ann Pharmacother*. 2003;37:640–645.
8. Alderman CP. Adverse effects of the angiotensin-converting enzyme inhibitors. *Ann Pharmacother*. 1996;30:55–61.
9. Wood RI. Bronchospasm and cough as adverse reactions to the ACE inhibitors captopril, enalapril and lisinopril. A controlled retrospective cohort study. *Br J Clin Pharmacol*. 1995;39:265–270.
10. Dubey NK, Kumar R, Tripathi P. Global promotion of herbal medicine: India's opportunity. *Curr Sci*. 2004;86:37–41.
11. Somanadhan B, Varughese G, Palpu P, et al. An ethnopharmacological survey for potential angiotensin converting enzyme inhibitors from Indian medicinal plants. *J Ethnopharmacol*. 1999;65:103–112.
12. Wagner H, Elbl G, Lotter H, Guinea M. Evaluation of natural products as inhibitors of angiotension I — converting enzyme (ACE). *Pharmacol Lett*. 1991;1: 15–18.
13. Machado TB, Pinto AV, Pinto MC, et al. In vitro activity of Brazilian medicinal plants, naturally occurring naphthoquinones and their analogues, against methicillin-resistant Staphylococcus aureus. *Int J Antimicrob Agents*. 2003;21: 279–284.
14. Dewanto V, Wu X, Liu RH. Processed sweet corn has higher antioxidant activity. *J Agric Food Chem*. 2002;50:4959–4964.
15. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal*. 2002;10:178–182.
16. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. *Biochem Pharmacol*. 1971;20: 1637–1648.
17. Huggins CG, Thampi NS. A simple method for the determination of angiotensin I converting enzyme. *Life Sci*. 1968;7:633–639.
18. Carmel A, Yaron A. An intramolecularly quenched fluorescent tripeptide as a fluorogenic substrate of angiotensin-I-converting enzyme and of bacterial dipeptidyl carboxypeptidase. *Eur J Biochem*. 1978;87:265–273.
19. Holmquist B, Bünning P, Riordan JF. A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal Biochem*. 1979;95:540–548.
20. Wu J, Aluko RE, Muir AD. Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions. *J Chromatogr A*. 2002;950:125–130.
21. Jimsheena VK, Gowda LR. Colorimetric, high-throughput assay for screening angiotensin I-converting enzyme inhibitors. *Anal Chem*. 2009;81:9388–9394.
22. Chen J, Liu S, Ye R, Cai G, Ji B, Wu Y. Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: purification and characterization. *Journal of Functional Foods*. 2013;5:1684–1692.
23. Priyanto AD, Doerksen RJ, Chang CI, et al. Screening, discovery, and characterization of angiotensin-I converting enzyme inhibitory peptides derived from proteolytic hydrolysate of bitter melon seed proteins. *Journal of proteomics*. 2015;128:424–435.
24. Liu JC, Hsu FL, Tsai JC, et al. Antihypertensive effects of tannins isolated from traditional Chinese herbs as non-specific inhibitors of angiontensin converting enzyme. *Life Sci*. 2003;73:1543–1555.
25. Stanton A. Therapeutic potential of renin inhibitors in the management of cardiovascular disorders. *Am J Cardiovasc Drugs*. 2003;3:389–394.
26. Unger T, Gohlke P. Converting enzyme inhibitors in cardiovascular therapy: current status and future potential. *Cardiovasc Res*. 1994;28:146–158.
27. Elbl G, Wagner H. A new method for the in vitro screening of inhibitors of angiotensin-converting enzyme (ACE), using the chromophore-and fluorophore-labelled substrate, dansyltriglycine. *Planta Med*. 1991;57:137–141.
28. Dorer FE, Kahn JR, Lentz KE, Levine M, Skeggs LT. Hydrolysis of bradykinin by angiotensin-converting enzyme. *Circ Res*. 1974;34:824–827.
29. Jaspard E, Wei L, Alhenc-Gelas F. Differences in the properties and enzymatic specificities of the two active sites of angiotensin I-converting enzyme (kininase II). Studies with bradykinin and other natural peptides. *J Biol Chem*. 1993;268:9496–9503.
30. Campbell DJ. Angiotensin converting enzyme (ACE) inhibitors and kinin metabolism: evidence that ACE inhibitors may inhibit a kininase other than ACE. *Clin Exp Pharmacol Physiol*. 1995;22:903–911.
31. Segel IH. *Enzyme Kinetics*. New York: Wiley; 1975.
32. Matthews BW, Weaver LH, Kester WR. The conformation of thermolysin. *J Biol Chem*. 1974;249:8030–8044.
33. Webb JL. *Enzyme and Metabolic Inhibitors*. New York, NY, USA: Academic Press; 1963:149–188.
34. Bachmetov L, Gal Tanamy M, Shapira A, et al. Suppression of hepatitis C virus by the flavonoid quercetin is mediated by inhibition of NS3 protease activity. *J Viral Hepat*. 2012;19:e81–e88.
35. Yadav JP, Arya V, Yadav S, Panghal M, Kumar S, Dhankhar S. Cassia occidentalis L.: a review on its ethnobotany, phytochemical and pharmacological profile. *Fitoterapia*. 2010;81:223–230.
36. Rai RV, Rajesh PS, Kim HM. Medicinal use of Coscinium fenestratum (Gaertn.) Colebr.: an short review. *Oriental Pharmacy and Experimental Medicine*. 2013;13:1–9.
37. Chen CH, Lin JY, Lin CN, Hsu SY. Inhibition of angiotensin-I-converting enzyme by tetrahydroxyxanthones isolated from Tripterospermum lanceolatum. *J Nat Prod*. 1992;55:691–695.
38. Lacaille-Dubois MA, Franck U, Wagner H. Search for potential angiotensin converting enzyme (ACE)-inhibitors from plants. *Phytomedicine*. 2001;8: 47–52.
39. Balasuriya BN, Rupasinghe HV. Plant flavonoids as angiotensin converting enzyme inhibitors in regulation of hypertension. *Functional Foods in Health and Disease*. 2011;1:172–188.
40. Guerrero L, Castillo J, Quiñones M, et al. Inhibition of angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. *PLoS One*. 2012;7. e49493.
41. Actis-Goretta L, Ottaviani JI, Fraga CG. Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *J Agric Food Chem*. 2006;54:229–234.
42. Al Shukor N, Van Camp J, Gonzales GB, et al. Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: a study of structure activity relationships. *J Agric Food Chem*. 2013;61:11832–11839.
43. Thippeswamy BS, Mahendran S, Biradar MI, et al. Protective effect of embelin against acetic acid induced ulcerative colitis in rats. *Eur J Pharmacol*. 2011;654: 100–105.
44. Liu P, Kallio H, Lü D, Zhou C, Yang B. Quantitative analysis of phenolic compounds in Chinese hawthorn (Crataegus spp.) fruits by high performance liquid chromatography–electrospray ionisation mass spectrometry. *Food Chem*. 2011;127:1370–1377.
45. Brain KR. Accumulation of L-DOPA in cultures from *Mucuna pruriens*. *Plant Sci Lett*. 1976;7:157–161.
46. Machado TD, Leal IC, Amaral AC, Santos K, Silva MG, Kuster RM. Antimicrobial ellagitannin of Punica granatum fruits. *J Braz Chem Soc*. 2002;13:606–610.
47. Ercisli S, Orhan E. Chemical composition of white (Morus alba), red (Morus rubra) and black (Morus nigra) mulberry fruits. *Food Chem*. 2007;103: 1380–1384.
48. Ali I, Khan FG, Suri KA, et al. In vitro antifungal activity of hydroxychavicol isolated from *Piper betle L*. *Ann Clin Microbiol Antimicrob*. 2010;9:7.
49. Zhu X, Zhang H, Lo R. Phenolic compounds from the leaf extract of artichoke (Cynara scolymus L.) and their antimicrobial activities. *J Agric Food Chem*. 2004;52:7272–7278.
50. Kim IS, Yang M, Lee OH, Kang SN. The antioxidant activity and the bioactive compound content of Stevia rebaudiana water extracts. *LWT-Food Science and Technology*. 2011;44:1328–1332.