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Research Article

Antioxidant and Angiotensin Converting Enzyme Inhibition Activity of *Landophia owariensis*

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ABSTRACT

High blood pressure is a common progressive disorder leading to various chronic diseases such as cardiovascular disease (CVD), stroke, renal disease and diabetes. In recent years, there has been increased interest in the possible health benefits of antioxidants due to the role they play against cardiovascular ailments and their ability to scavenge free radicals. Many medicinal plants contain large amount of antioxidants. The present study evaluates the antioxidant and angiotensin converting enzyme (ACE) inhibitory properties of *Landolphia owariensis, a* common Nigerian plant used traditionally for the treatment of hypertension: The phenolics in the extracts were identified and quantified by HPLC method. ABTS and DPPH were used to evaluate the antioxidant activities of the extracts in vitro. A comprehensive assessment of the ACE inhibition properties of the extract was also evaluated. From the results, total phenolic content increases with increasing polarity of the extracting solvent with water extract showing the highest total phenolic contents (154.02mg.GAE/g,) and hexane extract the lowest (13.05 mg.GAE/g). A linear regression analysis showed that antioxidant activity of the extracts correlated very well with the total polyphenol content (R^2 = 0.975) for DPPH, (R^2 = 0.942) for TEAC. The HPLC analysis indicates the presence of gallic acid, p-coumaric acid, quercetin and apiginine in substantial quantity only in polar solvents. ACE inhibition activity at different concentrations (0.25-4.0 mg/mL) showed varied 87.77% inhibitory activity at 4.0 mg/mL, which is comparable to Captopril standard drug with inhibitory activity of 90.07%. The molecular docking study also proved the isolated compounds from the extract exhibit good binding affinity and intermolecular interactions with different amino acids at the subsite of ACE.

Keywords: phenolics, antioxidant activity, *Landolphia owariensis*, docking, angiotensin converting enzyme.

INTRODUCTION

High blood pressure is a common progressive disorder leading to various chronic diseases such as cardiovascular disease (CVD), stroke, renal disease and diabetes. It has been reported that One-quarter of the world's adult population is afflicted by hypertension, and the trend is predicted to increase by 29% of the population in year 2025[1]. Though changes in life style, physical exercise, intake of healthy diets are some common practices associated with reducing the risk of hypertension. However, at critical stages, the administration of drugs becomes necessary. Therefore, it is of great importance to discover natural therapeutics for prevention and cure. Data from epidemiological studies showed that normalization of blood pressure result in significant reduction of major cardiovascular complications[2]. Most patients in developing countries with cardiovascular ailments use botanical drugs due to easy access and affordability[3]. Several botanicals have been used for the treatment of hypertension. The rennin-angiotensin-aldosterone system (RAAS) comprises of an enzymatic reactions sequence, which plays a fundamental role in controlling and regulating diverse metabolic processes necessary in the cardiovascular system[4]. The reaction sequence starts with the proteolytic breakdown of angiotensinogen by renin, an aspartyl protease synthesized in the kidney, resulting in the conversion of angiotensin I (Ang I). Angiotensin I is the main substrate of the angiotensin-converting enzyme (ACE). The action of ACE on Ang I lead to the conversion of inactive Angiotensin I to the active Angiotensin II, a potent vasoconstrictor that has been implicated in the development of important cardiovascular risk factors like hypertension[5,6]. The association of Ang II with cardiovascular disease has lead to various researches in area of developing potent drug inhibitors of the angiotensin-converting enzyme (IACEs). These inhibitors are designed to reduce the concentration of Ang II and, hence, its negative actions upon the cardiovascular system. Existing drug inhibitors in the market such as Captopril, Benzazepril, Enalapril and many more are widely used in

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hypertension treatment. However, some undesirable side effects limit their potency[7], which has led to research into the development of new, safer and more effective drugs, very often based on knowledge of traditional medicine. The use of medicinal plants in the treatment of various diseases has been on for decades, in most cases without a scientific background supporting their use. Recent days has witnessed increasing emphasis on determining the scientific evidence and rationale behind the use of extracts from medicinal plants. Various anatomical segments of *Landolphia owariensis* commonly known as white rubber vine or vine rubber, have been reported to possess anti-inflammatory, analgesic, antioxidant and hepatoprotective effects attributed to the antioxidant constituents of the herb; and have been used in ethno medicinal management of some infective conditions associated with pro-oxidation[8,9]. However, the scientific bases for the use of this herb by the traditional healers in Nigeria for the treatment of chest pains and even stroke have not been evaluated. Also the quantitative data on the phenolics content in the specie is still missing. We anticipate that if the therapeutic claim of the efficacy of this plant in treating cardiovascular diseases is true, it is necessary that this herb might be good inhibitors of angiotensin-converting enzymes. The objectives of this study were to: (1)To determine the total polyphenolic content, compare and evaluate total antioxidant capacity of the specie by two common antioxidant activity methods (2) identify and quantify major phenolic compounds present in this specie by HPLC; (3) determine the relationship between antioxidant activity and phenolic compounds to confirm that phenolic constituents are responsible for antioxidant activity of the plants and (4) Evaluate the ACE inhibition activity of the phenolic compounds present with reference to standard ACE inhibition drug.

MATERIALS AND METHODS

1, 1-Diphenyl, 2-picrylhydrazyl (DPPH), Gallic acid, P-coumaric acid, vanillic acid, Caffeic acid, P-hydroxybenzoic acid, ferulic acid, (+)-catechin, quercetin, glacial acetic acid, ABTS (2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid), Acetonitrile (HPLC), methanol (HPLC), acetic acid (HPLC), Acetylcholine, cyanuric chloride, dimethyl sulphoxide (DMSO) and hippuryl-l-histidyll-leucine (HHL), Folin-Ciocalteu reagent were obtained from Sigma Aldrich (Steinheim Germany), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Ascorbic acid were obtained from Merck (Germany)

Preparation of Plant Extract Plants materials used in this study were ethanol, methanol, water, hexane and acetone leaf extracts of *Landophia owariensis*. The plants materials were obtained from Benin City and authenticated by Professor Ozekwe of the department of Botany, university of Benin. The voucher specimen No 37332 was deposited at the herbarium of University of Benin.

The plant material was coarsely grounded in an electric grinder and soaked in aqueous methanol (30: 70), Ethanol (30:70), Hexane (30:70) acetone (30:70) and water (100) at room temperature with occasional stirring for three days. It was filtered through muslin cloth and then through Whatman grade 1 filter paper. The procedure of soaking and filtration was repeated with the residue using fresh solvent for two more times. All the three filtrates were combined and evaporated using rotary evaporator (Heidolph Laborota-efficient-4000, Germany) under reduced pressure at temperature ranging between 40-50°C (for methanol, ethanol, hexane, acetone) and 140^0C for water to obtain a crude extract thick semisolid paste of greenish brown colour for water, methanol and ethanol, and greenish brown semisolid paste for hexane and acetone and. Each extract was solubilized in 10% DMSO for ACE inhibitory assay.

Determination of total phenolic content

The total phenolic content of the crude extract of each plant was determined by Folin-Ciocaltu method[10] with slight modification. Briefly, 5 ml of Folin-Ciocaltu reagent (1:10 v/v distilled water) was mixed with 1.0 ml of each ethanol extract (1 mg/ml) and 4 ml (75 g/l) of Sodium carbonate. The resulting mixture was vortexed for 15 seconds and then allowed to stand for 30 min at 40°C for colour development. The absorbance was measured at 765 nm using Shimadzu UV–VIS spectrophotometer. and the total phenolic content was expressed as mg of gallic acid equivalent per gram using equation obtained from a standard gallic acid calibration curve y = 6.2548 x 0.0925, $R^2 = 0.9962$.

DPPH Radical Scavenging Activity Assay

The DPPH assay was determined according to Silva *et al.*[11]. An aliquot of the samples was mixed with DPPH solution (5 mL, 23.6 µg/mL in ethanol), followed by incubation of 30 min. The absorbance of each sample was read at 517 nm. Ascorbic acid (0.9, 1.9, 3.9, 4.9, 6.9 µg/mL) was used as positive reference. The percentage of scavenged DPPH was obtained from the equation

DDPH scavenging activity = $100 \times (Ac - As)/Ac$

where *A*c is the absorbance of the control and *as* is the absorbance of the sample. IC_{50} values calculated denote the concentration of the sample required to decrease the absorbance at 517 nm by 50%. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g.

Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The ABTS free radical-scavenging activity of each sample was determined according to the method described by RE *et al.*[12]. The radical cation $ABTS^+$ was generated by persulfate oxidation of ABTS. A mixture of ABTS (7.0 mM) and potassium persulfate (2.45 mM) was allowed to stand overnight at room temperature in the dark to form radical cation $ABTS^+$, 12–16 h prior to use. The solution was diluted with ethanol and absorbance measured, at 734 nm. An aliquot of each sample was mixed with the solution of the radical cation $ABTS^+$ (5 mL), and the decrease of absorbance was measured at 734 nm after 10 min. Trolox (1.1, 1.7, 2.3, 2.9, 3.5 µg/mL) was used as positive reference. IC_{50} values calculated denote the concentration of the sample required to decrease the absorbance at 517 nm by 50%.

All experiments were performed in triplicate. The DPPH and TEAC data were expressed as IC_{50} (mg/mL).

Reducing power

Table 1: Total polyphenol contents, DPPH radical scavenging activity and Trolox equivalent antioxidant capacity (TEAC) of *Landophia oweriensis*.

Extract	Assay		
	Total Phenol Contents (mg·GAE/g)	*DPPH* (IC50—mg/mL)	*TEAC* (IC50—mg/mL)
HE	13.05 ± 0.87	92.9 ± 0.19	47.05 ± 0.47
EE	99.05 ± 0.23	32.10 ± 0.13	13.33 ± 0.22
WE	154.05 ± 0.90	19.30 ± 0.33	8.30 ± 0.26
ME	120.05 ± 0.17	27.20 ± 0.26	17.43 ± 0.35
AE	1 9.05 ± 0.25	88.70 ± 0.33	40.44 ± 0.27
Positive reference	-	37.40 ± 0.29	13.45 ± 0.31

Data represented as Mean ± SD (n = 3). Hexane Extract (HE), Ethanol extract (EE), Water extract (WE), Methanol extract (ME), acetone extract (AE), Positive reference DPPH: ascorbic acid. Positive reference TEAC: Trolox

The determination of the reducing power of the water extract of the plant was carried out following the method of Oyaizu[13]. The crude extract of different concentration (100 µg – 1000 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%), followed by incubation of the mixture at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml) of the upper layer of the solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$. The absorbance of the resulting solution was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of three parallel experiments was taken and is expressed as mean ± standard deviation.

High Performance Liquid Chromatography (HPLC) Analysis of the Extract

Chromatographic analyses were carried out on a C18 Prevail column (150 · 2.1 mm, 2 lm) Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC (RSLC) systems (Thermo Fisher Scientific Inc., MA, USA), coupled to a rapid separation pump (LPG-3400RS), Ultimate 3000RS auto sampler (WPS-3000) and rapid separation diode array detector (DAD-3000RS). Data acquisition, peak integration, and calibrations were performed with Dionix Chromeleon software (Version 6.80 RS 10).

Chromatographic conditions

The method of Chuanphongpanich[21] was used to determine the phenolic composition of the ethanolic extract of *Landophia oweriensis* using HPLC. The mobile phase consisted of solvent A (acetonitrile), solvent B (acetic acid solution at pH 3.0), and solvent C (methanol). The system was run with the following gradient elution program: 0 min, 5%A/95%B; 10 min, 10%A /80%B /10%C; 20 min, 20%A /60%B /20%C and 30min, 100%A. The initial condition for equilibration of the column was carried out on a 5 min post run. The analysis was carried out at constant flow rate at 1 ml/min and the injection volume was 20 µl. For UV detection, the optimization of the wavelength program was carried out to monitor phenolic compounds at their respective maximum absorbance wavelengths as follows: λ 280 nm held for 18.0 min, changed to λ 320 nm and held for 6 min, and finally changed to λ 380 nm and held for the rest of the analysis and the diode array detector was set at an acquisition range from 200 nm to 700 nm. The detection and quantification of Gallic acid, (+)-Catechin, Vanillic acid, and Caffeic acid was done at 280 nm, of P-coumaric acid, Apigenine, and rutin hydrate at 320 nm, and of Quercetin, at 380 nm, respectively.

Standard and sample preparation

An approximate weight of 0.005 g of the analyte in methanol was weighed into the 50 ml volumetric flask to prepare the standard solution (100µg/ml) of each phenolic compound. The mixed standard solution was prepared by dilution the mixed stock standard solutions in methanol to give a concentration of 10 µg/ml for each polyphenols. All standard solutions were stored in the dark at 5°C and were stable for at least one month. Serial dilution of the stock standards with methanol was used to prepare the calibration curves to yield 1.25 - 20 µg/ml for Gallic acid, (+)-Catechin, Vanillic acid, P-coumaric acid, rutin hydrate, EA; 0.5 - 8.0 µg/ml for Caffeic acid, and 0.375 - 6.0 µg/ml for Quercetin. The calibration curves were constructed from chromatograms as peak area vs. concentration of standard. Solution of each extract of *Landophia owariensis* at a concentration of 5 mg/ml was prepared in ethanol by vortex mixing (Branson, USA) for 30 min. The samples were stored in the dark at low temperature (5°C). Spiking the sample solution with phenolic standards was done for additional identification of individual polyphenols.

Acute toxicity test

The acute toxicity dosage was studied using Albino mice, which were divided into different groups of five animals in each group. Increasing doses of the extract (1, 2, 3 and 5 g/Kg) were given orally in 10 mL/Kg volumes, to different groups serving as test groups. Another group was given normal saline (10 mL/Kg) as negative control. The animals were allowed food and water ad libitum and kept under regular observation for 6 h and then lethality was noted after 24 h[15].

Angiotensin converting enzyme (ACE) inhibitory assay

The determination of the ACE activity was carried out using hippuryl-l-histidyl-l-leucine (HHL) as the substrate in the presence of boric acid/NaOH buffer containing NaCl. The enzyme hydrolyzes this molecule to give hippurate. The final concentrations in the incubation

Table 2: HPLC isolated phenolic content in *L.oweriensis* extracts using different solvents

	Standards							
Sample	GA	PCA	VA	CA	Qu	(+)-CA	RH	AP
EE	3.4 ± 0.18	27.6 ± 0.27	1.2 ± 0.18	1.7± 0.07	45.1± 0.27	6.3 ± 0.15	ND	3.9 ± 0.11
HE	0.7 ± 0.28	5.5 ± 0.19	ND	2.3± 0.22	0.9± 0.20	ND	ND	2.09 ± 0.17
ME	7.2 ± 0.15	31.9 ± 0.22	4.9 ± 0.1	4.9 ± 0.18	50.3± 0.13	9.2 ± 0.29	ND	8.2 ± 0.33
WE	13.7 ± 0.17	33.3 ± 0.31	11.2 ± 0.8	9.1± 0.26	61.5± 0.19	11.5± 0.31	ND	9.2 ± 0.16
AE	1.1 ± 0.30	7.3 ± 0.13	ND	4.5± 018	1.1± 0.20	ND	ND	3.7 ± 0.17

Data represented as Mean ± SD (n = 3). Hexane Extract (HE), Ethanol extract (EE), Water extract (WE), Methanol extract (ME), Aceton extract (AE), GA: gallic acid, PCA: P-coumeric acid, VA: vanillic acid, CA: Caffeic acid , QU: quercetin, CA: (+)-Catechin, RH: rutin hydrate and AP:apigenine

Table 3: Angiotensin converting enzyme (ACE) inhibitory activity of the crude water extract of *Landophia oweriensis*, standard phenolic compounds and standard ACEI drug (captopril)

Extract or drug conc.	% Inhibition of ACE activity
Captopril (5 µM)	90.07 ± 2.15
L.O (0.25 mg/ml)	19.33 ± 2.15
L.O (0.50 mg/ml)	31.75 ± 2.07
L.O (1.00 mg/ml)	49.34 ± 2.18
L.O (2.00 mg/ml)	75.92 ± 1.81
L.O (4.00 mg/ml)	87.77 ± 1.08
PCoumeric acid (4.00 mg/ml)	16.79 ± 0.97
Quercetin (4.00 mg/ml)	40.11 ± 1.51
Apigenin (4.00 mg/ml)	26.27 ± 1.32
(+)-Catechin (4.00 mg/ml)	31.11 ± 0.89
Gallic acid (4.00 mg/ml)	34.27 ± 1.74
Vanillic acid (4.00 mg/ml)	29.18 ± 1.55

The values are mean ± SEM of three determinations. L.O: *L.oweriensis*

Table 4: Effect of *L.oweriensis* extract on growth and serum parameters level in SHRs

Experimental group	Control group	
Final body weight (g)	243 ± 3.0	238 ± 3.0
Food intake (g)	362 ± 4.0	359 ± 4.0
Serum parameters		
Triglyceride (mg/dl)	75.1 ± 7.2	91.0 ± 6.8
Cholesterol (mg/dl)	71.2 ± 2.4	74.1 ± 2.0
Glucose (mg/dl)	171.0 ± 2.0	160.1 ± 3.0
Insulin (ng/ml)	5.6 ± 0.3	5.5 ± 0.3

mixture were 80 mmol/L boric acid (adjusted to pH 8.3 with 5 M NaOH), 800 mmol/L NaCl and 4 mmol/L of HHL. The liberated hippurate was reacted with cyanuric chloride/dioxane (9 g/L), in the presence of phosphate buffer (200 mmol/L, pH 8.3), to yield a chromogen, which was quantified from its absorbance at 382 nm[22]. Rat blood was obtained through cardiac puncture under ketamine-diazepam anesthesia and allowed to clot for 15 min at room temperature. The serum was separated by centrifugation for 15 min at the speed of 5000 rpm and was used as source of ACE. Briefly, 0.1 mL of borate buffer was mixed with 0.05 mL of the extract solution and 0.05 ml of rat serum as source of ACE. After incubation at 37°C for 10 min, 0.05 mL of HHL solution (20 mM) warmed at 37°C was added to the reaction mixture and the reaction was allowed to proceed for 60 min at 37°C. The reaction was terminated with 0.25 mL of 1 M HCl solution and 30 sec later, neutralized with same volume of 1 M NaOH solution. Then 1 ml of phosphate buffer and 0.75 mL of coloring reagent were added, followed by mixing vigorously using vortex mixer (SeouLin Bioscience, Japan) for 30 sec in bursts of 5-10 sec, and allowed to stand for 5 min, vortex-mixed again, and then, centrifuged at 3000 rpm for 10 min in a bench centrifuge to remove denatured proteins and excess cyanuric chloride. Absorbance of the clear supernatant solution was measured by spectrophotometer (Model U220, Thermo scientific Japan) For positive control, 10% DMSO was used instead of extract solution; and for negative control, terminating and neutralizing solutions were added just after serum before the substrate. Captopril (2 µM) was used as standard ACE inhibitor for comparison. All determinations were performed in triplicate. The percentage inhibition was calculated by using the formula:

ACE Inhibition (%) = $[(A_{\text{positive control}} - A_{\text{sample}}) \times 100] / [(A_{\text{positive control}} - A_{\text{negative control}})]$, where "A" is the absorbance of respective solution at 382 nm.

Measurement of Blood Pressure in Spontenously hypertensive rat (SHR)

A three week study was carried out on 12 SHR (obtained from the University of Witwatersrand), Johannesburg. Each rat was kept in metal cages within a temperature controlled room (27^0C) under a 12hrs light/dark cycle. The rats were divided into two groups (n=6) after two weeks of adaptation period. The control group (CG) were administered with semi-synthetic diet having weight compositions as follows: Casein, 20%; corn oil, 8%, corn starch, 14%; DL-methionine, 0.3%; Cellulose, 5%; choline bitartrate, 0.1% and sucrose, 46% for a period of three weeks. The experimental group (EG) received semi-synthetic diet supplemented with 4% extract of *landophia oweriensis* in place of sucrose for the same period of time. The *L. Oweriensis* extract has weight composition as: carbohydrate, 63.7%; protein, 21.2%; fat, 1.9%, Ash, 0.4%; and moisture, 4.9%. The indirect tail cuff method was used to monitor the systolic blood pressure of each group on weekly basis using BP 200 BP monitor for rat and mice (Iprecio Italy) according to manufacturer's instruction. A total of five readings were obtained from each rat and average of the readings was taking, after excluding the highest and the lowest values. The study was approved by the Animal and Ethic Committee of University of Witwatersrand (approval no. AEC07/14) and

Table 5: Effects of the crude extract of *L. oweriensis* on urine output in Sprague-Dawley rats.

Group	Urine volume (ml/100g/6h)	Na^+ concentration (mmol/L)	K^+ concentration (mmol/L)	Na^+/K^+
N.C (10 ml/kg)	1.51 ± 0.14	65.22 ± 1.21	15.71 ± 1.33	4.21 ± 0.18
F.G (10 ml/kg)	5.64 ± 0.22	127.15 ± 1.36	29.92 ± 2.01	4.15 ± 0.27
L.O (10 ml/kg)	1.86 ± 0.17	68.37 ± 1.71	17.24 ± 1.08	3.91 ± 0.39
L.O (20 ml/kg)	2.43 ± 0.11	75.22 ± 0.98	17.03 ± 1.42	4.30 ± 0.45
L.O (40 ml/kg)	3.06 ± 0.17	86.09 ± 1.51	25.92 ± 1.33	3.73 ± 0.39

N.C: Normal control, F.G: Frusemide treated group, L.O: *Landophia oweriensis.* The values are mean ± SEM of five determinations. P-values are compared with control (N.C.) group

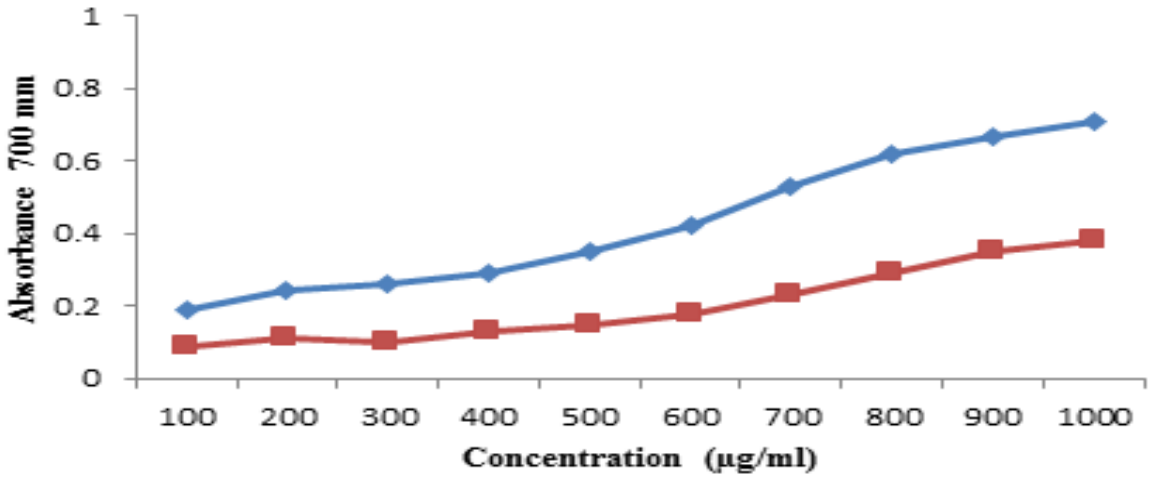

Figure 1: reducing activity of the leaf extracts of *L. oweriensis*. AA: Ascorbic acid, LO: *Landophia oweriensis*.

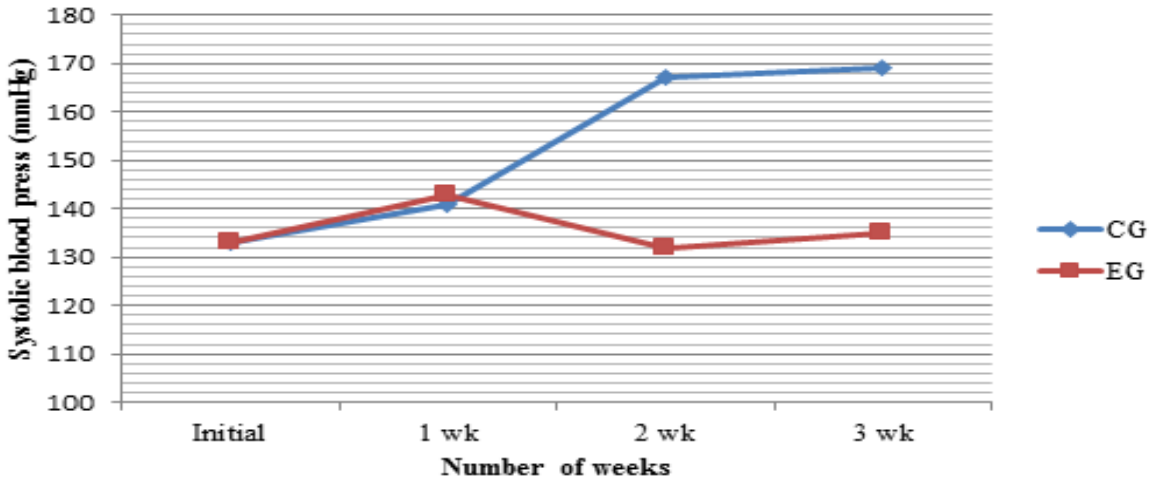

Figure 2: Effects of L. oweriensis extract on systolic blood pressure in spontenous hypertensive rats. CG: control group, EG: experimental group. Values are given as mean ± standard deviation. (n=6). Asterisks showed significant differences at $p < 0.05$

conducted according to international guidelines for laboratory animal use and care.

Diuretic assay

The diuretic assay was determined following the method of Jabeen *et al.*[16]. Briefly, Sprague-Dawley rats were randomly grouped into five groups of six animals each. The control group received normal saline (10 mL/Kg, IP). frusemide (10 mg/Kg, IP) was fed to another group of animals as standard diuretic. The treated groups of animals were intraperitoneally injected with different doses of extract. Immediately after dosing, animals were individually housed in metabolic cages (Techniplast, Japan) and the urine was collected for 6 h. Total urine volume was calculated and Na+ and K+ urinary concentrations were measured by using clinical flame photometer (Model 240C, Technoscientific, South Korea).

Molecular docking and structure activity study

Preparation of 3D structures

AutoDock-vina was used to understand the interaction between the ligands (natural product isolates and standard drugs) and the receptor testicular Angiotensin Converting Enzyme (tACE). 3-D atomic coordinates of tACE (PDB: 1UZF) were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB), Protein Data Bank

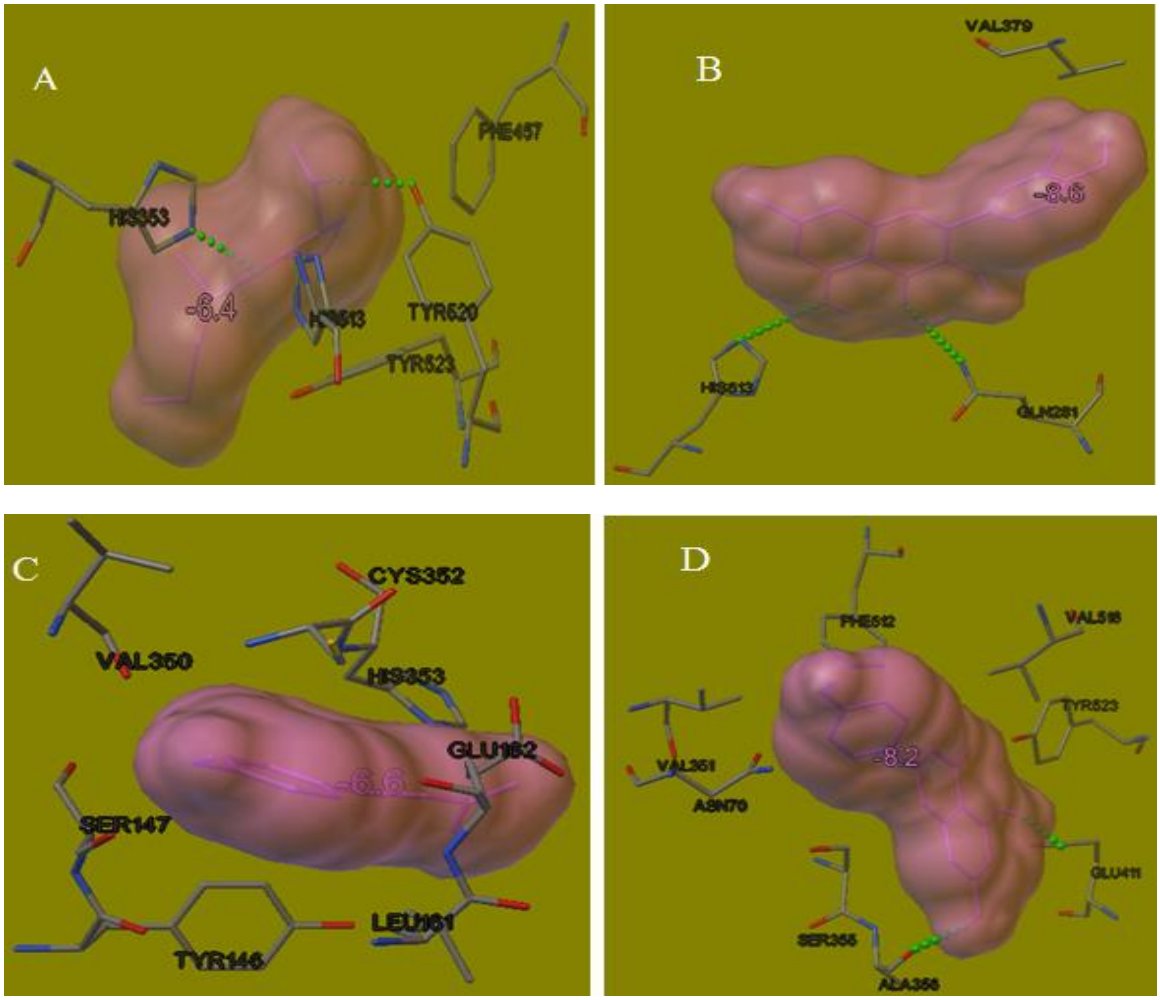

Figure 3: Docking of (A) Quercetin, (B) Captopril standard drug, (C) f P-coumaric Acid (D) Apigenin in the binding cavity of tACE binding site, showing interactions with amino acids in the active site. The ligands are shown in pink inside the cavity and the receptors outside the cavity. Green dashed balls are used to indicate hydrogen bonds.

(PDB) Database and prepared for docking. The ligands and the receptor protein were subjected to protein and ligand preparations to simulate the conditions used in the in vitro analysis (pH 8.3) using the Prepare Protein and Prepare Ligand protocols. Briefly, all hetero-molecules in the initial co-ordinates of tACE were deleted with UCSF Chimera 1.9 and polar hydrogens only were added with MGLTools-1.5.6[17]. Grid box sizes of 50 x 50 x 55 Å and centre of 117.66 x 105.967 x -140.421 Å for AChE were applied to the receptor at 1.0 Å using MGLTools-1.5.6.

Captopril standard drugs were manually curated[18]. The standard drug was filtered with drug bank database (available at http://www.drugbank.ca) to obtain only approved drug while discarding drugs that are experimental, investigational and withdrawn. The approved drug was treated with all hydrogen and the Gasteiger charges added with MGLTools-1.5.6[17].

Virtual Screening

Autodock was used tool to perform molecular docking of protein with virtually screened ligands and to compute the binding energy of docked complexes. The 3D structure of receptors and ligands were used in PDBQT format. The docking process or virtual screening to calculate the binding energies of the docked complexes were performed in quadruplet using AutoDock Vina[19]. The binding affinities were calculated and reported as mean ± SD. Virtualization and analysis of the molecular docking results were achieved with MGLTools-1.5.6 and UCSF Chimera 1.9.

Lead Identification/Optimization

An in-house database was created using the Molinspiration web based software available at http://www.molinspiration.com and ZINC ® Database and was used to optimize the lead anti-neurodegenerative agents in (plant) using Lipisinki Rule of Five[20] and bioactivity scores. Briefly, SMILES file format and physicochemical properties (Partition coefficient; Hydrogen Bond Donor; Hydrogen Bond Acceptor; Topological Polar Surface Area; Molecular Weight; Number of Rotatable bond**)** of the ligands were obtained from the ZINC ® Database at pH 7.0 ((John and Brian, 2005). Also, the Molinspiration software were used to predict the biological activities (G Protein Coupled-Receptor; Ion Channel Modulator; Kinase Inhibitor; Protease Inhibitor; Enzyme Inhibitor**)** of the ligands using the SMILES file format. Thereafter, ligands were ranked

according to their affinities for tACE and those that showed better or comparable affinities were identified and further characterized according to the Lipisinki Rule of Five[20] and bioactivity scores available at http://www.molinspiration.com using the in-house database.

RESULT AND DISCUSSION

Extraction and total Phenolic content

The solubility of phenolic compounds was monitored using five solvents of varying polarity. The result showed that phenolic content increases with increase in polarity of the solvent (Table 1) with water having the maximum extractable total phenolic content (154.05 mg·GAE/g), followed by methanol (120.05 mg·GAE/g).

This result is consistent with some literature reports that water is the most suitable solvent for extraction of phenolic compounds[21,22].

Free radical scavenging activity

Plant Phenolic compounds have been reported as good scavengers of singlet oxygen and free radicals, resulting to potent antioxidant activity in biological systems[23].

From the IC_{50} values of the extracts (Table1), it can be seen that methanol and water extracts with the highest amount of total phenolic content, also exhibit stronger radical scavenging activity while hexane and acetone extracts recorded lower antioxidant activity. It has been reported by various author that free radical scavenging and antioxidant activity of plant extracts is directly related with the total phenolic content present[24,25]. Furthermore, the antioxidant activity of plant extracts containing phenolic compounds has been attributed to their ability to donate hydrogen atoms or electrons and to quench the rampaging free radicals[26].

Reducing power capacity

The reducing power is one vital method for the determination of the antioxidant activity of any compound. The reducing ability has been associated with the development of reductones, which has been shown to exhibit antioxidant activity by termination of free radical chain reaction[27]. The result of reducing power capacity of the crude water extract showed that the reducing power increases in concentration dependent manner compared to standard ascorbic acid (figure 1). The increase in absorbance in the reducing power assay indicates the ability of the extracts to donate hydrogen atom in a dose dependent manner. The gradual increase in absorbance in the assay indicates the capacity of the extract to donate hydrogen atom in a dose dependent manner[28].

Separation and quantification of phenolic compounds by HPLC

In this study, we employed seven different standard phenolic compounds in order to compare with the chromatogram of *landophia oweriensis* extract. The result of the hplc analysis (Table 2) shows the presence of gallic acid, P-coumeric acid, vanillic acid, Caffeic acid, quercetin, (+)-Catechin, and apigenine in polar solvents extract. The same set of compounds with the exception of vanillic acid and (+) - Catechin were also found in the non polar solvents though in lower quantities than that of the polar solvents. Plants with phenolic constituents can be potential therapeutic drug because of their free radical scavenging ability. All the phenolic compounds found in this study have been reported to possess good antioxidant ability[29]; suggesting that the plant may have protective effects on cardiovascular system. The beneficial effect of quercetin and p-coumeric acid as potent antioxidant, anti-ageing, with ability to prevent cardiovascular complications has been reported[31-33]. Indeed, the water extract with highest concentration of quercetin and p-coumeric acid also showed the maximum antioxidant activity with IC_{50} of 19.3 ± 0.33 mg/ml.

Acute toxicity test

The acute toxicity test of *l. oweriensis* extracts showed that the extract were safe at the tested doses of 1, 2, 3 and 5 g/Kg orally.

I*nhibition of Angiotensin Converting Enzyme (ACE)*

Recent years, has witnessed a rising interest in plant extracts with phenolic constituents with regards to their potential for cardiovascular protection. In fact, many epidemiological studies associate the reduced risk of cardiovascular disease with an increased consumption of foods and beverages rich in phenolic compounds[34-36]. Furthermore, several of these phenolics or their derivatives are widely used as pharmaceutical agents for their vasoprotective properties. The ACE inhibitory activity of crude water extract of *l. oweriensis* was evaluated *in-vitro* at the concentrations of 0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml. The percentage inhibitions of serum ACE activity by different concentrations of the extract was tested and compared to standard ACE inhibition drug (captopril) as shown in Table 3. Also, the ACE activity of individual phenolic compounds present in the extract was investigated using standard phenolic compounds. It was observed that the extract at the maximum tested dose was able to inhibit ACE by 87.77%, which is comparable to the activity of standard drug (90.07%). The individual phenolic compounds also showed variable inhibitory activity ranging from 16.79 - 40.11%, with quercetin showing the highest inhibitory activity (40.11%), followed by garlic acid (34.27%). The pathogenesis of hypertension has been attributed to the conversion of angiotensin 1 to angiotensin 11 by the angiotensin converting enzyme. Therefore, one of the important therapeutic strategies for normalizing blood pressure in hypertensive patients is the use ACE inhibitors, which inhibit the conversion of angiotensin 1 to angiotensin 11[37]. It has been reported that one essential factor for inhibition of the zinc metalloproteinases is the presence of several hydroxyl groups in the phenolic compounds[38]. Additionally, the exact position of this group has been revealed to be very important for ACE inhibition. Also, the presence of a catechol group in the B ring (3', 4'-dihydroxy) and Hydroxylation at the 4'-position of the B ring has been reported to be essential in achieving an increased ACE inhibitory activity[38]. Indeed, the highest ACE inhibitory activity of individual phenolics was found in quercetin, which possesses these essential structural requirements.

Hypotensive effect of the extract on SHRs

One of the most widely studied model for evaluation of hypotensive effect of drug and dietary component *in vivo* is the SHRs[39,40]. In our study, the food intake, final body weight or level of serum parameters between the control and the experimental group did not show any significant difference (Table 4). The systolic blood pressure was found to increase during the onset. However, there was significant lowering of BP in the experimental group from the two week feeding point (Figure 2). Various literatures have reported that consumption of diet rich in ACE inhibitors, results in significant reduction in blood pressure in vivo[41-43]. Previous report has shown that quercetin has strong dose dependent hypotensive effect in SHRs[42]. The crude extract of *l. oweriensis* used in our study contained high percentage of quercetin; hence we anticipate that the antihypertensive effect of the extract may be through the inhibition of ACE activity in SHRs.

Diuretic assay

Table 5 showed the results of diuretic assay. In this study, Frusemide (10 mg/Kg), which has be known to increased urine volume and urinary electrolytes was used as standard diuretic drug. After 6hrs interval, a comparison of the volume of urine excreted by both the normal control and experimental rats per 100 g of the body weight was carried out. It was observed that intraperitoneal administration of the crude extract of *l. oweriensis* at the doses of 10, 20 and 40 mg/Kg increased the urine volume in rats. However, the increase in urine and electrolytes output was only significant at the doses of 20 and 40 mg/Kg, but insignificant at the dose of 10 mg/Kg. Further increase in dose did not increase the urine output in rats (data not shown). Frusemide was found to have higher diuretic effect than the extract at the tested doses. The ratio Na+/K+ is a measure of natriuretic activity and value greater than 2 indicates favourable natriuretic effect[44]. The ratio of Na+/K+ was found within the range of 3.73 ± 0.39 to 4.30 ± 0.45, indicating a favourable natriuretic effect of the extract.

It is a well known fact that diuretics decrease plasma volume, followed with a subsequent venous return to the heart; *i.e*. preload. As a result of this, there is decrease in cardiac workload, oxygen demand and plasma volume, thus decreasing the blood pressure. Diuretics play an important role in volume overloaded and salt sensitive hypertensive patients[45]. Many studies have shown that several secondary metabolites of plants such as alkaloids, tannins, saponins, terpenoids, organic acids and or phenolic compounds could be responsible for the diuretic effects[46]. The extract of *l. oweriensis was* found to contain many phenolic compounds, which may be responsible for the diuretic activity of the medicinal plant.

Molecular modelling and docking study

To probe further into the binding mode between the isolated compounds and ACE, the compounds were docked on active site of tACE (PDB code: 1O86) using Autodocks software. The predicted poses of the isolated compounds and their intermolecular interactions with tACE binding site residues is shown in figure 3. The report of captopril bound–ACE complex has given more insight for development of better binding ACE inhibitors[47] It is a well known fact that hydrogen bonding plays significant role in the formation of ligand-protein complex. The obtained result of the docking showed the hydroxyl group on quercetin positioned with strong hydrogen bond from His353 (2.67 Å) and Tyr620 (2.35 Å) in the subsite. Apiginine also showed strong hydrogen bond from Ala356 (2.39 Å) and Glu411 (2.30 Å) in the subsite. These interactions showed close relation with the Captopril standard drug and reported ACE-complex of Lisinopril[48]. However, no intermolecular hydrogen bonding was observed in P-coumeric acid. It is pertinent to note that most of the functional group of the isolated compounds were positioned with either hydrogen bonding or other non-covalent lipophilic interactions, which is very important in exerting greater affinity towards tACE enzyme and then ACE inhibition activity.

The affinity binding energy calculation showed that Captopril a theoretical higher energy than quercetin, p-coumeric acid and apiginine (Fig 3), indicating a lower binding affinity to tACE than the isolated compounds.

CONCLUSION

In conclusion *landophia oweriensis* extract was found to possess good antioxidant and polyphenolic content with high percentage inhibitiory activity against ACE, indicating that this edible plant could be a potential target for development of new ACE inhibition drugs

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