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Mobola plum seed methanolic extracts exhibit mixed type inhibition of angiotensin I-converting enzyme *in vitro*

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ABSTRACT

Objective: To explore the possible inhibitory potentials and mechanism by Mobola plum (*Parinari curatellifolia*) seeds crude methanol (CE) and flavonoid-rich (FE) extracts on angiotensin-1-converting enzyme (ACE I).

Methods: The sensitivity and kinetic model of inhibition of CE and FE on ACE I using N-[3-(2-furyl)-acryloyl]-Phe-Gly-Gly as enzyme substrate for ACE I was evaluated by Michealis Menten approach. The inhibition mechanism was explored from Lineweaver–Burk model and IC_{50} was determined from Cheng–Prusoff empirical analysis.

Results: The IC_{50} of CE and FE were 13.54 and 39.38 μg/mL, respectively. Both extracts exhibited mixed type inhibition with the inhibitory constant (K_i) of CE was between 0.38 and 0.37 μg/mL while that of FE showed a two-fold increase (1.62 μg/mL and 0.28 μg/mL). FE on ACE I demonstrated positive cooperativity with a Hill's coefficient of 1.89.

Conclusions: The study reveals the superior ACE I inhibitory potential of CE over FE and suggest that mixed inhibition pattern of the enzyme might be the underlying mechanism of antihypertensive activity.

1. Introduction

Angiotensin-I-converting enzyme (peptidyl dipeptide hydrolase, kininase II, EC 3.4.15.1; ACE I) plays an important role in the regulation of blood pressure [1]. It is an important blood pressure regulator that catalyzes the release of His–Leu from the carboxyl terminal of angiotensin I. This consequently, generates a potent vasopressor octapeptide, angiotensin II. ACE I is also involved in the degradation of vasodilator bradykinin [2]. The vasoconstriction potentials of angiotensin II suggest the relationship between the peptides and the development of cardiovascular risk factors such as hypertension [3]. Hypertension has been recognized as the leading cause of death worldwide [4]. In order to control it, various drugs have been developed, which inhibit renin angiotensin system commonly called angiotensin-converting enzyme inhibitors [5]. Despite their effectiveness, complications or side effects due to their administration have necessitated the development of new, safer and more effective drugs. In Western medicine, drug development has become increasingly more mechanistic in the focus of excluding unwanted side-effects [2]. The rationale behind this approach is to identify a molecular target (receptor or enzyme) which has an essential role both in the regulation of the disease and in search for ligands, substrates or inhibitors of the target. In the treatment of hypertension, inhibition of the ACE I is established as one of the current therapeutic principles [2]. Medicinal plants have been used over the years in the management of diseases. Recently, plants used in the management of hypertension in traditional medicine in different regions around the world are being explored in order to discover potential sources of antihypertensive drugs [6]. Most of the researches have been targeted at bioactive compounds from natural resources. Notable examples are peptides [7], anthocyanins [8], flavonols [9] and triterpenes [10]. *Parinari curatellifolia* (*P. curatellifolia*) (Chrysobalanceae) ex Benth, popularly known as Mobola plum in the Southwestern part of Nigeria, is an evergreen shrub which grows in the

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Savannah area. Its ethnopharmacological uses include treatment of fever, body pain, diabetes, pile and hypertension *etc*. The family of Chrysobalanceae is rich in flavonols especially quercetin, myricetin, and kaempferol [11]. We have previously demonstrated the antihypertensive potentials of the seed extract in salt-induced hypertension [12]. However, the angiotensin-converting enzyme inhibitory property has not been investigated. The present study therefore sought to compare the effect of the crude methanolic and flavonoid-rich extracts of this plant on ACE I activity and determine the mode of inhibition.

2. Materials and methods

2.1. Materials

Angioensin-1 converting enzyme (ACE I) and N-[3-(2-furyl) acryloyl]-Phe-Gly-Gly (FAPGG) were purchased from Sigma–Aldrich, MO, USA. Bradford Reagent and BSA were obtained from Bio-Rad Laboratories, Hercules, CA, USA. All other reagents used were of analytical grade.

2.2. Plant material and preparation of crude extract

The seeds of *P. curatellifolia* were purchased at the King's market in Akure, Nigeria, in June, 2016. Botanical identification and authentication were carried out and the voucher specimen (PC005) was deposited at the Department of Biochemistry, Federal University of Technology Akure, Nigeria. The seeds were air dried, pulverized and stored in air-tight containers. The crude extraction of the seeds was carried out with 80% methanol as described elsewhere [12] using a Soxhlet apparatus. The solvent evaporated to dryness, referred to as crude extract, was stored in an airtight bottle at 4 °C until need.

2.3. Preparation of flavonoid-rich extract

Flavonoid-rich extract was prepared using the method of [13] with slight modification.

2.4. Determination of ACE1 inhibitory activity

ACE I catalyzed the hydrolysis of FAPGG to furyl–acryloyl–phenyl–alanine and glycyl–glycine. Hydrolysis of FAPGG resulting in a decrease in absorbance was monitored at 345 nm, 25 °C using Shimadzu 1800 double beam UV–visible spectrophotometer. The molar extinction coefficient difference between substrate and product of 517 $cm^{-1}\cdot M^{-1}$ was used to calculate activity. The ACE I inhibitory activity was measured according to the method of Homoliquist *et al.* [14] with some modifications. The ACE I activity was expressed as ΔA 345 nm and the ACE I inhibition (%) was calculated using the equation:

$$\left[1-\frac{\Delta A\ inhibitor}{\Delta A\ control}\right]\times 100$$

IC_{50} was defined as the concentration of sample required to inhibit 50% of ACE I activity under these conditions. The molar extinction coefficient between substrate and product was assumed to be 517 $cm^{-1}\cdot M^{-1}$ at 345 nm [15]. Protein content was determined using Bradford method [16].

2.5. Determination of the kinetic properties of ACE1 inhibition by extracts

The kinetic properties of ACE I (20 mU) without or with crude (6 and 12 μg/mL) and flavonoid-rich extracts (30 and 60 μg/mL) in total volume of 1.22 mL were determined using different concentrations of FAPGG as substrates (0.1–0.5 mM). The K_M (without extracts) and K_M' (extracts) were calculated from linearization of Michaelis–Menten's equation:

$$\frac{1}{Vo}=\left(\frac{\alpha K_m}{V_{\max}}\right)\left[\frac{1}{S}\right]+\alpha'/V'_{\max} \quad (1)$$

where V is the volumetric reaction rate, S is the substrate concentration, K_M is the constant of Michaelis–Menten and V_{max} is the maximum reaction rate at infinite reactant concentration.

The determination of inhibition constant (K_i) was performed using the following equations:

$$\alpha=1+[I]./K_i \quad (2)$$

$$\alpha'=1+[I]./K_i' \quad (3)$$

where I is the inhibitor concentration, K_i is the inhibition constant, α is the factor by which K_M changes when I occupies the enzyme and α′ is the factor by which apparent K_M changes when I occupies the enzyme. The determination of dissociation constant (K_d) was performed using the Cheng–Prusoff's equation:

$$K_i=\frac{IC_{50}}{1+([L]./K_d)} \quad (4)$$

where K_i is the inhibition constant, IC_{50} is the concentration that inhibits 50% of the enzyme, L is the free Inhibitor and K_d is the dissociation constant.

The inhibition data of flavonoid-rich extract was sigmoidal and was fitted to a Hill's plot rather than Michaelis–Menten plot. This sigmoidal curve required application of an allosteric enzyme model for its interpretation and was analyzed by Hill's equation:

$$V_o=\frac{V^{n_H}_{max[S]}}{K^{n_H}_M}+[S].^{n_H}$$

where n_H is a qualitative indication of the level of cooperativity.

2.6. Statistical analysis

The data were expressed as the mean of the triplicate determinations and the standard deviation or standard error of measurements. Statistical significance was evaluated using ANOVA followed by Tukey's test for multiple comparisons. The data were analyzed using GraphPad Prism 5 (GraphPad software, Inc., La Jolla, CA, USA). Data were fitted to either equations (1)–(3) or the Michaelis–Menten equation using KaleidaGraph 4.5 (Synergy Software, MA, USA).

3. Results

3.1. Inhibitory plot

Figure 1a and b showed inhibitory potential of *P. curatellifolia* extracts on angiotensin-1-converting enzyme activity. The result revealed that extracts inhibited the activity of ACE activity in a dose dependent manner. The IC_{50}, the

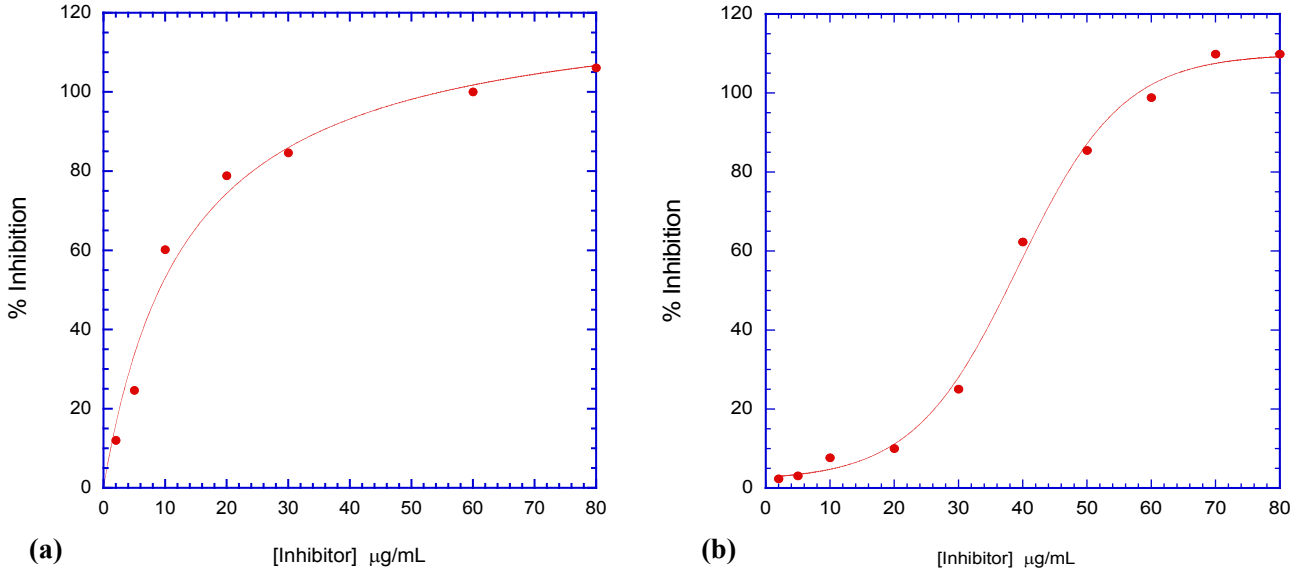

Figure 1. Inhibitory plot of crude (a) and flavonoid-rich extracts (b) of *P. curatellifolia* on ACE I activity.

concentration of crude and flavonoids-rich extracts that gave 50% inhibition was 13.54 and 39.38 µg/mL, respectively.

3.2. Lineweaver–burk plots

Figures 2 and 3 showed the Lineweaver–Burk plots (a, b, c) of angiotensin-1-converting enzyme (20 íu) without or with crude and flavonoid-rich extract of *P. curatellifolia* respectively.

The result showed that the extracts have different K_M and V_{max}. In addition, the V_{max} increased with the increase of concentration. The V_{max} of the extract was higher than the reaction without inhibitor. From the results, it showed that extract exhibited mixed type of inhibition.

3.3. Kinetic parameters of inhibitory potential

Table 1 showed the Kinetic parameters of the inhibitory potential of crude and flavonoid-rich extracts of *P. curatellifolia* against ACE activity. The result showed that the apparent V_{max} for crude extract (6 and 12 µg/mL) were 63.46 and 90.36 µM/min respectively; V'_{max} were 59.70 and 127.91 µM/min respectively; and flavonoid-rich extracts (30 and 60 µg/mL) were 416.75 and 494 µM/min respectively. Also, the apparent K_M for crude extract (6 and 12 µg/mL) were 31.73 and 63.95 µM respectively; and K_M'were 29.85 and 63.95 µM respectively; while that of flavonoid-rich extract (30 and 60 µg/mL) were 36.89 and 56.54 µM respectively; and apparent K_M' were 208.37 and 247.23 µM respectively. The binding constant of the extracts were concentration dependent.

3.4. Kinetic parameters

Table 2 showed the kinetic parameters obtained from Dixon adjustment for inhibition of ACE I by crude (CE) and flavonoid-rich (FE) extracts. The K_i and K_i' values were calculated. The CE has a K_i (competitive binding mode) values of 0.38 and 0.53 µg/mL

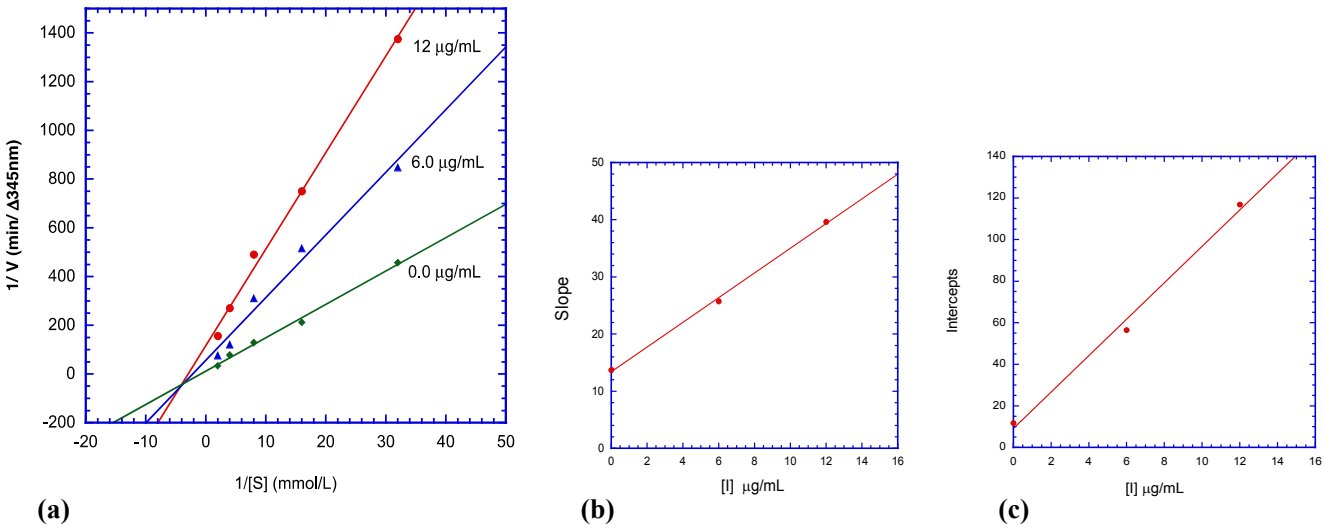

Figure 2. Lineweaver-Burk plots of angiotensin-1-converting enzyme (20 íu) without or with crude extract of extracts of *P. curatellifolia.* (a) Lineweaver-Burk plots (b) The secondary plot of the plot of the slope from the Lineweaver–Burk plot against 1/[*P. curatellifolia* extracts]. (c) The secondary plot of the plot of the Intercept from the Lineweaver–Burk plot against 1/[*P. curatellifolia* extracts]. All parameters are the average of two or three independent determinations, with SD less than ± 5%.

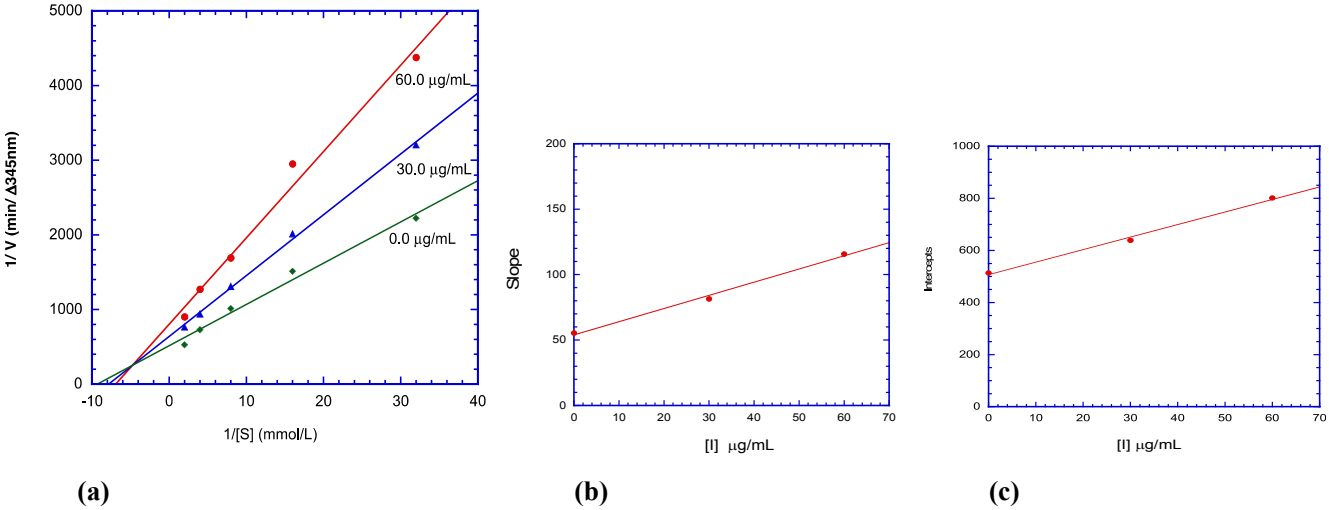

Figure 3. Lineweaver-Burk plots of angiotensin-1-converting enzyme (20 íu) without or with flavonoid-rich extracts of *P. curatellifolia.* (a) Lineweaver-Burk plots (b) The secondary plot of the plot of the slope from the Lineweaver–Burk plot against 1/[flavonoid rich *P. curatellifolia* extracts]. (c) The secondary plot of the plot of the Intercept from the Lineweaver–Burk plot against 1/[flavonoid rich *P. curatellifolia* extracts]. All parameters are the average of two or three independent determinations, with SD less than ± 5%.

Table 1

Kinetic parameters obtained from lineweaver–burk adjustment for ACE I Inhibition by crude and flavonoid-rich extracts.

Extracts	V_{max} (µM/min)	K_m (µM)	α	α′
No extract	0.039 ± 0.000^a	0.220 ± 0.020^a	1.000 ± 0.010^a	1.000 ± 0.010^a
CE (6 µg/mL)	3.870 ± 0.080^a	1.930 ± 0.040^b	16.700 ± 2.540^b	15.710 ± 3.540^b
CE (12 µg/mL)	3.870 ± 0.060^a	1.930 ± 0.010^b	23.780 ± 1.820^c	33.660 ± 3.730^c
FE (30 µg/mL)	3.870 ± 0.920^b	1.930 ± 0.000^b	19.420 ± 0.380^b	109.670 ± 5.630^d
FE (60 µg/mL)	3.870 ± 0.060^b	1.930 ± 0.000^b	29.760 ± 2.360^c	130.140 ± 8.630^e

CE: crude extract, FE: flavonoid-rich extract. Values are expressed as mean ± SD. Values with different superscripts are significantly ($P < 0.05$) different from each other down the column.

Table 2

Kinetic parameters obtained from dixon adjustment for inhibition of ACE I by crude and flavonoid-rich extracts.

Extracts	K_i	K_i'	K_d'	K'_d'
CE (6 µg/mL)	0.38 ± 0.04^a	0.37 ± 0.07^a	0.30 ± 0.00^a	0.30 ± 0.01^a
CE (12 µg/mL)	0.53 ± 0.06^b	0.40 ± 0.05^a	0.86 ± 0.09^b	0.64 ± 0.02^b
FE (30 µg/mL)	1.62 ± 0.07^c	0.28 ± 0.03^a	1.44 ± 0.14^c	0.23 ± 0.02^a
FE (60 µg/mL)	2.09 ± 0.07^d	0.47 ± 0.04^b	3.76 ± 0.27^d	0.81 ± 0.17^c

CE: crude extract, FE: flavonoid-rich extract. Values are expressed as mean ± SD. Values with different superscripts are significantly ($P < 0.05$) different from each other down the column.

and K_i' (an uncompetitive binding mode) values of 0.37 and 0.40 µg/mL while the K_d (competitive binding mode) values of 0.30 and 0.86 µg/mL and K_d' (an uncompetitive binding mode) values of 0.3 and 0.64 µg/mL. The FE has a K_i (competitive binding mode) values of 1.62 and 2.09 µg/mL and K_i' (an uncompetitive binding mode) values of 0.28 and 0.47 µg/mL while the K_d (competitive binding mode) values of 1.44 and 3.76 µg/mL and K_d (an uncompetitive binding mode) values of 0.23 and 0.81 µg/mL.

The Hill's plot for ACE binding to flavonoids rich extract gave a Hill coefficient (n_H) of 1.89. This indicates positive cooperativity of the inhibition at site outside the active site of ACE. The CE had a K_i (competitive binding mode) value of 6.19 µg/mL and K_i' (an uncompetitive binding mode) value of 1.04 µg/mL. The K_i and K_i' values for FE were 53.62 µg/mL and 105.52 µg/mL, respectively.

4. Discussion

Inhibition of angiotensin-converting enzyme is considered to be a target for discovery of lead antihypertensive agents and an important therapeutic approach in the management of high blood pressure. The inhibitory potential of crude methanolic extract of *P. curatellifolia* and its corresponding flavonoid rich extract on the catalytic activity of angiotensin-1-converting enzyme and ACE I on its physiological substrate FAPGG observed in this study may be due to formation of chelate complexes with Zn atom at the active center of zinc-dependent metallopeptidase or possibly by the formation of hydrogen bridges between inhibitor and amino acids near the active site [8]. Medicinal plants ACE Iwith high inhibitory potentials have been reported to possess antihypertensive potentials [17]. ACE I inhibitory potentials of flavonoids have also been documented [8,18]. The ACE I inhibitory potentials of the plant might be responsible for the antihypertensive potential earlier reported [12]. Hyperbolic inhibition kinetics was obtained with crude extract and was fitted to Michaelis–Menten plot. The IC_{50}, the concentrations of inhibition that gave 50% inhibition were 13.54 µg/mL and 39.38 µg/mL for crude methanolic extract and flavonoid-rich extract, respectively. With this, crude methanolic extract intensely inhibited ACE I *in vitro* more than flavonoid-rich extract. The IC_{50} values of both extracts were higher than *Hibiscus sabdariffa* [8] and *Malvis domestica* (flavonol-rich extract

of apple skin) [19]. This indicated that the CE is more active than flavonoid-rich extract. Olaleye *et al.* reported that crude extract of *P. curatellifolia* is rich in some phytoconstituents aside flavonoids such as alkaloids, terpenoids, saponins [12]. These constituents have been reported to possess ACE I inhibitory potentials [20,21]. Hence the effectiveness of CE could be as a result of the additive effect of these constituents.

Inhibition mechanism of angiotensin-1-converting enzyme by *P. curatellifolia* extracts detailed analyses with determination of the inhibition mechanism were performed for both crude extract and flavonoid extracts. The factor α describes the effect of the inhibitor on the affinity of the substrate towards the enzyme and the effect of substrate on the inhibitor affinity for the enzyme. The α for the crude and flavonoid-rich extracts of *P. curatellifolia* were 0.254 and 0.005, respectively. In general, there was partially mixed inhibition when $\alpha < 1$. Complete uncompetitive inhibition was characterized by $\alpha \ll 1$. The kinetic parameters obtained from Lineweaver–Burk adjustment for ACE I inhibition by crude and flavonoid-rich extracts of *P. curatellifolia* was carried out to reveal the maximum rate of substrate hydrolysis (V_{max}) and apparent Michaelis–Menten constant (K_M) and to characterize the kind of inhibition exerted by the extracts [22]. From this study, the V_{max} and K_M were significantly increased by the extract as compared with the control indicating a mixed type of inhibition. Also, it could be as a result of inhibitors structurally attacking the enzyme active site due to the affinity of the inhibitors to form inactive complexes (non-productive K_i'ESI) and attack other enzyme centers (EI has a lower affinity than E for the substrate). This can be seen in the variation that affects both kinetic parameters (K_M and V_{max}). However, the K_i values and inhibition pattern of methanolic extract and flavonoid rich extract on ACE I activity were determined and the representative Lineweaver–Burk. The double reciprocal plot of ACE catalyzed reactions in the absence and presence of CE and FE indicated mixed type inhibition. The mixed type inhibition suggested they bind to the enzyme and enzyme substrate complex. Mixed type inhibition was characterized by increased K_M and decreased V_{max}. CE had linear intersection at the 3rd quadrant and FE at 2nd quadrant. The K_i value (EI) and K_i' were determined from their respective secondary plots. The value of K*i* and K_i' were not in the same range which argued for a mixed model inhibition CE having a clear preference for uncompetitive inhibition than for competitive inhibition in the mixed competitive inhibition. With FE, the preference was for competitive inhibition in the mixed inhibition. The inhibition constant (K_i), which was the measure of the inhibition binding strength to ACE I indicating that CE showed a stronger binding than FE.

Also, the inhibitory constant for the binding (K_i) of inhibitor to ACE I, revealed that the active constituents of the extracts bind effectively to inhibit the enzyme at a lower concentration. Mixed type inhibition is typical of medicinal plants due to the presence of an array of constituents which act differently [23,24]. The observed dissociation constant (K_d) was determined from Cheng–Prusoff's equation. K_d measured the ability of the inhibitor to dissociate from either enzyme–inhibitor or enzyme–substrate–inhibitor complex. The higher the K_d the faster the dissociation. From the results, it was discovered that as concentration increases, the dissociation constant also increases. $K_d < 1$ showed a stronger binding and this corroborated the result obtained for K_i.

In summary, our current study represents the first investigation on the potential inhibitory effect and mechanism of *P. curatellifolia* seeds on angiotensin-converting enzyme inhibitors and raises awareness of the mechanism of inhibition. This could be one explanation of the pharmacological effect of the extract on the cardiovascular system.

Conflict of interest statement

There is no conflict of interest by the authors.

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