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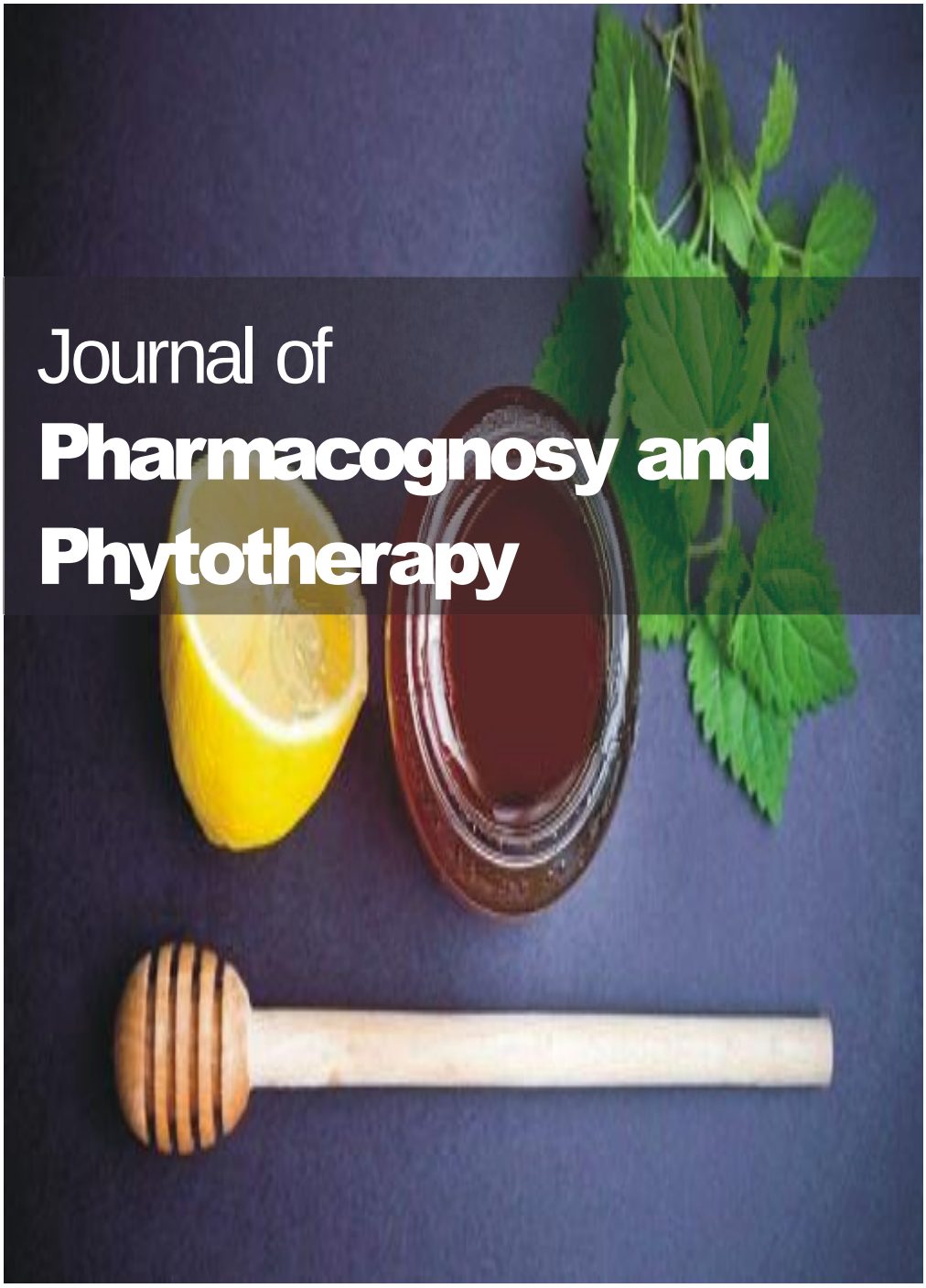

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Full Length Research Paper

UHPLC-DAD characterization of bioactive secondary metabolites from *Ocimum americanum* and *Pupalia lappacea* extracts: Antioxidant activity and antihypertensive effects on L-NAME-induced hypertensive rats

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Ocimum americanum* L. (Lamiaceae) and *Pupalia lappacea* (L) Juss. (Amaranthaceae) are two plants used in Bénin to manage hypertension. Little scientific data is available on the antihypertensive properties of these plants. Therefore, we investigated the antihypertensive potential of ethanolic extracts of *O. americanum* and *P. lappacea* on L-NAME-induced hypertensive rats. The DPPH free radical scavenging potential, Fe^{3+} reducing capacity, superoxide anion radical and hydrogen peroxide scavenging were assessed. Extracts were also screened for their active compounds using ultimate high-performance liquid chromatography 3000. CODA™ non-invasive blood pressure system was used to record blood pression parameters. Both extracts induced significant decrease of systolic, diastolic blood pressure and mean arterial pression. *O. americanum* extract at 250 mg/kg body weight, decreased mean blood pressure (MBP) from 146 ± 4.80 to 98.4 ± 9.44 mmHg and *P. lappacea* extract from 154.4 ± 11.28 to 111.8 ± 9.44 mmHg. A significant decrease of MBP was also observed with Losartan and Captopril at 100 mg/kg body weight. *P. lappacea* showed the higest ferric reducing/antioxidant power 4905 ± 87.79 µmol AAE g^{-1}. Superoxide anion and hydrogen peroxide scavenging activities showed higher activity, 68.42 ± 3.68 and 38.68 ± 4.18%, respectively for *O. americanum*. The chromatography analysis of extracts suggested the presence of ferulic acid, chlorogenic, tannic, ellargic, caffeic acids and chrysin, rutin and isorhamnetin. The obtained results justify the traditional use of *O. americanum* and *P. lappacea* in management of hypertension in southern Bénin.

Key words: *Ocimum americanum*, *Pupalia lappacea*, antihypertensive activity, antioxidant activity.

INTRODUCTION

Hypertension is reported next to many infectious diseases as most serious health problems in developing tropical countries (Orch et al., 2015). The reasons for increasing prevalence of hypertension can be correlated to exposure to persistent stress, excessive alcohol consumption, use of tobacco unhealthy diet, physical

consumption, use of tobacco unhealthy diet, physical inertness, excess weight and ageing (WHO, 2013). Oxidative stress is also one of the reasons for the occurrence of hypertension. Reactive oxygen species (ROS) are associated with many vascular risk factors, including hypertension (Amoussa et al., 2015). Thus, the control of hypertension becomes imperative given the high mortality and morbidity associated with its complications (WHO, 2009). According to the World Health Organization, more than 80% of people in developing countries still depend on local medicinal plants to fulfill their primary health needs (WHO, 2009). In Bénin, medicinals plants are used in the treatment of various pathology among which the arterial hypertension (HTA). Over the last three decades, many collaborative efforts have been devoted to research on local plants with hypotensive and antihypertensive effects (Raji et al., 2013). Today, research on therapeutic potential of medicinal plants has become a global issue, as medicinal plants are the richest source of medicines in traditional medicine systems, modern medicines, nutraceuticals, dietary supplements, traditional medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Pandey and Tripathi, 2014). Also, the importance of plants in medicine remains even of greater relevance with the current global shift to obtain drugs from plants sources, as a result of which, attention has been given to the medicinal value of herbal remedies for safety, efficacy and economy (Tsobou et al., 2015). In the field of hypertension, several plants are used in traditional medicine. *Ocimum americanum* is widely used alone or in combination to treat many diseases such as hypertension, skin disease, dysentery, digestive, stomachic, genitorurinary, lowering blood glucose and also treats cold, fever, parasitic infestations, inflammation of joints and headaches (Karou et al., 2011; Lagnika et al., 2016). Antibacterial, antimalarial, antioxidant and insecticidal activities have been also reported (Ntonga et al., 2014). *Pupalia lappacea* is reported in folk medicine for various purposes. It is used to treat urethra pain, leprosy, fractured bone, endometritis, cystitis and leucorrhoea and as laxative, purgative, anti vomitory, antisterility, anti emetic and antalgic (Naidu et al., 2014; Srinivas, 2015). Many studies have demonstrated its anticonceptive, hepatoprotective, antipyretic, anti-inflammatory properties and antimicrobial, antidiarrhoeal activity (Mehnoor and Chakrapani, 2015; Naidu et al., 2014; Apenteng et al., 2014; Hoekou et al., 2012). Despite their traditional use, there is little or no information to confirm the antihypertensive properties attributed empirically to these plants. Therefore, this study is designed to investigate the effects of chronic administration of ethanolic extrats of *O. americanum* (OAE) and *P. lappacea* (PLE) to L-NAME-induced hypertensive Wistar rats, to evaluate the antioxidant potential and quantify phenolic compounds of each extract.

MATERIALS AND METHODS

Plant materials

Fresh samples of *O. americanum* and *P. lappacea* were collected in Southern Bénin in July 2015. The plants were identified and authenticated at the National Herbarium of the University of Abomey-Calavi where the voucher specimens were deposited; *O. americanum* (YH 277/HNB) and *P. lappacea* (YH 234/HNB).

Preparation of extracts

Selected plants were dried in laboratory under air-conditioned (22 ± 2°C) and then reduced to powder using an electric grinder (MARLEX Electroline Excella). 300 g of each plant were extracted with 1 L of ethanol under stirring for 24 h. The macerate was filtered through a Whatman No.1 paper filter and concentrated using a rotary evaporator (BUCHI Rotavapor RII). The extraction process was repeated three times. The obtained extracts were stored at 4°C for assay.

Phytochemical investigation

Thin layer chromatography (TLC) and colorimetric methods tandem with high pression liquid chromatography analysis were used.

Qualitative phytochemical assay

Flavonoids, tannins, alkaloids, triterpenes, coumarins, saponins, essentials oils, lignans, pigments, naphthoquinones, anthracene derivatives and cardiac glycosides were characterized according to standard methods using TLC (Wagner and Bladt, 2001) and colorimetric reaction (Shah and Hossain, 2010).

Total phenolic contents

Total phenolics of extracts were determined according to methods used previously (Amoussa et al., 2015). Total phenolic content was measured spectrophotometrically using Folin–Ciocalteau reagent. The absorption of sample was read at 765 nm against a blank and gallic acid was used as the standard. The total phenolic was calculated using the equation of the calibration curve of gallic acid and expressed as gallic acid equivalent (mg GAE g^{-1} dry weight).

Total flavonoid content

The total flavonoid of extracts was determined by a colorimetric assay using aluminium chloride, and the absorbance was read at 415 nm (Amoussa et al., 2015). Quercetin was used as reference. Total flavonoid was expressed as quercetin (mg/g) using the equation of the calibration curve of quercetin. Total flavonoids are

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reported as milligrams of quercetin equivalent (QE) per 100 g of extract.

Identification and quantification of active compounds by U-HPLC 3000

Preparation of samples

Stock solutions at 100 µg/ml in methanol of thirteen standards phenolic compounds (ferulic acid, caffeic acid, chlorogenic acid, tannic acid, ellargic acid, gallic acid, syringic acid, luteolin, chrysin, rutin, hyperoside, quercétol and and isorhamnetin) were prepared and stored at 4°C. Appropriate dilutions were performed prior to analysis. The ethanolic extract of each plant was also prepared at 1 mg/ml in methanol. All samples and standards solutions were filtered with 0.2 µm pore sizes filters.

U-HPLC 3000 analysis

The standard phenolic compounds analysis and quantification were carried out using U-HPLC 3000 liquid chromatograph system, equipped with a degasser, binary gradient pump, a UV multiwavelength detector (DAD - 3000 RS and MWD - 3000 RS) and a C_{18} reversed phase column (150 × 4.6 mm, 5 µm Hypersil BDS) at ambient temperature. The mobile phases consisted of water (A) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid. The elution gradient (0-20 min, 20-50% B; 20-25 min 50-70% B; 25-30 min, 70-80% B; 30-35 min, 80-20% B; 35-40 min, 20% B). The flow rate was 1 ml/min and injection volume 20 µl. Data analysis was performed using Chromleon v.6.80 Software (Dionex, Thermo Fisher Scientific). Phenolic compounds in extracts were identified according to their retention times, UV-Vis spectra and comparison with standard compounds.

Antioxidant activity

Antioxidant activity of OAE and PLE was evaluated using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging, ferric-reducing antioxidant power (FRAP) assay, superoxide anion scavenging activity and hydrogen peroxide scavenging assay.

DPPH radical-scavenging activity

The free radical scavenging capacity of extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the method described by Talbi et al. (2015). The extracts were solubilized in dimethyl Sulfoxide (DMSO) and then diluted to obtain a stock solution at 100 µg/ml which is subjected to two-fold dilutions to make eight concentrations. The test consists of 1.5 ml of the freshly prepared 2% DPPH methanolic solution and 0.75 ml of each extract. Methanolic solutions of DPPH and ascorbic acid were used as blank and reference, respectively. After 15 min incubation in darkness, absorbance was measured at 517 nm using spectrophotometer (VWR UV-1600 PC). All experiments were performed in triplicate. The inhibition power of the DPPH radical, expressed as a percentage, is calculated according to the formula below:

$$\text{Inhibition (\%)} = [(AB - As)/ AB] \times 100] \quad (1)$$

Where: As; tested extract absorbance and AB; blank absorbance.

Ferric-reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the method of Saeed et al. (2012). The test mix consists of 2 ml of extracts (100 µg/ml) in ethanol, 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 min followed by addition of 2 ml of trichloroacetic acid (100 mg/ml). The obtained solution was centrifuged at 3000 rpm for 10 min. 2 ml of supernatant were mixed with 2 ml of distilled water and 0.4 ml of 0.1% fresh ferric chloride (w/v). After 10 min incubation, the absorbance was read at 700 nm. Ascorbic acid was used to produce the calibration curve. Assay was performed in triplicate and expressed in µMol Ascorbic Acid Equivalent (AAE)/g of extract.

Superoxide anion scavenging activity

A modified version of the method described by Kumar was used (Kumar et al., 2012). The superoxide radicals were generated by alkaline DMSO. All extracts and nitro blue tetrazolium (Sigma, N6639) were prepared in DMSO at 100 µg/ml and 1 mg/ml respectively. The test mixture consist of 50 µl of extract was mixed with 170 µl of alkaline DMSO and 30 µl of nitro blue tetrazolium (NBT). After 5 min incubation at laboratory temperature (22°C ± 2), the absorbance was measured at 630 nm against blank using microplate reader (Rayto R 6500, China). The blank consist of the reaction mixture without extract. Quercetin was used as standard. All the experiments were performed in triplicate. Superoxide anion scavenging percentage (SP) was calculated as follow:

$$\text{SSP} = [(AB - As) / AB] \times 100] \quad (2)$$

Where: SSP; Superoxide scavenging percentage, As; extract absorbance and AB; blank absorbance.

Hydrogen peroxide scavenging assay

Hydrogen peroxide (H_2O_2) scavenging potential was determined using the method of Mohan et al. (2012). A solution of hydrogen peroxide (100 mM) was prepared in phosphate buffer (0.1 mM, pH 7.4). The reaction mixture consists of 0.5 ml of extract at 100 µg/ml diluted in distilled water and 1.5 ml of hydrogen peroxide solution at 40 mM. After 10 min, the absorbance was measured at 295 nm using spectrophotometer (VWR UV-1600 PC). The phosphate buffer solution and gallic acid were used as blank and standard respectively. Assay was performed in triplicate. The H_2O_2 radical scavenging was calculated as bellow:

$$\text{HSP} = [(AB - As) / AB] \times 100] \quad (3)$$

Where: HSP; Hydrogen peroxide scavenging percentage, As; extract absorbance and AB; blank absorbance.

Blood pressure measurement

Animal

Male Wistar rats weighing 200 to 250 g obtained from the Laboratory of Human Biology, Faculty of Health Sciences, University of Abomey-Calavi were used. The selected animals were maintained under laboratory conditions (24 ± 2°C), exposed to 12 h day/night cycle, and free access to a diet and water. They were subjected to experimental conditions for two weeks in order to accustom them to blood pressure measure equipment and then minimizing stress during the experiment. Blood pressure was measured by the tail-cuff method using a non-invasive blood

pressure system for rats (Kent Scientific CODA™ 20942). The study was done in accordance with the guidelines for the care and use of laboratory animals of the Faculty of Health Science and Faculty of Sciences and Technologies of University of Abomey-Calavi.

Hypertension induction and treatment

Hypertension was induced in rats by administration of N(G)-Nitro-L-Arginine-Methyl Ester (L-NAME). After confirmation of the hypertensive status, animals were treated with the ethanolic extracts and the reference drugs; losartan and captopril. Fourty (40) rats divided into eight (8) groups of five (5) animals are used. The first group received distilled water from day 1 to 28. Groups 2 to 8 received L-NAME at 40 mg/kg body weight from day 1 to 14. These groups were subsequently treated with reference drugs and ethanolic extracts from day 14 to 28. Group 2 received distilled water. Groups 3 and 4 received respectively, losartan and captopril at 100 mg/kg body weight. Groups 5 and 6 respectively received OAE at 250 and 500 mg/kg body weight, whereas Groups 7 and 8 received respectively, PLE at 250 and 500 mg/kg body weight. All substances and extracts (L-name, losartan, captopril and crude extracts) were prepared in distilled water and administrated orally to rats.

Antihypertensive evaluation

At the end of the treatment, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR) were measured to assess the antihypertensive effect of extracts. All parameters are measured on days 1, 8, 15, 22 and 29 using the CODA™ non-invasive blood pressure system (Kent Scientific Corporation) which is based on blood flow to measure systolic and diastolic blood pressure in the tail of animal (Sung et al., 2013). Animals were placed in their sockets on a heated platform to improve blood flow to the tail and minimize movements when taking measurements. During each experiment, blood pressure was measured 20 times including five (5) initial acclimation measurements and 15 experimental measurements. Among the experimental measurements, at least seven (7) were considered valid by the CODA ™ system. Valid measurements were used for statistical analysis.

Ethical consideration

The experimental protocols used in this study were approved by the scientific committee of the Doctoral School of Life Sciences and Earth at University of Abomey-Calavi (UAC/FAST/EDSV/ 10132309).

Statistical analysis

All the results obtained are presented as mean ± standard deviation form. Results of the antihypertensive activity were analyzed using STATA version 14.0 software. Linear regression was used to evaluate the degree of significance of the induction of arterial hypertension and the effect of extracts. The level of significance was set at 0.05.

RESULTS

Phytochemical analysis

A similarity was noticed within secondary metabolites of both plants. Flavonoids, triterpenes, coumarins, lignanes, anthocyanines and essential oils are the secondary metabolites identified in both extracts. Contrary to *P. lappacea*, tannin, alkaloids, naphthoquinones and anthracene derivatives were also detected in *O. americanum*.

Total phenolic and flavonoids contents

The total phenolic of *O. americanum* (28.15 ± 0.23 mg EAG/100 mg) is higher than *P. lappacea (*13.77 ± 1.12 mg EAG/100), whereas total flavonoids are comparable in both extracts with 48.06 ± 0.82 mg EQ/100 mg for *O. americanum* and 49.90 ± 1.85 mg EQ/100 mg for *P. lappacea*.

Identification and quantification of actives compounds using U-HPLC 3000

Analysis of ethanolic extracts allowed to identified ferulic acid, caffeic acid, chlorogenic acid, tannic acid, ellargic acid, chrysin and rutin in OAE while ferulic acid, chlorogenic acid, tannic acid, ellargic acid, chrysin and rutin were identified in PLE. The results of the quantitative analysis by HPLC were presented in Table 1. Other unidentified molecules also appeared in the extracts.

DPPH radical-scavenging activity

The antioxidant activity of OAE and PLE are dose-dependent as described in Figure 1. At 100 µg/ml, DPPH scavenging activity of *O. americanum* (43.50 ± 2.12%) is higher than that of *P. lappacea* (25.52 ± 1.54%). Compared to ascorbic acid (99.46 ± 0.38%) used as the control, the plant extracts had moderate activity.

Ferric-reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay is based on the reduction of ferricyanide complex (Fe^{3+}) to ferrous form (Fe^{2+}) by antioxidant metabolite in extracts. Ethanolic extract of *P. lappacea* showed the highest ferric reducing power (4905 ± 87.79 µmol AAE g^{-1}) compared to *O. americanum* (4745.9 ± 113.39 µmol AAE g^{-1}) as shown in Table 2.

Superoxide anion and hydrogen peroxide scavenging activities

The superoxide anion and hydrogen peroxide scavenging activitiies of *O. americanum* and *P. lappacea* extracts are shown in Table 2. In superoxide anion assay, *O.*

Table 1. Actives phenolic compounds identified in *O. americanum* and *P. lappacea* extracts by U-HPLC 3000.

Standard	Retention time (min)	Amount (mg/g)	
		O. americanum	***P. lappacea***
Chlorogenic acid	7.19	0.089	0.051
Caffeic acid	7.47	0.031	nd
Tannic acid	10.11	6.349	0.001
Ferrulic acid	12.21	0.043	0.031
Rutin	17.87	0.505	0.001
Ellargic acid	18.63	0.200	0.040
Isorhamnetin	27.07	0.549	nd
Luteolin	nd	nd	nd
Chrysin	28.05	0.478	0.011
Hyperoside	nd	nd	nd
Gallic acid	nd	nd	nd
Syringic acid	nd	nd	nd
Quercetin	nd	nd	nd

nd: not detected.

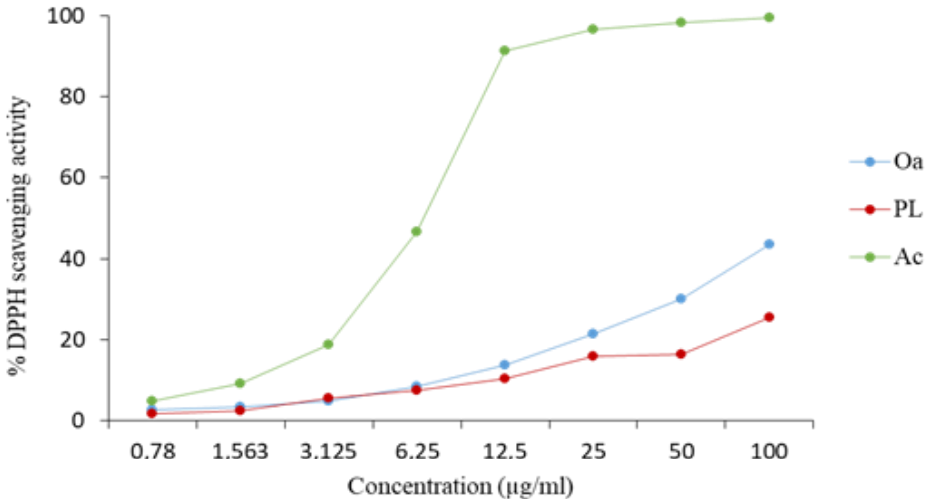

Figure 1. Radical DPPH scavenging activities of *O. americanum* and *P. lappacea* extracts. Oa: *O. americanum*; PL: *P. lappacea*; Ac: Ascorbic acid.

Table 2. Superoxide anion, hydrogen peroxide scavenging and ferric reducing antioxidant activities of *O. americanum* and *P. lappacea*

Extract	Superoxide anion (%)	Hydrogen peroxide (%)	Ferric reducing antioxidant potential (µmol AAE g^{-1})
O. americanum	68.42 ± 3.68	38.68 ± 4.18	4905 ± 87.79
P. lappacea	58.76 ± 3.42	32.67 ± 2.45	4745.9 ± 113.39
Quercetin	83.48 ± 1.21	na	na
Gallic acid	na	73.89 ± 1.93	na

na : not applicable.

americanum showed the scavenging percentage of 68.42 ± 3.68%, wereas *P. lappacea* extracts showed 58.76 ± 3.42%. Regarding hydrogen peroxide assay, both extracts showed moderate activity with 38.68 ± 4.18 for

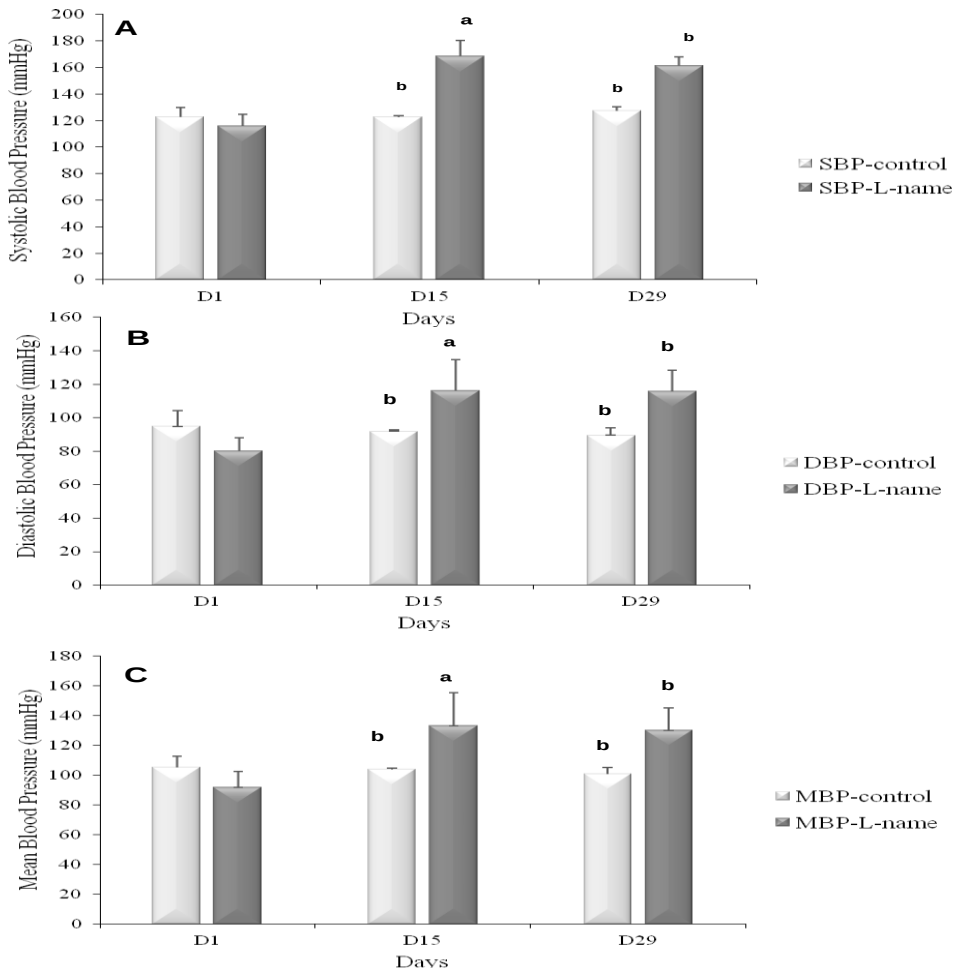

Figure 2. Effect of L-NAME on systolic (A), diastolic (B) and mean blood pressure (C). SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MBP: Mean blood pressure. Day 1 to 14: L-NAME administration; day 15 to 28: Distilled water administration. Data on day 15 were compared to those of day 1 to confirm induction of hypertension and perform statistical analyzes. Same procedures were used for the comparison of data between day 15 and 29. [a] ($p < 0.05$): significant change after L-NAME administration; [b] ($p > 0.05$): No significant change after L-NAME administration.

O. americanum against 32.67 ± 2.45 for *P. lappacea*.

Effet of extracts on blood pressure and heart rate

During the twenty-eight days, blood pressure was measured weekly using non-invasive method. No significant changes in mean blood pressure (MBP) were observed in the control group (105.2 ± 7.44 to 101 ± 4.4 mmHg) during the four weeks of experimentation. A significant increase of SBP, DBP and MBP was observed in animals that received L-NAME daily for fourteen (14) days as shown in Figure 2. Administration of OAE and PLE at 250 and 500 mg/kg body weight for fourteen days following L-NAME administration induced a significant decrease in blood pressure. OAE and PLE at 250 mg/kg bw induced significant decrease in SBP, DBP and MBP of all the groups and there is no significant change between results obtained at 250 and 500 mg/kg bw. From day 15 to 29, *O. americanum* decreased MBP from 118.2 ± 00.32 to 76.80 ± 3.36 mmHg and *P. lappacea* from 143.0 ± 12.40 to 101.8 ± 8.24 mmHg. Similar results were obtained with losartan and captopril which respectively reduced blood pression from 122.2 ± 06.16 to 83.00 ± 4.80 mmHg and 127.4 ± 03.52 to 87.20 ± 3.36 mmHg as presented in Tables 3 and 4. Contrary to blood pressure, administration of L-NAME (40 mg/kg/day) to animals for two weeks induced a significant decrease in the heart rate as shown in Table 5. Administration of OAE, PLE and reference drugs (Losartan and Captopril) restored the heart rate (HR). The significant increase ($p < 0.05$) of heart rate was observed at 500 mg/kg/bw as shown in Table 5.

Table 3. Effect of ethanolic extracts of *O. americanum* and *P. lappacea* on systolic and diastolic blood pressure in L-NAME-induced hypertensive Wistar rats.

Treatment	[C] (mg/kg.bw)	Systolic blood pressure			Diastolic blood pressure		
		Day 1	Day 14	Day 28	Day 1	Day 14	Day 28
Control (H_20)	-	122.8 ± 7.04	122.8 ± 0.8	127.4 ± 3.04	94.8 ± 9.44	91.8 ± 1.04	89.4 ± 4.64
O. americanum	250	120.8 ± 4.25	156.8 ± 11.52[a]	129,0 ± 06.16[a]	73.8 ± 03.76	118.2 ± 00.32[a]	76.80 ± 3.36[a]
	500	124.6 ± 6.08	159.0 ± 08.40[a]	118,0 ± 07.60[a]	92.6 ± 07.04	101.2 ± 17.52[b]	82.00 ± 7.60[b]
P. lappacea	250	119.8 ± 8.64	179.2 ± 08.64[a]	133.8 ± 12.24[a]	79.8 ± 10.64	143.0 ± 12.40[a]	101.8 ± 8.24[a]
	500	131.4 ± 1.68	167.8 ± 09.44[a]	140.0 ± 08.00[a]	96.4 ± 02.88	121.4 ± 07.28[a]	104.6 ± 6.72[a]
Losartan	100	122.2 ± 1.92	168.0 ± 05.60[a]	114.2 ± 04.32[a]	91.0 ± 00.80	122.2 ± 06.16[a]	83.00 ± 4.80[a]
Captopril	100	115.6 ± 3.68	167.8 ± 04.16[a]	116.8 ± 03.44[a]	79.6 ± 04.72	127.4 ± 03.52[a]	87.20 ± 3.36[a]

Day 1 to 14: L-NAME administration; day 15 to 28: Extracts administration; [C] (mg/kg.bw): Concentration of extracts mg per kg of body weight of rats; [a]($p < 0.05$): Significant change of SBP or DBP after L-NAME and extracts administration; [b] ($p > 0.05$): No significant change of DBP after L-NAME or extracts administration.

Table 4. Effect of ethanolic extracts of *O. americanum* and *P. lappacea* on mean blood pressure in L-NAME-induced hypertensive Wistar rats.

Treatment	[C] (mg/kg.bw)	Day 1	Day 15	Day 28
Control (H20)	-	105.2 ± 7.44	104 ± 0.8	101 ± 4.4
O. americanum	250	88.60 ± 3.12	138.4 ± 07.52[a]	91.80 ± 6.56[a]
	500	102.0 ± 5.60	119.0 ± 13.60[a]	91.20 ± 5.04[a]
P. lappacea	250	92.80 ± 9.84	154.4 ± 11.28[a]	111.8 ± 9.44[a]
	500	107.8 ± 2.24	136.4 ± 08.08[a]	116.0 ± 7.20[a]
Losartan	100	101.0 ± 0.80	135.8 ± 05.52[a]	93.20 ± 4.72[a]
Captopril	100	92.20 ± 3.84	136.0 ± 09.28[a]	92.80 ± 1.84[a]

Day 1 to 14: L-NAME administration; day 15 to 28: extracts administration; [a] ($p < 0.05$): Significant change of MBP after L-NAME and extracts administration.

Table 5. Effect of ethanolic extracts of *O. americanum* and *P. lappacea* on heart rate in L-NAME-induced hypertensive Wistar rats**.**

Treatment	[C] (mg/kg.bw)	Day 1	Day 14	Day 28
O. americanum	250	322.20 ± 10.24	261.40 ± 03.12[a]	275.80 ± 25.76[b]
	500	276.20 ± 35.84	249.20 ± 41.44[b]	330.80 ± 12.64[a]
P. lappacea	250	304.80± 11.44	211.80 ± 33.76[a]	251.00 ± 37.60[b]
	500	269.40 ± 32.88	182.80 ± 33.44[a]	274.40 ± 40.48[a]
Losartan	100	294.00 ± 36.80	205.00 ± 25.60[a]	264.00 ± 22.00[a]
Captopril	100	308.00 ± 28.00	247.00 ± 30.40[a]	310.60 ± 02.72[a]
L-NAME	40	266.00 ± 34.40	189.40 ± 42.64[a]	304.80 ± 15.84[a]
Control	-	277.80 ± 32.24	267.80 ± 36.24[b]	307.40 ± 24.32[b]

Day 1 to 14: L-NAME administration; day 15 to 28: Extracts administration. [a] ($p < 0.05$): Significant change of HR after L-NAME and extracts administration; [b] ($p > 0.05$): No significant change of HR after L-NAME and extracts administration.

DISCUSSION

In Benin, the use of medicinal plants for primary health care and the management of various diseases remains a reality. The management of hypertension does not escape this tradition and this has been confirmed by the results of recent surveys on the use of medicinal plants by hypertensives (Lagnika et al., 2016). In this study, we investigated the phytochemical constituents, total phenolic and flavonoids contents, antioxidant effect and the capacity of ethanolic extracts of *O. americanum* (OAE) and *P. lappacea* (PLE) to reduce blood pressure.

The phytochemical analysis of ethanolic extracts of OAE and PLE revealed the presence of various phytoconstituents. A large similarity was observed with previous published data about *O. americanum* (Birari and Dhulgande, 2010; Sarma and Venkata, 2011). However, differences were observed with coumarins (Dibala et al., 2016), alkaloids (Enemali and Udedi, 2018) and tannins (Elya et al., 2015). Regarding *P. lappacea*, a similitude was also observed with previous study (Udegbunam et al., 2014; Hoekou et al., 2012). Contrary to our results, alkaloids and tannins have been detected in ethanolic extract. These differences could be due to the phyto-geographical distribution, the phenology, the physiological stage of the species, the extraction method and/or solvents (Goli et al., 2005; Tarnaud et al., 2010).

A similarity was noted within secondary metabolites detected in OAE and PLE. The flavonoids, triterpenes, coumarins, essential oils, lignanes and anthocyanines are the secondary metabolites detected. Some of these phytoconstituents are well known for their antioxidant activity and their capacity to decrease high blood pressure (Oh et al., 2008). The antioxidant properties of tannins, flavonoids and coumarins make them protective molecules against free radicals that play an important role in the occurrence of more than 200 diseases such as cardiovascular diseases, cancer, hypertension, arthritis (Adjatin et al., 2013; Amoussa et al., 2015; Lajous et al., 2016; Bekoe et al., 2017). In general, flavonoids intake has been reported to have an inverse relation with cardiovascular disease and polyphenols have vascular protective effect (Siti et al., 2015).

Chronic administrations of L-NAME to rats significantly increase SBP, DBP and MBP when compared to control group. It is known that L-NAME administration causes a chronic increase in blood pressure in rats model (Gardiner et al., 1990; Babál et al., 1997). The increase in blood pressure could be explained by inhibition of nitric oxide (NO) synthesis by L-NAME (Kimura et al., 2017). Thus, a sufficient amount of NO is associated with normal vasodilatation and normal blood pressure, whereas inhibition of NO production may lead to hypertension (Nyadjeu et al., 2013; Sung et al., 2013). These data clearly justify the significant increase in SBP, DBP and MBP after L-NAME administration in our study. The increase in blood pressure during treatment with L-NAME may be associated with NO deficiency and alterations in various blood pressure regulation systems. Many studies reported that chronic blockade of NO synthesis by NOS inhibitors like L-NAME lead to endothelial dysfunction, significant increase in blood pressure and further pathological injuries to the cardiovascular system and kidneys, which may lead to aggravation of hypertension (Graciano et al., 2004). Evidence have been also provided that chronic inhibition of NO synthesis in rats leads to elevations of systemic blood pressure and peripheral vascular resistance with alteration of vascular responsiveness. These vascular alterations were associated with marked oxidative stress (Veerappan and Senthilkumar, 2015). These data clearly justify the significant increase in SBP, DBP and MBP after L-NAME administration in our study.

Chronic oral administration of ethanol extracts of *O. americanum* and *P. lappacea* to L-NAME-induced hypertensive rats caused a significant decreased in SBP, DBP and MBP. Same results were observed for losartan and captopril used as standard drugs. The decrease of blood pressure by extracts may be associated with the regulation of oxidative stress associated with endothelial dysfunction after L-NAME administration. Previous reports showed that plant extracts containing flavonoids and/or triterpenes exert antihypertensive effects through the combination of the vasodilatory and antioxidant activities (Oh et al., 2008; Curin and Andriantsitohaina, 2005). Antioxidants are also reported to be beneficial in preventing endothelial dysfunction by scavenging superoxide and peroxynitrite (Kang et al., 2015). In this study, the analysis of ethanol extracts of *O. americanum* and *P. lappacea* allowed identification of phenolic acids and flavonoids which could contribute to their antioxidant and antihypertensive activity. The antioxidant activity of *O. americanum* (Shobo et al., 2015; Dinata et al., 2015; Enemali and Udedi, 2018) and *P. lappacea* (Apenteng et al., 2014; Prasad et al., 2014; Jazy et al., 2018) have been reported previously; thus, the decrease of MBP might be associated with the phenolic acids and flavonoids identified in extracts. Indeed, ferulic acid is one of these phenolic acids endowed with varied biological potential such as antioxidant, increase NO synthesis, free radical scavenger activity and vasodilatory effect (Kumar and Vikas, 2014; Drăgan et al., 2018). Chrysin have been showed to possess an antihypertensive effect by lowering blood pressure, lipid peroxides and improved antioxidant status. The suggested pharmacological mechanism is that chrysin inhibits production of superoxide and hydroxyl free radicals in enzymatic and nonenzymatic systems (Veerappan and Thekkumalai, 2018). Rutin identied in extract was showed to increase NO production in human endothelial cells and improved endothelial functions (Ugusman et al., 2014; Aditya and Ajay, 2017). It has also been reported that ellagic acid attenuates hypertension and possibly improving nitric oxide bioavailability. Likewise, caffeic acid and chlorogenic acid

decreased blood pressure and improved nitric oxide (NO) bioavailability by reducing activities of key enzymes linked to the pathogenesis of hypertension in cyclosporine-induced rats. These might be possible suggested mechanism of action of the phenolic acids and flavonoids identified in studied plants (Chiou et al., 2017; Agunloye et al., 2019).

Moreover, it is known that elevation of vasoconstriction and attenuation of vasorelaxation was observed in different parts of the vascular tree and increased sympathetic activity and alterations in renin-angiotensin system in L-NAME treated rats (Rossoni et al., 2007). The imbalance of vasoconstrictor and vasodilator systems caused an elevation of blood pressure in hypertension. Likewise, the reduction of NO dependent vasodilation (vasonstriction) is the result of NO deficient hypertension which is followed by L-NAME adminitration (Zicha et al., 2006). Thus, the effect of plants studied might be associed with responses of balance between vasodilatation and vasoconstriction of blood vessels. Previous studies have demonstrated that ellargic acid improved vascular response affected by hypertension (Jordão et al., 2017). Several authors have also suggested that decrease in blood pressure and vasodilator effect could be created by phenolic acids such as tannic acid or ferulic acid (Turgut et al., 2015; Porteri et al., 2010). Taking into account these phenolic acids identified in the ethanolic extracts of *O. americanum* and *P. lappacea*, we could suggest that the effect of OAE and PLE against high blood pressure could be expained by their vasorelaxant property (Drăgan et al., 2018). The antihypertensive effect of plants could also be due to their ability to reduce the peripheral resistance via their vasodilating activities (Sung et al., 2013; Bilanda et al., 2017). In this part, we could suppose that OAE and PLE had direct effect on vascular smooth muscle cells.

Inhibition of NO production by L-NAME may have increased the effect of reactive oxygen species (ROS) generated by vascular NADPH oxidase, resulting in endothelial dysfunction (Sung et al., 2013). NADPH oxidase is critically involved in increased blood pressure, O_2 production, vascular hypertrophy, inflammation and endothelial dysfunction in experimental and clinical hypertension (Lodi et al., 2006; Beswick et al., 2001). Previous report showed that administration of ellagic acid to L-NAME induce-hypertensive rats reduced blood pressure and attenuates hypertension by reducing NADPH oxidase subunit $p47^{phox}$ expression, which prevents oxidative stress and restores NO bioavailability (Thewarid et al., 2015). The decrease in blood pressure after administration of OAE and PLE could be related to the inhibition of NADPH oxidase activity.

Conclusion

Overall, the present study demonstrated that OAE and PLE at a dose of 250 mg/kg bw exhibited antihypertensive effect by lowered blood pressure. The interesting antioxidant and antiradicals potential and phenolic contents were described. The presence of phenolic acids and flavonoids contributed to the biological effects observed. These results may partially justify the traditional use of studied plants for the management of hypertension; however, further studies need to be done to determine the mechanism of action of the extracts and to consider the valorization of these plants as part of the managment of hypertension.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

The effect of blending of extracts of Sudanese *Adansonia digitata* and *Tamarindus indica* on their antioxidant, anti-inflammatory and antimicrobial activities

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***Adansonia digitata* (Bombacaceae) *and Tamarindus indica* (Fabaceae) are the most popular herbal products in Sudan; they are used as food ingredients and in traditional treatments of various diseases. The aim of this study is to investigate phytochemical contents, antioxidant, anti-inflammatory and antimicrobial activities of plants fruit, leaf and bark ethanolic extracts. The phytochemical screening of different extracts revealed the presence of alkaloids, flavonoids, sterols/triterpens, tannins, saponins, coumarins, glycosides, reducing sugar, lignin and carbohydrates. The results indicate that all the extracts have reducing power DPPH radical scavenging abilities. The highest antioxidant activity showed in *A. digitata* parts. The fruit extracts of both plants showed the highest antioxidant activity (84.07 and 83.98% for *A. digitata* and *T. indica,* respectively). The *in vitro* anti-inflammatory effects have been studied by human albumin denaturation, and both plant extracts showed remarkable activity. Leaf extracts showed highest anti-inflammatory activity (76.714, 62 and 82.71% for *A. digitata*, *T. indica* and mixture of both respectively). The results of antimicrobial activity showed the effectiveness of mixture extracts against tested standard pathogens. Fruit mixtures showed the highest activity against *B. subtilis* (19 mm), *S. aureus* (35 mm) and *S. typhi* (21 mm), while the mixture of bark extracts showed the highest activity against *E. coli* (19 mm). It is noteworthy that higher antioxidant, anti-inflammatory and antimicrobial activities have been observed by blends in the ratio 1:1 of fruit, leaf and bark extracts of both plants.**

Key words: *Adansonia digitata*, *Tamarindus indica*, antioxidant, anti-inflammatory, antimicrobial, combination.

INTRODUCTION

Sudan traditional medicine is characterized by a unique combination of Islamic, Arabic, and African cultures. In

poor communities, traditional medicine has remained as the most reasonable source of treatment of several diseases and microbial infections. Although the traditional medicine is accepted in Sudan, to date there is no updated review available which focuses on most effective and frequently used Sudanese medicinal plants (Karar and Kuhnert, 2017) .

Adansonia digitata (Bombacaceae) known as Baobab is an important plant used in Sudanese traditional medicine; it is widespread throughout the hot, drier regions of tropical Africa. It extends from northern Transvaal and Namibia to Ethiopia and Sudan. In Sudan, the baobab is most frequently found on sandy soils and by seasonal streams Khors in short grass savannas. It forms belts in Central Sudan, Kordofan, Darfur and Blue Nile (Dabora, 2016). The Baobab fruit pulp is an important foodstuff used as a drink, a sauce for food and as a fermenting agent in local brewing (Gebauer et al., 2002). Different parts of the plant are used to treat many diseases. The alkaloid 'adansonin' in the bark is thought to be the active principle for treatment of malaria and other fevers (De Caluwé et al., 2010). The plant is: Anti-oxidizing agent including; polyphenolic compounds, vitamins E and C, cardiovascular diseases, cancer and aging related disorders (De Caluwé et al., 2010); antiviral activity against Herpes simplex, Sindbis and Polio (Anani et al., 2000); anti-inflammatory and antipyretic activity (Kaboré et al., 2011); anti-microbial activity and anti-trypanosoma activity (Varudharaj et al., 2015). *Tamarindus indica* (Fabaceae) grows wild in Africa in locales as diverse as Sudan, Cameroon, Nigeria, and Tanzania (Havinga et al., 2010). Tamarind fruit pulp is used for seasoning as a food component to flavour confections, curries and sauces, and is a main component in juices and certain beverages. Tamarind fruit pulp is eaten fresh and often made into a juice, infusion or brine, and can also be processed into jam and sweets (Hassan, 2014). The plant is widely used in African traditional medicine for treatment of many diseases such as fever, dysentery, jaundice, gonococci and gastrointestinal disorders (Lawal et al., 2010). Phytochemical investigation carried out revealed the presence of many active constituents, such as phenolic compounds, cardiac glycosides , L-(-)mallic acid, tartaric acid, the mucilage and pectin, arabinose, xylose, galactose, glucose, and uronic acid. The ethanolic extracts showed the presence of fatty acids and various essential elements like arsenic, calcium, cadmium, copper, iron, sodium, manganese, magnesium, potassium, phosphorus, lead, and zinc (Bhadoriya et al., 2011). Many studies have been performed on skin, eye and respiratory tract irritation mediated by complex mixtures, but only few studies allows a quantitative evaluation of the modulating effects of the combination of single chemicals. Further studies on the modulating effects of the combination of chemicals concerning skin, eye and respiratory tract irritation will be required in order to evaluate the possibilities for synergistic or antagonistic effects being mediated by mixtures of chemicals (Doty et al., 2004). The present study reports the effect of blending equal amounts of *A. digitata* (Bombacaceae) and *T. indica* (Fabaceae) fruit, leaf and bark 96% ethanolic extract on their antioxidant, anti-inflammatory and antimicrobial activities.

MATERIALS AND METHODS

Preparation of plants extracts

The fruit, leaves and bark of *A. digitata* and *T. indica* were collected from Abu karshola,West Kordofan State, Sudan, in December, 2017. The specimens were deposited in the herbarium of medicinal and aromatic plants institute, Khartoum, Sudan. The fresh samples were cleaned, air dried and ground to powder using a pestle and mortar. Fifty grams of each powdered sample was extracted with 96% ethanol at room temperature for 72 h, filtered through Whatman number 4 filter paper and concentrated in a rotatory evaporator under reduced pressure.

Phytochemical analysis

Qualitative preliminary phytochemical analysis was performed with different chemical reagents to detect the nature of phytoconstituents and their presence in the samples. The presence of sterols/terpenes, flavonoids, tannins, alkaloids, lignins, saponins and coumarins was evaluated by standard qualitative methods (Hameed, 2012).

Antioxidant activity

The DPPH radical scavenging was determined with some modification (Shimada et al., 1992). In 96-wells plate, the test samples were allowed to react with 2,2-Di (4-tert-octylphenyl)-1-picryl-hydrazyl stable free radical (DPPH) for half an hour. The concentration of DPPH was kept as 300 µl. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using Shimadzu UV spectrophotometer double beam. Ascorbic acid was used as standard. The ability to scavenge of the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging activity } (\%) = \frac{(\text{A0} - \text{A1})}{\text{A0}} \times 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the sample.

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Table 1. Yield percentage of different parts extracts of *A.digitata* and *T.indica.*

Sample	Yield (%)	
	A. digitata	***T.indica***
Fruit	4.64	22.84
Leave	14.7	14.5
Bark	1.9	5.08

Anti-inflammatory activity

Inhibition of albumin denaturation

Inhibition of protein denaturation was evaluated by the method of Mizushima and Kobayashi with slight modification: 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract with different concentrations. This mixture was kept at room temperature for 10 min, followed by heating at 51°C for 20 min. The absorbance was recorded at 660 nm. (Chandra et al., 2012). Percent inhibition for protein denaturation was calculated using:

$$\% \text{ Inhibition} = \frac{(\text{A0} - \text{A1})}{\text{A0}} \times 100$$

Where A0 is the absorbance of the control and A1 is the absorbance of the sample.

Antimicrobial activity

Test microorganisms

Bacteria organisms used were *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi*. The fungal organisms used were *Candida albicans* and *Aspergillus niger*. Standard strains of microorganism used in this study were obtained from medicinal and aromatic plants research institute.

Antibacterial assay

The disc-diffusion assay with some modifications was employed to investigate the inhibition of bacterial growth by plants extract (Kil et al., 2009). Extract solution (20 mg/ml) was prepared by diluting with dimethyl sulfoxide (DMSO) 30%. Base plates were prepared by pouring 10 ml Mueller-Hinton (MH) agar into sterile Petri dishes. About 0.1 ml of the standardized bacterial stock suspension 10^8 to 10^9 C.F.U/ ml were streaked on Mueller Hinton agar medium plates using sterile cotton swab. Sterilized filter paper disc (6 mm diameter) were soaked in the prepared extracts, and then were placed on the surface of the test bacteria plates. The plates were incubated for 24 h and the diameters of the inhibition zones were measured.

Antifungal assay

The same method described for bacteria was employed to assess antifungal activity, Sabouraud Dextrose Agar was used. The inoculated medium was incubated at 25°C for two days for the *C. albicans* and three days for *A. niger*.

RESULTS AND DISCUSSION

Extraction of the plants

Among the extracts obtained using 96% ethanol, *T. indica* fruits gave the highest yield of 22.84% as shown in Table 1, leaf extracts of both plants gave the same yield of 14%, while the bark extract of *A. digitata* gave the lowest yield of 1.9%.

Phytochemical screening of *T. indica* and *A. digitata*

The extracts of *T. indica* contained some secondary metabolites; most of them present in leaves and bark including alkaloids, flavonoids, glycosides, terpenoids, coumarins, tannins, saponins, reducing sugars and carbohydrates. Antibacterial activity of Tamarind leaf extract was attributed to the presence of flavonoids, alkaloids, tannins, cyanogenic glycosides and anthraquinones. It is known that these phytochemicals and some other aromatic secondary metabolites may serve as natural agents that protect plants against microbial pathogens and insect predators. Phytochemicals may act like antioxidants to facilitate, protect and regenerate essential nutrients and/or work to deactivate cancer-causing substances (Gomathi et al., 2017). The results of the phytochemical screening showed that *A. digitata* fruit, leaf and bark extracts were rich in alkaloids, flavonoides, sterols, triterpines, tannins, saponins, coumarins, glycosides, reducing sugar and carbohydrates in all parts, but anthraquinones were not detected (Table 2). Many of these are known to provide protection against insects' attacks and plant diseases. The results obtained seems to justify the use of leaf of *A. digitata* in African dishes as it contained appreciable amount of some important compounds such as phenols, saponins, flavonoids, alkaloids. It is also possible that these plant species could have allelopathy effect on other organisms in their ecotype since these bioactive substances are responsible for such actions. Cardiac glycoside detected in this plant indicated that the plant could be a good source for birds and insect repellants (Lock et al., 2016).

Table 2. Phytochemical screening of *T. indica* and *A. digitata* parts extracts.

Test	Specific test	Fruit		Leave		Bark	
		T. indica	***A. digitata***	***T. indica***	***A. digitata***	***T. indica***	***A. digitata***
Alkaloids	Wagner's	-ve	-ve	-ve	+ve	+ve	-ve
	Mayer's	-ve	-ve	+ve	-ve	-ve	-ve
	Dragendroff's	-ve	-ve	+ve	-ve	+ve	-ve
Flavonoids	$FeCl_3$	-ve	+ve	-ve	-ve	+ve	+ve
	Lead acetate	+ve	-ve	-ve	+ve	+ve	+ve
Sterols	Salkowski	+ve	+ve	+ve	+-ve	+ve	+ve
	Lebermann	-ve	+ve	+ve	+ve	-ve	+ve
Triterpines	Salkowski	-ve	-ve	-ve	-ve	-ve	-ve
	Leberman	-ve	+ve	-ve	-ve	+ve	+ve
Tannins	Gelatin	+ve	-ve	+ve	+ve	+ve	+ve
	lead acetate	+ve	-ve	-ve	-ve	-ve	-ve
Saponins	Foam test	-ve	+ve	+ve	-ve	-ve	+ve
Coumarin	UV lamp	-ve	+ve	-ve	-ve	+ve	-ve
Glycosides	Keller kiliani	+ve	+ve	+ve	+ve	+ve	+ve
	Kedd's	-ve	-ve	-ve	-ve	+ve	+ve
Reducing sugar	Fehlings	+ve	+ve	-ve	-ve	+ve	-ve
Lignin	Labat test	-ve	-ve	+ve	-ve	-ve	-ve
Carbohydrate	Molich	+ve	+ve	+ve	+ve	+ve	+ve

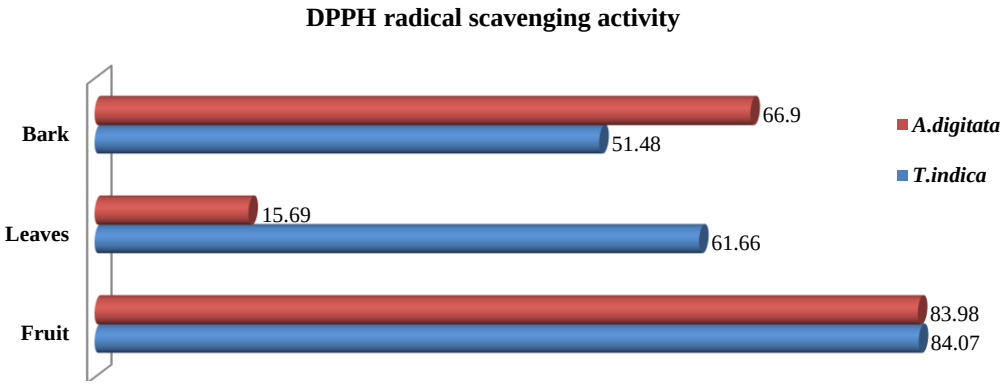

Figure 1. Antioxidant activity of *T. indica* and *A. digitata.*

Antioxidant activity

DPPH radical scavenging activity

The *in vitro* antioxidant activity of fruit, leaf and bark ethanolic extracts of *T. indica* and *A. digitata* was evaluated using DPPH assay. Results are shown in Figure 1. The highest result of antioxidant activity by DPPH scavenging assay in fruit extract in Tamarind (84.07%) followed by Baobab (83.98%). The result is very high compared with ascorbic acid (93.5%) as antioxidant standard. The leaves of Tamarind showed

Table 3. Inhibition of albumin denaturation**.**

Sample	*T. indica* (% inhibition)	*A. digitata* (% inhibition)	Mixture (% inhibition)
Fruit	55.85	50	73.42
Leave	76.714	62	82.71
Bark	57.642	54.92	59.14

Table 4. Antimicrobial activity of plant extracts against bacteria pathogens.

Part of plant	Sample	*Bacteria strain* (M.D.I.Z)*			
		B. subtilis	*S. aureus*	*E. coli*	*S. typhi*
Fruit	*A. digitata*	12 ±0.00	14±1.41	11±1.41	13±0.70
	T. indica	12± 1.41	11±0.70	10±0.70	11±1.41
	Mixure	19± 0.00	35±0.00	17±2.82	21±0.70
Leave	*A. digitata*	11±2.12	11±1.41	10±0.70	11±2.21
	T. indica	12± 0.70	10±0.00	9±0.70	12±0.70
	Mixure	18± 0.00	23±0.00	15±0.00	20±0.70
Bark	*A. digitata*	11±0.00	9±0.70	10±0.70	10±0.00
	T. indica	12± 0.70	9±1.41	10±0.00	9±1.41
	Mixure	16±0.00	12±0.70	19±0.70	12±0.70

*M. D. I . Z ., Mean diameter of growth inhibition zone in mm.

higher activity (61.66%) than Baobab (15.69 %), whereas the bark showed 51.48 and 66.90% in Tamarind and Baobab respectively. Several reports indicated that the antioxidant potential of medicinal plants may be related to the concentration of their phenolic compounds which include phenolic acids, flavonoids, anthocyanins and tannins (Djeridane et al., 2006; Wong et al., 2006). The health benefit of fruits are mainly attributed to phenolic compounds and vitamins, which enhance their antioxidant, anticancer, anti-mutagenic, antimicrobial, anti-inflammatory and neuroprotective properties. Bio-guided fractionation of extracts might promote the development of alternative therapeutic compounds for the prevention and treatment of various diseases and disorders.

Anti-inflammatory activity

Inflammation is defined as the local response of living mammalian tissues to injury due to any agent. The results showed both ethanolic extracts leaves parts represent the highest anti-inflammatory activity even in mixture form, followed by fruits, then bark, while in individual manner bark extracts represent higher activity than fruits as shown in Table 3. The results showed a concentration-dependent inhibition of protein (albumin) denaturation by *A. digitata* and *T. indica* extracts. *T. indica* extracts expressed a higher activity compared to *A. digitata*. Previous study by Osman and Idrees (2017) reported that both *A. digitata* and *T. indica* extracts exerted marked anti-inflammatory properties.

Antimicrobial activity

Antibacterial activity

Diseases such as bacterial, fungal and infectious parasitic are mainly controlled by chemotherapeutics and antibiotics in aquaculture. Nevertheless, the uses of antibiotics and chemotherapy have been strongly criticized as they created problems with drug resistance bacteria (Harikrishnan et al., 2011). All tested extracts showed various degrees of biological activity on the tested pathogens. All plants parts combinations showed remarkable activity against tested bacteria and fruit mixtures showed the highest activity against *B. subtilis* (19 mm), *S. aureus* (35 mm) and *S. typhi* (21 mm), while the mixture of bark extracts showed high activity against *E. coli* (19 mm). The results are presented in Table 4. Plant extracts are an important part in agroecology, as they benefit the environment in combating pathogenic organisms, without resorting to synthetic chemicals. (Rivera et al., 2014). Previous study showed that the significant differences observed in antibacterial activities

Table 5. Antifungal activity of parts extracts against *C.albicans* and *A.niger.*

Part of plant	Sample	*Fungi* (M.D.I.Z)*	
		C. albicans	***A. niger***
Fruit	*A. digitata*	15±1.41	13±0.70
	T. indica	18±0.70	16±0.70
	Mixure (1:1)	32±0.70	28±0.70
Leaves	*A. digitata*	16±0.70	21±0.70
	T. indica	13±1.41	17±0.00
	Mixure (1:1)	28±1.41	36±0.00
Bark	*A. digitata*	20±0.00	14±0.70
	T. indica	15±1.41	16±1.41
	Mixure (1:1)	30±0.00	33±1.41

*M. D. I . Z ., Mean diameter of growth inhibition zone in mm.

suggest that extract mixtures affect in a different way each of the tested food-borne pathogen bacteria; while differences among extract mixtures suggest that at least one extract mixture affect in a different way the bacterial growth. *A. digitata* leaves contain active ingredients against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *S. typhi*, *C. albicans, A.niger*, and *P. rotatumat* (Abiona et al., 2015).

Antifungal activity

All extracts and blends of the two plants were tested for their antifungal activity and the results are shown in Table 5. The inhibitory percentage of extracts and their combinations against *C. albicans* ranged from 13-32 mm and 13-36 mm against *A. niger*. The blends of all plant extracts of the same part showed higher activity than original extracts of the same plant.

Remarkable synergistic effect of plant extracts was observed because the plants have the same pharmacological activity, and the active compounds present in each extract act together to targeted receptors, therefore producing higher effect than using any plant extract alone. From other point of view, some of the plants compounds may not have pharmacological activity but act as a potentiated agents to improve the activity of the compounds found in the other plant extract with pharmacological activity (Atanasov et al., 2015).

Conclusion

In this study different part of *A. digitata* and *T. indica* were used. The plants are rich sources of chemical and bioactive compounds including therapeutic and dietary constituents. The present study suggests that blending of the plants parts of *A. digitata* and *T. indica* could be a potential source of natural antioxidant and anti-inflammatory properties that could have great importance in the inhibition of inflammation as well as against bacterial and fungal infections. The findings of this study suggest that the tested plants and their combinations can be developed as effective herbal phyto-pharmaceutical drugs for treatment and nutrition. Based on the research outcomes we recommend conducting more experiments on the combination such as anticancer activity, bacterial and fungal infections and virus infections.

CONFLICT OF INTERESTS

The authors have not declared any conflict of Interests.

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Full Length Research Paper

Immunomodulatory activities of polysaccharides isolated from plants used as antimalarial in Mali

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Medicinal plants used against malaria in Mali have previously been tested for their antiplasmodial activities using their organic solvent and water extracts. As the healers mainly use the water extracts for their treatments of malaria-patients, our aim was to study the water-soluble components from Malian plants used for treatment of malaria. *Argemone mexicana* (aerial parts), *Sarcocephalus latifolius* (root bark), *Vitex doniana* (leaves), and Malarial-5® (an improved traditional medicine (ITM) in tea) were the objects of our studies. Water extracts of these plants contained primarily polysaccharides. Due to this, the studies focused on the determination of the monosaccharide composition of the polymers present as well as assessing the immunomodulatory properties of the polysaccharide fractions isolated from these plants. Each plant material was extracted sequentially with dichloromethane, 80% ethanol and water at 100°C. The polysaccharides were obtained using gel filtration of the aqueous extracts and their monosaccharide compositions were determined using gas chromatography. Immunomodulatory effects were assessed using the complement fixation test and macrophage stimulation. All aqueous extracts from the four samples contained polysaccharides. The monosaccharide compositions vary between the plants. Arabinose, rhamnose, galactose, glucose and galacturonic acid were present in all samples, glucose being the main monomer. These polysaccharides showed complement fixing activity and induced nitrite oxide release from macrophages in a dose dependent manner. The polysaccharide fractions of *A. mexicana* (Am1) and *V. doniana* (Vd1) showed the most potent activities. These two fractions had an ICH_{50} of 2.4 and 6.3 µg/mL respectively in the complement fixation assay. The same two fractions induced a dose dependent release of nitrite oxide from macrophages. The results demonstrated that antimalarial plants contain polysaccharides with immunomodulatory properties. This preliminary work constitutes a new approach of antimalarial studies.

Key words: Polysaccharides, immunomodulatory effects, antimalarial plants, Mali.

INTRODUCTION

In 2017, World Health Organization (WHO) estimated 219 million cases of malaria in 90 countries with 435,000 deaths worldwide. The African region registered 92% of the malaria cases and 93% of the deaths (WHO, 2017).

This infectious disease caused by parasites is transmitted to people through the bites of infected female anopheles mosquitoes (WHO, 2017). *Plasmodium falciparum* is mainly responsible for the enormous deaths (99%), rarely caused by other *Plasmodium* species (Belachew, 2018). Malaria is the primary cause of morbidity and mortality in Mali, particularly among children under the age of five (Anonymous, 2018). The entire population of Mali is at risk for malaria. The disease is endemic in the central and southern regions where more than 90 percent of the population lives, and it is also epidemic in the north (Anonymous, 2018).

In Mali, the current health system is decentralized, and is composed of three levels, which involves an integrated community case management package at the community level (Anonymous, 2018). Most of the population is still using traditional medicines for their primary health care. Plants have been used to treat malaria for thousands of years and are the source of the two main groups of modern antimalarial drugs (quinine and artemisinin derivatives) according to Willcox and Bodeker, (2004). RTS,S/AS01 (RTS,S) also known as Mosquirix® is an injectable vaccine that offers protection against malaria in young children, but it is unavailable for the population where malaria is endemic (Anonymous, 2018). The problems of increasing levels of artemisinin-resistant parasites encourage researchers for finding a new source of anti-parasitic drugs. Since developing countries have difficulties in affording and accessing effective antimalarial drugs, traditional medicines could be an important and sustainable source of treatment (Willcox and Bodeker, 2004). Therefore the exigent need of effective molecules remains a huge challenge for scientists. Most of the recent investigations on antimalarial plants have been focused on organic solvent extracts. But in Mali, the department of traditional medicine (DMT) in order to supersede its first Malian improved traditional medicine for malaria (Malarial-5®) has demonstrated the efficacy and safety of aqueous extracts through preclinical and clinical studies including *Sumafura Tiemoko Bengalyan* herbal tea based on *Argemone mexicana* that came out from a retrospective treatment-outcome study (Diallo et al., 2007; Willcox et al., 2007; Sanogo et al., 2008; Graz et al., 2010; Willcox et al., 2011). The phytochemical analysis and biological activities on *Sumafura Tiemoko Bengaly* an herbal tea led to formulate syrups for an efficient utilisation and its standardization (Sanogo et al., 2012, 2014). This new antimalarial phytomedicine made by the department of traditional medicine of Mali retrieves its name from one traditional healer, *Tiemoko Bengaly*, who has participated in its development (Willcox, 2011). This author reported that several alkaloids including berberine, protopine, and allocryptopine from *A. mexicana* exhibited *in vitro* antimalarial effect while animal studies suggest that the crude aqueous extract is not effective against *Plasmodium berghei*, and berberine also is not well absorbed orally (Willcox, 2011). Thus, some investigations were underway to identify which compounds are active in humans. Most of traditional healers in Mali are thinking that plants mainly should be taken as water extract. Therefore a new approach is urgently needed to find a product for prevention and treatment of malaria. Although parasites have their own ways to develop resistance against drugs, the immune system has naturally evolved to arm the host against pathogens, including parasites. Both innate and adaptive immune responses selectively recognize pathogens and help the host to get rid of many of them at first sight (Coban and Yamamoto, 2018). Due to this, our theory is that the healing effect observed with patients using aqueous plant extracts could partly be due to their stimulating activity of the immune system.

In Malian traditional medicine, water decoction is the most popular mode of preparation of plants remedies and polysaccharides isolated from those crude water extracts have shown effects related to the immune system through various *in vitro* and *in vivo* tests (Paulsen and Barsett, 2005). Diallo and coworkers showed that the pectic polysaccharides isolated from the leaves of *Trichilia emetica* (Meliaceae), a plant used in traditional medicine in Mali, activated the complement system and induced the proliferation of T and B-lymphocytes (Diallo et al., 2003). *Biophytum petersianum*, traditionally used in Mali for wound healing contains polysaccharides with complement fixating activity (Inngjerdingen et al., 2006; Inngjerdingen et al., 2008; Grønhaug et al., 2011). *Opilia celtidifolia* used traditionally against skin diseases and malaria is also known as appetizer plant in Mali. Polysaccharide fractions from that species exhibited complement fixation and macrophage stimulation activities (Diallo et al., 2003; Togola et al., 2008; Šutovská et al., 2009). Investigations on the roots of *Vernonia kotschyana* used to produce Gastrosedal, an improved traditional medicine in Mali, revealed that its polysaccharides possessed a complement fixation activity (Nergard et al., 2005; Inngjerdingen et al., 2012). Recently, two other Malian medicinal plants, *Parkia biglobosa* and *Terminalia macroptera* were reported to contain polysaccharides having complement fixing and macrophage stimulating effects (Zou et al., 2014a, b). Often, pectic extracts prepared using hot water, were found to be active on the complement system. Aboughe-Angone et al. (2011) reported that plant water soluble

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compounds like pectic and hemicellulosic polysaccharides have immunomodulatory and mitogenic (proliferation of B-lymphocytes) properties. Plant polysaccharides can directly activate the immune function of macrophages, T or B lymphocytes, natural killer cells, and complement (Yu et al., 2017). Macrophages stimulated with lipopolysaccharides (LPS) or an immunomodulatory compound produce large amounts of free radicals such as nitric oxide (NO), which effectively suppressed the blood stage of the malarial parasite (Awasthi et al., 2003). Thus, the present study aimed to investigate the components and the immunomodulatory effects of polysaccharides from antimalarial plants used in Mali.

MATERIALS AND METHODS

Plant material

Three plants and one improved traditional medicine used frequently in Mali against malaria without prior knowledge on their immunodulatory properties were selected for the present study. The plant materials were *A. mexicana* L., Papaveraceae (aerial parts), *Sarcocephalus latifolius* (Sm.) E.A. Bruce, Rubiaceae (root barks), *Vitex doniana* Sweet, Lamiaceae (leaves), and Malarial-5® (improved traditional medicine containing *Senna occidentalis* (L.) Link. (syn. *Cassia occidentalis* L.) (leaves), *Lippia chevalieri* Moldenke (leaves) and *Acmella oleracea* (L.) R.K.Jansen. (syn. *Spilanthes oleracea* L.) (flowers) presented as herbal tea. *A. mexicana, S. latifolius* and *V. doniana,* were bought at the market of Medine in Bamako, Mali, in 2012, identified by Professor Drissa Diallo, Department of Traditional Medicine, Bamako, Mali, and voucher specimen were deposited at the herbarium of the DMT (Voucher No 2948 / DMT, 2198 / DMT, 2008 / DMT respectively). The Plant List website (www.theplantlist.org) was accessed in February 2019 for correct Latin names of the plants. The plant materials were air dried at room temperature and pulverized into fine powder by a mechanical grinder. The tea form of Malarial-5®, which is an improved traditional medicine of DMT, was provided by this institution. The powders and the herbal tea were used for the extraction.

Method of extraction

The powdered leaves, root bark, aerial parts and the formulated herbal tea (50 g of each) were extracted with dichloromethane using the Soxhlet system, followed by maceration in 80% ethanol in order to remove lipophilic compounds and colored materials. The residues were then extracted with water at 100 °C for 1 h, filtered through glass fiber filter, and concentrated at 40°C under vacuum with an evaporator. The concentrated solutions were frozen to give the crude water extracts called Vd, Sl, Am and Ma respectively for *V. doniana, S. latifolius, A. mexicana* and Malarial-5®.

Fractionation of polysaccharides by chromatography on Biogel P6

The aqueous extracts (Vd, Sl, Am and Ma) were dissolved in distilled water, centrifuged, filtered through a Millipore filter (5 µm) and gel filtered on a Biogel P6 column (5 cm × 60 cm) using distilled water as the mobile phase. The fractions, from high to low molecular weights, were identified based on their elution profiles as tested by the phenol sulphuric acid method (Dubois et al., 1956). Three fractions (1, 2 and 3) of Vd, Sl and Am; and two fractions (1 and 2) of Ma contained high molecular weight material. Each fraction was pooled, concentrated and freeze-dried to give polysaccharide fractions. The samples retained for further studies were called Vd1, Vd2, Vd3, Sl1, Sl2, Sl3, Am1, Am2, Am3, Ma1 and Ma2.

Determination of monosaccharide composition of the fractions

One milligram of the lyophilized polysaccharide of each sample was subjected to methanolysis for 24 h (80°C) using water free 3 M HCl in MeOH (Sigma–Aldrich) (Chambers and Clamp, 1971). Hundred microliters of mannitol (1 mg/mL) were added as an internal standard. After 24 h reaction time, the reagents were removed with nitrogen and the methyl-glycosides dried in vacuum over P_2O_5 for 1 h prior to their conversion into the corresponding trimethyl silylethers (TMS-derivates). The samples were analyzed by capillary gas chromatography (30 m × 0.32 mm, J and W Scientific Inc.) on a Carlo Erba 6000 Vega Series 2 gas chromatograph with an ICU 600 programmer (Chambers and Clamp, 1971; Barsett et al., 1992). The injector temperature was 250°C, the detector temperature 300°C and the column temperature was 140°C when injected, then increased with 1°C/mn to 170°C, followed by 6°C/mn to 248°C and then 30°C/mn to 300°C. Helium was used as carrier gas with a flow rate adjusted to a retention time of 33 min for the internal standard. Based on standards for all the monomers present, the monosaccharides were identified and quantified.

Immunomodulatory activities

The complement fixation test

The complement fixation test is based on inhibition of haemolysis of antibody sensitized sheep red blood cells (SRBC) by complement from human sera as described by Michaelsen et al. (2000). Sheep erythrocytes were washed twice with 9 mg/mL NaCl and once with veronal buffer (VB) pH 7.2 containing 2 mg/mL bovine serum albumin (BSA) and 0.02 % sodium azid (VB/BSA) and sensitized with rabbit anti-sheep erythrocyte antibodies (Viron amboceptor 9020, Ruschlikon, Switzerland). After incubation at 37°C for 30 min, the cells were washed as described above, and a 1% cell suspension in veronal buffer was prepared. The serum was diluted with VB/BSA to a concentration giving about 50% haemolysis. Samples were dissolved in VB/BSA (500 µg/mL) and a 4-fold dilution made. Sample dilutions (50 µL) and serum dilution (50 µL) were added in duplicates into the wells of a round bottom microtiter plate and incubated on a shaker at 37°C. After 30 min, the sensitized sheep erythrocytes (50 µL) were added and the microtiter plate incubated again as earlier described. After centrifugation at 1000 x *g* for 5 min, 100 µL of the supernatants were transferred to a flat bottom microtiter plate and the absorbance was read at 405 nm using a microplate reader. Hundred percent of lysis were obtained with distilled water and sensitized sheep erythrocytes. VB/BSA, serum and sensitized sheep erythrocytes were the control of the medium, and the pectin fraction, BPII from the leaves of *B. petersianum* was used as positive control. The prevention or inhibition of lysis induced by the test sample was calculated by the following formula:

$$[(A_{control}-A_{test})/A_{control}] \times 100\%$$

$A_{control}$ is the absorbance of control and A_{test} is the absorbance of test sample.

From these data, a dose-response curve was constructed and the concentration of test sample giving 50% inhibition of haemolysis

(ICH_{50}) was calculated. A low ICH_{50} value means a high complement fixing activity. This biological test system can have some day to day variations, and thus, the ration ICH_{50}-BPII / ICH_{50}-sample was calculated. A high ratio means high complement fixing activity.

Analysis of nitric oxide (NO) production

Nitric oxide (NO) released by activated macrophages is broken down to nitrite (NO_2^-) in the medium, which can be measured in a colorimetric assay using the Griess reagents. The mouse macrophage cell line Raw 264.7 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics, L-glutamine, and $5x10^{-5}$M 2-mercaptoethanol, and split every second day. Macrophages at a density of $5x10^5$ cells/mL were seeded into 96-well flat-bottomed plates, and stimulated for 22 h in duplicates with increasing concentrations (1,10,100 µg/mL) of samples (Vd1, Vd2, Sl1, Am1, Am2 and Ma1 selected from their effect in the complement fixing test), LPS (from *P. aeruginosa* 10, Sigma–Aldrich) and the pectic polysaccharide Oc50A1.I.A, from *O.celtidifolia* (Grønhaug et al., 2010) as positive controls, or medium alone. Nitrite was then determined in cell-free supernatants. The supernatant (50 µL) was mixed with an equal volume of Griess reagent A (1% [w/v] sulfanilamide in 5% [v/v] phosphoric acid) and incubated at room temperature in the dark for 10 min. After addition of 50 µL 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in water (Griess reagent B) the absorbance was read at 540 nm. A dilution series of $NaNO_2$ was used as a standard reference curve. The experiment was repeated two times, and the results shown are expressed as the mean ± SEM.

RESULTS AND DISCUSSION

Polysaccharide fractions

Eleven polysaccharide fractions Vd1, Vd2, Vd3, Sl1, Sl2, Sl3, Am1, Am2, Am3, Ma1 and Ma2 (Vd, Sl, Am and Ma indicate the fractions respectively for *V. doniana, S. latifolius, A. mexicana* and Malarial-5®) were extracted as presented in Table 1. The fraction Vd3 presented the highest yield while Ma1 showed the lowest amount. Based on literature, this is the first time to extract polysaccharides from these plant species. These fractions with undetermined different molecular weights were the objects for further studies. These fractions could contain both neutral and acidic polysaccharides as they were not separated. This was not done as it was important to have all the water soluble materials present in the fractions that were tested for bioactivities, as those were as close as possible to those extracts prepared by the traditional healers.

Carbohydrate composition of the fractions

The monosaccharide compositions of the eleven fractions were determined and the results are presented in Table 2. The monosaccharide composition is typical for pectins, additionally, glucose was identified often present in plant material derived from water-soluble, neutral polysaccharides like starch. Glucose was present in high amounts in all fractions. The other monosaccharides, galacturonic acid (Gal A), rhamnose (Rha), arabinose (Ara) and galactose (Gal), are all recognized as typical constituents in pectic polysaccharides (Inngjerdingen et al., 2012). The monosaccharides were mainly Gal A (39.9%), Ara (14.7%) and Gal (13.5%) for Vd1, while Vd2 had Gal (15.1%) as the major. The polysaccharide fraction Sl1 had Gal (14.8%) and Ara (14.4%) as the major pectic monomers, Am1 had Gal (39.8%) and Ara (22.3%), while Am2 was rich in GalA (12.0%) and Rha (10.3%). The major pectic monosaccharides of the polysaccharide fraction Ma1 were Gal A (23.8%), Ara (23.7%) and Gal (22.5%). The presence of these monosaccharides proved polysaccharide existence in the investigated antimalarial plants and also that they could be of the pectic type polysaccharides (Inngjerdingen et al., 2012). The high content of glucose after methanolysis could be related to starch (Inngjerdingen et al., 2012). In addition the presence of fair amount of xylose in some samples could explain that some of the glucose also could be due to xyloglucan. All fractions contained arabinose and galactose, indicating the presence of arabinogalactans, polymers which are commonly present in pectin as side chains on the main core. In addition, the presence of galacturonic acid and rhamnose could indicate that the polymers may contain a main core consisting of a rhamnogalacturonan (indicative of RG I) linked with longer chains of homogalaturonan as noted by Braünlich et al. (2018).

Immunomodulatory activities

Complement fixation activity

The ICH_{50} values of the polysaccharide fractions and the ratio ICH50 BPII/ICH50 sample are given in Table 3 and Figure 1 respectively. The polysaccharide fraction (Am1) was the fraction with the highest activity, more potent than all the other polysaccharide fractions and approximately 8.3 times stronger than the standard polysaccharide (BPII a pure pectic AGII type polysaccharide isolated from *B. petersianum*). It is interesting to also note that the polysaccharide fraction (Vd1) had a relative high activity, approximately 3 times stronger than the standard polysaccharide (BPII). The fraction Ma1, isolated from the product Malarial-5®, showed an effect in the complement assay twice times more than the one of the standard BPII, and Sl1 had similar activity to the standard. Earlier investigations have shown that polysaccharides from Malian medicinal plants activated the complement system (Diallo et al., 2003; Inngjerdingen et al., 2006 and 2012; Togola et al., 2008; Austarheim et al., 2012; Zou et al., 2014a,b). It has also been shown that the ethyl acetate extract of *Biophytum umbraculum* (syn. *B. petersianum*) showed in vitro antiplasmodial effect and also an effect in the complement

Table 1. Yields of polysaccharides from the extractions.

Plant material	Polysaccharide	Yield (%)
Vitex doniana(leaves)	Vd1	0.3
	Vd2	0.5
	Vd3	4.2
Sarcocephalus latifolius (root bark)	Sl1	0.4
	Sl2	3.9
	Sl3	0.4
Argemone mexicana (aerial parts)	Am1	0.8
	Am2	0.3
	Am3	0.3
Malarial-5® (herbal tea)	Ma1	0.1
	Ma2	0.4

Vd : *Vitex doniana*, Sl : *Sarcocephalus latifolius*, Am : *Argemone mexicana*, Ma : Malarial-5®.

Table 2. Monosaccharide compositions (mol%) of polysaccharide fractions obtained from aqueous extracts of three antimalarial plants and the improved traditional medicine Malarial-5®.

Monosaccharide composition	Fraction										
	***V. doniana* (Vd)**			***S. latifolius* (S)**			***A. mexicana* (Am)**			**Malarial-5® (Ma)**	
	Vd1	**Vd2**	**Vd3**	**Sl1**	**Sl2**	**Sl3**	**Am1**	**Am2**	**Am3**	**Ma1**	**Ma2**
Ara	14.7	6.6	1.1	14.4	3.1	0.8	22.3	7.2	2.5	23.7	Traces
Rha	5.7	6.5	4.0	4.5	2.2	4.1	12.9	10.3	9.1	7.5	28.9
Xyl	1.8	7.2	2.7	2.4	3.1	1.8	2.8	9.4	17.8	2.0	3.4
Man	4.8	7.4	Traces	1.5	Traces	0.2	5.6	6.5	5.4	4.4	Traces
Gal	13.5	15.1	10.0	14.8	8.3	1.4	39.8	8.5	3.0	22.5	7.3
Glc	19.6	50.4	69.1	52.9	69.6	79.3	10.1	44.3	62.2	14.1	60.4
GlcA	-	-	-	0.9	-	1.8	-	1.8	-	2.0	-
GalA	39.9	6.8	13.1	8.6	13.7	10.6	6.5	12.0	Traces	23.8	Traces

Vd: *Vitex doniana*, Sl: *Sarcocephalus latifolius*, Am: *Argemone mexicana*, Ma: Malarial-5®.

Table 3. ICH_{50} values of the test samples.

Sample	BPII	Vd1	Vd2	Vd3	Sl1	Sl2	Sl3	Am1	Am2	Am3	Ma1	Ma2
ICH_{50} (µg/mL)	19.9	6.3	38.9	150.6	22.4	186.3	54.9	2.4	27.1	63.3	10.5	77.5

assay (Austarheim et al., 2016).

Glucans are often recognized by their immuno-stimulatory activity and complement receptor type 3 (CR3, also CD11b/CD18) is a prime candidate as β-D-glucan receptor on human monocytes, neutrophils and NK cells (also dectin-1) according to Vannucci et al. (2013). The immunostimulatory activity of various polysaccharides include glucans, pectic polysaccharides, mannans, arabinogalactans, fucoidans, galactans, hyaluronans, fructans, and xylans as reported by Ferreira et al. (2015). The complement system is a potent player in innate immunity and a major effector arm of humoral immunity. Complement activation is linked to cellular responses by the recognition of cleaved complement protein fragments by receptors on leukocytes and vascular cells. The three primary roles of complement in host defense against infection are to (1) activate an inflammatory response; (2) opsonize microbial pathogens for immune adherence; and (3) damage membranes, including lysis of susceptible organisms (Atkinson et al., 2019). Complement fixating activity has previously shown to be a good indicator for effect in the immune system by plant polysaccharides (Inngjerdingen et al., 2012). This test does not distinguish between activation and inhibition of the complement system, so we do not know if the samples have inhibited or activated the complement

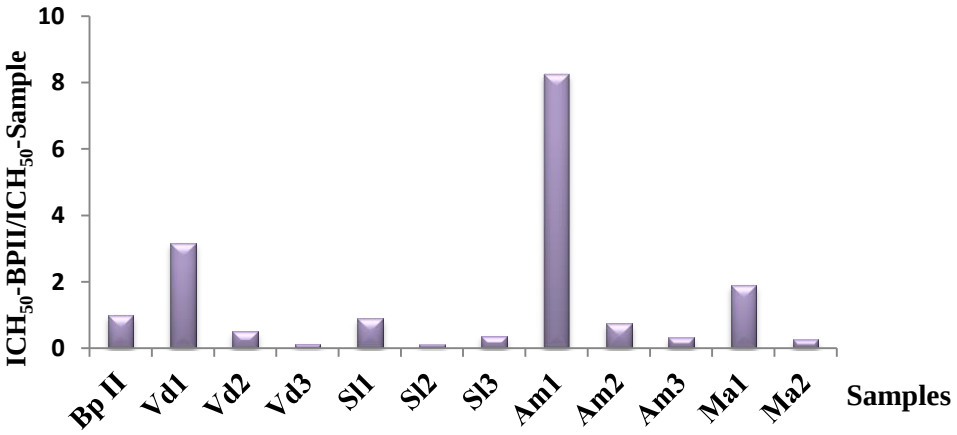

Figure 1. The complement fixation activity of the polysaccharide fractions, assayed as ICH_{50} values of a polysaccharide standard BPII from *Biophytum petersianum* relative to ICH_{50} of the polysaccharide tested (ICH_{50}-BPII / ICH_{50}-sample).

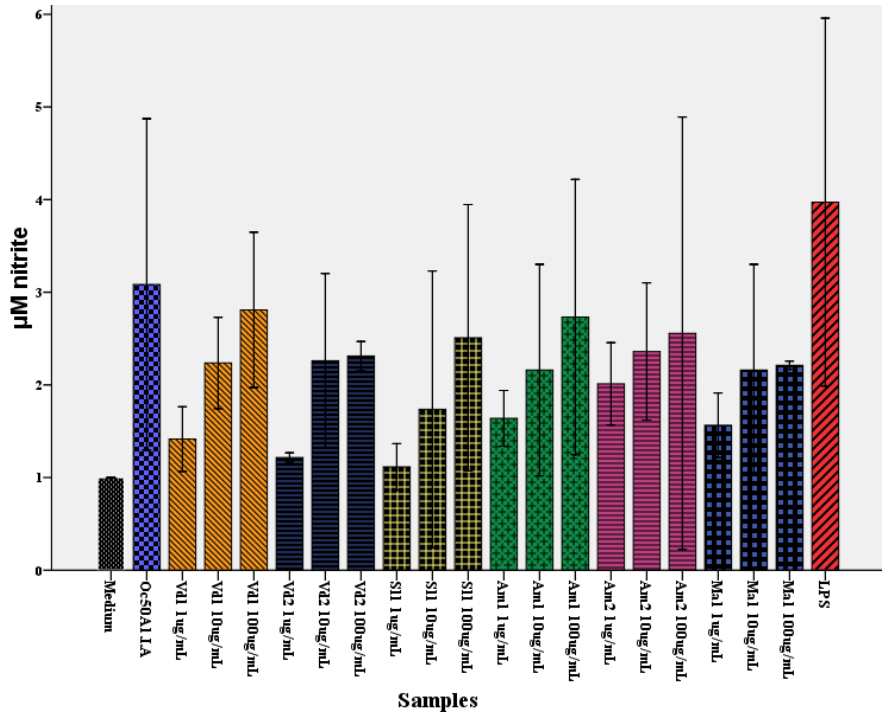

Figure 2. Stimulation of macrophages. A representative result is given as mean ± SEM. LPS and Oc50A1.I.A from *O. celtidifolia* are present as positive controls.

system. Medicinal plants traditionally used against inflammatory diseases and wounds containing complement inhibitors have been reported (Cazander et al., 2012).

Measurement of nitrite oxide (NO) released from stimulated macrophages

The ability of the polysaccharide fractions to stimulate mouse macrophages to produce NO is shown in Figure 2. NO is a good marker for macrophage activation, and its stable breakdown product nitrite can easily be detected in culture supernatants. All fractions induced a dose dependent release of NO, as measured by the quantification of its breakdown product nitrite. Among the tested samples *A. mexicana* (Am1) and *V. doniana* (Vd1) showed the highest activities by inducing the release of 2.7 and 2.8 µM of nitrite from macrophages respectively at a dose of 100 µg/mL, while the positives controls Oc50A1.I.A and LPS gave a release of 3.1 and 4.0 µM of

nitric oxide respectively. One of the important anti-parasitic chemicals generated by macrophages is nitric oxide (NO) during innate immune responses (Awasthi et al., 2003).

Plant-derived polysaccharides are potent immuno-modulatory substances, and have been shown to be clinically therapeutics eg, lentinan. Previous authors reported that a variety of beneficial pharmacological effects of plant polysaccharides were attributed to their ability to modulate macrophage immune function (Yu et al., 2017). Some earlier studies supported the proposition that the production of nitric oxide by macrophages plays a crucial role in the control of parasitaemia at the initial periods of blood stage malarial infection (Awasthi et al., 2003). However, the ethyl acetate extract of *B. umbraculum* which revealed *in vitro* antiplasmodial effect, but gave an inhibition of macrophage activation (Austarheim et al., 2016).

Conclusion

All polysaccharide fractions from *A. mexicana, S. latifolius, V. doniana* and Malarial-5® contain pectic type polymers as well as glucans. These polysaccharides displayed immunomodulatory properties primarily as determined by the complement assay. The characterization of the polysaccharides acting on the immune system explains more the effectiveness of aqueous extracts and gives an additional justification for the traditional form. These results could be used as a new approach in the management of malaria. Therefore further investigations will be undertaken on *A. mexicana* that showed the highest immunomodulatory activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Phytochemistry, acute toxicity and blood profile of albino rats treated with fruit extract of *Solanum macrocarpon*

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Phytochemical screening of the methanolic fruit extract of *Solanum macrocarpon* was performed using standard method. Eighteen male albino mice, assigned into six groups (n=3) were used to determine the acute toxicity (LD_{50}) of the extract. Haematological effect of the extract was determined using forty eight adult male rats assigned into four groups (A-D; n=12). The treatment groups received daily oral administration of the extract at doses of 400, 800 and 1600 mg/kg of body weight (bw) respectively for 21 days. The phytochemical screening of the extract revealed the presence of flavonoids, saponins, alkaloids, phenols, phytates, tannins, cyanides and terpenoids. The extract showed no mortality even at the dose of 5000 mg/kg bw. The highest treatment dose (1600 mg/kg) showed significant reduction in the white blood cell (WBC) count compared to rest of the treatment groups. There were no significant difference ($p<0.05$) in red blood cell (RBC), packed cell volume (PCV) and haemoglobin (Hb) levels of the treatment groups compared to control. Similarly, the mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) results showed no significant difference from days 0 to 21 in all the treatment groups. Findings from this study suggest that except for the fact that the high dose of the extract antagonizes immunity; it has no serious adverse effect on the various haematological parameters, especially as it improves haemoglobin levels on prolonged administration.

Key words: Acute toxicity, blood profile, phytochemistry, *Solanum macrocarpon.*

INTRODUCTION

The usage of medicinal plants in West Africa is probably as old as the duration of human settlement in the region (Abdulrahman et al., 2010). As estimated by the World Health Organization, 80% of the global population in developing countries depends on traditional medicines mainly of plant origin with the steadily growing global market for traditional therapies standing at US$ 60 billion a year(World Health Organization, 2002).

According to Kariuki and Njoroge (2011),

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approximately 25% of all pharmaceutical products worldwide originated from traditional medicinal knowledge. In Nigeria, several thousands of plant species have been claimed to possess medicinal properties and have been employed in the treatment of many ailments (Okigbo and Mmeka, 2006).

Solanum macrocarpon, a tropical perennial plant of African ancestry belonging to the family Solanaceae. The genus *Solanum* is well known in traditional medicine (Burkhill, 2000; Grubben and Denton, 2004). The cultivated form of *S. macrocarpon*, known as 'gboma' in West Africa constitutes an important fruit and leaf vegetable. Local cultivars grown for the leaves are common throughout west and central Africa while the fruit types are mainly restricted to the humid coastal areas of West Africa (Bukenya-Ziraba and Bonsu, 2004). The leaves and fruits are cooked and consumed as a vegetable in soups and sauces and the taste is more or less bitter (Gbile and Adesina, 1988). The plant also serves as foliage for feeding livestock and is occasionally grown as an ornamental (Bukenya-Ziraba and Bonsu, 2004; Schippers 2001). Hematological parameters are important indices of physiological and pathological status for both animals and humans, thus playing a major role in disease investigation and diagnosis (Malomo, 2000; Adeneye et al., 2006). It has been documented that ingestion of some plant materials (either in the raw form or their extracts) having useful medicinal properties may cause anemia resulting from the sequestration of red blood cell (RBC) in spleen, impaired RBC production or primary bone marrow dysfunction (Cheeke, 1998; Mishra and Tandon, 2012). Therefore, this study was designed to investigate phytochemical constituents, acute toxicity and possible hematological effect of the methanolic *S. macrocarpon* fruit extract in order to ascertain its safety as a medicinal agent.

MATERIALS AND METHODS

Procurement of plant sample

The plant was cultivated at the agricultural farm of the University of Nigeria, Nsukka, and at maturity, unripe fruits were harvested and extracted in methanol for the study. The plant was identified at the herbarium of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Preparation of *S. macrocarpon* fruit extract

The method of extraction was adapted from Uhegbu and Ogbuehi (2004) and Anaga et al. (2010). A total of 250 g of the powdered plant material was soaked in 500 ml of 99% methanol, stirred thoroughly and left to stand for 48 h. Afterwards, the mixture was filtered and evaporated to dryness at room temperature giving a 7.8% yield.

Phytochemical screening of extract (Qualitative and quantitative)

The flavonoids and cyanides contents of the extract were determined using the method described by Onwuka (2005) while that of alkaloids followed the gravimetric method of Harbone (1973). Phenols and saponins were determined using the method described by Obadoni and Ochuko (2001). The phytates was determined using the method described by Oberleas (1973). The method used for tannins determination followed that described by Pearson (1976).

Determination of terpenoids was done as described by Ezeonu and Ejikeme (2016). Briefly, two grams of the sample was weighed into a conical flask and 40 ml of chloroform solution was added and allowed to stand for 4 h. Thereafter the solution was filtered into a weighed crucible and oven dried. Then the weight of crucible with the extract was taken. Then 40 ml of absolute methanol was added and allowed for 2 h and then filtered into a weighed crucible, oven dried and re-weighed. The weight of the terpenoids was quantified in percentage as follows:

Terpenoid (%) = × 100

Determination of steroids was done as described by Mujeeb et al. (2014). Briefly, two grams of the sample was put into a test tube, and 50 ml of ethyl acetate was added and placed in a boiling water bath for 5 min. After cooling, the solution was filtered and mixed with an equal volume of chloroform which formed two layers. Two millilitres of the chloroform layer was pipetted into a test tube and 5 ml of water was added and adjusted to a pH of 7.0 using 0.1N NH_4OH. Then the solution was filtered and allowed to stand for 5 min after which the absorbance was measured at 240 nm. Where: 2550 = extinction coefficient for steroids, df = dilution factor.

Acute toxicity studies of the plant extract

This was carried out by the method of Lorke (1983). The mice were separated into six groups (A, B, C, D, E and F) of three mice each. The first three groups (A, B, C) were administered the extract in graded doses of 200, 400 and 800 mg/k per kg body weight respectively in the first phase and observed for 24 h for signs of toxicity. Thereafter, the groups D, E, F were administered the extract at doses of 1000, 3000 and 5000 mg per kg body weight respectively and observed for signs of toxicity within the next 24 h.

Procurement and management of experimental animals

A total of eighteen male albino mice weighing 20-30 g were procured for the acute toxicity studies. On the other hand, forty eight male albino rats weighing 130 - 220 g were used to determine the effect of the extract on haematology. All the animals were procured from the Zoological Garden of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. The animals were such as have no history of drug consumption and they were housed in clean rat cages under hygienic conditions in the experimental animal house. The animals had unrestricted access to feed (commercial growers mash Vital Feeds Nigeria) and clean water throughout the experiment. The animals were left for 14 days to acclimatize before the start of each experiment.

Experimental design

The study adopted an experimental approach and was approved by Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (FVM-UNN-IACUC-2019-042). The acute toxicity study of the extract was carried out by the method of Lorke (1983). The mice were separated into six groups (A, B, C, D, E and F) of three mice each. The first three groups (A, B, C) were administered the extract in

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To study the effect of the extract on the haematological profile, the procured albino rats were divided into a control group (A) and three treatment groups (B, C and D) of twelve rats each. The treatment groups (B, C and D) received daily oral administration of the methanolic *S. macrocarpon* fruit extract at doses of 400, 800 and 1600 mg/kg of body weight respectively, while the control group received 1 ml/kg body weight of distilled water for the 21 days of the experiment.

Collection of blood samples

Blood samples were collected from each group before the start of the experiment (day 0) and at weekly intervals during treatment for the biochemical analyses. The blood samples were collected through the retro orbital plexus as described by Hoff (2000) and allowed to clot for about 30 min and afterwards centrifuged at 2000 rpm for 10 min. The sera obtained were then used to determine the biochemical parameters.

Determination of white blood cell (WBC)

This was determined using the method described by Sood (2006). The blood was sucked slowly and carefully up to 0.5 mark in the red diluting pipette. Immediately, the pipette was plunged into the diluting fluid and sucked up to 11 marks. Then, the ends of the pipette were gripped between the finger and the thumb and shaken thoroughly for 3 min. The diluted blood sample was loaded on a Neubauer counting chamber and all the cells in the four corner squares of the Neubauer chamber was counted using a light microscope at a high magnification of x 100. The number of white cells counted for each sample was calculated using the formula:

$$WBC(mm^3) = \frac{cellcounted \times dilutionfactor}{volumecountedinmm^3}$$

Where: dilution factor = 1: 20, and Number of large squares counted = 4

$$WBC/mm^3 = \frac{numberofcellcounted \times 20}{0.1 \times numberoflargesquarecounted}$$

$$\text{WBC}/mm^3 = cellcounted \times 50$$

Determination of red blood cell count (RBC)

The method of RBC count was according to Sood (2006). The blood was sucked slowly and carefully up to 0.5 mark in the red cell diluting pipette. Immediately, the pipette was plunged into the diluting fluid and sucked up to 101 marks. Then, the ends of the pipette were gripped between the finger and the thumb and shake thoroughly for 3 min. The diluted blood sample was loaded on a Neubauer counting chamber and all RBC in the five groups of 16 small squares in the central area of the Neubauer chamber were counted using a light microscope at a high magnification of x 100. The number of red cells counted for each sample was calculated using the formula:

$$RBC(mm^3) = \frac{cellcounted \times dilutionfactor}{volumecountedinmm^3}$$

Where: dilution factor = 1: 200 and Volume counted = $0.02mm^3$

$$\text{RBC}\ (mm^3) = \frac{numberofcellcounted \times 200}{0.02mm^3}$$

Then,

$$\text{RBC}\ (mm^3) = cellcounted \times 10^4$$

Blood haematocrit estimation (PCV)

This was determined by the microhaematocrit method as described by Coles (1986). A heparinized capillary tube was filled to approximately three fourth (3/4) of its length with well-mixed anticoagulated blood. The coloured end of the capillary tube was sealed with plasticin. The capillary tube was then placed into a microhaematocrit centrifuge set at 10,000 revolutions per minute (rpm) for 5 min. The height of the packed cell as well as the total height in millimeter was measured using the haematocrit reader. The packed cell volume was then calculated using the formula:

$$\text{PCV}\ (\%) = \frac{Heightofredcell\ (mm)}{Total\ height\ (mm)} \times 100$$

Haemoglobin estimation

Haemoglobin concentration was determined using the method of described by Sood (2006). The graduated tube was filled to the mark 2 on the red graduations with N/10 HCL using a dropper. Blood sample was sucked into the capillary pipette to 20 cm mark; the end of the pipette was wiped and blown into the acid in the mixing tube. The pipette was then rinsed by sucking up the acid and blowing it out back into the tube 3 - 4 times and then allowed to stand for 10 min for formation of acid haematin. Similarly, the acid haematin was diluted with distilled water in drop wise manner, mixed and the colour was compared with the standard by holding the comparator towards light. This was repeated until the colour of the diluted fluid matched with that of the standards in the comparator. From the level of the fluid in the mixing tube, the Hb was read out in gram percent.

Estimation of mean cell volume (MCV)

The mean cell Volume (MCV) is the mean volume of the red cells. It was determined using the method as described by Baker et al. (2001). It was derived by calculation from packed cell volume (PCV) and RBC count as follows:

$$MCV\ (\mu^3) = \frac{PCV(\%) \times 10}{RBC\ /mm^3\ inmilions}$$

Estimation of mean cell haemoglobin (MCH)

This refers to the amount of haemoglobin present in the average erythrocyte. It was determined using the method as described by Baker et al. (2001). It was derived by calculation from the ratio of haemoglobin (Hb) and RBC count according to the following formula:

$$MCH\ (pgHb) = \frac{Hbg/100\ ml \times 10}{RBC\ /mm^3\ inmilions}$$

Estimation of mean cell haemoglobin concentration (MCHC)

The MCHC refers to the amount of haemoglobin in 100 ml of PCV,

Table 1. Phytochemical composition of methanolic *S. macrocarpon* fruit extract.

S/N	Phytochemicals	Qualitative Analysis	Quantitative Composition (%)
1.	Saponin	+ + + +	35.09 ± 0.040
2.	Phenol	+ +	8.13 ± 0.057
3.	Tannin	+	0.11 ± 0.001
4.	Phytate	+	2.19 ± 0.625
5.	Flavonoids	+ + + +	40.90 ± 0.450
6.	Alkaloids	+ + +	24.01 ± 0.006
7.	Steroid	–	0.00 ± 0.002
8.	Terpenoid	+	1.69 ± 0.100
9.	Cyanide	+	0.11 ± 0.013

– negligible, + slightly present, + + moderately present, + + + and above highly present.

as opposed to the amount of haemoglobin in whole blood. It was determined using the method as described by Baker et al. (2001). It was derived by calculation from the ratio of hemoglobin (Hb) and PCV count according to the following formula:

$$MCHC\ [\%(g/100\ ml)] = \frac{Hbg/100\ ml \times 100}{PCV}$$

Statistical analysis

The statistical analysis of data collected was done using the Statistical Package for Social Sciences (SPSS v.16). Analysis of Variance (ANOVA) was used to analyze the data while the Duncan Multiple Range Test (DMRT) was used to compare means. The results were presented as mean± S.EM with the level of significance set at $p<0.05$.

RESULTS

Result of phytochemical screening of the methanolic *S. macrocarpon* fruit extract

Table 1 shows the result of the qualitative and quantitative determination of phytochemicals present in the methanolic fruit extract of *S. macrocarpon.* It was observed that the quantity of flavonoids was highest in the plant extract followed by saponins and alkaloids. Phenols were moderately present, while phytate, tannin, cyanide and terpenoid were slightly present and steroids were negligible.

Acute toxicity studies of methanolic *S. macrocarpon* fruit extract

There was no mortality observed at the initial doses of 200, 400 and 800 mg/kg of the fruit extract after 24 h in phase one. Similarly, at higher dose levels of 1,000, 3,000 and 5,000 mg/kg (phase two), no death was recorded after 24 h, although some cage side behaviors such as restlessness and shivering were observed in the animals.

Effects of the methanolic fruit extract of *S. macrocarpon* on haematological profile

The result of the effects of the methanolic fruit extract of *S. macrocarpon* on blood picture of the albino rats is presented in Table 2. The result showed that there was no significant difference ($p>0.05$) in the WBC counts of the treated rats in all the weeks when compared with the control. However, in week 3, whereas the 400 and 800 mg/kg treatment doses showed significantly higher values of 1.78±0.10 and 1.80±0.06 respectively, the 1600 mg/kg treatment dose showed a significantly lower value of 0.90±0.10 when compared with the control. Considering the result across the weeks, whereas the rats administered 400 mg/kg of the extract showed no significant difference ($p>0.05$) in their WBC values from weeks 1 to 3 when compared with the value at week 0, those given 800 mg/kg showed a significant increase in week 3 (1.80±0.06) while those that received 1600 mg/kg had a significantly decrease value at week 2 (0.80±0.17).

There was no significant difference ($p>0.05$) in the RBC of the treated rats when compared with the control in all the weeks. However, the treated rats were observed to obtain higher RBC levels than that of the control in week 3, with the highest treatment dose (1600 mg/kg) showing the highest RBC value (7.17±0.17). On the other hand, there was a general non-significant ($p>0.05$) decrease in the RBC of the treated rats across the treatment period. However, in week 1, the RBC of the rats administered 800 mg/kg of the extract decreased significantly ($p<0.05$) from its value in week 0, while that of the rats administered 1600 mg/kg increased significantly ($p<0.05$) in week 3 from its value in week 2.

There was no observed dose-dependent significant difference ($p>0.05$) in the PCV of the treated rats when compared with those of the control in all the weeks. However, it was noticed that the PCV of the rats administered 1600 mg/kg decreased minimally from that of the control in week 2. On the other hand, whereas there was an overall non-significant decrease ($p>0.05$) in the PCV of the treated rats across the treatment period, the 800 mg/kg dose level produced a significant decrease

Table 2. Effects of methanolic fruit extract of *S. macrocarpon* on haematological parameters of albino rats.

Parameter	Treatments	Duration (Days)			
		0	7	14	21
WBC ($x10^3/mm^3$)	Control	1.60±0.08^{a1}	1.60±0.12^{a1}	1.32±0.19^{ab1}	1.33±0.11^{b1}
	400 mg/kg	1.42±0.13^{a1}	1.15±0.28^{a1}	1.25±0.10^{ab1}	1.78±0.10^{c1}
	800 mg/kg	1.43±0.06^{a1}	1.30±0.13^{a1}	1.50±0.15^{b1,2}	1.80±0.06^{c2}
	1600 mg/kg	1.34±0.15^{a2}	0.95±0.08^{a2}	0.80±0.17^{a1}	0.90±0.10^{a1,2}
RBC ($x10^6/mm^3$)	Control	6.86±0.80^{a1}	7.11±0.52^{a1}	6.85±0.41^{a1}	6.35±0.53^{a1}
	400 mg/kg	7.54±0.39^{a1}	7.08±0.15^{a1}	6.72±0.29^{a1}	6.89±0.21^{a1}
	800 mg/kg	7.90±0.38^{a2}	6.53±0.33^{a1}	6.89±0.32^{a1,2}	6.89±0.10^{a1,2}
	1600 mg/kg	7.35±0.21^{a2}	6.89±0.32^{a2}	5.88±0.27^{a1}	7.17±0.17^{a2}
PCV (%)	Control	37.33±4.33^{a1}	39.33±2.19^{a1}	37.33±2.19^{ab1}	34.67±2.40^{a1}
	400 mg/kg	41.00±2.08^{a1}	38.67±1.86^{a1}	36.67±1.33^{ab1}	37.67±1.76^{a1}
	800 mg/kg	43.00±2.08^{a2}	35.67±2.33^{a1}	37.67±1.45^{b1,2}	37.67±1.45^{a1,2}
	1600 mg/kg	40.00±1.16^{a2}	37.67±2.19^{a2}	32.00±1.16^{a1}	39.00±1.16^{a2}
Hb (g/dl)	Control	12.44±1.44^{a1}	13.52±0.34^{a1}	12.56±0.62^{a1}	11.50±0.76^{a1}
	400 mg/kg	12.56±0.49^{a1}	12.89±0.11^{a1}	12.23±0.55^{a1}	12.49±0.25^{ab1}
	800 mg/kg	14.33±0.69^{a2}	11.90±0.65^{a1}	12.50±0.64^{a1,2}	12.50±0.18^{ab1,2}
	1600 mg/kg	13.34±0.38^{a2}	12.52±0.91^{a2}	10.68±0.37^{a1}	13.01±0.26^{b2}
MCV (fl)	Control	54.42±0.01^{a1}	55.45±1.06^{a1}	54.49±0.12^{a1}	55.43±6.25^{a1}
	400 mg/kg	54.40±0.03^{a1}	54.61±2.35^{a1}	54.62±0.49^{a1}	54.64±1.51^{a1}
	800 mg/kg	54.41±0.02^{a1}	55.11±5.52^{a1}	54.73±0.95^{a1}	54.75±2.84^{a1}
	1600 mg/kg	54.42±0.01^{a1}	54.75±2.74^{a1}	54.84±4.51^{a1}	54.40±0.33^{a1}
MCH (pg)	Control	18.14±0.00^{a1}	19.15±1.00^{a1}	18.35±0.21^{a1}	18.48±2.47^{a1}
	400 mg/kg	16.80±1.35^{a1}	18.22±0.40^{a1}	18.22±0.58^{a1}	18.14±0.25^{a1}
	800 mg/kg	18.14±0.00^{a1}	18.27±1.07^{a1}	18.14±0.36^{a1}	18.14±0.01^{a1}
	1600 mg/kg	18.15±0.01^{a1}	18.12±0.51^{a1}	18.18±0.21^{a1}	18.16±0.25^{a1}
MCHC (%)	Control	33.33±0.00^{a1}	34.49±1.13^{a1}	33.67±0.32^{a1}	33.21±0.91^{a1}
	400 mg/kg	30.88±2.47^{a1}	33.47±1.41^{a1}	33.34±0.83^{a1}	33.23±0.89^{a1}
	800 mg/kg	33.33±0.01^{a1}	33.54±2.23^{a1}	33.18±1.19^{a1}	33.31±1.79^{a1}
	1600 mg/kg	33.35±0.0 ^{a1}	33.29±2.16^{a1}	33.55±2.37^{a1}	33.38±0.55^{a1}

Values with the same alphabetical superscript in the same column are not significantly different (p>0.05) and values with the same number superscript in the same row are not significantly different (p>0.05)

(p<0.05) in the PCV of the rats in week 1, while the 1600 mg/kg dose level showed a significant increase (p<0.05) in week 3.

There was no observed dose-dependent, significant difference (p>0.05) in the haemoglobin of the treated rats when compared with control in all the weeks. However, the 1600 mg/kg treatment dose showed a significantly higher (p<0.05) haemoglobin value (13.01±0.26) than control in week 3. Whereas, the result showed an overall non-significant decrease (p>0.05) in the haemoglobin levels of the treated rats across the weeks, the 800 mg/kg treatment dose showed a significant decrease in haemoglobin levels in week 1, while the 1600 mg/kg dose showed a significant decrease in week 2 followed by a significant increase in week 3.

It was observed that the MCV of the treated rats was not significantly different (p>0.05) when compared with the control in all the weeks. Similarly, there was no significant difference (p>0.05) in MCV of the various treatment doses across the weeks. However, the observations show an increasing trend in the MCV of the treated rats across the duration of treatment with the 800 mg/kg treatment dose showing the highest increase from 54.41±0.02 in week 0 to 54.75±2.84 in week 3. The result

showed that there was no significant difference (p>0.05) in the MCH of the treated rats when compared with the control in all the weeks. Similarly, there was no significant difference (p>0.05) in MCH of the various treatment doses across the weeks. However, whereas the 800 mg/kg treatment dose showed an almost constant value from 18.14±0.00 in week 0 to 18.14±0.01 in week 3, the 400 and 1600 mg/kg treatment doses showed a fluctuating non-significant (p>0.05) increases from 16.80±1.35 in week 0 to 18.14±0.25 in week 3 and from 18.15±0.01 in week 0 to 18.16±0.25 in week 3 respectively.

Furthermore, there was no significant difference (p>0.05) in the MCHC of the treated rats when compared with the control in all the weeks. Similarly, there was no significant difference (p>0.05) in MCHC of the various treatment doses across the weeks. However, whereas the 400 mg/kg treatment dose showed a progressive non-significant (p>0.05) decrease from 30.88±2.47 in week 0 to 33.23±0.89 in week 3, the 800 and 1600 mg/kg treatment doses showed a fluctuating non-significant (p>0.05) decrease from 33.33±0.01 in week 0 to 33.31±1.79 in week 3 and a non-significant (p>0.05) increase from 33.35±0.0 in week 0 to 33.38±0.55 in week 3 respectively.

DISCUSSION

Phytochemicals are substances found in edible fruits and vegetables that exhibit a potential for modulating human metabolism in a manner beneficial for the prevention of chronic and degenerative diseases (Tripoli et al., 2007). Thus the medicinal value of plants lies in the presence of these chemical substances that have a definite physiological action in the human body. The phytochemical analysis of the methanolic fruit extract of *S. macrocarpon* established the presence of such bioactive substances as flavonoids, saponins, alkaloids, phenols, phytates, tannins, cyanides, terpenoids and steroids in decreasing order of abundance. Sodipo et al. (2008) in their phytochemical analyses of *S. macrocarpon* L. aqueous fruit extract, reported copious presence of alkaloids and flavonoids, and slight presence of tannins, similar to the result of this present study. They, however, reported moderate presence of saponins in contrast to the result obtained in this study. Similarly, Dougnon et al. (2012), who carried out their study in Cotonou (Republic of Benin), reported a high presence of alkaloids, moderate presence of tannins, but absence of flavonoids and saponins in fruits of *S. macrocarpon* (Solanaceae) contrary to the results of this study.

The observed disparities in comparison with these past works could be explained on the basis of differences in the solvent of extraction (Parekh et al., 2005; Tatiya et al., 2011; Dent et al., 2013). It may also be as a result of differences in the geographical and environmental peculiarities. This is more so as past studies have implicated such factors in affecting the abundance and activities of the phytochemicals (Borokini and Ayodele, 2012; Ubani et al., 2012; Ullah et al., 2012). Flavonoids, saponins and alkaloids have been reported to exert a wide range of biological effects (Sandhar et al., 2011). According to Sudheesh et al. (1997), flavonoids extracted from *Solanum melongena* fruits showed significant hypolipidaemic action in normal and cholesterol-fed rats. Similarly, antioxidant, anti-inflammatory, hypocholesterolaemic and antimicrobial effects have been reported of flavonoids and saponins (Vinson et al., 1998; Francis et al., 2002; Cushnie and Lamb, 2005; Soetan et al., 2006; Chinedu et al., 2011; Mir et al., 2013). The bitter taste of *S. macrocarpon* and other eggplants is attributed to the presence of alkaloids, mainly glycoalkaloids (Chinedu et al., 2011). Alkaloids have several pharmacological activities including antihypertensive, antiarrhythmic, antimalarial, anticancer and antiseptic effects (Roberts and Wink, 1998; Soetan, 2008). Vohora et al. (1984) observed significant analgesic effect and some central nervous system depression with crude alkaloidal fraction isolated from *S. melongena* leaves.

Drugs and plant products are usually subjected to toxicity tests with experimental animals in order to predict safety or toxicity, and to provide guidelines for selecting safe doses in humans. Signs of toxicity include convulsions, tremors, protrusion of eye ball, and mortality (Rajurker et al., 2009). However, as no such signs were observed, the result of this present study, therefore, indicated no toxicity of the methanolic *S. macrocarpon* fruit extract. This confirms the safety of the plant for consumption as reported by Sodipo et al. (2011) who administered aqueous fruit extract of *S. macrocarpon* to hyperlipidemic rats. Nevertheless, cases of toxicity have been reported in tuberous *Solanum* spp. such as *Solanum tuberosum* (potato) due to the high presence of the glycoalkaloids, alpha-solanine and alpha-chaconine (Schippers, 2001; Langkilde et al., 2008).

Hematological parameters are important indices of physiological and pathological status for both animals and humans, thus playing a major role in disease investigation and diagnosis (Malomo, 2000; Adeneye et al., 2006). White blood cells (WBCs) are an important component of the host defence system, responsible for protection against bacteria, fungi, viruses, and other exogenous substances. The host defense system consists of an intricate cytokine network and progenitor cells, which maintain baseline myelopoiesis (formation of WBCs) and allow rapid adjustment in the rates of production of WBCs in response to acute and chronic stress (Ogawa, 1993; Stock and Hoffman, 2000). Increase in WBC is usually considered as a defensive mechanism by the immune system (Duru et al., 2013).Thus, the observed increases in WBC counts of the treated rats could be as a result of normal response

of the animals' defense system to foreign substances, in this case the plant extract. This is in line with the findings of Duru et al. (2013) who also observed an increase in WBC levels. However, it is possible that the extract had a suppressive effect on the WBCs at the highest dose. This is more so because while at dose levels of 400 and 800 mg/kg the WBC of the treated rats increased from 1.42±0.13 to 1.78±0.10 and 1.43±0.06 to 1.80±0.06 from weeks 0 to 3 respectively, at the 1600 mg/kg dose level, the WBC decreased progressively from 1.34±0.15 to 0.90±0.10.

The observations on the RBC count, PCV and haemoglobin (Hb) in this present study indicated no overall significant effect of the methanolic *S. macrocarpon* fruit extract on haematological parameters, although there are indications that there could be improvement with prolonged administration of the extract at high doses. This is because the highest dose (1600 mg/kg) administered, showed the highest RBC, PCV and Hb values in week 3.However, the results of this study portray the fact that the balance between the rate of production (erythropoiesis) and destruction of RBCs was not altered. Furthermore, as no adverse effect on serum bilirubin was observed, it is in agreement with the observation on RBC, and further confirms the absence of any haemolytic effect of the extract. In addition, the extract did not seem to stimulate erythropoietin release from the kidneys, which is the humoral regulator of RBC production (Oyedeji and Bolarinwa, 2012). RBCs and Hb are very important in the transfer of respiratory gases (Oyedeji et al., 2012). Hence the plant extract did not appear to affect the oxygen-carrying capacity of the blood. Low values of RBC and associated parameters (Hb and PCV) are indicative of anaemic conditions while very high values predict polycythemia. Thus, the present observation shows that the methanolic fruit extract of *S. macrocarpon* may not have the potential to induce anemia or polycythemia. Substances which significantly affect the values of RBC and associated parameters have effects on the bone marrow, kidney and haemoglobin metabolism (Young and Maciejewski, 1997; Oyedeji and Bolarinwa, 2012). Therefore, the treatment with the extract may not have had adverse effects on the bone marrow, kidney and haemoglobin metabolism, which is in line with the observations on the parameters studied. However, contrary to the findings of this study, Sodipo et al. (2012) reported a significant increase in haemoglobin (Hb), RBC count and PCV in triton-induced hyperlipidaemic rats treated with aqueous fruit extract of *S. macrocarpon*. Similarly, Duru et al. (2013) observed a significant increase in Hb and PCV when they incorporated powdered *S. macrocarpon* fruit into rat feed. Therefore, we can infer that the *S. macrocarpon* fruit in aqueous medium or in powdered form is more effective in improving haematological parameters than the methanolic extract.

The observed no significant effect of the methanolic *S. macrocarpon* fruit extract on the red cell indices (MCV, MCH and MCHC) were similar to those of Sodipo et al. (2012) and Duru et al. (2013), and implies that the incorporation of haemoglobin into RBC as well as the morphology and osmotic fragility of the RBC were not altered. Haemoglobin, RBC and PCV are associated with the total population of RBC while MCHC and MCH relate to individual RBC (Adebayo et al., 2010). Increased MCV and MCH values are indicative of macrocytic anaemia (Oyedeji et al., 2012). Thus, in addition, the methanolic *S. macrocarpon* fruit extract did not seem to have the potential to cause macrocytic anaemia in the treated animals.

In conclusion, the phytochemicals detected in this plant extract attest to the tremendous potentials of the plant. The acute toxicity study confirms the safety of its consumption. The results of this present study herein presented, revealed that the methanolic fruit extract of *S. macrocarpon*, at the administered doses and duration did not cause any serious adverse effect on haematological parameters. Thus, the extract did not impair the functional capabilities of the blood cells; hence we can assert that the fruits of the plant *S. macrocarpon* may not have any harmful effect on the consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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