

Original Research Article

PARTIAL-PURIFICATION, CHARACTERIZATION AND INHIBITION PATTERN OF ACE INHIBITORY PROTEINS FROM THE LEAVES AND SEEDS OF *MORINGA* *OLEIFERA*

ABSTRACT

Aim: To partially-purify, characterize and investigate the inhibition pattern of proteins obtained from the leaves and seeds of *Moringa Oleifera*

Methodology: Crude proteins from the leaves and seeds were partially-purified by precipitating with cold acetone 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; and the mechanism of inhibition 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 inhibitor was 8.2 and 37.6⁰C, for leaves and 7.8 and 39⁰C, for seeds, respectively. Kinetic studies of the partially-purified ACE inhibitors exhibited a mixed type of inhibition. For the leaves, K_m increased from 3.51 mM in the absence of the inhibitor to 4.79 mM and 5.62 mM $\mu\text{mol}/\text{min}$ in the presence of 0.5 and 1mg/ml of the inhibitor, respectively, while V_{max} decreased from 0.953 $\mu\text{mol}/\text{min}$ without inhibitor to 0.784 $\mu\text{mol}/\text{min}$ and 0.629 $\mu\text{mole}/\text{min}$, respectively, in the presence of 0.5 and 1mg/ml of the inhibitor with a K_i of 3.51 mM. For the seeds, the K_m increased from 4.367 to 5.747 mM and V_{max} decreased from 0.780 to 0.628 $\mu\text{mol}/\text{min}/\text{ml}$ for 0.5 and 1mg of the inhibitor, respectively with a K_i of 1.58 mg/ml. Digestive

enzymes, pepsin and trypsin reduced the activity of the partially-purified inhibitors from both the leaves and seeds compared to captopril. In conclusion, ACE inhibitory proteins from *M. oleifera* may be beneficial as nutraceutical or drug for blood level regulation in hypertensive patients.

Keywords: Angiotensin Converting Enzyme, Hypertension, *Moringa Oleifera*

1.0 INTRODUCTION

Hypertension, a condition characterized by a systolic blood pressure $\geq 140\text{mmHg}$ and diastolic blood pressure $\geq 90\text{mmHg}$ [1] is one of the most common cardiovascular disease and to this day has 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 angiotensin converting enzyme 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]. ACE 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] therefore, the inhibition of ACE is considered to be useful in the prevention and treatment of hypertension and related diseases [9]. Captopril, enalapril, lisinopril and alacepril are synthetic ACE inhibitors that are remarkably effective at

regulating blood pressure [10], however, they have demonstrated diverse side effects, such as allergic reactions, skin rashes, cough, taste disturbances [9], hence interest in finding natural, nontoxic, safer, economical, and effective ACE inhibitors for the control and treatment of hypertension. A study by Govetta *et. al.* [11] demonstrating that plants known to be potent antioxidants also possess ACE inhibitory activity has increased the search for potent ACE inhibitors within plants with strong antioxidant activities, such as *Moringa oleifera*.

Moringa oleifera belongs to a monogenetic family called Moringaceae. It is commonly called “drumstick tree” in English and “Zogele” amongst the Hausas in northern Nigeria where it is commonly used. In last few years, the cultivation and consumption *M. oleifera* has increased markedly due to its potential health benefits and high nutritive value. The leaves have been reported to have antihypertensive, hypocholesterolemia, anti-ulcer 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 component 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. Thus, 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 seeds and leaves of *Moringa oleifera*, characterize, investigate the effect of some gastrointestinal enzymes on its activity, as well as determine its pattern of inhibition.

2.0 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.

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98 **2.3 Partial-purification of ACE inhibitory proteins from *Moringa oleifera***

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

102

103 **Cold acetone precipitation**

104 Supernatants obtained from both seeds and leaves were precipitated using cold acetone at a ratio
105 of 1:4, the test tubes containing the precipitated proteins were vortexed and incubated at -20°C for
106 60 minutes before centrifuging for 10 minutes at 4°C and 10000xg. The pellets were
107 reconstituted in 5 ml phosphate buffer (pH 7.4) to determine the protein content and ACE
108 inhibitory activity.

109

110 **Gel filtration using sephadex G-75**

111 The reconstituted proteins were then subjected to gel filtration chromatography with sephadex G-
112 75. The gel was soaked overnight in phosphate buffer (pH 7.4). Two columns were packed with
113 the gel and washed with the same buffer before introducing proteins from the seeds in one
114 column and that from the leaves in the second column. The proteins were allowed to elute at a
115 flow rate of 0.25ml/min. Twenty (20) fractions of 5 ml each was collected separately for the
116 leaves and seeds. The protein content and ACE inhibitory activities of each fraction was
117 determined and fractions with high ACE inhibitory activity were pulled together and
118 subsequently used to determine the pattern of inhibition and investigate the effect of
119 gastrointestinal enzymes.

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123 **2.4 Protein Estimation**

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

127

128 **2.5 Determination OF ACE Inhibitory Activity**

129 Angiotensin converting enzyme converts hippuryl-histidyl-leucine (HHL) to hippuric acid and
130 Histidyl-leucine. The assay for ACE inhibitory activity was determined using the Cushman and
131 Cheung method [18] with some modifications on the assay conditions. The protein-rich fractions
132 were added to a test-tube containing 5 mM hippuryl-histidyl-leucine (HHL) previously dissolved
133 in 0.1M potassium phosphate buffer containing 0.3 M NaCl (pH 8.3). Then the enzyme, ACE
134 was added to the mixture and incubated at 37⁰C for 30 minutes. The reaction was terminated by
135 adding 0.25 ml of 1 M HCl, and then 1.5 ml ethyl acetate was added to extract the hippuric acid
136 formed by the action of ACE. The ethyl acetate was removed by heat evaporation, residual
137 hippuric acid (HA) was dissolved in 3 ml of deionized water, and absorbance of the solution was
138 taken at 228 nm to determine the hippuric acid concentration. The % inhibition was calculated
139 from the equation:

$$140 \frac{E_c - E_s}{E_c - E_b} \times 100$$

$$141 E_c - E_b$$

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

144

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*

Effect of pH on ACE Inhibitor

The effect of pH on the ACE inhibitors from the leaves and seeds was determined by varying the pH of the reaction mixture from 3 to 10 and the activity of inhibitor determined.

Effect of temperature on ACE Inhibitor

The influence of temperature on the inhibitor 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 5 mM) was incubated with ACE solution in the presence and absence of 0.5 and 1 mg/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 25°C for trypsin for 12 h. The reaction was then stopped by standing the mixture in a hot water bath for 10 min, centrifuged at 2800xg and assayed for ACE inhibitory activity.

3.0 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 inhibitor 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 9.82 mg/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 0.917 U/mg and purification fold of 8.41 (Table 1).

The crude proteins extracted from *M. oleifera* seeds was estimated to be 5.5 mg/ml with a specific activity of 0.04 U/mg. Precipitation of the crude protein inhibitor 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	0.67	2.01	4.01	1.99	12.44	35.18
Filtration						

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 (Figure 1).

The temperature-dependent effect of the partially-purified inhibitors is shown in figure 2. The activity of the partially-purified inhibitor increased with increase in temperature, until a peak was obtained at 37.6⁰C and 39⁰C for leaves and seeds of *M. oleifera*, respectively. Further increase in temperature caused a decrease in inhibitory activity.

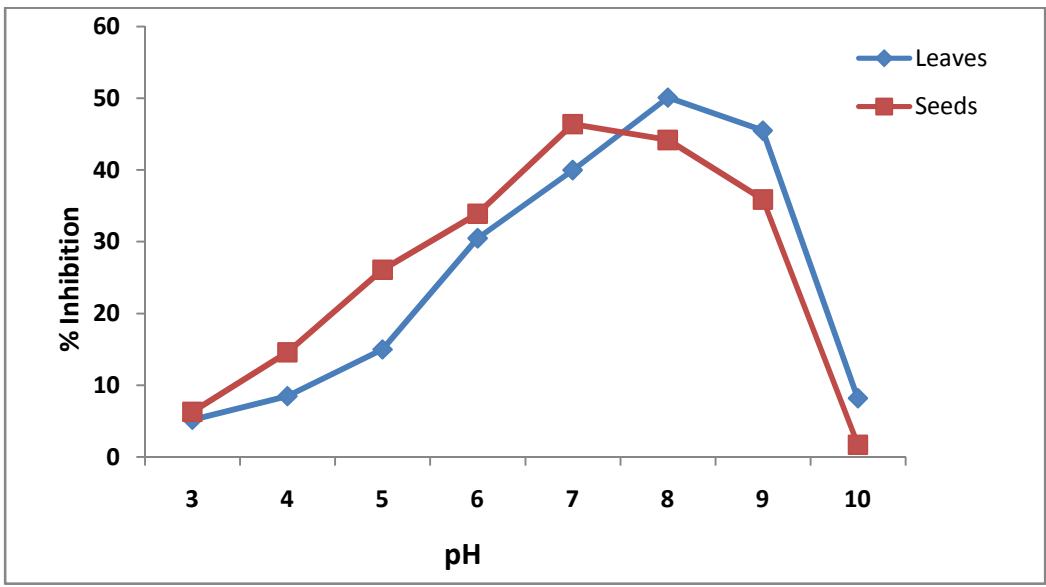


Figure 1: Effect of pH on the inhibitory activity of partially-purified ACE inhibitory proteins from leaves and seeds of *Moringa oleifera*.

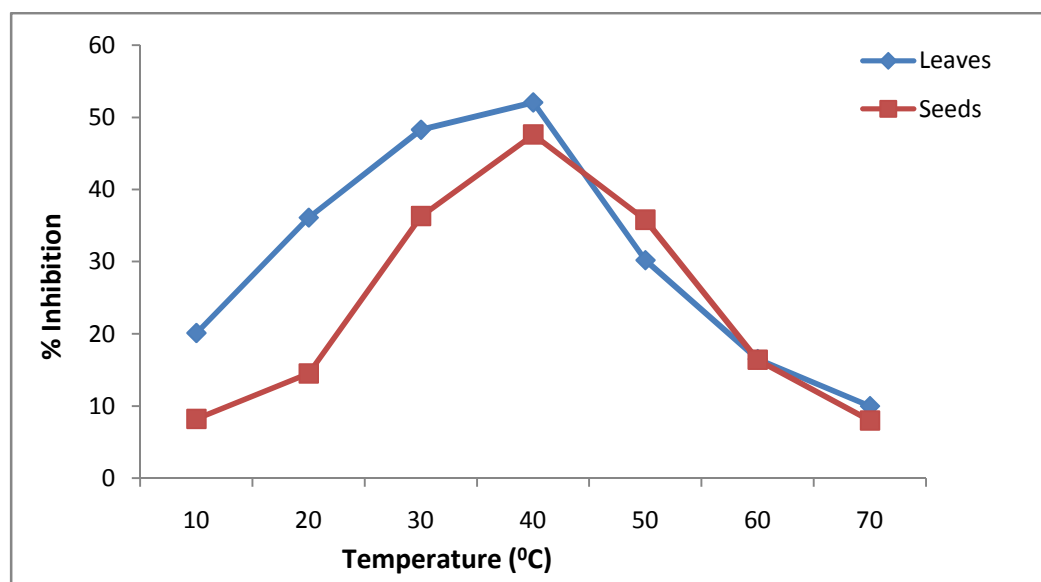


Figure 2: Effect of temperature on inhibitory activity of partially-purified ACE inhibitory proteins from leaves and seeds of *Moringa oleifera*.

3.3 Determination of ACE inhibitory pattern of Partially-purified ACE inhibitors from *M. oleifera*

The ACE inhibition pattern of the partially-purified proteins from leaves and seeds of *M. oleifera* was found to be a mixed type of inhibition (Figure 3), with an increase in K_m and decrease in V_{max} as concentration increased. The value of K_m in the absence of inhibitory proteins from the leaves was found to be 3.51 mM, while at 0.5 and 1.0 mg/ml of the inhibitor, the K_m was 4.79 and 5.62 mM, respectively. Furthermore, the values of V_{max} in the absence and presence (0.5 and

0.1 mg/ml) of the inhibitor was 0.953, 0.784 and 0.629 $\mu\text{mol}/\text{min}$, respectively. The K_i was found to be 1.54 mg/ml.

For the partially-purified ACE inhibitory proteins obtained from the seeds of *M. oleifera*, the K_m increased from 2.89 mM in the absence of inhibitor to 4.367 and 5.747 mM at 0.5 and 1.0 mg/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 $\mu\text{mol}/\text{min}$ at 0.5 and 1.0 mg/ml concentration of inhibitor, respectively. The K_i was found to be 1.58 mg/ml (Figure 4).

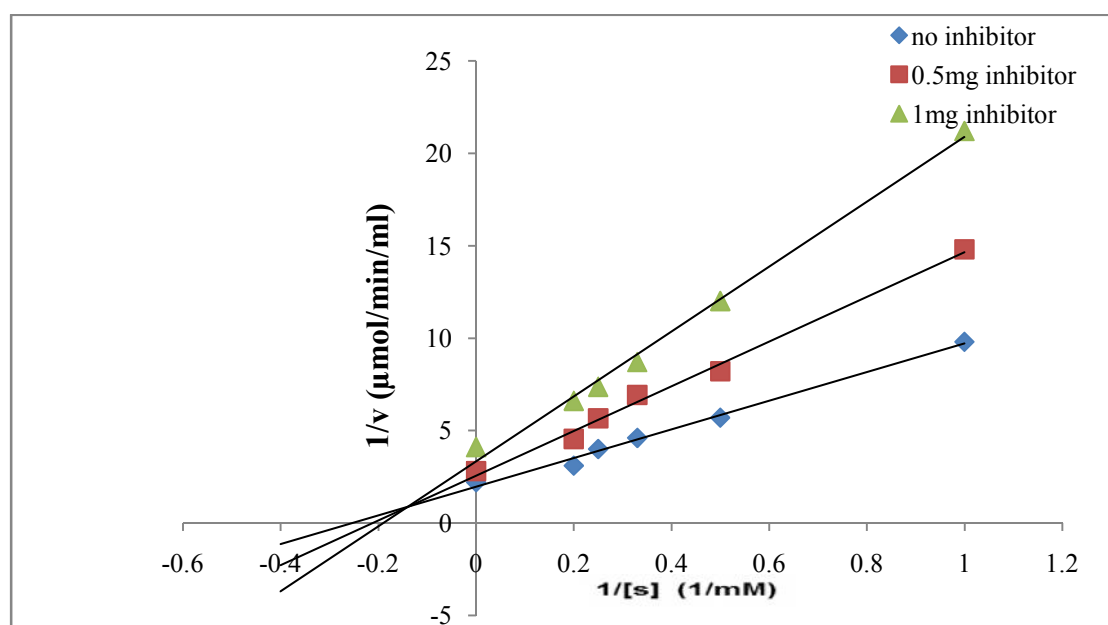


Figure 3: Lineweaver–Burk plots for the inhibition of angiotensin-converting enzyme by the partially-purified ACE inhibitory proteins from the leaves of *M. oleifera*.

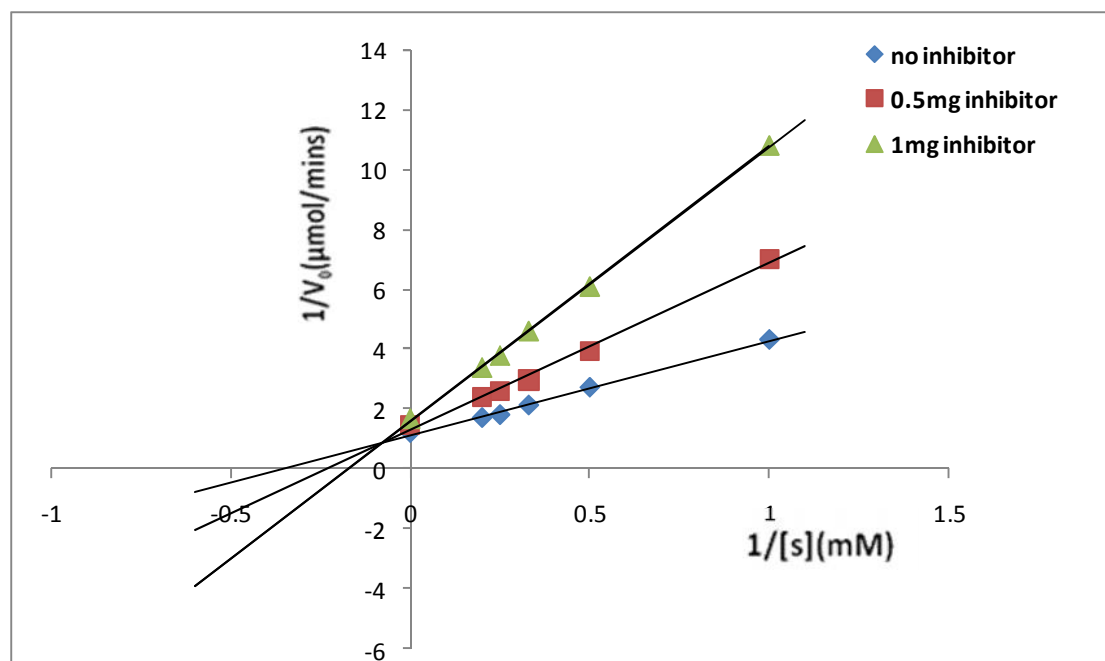


Figure 4: Lineweaver–Burk plots for the inhibition of angiotensin-converting enzyme by the partially-purified ACE inhibitory proteins from the seeds of *M. oleifera*.

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 inhibitors decreased significantly ($P < 0.05$) when treated with gastrointestinal enzymes, pepsin and trypsin compared to the untreated inhibitors (Figure 4). The IC_{50} at which the untreated (control) inhibitors obtained from the leaves and seeds (5.09 ± 0.41 and 4.01 ± 0.63 U/ml, respectively) inhibited 50% activity of the enzyme was significantly ($P < 0.05$) lower compared to inhibitors 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 (Figure 4).

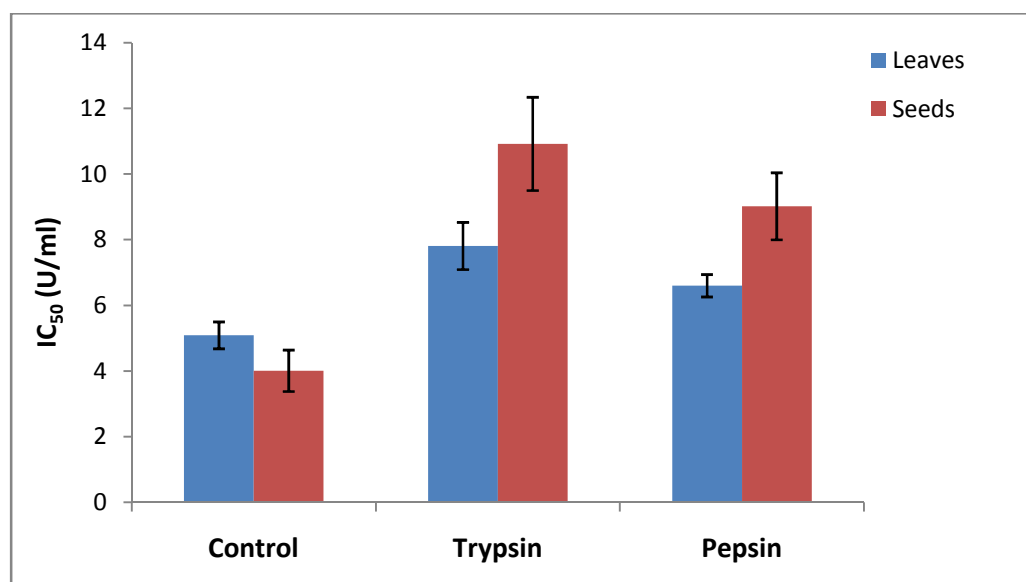


Figure 4: Effect of some gastrointestinal enzymes on the ACE inhibitory activity of the partially-purified proteins obtained from the leaves and seeds of *M. oleifera*.

Values are mean $\pm$ standard deviation.

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

IC₅₀ defined as the concentration which inhibits 50% of the Angiotensin converting enzyme activity

4.0 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 ± 1.63 U/ml, respectively) was significantly ($P < 0.05$) higher than that obtained for *Hibiscus rosasinensis* (0.43 ± 0.04 U/ml) and Vinica leaves (1.37 ± 0.06 U/ml) [23]. This may be due to the source of ACE, differences in assay condition, and substrate used. Purification of ACE inhibitor via acetone precipitation and gel filtration increased the specific activity of the inhibitor 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 partially-purified ACE inhibitor at high temperatures (Figure 2) indicate that it 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. This demonstrates that the inhibitors would be affected when exposed to strong acidic or alkaline conditions. Although, the partially-purified inhibitors still maintained their ACE inhibitory activity ($>10\%$) at temperatures between 10 and 60°C and pH 5 and 9, they may be 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 a lot of peptides and other components, some of which may not possess ACE inhibitory activity, and so may interact with the activities of the inhibitors [24].

From the kinetic studies using Lineweaver-Burks' plot, 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]. There are different reports on the inhibition patterns of different ACE inhibitors isolated from different plants. ACE inhibitors from *Grifola frondosa* [27], *T. giganteum* [19] and *Peristrophe bicalyculata* [28] were demonstrated to exhibit a competitive pattern of inhibition. In other studies, the ACE inhibitors isolated from algae protein waste [20] and lentils [29] were found to be non-competitive.

The effect of gastrointestinal enzymes on ACE inhibitory peptides is important because after ingestion, the enzymes may break up peptides, thereby increasing or decreasing their activity. Thus, it is expected that hydrolysates of the inhibitor obtained after treating with the 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 ± 0.21 mg/ml to 6.60 ± 0.04 and 7.81 ± 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]. It has been reported that even if the ACE inhibitory activity of an inhibitor decreases after treatment with gastrointestinal enzymes, the peptides may be effective after oral administration because they still maintained their inhibitory activity [27]. Studies by Fujita and Yoshikawa [30] using spontaneously hypertensive rats showed 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, even if the partially-purified inhibitor obtained from the leaves of *M. oleifera* were affected by the enzymes, they may still be effective in reducing blood pressure.

5.2 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.

5.3 Recommendation

In vivo tests should be carried out to investigate whether the *in vitro* results reported translate into activities that might support the traditional uses of these plants. Amino acid sequencing and molecular weight determination of the inhibitor should be carried out

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