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Modulatory effect of ethylacetate fraction from *Theobroma* cacao pod husk on blood pressure in L-NAME-induced hypertensive rats using in *vivo* and *in silico* approaches

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ABSTRACT

Hypertension related diseases are public health concerns due to their high incidence and mortality. However, the inability of the synthetic drugs to normalize blood pressure (BP) to acceptable range has motivated the search for safer and effective alternatives. This study investigated the antihypertensive activity of ethyl acetate fraction of *Theobroma cacao* pod husk (EAF-TCPH) in L-nitro-arginine-methyl-ester (L-NAME)-induced hypertensive male rats. Methanol extract of *Theobroma cacao* pod husk (TCPH) was partitioned to obtain the EAF-TCPH fraction. Hypertension was induced by oral administration of 40 mg/kg body weight (bw) L-NAME; while BP was checked by tail plethysmography. Wistar rats were assigned into groups 1–6. Group 1 received 1.0 ml of paraffin oil, while groups 2–6 were hypertensive rats and received 1.0 ml of paraffin oil, 12.5, 25 and 50 mg/kg bw of the EAF-TCPH and 25 mg of captopril respectively for 6 weeks. The compounds in EAF-TCPH were identified using high performance liquid chromatography and were docked against angiotensin converting enzyme (ACE). Elevated BP of 180.17 (systolic) and 125.79 mmHg (diastolic) in the hypertensive rats was lowered to 125.33 and 88.00 mmHg, respectively in the 25 mg/kg bw EAF-TCPH treated group. The major compounds in EAF-TCPH were theaflavin, epigallocatechin, procyanidin, epicatechin and cianidanol. Theaflavin binds to ACE better than captopril (standard drug) with binding energy of −10.8 and −5.8 kcal/mol, respectively. This study therefore suggests that EAF-TCPH could have demonstrated potential antihypertensive activities in L-NAME-induced hypertensive rats due to the available bioactive components in EAF-TCPH that probably interacted in a beneficial way with ACE.

INTRODUCTION

There is a global increase in chronic diseases such as hypertension, obesity, diabetes, and cancer [1]. Hypertension is the most prevalent cardiovascular disease (CVD) with a serious public health concern in both industrialized and developing countries [2]. It is characterized by an increase in blood pressure (BP) in the arteries. The normal systolic and diastolic BP during rest are 100–140 and 60–90 mmHg, respectively. BP that is consistently above 140/90 mmHg indicates hypertension [3]. It has also been implicated to be responsible for other medical conditions such as myocardial infarction, hypertensive retinopathy, convulsion, elevated sugar level [4]. According to previous study, CVDs is responsible for 5% of disability-adjusted life years [2,5]. Many emerging countries, particularly urban populations, are claimed to have prevalence levels that are comparable to those seen in affluent ones [3].

Cocoa (*Theobroma cacao* L.) is a member Sterculiaceae family and is native to the tropical rain forest of the eastern Andean slopes [6]. It is a perennial crops principally utilized in the manufacturing of chocolate. Cocoa pod husk

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(CPH) accounts for more than 70% of the total weight. Cocoa is rich in polyphenols such as flavonoids. Chocolate flavonoids boost nitric oxide (NO) production, increase vasodilation, and minimize endothelial dysfunction. Hence, they are beneficial to hypertensive [7].

Due to the concerns about the cost and negative effects of long-term use of synthetic substances, there is increased interest in natural compounds and their derivatives as safer alternatives as functional foods or nautraceuticals [4]. Plants rich in polyphenols have been reported to contain biologically active compounds which offer beneficial protection against chronic degenerative diseases [8]. Cocoa extract has been reported to contain gamma-aminobutyric acid which is a natural amino acid that aids in reducing BP and calming of nerves [9]. However, BP lowering properties of CPH polyphenol has not been scientifically validated. As a result, the aim of this research was to investigate antihypertensive activity of polyphenol-rich fraction of *T. cacao* pod husk in L-nitro-arginine-methyl-ester (L-NAME)-induced hypertensive male rats as well as appraising computational prediction of drug-like bioactive lead in ethyl acetate fraction of *Theobroma cacao* pod husk (EAF-TCPH).

MATERIALS AND METHODS

Chemicals and reagents

Captopril was procured from Bristol Pharmaceutical Company (BPC, UK). L-NAME from ElabScience, Texas, USA. Electrosphygmomanometer was a product of Kent Scientific (CODA, Kent Scientific, USA). All other reagents utilised in this research were of analytical grade and were prepared in all-glass apparatus with distilled water.

Collection of sample

The freshly processed CPHs were obtained/collected from a Cocoa plantation at Oko, Surulere LGA, Oyo State, Nigeria. The voucher samples were authenticated at the University of Ilorin, Plant Biology's Herbarium Unit where a sample was deposited and assigned voucher number UIH 001/786.

Methods used

Preparation of sample extract

Fresh CPHs were air-dried for 15 days to prevent loss of bioactive agents due to irradiation and then pulverized to powdered form using mechanical blender. The sample (100 g of CPH) was soaked in 80% methanol (1:10 *w*/*v*) for 48 hours. Then, mixture was filtered and the filtrate obtained was concentrated using rotary evaporator, dried with water-bath at 45°C, and kept in the refrigerator [i.e., methanolic extraction of cocoa pod husk (MCPH)] (Sample 1).

Solvent fractionation of methanol extract of Theobroma cacao pod husk (TCPH)

The sample 1 (above) was subjected to exhaustive solvent/solvent partitioning using organic solvents (n-hexane and ethylacetate) in order of their polarity index. Briefly, 10 g of the dried MCPH extract was dissolved in 20 ml distilled water (1/2 *w*/*v* %). Thereafter, the mixture was poured into a

separating funnel, this was followed by the addition of 100 ml of n-hexane. The mixture was agitated and set aside until the mixture was separated into three partitioned layers of n-hexane extract, water extract and crude residue of the MCPH extract in the funnel. Each layer was collected into different conical flask. The residue of the crude extract was then again poured into the funnel and 100 ml of n-hexane was again added to continue the partitioning. The process was repeated continuously with the n-hexane solvent until a clear n-hexane solvent appeared. The entire residue obtained from the n-hexane partitioning was further partitioned with ethylacetate solvent, as done with the n-hexane solvent until a clear ethyl acetate solvent appeared in the separating funnel. Each of the portioned fractions (n-hexane and ethylacetate) obtained was then concentrated using a rotary evaporator to remove the solvents. The ethyl acetate yield was subjected to high performance liquid chromatography (HPLC) in order to profile the bioactive components of the sub-fraction (EAF-TCPH) as described by Afolabi *et al*. [10]. The yields of the fractions were calculated with the ethylacetate fraction having lesser yield (%) than the hexane fraction.

Animals' treatment protocol

Thirty-six Healthy male Wistar rats weighing 200 ± 1.20 g used for this research were obtained from the Animal Housing Unit of the Department of Biochemistry, University of Ilorin, Ilorin, Kwara State, Nigeria. They were kept in plastic cage in a well-ventilated house condition with free access to rat pellets and water *ad libitum*. They were allowed to acclimatize for 1 week prior the commencement of the study.

Ethical clearance

Ethical clearance on the use and proper handling of laboratory animals in line with United Nation guidelines was approved by the University of Ilorin Ethical Review Committee (UERC) with the number UERC/ASN/2018/1380.

Induction of hypertension

High BP was induced in the Wistar rats following the exposure to oral dose of 40 mg/kg body weight (bw) L-Name for 3 weeks (21 days). Hypertension was confirmed in rats in the fourth week by measuring an increase in the systolic and diastolic BP in the rats by tail plethysmography using an electrosphygmomanometer. Experimental rats were considered hypertensive at ≥140 mmHg systolic and ≥90 mmHg diastolic.

Animal grouping

The animals used (36 male Wistar rats) in this study were randomly grouped into six groups of six (*n* = 6) rats as follows; and the choice of extract dosing regimen was informed based on the pilot studies carried out prior the study.

Group 1: Control group (Paraffin oil only);
Group 2: L-NAME induced untreated (Positive control);
Group 3: L-NAME induced + 12.5 mg/kg EAF-TCPH;
Group 4: L-NAME induced + 25 mg/kg EAF-TCPH;
Group 5: L-NAME induced + 50 mg/kg EAF-TCPH;
Group 6: L-NAME induced + 25 mg/kg Captopril.
Note: *The doses of EAF-TCPH used were determined during the pilot study and were calculated based on the individual rat's bw in relation to the chosen concentration.*

Molecular docking

The HPLC-identified compounds and Captopril (PDB: 44093) were subjected to docking with the PubChem CID: ACE proteins (PDB: 3BKK) obtained from the protein database. This docking process was performed using Schrodinger's Grid-Based Ligand Docking with Energetics software, as outlined in the study by Friesner *et al.* [11] and Mahmoud *et al.* [12]. The X-ray crystal structure of ACE was acquired from the Protein Data Bank (https://www.rcsb.org/), and subsequently processed using Glide's protein preparation wizard [13].

Statistical analyses

Data were analyzed using one-way ANOVA, followed by Tukey's test for *post-hoc* analysis and graphical representation of results performed using GraphPad Prism 8.5 version (GraphPad Software, San Diego, CA). All values were expressed as mean ± SEM ($n = 6$). Statistical differences were considered at $p < 0.05$.

RESULTS

Effect of the administration of EAF-TCPH on systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial volume of L-NAME-induced hypertensive rats

The effect of administration of various doses of sub-fraction of EAF-TCPH on the SBP, DBP and mean arterial volume of hypertensive rats is displayed in Table 1. There was a significant increase ($p < 0.05$) in the BP of rats administered 40 mg/kg bw of L-NAME for 3 weeks, while there was a significant decrease ($p < 0.05$) in the SBP, DBP and mean arterial volume in the hypertensive rats following 6 weeks of treatment with the various doses of sub-fraction of EAF-TCPH.

HPLC-identification of bioactive components of EAF-TCPH

The chromatogram of sub-fraction of EAF-TCPH is represented in Figure 1. Theobromine, caffeine, resveratrol, flavanol, flavan-3-ol, procyanidin, catechin, epicatechin, epigallocatechin and theaflavin were the compounds identified.

Theaflavin has the highest retention time, while catechin has the highest area (Table 2).

In silico **analysis of angiotensin-1 converting enzyme (PDB: 3BKK) in complex with captopril, theaflavin and procyanidin**

Figures 2 and 3 shows hydrogen bonding, hydrophobic and binding energy interacion of theaflavin and capropril (standard drug) with angiotensin-1-converting enzyme (ACE) respectively. There were several hydrogen interactions observed in theaflavin at Ser[355], His[387], Tyr[66], Glu[143] of ACE. His[387], Val[518], Val[63] are involved in the formation of hydrophobic interactions. Val[518] in theaflavin were involved in π-π stack interactions. Gln[281], His[513], Phe[457], His[383], Tyr[523] and Phe[527], His[383], Tyr[523] and Phe[527] are involved in hydrogen bonding. Molecular docking of sub-fraction of TCPH using AutoDock Vina scoring function algorithm shows that procyanidin and theaflavin had the highest binding energy at the binding pocket of angiotensin-1 converting enzyme when compared to the co-crystalized ligand captopril (Table 3).

DISCUSSION

The importance of cocoa in vasodilation, which aids in the management of hypertension by lowering BP has been well-reported previously [14]. These earlier reports emphasized on the presence of bioactive components in cocoa as the basis for this acclaimed activity [15]. Also, health benefit from the intake of tea or chocolate has also been attributed to the consumption of flavan-3-ols and flavonoids. Numerous studies have suggested that the intake of flavan-3-ols attenuates the risk of CVDs, which included; hypertension, coronary heart diseases, myocardial infarction and stroke. Mechanistic *in vitro* studies confirmed that flavan-3-ols modulate NO production or breakdown, lipid metabolism and platelet function [16–18]. In our study (Table 1), a noteworthy decrease in both SBP and diastolic blood pressure (DBP) was observed in rats administered 12.5 and 25 mg/kg bw of a sub-fraction from EAF-TCPH. This reduction was comparable to the group that received 25 mg/kg bw of captopril. This effect may be attributed to the high polyphenol content found in this sub-fraction (Fig. 1 and Table

Table 1. Effect of EAF-TCPH on the SBP, DBP and mean arterial volume of L-NAME- induced hypertensive rats.

| Parameters | Groups | | | | | |
	1	2	3	4	5	6
SBP$_1$	111.66 ± 0.88	115.24 ± 1.42[a]	101.11 ± 0.91[b]	100.99 ± 0.21[b]	108.45 ± 0.65[c]	121.11 ± 1.81[d]
DBP$_1$	76.11 ± 0.12	80.11 ± 1.10[a]	88.72 ± 0.09[b]	81.12 ± 0.63[c]	78.99 ± 0.13[d]	92.12 ± 0.15[e]
Mean$_1$	88.25 ± 0.21	89.92 ± 0.45[a]	84.55 ± 0.11[b]	83.91 ± 1.00[b]	85.34 ± 0.19[c]	92.28 ± 0.01[d]
SBP$_2$	121.66 ± 0.56	179.33 ± 1.76[a]	164.33 ± 2.18[b]	167.00 ± 3.05[b]	173.00 ± 2.51[c]	183.00 ± 1.00[d]
DBP$_2$	81.66 ± 2.96	127.00 ± 4.04[a]	108.66 ± 7.17[b]	116.66 ± 1.85[b]	115.66 ± 3.66[b]	144.00 ± 1.15[c]
Mean$_2$	91.66 ± 1.85	144.00 ± 3.0[a]	127.00 ± 4.00[b]	133.00 ± 2.30[b]	134.33 ± 3.33[b]	156.66 ± 0.88[c]
SBP$_3$	115.0 ± 2.30	180.17 ± 2.55[a]	126.00 ± 1.52[b]	125.33 ± 3.17[b]	139.66 ± 6.56[c]	121.00 ± 2.00[d]
DBP$_3$	81.66 ± 3.38	125.79 ± 3.11[a]	77.00 ± 4.16[b]	88.00 ± 1.00[c]	96.66 ± 5.17[d]	91.00 ± 4.00[d]
Mean$_3$	92.66 ± 2.96	143.98 ± 1.99[a]	93.33 ± 2.60[b]	90.33 ± 1.76[c]	101.00 ± 5.29[b]	93.00 ± 2.00[b]

Values are means of eight readings ± SEM.
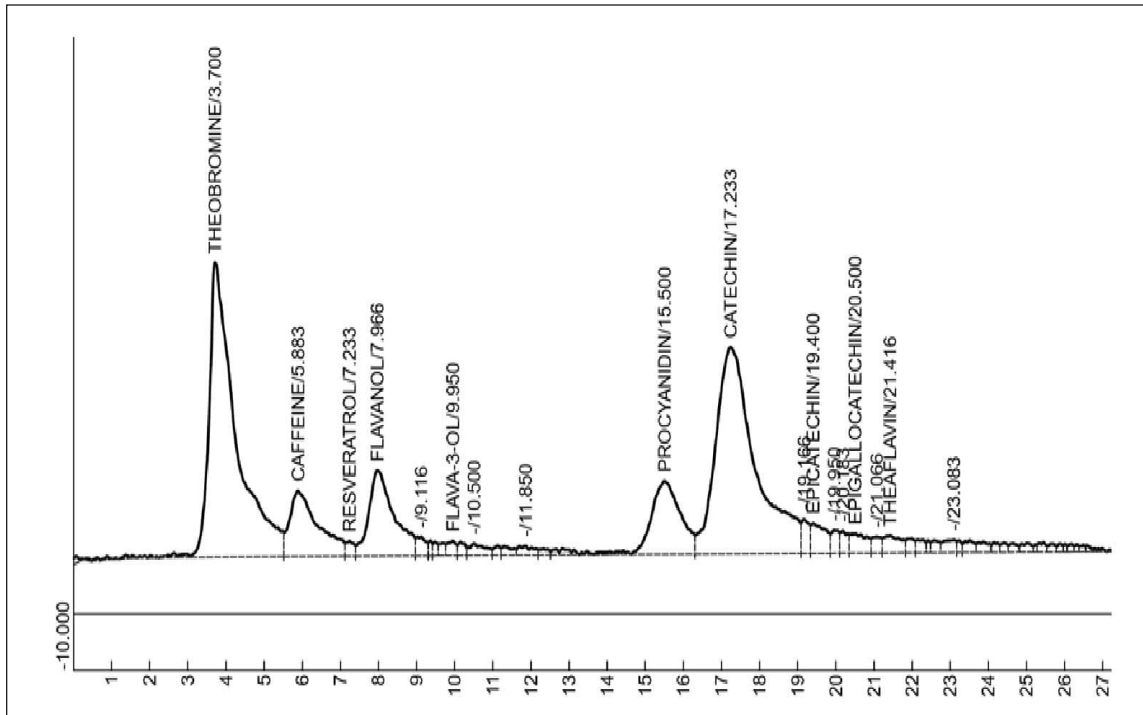Alphabets (a–e) indicate significant ($p < 0.05$) difference compared to control.

Figure 1. HPLC-fingerprints of identified bioactive constituents of EAF-TCPH.

Table 2. HPLC-identified phytoconstituents of EAF-TCPH.

S/N	Name of compound	Retention time	Area under the curve (%)
1	Theobromine	3.70	34.22
2	Caffeine	5.88	8.00
3	Resveratrol	7.23	0.53
4	Flavanol	7.97	9.24
5	Flava-3-ol	9.95	0.54
6	Procyanidin	15.50	8.85
7	Catechin	17.23	35.05
8	Epicatechin	19.40	1.86
9	Epigallocatechin	20.50	1.37
10	Theaflavin	21.42	1.29

2), specifically the flavanol monomer/polymer, as indicated by Żyżelewicz *et al*. [19]. These findings are supported by a prior study which demonstrated that compounds like catechin and epicatechin have vasodilatory effects by stimulating NO-synthase, leading to an increase in NO and subsequently soluble guanylate cyclase (SGC) activity in the blood vessels [20].

The process by which guanosine triphosphate is transformed into cyclic guanosine-3′,5′-monophosphate is expedited by SGC, thereby instigating the expansion of blood vessels and enhancing blood flow [21]. In a similar vein, polyphenols found in CPHs are recognized for their role in safeguarding against oxidative stress, inhibiting platelet aggregation, promoting insulin sensitivity, and reducing lipid peroxidation [22]. Collectively, these mechanisms may contribute to the reduction of BP and the potential mitigation of CVDs. The major bioactive compounds, including theobromine, caffeine, resveratrol, flavanol, flavan-3-ols, procyanidin, catechin, epicatechin, epigallocatechin, and theaflavin, found in the methanol extract of sub-fractions from TCPH, have been documented for their ability to enhance antioxidant enzymes. Additionally, they have shown a capacity to reduce malondialdehyde levels, thereby playing a crucial role in combating reactive oxygen species, which have been linked to the development of various diseases, including hypertension [23]. The observed reduction in BP attributed to the bioactive components within the EAF-TCPH sub-fraction of TCPH may also be attributed to their ability to inhibit pro-inflammatory cytokines [24].

Acute intake of caffeine has been associated with elevation of BP both in the normotensive and hypertensive individuals [25]. In contrast chronic intake has been associated with protective effects in the development of insulin resistance, glucose intolerance and hypertension [26,27]. Resveratrol, a naturally occurring flavonoid found in EAF-TCPH has previously been reported to have cardioprotective, vasoprotective, antioxidant, anti-vasoconstriction, antidiabetic and neuroprotective properties [28]. Resveratrol has been reported to increase the synthesis of NO (a vasodilator) via the activation protein kinase. Vasodilation lowers BP by lowering peripheral resistance [29]. Theobromine on the other hand has also been reported to have vasodilating properties; thus, playing crucial roles in hypertension. Mechanistically, theobromine was reported to inhibit phosphodiesterase and block the activities of adenosine receptors [30,31]. This mechanism has been reported to be beneficial in hypertensives by preventing obstruction of blood vessels and vascular resistance [32].
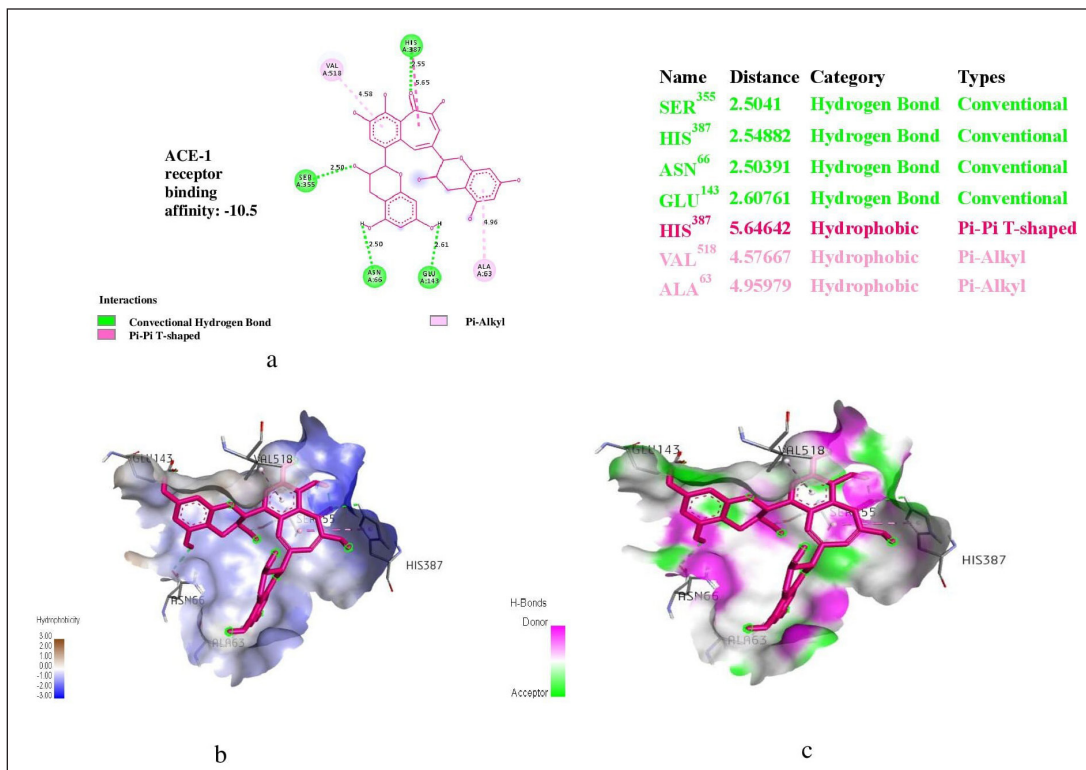
Figure 2. (a) Binding energy. (b) hydrophobic, and (c) hydrogen bonding interactions of theaflavin with angiotensin-1 converting enzyme.
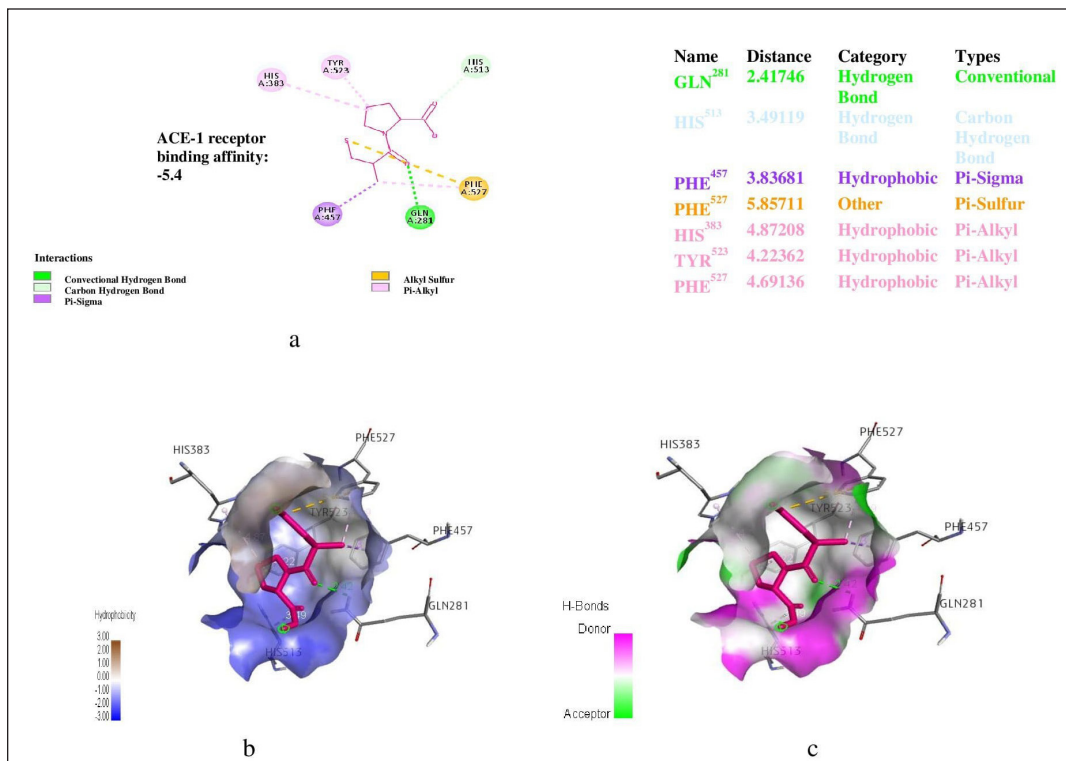

Figure 3. (a) Binding energy, (b) hydrophobic; and (c) hydrogen bonding interactions of captopril with angiotensin-1 converting enzyme.

Table 3. Binding affinities of HPLC-identified compounds from EAF-TCPH and Captopril using AutoDock Vina scoring function algorithm.

Ligand	Binding affinity (ΔG)
Captopril	−5.8
Caffeine	−6.1
Theobromine	−6.1
Cianidanol	−9
Epicatechin	−9
Epigallocatechin	−9.3
Procyanidin	−10.3
4-Flavanol	−7.9
Resveratrol	−7.7
3-Flavanol	−8
Theaflavin	−10.8

Similarly, the interactions of these bioactive compounds in lowering BP were also investigated in this study (Figs. 2 and 3; Table 3). The binding sites of the ligands were predicted using P2Rank, a machine learning method that predicts local chemical neighbourhood ligand ability based on points placed on a solvent-accessible protein surface. Points that has high ligand ability score were used to create ligand binding sites. In this study, the first ranked binding pocket of ACE1 has probability of 0.988 with a score of 89.84 compared to the second ranked pocket which has a low probability of 0.279 and a score of 8.27. This implies that the first ranked pocket is the most likely binding site of ligands.

The ligand-based and structural-based (receptor-based) are virtual screening approaches commonly utilized in the discovery of prospective lead compounds against a potential target [33,34]. In this study, all the identified compounds have higher binding affinities than captopril (standard drug); however, two lead compounds (procyanidin and theaflavin) of the 10 compounds had extremely high binding affinities. This implies that the selected designed compounds would be a potential target for drug design against hypertension compared to captopril already available in markets.

The amino acid residues involved in ligand interactions of captopril after molecular docking simulation study are Gln[281], His[513], Phe[457], His[383], Tyr[523] and Phe[527], His[383], Tyr[523] and Phe[527]. Hydrogen bonds are the most common directed intermolecular interactions in biological complexes, and they account for the majority of molecular recognition specificity. Because of their severe distance and geometric restrictions, hydrogen bonds are used in medication design to gain specificity. The binding of captopril to ACE-1 resulted in Gln[281] hydrogen-donating interaction. Previous study showed that the contribution of hydrogen bonds to free energy is dependent on the local environment. Solvent-exposed hydrogen link contributes more to net interaction energy than a concealed hydrophobic pocket hydrogen bond [35]. Thus, the increased number of hydrogen interactions observed in theaflavin (Ser[355], His[387], Tyr[66],Glu[143]) may greatly contribute or explained the higher binding affinities or free energy observed in both of the ligands compared

to captopril. In previous studies, heavy atoms in N–H…O, N–H…N, and O–H…O hydrogen bonds were shown to be separated by similar median lengths of roughly 3.0. The bond distances for lead complexes were investigated in this study. Theaflavin (Ser[355]: 2.5041, His[387]: 2.54882, Tyr[66]: 2.50391,Glu[143]: 2.60761) are within the required threshold of median distance of 3.0 Å [36].

Hydrophobic contacts are by far the most common interactions in protein–ligand complexes, they account for 66, 772 connections. The most popular group is formed by an aliphatic carbon in the receptor and an aromatic carbon in the ligand, accounting for about 42, 000 interactions [37]. This shows that aromatic rings frequently inhibits small molecules. In fact, 76% of marketed medications have one or more aromatic rings, with benzene as the most frequent ring system [38]. Leucine side-chains are the most frequent involved in hydrophobic interactions, followed by valine, isoleucine, and alanine side-chains [36]. In this study, theaflavin (His[387],Val[518], Val[63]) are involved in the formation of hydrophobic interactions which precisely entails alkyl, π-alky and π-π alkyl types of bond of interactions which are responsible for 3-dimensional structural shape of the complex. More precisely, the π-π stack are the third most common protein-ligand interactions involving aromatic rings. The amino acid residue Val[518] in theaflavin involved in π-π stack interactions has been indicated in previous works to enormously contribute to protein-ligand recognition, thus aiding drug design [39,40].

CONCLUSION

The findings from this research highlight the potential therapeutic value of sub-fraction of ethylacetate from the methanol extract of *Theobroma cacao* pod husk (EAF-TCPH) and the computer-aided interactions of its bioactive compounds (such as theaflavin, epigallocatechin, procyanidin, and epicatechin). The EAF-TCPH showed remarkable antihypertensive and antioxidant properties in both *in vitro* experiments and L-NAME induced hypertensive rats which could possibly be credited to the presence of HPLC-identified constituents present in the extract. Therefore, EAF-TCPH might be useful as alternative in the management of hypertension.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

Ethical clearance on the use and proper handling of laboratory animals in line with United Nation guidelines was approved by the University of Ilorin Ethical Review Committee (UERC) with the number UERC/ASN/2018/1380.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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