Onions tunic: A flavonol rich competitive inhibitor of key enzyme (Angiotensin-1 converting enzyme) linked hypertension

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Abstract— Onion (*Allium cepa*) is popularly known for its organosulphur and flavonoid contents and its tunic has been demonstrated to possess hypotensive effect. In this study, a flavonol-rich extract of onion tunic (FREOT) was investigated for its antioxidant potential and effect on angiotensin-1 converting enzyme (ACE) *in vitro*. The extract showed remarkable antioxidant activity when compared with the standard flavonoid compound, quercetin. The ACE inhibitory activity of the extract (IC_{50}: 0.36 ± 0.04 µg/ml) was comparable to that of quercetin (IC_{50} 0.34 ± 0.03 µg/ml) ($p > 0.05$). Studies on the inhibition pattern revealed that the extract inhibited ACE by competing with the substrate, N-[3-(2- furyl) acryloyl]-L-phenylalanylglycylglycine, for the active site. These results indicate that the FREOT exhibited its hypotensive effects via antioxidant and ACE inhibitory mechanisms.

Index Terms— Angiotensin converting enzyme, *Allium cepa*, Flavonoids, Hypotensive, Tunic.

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1 INTRODUCTION

The renin angiotensin Aldosterone system (RAAS) is the major target in the regulation of blood pressure, fluid, and electrolyte balance [1]. This realization has led to exploration of the renin angiotensin system and the possible ways through which its actions could be inhibited.

Angiotensin converting enzyme (ACE), an important enzyme in the regulation of the renin angiotensin system, is a zinc-containing peptidyl dipeptide hydrolase whose active site consists of three parts: a carboxylate binding moiety such as the guanidinium group of arginine, a pocket that accommodates a lipophilic side chain of amino acid residues at C-terminal and a Zn ion. The Zn ion binds to the carbonyl group of the penultimate peptide bond of the substrate, thereby making the carbonyl group polarized and subjecting it to a nucleophilic attack [2].

Angiotensin I is relatively inactive and is activated by being turned into angiotensin II by angiotensin-1 converting enzyme. Angiotensins II causes vasoconstriction (constriction of the blood vessels) and stimulation of the synthesis of aldosterone by the adrenal cortex [3]. The research on potential ACE inhibitors is expanding broadly and most are focused on natural product derivatives such as polyphenolics, terpenes, and peptides [4].

Onions (*Allium cepa*) is widely known for its organosulphur and flavonoid compounds [5]. Its tunic which are mostly discarded had been previously used in our laboratory for its effects on hemodynamic and antioxidant parameters in rats. This research work was designed to investigate the possible mechanism through which onion tunic exhibited its hypotensive properties as reported by our previous work.

2 Materials and methods

2.1 Chemicals

Furnacryloyl-l-phenylalanylglycylglycine (FAPGG) and quercetin were purchased from Sigma Chem., Co. (London, UK). Angiotensin converting enzyme was extracted from rabbit lung (EC.3.4.15.1). All other chemicals were of analytical grade and were either obtained from Sigma–Aldrich or British Drug Houses, (Poole,UK).

2.2 Plant material

Red onions (*A. cepa*) were bought from Shasha market, out-skirt Akure metropolis, Nigeria, in the month of February 2013. Botanical identification and authentication were carried out at the herbarium of the Forestry Research Institute of

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2.3 Preparation of the flavonol rich extract of onions (A.cepa) tunic

This was carried out according to the method of Olayeriju et al., [14]. Briefly, the tunics of onions were removed, cleaned, air-dried and pulverized. The powdered sample (80 g) was macerated in 600 mL of ethyl acetate (Sigma Chemicals; USA) for 4 h at 40◦C, with constant agitation. The mixture of the solvent and the ground sample was filtered first with mesh cloth, and then with filter paper (Whatman No. 1) and concentrated using a rotary evaporator. The residue was kept in a refrigerator at 4°C until further uses.

2.4 Angiotensin converting enzyme inhibition assay

The angiotensin converting enzyme inhibitory activity was carried out by using n-furnacryloyl-lphenylalanylglycylglycine (FAPGG) as the substrate [7]. Twenty microliters (20 µU) of extracted ACE (1 unit/µL, rabbit lung) Sigma-Aldrich was mixed with 200 µL of different concentration of the extract and standard quercetin (0.05 µg/ml to 1 µg/ml), and then 1000 µL of 5×10^{-4} M [FAPGG, dissolved in 50 mM Tris- HCl buffer (pH 7.5) containing 0.3 M NaCl] was added. The decreased absorbance at 345 nm (ΔA inhibitor) was recorded during 10 min at room temperature using T 70^{+} UV-Visible spectrometer, PG instruments Ltd. 50 mM Tris- HCl buffer (pH 7.5) containing 0.3 M NaCl was used instead of sample solution for control experiments (ΔA control). The ACE activity was expressed as ΔA 345 nm, and the ACE inhibition (percent) was calculated as follows: [1 - (ΔA inhibitor /ΔA control)] × 100. Means of triplicates were determined. The 50% inhibition (IC_{50}) of ACE activity was calculated as the concentrations of samples that inhibited 50% of ACE activity under these conditions.

2.5 Determination of the kinetic properties of ACE inhibition

The kinetic properties of ACE (20 µU) without or with extract (0.1 µg/mL and 0.3 µg/mL) in total volume of 1220 µL were studied with 0.0625, 0.125, 0.25 and 0.5 mM substrate (FAPGG) concentrations.

2.6 Kinetic calculations

The mode of ACE inhibition was determined from the Lineweaver-Burk plots while kinetic parameters (*Vmax* and *Km*) were estimated from non-linear regression fit of the data to the Michaelis-Menten equation using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Inhibition constant (*Ki*) was calculated as the x-axis intercept from a plot of the slope of the Lineweaver-Burk lines against sample concentration using the equation: mi = m ((1 + [I])/Ki), where mi = slope of lineal plot from inhibited reaction, m= slope of lineal plot from reaction without inhibitor, [I] = nanogram per ml concentration of inhibitor, and Ki = inhibitory constant. The catalytic efficiency (CE) was calculated from *Vmax*/*Km* ratio.

2.7 Reducing power ability

Reducing power ability was measured by mixing 1 ml extract of various concentrations (10-400 µg/ml) prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. Aliquot of 2. 5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged for 10 min at 3000 rpm, 2.5 ml from the upper part was diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1% ferric chloride. The absorbance was measured at 700 nm. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as positive control [8].

2.9 Nitric oxide radical scavenging activity

Various concentrations of the extracts (6.25-200 µg/ml) and sodium nitroprusside (5 mM) in phosphate buffer saline (0.025 M, pH 7.4) in a total volume of 3 ml was incubated at room temperature for a period of 150 min. After which, 0.5 ml of the incubated solution and 0.5 ml Griess' reagent (1% sulphanilamide, 2% O-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) were added together and allowed to react for 30 min. Control samples without the test compounds but with equal volume of buffer was prepared in a similar manner as done for the test. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and successive coupling with naphthyethylene diamine dihydrochloride was measured at 546 nm. The experiment was carried out using ascorbic acid as positive control [9]. The percentage inhibition of the extract and standard was calculated as:

$$\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \text{ X } 100$$

2.10 DPPH free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical was evaluated as described by Gyamfi et al [10]. Briefly, appropriate dilution of the extracts (1 mL) was mixed with 1 mL, 0.4 mmol/L methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance was taken at 516 nm. The DPPH free radical

scavenging ability was subsequently calculated as:

$$\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \text{ X } 100$$

2.11 Assessment of inhibition of lipid peroxidation

Inhibitions of lipid peroxidised were assessed according to the method of Ohkawa et al., [11]. Wistar albino rat was sacrificed by cervical dislocation. The heart was carefully excised and washed in ice cold 1.15% potassium chloride solution, blotted with filter paper and weighed. The heart was then chopped into bits and homogenized in four volumes of the homogenizing buffer which is 0.1 M phosphate buffer (pH 7.4) using Teflon homogenizer. Each of the resulting homogenates was centrifuged separately at 3000 rpm for 10 minutes to obtain the supernatant. A 200 µl aliquot of each of the supernatant was mixed with 60 µl 0.1M Tris HCl buffer (pH7.4) and plant extracts (1000 µl) of various concentrations (10-200 µg/ml) followed by the addition of 100 µl of 50 mM $FeSO_4$ and 240 µl of distilled water. The resultant solutions were incubated at 37^0C for 1 h. Then 600 µl of 8.1% SDS was added followed by the addition of 1200 µl of 1.3 M Acetate buffer (pH3.4) and 1200 of µl 0.8% Thiobarbituric Acid (TBA). The mixture was heated at 100^0C for 1hr to complete the reaction. Then the samples were cooled, centrifuged at 3000 rpm for 10 mins. The intensity of pink coloured complex was measured at 532nm.

Percent inhibition of lipid peroxidation was calculated as:

$$\frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \text{ X } 100$$

2.12 Statistical analysis

All values are expressed as mean ± SD of five animals. Statistical evaluation was done using One Way Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test. The significance level was set at $P < 0.05$.

3.0 Results

3.1 Inhibitory effects of FREOT on ACE activity

The inhibitory effect of FREOT on ACE activity when FAPGG was used as substrate is presented in Fig. 1. FREOT inhibits angiotensin-1 converting enzyme in a concentration dependent manner which compares well with the standaed quercetin. The IC_{50}, 50% inhibition of ACE activity was calculated to be 0.36 µg/ml when FREOT was used as an inhibitor. This compares favourably well with the standard quercetin, with IC_{50} of 0.34 µg/ml.

3.2 kinetics of ACE inhibition

The Lineweaver-Burk plots of ACE-catalyzed reactions in the absence and the presence of FREOT (0.1- and 0.3 µg/ml) indicates a competitive type inhibition as shown in Fig. 2. The kinetic parameters as shown in Table 1 revealed that the Vmax was 0.02 mM/min for ACE activity with or without FREOT, with an increased Km from 0.05 mM to 0.07-, and 0.4 mM when 0.1-and 0.3 g/ml of FREOT was used as inhibitors respectively. The catalytic efficiency also decreased from 0.4 to 0.29 and 0.05 in the presence of 0.1- and 0.3 µg/ml FREOT respectively. The inhibitory constant was 15.60 and 9.23 µg/ml for the respective concentrations used.

3.3 *In vitro* antioxidant activities of FREOT

The reductive potential activity of FREOT as presented in Table 2 showed that there was a concentration dependent reduction $p<0.05$ of ferric complex to ferrous form which compares favorably well with the standard quercetin used. The nitric oxide radical scavenging ability, DPPH radical scavenging and inhibition of lipid peroxidation induced by ferrous ion are presented in Table 3. FREOT was able to scavenge nitric oxide radical with IC_{50} of 60.26 µg/ml, DPPH radical with IC_{50} of 33.39 µg/ml, and inhibit lipid peroxidation with IC_{50} of 7.45 µg/ml.

Table 3: In vitro antioxidant activity of FREOT and standard

Antioxidants	IC50 (µg/ml) FREOT	IC_{50} (µg/ml) Ascorbic acid	IC_{50}(µg/ml) Quercetin
%NO scavenging	60.26 ± 0.24	59.16 ± 0.74	
%DPPH Scavenging	33.39 ± 0.24*		57.91 ± 0.18
% inhibition of LPO	7.45 ± 0.50*		13.19 ± 0.50

Values are expressed as mean ± standard deviation (n=3).

*P < 0.01 when compared with standard.

DPPH: 1,1-diphenyl-2 picrylhydrazyl, FREOT: Flavonol Rich Extract of Onions Tunic, LPO: Lipid Peroxidation, NO: Nittric oxide.

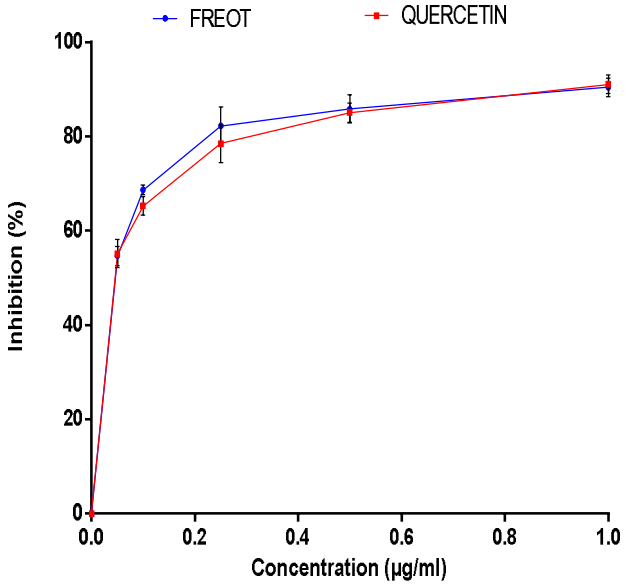

Fig. 1: Inhibitory Effect of FREOT and Quercetin on Angiotensin-I Converting Enzyme Activity

FREOT: Flavonol Rich Extract of Onions Tunic

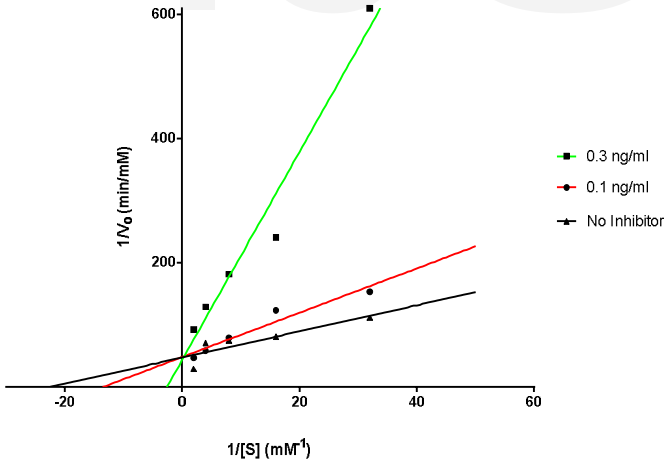

Fig. 2: Lineweaver-Burk Plots of Angiotensin-I Converting Enzyme (ACE (20 *i*U)) without or with Inhibitor (FREOT 0.1- and 0.3 µg/ml)

ACE: Angiotensin-1 converting Enzyme, FREOT: Flavonol Rich Extract of Onions Tunic

4.0 Discussion

Angiotensin converting enzyme (ACE), a crucial enzyme in the regulation of the renin angiotensin system, ACE plays a significant role in RAAS, by converting the precursor angiotensin I into angiotensin II which is the peptide responsible in triggering blood pressure increasing mechanisms [12].

The inhibition of ACE activity is generally recognized to result in the reduction of blood pressure, thereby being considered to be one of the effective measures to improve the conditions of patients with hypertension [13]. Studies from our laboratory had shown that FREOT possesses hypotensive effect on wistar albino rats [14].

In this present study, FREOT inhibited the activity of angiotensin-1 converting enzyme in a concentration dependent manner; with IC_{50} which compares favourably well with the standard quercetin. Olayeriju *et al* [14] reported the presence of flavonols: kaempferol, quercetin and quercetin glycosides (isoquercitrin, quercitrin, and rutin) in the FREOT.

Flavonoids are a group of polyphenolics compounds, which have been reported to possess ACE inhibitory activity [15] and the activity of flavonoids and other polyphenols may be due to the formation of chelate complexes with the zinc atom within the active centre of zinc-dependent metallopeptidases or possibly by the formation of hydrogen bridges between the inhibitor and amino acids near at the active site of ACE [16].

In order to characterize the inhibitory effect of FREOT on ACE activity, the kinetic properties of the enzyme inhibition were analyzed using a Lineweaver-Burk plot, which is shown to have a competitive inhibition of ACE activity under the assay conditions used.

In general, the Vmax was not modified significantly with respect to FAPGG, which indicates that FREOT competes with the substrate for the active site on the enzyme. Also, FREOT exhibited a dose dependent inhibitory effect on ACE, as it increased the Km of ACE activity, which is also reflected in the decreased Catalytic Efficiency (CE). The inhibiton constant (k_i) is defined as a measure of the strength of the inhibitor binding to ACE; therefore, low values indicate stronger binding affinity when compared to high values. FREOT was observed to have a stronger binding affinity for the enzyme when higher concentration was used. Flavonols: Kaempferol, rutin, and quercetin have been shown to be competitive inhibitors of ACE [17], [18].

Angiotensin II does not only stimulate vasoconstriction, but also oxidative stress, which has been shown to play a major role in the development of hypertension [19]. The antioxidative effects of ACE inhibitors have been known to contribute to their therapeutic effects in patients suffering

from cardiovascular complications [20]. From this study, the antioxidative properties of FREOT as revealed by their ability to reduce ferric complex to ferrous form was concentration dependent. The reducing power ability of a compound usually depends on the existence of reductones, which break free radical chain reactions by donating protons [21].

Also, the ability of FREOT to scavenge nitric oxide radicals, DPPH radicals, and the ability of the extract to inhibit lipid peroxidation induced by ferrous ion, can all be attributed to the high antioxidant content of the extract.

The antioxidant activities of onions have been documented [22], [23] and had been ascribed to its phenolics and flavonoids contents. This finding gives a pointer to the flavonol constituents in the thin back of onions as a potent antioxidant agreeing with previous studies and its inhibitory potentials on angiotensin-1 converting enzymes which play a major role in regulation of hypertension.

5.0 Conclusion

The inhibition of ACE activities, as well as the anti-oxidative abilities of the Flavonoid Rich Extract of Onions (Allium cepa) Tunic (FREOT) could be part of the mechanisms by which the extract exhibited its earlier reported hypotensive effects from previous studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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