CHAPTER II

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Materials

Angiotensin converting enzyme (ACE) from rabbit lung and porcine kidney, hippuryl-histidyl-leucine acetate salt (HHL), lisinopril, Sepharose CL-4B, 1,4-butanediol diglycidyl ether, HEPES buffer, Nonidet P-40 and dialysis membrane (dialysis tubings) were purchased from Sigma-Aldrich Chemical Laboratories (St. Louis, MO, USA). Sepharose G-200 superfine was purchased from Pharmacia Chemical Company, Uppsala, Sweden. L-Phe-D-His-L-Leu was custom-synthesized from Genemed Synthesis, Inc., San Antonio, TX, USA. Gly-Cys-Phe was a kind gift from Prof. R. Manjunath Kini, Professor, Department of Biological Sciences, National University of Singapore, Singapore. TLC Silica gel 60 F254 plates and silica gel (60-120 and 100-200 mesh) for column chromatography were purchased from Merck Specialities Pvt. Ltd., Mumbai, India. Hippuric acid was purchased from SRL chemical company, Mumbai, India. Porcine kidney used as ACE source in the initial screening studies was obtained from the local pork stall, Mysore. All other chemicals and reagents used in this study were of analytical grade or better. Solvents were redistilled before use.

Plant materials

The leaves of Artocarpus altillis, Azadirachta indica, Catharanthus roseus and Pongamia pinnata were collected from the local areas of Mysore, Karnataka, India. These plants were authenticated by Prof. G. R. Shivamurthy, Professor, Department of Studies in Botany and Dr. P. Sharanappa, Assistant Professor, Department of Studies in Bioscience, University of Mysore, Mysore, India. The voucher specimens were deposited in the herbarium, Department of Studies in Botany, and Department of Studies in Bioscience, University of Mysore, India. The seeds of Tamarindus indica were purchased from herbal products shop while the seeds of Trigonella foenum-graecum were purchased from local provision store, Mysore, India. All the plant materials were washed under running tap water and dried under mild sunshine. The dried samples (250 g each) were powdered using conventional mixer, sieved, and stored in air tight brown bottles at 4°C until further use.
Methods

Purification of ACE

Preparation of porcine kidney acetone extract

The porcine kidney acetone powder was prepared according to the method of Vermeirssen et al., (2002), with some modifications. The porcine kidney cortex was chopped into small pieces and suspended in chilled acetone for 30 min. The cortex was homogenized with chilled acetone using conventional mixer grinder. Thus obtained homogenate was air dried and powdered. The acetone powder was dissolved in 100 mM phosphate buffer (pH 8.3) in the ratio of 1:10, stirred using magnetic stirrer overnight at 4°C, centrifuged at 40,000 g for 45 min. The supernatant obtained served as crude enzyme source of ACE.

Ammonium sulphate precipitation of crude enzyme

The crude enzyme extract (10 g) was subjected to ammonium sulphate precipitation (20%, 40% and 60%), using magnetic stirrer and centrifuged at 10,000 g for 15 min. The pellets (20%, 40% and 60%) and the supernatant (60%) were dialyzed against 10 mM phosphate buffer (pH 8.3) using 12 kDa cut off dialysis membrane. The supernatant fraction was lyophilized in order to reduce the volume prior to dialysis. All the dialyzed fractions were subjected to protein estimation and evaluated for the ACE activity.

Sepharose G-200 column chromatography

The lyophilized ammonium sulphate precipitate fraction (20% pellet) with higher ACE activity was fractionated on Sepharose G-200 column, with some modifications to the method described by Bull et al., (1985). Hundred milligrams of ammonium sulphate precipitate (20%) fraction was dissolved in 1mL of 10 mM phosphate buffer (pH 8.3), centrifuged at 5000 g for 10 min. The supernatant was loaded on to Sepharose G-200 column (1.2 cm x 120 cm), pre-equilibrated with the same buffer. The fractionation was carried out at 4°C. The flow rate was adjusted to 10 mL/h and 1 mL fractions were collected at 6 min intervals. The protein elution was monitored at 280 nm using UV-visible 1601 Shimadzu spectrophotometer. The peak fractions were assayed for ACE activity. The fractions having enzyme activity were pooled, dialyzed, lyophilized and stored at -4°C.
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_Affinity chromatography_

_Preparation of adsorbent for affinity column chromatography_

Epoxy-activated Sepharose CL-4B was prepared as described by Sundberg & Porath (1974). A slurry consisting of 100 mL of packed Sepharose CL-4B, 100 mL of 1,4-butanediol diglycidyl ether (as a 70% nominal solution), and 100 mL of 0.6 M NaOH containing 2 mg/mL sodium borohydride was mixed using an overhead stirrer for 8 h at 23°C. The resulting epoxy-activated Sepharose CL-4B was washed exhaustively on a coarse scinttered glass funnel with 10 L of water. Lisinopril was coupled to the epoxy-activated Sepharose CL-4B by combining the suction-dried cake (approximately 67 g) with 100 mL of a solution containing 2.2 mM lisinopril in 0.3 M potassium carbonate adjusted to pH 11.0 with diluted HCl. This slurry was gently stirred on a rotator for 3 days at 37°C. The product was washed briefly, and any residual epoxide was blocked by reaction with 100 ml of 1 M glycine at pH 10.0 overnight at 37°C. The affinity gel was then washed completely with 1 M NaCl followed by distilled water, and it was stored in 0.1 M sodium bicarbonate containing 100 µM EDTA to inhibit bacterial growth.

_Fractionation of the G-200 peak-II fraction_

The lyophilized peak-II fraction was further fractionated on affinity column, according to the method described by Bull et al., (1985). The affinity column was pre-equilibrated with 0.01 M HEPES buffer (pH 7.5) containing 0.3 M KCl, 100 µM ZnCl<sub>2</sub> and 0.5% Nonidet P-40 and the lyophilized peak-II fraction (50 mg) dissolved in pre-equilibration buffer was centrifuged at 5000 g for 10 min and loaded on to the gel bed (1 cm x 22 cm). Before elution, the column was equilibrated with the same solution without Nonidet-P40. Detergent rinse removes most of the entrapped protein from the column, and there is no detectable protein in the effluent at completion of the detergent-free rinse. Later, the enzyme was eluted from the rinsed column by including 10 µM free lisinopril in the above rinse solution, at a flow rate of 14 mL/h. The elution of enzyme protein occurred after one bed volume of the eluent passed through the affinity column. The fractionation was carried out at 4°C and 2 mL fractions were collected at 7 min intervals. The protein elution was monitored at 280 nm using UV-visible 1601 Shimadzu spectrophotometer. The enzyme protein fractions were pooled and dialyzed against 0.002 M HEPES buffer (pH 8.0).
containing 0.1 M NaCl, and 10 μM EDTA, in order to remove lisinopril. The dialyzed sample was centrifuged at 10,000 g for 30 min to remove minor precipitate that appeared during dialysis.

**SDS-PAGE**

SDS-PAGE was carried out according to the method of Laemmli (1970). The homogeneity of purified ACE was checked on 10% resolving polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at constant voltage of 100 volts for 2 h. After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue R-250 and protein band was visualized after destaining with methanol, acetic acid and water (30:10:60 v/v).

**Protein estimation**

The protein content of test samples was estimated according to the method of Lowry et al., (1951) using bovine serum albumin (BSA) as standard (75 μg/mL). Aliquots of BSA standard solution (0-1 mL) were taken in clean and dry test tubes. Lowry’s reagent (5mL) was added and kept for 15 min. To this mixture, 0.5 mL of 1:1 diluted Folin-Ciocalteu’s phenol (FC) reagent was added and allowed to stand for 30 min. The colour developed was measured at 660 nm. In the same manner, different proportions of test samples were taken and the experiment was done as described above. The protein concentration in the sample was determined by comparing with the standard graph obtained.

**ACE assay**

ACE activity was measured by the spectrophotometric assay of Cushman & Cheung (1971), with some modifications. In the assay, ACE was incubated in 125 μL reaction mixture of 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl and 12.5 mM substrate (HHL), for 30 min at 37°C. The reaction was terminated by the addition of 125 μL of 1 M HCl. The hippuric acid formed was extracted with 1 mL of ethyl acetate. After centrifugation (800 g; 15 min), 0.5 mL of the upper layer was transferred to another set of test tubes, heat-evaporated to dryness and re-dissolved in 1.5 mL of distilled water. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm using UV-visible 1601 Shimadzu spectrophotometer.
The concentration of hippuric acid in the test reaction compared to control reaction was expressed as percentage of ACE activity. The activity of enzyme is expressed as nmoles of hippuric acid released/30 min/mg protein at 37°C and the specific activity of enzyme is expressed as nmoles of hippuric acid released/min/mg protein at 37°C.

**Preparation of plant materials by Soxhlet method**

The pulverized powder of each plant materials (100 g) were packed in Whatman No.1 filter paper respectively. The packed plant materials were extracted successively by Soxhlet method using solvents with increasing polarity; hexane, petroleum ether, ethyl acetate, n-butanol, acetone, ethanol, methanol and water (in the ratio of 50 g/400 mL (w/v)). The plant extract samples obtained were concentrated under vacuum at 40°C. Thus obtained gummy residues of all the plant leaf materials were again dissolved in respective solvents and separately passed through a column (1 cm x 10 cm) packed with activated charcoal, in order to remove chlorophyll pigments. For a second time, these leaf extracts were concentrated under vacuum at 40°C and stored in air tight brown bottles at room temperature. The yield was calculated and expressed as % w/w. For further studies the extracts were dissolved in suitable solvents.

**Preparation of plant materials by cold (magnetic stirring) method**

The pulverized powder of *A. altilis* leaf material (100 g) was taken in a beaker and extracted sequentially using solvents with increasing polarity; hexane, petroleum ether, ethyl acetate, n-butanol, acetone, ethanol, methanol and water. The extraction was carried out by stirring plant leaf material taken in beaker, using magnetic stirrer under cold condition for 6 h each. Each time, the extracts were centrifuged at 5,000 g for 10 min and filtered through Whatman No.1 filter paper. The residue obtained after each solvent extraction was re-extracted sequentially using solvents with increasing polarity, as described above. The extracted samples were concentrated under vacuum at 40°C and the obtained gummy residues were again dissolved in respective solvents and passed through a column (1 cm x 10 cm) packed with activated charcoal, in order to remove chlorophyll pigments. For a second time, these leaf extracts were concentrated under vacuum at 40°C. Thus obtained, gummy residues were stored in
air tight brown bottles at room temperature. The yield was calculated and expressed as % w/w. For further studies the extracts were dissolved in suitable solvents.

**Phytochemical analysis**

**Tannins:** Three drops of 5% ferric chloride was added to 1 mL of plant extract. Greenish black precipitate indicated the presence of tannins (Kolawole et al., 2006).

**Phenolics:** The phenolic content of plant extract was determined according to the method of Singleton et al., (1999) with slight modifications. The plant extract (2 mL) was mixed with 0.5 mL of 5% sodium carbonate. To this mixture, 0.25 mL of FC reagent (1:1 diluted with water) was added, mixed thoroughly and incubated for 60 min at room temperature. After incubation, absorbance was measured at 725 nm using spectrophotometer. The increase in optical density of test samples when compared to blank was taken as indication of the presence of phenolics. Gallic acid, a known phenolic was taken as standard.

**Alkaloids:** To about 5 mL of plant extract, 1.5 mL of 10% HCl was added. The mixture was heated for 20 min, cooled and filtered. Thus obtained filtrate (1 mL) was tested for the presence of alkaloids with 5 drops of Draggendorff’s reagent. Reddish precipitate indicated the presence of alkaloids (Kolawole et al., 2006).

**Glycosides:** Ten milliliter of 50% HCl was added to 2 mL plant extract and the mixture was heated in boiling water for 30 min. Fehling’s solution (5 mL) was added and the mixture was boiled for 5 min. Brick-red precipitate indicated the presence of glycosides (Kolawole et al., 2006).

**Saponins:** The plant extract (2 mL) in a test tube was vigorously shaken for 2 min. The persistent frothing for 5 min and the frothing observed after heating on water bath also indicated the presence of saponins (Kolawole et al., 2006).

**Steroids:** The presence of steroids was determined by Liebermann’s Burchard test. The plant extract (1 mL) was dissolved in 0.5 mL of acetic anhydride and cooled under ice bath. This was mixed with 0.5 mL of chloroform and then 1 mL of concentrated \( \text{H}_2\text{SO}_4 \) was carefully added by means of a pipette, through the sides of test tube. At the junction of two liquids, appearance of reddish-brown ring indicated the presence of steroids (Kolawole et al., 2006).
**Terpenoids:** One milliliter of mixture containing 0.5 g of 2,4-dinitrophenylhydrazine in 100 mL of 2 M HCl was added to 2 mL plant extract. Yellow-orange colouration indicated the presence of ketonic terpenoids (Kolawole et al., 2006).

**Flavonoids:** The presence of flavonoids was determined by Shibata’s reaction method. The plant extract (3 mL) was warmed with three pieces of magnesium turnings and mixed with 3 drops of concentrated HCl. Orange pink colouration indicated the presence of flavonoids (Kolawole et al., 2006).

**Anthraquinones:** The presence of anthraquinones was determined by Borntrager’s test. The plant extract (5 mL) was dried and mixed with 3 mL petroleum ether. The filtrate was added to 2 mL of 25% ammonia solution. The mixture was shaken well and red colouration indicated the presence of anthraquinone (Kolawole et al., 2006).

**Peptides/proteins:** The peptide/protein content was determined according to Biuret method (Weichselbaum, 1946). Different proportion of water and methanol extracts of plant materials were mixed with 5 mL of Biuret reagent and incubated for 20 min at room temperature. After incubation, absorbance was measured at 540 nm. The peptide/protein content was determined using gelatin as a standard.

**High performance liquid chromatography (HPLC)**

The HPLC (Shimadzu Corporation, Kyoto, Japan) was carried out using 1000 ppm solutions of hot-ethanol, -methanol and -ethyl acetate leaf extracts of *A. altilis* on a reverse phase packed column (RP C-18 column; Luna 5 µm C18 (2) 100A, Phenomenex, CA, USA; 250 mm x 4.6 mm; particle size 5 µm) using gradient elution. The gradient elution was performed using acetonitrile (Solvent A) and 0.1% trifluoroacetic acid in water (Solvent B), at a total flow rate of 1 mL/min with a run time of 30 min and the elution was monitored by PDA detector at 210 nm. The gradient composition: 0 min, 20% Solvent A; 5 min, 40% Solvent A; 8 min, 75% Solvent A; 12 min, 90% Solvent A; 15 min, 95% Solvent A; 25 min, 95% Solvent A; 27 min, 20% Solvent A; 30 min, 20% Solvent A was used.

**Bio-guided assay fractionation and isolation of ACE inhibitory compound(s)**

**Fractionation of crude extracts**

The hot ethanol, -methanol and -ethyl acetate extracts of *A. altilis* leaf were concentrated to dryness under vacuum and this resulted in gummy residues. All these
gummy residues were individually fractionated by partition method using methanol, water, ethyl acetate and chloroform, followed by acid-base extraction (Moqbel et al., 2011). The dried leaf extracts were fractionated by dissolving in methanol:water (4:1), evaporated to one tenth the volume and filtered using Whatman No.1 filter paper. The residues obtained were further dissolved in ethyl acetate and filtered to obtain two fractions i.e, ethyl acetate insoluble fraction (F1) and ethyl acetate soluble fraction (F2). The filtrates obtained were further extracted using acid-base extraction. The filtrates were first acidified using 5% H$_2$SO$_4$ (pH 2.0) and extracted twice with chloroform. The remaining aqueous layer was made alkaline using 5% NaOH (pH 10.0) and extracted twice using chloroform:methanol (3:1). The remaining aqueous layer was neutralized using 5% H$_2$SO$_4$. The five fractions obtained were ethyl acetate insoluble fraction (F1), ethyl acetate soluble fraction (F2), chloroform fraction (F3), the basic fraction (F4) and neutral fraction (F5).

**Silica gel column chromatography**

The F3 fractions of hot ethanol, -methanol and -ethyl acetate extracts of *A. altilis* were subjected to silica gel column (2.2 cm x 36 cm) chromatography for the separation of compounds by gradient elution method. The F3 fractions were eluted from a column of silica gel (60-120 mesh) using solvent mixtures prepared by mixing different ratios of n-hexane: chloroform, chloroform: methanol (step-wise gradient). The fractions of 10 mL were collected and monitored using silica gel thin layer chromatography (TLC); the visualization of spots was under UV light and iodine vapours. The fractions with similar spots were pooled, concentrated and analyzed for the ACE inhibitory activity. The fractions exhibiting ACE inhibitory activity were again pooled and concentrated under vacuum at 40°C.

The concentrated samples were re-chromatographed on column (0.8 cm x 22 cm) packed with silica gel (100-200 mesh). The elution was carried out using eluents of increasing polarity of solvent mixtures including the different ratios of n-hexane: chloroform, chloroform: methanol, but with narrow range of ratios (step-wise gradient). The fractions of 5 mL were collected and monitored through silica gel TLC, the visualization of spots was under UV light and iodine vapours. The fractions with similar spots were pooled, concentrated and analyzed for the ACE inhibitory activity.
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The fractions exhibiting ACE inhibitory activity were again pooled and concentrated under vacuum at 40°C.

The separation was repeated over a column packed with silica gel column (100-200 mesh) using different bed volumes of column, in order to isolate ACE inhibitory compounds from F3 fractions of hot ethanol, -methanol and -ethyl acetate extracts, respectively. Each time, TLC was carried out to analyze the banding pattern and observed under UV light and iodine vapours. Finally, the fraction showing single spot was further characterized by liquid chromatography (LC), quadrupole time-of-flight (Q-ToF) mass spectrometry, fourier transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy.

Liquid chromatography (LC)

The LC system used was Thermo Finnigan Surveyor liquid chromatograph (San Jose, CA, USA) equipped with an isocratic pump and an auto-sampler. The used stationary phase for HEaFr-3 of A. altilis leaf analytical run was C18 packed in a (250 mm x 4.6 mm) with 5 μm particle size column from BDS HYPERSIL, Thermo Electron Corporation, San Jose, CA, USA. The chromatographic procedure was carried out in the isocratic mode at room temperature. The mobile phase for the chromatographic run was a solution of acetonitrile: methanol (95:5; v/v), pumped at a flow rate of 0.15 mL min⁻¹. The injection volume was 10 μL and the total run time is set for 60 min. The elution was monitored by PDA detector (UV, Channel A) at 214 nm.

Quadrupole-time of flight (Q-ToF) mass spectrometry

Quadrupole-Time of Flight mass spectrometric analysis was performed using a Micromass® Q-ToF mass spectrometer, Micromass UK Ltd. UK., working with ES interface in the positive ion mode (volts 230).

Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopic analysis was performed in the mid IR region of 400~4,000/cm with 16 scan speed using Shimadzu FTIR 8400S. The pellet was prepared using spectrosopically pure potassium bromide (5:95) and fixed in a sample holder.
Nuclear magnetic resonance (NMR) spectroscopy

Proton (1H) NMR (400 MHz) spectra was recorded on Methanol-d4 and DMSO-d6 solution in a 5 mm tube on a BRUKER amx 400 MHz Fourier transform spectrophotometer with tetramethylsilane (TMS) as internal standard. The spectrophotometer was internally locked to deuterium frequency of the solvent chemical shifts were recorded in ppm relative to TMS.

Carbon-13 ($^{13}$C) NMR spectra were recorded on a Bruker 100 MHz spectrometer in Methanol-d4 solution in a 5 mm tube on a BRUKER amx 100 MHz Fourier transform spectrophotometer with TMS as internal standard. The spectrophotometer was internally locked to deuterium frequency of the solvent and chemical shifts were recorded in ppm relative to TMS.

Distortionless enhancement by polarization transfer (DEPT) 90 and DEPT 135, two dimensional heteronuclear single quantum coherence (2D HSQC) and two dimensional heteronuclear multiple-bond correlation (2D HMBC) NMR spectroscopy experiments were carried out to confirm the structure of the isolated ACE inhibitory compound. HOMO-COSY spectroscopy experiment was carried out to identify the presence of AB and ABX spin system for aromatic ring.

Elemental (CHNOS) analysis

CHNS elemental analysis was carried out using CHNOS elemental analyzer, Vario EL III, Serial No: 11014041. Elementar Analysensysteme GmbH, Donaustraße 7, D-63452, Hanau-Germany.

ACE inhibition assay

ACE inhibitory activity of plant extracts/ fractions/ isolated compounds/ tripeptides was measured by spectrophotometric assay of Cushman & Cheung (1971), with some modifications. In the assay, porcine kidney/rabbit lung ACE (5 mU) with and without different concentrations of test samples were pre-incubated for 10 min. The substrate (12.5 mM HHL) was added to pre-incubated samples and incubated for 30 min at 37°C after adjusting the reaction volume to 125 μL with 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl. The reaction was terminated by the addition of 1 M HCl (125 μL). The hippuric acid formed was extracted with 1 mL of ethyl acetate. After centrifugation (800 g; 15 min), 0.5 mL of the upper layer was
transferred to another set of test tubes, heat-evaporated to dryness and re-dissolved in 1.5 mL of distilled water. The absorbance of hippuric acid released by the action of ACE was measured at 228 nm using UV-visible 1601 Shimadzu spectrophotometer.

The decreased concentration of hippuric acid in test reaction compared to control and enzyme activity reaction was expressed as percentage of inhibition. The activity of each sample was tested in triplicates. Lisinopril, a known ACE inhibitor was used as positive control. The control experiments were performed with respective solvents. The IC$_{50}$ values were calculated by Boltzmann’s dose response analysis using Origin 6.1 and 8 software versions.

**ACE inhibitory kinetics**

In the inhibitory kinetics assay, porcine kidney ACE (5 mU) was pre-incubated with and without inhibitors (two different concentrations) for 10 min. Various substrate concentrations (0.5-5 mM HHL) in 50 mM HEPES buffer (pH 8.3) containing 300 mM NaCl (reaction volume of 125 µL) was incubated for 30 min at 37°C. The reaction was terminated by the addition of 1 M HCl (125 µL) and the absorbance of hippuric acid released by the action of ACE was measured at 228 nm. The decreased concentration of hippuric acid in the test reaction compared to control and enzyme activity reaction was used to calculate the extent of inhibition. A calibration curve for standard hippuric acid was constructed for reference. The initial reaction velocities were calculated from hippuric acid released. The type of inhibition was determined from a Lineweaver-Burk plot (Lineweaver & Burk, 1934).

**Intrinsic fluorescence interaction study**

The relative intrinsic fluorescence intensity of ACE with and without inhibitors was monitored using Shimadzu spectrofluorimeter. A reaction mixture of 2 mL in 1 cm path length quartz cuvette containing 10 mM potassium phosphate buffer (pH 7.4), ACE (25 µg/mL) and various concentration of inhibitors. Fluorescence spectra were measured between 300 and 400 nm after excitation at 280 nm. The non-specific quenching of spectra due to internal absorption and filtration by inhibitors was corrected empirically using a tryptophan standard.
Far ultraviolet-circular dichroism (Far UV-CD) spectroscopy

Far UV-CD spectra were obtained in absence or presence of IC50 concentration of inhibitors with ACE (250 µg/mL in 10 mM potassium phosphate buffer, pH 7.4) using Jasco J715 Spectropolarimeter. The spectra were recorded at room temperature between 200-260 nm using quartz cuvette with path length of 2 mm. The bandwidth was 1 nm and response time was 2 s. The final spectra were cumulative of ten scans. The protein spectra were corrected by subtracting a blank solution (10 mM potassium phosphate buffer, pH 8.3). Secondary structure contents from CD spectra were calculated using K2D2 software.

Molecular modeling and docking

All docking calculations and molecular graphic images were performed using Molegro virtual docker 2008.3.0.0. The ligand design and energy minimization was done using the Dundee PRODRG2 server (Schuettelkopf & van Aalten, 2004) and saved as a PDB file-type and thus prepared for import into the molecular docking platform Molegro virtual docker (http://www.molegro.com) based on the MolDock molecular docking algorithm. The coordinates for the crystal structure of human ACE (PDB: 108A) was imported into Molegro platform following subsequent removal of all crystallographic water molecules and other heteroatoms (except zinc and chloride ions) were removed from the PDB file and assignment of bonds, bond order and hybridization, explicit hydrogens and protein charges. The Molegro docking wizard was used to simulate in silico docking of ligands into ACE using a MolDock scoring function of grid resolution of 0.30 Å and search algorithm MolDock SE was used, 10 independent runs per ligand were performed for better docking result. A value of population size and maximum interactions 100 and 10,000 respectively were used for each run and 5 best poses were retained for each ligand. Molecular visualization was done using Molegro molecular viewer 2008.1.2.0 version.

Statistical analysis

The results were presented as mean ± SD of three determinations. The IC50 values were calculated by Boltzmann’s dose response analysis using Origin 6.1 and 8 software versions. Statistical differences were calculated by Duncan’s multiple range tests at p<0.05 using ANOVA of SPSS 11.0 statistical software.