Role of selective Bioactive Compounds as an Angiotensin Converting Enzyme Inhibitor

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Abstract

Hypertension is one of a major reason of mortality and morbidity and it is associated with heart and renal disease. The aim of this study is to find out the antihypertensive role of bioactive compounds from selected medicinal plants targeting ACE molecule which so far is not known. The plants taken in this study were *Moringa oleifera*, *Azadirachta indica*, and *Hibiscus sabdariffa*. The nitric oxide and superoxide scavenging property vary from 39.50% to 68% and 37.67 % to 75.50 %. respectively. The inhibition of ACE activity was found maximally in methanolic extract of *A. indica* (74 %), followed by *H. sabdariffa* (73.4%), and least in *M. oleifera* (71.8 %). The bioactive chloroform fraction was characterized for the presence of compound using standard techniques such as LCMS and NMR (^{13}C -NMR ^1H -NMR). The results revealed the presence of beta-sitosterol in *M. oleifera*, azadiradionolide in *A. indica* and hibiscitrin in *H. sabdariffa*. The compounds have shown significant low binding energy for hibiscitrin (-12.3kcal/mol), beta-sitosterol (-11.2kcal/mol) and azadiradionolide (-11.3kcal/mol) indicating the high efficacy of binding on the enzyme. While, binding energy of drug captopril was -5.6kcal/mol & enalapril - 8.1kcal/mol in the same pocket of the ACE molecule. Upon subjecting molecular dynamic simulation results indicated that beta sitosterol complex provided more compactness than the hibiscitrin and azadiradionolide compounds. The current study delivers a new perspective for the drug development against systolic blood pressure regulation and also opens new horizons for considering alternate highly potent drug target for hypertension.

Keyword: Systolic blood pressure, *Hibiscus sabdariffa*, Angiotensin converting enzyme, beta sitosterol, Nitric oxide, *Moringa oleifera*

Introduction

Hypertension or high blood pressure is acknowledged as one of the leading factors for human illness and impermanence. This is a persistent medical condition and is the utmost common life threatening non-communicable disease (Gavras, 2009). With the rise of urbanization, there is a

change in lifestyles such as smoking, lack of physical activity, unhealthy diet, alcohol, which are major risk factors of hypertension (Alwan, 2010). Many antihypertensive agents, which include potassium-sparing diuretics, central α 2-adrenergic agonists, peripheral α 1-adrenergic antagonists, central/peripheral adrenergic neuronal-blocking agents, diuretics, β -blockers, calcium-channel blockers, and blockers of the rennin-angiotensin system, such as angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor blockers are used alone or in combination to treat hypertension. But antihypertensive drugs have many side-effects such as dry cough, muscle cramps, dizziness, extreme tiredness, dehydration, blurred vision, abnormal heart rate, skin rash, hence, the management of hypertension by herbal medicine is an alternative approach (Vardanyan and Hruby, 2017; McLaughlin *et al.*, 1998).

The renin angiotensin system (RAS) is an important regulator of blood pressure and plays a critical role in the pathogenesis of hypertension, renal and cardiovascular disease. The RAS consist of many components including renin, angiotensinogen (AGT), angiotensin converting enzyme (ACE), ACE2, angiotensin subtype2 receptor (AT2R) and angiotensin subtype 1 receptor (AT1R) (Barnaset *al.*, 1997). The ACE (EC 3.4.15.1) converts Ang I to Ang II, and bradykinin to inactive components. The ACE generated potent Ang II peptide exert effect via predominantly activation of AT1R a vasoconstrictor, plays an important role in the pathogenesis of hypertension, suggesting ACE inhibition as a critical target for controlling systolic blood pressure. Consequently, various compounds from medicinal plants had been explored as ACE inhibitors. *Eleusine indica* leaves showed a role in inhibiting ACE activity (Tutor *et al.*, 2018). The *Chrysophyllum cainito* controls blood pressure due to the presence of phenolic compounds (Mao *et al.*, 2015). Quercetin glycoside a predominant bioactive compound present in citrus fruit, buckwheat and onion was reported as a potential ligand of ACE (Muhammad and Fatima, 2015). The occurrence of secondary metabolites i.e. flavonoids, alkaloids, glycosides, lipids and tannins in plant extract reported to play an important role in ACE inhibition (Reddy *et al.*, 2018). Several, *in silico* studies have also suggested that bioactive compounds from medicinal plants and herbs are potential drugs to target the RAS components including ACE (Khan *et al.*, 2019). Therefore, there is a significant scope to explore the natural compounds as a mediator in controlling blood pressure. The selected plants used in the study were based on literature survey on ethnopharmacology of *Moringa oleifera*, *Hibiscus sabdariffa*, and *Azadirachta indica*. The compounds from *Moringa oleifera* (Niaziminin-A) (Anwaret *al.*, 2007), *H. sabdariffa* (Delphinidin-3-sambubioside) (Ojeda *et al.*, 2010), and A.

indica (Nimbidin) (Shori and Baba, 2012) were reported to have antihypertensive activity but their mechanism for targeting ACE molecule is unclear. Hence, the aim of this study is to evaluate ACE inhibitory activity of the compounds from these plant extracts.

Material and Methods

Sample Collection and preparation

Leaves of *Moringa oleifera*, *Hibiscus sabdariffa*, and *Azadirachta indica* were collected from the campus of Shoolini University, Solan, Himachal Pradesh (India) and verified with the Botanical Survey of India, Dehradun, Uttarakhand (India) for herbarium No: (271, 272 and 276). The plant leaves were washed and sterilized by mercuric chloride (HgCl₂) and air dried for 4-5 days. Subsequently, leaves were pulverized by pestle motor and used for extract preparation in methanolic solvent by Soxhlet method. The extract was weighed and stored in a tube,

Fractionation of the extract

Ten grams of leaves extracts of *Moringa oleifera*, *Hibiscus sabdariffa*, and *Azadirachta indica* were re-suspended in 300 ml lukewarm distilled water and then transferred into a separating funnel. Chloroform (50 ml x 3 times) was taken in the separating funnel, observed monitored by mild shaking (1-2 min) and the chloroform fraction was collected. Similar process of fractionation was repeated for n-butanol and ethyl acetate solvents to collect fractions of different polarity by different solvents (Matkowski et al., 2008).

Characterization by LCMS and NMR

The LC-MS analysis was carried out on X-Bridge C18 (2.1 x 50 mm, 3.5 µm) column fitted to an LC-MS 6320 Ion Trap instrument (Agilent Technologies). A gradient of 50 to 100% acetonitrile in 25 mM ammonium acetate buffer was used as a mobile phase and the flow rate was set at 0.4 ml/min. Injection volume was 5 µl. ¹H & ¹³C NMR spectra were recorded on 400 & 100 MHz cryo-magnet spectrometer (Bruker), respectively (Wolfender et al., 2000).

Nitric oxide (NO) free radical scavenging activity

In nitric oxide assay, 2 ml of 10 mM sodium nitroprusside in 0.5 ml of phosphate buffer saline was mixed with 0.5 ml of plant extract at different concentrations (100-500 µg/ml) and the mixture was incubated for 150 min at 23°C. Of this incubating solution, 0.5 ml was added into 1 ml of sulfanilic acid reagent (20% glacial acetic acid) and incubated for 5 min at 23°C. Thereafter, 1 ml of

Naphthylethylene diamine dihydrochloride was added and incubated for 30 min at room temperature. Absorbance was read at 540 nm with spectrophotometer (Rao et al., 2016).

The NO radical scavenging activity was calculated according to the following equation: -

$$\% \text{ NO inhibitory activity} = \frac{A_o - A_s}{A_o} \times 100$$

Where A_o = absorbance of the control (blank, without extract); A_s = absorbance in the presence of the extract.

Superoxide (O_2^-) radical scavenging activity

The superoxide radical scavenging assay was performed as described by Khatua and Acharya, 2016. The 2mM methionine was prepared by dissolving 0.076 gm in 5 ml of distilled water and 0.1 mM EDTA was prepared by 0.004gm in 10 ml of distilled water. Riboflavin was prepared by 0.004 gm in 5 ml of water and 50Mm sodium phosphate buffer of pH 7.8. The reaction was containing 50 μ l of sodium phosphate buffer (pH-7.8), 83 μ l plant extract in various concentrations (100-500 μ g/ml), methionine 26 μ l, EDTA 20 μ l, Nitrobluetetrazolium 17 μ l and riboflavin 4 μ l. The 96 plate was shaken for 15 seconds and initial absorbance was recorded at 595 nm. The plate was kept under in 15 W light and kept at room temperature for 10 minutes to start reaction. After the incubation the reading was taken at the 595 nm. Ascorbic acid was used as Positive control in different concentration.

The superoxide radical (O_2^-) scavenging activity

$$\% \text{ Superoxide (O}_2^-) \text{ inhibitory activity} = \frac{A_o - A_s}{A_o} \times 100$$

A_o = absorbance of the blank without extract (control); A_s = absorbance in presence of extract.

Angiotensin converting enzyme inhibition assay

The inhibition of angiotensin converting enzyme by substrate Hippuryl-histidyl-leucine (HHL) (Sigma- H1635) with benzene sulfonyl chloride and pyridine to convert hippuric acid and histidyl-

leucine (Tutor and Chichioco-Hernandez, 2018). The positive control used in this method was captopril.

Forty µl of (100-500µg/ml) plant extract, 20µl of 5mM Hippuryl-histidyl-leucine (Sigma- H1635) and 20µl of angiotensin converting enzyme (Sigma A6678) from rabbit lungs were mixed with 40 µl of sodium borate buffer (pH-8.2) having 0.3 M NaCl. The reaction was start with 15 min pre-incubation of angiotensin converting enzyme with inhibitor at 37 °C for establish contact. After incubation, substrate 20µl of 5mM HHL was further to start Angiotensin converting enzyme reaction. Then incubated mixture aimed at 30 min at 37 °C and then experiment was finished by addition 50 µl of 1m HCl. After this instantaneously by the adding of 100 µl pyridine and 50µl benzenesulfonyl chloride to grow the yellow color. Absorbance was taken at 410 nm using microplate reader

The following formula was used to determine percent inhibition:

$$\% \text{ inhibition} = 1 - \frac{[A - B]}{C} \times 100$$

A: Absorbance of Inhibited sample

B: absorbance of Initial sample

C: Absorbance of Uninhibited sample

***In silico* molecular docking analysis**

Crystal structure of C-domain (1O86) with resolution 2 Å of Angiotensin converting enzyme was downloaded from RCSB protein data bank (Corradi HR et al., 2016; Natesh R et al., 2003). The format of the crystal structure of enzyme was changed from .pdb to. pdbqt via AutodockTools-1.5.6. The receptors were further modified by adding polar hydrogens and converting to. pdbqt files used for molecular docking analysis (Muhammad and Fatima, 2015). Chemical structure of isolated phyto-constituents found in methanolic leaf extract of *Moringa oleifera*, *Hibiscus sabdariffa*, and *Azadirachta indica* were drawn in chemdraw software where 2 D structures were converted into 3 D structure which were further saved as .pdb files (Kashyap et al., 2017). The files were further optimized and converted to. pdbqt files for molecular docking by Auto Dock tools 1.5.6 (Gheri D and Singh M, 2014). Molecular docking was carried out to evaluate the potential of three compounds against ACE which play an important role in Hypertension. AutoDock Vina achieves a nearly two orders of extent speed-up compared to the other molecular

docking software like AutoDock 4 (Hisle et al., 2018; Trott and Olson, 2010). The results of docking are analysed by the association of binding energy with the binding or inhibiting capacity of the ligand for any particular receptor. After docking, the resultant receptor-ligand complexes were generated by UCSF chimera 1.9 which is a highly extensible program for interactive visualization and analysis of molecular structures and docking results to know whether the ligands were bound to active sites of the drug target (Pettersen *et al.*, 2004). The minimum binding energy of bioactive compounds specified potent drug targets for ACE. The best docking compound was selected on the basis of hydrogen bonding with the target. The toxicity of the compounds was predicted by ADME - toxicity (<http://lmmd.ecust.edu.cn:8000/predict/>). The all isolated compounds i.e. beta-sitosterol, hibiscitrin and azardinolide from *M. oleifera*, *H. sabdariffa*, and *A. indica* respectively were used ADME-toxicity server (Khan *et al.*, 2019).

Molecular dynamic simulation analysis

All the MD simulations was performed using GROMACS (Wang and Chou, 2009; Sheng-Xiang, 2013) Bio-Simulation package installed in Multi-core enabled Linux Ubuntu system. The force field CHARMM27 (Berendsen, 2005) was used to determine the dynamic behavior and different calculations of proteins (de Souza *et al.*, 1999). The water model SPC216 was used to solvate the system (Angiotensin converting enzyme) and prepare the different using different natural compounds to see the inhibitor of Angiotensin converting enzyme. As a control, MD simulation of 30 ns was carried in water at 300 K. All atoms of Angiotensin converting enzyme and include compounds Beta-sitosterol, Hibiscitrin and Azadiradionolide were equilibrated in cubic box, having size of approximately $73.3 \times 73.3 \times 73.3$ Å. Protein was immersed into water and well equilibrated and overlapping molecules were deleted. Each system was energy minimized with steepest descent, up to a tolerance of 100 kJ mol^{-1} Each system was energy minimized with steepest descent, up to a tolerance of $100 \text{ kJ mol}^{-1} \text{nm}^{-1}$ to remove all bad contacts. The overall charge on the system was neutralized with addition of cationic and anionic concentration of Na^+Cl^- . To run the simulation, based on the size and 3D spatial orientation of protein, the dimension (x, y and z) of simulation box was defined. All the systems wer prepared in define box, and protein was placed in centre of box, padded around with water and co-solvents. The steepest-descent algorithm along with conjugate gradient was used for the energy minimization process. The system was

equilibrated with two ensemble processes, NVT and NPT. Before we start production run, we have to prepared or established the pre-define condition like pH, Temperature. All these information is encapsulated in NVT, NPT and MD parameter files. These physiochemical conditions play a vital role for protein structural and conformational changes. Once, the system is prepared the system is now ready to run.

Cell toxicity MTT assay

Baby hamster Kidney (BHK-21) cells were cultured at 37°C with 5% CO₂ in DMEM medium, supplemented with 10% heat inactivated fetal bovine serum. Remove and discard culture medium and then rinse with PBS for removal of extra medium in flask. Briefly rinse the cell layer with trypsin for 2-3 min and incubate in CO₂ incubator for 2-3 min. Trypsin poured is around 2-3ml and take cells out containing trypsin to observe cells whether they are dispersed (de-adhered) or not. Pour FBS to neutralize the effect of trypsin. Add cell to falcon and centrifuge at 1000 rpm for 5 min to remove debris and get pellet containing cells. Remove supernatant and resuspended pellet in new media. Along the experiments, the cells were monitored by microscopic observation in order to detect any morphological changes. Cells were seeded in triplicate at 10⁴ cells/200 µL per well in a sterile 96- well plate. The cells were grown in an incubator (5 % CO₂ and 37°C) for 24 hours. After the first incubation, culture medium was replaced by 15 µL of variable concentrations of plant extract (0.12 µg/mL – 0.97 µg/mL). The cells were incubated for the second time for 24 hours. After 24 hours 150ul of cell culture media is removed and replaced by 100ul of DMSO and further incubated for 15 minutes. After 15 minutes reading was taken at 490nm (Srivastava et al., 2009).

Results and Discussion

Nitric oxide (NO) free radical scavenging activity and Superoxide (O₂⁻) radical scavenging activity

The nitric oxide free radical scavenging assay estimation was done by method described by Parul *et al.*, 2013. Among the plant extracts undertaken the *Hibiscus sabdariffa* showed percentage inhibition of 68% (IC₅₀ value 317.74 µg/mL) followed by the plant extract of *Azadirachta indica* inhibition of 62% and IC₅₀ value (320.7473 µg/mL), and *Moringa oleifera* inhibition of 48.50% (IC₅₀ value 350.618 µg/mL), (Table-1). While the ascorbic acid (positive control) showed

inhibition of 73.50% (IC₅₀ value 302.222 µg/mL) (Figure-1 and Table-1). Based on the free radical scavenging activity the IC₅₀ value of *Hibiscus sabdariffa* (317.74 µg/mL) were observed closed to IC₅₀ of positive control (302.222 µg/mL). The need of the hour is to harness and explore novel plant sources having appreciable bioactivity against NO radical accumulation and the accompanying inflammatory reaction. In the present work, *Azadirachta indica* and *Moringa oleifera* display higher NO free radical scavenging activity as compared to *Hibiscus sabdariffa*. One of the main enzymes involved in NO production is nitric oxide synthase (NOS) (Ondua et al., 2019). NO scavenging activity of the three plants was evaluated spectrophotometrically at 595 nm. The variations in optical density give a fair assessment of the NO scavenging potency of diverse plant extracts. The phytochemical profile of these plant extracts exhibited the presence of flavanoids and phenolic compounds which are important phytobioactives known for their appreciable radical scavenging potential (Kaurinovic and Vastag, 2019). All the three plants analyzed in the present investigations demonstrate ample NO free radical eradication ability and thus exemplify their intrinsic medicinal potential. Superoxide radicals play a key role in signal transmission at the cellular level (Luis et al., 2018). However, their production beyond a certain threshold in cells has proven to be counterproductive (Basu and Hazra, 2006). This ultimately produces a deleterious effect on biomolecules like proteins, lipids and nucleotides (Engwa, 2018). The most potent superoxide inhibitor proved to be *Moringa oleifera* followed closely by *Azadirachta indica*. These plants were traditionally incorporated in folk medications and hence it becomes imperative to prepare a comprehensive biochemical and antioxidant profile so as to harness their antioxidative stress release potential. These plants also displayed higher radical scavenging activity than the positive control used in these experiments. This further underscores the superoxide radical neutralization effect of these medicinal plants capable enough to prevent cell damage. A wide variety of superoxide scavenging phytoconstituents have been identified and characterized which include mangiferin, naringin, quercetin, myricetin among others (Septiana et al., 2010). The elucidation of optimum concentration of these natural superoxide deradicalizers is a key aspect in their use as efficient therapeutic agents.

LC-MS analysis of active Fractions of *Moringa oleifera*, *Azadirachta indica*, and *Hibiscus sabdariffa*

While doing the LC-MS analysis of the isolated molecule from chloroform fraction of *Moringa oleifera*, we found a peak of 274 m/z in the mass spectrum (Figure 2). Which was assumed to be a fragmentation of beta-sitosterol 414 m/z. Hence, the primary conformation of the isolated molecule was beta sitosterol (414 m/z). For further conformation we have done ^1H -NMR and ^{13}C -NMR.

^1H -NMR and ^{13}C -NMR spectra were done using CDCl_3 as solvent on Bruker Advance II 400 NMR spectrometer at the department of Pharmacy at Guru Jambheshwar University Hisar, Haryana. The ^1H -NMR spectrum of compound varied between δ 0.5 to 1 ppm. This spectrum shown fifteen protons on carbon C_{18} , C_{21} , C_{26} , C_{27} and C_{29} position in the compound structure (Supplementary Figure -1 and Table-1). proton signal between δ 2.6-4.2 ppm at C_4 , C_7 , C_{25} and 1.2 to 2.5 ppm 47 proton present at different-different carbon position. Chemical shift of δ 5.3 corresponds to olefinic proton. ^{13}C NMR spectrum of Compound (1) shows signal at C_3 β -hydroxyl group 17.064 and 11.0 for angular methyl carbon atoms for C_{19} and C_{18} respectively. The C_5 , C_6 , C_{22} and C_{23} appeared to be alkene carbons. The value at 17.064 ppm corresponds to angular carbon atom (C_{19}). Spectra show twenty-nine carbon signal including six methyls, nine methylenes, eleven methane and three quaternary carbons (Supplementary Figure-2 and Table-2).

While doing the LC-MS analysis of the chloroform fraction of *Azadirachta indica*, we found a peak of 425.19 m/z. Which was assumed to be a fragmentation of Azadiradionolide m/z 480.574g/mol. The *Moringa oleifera* compound i.e. beta sitosterol, similar to cholesterol was also found in fungi, animal and plants. Beta- sitosterol belong to phytosterol which play role in heart disease, antioxidant, diabetes. These are mainly found in the mixture of stigmasterol. The difference between β - sitosterol and stigmasterol is single bond and double bond respectively. Study done by Pierre et al., (2015) found β -sitosterol from *Odontonema strictum*. Hence, the primary conformation of the isolated molecule was Azadiradionolide, which was 425.19 m/z (Figure-3) (274 m/z). The ^1H NMR spectrum of compound varied between δ 0.5 to 1.75 showed the 15 proton at C_{18} and C_{19} methyl group carbon (supplementary file Figure-3 and Table-3). In δ 2.30 ppm shown 7 protons at C_6 , C_9 , C_{11} and C_{12} , δ 3.72 shown 3 protons, δ 3.30-7.55 shown 8 protons at C_{17} , C_{23} , C_{15} , C_2 , C_1 and C_{21} (supplementary file Figure-4 and Table-4).

The LC-MS analysis of the chloroform fraction of *Hibiscus sabdariffa*, we found a peak of 274.25 m/z. Which was assumed to be a fragmentation of Hibiscitrin m/z 496.377g/mol

(Figure-4). As the molecule was peak of Hibiscitrin after fragmentation is 274.25 m/z (Figure-4). By ^1H NMR analysis δ 1.01-1.47 ppm shown 12 protons at C₂₆, C₂₇, C₂₈ and C₃₁ and δ 2.3-2.8 shown 4 protons at C₂₃, C₂₄ and C₂₅ (supplementary file Figure-5 and Table-5). At δ 3.68-7.28 ppm shown also shown 4 protons at C₁₃, C₆ and C₂₃. Methylene group shown at C₂₈ (Figure-6 and Table-6).

Angiotensin converting Enzyme Inhibitory Assay

The six plants extract under this study subjected to ACE inhibitory activity. The percentage inhibition of *Azadirachta indica*, *Hibiscus sabdariffa*, *Moringa oleifera*, *Punica granatum*, and *Allium sativum* were showed 74.00±0.7937 %, 73.47±1.434 %, and 71.80±2.650 %, (Figure-5). The lowest IC₅₀ activity were showed by *Azadirachta indica* (255.991 µg/ml) followed by *Hibiscus sabdariffa* (267.722 µg/ml), and *Moringa oleifera* (294.397 µg/ml) (Table-2). Among them based on the IC₅₀ the following plants extracts (*Azadirachta indica*, *Hibiscus sabdariffa*, and *Moringa oleifera*) were used for fractionation. The *Azadirachta indica* fraction i.e. n- butanol, ethyl acetate and chloroform showed the percentage inhibition and IC₅₀ value were 42.45±1.050% (IC₅₀ value 508.5438 µg/mL), 53.25±1.750 % (IC₅₀ 373.0502 value µg/mL), and 68.45±0.550 % (IC₅₀ value 264.4097 µg/mL) respectively. The *Hibiscus sabdariffa* fraction i.e. n- butanol, ethyl acetate and chloroform showed the percentage inhibition and IC₅₀ value were 28.67±1.167 % (IC₅₀ value 550.3377 µg/mL), 47.17±1.424 % (IC₅₀ value 340.875 µg/mL), and 62.63±1.660 % (IC₅₀ value 304.246 µg/mL) respectively. The *Moringa oleifera* fraction i.e. n- butanol, ethyl acetate and chloroform showed the percentage inhibition and IC₅₀ value were 32.00±0.981 % (IC₅₀ value 461.9108 µg/mL), 43.57±0.263 % (IC₅₀ value 338.2692 µg/mL), and 58.50±0.458 % (IC₅₀ value 296. 7497 µg/mL) respectively. The percentage inhibition and IC₅₀ of positive control (captopril) was 81.13±0.753% (IC₅₀ value 203.2158 µg/mL) (Figure-5). Among those fractions the chloroform fractions showed best inhibition activity. Tutor JT and Chichioco-Hernandez CL, 2018 worked on plant extract and ethyl acetate fraction of *Eleusine indica* and calculated percentage inhibition is 68.84% and 51.51% respectively. In our study n-butanol, ethyl acetate, chloroform fraction and extract of *A. indica* shows 30.8%, 45.5%, 63.9% and 67.0% inhibition respectively. The positive control captopril shows 76.0% inhibition for ACE. Deo et al., 2016 and Khan et al., 2019 reported that the 50% inhibition of enzyme by plant extract and fraction consider is active.

ACE is a significant enzyme playing a critical role in regulation of blood pressure. Various plant constituents have the ability to inhibit the action of ACE. The active site of ACE is influenced by the phytochemicals present in neem plant. Flavonoids, terpenoids and phenolic compounds have the ability to forge chemical bonds with the amino acids present in close proximity with the active centre of ACE. These new chemical interactants tend to produce distortions in the catalytic centre of ACE thus causing inhibition. Since ACE is a metalloenzyme having zinc atom at its active site, a majority of hydrogen bonds and chemical bridges involve not just conserved amino acids of the active site but also zinc atom (Yusuf et al., 2000). In this study the ACE inhibiting compound is identified as azadiradionolide. It is a terpenoid comprising multiple rings. The tetracyclic nature of azadiradionolide is responsible for high ACE inhibitory activity of neem extract. The presence of carbonyl, lactones and methyl groups in azadiradionolide is responsible for enhancing the potency of neem plant extract against ACE. These chemical groups may be acting as causative chelating agents for the zinc atom located at the nerve centre of ACE. The *in silico* studies reveal that azadiradionolide due to its unique structure is able to gain access into the active site of ACE, establish chemical interactions simultaneously with amino acids and zinc atom and causing alterations in the 3-D structure of catalytic protein thus proving to be a successful ACE inhibitor.

***In silico* study of antihypertensive bioactive compounds against Renin angiotensin components**

The *in silico* study is based on the lock and key theory for drug design. This is used for check the biological activity of the natural and synthetic compound and it is time consuming, reasonably and accurate method. The docking of the ligand and enzyme was done by autodock vina software and visualize the hydrogen bonding by chimera and PyMOL. The hydrogen bonding of enzyme and ligand is the important aspect for ligand and enzyme complex formation. In the present study natural isolated compounds targeted the ACE enzyme which has a role in blood pressure regulation. The Angiotensin-converting enzyme converts angiotensin I to angiotensin II a potent peptide hormone exerts its effect predominantly via AT1R (Donoghue et al., 2000). The bioactive molecule reduces the action of angiotensin converting enzyme and control the systolic blood pressure. The fractions of the selected plants were subjected to LCMS and ¹H-NMR and ¹³C-NMR and following compounds were analyzed. Beta- sitosterol was determined in chloroform fraction of *Moringa oleifera*, Azadiradionolide was determined in chloroform fraction of *Azadirachta*

indica and Hibiscitrin found in chloroform fraction of *Hibiscus sabdariffa*. All three compounds were docked with one of the major cascade renin angiotensin component i.e. angiotensin converting enzyme. The compounds i.e Hibiscitrin, beta-sitosterol, and azadiradionolide were showed very low binding energy i.e. -12.3kcal/mol, -11.2kcal/mol and -11.3kcal/mol binding energy with ACE crystal structure respectively. The standard drugs are used in this study is captopril and enalapril which shown -5.6kcal/mol and -8.1kcal/mol binding energy with ACE. The Hibiscitrin compound was interacting with six amino acid residues i.e. Asn-263, Gly-254, Thr-358, His-331, Tyr-498, Lys-489 (Table-3, Table-4). This Hibiscitrin compound shown hydrophobic interaction with ten amino acid residues i.e. His 491, Ala322, Gln 259, Asp255, Thr 144, Glu 262, Glu 431, Ser 357, Phe 435, Phe 505. The beta-sitosterol shown hydrophilic interaction with Asn 145, Gly 254 and Thr 144 and hydrophobic interaction with Thr 352, Asp 255, Asp 340, Leu 139, His 331, Gln 355, and Asp 354 amino acids residues. The compound isolated from neem i.e. azadiradionolide has shown hydrophilic interaction with Asn 145, Asn 263, Thr 144 and hydrophobic interaction Asp 354, Gly 254, Ala 148, Asp 255, Leu 147, Leu 139, Asp 140, His 331, Gln 355, and Thr 352 amino acid residues. It can be observed that compounds Hibiscitrin a greater number of hydrogen bond interactions with various amino acid residues of the Angiotensin converting enzyme residues. We believe that these hydrogen bond interactions play an important part in the inhibition of the Angiotensin converting enzyme residues, and ultimately better anti-inflammatory activity of the compounds Hibiscitrin (Figure-6). An overlay of the docked pose of Azadiradionolide and Beta-sitosterol also exhibited that these compounds are superimposable with each other (Figure-7, Figure-8.). This indicates that the active site of the Angiotensin converting enzyme residues can accommodate these compounds, which synergizes the Angiotensin converting enzyme residues inhibitory activity of the compounds Azadiradionolide and Beta-sitosterol. All these three compounds have shown better binding affinity in comparison to standard drugs (captopril and enalapril). Further these compounds are used for toxicity of the molecule by ADMETSAR software. The toxicity of the compound is the main reason of drug failure in the drug development field (Table-5).

Molecular Dynamic Simulation Study

MD simulation study has been also executed to investigate the conformational stability of Beta-sitosterol, Hibiscitrin and Azadiradionolide in active site of Angiotensin converting enzyme in

water at 300 K. To examine the structural stability of Angiotensin converting enzyme and complex with beta-sitosterol, hibiscitrin and Azadiradionolide. We monitored the time evolution plot of all C_{α} atom RMSD, R_g , RMSF and SASA. In the fourth parameter the angiotensin converting enzyme with beta sitosterol complex has shown a reduced SASA from 310 nm² to 285 nm² up to 5 ns. The solvent accessibility surface area for the of angiotensin converting with beta sitosterol complex indicated a more compact size at the end of the simulation period. SASA. In this case of hibiscitrin (depicted in green), was reduced from 310 nm² to 290 nm² and in the case of azadiradionolide reduced from 310 nm² to 280 nm². This indicates that beta sitosterol complex will provide more compactness than the hibiscitrin and azadiradionolide, and aromatic ring has less exposed the later on complex. We have detected that complex of protein and compounds shows relatively higher SASA value than wild type protein this could be explain by the presence of relatively larger hydrophobic packed core region in native protein as compare to protein compound complex. In one such similar studies these parameters (RMSD, R_g , SASA and RMSF) were studied by Fang *et al.*, (2019) for ACE that support that support our study.

Radius of gyration

Another important parameter, radius of gyration (R_g) is used to determine the dynamic adaptability of A CE in water and three different compounds which namely, beta sitosterol, hibiscitrin and azadiradionolide. A time evolution R_g plot of backbone atoms (Figure-9 A) shows that the native conformation of ACE is retained in water R_g trajectory in water (Black) quickly achieves the stable equilibrium in few nanoseconds. The average R_g values of 2.37 ± 0.01 nm is maintained throughout the simulation period. In the presence of beta sitosterol (depicted in red), 298 K temperature, the system is decreased in the fluctuation of trajectory is suggested that initial compaction in structure with comparison to water (depict in black) an average value is 2.31 ± 0.02 . while the previous condition is remaining same and only change the compound instead of Beta sitosterol to used hibiscitrin (depicted in green). Now, the system is gradually increase the fluctuation throughout the simulation relatively less stable than Beta sitosterol, and the average R_g values of 2.34 ± 0.04 which indicates a less stable structure and system is expanded. In the presence of Azadiradionolide, 298 K temperature, the Angiotensin converting enzyme is more stabilized than other three compounds. In R_g trajectory of Azadiradionolide (Blue line) depicted that quickly achieves stability at 5 ns and system remain stable and the average R_g values of 2.28 ± 0.01 . nrg for native

protein is comparatively more than protein compound complex. The Rg results shows that native protein was more compact after the binding of compound.

Root means square distance (RMSD)

RMSD are calculated as a function of time, with respect to the initial conformation and its shown in Figure-9 B. Visual inspection of this plot show that Angiotensin converting enzyme in water (black) is unstable during the 0-10 ns time interval and after 10-30 ns system have attained the equilibrium is maintained till the end of simulation of 30 ns. Addition of in the presence of Beta sitosterol (depicted in red), 298 K temperature to the system resulted in structural perturbation is slightly come into picture at time 0–10 ns which is relatively more than water and the simulation end up with an increase in RMSD of ~0.012 nm as compare to native condition. The remaining condition is remain intact only compound is change Hibiscitrin (depicted in green) the morphology of structure is change more than Beta sitosterol and the system remain unstable during the entire time evolution of simulation. The presence of azadiradionolide (depicted in blue) at 298 K temperature, the ACE in presence of azadiradionolide the system is unstable during the 0-10 ns time interval and after 10-30 ns system. While the behavior of morphology is in between the Beta sitosterol and Hibiscitrin.

Root means square fluctuation (RMSF)

To capture the dynamics progression of Angiotensin converting or flexibility of structure in water and three different complex with compounds of the Beta-sitosterol (depicted in red), Hibiscitrin (depicted in green) and are Azadiradionolide (depicted in blue) are depicted in Figure-10 A. RMS fluctuations showed that the most flexible residues 291–301, 338-354 are located in loop regions and 324–347 are located in the loop regions, which connect the Beta-sheets to alpha-helices. RMS fluctuation is more in the of Angiotensin converting +Azadiradionolide (depicted in blue) complex compared to the protein +beta sitosterol (depicted in red) complex. Comparatively, Angiotensin converting +Hibiscitrin (depicted in green) showed high fluctuations at 53–65, 66–144, 145–192 positions, these amino acids mostly constituting the loop region, alpha helices played no role in Azadiradionolide (depicted in blue) binding.

Solvent accessible surface area (SASA)

The SASA for Angiotensin converting + water (in depicted black colour) was 190nm² at the beginning of the simulation and retained the area throughout the simulation period shown in the

Figure 10 B Which is quite stable, apart from this addition of in the presence of Beta sitosterol (depicted in red), 298 K temperature to the system the solvent accessible surface area (SASA) for Angiotensin converting + beta sitosterol was observed to be 310 nm² at the start of simulation; as dynamics proceeded, it continuously decreased to 285 nm². The Angiotensin converting + beta sitosterol complex has shown a reduced SASA from 310 nm² to 285 nm² up to 5 ns.

The solvent accessibility surface area for the of Angiotensin converting + beta sitosterol complex indicated a more compact size at the end of the simulation period. SASA. In this case of Hibiscitrin (depicted in green), was reduced from 310 nm² to 290 nm² and in the case of azadiradionolide reduced from 310 nm² to 280 nm². This indicate that beta sitosterol complex is provide more compactness than the Hibiscitrin and azadiradionolide, and aromatic ring has less exposed the later on complex. We have detected that complex of protein and or compounds shows relatively higher SASA value than wild type protein this could be explain by the presence of relatively larger hydrophobic packed core region in native protein as compare to protein compound complex.

Cell toxicity assay

To determine the optimum concentration of the fractionated compounds for biological assays the cell toxicity was determined by MTT assay. The chloroform fraction containing predominantly beta-sitosterol, Hibiscitrin and azadiradionolide compounds were subjected to MTT assay on BHK-21 cell lines. The results revealed that with the treatment of bioactive fraction bearing hibiscitrin (HS) compound showed 99.34% viability at 970ug/ml followed by beta sitosterol (BS) with 99.13% viability at 970ug/ml and azadiradionolide (AZ) with 98.67% viability at 970ug/ml. Thus, results indicated that these compounds were least toxic to the cells, further suggesting these compounds are biocompatible and can be utilized as a drug (Figure-11). The Nemudzivhadi and Masoko (2014) showed the 90% cell viability of bioactive compound from *Ricinus communis*. In another study by Raiola *et al.*, (2016) found the 80% cell viability on HEK- 293 cell lines by the extract of tomatoes which is lower from the present study. In support to our findings Arevalo *et al.*, (2018) found 50% cell viability by the extract of *M. oleifera* and *A. indica* on MDCK cell lines.

Summary

The present study suggested the consideration of medicinal plants of Himachal Pradesh for targeting the role in hypertension. The isolated and characterized bioactive molecule from *Moringa oleifera*, *Hibiscus sabdariffa* and *Azadirachta indica* were found to be beta-sitosterol, hibiscitrin

and azadiradionolide respectively. The most potent molecule to inhibit the ACE of these was hibiscitrin. So this compound could play a significant role in inhibiting the conversion of angiotensin I to angiotensin II to control the systolic blood pressure. The current study delivers a new perspective for the drug development against systolic blood pressure regulation and also opens new horizons for considering alternate highly potent drug target for hypertension.

References

1. Agrawal DP. Medicinal properties of neem: new findings. History of Indian science and. 2001.
2. Al Disi SS, Anwar MA, Eid AH. Anti-hypertensive herbs and their mechanisms of action: part I. Frontiers in pharmacology. 2016 Jan 19; 6:323.
3. Al-Hashemi ZS, Hossain MA. Biological activities of different neem leaf crude extracts used locally in Ayurvedic medicine. Pacific Science Review A: Natural Science and Engineering. 2016 Jul 1;18(2):128-31.
4. Biswas K, Chattopadhyay I, Banerjee RK, Bandyopadhyay U. Biological activities and medicinal properties of neem (*Azadirachta indica*). CURRENT SCIENCE-BANGALORE-. 2002 Jun 10;82(11):1336-45.
5. Cooper R, McFarlane-Anderson N, Bennett FI, Wilks R, Puras A, Tewksbury D, Ward R, and Forrester T. ACE, angiotensinogen and obesity: a potential pathway leading to hypertension. J Hum Hypertens. 1997; 11:107-11.
6. Corradi HR, Schwager SL, Nchinda AT, et al. Crystal structure of the N domain of human somatic angiotensin I-converting enzyme provides a structural basis for domain-specific inhibitor design. J Mol Biol 2006; 357: 964-974.
7. Delmondes P, Benício R. In silico study of the natural compounds inhibiting angiotensin converting enzyme II, 2017.
8. 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 medica. 1991 Apr;57(02):137-41.
9. Engwa GA. Free radicals and the role of plant phytochemicals as antioxidants against oxidative stress-related diseases. Phytochemicals: source of antioxidants and role in disease prevention. 2018 Nov 7:49-73.

10. Ghersi D, Singh M. mol BLOCKS: decomposing small molecule sets and uncovering enriched fragments. *Bioinformatics* 2014; 30: 2081-3.
11. Ghimeray AK, Jin CW, Ghimire BK, Cho DH. Antioxidant activity and quantitative estimation of azadirachtin and nimbin in *Azadirachta indica* A. Juss grown in foothills of Nepal. *African Journal of Biotechnology*. 2009;8(13).
12. Gupta R. Trends in hypertension epidemiology in India. *J Hum Hypertens* 2004; 18:73–78.
13. Hafeez A, Askari H, Khan N, Akram M, Hanif S, Urooj N. Molecular modeling of plant flavonoids as angiotensin converting enzyme (ACE) inhibitors in hypertension: A docking study. *World Journal of Pharmaceutical Sciences*. 2014;2(5):499-506.
14. Hashmat I, Azad H, Ahmed A. Neem (*Azadirachta indica* A. Juss)-A nature's drugstore: an overview. *Int Res J Biol Sci*. 2012 Oct;1(6):76-9.
15. Hisle MS, Meier MS, Toth DM. Accelerating AutoDock Vina with Containerization. In *Proceedings of the Practice and Experience on Advanced Research Computing* 2018.
16. Kaminidevi S. Antimicrobial activity of methanolic extracts of indigenous traditional Indian folk Medicinal Plant, *Gnaphalium polycaulon*. *International Journal of Green Pharmacy (IJGP)*. 2015 Mar 17;9(1):39-44.
17. Kashyap M, Jaiswal V, Farooq U. Prediction and analysis of promiscuous T cell-epitopes derived from the vaccine candidate antigens of *Leishmania donovani* binding to MHC class-II alleles using *in silico* approach. *Infect, Genet and Evol*. 2017; 53: 107-15.
18. Kaurinovic B, Vastag D. Flavonoids and phenolic acids as potential natural antioxidants. *In Antioxidants* 2019 Feb 1. IntechOpen.
19. Khatua S, Ghosh S, Acharya K. A simplified method for microtiter based analysis of in vitro antioxidant activity. *Asian Journal of Pharmaceutics*. 2017 Apr 1;11(2):S327-35.
20. Khatua, Somanjana & Ghosh, Sandipta & Acharya, Krishnendu. (2017). Simplified methods for microtiter based analysis of in vitro antioxidant activity. *Asian Journal of Pharmaceutics*. 11.
21. Kumari R, Dubey RC, Kumar S. Antibacterial Efficacy of Acetone Soluble Oil of *Azadirachta indica* on Some Bacterial Strains. *J Plant Pathol Microbiol*. 2018;9(447):2.
22. Laskar, Monjur & Duttachoudhury, Manabendra. (2014). Screening of Some Triterpenoids Isolated from *Terminalia arjuna* as Inhibitors of Angiotensin Converting Enzyme to

- Prevent Cardiovascular Diseases. Research & Reviews: A Journal of Drug Design & Discovery (ISSN: 2349-9036). 1. 12-19.
23. Leeder S, Raymond S, Greenberg H, Liu H. A race against time. The challenge of cardiovascular disease in developing economies. New York: Columbia University; 2004.
24. Lloyd-Sherlock P, Beard J, Minicuci N, Ebrahim S, Chatterji S. Hypertension among older adults in low-and middle-income countries: prevalence, awareness and control. International journal of epidemiology. 2014 Feb 1;43(1):116-28.
25. Luis A, Corpas FJ, López-Huertas E, Palma JM. Plant superoxide dismutases: function under abiotic stress conditions. In Antioxidants and antioxidant enzymes in higher plants 2018 (pp. 1-26). Springer, Cham.
26. Matkowski A, Tasarz P, Szypuła E. Antioxidant activity of herb extracts from five medicinal plants from Lamiaceae, subfamily Lamioideae. Journal of Medicinal Plants Research. 2008 Nov 30;2(11):321-30.
27. Muhammad SA, Fatima N. *In silico* analysis and molecular docking studies of potential angiotensin-converting enzyme inhibitor using quercetin glycosides. Pharmacog Mag 2015; 11: 123.
28. Muhammad SA, Fatima N. *In silico* analysis and molecular docking studies of potential angiotensin-converting enzyme inhibitor using quercetin glycosides. Pharmacognosy magazine. 2015 May;11(Suppl 1):S123.
29. Natesh R, Schwager SL, Sturrock ED, et al. Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. Nature 2003; 421: 551.
30. Ondua M, Njoya EM, Abdalla MA, McGaw LJ. Anti-inflammatory and antioxidant properties of leaf extracts of eleven South African medicinal plants used traditionally to treat inflammation. Journal of ethnopharmacology. 2019 Apr 24;234:27-35.
31. Peach MJ. Renin-angiotensin system: biochemistry and mechanisms of action. Physiological reviews. 1977 Apr;57(2):313-70.
32. Pescatello LS, Franklin BA, Fagard R, Farquhar WB, Kelley GA, Ray CA. Exercise and hypertension. Medicine & Science in Sports & Exercise. 2004 Mar 1;36(3):533-53.
33. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera-a visualization system for exploratory research and analysis. J Comput Chem 2004; 25: 1605-1612.

34. Rao UM, Ahmad BA, Mohd KS. In vitro nitric oxide scavenging and anti-inflammatory activities of different solvent extracts of various parts of *Musa paradisiaca*. *Malaysian J. Anal. Sci.* 2016;20(5):1191-202.
35. Richardson DW, Wyso EM, Magee JH, Cavell GC. Circulatory effects of guanethidine: Clinical, renal, and cardiac responses to treatment with a novel antihypertensive drug. *Circulation.* 1960 Aug 1;22(2):184-90.
36. Sahrawat A, Sharma J. Phytochemical analysis and Antibacterial properties of *Azadirachta indica* (Neem) leaves extract against *E. coli*. *Journal of Pharmacognosy and Phytochemistry.* 2018;7(4):1368-71.
37. Septiana I, Nguyen TT, Lim S, Lee S, Park B, Kwak S, Park S, Kim SB, Kim D. Enzymatic synthesis and biological characterization of a novel mangiferin glucoside. *Enzyme and Microbial Technology.* 2020 Mar 1;134:109479.
38. Sridevi P, Prashanth KS, Bhagavan MR. Angiotensin converting enzyme: A target for anti-hypertensive drugs. *Int J Res Pharm Biomed Sc.* 2011; 2:63-72.
39. Subapriya R, Nagini S. Medicinal properties of neem leaves: a review. *Current Medicinal Chemistry-Anti-Cancer Agents.* 2005 Mar 1;5(2):149-56.
40. Sultana B, Anwar F, Przybylski R. Antioxidant activity of phenolic components present in barks of *Azadirachta indica*, *Terminalia arjuna*, *Acacia nilotica*, and *Eugenia jambolana* Lam. trees. *Food Chemistry.* 2007 Jan 1;104(3):1106-14.
41. Swales JD. Renin-angiotensin system in hypertension. *Pharmacology & therapeutics.* 1979 Jan 1;7(1):173-201.
42. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* 2010; 31: 455-61.
43. Tutor JT, Chichioco-Hernandez CL. Angiotensin-Converting Enzyme Inhibition of Fractions from *Eleusine indica* Leaf Extracts. *Pharmacognosy Journal.* 2018;10(1).
44. Van Bortel LM, Struijker-Boudier HA, Safar ME. Pulse pressure, arterial stiffness, and drug treatment of hypertension. *Hypertension.* 2001 Oct 1;38(4):914-21.
45. Wolfender JL, Terreaux C, Hostettmann K. The importance of LC-MS and LC-NMR in the discovery of new lead compounds from plants. *Pharmaceutical biology.* 2000 Dec 1;38(sup1):41-54.

46. Basu S, Hazra B. Evaluation of nitric oxide scavenging activity, in vitro and ex vivo, of selected medicinal plants traditionally used in inflammatory diseases. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*. 2006 Oct;20(10):896-900.
47. Srivastava P, Kasoju N, Bora U, Chaturvedi R. Dedifferentiation of leaf explants and cytotoxic activity of an aqueous extract of cell cultures of *Lantana camara* L. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 2009 Oct 1;99(1):1.
48. Khan H, Jaiswal V, Kulshreshtha S, Khan A. Potential Angiotensin Converting Enzyme Inhibitors from *Moringa oleifera*. *Recent Patents on Biotechnology*. 2019 Sep 1;13(3):239-48.
49. Nemudzivhadi V, Masoko P. In vitro assessment of cytotoxicity, antioxidant, and anti-inflammatory activities of *Ricinus communis* (Euphorbiaceae) leaf extracts. *Evidence-Based Complementary and Alternative Medicine*. 2014; 1-8.
50. Raiola A, Del Giudice R, Monti D, Tenore G, Barone A, Rigano M. Bioactive compound content and cytotoxic effect on human cancer cells of fresh and processed yellow tomatoes. *Molecules*. 2016; 21(1):33.
51. Arevalo-Híjar L, Aguilar-Luis MÁ, Caballero-García S, Gonzáles-Soto N, Valle-Mendoza D. Antibacterial and Cytotoxic Effects of *Moringa oleifera* (Moringa) and *Azadirachta indica* (Neem) Methanolic Extracts against Strains of *Enterococcus faecalis*. *International Journal of Dentistry*. 2018; 1(5).
52. Fang L, Geng M, Liu C, Wang J, Min W, Liu J. Structural and molecular basis of angiotensin-converting enzyme by computational modeling: Insights into the mechanisms of different inhibitors. *PloS one*. 2019; 14(4):0215609.
53. Pierre LL, Moses MN. Isolation and characterisation of stigmasterol and β -sitosterol from *Odontonema strictum* (acanthaceae). *Journal of Innovations in Pharmaceuticals and Biological Sciences*. 2015;2(1):88-95.
54. Khan MY, Kumar V. Mechanism & inhibition kinetics of bioassay-guided fractions of Indian medicinal plants and foods as ACE inhibitors. *Journal of Traditional and Complementary Medicine*. 2019 Jan 1;9(1):73-84.
55. Yusuf S, Sleight PE, Pogue JF, Bosch J, Davies R, Dagenais G, Heart Outcomes Prevention Evaluation Study Investigators. Effects of an angiotensin-converting-enzyme inhibitor,

ramipril, on cardiovascular events in high-risk patients. The New England journal of medicine. 2000 Jan 20;342(3):145.

56. Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE. A novel angiotensin-converting enzyme–related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circulation research. 2000 Sep 1;87(5):e1-9.

Table 1: IC₅₀ value of Nitric oxide (NO) free radical scavenging activity and Superoxide (O₂⁻) radical scavenging activity

S.NO	Sample	Nitric oxide (NO) free radical scavenging activity (IC ₅₀)	Superoxide (O ₂ ⁻) radical scavenging activity (IC ₅₀)
1	<i>Azadirachta indica</i>	352.7473	269.7179
2	<i>Moringa oleifera</i>	350.618	271.5385
3	<i>Hibiscus sabdariffa</i>	317.74	251.8527
4	Ascorbic acid (Positive control)	302.2222	243.3071

Table 2: IC₅₀ value of plant extract

S.No	Plants	IC ₅₀ µg/mL
1	<i>Moringa oleifera</i>	294.397
2	<i>Hibiscus sabdariffa</i>	267.722
3	<i>Azadirachta indica</i>	255.991

Table 3: *In silico* study of isolated compounds with angiotensin converting enzyme

S.No	Plant name	Chemical compounds	Type of compound	Molecular formula	Molecular weight	Binding affinity
1.	<i>Hibiscus sabdariffa</i>	Hibiscitrin	Natural	C ₂₁ H ₂₀ O ₁₄	496.4g/mol	-12.3 kcal/mol
2.	<i>Azadirachta indica</i>	Azadiradionolide	Natural	C ₂₈ H ₃₄ O ₆	466.6g/mol	-11.3 kcal/mol
3.	<i>Moringa oleifera</i>	Beta sitosterol	Natural	C ₂₉ H ₅₀ O	414.7g/mol	-11.2 kcal/mol
6.	Positive control	Enalapril (standard drug)	Synthetic	C ₂₀ H ₂₈ N ₂ O ₄	376.453g/mol	-8.1kcal/mol
		Captopril (standard drug)	Synthetic	C ₉ H ₁₅ NO ₃ S	217.283g/mol	-5.6kcal/mol

Table 4: Hydrogen bonding interaction of the compounds with active amino acid residues of angiotensin converting enzyme

S.No	Compound	Active residues	Hydrogen Bonding length	Average bond length
1	Hibiscitrin	TYR (498) LYS (489) HIS (263) TYR (358) SER (260) ASN (263) GLY (254)	3.21 3.24 3.20 3.09 2.92 2.95 2.71	2.9
2	Azadiradionolide	THR (144) ASN (263) ASN (145)	3.00 2.80 3.09	2.6
3	Beta sitosterol	TYR (372), ASP (366), HIS (388), GLY (382)	2.70 3.01 3.09 2.74	2.8
13	Captopril	ARG (522) GLU (411)	1.75 2.85	2.1
14	Enalapril	TYR (523) HIS (353)	2.55 2.87	2.7

Table 5: Toxicity Profile of the Potential Compounds (WI: Weak inhibitor, NI: Non-inhibitor, NT: Non-toxic, T: Toxic NC: Non-carcinogen, C: Carcinogen, HT: High toxic, LT: Low toxic, NRB: Not readily biodegradable)

Parameters ↓ Compounds	Human Ether-a-gogo- Related Gene Inhibition	AMES Toxicity	Carcinogens	Fish Toxicity	Tetrahymena Pyriformis Toxicity	Honey Bee Toxicity	Acute Oral Toxicity
Beta-sitosterol	WI	NT	NC	HT	HT	HT	IV
Hibiscitrin	WI	NT	NC	HT	HT	HT	I
Azadiradionolide	WI	NT	NC	HT	HT	HT	III

Figure 1: A) percentage inhibition of nitric oxide B) percentage inhibition of SOD

Figure:2 Mass spectrum of different chromatographic peaks of *Moringa oleifera* fraction

Figure 3: Mass spectrum of different chromatographic peaks of *Azadirachta indica* fraction

Figure 4: Mass spectrum of different chromatographic peaks of *Hibiscus sabdariffa* fraction

Figure: 5 Percentage inhibition of *Moringa oleifera* MO, *Azadirachta indica* (AI), *Hibiscus sabdariffa* (HS), and Positive control (PC)

Figure 6: Ligplot of Hibiscitrin ligand from *Hibiscus sabdariffa* fraction with active amino acid residue of ACE

Figure 7: Ligplot of beta sitosterol ligand from *Moringa oleifera* fraction with active amino acid residue of ACE

Figure 8: Ligplot of Azadiradionolide ligand from *Azadirachta indica* fraction with active amino acid residue of ACE

Figure 9: Radius of gyration, RMSD analysis of ACE and bioactive compounds over 30ns simulation

Figure 10: RMSF of ACE with active amino acid residues and SASA of ACE and bioactive compounds over 30ns

Figure 11: Percentage viability of the compounds Hibiscitrin (HS) beta-sitosterol (BS), and Azadiradionolide (AZ)

Supplementary file

Figure 1: ^1H NMR spectrum of different chromatographic peaks of *Moringa oleifera* fraction

Table 1: Proton shift position on carbon atom of *Moringa oleifera* fraction

Sr. No	Carbon	Proton shift (ppm)
1	C ₁	1.39, 1.38
2	C ₂	1.579, 1.307
3	C ₃	3.658
4	C ₄	2.291, 2.068

5	C ₅	No proton
6	C ₆	5.386
7	C ₇	2.068, 1.794
8	C ₈	1.459
9	C ₉	No proton
10	C ₁₀	1.600, 1.367
11	C ₁₁	1.547; 1.367
12	C ₁₂	1.563
13	C ₁₃	No proton
14	C ₁₄	1.403
15	C ₁₅	1.600, 1.307
16	C ₁₆	1.600, 1.307
17	C ₁₇	1.47
18	C ₁₈	No proton
19	C ₁₉	1.30
20	C ₂₀	1.459
21	C ₂₁	No proton
22	C ₂₂	1.647
23	C ₂₃	1.307
24	C ₂₄	1.307
25	C ₂₅	1.459
26	C ₂₆	1.794
27	C ₂₇	1.307
28	C ₂₈	1.307
29	C ₂₉	1.563
30	C ₃₀	1.307
31	C ₃₁	1.459
32	C ₃₂	1.411
33	C ₃₃	1.403

Figure 2: ^{13}C NMR spectrum of different chromatographic peaks of *Moringa oleifera* fraction

Table 2: Carbon shift position on carbon atom of *Moringa oleifera* fraction

Sr. No.	Carbon	Carbon shift (ppm)
1	C ₁	34.88
2	C ₂	31.92
3	C ₃	64.11
4	C ₄	34.88
5	C ₅	34.88
6	C ₆	64.11
7	C ₇	31.92
8	C ₈	31.92
9	C ₉	62.78
10	C ₁₀	25.62
11	C ₁₁	25.62
12	C ₁₂	62.78
13	C ₁₃	34.88
14	C ₁₄	34.88
15	C ₁₅	20.55
16	C ₁₆	62.78
17	C ₁₇	34.88
18	C ₁₈	No carbon
19	C ₁₉	13.70
20	C ₂₀	19.3
21	C ₂₁	19.3
22	C ₂₂	34.88
23	C ₂₃	34.42
24	C ₂₄	22.69
25	C ₂₅	34.88
26	C ₂₆	30.72
27	C ₂₇	20.55

28	C ₂₈	22.69
29	C ₂₉	22.69
30	C ₃₀	13.70
31	C ₃₁	No carbon
32	C ₃₂	No carbon
33	C ₃₃	No carbon

Figure 3: ¹H NMR spectrum of different chromatographic peaks of *Azadirachta indica* fraction

Table 3: Proton shift position on carbon atom of *Azadirachta indica* fraction

Sr. No.	Carbon neem	Proton shift (ppm)
1	C₁	No proton
2	C₂	No proton
3	C₃	No proton
4	C₄	No proton
5	C₅	No proton
6	C₆	No proton
7	C₇	No proton
8	C₈	No proton
9	C₉	No proton
10	C₁₀	No proton
11	C₁₁	No proton
12	C₁₂	No proton
13	C₁₃	No proton
14	C₁₄	1.153, 1.267
15	C₁₅	1.759, 1.482
16	C₁₆	1.153, 1.267
17	C₁₇	No proton
18	C₁₈	2.720
19	C₁₉	1.267
20	C₂₀	1.267
21	C₂₁	5.908
22	C₂₂	4.240
23	C₂₃	1.267
24	C₂₄	No proton
25	C₂₅	2.720
26	C₂₆	5.908
27	C₂₇	No proton
28	C₂₈	No proton

29	C₂₉	No proton
30	C₃₀	No proton
31	C₃₁	4.240
32	C₃₂	1.482
33	C₃₃	1.609
34	C₃₄	1.482
35	C₃₅	1.267
36	C₃₆	1.267
37	C₃₇	2.222

Figure 4: ¹³C NMR spectrum of different chromatographic peaks of *Azadirachta indica* fraction

Table 4: Carbon shift position on carbon atom of *Azadirachta indica* fraction

Sr. No.	Carbon	Carbon shift (ppm)
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1	C ₁	No carbon
2	C ₂	No carbon
3	C ₃	No carbon
4	C ₄	No carbon
5	C ₅	No carbon
6	C ₆	No carbon
7	C ₇	38.96
8	C ₈	38.96
9	C ₉	38.96
10	C ₁₀	38.96
11	C ₁₁	38.96
12	C ₁₂	76.71
13	C ₁₃	77.53
14	C ₁₄	15.14
15	C ₁₅	29.10
16	C ₁₆	29.68
17	C ₁₇	38.96
18	C ₁₈	38.96
19	C ₁₉	15.14
20	C ₂₀	19.74
21	C ₂₁	77.53
22	C ₂₂	77.53
23	C ₂₃	22.67
24	C ₂₄	77.53
25	C ₂₅	77.53
26	C ₂₆	77.53
27	C ₂₇	77.53
28	C ₂₈	77.53
29	C ₂₉	77.53
30	C ₃₀	77.53

31	C ₃₁	76.71
32	C ₃₂	No carbon
33	C ₃₃	No carbon
34	C ₃₄	19.74
35	C ₃₅	22.67
36	C ₃₆	22.67
37	C ₃₇	22.67

Figure 5: ^1H NMR spectrum of different chromatographic peak of *Hibiscus sabdariffa* fraction

Table 5: Proton shift position on carbon atom of *Hibiscus sabdariffa* fraction

Sr. No.	Carbon	Proton shift (ppm)
1	C ₁	No proton
2	C ₂	No proton
3	C ₃	No proton
4	C ₄	No proton
5	C ₅	No proton

6	C_6	5.423
7	C_7	No proton
8	C_8	No proton
9	C_9	No proton
10	C_{10}	No proton
11	C_{11}	No proton
12	C_{12}	No proton
13	C_{13}	5.423
14	C_{14}	No proton
15	C_{15}	No proton
16	C_{16}	No proton
17	C_{17}	5.423
18	C_{18}	5.350
19	C_{19}	5.350
20	C_{20}	5.350
21	C_{21}	No proton
22	C_{22}	No proton
23	C_{23}	5.423
24	C_{24}	3.710
25	C_{25}	3.688
26	C_{26}	3.688
27	C_{27}	3.757
28	C_{28}	3.757; 2.828
29	C_{29}	2.206
30	C_{30}	3.688
31	C_{31}	2.828
32	C_{32}	2.306
33	C_{33}	5.350
34	C_{34}	5.350
35	C_{35}	5.350

8	C ₈	128.30
9	C ₉	131.97
10	C ₁₀	No carbon
11	C ₁₁	No carbon
12	C ₁₂	127.12
13	C ₁₃	127.12
14	C ₁₄	127.12
15	C ₁₅	130.23
16	C ₁₆	131.97
17	C ₁₇	77.33
18	C ₁₈	No carbon
19	C ₁₉	No carbon
20	C ₂₀	No carbon
21	C ₂₁	No carbon
22	C ₂₂	No carbon
23	C ₂₃	127.12
24	C ₂₄	76.69
25	C ₂₅	77.01
26	C ₂₆	76.69
27	C ₂₇	60.04
28	C ₂₈	60.04
29	C ₂₉	No carbon
30	C ₃₀	No carbon
31	C ₃₁	No carbon
32	C ₃₂	No carbon
33	C ₃₃	No carbon
34	C ₃₄	No carbon
35	C ₃₅	No carbon