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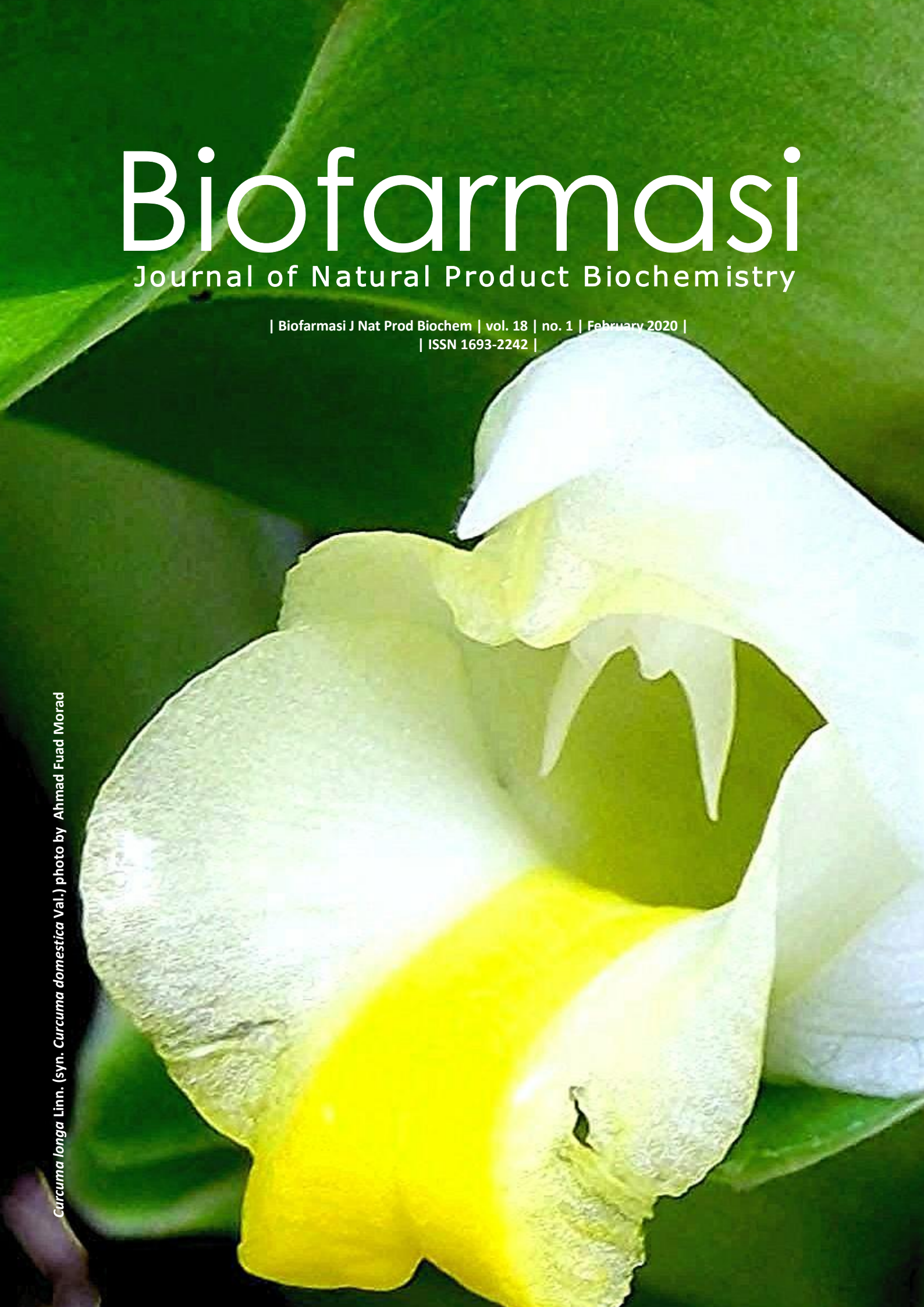

Curcuma longa Linn. (syn. *Curcuma domestica* Val.) photo by Ahmad Fuad Morad

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Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Cytotoxic and genotoxic effects of the *ent*-kaurenoic acid and *ent*-kaurenoic acid enriched *Mikania glomerata* extract in V79

NATÁLIA HELEN FERREIRA, ARTHUR BARCELOS RIBEIRO, MARIÂNGELA DIAS DE MORAIS, ALINE DE MORAIS PEIXOTO, MARCELA ARAÚJO BERNARDINO, MONIQUE RODRIGUES MOREIRA, ANA CAROLINA FERREIRA SOARES, VLADIMIR CONSTANTINO GOMES HELENO, RODRIGO CASSIO SOLA VENEZIANI, DENISE CRISPIM TAVARES♥

Universidade de Franca. Avenida Dr. Armando Salles de Oliveira, 201, 14404-600, Franca, São Paulo, Brazil.
Tel.: +55-16-3711-8871, Fax: +55-16-3711-8878, ♥email: denisecrispim2001@yahoo.com

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Abstract. *Ferreira NH, Ribeiro AB, Morais MD, Peixoto AM, Bernardino MA, Moreira MR, Soares ACF, Heleno VCG, Veneziani RCS, Tavares DC. 2020. Cytotoxic and genotoxic effects of the* ent-*kaurenoic acid and* ent-*kaurenoic acid-enriched* Mikania glomerata *extract in V79. Biofarmasi J Nat Prod Biochem 18: 1-4.* The *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel effectively inhibited the formation of *Streptococcus mutans* biofilm. Given the biological potential of this extract and its major component, the present study was carried out to evaluate the safety of the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel and *ent*-kaurenoic acid alone in an in the vitro test system. The results showed that the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel was cytotoxic at concentrations up to 40.0 µg/mL. Genotoxic effects were observed in cell cultures treated with the highest concentrations tested of *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel (10.0 and 15.0 µg/mL) and *ent*-kaurenoic acid alone (2.5, 5.0 and 7.5 µg/mL) when compared to the control group. Therefore, the *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel demonstrated cytotoxicity and genotoxicity effects at the highest concentrations tested, while *ent*-kaurenoic acid showed to be genotoxic at the same concentrations present in the an *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel in V79 cells. These results demonstrate that the *ent*-kaurenoic acid should be partly responsible for the genotoxicity of *ent*-kaurenoic acid-rich extract from *Mikania glomerata* Sprengel.

Keywords: Clonogenic assay, cytotoxicity, *ent*-kaurenoic acid, genotoxicity, *Mikania glomerata*

INTRODUCTION

In a recent study, our research group described the *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel (KAMg) as a new proposal for antimicrobial products for oral care. The results presented by the authors showed that KAMg was effective in inhibiting the formation of *Streptococcus mutans* biofilm (Moreira et al., 2016). Given the biological potential of this extract and its major component (*ent*-kaurenoic acid - KA – Figure 1), the present study was carried out to evaluate the cytotoxic potential of KAMg through the clonogenic efficiency assay and the genotoxic potential of KAMg and KA in the V79 (Chinese hamster lung fibroblasts) cell line through the cytokinesis-block micronucleus assay.

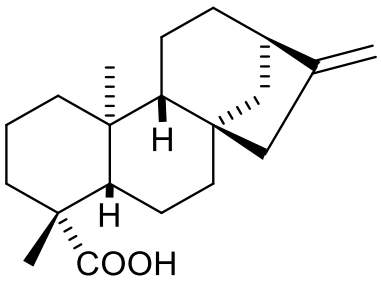

Figure 1. Chemical structure of *ent*-kaurenoic acid

MATERIALS AND METHODS

Procedures

*Obtention of the **ent**-kaurenoic acid-rich **M. glomerata** extract and **ent**-kaurenoic acid*

KAMg was prepared and characterized as described by Moreira et al. (2016). The KA content in the extract was determined after developing and validating a reverse-phase HPLC analytical method, which corresponded to about 48% of KA content in KAMg (Moreira et al., 2016). The KA samples used in this work were obtained from the leaves of *M. glomerata*, just as described by Soares et al. (2019).

Cell line and culture conditions

The Chinese hamster lung fibroblasts (V79 cell line) were employed for experimental assays. The cell line was maintained as monolayers in plastic culture flasks (25 cm2) in HAM-F10 plus DMEM (1:1; Sigma-Aldrich) culture medium supplemented with 10% fetal bovine serum (Nutricell), antibiotics (0.01 mg/mL streptomycin and 0.005 mg/mL penicillin; Sigma-Aldrich), and 2.38 mg/mL HEPES (Sigma-Aldrich), at 37°C in a humidified 5% CO2 atmosphere. The V79 cells were used from the 4th until the 12th passage. This cell line has an average cycle of 12 h in the experimental conditions described.

Cytotoxicity assessment

The clonogenic efficiency assay was used to determine the cytotoxicity of KAMg. For this, cells were treated for 3 h with KAMg concentrations ranging from 0.3 to 160 µg/mL. Negative (no treatment), solvent (Methanol, MeOH, 0.4%), and positive (methyl methanesulfonate, MMS, Sigma-Aldrich, 110 µg/mL) controls were also included. Following, the cultures were trypsinized, and 300 cells were seeded per culture flask (three flasks per concentration). The experiments were carried out for 10 days. The culture medium was then removed, and the colonies were washed with PBS (phosphate-buffered saline), fixed (1:1:9 of methanol/acetic acid/distilled water), and stained with Giemsa (1:20 in phosphate buffer, pH 7.0) for 20 minutes. Individually stained colonies were counted, and the colony-surviving fraction was calculated as follows (adapted from Franken et al. 2006):

$$SF\ (\%) = (A/B) \times 100$$

Where; *A* corresponds to the number of colonies found in the corresponding treatments and *B* to the number of colonies found in the negative control.

Genotoxicity assessment

The cytokinesis-block micronucleus assay was used to determine the genotoxicity of KAMg and KA. The concentrations of KAMg were chosen based on the results of the clonogenic efficiency assay, using cytotoxicity as selection criteria, according to *OECD 487* (2016). The concentrations of KA were chosen according to the content of KA in KAMg, which corresponded to 48%. To assess the genotoxicity, $1x10^6$ cells were seeded in culture flasks, incubated for two cycles (24 h) in 5 mL of complete HAM-F10/DMEM medium, washed with PBS, pH 7.4, and then submitted to the treatment in serum-free medium for 3 h with KAMg (2.5, 5.0, 10.0 and 15.0 µg/mL) or KA (1.25, 2.5, 5.0 and 7.5 µg/mL). Negative control (no treatment), solvents (MeOH, 0.4% for KAMg and dimethylsulfoxide, DMSO, Sigma-Aldrich, 1% for KA), and positive (MMS, 44 µg/mL) controls were also included. At the end of this period, cells were washed twice with PBS. A fresh serum-supplemented medium containing 3 µg/mL cytochalasin-B (Sigma-Aldrich) was added, and the cells were incubated for an additional 17 h. At harvest, the cells were trypsinized (0.025%), and a hypotonic solution of 1% sodium citrate at 37°C was added. The cells were then fixed in methanol: acetic acid (3:1), and the slides were stained with 3% Giemsa for 5 minutes.

The criteria established by Fenech (2000) were used to analyze micronuclei and binucleated cells. A total of 3000 binucleated cells were scored per treatment, corresponding to 1000 cells/treatment/repetition. For the evaluation of cytotoxicity of the treatments was analyzed 1500 cells analyzed per treatment, for a total of 500 cells per repetition, and the nuclear division index (NDI) was calculated. Cells with well-preserved cytoplasm containing 1-4 nuclei were scored. The NDI was calculated using the following formula (Kirsch-Volders et al. 2004):

$$NDI = (M1 + 2 \times M2 + 3 \times M3 + 4 \times M4) / N$$

Where; *M1-M4* is the number of cells with 1, 2, 3, and 4 nuclei, respectively, and *N* is the total number of viable cells.

Additionally, the cytotoxicity index (CI) of KAMg and KA was calculated as described by Kirsch-Volders et al. (2004):

$$CI = 100 - 100 \times [(NDIt - 1) / (NDIc - 1)]$$

Where; *NDIt* is the NDI value found for the different treatments, and *NDIc* is the NDI value of the negative control.

Data analysis

Data were statistically analyzed by analysis of variance (ANOVA) for completely randomized experiments; the statistical F and its respective "*p-value*" were calculated. The Tukey method compared the treatment means in cases where $p < 0.05$, and the minimum significant difference was calculated for $\alpha = 0.05$. All statistical analyses were performed using the GraphPad Prism 5.0 program (GraphPad Sofware, San Diego, CA, USA).

RESULTS AND DISCUSSION

Cytotoxicity assessment

Dose-dependent changes in the viability of KAMg-treated cells were evaluated using the clonogenic efficiency assay. Each survival curve represents the mean of three independent experiments, and the bars indicate the standard error of the mean (Figure 2). Cultures treated with KAMg concentrations above 20.0 µg/mL showed significant reductions in cell viability compared to the control cultures, indicating a cytotoxic effect. Therefore, KAMg concentrations of 2.5, 5.0, 10.0, and 15.0 µg/mL were chosen for further analysis in the genotoxic assay.

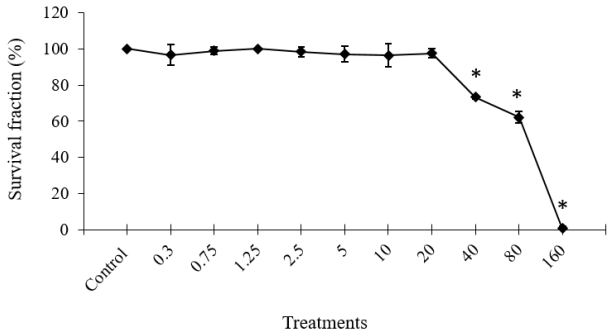

Figure 2. Cell survival means percentages obtained after exposure of V79 cells to *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel (KAMg) concentrations ranging from 0.3 to 160.0 µg/mL. MeOH: methanol (0.4%); MMS: methyl methanesulfonate (110 µg/mL). *Significantly different from the control group ($P<0.05$).

Table 1. Mean frequencies of micronuclei (MN), nuclear division index (NDI), and cytotoxicity index observed in V79 cell cultures treated with different concentrations of *ent*-kaurenoic acid-rich extract from *M. glomerata* Sprengel, and their respective controls

Treatment (µg/mL)	MN frequency	NDI[b]	Cytotoxicity index
	Mean ± SD	Mean ± SD	(%)
Control	5.33 ± 1.15	1.74 ± 0.04	-
MeOH	4.33 ± 1.15	1.69 ± 0.08	6.76
2.5	9.00 ± 2.00	1.66 ± 0.09	10.82
5.0	11.33 ± 5.86	1.63 ± 0.10	14.87
10.0	16.00 ± 2.65[c]	1.60 ± 0.05	18.92
15.0	17.33 ± 2.52[c]	1.35 ± 0.03[c]	52.71
MMS	62.33 ± 1.53[c]	1.60 ± 0.07	18.92

Note: MeOH: methanol (0.4%); MMS: methyl methanesulfonate (44 µg/mL). [a]A total of 3000 binucleated cells was scored per treatment, corresponding to 1000 cells/treatment/repetition for the determination of micronucleus frequency. [b]A total of 1500 cells was analyzed per treatment group, corresponding to 500 cells/treatment/repetition for calculation of NDI. [c]Significantly different from the control group (P<0.05).

Table 2. Mean frequencies of micronuclei (MN) and nuclear division index (NDI) were observed in V79 cell cultures treated with different concentrations of kaurenoic acid and their respective controls.

Treatments	MN frequency	NDI[b]
(µg/mL)	Mean ± SD	Mean±SD
Control	6.67 ± 2.08	1.66 ± 0.02
DMSO	6.33 ± 1.15	1.70 ± 0.12
1.25	14.67 ± 2.08	1.64 ± 0.06
2.5	20.00 ± 5.29[c]	1.65 ± 0.09
5.0	17.67 ± 2.31[c]	1.76 ± 0.14
7.5	17.67 ± 4.04[c]	1.72 ± 0.13
MMS	62.33 ± 1.53[c]	1.68 ± 0.08

Note: DMSO: dimethylsulfoxide (1%); MMS: methyl methanesulfonate (44 µg/mL). [a]A total of 3000 binucleated cells was scored per treatment, corresponding to 1000 cells/treatment/repetition for micronucleus frequency determination. [b]A total of 1500 cells was analyzed per treatment group, corresponding to 500 cells/treatment/repetition for NDI calculation. [c]Significantly different from the control group (P<0.05).

Genotoxicity assessment

Table 1 shows the mean frequencies of binucleated cells with micronuclei and the mean standard deviation after exposure to KAMg. There was no significant difference in micronuclei induction observed between cultures treated with KAMg concentrations of 2.5 and 5.0 µg/mL and the control group. However, a significant micronuclei frequency increase was observed in cultures treated with 10.0 and 15.0 µg/mL of KAMg compared to the control, demonstrating genotoxicity for these concentrations. In relation to NDI, the cultures treated with the highest concentration of KAMg tested (15.0 µg/mL) showed significantly lower indexes when compared to the cultures of the control group, with a cytotoxicity index equal to 52.71%. In this way, KA (in a percentage corresponding to KAMg concentrations) treatment groups were included to assess whether the genotoxic effect of KAMg at the highest concentrations was due to the presence of KA.

Table 2 shows the mean frequencies of binucleated cells with micronuclei and NDI after exposure to KA. The lowest tested concentration of KA did not significantly increase the micronuclei frequency compared to the control group. On the other hand, the results showed a significant increase in micronuclei frequency at concentrations of 2.5, 5.0, and 7.5 µg/mL, compared to the control group, demonstrating genotoxic activity. The results of NDI (nuclear division index) showed no significant differences for any KA concentration compared to the control group, revealing the absence of cytotoxicity.

Discussion

The present study aimed to investigate the cytotoxic and genotoxic potential of KAMg in V79 cells. The results demonstrated cytotoxic and genotoxic effects of KAMg at the highest concentrations tested through the clonogenic efficiency assay (above 20 ug/mL) and the micronucleus assay (10 and 15 ug/mL), respectively. In order to investigate the influence of KA on the genotoxicity of the extract, the genotoxic potential of this diterpene was evaluated in the concentrations present in KAMg. Except for the lowest KA tested concentration, all others revealed genotoxic effects. These results demonstrate that KA should be responsible, at least in part, for the genotoxicity of KAMg.

Some studies have already demonstrated the genotoxic potential of *M. glomerata* extract and KA. Dalla Nora et al. (2010) observed the genotoxic and antiproliferative effects of *M. glomerata* leaves extract on *Allium cepa* cells. The genotoxicity of the *M. glomerata* leaves extract was also reported by Costa et al. (2008) using murine hepatoma cells (HTC).

Cavalcanti et al. (2006) demonstrated the genotoxic effect of KA at concentrations of 30 and 60 µg/mL in V79 cell lines using the comet and micronucleus assays. However, Cano et al. (2017) investigated the genotoxicity of KA in the CHO-K1 (Chinese hamster ovary) cell line using the micronucleus assay, and this diterpene showed no genotoxicity at concentrations ranging from 7.36 to 30.25 µg/mL. O'Donovan (1990) and Erexson et al. (2001) reported that CHO and V79 cell lines vary significantly in their ability to metabolize endotoxins. This difference in cellular metabolic capacity may explain the results difference obtained in relation to the genotoxicity of KA.

In relation to the genotoxicity of KAMg, it is important to note that extracts are complex mixtures of biologically active substances that may act synergistically or antagonistically. All these effects may lead to attenuation or accentuation of genotoxic and or cytotoxic effects caused by some specific substance. The enrichment of *M. glomerata* extract with KA may have contributed to the genotoxicity of the KAMg, since KA showed to be genotoxic at the same concentrations present in the enriched extract.

Cavalcanti et al. (2010) demonstrated that the genotoxic and mutagenic properties of KA are due to clastogenic damage, probably derived from DNA double-strand breaks and/or inhibition of topoisomerase I. In addition, the authors confirmed that the exocyclic double bond (C16) in the chemical structure of KA is an important element in its genotoxicity and mutagenicity.

Despite the genotoxicity of the extract observed in the in vitro test system, KAMg did not show genotoxic or cytotoxic activity in Swiss mice bone marrow by the micronucleus test (Moreira et al. 2016). Factors such as metabolism, pharmacokinetics, and pharmacodynamics alter compounds' distribution and concentration in more complex organisms than cellular units (Singulani et al., 2018).

The oral bioavailability of numerous drugs is greatly reduced before reaching systemic circulation (Kuppens et al. 2005). The first pass effect reduces the concentration of a drug to such an extent that only a small amount of active drug emerges from the liver to the systemic circulation and the target tissue, thus impacting its biological effect on in vivo systems (Sousa & Bernkop-Schnürch 2014).

Jiang et al. (2019) carried out the pharmacokinetic analysis of KA after oral administration of 10, 20, and 40 mg/kg in rats to 36 h after the treatment. The pharmacokinetic parameters indicated that KA was widely distributed in body tissues with high Vz/F values (apparent volume of distribution during terminal phase after non-intravenous administration) and experienced slow in vivo elimination after oral administration with terminal phase half-life (t½z) > 10 h values for all doses.

The absence of genotoxicity of KAMg in in vivo test system may be related to the high liposolubility of KA. The high value in the partition coefficient of KA (5.4) may influence a higher concentration in adipose tissues and a slow concentration released into the bloodstream that exerts genotoxic effects on the bone marrow.

Under the experimental conditions used, KAMg demonstrated cytotoxicity as well as genotoxicity at the highest concentrations tested. At the same time, KA showed to be genotoxic at the same concentrations present in KAMg in V79 cells. These results demonstrate that KA should be responsible, at least in part, for genotoxicity KAMg.

ACKNOWLEDGEMENTS

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Protective effects of *Curcuma longa* rhizomes ethyl acetate extract against alcohol induced oxidative stress and nephrotoxicity in female Wistar rats

O.E. ETENG[1♥], C.A. MOSES[1], J. ENOBONG[1], A.J. AKAMO[1], D.I. AKINLOYE[1], R.N. UGBAJA[1], O.A. AKINLOYE[2]

[1]Department of Biochemistry, Federal University of Agriculture. Abeokuta, Ogun State, Nigeria. ♥email: ofemeffiom@gmail.com
[2]Department of Biochemistry, University of Calabar. Calabar, Nigeria

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Abstract. *Eteng OE, Moses CA, Enobong J, Akamo AJ, Akinloye DI, Ugbaja RN, Akinloye OA. 2020. Protective effects of* Curcuma longa *rhizomes ethyl acetate extract against alcohol induced oxidative stress and nephrotoxicity in female Wistar rats. Biofarmasi J Nat Prod Biochem 18*: *5-12.* This study aimed to evaluate the protective effect of *Curcuma longa* Linn. (syn. *Curcuma domestica* Val.) rhizomes ethyl acetate extract (CLREAE) facing alcohol-induced oxidative stress and nephrotoxicity. Thirty female (30) Wistar rats were categorized randomly into six groups. Groups 1, 2, 3, 4, 5 and 6 were treated with normal saline; 20% ethanol; 100 mg of CLREAE + 20% ethanol; 200 mg of CLREAE + 20%; 350 mg of CLREAE + 20% ethanol and 350 mg of CLREAE respectively for 14 days. A significant ($p<0.05$) decrease in the SOD, CAT, and GPx activities and GSH concentration of rats treated with only 20% ethanol were found when compared to the normal control group, whereas a significant ($P<0.05$) increase in the groups pretreated with different doses of the CLREAE were also found when compared to groups with only 20% ethanol treatment. Thus, compared to the normal control group, treatment with the CLREAE fetched a significant ($p<0.05$) decrease in the renal biomarkers (creatinine and urea). Whilst, compared to the groups with 20% methanol treatment, a significant ($p<0.05$) increase happened in the groups pretreated with different doses of the CLREAE. There was a significant ($p<0.05$) decrease in Kidney MDA levels in rats pretreated with different doses of CLREAE compared with the normal control. The histology results showed a physiologic recovery in the kidney tissues as groups were treated with different doses of the CLREAE. This is evidenced by reduced necrosis of tubular and glomerular epithelial; the signs of protection against toxicity were found in the rats. The study suggested that through *in vivo* free radical scavenging ability, the CLREAE has protective effects against alcohol-induced oxidative stress and nephrotoxicity in female Wistar rats.

Keywords: Nephrotoxicity, *Curcuma longa*, oxidative stress, alcohol, ethyl acetate

INTRODUCTION

Nephrotoxicity is the toxicity in the kidneys, and it is caused by toxic chemicals or medications having poisonous effects on kidney function. Several studies have identified that risk factors for nephrotoxicity involve alcoholism, high blood pressure, diabetes mellitus, exposure to occupational nephrotoxins, and chronic use of analgesics (Knott et al. 1996). Alcohol and its metabolites go through the kidneys and are excreted into the urine, and its content in the urine is higher than that of the blood and the liver. The kidney is often involved in developing, maintaining, and counter-regulation of complex electrolyte disturbances (Das et al. 2008).

As a risk factor for kidney damage, blood pressure is being raised by regular alcohol consumption. Abnormalities in kidney structure and function are reported to increase the frequency of fetal alcohol syndrome, as seen in children prenatally exposed to ethanol. The kidney is an organ that is efficiently designed to perform two main tasks in the body, namely the excretion of metabolic end products and the proper regulation of fluid body constituents (Guyton 1992). When the tasks are completed, the kidney forms and collects urine, passing through the ureter into the bladder (Rhoades and Pffanzer 1992).

Nephrotoxicity determines xenobiotic damaging effects on the kidneys. Alcohol consumption has been related to an aloft incidence of coronary heart disease (CHD) and chronic kidney disease (CKD) (Parekh et al. 2001). Alcohol toxicity is linked to metabolism through alcohol dehydrogenase (ADH), which alters ethanol into toxic acetaldehyde, eventually oxidized to acetate through aldehyde dehydrogenase (ALDH). Acetaldehyde is a toxic by-product of ethanol metabolites that causes liver defects (Purohit et al. 2003). Acute alcohol poisoning or chronic alcoholism causes kidney disablement (Knott et al. 2015). Other studies also denounce similar conclusions, which show that the incidence of kidney disablement is comparable to or even lower in heavy drinkers (more than 210 g/week of alcohol consumption) than those in moderate drinkers (70-210 g/week of alcohol consumption) (Koning et al. 2015). On the contrary, several other studies have found that large amounts of alcohol consumption can predict worse outcomes in patients with chronic kidney sickness (White et al. 2009). For example, White and colleagues (Latchoumycandane et al. 2015) informed that heavier drinkers (those who consumed more than 30 g of alcohol/week) had a higher risk of developing

albuminuria, which is usually a symptom of kidney sickness. There is increasing evidence that chronic ethanol exposure can cause functional conversions and structural disablement in the kidneys (Latchoumycandane et al. 2015).

Previous studies have observed that, from a functional perspective, after ethanol exposure, proximal tubular disorganization, microvilli disorientation, luminal casts, glomerular mesangial matrix expansion, and convoluted proximal tubule occlusion accompanied by partial degeneration were of proximal tubular cells, and reduced cell height cell will occur. While the mechanisms of alcohol-induced cell lesions and sicknesses are still being examined, recent research suggests that reactive oxygen species (ROS) may have a significant role. Reactive oxygen species can lead to various cellular wounds, such as DNA breakage, lipid peroxidation, and protein alteration. The cellular system is protected from ROS-generated cell wounds by a series of defenses consisting of various anti-oxidants with distinct functions. The presence of ROS in cellular systems will defeat the defense system, and they will generate oxidative stress or cell wound, which leads to the development of disease (Deodhar et al. 1980). ROS production is usually accompanied by the presence and cellular localization of anti-oxidant enzymes and thiol, such as superoxide dismutase (SOD), CAT, glutathione peroxidase (Gpx), and glutathione (GSH). The synthesis of GSH depends on ATP, but its reducing power depends on NADPH and the pentose phosphate pathway (Lu et al. 2009). In vivo studies have discovered that accumulation of oxidative breakage emerging from lessened endogenous anti-oxidant degrees somewhat increases ROS production (Meng et al. 2007). Plant extracts containing natural antioxidants are recommended to improve kidney health status and lessen the risk of oxidative stress-based sicknesses.

Consequently, research has been carried out to reveal potential new sources of natural plant material. Over the years, many studies based on the utilization of natural compounds derived from plants as potential therapeutic agents for many sicknesses in humans have been carried out. Curcumin is a phenolic compound extracted from *Curcuma longa* Linn. (syn. *Curcuma domestica* Val.) rhizomes commonly utilized in Asia as seasonings, pigments, and additives. Several studies have reported that curcumin owns numerous biological functions, especially as antioxidants and anti-inflammation. Actually, it has been determined that curcumin is a bifunctional antioxidant; it mobilizes antioxidant activity directly and indirectly by scavenging reactive oxygen species and generates an antioxidant response severally. The renoprotective effect of curcumin has been assessed in numerous experimental models, including diabetic nephropathy, chronic kidney disablement, ischemia and reperfusion, and nephrotoxicity generated by compounds such as gentamicin, adriamycin, chloroquine, iron nitrilotriacetate, sodium fluoride, hexavalent chromium, and cisplatin. It has been reported lately in models of chronic kidney disablement that curcumin provides a therapeutic effect; indeed, it recovers systemic changes and glomerular hemodynamic alterations. Another recent finding indicates that the renoprotective effect of curcumin is related to the maintenance of mitochondrial function and redox balance. Together, these studies link the protective effects of curcumin in the kidney with the generation of the main regulator of the antioxidant response nuclear factor erythroid-derived 2 (Nrf2), restraint of mitochondrial dysfunction, weakening of the inflammatory response, keeping of antioxidant enzymes, and the avoidance of oxidative stress.

MATERIALS AND METHODS

Apparatus and Biochemical instruments

As equipment and biochemical instruments, the experiment utilized micropipettes, cotton wool, tissue paper, heparinized tubes, beakers, Eppendorf tubes, measuring tubes, conical flasks, disposable gloves, surgical kits, needles and syringes (5 mL and 10 mL), scales balance, spectrophotometer (Spectrum 23A), centrifuge, homogenizer, chloroform-methanol (2: 1v/v), diethyl ether, normal saline, distilled water, 0.05 M potassium chloride (KCl), creatine reagent kit, reagent kit all creatinine was purchased at the Libertas, Camp, Abeokuta laboratory services.

Animals

This study utilized thirty (30) female Wister mice weighing 150-220 g as experimental animals, which were obtained from the Tayo farm, Ajibode, Ibadan University, Ibadan. These animals were placed in well-ventilated wooden cages at room temperature (28-30°C), with good lightening and humidity. Here, they were allowed to have free access to maximum feeding of animal feed and water *ad libitum.* They were acclimatized for two weeks before the experiment began. The animals were randomly separated into six groups of five animals each, as shown in Table 1. The administration was carried out using oral cannulas.

Collection and preparation of plants

Curcuma longa rhizomes were collected from the Ajasa farm, Idi-Ori Village, Ile-Ise Awo, Abeokuta, Nigeria. Plant specimens were validated by the Department of Botany, Federal Agricultural University, Abeokuta, as *Curcuma longa* (Family: Zingiberaceae). Plant specimens matched Herbarium specimens no: FUNAAB H-0065 (Meng et al. 2007). Turmeric has long been known as a spice, medicine, and coloring agent, and since 1280, Marco Polo has mentioned turmeric in his travels around China and India.

Table 1. Animal grouping

Groups	Treatment
1	Control (normal saline)
2	20% Ethanol only
3	100 mg/kg body weight of extract + 5.22 mg/kg body weight 20% ethanol
4	200 mg/kg body weight of extract + 5.22 mg/kg body weight 20% ethanol
5	350 mg/kg body weight of extract + 5.22 mg/kg body weight 20% ethanol
6	35 0mg/kg body weight of extract

Figure 1. Turmeric rhizomes in (A) natural form and (B) grounded form

Extraction of plant material

The rhizomes were thoroughly rinsed, cut into small sizes, and dried with air to remove the moisture inside. After air-drying, the rhizomes were ground into powder using a mechanical blender. 1000 mg of turmeric powder was weighed using analytical scales and treated with an appropriate solvent, ethyl acetate, measured to 2000 mL. The mixture was left for 3 days in a shaker at room temperature. After that, the solution was filtered using Whatman No.1 filter paper. Next, the solvent was evaporated using a rotary evaporator under reduced pressure at a controlled temperature. The extract was stored for subsequent biochemical analysis.

Experimental design

The animals were separated into six groups which contained five animals each.

Sacrifice

Feed and water are taken from animals 24 hours before sacrificing process. Mice were lightly anesthetized with diethyl ether in a desiccator and then sacrificed.

Blood collection

Blood was collected from the inferior vena cava of the animal's heart, poured into a plain centrifuge tube, and left to stand for 1 hour. The serum was prepared by centrifugation at 4000 rpm for 10 minutes in a centrifuge. For other biochemical tests, the clear supernatant was stored at -4°C.

Organ Collecting

The mice were then dissected from the abdomen to the thoracic area using surgical scissors and forceps. Important organs (kidneys) are then collected from mice, rinsed in normal saline, and stored in an ice cooler box.

Homogenization of organs

The collected kidneys were sliced to a weight of 0.2 g and homogenized in 1.8 mL of Sucrose-Tris-EDTA buffer. These were then centrifuged at 4000 rpm for 10 minutes, after which the supernatant was collected into an Eppendorf tube and stored in a cooler box.

Statistical analysis

Quantitative data were calculated using a one-way analysis of variance (ANOVA), followed by a post hoc test (Duncan) for significant values. A P-value of <0.005 was deemed statistically significant. Statistical analysis was done using statistical package software for social science (SPSS) version 20, while the graphs were plotted using Microsoft-Excel 08 software. Data were expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

The effect of ethyl acetate extract of *C. longa* on antioxidant parameters, namely GSH, GPx, catalase, and SOD, in the kidney of alcohol-generated female Wistar mice was shown in Table 2. Meanwhile, Figures 8-13 showed severe kidney necrosis led by treatment.

The level of MDA in plasma was significantly ($P < 0.05$) declined in the groups treated with different doses of ethyl acetate extract of *C. longa* before treating 20% ethanol compared to animals with only 20% alcohol administration. Still, when it was compared to the normal control group, there was a significant ($P < 0.05$) increase in MDA level (Figure 2).

The MDA level in the kidney significantly ($P < 0.05$) declined in the group pretreated with different doses of *C. longa* ethyl acetate extract before 20% ethanol treatment compared to animals treated with 20% alcohol alone, but there was a significant ($P < 0.05$) increase when compared to the normal control group (Figure 3).

Treatment with extract caused a significant raise ($P < 0.05$) of creatinine level in the kidney in the group which was given 20% ethanol only compared to the normal control group, but a significant decline ($P < 0.05$) happened in the group given by initial treatment with different extract

doses when compared to the group given only 20% ethanol (Figure 4).

Treatment with extract caused a significant decline in plasma level of creatinine (P <0.05) in the group administered only with 20% ethanol compared to the normal control group. Still, there was a significant raise (P <0.05) in the group initially administered with various extract doses when compared to the group that was only given 20% ethanol (Figure 5).

Table 2. Effect of ethyl acetate extract of *Curcuma longa* on antioxidant parameters, i.e., GSH, GPx, catalase, and SOD, in the kidney of alcohol induced female Wistar rats

Groups (n=5)	GSH (µmol/mg protein)	GPx (U/mg protein)	SOD (U/mg protein)	Catalase (U/mg protein)
1	1.96 ± 0.06	0.93 ± 0.04	25.74 ± 1.17	0.47 ± 0.05[b]
2	1.29 ±0.06[a]	0.71 ± 0.03	15.59 ± 0.40[a]	0.36 ± 0.02[a]
3	1.77 ± 0.08	0.84 ± 0.06[b]	15.80 ± 0.95[a]	0.36 ± 0.03[a]
4	1.65 ± 0.07	0.81 ± 0.02[b]	19.06 ± 0.63[b]	0.45 ± 0.06[b]
5	1.50 ± 0.07[b]	0.77 ± 0.08[a]	21.31 ± 0.74	0.44 ± 0.06[b]
6	2.29 ± 0.04	0.79 ± 0.06[a]	23.96 ± 1.28	0.99 ± 0.14

Note: Results are mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups. p<0.05

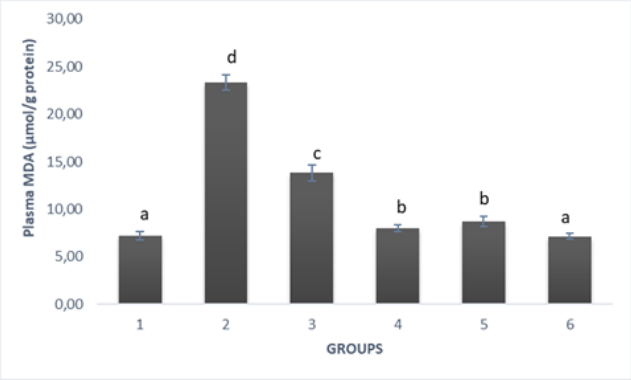

Figure 2. Effect of ethyl acetate extract of *Curcuma longa* on malondialdehyde in the plasma of alcohol-induced female Wistar rats. Results are Mean ± SEM a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups. p<0.05

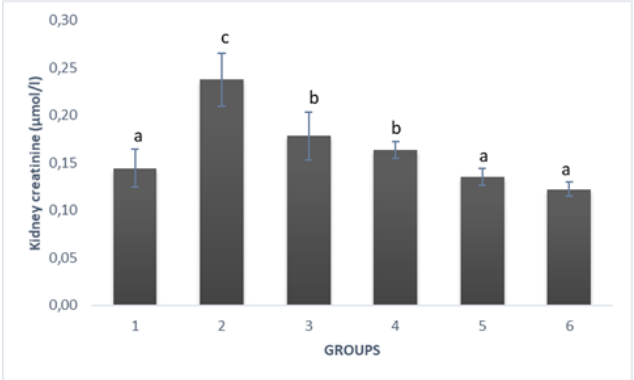

Figure 4. Effect of ethyl acetate extract of *Curcuma longa* on creatinine level in the kidney of alcohol-induced female Wistar rats. Results are Mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups. p<0.05

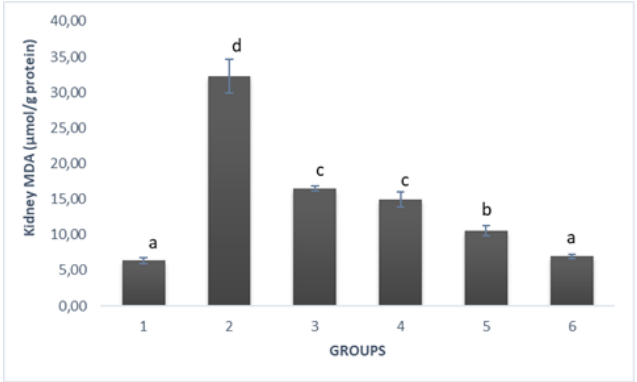

Figure 3. Effect of ethyl acetate extract of *Curcuma longa* on malondialdehyde in the kidney of alcohol-induced female Wistar rats. Results are Mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups. p<0.05

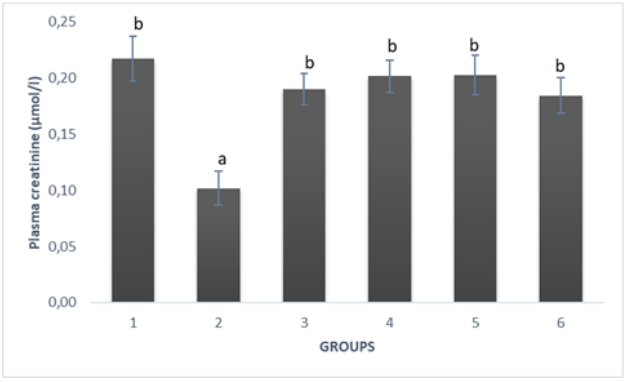

Figure 5. Effect of ethyl acetate extract of *Curcuma longa* on creatinine in the plasma of alcohol-induced female Wistar rats. Results are Mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups P <0.05

Extract administration led to a significant raise (P <0.05) in plasma level of urea when the group was given 20% ethanol only compared to the normal control group, but there was a significant decline (P <0.05) in the group initially treated with various extract doses when compared to the group that was only given by 20% ethanol (Figure 6).

Treatment with extracts led to a significant decline (P <0.05) in kidney level of urea when the group was given 20% ethanol only compared to the normal control group, but there was a significant raise (P <0.05) in the group pre-administered with various extract doses when compared to the group that was only given by 20% ethanol (Figure 7).

Discussion

Medicinal plants have therapeutic and pharmacological interest values, remaining the main source of active medicine from natural sources. Studies show that functional groups related to *C. longa*'s chemical structure, including bis-α, β-unsaturated β-dicone, two methoxy groups, two phenolic hydroxy groups, and two conjugated bonds, could have an important role in antiproliferative and anti-inflammatory activities. *C. longa* holds a keto-enol tautomer, in which the keto form is the predomination in acidic and neutral solutions, and enols form is the predomination in alkaline solutions. In this study, ethanol treatment significantly declined (P <0.05) in Reduced GSH content and SOD, GPx, and CAT activity in rat kidneys compared to the control group. These changes are significantly reversed with the treatment of *C. longa* extract.

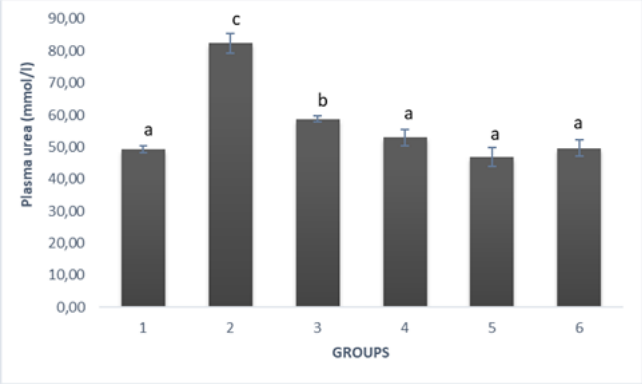

Figure 6. Effect of ethyl acetate extract of *Curcuma longa* on urea in the plasma of alcohol-induced female Wistar rats. Results are Mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups. P<0.05

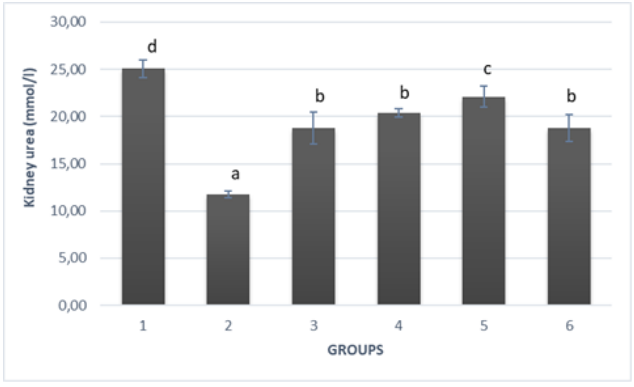

Figure 7. Effect of ethyl acetate extract of *Curcuma longa* on urea in the plasma of alcohol-induced female Wistar rats. Results are Mean ± SEM. a Controls are compared with ethanol-treated groups. P <0.05, b the ethanol-treated group is compared with the *C. longa* extract + ethanol-treated groups P <0.05

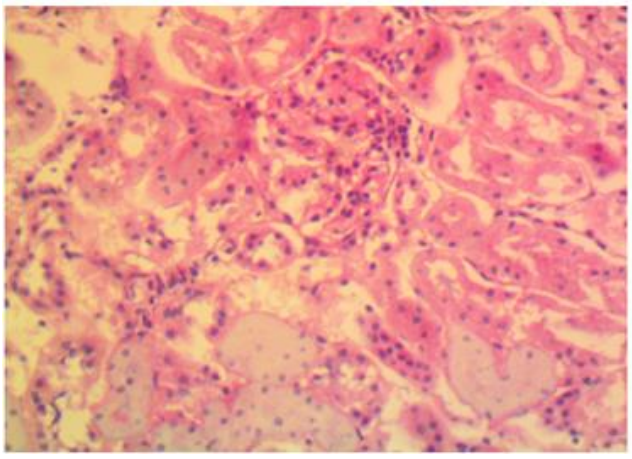

Figure 8. Section of the kidney showing moderate necrosis of tubular (arrow) and glomerular (arrowhead) epithelial cells and proteinaceous materials in the tubules (x400; H & E)

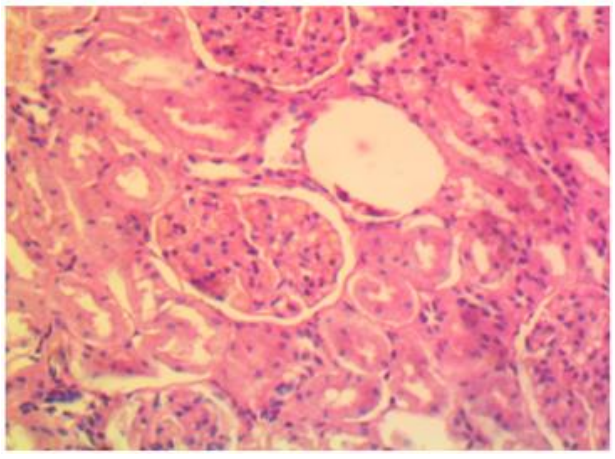

Figure 9. Section of the kidney showing severe necrosis of tubular (arrow) and glomerular epithelial cells (arrowhead) (x400; H & E)

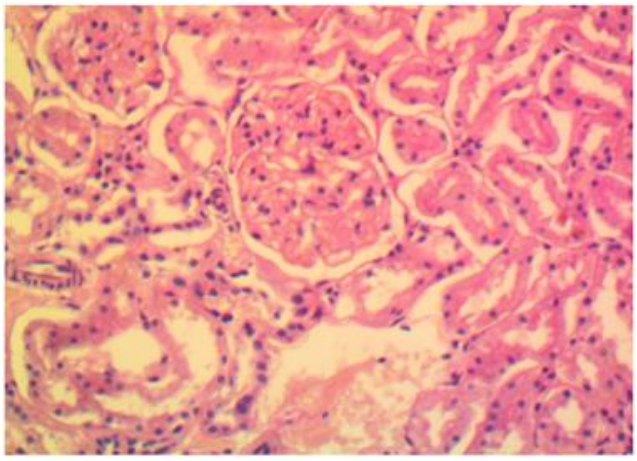

Figure 10. Section of the kidney showing mild necrosis of tubular (arrow) and glomerular (arrowhead) epithelial cells (x400; H & E)

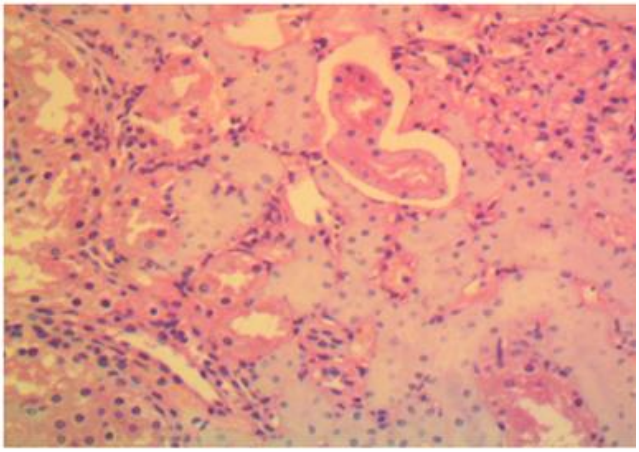

Figure 11. Section of a kidney showing moderate necrosis of tubular (arrow) and glomerular (arrowhead) epithelial cells (x400; H & E)

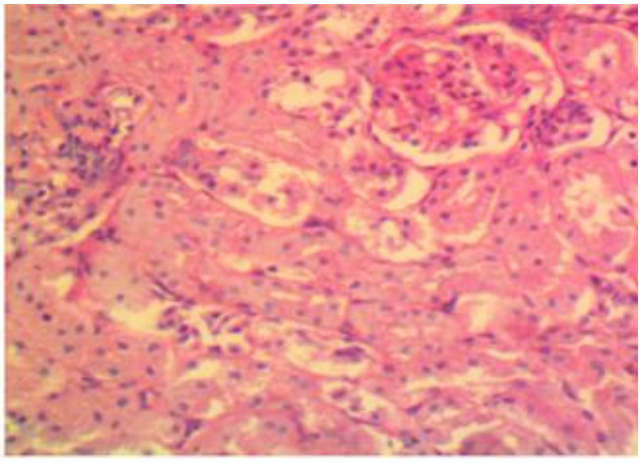

Figure 12. Section of the kidney showing moderate necrosis of tubular (arrow) and glomerular (arrowhead) epithelial cells (x400; H & E)

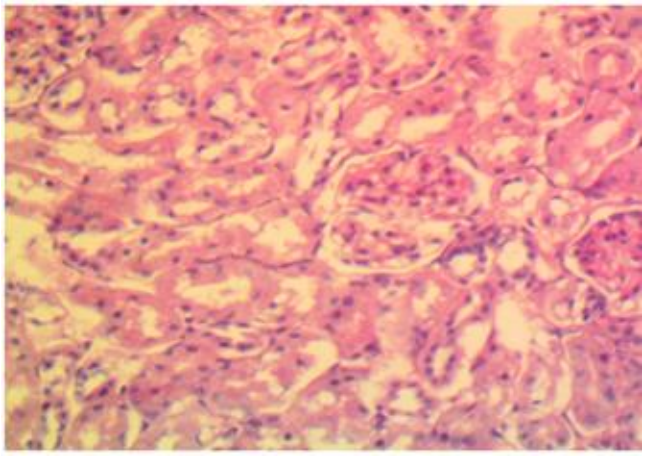

Figure 13. Section of the kidney showing moderate necrosis of tubular (arrow) and glomerular (arrowhead) epithelial cells (x400; H & E)

The deterrence of their synthesis may cause the activity reduction of these antioxidant enzymes by several reactive molecules produced during ethanol metabolism. Also, it could be the result of enzymatic protein oxidation by the induced reactive oxygen species. GSH plays an important role in flushing reactive oxygen species (ROS) and xenobiotic detoxification (Sen, 1997). The decline of endogenous antioxidants is clearly related to ethanol-induced oxidative stress, which is indicated by the cell's formation of toxic acetaldehyde and other reactive molecules. The attained results agree with Hussain et al. (2001) and Molina et al. (2011), showing that chronic ethanol treatment led to a significant decline in the GSH levels of kidneys. The monitored raise in declining GSH levels of mice treated with both *C. longa* and ethanol extracts is probably led by the integrated protective effect of the extract and endogenous GSH. It might also result from the generation of glutathione reductase, which has an important role in lessening oxidized glutathione to decrease at the expense of the NADPH and GSH-GSSG cycles in cells.

The extract administration also yielded a significant decline ($P < 0.05$) in kidney activities of renal biomarkers, i.e., creatinine and urea, when the group was given by only 20% ethanol compared to the normal control group. Still, there was a significant raise ($P < 0.05$) in the group treated with various dosages of extracts when compared to the group given 20% ethanol only. *C. longa* has been indicated to demonstrate strong anti-oxidants (Choi 2009). This might explain its anti-oxidant properties, which enable it to protect the kidneys against the adverse effects of free radicals and reactive oxygen species (ROS). Several systems that produce reactive aldehyde species and reactive oxygen species are activated by chronic alcohol consumption (Maher 1997). These results indicate that *C. longa* extract significantly impedes kidney disorders caused by ethanol. The plants under investigation are rich in

polyphenol compounds, such as flavonoids, as part of their secondary metabolites (Ighodano et al. 2009). This might be explained by its antioxidant property, which prevents the kidney from the dangerous effects of free radicals and reactive oxygen species (ROS). Oxidative stress results from an intrusion of balance between the produced oxidant and the anti-oxidant that supports the oxidant. This is frequently generated by a raise in the production of reactive oxygen species (ROS) and declined activity of the anti-oxidant system (Reddy and Lokesh 1994). Based on Albano's (2002) and Cederbaum et al. (2009) opinions, chronic alcohol consumption induces the creation of free radicals and alters the level of enzymatic and non-enzymatic endogenous antioxidant systems. This leads to oxidative stress with a cascade of effects, thus, affecting the functional and structural integrity of cells and organelle membranes (Das et al. 2008). The tested ethyl acetate extract of *C. longa* has the ability to squelch alcohol-induced oxidative stress in mice.

The MDA level in the kidney significantly ($P < 0.05$) declined in the group administered with various dosages of *C. longa* ethyl acetate extract before being given with 20% ethanol as compared to animals given with only 20% alcohol, but there was significant ($P < 0.05$) raise when compared to the normal control group. Several systems that produce reactive aldehyde species and reactive oxygen species are triggered by chronic alcohol consumption (Maher 1997). This is in accordance with the findings in this study which exhibit a significant raise ($P < 0.05$) in kidney malondialdehyde concentrations in mice administered with ethanol relative to controls. Alcohol metabolism that happens mainly in the liver and kidneys is indicated by the formation of free radicals and reactive oxygen species. Thus, the high level of MDA in the kidney could be featured in the production of free radicals, which generate membrane lipid peroxidation. In addition, the enzyme alcohol dehydrogenase (ADH) is included in the main pathway for alcohol metabolism (Pronk et al. 2002). ADH alters alcohol into toxic acetaldehyde, whose interactions with cell proteins and lipids can lead to free radical formation and reno-cellular detriment. In contrast, compared to mice treated with ethanol only, a marked decrease ($P < 0.05$) in kidney MDA levels is due to the co-treatment from 100 mg/kg bw/day to 350 mg/kg bw/day from *C. longa* extract with ethanol. According to the report of Kumar et al. (2004) and Kassuya et al. (2005), the decline of lipid peroxidation (LPO) levels is caused by the array of antioxidant phytochemicals found in plant extracts. In this study, the free radical scavenging activity shown by the extract is led by the antioxidant molecules in *C. longa* (Khatoon et al. 2005). A tremendous alteration of renal architecture is revealed by histopathological examination of the kidney section of the rats in the ethanol-treated group. Kidney cells were spoiled in mice treated with ethanol, while negligible detriment was seen in mice treated with extracts. The histological results show that it is a physiological recovery in renal tissue when groups treated with various extract doses show signs of protection against toxicity. Evidence can be seen from lessened tubular and glomerular epithelial necrosis.

In conclusion, from this study, it can be suggested that *C. longa* extract provides protection against alcohol-induced nephrotoxicity and oxidative detriment in mice, presumably by acting as an *in vivo* as scavengers of free radicals in vivo or by inducing antioxidant enzymes, drugs detoxifying enzymes, and hindering excessive stimulation from lipid peroxidation.

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Consumption patterns, perceptions and total carotenoids, iron and zinc contents of yellow flesh cassava

ELIZABETH AFRIYIE DUAH, MATILDA STEINER-ASEIDU♥, ANGELINA O. DANQUAH
Department of Nutrition and Food Science, University of Ghana, Legon, Accra, Ghana. ♥email: tillysteiner@gmail.com

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Abstract. *Duah EA, Steiner-Aseidu M, Danquah AO. 2020. Consumption patterns, perceptions, and total carotenoids, iron, and zinc contents of yellow flesh cassava. Biofarmasi J Nat Prod Biochem 18: 13-28*. The main objective was to investigate consumption patterns of white cassava, knowledge, perceptions, and the nutritional profiles of yellow flesh cassava roots and leaves and their relationship to the recommended daily allowance for vitamin A. Total carotenoids, iron and zinc contents in fresh yellow cassava leaves, roots and products (gari, konkonte, boiled cassava roots, and leaves), antioxidant activity, and the in vitro bioaccessibility of the carotenoids were determined using standard methods. A cross-sectional survey was carried out to identify the consumption patterns of white cassava, the knowledge, and the perception of yellow flesh cassava among Ghanaians. Total carotenoids (T.C), iron (Fe) and zinc (Zn) for fresh roots ranged from 4.73±0.11 to 10.11±0.18 µg/g; 87.35±3.18 to 146.25±1.20 mg/100g; 0.30± 0.01 to1.55±0.07 mg/100g, respectively. T.C, Fe, and Zn for fresh leaves ranged from 792.93±0.98 to 2649.20±29.10 µg/g; 118.35±0.070 to 07182.05± mg/100g; 3.75±0.64 to 15.50±0.14 mg/100g respectively. T.C for gari was between 3.21±2.79 and 7.39±1.06 µg/g, iron 118.75±0.64 to 181.85±2.05 mg/100g and zinc, 0.25±0.07 to 0.80±0.14 mg/100g. Iron and zinc contents for the kokonte samples ranged from 101.45±0.64 to 116.30± 0.14 mg/100g and 0.15±0.07 to 0.70±0.01 mg/100g; however, T.C was not detectable. T.C for boiled roots were between 1.22±0.05 and 2.14±0.11µg/g. Iron content for the samples ranged from 118.75±0.64 to 181.85±2.05 mg/100g, and for zinc, 0.60±0.01 to 1.30±0.01 mg/100g. T.C for boiled leaves was between 524.39±9.89 and 1323.5±15.6 µg/g, iron; 95.90±0.01 and 148.75±2.76 mg/100g, and zinc 0.60±0.01-1.30±0.01mg/100g. In vitro bio-accessibility of carotenoids for boiled roots had the highest of 104.42±0.88%, gari had the highest value of 57.22±9.01%, and boiled leaves 0.28±0.01%. Gari recorded the highest frequency of consumption. 36.6% of the respondents knew yellow flesh cassava. 51.2% were willing to accept yellow flesh cassava. Fermentation, drying, roasting, and boiling retained some carotenoids after processing, but solar drying over a long period completely degraded carotenoids in yellow cassava roots. Cassava leaves had higher retention of carotenoids, but cassava roots had more bio-accessible carotenoids. Carotenoids in yellow flesh cassava leaves had antioxidant properties that have the potential to help combat free radicals in the body. Ghanaians had insufficient knowledge and "willingness to accept" yellow cassava. Yellow cassava will serve as a promising source of provitamin A.

Keywords: Carotenoid, cassava, consumption

INTRODUCTION

Cassava is a relevant root tuber, which is typical food for several populations in the tropical and sub-tropical regions of the world (Bradbury and Holloway, 1988). It is the sixth major staple crop after rice, wheat, maize, Irish potato, and sweet potato globally (FAO 2003). Cassava is a crop that grows on soils with marginal nutrition (Aerni 2006). Cassava roots contain small amounts of zinc, iron, and β-carotene; however, it is the primary essential crop of more than 250 million Africans (Gegios et al. 2010); it also reported that consumers who often eat cassava are at higher risk for malnutrition, especially deficiencies in iron, vitamin-A, and zinc than people who consume other diets, particularly those that are predominantly cereal.

Micronutrient deficiencies are becoming more endemic, specifically in West Africa. The most vulnerable in this population include children, juveniles, women in their reproductive years, breastfeeding mothers, and people who have suffered from war and starvation. Iodine, vitamin A and iron deficiencies are of significant public health importance in several places in Africa and are responsible for the increased mortality and morbidity in children in Africa (Black et al. 2008). Micronutrient malnutrition, also known as hidden hunger, reduces the productivity rate and income of individuals and governments in Africa. It also decreases psychological development, decreases working capacity, hinders growth, lowers how immune a person is, causes several pregnancy complications, blindness, and goiter, and increases the possibility of mortality (Darnton-Hill et al. 2005).

The National and Regional Estimates of the prevalence of anemia are 65.7% for children between 6-59 months and 42.4% for women between 15-49 years in Ghana, and vitamin A deficiency is also quite high and of public health concern even though various intervention strategies are being used to combat these micronutrient deficiencies. Cassava is very important to the food security status of most Ghanaian households because it is one of the significant staples. It is consumed in various ways such as cooked fresh roots (that include pounded fresh cassava, known as fufu in Ghana), flour (fermented and un-fermented), granulated, roasted cassava (known as gari), fermented pastes, known as agbelima (MoFA 2009).

Bio-fortification is the creation of essential nutrient-dense staple crops using the best traditional propagation

methods and modern biotechnology (Nestel et al. 2006). Biofortification gives a convenient way of getting to people in the rural and remote areas that are malnourished but do not have access to commercially available fortifies (Graham, 1996). Several staple foods are being fortified. These include rice, sweet potato, maize, and cassava. The International Institute of Tropical Agriculture (IITA), with several partners like HarvestPlus over a while, has developed several bio-fortified cassava genotypes popularly known as yellow flesh cassava, which contains predominantly β-carotene, a provitamin A. Yellow flesh cassava proposes to be an avenue to solving micronutrient deficiencies in Africa. The International Institute of Tropical Agriculture (IITA), in conjunction with the CSIR-Crop Research Institute of Ghana, is currently breeding some cassava genotypes in Ghana.

The objectives of the study were to determine micronutrient composition (carotenoids, iron, and zinc) in the yellow flesh cassava genotypes (fresh roots and leaves, boiled leaves and roots, *gari* and *kokonte*), to determine the effect of processing on retention of micronutrient composition (carotenoids, iron, and zinc) in the yellow flesh cassava genotypes, to ascertain the in-vitro bioaccessibility of the carotenoids in cassava products (boiled leaves and roots, *gari* and *kokonte*), to determine antioxidants properties of the carotenoids in yellow flesh cassava genotypes, to assess the consumption patterns of the white cassava and products and as well as perception and knowledge of yellow flesh cassava among consumers of cassava, to determine the contribution of yellow cassava products to the RDA of vitamin A.

MATERIALS AND METHODS

Laboratory study

The laboratory study comprised moisture analysis, total carotenoids, iron and zinc determination, antioxidant activity, and in vitro bio-accessibility of carotenoids.

Sample collection and preparation

Fourteen yellow flesh cassava genotypes were planted by IITA in Crops Research Institute (CRI) at Pokuase in the Greater Accra Region of Ghana. Before processing, moisture contents, total carotenoids, iron, and zinc determination were determined for all fourteen genotypes. Out of these fourteen genotypes, five were selected by IITA to study the effects of processing on the total carotenoid retention, beta carotene concentration, in-vitro bioaccessibility, and iron and zinc concentration of the cassava genotypes. Reasons for the selected five were because previous trials concluded that these genotypes (01, 03, 05, 07, and 15) had sensory and textural characteristics preferred by farmers as well as high contents of total carotenoids. Yellow flesh cassava samples were collected at the Crops Research Institute (CRI) at Pokuase. Samples were processed and bagged in the Nutrition and Food Science Department, Legon University of Ghana.

Preparation of gari

Cassava roots were peeled, washed, and grated. The grated cassava was put into perforated jute bags, fermented for 24 hours, and dewatered. The grated cassava was then sifted over a raft and roasted over minimal heat till crispiness was achieved to avoid loss and degradation of carotenoids in the cassava. Temperature and lighting were not strictly controlled to mimic the garification methods used among gari processors in Accra and quantify carotenoids lost during the process. The resulting product, *gari*, was allowed to cool and packaged in an airtight bag, and stored in a cool dark place to help prevent carotenoid loss and degradation.

Preparation of kokonte

The cassava roots were harvested from the farm and peeled. The peeled roots were washed and chopped into bigger chunks. The chopped roots were dried evenly in a solar dryer for five days, after which they were milled and packaged in black polythene bags and stored in a cool, dry place for further analysis.

Preparation of boiled roots and leaves

Fresh roots and leaves samples were chopped separately into small pieces, and 5 grams were weighed into a plastic bag, cooked in a stainless-steel pan on an electric cooker for fifteen minutes, and cooled for further analysis.

Determination of moisture content

Moisture content was determined by weighing 2g of the sample (fresh leaves and roots, *gari, kokonte,* boiled leaves, and roots separately) and dried in an air oven at a temperature of 105°C for 24 hours. The samples were removed, cooled over a desiccant for 15 minutes, weighed, and the loss in weight of the sample after drying was calculated as percent moisture (AOAC 2005).

Quantification of Beta carotene in yellow cassava genotypes

Beta carotene in yellow cassava genotypes and their products (fresh roots and leaves, *gari, kokonte,* boiled leaves, and roots) were assessed using high-performance liquid chromatography (HPLC). Five grams (5g) of each sample was weighed on a digital balance (Professional electronic balance) into a mortar and ground with pyrogallol to prevent oxidation of the carotenoids. The carotenoids were extracted by successive additions of 25 mL of acetone to the weighed sample. The final solution was transferred into a sintered funnel (5μm) coupled to a 250 mL Buchner flask and filtered under a vacuum. This procedure was repeated three times, or until the sample became colorless. The obtained extract was transferred to a 500 mL separatory funnel containing 20ml of petroleum ether. The acetone was removed by slowly adding ultrapure water to prevent emulsion formation, and the aqueous phase was discarded. This procedure was repeated four times until no residual solvent remained. The extract was then transferred to a 50 mL volumetric flask through a funnel. The sample was evaporated with a rotary vacuum evaporator to dryness. The concentrate was reconstituted

with 2ml of acetone and put into vials for HPLC analysis (Rodriguez-Amaya and Kimura 2004).

Determination of total carotenoids in fresh yellow cassava roots

The iCheck ™ carotene measures total carotenoids in vitamin premix, food, and biological fluids. iCheck Carotene measures the color in the test vial and calculates the total carotenoid content in mg/L. The principle of the method is photometric, and its sample volume per analysis is 400 µL (0.4 mL). It works in a linear range of 0.15-25.0 mg/L. The device's time per report is < 2 min, and its variation is < 10%. ICheck™ carotene has been validated against HPLC (Bioanalyt 2014).

Fresh yellow cassava roots

About 5g of the fresh yellow cassava roots were weighed and ground to a smooth paste to help solubilize all the carotenoids present in the sample and transferred into a falcon tube and made to 25ml mark with distilled water. 0.4ml of the diluted sample was injected into the reagent vial. The vial was vigorously shaken for 10 seconds till the vial's content appeared as one uniform solution. The vial was made to stand still for 5 minutes until the solution in the vial appeared in two distinct phases. The vial with the sample was measured with iCheck™ Carotene earliest 5 minutes and the latest 1 hour after sample injection. The total carotenoid was calculated by multiplying the iCheck™ reading with the dilution factor (Bioanalyt 2014).

Fresh yellow cassava leaves

The procedure was modified for the leaves because they contained excessive amounts of carotenoids, which was above the working range of the check device; thus, 0.5g of the fresh yellow cassava leaves were weighed and ground to a smooth paste and transferred into a falcon tube and made to the 50 ml mark with distilled water. 0.4 ml of the diluted sample was injected into the reagent vial. The vial was vigorously shaken for 10 seconds till the vial's content appeared as one uniform solution. The vial was made to stand still for 5 minutes until the solution in the vial appeared in two distinct phases. The vial with the sample was measured with iCheck™ Carotene earliest 5 minutes and the latest 1 hour after sample injection. The total carotenoid was calculated by multiplying the iCheck™ reading with the dilution factor **(**Bioanalyt 2014).

Kokonte made from yellow flesh cassava

Two grams (2g) of the sample were weighed and ground to a smooth paste, transferred into a falcon tube, and made a 30 ml mark with distilled water. 0.4 ml of the diluted sample was injected into the reagent vial. The vial was vigorously shaken for 10 seconds until the vial's content appeared as one uniform solution. The vial was made to stand still for 5 minutes until the solution in the vial appeared in two distinct phases. The vial with the sample was measured with iCheck™ Carotene earliest 5 minutes and the latest 1 hour after sample injection. The total carotenoid was calculated by multiplying the iCheck™ reading with the dilution factor (Bioanalyt 2014). The procedure was modified for kokonte because it had a high swelling capacity, which blocked the syringe during the injection into the vials of the icheck device.

Yellow cassava gari

The procedure was modified for gari because it had a high swelling capacity which blocked the syringe during the injection into the vials of the iCheck device; thus, two grams (2g) of the sample was weighed and ground to a smooth paste and transferred into a falcon tube and made to 30 ml mark with distilled water. 0.4ml of the diluted sample was injected into the reagent vial. The vial was vigorously shaken for 10 seconds till the content of the vial appeared as one uniform solution, and the vial was made to stand still for 5 minutes until the solution in the vial appeared in two distinct phases. The vial with the sample was measured with iCheck™ carotene earliest 5 minutes and the latest 1 hour after sample injection. The total carotenoid was calculated by multiplying the iCheck™ reading with the dilution factor (Bioanalyt 2014).

Determination of iron and zinc contents

Iron and zinc contents in yellow cassava products (fresh and boiled roots and leaves, gari, and kokonte) were assessed using AOAC (2005) method. Wet digestion was used to digest samples. The weight of 0.1g of each sample (*fresh and boiled roots and leaves, gari, and kokonte)* was digested in 4ml of concentrated sulphuric acid and heated in a fume hood. Ten drops of H_2O_2 were added to the digest and heated from black to dark brown. Six drops of hydrogen peroxide (H_2O_2) were added and heated until the digest was colorless. It was then transferred to a 100 ml volumetric flask and made up to the 100ml mark with deionized water. The wet digest solutions were prepared on the same day for each sample as the check sample and duplicate blanks. The Atomic Absorption Spectrophotometer determined iron and zinc contents (Analyst 400 Perkin Elmer).

Determination of antioxidants properties of carotenoids in yellow flesh cassava

Antioxidant assays were based on measuring the loss of 2, 2-Diphenyl 1, 1-picrylhydrazyl (DPPH) color at 515 nm after reaction with test samples, and the reaction was monitored by a spectrophotometer. The percentage of the DPPH remaining was proportional to the antioxidant concentration of the test samples. One gram (1g) of each sample (fresh and boiled roots and leaves, *gari, and kokonte)* was ground, and carotenoids were extracted using 50ml of 95% methanol as solvent. The solution was filtered using a Whatman filter paper (grade F101 with a pore size of 125mm) till the residue was white. 3ml of the filtrate was added to 3ml of DPPH, vortexed, and allowed to stay in the dark for 30 minutes. The absorbance was read using the Atomic absorption spectrophotometer (T80 UV/VIS spectrophotometer-PG instruments Ltd) using a wavelength of 450nm (Bondet et al. 1997).

Determination of carotenoid retention after processing

For the estimation of true carotenoid retention, the following formula was used:

$$\% \text{ True Retention} = \frac{c_1 \times w_b}{c_2 \times w_{ub}} \times 100$$

Where: c_1 is the carotenoid content in the processed sample, c_2 is the carotenoid content in the fresh (unprocessed) sample, w_b is the weight of the processed sample, and w_{ub} is the weight of the fresh (untreated) sample. Carotenoid content (µg/g) was calculated on a dry matter basis for both the processed and unprocessed root samples (Murphy et al. 1975).

Determination of in vitro bio-accessibility of carotenoids in yellow flesh cassava

The method was adapted from Hedre´n et al. (2002) with some modifications. Three grams (3g) of the cassava sample were weighed and ground in a mortar. For the oral phase of the digestion, 0.003grams alpha-amylase was added to 0.005M saline solution (NaCl). About 7.5 ml of the saline solution was added to the sample and sonicated in a water bath at a temperature of 37ºC and a pH of 6.8. After 10 minutes, the sample solution was acidified to a pH of 3 using 0.01M HCl to stimulate the gastric phase. 1ml of porcine pepsin (40mg/ml) was added to the sample solution at pH three and incubated for an hour at 37ºC, after which pH was brought up to 7 to stimulate the intestinal phase of the digestion. About 4.5 ml of a mixture of porcine pancreatin (4mg/ml) and porcine bile extract (24 mg/ml) was added to the simulated gastric fluid, and 1% (w/v) alpha-tocopherol. The final volume was adjusted to 25ml with a physiological saline solution and incubated in a shaking water bath (100 rpm at 37ºC) for two hours. It was then centrifuged at 5,000 g for 45 minutes at 4ºC. The supernatant was collected with a plastic pipette into a separatory funnel containing 20 ml of 25% (w/v) NaCl. 20 ml of 95% ethanol and 15 ml of petroleum ether were added and allowed to separate. The solution was washed with water, the organic phase was collected, and the absorbance was read with an Atomic Absorption spectrophotometer at 450nm.

Contribution of yellow cassava to recommended daily allowances for under-five children

The contribution of yellow cassava to the recommended daily allowance for vitamin A was calculated first using a conversion factor of 1µg/g of beta carotene =3.3IU of vitamin A (Bioanalyt, 2015) to get the amount of vitamin A in the food. After that,

An example of one of the samples is shown below:

1µg/g of beta carotene in food sample =3.3IU of vitamin A 3.439ug /g of food sample =3.3IU *3.439

= 11.348 IU

Thus if every 1g of food contained 11.34IU of vitamin A, then an allowance of 200IU, which is the upper limit, will be:

=2000/11.35

=176g of food

Thus, for every 100grams = (2000*100)/176

=1136.36 IU

To find the %RDA= (1136.36/2000)*100

This was also done using the lower limit and an average to give the final % RDA

Cross-sectional study

A survey was conducted to describe white cassava consumption patterns and the perception and knowledge of participants on yellow flesh cassava. A pretested semi-structured questionnaire was used to interview adult Ghanaians. Data on their socio-demographic characteristics were also collected.

Study area and population

The study area was selected in the Greater Accra region and was chosen for convenience and because the cassava genotypes were planted in Greater Accra. The study population comprised individuals above 18 years of age who consumed cassava. This population was chosen as representative of cassava consumers.

Sample size determination and sampling technique for the survey

Simple random sampling was employed in subject selection for the survey, and a sample size of 287 was used based on the formulae by

$n_0 = Z^2\, pq/E^2$

n_0= sample size, Z= confidence interval (1.96), p=α level (0.5), q=1-p, E= margin of error(0.05) (Cochran 1963).

Data collection

Data were collected on background information, consumption, usage of cassava, knowledge, and the perception of yellow flesh cassava using a pretested questionnaire.

Socio-demographic characteristics

Data were collected on participants'" age, marital status, educational level, occupation, residential status, income, and household size.

Consumption and usage of white cassava

Data were collected on the frequency of cassava and cassava products consumed, time of consumption, reasons for cassava consumption, and knowledge of the nutritional value of white cassava.

Knowledge and perception of yellow flesh cassava

Participants were interviewed on knowledge about yellow cassava, source of information about yellow flesh cassava, willingness to accept yellow flesh cassava, and perceived usage of yellow cassava.

Data analyses

The data analysis tools used were Statistical Package for Social Sciences version 16(SPSS), MINITAB version 17, Statistical Analysis System version 9.01 (SAS), and Microsoft Excel.

Laboratory data

Means and standard deviations were computed using Microsoft Excel. Analysis of variance was used to compare means for laboratory analyses.

Survey data

Descriptive statistics were computed using SPSS version 17 for demographic data and consumption patterns of knowledge on yellow cassava. Logistic regression was computed using the Statistical Analysis System version 9.01. Multinomial regression was used, and this provided the opportunity to see the interaction between more than two categories of an outcome (knowledge and "willingness to accept" yellow flesh cassava) and predictors (in this case, selected socio-demographic factors). The socio-demographic factors, education status, marital status, age, and gender were considered independent variables.

Ethical consideration

Ethical clearance was sought from the College of Basic and Applied Science (CBAS) ethical clearance board. Informed consent was also sought from all participants interviewed.

RESULTS AND DISCUSSION

This study was primarily in two sections; laboratory analysis and a survey. The laboratory analysis focused on determining the total carotenoids, iron, and zinc of fourteen yellow flesh cassava genotypes. We also determined the effect of processing on the nutrients and the antioxidant activity of the carotenoids present in the roots and leaves of the selected yellow cassava genotypes. The same analysis was carried out on some selected yellow cassava products (gari, boiled cassava roots, leaves, and kokonte). The breeder's names of the cassava genotypes are in Appendix III. The survey provided information on the consumption patterns of white cassava among Ghanaian consumers and their willingness to accept the yellow flesh cassava. The survey also determined associations between socio-demographic characteristics and consumption patterns.

Laboratory analysis

Fourteen yellow flesh cassava genotypes were planted by IITA in Crops Research Institute (CRI) at Pokuase in the Greater Accra Region of Ghana. Before processing, moisture contents, total carotenoids, iron, and zinc determination were determined for all fourteen genotypes. Out of these fourteen genotypes, five were selected by IITA to study the effects of processing on the total carotenoid retention, beta carotene concentration, in-vitro bioaccessibility, iron, and zinc concentration of the cassava genotypes. Iron, zinc, and carotenoid contents were reported on a dry matter basis.

Total carotenoids, iron, and zinc contents of fresh yellow cassava roots

Fresh yellow cassava roots had a moisture content between 67.05±1.09% (sample 08) and 82.123±0.76% (sample 15) (Table 1). Total carotenoids ranged from 4.73±0.11 µg/g (sample 07) to 10.11±0.18µg/g (sample 09). Iron content for the samples ranged from 87.35±3.18 to 146.25±1.20 mg/100g (samples 06 and 03), and for zinc, 0.30±0.01 to 1.55±0.07 mg/100g (samples 06 and 14). There were significant differences among all samples ($p<0.05$) for dry matter, total carotenoids, iron, and zinc.

Total carotenoids, iron, and zinc contents of fresh yellow cassava leaves

The moisture content was between 56.06±0.33% (sample 09) and 81.62±0.52% (sample 10) (Table 2). T.C ranged from 792.93±0.98 µg/g (sample 08) to 2649.20±29.10 µg/g (sample 07). Iron and zinc values ranged between 118.35±0.07 and 182.05±0.07 mg/100g (sample 08 and sample 01) and 3.75±0.64 and 15.50±0.14 mg/100g (sample 08 and 10). There were significant differences among all samples for each parameter measured ($p<0.05$).

Table 1. Total carotenoids, iron, and zinc contents of fresh yellow flesh cassava roots (d.m.b)

Sample	Moisture content (%)	Total carotenoid (µg/g)	Iron (mg/100g)	Zinc (mg/100g)
01	78.30 ± 0.96^{abc}	7.85 ± 0.07^{d}	76.10 ± 1.27^{g}	0.45 ± 0.07^{def}
02	76.17 ± 0.25^{bcde}	4.67 ± 0.03^{g}	98.70 ± 0.85^{d}	0.70 ± 0.14^{bcdef}
03	71.60 ± 1.15^{efgh}	5.77 ± 0.07^{f}	146.25 ± 1.20^{a}	0.85 ± 0.070^{bcde}
05	68.40 ± 1.74^{gh}	5.69 ± 0.04^{f}	112.20 ± 0.14^{d}	0.95 ± 0.07^{bc}
06	79.08 ± 0.32^{abc}	5.02 ± 0.07^{g}	87.35 ± 3.18^{f}	0.30 ± 0.01^{f}
07	76.21 ± 1.04^{bcd}	4.73 ± 0.11^{g}	111.00 ± 0.42^{d}	0.60 ± 0.14^{cdef}
08	67.05 ± 1.09^{h}	6.57 ± 0.28^{e}	131.05 ± 1.06^{b}	0.60 ± 0.14^{cdef}
09	75.55 ± 1.99^{cdef}	10.11 ± 0.18^{a}	88.65 ± 0.21^{f}	0.40 ± 0.01^{ef}
10	70.22 ± 0.57^{gh}	9.21 ± 0.14^{b}	121.90 ± 0.01^{c}	1.05 ± 0.07^{bc}
11	71.39 ± 2.32^{fgh}	6.46 ± 0.04^{e}	123.85 ± 2.76^{c}	0.90 ± 0.01^{bcd}
12	72.76 ± 0.42^{defg}	6.43 ± 0.14^{e}	143.45 ± 2.33^{a}	0.70 ± 0.28^{bcdef}
13	80.33 ± 0.58^{ab}	8.43 ± 0.07^{c}	121.55 ± 0.21^{c}	1.10 ± 0.14^{ab}
14	70.98 ± 0.19^{fgh}	6.16 ± 0.04^{ef}	126.15 ± 0.49^{bc}	1.55 ± 0.07^{a}
15	82.12 ± 0.76^{a}	7.69 ± 0.04^{d}	110.25 ± 0.07^{d}	0.70 ± 0.01^{bcdef}
P-value	0.00	0.00	0.00	0.00

Note: Means with different superscripts on the same column are significantly different at $P \leq 0.05$.

Table 2. Total carotenoids, iron, and zinc contents of fresh yellow cassava leaves (d.m.b)

Sample	Moisture content (%)	Total carotenoids (µg/g)	Iron (mg/100g)	Zinc (mg/100g)
01	63.65±0.89cde	2224.51±2.84b	182.05±0.07a	6.85±0.07h
02	63.65±0.89ef	2223.80±28.90b	147.20±0.28ab	8.95±0.07def
03	61.82±0.31f	862.20±21.50g	177.3±46.60a	7.80±0.70gh
05	70.69±0.55b	2034.74±8.23c	147.6±25.00ab	13.42±0.16bc
06	69.70±0.49b	1798.76±2.76d	163.85±1.34f	8.55± 0.07efg
07	67.37±0.58bcd	2649.20±29.10a	181.55±0.63d	4.25±0.21i
08	61.01±1.43f	792.93±0.98g	118.35±0.07b	3.75±0.64i
09	56.05±0.32g	2593.90±101.80a	181.75±0.64a	12.75±0.07c
10	81.62±0.52a	2312.53±12.56b	143.40±0.14ab	15.50±0.14a
11	60.79±1.40f	991.85±8.86f	165.25±0.35ab	9.75± 0.07d
12	68.21±1.40bc	2042.74±1.86c	152.90±0.14ab	8.25±0.07fg
13	62.60±0.45ef	1810.28±4.86d	174.40±0.42ab	7.50±0.28gh
14	65.92±0.10cde	1171.15±0.95e	168.10±0.14ab	9.40±0.01de
15	64.12±1.05def	1133.16±1.92e	129.65±0.21ab	14.30±0.14b
P-value	0.00	0.00	0.00	0.00

Note: Means with different superscripts on the same column are significantly different at $P \leq 0.05$

Total antioxidant activity of fresh yellow cassava roots and leaves

The overall antioxidant activity of fresh roots (Table 3) ranged from non-detectable (ND) to 86.28±0.10% (sample 02). Fresh leaves had total antioxidant activity between 17.39±0.10% for (sample 03) and 70.96±0.10% (for sample 12). The antioxidant activity was also expressed in Alpha-Tocopherol units. For fresh leaves, antioxidant activity ranged from 13.64±0.05 Alpha Tocopherol units (sample 12) to 38.81±0.05 Alpha Tocopherol units (sample 03). For cassava roots, values ranged between ND to 8.53±0.05 Alpha Tocopherol units (sample 02). There were significant differences among all samples for each parameter measured *(p<0.05).*

Total carotenoids, iron, and zinc contents of yellow flesh cassava products

Five out of the fourteen genotypes were selected based on farmer preferences and previous trials by IITA to study further the effect of processing on total carotenoids, iron and zinc content, antioxidant activity, in-vitro bioaccessibility, and beta-carotene concentration. The selected genotypes were 01, 03, 05, 07, and 15 were made into gari, kokonte, boiled, and boiled leaves.

Total carotenoids, iron, and zinc contents of yellow flesh gari

Dry matter content was between 94.74±0.07% and 97.12±0.09% for samples 05 and 07, respectively. Total carotenoids were between 7.39±1.06 µg/g (sample 07) and 3.21±2.79 µg/g (sample 05). Iron content for the samples ranged from 118.75±0.64 to 181.85±2.05 mg/100 g (samples 07 and 15), and for zinc, 0.25±0.07 to 0.80±0.14 (samples 01 and 15). There were significant differences among all samples (p<0.05) for dry matter, total carotenoids, iron, and zinc (Table 4).

The nutrient profile of yellow flesh kokonte

Table 5 shows the Total carotenoids, iron, and zinc contents of *kokonte*. Moisture content ranged from 11.30±0.15% (sample 07) to 13.79±0.46% (sample 05). Dry matter content ranged between 86.21±0.46% (sample 05) and 88.56±0.28% for sample 01. Iron content ranged from 101.45±0.64 to 116.30±0.14 mg/100 g (samples 01 and 07) and for zinc, 0.15±0.07 to 0.70±0.01 mg/100 g (samples 07 and 03). Total carotenoids were not detectable (ND) in all *kokonte* samples. There were significant differences among all samples *(p<0.05)* for dry matter, total carotenoids, iron, and zinc.

Total carotenoids, iron, and zinc contents of boiled yellow flesh cassava roots

Dry matter content was between *23.20±0.60%* (sample 05) and *30.58±0.81%* (sample 07). Moisture content ranged between *69.43±0.81*% (sample 07) and *76.80±0.60%* (sample 05) (Table 6). Total carotenoids were between *1.22±0.05* µg/g (samples 05) and *2.14±0.11*µg/g (sample 15). Iron content for the samples ranged from *118.75±0.64* to *181.85±2.05* mg/100 g (samples 07 and 15), and for zinc, *0.60±0.00* to *1.30±0.00* (samples 07 and 03). There were significant differences among all samples for each parameter measured (p<0.05).

Total carotenoids, iron, and zinc contents of boiled yellow flesh cassava leaves

Samples recorded moisture content of between 72.00±2.97% (sample 15) and 77.80±0.28% (sample 03) (Table 7). Dry matter content ranged between 28.00±2.97% (sample 15) and 22.200±0.28% (sample 03). Total carotenoids were between 524.39±9.89 µg/g (sample 07) and 1323.5±15.6µg/g (sample 03). Iron values were between 95.90±0.01 mg/100g for sample 05 and 148.75±2.76 mg/100g for sample 15. Zinc values 0.60±0.01mg/100 g for sample 07 and 1.30±0.01mg/100 g for sample 03 respectively. There were significant differences among all samples *(p<0.05)* (Table 8).

Table 3. Total antioxidant activity of fresh yellow cassava roots and leaves (d.m.b)

Sample	Fresh roots (%)	Fresh roots (AT units)	Fresh leaves (%)	Fresh leaves (AT units)
01	81.85±0.10^{a}	8.53±0.05^{a}	62.45 ±0.10^{b}	17.64 ±0.05^{m}
02	86.28±0.10^{b}	6.44±0.05^{b}	51.03±0.01^{e}	23.00±0.01 ^{j}
03	*ND^{c}	*ND^{c}	17.39±0.10^{n}	38.81±0.05^{a}
05	*ND^{c}	*ND^{c}	26.31±0.27^{m}	34.61±0.13^{b}
06	*ND^{c}	*ND^{c}	50.44±0.21^{f}	23.28±0.10^{i}
07	*ND^{c}	*ND^{c}	41.63±0.01^{j}	27.42±0.01^{e}
08	*ND ^{c}	*ND ^{c}	37.67±0.27^{l}	29.28±0.13^{c}
09	*ND ^{c}	*ND ^{c}	57.13± 0.10^{d}	20.14±0.05^{k}
10	*ND^{c}	*ND^{c}	48.20±0.01^{g}	24.33±0.01^{h}
11	*ND^{c}	*ND^{c}	44.47±0.01^{h}	26.08±0.01^{g}
12	*ND^{c}	*ND^{c}	70.96±0.10^{a}	13.64±0.05^{n}
13	*ND^{c}	*ND^{c}	38.97±0.18^{k}	28.67±0.08^{d}
14	*ND ^{c}	*ND ^{c}	43.64±0.10^{i}	26.47±0.05^{f}
15	*ND^{c}	*ND^{c}	59.85±0.20^{i}	18.86±0.10^{l}
P-value	0.00	0.00	0.00	0.00

Note: *ND means not detectable; means with different superscripts on the same column are significantly different at P ≤ 0.05. AT-alpha-tocopherol units

Table 4. Total carotenoids, iron, and zinc contents of yellow flesh gari (d.m.b)

Sample	Moisture content (%)	Dry matter (%)	Total carotenoids (g/100g)	Iron (mg/100g)	Zinc (mg/100g)
01	3.36±0.11^{c}	96.64±0.12^{b}	7.37±0.51^{a}	143.40±0.57^{c}	0.25±0.07^{b}
03	3.19±0.02^{c}	96.81±0.02^{b}	6.00±0.45^{ab}	156.10±1.13^{b}	1.10±0.14^{a}
05	5.25±0.07^{a}	94.75±0.07^{d}	3.21±2.79^{b}	125.50±0.28^{d}	0.80±0.01^{a}
07	2.88±0.09^{d}	97.12±0.09^{a}	7.39±1.06^{a}	118.75±0.64^{e}	0.35±0.07^{b}
15	4.01±0.08^{b}	95.99±0.08^{c}	6.96±0.33^{a}	181.85±2.05^{a}	0.80±0.14a
P-value	0.00	0.00	0.00	0.00	0.00

Note: P-value <0.05; Means that do not share a letter are significantly different

Table 5. Total carotenoids, iron, and zinc contents of yellow flesh kokonte (d.m.b)

Sample	Moisture content (%)	Dry matter (%)	Total carotenoids (µg/g)	Iron (mg/100g)	Zinc (mg/100g)
01	11.44±0.28^{b}	88.56±0.28 ^{a}	ND*	101.45±0.64^{c}	0.30±0.01^{c}
03	11.71±0.145^{b}	88.29±0.15^{a}	ND*	113.35±0.21^{b}	0.70±0.01^{a}
05	13.80±0.46 ^{a}	86.21±0.46^{b}	ND*	112.10±0.42^{b}	0.50±0.01^{c}
07	11.53±0.11^{b}	88.46±0.11^{a}	ND*	116.30±0.14 ^{a}	0.15±0.07^{b}
15	11.30±0.152^{b}	88.70±0.15 ^{a}	ND*	116.40 ±0.00^{a}	0.15±0.07^{c}
P-value	0.00	0.00	0.00	0.00	0.00

Table 6. Total carotenoids, iron, and zinc contents of boiled yellow flesh cassava roots (d.m.b)

Sample	Moisture content (%)	Dry matter (%)	Total carotenoids (µg/g)	Zinc (mg/100g)	Iron (mg/100g)
01	71.60±1.09b	28.40±1.09^{a}	2.12±0.163^{a}	0.65±0.07^{a}	143.40±0.57^{c}
03	70.90±1.09 ^{b}	29.10±1.09^{a}	1.35±0.05^{b}	1.30±0.01^{ab}	156.10±1.13^{b}
05	76.80±0.60^{a}	23.20±0.60^{b}	1.22±0.05^{b}	1.15±0.21^{bc}	125.50±0.28^{d}
07	69.43±0.81^{b}	30.57±0.81^{a}	2.07±0.05^{a}	0.60±0.01^{c}	118.75±0.64^{e}
15	72.59±0.52^{b}	27.41±0.52^{a}	2.14±0.11^{a}	0.80±0.01^{c}	181.85±2.05^{a}
P-value	0.00	0.00	0.00	0.00	0.00

Note: *ND means not detectable; means with different superscripts on the same column are significantly different at P ≤ 0.05.

Table 7: Total carotenoids, iron, and zinc content of boiled yellow flesh cassava leaves (d.m.b)

Sample	Moisture content (%)	Dry matter (%)	Total carotenoids (µg/g)	Iron (mg/100g)	Zinc (mg/100g)
01	72.40±1.27^{a}	27.60±1.27^{a}	633.23±3.62^{c}	14.80± 0.14^{d}	0.65±0.07^{c}
03	77.80±0.28^{a}	22.20±0.28^{a}	1323.50±15.60^{a}	148.25±0.07^{b}	1.30±0.01^{c}
05	75.55±0.35^{a}	24.85±0.35^{a}	662.40±44.10^{c}	95.90±0.01^{c}	1.15±0.21^{ab}
07	72.95±1.06^{a}	27.05±1.06^{a}	524.39±9.89^{d}	113.10±1.13^{a}	0.60±0.01^{a}
15	72.00±2.97^{a}	28.00 ±2.97^{a}	1194.79±10.01^{b}	148.75±2.76^{a}	0.80±0.01^{bc}
P-value	0.00	0.00	0.00	0.00	0.00

Note: Means with different superscripts on the same column are significantly different at P ≤ 0.05.

Table 8. Beta carotene in selected fresh yellow cassava and their products (d.m.b)

Sample	Fresh cassava roots (µg/100g)	Boiled Cassava roots (µg/100g)	Gari (µg/100g)	Kokonte (µg/100g)	Fresh cassava leaves (µg/100g)	Boiled cassava leaves (µg/100g)
01	343.99	79.05	0.54	*ND	691.86	12.78
03	72.26	40.13	3.02	*ND	*ND	417.85
05	6.74	14.88	ND	3.94	1911.91	1875.15
07	20.24	104.99	0.10	*ND	1213.78	1190.45
15	160.04	21.42	1.65	*ND	97.47	95.60

Note: *ND means not detectable

Total antioxidant activity of yellow flesh cassava products

Total antioxidant activity for boiled roots, *kokonte*, and *gari* were not detectable. For boiled leaves, values ranged between not detectable and 97.52±0.47%. There was a significant difference among all samples ($p<0.05$) (Table 9). The antioxidant activity was also expressed in Alpha-Tocopherol units. For boiled leaves, antioxidant activity ranged from 1.17±0.22 to 6.94±0.05 Alpha Tocopherol units. There was a significant difference among all samples ($p<0.05$) (Table 10).

Total carotenoid retention in yellow cassava products after processing

Table 11 shows carotenoid retention in yellow cassava products after processing. *Gari* had retention between 33.88±1.73% and 61.15±0.01% for samples 05 and 07, respectively. Carotenoid retention in boiled roots was between 23.47±1.32% (sample 03) and 43.89±0.01% (sample 07). Boiled leaves showed retention of between 19.79±0.16% and 153.52±2.02%. There was a significant difference among all samples ($p<0.05$).

Effect of processing and variety on total carotenoids, iron and zinc contents of yellow flesh cassava

Cassava processing had a significant effect on dry matter and the total carotenoids *($p<0.05$)* but had no significant effect on iron and zinc contents *($p<0.25$)*, as presented in (Table 12). The type of cultivar, on the other hand (Table 13), had a significant effect ($p<0.05$) on the dry matter, iron, and zinc contents but had no significant effect on the total carotenoids ($p>0.05$).

Table 9. Total antioxidant activity (%) of yellow flesh cassava products

Sample	Boiled roots	Boiled leaves	Kokonte	Gari
01	*ND	90.36 ±0.10^{a}	*ND	*ND
03	*ND	96.03 ±0.72^{b}	*ND	*ND
05	*ND	0.00±0.01^{c}	*ND	*ND
07	*ND	97.51±0.45^{d}	*ND	*ND
15	*ND	85.26±0.03^{e}	*ND	*ND
P-value	0.00	0.00		0.00

Note: *ND means not detectable; means with different superscripts on the same column are significantly different at $P \leq 0.05$.

Table 10. Total antioxidant activity in yellow cassava products in alpha-tocopherol units

Sample	Boiled leaves	Boiled roots	Kokonte	Gari
01	4.53±0.05^{b}	*ND	*ND	*ND
03	1.86±0.34^{c}	*ND	*ND	*ND
05	*ND^{e}	*ND	*ND	*ND
07	1.17±0.22^{d}	*ND	*ND	*ND
15	6.94±0.05^{a}	*ND	*ND	*ND
P-value	0.00	0.00	0.00	0.00

Note: *ND means not detectable; means with different superscripts on the same column are significantly different at $P \leq 0.05$.

Table 11. Total carotenoid retention (%) in yellow cassava products after processing

Sample code	Boiled roots	Gari	Kokonte	Boiled leaves
01	26.96±1.79	39.08±0.43	*ND	28.47±0.20
03	23.48±1.32	39.85±0.82	*ND	153.52±2.02
05	21.44±1.09	33.88 ±1.73	*ND	32.56±2.30
07	43.89±0.01	61.15±0.01	*ND	19.79±0.16
15	27.77±1.55	35.17±0.19	*ND	105.44±0.70

Note: *ND means not detectable; means with different superscripts on the same column are significantly different at $P \leq 0.05$.

Table 12. Statistical effect of processing on total carotenoids, iron and zinc contents of yellow flesh cassava

Sample	Mean total carotenoids(µg/g)	Mean dry matter (%)	Mean iron (mg/100g)	Mean zinc (mg/100g)
Fresh roots	6.40^{c}	37.52^{c}	114.18^{a}	0.78 ^{a}
Gari	6.20^{c}	96.26^{a}	145.12^{a}	0.66 ^{a}
Kokonte	0.00^{c}	88.05^{b}	111.92a	0.36 ^{a}
Boiled roots	1.80^{c}	27.70^{d}	104.20^{a}	0.90 ^{a}
Boiled leaves	867.70^{b}	26.18^{d}	104.20^{a}	0.90 ^{a}
Fresh leaves	1780.80^{a}	33.99^{cd}	159.52^{a}	9.36^{a}
P-Value	0.01	0.01	0.25	0.25

Note: Means with different superscripts on the same column are significantly different at P ≤ 0.05.

Table 13. Statistical effect of cultivar on total carotenoids, iron and zinc contents of yellow flesh cassava

Cultivar	Mean total carotenoids (%)	Mean dry matter (%)	Mean iron (mg/100g)	Mean zinc (mg/100g)
01	442.90a	53.14b	88.8b	1.53a
03	338.80a	54.11b	148.24a	2.18a
05	416.80a	51.88b	114.87ab	2.99a
07	491.00a	53.58b	125.63ab	1.09a
15	361.30a	62.52a	141.16a	1.86a
P-value	**0.74**	**0.03**	**0.00**	**0.00**

Note: Means with different superscripts on the same column are significantly different at P ≤ 0.05.

Effects of processing and cultivar on antioxidant activity of yellow flesh cassava

Processing and cultivar significantly affected the antioxidant activity of yellow flesh cassava (p-value 0.0001) (Tables 14 and 15).

Contribution of yellow flesh cassava to RDA of vitamin A for under-5 children

The percentage of recommended daily allowance (RDA) met for vitamin A per 100 grams of fresh yellow cassava for children under five years was between 1.39% (sample 05) and 70.95% for sample 01. The percent RDA was between 3.07% and 21.66% for boiled roots for samples 05 and 07, respectively. *Kokonte* had an RDA of between *ND* to 0.81%; fresh leaves reported values between 20.11% and 394.39% for samples 15 and 05, respectively. Boiled leaves also had values between 2.64% for sample 01 and 245.56% for sample 05 (Figure 1).

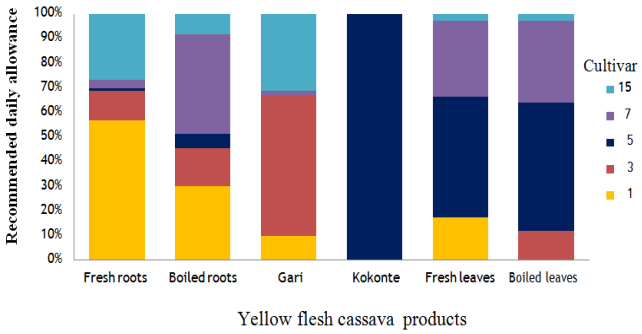

Figure 1. Contribution of yellow flesh cassava to recommended daily allowance (RDA) of vitamin A for children under-5

Comparison of icheck^{TM} carotene and spectrophotometric method for carotenoid determination

The spectrophotometric method for determining total carotenoids compared to the icheck^{TM} carotene method for fresh roots and gari. Regression (R^2) of 96.49% and 95.31% were obtained (Figures 2 and 3).

Table 14. Statistical effects of processing on antioxidant activity of yellow flesh cassava

Treatment	Mean total antioxidant activity (%)
Fresh roots	16.37c
Gari	0.00d
Kokonte	0.00d
Boiled roots	0.00d
Boiled leaves	37.88b
Fresh leaves	73.83a
P-value	0.01

Note: Means with different superscripts on the same column are significantly different at P ≤ 0.05.

Table 15. Statistical effect of cultivar on antioxidant activity of yellow flesh cassava

Cultivar	Mean total antioxidant activity (%)
01	43.99a
03	21.27b
05	4.93c
07	26.09b
15	23.79b
P-value	0.01

Note: Means with different superscripts on the same column are significantly different at P ≤ 0.05.

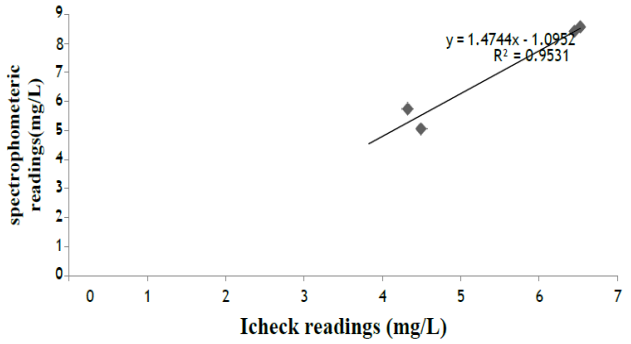

Figure 2. Relationship between iCheck™ carotene and spectrophotometric method (*Gari)*

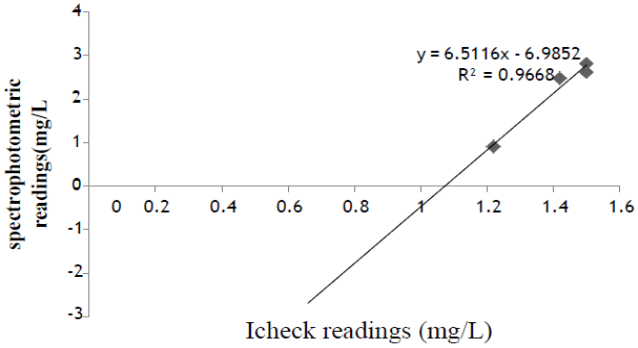

Figure 3. Relationship between iCheckTM carotene and spectrophotometric method (Fresh roots)

In vitro bio-accessibility of carotenoids in yellow flesh cassava

Table 16 shows the in vitro bio-accessibility of boiled yellow cassava roots, *gari*, and boiled yellow cassava leaves. In vitro bio-accessibility of carotenoids for boiled roots ranged between not detectable to 104.42±0.88%, and for *gari*, values ranged between not detectable (ND) to 57.22±9.01%. Values ranged between not detectable (ND) to 0.28±0.01% for boiled leaves. There was a significant difference between all samples (P<0.000).

Effect of processing and cultivar on the in vitro bio-accessibility of carotenoids

Processing significantly affected the in-vitro bio-accessibility of the carotenoids, with a p-value of 0.0299. The cultivar, however, did not have a significant effect on the in vitro bioaccessibility of carotenoids present in the yellow flesh cassava genotypes (Tables 17 and 18).

Survey on consumption patterns of cassava

This section of the results describes the consumption patterns of cassava and then probes into the attitudes toward the adoption of yellow flesh cassava in the Ghanaian diets.

Socio-demographic characteristics of the study population

The socio-demographic characteristics of respondents are presented in Table 19. Two hundred and eighty-seven (287) respondents were interviewed for the survey, 135(47%) being males and 152(53%) females.

Consumption patterns of white cassava

Cassava product consumption patterns data indicated that *gari* had the highest frequency of consumption, followed by other cassava products, cassava dough, *kokonte*, boiled cassava, and, lastly, boiled leaves (Figure 4).

Reasons for cassava consumption among Ghanaians

Reasons given for the consumption of cassava included it being a staple food, affordable, available, and having lots of uses. Other purposes stated were that cassava could be used as a source of nutrients and easy to prepare. The frequencies of responses are shown in Figure 5.

Knowledge of the nutritional value of white and yellow flesh cassava

The survey also showed that 70.4% of respondents knew the nutritional value of white cassava. Out of this population, 63.9% knew white cassava to be a major source of macronutrients, but only 6.5% of this same population knew it to be a source of micronutrients. 36.6% of the respondents knew about yellow flesh cassava, and 13% of this population knew its nutritive value as it is a source of carbohydrates and vitamin A (Figure 6).

Table 16. In-vitro bio-accessibility (%) of carotenoids in yellow flesh cassava

Sample	Boiled roots	Gari	Boiled leaves
01	21.49±3.53b	NDc	0.28±0.01a
03	4.10±1.42c	29.25±0.49b	0.04±0.01d
05	NDc	57.22±9.01a	NDe
07	NDc	0.617±0.01c	0.13±0.01b
15	104.42±0.88a	NDc	0.08±0.01c
P-Value	0.00	0.00	0.00

Note: Means that do not share a letter are significantly different

Table 17. Effect of processing on the in vitro bioaccessibility of carotenoids

Sample	Mean (%) in vitro bio-accessibility
Gari	26.000a
Boiled roots	17.416c
Boiled leaves	0.104b
P-Value	0.03

Note: Means that do not share a letter are significantly different

Table 18. Effect of cultivar on the in vitro bioaccessibility of carotenoids

Sample	Mean % in vitro bio-accessibility
01	7.25b
03	11.13ab
05	19.07ab
07	0.25b
15	34.83a
P-Value	0.07

Note: Means that do not share a letter are significantly different

Table 19. Socio-demographic characteristics of the study population (N=287)

Variable	n (%)
Age (years)	
18-39	262(94)
40-59	19 (4)
Above 60	6(1.9)
Sex	
Male	135(47)
Female	152(53)
Marital status	
Single	224(78)
Married/Cohabitating	50(17.4)
Divorced	8(2.8)
Widow	5(1.7)
Educational level	
No formal education	8 (2.8)
Primary/JHS/ middle school	53(18.5)
SHS/secondary	30(10.5)
Post-secondary	33(11.5)
Tertiary	163(56.8)
Occupation	
Student	131(45.6)
Fixed income	64(22.3)
*Non-fixed income	77(26.2)
Average monthly income (GHC)	
0-100	104(36.2)
100-1000	143(49.8)
1000-2000	14(4.9)
2000 and above	26(9.1)
Number of households with children under 15 years	
Yes	170(59.2)
No	117(40.8)
Residential status	
Own house	57(19.9)
Family house	105(36.6)
Rented house	104(36.2)
Company/mission house	2(0.7)
Government house	12(4.2)
Caretaker	4(1.4)
Squatter	3(1.0)

Note: *Non fixed income-(beautician, carpentry, caterer, Craftsmanship, driver, hairdressing, marketer, self-employed, sound engineer, steel bender, petty trading, farming, dressmaking)

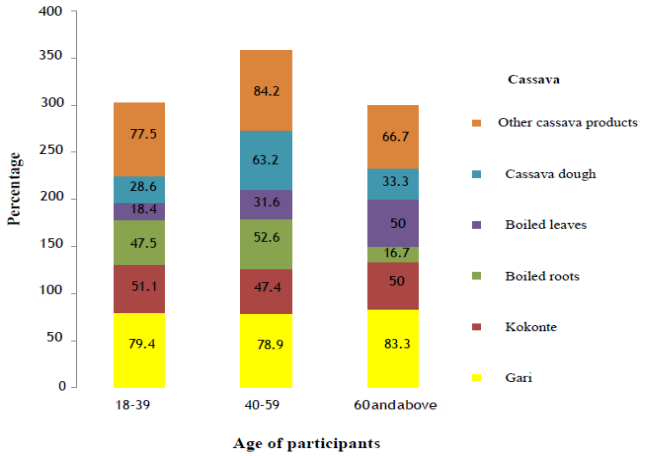

Figure 4. Consumption frequency of cassava products. Note: other cassava products (fufu, fried cassava balls, and chips, tapioca, yakeyake)

Acceptability of yellow flesh cassava

Of the total population, 51.2% were willing to accept yellow flesh cassava. The reasons given for "willingness to accept" were diet diversity and curiosity. The unwillingness to accept yellow flesh cassava was due to a lack of knowledge about the yellow cassava (Figure 7).

Perceived usage of yellow flesh cassava

Some of the respondents (64.3%) perceived that yellow flesh cassava could be used for *gari*, cassava dough, starch, and other cassava products that white cassava is being used for. The remaining 35.7% did not know what yellow flesh cassava could be used for (Figure 8).

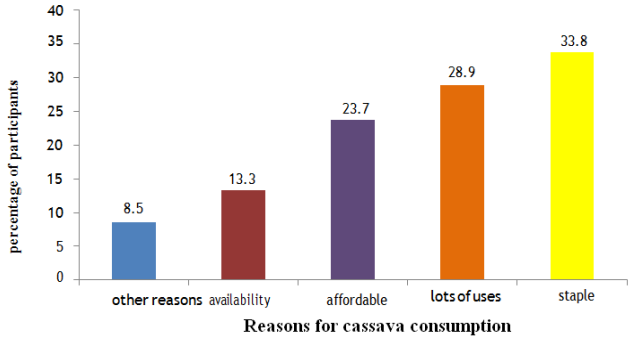

Figure 5. Reasons for cassava

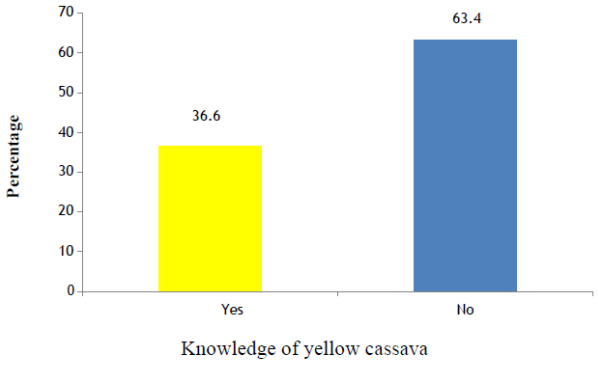

Figure 6. Knowledge of yellow flesh cassava

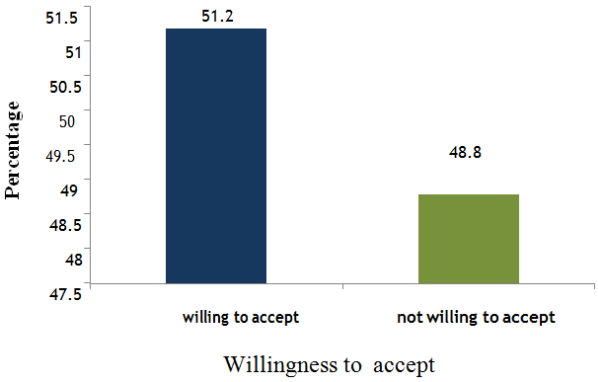

Figure 7. Acceptability of yellow flesh cassava

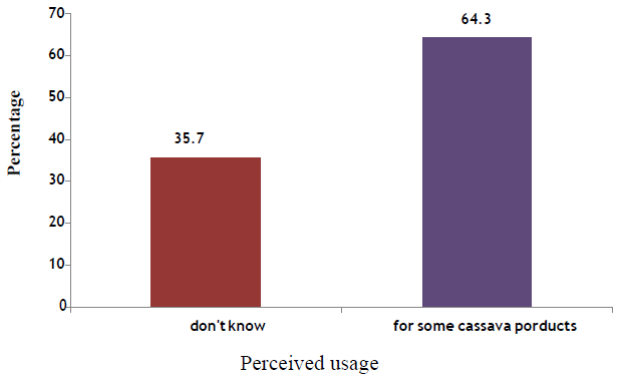

Figure 8. Perceived usage of yellow flesh cassava

Predictors of knowledge and willingness to accept yellow flesh cassava

Results of the logistic regression (Table 20) indicate that compared to people between 18-39 years (the reference category), those aged between 40 to above 59 were four times more likely to have knowledge about yellow flesh cassava. Married people were two times more likely to "willingly accept" yellow flesh cassava.

Discussion

Nutrient profiles of yellow flesh cassava roots and leaves

Moisture content is an essential factor in the storage of cassava and its products; very high levels greater than 12% are reported, which allows for microbial growth (Padonou et al. 2010). Low levels are more favorable because they give a relatively longer shelf life (Harris and Koomson 2011). Fresh yellow cassava roots had a moisture content between (67.053±1.09) and (82.123 ±0.76) % and dry matter content of (17.877±0.760) and (32.947±1.09) %. The United States Department of Agriculture (2009) reported 45.9 to 85.3% for moisture content and (29.8 to 39.3%) dry matter content for yellow flesh cassava roots (Zvinavashe et al. 2011) reported moisture content for cassava roots to be between 60.3% to 87.1%. Chávez et al. (2008) and (Teye et al. 2011) also recorded values ranging from 29.8% *to* 40.7%, with an average of 34.5% for dry matter content. The moisture content of between 57.5 and 54.8 % for yellow flesh cassava roots was reported by (Ceballos et al. 2013), and 69.1±4.17% was reported by (Maziya-Dixon and Dixon, 2015). The moisture content of the leaves was between 56.06±0.33% and 81.62±0.52%, as reported by (Wobeto et al. 2006). The moisture content of 64.8 to 88.6% was also reported by (USDA 2009). This trend from the various studies generally indicates that yellow flesh cassava is very susceptible to post-harvest deterioration and therefore needs to be processed immediately after harvesting. However, (Morante et al. 2010) reported that the presence of the beta carotene trait, which has an oxidative nature, influences the reduction of the deterioration of harvested cassava roots.

Total carotenoids ranged from 792.93±0.98 µg/g and 2649.20±29.10 µg/g for fresh leaves and between 4.73±0.11 µg/g and 10.11±0.18 µg/g for fresh roots. This is in agreement with reports by (Vimala et al. 2008), who reported a total of 3.6-6.4µg/g (fresh weight.) carotenoids for yellow flesh cassava roots. Iglesias et al. (1997) also reported 7.7 to 46.9 mg/g (dry matter basis) for yellow flesh cassava roots, similar to results by (Omodamiro et al.2012) and (Esuma et al. 2012). Total carotenoid content differences could result from factors such as variety, stage of maturity, geographic site of production, part of the plant which is used, conditions during planting, post-harvest handling, processing, and storage conditions (Rodriguez-Amaya and Kimura 2004).

Table 20. Predictors of Knowledge and 'willingness to accept' Yellow Flesh Cassava (N=284)

Variable	Knowledge of yellow flesh cassava O.R(C.I)	Willingness to accept yellow cassava O.R(C.I)
Age		
18-39	1.00	1.00
18-39 versus 40-59	4.44(1.63-12.07)*	2.18(0.81-5.92)
18-39 versus above 60	4.09(0.74-22.80)	0.50(0.09-2.80)
Gender		
Male	1.00	1.00
Male versus female	0.72(0.45-1.12)	0.65(0.41-1.04)
Marital Status		
Single	1.00	1.00
Single versus Married	1.81(0.95-3.47)	2.49(1.26-4.94)*
Single versus Cohabitating	1.38(0.23-8.44)	3.38(0.67-17.09)
Single versus Divorced	2.07(0.50-8.51)	0.281(0.03-2.56)
Single versus Widow	3.10(0.51-18.98)	0.75(0.12-4.58)
Educational status		
No formal education	1.00	1.00
No formal education versus up to middle school	2.98(0.64-13.88)	1.94(0.43-8.70)
No formal education versus secondary	1.11(0.22-5.54)	1.50(0.31-7.19)
No formal education versus Post-secondary	0.56(0.11-2.86)	0.52(0.11-2.51)
No formal education versus Tertiary	0.65(0.15-2.82)	0.90(0.21-3.70)

Note: Reference category * means p-value<0.05

Iron and zinc contents in yellow flesh cassava were reported by (Wobeto et al. 2006) to be between 20.29-22.5 mg/100g and 4.20-5.16 mg/100g. (Umuhozariho et al., 2014) also reported 5.9 to 7.62 mg/100g for zinc and iron of 21.5-27.8 mg/100g. These reported values from the literature are lower than the results from the study with iron (87.35±3.18 to 146.25±1.20 mg/100g) and higher for (zinc) 0.30±0.00 to 1.55±0.07 mg/100g for fresh yellow cassava roots. Iron (23.0-27.8 mg/100g) and zinc contents (6.4-7.6 mg/100g) were reported in fresh cassava leaves (Umuhozariho et al. 2014), and (Balal et al. 2013) reported iron contents of 7.4 mg/100g for fresh white cassava leaves. According to (Graham and Rosser 2000) and (Hess et al. 2005), Cassava roots with provitamin A carotenoids have a mutually stimulating effect on iron and zinc, which could have accounted for the higher iron and zinc contents in the study. Fresh leaves, on the other hand, contained 118.35±0.07 and 182.05±0.07 mg/100g (iron) and 3.75±0.64 and 15.50±0.14 mg/100g (zinc). The pH of the soil where the cassava was planted also has a high impact on Fe and Zn contents in the roots (Bortey-Sam et al., 2015).

Effect of processing on total carotenoids, iron, and zinc contents of yellow cassava Cassava must be modified into different products to promote a good shelf life, improve transportation and marketing, decrease cyanide content and improve acceptability (Nyirenda et al. 2011). Cassava products include *gari, kokonte*, cassava dough, and *fufu*, among others. Cassava processing methods involve many operations that are done in various phases. Such activities are taking off the peels, cutting them into chips or slices, mashing, milling into finer particle sizes, and grating, among others (FAO 2001).

Carotenoids are highly unsaturated and therefore are more susceptible to isomerization and be oxidization (Rodriguez-Amaya 2002). This occurs when carotenoids are exposed to heat, acids, and light (Maziya-Dixon et al., 2015). Carotenoid loss happens during processing through peeling, isomerization, and enzymatic or non-enzymatic oxidation (Rodriguez-Amaya et al. 1997).

Gari is a moisture-free, brittle but tender, creamy-white, grainy, and fermented food made from cassava (James et al. 2012). It is the most popular form of processed cassava presently eaten in West Africa (Ukenye et al., 2013). Drying causes a reduction in moisture, volume, and cyanide content of roots, thereby prolonging product shelf life (Westby 2002). Osunde and Fadeyibi (2011) reported that the moisture content of gari for safe storage should be below 12.7%, and their findings agree with the results, which reported moisture content of 2.88±0.09 to 5.25±0.07%. The total carotenoid from the study was similar to findings from (Maziya-Dixon et al. 2015) for gari, who reported total carotenoid content of 3.11-15.9 µg/g. Iron content for the boiled leaves samples was not significantly different from that of the fresh leaves and was not affected much by the processing. Fermentation, a process used in making gari, is also one of the oldest and most important traditional food processing and preservation techniques (Cardoso et al. 2005). Fermentation is reported by (Achinewhu et al. 1998) to enhance the nutrient content of foods through the biosynthesis of vitamins, fiber digestibility as well as enhancing micronutrient bioavailability. This could have accounted for the high carotenoid content in the yellow cassava gari, as well as iron and zinc contents. The observed increase in total carotenoid concentration after processing into gari, according to (Rodriguez-Amaya 2004), could be because carotenoids in processed foods can be obtained more quickly than with those in an uncooked form where the provitamin A are shielded and/or in combination with other food compounds that hinder solvent entry and extraction. The higher amounts may also result from unaccounted losses of moisture and solids, which would increase the retention and the total carotenoid per unit weight of the processed food (Maziya-Dixon et al., 2015).

Boiling has been implicated in losses of certain micronutrients in food, including iron, copper, and zinc (Ahenkora et al. 1996). Boiled roots have lower total carotenoid concentration than that in gari, even though some studies have reported that boiling retains more carotenoids than the roasting (Iglesias et al.1997; Chavez et al. 2005). This could be because wet heat treatments are more destructive to nutrients than dry heat treatments. Boiled leaves also had a reduction in total carotenoids; however, the location of carotenoids in the leaves makes them more protected from heat than the roots (Coleman et al. 2010).

Drying in a solar dryer can appreciably reduce losses of carotenoids by protecting the food from direct sunlight. However, this depends on the length of the drying period. Longer drying periods in the solar dryer after five days degrade the carotenoids instead of being protective (Rodriguez-Amaya and Kimura 2004). Vimala et al. (2011) also reported that the drastic reduction of carotenoids in this process might be due to the destructive effect of sunlight on the stability of carotenoids. *Kokonte* used in the study was solar-dried during the rainy season, which elongated the drying period to 7 days. This could have accounted for the degradation of the carotenoids, making it not detectable. The cultivar or genotype of cassava also affected the total carotenoid content after processing (Iglesias et al. 1997).

Factors affecting carotenoid retention

Nutrient retention is defined as the measure of the number of nutrients left in processed food concerning the nutrient originally present in the raw food (USDA 2009). Smolin et al. (2003) report that the time, method, ad temperature for cooking are some factors that affect nutrient retention. The retention of carotenoids also decreased with longer processing time and after a higher processing temperature, also when food is blended (Rodriguez-Amaya 2002). *Gari* had the highest retention of the carotenoids for the products made from the roots. The carotenoid loss during gari processing was much lower in yellow gari. On the other hand, boiled roots retained the lower amounts of carotenoids, while kokonte retained no carotenoids at all after processing. Boiled leaves retained the highest amounts of carotenoids after processing. Carotenoids in green leafy vegetables are

located in the chloroplasts and also bound to protein complexes, which protect the carotenoid, thus giving room for more retention (Canene-Adams and Erdman 2009). Carotenoids in the roots, however, are within membranes of large proteins, which are crystalline. These membranes are easily broken on exposure to heat, making them more liable for the carotenoids to be lost (Serrano et al. 2005). Retention varies between 10% for cassava products processed to a greater extent to 87% for boiling (Maziya-Dixon et al. 2000; Thakkar et al. 2009). This was in agreement with the findings from the study.

In vitro bio-accessibility of carotenoids in yellow cassava

Processing methods using heat are said to enhance the bio-accessibility of beta carotene by unbinding the matrix, thus facilitating its assimilation (Veda et al. 2006). Carotenoids present in green leafy vegetables are located in the chloroplasts and form bonds to protein complexes, which are not easily digestible. On the other hand, carotenoids in the roots are within membranes of large proteins, which are crystalline, making them more available (Canene-Adams and Erdman 2009). This could explain why cassava root products were more bio-accessible than the leaves. Inhibitors such as gels and cellulose fiber inhibit absorption by sustaining bile salts and preventing micelle formation, which will aid at the point of absorption (Serrano et al. 2005). In vitro bioaccessibility of the carotenoids was comparable to studies reported by Failla et al. (2009).

Contribution of yellow flesh cassava to RDA of vitamin A under-5 children

Boiled roots contributed between 4 and 21% of the RDA for children less than five years, and the boiled leaves contributed between 2.6 to 386% of the RDA for every 100 grams of food consumed, which showed more potential to meet the RDA. Gari and kokonte, however, provided less than 1% of the RDA for every 100 grams of cassava. A greater amount of the food must be consumed to meet a more significant amount of the RDA. Notwithstanding, a positive outcome of some contribution to RDA implies that processors should put some viable measures to help reduce carotenoid losses in these products, especially in gari, because it is the most frequently consumed cassava food.

Effect of processing and cultivar on antioxidant activity of carotenoids

Processing methods such as dehydration, blanching, and canning also affect the antioxidant property of the carotenoids of some dietary plants (Van Het Hof et al. 2000). Vegetables, such as carrots, mushrooms, peppers, potatoes, sweet potatoes, cabbage, and tomatoes, increase their antioxidant activity when these vegetables are boiled or steamed (Halvorsen et al. 2006). Antioxidant activity of the cassava leaves also showed similar trends of increasing after boiling. However, the antioxidant activity of the roots was destroyed after processing.

Comparison of icheck and spectrophotometric method for carotenoid determination

The study showed that the icheck method for carotenoid determination was highly comparable to the spectrophotometric method, with regression (R^2) of 95.3% and 96.68%, respectively. This was comparable to the regression results (Islam and Schweigert, 2015) that compared the two methods using carotenoids from egg yolk. This comparison was made to validate the accuracy of total carotenoid content in this study because a quite recent method of carotenoid analysis was used.

Socio-demographic characteristics

The majority of the study participants were between 18-39 years. This population represents the highly reproductive part of the population who are also vulnerable to vitamin A deficiency (WHO 2001). The majority of the participants had up to tertiary education. However, their level of education did not predict the corresponding level of knowledge about yellow flesh cassava. Again, most of the population had children less than 15 years in their households, another vulnerable group to vitamin A deficiency (West 2002). This suggests an early adaptation to yellow cassava would be a good step in curbing the prevalence of vitamin A deficiency.

Consumption patterns of white cassava

Participants between 18 - 39 years consumed gari and boiled leaves the least. Gari has a longer shelf life and thus may be a factor in it being the most consumed food for that population. Onyemauwa (2010) also reported gari to be the highest consumed cassava product making it a more viable vehicle for introducing yellow cassava to the Ghanaian populace. However, for the participants between 40-59 years, the most consumed cassava foods were other cassava products, including fufu. Fufu is known to be more energy-dense because of the addition of cocoyam, plantain, or yam. This age group preferred more energy-dense foods probably because of their family size and economic status.

Acceptability of yellow flesh cassava

Nutrition education is a vital tool in communicating biofortified crops' nutritional and health benefits and an important factor affecting the acceptability of bio-fortified crops (Tanumihardjo 2008). Chowdhury et al. (2011) reported that mothers in Uganda easily adopted bio-fortified foods after receiving nutrition education. From the present study, most of the consumers who took part in the survey were not willing to accept yellow flesh cassava because they did not know about it, which is similar to studies on the acceptability of bio-fortified foods. Consumer preferences for bio-fortified foods have also been influenced by providing nutritional information in Ghana (De Groote et al. 2010). When given the same information on the willingness to pay for orange sweet potato, orange maize, and yellow cassava, research showed that consumers liked the organoleptic traits of the biofortified crops and were more likely to pay more money for high provitamin A genotypes than for white genotypes

(Oparinde et al. 2012, Chowdhury et al. 2011; Meenakshi et al. 2012).

Determinants of knowledge and willingness to accept yellow flesh cassava

Socio-economic characteristics of poor consumers, such as their income, amount of land owned, age, education level, household size, and the number of young minors they have in their household, might play a vital role in the success of a bio-fortification program (Etumnu 2016). The study showed that socio-demographic characteristics such as age and marriage significantly affected a person's knowledge and willingness to accept yellow cassava.

Limitations of the study

Although iron and zinc values were very high, their bioavailability was not estimated and thus was not compared to the recommended daily allowances (RDA). RDA for vitamin A could not be computed after the in vitro bio-accessibility of carotenoids because of the unavailability of an HPLC to quantify beta-carotenes at the time of the study. Micellization efficiency was not computed for this study's in vitro bio-accessibility model; thus, the bioavailability could not be calculated.

Conclusion

Fermentation, drying, roasting, and boiling retained some carotenoids after processing, but solar drying over a long time completely degraded carotenoids in yellow cassava. Cassava leaves had higher retention of carotenoids, but cassava roots were more bioaccessible. Carotenoids in yellow flesh cassava had antioxidant properties and thus had the potential to help combat free radicals in the body. The antioxidant activity in the yellow flesh cassava roots was destroyed after processing into gari, kokonte, and boiled roots but was higher in leaves after boiling. Yellow cassava would provide a viable source of provitamin A. The knowledge and "willingness to accept" yellow cassava was low among Ghanaians in the Greater Accra region.

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Phytochemical composition and antimicrobial activity of *Ochna thomasiana*

MBITHI JUSTUS MUEMA[1], ALEX K. MACHOCHO[2,♥], NICHOLAS K. GIKONYO[1]
[1]Department of Pharmacy and Complementary/Alternative Medicine, Kenyatta University. Mfangano Island, Kakamega Centre, Suba North, Kenya
[2]Department of Chemistry, Kenyatta University. Mfangano Island, Kakamega Centre, Suba North, Kenya. ♥email: machocho.alex@ku.ac.ke

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Abstract. *Muema MJ, Machocho AK, Gikonyo NK. 2020. Phytochemical composition and antimicrobial activity of* Ochna thomasiana. *Biofarmasi J Nat Prod Biochem 18: 29-41.* Infectious diseases are the leading cause of mortality worldwide despite the vigorous campaigns that have been made to combat them. This study aimed to determine and evaluate the biological activities of *Ochna thomasiana* Engl. & Gilg ex Gilg. Here, we screened the plant extracts and tested them for their antibacterial activity against *Salmonella typhi* (clinical isolate), *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. Various chromatographic techniques separated and isolated the active compounds from this plant. The extracts were purified using silica gel, column chromatography (CC), Sephadex gel, and preparative thin-layer chromatography (PTLC). Structure characterization was determined using standard spectroscopic methods: Infrared (IR), ultraviolet (UV) spectroscopy, mass spectroscopy (MS) and proton nuclear magnetic resonance (^{1}H NMR), distortionless enhancement by polarization transfer (DEPT), carbon-13 nuclear magnetic resonance (13C NMR), coherence spectroscopy (COSY), heteronuclear multiple bond coherence (HMBC) and heteronuclear single quantum coherence (HSQC). Compounds identified from the extracts are Lophirone A (18), afzelone D dimethyl ether (20), calodenone (17), a mixture of stigmasterol (74), and β-sitosterol (23) and 3β-acetyl-24-ethylfriedelane (75). Methanol crude extracts' stem and root bark showed high activity against the Gram-positive bacteria. Lophirone A, afzelone D dimethyl ether, and 3β-acetyl-24- ethylfriedelane showed high activity against *S. aureus.* The results showed that the root of O. thomasiana contains biflavonoids. Some sterols as their constituents and antimicrobial activity are significant and lead to the development of antimicrobial agents. The essential bioactive compounds and the antimicrobial activity of the crude extracts of this plant confirms its use in traditional medicine. However, there is a need to test the effectiveness of the crude extracts and isolated compounds via in vivo and in vitro evaluation. The plant species should be cultivated using good agricultural practices for medicinal plants for the future assessment of their activity against pathogens.

Keywords: Antimicrobial, *Ochna thomasiana*, phytochemistry

INTRODUCTION

The use of plants in indigenous cultures is multiple and very diverse. They form an important economic basis and consume as food, medicine, construction material, dyes, firewood, ritual paraphernalia, and ornaments. Plants have been the foundation of traditional medicine systems for thousands of years, where the knowledge of the plants has been passed on from one generation to another generation (Koehn and Carter 2005). The abundance of plants on the earth's surface has increased interest in investigating distinct extracts obtained from traditional medicinal plants as potential sources of new antimicrobial agents (Bonjar and Farrokhi 2004). Because people have used plants for millennia, much information on plants' therapeutic uses has accumulated, especially in the tropical parts of the world where plant diversity is much higher. In African, Chinese, and Indian communities, plants have become the main ingredient of traditional medicines (Gurib- Fakim 2006; Magassouba et al. 2007).

Medicinal plants are gifts of nature to cure a limitless number of diseases among human beings (Bushra and Ganga 2003). More than 66% of the population residing in developing countries was predicted by WHO (2008) to be depending directly on plants for their primary medical requirements (WHO 2008). This phenomenon is attributed to the fact that plant-derived medicines are readily accessible and relatively cheaper than pharmaceutical drugs (Amin and Mousa 2007; Ramawat and Goyal 2008; WHO 2008). Even the society of the developed world relies on their health care for plants directly or indirectly. In the United States, community pharmacies prescribe 11% of medicine consisting of plant extracts or active ingredients of plant origin (Cragg and Newman 2005). As many as 21% of patients in Dar es Salaam, Tanzania, who visited public hospitals had consulted a traditional healer before going to the hospital (de Boer et al. 2005). Medicine derived from the plant is taken in the form of teas, tinctures, poultices, and powders, depending on the knowledge of the use and application method (Fennell et al. 2004; Balunas and Kinghorn 2005).

Plants produce diverse compounds as a means of defense against fungi, bacteria, pests, as well as predators. Therefore, plants are efficient natural chemical factories, creating various structures of compounds that result in different physiological effects in the body upon ingestion (Edeoga et al. 2005). Substances isolated from plants were utilized as important drugs in one or more countries. Moreover, 60% of these substances were identified as a

result of phytochemical studies on plants used for medicinal purposes (Farnsworth et al. 1985).

The natural products of the plant kingdom, primarily from higher plants may serve as a candidate for a new source of antimicrobial agents with possibly novel mechanisms of action (Shahidi 2004; Runyoro et al. 2006). The provision of safe and effective medicines might become a valuable tool to improve access to health care (WHO 2002; Duraipandiyan and Ignacimuthu 2007). Consequently, this has necessitated the investigation of plants as other potential sources of effective, safe, and cheap antimicrobial drugs (Thangadurai et al. 2004).

The objectives of this research were (i) To evaluate antimicrobial activities of the EtOAc, MeOH, and DCM of crude extracts of *O. Thomasiana*, (ii) To elucidate the structures of the isolated compounds from the acquired spectrum using spectroscopic techniques, (iii) To evaluate antimicrobial activities of isolated pure compounds from *O. thomasiana.*

MATERIALS AND METHODS

Plant collection and processing

Plant samples (root and stem barks) were acquired from Arabuko-Sokoke, a forest in Malindi district, Kilifi County of Kenyas' coast province. The wood is located about 120 Km north of Mombasa town and transverses Kilifi County (ASFMT 2002). The plant identity was authenticated by a taxonomist of Kenyatta University, School of Medicine, Department of Pharmacy and Complementary/Alternative Medicine (Mr. Lucas Karimi) and confirmed at the herbarium of the Kenya National Museums where the Voucher specimen (number MM/OH/001/06) was submitted at Kenyatta University Botany Herbarium. The samples were air-dried for two weeks in the shade at room temperature (25°C ± 3°C) away from direct sunlight. The dry material was ground into a fine powder using a KHD Humboldt Wedag AG Electric grinding mill and then weighed.

Reagents

All reagents were purchased from Kobian, Nairobi, Kenya. Organic solvents used in this study include acetone, n-hexane, dichloromethane, ethyl acetate, and methanol. All solutions used for the extraction, fractionation, and crystallization were distilled two times before use and kept in amber bottles.

Sequential extraction of root and stem barks

The extraction protocol was based on the National Cancer Institute (NCI) protocol extraction of bioactive molecules (McCloud et al. 1998). Briefly, powdered material of *O. thomasiana* stem barks (6.43 kg) were soaked in 10 liters DCM for three days. The solvent must be sufficient enough to saturate plant material for thorough extraction thoroughly. Sequential extraction was done repeatedly with solvents of increasing polarity, starting with 8 liters ethyl acetate (EtOAc) and finally 7 liters methanol (MeOH) for three days, each with occasional swirling to ensure thorough extraction. Similarly, the sequential extraction of 4.01 kg of powdered material *(O. thomasiana* root barks) was done using 8 liters DCM, 7 liters EtOAc, and finally 6 liters MeOH for three days each.

The extracts were filtered using a Buchner funnel using reduced pressure of a vacuum pump through filter paper (40 mm Whatman), the residue was soaked in solvent again for three days. The extraction resulted in a clear extract after three times of repetition. In each step, plant residue (material) was allowed to dry before appliying the next solvent. All the combined filtrates were concentrated using a rotary evaporator under reduced pressure at a temperature of 45 ^{o}C to recover back solvents. The crude extracts were kept in the desiccator to evaporate the excess solution and after that wrapped in aluminum foil and stored in the deep freezer to avoid decomposition of compounds. A portion of each crude extract (2 g) was used for bioassay.

Thin-layer chromatography (TLC)

Analytical TLC was done on a 0.25 mm thickness of Silica gel 60 F_{254} (Macherey-Nagel) plates. The spots were visualized on the chromatograms using a multi-band ultraviolet light (254/365 nm) lamp (UV GL-58). The TLC plates were sprayed with *p*- anisaldehyde solution and stored in the oven at 110°C until the spots appeared. Anisaldehyde was used as a developing reagent. The reagent contains a solution of 0.5 mL *p*-anisaldehyde combined with 10 mL glacial acetic acid 85 mL, ice-cold methanol or ethanol, and 98% sulphuric acid (Randerath et al. 1968). Plants extracts were tested for content of the steroids/terpenes and flavonoids. The TLC plates were sprayed with the *p*-anisaldehyde solution and kept in the oven at 110°C until the spots appeared. Visualization of spots on developed TLC plates was done using long and short wavelengths (λ) (365 and 254 nm respectively), on an ENF-240 C/F UV lamp (Spectronics Co., Westbury, UK).

Melting point

Uncorrected melting points were documented using open capillary tubes using a Gallenkamp melting point apparatus (Sanyo, West Sussex, and the UK).

Infrared (IR) spectroscopy

IR spectra were measured on an FTIR 8400S (Shimadzu, Japan). The spectra were calculated using potassium bromide (KBr) pellets in the Chemistry research laboratory of Jomo Kenyatta University of Agriculture and Technology (JKUAT). A portion of the sample (2 mg) was added and ground with KBr (100 mg) in a mortar to a fine powder until the sample was uniformly mixed. Then the mixture was pressed in a vacuum die to produce a transparent disk (pellet). The sample was later placed in a sample holder that was inserted in the spectrophotometer.

Ultraviolet (UV) spectroscopy

The UV spectra were determined on an ENF-240 C/F UV lamp (Spectronics Co., UK) in the Kenyatta University research laboratory instrument room. A tiny amount of the sample was dissolved in MeOH and chloroform, for only those samples that were soluble in them. This spectroscopic

technique is *ratio recording*; where the ratio between the reference beam and the sample beam intensities (I_0/I) was fed to a pen recorder. The recorder trace is set absorbance (A) invariably against wavelength (λ).

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H (1D, 2D COSY) and ^{13}C spectra were recorded in $CDCl_3$ and CD_3OD as solvent with Tetramethylsilane (TMS) as internal standard on a Bruker (400 MHz and 600 MHz) spectrometer operating at 400 and 600 MHz (^{1}H-NMR), 100 and 150 MHz (^{13}C NMR) machine at Kwazulu Natal University, South Africa and Nagasaki University, Japan Chemistry research laboratories, respectively. The peaks on ^{1}H NMR were determined as singlet (*s*), doublet (*d*), doublet of doublet (*dd*), triplet (*t*), quartet (*q*), multiplet (*m*) and (or) broad (*b*) by employing TMS as reference. DEPT experiments determined the ^{13}C NMR multiplicity that gave chemical shift values for assignment. Chemical shifts were calculated in δ (ppm) and coupling constants, *J*, in hertz (Hz). The sample was dissolved in CD_3OD in a 5 mm NMR tube and stirred thoroughly. The solution was then transferred into an NMR tube and the spectrum recorded.

Two-dimensional NMR techniques may be utilized to access the correlations between nuclear in a compound. *J*-resolved 2D NMR experiments are useful in the measurement of ^{13}C–^{1}H coupling in complex spin systems because they can separate overlapping multiplets. Correlated 2D tests applied to homonuclear spin systems include COSY (Correlated spectroscopy) while NOESY (Nuclear overhauser enhancement spectroscopy) is applied in the analysis of NMR spectra of large molecules (Abraham et al. 2003). HETCOR and HMQC displayed all the protons to which carbon atoms are attached although HMQC has higher sensitivity. On one axis the ^{1}H NMR spectrum is displayed while the other axis has the ^{13}C NMR spectrum, and thereby, signals indicate a direct coupling of the protons with the carbons (Silverstein et al. 2005). HMBC (Heteronuclear Multiple Bond Correlation) is a long-range correlation experiment which gives information about carbons bonded to proton which are 2-3 bonds away. HMBC has applications in every field for identification and characterization of the structure of compound (Vasavi et al. 2011).

Mass Spectroscopy (MS)

Mass spectrometers use the ratio difference in mass to charge (m/z) of ionized atoms or molecules to separate them. Mass spectroscopy allows the quantization of atoms or molecules and provides structural information by the identification of different fragmentation patterns (Uggerud et al. 2003). The analysis was done out at Chemistry research laboratory of Jomo Kenyatta University of Agriculture and Technology (JKUAT) using Shimadzu Gas Chromatography-Mass Spectrometry (GCMS). A small amount of a compound, approximately one micromole/ microgram or less was injected in the spectrometer and evaporated with vapor leaking into the ionization chamber set at a given pressure of about 10-7 mb.

Antibacterial tests

Bacterial strains tests

Plants extracts were screened in Mycology laboratory center for microbiology research at Kenya Medical Research Institute (KEMRI) against two standard strains of bacteria and two local clinical isolates from patients. The standard strains were obtained from the American Type Culture Collection (ATCC). The strains used were collected from Kenya National Public Health Laboratories (KNPHL), (KEMRI) and Plant and Microbial laboratory of Kenyatta University. Strains that were used included: Two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853), a reference strain, *Salmonella typhi* (Type K [I]) clinical isolate was also used. Also, two Gram-positive bacteria, *Staphylococcus aureus,* (ATCC 20572), a reference strain, *and Bacillus subtilis* (Type K [11]), clinical isolate. These microorganisms were cultivated in diagnostic sensitivity test agar (DST) and Muller–Hinton broth (MHB-Merck Germany).

Preparation of media

DST agar was according to the manufacturer instruction and dissolved in the recommended quantities of distilled water. MHB was made by using 28 g of the media, dissolved in 1 liter of distilled water and boiled. The media were sterilized separately by autoclaving at 15 atmospheres pressures and 121°C for 15 minutes, before used.

Screening procedure

The disc diffusion method was used for the initial activity of the tests on the plant extracts at concentrations of 450 µg/disc. Concentrations of sample solutions were made by dissolving 1g of extract in 1 mL of the solvent that had been used in their extractions, then followed by appropriate dilutions to the required concentrations. The resulting solution was then used to prepare the discs by the soaking method. Each disc was inoculated with of bacterial (0.1 mL) and yeast culture directly from the 24 h broth culture which was diluted to match 0.5 and 1.0 MacFarlands standard, respectively (10^8 Colony Forming Units (CFU)/mL) and fungi diluted to match 1.0 MacFarland standard (10^8 spores/mL). The discs loaded with the extracts were then put onto the seeded plates (Figure 1).

The bacterial and yeast cultures were incubated for 24 and 48 hours, respectively at 37 °C, while fungi were incubated at 25 °C for five days. The zones of inhibition were measured and recorded in mm, as described earlier (Elgayyar et al. 2000). Negative control plates were discs with sterile distilled water and methanol. Growth of the organism and sterility of media was controlled by use of broth only in negative control tubes, while broth plus microorganism in question in positive control tubes. Tetracycline served as the standard for positive control. All the controls were maintained at the same conditions as the tested samples (Figure 2).

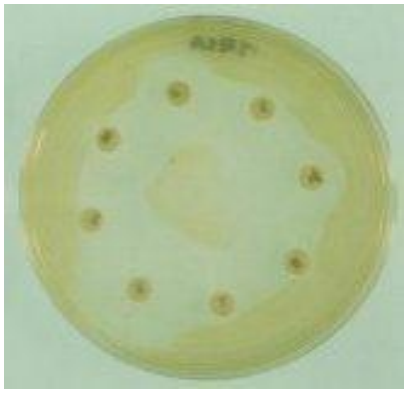


Figure 1. Photograph of petri dish showing disk diffusion method

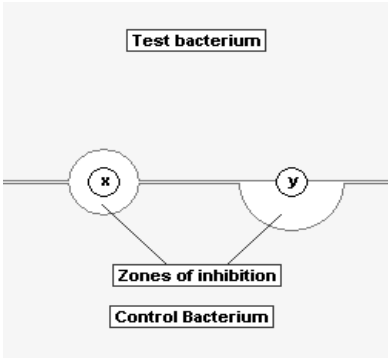


Figure 2. Test of the bacterium

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

Minimum inhibitory concentrations (MICs) are known as the least concentration of an antimicrobial substance that will inhibit the visible growth of a microorganism after overnight incubation. Minimum bactericidal concentrations (MBCs) is the lowest concentration of an antimicrobial that will prevent the growth of an organism after subculture on to antibiotic-free media (Andrews 2001).

The active extracts were tested for MICs and MBCs. The MICs were set using a two-fold serial dilution method in a peptone water solution for bacterial of the active extracts to give a final extract with a concentration of between 1.74 and 8000 µg/mL. Each tube was inoculated with 0.1 mL of standardized bacterial suspension (1×10^8 CFU/mL). The cultures were incubated for 24 hrs at 37°C for bacteria. The first tube displaying no growth was taken as the MICs. MBCs were determined by sub-culturing all the tubes (0.1 mL) showing no growth on Nutrient Agar (NA) for bacteria. The first plate showing no growth was considered as the MBC after 48 hrs at 37°C (Michael et al. 2003).

Disc diffusion and MIC ratings of the extracts

The negative controls showed no inhibition as opposed to the positive control using the standard antibiotic discs (Oxoid). Three replicates represent the mean zone of inhibition. A clearing zone of 9 mm or higher for either Gram-positive and gram-negative bacteria was used as the criterion for designating significant antibacterial (Faizi et al. 2003). The extracts that showed MIC lower than 100 µg/mL were considered as having very high antimicrobial activity (AA); from 100-500 µg/mL has high AA; 500-1000 µg/mL has moderate; 1000-4000 µg/mL, has low AA and anything above this range, the extracts were considered inactive for bacteria.

Isolation of compounds from *Ochna thomasiana*

The extracts of DCM and EtOAc from root bark were fractioned by CC of 4.75 cm, and 5.75 cm in diameter, respectively with each height measured 70 cm. The smaller CC of 2.75 cm in diameter and a height of 40 cm on silica gel 60 mesh 0.6 nm (230-400) was used for further purification, and the samples were eluted with varying concentrations of hexane, DCM, EtOAc, and MeOH mixtures until 100 % of each solvent was achieved.

Packing of both types of columns was made using the slurry method with silica gel suspended in the less polar solvent in the solvent system. The mass of the silica gel packed measured in the ratio of 30:1 to the mass of the extract to be loaded. The extract was dissolved in the minimum possible solvent that dissolved it, then mixed with an equal amount of the silica gel used in the CC, ground into fine powder form and dry up by evaporating in a vacuum evaporator to remove the excess solvent. The powder was loaded at the top of the column, covered by a small amount of dry silica gel and covered with a piece of cotton wool to reduce the disturbance of the sample when the eluting solvent is added. The eluting solvent was allowed to trickle down dropwise from a separating funnel to the column to avoid pouring of the solvents directly into the column.

Analytical TLC 60 F_{254} plates were used throughout the purification process. These were mainly for the establishment of optimum solvent systems for the separations and purification of isolated compounds. Spots on the chromatograms were visualized under UV light at λ 254 and 365 nm for UV active compounds and visualized upon development by spraying *p*-anisaldehyde separately and heating for about 10 min at 110 °C in an oven. Fractions that had same Rf were combined and concentrated at the same time to give pure compounds or semi-pure compounds for further purification. Further purifications steps included centrifuging, recrystallization, Sephadex column, and PTLC. The Sephadex (LH-20) columns were run using 1:1 (DCM: MeOH).

DCM extract from the root (9.65 g) of the *O. thomasiana* proceed to fractionation by column chromatography on silica gel with a Hex:DCM-DCM: MeOH gradient (100:0-0:100) to yield 209 x 25 mL fractions. Three fractions were acquired based on R_f portrayed on the TLC profiles. Fractions 1-167 were pooled together since they gave a purple color on spraying with *p*-anisaldehyde followed by heating in the oven for 10 minutes at 110°C. These fractions were generated at different solvent mixtures of hexane and DCM that range from 4:1 (Hex: DCM) to 100% DCM. Fractions 168-181 were pooled at the same time and was obtained at 19:1

(DCM: MeOH). These fractions had purple and red-brown pigmentation after spraying with spraying reagent.

Fractions 182-209 were pooled together to form the third fraction which had yellow and red-brown pigmentation after spraying the TLC plate with *p*-anisaldehyde followed by heating in the oven for 5-10 minutes. This fraction was obtained at 9:1 of DCM: MeOH. The first fraction (5-167) was rinsed with MeOH to remove brown coloration obtained from the column followed by hexane to make the crystals clean. Three other fractions were collected, but based on the TLC profile they all had two spots each. The white crystals obtained were dissolved in chloroform and then recrystallized. Decanting was done leaving behind the sharp white crystals of the compound of MJ/RD/OT0$_1$ (75) of 3 g. PTLC was done on one of the resulting three fractions to isolate 14.5 mg of MJ/RD/OT0$_{10}$ (a mixture of 23 and 74) at 100% DCM. The other two portions were separated using Sephadex (L-20) column 1:1 (DCM: MeOH) and fractions 30 and 40were obtained. A PTLC was done on these combined fractions from the Sephadex column using 10% MeOH/DCM, resulting in a white cream compound MJ/RD/OT0$_2$ (17).

Ethyl acetate extract (18.6 g) of the root bark was proceeded to regular CC on silica gel with a DCM:EtOAc: MeOH gradient (100:0-0:100) to obtain 43 x 200 mL fractions. The fractions 1-5 obtained at 43:7 (DCM: EtOAc), 6-9 obtained at 5:3 (DCM: EtOAc), ten obtained at 11:9 (DCM: EtOAc), 11-15 obtained at 23:27 (DCM: EtOAc) and 16-43 were not incorporated because tailing produced in the TLC profiles. Pigmentation created after spraying with the *p*-anisaldehyde was dark brown. The fraction 11-15 of 9.36 g was proceeded to a smaller CC to yield four other smaller fractions. The fractions 1-4 isolated at 19:1 (DCM: EtOAc), portion 5-11 obtained at 7:3 (DCM: EtOAc), portion 12-15 obtained at 5:3 (DCM: EtOAc), was separated further using Sephadex (L-20) column 1:1 (DCM: MeOH) to receive 21 fractions which were of the same Rf. A PTLC was developed for the combined portion from the Sephadex using 9:1 (DCM: MeOH), and 15.3 mg of compound 18 (MJ/RE/OT0$_6$) was obtained. The same procedure above was used for fractions 6-9 to acquired 13.2 mg of compound 20 (MJ/RE/0T0$_5$) and 14.5 mg compound 17 (MJ/RE/0T0$_2$). Compound 17 was obtained from both DCM and EtOAc root bark extracts.

Physical and spectroscopic data of isolated compounds

Compound 18*; Lophirone A*

Cream white crystals (MeOH), Mp 211-213°C; UV (MeOH) λ_{max} 237, 335 nm; ^{1}H NMR (600 MHz, MeOD): δ 8.25 (1H, *s*, β_1), 6.68 (1H, *d*, *J*= 2.2, B_1H_3),7.87 (1H, *d*, *J*=8.9Hz, B1H6),6.83 (1H, *d*d, *J*=2.2, 8.9, B1H5), 5.97 (1H, *d*, *J*= 12.16, H-α_1), 4.67 (1H, *d*, *J*=12.16, H-β_1),6.59 (4H, d, *J*=8.4, H-C3, A1C5, A2,7.16 (4H, d, *J*=8.4, *H-C2, A1 C6, A2), 6.33 (1H, dd, J*=2.3, *9.0, B2H5),6.12 (1H, d, J*=2.3, B2H3),8.14 (1H, *d*, *J*=9.0, B_2H_6). ^{13}C NMR (150 MHz, MeOD): δ 157.4 (β_1), 122.6 (α_1), 177.1 (s, C_1), 117.1 (s, B_1C_1), 103.5 (*d*, B_2C_3), 164.7 (B_1C_4),159.4 (s, B1C6),116.5 (*d*, A_1C_5), 128.2 (*d*, A_1C_2),54.3 (*d*, β_1),44.7 (α2), 204.8 (s, C_2), 135.9 (s, A_1C_1), 129.8 (*d*, A_2C_2), 116.0 (*d*, A_2C_3), 156.7 (s, A_1C_4), 134.9 (s, A_2C_1), 130.5 (*d*, A_2C_6), 116.1 (*d*, A_2C_5),114.3 (s, B2C1),159.4 (B1C6), 166.9 (*s*, B_2C_2), 103.5 (*d*, B_2C_3), 166.8 (s, B_2C_4), 109.2 (*d*, B_2C_5), 134.4 (*d*, A_2C_1); IR (KBr) nmax 3751, 3368, 2728, 1626, 1510, 1454, 1364, 1238, 831 cm^{-1} (Tables 1-10).

Compound 20*; Afzelone D dimethylether*

Yellow crystals (MeOH), Mp 172-175°C; Formula: $C_{33}H_{28}O_8$, ^{1}H NMR (400 MHz, MeOD): δ 8.22 (1H, s, β_1), 6.69 (1H, *d*, *J*= 2.2, B_1H_3),7.85 (1H, d, J=8.9 Hz, B1H6),6.83 (1H, *dd*, *J*=2.2, 8.9, B_1H_5),7.10 (4H, *d*, *J*=8.4, H-C2, A1C6, A2),6.02 (1H, *d*, *J*= 12.2, H-α_1), 4.70 (1H, *d*, *J*=12.2, H-β_1), 6.56 (4H, *d*, *J*=8.4, H-C_3, A_1C_5, A_2), 6.44 (1H, *d*d, *J*=2.3, 9.0, B2H5),6.28 (1H, *d*, *J*=2.3, B2H3),8.14 (1H, *d*, *J*=9.0, B_2H_6), 3.76 (3H, A_1C_4, OMe), 3.75 (3H, A_2C_4, OMe), 3.59 (3H, OMe B_2C_4). ^{13}C NMR (100 MHz, MeOD): δ 157.4 (β_1), 177.1 (*s*, C_1), 117.1 (*s*, B_1C-1),122.6 (α1),159.4 (*s*, B_1C_6), 103.5 (*d*, B_2C_3), 164.7 (B_1C_4), 116.5 (*d*, A_1C_5),135.9 (s, A1C1),128.2 (*d*, A_1C_2), 44.7 (α_2), 54.3 (*d*, $C_{\beta1}$), 204.8 (*s*, C_2), 129.8 (d, A_2C_2), 116.0 (*d*, A_2C_3), 156.7 (s, A_1C_4),130.5 (d, A2C6),134.9 (s, A2C1), 116.1 (*d*, A_2C_5), 159.4 (B_1C_6), 114.3 (*s*, B_2C_1),166.8 (s, B2C4), 166.9 (*s*, B_2C_2), 103.5 (*d*, B_2C_3), 109.2 (*d*, B_2C_5), 134.4 (*d*, A_2C_1), 56.0 (*q*, A_2C_4 OMe), 56.1 (*q*, A_1C_4 OMe), 55.5 (*q*, B_2C_4 OMe); IR (KBr) nmax 3855, 3443, 2933, 1611, 1510, 1452, 1365, 1256, 1026, 831 cm^{-1} (Table 6).

Compound 17*; Calodenone*

Cream white crystals (MeOH), Mp 172-175°C; Formula: $C_{31}H_{24}O_8$, ^{1}H NMR (400 MHz, MeOD): δ 8.22 (1H, *s*, β_1), 6.83(1H, *d*, *J*= 2.2, B_1H_3), 7.87 (1H, d, J=8.9Hz, B1H2),6.72 (1H, *dd*, *J*=2.2, 8.9, B_1H_5),6.03 (1H, *d*, *J*= 12.2, H-α_2), 4.67 (1H, *d*, *J*=12.2, H-β_2), 7.15 (4H, *d*, *J*=8.4, H-C_2, A_1C_6, A_2), 6.55 (4H, *d*, *J*=8.4, H-C_3, A_1C_5, A_2), 6.60 (1H, *dd*, *J*=2.3, 9.0, A1H5),6.29 (1H, *d*, *J*=2.3, B2H3),8.21 (1H, *d*, *J*=9.0, B_2H_6), 3.76 (3H, OMe A_2C_4). ^{13}C NMR (100 MHz, MeOD): δ 157.4 (β_1), 122.6 (α_1), 177.1 (s, C_1), 117.1 (s, B_1C_1),164.7 (B1C4),159.4 (s, B_1C_6), 103.5 (d, B_2C_3), 116.5 (*d*, A_1C_5), 128.2 (*d*, A_1C_2), 44.7 (α_2), 54.3 (d, $C_{\beta1}$), 204.8 (s, C_2),156.7 (s, A1C4),135.9 (s, A_1C_1), 129.8 (*d*, A_2C_2), 116.0 (*d*, A_2C_3), 134.9 (s, A_2C_1), 130.5 (*d*, A_2C_6), 159.4 (B1C6),116.1 (*d*, A_2C_5),114.3 (*s*, B_2C_1), 166.9 (*s*, B_2C_2), 103.5 (*d*, B_2C_3), 166.8 (s, B_2C_4),134.4 (d, A2C1),109.2 (d, B2C5), 56.1 (*q*, A_1C_4 OMe); IR (KBr) nmax 3753, 3423, 2747, 1626, 1510, 1454, 1369,1253, 1024, 749, 829 cm^{-1} (Table 7).

Compound 23*; β-sitosterol*

White amorphous solid ($CHCl_3$) Mp 130-134°C; Mol. Wts: 415 and Mol. Formula: $C_{29}H_{50}O$ ($CHCl_3$). IR (KBr) ν_{max}: 3400 cm^{-1} (OH- stretching), 2899 cm^{-1} (CH-stretching), 1679 cm^{-1}, 1460 cm^{-1} C=C stretching, 1041 cm^{-1} C-O-C stretching. Mass spectra: m/z: 415 (M+, $C_{20}H_{50}O$), 399 (M+-CH_3),381 (M+ CH, OH),376 (M+ H2O), 320, 303, 273, 255. ^{1}H NMR: ($CDCl_3$, 400 MHz) 0.68 ppm (3H, *s*, C_{18}H), 0.85 (H_{26}), 0.81 (H29),0.80 (H27),1.01 (H_{19}), 1.02 (3H, *s*, C_{21}H), 3.53 (1H, *m*, C_3H), 5.36 (1H, *t*, *J*=6 Hz, C_6H). Other peaks are observed at δ 0.80- δ 2.4. ^{13}C NMR ($CDCl_3$, 100 MHz) of **23**: ^{13}C NMR has given signal

at 140.8 (C_5), 121.7(C_6), 33.7 (C_{22}), 26.1 (C_{23}), 56.9 (C_{14}),56.1(C17), 71.8 (C_3), 42.3 (C_{14}), 50.2 (C_9), 36.2 (C_{20}), 39.8 (C_{12}), 42.3 (C_{13}), 42.3 (C_4), 37.2 (C_1), 30.5 (C_{10}), 31.9 (C_8),33.7 (C22),36.2 (C20), 31.9 (C8),31.7 (C7), 29.2 (C_{25}), 28.2 (C_{16}), 31.7 (C_2), 24.3 (C_{15}), 23.1 (C_{28}), 21.1 (C_{11}), 21.2,19.4 (C19),19.0 (C27), 18.8 (C_{21}), 11.9, 12.0 (C_{18}), 12.0, 11.7 (C_{29}) (Table 8).

Compound 74*; Stigmasterol*

White amorphous solid ($CHCl_3$) Mp 130-134°C; Mol. Wts: 413 and Mol. Formula: $C_{29}H_{48}O$ ($CHCl_3$). IR (KBr) ν_{max}: 3400 cm^{-1} (OH- stretching), 2899 cm^{-1} (CH-stretching), 1679 cm^{-1}, 1460 cm^{-1} C=C stretching, 1041 cm^{-1} C-O-C stretching. Mass spectra: m/z: 414 (M+, $C_{20}H_{50}O$), 399 (M+-CH_3), 376 (M+ H_2O), 381 (M+ CH, OH), 320, 303, 273, 255. ^{1}H NMR: ($CDCl_3$, 400 MHz) 0.68 ppm (3H, *s*, C_{18}H), 0.85 (H_{26}), 0.81 (H29),0.80 (H27),.01 (H_{19}), 3.53 (1H, *m*, C_3H), 5.36 (1H, *t*, *J*=6 Hz, C_6H),1.02 (3H, s, C21H), 02 (1H, *m*, H_{23}), 5.15 (1H, *m*, H_{22}). Other peaks appeared at δ 0.80- δ 2.4. ^{13}C NMR ($CDCl_3$, 100 MHz) of **74**: ^{13}C NMR has given signal at 140.8 (C_5),138.3(C22),121.7(C_6), 129.3(C_{23}), 56.8(C_{14}) 71.8 (C_3), 42.3 (C_{14}), 56.1(C_{17}), 50.2 (C_9), 40.5 (C_{20}), 39.7 (C_{12}), 42.3 (C_{13}), 42.3 (C_4), 37.2 (C_1), 31.9 (C8),30.5 (C10),40.5 (C_{20}), 31.7 (C_7), 31.9 (C_8), 31.9 (C_{25}), 28.7 (C_{16}), 31.7 (C_2), 24.3 (C_{15}), 25.4 (C_{28}),21.2 (C27),21.1 (C_{11}), 19.4 (C_{19}), 21.1 (C_{21}), 11.9 (C_{18}), 12.0 (C_{29}) (Table 8).

Compound 75*; 3β-acetyl-24-ethylfriedelane*

White amorphous solid ($CHCl_3$), Mp 266–267°C; IR ((KBr) ν_{max}: 3619, 3471, 2725, 2869, 1448, 1384, 1360, 1172, 1089, 1020, 1000, 979, and 720; ^{1}H-NMR (600 MHz; $CDCl_3$; ppm) δ 3.70 (l s; H-3; 1H) and 0.83-0.97 (superposed signals) ^{1}H NMR ($CDCl_3$, 600 MHz), 0.82 (3H, *s*, CH_3-25), 0.86 (3H, *s*, CH_3-24), 0.93 (3H, *s*, CH_3-28),1.03 (6H, *s*, CH_3-27, CH3-28),0.97 (3H, s, CH_3-32), 1.00 (3H, *s*, CH_3-23), 1.23 (3H, *s*, CH_3-30), 2.00 (3H, *s*, CH_3), 4.10, (1H, *d*,14.0, H_3). ^{13}C-NMR (150 MHz; $CDCl_3$; ppm) δ 72.8 (C_3), 61.3 (C_{10}), 53.2 (C_8), 37.1 (C_4),60.4 (C5),42.8 (C_{18}), 41.7 (C_6), 39.7 (C_{14}), 39.3 (C_{22}), 38.4 (C_{13}), 37.1 (C_{32}), 37.8(C_9),35.6 (C_{16}), 35.3 (C_{11}), 35.2 (C2), 35.2 (C_{19}), 35.0 (C_{30}), 32.8 (C_{21}), 32.3 (C_{15}), 32.1 (C_{28}), 31.8 (C_{29}), 30.6 (C_{12}), 30.0 (C_{17}), 28.2 (C_{20}), 20.1 (C_{26}), 18.2 (C25),18.6 (C27),17.5 (C_7), 16.4 (C_{24}),14.2 (31),15.8 (C_1), and 11.6 (C_{23}) (Table 9).

RESULTS AND DISCUSSION

Plant material yield of extracts

The MeOH extracts of root and stem recorded the highest return (Table 1). The root bark of *O. thomasiana* was more abundant in metabolites that were soluble in the MeOH solvents used in the extraction compared DCM and EtOAc solvents. It also showed that the yield increased with increased polarity, as in the case of the root bark extract, which increased from 1.15 % yield with DCM as a solvent to 27.0 % with MeOH as the solvent. This suggests that both the stem and root bark of MeOH extracts contain more polar compounds. Before embarking on fractionation of the crude extracts, these extracts were subjected to various antibacterial tests. The total yield of the extract from the stem bark was 22.45 % while that of the root bark was 34.6%.

Antibacterial disc diffusion screening test for the *O. thomasiana* extracts

The DCM extracts

The stem DCM extracts did not show any activity against *P. aeruginosa, S. typhi,* and *S.aureus.* The root DCM extracts showed slight activity against *B. subtilis*, but no activity on *P. aeruginosa*, S. typhi, and *S. aureus* (Table 2).

The ethyl acetate extracts

The root and the stem bark extracts of *O. thomasiana* exhibited moderate activity against *S. aureus* and *B. subtilis* (Table 2). There was no antibacterial activity observed for the acetate extracts of the plant against *S. typhi* and *P. aeruginosa*.

The methanol extracts

There was high activity against *B. subtilis* and *S. aureus* for the root bark extracts of *O. thomasiana* (Table 2). The root and stem methanol extracts for the plant were highly active against *B. subtilis.* Methanol extracts demonstrated broad spectra of activity against the tested organisms, except for *P. aeruginosa and S.typhi*. The MeOH root extract of the plant had moderate activity at the concentrations tested with a mean zone of inhibition diameter greater than or equal to 15 mm.

The selective effects of the extracts *S. aureus and B. subtilis* suggest that the extracts may serve as a source of compounds that can be used to combat infection caused by these organisms (Tania et al. 2000).

Table 1. Plant material yield of *Ochna thomasiana* extracts

Plant part	Extract	Mass of extract(g)	% yield
	DCM	71	1.10
Stem bark	EtOAc	100	1.65
	MeOH	741	19.7
	DCM	46	1.15
Root bark	EtOAc	167	6.65
	MeOH	631	27.0

Table 2. The inhibition zones (in mm) of crude extracts of *Ochna thomasiana*

Extract	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*S. typhi*
EtOAc stem	9	11	6	6
MeOH stem	10	14	6	6
EtOAc root	9	11	6	6
MeOH root	15	20	6	6
Control experiment (+ve)	20 (Tet)	18 (Tet)	19 (Tet)	15 (Tet)

Note: *Bacillus subtilis* ATCC 25726, *Staphylococcus aureus* ATCC 25724, *Pseudomonas aeruginosa* ATCC 25723, *Salmonella typhi* (clinical isolate), Tet: tetracycline

Table 3. The MIC and MBC (µg/mL) of crude extracts of *Ochna thomasiana*

Extract	µg/mL	*S. aureus*	*B. subtilis*
EtOAc stem	MIC	1000	1000
	MBC	8000	8000
MeOH stem	MIC	1000	1000
	MBC	8000	8000
EtOAc root	MIC	1000	1000
	MBC	8000	8000
MeOH root	MIC	500	500
	MBC	8000	8000

MIC and MBC of *Ochna thomasiana*

The MIC and MBC results are also given in Table 3. MeOH root bark recorded *the lowest MIC* of 500 µg/mL against both *B. subtilis* and *S. aureus*. Both stem and root extract of EtOH and EtOAc respectively showed the highest MIC of 1000 µg/mL against *B. subtilis*. The activity against all the Gram-positive bacteria used was high in the stem and root extracts because all of them had MICs of 500-1000 µg/mL. The MBCs for all extracts were observed to be about 8000 µg/mL; confirming that *O. thomasiana* had considerable antimicrobial activity against gram-positive tested microorganisms. The MeOH and EtOAc extract showed more activity against the tested organisms, as indicated by the susceptibility, MIC, and MBC tests. The result suggests that more of the bioactive chemical constituents present in the MeOH extracts. These could probably be polar or moderately-polar compounds such as flavonoids. All the EtOAc and MeOH extracts had no activity against all the Gram-negative bacteria tested.

Structure elucidation

The structure elucidation of compounds 17 to 75 employed IR spectroscopic, mass spectrometric spectral data, and interpretive NMR obtained for the purified compounds in comparison with literature values.

Compound 18

Compound 18 was isolated as a cream-white amorphous solid (15.3 mg) from a MeOH soluble portion of EtOAc root bark extract (MeOH/DCM; 1:9) and with an mp of 211- 213°C. It had fluorescing characteristic under UV-light (254 nm) and produced a characteristic red colored spot when sprayed with *p*-anisaldehyde followed by heating at 110°C in the oven for 5-10 minutes. The UV in MeOH displayed absorptions 237 and 335 nm suggestive of an isoflavonoid nucleus (Enas et al. 2012). The IR spectrum, comprised of absorption bands at 3252 cm^{-1} (C-H aromatic stretching), 831^{-1} (out of plane bend), 1625 cm^{-1} (aromatic C=C). The IR spectrum produced a broad O-H absorption band at 3368 cm^{-1} and two carbonyl bands at 1625 cm^{-1}.

The ^{1}H, ^{1}H COSY, and the ^{1}H NMR spectra displayed the presence of two *p*-substituted aromatic rings plus two trisubstituted phenyl ring systems. A singlet at δ 8.25 is characteristic of H-β_1 of a 3-substituted benzopyran- 4-one moiety and the peaks at δ 6.01 and 4.67 showed the presence of an AX system. The large coupling (*J*=12.2 Hz) seen between the latter two protons suggested that they are in an antiperiplanar (Table 4) (Mabry et al. 1970; Pegnyemb et al. 2003).

In the ^{13}C NMR spectrum, 22 peaks are corresponding to 26 carbon atoms with chemical shifts between δ 100 to 170 indicating the presence of four aromatic ring systems in addition to the pyran-4-one ring (Markham et al. 1978). In the HSQC spectrum, the methine carbon atoms at δ 54.3 and 44.7 were positively correlated to protons at δ 4.67 and 6.01, respectively. The two carbonyl carbon atoms resonated at δ 204.6 and 177.1 and the downfield resonance of the later carbonyl carbon atom indicated that it is part of a 4-pyrone system.

The position of the two *p*-substituted aromatic ring systems (AA˝XX') is assigned by the long-range heteronuclear correlation [HMBC spectrum between H-β_2 at δ 4.67 and A_2C_1 and A_1C_1 at δ 134.9 and 129.7 of ring A_1 and A_2, respectively. The result is indicative of the connection of both rings to C-β_2; therefore, validating partial structure compound 18 (Figure 3). The downfield resonance of B_1H_6 at δ 7.89 shows its proximity to the carbonyl and therefore, the second trisubstituted aromatic ring (appeared as ring B_1) is not substituted at C_5 (Abraham et al. 2003).

The presence of a singlet at around δ 8.25 evidenced that C_2 of the benzopyran moiety is not substituted. The long-range correlation between H-α_2 and C-α_1 confirms that C-α_2 linked to the benzopyran moiety through C-α_1. The long-term relationships between B1H6 confirmed the position of the first trisubstituted aromatic system and the carbonyl at δ 177.1 suggesting that this particular ring B_1 is part of a benzopyran moiety and therefore forms ring A of a flavonoid skeleton. Long-range correlations between B_2H_6 and a carbonyl at δ 204.7 (C_2) suggested the relationship of the second trisubstituted aromatic ring to this carbonyl, which in turn was also correlated to H-α_2 as shown in partial structure and Table 5.

The assignment of compound 18 was with an agreement with the previously isolated from the stem bark of *Lophira lanceolata* (Ochnaceae) that has a molecular formula of C30H22O8 (Ghogomu et al. 1987). The NMR data of compound 18 were in agreement with those reported for the compound. The compound was confirmed to be a biflavonoid; Lophirone A and with the same structure as compound 18 based on the analysis of data above, and it is the first time the compound was isolated from this plant (Figure 5).

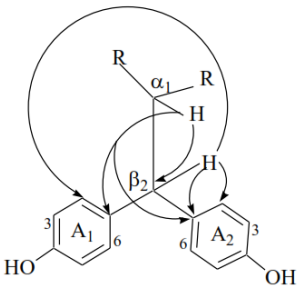


Figure 3. HMBC correlations in partial structure 18

Table 4. ^{1}H NMR (600 MHz, CD_3OD) and COSY for compound 18

Position	δObs	Multiplicity	J_{Obs}(Hz)	COSY	δ Lit.
β_1	8.25	*s*			8.27
B_13	6.85	*dd*	8.9, 2.2		6.72
B_15	6.72	*d*	2.2		6.96
B_16	7.89	*d*	8.9	B_2H_3	7.74
α_2	6.01	*d*	12.2	α_2	6.01
B_23	6.14	*d*	2.3		6.19
B_25	6.34	*dd*	9.0, 2.2		6.44
B_26	8.15	*d*	8.9		8.34
β_2	4.67	*d*	12.2		4.79
A_12	7.15	*dd*	19.5, 8.4	A_1H_3	7.26
A_13	6.55	*d*	3.1		6.65
A_15	6.60	*d*	8.5	A_1H_6	6.65
A_16	7.15	*dd*	19.5, 8.4		7.26
A_22	7.15	*dd*	19.5, 8.4	A_2H_3	7.26
A_23	6.60	*dd*	9.0, 2.3		6.61
A_25	6.60	*dd*	9.0, 2.3		6.61
A_26	7.15	*dd*	19.5, 8.4		7.26

Note: Literature data (400 MHz CD_3OD) derived from Ghogomu et al. (1987)

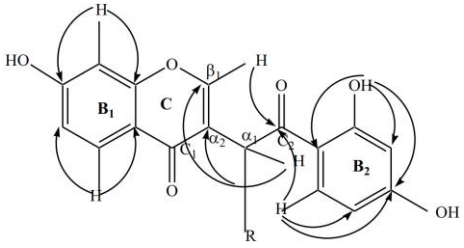


Figure 4. HMBC correlations in the partial structure compound 18

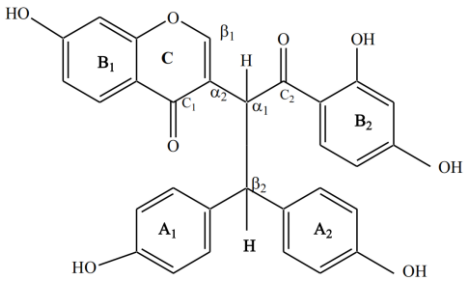


Figure 5. Structure for Lophirone A (18)

Table 5. ^{13}C NMR (150 MHz, CD_3OD), DEPT, HSQC and HMBC of 18

Position	δObs	DEPT	HSQC(δ_H)	HMBC	δ Lit.
β_1	157.4	CH	8.25		156.4
α_1	122.6	C		α_2H	122.1
C_1	177.1	C		α_2H	175.4
B_11	117.0	C			117.2
B_12	159.2	C		B_1H_2	158.5
B_13	116.0	CH	6.85		115.9
B_14	164.7	C		B_1H_2	163.4
B_15	103.3	CH	6.72		103.2
B_16	128.2	CH	7.89		128.2
α_2	44.7	CH	6.01	β_2H	43.9
C_2	204.6	C		α_2H	204.5
B_21	114.3	C			114.1
B_22	166.9	C			166.8
B_23	103.5	CH	6.14		103.5
B_24	166.8	C			166.1
B_25	109.2	CH	6.34		109.0
B_26	134.4	CH	8.15		134.4
β_2	54.3	CH	4.67	α_2H	53.4
A_21	134.9	C			134.6
A_22	130.5	CH	7.15		130.0
A_23	116.1	CH	6.60		115.8
A_24	156.7	C			156.4
A_25	116.1	CH	6.60		115.8
A_26	130.5	CH	7.15		130.0
A_11	129.7	C		A_1H_3, A_1H_5	129.6
A_12	129.8	CH	7.15	A_1H_6	129.4
A_13	116.1	CH	6.55		115.9
A_14	156.7	C		A_1H_6	156.5
A_15	116.5	CH	6.60		115.9
A_16	129.8	CH	7.15		129.4

Note: Literature data derived from Ghogomu et al. (1987); Pegnyemb et al. (2003); Anuradha et al. (2006)

Compound 20

Compound 20 was identified as an amorphous cream white powder from MeOH soluble fraction of EtOAc root bark. It gave a red color when sprayed with *p*-anisaldehyde and heated to a temperature of 110 ^{o}C for 5 to 10 minutes and fluoresced under UV-light (254 nm) suggesting the structure of a biflavonoid. Its mp was 172-176 ^{o}C.

The IR spectrum (KBr), V_{max} cm^{-1} comprised of absorption bands at 3026 cm^{-1} (C-H aromatic ring str), 957 cm^{-1} (out of plane bend), 1510 cm^{-1} and 1452 cm^{-1} (aromatic C=C- C str). Absence of absorption band at 1620 cm^{-1} to 1670 cm^{-1} suggests compound as a chalconoid or an isoflavonoid (Mabry et al. 1970; Peng et al. 2006). The IR spectrum showed a broad O-H absorption band at 3256 cm^{-1} and aromatic ring stretch at 1611 cm^{-1}. It also exhibited an OCH_3 group at 2841 cm^{-1} and vicinal C-H bands at 2715 cm^{-1}. The IR spectrum of 20 and 18 are similar in almost all aspects.

The ^{1}H-NMR spectrum exhibited signals typical of a 1,2,4-trisubstituted benzene ring representing B1H6 as indicated by a set of the meta-coupled proton at δ 6.8, δ 6.66 and an ortho-coupled proton downfield at δ 7.8_6 (Mabry et al. 1970). The proton resonance which seen as a singlet further downfield at δ 8.22 presumably due to the influence of a keto group suggests an H- β_1 proton as in an isoflavone system (Mabry et al. 1970; Pegnyemb et al. 2003). 1H NMR resonances represent another set of the 1,2,4-trisubstituted benzene ring system at δ6.2, δ6.43, and an ortho-coupled proton downfield at δ 7.74 (Pegnyemb et al. 2003a; Anuradha et al. 2006). This ring substructure is near a carbonyl group similar to Lophirone A (18). The ^{1}H NMR spectrum also appeared as two closely *para* overlapping 1,4-disubstituted benzene rings. The protons integrated for 8 hydrogens and these 2 systems (AA"XX') comprise of *ortho*-coupled protons at δ 7.21, assignable to A_1H_2 and A_1H_6 and δ 6.60 assignable to A_1H_3 and A_1H_5

as well as δ 7.15 assignable to A_2H_2 and A_2H_6 and δ 6.60 assignable to A_2H_3 and A_2H_5 of ring A_2, respectively.

The ^{1}H NMR spectrum of compound 20 resembles that of Lophirone A (18) and Afzelone D (19) (Pegnyemb et al. 2003a). It showed the same signal for protons on all the rings present as well as the ring system of two aliphatic protons. Differences observed between compound 18 (Lophirone A) and 20 included modification of the chemical shifts of the ring A_1, A_2, and B_2 protons. Also, the presence of two OMe signals at δ 3.76 (a long singlet peak, double integrated, 6H) and one OMe signal at δ 3.59 in compound 20 (Table 6).

The double integrated singlet peak suggested that the presence of two symmetrically positioned methoxyl groups, which strongly indicated that compound 20 is a derivative of compounds 18 and 19 (Ghogomu et al. 1987; Messanga et al. 2001; Pegnyemb 2003a; Anuradha et al. 2006; Abdullahi et al. 2014). The ^{13}C NMR spectrum exhibited signals for 33 carbon atoms, including the intensely overlapping signals at δ 129.8 (A_1C_2 and A_1C_6), 130.5 (A_2C_2 and A_2C_6), 116.1 (A2C3 and A2C_5) and 116.1 (A1C3 and A1C_5) (Markham et al. 1978). The carbon chemical shift values displayed 8 of the carbon atoms are oxygenated. The three singlet signals at δ 56.1, 56.0 and 55.5 were assignable to methoxy (OCH_3) group attached to the aromatic ring system. The summary of the 1D of compound 20 in comparison with 1D and 2D results of Lophirone A (18) established and characterized the structure of compound 20 as Afzelone D dimethyl ether (Trimethoxy Lophirone A) (20) (Figure 6).

Compound 17

Compound 17 was isolated as an amorphous cream white powder from MeOH soluble fraction of both DCM and EtOAc root bark. It gave a red color when sprayed with *p*- anisaldehyde and heated to a temperature of 110°C for 5 to 10 minutes and fluoresced under UV-light (254 nm) suggesting the structure of a biflavonoid. The IR spectrum exhibited a broad O-H absorption band at 3423 cm^{-1} and two conjugated carbonyl groups at 1626 cm^1 due to aromatic rings and conjugated double bonds. It also presented an O- CH_3 group and vicinal C-H absorption bands at 2850 and 2757 cm^{-1}, respectively.

The ^{1}H NMR spectrum of compound 17 was almost similar in many aspects to that of Lophirone A (18), Afzelone D (19) and Afzelone D dimethyl ether (20) (Pegnyemb et al. 2003a). It showed the same signal for protons on all the rings existed as well as the ring system of two aliphatic protons. The only difference noted arises in the modification of the chemical difference of the ring A protons and the existence of one OMe signal at δ 3.76 integrating for 3H (s) in compound 17 (Table 7). The OMe group can be assigned equally to positions A_1C_4 and A_2C_4 because its protons are equivalent to those of ring A of compound 20.

These results strongly suggest that compound 17 is a derivative of Lophirone A and Afzelone D (Ghogomu et al. 1987; Pegnyemb et al. 2003a). The spectral data of compound MJ/RE/$OT0_2$ were similar to those reported for Calodenone (17), a compound extracted from the stem bark of *Ochna calodendron* that is a derivative of lophirone A (Messanga et al. 1992; Pegnyemb et al. 2003a; Anuradha et al. 2006). Compound 17 was therefore recognized to be Calodenone (17) corresponding to the molecular formula $C_{31}H_{24}O_8$ and a molecular weight of 524 (Figure 7), and it is the first time to be reported from this plant.

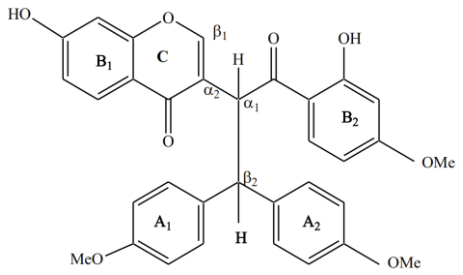


Figure 6. Structure for compound 20

Table 6. ^{1}H (400MHz) and ^{13}C NMR data for 20 (100 MHz, CD_3OD)

Position	δObs	δH (ppm), *m* (*J* (Hz))	δ Lit.
β_1	157.3	8.22 (1H, *s*)	156.4
α_1	122.4		122.1
C_1	177.0		175.4
$B_1$1	117.0		117.2
$B_1$2	128.2		128.2
$B_1$3	116.0	6.82 (1H, *dd*, *J*= 2.2 Hz)	115.9
$B_1$4	164.7		163.4
$B_1$5	103.3	6.66 (1H, *dd*, *J*= 2.2, 8.6 Hz)	103.2
$B_1$6	159.4	7.86 (1H, *d*, *J*= 8.7 Hz)	158.5
α_2	44.6	6.04 (1H, *d*, *J*= 12.2 Hz)	43.9
C_2	205.2		204.5
$B_2$1	114.3		114.1
$B_2$2	166.9		166.8
$B_2$3	103.5	6.27 (2H, *d*, *J*=8.4 Hz)	103.5
$B_2$4	166.8		166.1
$B_2$5	109.2	6.43 (*dd*, *J* = 2.5 Hz, 9.1 Hz)	109.0
$B_2$6	134.4	7.74 (4H, *d*, *J*= 9.1 Hz)	134.4
β_2	54.3	4.69 (2H, *d*, *J*= 12.2 Hz)	53.4
$A_2$1	134.9		134.6
$A_2$2	130.5	7.15 (2H, *d*, *J*=8.4 Hz)	130.0
$A_2$3	116.1	6.56 (4H, *d*, *J*=8.4 Hz)	115.8
$A_2$4	156.7		156.4
$A_2$5	116.1	6.60 (4H, *d*, *J*=8.4 Hz)	115.8
$A_2$6	130.5	7.15 (2H, *d*, *J*=8.4 Hz)	130.0
$A_1$1	129.7		129.6
$A_1$2	129.8	7.21 (1H, *d*, *J*=9.1 Hz)	129.4
$A_1$3	116.1	6.59 (1H, *d*, *J*=2.4 Hz)	115.9
$A_1$4	156.7		156.5
$A_1$5	116.5	6.60 (1H, *dd*, *J*=2.4 Hz, 9.1 Hz)	115.9
$A_1$6	129.8	7.21 (1H, *d*, *J*=9.1 Hz)	129.4
A_1-4 - OCH_3	56.1	3.76 (3H, *s*)	56.0
A_2-4 - OCH_3	56.0	3.75 (3H, *s*)	56.4
B_2-4 - OCH_3	55.5	3.59 (3H, *s*)	55.5

Note: Literature data derived from Messanga et al. (2001); Pegnyemb et al. (2003); Abdullahi et al. (2014)

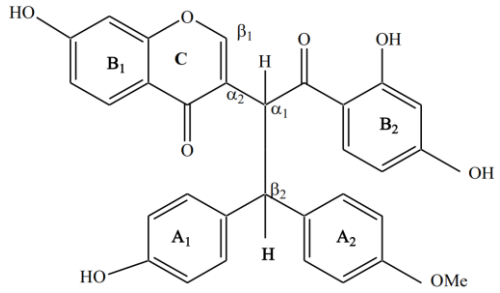


Figure 7. Structure of compound 17

Table 7. ^{1}H (400MHz) and ^{13}C NMR data for 17 (100 MHz, CD_3OD)

Position	δObs	δH (ppm), m (J (Hz))	HMBC	δ Lit.
β_1	157.3	8.22 (1H, *s*)		156.4
α_1	122.4		α_2H	122.1
C_1	177.1		α_2H	175.4
B_11	117.0			117.2
B_12	128.2	7.87 (1H, *d, J*=8.7 Hz)		128.2
B_13	116.0	6.83 (1H, *dd, J*=2.1 Hz)		115.9
B_14	164.7		B_1H_2	163.4
B_15	103.3	6.72 (1H, *dd, J*=2.1, 8.8 Hz)		103.2
B_16	159.4		B_1H_2	158.5
α_2	44.6	6.03 (1H, *d, J*=12.2 Hz)	β_2H	43.9
C_2	205.3		α_2H	204.5
B_21	114.3			114.1
B_22	166.9			166.8
B_23	103.5	6.14 (2H, *d, J*=8.4 Hz)		103.5
B_24	166.8			166.1
B_25	109.2	6.29 (*d, J* = 2.1Hz)		109.0
B_26	134.4	8.21 (4H, *d, J*=9.0Hz)		134.4
β_2	54.3	4.67 (1H, *d, J*=12.2 Hz)	α_2H	53.4
A_21	134.9			134.6
A_22	130.5	7.15 (2H, d, J=8.4 Hz)		130.0
A_23	116.1	6.58 (4H, d, J=8.4 Hz)		115.8
A_24	156.7			156.4
A_25	116.1	6.60 (4H, d, J=8.4 Hz)		115.8
A_26	130.5	7.15 (2H, d, J=8.4 Hz)		130.0
A_11	129.7		A_1H_3, A_1H_5	129.6
A_12	129.8	7.15	A_1H_6	129.4
A_13	116.1	6.55 (1H, d, J=2.4 Hz)		115.9
A_14	156.7		A_1H_6	156.5
A_15	116.5	6.60 (1H, dd, J=2.4, 9.1 Hz)		115.9
A_16	129.8	7.15 (1H, d, J=9.1 Hz)		129.4
A_14 - OCH_3	56.1	3.76 (3H, s)		56.0

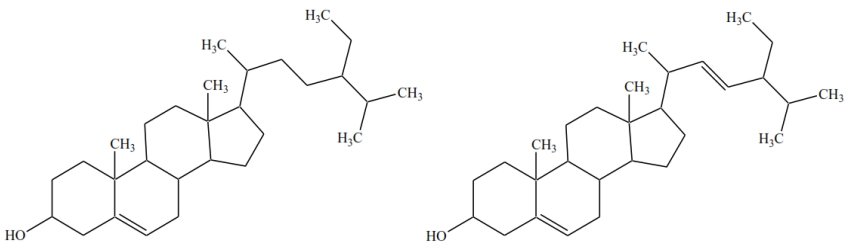
Note: Literature data derived from Messanga et al. (1992); Pegnyemb et al. (2003); Anuradha et al. (2006)

Compound 23 and 74

This compound was characterized as white amorphous solid in CHCl3 soluble fraction of DCM root bark with a melting point 130-134°C. It gave purple color on spraying in p-anisaldehyde and did not fluoresce in UV suggesting that the compound had steroidal structure. The mass spectra showed a molecular ion peak at m/z 415 [M+H] + and m/z 413 [M+H] + which corresponded to the molecular formula C29H50O and C29H48O, respectively. The other obvious fragments showed at m/z 396, 351, 300, 271, and 255 On subjecting to IR spectroscopic analysis, the observed absorption bands are 3459.1 cm-1 that is characteristic of O-H stretching and tri-substituted double bonds. Absorption at 2939.3 cm-1 and 2734.9 cm-1 is due aliphatic C-H stretching. Other absorption frequencies include 1647.1 cm^{-1}; as a result of C=C stretching; however, this band is weak. At 1463.9 cm^{-1} is a bending frequency for cyclic (CH2)n and 1381.6 cm^{-1} is for $-CH_2(CH_3)_2$. The absorption frequency at 1053.1 cm^{-1} signifies cycloalkane. The out of plane C-H vibration of the unsaturated part was observed at 881.4 cm^{-1}.

The 1H NMR of compounds 23 and 74 revealed signals for two singlet methyls at δ 1.01 and 0.68, four doublet methyls at δ 1.02, 0.85, 0.81 and 0.8. There were three vinylic proton signals at δ 5.3, 5.15 and 5.02 and an OH proton signal at δ 3.5. The remaining proton signals were at δ 0.8-2.4. Direct comparison of the 13C NMR data of 23 and 74 with those reported in the literature (Kovganko et al. 1999; Subhadhirasakul and Pechpongs 2005; Kamboj and Saluja 2011) (Table 9) exhibited they were identical. Thus, 23 and 74 was identified as a mixture of two identical compounds. From the 1H NMR spectra, integration of proton signals at δ 5.3, 5.15, 5.02 and 3.53 were in the ratio 2:1:1:2. Thus, compounds 23 and 74 was confirmed to be a mixture of two of β-sitosterol (23) and Stigmasterol (74) (Figures 8.A and 8.B, respectively) and approximately in the ratio 1:1.

The 13C NMR spectrum showed a total of 47 carbon signals, among them four olefinic carbon signals and one monoxygenated carbon signal was observed. The13C NMR gave a signal at 140.8 and 121.7 ppm for C5=C6 double bond and 138.3 and 129.3 ppm for C22=C23 double bond, respectively for compound 74 and at 140.8 and 121.7 ppm for C5=C6 double bond for compound 23 (Table 8) which demonstrated a high level of saturation in the carbon-proton linkages in its structures. The remaining carbons displayed signals having chemical shifts between 11 and 57 ppm. From the above spectroscopic data, it appeared to be a mixture of almost related compounds.



A **B**

Figure 8. A. β-sitosterol (23), B. Stigmasterol (74)

Table 8. 13C NMR (100 MHz, CDCl3), of compounds 23 and 74

Position	Group	δObs (74)	δObs (23)	δLit.(74;23)	Major δ¹H
1	CH_2	37.2	37.2	37.3	
2	CH_2	31.7	31.7	31.6	
3	CH	71.8	71.8	71.8	3.53 (m, H_3)
4	CH_2	42.3	42.3	42.3	
5	C	140.8	140.8	140.1	
6	CH	121.7	121.7	121.7	5.30 (d, H_6)
7	CH_2	31.7	31.7	31.9, 31.6	
8	CH	31.9	31.9	31.9	
9	CH	50.2	50.2	50.1, 50.1	
10	C	30.5	30.5	30.5	
11	CH_2	21.1	21.1	21.1	
12	CH_2	39.7	39.8	39.7, 39.8	
13	C	42.3	42.3	42.3	
14	CH	56.8	56.9	56.7, 56.9	
15	CH	24.3	24.3	24.3, 24.3	
16	CH_2	28.7	28.2	28.7, 28.2	
17	CH	56.1	56.1	56.7, 56.1	
18	CH_3	11.9	12.0	11.8, 12.0	0.68 CH_3
19	CH_3	19.4	19.4	19.4	1.01 CH_3
20	CH	40.5	36.2	40.5, 36.1	
21	CH_3	21.1	18.8	21.1, 18.8	1.02 CH_3
22	CH, CH_2	138.3	33.7	137.3, 33.7	5.15
23	CH, CH_2	129.3	26.1	129.3, 26.1	5.02
24	CH	51.2	45.8	51.2, 45.8	
25	CH_2	31.9	29.2	31.9, 29.2	
26	CH_3	20.0	19.8	19.0, 19.8	0.85 CH_3
27	CH_2	21.2	19.0	21.2, 19.0	
28	CH_3	25.4	23.1	25.4, 23.1	0.81 CH_3
29	CH_3	12.0	11.7	12.0, 12.0	0.86 CH_3

Note: Literature data derived from Kovganko et al. (1999); Subhadhirasakul and Pechpongs (2005)

Table 9. 13C NMR (150 MHz, CDCl3), of compound 75

Position	Group	δObs	δLit.	Position	Group	δObs	δLit.
1	CH_2	15.8	15.8	18	CH	42.8	42.9
2	CH_2	35.2	35.2	19	CH_2	35.3	35.6
3	CH	72.8	72.8	20	C	28.2	28.2
4	CH	49.2	49.1	21	CH_2	32.8	32.9
5	C	60.4	59.0	22	CH_2	39.3	39.3
6	CH_2	42.8	41.8	23	CH_3	11.6	11.6
7	CH_2	17.5	17.5	24	CH_3	16.4	16.4
8	CH	53.2	53.1	25	CH_3	18.2	18.2
9	C	37.8	37.8	26	CH_3	18.7	18.6
10	CH	61.3	61.3	27	CH_3	20.1	20.1
11	CH_2	35.6	35.6	28	CH_3	32.1	32.1
12	CH_2	32.8	32.9	29	CH	31.8	31.8
13	C	38.3	38.2	30	CH_3	35.0	35.0
14	C	39.7	39.7	31	CH_2	14.2	14.1
15	CH_2	30.6	30.6	32	CH_3	37.1	37.1
16	CH_2	36.1	36.1	$OCOCH_3$-		171.2	171.1
17	C	30.0	29.3	$OCOCH_3$-		21.1	21.4

Note: Assignments according to Aragao et al. (1990); Costa and Carvalho (2003)

Compound 75

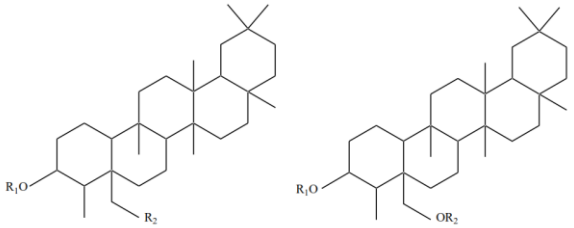
This compound was extracted as white amorphous solid in CHCl3 soluble fraction of DCM root bark with a melting point 266-267°C. It gave purple color on spraying in p-anisaldehyde and did not fluoresce in UV suggesting that the compound had a triterpenoid skeleton. The UV in CDCl3 showed one absorption band at 231.5 nm suggestive of a triterpenoid nucleus (Enas et al. 2012). The calculated molecular mass was 500.4, which corresponded to the molecular formula C34H60O2. IR (KBr) spectrum peak shows an intense C-H stretch at 2936 cm^{-1}, 2866 cm^{-1} indicates the presence of –CH3 and –CH2, CH antisymmetric/symmetric stretch. The C-O-C stretching frequency was attributed to 1226 cm^{-1}. At δ 171.2, an acetyl group is linked to C3, supported by the appearance of a peak in the IR spectrum at 1707 cm^{-1}. As a result, ester carbonyl stretch (Houghton and Lian 1986).

Structural determination of 75 was achieved by careful analysis of 1D (1H, 13C, and DEPT) NMR spectra. The 1H NMR spectrum of compound 75 appeared as singlet signals for nine methyl groups of a pentacyclic triterpenoid, and a signal at δ 2.00 is characteristic of an acetyl group. The 1H NMR data of 75 showed methyl protons at δ 0.835-0.982, one C3 proton at δ 3.70 are typical of ester protons, and δ 4.08 is characteristic of two methylene hydrogens. The 1H NMR data did not allow much analysis of the hindered signals. However, we observed a doublet (J = 7.8 Hz) centered at δ 0.835 ppm characteristic of a methyl group at position C23 of friedelane compounds (Lopez-Perez et al. 2007).

Comparing the 1H and 13C NMR data of compound 75 with that of 3β,24- diacetylfriedelane (76) implied that compound 75 was similar to 76 in rings A, B, C and D (Figure 9). It suggests that the acetyl group is bound to C3, which was supported by the HMBC correlations of the latter (Mahato and Kundu 1994; Carvalho et al. 1995). The DEPT 13C NMR spectra were used to recognize the signals corresponding to six quaternary carbons, one monoxygenated carbon at δ 72.8 besides signals of nine methyls, twelve methylene, six methylene groups and one acetyl group (δ 171.2, 21.1). From the DEPT spectra, the compound has 34 carbons, of which 27 bear proton. Compound 75 was given the name 3β-acetyl-24-ethylfriedelane.

Antibacterial activity test for the isolated compounds

The activity of isolated compounds 17 to 75 was measured using the same procedure as discussed for the crude extracts, based on the method used by Chhabra and Usio (1991). The inhibition zone was determined as in the crude extracts using the same pathogens except for *E. coli*. An isolated compound showing a clear area of 9 mm or higher was considered to have significant activity. The antimicrobial investigations suggest the compounds have activity against *S. aureus* and *S. typhi*, however, were not active at the tested *E. coli* (Table 10). The isolated compounds displaying zones of inhibition below 9 mm were considered to be inactive (Faizi et al. 2003). All the compounds showed activity against all indicator strains, but compound mixture 23 and 74 had moderate activity as presented (Table 10).



R1 = Ac **R2** = Et **R1** = **R2** = Ac

A **B**

Figure 9. Structure for A. 75, B. 76

Table 10. Inhibition zones (in mm) of antibacterial activity of isolated compounds

Compound	*S. aureus*	*E. coli*	*S. typhi*
Compound 75	16	6	12
Compound 17	12	6	14
Compound 20	14	7	14
Compound 18	18	6	12
Compound 23 and 74	10	6	6
Chloramphenicol (+ve)	25	24	23
DMSO (-ve)	6	6	6

Note: Staphylococcus aureus ATCC 25724, Escherichia coli ATCC 25723, Salmonella. typhi (clinical isolate)

The study supports the finding that biologically-active compounds and potential pharmaceuticals can be isolated from medicinal plants. As was found from other Ochnaceae species, the majority of the compounds isolated from the plant were biflavonoids. This project aimed at investigating and establishing the presence of antimicrobial activity in Ochna thomasiana based on its use in traditional medicine against microbial infections. The following was found: (i) The plant crude extracts had appreciable yields, especially the MeOH extracts. (ii) There was a notable activity for the root and stem bark MeOH extracts with MICs against the tested Gram-positive bacteria fell between 500 and 1000 µg/mL. (iii) The MeOH root bark extract showed the greatest activity against Gram-positive bacteria (iv). All the extracts had no activity against the Gram-negative bacteria strains. (v) We isolated and characterized three biflavonoids and three sterols. (vi) Compounds 18, 20, and 75 from the plant had fairly noticeable activity against Sa. typhi and S. aureus ranging between 12-18 mm. However, all the isolated compounds had no activity against *E. coli*.

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Anti-inflammatory activities of ethanol leaves extract and solvent fractions of *Zehneria scabra* (Cucurbitaceae) in rodents

REDIET BELAY, EYASU MAKONNEN ♥
Department of Pharmacology, School of Medicine, College of Health Sciences, Addis Ababa University. Addis Ababa, Ethiopia,
♥email: eyasu.makonnen@aau.edu.et

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Abstract. *Belay R, Makonnen E. 2020. Anti-inflammatory activities of ethanol leaves extract and solvent fractions of* Zehneria scabra *(Cucurbitaceae) in rodents. Biofarmasi J Nat Prod Biochem 18: 42-56. Zehneria scarba* (L.f.) Sond is one of the medicinal plants used in folkloric medicine of Ethiopia for years to treat various inflammatory disorders. The present study was aimed to validate the anti-inflammatory activity of crude 70% ethanol leaves extract (70EE) against a sub-acute model and further evaluate the solvent fractions (AF, BF, and CF) in an acute (carrageenan-induced paw edema), sub-acute (formaldehyde induced arthritis) and chronic (cotton pellet induced granuloma) inflammatory models. The 70EE was first prepared by maceration, and the fractions were obtained by sequential partitioning with chloroform and n-butanol from the aqueous suspension of crude 70EE. The test groups then received 100, 200, and 400 mg/kg of the crude 70EE or the fractions (AF, BF, and CF) at the same dose levels. In contrast, positive controls received aspirin (200 mg/kg) or dexamethasone (0.5mg/kg) and negative controls received vehicle (2% tween 80 or distilled water, 10 mL/kg). All tested doses of the crude 70EE showed significant inhibition of formaldehyde-induced arthritis on the 10th day of treatment. The 400 mg/kg dose showed the maximum anti-arthritic effect (%A = 60.5; $p < 0.001$). In the carrageenan-induced paw edema, all three fractions showed a statistically significant effect, in fact, with different onset and magnitude. In this model, the AF was found to be the most active fraction, and the 400mg/kg dose demonstrated the maximum effect (%A = 76.25; $p < 0.001$) at 5h post-induction, which is much better than the effect of aspirin at the dose employed. The overall order of efficacy in inhibiting the exudative component of carrageenan-induced paw edema was found to be AF> BF> CF. The AF was also the most active fraction in inhibiting the exudative component of chronic inflammation in the cotton pellet-induced granuloma model, where the maximum effect (%A = 43.10, $p < 0.001$) was exhibited by a dose of 400mg/kg. The AF was also the most active fraction in inhibiting formaldehyde-induced arthritis, in which the BF and CF relatively showed a comparable effect throughout days 4-10. On the contrary, in the cotton pellet-induced granuloma model, the CF was the most active fraction in inhibiting the proliferative and granulomatous component of chronic inflammation. The overall order of effectiveness was found to be CF> AF> BF. Besides, 400mg/kg of CF demonstrated the maximum inhibition of granuloma formation (%A = 55.52; $P < 0.001$). The phytochemical analysis revealed the differential distribution of secondary metabolites into the three fractions, which either singly or in concert appeared to be responsible for the observed effects. The data obtained from the present study collectively indicate that the extract and fractions of leaves of *Z.scabra* possessed a significant anti-inflammatory activity, upholding the folkloric use of the plant.

Keywords: Anti-inflammatory, arthritis, granuloma, phytochemical, *Zehneria scarba*

INTRODUCTION

Inflammation is central to many diseases that affect both developed and emerging nations. Virtually all acute and chronic diseases are either driven or modulated by inflammation (Vodovotz et al., 2010). Despite this fact, the complex interplay between beneficial and harmful arms of the inflammatory response underlies the lack of safe and fully effective therapies for many pathologies (Vodovotz et al., 2009). On top of this, most existing drugs suffer from diverse adverse events, especially at higher doses and longer duration of therapy (Spies et al., 2011).

Non-steroidal anti-inflammatory drugs (NSAIDs), for example, are associated with the development of gastric or duodenal ulceration, nephrotoxicity, bronchospasm, exacerbation of symptoms of asthma, an increase in blood pressure, and increased incidence of myocardial infarction and stroke (Ong et al. 2007; Stanos 2013). On the other hand, corticosteroids are associated with numerous side effects, such as diabetes mellitus/glucose intolerance, hypertension, obesity, osteoporosis, immune suppression, glaucoma, and growth retardation in children (Spies et al. 2011; Rhen and Cidlowski 2005). Because of this, WHO advocates the inclusion of herbal medicines with proven safety and efficacy in healthcare programs because of the high potential they hold in combating various diseases (WHO 2002).

Z.scabra is one of these plants whose 80% methanol leaf extract has been proven anti-inflammatory and analgesic activity. Such a plant exhibiting anti-inflammatory, analgesic (Akele 2012), antifungal (Arulappan et al. 2015), and antibacterial activity (Anand et al. 2012) would improve patient compliance and has economic importance. However, there is a shortage of scientific evidence to substantiate further the plant's therapeutic potential in different inflammatory models.

Hence, this study focuses on an *in-vivo* anti-inflammatory activity of 70EE, and solvent fractions of *Z. scabra* leave using acute (carrageenan-induced paw edema), sub-acute (formaldehyde induced arthritis), and

chronic (cotton pellet induced granuloma) models of inflammation to validate its acclaimed use by the traditional practitioners.

Moreover, it also tries to identify the most active fraction to give a clue for further investigation in search of the specific agent(s) responsible for the anti-inflammatory effect of the plant. Besides, the finding of this study might provide a clue about the possible mechanisms of anti-inflammatory action of the plant. It might serve as baseline information for the scientific community to further investigate the plant by initiating advanced studies on molecular mechanisms to identify the specific agent(s) responsible for the anti-inflammatory effect of the most active fraction.

The aims of this research were (i) To assess the acute toxicity of 70EE and solvent fractions of *Zehneria scabra* leaves in mice, (ii) To evaluate the effect of crude 70EE of *Zehneria scabra* leaves on formaldehyde-induced arthritis in mice, (iii) To assess the effect of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves on carrageenan-induced paw edema in mice, (iv) To evaluate the effect of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves on formaldehyde-induced arthritis in mice, (v) To assess the impact of chloroform, n-butanol and aqueous fractions of *Zehneria scabra* leaves using cotton pellet induced granuloma in rats, (vi) To determine the phytochemical constituents of the 70EE and solvent fractions of *Zehneria scabra* leaves.

MATERIALS AND METHODS

Drugs and chemicals

Aspirin active (EPHARM, Ethiopia), Dexamethasone (Medico Labs, Lot E6A00, Syria), Carrageenan (Sigma Aldrich, Germany), thiopental sodium (NEON Labs, India); ethanol (Lot 30320601EX) and formaldehyde (Research-Lab Fine Chem Industries- India); n-butanol (Lot 10061), chloroform (Lot 10077), and glacial acetic acid (Lot MR0478) (BDH chemical LTD Poole, England); acetic anhydride (Lot A13/45/67/A) and Mayer's reagent (May & Baker LTD Dagenham, England); Dragendroff's reagent and sulfuric acid (Lot 30326) (Fisher Scientific, UK); ammonia, hydrochloric acid, and ferric chloride (Lot 10111) (BDH Laboratory Supplies Poole, England); normal saline (Addis Pharmaceutical Factory, Ethiopia), distilled water (Biochemistry Laboratory of AAU, Ethiopia), tween 80 (UNI-CHEM Chemical Reagents, India), were used in the study and all were of analytical grade.

Materials and instruments

Rotary evaporator (Heidolph, Germany), lyophilizer (OPERON, OPR-FDU-5012, Korea), digital plethysmometer (Ugo Basile-Cat no 7140, Italy), electronic balance (KERN-ALJ 220-4, Germany), mini orbital shaker (SSM1-STUART), Tissue Drying Oven (Medite - Medizin Technik, Germany), separatory funnel, flasks, Cotton pellets, syringes with needles, feeding tube, blunt forceps, scissors, suturing set.

Plant material collection and authentication

The fresh leaves of *Zehneria scabra* were collected in the Lideta sub-city, Addis Ababa, Ethiopia, in December 2014. Identification and authentication of the plant specimen were made at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, and a voucher specimen was deposited with voucher number RB 001/2014 for future reference. The leaves were washed gently by rinsing with running water to remove dust particles, air-dried under the shade, and then reduced into a coarse powder with mortar and pestle.

Experimental animals

Healthy male (for the anti-inflammatory test) and female (for acute toxicity test) Swiss albino mice weighing 20-30 g and male albino Wistar rats weighing 180-220 g were obtained from the animal house of the Ethiopian Public Health Institute and Department of Pharmacology, School of medicine, Addis Ababa University were used. They were kept in plastic cages at room temperature on a 12 h light-dark cycle with free access to pellet food and water *ad libitum*. The animals were acclimatized to laboratory conditions for one week before the commencement of the experiments. All experiments were carried out during the light period of the day (9:00 a.m. - 5:00 p.m.) and followed the guideline for the care and use of laboratory animals (Institute for Laboratory Animal Research 1996; OECD 2008). The study was conducted after approval by Addis Ababa University, College of Health Sciences Institution Review Board (IRB).

Preparation of crude extracts and solvent fractions of *Z. scabra* leaves

The air-dried and powdered leaves of *Z. scabra* were first defatted by macerating with petroleum ether for 24 hours at room temperature and occasionally shaking, followed by filtration. The solvent (petroleum ether) was removed from the residue by exposing it to open air. Then the defatted coarse powder was divided into two halves of 665gm each for the extraction process. The first half (665gm of the leave) was macerated in a flask containing 70% ethanol (1:5 w/v) for 72 hrs, and the other half was macerated with distilled water with the same ration and for the same period. The maceration was undertaken with occasional shaking using a mini orbital shaker tuned to 120 rpm for 72 hrs at room temperature. Then, the extract was filtered first using a muslin cloth and then using Whatman filter paper (No 1), and the marc was re-macerated for a second and third time by adding another fresh solvent.

The three batches of the 70EE filtrates were combined and concentrated in a rotary evaporator with a temperature of 40 °C. The concentrate was then placed in a deep freezer operating at negative 20°C until it formed a block of ice, and then the remaining solvent (water) was removed using a lyophilizer. After water removal, a black powder residue weighing 88 gm was obtained, giving rise to a percentage yield of 13.23%. The filtrates from the three batches of the aqueous extract were also combined, placed in a deep freezer at -20°C to form ice, and lyophilized. The water extract yields 39.34 gm of black powder (5.92% w/w). The

powders were kept in tightly stoppered bottles and stored in a deep freezer at -20°C until the commencement of the pilot study.

The hydroalcoholic extract (70EE) (which had a better activity) was selected for further fractionation based on the pilot experiment performed. A total of eighty grams of the powdered residue of the 70EE was divided into four equal parts (20gm each) and then employed for the fractionation. At a time, the 70EE (20 g) aliquot was suspended in distilled water (100 mL) and then sequentially partitioned with chloroform and *n*-butanol at room temperature using a separatory funnel. Partitioned layers of each solvent were pooled together and concentrated on a rotary evaporator at 40^0C, followed by an oven at room temperature for 48 h, yielding 13.4 gm of black gummy residue from the chloroform fraction (16.75% w/w), and 16.73 gm of light brown slightly hygroscopic powder from the *n*-butanol fraction (20.91% w/w). The aqueous residue was lyophilized to give 38.85gm of black dried powder (48.56% w/w) fraction. The fractions were kept in tightly stoppered bottles and stored in a deep freezer at -20°C until the commencement of the actual experiment. Finally, the portions were reconstituted in distilled water/2% tween 80 at appropriate concentrations for the various trials.

Acute oral toxicity test

An acute oral toxicity test for 70EE and solvent fractions of the leaves of *Z. scabra* was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 425; "Limit Test at 2000 mg/kg" (2008). Five female albino mice of 6-8 weeks were used for each test. All mice fasted for four h before and two h after administering the extract/fractions. First, a sighting study was performed to determine the starting dose, in which a single female mouse was given 2000 mg/kg of the extract/fractions as a single dose by oral gavage. Since no death was observed within 24 h, four additional mice were used for each extract and fraction and administered the same dose of extract/fractions. The animals were observed continuously for 4 h with 30 min intervals and then for 14 consecutive days with a range of 24 h for the general signs and symptoms of toxicities such as changes in skin and fur, eyes and mucous membranes, somatomotor activity, and behavioral pattern, salivation and diarrhea, weight loss, tremor and convulsions, lethargy and paralysis, food and water intake and mortality.

Pilot study

The pilot study was done using the Carrageenan-induced paw edema acute model of inflammation on 70% ethanol leave extract (70EE), aqueous leave extracts (AE), and petroleum ether extract (PE) of *Z. scabra.* All three extracts were administered at doses of 100, 200, and 400mg/kg, and three animals per group were used at all the dose levels. The result indicated that both the AE and 70EE have anti-inflammatory activity; even though the 70EE had shown a better anti-inflammatory effect (%A) at all employed doses (maximum %A at the 5^{th} h = 37% for 100mg/kg, 45% for 200mg/kg, and 52% for 400mg/kg) than the AE (maximum %A at the 5^{th} h = 19% for 100mg/kg, 28% for 200mg/kg, and 41% for 400mg/kg). On the other hand, the PE extract failed to demonstrate anti-inflammatory activity at all employed doses. Hence, the 70EE opted for further study and fractionation.

Animal grouping and dosing

The animals were randomly assigned into twelve groups of six to perform the anti-inflammatory activity test in three models. The first two groups served as negative controls, and the vehicles for the fractions (distilled water and 2% tween 80 at a dose of 10 mL/kg) were administered. The third group served as a positive control. The standard drugs aspirin (200mg/kg p.o. in the acute and sub-acute models) or dexamethasone (0.5mg/kg p.o. in the chronic model) was administered to this group. The first three test groups (4-6) received three doses (100, 200, and 400mg/kg) of the aqueous fraction. The next three test groups (7-9) received n-butanol fraction at doses of 100, 200, and 400mg/kg, while the final three test groups (10-12) received the chloroform fraction at the same three dose levels. The same dose levels were also applied during an anti-inflammatory test of the crude extract using formaldehyde-induced arthritis in mice, during which a total of five groups, i.e., two control groups (positive and negative) and three test groups of 70EE (100, 200, and 400mg/kg) were used. The dose levels (100, 200, and 400mg/kg) were selected based on the acute oral toxicity test results and pilot study.

Determination of anti-inflammatory activity

Carrageenan-induced paw edema

The method described by Mequanint et al. (2011) was followed in this model to study the effect of solvent fractions of *Z. scabra* leaves on acute inflammation. Mice fasted for 12 h with free access to water until the experiment started and grouped and treated by oral gavage as described under section 3.8. Aspirin 200mg/kg p.o. was administered as a standard drug. The right hind paw was marked with ink at the level of lateral malleolus so that it could always be immersed to the same extent in the measurement chamber of the plethysmometer.

An hour later, edema was induced by injecting 0.05 mL of 1% w/v carrageenan in normal saline into the right hind paw of each mouse. The increased volume of the right hind paws was taken to signify paw edema. Paw volume was determined by volume displacement technique using the Ugo-Basile plethysmometer just before carrageenan injection (initial amount (v_0)), and 1, 2, 3, 4, and 5 h after carrageenan injection (final volumes (v_f)). The degree of swelling, i.e., edema, was evaluated by the delta volume (v_f - v_0) (Sanusi et al., 2013).

$$\text{Oedema (I)} = V_f - V_0$$

Where: V_0 is the paw volume before the carrageenan injection, and V_f is the paw volume after the carrageenan injection at a given time.

Also, the anti-inflammatory effect of the fractions expressed in percentage (%A) was calculated according to the formula given by Sanusi et al. (2013):

$$\% \text{ inhibition } (\%A) = \left(1 - \frac{It}{Ic}\right) x\ 100$$

Where: It and Ic are the mean inflammation (Oedema) values reached at a given time in treatment and control groups, respectively.

Formaldehyde induced arthritis

The method described by Mehta et al. (2012) and Cui et al. (2014) was used as a subacute inflammation model. Mice (20-30 g) fasted for 12 h with free access to water until the commencement of the experiment. The right hind paw was marked with ink at the level of lateral malleolus so that it could always be immersed to the same extent in the measurement chamber of the plethysmometer. The control, standard, and test groups of mice received distilled water (2% tween 80 in case of chloroform fraction), Aspirin (200mg/kg p.o.), and extract/fractions, respectively, as described in section 3.8. On the first day, the basal paw volume (V_0) of the right hind paw of each mouse was measured using a plethysmometer. On day one and day 3, mice were injected into the sub-plantar region of the right hind paw with 0.05 mL of 2 % v/v formaldehyde in normal saline. Dosing with vehicles, standard drug (Aspirin), and extract/fractions was started on the same day an hour before induction of arthritis and continued for ten consecutive days by oral gavage. The mice paw volume was recorded daily using a plethysmometer after one h of drug administration, but on day one, the measurement was taken three h after formaldehyde injection. Finally, the percentage of inhibition of edema was calculated, as described in section 3.9.1.

Cotton pellet induced granuloma method

The method previously described by Afsar et al. (2013) was used to assess chronic inflammation's transudative and proliferative (granulomatous) components. Male albino Wistar rats (180-220 g) fasted for 12 h with free access to water until the commencement of the experiment. The control, standard, and test groups of rats received distilled water (2% tween 80 in case of chloroform fraction), dexamethasone (0.5 mg/kg p.o.), and fractions, respectively, as described in section 3.8.

Cotton pellets weighing ten ± 1 mg were sterilized in an autoclave for 30 min at 120 C^0 under 15Ib pressure. Twenty minutes after treatment with the standard drug and fractions, the rats were anesthetized with thiopental sodium (25 mg/kg, i.p.). The subcutaneous tunnel was made aseptically using blunted forceps on both sides of each rat's previously shaved groin region. Two sterilized cotton pellets weighing 10±1 mg each were implanted bilaterally in the subcutaneous tunnel and sutured with chromic catgut (0/4metric-1/2 Circle). Treatment with the standard drug (dexamethasone) and fractions continued for seven consecutive days (p.o., once a day). On the 8th day, the rats were sacrificed with ether anesthesia; then, the pellets surrounded by granuloma tissue were dissected carefully and freed from extraneous tissue. The wet weight of the cotton was taken immediately after removal and then dried up to constant weight at 60^0C for 24hrs, and the net dry weight, that is, after subtracting the weight of the cotton pellets.

The exudate amount (mg), granulation tissue formation (mg), and percent inhibition of exudate and granuloma tissue formation were calculated according to the formula given by Aziz et al. (2014):

$$\text{Exudate inhibition } (\%) = (1 - \frac{\text{Exudate in treated group}}{\text{Exudate in controls}})X100$$

$$\text{Granuloma inhibition } (\%) = (1 - \frac{\text{Granuloma in treated group}}{\text{Granuloma in controls}})X\ 100$$

Where:

The measure of exudate formation = immediate wet weight of pellet - Constant dry weight of the pellet

The measure of granuloma tissue formation = Constant dry weight - Initial weight of the cotton pellet.

Preliminary phytochemical screening

The initial phytochemical screening of secondary metabolites of 70EE, and chloroform, n-butanol, and aqueous fractions of leaves of *Z.scabra* were carried out using standard tests (Debella 2002; Sasidharan et al. 2011).

Test for saponins

To 0.25 g of 70EE and each fraction (AF, BF, and CF), 5 mL of distilled water was added to a test tube. Then, the solution was shaken vigorously and observed for a stable, persistent froth. The formation of a stable froth that persisted for about half an hour indicated the presence of saponins.

Test for terpenoids

To 0.25 g of 70EE and each fraction, 2 mL of chloroform was added. Then, 3mL concentrated sulfuric acid was carefully added to form a layer. A reddish-brown coloration of the interface indicated the presence of terpenoids.

Test for tannins

About 0.25 g of 70EE, each fraction was boiled in 10 mL of water in a test tube and filtered with filter paper (Whatman No. 1). A few drops of 0.1% ferric chloride were added to the filtrate. A brownish-green or a blue-black precipitate indicated the presence of tannins.

Test for flavonoids

About 10 mL of ethyl acetate was added to 0.2 g of 70EE, and each fraction and heated in a water bath for 3 min. The mixture was cooled and filtered. Then, about 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. The layers were allowed to separate, and the yellow color in the ammonia layer indicated the presence of flavonoids.

Test for cardiac glycosides

To 0.25 g of 70EE and each fraction diluted with 5 mL of water, 2 mL of glacial acetic acid containing one drop of

ferric chloride solution was added. This was underlaid with 1 mL of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.

Test for steroids

Two mL of acetic anhydride was added to 0.25 g of 70EE and each fraction with 2 mL sulfuric acid. The color change from violet to blue or green in some samples indicated the presence of steroids.

Test for alkaloids

0.5 g of 70EE, and each fraction was diluted to 10 mL with acid alcohol, boiled, and filtered. To 5 mL of the filtrate, 2 mL of dilute ammonia and 5 mL of chloroform were added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 mL of acetic acid. This was divided into two portions. Mayer's reagent was added to one part, and Dragendorff's reagent to the other. The formation of cream (with Mayer's reagent) or reddish-brown precipitate (with Dragendorff's reagent) was regarded as positive for the presence of alkaloids.

Statistical analysis

The data were analyzed using SPSS version 16.0 for Windows. The experimental results are expressed as mean ± standard error of the mean (SEM), and statistical significance was carried out by employing a one-way analysis of variance (ANOVA) followed by the Tukey post hoc test for multiple comparisons to compare results among groups, where p values < 0.05 were considered statistically significant. The analyzed data were then presented using tables. Linear regression was also used where appropriate.

RESULTS AND DISCUSSION

Acute oral toxicity test

The acute oral toxicity test of 70EE and fractions (AF, BF, and CF) of leaves of *Z. scabra* showed that neither the 70EE nor the solvent fractions caused gross behavioral changes, toxic effects, or mortality within 24 h and in the next 14 days. According to the "Limit Test at 2000 mg/kg" of OECD guideline 425 (2008), it can be concluded that the oral LD_{50} of both the crude 70EE and solvent fractions are more significant than 2000 mg/kg in mice.

Carrageenan induced paw edema

Subplantar injection of 0.05 mL of 1% carrageenan to the mice's hind paw produced a progressive increment of paw thickness that reached its maximum value after 3h of induction in both distilled water and 2% tween vehicle controls (Table 1). All tested doses of the aqueous fraction (100, 200, and 400mg/kg of AF) showed significant inhibition of paw edema that started from 1 h ($p < 0.01$), and the effect lasted till 5 h post-induction ($p < 0.001$ from 2^{nd} - 5^{th} h) as compared to the distilled water vehicle control.

Maximum anti-inflammatory effect (%A) by the 100, 200 and 400 mg/kg of AF was observed at 5 h post-induction, with respective values of 50.97%, 72.02 %, and 76.25 %, and the effect at this hour was found to increase in dose-dependent manner ($R^2 = 0.721$). Intergroup comparison among doses of the AF also showed a statistically significant different effect in both 200 versus 100mg/kg ($p < 0.05$ at 4h and 5h), and 400 versus 100mg/kg ($p < 0.05$ at 4 h, and $p < 0.01$ at 5 h).

Unlike the AF, only the higher doses of butanol fraction (200mg/kg and 400mg/kg of BF) showed statistically significant inhibition of paw edema at 1h post-induction as compared to distilled water vehicle control, with $p < 0.05$ and $p < 0.01$, respectively. The effect in both dose levels then persisted from the 2^{nd} - 5^{th} h post-induction with $p < 0.001$, except at the 3^{rd} h where $p < 0.01$ for 200mg/kg compared to the negative control. However, the 100mg/kg of BF failed to demonstrate statistically significant inhibition of paw edema compared to the negative control except at two h post-induction, where $p < 0.05$.

Like the AF, maximum percent inhibition by the 100, 200, and 400 mg/kg of BF was observed at five h post-induction, with respective values of 19.93%, 43.29%, and 51.33%, and the anti-inflammatory effect was found to increase in a dose-dependent manner ($R^2 = 0.799$). Intergroup comparison among doses of the BF also showed a statistically significant different effect in both 200 versus 100 mg/kg (5h, $p < 0.05$) and 400 versus 100 mg/kg (2 h, $p < 0.05$ and 3 - 5 h, $p < 0.01$).

The higher doses of chloroform fraction (CF), on the other hand, significantly inhibited paw edema as compared to the 2% tween vehicle control only late at the 4^{th} h ($p < 0.05$ for 200mg/kg; $p < 0.01$ for 400mg/kg), and 5^{th} h ($p < 0.01$ for 200mg/kg; $p < 0.001$ for 400mg/kg) post induction. The 100mg/kg of CF, however, did not show significant inhibition of paw edema as compare to the negative control throughout the observation period. Interestingly, no significant difference was noted among the doses of CF, except at 5h post induction where 400mg/kg showed a statistically significant inhibition ($p < 0.05$; %A = 39.31) as compared to 100mg/kg (%A = 12.63), and the effect at this hour was found to increase dose dependently ($R^2 = 0.931$).

Significant inhibition of paw edema occurred with 200mg/kg of aspirin from the 1^{st} h ($p < 0.01$) till the 5^{th} h after carrageenan injection ($p < 0.001$ from 2^{nd} - 5^{th} h) as compared to the negative control. Moreover, no difference in onset and duration of action was observed among all tested doses of the AF, 200 and 400mg/kg of the BF, and 200mg/kg of aspirin, as all showed significant inhibition of paw edema from the 1^{st} h till the 5^{th} h post-induction. Nevertheless, 200 and 400mg/kg of the AF showed a higher anti-inflammatory effect (%A) than that of 200mg/kg of aspirin throughout the observation period, whereas 100mg/kg of AF had shown a comparable anti-inflammatory impact with 200mg/kg of aspirin (Table 1).

Therefore, it can be concluded that the AF was the most active fraction in terms of anti-inflammatory effects on carrageenan-induced mice paw edema. This is evidenced by the higher percent inhibition (%A) values of all tested doses of AF compared to the equivalent doses of the BF and CF.

The reference drug, 200mg/kg of aspirin, showed significant inhibition of paw edema beginning from day 2 of treatment. The effect lasted till day 10 with $p < 0.001$, but on day 4 $p < 0.01$compared to the negative control. Maximum percentage inhibition (57.44%) by 200mg/kg of aspirin was noted on day 3 of treatment. Moreover, no significant difference in onset of action was observed among aspirin; all three doses of AF, 200 and 400 mg/kg of BF, and the highest dose (400mg/kg) of CF all showed a statistically significant inhibition starting from the 2nd day of treatment. Furthermore, 100mg/kg of AF and the BF at 200 and 400mg/kg showed a comparable anti-inflammatory effect (%A) with 200mg/kg of aspirin. The 200 and 400mg/kg of AF, on the other hand, exhibited a significantly higher anti-inflammatory effect than 200mg/kg aspirin throughout days 3 - 10, as shown in Table 3.

Even though all three fractions, of course, showed significant inhibition of paw edema at different doses compared to their respective negative controls, the AF was found to be the most active fraction in terms of the anti-inflammatory effect on formaldehyde-induced arthritis in mice. This is evidenced by the higher percent inhibition value of the AF throughout the observation period compared to the equivalent doses of BF and CF.

Cotton pellet induced granuloma

Subcutaneous implantation of two pellets of cotton, each weighing ten ± 1 mg in the groin region of rats, induced granulomatous inflammation with a maximum granuloma weight and exudates observed in distilled water and 2% tween 80 received negative controls as shown in Table 4. The aqueous fraction (AF) at all tested doses significantly inhibited the formation of inflammatory exudates ($p < 0.001$) and granuloma mass ($P < 0.01$ for 100mg/kg; $p < 0.001$ for 200 and 400mg/kg) as compared to the negative control.

Intergroup comparisons among doses of the AF revealed a statistically significant different effect in 400 versus 200mg/kg ($p < 0.05$ in exudates inhibition; $p < 0.001$ in granuloma inhibition), 400 versus 100mg/kg ($P < 0.001$ in both exudates and granuloma inhibition), and 200 versus 100mg/kg ($p < 0.001$ in exudates inhibition). Furthermore, the anti-inflammatory effect of the AF was found to increase in dose dependent manner ($R^2 = 0.829$ for exudates inhibition; $R^2 = 1$ for granuloma inhibition).

All tested doses of the chloroform fraction (CF) significantly ($p < 0.001$) inhibited the formation of both inflammatory exudates and granuloma mass as compared to the 2% tween negative controls. Comparison among doses of the CF revealed a statistically significant different effect in 200 versus 100mg/kg ($p < 0.01$ in exudates inhibition), 400 versus 200mg/kg ($P < 0.01$ in granuloma inhibition), and 400 versus 100mg/kg ($p <0.001$ in both exudates and granuloma inhibition). Besides, the anti-inflammatory effect of the CF was ascertained to increase in a dose-dependent manner ($R^2 = 0.928$ for exudates inhibition; $R^2 = 0.998$ for granuloma inhibition). Furthermore, the maximum anti-proliferative effect (peak percentage inhibition of granuloma formation, 55.52%) was shown at 400mg/kg of CF compared to all other CF, BF, and AF doses.

The butanol fraction (BF), on the other hand, showed significant inhibition of both inflammatory exudates ($p < 0.001$) and granuloma mass ($p < 0.01$) only at the highest tested dose (400mg/kg) as compared to the distilled water vehicle control. The 200mg/kg of BF showed a significant ($p < 0.05$) inhibition of exudate formation, but no considerable protection against granuloma formation was noted compared to the negative control.

The 100mg/kg of BF, on the other hand, failed to demonstrate significant inhibition of both exudates and granuloma formation as compared to the negative control. Intergroup comparisons among doses of the BF revealed a statistically significant different protection against exudates formation in 400 versus 200mg/kg ($p < 0.05$) and 400 versus 100mg/kg ($p < 0.01$). Still, no significant difference was observed among all three doses of BF regarding inhibition of granuloma mass formation.

The reference drug, 0.5mg/kg of dexamethasone, significantly ($p < 0.001$) inhibited the formation of both exudates (%A = 45.07) and granuloma mass (%A = 65.99) as compared to the negative control. The highest tested dose (400mg/kg) of both the AF and CF showed a comparable inhibition of exudate formation with the reference drug. But a significant ($P < 0.01$ and $P < 0.001$) difference was noted when all doses of the three fractions were compared with dexamethasone in terms of granuloma inhibition (Table 4).

As the results of this model revealed, the AF and CF were comparably effective at all tested doses in inhibiting cotton pellet induced exudates formation, whereas the CF was the most active fraction in inhibiting the formation of granuloma mass, as evidenced by the higher percentage of granuloma inhibition as compared to the respective doses of the AF and BF.

Phytochemical screening

Preliminary phytochemical screening for secondary metabolites was carried out on the crude 70EE, and solvent fractions of *Z. scabra*. The result revealed a differential distribution of secondary metabolites in the solvent fractions, as shown in Table 5.

Discussion

Zehneria scabra has been used in the folk medicine of Ethiopia for the management of different inflammatory pathologies. Its use in various inflammatory conditions is recorded in ethnobotanical studies of Ethiopia with a high fidelity rate; e.g., FL= 100% for febrile conditions by the ethnic groups of Gondar Zuria district (Birhanu 2013); 86% for 'mich' by people in Zegie Peninsula (Teklehaymanot and Giday 2007); 95% for febrile conditions by people of Ankober District, North Shewa Zone (Lulekal et al. 2013) and people of Bahirdar Zuria district (Ragunathan and Abay 2009).

Table 1. Effects of the solvent fractions of *Zehneria scabra* on carrageenan-induced mouse paw edema

Treatment group	Mean increase in paw Volume ± S.E.M and [% Inhibition (%A)]				
	1hr	2hr	3hr	4hr	5hr
Distilled H_20	0.490 ± 0.037	0.560 ± 0.020	0.598 ± 0.028	0.5167 ± 0.030	0.4350 ± 0.024
2% Tween 80	0.537 ± 0.025	0.563 ± 0.027	0.610 ± 0.025	0.543 ± 0.023	0.475 ± 0.026
ASA200mg/kg	0.343 ± 0.025^{a2} [29.94]	0.355 ± 0.026^{a3} [36.61]	0.388 ± 0.033^{a3} [35.10]	0.282 ± 0.023^{a3} [45.48]	0.180 ± 0.023^{a3} [58.62]
AF 100mg/kg	0.345 ± 0.023^{a2} [29.59]	0.338 ± 0.022^{a3} [39.59]	0.378 ± 0.022^{a3} [36.77]	0.303 ± 0.024^{a3d1e1} [41.3]	0.213 ± 0.020^{a3d1e2} [50.97]
AF 200mg/kg	0.318 ± 0.021^{a2} [35.04]	0.277 ± 0.027^{a3} [50.59]	0.288 ± 0.028^{a3} [51.81]	0.185 ± 0.024^{a3} [64.2]	0.122 ± 0.012^{a3} [72.02]
AF 400mg/kg	0.317 ± 0.024^{a2} [35.37]	0.272 ± 0.015^{a3} [51.48]	0.267 ± 0.034^{a3b1} [55.42]	0.176 ± 0.026^{a3b1} [65.8]	0.103 ± 0.014^{a3} [76.25]
BF 100mg/kg	0.428 ± 0.023 [12.59]	0.465 ± 0.018^{a1b1e1}[16.96]	0.493 ± 0.022^{b1e2}[17.55]	0.433 ± 0.023^{b2e2} [16.14]	0.348 ± 0.026^{b3d1e2} [19.93]
BF 200mg/kg	0.377 ± 0.022^{a1} [23.12]	0.403 ± 0.030^{a3} [27.98]	0.425 ± 0.028^{a2} [28.97]	0.333 ± 0.035^{a3} [35.49]	0.247 ± 0.030^{a3} [43.29]
BF 400mg/kg	0.350 ± 0.018^{a2} [28.57]	0.357 ± 0.015^{a3} [36.30]	0.363 ± 0.011^{a3} [39.28]	0.272 ± 0.016^{a3} [47.42]	0.212 ± 0.013^{a3} [51.33]
CF 100mg/kg	0.495 ± 0.023^{b2} [7.77]	0.518 ± 0.026^{b2} [7.99]	0.567 ± 0.029^{b2} [7.10]	0.490 ± 0.028^{b3} [9.81]	0.415 ± 0.024^{b3e1} [12.63]
CF 200mg/kg	0.480 ± 0.031^{b1} [10.60]	0.512 ± 0.038^{b2} [9.16]	0.537 ± 0.044^{b1} [12.02]	0.427 ± 0.025^{f1b2} [21.50]	0.343 ± 0.025^{f2b2} [27.73]
CF 400mg/kg	0.482 ± 0.031^{b1} [10.20]	0.500 ± 0.022^{b1} [11.20]	0.488 ± 0.025 [19.95]	0.403 ± 0.036^{f2b1} [25.80]	0.288 ± 0.030^{f3b1} [39.31]

Note: Values are expressed as Mean ± S.E.M.; n = 6; Values in parenthesis shows % inhibition of paw edema; [a] compared with distilled H_20, [b] compared with ASA 200mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction.

Table 2. Effects of crude 70EE of *Zehneria scabra* on formaldehyde-induced arthritis in mice

Treatment group	Mean increase in paw volume (mL) ± S.E.M and [% Inhibition (%A)]				
	Day 1	Day 2	Day 3	Day 4	Day 5
Distilled H_20	0.450 ± 0.029	0.533 ± 0.023	0.645 ± 0.024	0.878 ± 0.025	0.887 ± 0.028
ASA 200mg/kg	0.347 ± 0.029^{a1} [22.89]	0.295 ± 0.031^{a3} [44.65]	0.238 ± 0.025^{a3} [63.10]	0.597 ± 0.037^{a3} [32.00]	0.550 ± 0.028^{a3} [37.99]
70EE 100mg/kg	0.367 ± 0.024 [18.44]	0.436 ± 0.027^{b2} [18.20]	0.480 ± 0.024^{a2b3d1e2}[25.58]	0.767 ± 0.029^{b2e2} [12.64]	0.748 ± 0.020^{a1b3e2} [15.67]
70EE 200mg/kg	0.353 ± 0.010 [21.56]	0.398 ± 0.016^{a1} [25.33]	0.368 ± 0.025^{a3b1} [42.95]	0.666 ± 0.031^{a3} [24.15]	0.656 ± 0.030^{a3} [26.04]
70EE 400mg/kg	0.332 ± 0.026^{a1} [26.22]	0.363 ± 0.033^{a2} [31.89]	0.307 ± 0.031^{a3} [52.40]	0.607 ± 0.023^{a3} [30.87]	0.578 ± 0.032^{a3} [34.84]
	Day 6	**Day 7**	**Day 8**	**Day 9**	**Day 10**
Distilled H_20	0.808 ± 0.031	0.693 ± 0.031	0.642 ± 0.033	0.566 ± 0.035	0.477 ± 0.034
ASA 200mg/kg	0.512 ± 0.025^{a3} [36.63]	0.452 ± 0.026^{a3} [34.85]	0.400 ± 0.025^{a3} [37.69]	0.310 ± 0.029^{a3} [45.23]	0.232 ± 0.028^{a3} [51.40]
70EE 100mg/kg	0.713 ± 0.030^{b3e3}[11.76]	0.610 ± 0.031^{b2e3} [12.02]	0.502 ± 0.030^{a2e3} [21.81]	0.455 ± 0.026^{a1b2e3} [19.61]	0.382 ± 0.015^{a1b2e3}[19.92]
70EE 200mg/kg	0.607 ± 0.018^{a3e1}[24.88]	0.518 ± 0.014^{a2e1} [25.24]	0.460 ± 0.016^{a3e2} [28.35]	0.383 ± 0.009^{a3e1} [32.33]	0.308 ± 0.016^{a3e1} [35.33]
70EE 400mg/kg	0.487 ± 0.033^{a3} [39.73]	0.407 ± 0.027^{a3} [41.34]	0.322 ± 0.018^{a3} [49.84]	0.257 ± 0.017^{a3} [54.59]	0.188 ± 0.019^{a3} [60.50]

Note: Values are expressed as Mean ± SEM; n = 6; Values in parenthesis shows % inhibition of paw edema (%A); [a] compared with distilled H_20, [b]compared with 200mg/kg ASA, [d]compared

Table 3. Effects of the solvent fractions of *Zehneria scabra* on formaldehyde-induced arthritis in mice

Treatment group	Mean increase in paw volume (mL) ± S.E.M and [% Inhibition (%A)]				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
Distilled H_20	0.482 ± 0.026	0.558 ± 0.023	0.627 ± 0.025	0.832 ± 0.017	0.855 ± 0.015
2% Tween	0.515 ± 0.031	0.615 ± 0.030	0.640 ± 0.019	0.812 ± 0.024	0.827 ± 0.021
ASA200mg/kg	0.363 ± 0.027 [24.58]	0.308 ± 0.032^{a3} [44.78]	0.267 ± 0.023^{a3} [57.44]	0.643 ± 0.025^{a2} [22.65]	0.563 ± 0.026^{a3} [34.12]
AF100mg/kg	0.383 ± 0.029 [20.43]	0.262 ± 0.028^{a3} [53.13]	0.192 ± 0.031^{a3} [69.41]	0.600 ± 0.045^{a3e2} [27.86]	0.580 ± 0.047^{a3d2e3}[32.16]
AF200mg/kg	0.408 ± 0.045 [15.24]	0.258 ± 0.026^{a3} [53.73]	0.153 ± 0.025^{a3b1} [75.54]	0.492 ± 0.026^{a3b1} [40.88]	0.402 ± 0.046^{a3b1} [53.02]
AF400mg/kg	0.393 ± 0.025 [18.35]	0.235 ± 0.031^{a3} [57.91]	0.138 ± 0.022^{a3b1} [77.93]	0.447 ± 0.027^{a3b2} [46.29]	0.333 ± 0.026^{a3b2} [61.02]
BF100mg/kg	0.425 ± 0.031 [11.77]	0.372 ± 0.034^{a2} [33.42]	0.323 ± 0.037^{a3} [48.41]	0.715 ± 0.038^{e1} [14.03]	0.768 ± 0.043^{b2d1e3} [10.14]
BF200mg/kg	0.400 ± 0.030 [16.96]	0.345 ± 0.029^{a3} [38.21]	0.280 ± 0.025^{a3} [55.32]	0.662 ± 0.026^{a2} [20.44]	0.617 ± 0.026^{a3e1} [27.87]
BF400mg/kg	0.387 ± 0.030 [19.72]	0.288 ± 0.028^{a3} [48.36]	0.222 ± 0.026^{a3} [64.62]	0.578 ± 0.034^{a3} [30.47]	0.492 ± 0.033^{a3} [42.49]
CF100mg/kg	0.455 ± 0.016 [11.65]	0.525 ± 0.023^{b3} [14.63]	0.497 ± 0.026^{f2b3} [22.39]	0.737 ± 0.022 [9.24]	0.720 ± 0.030^{b2e2} [12.91]
CF200mg/kg	0.445 ± 0.032 [13.59]	0.522 ± 0.030^{b3} [15.17]	0.472 ± 0.031^{f2b3} [26.3]	0.695 ± 0.029^{f1} [14.38]	0.672 ± 0.029^{f2} [18.75]
CF400mg/kg	0.423 ± 0.023 [17.81]	0.468 ± 0.029^{f1b2} [23.85]	0.388 ± 0.034^{f3b1} [39.33]	0.627 ± 0.037^{f2} [22.79]	0.567 ± 0.033^{f3} [31.45]
	Day 6	**Day 7**	**Day 8**	**Day 9**	**Day 10**
Distilled H_20	0.797 ± 0.022	0.715 ± 0.027	0.610 ± 0.039	0.522 ± 0.034	0.450 ± 0.035
2% Tween	0.758 ± 0.020	0.698 ± 0.016	0.600 ± 0.017	0.553 ± 0.023	0.492 ± 0.031
ASA200mg/kg	0.498 ± 0.026^{a3} [37.45]	0.442 ± 0.022^{a3} [38.22]	0.370 ± 0.019^{a3} [39.34]	0.327 ± 0.027^{a3} [37.38]	0.262 ± 0.024^{a3} [41.84]
AF100mg/kg	0.505 ± 0.048^{a3d2e3} [36.61]	0.423 ± 0.049^{a3d2e3} [40.80]	0.353 ± 0.039^{a3d2e3} [42.08]	0.273 ± 0.036^{a3d2e2} [47.61]	0.201 ± 0.036^{a3d1e2} [55.18]
AF200mg/kg	0.315 ± 0.037^{a3b2} [60.46]	0.227 ± 0.032^{a3b2} [68.29]	0.172 ± 0.027^{a3b2} [71.85]	0.122 ± 0.020^{a3b3} [76.67]	0.078 ± 0.015^{a3b3} [82.60]
AF400mg/kg	0.243 ± 0.027^{a3b3} [69.46]	0.185 ± 0.017^{a3b3} [74.13]	0.132 ± 0.013^{a3b3} [78.41]	0.097 ± 0.011^{a3b3} [81.46]	0.052 ± 0.010^{a3b3} [88.51]
BF100mg/kg	0.675 ± 0.047^{b2e3} [15.28]	0.590 ± 0.041^{b1d1e3} [17.48]	0.518 ± 0.041^{b1d1e3} [15.03]	0.427 ± 0.037^{e2} [18.21]	0.347 ± 0.037^{e2} [22.96]
BF200mg/kg	0.540 ± 0.028^{a3} [32.22]	0.457 ± 0.029^{a3e1} [36.13]	0.373 ± 0.033^{a3} [38.8]	0.316 ± 0.038^{a2} [39.29]	0.262 ± 0.030^{a2} [41.84]
BF400mg/kg	0.408 ± 0.036^{a3} [48.75]	0.330 ± 0.028^{a3} [53.85]	0.265 ± 0.025^{a3} [56.56]	0.220 ± 0.022^{a3} [57.83]	0.168 ± 0.017^{a3} [62.6]
CF100mg/kg	0.657 ± 0.031^{b2e2} [13.40]	0.592 ± 0.038^{b2e2} [15.27]	0.483 ± 0.037^{f1b1e3} [19.45]	0.422 ± 0.034^{f1e3} [23.78]	0.346 ± 0.031^{f2e3} [29.49]
CF200mg/kg	0.597 ± 0.035^{f2e1} [21.31]	0.503 ± 0.034^{f3} [27.92]	0.420 ± 0.034^{f2e1} [30.00]	0.368 ± 0.035^{f2e1} [33.44]	0.302 ± 0.027^{f3e2} [38.64]
CF400mg/kg	0.473 ± 0.028^{f3} [37.58]	0.406 ± 0.026^{f3} [41.76]	0.286 ± 0.020^{f3} [52.22]	0.227 ± 0.021^{f3} [59.03]	0.150 ± 0.015^{f3b1} [69.49]

Note: Values are expressed as Mean ± SEM; n = 6; Values in parenthesis shows % inhibition of paw edema; [a] compared with distilled H_20, [b] compared with ASA 200mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% Tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction

Table 4. Effects of solvent fractions of *Zehneria scabra* on cotton pellet induced granuloma in rats

Treatment group	Mean weight of exudates (mg) ± S.E.M and [% inhibition]	Mean weight of granuloma (mg) ± S.E.M and [% inhibition]
Distilled H_20	159.28 ± 1.93	32.61 ± 0.76
2% Tween	152.72 ± 2.08	34.2833 ± 0.44
Dexa 0.5mg/kg	87.50 ± 2.05^{a3} [45.07]	11.0917 ± 0.44^{a3} [65.99]
AF 100mg/kg	$122.02 \pm 2.37^{a3b3d3e3}$ [23.39]	$28.9333 \pm 0.83^{a2b3e3}$ [11.27]
AF 200mg/kg	99.81 ± 1.91^{a3b2e1} [37.34]	$26.3833 \pm 0.66^{a3b3e3}$ [19.09]
AF 400mg/kg	90.63 ± 2.34^{a3} [43.10]	21.3250 ± 0.50^{a3b3} [34.61]
BF 100mg/kg	152.23 ± 1.77^{b3e2} [4.43]	30.6750 ± 1.09^{b3} [5.93]
BF 200mg/kg	150.82 ± 1.58^{a1b3e1} [5.31]	29.6917 ± 0.86^{b3} [8.95]
BF 400mg/kg	142.21 ± 1.29^{a3b3} [10.72]	27.7483 ± 0.65^{a2b3} [14.91]
CF 100mg/kg	$109.07 \pm 3.09^{f3b3d2e3}$ [28.58]	21.01 ± 0.84^{f3b3e3} [38.72]
CF 200mg/kg	97.40 ± 2.38^{f3b1} [36.22]	18.85 ± 0.59^{f3b3e2} [45.02]
CF 400mg/kg	88.61 ± 0.95^{f3} [41.98]	15.25 ± 0.66^{f3b2} [55.52]

Note: Values are expressed as Mean ± S.E.M; n = 6; Values in parenthesis shows % inhibition (%A); [a] compared with distilled H_20, [b] compared with dexa 0.5 mg/kg, [d] compared with 200 mg/kg of respective fraction, [e] compared with 400 mg/kg of respective fraction, [f] compared with 2% Tween 80; [1]*p<0.05*, [2]*p<0.01*, [3]*p<0.001*; AF: aqueous fraction; BF: butanol fraction; CF: chloroform fraction; Dexa: dexamethasone.

Table 5. Preliminary phytochemical screening of 70EE and solvent fractions of *Zehneria scabra*

Secondary metabolites	Crude 70EE	AF	BF	CF	Distilled water	2% Tween 80
Alkaloids	+	++	+	-	-	-
Tannins	+	+	++	-	-	-
Saponins	+	++	-	-	-	-
Terpenoids	+	++	+	-	-	-
Steroids	+	-	-	++	-	-
Flavonoids	+	-	-	++	-	-
Anthraquinones	-	-	-	-	-	-
Cardiac glycosides	++	+	+	-	-	-

Note: (+, Present); (-, Abscent); (++, relatively concentrated as compared to the crude extract)

In line with this notion, Akele (2012) has elucidated the anti-nociceptive and acute anti-inflammatory activity of the hydro-alcoholic crude leave extract of *Z. scabra* using the acetic acid-induced writhing test and carrageenin-induced edema model, respectively. Despite this, the literature recorded no reports about the anti-inflammatory activity of the crude extract and further fractions in experimental models of sub-acute and chronic inflammation. Thus, the present study is the first one to demonstrate the anti-inflammatory activity of 70% ethanol leave extract and solvent fractions of *Z. scabra* using a battery of pharmacological inflammatory models ranging from acute to chronic ones.

The inflammatory response is a polyphasic tissue reaction, ranging from a temporary increase in vascular permeability to a prolonged cellular infiltration and proliferation. So, it is essential to evaluate the performance of agents claimed for anti-inflammatory effects via a battery of tests valid for various phases of inflammation (Kumar et al. 2012).

The carrageenan-induced paw edema model is a prototype of the exudative phase of acute inflammation (Divakar et al., 2010; Sarkar, 2015). As a phlogistic agent, Carrageenan is not antigenic and is devoid of apparent systemic effect (Igbe and Inarumen 2013). Hence, carrageenan injection induces localized inflammation in two different phases through the subsequent release of several mediators. The initial period, which occurs between 0 and 2.5 h after carrageenan injection, has been attributed to the action of mediators such as histamine, serotonin, and bradykinin on vascular permeability. Histamine and serotonin are mainly released during the first 1.5 h, while bradykinin is released from 1.5 to 2.5 h after carrageenan injection (Masresha et al. 2012).

The second phase (2.5 - 6 h post-carrageenan injection) is a result of the overproduction of COX-2 and its pro-inflammatory PG products, with infiltration of polymorphonuclear leukocytes (neutrophils) (Dawson et al. 1991; Coura et al. 2015). The peak inflammation is usually seen approximately 2 - 3 h post carrageenan injection and is attributed to PG release (Kumar et al., 2012; Silva-Neto et al., 2014). Hence, this second phase appears to be the most exciting in terms of inflammatory processes. The carrageenan-induced paw edema is known to be sensitive to COX inhibitors but not to 5- LOX inhibitors and hence has been used to evaluate the effect of NSAIDs, which primarily inhibit the COX pathway, i.e., single-action NSAIDs such as aspirin. It has been demonstrated that the suppression of carrageenin-induced hind paw edema after the 3rd h correlates reasonably with therapeutic doses of most clinically useful anti-inflammatory agents (Panthong et al., 2007; Mathew et al., 2014). Thence, in this study, carrageenan-induced hind paw edema was used as an appropriate acute inflammation model, and aspirin has opted as a reference drug.

The formaldehyde-induced arthritis model, on the other hand, represents a sub-acute phase characterized by increased migration of leukocytes and phagocytes to the area of inflammation (Divakar et al., 2010). Hence, the inhibition of formaldehyde-induced edema is one of the

most suitable methods for evaluating the antiproliferative activity and screening anti-arthritic agents. Injection of formaldehyde into mice's hind paws produces localized inflammation and pain, which is biphasic. During the first neurogenic phase (0-5 min), pain is initiated due to the direct chemical stimulation of nociceptors. It is thought to be mediated by substance-P and bradykinin (Kaithwas et al., 2012; Sanusi et al., 2013). The second phase (15 min post-induction) appears to be an inflammatory phase during which histamine, serotonin, prostaglandin, and bradykinin become key mediators and thus could be inhibited by peripherally acting anti-inflammatory drugs such as aspirin. Leukocyte migration into the inflamed site will commence late in this phase (2.5 - 6 h) and is considered the most crucial inflammatory response process (Silva-Neto et al., 2014; Cui et al., 2014). In line with this notion, on the 1st day of induction, paw edema was determined three h following formaldehyde injection.

The cotton pellet-induced granuloma model is one of the most commonly employed models in animal research to screen for the chronic anti-inflammatory activity of drugs and novel natural products (Roome et al., 2014). In this model, the transudative phase causes an increase in the wet weight of the cotton pellet while hosting an inflammatory response to the implanted cotton between days 3 – 6, causing granuloma formation due to the proliferation of fibroblasts and infiltration of modified macrophages and the lymphocytes. Hence, the increase in dry weight is considered a measure of chronic inflammation's proliferative component (Bagad et al., 2013). Therefore, this model was used to verify further the anti-inflammatory activity of solvent fractions of *Z. scabra* on the transudative and proliferative components of chronic inflammation. Steroidal anti-inflammatory drugs demonstrated higher activity in this model (Andrade et al. 2007), and hence dexamethasone was opted as a reference drug.

The anti-inflammatory activity of hydroalcoholic crude leaf extracts of *Z. scabra*, in carrageenan-induced paw edema was reported by Akele (2012). In the present study, the anti-inflammatory potential of 70% ethanol crude leaves extract of *Z. scabra* was first tested against formaldehyde-induced arthritis, which is one of the most suitable test procedures to screen for the anti-arthritic and sub-acute anti-inflammatory activity of natural products as it closely resembles human arthritis (Deshpande et al. 2011). In this model, the crude 70EE at 200 and 400mg/kg doses demonstrated statistically significant inhibition of paw edema from day two to day 10 of treatment compared to the negative control (Table 2). In addition, 400mg/kg of 70EE showed a comparable anti-inflammatory effect (%A) with 200mg/kg of aspirin. This could be due to certain alterations in the inflammatory response with possible anti-arthritic potential comparable with the standard drug aspirin.

Furthermore, the result was also in agreement with the anti-inflammatory effects of the hydroalcoholic crude extract on carrageenan-induced acute inflammation, as reported by Akele (2012), and expands the evidence of its anti-arthritic and anti-proliferative efficacy on the sub-acute model of inflammation. The result obtained for the 70EE in formaldehyde induced arthritis model was also in line with those reported elsewhere (Umukoro and Ashorobi 2006; Deshpande et al. 2011; Reddy et al. 2015), where extracts of *Momordica charantia, Coccinia grandis,* and *Momordica cymbalaria* showed statistically significant inhibition of formaldehyde induced paw edema, signifying the potential anti-arthritis and anti-proliferative activity of plants in the Cucurbitaceae family.

Besides, for further evaluation of the nature of the active constituents and to state the possible mechanism(s) of the anti-inflammatory activity of the plant, the 70EE was successively fractionated by partitioning into solvents of differing polarity, and the anti-inflammatory activity of the fractions was evaluated by using acute, sub-acute and chronic models of inflammation.

The aqueous fraction (AF), at all tested doses, showed statistically significant inhibition of inflammatory parameters in all three employed models of inflammation. In the acute model (carrageenan-induced paw edema), all employed doses of the AF showed a significant ($p < 0.01$ and $p < 0.001$) inhibition of paw edema starting from 1h post-induction, and the effect was maintained till the 5th h (Table 1). The significant anti-inflammatory activity of this fraction during the first 2 hours of the initial phase of inflammation could be due to the inhibitory effect on mediators like histamine and 5-HT. This is further evidenced by the higher inhibitory effect of all doses of the AF compared to the particular time the inhibitory effect of aspirin, which like most other NSAIDs, has less effect on the first phase of carrageenan-induced inflammation. No significant increment in percent inhibition values of all tested doses of the AF was noted from the 2nd to the 3rd h. As noted above, this is the period where the release of the mediator bradykinin reaches its peak. Hence, it can be generalized that the AF may not have a significant inhibitory effect on the release or activity of bradykinin.

Furthermore, all tested doses of the AF showed more pronounced edema inhibition in the second phase of inflammation on the 4th and 5th h, compared to their respective inhibitory values in the first phase (0 - 2.5 h), achieving the maximum anti-inflammatory effect on the 5th h. This indicates that the primary mechanism of the anti-inflammatory effect of the AF could be via inhibition of COX and its pro-inflammatory metabolites, such as PGs. This is substantiated by the fact that even the lower employed dose of the AF (100mg/kg) showed a comparable inhibitory effect with that of aspirin, which like most other NSAIDs, exert a more pronounced effect on the second phase (Panthong et al. 2007; Mathew et al. 2014). Moreover, the higher employed doses, 200 and 400mg/kg, of the AF even showed a better inhibitory effect than aspirin in this late phase of inflammation, which is primarily mediated through products of the inducible COX.

This finding is also in line with Akele's (2012) previous report. The crude hydroalcoholic extract of *Z.scabra* showed significant inhibition of carrageenan-induced paw edema in the second phase of inflammation, with the maximum effect observed at the 3rd h. In comparison with Akele's (2012) report on the crude extract, it could be concluded that the phytochemicals responsible for the

pronounced anti-inflammatory effect on the second phase of acute inflammation could be highly concentrated in the AF. This could be explained since the equivalent doses of the crude extract showed a better percent inhibition value in the first phase (~ up to the 3rd h) than treatments of the AF at a given point of time; whereas from the 3rd to 5th h of the second phase, all tested doses of the AF showed a remarkably higher percent inhibition than the equivalent doses of the crude extract at the respective point of time. This could be possibly due to preferential partitioning of the secondary metabolites responsible for a better anti-inflammatory effect on the second phase, perhaps through COX inhibition, into the AF.

The results from the sub-acute and chronic models also revealed the anti-inflammatory effects of this fraction. For example, in the cotton pellet-induced granuloma model, all tested doses of the AF showed statistically significant inhibition of both exudates and granuloma formation. In this model, the vital ($P < 0.001$) inhibitory effect of the AF on the formation of exudates (Table5) substantiates the finding of the carrageenan-induced acute model, i.e., both findings solidify the effectiveness of the AF in inhibiting the exudative and transudative component of inflammation. On the other hand, the statistically significant ($P < 0.01$ and $P < 0.001$) inhibition of granuloma formation justifies the effectiveness of this fraction in inhibiting the proliferative phase of inflammation. This could also be ascertained from the findings of the formaldehyde-induced arthritic model, in which all tested doses of the AF significantly ($p < 0.001$) inhibited the development of arthritis from day 2 to 10 of treatment (Table 3). Moreover, the AF at all tested doses demonstrated a considerably better inhibitory effect on formaldehyde-induced arthritis as compared to equivalent doses of the crude 70EE (Table 2) from day two throughout to day 10 of treatment. This could be possibly due to preferential partitioning of the secondary metabolites responsible for a better anti-arthritic activity in the AF.

Phytochemical screening for secondary metabolites revealed the presence of alkaloids, saponins, tannins, and terpenoids in the aqueous residue. The anti-inflammatory activity of this fraction could emanate from the presence of these secondary metabolites whose protection against inflammation is also reported elsewhere; alkaloids (Küpeli et al. 2002; Souto et al. 2011), saponins (Navarro et al. 2001; Ahn et al. 2005; Chen et al. 2014), and terpenoids (Heras and Hortelano 2009; Bellik et al. 2012; Ku and Lin 2013).

The mechanism of the anti-inflammatory effect of these different secondary metabolites is also documented in the literature. Various terpenoids were reported to exert their anti-inflammatory influence by inhibiting PLA2 activity, inhibition of TNF-α production, inhibition of iNOS expression, inhibition of COX-2 expression, and inhibition of NF-κB activation (Bellik et al. 2012). Saponins were also reported to exert an anti-inflammatory effect by inhibiting iNOS expression, COX-2 expression, and subsequent production of PGE2 (Ahn et al. 2005). The anti-inflammatory activity of different alkaloids was also reported to be mediated through inhibition of COX expression and production of PGE2 (Fukuda et al. 1999; Kuo et al. 2004), inhibition of pro-inflammatory cytokines production (IL-1β, IL-6, TNF-α) and inhibition of the expression of ICAM-1 and VCAM-1 adhesion molecules (Bellik et al. 2012).

From the results of the phytochemical screening, terpenoids were found to be highly concentrated in the AF, less in the BF, and none in the CF. Polar solvents such as ethanol and water led to the extraction of highly oxygenated polar triterpenes and triterpenoid glycosides. In contrast, non-polar solvents such as chloroform and petroleum ether extracted most other lipid-soluble terpenoids such as Sesquiterpene lactones, diterpenes, and sterols (Citoglu and Acikara 2012). From this fact and the findings of the phytochemical screening, highly oxygenated polar triterpenes or triterpenoid glycosides could be the significant terpenoids concentrated in the AF and responsible for their anti-inflammatory activity. This could further be substantiated by a lack of activity of the petroleum ether extract in the pilot study and the negative terpenoid test result of the CF for the lipid-soluble terpenoids.

Other plants in the Cucurbitaceae family such as *Cayaponia tayuya* (Escandell et al. 2007), *Cucurbita andreana* (Jayaprakasam et al. 2003), *Picrorhiza scrophulariaeflora* (Smit et al. 2000), *Wilbrandia ebracteata* (Peters et al. 1999), and *Citrullus lanatus* (Abdelwahab et al. 2011), were also reported to contain highly oxygenated triterpenoid molecules called cucurbitacins, which were proved to have potent anti-inflammatory and anti-proliferative activities (Wakimoto et al. 2008; Duangmano et al. 2012).

All in all, from the results of the acute (Table 1) and sub-acute (Table 3) models of inflammation and percentage inhibition values of exudate formation in the chronic model (Table 4), the AF was found to be the most active fraction in inhibiting the exudative and transudative component of inflammation. It was also found to exhibit significant anti-proliferative activity, as revealed by the results of the sub-acute model (Table 3) and percentage inhibition values of granuloma formation in the chronic granulomatous inflammatory model (Table 4).

Moreover, the probable mechanism of the anti-inflammatory effect of the phytochemicals concentrated in the AF could be explained by inhibiting mediators like histamine, serotonin, and products of COX, such as pro-inflammatory PGs. The latter mechanism, i.e., inhibition of COX, could be the primary mechanism of the anti-inflammatory effect of this fraction since it showed a more significant effect during the second phase of the acute model of inflammation. This is further substantiated by the type of secondary metabolites found in this fraction, most of which were proved to possess COX inhibitory activities.

The butanol fraction (BF), at doses of 200 and 400mg/kg, significantly inhibited mice paw edema in both acute (Table 1) and sub-acute (Table 3) models of inflammation. In the cotton pellet-induced granuloma model, on the other hand, only the highest tested dose (400mg/kg) showed statistically significant inhibition of both inflammatory exudates ($P < 0.001$) and granuloma

formation ($P < 0.01$). A phytochemical test (Table 5) of this fraction revealed the presence of tannins, alkaloids, and terpenoids, albeit the last two secondary metabolites with a relatively lower concentration than the AF.

The moderate anti-inflammatory effect of the BF in the acute and sub-acute model, compared to the AF, could be accounted for by the presence of alkaloids and terpenoids, which were the main constituents of the most active AF. The anti-inflammatory effect of the BF could also emanate from tannins, the primary concentrate. The anti-inflammatory effect of tannins was reported to be mediated through inhibition of leukocyte migration by their well-known astringent properties, which cause precipitation of cell membrane proteins and hence affect cellular movements, recruitment, and membrane permeability (Mota et al. 1985) and/or through inhibition of expression of pro-inflammatory cytokines and chemokines by blocking of transcription factors, NF-κB and AP-1 (Erdèlyi et al. 2005). These different mechanisms underlying tannins may at least partly be responsible for the moderate anti-inflammatory effect observed for higher doses of the BF in inhibition of edema (carrageenan and formaldehyde-induced edema models) and cellular proliferation in the chronic model.

Unlike the AF and BF, the higher doses of chloroform fraction (200 and 400mg/kg CF) showed a statistically significant anti-inflammatory effect only during the second phase (4^{th} and 5^{th} h) of carrageenan-induced paw edema (Table 1). Similarly, the anti-inflammatory effect of this fraction was delayed until the 3^{rd} day of formaldehyde-induced arthritis. This could probably be due to the specific inhibitory effect of phytochemicals in the CF on the synthesis or outcome of pro-inflammatory PGs, which mainly mediate the late phase of carrageenan-induced paw edema.

In the cotton pellet-induced granuloma model, on the other hand, the CF at all employed doses showed a comparable inhibitory effect on exudate formation with the equivalent doses of the AF (Table 4). Moreover, in this model, the CF at all treatments was found to be the most active fraction of inhibition of granuloma formation, the maximum inhibitory effect ($\%A = 55.52$, $p < 0.001$) being observed at a dose of 400mg/kg. From these findings, i.e., the delayed anti-inflammatory effect in the acute and sub-acute model and its profound inhibitory effect on granuloma formation in the chronic model, it can be concluded that the CF's phytochemicals may be most effective in inhibiting pro-inflammatory prostanoids and cytokines induced cellular proliferation.

The results of the phytochemical screening revealed the preferential partitioning of steroids and flavonoids into the CF. Different kinds of literature support the anti-proliferative efficacy of these phytochemicals; Loizou et al. (2009) and Bigoniya et al. (2013), for example, showed phytosterols to inhibit TNF-α induced endothelial activation and expression of ICAM-1 and VCAM-1 adhesion molecules which mediate cellular recruitment to sites of inflammation. Hernández-Valle et al. (2014), Han et al. (2015), and Wagle et al. (2016), on the other hand, showed phytosterols to inhibit the inflammatory cytokines IL-6, IL-1β, TNF-α, and on the contrary induce the production of anti-inflammatory cytokines IL-4 and IL-10.

On the other hand, Flavonoids were reported to exhibit anti-inflammatory and anti-proliferative effects through selective inhibition of 5-LOX (Schewe et al. 2002; Redrejo-Rodriguez et al. 2004), COX-2, and/or iNOS (Tunon et al. 2009). Aquila et al. (2009) further reported the anti-inflammatory effect of flavonoids from *Cayaponia tayuya* (Cucurbitaceae) to be mediated through inhibition of COX-2 and iNOS induction. The possibility of selective COX-2 inhibition by flavonoids in the CF is further evidenced by the delayed anti-inflammatory effect shown in the second phase of the acute model.

The major products of the 5-LOX pathway, LTB4, and CysLTs, in concert with the adhesion molecules ICAM-1 and VCAM-1, are known to mediate chemokinesis, firm adhesion, and subsequent extravasation of leucocytes to sites of inflammation (Werz et al. 2002; Pelletier et al. 2003; Weber et al. 2007; Afonso et al. 2012). On the other hand, products of the COX pathway, especially PGE2 and PGI2, are well known for their potent vasodilatory actions and increasing vascular permeability and leukocyte infiltration (Pelletier et al., 2003; Smyth et al., 2009). Hence, the effectiveness of the CF in inhibiting the formation of inflammatory exudates and, more profoundly, granuloma in the chronic model could emanate from the possible inhibitory effect of the flavonoids and phytosterols on the COX and 5-LOX pathways and/or inhibition of inflammatory cytokines induced endothelial activation and expression of ICAM-1 and VCAM-1.

All in all, the results of this study revealed that the crude extract (70EE) and solvent fractions of *Z.scabra* leaves possess anti-inflammatory activities. The overall order of efficacy in inhibiting the exudative phase of acute inflammation, as evidenced by the percentage inhibition of paw edema in the critical model, was found to be AF > BF > CF. Hence the phytochemicals in the AF could be most effective in inhibiting the acute phase of inflammation. While on the contrary, the overall order of effectiveness in inhibiting the proliferative phase of chronic inflammation, as evidenced by the percentage of granuloma inhibition in the chronic model, was found to be CF > AF > BF. This indicates that the phytochemicals concentrated in the CF may be specifically useful in inhibiting the cellular response of the proliferative phase of inflammation.

Such disparity in order of effectiveness of the fractions in modulating the acute and chronic phases of inflammation could be due to the differential partitioning of phytochemicals into the three portions (Table 5) and the associated difference in the mechanism of action of the secondary metabolites. Furthermore, the greater efficacy of the AF in comparison with other fractions in inhibiting the acute phase of inflammation supports the traditional method of extraction of the leaves of *Z.scabra* in Ethiopia, where the leaves are boiled in water, and the vapor inhaled, or they leave juice is given orally for the treatment of inflammatory conditions (Teklehaymanot and Giday 2007; Ragunathan and Abay 2009; Birhanu 2013).

From the overall results of this study, it can be postulated that *Z. scabra* is rich in secondary anti-

inflammatory metabolites that act by different mechanisms to inhibit acute, sub-acute, and chronic inflammatory conditions. Moreover, the potent anti-inflammatory activity of the 70EE and solvent fractions of *Z. scabra* in this study as compared to the standard drug aspirin may be due to the cumulative effects of different active constituents in reducing the synthesis, release, and/or action of various inflammatory cytokines, chemokines and mediators such as histamine, serotonin, prostaglandins, and Leukotrienes. This notion is in line with the proven concept that medicinal plants possess a combination of phytoconstituents with different anti-inflammatory mechanisms, offering synergistic or additive effects (Liu 2003; Csaki et al. 2009).

The phytochemical screening in the present study revealed the presence of secondary metabolites such as alkaloids, tannins, saponins, terpenoids, steroids, flavonoids, and cardiac glycosides in the crude 70EE and their differential partitioning into employed solvents of differing polarity (Table 5). Tesfaye and Alamneh (2014) reported the absence of saponins in the crude 80% methanol extract of the leaves of *Z. scabra*. However, the test for other secondary metabolites was in line with the present study's findings. On the other hand, Tadesse et al. (2014) reported the presence of saponins, which is in agreement with the results of the present study, while Alkaloids and steroids were tested negative in 80% methanolic leaves extract of *Z. scabra*. Such discrepancy in the presence and/or absence of secondary metabolites could be accounted for by seasonal and geographical variations, which are known to influence the expression of phytochemical constituents in plants (Jayanthy et al., 2013).

Arulappan et al. (2015), conversely, showed the presence of phenolic compounds, steroids, and glycosides and the absence of tannins, flavonoids, alkaloids, and saponins in the ethanolic (absolute) and aqueous tuber extract of *Z.scabra*. Such disagreement with the present study results could be due to the differential distribution of the secondary metabolites into different parts of the plant, i.e., the tuber and leaves, on top of the geographic variation and the difference in the extraction solvents.

In this study, ethanol specifically was chosen as the solvent of extraction as it can extract a wide variety of polar and nonpolar phytochemical constituents in medicinal plants (Herman et al., 2013; Tatke and Rajan, 2014). Hydroalcoholic solvents (a mixture of alcohol and water in varying proportions) are generally considered to give high extraction yields, owing to their expanded polarity range (Gupta et al. 2012). It is also hypothesized that alcoholic solvents efficiently penetrate the cell membranes, permitting the extraction of high amounts of endocellular components, including phytochemicals produced in plants. Furthermore, ethanol is widely used to obtain crude extracts of phytochemicals in the herbal medicine industry for therapeutic applications due to its relative safety (Wendakoon et al., 2012). Hence, 70% v/v ethanol was the solvent of choice in the present study for extracting the leaves of *Z. scabra.*

Moreover, male mice and rats were preferably used in this study for all the anti-inflammatory models. The rationale for using the male sex in inflammation models is because estrogen, the primary female sex hormone, has been confirmed to possess anti-inflammatory activity by a line of evidence (Miyamoto et al. 1999; Cuzzocrea et al. 2000; Vegeto et al. 2002).

The results of phytochemical tests revealed that leaves of *Z.scabra* are chemically enriched with alkaloids, tannins, saponins, terpenoids, steroids, flavonoids, and cardiac glycosides. The results from the pharmacological tests further confirmed the aqueous fraction to be the most efficient fraction in inhibiting the exudative component of acute inflammation. In contrast, the chloroform fraction showed the highest activity in inhibiting the cellular response of the proliferative part of chronic inflammation. On the other hand, the butanol fraction was found to possess a moderate activity in both the acute (carrageenan-induced) and sub-acute (formalin-induced) paw edema models and was found to be the least active in the cotton pellet-induced chronic granuloma model.

In conclusion, the data obtained in this study demonstrated that the leaves of *Z.scabra* possess different secondary metabolites, which, by acting through an array of possibly different mechanisms, are useful in treating both acute and chronic inflammatory conditions. The current findings also demonstrated the scientific rationale for the traditional use of this plant in different inflammatory conditions. It also confirms the presence of biologically active components, which are worth further investigation and elucidation.

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