SUMMARY AND CONCLUSION
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Angiotensin converting enzyme (ACE) plays a critical role in the control of blood pressure by virtue of its participation in the renin-angiotensin system. ACE hydrolyzes angiotensin (Ang) I to generate an active vasoconstrictor Ang II. In addition, it also degrades and inactivates the vasodilator peptide bradykinin, thereby increasing the risk of hypertension. Since, the suppression of ACE activity results in decreased formation of Ang II and decreased inactivation of bradykinin, ACE inhibition has been considered to be one of the effective therapeutic approaches for the management of hypertension and other cardiovascular diseases.

From the clinical studies, it is evident that ACE inhibitors are effective antihypertensive agents with favorable metabolic and cardioprotective benefits. However, despite their beneficial effects, these drugs are associated with side effects including angioedema, dry cough, hyperkalemia, headache, dizziness, fatigue, nausea, renal dysfunction, skin rashes, taste disturbances, hypotension, impotence, allergic reactions, and increased inflammation-related pain. Therefore, scientists and researchers from different parts of the world are making efforts to develop new ACE inhibitors, either targeting chemical synthesis procedure or isolating from the natural sources, with improved safety and efficacy.

From ancient times, rural and tribal population around the world use plant extracts as traditional medicine for controlling high blood pressure. A number of inhibitors have been isolated from different natural sources including marine organisms, microorganisms, animals, plants and functional foods. In addition, quite a number of synthetic and natural products are at different stages of clinical trials and also available in the market. Screening studies of medicinal plants have shown that the antihypertensive effect of majority of plants is mediated through ACE inhibition. A large number of plants with antihypertensive activity have been reported from different parts of the world. In addition, different class of compounds exhibiting ACE inhibitory activity have been isolated from plants including phenolics, glycosides, tannins, flavonoids, alkaloids, xanthones, terpenes, peptides and anthraquiniones.

All these attempts indicate the need for an effective and safe ACE inhibitor, which can serve a role as that of the existing chemically synthesized ACE inhibitors. In spite of extensive work on plants, there is no information available with respect to ACE inhibitory activity of medicinal plants such as *Artocarpus altillis, Azadirachta*
indica, Catharanthus roseus, Pongamia pinnata, Tamarindus indica and Trigonella foenum-graecum, which have been traditionally used for the treatment of hypertension. Similarly, there are no reports on ACE inhibitory activity of structure-based designed synthetic peptides; L-Phe-D-His-L-Leu and Gly-Cys-Phe. Therefore, the above said plants and peptides were chosen for the present thesis work. The plants were processed and extracted for evaluating ACE inhibitory activity. The peptides were designed and synthesized in order to evaluate for their ACE inhibitory potential, based on the available structure-function relationship.

ACE was purified from porcine kidney cortex by employing various purification techniques and ACE assay was standardized to screen in vitro ACE inhibition by plant extracts/fractions/isolated compounds/peptides. Among the plant extracts tested, ethanol and methanol extracts of A. altillis leaf exhibited potent ACE inhibitory activity at 100 μg/125 μL concentration (IC\textsubscript{50} of 0.125±1.93 mg/mL and 0.125±4.73 mg/mL respectively) followed by methanol and ethanol extracts of T. foenum-graecum, T. indica, A. indica, C. roseus and P. pinnata with ~60%-70% ACE inhibition at 200 μg/125 μL concentration (varied IC\textsubscript{50} values). Further, correlation between ACE inhibitory activity and phytochemical analysis revealed the possible involvement of phenolics, tannins and terpenoids in exerting potent ACE inhibitory activity.

A. altillis with potent ACE inhibitory activity was considered for isolation and characterization of metabolites that account for the inhibition of ACE. Between hot and cold methods of extraction, the percent yield and ACE inhibitory potency of extracts was found to be high in hot extraction procedure, with few exceptions. Hot ethanol, cold and hot ethyl acetate, hot and cold methanol as well as cold n-butanol extracts of A. altillis leaf exhibited potent ACE inhibitory activity (~94-98%) at 80 μg/125 μL concentration. In contrast, cold ethanol, -acetone and -water extracts exhibited fairly good inhibitory activity (64-84%) at 100 μg/125 μL concentration while hot acetone and -water extracts did not show significant inhibition of ACE.

Among these extracts, hot ethanol extract exhibited a potent ACE inhibitory activity with IC\textsubscript{50} value of 54.08±0.29 μg/mL followed by cold ethyl acetate extract (IC\textsubscript{50} of 85.44±0.85 μg/mL). In contrast, hot water extract showed minimum inhibition with IC\textsubscript{50} value of 765.52±11.97 μg/mL at the maximum concentration tested. The correlation between phytochemical analysis and ACE inhibitory activity
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of A. altilis leaf extracts suggested that, the high content of phenolic and glycosidic compounds could be involved in exerting ACE inhibitory activity. Since, the percent yield and ACE inhibitory activity was more pronounced in hot extracts, hot extraction method alone employed for further isolation and characterization of ACE inhibitory compound(s) from A. altilis leaf extracts.

Furthermore, analytical HPLC chromatogram of hot ethanol extract showed three major peaks eluting at 2.212, 3.037 and 6.588 min, whereas hot methanol extract exhibited three major peaks eluting at 2.645, 2.774 and 2.928 min. On the other hand, hot ethyl acetate was found to contain eight major peaks eluting at 2.759, 2.988, 3.221, 3.595, 3.756, 3.944, 4.740 and 5.174 min. The comparison of HPLC chromatogram of these extracts suggested that, the potent ACE inhibitory activity of hot ethanol extract could be due to minimum number of constituents compared to hot ethyl acetate extract and large quantity of the constituents when compared to hot methanol extract.

Hot ethanol, -methanol and -ethyl acetate extracts of A. altilis leaf were concentrated and were individually fractionated by partition method using methanol, water, ethyl acetate and chloroform, followed by acid-base extraction. This resulted in five fractions including ethyl acetate insoluble fraction (F1), ethyl acetate soluble fraction (F2), chloroform fraction (F3), the basic fraction (F4) and neutral fraction (F5). Among these fractions, F3 and F4 of hot ethanol, -methanol and -ethyl acetate extracts exhibited significant ACE inhibitory activity but not the other fractions.

Based on their ACE inhibitory activity and banding pattern on silica gel TLC, F3 and F4 of individual extracts were pooled, concentrated and fractionated individually on silica gel column (60-120 and 100-200 mesh) by gradient elution method using eluents of increasing polarity of solvent mixtures, including the different ratios of n-hexane: chloroform, chloroform: methanol. The repeated fractionation over different bed volumes of silica gel with different ratios eluents resulted in six fractions each.

Among the fractions obtained, fraction (Fr) 5 of hot ethanol extract (HEFr-5), 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 25 µg/125 µL. Furthermore, silica gel TLC was carried out to
analyze the banding pattern of these fractions. 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, indicating the homogeneity of HEaFr-3. Based on the LC analysis result, FT-IR spectroscopic analysis was carried out for 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. 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.

Furthermore, \(^1\)H, \(^{13}\)C, DEPT 90, DEPT 135, 2D HSQC, 2D HMBC and HOMO-COSY NMR spectroscopic analysis of HEaFr-3 were carried out to elucidate the structure of HEaFr-3. 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 indicated that HEaFr-3 of A. altilis leaf is \(\text{1-}(2,4\text{-dihydroxyphenyl})-3\text{-}(2\text{-}(4,10\text{-dimethylundeca-3,9-dienyl})-3,5\text{-dihydroxyphenyl})\text{propan-1-one (C}_{28}\text{H}_{36}\text{O}_5)\) with m/z 452.00 [M\(^+\) ion; ~17% relative abundance].

The chemical name and structure of \(\text{1-}(2,4\text{-dihydroxyphenyl})-3\text{-}(2\text{-}(4,10\text{-dimethylundeca-3,9-dienyl})-3,5\text{-dihydroxyphenyl})\text{propan-1-one was compared with existing literature on compounds from A. altilis and found that it is for the first time, we have isolated and characterized a new ACE inhibitory polyphenolic compound. 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. Based on its isolation from A. altilis and polyphenolic nature, the common name was assigned to as ‘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. The IC₅₀ 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 IC₅₀ value of 60.12±0.223 μM. Further, the type of enzyme inhibition as determined by Lineweaver-Burk (LB) plot indicated that altilisol is non-competitive inhibitor of ACE. Furthermore, altilisol quenched the relative fluorescence intensity of ACE in a dose dependent manner and exhibited apparent shift in the far UV-circular dichroism (CD) spectra of ACE, indicating a direct interaction with the enzyme. These data encourage developing altilisol into a new antihypertensive drug with further clinical experiments.

Apart from this, the peptidomimetic L-Phe-D-His-L-Leu was custom-synthesized from Genemed Synthesis, Inc., USA. C-terminal amino acid sequence of Ang I, where the cleavage occurs by the action of ACE was considered and modified the stereochemistry of histidine residue. Thus obtained tripeptide was evaluated in order to confirm whether the modified stereochemistry of peptide bond between L-phenylalanine and D-histidine is helpful in ACE inhibitory activity. L-Phe-D-His-L-Leu exhibited potent ACE inhibitory activity with up to 90% decrease in enzyme activity at 200 nM concentration. The concentration-dependent inhibition was observed with significantly lower IC₅₀ value of 53.32±0.13 nM when compared to most of the earlier reported ACE inhibitory peptides.

Further, the mechanism of inhibition as determined by Lineweaver-Burk plot indicated its competitive inhibitory nature. L-Phe-D-His-L-Leu quenched the relative fluorescence intensity of ACE in a dose dependent manner. In the presence of L-Phe-D-His-L-Leu, apparent shift in the far UV-CD spectra of ACE was observed, indicating a direct interaction with the enzyme. In silico molecular modeling study displayed high negative values of MolDock score, Rerank score and binding energy, indicating higher binding affinity of L-Phe-D-His-L-Leu towards ACE. In addition, it also suggested putative binding sites for the interaction by forming hydrogen bonds between L-Phe-D-His-L-Leu and Tyr⁵²⁰, Tyr⁵²₃, Asp⁴¹⁵ and Gln²₈¹ residues of ACE. These findings indicate that inhibition of ACE by L-Phe-D-His-L-Leu is mediated by direct interaction with the enzyme. Hence, the strategy of incorporation of D-amino
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... acids in ACE inhibitory peptides could be useful for designing potent drug molecules for the management of hypertension.

Gly-Cys-Phe (GCF) is another tripeptide designed based on the available structure-function activity relationship information. It exhibited concentration-dependent enzyme inhibition with up to 69% decrease in enzyme activity obtained at a concentration of about 800 nM. GCF also exhibited a potent ACE inhibitory activity with significantly lower IC₅₀ value of 369.43±0.1 nM when compared to most of the earlier reports on ACE inhibitory peptides. Further, GCF was found to be non-competitive inhibitor, as evident from Lineweaver-Burk plot with an intersection on 1/S axis. Biophysical interaction of GCF with ACE was studied by fluorescent spectral analysis and circular dichroism studies. GCF enhanced the fluorescence intensity of ACE in a dose dependent manner. In addition, apparent shift in the far UV-CD spectra of ACE with GCF indicated changes in secondary structure of ACE. Thus, these spectral results suggested its direct interaction with the enzyme.

Furthermore, in silico molecular modeling study indicated the direct interaction of GCF with ACE by forming hydrogen bonds with amino acid residues of ACE that are resided away from the active site. GCF formed hydrogen bonds with Tyr⁵²³, Ala⁵₅⁶, Ser⁵⁵⁵, Asn⁷₀ and Glu¹⁴₃ residues with high negative values of MolDock score, Rerank score and binding energy, indicating its higher binding affinity and interactions with ACE. These data suggest that the tripeptide GCF is potent non-competitive inhibitor of ACE with IC₅₀ value of 369.43±0.1 nM. Thus, the strategy of structure-based designing of peptides could be used for the development of therapeutically important ACE inhibitors.
MAJOR OUTCOMES OF THE THESIS:

- Ethanol and methanol extracts of *A. altilis* leaf exhibited potent ACE inhibitory activity, followed by methanol and ethanol extracts of *T. foenum-graecum* seed, *T. indica* seed coat, *A. indica*, *C. roseus* and *P. pinnata* leaves.
- The phytochemical analysis of above plant extracts revealed the possible involvement of phenolics, tannins and terpenoids in exerting potent ACE inhibitory activity.
- Among the cold and hot extracts of *A. altilis* leaf, hot ethanol, cold and hot ethyl acetate, hot and cold methanol as well as cold n-butanol extracts showed potent ACE inhibitory activity.
- 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.
- Analytical HPLC chromatogram of these extracts suggested that, the potent ACE inhibitory activity of hot ethanol extract could be due to minimum number of constituents compared to hot ethyl acetate extract and large quantity of the constituents when compared to hot methanol extract.
- Chloroform fraction (F3) and basic fraction (F4) obtained from hot -ethanol, -methanol and -ethyl acetate extracts showed strong ACE inhibitory activity.
- HEaFr-3 and HEFr-5 exhibited potent ACE inhibitory activity when compared to HMFr-5 and HMFr-6.
- Structural elucidation based on the data obtained from TOF MS ES+ spectrometric analysis, FT-IR, CHNOS analysis, $^1$H, $^{13}$C, DEPT 90, DEPT 135 and HOMO-COSY NMR spectroscopic analysis indicated that HEaFr-3 of *A. altilis* leaf is 1-(2,4-dihydroxyphenyl)-3-(2-(4,10-dimethylundeca-3,9-dienyl)-3,5-dihydroxyphenyl)propan-1-one (chemical formula: C$_{28}$H$_{36}$O$_5$) with m/z 452.00 [M$^+$ ion; ~17% relative abundance]. The common name was coined as ‘altilisol’.
- Altilisol exhibited potent ACE inhibitory activity and the inhibition was found to be concentration-dependent with an IC$_{50}$ value of 60.12±0.223 μM.
- The mechanism of inhibition of ACE by altilisol was found to be non-competitive in nature.
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- The fluorescent interaction study and far UV-CD spectral analysis indicated that altilosol interacts directly with ACE.
- The outcomes of the present study encourage developing altilosol into a new antihypertensive drug with further clinical experiments.
- L-Phe-D-His-L-Leu and Gly-Cys-Phe exhibited potent ACE inhibitory activity and the inhibition was found to be concentration-dependent with significantly lower IC₅₀ value of 53.32±0.13 nM and 369.43±0.1 nM respectively.
- The mechanism of inhibition of ACE by L-Phe-D-His-L-Leu was found to be competitive in nature while Gly-Cys-Phe was found to be non-competitive inhibitor of ACE.
- The fluorescent interaction study and far UV-CD spectral analysis indicated that both L-Phe-D-His-L-Leu and Gly-Cys-Phe interact directly with ACE.
- In silico molecular modeling studies displayed high negative values of MolDock score, Rerank score and binding energy, indicating higher binding affinity of L-Phe-D-His-L-Leu and Gly-Cys-Phe towards ACE.
- The combination of in vitro ACE inhibitory and in silico molecular docking results indicated that these molecules could be developed into potential antihypertensive lead molecules.
- Strategy of incorporation of D-amino acids in ACE inhibitory peptides and structure-based designing of peptides could be helpful in development of potent drug molecules for the management of hypertension.