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Angiotensin converting enzyme inhibitory effect of *Medicago sativa* root extract

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Abstract : Angiotensin-converting enzyme plays an important role in treatment of hypertension. The purpose of present study is to estimate *in vitro*(angiotensin converting enzyme) ACE inhibitory activity of *Medicago sativa* Linn.Root. The ability to inhibit ACE was measured by direct spectrophotometric method. ACE inhibitory study was evaluated by using Lisinopril as standard. Extract were prepared by successive maceration with toluene, chloroform, methanol and water. Among all extract tested of root methanol extract showed the maximum and dose dependent ACE inhibitory activity at IC_{50} value of 34.43±0.31 µg/ml concentration. Standard Lisinopril possess inhibition of ACE at IC_{50} value of 0.2599±0.02ng/ml. Present study shows root methanol extract of *Medicago sativa* as potential ACE inhibitor. *Medicago sativa* can further explored for active constituent responsible for ACE inhibitory activity.

Keywords : Hypertension,Alfalfa, Angiotensin converting enzyme, *Medicago sativa.*

Introduction

Globally cardiovascular disease accounts for approximately 17 million deaths a year, nearly one third of the total, 9.4 million deaths occurs due to complications of hypertension every year. Hypertension is responsible for at least 45% of deaths due to heart disease[1]. There are a number of choices for the treatment of hypertension are available. Some treatments include diuretics, β-blockers, calcium channel blockers and angiotensin II receptor blockers, and one of the most effective medications for the treatment of hypertension is angiotensin converting enzyme inhibitors[2].However,these synthetic drugs are believed to havecertain side effects such as hypotension, reduced renal function, dry cough, skinrashes, taste disturbances and fetal abnormalities.[3]A numberof compounds and extracts from different plants and food havebeen identified to possess *in vitro* ACE inhibitory activity which can be safer and economical alternative for treatment of hypertension.[4-7]

Medicago sativa Linn. (leguminosae) is known as the "father of all foods" (*al-fal-fa*), a perennial herb plant species that originated in Asia. Pharmacological reports revealed that it is used as neuroprotective, hypocholesterolemic, antioxidant, antiulcer, antimicrobial, hypolipidemic, estrogenic, and in the treatment of atherosclerosis, heart disease, stroke, cancer, diabetes and menopausal symptoms in women.[8-12]According to literature survey *Medicago sativa* is a preventive of high bloodpressure.[13] Angiotensin-converting enzyme inhibitor plays an important role in treating hypertension by causing blood vessels to constrict by converting the precursor angiotensin I into angiotensin II which is the peptide responsible in triggering blood pressure increasing mechanisms. [14]According to literature survey *Medicago sativa* seed possess 69% Angiotensin

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converting enzyme inhibition at 0.33 mg/ml.[15]The literature survey revealed that root of *Medicago sativa* have not been evaluated for Angiotensin converting enzyme inhibition activity so far. The purpose of this study was to estimate ACE inhibition activity by using an *in vitro*direct spectrophotometric assay.The assay method is based on the hydrolysis of the substrate HHL(hippuryl-l-histidyl-l-leucine) by ACE, and measuring the amount released HA(Hippuric acid) by specific colorimetric reaction of HA with Benzenesulfonyl chloride (BSC) in the presence of Quinoline.

Figure 1.*Medicago sativa* root

Experimental

Chemicals and instruments

Angiotensin converting enzyme (ACE) and hippuryl-L-histidyl- L-leucine (HHL) substrate were purchased from Sigma Aldrich, USA. Lisinopril was obtained from Lupin Pharmaceuticals (India). Hydrochloric acid, Boric acid, Borax, Toluene, chloroform, methanol, Sodium hydroxide were purchased from Merck India limited. All other reagents used were of analytical grade. Doubledistilled water was used throughout. Instrument used for spectrophotometric analysis was Molecular devices SpectraMac M2e, SoftMax Pro7.

Crude drug collection

Medicago sativa root (Figure 1) was (12" to 18") collected from Than, District Rajkot in December & March 2016 before & after flowering respectively. A Herbarium was prepared and authenticated by Dr. Kunjal Soni and deposited in herbarium repository of School of Pharmacy SOP/COG/473/2016.

Extraction

Root of *Medicago sativa* were cleaned and dried under shade. Sliced root were than powdered. Three hundred gram of root powder was macerated with toluene for 24 hours at room temperature and extract filtered with the help of muslin cloth, marc produced were treated with chloroform, methanol and water subsequently by same procedure. Extracts were than subjected for evaporation on heating mantle below 40˚C, and further drying was done on water bath.1 mg/ml stock solution of *Medicago sativa* root Toluene, chloroform, methanol and water extracts were prepared using Dichloromethane as solvent.

ACE inhibition assay

ACE inhibitory activity was measured by direct spectrophotometry method.[16,17]For each assay, a sample solution ofACE inhibitor (*Medicago sativa* extract and Lisinopril) (20 µl) (at 8, 12, 36 and 108 µg/ml concentration) with 50 µl of 5mM HHL in 100mMsodium borate buffer (pH 8.3) containing 300mM Sodium chloride was preincubated at 37 ◦C for 5 min. The reaction was initiatedby the addition of 10 µl of ACE solution (0.1 U/ml),and the mixture was incubated at 37 ◦C for 30 min. The reaction was stopped by adding 100 µl of 1M Hydrochloric acid. The HA released from HHL by ACE was monitored according to the method of Wang et al.[16]Sodium borate buffer was then added to the reaction mixture to a volume of0.5 ml. After that 600 µl Quinoline and 200 µl Benzenesulfonyl chloride was added to reaction mixture, and Incubated at 30 ◦C for 30

min in the darkness. 3700 µl of Ethanol was added to reaction mixture and reaction mixture was incubated at 30 ◦C for 30 min in the darkness and then measured at 492 nm.

The extent of inhibition was calculated as follows:

$$\textbf{ACE inhibitory activity (\%)} = \frac{(B-A)}{(B-C)} \textbf{ X 100}$$

Where *B* is the absorbance of control (buffer added instead of test sample), *C* the absorbance of the reaction blank (HClwasadded before ACE), and *A* is the absorbance in the presence ofsample.

Statistical analysis

All the experiments were carried out in triplicate and results were expressed as mean ± SEM, n=3. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assayed conditions and determined via nonlinear regression analysis bylog (inhibition) versus response (three parameter)using Graph Pad Prism version 7.03.

Results and Discussion

The ACE inhibitory activity of four different root extracts of *Medicago sativa* were evaluated by spectrophotometric method.[16,17] Effect ofACE inhibitory activity was performed by *in vitro* method using HHL as a substrate. ACE converts HHL into Hippuric acid and Histidine-Leucine. This method relies on spectrophotometric determination of Hippuric acid (HA) content by specific colorimetric reaction of HA with Benzene Sulfonyl Chloride (BSC) in the presence of Quinoline. Lisinopril was used as the standard drug. The results demonstrated that Methanol extract of *Medicago sativa* root (MSRM) showed highest inhibition of ACE in dose dependent manner with IC_{50}value of 34.43±0.31 µg/ml. Lisinopril showed IC_{50}value of 0.2599±0.02ng/ml concentration.(Table 1 & Figure 2)Chloroform and Toluene also showed inhibition of ACE but lower than MSRM and not in dose dependent manner. (Table 2 and Figure 3)While root water extract showed only 31 % ACE inhibition at 108 µg/ml.

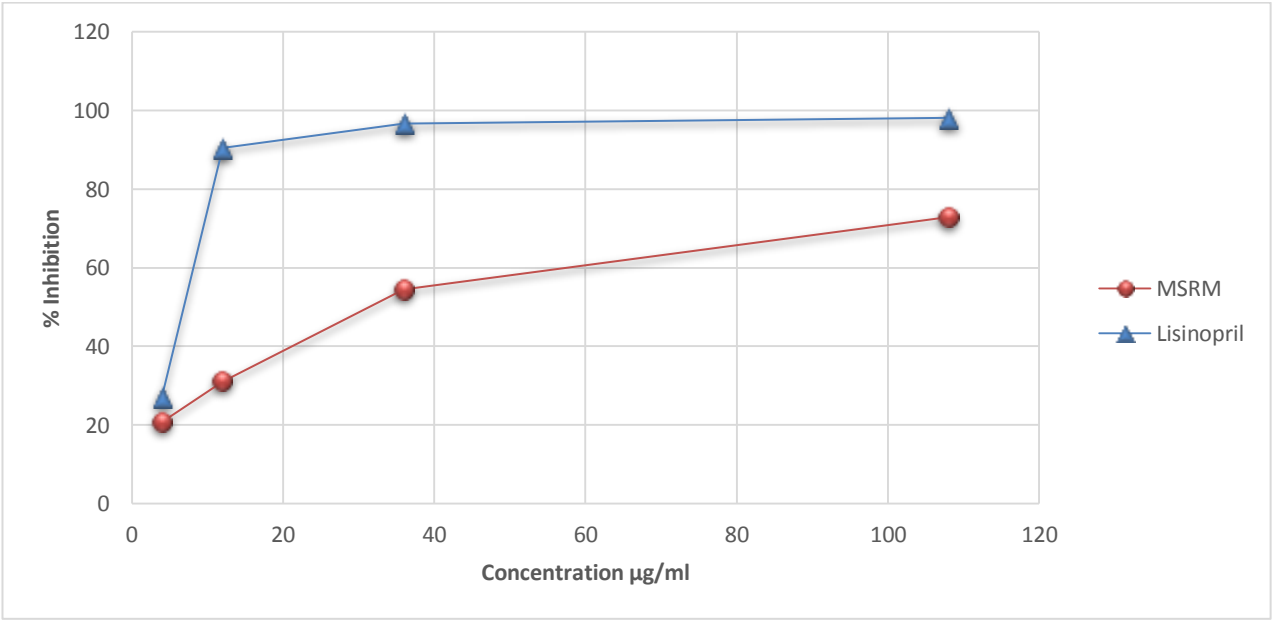

Figure 2: ACE inhibitory activity of methanol extract of *M. sativa* root and Lisinopril

Table 1. Percentage inhibition and IC_{50}values of Methanol extract of *M. sativa*

Extract/standard	Concentration (µg/ml)	Percentage Inhibition (%)	IC_{50}
Lisinopril	4	27.12±0.35	0.2599±0.02ng/ml
	12	90.46±0.57	
	36	96.73±0.76	
	108	98.14±0.34	
MSRM	4	20.72±0.64	34.43±0.31 µg/ml
	12	31.04±0.86	
	36	54.42±0.23	
	108	72.89±0.52	

All values are expressed as mean ± SEM.(n=3)MSRM- Medicago sativa root methanol extract

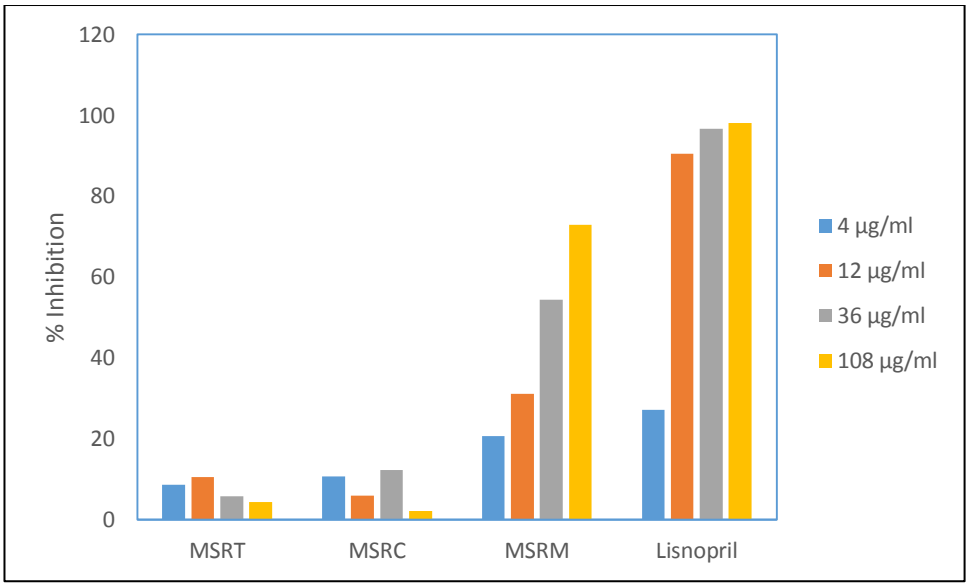

Figure 3: % Inhibition of ACE by MSRT, MSRC, MSRM and Lisinopril

Table 2. Percentage inhibition of chloroform and Toluene root extracts of *M. sativa*

Extract	Concentration (µg/ml)	Percentage Inhibition (%)
MSRT	4	8.65±0.82
	12	10.56±0.64
	36	5.68±0.57
	108	4.38± 0.38
MSRC	4	10.68±0.43
	12	5.85±0.32
	36	12.23±0.46
	108	2.14±0.23

All values are expressed as mean ± SEM, (n=3).MSRT- Medicago sativa root toluene extract, MSRC - Medicago sativa root chloroform extract

The present research suggests that among all other extract *Medicago sativa* root methanol extract possess active constituent responsible for ACE inhibitory activity and can be utilized for the treatment of hypertension. In addition, Antihypertensive activity is reported in traditional medicine and modern research.[13,15]Further investigations on the isolation of active compounds present in the methanol extract of root of *Medicago sativa* is necessary to identify a biologically active constituents for clinical use in the treatment of hypertension.

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