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IDENTIFICATION OF COMPOUNDS WITH POTENTIAL ANGIOTENSIN CONVERTING ENZYME INHIBITORY ACTIVITY IN *ADANSONIA DIGITATA L.* FRUIT PULP

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ABSTRACT

The study employed bioassay-guided fractionation to identify novel angiotensin-converting enzyme inhibitory compounds from *Adansonia digitata* fruit pulp, potentially responsible for its antihypertensive effects. *A. digitata* Fruit pulp was extracted using the solvents n-hexane, ethylacetate, methanol, and water. The percentage weight, *in vitro* Angiotensin Converting Enzyme (ACE) inhibitory activities and IC_{50} values of the extracts were determined. Fractionation and phytocompound identification were performed by thin layer chromatography (TLC), column chromatography techniques, and gas chromatography-mass spectrometry (GC-MS) techniques. The yields obtained from solvent extractions using n-hexane, ethylacetate, methanol, and water were 0.14%, 1.17%, 4.30%, and 21.80%, respectively. The IC_{50} values of Ramipril®, n-hexane, ethylacetate, methanol, and water extracts were found to be 101.40µg/ml, 116.70µg/ml, 40.53µg/ml, 47.25µg/ml, and 81.05µg/ml, in that order. The most potent ACE inhibitory activity was found in methanol extract of *A. digitata* Fruit (MEADF), which was chosen for bioassay-guided fractionation. From the assay, two fractions (Fraction I and III), among others, showed low IC_{50}s of 11.77±0.10 µg/ml and 11.96±0.16 µg/ml, respectively, and significant ACE inhibitory activity. The most relatively abundant compounds found in Fractions I and III) were 9, 12, octadecadienoic acid methyl ester (conjugated linoleic acid isomers), cis-Vaccenic acid, 3H-Pyrazol-3-one, 4-benzoyl-2, 4-dihydro-5-methyl-2-phenyl (a pyrazole), and Hexadecanoic acid methyl ester (aromatic acid ester).

The study reveals valuable compounds in the methanol extract of Baobab fruit pulp, which could be a promising source for developing antihypertensive foods.

Keywords: Angiotensin Converting Enzyme, Hypertension, cis-Vaccenic acid, 3H-Pyrazol-3-one, 4-benzoyl-2, 4-dihydro-5-methyl-2-phenyl and Bioassay-guided fractionation

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Introduction

It has been acknowledged that as societies developed, epidemiological shifts occurred as well. Unfortunately, the rising burden of non-communicable diseases (NCDs), particularly cardiovascular diseases (CVDs), cancer, and diabetes mellitus, has been the most internationally prevalent health shift during the second half of the twentieth century [1,2,]. Hypertension is one of the top five causes of death worldwide [3], as well as a key peril factor for more than half of all CVD-related deaths [4].

Hypertension (HP) is a progressive cardiovascular illness characterized by a sustained elevation of blood pressure (BP) of ≥ 140 mm Hg (systolic) or ≥ 90 mm Hg (diastolic) caused by a variety of complex and interconnected aetiologies with both structural and functional changes in the blood vessels and heart [5,6]. Thus, HP was found to be prevalent in20.5% of females and 24.0% of males worldwide; while Africa reported the prevalence of 29.5% and 29.7% for females and males, respectively. Similarly, in Nigeria, the reported prevalence was found to be 28.1% for males and 27.5% for females ([7]. The current worldwide hypertension figure of 1.13 billion people is expected to rise to 1.5 billion by 2025 ([8].

Although different categories of synthetic medications against hypertension (including angiotensin receptor blockers, diuretics, calcium channel blockers, angiotensin converting enzyme inhibitors, and vasodilators, among others) are available in modern medicine ([9, 10, 11,], majority of them are less effective (< 25% control Blood Pressure below the normal threshold) and those with better efficacy produce adverse effects [12]. Despite their current side effects such as dry cough, and angioedema; angiotensin converting enzyme inhibitors have become powerful, successful, and preferred therapeutic medicines in the treatment of hypertension. This has prompted a surge in commercial interest in the development of novel, safer antihypertensive medications based on angiotensin converting enzyme inhibitory activity derived from natural sources, particularly plant-based foods.

Adansonia digitata L. is an endangered member of the Bombacaceae family, which was labelled as a "Superfruit" by [13] due to its high phytochemical and nutritional contents. According to Ramadan *et al.* [14], Baobab fruit pulp is relatively safe, with an LD_{50} of 8000 mg/kg. Anti-hyperlipidaemic [15], anti-inflammatory [14], and anti-diabetic [16] potentials of fruit pulp of *A. digitata* were earlier reported. Although the antihypertensive potential of methanol extract of Baobab fruit pulp was reported by Liman *et al.* [5], the phytocompounds responsible for the bioactivity are yet to be identified. As a result, this work used a bioassay-guided fractionation technique to identify angiotensin converting enzyme inhibitory compounds from extracts of *A. digitata* fruit pulp that could be responsible for the fruit's antihypertensive effects.

Materials and Methods

Reagents

Angiotensin Converting Enzyme (Sigma Aldrich,USA), Hippuryl-L-Histidyl-Leucine (Sigma Aldrich,USA), Hippuric Acid (Sigma Aldrich,USA), n-hexane (Guangdong Guanghua SCi-Tech CO Ltd, China), Ethylacetate (Guangdong Guanghua SCi-Tech CO Ltd, China), Methanol (Guangdong Guanghua SCi-Tech CO Ltd, China), Sodium tetraborate (BDH, England), Boric acid (BDH, England), Sodium chloride (BDH, England), HCl (May and Baker Degenham, England), Ramipril® (Pfizer Laboratories Div Pfizer Inc), Deionized Distilled water (Pharmaceutical Chemistry Lab. ABU, Zaria, Nigeria), TLC Silica gel 60 F_{254} (Merck KGaA), Germany, Silica Gel 60-120 mesh for Column Chromatography (Lobal Chenie, India) and Sodium hydroxide (BDH, England).

Equipment

Spectrophotometer (SP-3000 Plus Optima Inc, Japan), Spectrophotometer (Carry 300 UV-VIS), Ultraviolet lamp (Slough, England) and TLC Chroma Tank (Shandon Souther).

Plant Material

Ripe baobab fruits were harvested from the wild around Buka village, Mokwa Local Government Area, Niger State, Nigeria, between January to March, 2019. The fruits were identified, authenticated and provided with a voucher number of 21512 at Herbarium Laboratory, Department of Botany, Ahmadu Bello University Zaria.

Extraction of Baobab Fruit pulp

The Baobab fruit shell was broken using Hammer and the pulp was removed from the seeds. The pulp was then milled and the powdered sample (1000g) was successively macerated with 5 litres each of *n*-hexane, ethylacetate, methanol and water. The crude extracts obtained from each step were filtered with muslin cloth and evaporated. Each extract was weighed and the corresponding percentage yield was calculated as follows:

$$\text{Percentage yield} = \frac{\text{weight of extract obtained (g)}}{\text{weight of baobab fruit pulp soaked (g)}} \times 100$$

Screening of Baobab fruit pulp extracts for inhibition of Angiotensin converting enzyme activity

Each of the four extracts was screened for Angiotensin-Converting Enzyme (ACE) inhibitory activity assay following the method of Cushman and Cheung [17]. The assay utilized hippuryl-histidyl leucine (HHL) as a substrate. At pH 8.3, the ACE hydrolyzed HHL into hippuric acid (HA). The HA produced was extracted into the ethylacetate layer by centrifugation at 800×g for 15min and 750µl aliquot upper organic stratum was collected into a clean tube by means of micropipette (0-1000µl). The ethylacetate aliquots were evaporated completely by heating at 95^0C for 30 minutes in a rotary evaporator. The hippuric acid was solubilized in 1.0 ml of water, thereafter; its absorbance was measured at 228 nm. At least triplicate assay for each was carried out. The percentage ACE inhibitory activity was computed using the formula:

$$\text{ACEI (\%)} = \frac{\text{Aa} - \text{Ab}}{\text{Aa} - \text{Ac}} \times 100$$

Where Aa is the absorbance of control (ACE + HHL solution), Ab is the absorbance of ACE + HHL + sample (inhibitor solution), and Ac is the absorbance of blank (HHL solution) only. The graph of percentage ACE inhibition against varying concentrations of extracts/fractions or Ramipril® were plotted using GraphPad Prism (version 8.0 software, USA) with their IC_{50} values interpolated.

Purification of the selected extract

Based on the ACE inhibitory assay, methanol extract (most active extract) was selected for purification and identification of bioactive compounds.

Five different solvent systems: 100% ethylacetate, ethylaceate/chloroform/methanol/water (15:8:4:1), chloroform/methanol system (1:1) butanol/acetic acid /water (4:1:1) and ethylacetate/methanol systems (7:3) were tested using Thin Layer Chromatographic (TLC) technique. Based on good separation and a greater number of spots observed using UV-lamp, two solvent combination system made up of ethylacetate and methanol was selected for column chromatography.

A glass column (of 60 cm length and 30 mm diameter) was packed with 100g Silica Gel 60-120 mesh particle size. Methanol extract of *A. digitata* fruit (5g) mixed with silica gel was transferred into the column until it reaches the desired column height (60cm). Once the sample matrix has settled, the ethylacetate was applied initially with two bed volumes and it was mixed with methanol to increase the polarity gradient and a total of 75 factions (50ml each) were collected. The flow rate was set at 10 drops per second. Each fraction collected was analysed in TLC for the compound separation. In the course of time, the polarity of the solvent was slowly increased as follows: 100% ethylacetate, ethylacetate/methanol (4:1), ethylacetate/methanol (3:2), ethylacetate/methanol (2:3), ethylacetate/methanol (1:4) and 100% methanol. Fractions were collected until TLC showed no more compound peaks. Fractions with similar retention time were pooled together to obtain six bulk fractions (I, II, III, IV, V and VI). An *in vitro* ACE inhibitory activity assay was carried out on each of the six bulk fractions. Fraction I and Fraction III were the most potent and were subjected to GC-MS analysis.

Identification of bioactive compounds by Gas Chromatography-Mass Spectrometry

The phytochemical investigation of fractions I and III of methanol extract of *A. digitata L.* fruit pulp was performed using a GC-MS equipment (Thermo Scientific Co). The oven temperature was maintained at 220ºC on a rate of 6ºC/min. The carrier gas with a flow rate of 1 ml/min, 1 µl volume of sample was injected using split sampling technique in 1:10 ratio. Retention indices (RI) of the compounds were obtained by comparing the retention times of various compounds' mass spectra and identification of each constituent was putatively confirmed by spectral matching with those of libraries (MassHunter, Library and NIST14.L).

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 20.0 and GraphPad prism were employed to analyse the data statistically. The mean of data was compared for significant

differences using One Way Analysis of variance (ANOVA) and Post-hoc Tukey Multiple Comparison Test was used to separation the mean to identify where the significant difference lies. In all cases, the $p<0.05$ was considered statistically significant.

Percentage Yield of *Adansonia digitata* fruit pulp extracts

As shown in Table 1, 0.14%, 1.17%, 4.30% and 21.80% yields of extracts were obtained from exactly 1000g of baobab fruit pulp by maceration using *n*-hexane, ethylacetate, methanol and water as solvents respectively.

Results

Table 1: Percentage Yield of Baobab Fruit Pulp Extracts

S/N	Crude Extract	Yield (%)
1	n-hexane	0.14
2	Ethylacetate	1.17
3	Methanol	4.30
4	Aqueous	21.80

ACE Inhibitory Activity (IC_{50}) of *Adansonia digitata* Fruit Pulp Extracts

Figure 1 showed the calculated IC_{50} values for each of the extracts. There was significant ($p<0.05$) difference in the IC_{50} values of the extracts and Ramipril®. Thus, Ramipril® was found to have IC_{50} value of 81.05±0.08µg/ml while *n*-hexane, ethylacetate, methanol and aqueous extracts had the IC_{50} values of 101.4±0.29µg/ml, 116.70±0.07µg/ml, 40.53±0.32µg/ml and 47.25±0.30µg/ml respectively. Based on the IC_{50} values obtained, methanol extract had the lowest value among all the extracts.

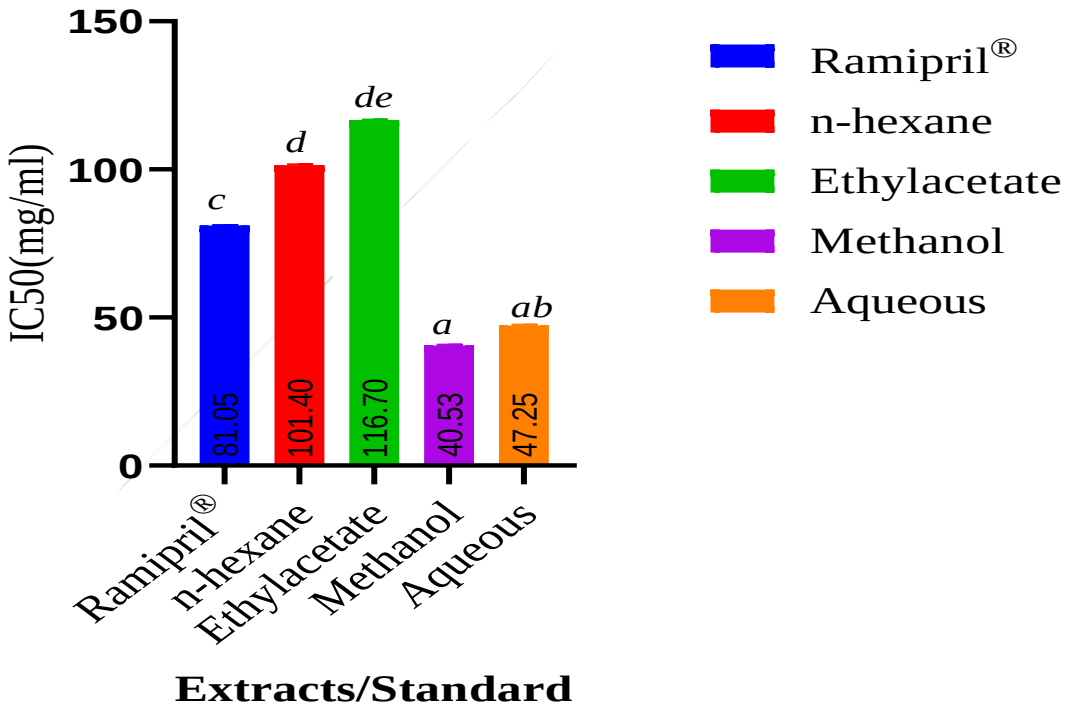

Figure 1: IC_{50} values of *A. digitata* fruit pulp extracts and Ramipril® (a standard reference ACE inhibitor), Mean ± standard deviation values with different superscript letters vary significantly at $P<0.05$,

Bioassay-guided fractionation of methanol extract of Baobab fruit pulp

The methanol extract which recorded the lowest IC_{50} value was selected for ACE inhibitory assay-guided fractionation. Five different solvent systems; 100% ethylacetate, ethylaceate/chloroform/methanol/water (15:8:4:1), butanol/acetic-acid/water (4:1:1), ethylacetate/methanol (7:3) and chloroform/methanol system (1:1) were tested using thin layer chromatography (TLC) and viewed under UV-lamp. Among the solvent systems tested, ethylacetate and methanol (7:3) had a better resolution, separation and a greater number of spots (Data not shown), thus, it was selected for column chromatography. From the column chromatography of MEADF pulp; 75 fractions (50ml each) were collected. Thin layer chromatography of the fractions as shown in Figure 1 led to the pooling together of fractions with similar retention times where six pooled fractions (I, II, III, IV, V and VI) were obtained. The IC_{50} values interpolated for each of the pooled fractions were shown in Figure 3. The IC_{50} values of the pooled fractions differ significantly ($p<0.05$) except between fractions III and I. Fractions I and III were found to have the lowest IC_{50} of 11.96±0.16µg/ml and 11.77±0.10µg/ml, respectively. Ramipril® (a reference ACE inhibitor) had an IC_{50} value of 81.05±0.08µg/ml. Fraction I and III with lower IC_{50} values were thus selected for Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

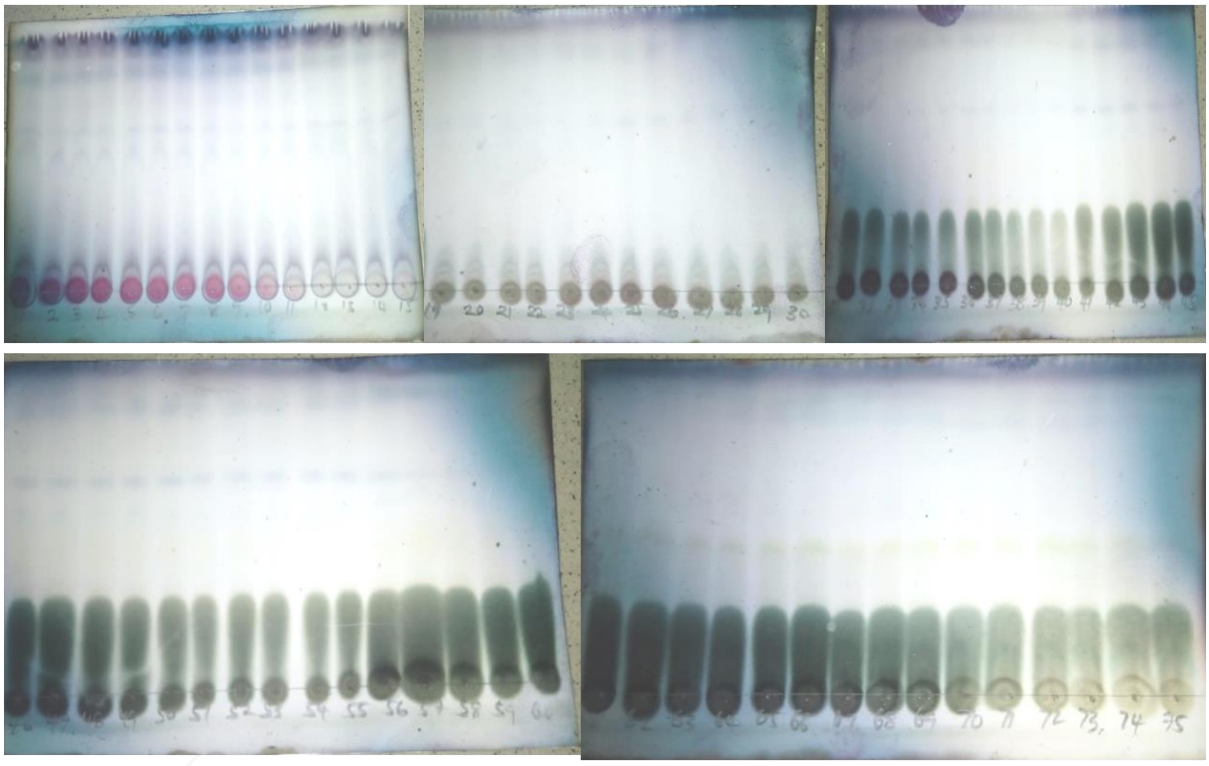

Figure 2: Thin Layer Chromatography plates of various fractions obtained

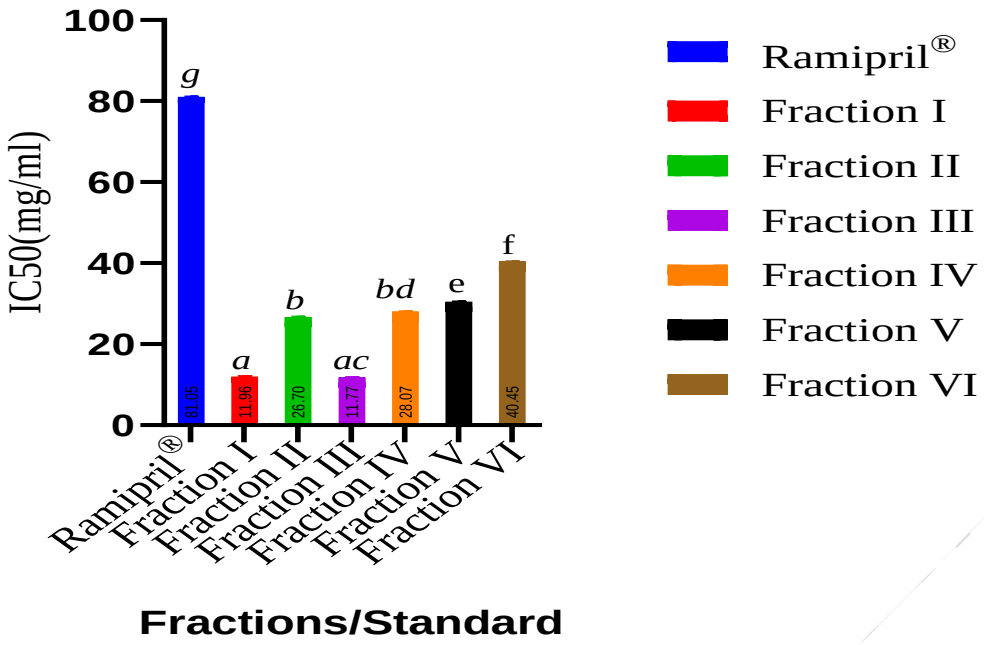

Figure 3: IC_{50} values of Pooled Fractions, Mean±standard deviation values with different superscript alphabets vary significantly at P<0.05

Gas chromatography-Mass spectrometry

As shown in Figure 4, the GC-MS chromatogram indicates the possibility of different constituents present in Fraction I of MEADF. The constituents which each peak indicate were putatively identified by spectral matching with the libraries (MassHunter, Library and NIST14.L) and the results were presented in Table 2. Peak 6-14 were predominantly constituted by conjugated linoleic acids and their isomers (9, 12 Octadecadienoic acid methyl ester, 10-Octadecenoic acid methyl ester, Octadecanoic acid, 9, 12-Octadecadienoic acid, Linoelaidic acid and Butyl 9, 12-octadecadienoate). The compounds in each of the peaks from 1-15 had percentage similarity index above 90% with those of the libraries. Other compounds identified with similarity index above 90% in this fraction are 4-hexadecyl ester (peak 1), 1-Octadecene (peak 2), Hexadecanoic acid methyl ester (peak 3), Dibutyl phthalate (peak 4), n-Hexadecanoic acid (peak 5) and Bis (2-ethylhexyl) phthalate and 11, 13-Dimethyl-12-tetradecen-1-ol acetate. Among the compounds identified in this fraction, 9, 12-Octadecadienoic acid (area % = 40.98) was found to be the major compound. Similarly, GC-MS analysis of Fraction III showed various peaks (Figure 5) indicating the presence of different compounds as listed in Table 3. From the table, 13 compounds predominantly constituted by various aliphatic carboxylic acids (Hexadecanoic acid methyl ester, Dodecanoic acid, Tetradecanoic acid, n-Hexadecanoic acid, Heptadecanoic acid 16-methyl-methyl ester, Octadecanoic acid), conjugated linoleic acid isomers (9, 12 Octadecadienoic acid, 9, 12-Octadecadienoic acid (Z,Z)-), Dibutyl phthalate, cis-Vaccenic acid, a pyrazole (3H-Pyrazol-3-one-4-benzoyl-2,4-dihydro-5-methyl-2-phenyl-) and aromatic acid ester (10-Octadecenoic acid, methyl ester), with spectral matching similarity of ≥90%. Thus, cis-vaccenic acid with retention time of 16.341min and 99% spectral matching similarity was the most abundant (% area = 15.10) compound identified in Fraction III of MEADF.

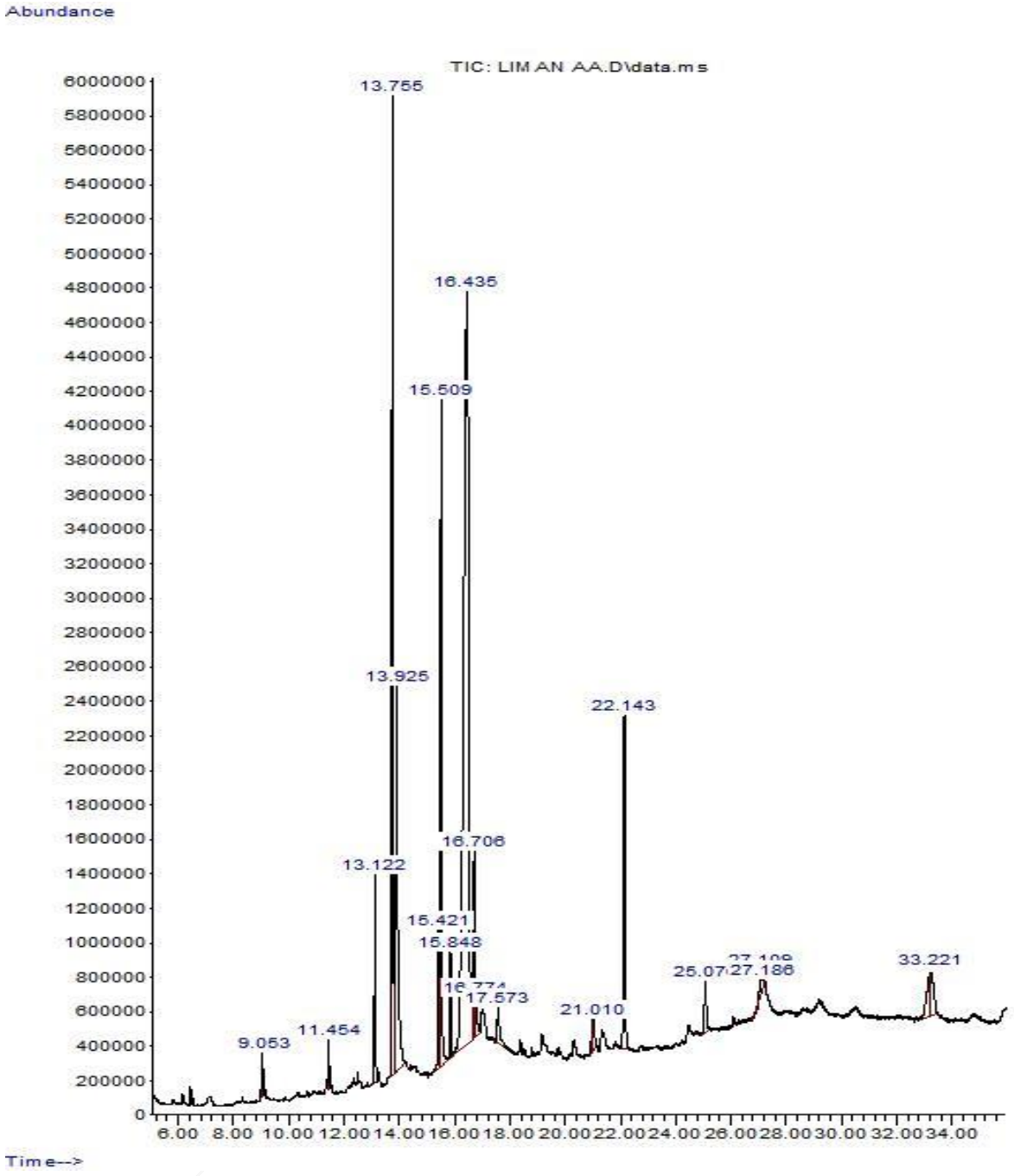

Figure 4: GC-MS chromatogram of Fraction I of methanol extract of*A. digitata* fruit pulp

Table 2: Some phytocompounds identified in Fraction I

Peak	Retention time (min)	Constituents	Area (%)	Similarity (%)
1	9.053	Dichloroacetic acid, 4-hexadecyl ester	0.46	94
2	11.454	1-Octadecene	0.50	99
3	13.122	Hexadecanoic acid, methyl ester	2.71	98
4	13.755	Dibutyl phthalate	14.39	97
5	13.925	*n*-Hexadecanoic acid	10.97	99
6	15.421	9,12-Octadecadienoic acid, methyl ester	1.95	99

7	15.509	10-Octadecenoic acid, methyl ester	9.63	99
8	15.848	Methyl stearate	1.54	99
9	16.435	9,12-Octadecadienoic acid	40.98	99
10	16.706	Octadecanoic acid	3.12	99
11	16.774	9,12-Octadecadienoic acid	0.69	95
12	17.573	9,12-Octadecadienoic acid	1.05	96
13	20.988	Linoelaidic acid	0.48	97
14	21.010	Butyl 9,12-octadecadienoate	0.62	95
15	22.143	Bis(2-ethylhexyl) phthalate	6.00	91
17	27.109	11,13-Dimethyl-12-tetradecen-1-ol acetate	0.44	91
18	27.186	11,13-Dimethyl-12-tetradecen-1-ol acetate	0.03	93

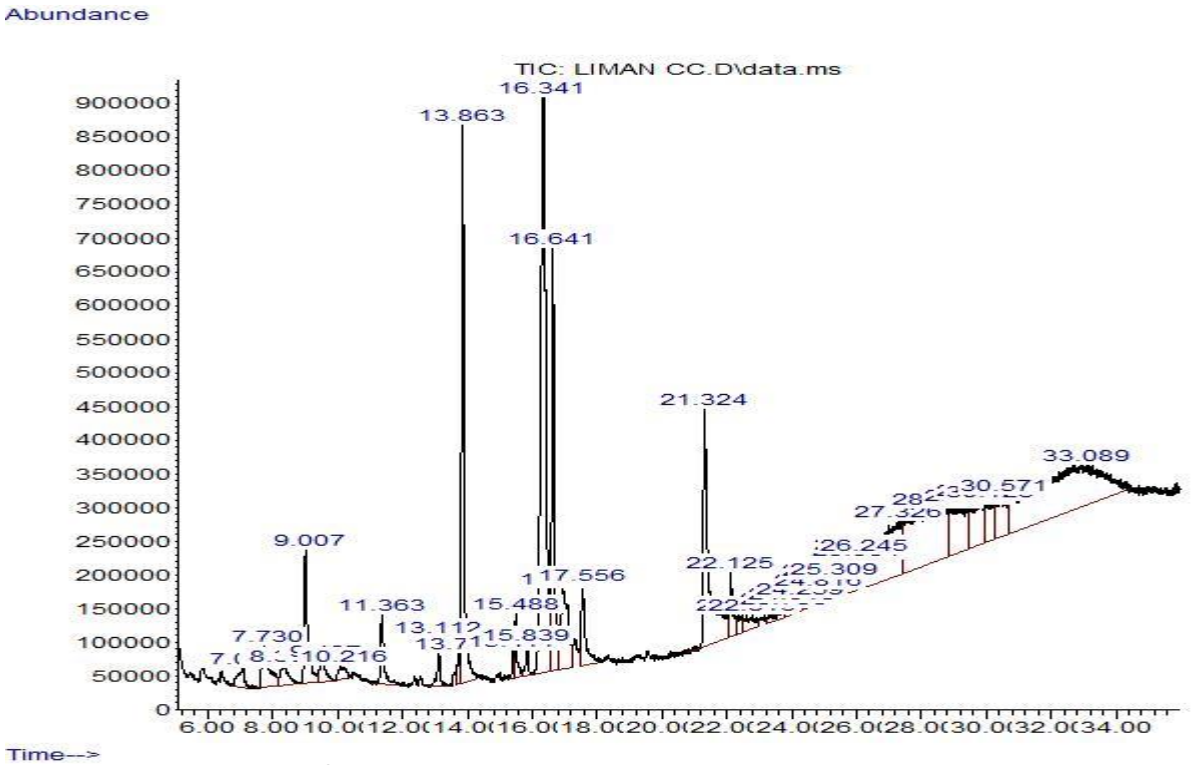

Figure 5: GC-MS chromatogram of Fraction III of methanol extract of *A. digitata* fruit pulp

Table 3: Some phytocompounds identified in Fraction III

Peak	Retention time (min)	Constituents	Area (%)	Similarity (%)
1	9.007	Dodecanoic acid	2.41	98
2	11.363	Tetradecanoic acid	1.18	98
3	13.112	Hexadecanoic acid, methyl ester	0.63	96
4	13.721	Dibutyl phthalate	0.23	91
5	13.863	n-Hexadecanoic acid	7.16	99
6	15.411	9,12-Octadecadienoic acid, methyl ester, (E,E)-	0.23	90
7	15.488	10-Octadecenoic acid, methyl ester	0.7	99
8	15.839	Heptadecanoic acid, 16-methyl-, methyl este	0.39	95
9	16.341	cis-Vaccenic acid	15.10	99
10	16.641	Octadecanoic acid	5.50	99
11	16.961	9,12-Octadecadienoic acid (Z,Z)-	3.15	98
12	17.556	9,12-Octadecadienoic acid (Z,Z)-	1.85	91

13	21.324	3H-Pyrazol-3-one-4-benzoyl-2,4-dihydro-5-methyl-2-phenyl-	6.78	91

Discussion

Synthetic medications for hypertension still created a slew of issues like decreased efficacy, scarcity, and adverse effects [18]. Natural, safe, and cost-effective medicines are strongly needed. Although Liman *et al*. [5] reported the *in vivo* hypotensive activity of the methanol extract of baobab fruit pulp; the bioactive compounds responsible for this activity are yet to be identified. This study investigated the *in vitro* hypotensive activity of baobab fruit pulp using an angiotensin-converting enzyme inhibitory activity assay, aiming to identify its constituents with antihypertensive potentials.

The study demonstrated that the increased percentage of ACE inhibitory activity of Ramipril® and those of *A. digitata* fruit pulp extracts were concentration-dependent. Methanol and aqueous extract due to their significantly lower IC_{50} compared to that of Ramipril® (a reference ACE inhibitor) may thus be considered as effective ACE inhibitors and potential antihypertensive agents. The phytochemicals with high antioxidant properties earlier reported in *A. digitata* fruit pulp might have constitute the methanol extract and also responsible for the observed ACE inhibitory activity. The findings provides additional support for Huang et al. [19]'s earlier report, which suggested that naturally occurring bioactive components from food sources, such as grains, dairy products, meats, fruits, vegetables, nuts, and foods with microbiological origin, may be used to treat or prevent hypertension. The methanol extract (40.53µg/ml) and aqueous extract (47.25µg/ml) IC_{50} values found in this investigation were comparable to those of bound phenolic extracts of *Parkia biglobosa* reported by Komolafe *et al*. [20]. Similar to *A. digitata* fruit pulp, which is also widely available in northern Nigeria, *Parkia biglobosa* fruit pulp is another naturally dehydrated fruit. Because methanol extract had the lowest IC_{50} value and a high ACE inhibitory efficacy, it was selected for use in bioassay-guided fractionation.

The study identified compounds in Fraction I and Fraction III of the MEADF, including conjugated linoleic acids and various aliphatic carboxylic acids. Conjugated linoleic acid has antioxidant [21], hypocholesterolaemic, anti-atherogenic, anti-inflammatory [22] and systolic blood pressure lowering activities [23, 24, 25,]. Several workers [26, 27, 28, 39, 30, 31,] have reported that conjugated linoleic acid lowers systolic blood pressure by decreasing body fat, improving insulin sensitivity, and increasing lipid profile through reduced adipocytokines, β-oxidation, and suppression of fatty acid synthesis. Hexadecanoic acid methyl esters, hexanoic acid, and palmitate have anti-inflammatory, antioxidant, antifibrinolytic, and hypocholesterolemic properties [32, 33]. Cis-Vaccenic acid, a pyrazole, and aromatic acid ester dominate Fraction III of the MEADF. Vaccenic acid is the only known dietary precursor of conjugated linoleic acid and was reported earlier by Diane *et al.* [34] to have antihyperlipidaemic and anti-inflammatory activities. These compounds may be responsible for the observed ACE inhibitory activities of Fraction I and Fraction III in this study. It may also explain the *in vivo* antioxidant, anti-inflammatory, antihyperlipidaemic, and hypotensive properties of a methanol extract of *A. digitata* fruit pulp observed by Liman *et al.* [5]. Among the compounds identified in this study, the blood pressure lowering effect of Cis vacenic acids and 3H-Pyrazol-3-one-4-benzoyl-2,4-dihydro-5-methyl-2-phenyl- is yet to be established, necessitating further study of their *in vivo* antihypertentensive activities.

Conclusion

The study employed a bioassay-guided fractionation to identify novel angiotensin-converting enzyme inhibitory compounds from *Adansonia digitata* fruit pulp. The most potent ACE inhibitory activity was found in the methanol extract *A. digitata* Fruit (MEADF) with the lowest IC_{50} value of 40.53±0.32µg/ml. Gas chromatography-mass spectrometry (GC-MS) of fraction I and III obtained from MEADF, putatively revealed the presence of 9,

12, octadecadienoic acid methyl ester (conjugated linoleic acid isomers), cis-Vaccenic acid, 3H-Pyrazol-3-one, 4-benzoyl-2, 4-dihydro-5-methyl-2-phenyl (a pyrazole), and Hexadecanoic acid methyl ester (aromatic acid ester). The study identifies Cis vacenic acids and 3H-Pyrazol-3-one, 4-benzoyl-2, 4-dihydro-5-methyl-2-phenyl as compounds with potential blood pressure lowering effects, but their *in vivo* antihypertensive study is yet to be established. Therefore, methanol extract of Baobab fruit pulp could be a promising source for the development of antihypertensive drug.

Declarations

Conflict of interest: We declare no conflict of interests

Funding: This study did not receive any specific grant from public, commercial and private sectors.

Authors' contributions: AAL carried out the laboratory experiments, analysed the data and wrote the first draft of the manuscript. AS conceived and designed the work accordingly, analysed the data, interpreted the results and edited the manuscript. All the authors holistically read and approved the final draft of this manuscript and are responsible for its contents.

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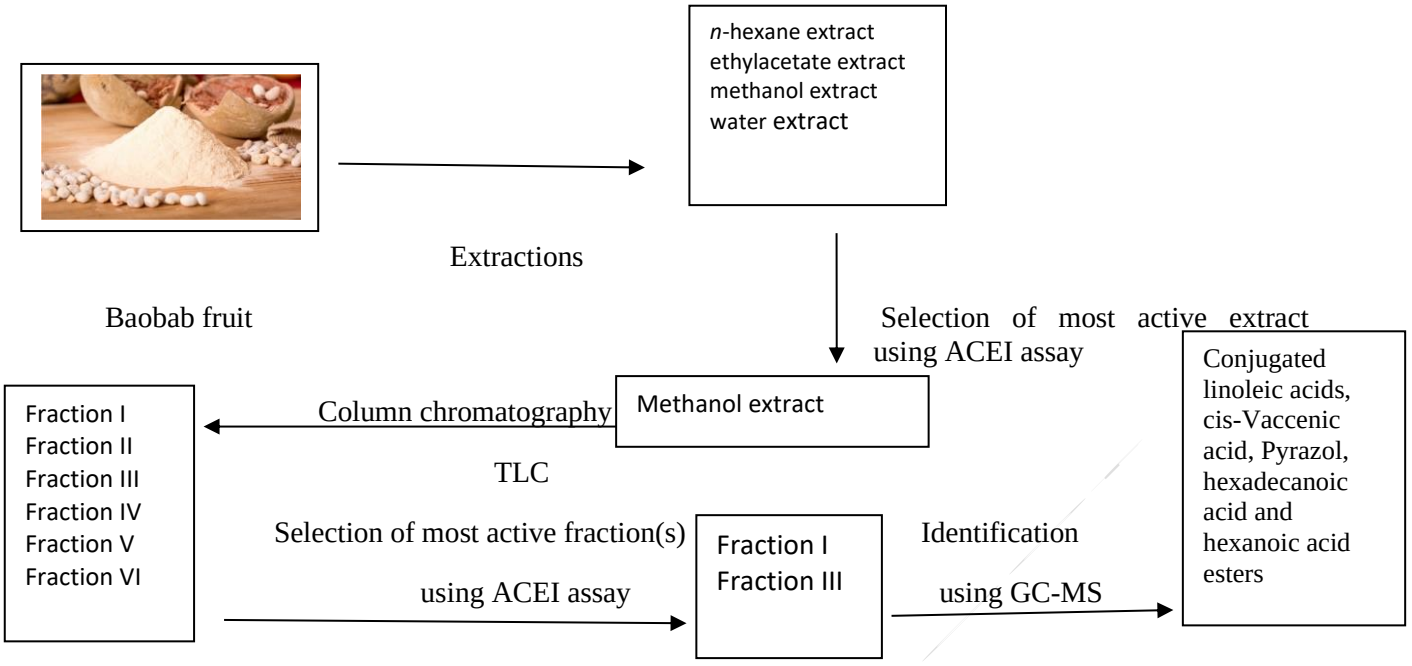

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