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Active Blood Pressure Lowering Fractions from the Aqueous Extract of the Leaves of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae)

Fabian C. Amaechina [1*], Eric K. Omogbai [1] Zuleika A. Nworgu [1], Enitome E. Bafor[1] and Buminiyan A. Ayinde [2].

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin.
[2]Department of Pharmacognosy, Faculty of Pharmacy, University of Benin.

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* *Corresponding Author:*
amaefabs@uniben.edu
https://orcid.org/0000-0003-4832-9748
+234 8023303138

ABSTRACT

Background: The blood pressure-lowering effect of aqueous leaves extract of *Phyllanthus amarus* has been reported in earlier work in normotensive albino rabbits. The effects of organic solvent fractions were evaluated in Wistar rats.

Methods: The aqueous fraction obtained from the crude extract of the leaves was evaluated for blood pressure-lowering effect in anaesthetized normotensive Wistar rats, at the graded doses of 2.5-40 mg/kg after which it was subjected to vacuum liquid chromatography (VLC) using combinations of chloroform, ethyl acetate, methanol and water. This yielded eleven fractions which were bulked into six samples (A-F) after thin layer chromatograph analysis, and samples A, B and C were evaluated for activities on the blood pressure of hypertensive Wistar rats at the graded doses of 5 to 20 mg/kg. Fraction "C" showed the highest blood pressure lowering effect and was further subjected to column chromatographic and VLC analysis, to obtain fraction (B_{VLC2}) which was evaluated for possible blood pressure lowering effect in hypertensive Wistar rats.

Results: The aqueous fraction caused a dose dependent decrease in the blood pressure of normotensive Wistar rats, decreasing the mean arterial blood pressure (MAP) from a basal level of 104.58 ± 8.29 mmHg to 45.22 ± 6.71 mmHg ($p<0.0001$; 66.4%) at the dose of 40 mg/kg. Fraction "C" caused significant decrease in the MAP from 116.99 ± 10.28 mmHg to 68.33 ± 6.78 mmHg ($p < 0.001$; 0.01%) at the dose of 20 mg/kg, while the fraction B_{VLC2} decreased the MAP from 146.11 ± 8.29 mmHg to 79.33 ± 6.18 mmHg (***$p< 0.001$) at the dose of 10 mg/kg, indicating a trend of increasing potency along the course of purification.

Conclusion: The aqueous fraction of the leaves of *Phyllanthus amarus* yielded column chromatographic sample (B_{VLC2}), that might possibly serve as a lead sample from which pure active hypotensive constituent(s) could be isolated.

1. Introduction

Hypertension is the most prevalent cardiovascular disease worldwide[1], and the most important risk factor for other cardiovascular complications like heart failure, myocardial infarction, and end organ damage[2]. Conventional drugs for the management of hypertension include diuretics (indapamide, furosemide, amiloride), sympathoplegic agents (clonidine, reserpine), renin inhibitor (aliskiren), angiotensin-converting enzymes (ACE) inhibitors (Enalapril, Captopril, Quinapril), angiotensin receptor blockers (ARBs—Losartan, Irbesartan, Olmesartan), calcium channel blockers (Nifedipine, Verapamil, Diltiazem), α-adrenergic blockers (Prazosin, Doxazosin), β-adrenergic blockers (Nebivolol, Atenolol) to vasodilators (Minoxidil, sodium nitroprusside)[3]. It is an important observation that with the long list of available antihypertensive drugs in clinics, elevated blood pressure is only adequately controlled in about 34% of hypertensive

patients[3].

This poor response of patients can be attributed to a lot of issues which include higher cost of antihypertensive dugs[4], problems with availability and accessibility of drugs[5], and intolerable side effects[6] that most times results in poor patient compliance as some patients tend to abandon their medication[8]. This situation would generally increase the risk of cardiovascular complications[2], thereby creating an urgent need for hypertensive patients to seek alternative intervention in herbal medicine to treat and control 3 their elevated blood pressure because medicinal plants have been used and are still being used for the healing and curing of human diseases from folk 8 medicine to date[8]. They are rich sources of phytochemicals, like alkaloids, tannins, flavonoids phenolic compounds, glycosides, and terpenoids which have sometimes become intermediate bioactive principles or even lead compounds in drug discovery[9].

Following an earlier report on the hypotensive effects of the aqueous extract of *Phyllanthus amarus* in normotensive albino male rabbits[10], this paper reports the blood pressure lowering effects of fractions and samples obtained from the phytochemical analysis of the aqueous fraction of the leaves of *Phyllanthus amarus*, carried out through solvent/solvent partition, vacuum liquid chromatography and column chromatography in hypertensive Wistar rats.

2. Materials and methods

2.1 Collection and preparation of plant extract

Phyllanthus amarus was collected around the premises of University of Benin, Benin City, Edo State, Nigeria between the months of May-November 2010, and was identified at the Forest Research Institute of Nigeria (FRIN) in Ibadan, Oyo state, with herbarium specimen with voucher number SH1107456. The plant was air-dried for 14 days, after which the leaves were separated from the stalk and put in an oven at about 40 °C for 30 min before they were pulverized with an electric milling machine. The powdered plant material (2.0 kg) was extracted with 3 L distilled water using Soxhlet apparatus. The extract was concentrated under pressure at 60 °C to give a yield of 347.78g (17.389%), with a Stuart rotary evaporator (complete set up of: Rotary apparatus of CAS no: RE300/MS, Digital Water Bath of CAS No: RE300/DB, and Pressure Vacuum Pump of CAS No:3022C) manufactured by Bibby Scientific Limited, Stone Staffordshire ST 15 DSA, UK.

2.2 Organic solvent partitioning of the aqueous extract

Weighed amount (300 g) of the aqueous extract was dissolved in water and subjected to exhaustive solvent/solvent partition with chloroform (250 mL x 5) to obtain the aqueous and the chloroform fractions which were concentrated using rotary evaporator. The fractions were weighed and stored in a refrigerator for further studies.

2.3 Sources and preparation of laboratory animals

Wistar rats (180-220g) of either sex were obtained from the animal house of the Department of Pharmacology and Toxicology, University of Benin, Benin City, Nigeria. The animals were cared for and handled for the experiments in line with the standard Guidelines for the care and use of laboratory animals[11]. In addition, ethical approval was obtained from the Faculty of Pharmacy, University of Benin Ethical Committee on the Use of Animals for Experiments (RE:EC/FP/011/04).

2.4 Evaluation of the aqueous and chloroform fractions for blood lowering effects in normotensive rats

Wistar rats were anaesthetized with a combination of urethane/thiopentone at the dose ratio of 1250/20 mg/kg. Under full anesthesia, the rats were placed in the supine position on the dissecting table with the limbs fastened to the table with aid of adhesive plaster. The trachea-cervical region was dissected and opened to expose the trachea which was isolated, cleared of adhesive tissues, and intubated with a polythene tube to assist respiration. The carotid artery was located, carefully separated from the vagus nerve and cleared of connective tissues. The portion leading to the head was ligated with surgical silk to prevent blood from flowing back, while the portion from the heart was nipped opened and intubated with heparinized saline-filled Teflon tubing (22 Ga. Lightweight; ID- 0.28", Wall-0.006"). The Teflon was connected via a three-way tap to an Ugo Basile Uni-channel recorder (model 7040), for blood pressure recording. The channel recorder was always calibrated before and after each experiment, using a mercury sphygmomanometer. When the animal had stabilized and all the measurable variables had remained constant, the chloroform and the aqueous fractions were evaluated for hypotensive effect at doses of 2.5-40 mg/kg. The fraction that demonstrated significant hypotensive effect was selected for further phytochemical purification.

2.5 Vacuum liquid chromatography (VLC) of the aqueous fraction

The aqueous fraction (140 g) was re-dissolved in methanol and adsorbed on coarse silica gel (60-120 mesh) and allowed to dry. The mixture was triturated in a mortar to obtain a fine powder which was loaded on a Sinta glass (Number 3). The glass was thereafter attached to a Buckner flask connected to a vacuum pump. The fraction was eluted with 300 mL of 100% $CHCl_3$, $CHCl_3$- $CH_3COOC_2H_5$ (1:1), $CH_3COOC_2H_5$ (100%), $CH_3COOC_2H_5$- CH_3OH (4:1; 3:2; 2:3; 1:4), 100% CH_3OH, CH_3OH- H_2O (4:1; 1:1) and 100% H_2O in succession.

2.6 Thin layer chromatography analyses of the various VLC fractions

Thin layer chromatography analyses of the various aqueous VLC fractions were carried out using pre-coated aluminum TLC plate of silica gel GF_{254} with CH_3OH- $CH_3COOC_2H_5$- H_2O (2:3:1) as the developing solvent system. The plates were sprayed with 10% concentrated sulphuric (H_2SO_4) in methanol and heated in an oven at 110°C for 5 minutes. The spots were noted and their respective R_f values recorded. Following their respective TLC profiles, the fractions were bulked together as A (fractions 1 and 2); B (3 and 4); C (5); D (6 – 8); E (9 and 10) and F (11).

2.7 Induction of renal hypertension in Wistar albino rats

Renal hypertension was induced in Wistar rats (180-220 g) of either sex according to the protocol earlier described[12]. Anesthesia was induced in the rats with thiopentone (45 mg/kg). The two sides of the abdomen were shaved and sterilized with a methylated spirit swab. After successful dissection, one kidney was excised and the base tightly secured with a surgical chromic catgut suture while a figure-eight ligature was placed on the other kidney to render it ischaemic, and the rats were placed on 0.9% physiological saline as drinking water for four weeks.

2.8 Evaluation of blood lowering effects of the VLC fractions in hypertensive rats

Each of the bulked VLC fractions A, B, C, D, E and -F were evaluated for probable blood lowering effects in renal induced hypertensive rats at doses of 5, 10 and 20mg/kg. The experiment was repeated five times.

2.9 Column chromatography of fraction C

Fraction C (27.6 g) was subjected to column chromatographic separation on silica gel 60-120 mesh using combinations of $CHCl_3$, $CH_3COOC_2H_5$ and CH_3OH (200 mL) beginning with $CHCl_3$, - $CH_3COOC_2H_5$ (10:90 %), and subsequently with 100% $CH_3COOC_2H_5$, and $CH_3COOC_2H_5$ - CH_3OH (90:10%) up to CH_3OH (100%) and later H_2O (100%). Out of 23 fractions obtained, fractions 3-8 were bulked together based on their TLC profile.

2.10 Statistical analysis

Results obtained were expressed as Mean ± SEM (Standard Error of Mean) in tables and continuous line graphs. Results were analyzed by student's t-test and one-way analysis of variance (ANOVA), and the Dunnets multiple comparison test with a confidence level set at 95%, while "n" is the sample number. The confidence level was adopted

3. Results

The chloroform fraction was observed to produce no remarkable effects on systolic blood pressure (SBP), but exhibited a non-dose dependent transient decrease in the Diastolic blood pressure (DBP), and Mean arterial blood pressures (Figure 1).

3.1 Effect of the aqueous fraction on the blood pressure of normotensive rats

The aqueous fraction produced remarkable dose-dependent reductions in systolic, diastolic, and consequently the mean arterial blood pressures. At a dose of 2.5 mg/kg, -40 mg/kg) decrease in blood pressure, with the dose of 40 mg/kg significantly reducing the systolic blood pressure from a basal level of 123.75 ± mmHg to 61.66 ± 6.29 mmHg ($p< 0.00001$) which is 37.75% reduction, the diastolic from a basal level of 95 ± 8.66 mmHg to 37 ± 8.26 mmHg ($p< 0.001$) which is 68.52% and the mean arterial blood pressure from 104.58 ± 8.29 mmHg to 45.22 ± 6.71 mmHg ($p<0.0001$) which also is 66.4% in normotensive rats (Figure 2).

3.2 Effect of VLC Samples A, B and C on the Blood Pressure of Hypertensive Wistar Rats

The results of the three VLC samples (A, B and C) on the blood pressure of Hypertensive Wistar rats are presented in figures 3, 4 and 5 below. The VLC procedure separated the aqueous fraction into bulked samples with varied effects on blood pressure as seen in Figures 3, 4, 5a and 5b.

VLCfraction (A) caused a graded dose-dependent reduction of the blood pressure in the blood pressure of the hypertensive Wistar rats, which were within the hypertensive values for the doses of 5 and 10 mg/kg. however, the dose of 20

mg/kg caused a significant decrease in blood pressure,

reducing the systolic BP from 170 ± 6.77 mmHg to 110.75 ± 13.06 mmHg, the diastolic BP from 125 ± 6.12 mmHg to 77 ± 4.36 mmHg and the MAP from 137.5 ± 6.4 mmHg to 88.25 ± 8.75 mmHg respectively ($p < 0.001$, $p < 0.01$ and $p < 0.05$).

VLC sample (B) demonstrated a first dose hypotensive effect, lowering the systolic, diastolic and mean arterial blood pressure marginally at 5mg/kg from the baseline values of 184.33 ± 7.98, 136.67 ± 8.1 and 152.56 ± 5.81 mmHg, to 164.33 ± 10.1, 118.33 ± 5.85 and 133.67 ± 8.23 respectively. The doses of 10 and 20 mg/kg caused elevation of blood pressure, with the dose of 20 mg/kg increasing the systolic, diastolic and the mean arterial pressure to 165 ± 7.02, 126.67 ± 3.9 and 139.44 ± 6.35 mmHg respectively (Figure 4).

The VLC sample (C) reduced the systolic, diastolic and the mean arterial pressures from the baseline values of 145 ± 12.85, 104 ± 9.14 and 116.99 ± 10.28 mmHg, to 98.6 ± 4.24, 54.0 ± 4.85 and 68.33 ± 6.78 mmHg respectively at the dose of 20 mg/kg (Figure 5a and b).

3.3 The effect of sample B_{VLC2} (4-6) on the blood pressure of hypertensive Wistar rats

Sample B_{VLC2} (4-6) demonstrated a greater blood pressure lowering potency along the course of purification. The least effective dose has decreased to 2.5mg/kg, while the highest tolerable dose of 10 mg/kg reduced the systolic, diastolic and the mean arterial blood pressure from the baseline values of 168 ± 8.34, 135 ± 5.72 and 146.11 ± 7.81 mmHg to 103.33 ± 5.94, 67.33 ± 6.73 and 79.33 ± 8.23 mmHg respectively, ($p < 0.001$).

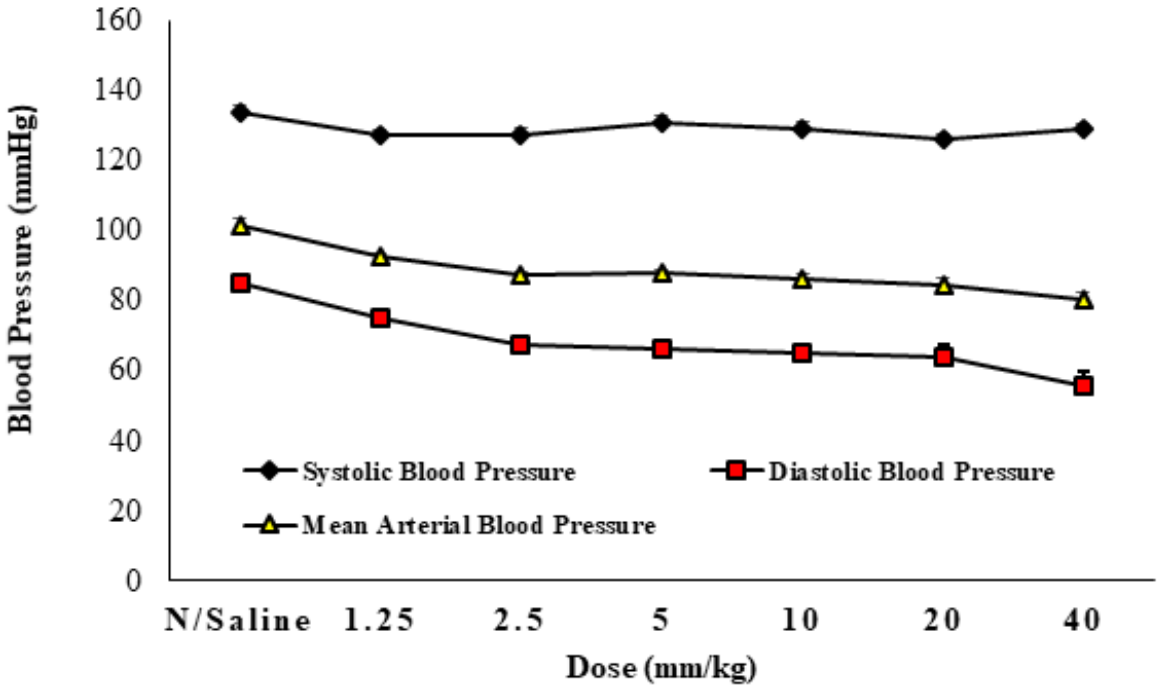

Figure 1 The effect of the Chloroform fraction from the aqueous extract of the leaves of *Phyllanthus amarus* on the blood pressure of normotensive Wistar rats. The chloroform fraction demonstrated transient marginal decrease in the diastolic and the mean arterial blood pressure which was not significant ($p > 0.05$) (n=5 Wistar rats).

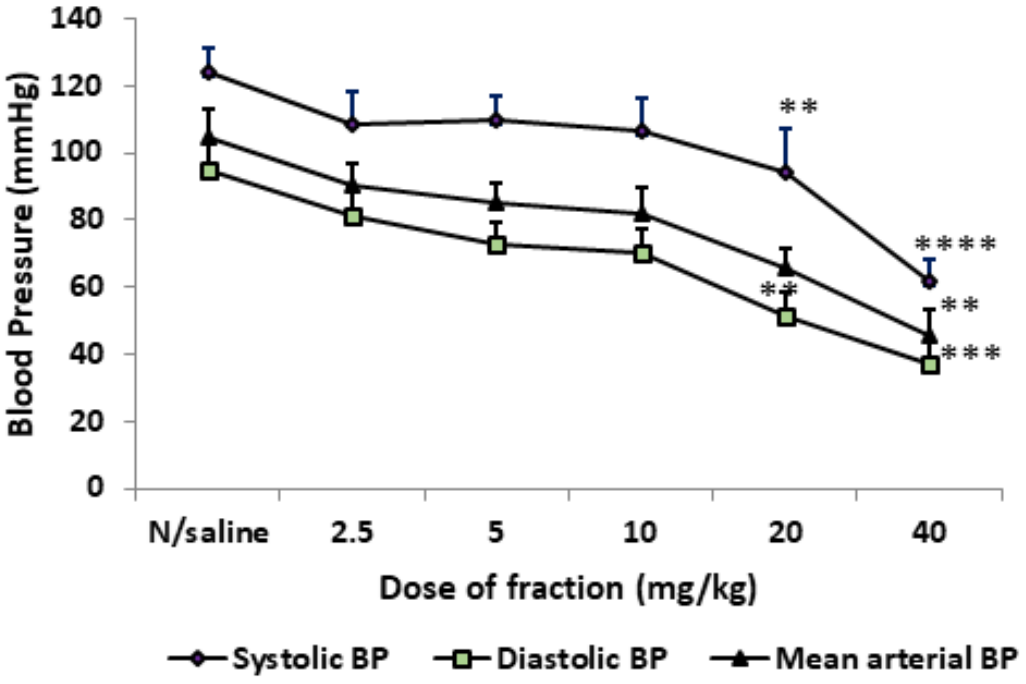

Figure 2 Hypotensive effects of the aqueous fraction of *Phyllanthus amarus* in normotensive Wistar rats.
The aqueous fraction caused a dose dependent (2.5-40 mg/kg)decrease in blood pressure, with the dose of 40 mg/kg significantly reducing the systolic blood pressure from a basal level of 123.75 ± 5.87 mmHg to 61.66 ± 6.29 mmHg ($p<0.00001$) which was 37.75% reduction, the diastolic from a basal level of 95 ± 8.66 mmHg to 37 ± 8.26 mmHg ($p<0.001$) about 68.52% and the mean arterial blood pressure from 104.58 ± 8.29 mmHg to 45.22 ± 6.71 mmHg ($p<0.0001$) which is 66.4% reduction in normotensive rats.

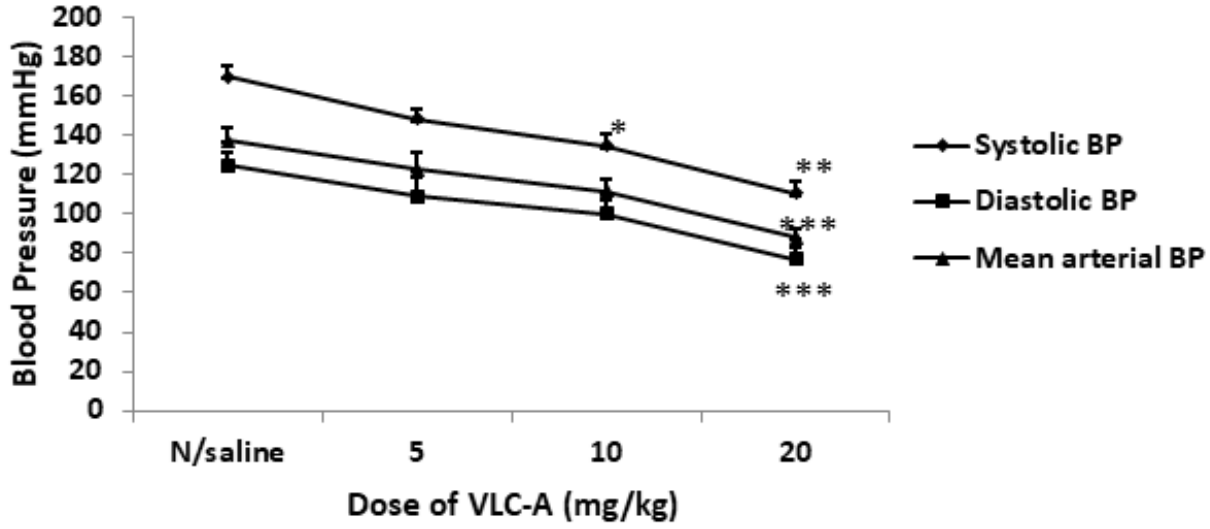

Figure 3 The effect of VLC (A) on the blood pressure of hypertensive rats. The fraction caused a graded dose dependent reduction of the blood pressure in renal-induced hypertensive rats. The dose of 20mg/kg caused a significant decrease in blood pressure, reducing the systolic BP from 170 ± 6.77 mmHg to 110.75 ± 13.06 mmHg, the diastolic BP from 125 ± 6.12 mmHg to 77 ± 4.36 mmHg and the MAP from 137.5 ± 6.4 mmHg to 88.25 ± 8.75 mmHg respectively (***$p < 0.001$, **$p <0.01$ and *$p <0.05$), n=4 animals.

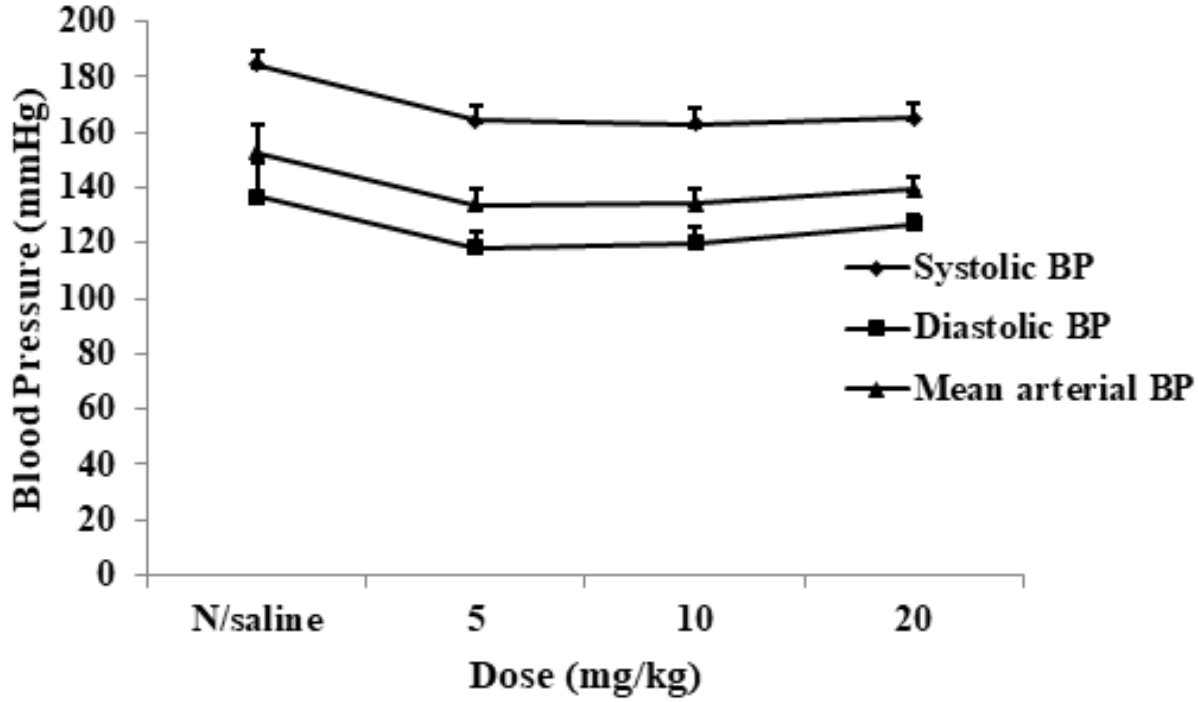

Figure 4 The of fraction VLC (B) on the blood pressure of renal-induced hypertensive rats.
The fraction demostrated a first dose hypotensive effect, lowering blood pressure marginally at 5mg/kg, but still within hypertensive range with higher doses causing increase in blood pressure. n=4 animals.

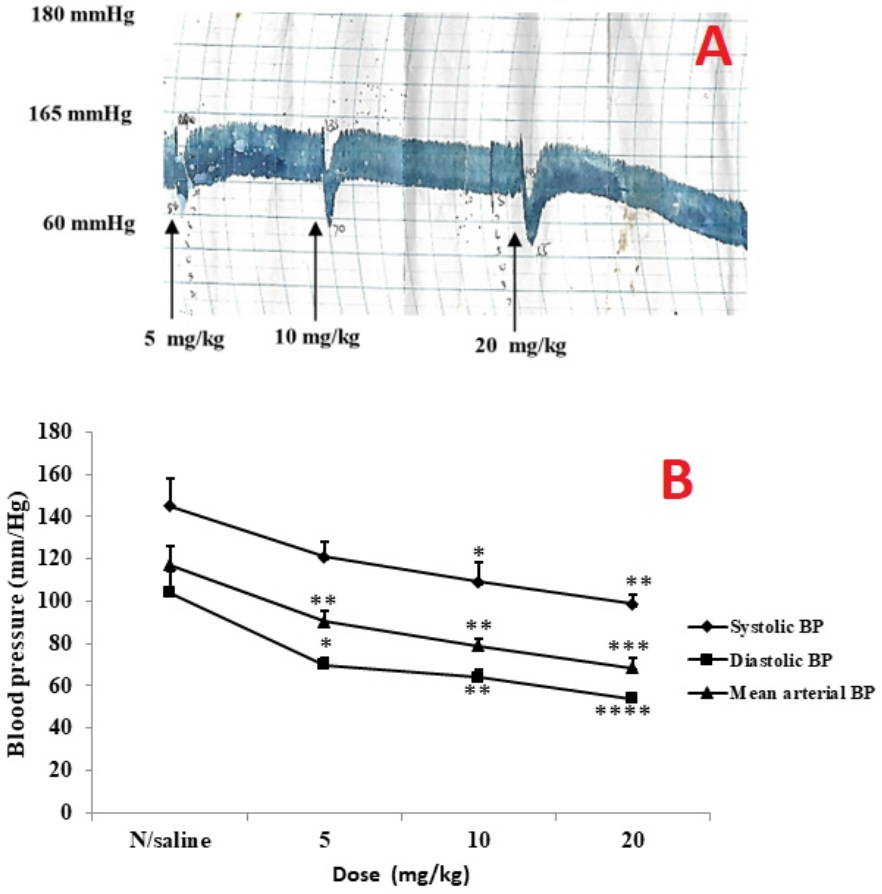

Figure 5 (A): A representative tracing of the effect of VLC (C) on the blood pressure of hypertensive Wistar rats.
Figure 5(A) shows the dose dependent blood pressure lowering pattern of fraction VLC (C).
Figure 5 (B): The line graphs showing the antihypertensive effect of the fraction on the blood pressure of hypertensive Wistar rats.
The sample reduced the systolic, diastolic and the mean arterial pressures from the baseline values of 145 ± 12.85, 104 ± 9.14 and 116.99 ± 10.28 mmHg respectively, to 98.6 ± 4.24, 54.0 ± 4.85 and 68.33 ± 6.78 mmHg respectively at the dose of 20 mg/kg.

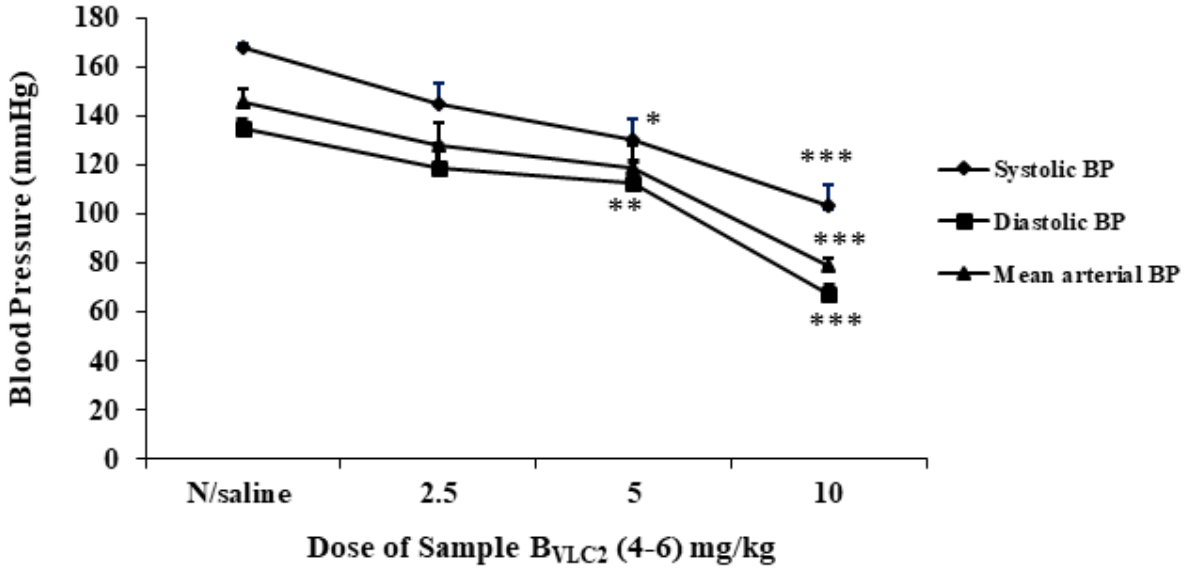

Figure 6 The effect of Sample B_{VLC2} (4-6) on the blood pressure of hypertensive Wistar rats.

The result showed that the sample demonstrated a greater blood pressure lowering potency along the course of purification. The least effective dose has decrease to 2.5mg/kg, while the highest tolerable dose of 10 mg/kg reduced the systolic, diastolic and the mean arterial blood pressure from the baseline values of 168 ± 8.34, 135 ± 5.72 and 146.11 ± 7.81 mmHg to 103.33 ± 5.94, 67.33 ± 6.73 and 79.33 ± 8.23 mmHg respectively, (***$p<0.001$), n = 4 animals.

4. Discussion

The wide range of pharmacological activities of medicinal plants constitute the basis of their recognition in medicine and pharmaceutical industry because they contain biologically active phytochemicals in their different parts[13]. Medicinal plants like *Phyllanthus amarus* would continue to be relevant to the Medical and Pharmaceutical industries because they provide unlimited opportunities for new drug leads[14]. Identification and purification of potential new drug leads from medicinal plants requires well organized systematic and chronological design of procedures that optimizes all aspect of phytochemical techniques, from extraction to the isolation of the bioactive constituent(s)[15] which in some instances may be guided by biological activity.

Employing the activity guided procedure, the aqueous fraction of the leaves of *Phyllanthus amarus* had hypotensive effects in normotensive Wistar rats, and lowered blood pressure in the same pattern with the aqueous extract of *Phyllanthus amarus* by depressing the diastolic blood pressure more than the systolic blood pressure as earlier reported[10]. The aqueous fraction reduced the mean arterial blood pressure from a resting value of 104.58 ± 8.29 to 82.08 ± 7.50 and 65.67 ± 7.47 mmHg respectively, at the doses of 10 and 20 mg/kg. The VLC

procedure sorted the aqueous fraction into three fractions of A, B and C with varied activity in hypertensive Wistar rats. Fraction 'A' had dose dependent blood pressure lowering effect which was however within the hypertensive range, and lowered the blood pressure parameters from the resting values of 170 ± 6.77, 125 ± 6.12 and 137.5 ± 6.40 to 148.5 ± 10.5, 109.75 ± 9.04 and 122.67 ± 9.21; and 134.5 ± 8.18, 100.25 ± 5.57 and 111.66 ± 6.41 mmHg for the systolic, diastolic and the mean arterial pressures at 5 mg/kg and 10 mg/kg respectively. Only the dose of 20 mg/kg reduced the blood pressure parameters to normal values of 110.75 ± 13.06, 77 ± 4.36 and 88.25 ± 5.61 mmHg for the systolic, diastolic and the mean arterial pressure.

Fraction B demonstrated blood pressure elevating properties, in which all the measurable parameters were elevated above the basal hypertensive values at the doses of 5, 10 and 20 mg/kg respectively (Fig. 4).

Fraction (C) demonstrated blood pressure lowering properties at all the doses used for the evaluation, and reduced all the measurable parameters to a normotensive value (Fig. 5a and 5b). Fraction 'C' reduced the MAP from the resting value of 104 ± 9.14, to 90.33 ± 7.67, 78.99 ± 3.6 and 68.33 ± 1.82 mm Hg for 5, 10 and 20 mg/kg respectively.

Fraction B_{VLC2} (4-6) was obtained from the column partitioning of the VLC fraction 'C'. Fraction B_{VLC2} (4-6) maintained a progressive increase in potency of hypotensive effects in hypertensive Wistar rats, by causing a decrease in blood pressure from the dose of 2.5 mg/kg through to the dose of 10 mg/kg. While the dose of 2.5 mg/kg was lower than the 5 mg/kg of VLC fraction 'C', the dose of 10 mg/kg of Fraction B_{VLC2} (4-6) caused greater blood pressure lowering effect than the 20 mg/kg dose of the VLC fraction 'C', by reducing the MAP from a resting value of 146.11 ± 3.64, to 79.33 ± 3.97 mmHg.

The aqueous fraction, VLC fraction 'C' and the Column partitioned Fraction B_{VLC2} (4-6) maintained the MAP at values above 60 mmHg. Demers and Wachs (2021)[16], reported that to ensure adequate perfusion of vital organs, the MAP must be maintained at the minimum value of 60 mmHg. This is clinically important because if the MAP falls below 60 mmHg for a prolong period, end-organ manifestation such as ischaemia and myocardial infarction will likely occur. In addition, perfusion of cerebral tissues would be compromised thereby resulting to loss of consciousness and hastened neuronal death[16]. All the fractions showed greater effect on the DBP than the SBP, which suggests that they might be useful in the management of isolated diastolic hypertension.

5. Conclusion

The results of the experiment carried out, show that the activity guided phytochemical purification of the aqueous fraction yielded fractions that have the potential to be used in alternative medicine to manage high blood pressure, and that fraction B_{VLC2} (4-6) that demonstrated effective antihypertensive activity in Wistar rats, and could serve as a precursor sample to the isolation of possible antihypertensive compounds.

Conflict of interest

The authors declare no conflict of interest whatsoever.

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