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Partial-purification and Characterization of Angiotensin Converting Enzyme Inhibitory Proteins from the Leaves and Seeds of *Moringa oleifera*

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Authors' contributions

This work was carried out in collaboration between all authors. Author AAM designed the study, wrote the protocol and supervised the work. Authors NTC and MI carried out all laboratories work and performed the statistical analysis. Author SAA managed the analyses of the study. Author CW wrote the first draft of the manuscript. Author AMW managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To partially-purify, characterize and investigate the inhibition pattern of proteins from the leaves and seeds of *Moringa oleifera.*
Methodology: Crude proteins obtained from the leaves and seeds were partially-purified by a two-step method: cold acetone precipitation and sephadex G-75 gel filtration chromatography and Angiotensin Converting Enzyme (ACE) inhibitory activities determined using Cushman and Cheung method with some modifications on the assay conditions. The effects of pH, temperature and some digestive enzymes on the inhibitory proteins were determined. The inhibition pattern was also

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investigated using Lineweaver-Burk plots.
Results: The ACE inhibitory proteins from the leaves and seeds were purified 8.41 and 12.40 folds with a yield of 38 and 35.18%, respectively on sephadex G-75 column. The optimum pH and temperature of the inhibitory proteins were 8.2 and 37.6°C, for leaves and 7.8 and 39°C, for seeds, respectively. The partially-purified ACE inhibitors exhibited a mixed type of inhibition. The K_m of inhibitory proteins obtained from the leaves increased from 3.51mM without the inhibitor to 4.79mM and 5.62mM in the presence of 0.5 and 1mg/ml of the inhibitor, respectively, while V_{max} decreased from 0.953µmol/min without inhibitor to 0.784µmol/min and 0.629µmol/min, in the presence of 0.5 and 1mg/ml of the inhibitor, respectively, with a K_i of 3.51mM. For the seeds, the K_m increased from 2.89mM (without inhibitor) to 5.747mM (at 1mg/ml inhibitor), while V_{max} decreased from 0.910 (without inhibitor) to 0.628µmol/min (at 1mg/ml inhibitor), with a K_i of 1.58mg/ml. The digestive enzymes, pepsin and trypsin significantly (P<0.05) reduced the activity of the partially-purified inhibitors from both leaves and seeds compared to captopril. In conclusion, ACE inhibitory proteins from *M. oleifera* may be beneficial as nutraceutical or drug for blood pressure regulation in hypertensive patients.

Keywords: Angiotensin converting enzyme; hypertension; Moringa oleifera, proteins

1. INTRODUCTION

Hypertension, a condition characterized by a systolic blood pressure ≥ 140mmHg and diastolic blood pressure ≥ 90mmHg [1] is one of the most common cardiovascular diseases and to this day remains one of the most important health problems [2]. Non-communicable diseases, such as cardiovascular diseases, have been shown to be a severe threat to global economic development due to the long-term cost of treatment and negative effects on productivity [3].Despite the large number and classes of drugs available for the treatment of hypertension, many more anti-hypertensive drugs, including angiotensin converting enzyme (ACE) inhibitors have been introduced within the last decade [4]. The guideline for the management of hypertension issued by World Health Organization and the International Society of Hypertension classify ACE inhibitors as the first line treatment together with diuretics and β-blockers [5].

Angiotensin converting enzyme is a zinc-dependent exopeptidase, that catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor [6]. It is an important drug target in the treatment of cardiovascular diseases due to its role as a key regulator of blood pressure in the renin-angiotensin-aldosterone and kallikrein-kinin systems [7]. Angiotensin converting enzyme also inactivates the vasodilator, bradykinin [8] thus, its inhibition is considered useful in the prevention and treatment of hypertension and related diseases [9]. Captopril, enalapril and lisinopril are synthetic ACE inhibitors known to regulate blood pressure [10], but have been demonstrated to have side effects, such as allergic reactions, skin rashes, cough and taste disturbances [9], hence interest in finding nontoxic, safe, and effective ACE inhibitors from natural sources. Studies have shown that plants with antioxidant properties possess potent ACE inhibitory activity. This has intensified the search for ACE inhibitory compounds from plants with strong antioxidant activities [11].

Moringa oleifera belongs to a monogenetic family called *Moringaceace*. It is commonly called “drumstick tree” in English and “Zogale” amongst the Hausas in northern Nigeria where it is commonly used. The cultivation and consumption of *M. oleifera* has increased markedly in last few years due to its potential health benefits and high nutritive value. The leaves have been reported to possess antihypertensive, hypocholesterolemic, antiulcer and wound healing properties [12-15]. Hydro-alcoholic extract of the plant has been shown to possess significant antioxidant potential by increasing the activities of catalase, glutathione peroxidase and glutathione reductase and decreasing hepatic malondialdehyde level [16]. However, there is paucity of information on the effect of the seeds and leaves on the activity of ACE.

In recent years, ACE inhibitors derived from food have been studied. Food intake is being increasingly considered as not only a source of nutrients but also a source of bioactive compounds, including bioactive peptides. These peptides may be present in food as natural

constinuents or be produced after enzymatic hydrolysis and are considered to be mild and safer without side effects, compared to synthetic drugs [17]. Although most ACE inhibitory peptides are present in an inactive form within the primary sequences of proteins, and have to be released and degraded in the animal or human body through either *in vitro* or *in vivo* proteolysis e.g. during gastrointestinal digestion or food processing, it is important that these peptides must be resistant to *in vivo* proteolysis once they are within the body in order to reach the desired target tissue or organ and exert an antihypertensive effect. Therefore, determining the effect of digestive enzymes on ACE inhibitors *in vitro* is an important means of over-coming poor solubility, absorption and availability *in vivo*. Thus, the present study is aimed at partially-purifying ACE-inhibitory proteins from the seeds and leaves of *Moringa oleifera*, characterize and investigate the effect of some gastrointestinal enzymes on its activity, as well as determine the pattern of inhibition of the proteins.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Pepsin, trypsin, hippury-histidine-leucine (HHL) and ACE (rabbit lungs), Pepsin (porcine stomach mucosa) and trypsin were obtained from Sigma Chemical Co. (St. Louis, MO, USA), sodium chloride, sodium diphosphate, sodium monophosphate, sodium hydroxide, hydrochloric acid, glycine, Bradford reagent, Bovine Serum Albumin, acetone, ethyl acetate, potassium chloride, potassium diphosphate, potassium monophosphate and all other reagents and chemicals used were of analytical grade.

2.2 Collection and Identification of Plant

Fresh leaves and seeds of *M. oleifera* were obtained from the National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria, and authenticated with the Botanist at the Department of Biological Sciences, Ahmadu Bello University Zaria, Kaduna State, Nigeria.

2.3 Partial-purification of ACE Inhibitory Proteins from *Moringa oleifera*

The fresh leaves and seeds of *Moringa oleifera* were washed under running water and macerated separately with cold distilled water at a ratio of 1:10. Both mixtures were then centrifuged at 4°C and 10000xg for 15 minutes to obtain the supernatants.

2.3.1 Cold acetone precipitation

Supernatants obtained from both seeds and leaves were precipitated using cold acetone at a ratio of 1:4, the test tubes containing the precipitated proteins were vortexed and incubated at -20°c for 60 minutes before centrifuging for 10 minutes at 4°C and 10000xg. The pellets were reconstituted in 5ml phosphate buffer (pH 7.4) to determine the protein content and ACE inhibitory activity.

2.3.2 Gel filtration using sephadex G-75

The reconstituted proteins were then subjected to gel filtration chromatography with sephadex G-75. The gel was soaked overnight in phosphate buffer (pH 7.4). Two columns were packed with the gel and washed with the same buffer before introducing proteins from the seeds in one column and that from the leaves in the second column. The proteins were allowed to elute at a flow rate of 0.25ml/min. Twenty (20) fractions of 5 ml each were collected, separately for leaves and seeds and assayed for ACE inhibitory activity and protein content.

2.4 Protein Estimation

Protein content of the clear supernatants was determined according to Bradford method using Serum Bovine Albumin (BSA) as standard and the concentration expressed in milligram per milliliter.

2.5 Determination of ACE Inhibitory Activity

Angiotensin converting enzyme converts hippuryl-histidyl-leucine (HHL) to hippuric acid and Histidyl-leucine. The assay for ACE inhibitory activity was determined using the Cushman and Cheung method [18] with some modifications on the assay conditions. The protein-rich fractions were added to a test-tube containing 5mM hippuryl-histidyl-leucine (HHL) previously dissolved in 0.1M potassium phosphate buffer containing 0.3M NaCl (pH 8.3). Then the enzyme, ACE was added to the mixture and incubated at 37°C for 30 minutes. The reaction was terminated by adding 0.25ml of 1M HCl, and then 1.5 ml ethyl acetate was added to extract the hippuric acid formed by the action of

ACE. The ethyl acetate was removed by heat evaporation, residual hippuric acid (HA) was dissolved in 3ml of deionized water, and absorbance of the solution taken at 228nm to determine the hippuric acid concentration. The % inhibition was calculated from the equation:

$$\frac{Ec - Es}{Ec - Eb} \times 100$$

Where Es is the absorbance of the reaction mixture, Ec is the absorbance of the buffer, Eb is the absorbance when the stop solution was added before the reaction occurred.

Inhibition was expressed as the concentration of inhibitor that inhibits 50% of ACE activity (IC_{50}), and 1 unit of ACE inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions.

2.6 Characterization of ACE Inhibitory Proteins from Seeds and Leaves of *M. oleifera*

2.6.1 Effect of pH on ACE inhibitor

The effect of pH on the ACE inhibitory proteins from the leaves and seeds was determined by varying the pH of the reaction mixture (from 3 to 10) and determining the activity.

2.6.2 Effect of temperature on ACE inhibitor

The influence of temperature on the inhibitory activity was determined by varying the incubation temperature from 10 to 70°C.

2.7 Determination of ACE Inhibitory Pattern of Partially-purified ACE Inhibitor

To characterize the ACE inhibitory mechanism of the purified peptide fraction from the leaves and seeds of *Moringa oleifera*, various concentrations of the substrate (0 to 5mM) were incubated with ACE solution in the presence and absence of 0.5 and 1mg/ml of the partially-purified protein inhibitor at 37°C. The Michaelis Menten constant, K_m, the inhibition constant, K_i, and the V_{max} were obtained from the Lineweaver-Burk plots.

2.8 Effect of Digestive Enzymes on the Inhibitor

This was determined as described by Lee et al. [19]. Briefly, the enzymes (pepsin and trypsin) were added to a 20mM phosphate buffer containing 1% (w/v) of ACE inhibitor. The reaction mixture was incubated at 37°C for pepsin and 25ºC for trypsin for 12h. The reaction was stopped by allowing the mixture to stand in a hot water bath for 10min. This was centrifuged at 2800xg and assayed for ACE inhibitory activity.

3. RESULTS

3.1 Partial Purification of ACE Inhibitory Proteins from Seeds and Leaves of *M. oleifera*

The steps involved in the partial-purification of ACE inhibitory proteins from leaves and seeds of *M. oleifera* are summarized in Tables 1 and 2 below. The crude proteins from the leaves and seeds were found to possess ACE inhibitory activity. The crude protein from the leaves of *M. oleifera* was estimated to be 9.82mg/ml with a specific activity of 0.027 U/mg, which increased to 0.109 U/mg at a recovery rate of 62% after precipitation with cold acetone. Passing the protein through sephadex-G75 further increased the specific activity to 0.917U/mg and purification fold of 8.41 (Table 1).

The crude proteins extracted from M. *oleifera* seeds was estimated to be 5.5mg/ml with a specific activity of 0.04 U/mg. Precipitation of the crude proteins with cold acetone increased the specific activity to 0.16 U/mg at a recovery rate of 53.68%. Gel filtration gave a specific activity of 1.99 U/mg, purification fold of 12.44 and recovery rate of 35.18% (Table 2).

Table 1. Purification profile of ACE inhibitory proteins from *M. oleifera* leaves

Purification step	Protein content (mg/ml)	Total protein content (mg)	Inhibitory activity (U)	Specific Inhibitory activity (U/mg)	Purification fold	Yield (%)
Crude	9.82	491	13.24	0.027	1	100
Acetone Precipitation	5.02	75.3	8.21	0.109	4.04	62
Gel Filtration	1.85	5.55	5.09	0.917	8.41	38

1 U of inhibitory activity was defined as the amount of the inhibitor that decreased the ACE activity by 50%

Table 2. Purification profile of ACE inhibitory proteins from *M. oleifera* seeds

Purification Step	Protein content (mg/ml)	Total Protein (mg)	inhibitory activity (U)	Specific inhibitory activity (U/mg)	Purification fold	Yield activity (%)
Crude Protein	5.50	275.00	11.40	0.04	1.00	100.00
Acetone Precipitation	2.53	37.95	6.12	0.16	4.00	53.68
Gel Filtration	0.67	2.01	4.01	1.99	12.44	35.18

1 U of inhibitory activity was defined as the amount of the inhibitor that decreased the ACE activity by 50%

3.2 Effect of pH and Temperature on Partially-purified ACE Inhibitors from *M. oleifera*

The inhibitory activity of the partially-purified proteins on ACE was optimal at pH 8.2 and 7.8 for the leaves and seeds, respectively. There was a complete cessation of activity below pH 2 for both inhibitors and above pH 10 for the partially-purified ACE inhibitors obtained from seeds (Fig. 1).

The temperature-dependent effect of the partially-purified proteins are shown in Fig. 2. The activity of proteins increased with temperature, until a peak was obtained at 37.6ºC and 39ºC for leaves and seeds of *M. oleifera,* respectively. Further increase in temperature caused a decrease in inhibitory activity.

3.3 Determination of Inhibitory Pattern of Partially-purified ACE Inhibitory Proteins from *M. oleifera*

The ACE inhibition pattern of the partially-purified proteins from leaves and seeds of *M. oleifera* exhibited a mixed type of inhibition (Fig. 3), with an increase in K_m and decrease in V_{max} as concentration of inhibitor increased. The K_m of the partially-purified proteins obtained from the leaves increased from 3.51mM (without inhibitor) to 4.79mM (at 0.5mg/ml inhibitor) and 5.62mM (at 1 mg/ml inhibitor), while V_{max} decreased from 0.953 (without inhibitor) to 0.784 and 0.629 µmol/min at 0.5 and 1.0 mg/ml of the inhibitor, respectively. The K_i was found to be 1.54mg/ml.

For the partially-purified ACE inhibitory proteins obtained from the seeds of *M. oleifera*, the K_m increased from 2.89mM without the inhibitor, to 4.367 and 5.747mM at 0.5 and 1.0mg/ml concentration of the inhibitor, respectively, while the V_{max} decreased from 0.910 in the absence of inhibitor to 0.780 and 0.628 µmol/min at 0.5 and 1.0 mg/ml concentration of inhibitor, respectively. The K_i was found to be 1.58 mg/ml (Fig. 4).

3.4 Effect of Digestive Enzymes on the Partially-purified ACE Inhibitors from *M. oleifera*

Results of the ACE inhibitory activities of the partially-purified proteins from the leaves and seeds of *M. oleifera* were expressed based on the inhibition concentration (IC_{50}) values, defined as the concentration of the inhibitor required to inhibit 50% activity. From the results, the activity of the partially-purified ACE inhibitory proteins decreased significantly (P<0.05) when treated with gastrointestinal enzymes, pepsin and trypsin compared to the standard drug, captopril (1.50±0.36 U/ml) and control groups (Fig. 5). The IC_{50} of the untreated (control) inhibitors obtained from the leaves and seeds (5.09±0.41 and 4.01±0.63 U/ml, respectively) increased significantly (P<0.05) when treated with pepsin (6.60±0.34 and 9.02±1.02 U/ml) and trypsin (7.81±0.72 and 10.92±1.42 U/ml) for the leaves and seeds of *M. oleifera*, respectively (Fig. 5).

4. DISCUSSION

Since the discovery of the first ACE inhibitor from snake venom, many ACE inhibitors have been isolated and characterized from various sources, such as *Tricholoma giganteum* [20], algae [19], eggs [21], *Pleurotus cornucopiae* [22] etc. In the present study, partially-purified proteins from seed and leaves of *Moringa oleifera* were found to inhibit ACE *in vitro*. The concentration at which the partially-purified inhibitors from the leaves and seeds of *Moringa oleifera* inhibit 50% ACE activity (5.09±0.21 and 6.01±1.63U/ml, respectively) was significantly (P<0.05) higher than that obtained for *Hibiscus rosasinensis* (0.43±0.04U/ml) and Vinica leaves (1.37±0.06U/ml) [23]. This may be due to the source of ACE, differences in assay conditions, and substrate used. Purification of ACE inhibitory proteins via acetone precipitation and gel filtration increased the specific activity from 0.109 to 0.917 U/mg and 0.16 to 1.99 U/mg in the leaves and seeds, respectively (Tables 1 and 2). This could be due to the removal of other

synergistically interacting components of the protein. Although the purification fold of the partially-purified ACE inhibitors obtained from the leaves and seeds increased, the low yield obtained indicates that the purification steps needs to be modified [24].

The low ACE inhibition rates of the partially-purified ACE inhibitory proteins at high temperatures (Fig. 2) indicate that they may undergo thermal denaturation at high temperatures [25]. The ACE inhibitors from the leaves and seeds of *M. oleifera* were most stable at pH 8.2 and 7.8, respectively, showing that the inhibitors may be affected when exposed to strong acidic or alkaline conditions. Although, the partially-purified inhibitors still maintained their ACE inhibitory activities (>10%) at temperatures between 10 and 60°C and pH 5 and 9, they may be regarded as unstable; unlike ACE inhibitors obtained from *Porphyra yezoensis* [25] and algae protein waste [20]. The instability of the inhibitors may be because they have not been completely purified, and so contain some peptides and components, that may interact with their activities [24].

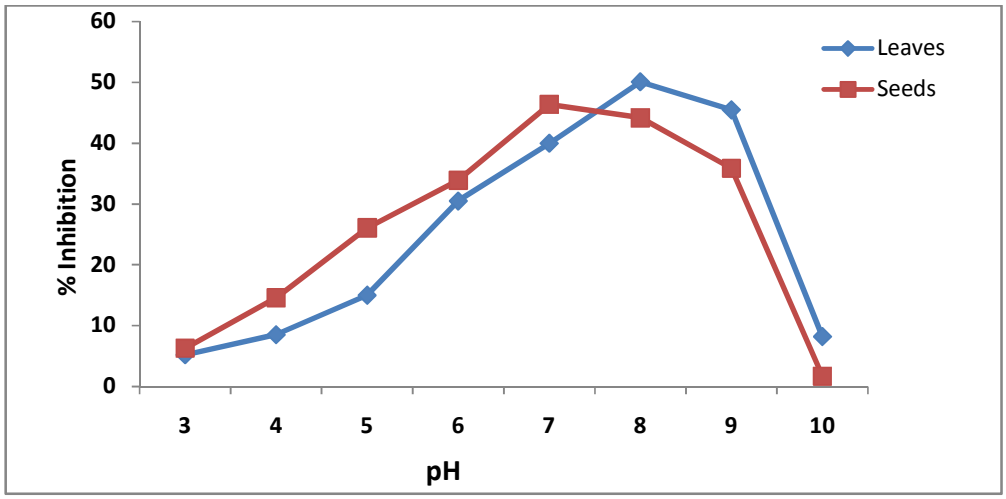

Fig. 1. Effect of pH on the ACE inhibitory activity of partially-purified proteins from leaves and seeds of *Moringa oleifera*

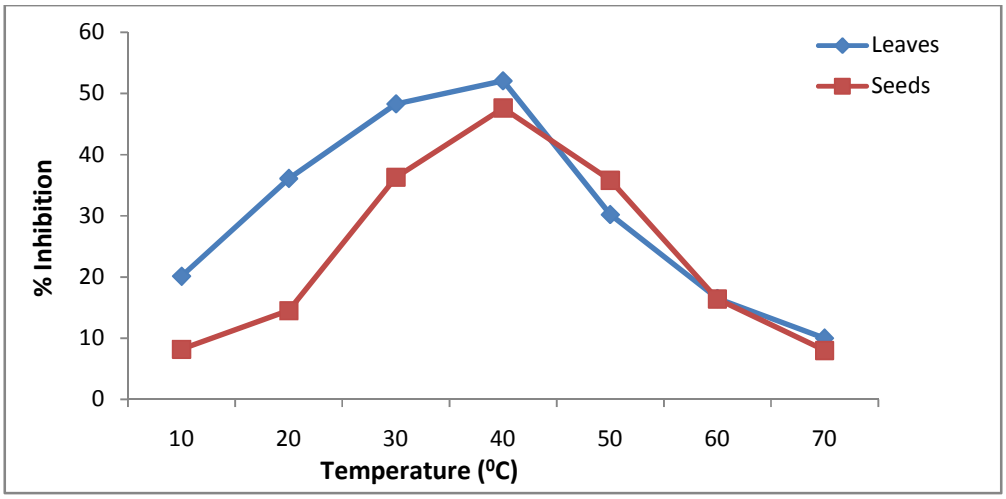

Fig. 2. Effect of temperature on ACE inhibitory activity of partially-purified proteins from leaves and seeds of *Moringa oleifera*

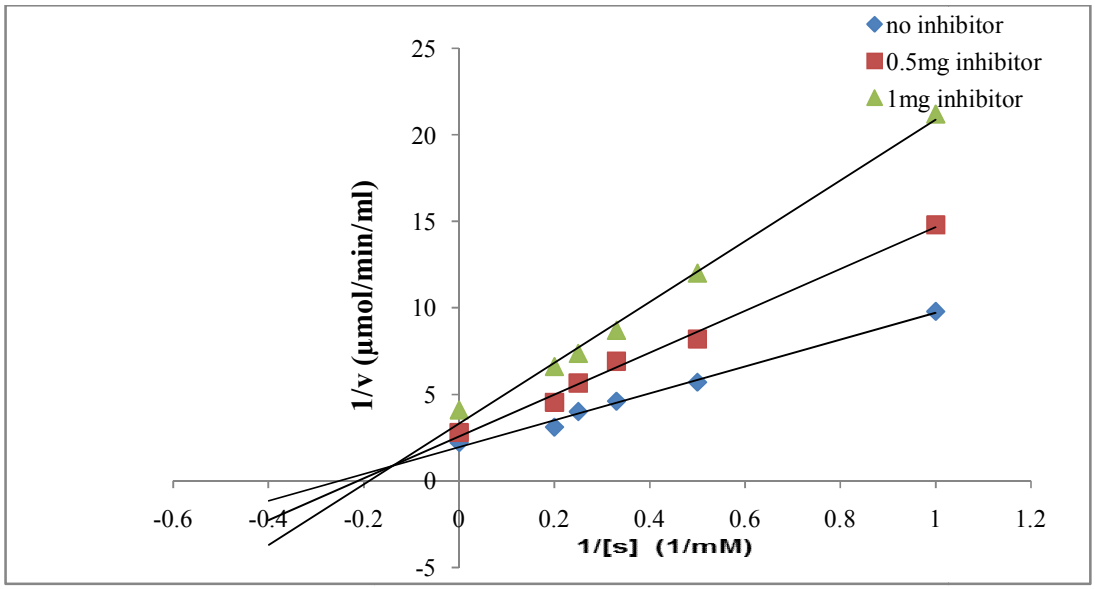

Fig. 3. Lineweaver–Burk plots for the inhibition of angiotensin-converting enzyme by the partially-purified proteins from the leaves of *M. oleifera*

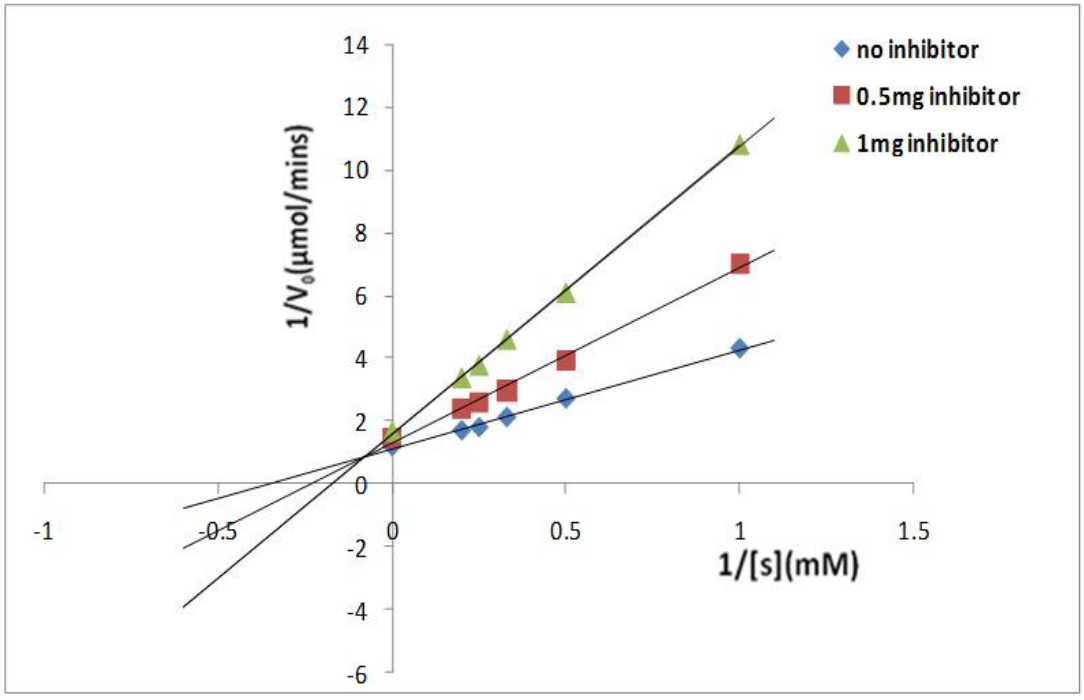

Fig. 4. Lineweaver–Burk plots for the inhibition of angiotensin-converting enzyme by the partially-purified proteins from the seeds of *M. oleifera*

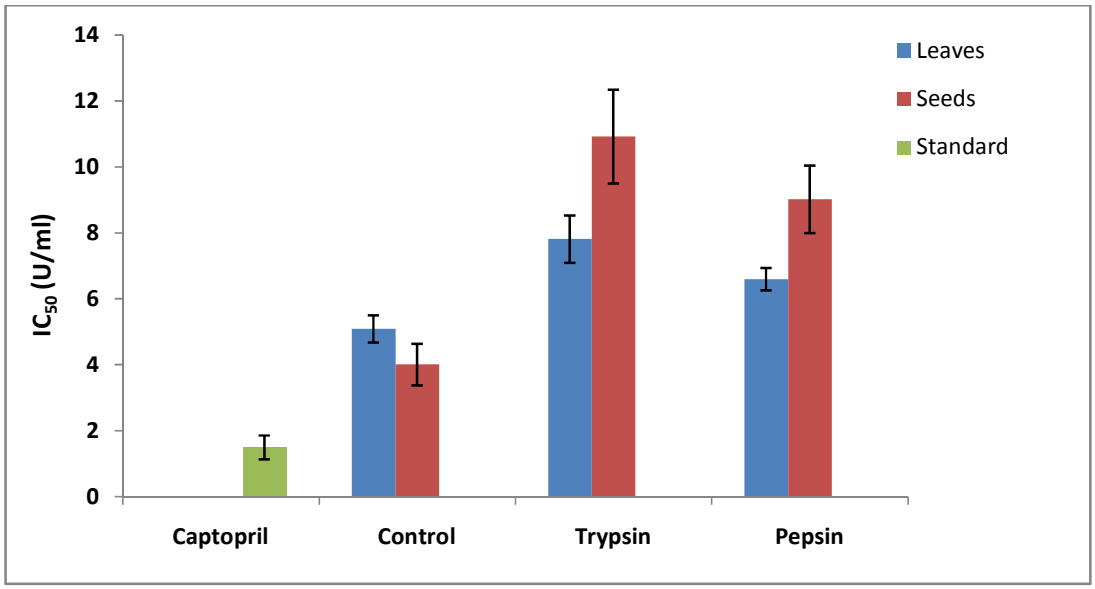

Fig. 5. Effect of some gastrointestinal enzymes on the ACE inhibitory activity of the partially-purified proteins obtained from the leaves and seeds of *M. oleifera*

Values are mean ± standard deviation

[a, b, c, d, e] Mean values having different superscript letters are significantly different (P<0.05)

IC_{50} defined as the concentration which inhibits 50% of the angiotensin converting enzyme activity

From the Lineweaver-Burks' plot, it was observed that the K_m increased as concentration of inhibitor increased, while V_{max} decreased, for both inhibitors, exhibiting a mixed type of inhibition. This implies that both ACE inhibitors from the leaves and seeds of *M. oleifera*, although away from the active site, alters the conformation of the enzyme and reduces its catalytic activity due to changes in the nature of the catalytic groups at the active site [26]. This finding does not conform with other reports where ACE inhibitors obtained from *Grifola frondosa* [27], *T. giganteum* [19] and *Peristrophe bicalyculata* [28] exhibited a competitive pattern of inhibition, while those isolated from algae protein waste [20] and lentils [29] were found to be non-competitive inhibitors.

The effect of gastrointestinal enzymes on ACE inhibitory peptides is important because after ingestion, they may be broken down and their activity affected. Thus, it is expected that hydrolysates of inhibitors obtained after treating with gastrointestinal enzymes would resist further *in vivo* gastrointestinal hydrolysis after oral intake and consequently increase the *in vivo* ACE inhibitory activity [29]. From the results obtained in this study, the enzymatic treatment of the partially-purified inhibitors significantly (P<0.05) decreased ACE inhibitory activity with IC_{50} values from 5.09±0.21mg/ml to 6.60±0.04 and 7.8±0.12 mg/ml in pepsin and trypsin hydrolysates, respectively. The different inhibitory effects obtained for the hydrolyzed samples using different enzymes may be attributed to variations in the type of ACE-inhibitory peptides liberated [29]. Studies have shown that even if the inhibitory activity of an ACE inhibitor decreases after treatment with gastrointestinal enzymes, the peptides may be effective after oral administration because they still maintained their inhibitory activity [27]. Fujita and Yoshikawa [30] using spontaneously hypertensive rats demonstrated that degradation of ACE inhibitory peptides by gastrointestinal enzymes *in vivo* may not affect its ability to reduce blood pressure because *in vivo* activity also depends on other factors such as routine of administration, food matrix and intestinal absorption. Therefore, it is possible that the partially-purified inhibitor obtained from the leaves of *M. oleifera,* though affected by digestive enzymes, may still be effective in reducing blood pressure.

5. CONCLUSION

This study has shown that ACE inhibitory proteins from *Moringa oleifera* may be beneficial as nutraceuticals or drugs for treatment of hypertension. However, further studies are necessary to purify the protein and establish its

stability at different pH, temperature and simulated gastrointestinal conditions for application in the medicinal industry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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