Chapter IV

ALTLISOL: A NEW ANGIOTENSIN CONVERTING ENZYME INHIBITORY POLYPHENOLIC COMPOUND FROM ARTOCARPUS ALTLIS LEAF
INTRODUCTION

ACE cleaves Ang I to generate an active vasoconstrictor Ang II and thus formed Ang II, stimulates vasoconstriction, secretion of aldosterone and antidiuretic hormone. Ang II also stimulates the hypothalamus to activate the thirst reflex and these multifunctions of Ang II lead to the elevation of blood pressure (Ondetti & Cushman, 1982). Apart from the conversion of Ang I to Ang II, ACE also degrades and inactivates the physiological vasodilatory peptide, bradykinin, thereby increasing the risk of hypertension (Zimmerman et al., 1984).

Currently there are number of ACE inhibitory drugs available in the market, which are primarily used for the treatment of hypertension. However, these drugs are found to be associated with some of the undesirable side effects including dry cough, hyperkalemia, headache, dizziness, fatigue, nausea, renal dysfunction, hypotension, angioedema, impotence, skin rashes, allergic reactions, increased inflammation-related pain and taste disturbances (Israili & Hall, 1992; Agustí et al., 2003; Rossi, 2006; Fein, 2009).

Therefore, the search for new and safe ACE inhibitors has promoted researchers to investigate the efficacy of the natural products, which are generally referred to have higher cultural acceptability and compatibility with the human body and lesser side effects (Kamboj, 2000). It has been widely shown that many plant-derived compounds present significant antihypertensive effects. For this reason, they represent potential molecules for the development of new drugs, especially designed for the treatment and/or control of hypertension (Herrera-Arellano et al., 2007; Siddesha et al., 2010).

Over many years, a significant amount of evidences have indicated that the chemically diverse classes of naturally occurring substances from the higher plants are of potential interest for the therapeutic interventions in hypertension. Most of the research and review articles focus on ACE inhibition, which is a major and primary target in the treatment of hypertension by a large range of plant-derived compounds (Barbosa-Filho et al., 2006; Hong et al., 2008).

Most of the researchers have isolated ACE inhibitory compounds from the plants by bioassay guided purification procedures as explained in the previous research articles (Li et al., 2004; Li et al., 2008; Lin et al., 2008; Wijesinghe et al., 2011). Our preliminary screening studies of six folk medicinal plants clearly inhibited
ACE activity. However, *A. altillis* alone has been considered for the isolation and characterization of ACE inhibitory compounds, based on its potent ACE inhibitory activity.

*A. altillis* commonly known as breadfruit is widely distributed in tropical and subtropical regions, including India. In folk medicine, decoction of the leaves has been used for the treatment of hypertension, high cholesterol and diabetes (Wang et al., 2006; Lans, 2006). The preliminary screening study of *A. altillis* leaf extracts has given promising experimental evidence of ACE inhibitory activity and justified the traditional use of *A. altillis* leaf for the treatment of hypertension (Siddesha et al., 2011).

However, characterization of the active principles responsible for the ACE inhibitory activity/antihypertensive effect has not yet been determined. The previous research on the chemical constituents of *A. altillis* has resulted in the isolation of several classes of compounds such as flavonoids, flavones and phenolic compounds (Patil et al., 2002; Wang et al., 2006; Wang et al., 2007; Shamaun et al., 2010) as well as triterpenoids (Altman & Zito, 1976). To our knowledge, no reports of ACE inhibitory phytochemicals from this plant have been recorded.

Therefore, the present investigation is carried out to isolate the ACE inhibitory compound(s) from *A. altillis* leaf extract, by employing bioassay-guided fractionation and isolation. Further, the physical and chemical characterization of potent ACE inhibitory compound(s) will be carried out. Further, the type of enzyme inhibition and biophysical mechanism of interaction will be established. In addition, molecular docking is also carried out *in silico* in order to establish its potency and usefulness as antihypertensive drug.
RESULTS AND DISCUSSION

In many countries all over the world, the plant extracts and their derived metabolites have been traditionally used against hypertension (Somanadhan et al., 1999; Barbosa-Filho et al., 2006; Ramesar et al., 2008; Hong et al., 2008; Liu et al., 2010; Anne et al., 2011; Nagai et al., 2011). At present many pharmaceutical companies are looking in this line for the development of new therapeutically important compounds, which are useful in the treatment of many diseases including hypertension and cardiovascular complications (Fabricant & Farnsworth, 2001; Wang et al., 2006; Herrera-Arellano et al., 2007; Maridass & De Britto, 2008). There has been a certain degree of scientific validation of several plants and their active compounds isolated against hypertension, targeting the rate-limiting enzyme ACE (Lin et al., 2008; Kwon et al., 2010; Ojeda et al., 2010; Siddesha et al., 2010; Yodjun et al., 2011; Anne et al., 2011). The search is still going on for the identification and isolation of the bioactive plant metabolites as such or as the input for further chemical modification to combat against this enzyme to treat hypertension.

In chapter III of this thesis, we have screened six medicinal plants that are used as folk medicine against hypertension. However, A. altilis with potent ACE inhibitory activity was considered for the further isolation and characterization of metabolites that account for the antihypertensive effect by mediating ACE inhibition. As a part of our work on isolation and characterization of ACE inhibitory compound(s), the powdered leaf sample of A. altilis was extracted successively with solvents of increasing polarity; hexane, petroleum ether, ethyl acetate, n-butanol, acetone, ethanol, methanol and water, independently by both hot (Soxhlet) and cold (magnetic stirring) methods.

The cold extraction was carried out sequentially using solvents of increasing polarity. The powdered leaf sample along with solvent (from non-polar to polar; sequentially) was taken in a beaker and the beaker was placed on a magnetic stirrer. The extraction was done by stirring on magnetic stirrer under cold condition for 6 hrs. The extracts were centrifuged at 5000 g for 10 min and filtered through Whatman No.1 filter paper. Similarly, the hot leaf extracts were prepared by successive extraction with solvents with increasing polarity using Soxhlet apparatus. The extracts obtained from both cold and hot methods were concentrated under vacuum at 40°C. Thus 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 and the obtained gummy residues were weighed to calculate the percentage yield from the initial weight of the leaf material. The yield of leaf extracts are expressed as percentage (w/w) and is given in Table 4.01. The yield obtained from these extracts was ranging from 0.4 to 9.15% (w/w).

The cold and hot methods of extraction were employed, in order to check the difference in both the extract yields and the extent of ACE inhibitory activity. The overall yield of extraction by the hot method was more when compared to the cold extraction, except for the acetone and n-butanol extracts. In the hot method of extraction, *A. altilis* leaf extracted with water gave maximum yield of 9.15% (w/w) followed by ethyl acetate (6.73% w/w), methanol (3.65% w/w), ethanol (3.24% w/w), hexane (3.17% w/w) and petroleum ether (0.98% w/w), whereas the cold-acetone and -n-butanol extracts showed maximum yield (2.28% w/w and 1.45% w/w respectively) compared to that of hot-acetone and -n-butanol extracts. This study suggested that, the hot method of extraction is more beneficial in terms of the yield compared to the conventional cold method.

The inhibition of ACE was assayed using the cold and hot extracts of *A. altilis* leaf at 50 μg/125 μL concentration. The leaf extracts tested showed inhibition to various extents. Out of the sixteen extracts tested, hot -ethanol, -methanol and -ethyl acetate extracts of *A. altilis* leaf showed potent ACE inhibitory activity followed by cold -n-butanol, -methanol and -ethyl acetate extracts of *A. altilis* leaf (Figure 4.01). Other than these extracts, cold -water, -ethanol and -acetone extracts exhibited fairly good ACE inhibitory activity while hot -acetone and -water extracts inhibited ACE activity to a lesser extent. However, hot n-butanol, cold hexane, hot hexane, cold petroleum ether and hot petroleum ether extracts did not inhibit ACE activity. The difference in ACE inhibitory activity of extracts could be attributed to the nature of phytochemicals present in them. The greater ACE inhibitory activity of ethanol, methanol and ethyl acetate extracts might be due to the presence of polar compounds while inability of hexane and petroleum ether extracts could be due to the presence of non-polar compounds.

Further, the concentration-dependent inhibition of ACE was studied using the cold and hot leaf extracts that exhibited ACE inhibitory activity. The concentration-
dependent ACE inhibitory activity was carried out by increasing the concentration up to 100 μg/125 μL for cold extracts while 50 μg/125 μL was retained for hot extracts in order to check the effect of increasing inhibitor concentrations. Among the extracts tested, hot ethanol, cold and hot ethyl acetate, hot and cold methanol as well as cold n-butanol extracts of A. altilis leaf exhibited potent ACE inhibitory activity (~94-98%) at 80 μg/125 μL concentration when compared to other plant extracts. In contrast, cold ethanol, acetone and -water exhibited fairly good inhibitory activity (64-84%) at 100 μg/125 μL concentration while hot acetone and -water did not show significant inhibition of ACE (Figure 4.02 and 4.03). Further, the differential inhibitory effect of leaf extracts on ACE activity was observed with varied IC₅₀ values. Among the extracts tested, hot ethanol extract exhibited a potent ACE inhibitory activity with IC₅₀ value of 54.08±0.29 μg/mL followed by cold ethyl acetate extract (IC₅₀ of 85.44±0.85 μg/mL). In contrast, hot water extract showed minimum inhibition with IC₅₀ value of 765.52±11.97 μg/mL at the maximum concentration tested. The IC₅₀ values of A. altilis leaf extracts are summarized in Table 4.02.

The extent of ACE inhibition was found to be similar between cold and hot ethyl acetate, methanol and acetone extracts whereas hot ethanol extract showed significantly lower IC₅₀ value compared to that of cold ethanol extract and cold aqueous extract showed significantly lower IC₅₀ value than that of its counterpart hot aqueous extract. In addition to these differences, cold n-butanol showed significant ACE inhibition with the lower IC₅₀ value while the hot n-butanol extract did not inhibit the ACE activity. The cold and hot extracts with hexane and petroleum ether did not inhibit the ACE activity even at the maximum concentrations tested. The differential ACE inhibitory activity exhibited by the cold and hot extracts suggested the benefit of both the extraction procedures with respect to the isolation of diverse bioactive molecules.

A number of different class of compounds with ACE inhibitory activity have been isolated from the plants including phenolics, glycosides, tannins, flavonoids, alkaloids, xanthones, terpenes, peptides/proteids/iridoids (Ueno et al., 1988; Ogino et al., 1988; Hansen et al., 1996; Kang et al., 2003; Oh et al., 2003a & 2004; Barbosa-Filho et al., 2006; Charles et al., 2009) and anthraquinones (Hyun et al., 2009). Therefore, the phytochemical analysis of leaf extracts was carried out to establish the
possible classes of compounds responsible for the ACE inhibitory activity. The phytochemical analysis of A. altilis leaf extracts indicated the varied distribution of tannins, phenolics, glycosides, saponins, steroids, terpenoids and anthraquinones in cold and hot leaf extracts, as shown in Table 4.03. Among these phytoconstituents, phenolics and glycosides showed significant correlation with the ACE inhibitory activity. However, steroids, terpenoids and anthraquinones can not be ruled out for their possible contribution to the ACE inhibitory activity. Unpredictably, none of the extracts tested in this study were positive for alkaloids and flavonoids.

The correlation between phytochemical analysis and ACE inhibitory activity of A. altilis leaf extracts suggested that, the high content of phenolic and glycosidic compounds could be involved in exerting ACE inhibitory activity. Furthermore, analytical high performance liquid chromatography (HPLC) was carried out for hot -ethanol, -methanol and -ethyl acetate leaf extracts on a reverse phase packed column (RP C-18 column) using gradient elution. The gradient elution was performed using acetonitrile and 0.1% trifluoroacetic acid in water 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.

HPLC chromatogram of hot ethanol extract was found to contain metabolites eluting between 2.212 min to 25.205 min, with three major peaks eluting at 2.212, 3.037 and 6.588 min (Figure 4.04). While, the HPLC chromatogram of hot methanol extract was found to contain metabolites eluting between 2.645 min to 24.168 min, with three major peaks eluting at 2.645, 2.774 and 2.928 min (Figure 4.05). On the other hand, the HPLC chromatogram of hot ethyl acetate was found to contain metabolites eluting between 2.759 min to 10.452 min, with eight major peaks eluting at 2.759, 2.988, 3.221, 3.595, 3.756, 3.944, 4.740 and 5.174 min (Figure 4.06). The comparison of HPLC chromatogram of these extracts suggested that, the potent ACE inhibitory activity of hot ethanol extract with lower IC$_{50}$ value could be due to the minimum number of constituents compared to hot ethyl acetate extract and abundance of the constituents when compared to hot methanol extract.

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 (Figure 4.07) (Moqbel et al., 2011). Initially, the gummy residues were dissolved 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\textsubscript{2}SO\textsubscript{4} (pH 2.0) and extracted thrice with chloroform (F3). The remaining aqueous layer was made alkaline using 5% NaOH (pH 10.0) and extracted twice using chloroform:methanol (3:1) (F4). The remaining aqueous basic layer was neutralized using 5% H\textsubscript{2}SO\textsubscript{4}, evaporated and extracted with methanol (F5). 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).

The fractions; F1, F2, F3, F4 and F5 of hot -ethanol, -methanol and -ethyl acetate extracts (50 µg each) were evaluated for their ACE inhibitory activity. Among the fractions tested, F3 and F4 of hot -ethanol, -methanol and -ethyl acetate extracts exhibited significant ACE inhibitory activity but not the other fractions (Figure 4.08). Therefore, based on their ACE inhibitory activity and banding pattern on silica gel thin layer chromatography (TLC), F3 and F4 of individual extracts were pooled and concentrated. The pooled fractions of hot -ethanol, -methanol and -ethyl acetate extracts were further fractionated individually on silica gel column (60-120 mesh) by gradient elution method using eluents of increasing polarity of solvent mixtures, including the different ratios of n-hexane: chloroform, chloroform: methanol. The fractions were monitored through silica gel TLC and 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.

The concentrated samples were re-chromatographed on column 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 process of monitoring fractions through silica gel TLC, pooling, concentrating and screening of the fractions for ACE inhibitory activity was carried out. The fractions with ACE inhibitory activity were again pooled and concentrated. Further, the fractionation was carried out repeatedly over a column packed with silica gel column (100-200 mesh), using different bed volumes of silica gel, in order to isolate ACE
inhibitory compound(s) from the F3+F4 fractions of hot ethanol, -methanol and -ethyl acetate extracts respectively. Each time, the process of monitoring fractions through silica gel TLC, pooling, concentrating and screening for the ACE inhibitory activity was repeated.

The repeated fractionation of F3+F4 fractions of hot ethanol, -methanol and -ethyl acetate extracts resulted in six fractions each and they were evaluated for ACE inhibitory activity. Among the fractions obtained, fraction (Fr) 5 of hot ethanol extract (HEFr-5) and Fr-3 of hot ethyl acetate extract (HEaFr-3) exhibited potent ACE inhibitory activity when compared to Fr-5 and Fr-6 of hot methanol extract (HMFr-5 and HMFr-6), Fr-2 of hot ethyl acetate extract (HEaFr-2) and Fr-6 of hot ethanol extract (HEFr-6) at concentration of 50 µg/125 µL. While, other fractions tested did not inhibit ACE activity (Figure 4.09). Silica gel TLC was carried out to analyze the banding pattern of these fractions and observed under UV light and iodine vapours. Finally, based on the purity on TLC plate and obtained percent yield of the fractions, HEaFr-3 alone 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.

The brown coloured semi-solid methanol-soluble HEaFr-3 fraction was subjected to LC analysis in order to check the purity. LC analysis showed single major peak eluting at 16.53 min with 100% relative abundance (Figure 4.10), indicating the homogeneity of HEaFr-3. Based on the LC analysis result, FT-IR spectroscopic analysis was carried out for the HEaFr-3. FT-IR analysis showed broad peak at 3434.84 cm\(^{-1}\) corresponding to phenolic OH stretching, sharp peak at 1611.36 cm\(^{-1}\) corresponding to C=O bond stretching, medium size peak at 2927.86 cm\(^{-1}\) corresponding to aromatic C-H bond stretching, medium size peak at 1467.08 cm\(^{-1}\) corresponding to aromatic ring C=C bond stretching frequencies (Figure 4.11). Further, Q-ToF mass spectrometry was carried out in positive ES mode. Q-ToF mass spectrum of HEaFr-3 indicated a number of ionic peaks from 199 m/z to 874 m/z (Figure 4.12).

Furthermore, \(^1\text{H}, \text{}^{13}\text{C}, \text{distortionless enhancement by polarization transfer (DEPT) 90 and DEPT 135 NMR spectroscopy, two dimensional heteronuclear single quantum coherence (2D HSQC) and two dimensional heteronuclear multiple-bond correlation (2D HMBC) NMR spectroscopy, as well as homonuclear correlation
spectroscopy (HOMO-COSY) analysis were carried out to elucidate the structure of isolated ACE inhibitory compound. The results of $^1$H NMR (400 MHz) spectrum in methanol-d$_4$ is summarized as: δ 2.86 (s, 6H, 2CH$_3$), 2.68 (s, 3H, 2CH$_3$), 2.86 (t, 2H, 1CH$_2$), 3.1 (t, 2H, 1CH$_2$), 2.68 (t, 2H, 1CH$_2$), 1.82 (m, 2H, 1CH$_2$), 1.48 (m, 2H, 1CH$_2$), 1.4 (m, 2H, 1CH$_2$), 1.45 (m, 2H, 1CH$_2$), 1.58 (t, 2H, 1CH$_2$), 3.62 (broad, 1H, vinyl-CH), 3.34(s, 1H, vinyl-C-H), 7.264 (d, 1H, 8.9), 6.56 (d, 1H, 2Hz), 6.57 (d,1H,2Hz), 6.308 (dd, 8.9Hz, 2.3Hz), 6.24 (d, 2.2Hz) (Figure 4.13). Phenolic OH at 12.604 was found when DMSO-d$_6$ was used for $^1$H NMR analysis (Figure 4.14).

The results of $^{13}$C NMR (100MHz) spectrum in methanol-d$_4$ are summarized as follows: C=0 at 209.9, seven aromatic quaternary carbons at 166.42, 166.31, 145.95, 131.3, 121.33, 120.059 and 114.15 (Figure 4.15). DEPT 90 experiment confirmed five aromatic CH’s at 133.8, 120.59, 114.15, 113.56 and 109.13 (Figure 4.16). DEPT 135 experiment confirmed eight CH$_2$’s at 45.16, 40.75, 39.64, 32.24, 30.68, 28.24, 20.69, and 19.49 (Figure 4.17). The comparison of these two DEPT experiments with $^{13}$C NMR showed the presence of two vinyl CH’s at 51.95 and 78.8ppm and also two quaternary carbons at 71.47 and at 77.09 ppm. 2D HSQC (Figure 4.18) and 2D HMBC (Figure 4.19) experiments were used to correlate $^{13}$C to $^1$H. HOMO-COSY (Figure 4.20) identified the presence of AB and ABX spin system for aromatic ring.

Thus, the structural elucidation was performed based on the data obtained from Q-ToF MS ES+ spectrometric analysis, FT-IR, CHNOS analysis, $^1$H, $^{13}$C, DEPT 90, DEPT 135, 2D HSQC, 2D HMBC and HOMO-COSY NMR spectroscopic analysis. The analysis of the above experiments revealed that HEaFr-3 of A. altillis leaf is 1-(2,4-dihydroxyphenyl)-3-(2-(4,10-dimethylundeca-3,9-dienyl)-3,5-dihydroxyphenyl)propan-1-one (C$_{28}$H$_{36}$O$_{5}$) with m/z 452.00 [M$^+$ ion; ~17% relative abundance] (Figure 4.21). Analytically calculated mass for C$_{28}$H$_{36}$O$_{5}$=452.58 and analytical percentages of elements are C=74.31; H=8.02; O=17.68 while the experimental percentages were found to be C= 74.21; H=8.06; O=17.73.

The chemical name and structure of 1-(2,4-dihydroxyphenyl)-3-(2-(4,10-dimethylundeca-3,9-dienyl)-3,5-dihydroxyphenyl)propan-1-one was compared with existing literature on compounds from A. altillis and found that it is for the first time, we have isolated and characterized a new ACE inhibitory polyphenolic compound. Based on its isolation from A. altillis and polyphenolic nature, the common name was
assigned to as ‘altilisol’. Till date, there is no report on ACE inhibitory compound from A. altilis as well as there is no report on ACE inhibitory activity of altilisol.

The concentration-dependent inhibition of ACE from porcine kidney was studied using varied concentrations of altilisol. It inhibited ACE activity with up to 99% decrease in enzyme activity at a concentration of 300 µM (Figure 4.22). The IC$_{50}$ value was calculated by Boltzmann’s dose response analysis using Origin 8 software. Lisinopril, a standard ACE inhibitor was used as the positive control for all the inhibitory assays in the study.

Altilisol exhibited a potent ACE inhibitory activity with an IC$_{50}$ value of 60.12±0.223 µM ($p$ value; 1.4275). The extent of potency in inhibiting ACE activity was found to be similar to most of the earlier reports on ACE inhibitory polyphenolic compounds (Oh et al., 2002; Kang et al., 2003a; Kang et al., 2003b; Gao et al., 2004; Li et al., 2008). In addition, in order to clarify the mechanism underlying inhibition at kinetic level, ACE inhibitory activity was evaluated by varying the substrate with indicated altilisol concentrations. Lineweaver-Burk (LB) plot for ACE with and without altilisol (40 µM and 80 µM) were determined by plotting the values of 1/S on x-axis and values of 1/v on y-axis. Thus obtained LB plot with an intersection on 1/S axis indicated that altilisol is noncompetitive inhibitor of ACE (Figure 4.23).

The active site of ACE is known to consist 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. The zinc ion coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack. Therefore, free hydroxyl groups of phenolic compounds were suggested to show in vitro activity via the generation of chelate complexes within the active center of ACE. Free hydroxyl groups of phenolic compounds are important structural moieties to chelate the zinc ions, thus inactivating the ACE activity (Oh et al., 2004). Since, altilisol contain aromatic hydroxyl groups, these hydroxyl groups may exhibit ACE inhibitory activity due to the generation of chelate complexes with zinc ions within the active center of ACE. Thus, altilisol seems to have similar or stronger levels of inhibitory activities toward ACE as compared to previously reported ACE-inhibitory phenolic compounds.
It is known that the alteration in intrinsic fluorescence reflects change in conformation of protein due to interaction with substrate or ligand (Nataraju et al., 2007). It was estimated that fluorescence measurement with ACE and altilisol would give information about the formation of enzyme-inhibitor complex. Therefore, we examined the ability of altilisol to interact with purified ACE from porcine kidney by fluorescence spectroscopy. Altilisol exhibited concentration-dependent reduction of relative fluorescence of ACE (Figure 4.24), which was observed by increased fluorescence with increasing concentration of altilisol (20 to 100 μM). ACE on addition of altilisol showed decrease in intensity of fluorescence when compared to native condition, indicating the direct interaction of altilisol with the enzyme to form enzyme-inhibitor complex.

Further evidence of the interaction between altilisol and ACE was carried out by circular dichroism (CD) spectroscopy. CD spectra of proteins in far-UV region are due to the optical transitions of amide bonds. This spectrum depicts orientation of peptide planes in the well-ordered secondary structural elements. Exploiting this phenomenon, the overall secondary structure of proteins in solution can be determined, disclosing the main conformational motifs such as α-helices, β-sheets, β-turns and random coil. These CD contributions are very sensitive to changes in the environment of the chromophore and are therefore well suited to follow changes in the secondary structure of protein as well as their binding to the ligands (Venyaminov & Yang, 1996).

In the present study, addition of altilisol changed the far UV-CD spectra of native ACE. In the absence of altilisol, ACE showed a prominent single large negative band, which had maximum absorbance at 206 and 225 nm in far UV-CD spectrum. The negative band of absorbance was diminished and the peak height was reduced compared to native protein band in presence of IC₅₀ concentration of altilisol. However, significant shifting and broadening of the band was not observed upon interaction (Figure 4.25). Altilisol significantly did not change the secondary structure of ACE, which is in parallel with CD spectral analysis results of lisinopril and captopril with ACE (Jones & Clarke, 2004). The change in percentage of secondary structures upon interaction of ACE with IC₅₀ concentration of altilisol is summarized in Table 4.04. Hence, from fluorescence and CD spectroscopic studies, it can be
concluded that inhibition of ACE by altilisol is due to direct interaction with the enzyme rather than with the substrate.

In conclusion, altilisol exhibited concentration-dependent ACE inhibitory activity with IC$_{50}$ value of 60.12±0.223 μM. It was found to be non-competitive inhibitor, as revealed by LB plot. These data encourage developing altilisol into a new antihypertensive drug with further clinical experiments.
**Figure 4.01:** ACE inhibitory activity of cold and hot extracts of *A. altilis* leaf: Rabbit lung ACE (5 mU) was pre-incubated with 50 μg of *A. altilis* leaf extracts for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).

**Figure 4.02:** Concentration-dependent inhibition of ACE by cold leaf extracts of *A. altilis*: Rabbit lung ACE (5 mU) was pre-incubated with different concentrations of *A. altilis* leaf extracts for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).
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*Altilisol: a new angiotensin...*

![Graph](image)

**Figure 4.03:** Concentration-dependent inhibition of ACE by hot leaf extracts of *A. altilis*: Rabbit lung ACE (5 mU) was pre-incubated with different concentrations of *A. altilis* leaf extracts for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).

![Graph](image)

**Figure 4.04:** HPLC chromatogram of hot ethanol extract of *A. altilis* leaf; HPLC chromatogram of hot ethanol extract, separated on a RP-C18 column (250 x 4.6 mm²; 5 μm) using gradient elution-acetonitrile and 0.1% trifluoroacetic acid in water at a total flow rate of 1 mL/min; gradient composition (min,% acetonitrile): 0; 20; 5; 40; 8; 75; 12; 90; 15; 95; 25; 95; 27; 20; 30, 20. The elution was monitored by PDA detector at 210 nm.
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Figure 4.05: HPLC chromatogram of hot methanol extract of A. altilis leaf: HPLC chromatogram of hot methanol extract, separated on a RP-C18 column (250 × 4.6 mm²; 5 µm) using gradient elution-acetonitrile and 0.1% trifluoroacetic acid in water at a total flow rate of 1 mL/min; gradient composition (min,% acetonitrile): 0, 20; 5, 40; 8, 75; 12, 90; 15, 95; 25, 95; 27, 20; 30, 20. The elution was monitored by PDA detector at 210 nm.

Figure 4.06: HPLC chromatogram of hot ethyl acetate extract of A. altilis leaf: HPLC chromatogram of hot ethyl acetate extract, separated on a RP-C18 column (250 × 4.6 mm²; 5 µm) using gradient elution-acetonitrile and 0.1% trifluoroacetic acid in water at a total flow rate of 1 mL/min; gradient composition (min,% acetonitrile): 0, 20; 5, 40; 8, 75; 12, 90; 15, 95; 25, 95; 27, 20; 30, 20. The elution was monitored by PDA detector at 210 nm.
Figure 4.07: Flow chart of fractionation of hot-ethanol, -methanol and -ethyl acetate extract of A. altilis leaf: Fractionation is carried out by partition method using methanol (MeOH), water (H₂O), ethyl acetate (EtOAc) and chloroform (CHCl₃) followed by acid-base extraction.
Figure 4.08: ACE inhibitory activity of fractions of hot -ethanol, -methanol and -ethyl acetate extracts of A. altillis leaf: Rabbit lung ACE (5 mU) was pre-incubated with 50 µg of fractions of hot -ethanol, -methanol and -ethyl acetate extracts of A. altillis leaf for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).

Figure 4.09: ACE inhibitory activity of chromatographic fractions of combined F3+F4 fractions of hot -ethanol, -methanol and -ethyl acetate extracts of A. altillis leaf: Rabbit lung ACE (5 mU) was pre-incubated with 50 µg of chromatographic fractions of combined F3+F4 fractions of hot ethanol, -methanol and -ethyl acetate extracts of A. altillis leaf for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).
Figure 4.10: Liquid chromatography (LC) chromatogram of HEaFr-3: LC chromatogram of HEaFr-3, separated on a RP-C18 column (250 × 4.6 mm²; 5 µm) using acetonitrile: methanol (95:5) at a total flow rate of 0.15 mL min⁻¹. The chromatographic procedure was carried out in the isocratic mode at room temperature. The elution was monitored by PDA detector (UV, Channel A) at 214 nm.

Figure 4.11: Fourier transform infrared spectrum of HEaFr-3: The analysis was performed in the mid infrared region of 400-4,000/cm with 16 scan speed using Shimadzu FTIR 8400S. The pellets were prepared using spectroscopically pure KBr (5:95) and fixed in a sample holder.
Figure 4.12: Quadrupole-time-of-flight mass spectrum of HEaFr-3: HEaFr-3 was subjected to Q-ToF mass spectrometry in positive ES mode.

Figure 4.13: $^1$H NMR spectrum of HEaFr-3 (methanol-d4): HEaFr-3 dissolved in methanol-d4 was subjected to $^1$H NMR spectroscopy using Bruker 400 MHz NMR instrument.
Figure 4.14: $^1$H NMR spectrum of HEaFr-3 (DMSO-d6): HEaFr-3 dissolved in DMSO-d6 was subjected to $^1$H NMR spectroscopy using Bruker 400 MHz NMR instrument.

Figure 4.15: $^{13}$C NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to $^{13}$C NMR spectroscopy using Bruker 100 MHz NMR instrument.
Figure 4.16: Distortionless enhancement by polarization transfer (DEPT) 90 NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to DEPT 90 NMR spectroscopy using Bruker 100 MHz NMR instrument.

Figure 4.17: Distortionless enhancement by polarization transfer (DEPT) 135 NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to DEPT 135 NMR spectroscopy using Bruker 100 MHz NMR instrument.
Figure 4.18: Two dimensional heteronuclear single quantum coherence (2D HSQC) NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to 2D HSQC NMR spectroscopy using Bruker 100 MHz NMR instrument.

Figure 4.19: Two dimensional heteronuclear multiple-bond correlation (2D HMBC) NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to 2D HMBC NMR spectroscopy using Bruker 100 MHz NMR instrument.
Figure 4.20: Homonuclear correlation spectroscopy (HOMO-COSY) NMR spectrum of HEaFr-3: HEaFr-3 dissolved in methanol-d4 was subjected to HOMO-COSY NMR spectroscopy using Bruker 100 MHz NMR instrument.

Figure 4.21: Chemical structure of 1-(2,4-dihydroxyphenyl)-3-(2-(4,10-dimethylundeca-3,9-dienyl)-3,5-dihydro-xyphenyl)propan-1-one (altilisol; C_{28}H_{36}O_{5}).
Figure 4.22: Concentration-dependent inhibition of ACE by altilisol: Porcine kidney ACE (5 mU) was pre-incubated with different concentrations of altilisol for 10 min. The substrate (12.5 mM HHL) was added 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 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 expressed as percentage of inhibition. Data represents the mean ± SD (n=3).

Figure 4.23: Lineweaver-Burk plot of porcine kidney ACE inhibition by altilisol: Porcine kidney ACE (5 mU) was pre-incubated with 40 and 80 μM altilisol for 10 min. The substrate (0.5-5 mM HHL) was added 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 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. The initial reaction velocities were calculated from hippuric acid released.
Figure 4.24: Intrinsic fluorescence spectra of porcine kidney ACE with altisol: The reaction mixture contained 25 μg of porcine kidney ACE in 10 mM potassium phosphate buffer, (pH 7.4), with increasing concentrations of altisol (from top to bottom direction). Fluorescence spectra were recorded after excitation at 280 nm. Emission spectral intensity was recorded from 300 to 400 nm. Black-ACE alone; blue-ACE+20 μM; red-ACE+40 μM; brown-ACE+60 μM; green-ACE+80 μM and grayish blue-ACE+100 μM altisol.

Figure 4.25: Far-UV-Circular dichroism spectra of ACE with altisol: Circular dichroism spectra were recorded for porcine kidney ACE in absence and presence of IC₅₀ concentration of altisol between 200 and 260 nm on Jasco J715 spectropolarimeter. The samples were dissolved in 10 mM potassium phosphate buffer, (pH 7.4). Quartz cuvette with path length of 2 mm was used. Measurements were made with bandwidth of 1 nm and response time of 2 s.


**Chapter IV  
Altilisol: a new angiotensin…**

**TABLES**

**Table 4.01:** Total percent yields of the cold and hot extraction of *A. altilis* leaf

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Cold extract</th>
<th>Hot extract</th>
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<tbody>
<tr>
<td>Hexane</td>
<td>0.98</td>
<td>3.17</td>
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<tr>
<td>Petroleum ether</td>
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<tr>
<td>Ethyl acetate</td>
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<td>Acetone</td>
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<td>n-Butanol</td>
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<tr>
<td>Ethanol</td>
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<td>3.24</td>
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<tr>
<td>Methanol</td>
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<td>3.65</td>
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<tr>
<td>Aqueous</td>
<td>6.98</td>
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Note: The yield was calculated compared to the initial weight of the leaf powder.

**Table 4.02:** IC\(_{50}\) values of *A. altilis* leaf extracts

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<th>Solvents</th>
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<tbody>
<tr>
<td>Ethyl acetate</td>
<td>85.44 ± 0.85</td>
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<tr>
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<td>441.68 ± 6.2</td>
<td>396.24 ± 3.67</td>
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<tr>
<td>n-Butanol</td>
<td>106.64 ± 0.63</td>
<td>NT</td>
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<tr>
<td>Ethanol</td>
<td>400.24 ± 3.23</td>
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<tr>
<td>Methanol</td>
<td>125.04 ± 0.37</td>
<td>106.56 ± 1.27</td>
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<tr>
<td>Water</td>
<td>210.32 ± 5.37</td>
<td>765.52 ± 11.97</td>
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*IC\(_{50}\) value is defined as the concentration of test sample (µg) required for inhibiting enzyme activity by 50% per milliliter of reaction mixture. The linear regression analysis of the linear portion of the dose dependent inhibition curve of ACE was used for the calculation of IC\(_{50}\).*

Note: NT= Not tested
### Table 4.03: Summary of the phytochemical analysis of *A. altilis* leaf extracts

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**Note:**  + = Present, - = Absent, NT= Not tested
**Table 4.04**: Effect of altilisol on secondary structure of porcine kidney ACE

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<thead>
<tr>
<th></th>
<th>ACE alone</th>
<th>ACE + Altilisol</th>
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<tr>
<td>α-helix</td>
<td>40.18 %</td>
<td>44.73 %</td>
</tr>
<tr>
<td>β-sheet</td>
<td>10.65 %</td>
<td>13.42 %</td>
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<tr>
<td>Others</td>
<td>49.17%</td>
<td>41.85%</td>
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Note: Secondary structure contents were calculated using K2D2 software.