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Javanese cardamom (Amomum compactum), photo by Jakasuryanta

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Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

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Chapter in the book:

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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]

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Information from the internet:

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. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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Hippocratea africana root extract and fractions ameliorated carbon tetrachloride-induced oxidative stress and kidney injuries in rats

KUFRE NOAH[1], UTIBE A. EDEM[1], UCHECHUKWU L. IYANYI[2,♥], DANIEL L. AJAGHAKU[3], JUDE E. OKOKON[1]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo. Uyo 520103, Nigeria
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Madonna University. Elele 511101, Nigeria.
Tel.: +234-810-452-7696, ♥email: uchechukwuiyanyi@yahoo.com
[3]Department of Pharmacology, Faculty of Pharmacy, Enugu State University of Science and Technology. PMB 01660, Agbani, Enugu State, Nigeria

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Abstract. *Noah K, Edem UA, Iyanyi UL, Ajaghaku DL, Okokon JE. 2024.* Hippocratea africana *root extract and fractions ameliorated carbon tetrachloride-induced oxidative stress and kidney injuries in rats. Asian J Nat Prod Biochem 22: 51-58.* A common cause of renal failure with potential side effects is drug-induced nephrotoxicity. Renal damage in experiments is frequently induced chemically using carbon tetrachloride (CCl_4), a well-known environmental contaminant with nephrotoxic effects. Intoxicated animals to CCl4 experience experimental oxidative stress in various physiological conditions that promote peroxidative degeneration in different tissues by binding to lipids, proteins, and DNA. The root of *Hippocratea africana* (Willd.) Loes. was investigated to confirm its nephron-protective potential in CCl_4-induced kidney injury in rats. The root extract at the concentrations range of 200-600 mg/kg and its fractions, dichloromethane (DCM) and aqueous at the concentration of 400 mg/kg, was evaluated concerning the kidney damage in the rats induced with carbon tetrachloride (1.5 mL/kg). The anti-oxidative stress and reno-protective potentials of root extract and fractions in rats were determined by observing histological alterations, parameters of kidney function, and oxidative stress markers. The administration of root extract and fractions caused a significant ($p < 0.05$-0.001) increase in oxidative stress markers (SOD, CAT, GPx, SOD, and GSH) in the kidney, while the MDA level was decreased. The root extract/fractions also caused a significant ($p<0.05$-0.001) reduction in serum levels of creatinine, urea, and electrolytes in the rats significantly ($p<0.05$). The kidney histology of the rats treated with *H. africana* extract showed fewer abnormal characteristics than the organotoxic group. The findings demonstrated that the *H. africana* root extract and fractions could preserve the nephron and prevent oxidative stress. They also prevented CCl_4-induced renal damage in rats, possibly due to the phytochemical content with antioxidant properties.

Keywords: Antioxidant, anti-toxicant, *Hippocratea africana*, kidney protective, oxidative stress

INTRODUCTION

Carbon tetrachloride (CCl_4) has been used to study the damage synthetic poisons cause to internal organs in animal models. Upon entering the body by ingestion or skin absorption, CCl_4 diffuses throughout the body. It frequently builds up in the liver, brain, kidney, muscle, fat, and blood (Agency for Toxic Substances and Disease Registry 2000). Animals are intoxicated with CCl_4 in an attempt to create oxidative stress under various physiological conditions. These radicals attach to lipids, proteins, and DNA, causing peroxidative degeneration of many tissues. According to several previous studies, CCl_4 is the most accurate model of the mechanism of Reactive Oxygen Species (ROS) produced in various tissues (Kamisan et al. 2014). After the administration of CCl_4, radicals such as hydroxyl radicals, superoxide anion, hydrogen peroxide, and other radicals are produced, leading to oxidative stress development (Ritter et al. 2004). Long-term exposure to CCl_4 causes necrosis, fibrosis, cirrhosis, inflammatory leukocyte infiltration, and other histological characteristics. It can potentially cause cancer (Qiu et al. 2005).

The kidney is the most significant excretory organ and is essential to preserving the equilibrium of the internal environment. Renal failure, which can be classified as acute or chronic, is a common pathophysiological disorder caused by CCl_4 and can result in death (Shirwaikar et al. 2004). The Reactive Oxygen Species (ROS) are produced due to oxidative stress and are one of the leading causes of acute kidney injury. Proximal tubular toxicity is caused by the direct nephrotoxic effects of toxins such as CCl_4, which include mitochondrial dysfunction, phospholipid damage, increased intracellular calcium concentration, and lysosomal hydrolase inhibition. These effects increase oxidative stress by forming ROS (Hosohata 2016); by encouraging inflammation, ROS either directly or indirectly advances fibrosis. Fibrosis and inflammation may increase the generation of ROS or promote the synthesis of growth factors and cytokines (Siddik 2003). As a result, the accumulation of free radicals within cells can cause lipid peroxidation, and the oxidative degradation of polyunsaturated fats in the membrane causes modifications to the permeability and viscosity of cell membranes (Baud and Ardaillou 1986). Following the in vivo and in vitro studies, CCl_4 raises lipid peroxidation, lowers oxidized glutathione levels in the renal cortex, and

lowers enzyme activity (Khan and Siddique 2012). CCl_4 can alter granular pneumocytes and sub-lethally cause proximal tubular injury in the kidney (Rajesh and Latha 2004).

Moreover, medicinal plants contain various complex chemical components, many of which are well-known for their curative qualities when applied to renal problems. One of the kidney diseases frequently encountered in clinical conditions is acute renal injury (Vijayan 2021). There are fewer effective therapy medications available, which raises the death rate in clinics (Yang et al. 2020; Vijayan 2021). Therefore, the creation of novel therapies or potent medications is desperately needed to treat acute kidney injuries that pose a severe threat to life. *Hippocrata africana* (Willd.) Loes. ex Engl. (Celastraceae) syn. *Loeseneriella africana* (Willd.) N. Hallé is a green forest perennial climber widely distributed in tropical Africa (Hutchinson and Dalziel 1963). It is commonly known as the African paddle-pod and '*Eba enang enang*' by the Ibibios of Nigeria. The Ibibios of Nigeria's Niger Delta region have historically employed the plant root in a variety of ways to treat illnesses like fever, convulsions, malaria, bodily aches, diabetes, and diarrhea (Okokon et al. 2006). Additionally, the plant's root is used for its potential as an antidote or anti-poison to cure liver conditions like jaundice and hepatitis (Etukudo 2000, 2003; Ajibesin et al. 2008). Previous reports showed that the root extract possesses antimalarial (Okokon et al. 2006, 2021), antioedema and antinociceptive (Okokon et al. 2008), antidiabetic and hypolipidemic (Okokon et al. 2021, 2022), antidiarrhoeal and antiulcer (Okokon et al. 2011), hepatoprotective, antileishmanial, cytotoxicity and cellular antioxidant (Okokon et al. 2013), antibacterial, anticonvulsant and depressant (Okokon et al. 2014), in vivo alpha-amylase and alpha-glucosidase inhibitory (Okokon et al. 2021) and in vitro antioxidant (Okokon et al. 2022; Umoh et al. 2021) activities. Earlier studies had reported the presence of spiro hexane-1-carboxylic acid, ethyl ester, 3-methoxy-2-methylphenol,2,3-benzofuran dione,6-hydroxy-4-(p-hydroxy benzyl), δ-3-Carene and α-terpineol in ethyl acetate fraction (Okokon et al. 2017) and the presence of monoterpenes (thujene, limonene, linalool, α-phellandrene, α-terpineol and sabinene) and sesquiterpenes (dehydromevalonic lactone), in the n-hexane fraction of the root extract (Okokon et al. 2013). Also, two xanthones, 1,3,6,7-tetrahydroxyxanthone and 1,3,6-trihydroxy-7-methoxyxanthone, have been isolated from the root of *H. africana* (Umoh et al. 2021). This study aims to determine the anti-oxidative stress and kidney protective properties of *H. africana* root extract and fractions against carbon tetrachloride-induced renal damage in rats.

MATERIALS AND METHODS

Plants collection

Fresh roots of *Hippocratea africana* (Willd.) Loes. roots were collected from bushes in the Uruan area of Akwa Ibom State, Nigeria, in November 2021. A taxonomist from the Department of Botany and Ecological Studies at the University of Uyo in Uyo, Nigeria, identified and verified the plant. The herbarium specimen was deposited in the Department of Pharmacognosy and Natural Medicine Herbarium, University of Uyo.

Preparation of extract and fractions

Fresh *H. africana* roots were cleaned, chopped into smaller pieces, and dried in a shaded area for two weeks. Dried roots were ground using an electric grinder. The root powder of *H. africana* (HAE) was immersed in 50% ethanol for 72 hours. The filtrate was concentrated at 40°C in a rotary evaporator. The crude extract (20 g) was dissolved in 500 mL of distilled water and partitioned with an equal volume of dichloromethane (DCM, 5×500 mL) till no colour change was observed to obtain dichloromethane and aqueous fractions. The extract and its fractions were refrigerated at 4°C until used in the following experiment.

Animals

This study used male Wistar rats. The animals were obtained from the University of Uyo Animal House and placed in plastic cages. They were fed standard pelleted Feed (Guinea feed) and given unlimited access to water. The College of Health Sciences Animal Ethics Committee at the University of Uyo approved the study.

Administration of ethanol root extract and fractions of *Hippocratea africana* on carbon-tetrachloride-induced toxicity in rat

In this model, eight (8) groups of five rats were randomly selected from forty (40) rats. Group 1 (the control or normal control group) was given 10 mL/kg of distilled water orally for eight consecutive days. The organotoxic group was represented by Group 2, which was orally given 10 mL/kg of normal saline for eight days. As the extract-treated groups, groups 3 through 5 received oral administration of 200, 400, and 600 mg/kg of root extract daily for eight days, respectively. Animals in groups 6 and 7 were given 400 mg/kg pretreatments of DCM and aqueous fractions for 8 days. Group 8 was the positive control group and received 100 mg/kg of silymarin orally for 8 days. On the eighth (8th) day, animals in groups 2-8 received carbon tetrachloride (1.5 mL/kg, i.p) dissolved in corn oil mixed at a ratio of 1:3. Finally, 24 hours after carbon tetrachloride administration, all animals were weighed again and sacrificed under light diethyl ether vapour.

Collection of blood samples and organs

After 8 days of treatment (24 hours after the last treatment), the rats were weighed again and sacrificed by light diethyl ether vapor.

The blood samples were collected by cardiac puncture into plain centrifuge tubes and used immediately. The blood in the centrifuge tubes was centrifuged immediately at 2,500 rpm for 15 minutes, and to avoid hemolysis, the serum was separated at room temperature and then used for biochemical assays. The kidneys were surgically removed, weighed, and fixed in 10% formaldehyde for histological process.

Biochemical analysis

Kidney function test

The test was conducted in the Chemical Pathology Department of the University of Uyo Teaching Hospital. It used diagnostic kits to determine the biochemical parameters as markers of kidney function, i.e., levels of electrolytes (Na, K, Cl, and HCO_3), creatinine, uric acid, and urea.

Preparation of renal homogenate

After the other kidney was removed, the fat and surrounding connective tissues were separated from the kidney. After longitudinally cutting each kidney, the renal cortex was isolated and maintained at -8°C. The renal cortex was then homogenized in 0.05 M, pH 7.4 cold potassium phosphate buffer. The renal cortical homogenates were centrifugated for 10 minutes at 4°C at 5,000 rpm. The resulting supernatant was used to measure the enzyme activities using colorimetric assay, i.e., glutathione peroxidase (GPx) (Lawrence and Burk 1976), reduced glutathione (GSH) (Ellman 1959), catalase (CAT) (Rasheed et al. 2020), superoxide dismutase (SOD) (Marklund and Marklund 1974), and malondialdehyde (MDA) content (Esterbauer and Cheeseman 1990). The anti-oxidative stress potentials of the extract were evaluated using these oxidative stress indicators.

Histopathological studies

The other removed kidneys were preserved for histopathology studies using 10% buffered formalin. Following standard protocols, they were processed and stained with hematoxylin and eosin (H&E) (Drury and Wallington 1980) at the Department of Chemical Pathology, University of Uyo Teaching Hospital, Uyo. Morphological changes were observed, and micrographs were made from histological images.

Statistical analysis

Data collected from this study were analyzed statistically using ANOVA (one–way) followed by a post-test (Tukey-Kramer multiple comparison test). Differences between means were considered significant at a 5% significance level, i.e., $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of *H. africana* root extract on kidney weights of rats induced by carbon tetrachloride

The administration of carbon tetrachloride, root extract, and fractions of *H. africana* did not significantly affect ($p>0.05$) the kidney weights of rats compared to the normal control and the organotoxic group (Table 1).

Effect of root extract and fractions of *H. africana* on kidney function parameters of rats with carbon tetrachloride-induced kidney injury.

The administration of 1.5 mL/kg of carbon tetrachloride to normal rats resulted in a significant ($p<0.05$-0.001) increase in blood urea, creatinine, and electrolytes (K^+, Na^+, Cl^-, and HCO^{-3}) compared to normal control. Rats treated with silymarin and root extract/fractions (200–600 mg/kg) showed elevated levels of serum urea and creatinine; however, electrolytes were significantly ($p<0.05$-0.001) decreased. The effect was not dose-dependent, with the DCM fraction having the highest impact. Furthermore, in comparison to the organotoxic group, the reduction in the level of Cl^- was only significant ($p<0.05$) in the groups treated with root extract (400 mg/kg) and silymarin, respectively (Table 2).

Table 1. Effect of *H. africana* root extract administration on organ weights of rats induced with carbon tetrachloride

Parameters/treatment	Dose (mg/kg)	Kidney
Normal control	-	1.06±0.06
Carbon tetrachloride	1.5mL	1.05±0.02
Silymarin+CCl4	100	1.10±0.09
Extract+CCL4	200	1.11±0.04
	400	1.01±0.08
	600	1.07±0.02
Aqueous fraction	400	1.00±0.06
DCM fraction	400	1.17±0.06

Note: Data were expressed as mean ±SEM. n = 5

Table 2. Effect of *H. africana* root extract and fractions on the parameters of kidney function of rats induced with carbon tetrachloride

Treatment	Dose (mg/kg)	Urea (mMol/L)	Creatinine (µmol/L)	Chloride (Mmol/L)	Potassium (mMol/L)	Sodium (mMol/L)	Bicarbonate (mMol/L)
Control (distilled water)	10 mL/kg	3.57± 0.17	77.75±3.42	39.25±1.70	3.57± 0.20	114.5± 1.65	20.02± 0.31
CCl4	1.5 mL/kg	7.87± 0.39^{c}	161.50±6.41^{c}	54.0±3.02^{c}	6.35± 0.50^{c}	168.25±6.86^{c}	35.30± 0.10^{a}
Crude extract	200	6.97±0.23^{f}	140.5± 4.55^{f}	48.0±1.22^{d}	5.40± 0.31^{e}	162.5±5.10^{c}	22.00± 0.28^{d}
	400	5.85±0.15^{b,d}	118.25±2.92^{c,e}	44.50±1.04^{d}	3.30± 0.23^{f}	116.25±4.53^{f}	21.10± 0.20^{d}
	600	5.70±0.67^{b,e}	121.25± 10.0^{b,f}	38.0±1.73^{f}	3.42± 0.24^{f}	111.75±3.35^{f}	20.55± 0.47^{d}
Aqueous Fraction	400	6.90±0.56^{b,f}	138.0±8.59^{f}	34.0±1.29^{f}	4.07± 0.34^{f}	129.5± 7.70^{f}	25.29± 1.24^{d}
DCM fraction	400	6.42±0.14^{f}	128.75±2.92^{a,f}	40.25±1.75^{f}	3.70± 0.24^{f}	122.25±5.79^{f}	21.18± 0.18^{d}
Silymarin	100	5.37±0.14^{d}	108.75±2.68^{c,f}	40.25±1.25^{f}	3.30± 0.14^{f}	113.0± 1.47^{f}	20.42± 0.56^{d}

Note: Data is expressed as MEAN ± SEM, Significant at $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ when compared to control; Significant at $^{d}p<0.05$, $^{e}p<0.01$, $^{f}p<0.001$ when compared to the organotoxic group. (n=5)

Table 3. Effect of *H. africana* root extract and fractions on oxidative stress markers in CCl_4-induced rat kidney

Treatment	Dose (mg/kg)	SOD (U/ml)	CAT (U/g of protein)	GPx (µg/ml)	GSH (µg/ml)	GST (µg/ml)	MDA (µMol/ml)
Control (distilled water)	10 mL/kg	1.31±0.15	1.85±0.11	0.072±0.008	0.35±0.03	0.45±0.06	0.56±0.02
CCl_4	1.5 mL/kg	0.45±0.19 [b]	0.33±0.01[b]	0.029±0.002[c]	0.16±0.01[b]	0.28±0.01[a]	1.38±0.04 [b]
Crude extract	200	1.09±0.06[f]	2.92± 0.26[f]	0.034±0.002[e]	0.79± 0.04	0.37±0.02[d]	0.50±0.01[f]
	400	0.87±0.01[d]	3.44±0.38[f]	0.041±0.001[f]	1.08± 0.03[f]	0.41±0.02[f]	0.44±0.02[f]
	600	0.99±0.02[d]	4.51± 0.20[f]	0.035±0.002[e]	0.99± 0.02[e]	0.40±0.03[f]	0.41±0.01[f]
Aqueous Fraction	400	0.76±0.02[d]	2.44±0.04[f]	0.046±0.006[f]	0.88± 0.02[e]	0.35±0.02[d]	0.42± 0.02[f]
DCM fraction	400	0.98±0.05[d]	2.68±0.24[f]	0.055±0.003[f]	1.18± 0.02[f]	0.44±0.01[f]	0.38± 0.02[f]
Silymarin	100	1.08±0.37[f]	2.47±0.01 [f]	0.045±0.001[f]	0.61±0.06[d]	0.46±0.01[f]	0.46±0.02 [f]

Note: Data is expressed as MEAN ± SEM, Significant at [a]p<0.05, [b]p<0.01, [c]p<0.001 when compared to control; Significant at dp<0.05, ep<0.01, fp<0.001 compared to the organotoxic group. (n=5)

Effect of *H. africana* root extract and fractions on kidney oxidative stress markers of rats induced with carbon tetrachloride

The administration of carbon tetrachloride significantly (p<0.05-0.001) lowers the levels of MDA and increases the levels of GSH, GPx, CAT, GST, and SOD significantly. The organ injuries of CCl_4-induced rats that were treated with root extract and fractions of *H. africana* and silymarin produced a significant (p<0.05-0.001) and non-dose dependent elevation in the levels of GSH, GPx, CAT, GST, and SOD compared to the organotoxic group. However, pretreatment of the rats with root extract and fractions of *H. africana* caused reductions in the levels of MDA of various treatment groups, which were only significant in the groups treated with the DCM fraction and silymarin (Table 3).

Effect of root extract and fractions of *H. africana* on histology of rat kidney in carbon tetrachloride-induced nephrotoxicity

Histological sections of rats' livers in Group 1 (normal control, A) treated with distilled water (10 mL/kg) at magnification (x400) stained with the H&E method showed normal renal tubules and glomeruli with no evidence of pathology. The organotoxic group (Group 2, B) treated with carbon tetrachloride (CCl_4) (1.5 mL/kg) showed normal renal tubules, glomeruli, and congested blood vessels compared to the control group (Figure 1). Group 3 (C) rats treated with 200 mg/kg of *H. africana* root extract and CCl4 showed normal renal tubules and glomeruli with no evidence of pathology (Figure 1). Rats in group 4 (D) treated with *H. africana* root extract (400 mg/kg) and CCl_4 showed normal renal tubules and glomeruli with no evidence of pathology. Group 5 (E) rats treated with *H. africana* root extract (600 mg/kg) and CCl_4 showed normal renal tubules, glomeruli, and congested blood vessels. Kidney sections of rats in group 6 (F) treated with an aqueous fraction (400 mg/kg) of *H. africana* root and CCl4 showed normal renal tubules, glomeruli, and congested blood vessels. Kidney sections of rats in group 7 (G) treated with dichloromethane fraction (400 mg/kg) of *H. africana* root and CCl_4 showed normal renal tubules and glomeruli, with no evidence of pathology. The silymarin-treated rats with carbon tetrachloride-induced toxicity (Group 8, H) had kidney sections that revealed normal renal tubules and glomeruli without any evidence of pathology (Figure 1).

Discussion

Acute kidney injury caused by chemicals and drugs is one of the leading causes of death globally. Acute kidney injury, which includes acute renal illnesses and disorders, is characterized by an abrupt and quick loss of excretory kidney function within a few hours or days (Dai et al. 2023). Carbon tetrachloride (CCl_4) is a substance commonly used in experiments to cause damage to the liver and kidneys (Azri et al. 1992) due to lipid peroxidation, producing free radicals and decreasing the activity of antioxidant enzymes (Brent and Rumack 1993). According to previous studies, exposure to CCl_4 damages the kidneys by increasing the generation of reactive oxygen species (Tirkey et al. 2005; Ganie et al. 2011).

Through metabolism, CCl_4 becomes CCl_3 or trichloromethyl free radical. The trichloromethyl peroxyl radical is formed when the trichloromethyl free radical reacts with proteins and lipids in cells and oxygen. It can potentially damage endoplasmic reticulum lipids more quickly than trichloromethyl free radicals (Recknagel and Glende Jr. 1973). Therefore, lipid peroxidation is caused by the trichloromethyl peroxyl free radical. The highly reactive trichloromethyl radical leads to auto-oxidation of the fatty acids in the phospholipids that make up the cytoplasmic membrane, which alters the morphology and function of the cell membrane, disrupts Ca^{2+} homeostasis and finally results in cell death (Recknagel and Glende Jr. 1973). Changes in the levels of several endogenous scavengers and lipid peroxidation are considered good, indirect markers of oxidative stress in vivo (Babu et al. 2001).

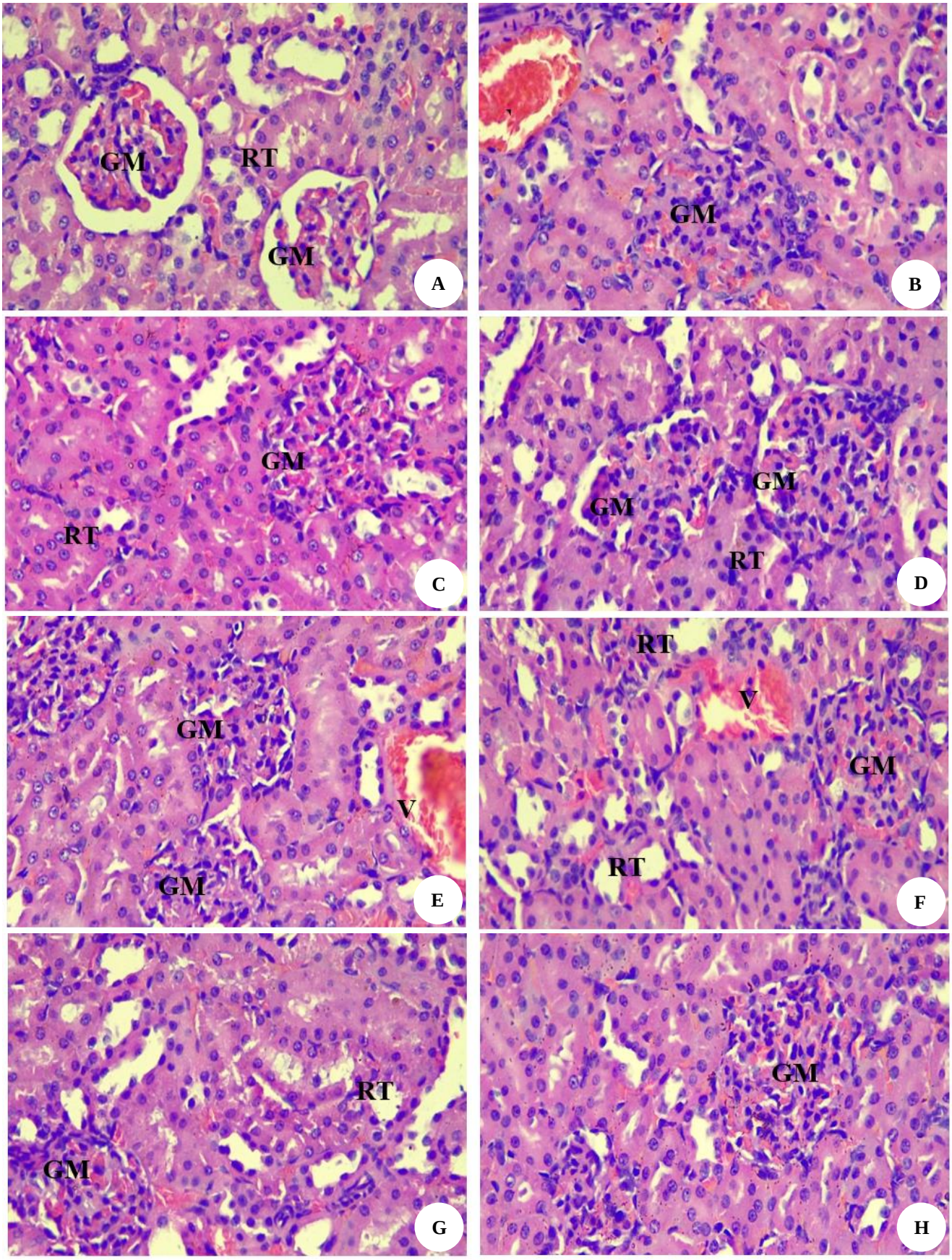

Figure 1. A. Kidney histological sections of rats treated with distilled water 10 mL/kg, B. Carbon tetrachloride 1.5 mL/kg, C. *H. africana* extract 200 mg/kg, D. 400 mg/kg, E. 600 mg/kg, F. Aqueous fraction, G. DCM fraction, H. Silymarin 100 mg/kg. RT: Showing normal renal tubules and GM: Glomeruli, and congested blood vessel with no evidence of pathological lesion

It has been known that several natural compounds have antioxidant activity and can prevent acute kidney injury by reducing the production of free radicals (Tirkey et al. 2005; Jayakumar et al. 2008). The current investigation assessed the anti-oxidative stress and nephroprotective properties of *H. africana* root extract and fractions against CCl_4-induced nephrotoxicity in rats. The administration of CCl_4 (1.5 mL/kg) caused renal function reduction and oxidative stress damage in renal tissues. The animals treated with CCl_4 showed significantly decreased serum concentrations of urea, creatinine, and electrolytes ($p<0.05$-0.001) higher than those of the normal group. The nitrogenous byproducts of blood metabolism, creatinine and urea, are dispersed throughout the body's fluids and are typically eliminated from the blood by the kidney (Safhi 2018). In contrast to the organotoxic group, pretreatment of the rats with the *H. africana* root extract and fractions considerably ($p<0.01$) reduced these parameters non-dose dependently, indicating the nephroprotective efficacy of the root extract and fractions. Oxidative stress was connected to increased urea and creatinine, indicating nephrotoxicity (Lakshmi and Sudhakar 2010).

Numerous investigations have shown that the primary cause of free radical production in various organs, including the liver, kidney, lungs, brain, and blood, is CCl_4 poisoning (Hamed et al. 2012). Additionally, it has been documented that following CCl_4 treatment in rats, the kidney has a higher concentration of CCl_4 than the liver (Sanzgiri and Bruckner 1997), likely due to the kidney's strong affinity for CCl_4 and its substantial cortical cytochrome P450 content. Glutathione (GSH) is a potent antioxidant that significantly prevents cellular damage caused by peroxides and free radicals (Kaur et al. 2003). According to Javed et al. (2011) and Shimeda et al. (2005), CCl_4 showed a significant decrease in GSH because it impairs Hydrogen peroxide (H_2O_2) clearance and encourages the generation of hydroxyl radicals (•OH), which causes oxidative stress. Following treatment with *H. africana* extract/fractions, there was a noticeable and significant ($p<0.05$-0.001) improvement in glutathione concentration and an efficient restoration of lipid peroxidation. The antioxidant enzyme Glutathione Peroxidase (GPx) helps the cell remove excess free radicals and lipid hydroperoxides. Next, a proton is added to GSH to transform it into glutathione disulfide (GSSG), and GR uses NADPH to convert GSSG back to GSH, maintaining the GSH pool in a reduced state. CCl_4 treatment has significantly reduced the concentration of GSH and altered the activity of several essential enzymes, including GPx. A decrease in GSH level may cause the decreased activity of glutathione-metabolizing enzymes in renal tissue. According to an earlier study, CCl_4 altered the activity of these enzymes, which are crucial for scavenging harmful free radicals (Ogeturk et al. 2005). After CCl_4 treatment, the activity of GPx was decreased; however, the kidney tissue treated with *H. africana* extract and fraction showed a considerable increase ($p<0.05$-0.001) in the activity of these enzymes.

The enzymatic antioxidant defense system relies heavily on the enzymes superoxide dismutase (SOD) and catalase (CAT), which work together to scavenge free radicals and transform them into stable molecules like hydrogen peroxide, thereby reducing the cell damage caused by free radicals (Curtis et al. 1972). Superoxide dismutation, which produces H_2O_2, is catalyzed by the metalloenzyme SOD (Freeman and Crapo 1982; McCord 1987). Following CCl_4 treatment, SOD activity was markedly reduced; however, SOD activity was recovered upon administration of the *H. africana* extract/fractions. Catalase reduces H_2O_2 to oxygen and water and protects cells from oxidative stress-related damage. CAT activity was considerably protected from CCl_4 treatment by *H. africana* extract and fraction. It suggests that in addition to significantly ($p<0.05$-0.001) increasing the activity of hepatic antioxidant enzymes, root extract may be lowering reactive free radicals due to the availability of antioxidant compounds, reducing oxidative damage to the tissues. It might be due to the root extract's or fractions' capacity to scavenge free radicals and its anti-oxidative stress activity (Okokon et al. 2013, 2021). It may be connected to the actions of its phytochemical constituents, which include the xanthones, monoterpenes, and sesquiterpenes that are contained in the root extract (Okokon et al. 2013, 2021; Umoh et al. 2021). One crucial indicator of oxidative stress is lipid peroxidation; one byproduct of polyunsaturated fatty acid peroxidation in cells is malondialdehyde (MDA), and increased free radicals lead to overproduction of MDA. After administration of CCl_4, it showed that the amount of MDA in kidney tissue had significantly ($p<0.05$-0.001) increased. Treatment with *H. africana* extract/fractions has significantly reduced MDA levels. It could be due to the antioxidant activity of *H. africana* to scavenge free radicals and prevent lipid peroxidation.

The mechanisms underlying the nephroprotective effects of the root extract and fractions could be caused by antioxidant activity, indicated by the elevation of GSH, GPx, SOD, and CAT, as well as the decrease in MDA levels in the kidney tissues of rats treated with extracts or fractions. These findings confirm the role of oxidative stress in CCl_4-induced nephrotoxicity. These imply that the kidney protective properties of the root extract may be attributed to the phytochemical content to overcome oxidative stress. Rat kidney weights were unaffected by the CCl_4 and extract/fractions treatment compared to the control group. The levels of urea, creatinine, sodium (Na), potassium (K), chlorine (Cl), and bicarbonate were also significantly reduced in the groups treated with root extract and fractions. Nephroprotective potentials may be caused by decreased ($p<0.05$-0.001) kidney function parameters (creatinine, urea, Na, K, Cl, and bicarbonate) after pretreatment with root extract/fractions. These outcomes strengthened the histology results, namely that the kidney tissues in the groups treated with extract and fractions remained comparatively intact compared to those in the organotoxic group. It suggests that the root extract and fractions protected the kidney tissues from the damaging effects of carbon tetrachloride. Histopathological examination of the organotoxic group confirmed the induction of kidney injuries by CCl4, indicated by severely congested blood vessels, as seen in the CCl_4-treated group.

In conclusion, the present study demonstrated that CCl_4 is a potent nephrotoxic substance, which leads to oxidative stress by depleting the activities of antioxidant enzymes, inflammatory cytokine production, and stimulating apoptosis. Treatments with the root extract and fractions of *H. africana* attenuated the CCl_4-induced renal toxicity. Hence, the root extract and fractions of *H. africana* possess nephroprotective and anti-oxidative stress activities against harmful substances due to the activities of its phytochemical constituents.

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Solanum anomalum leaf extract mitigated doxorubicin-induced kidney toxicity and oxidative stress in male rats

JUDE E. OKOKON[1,♥], JOHN AYOOLA ONUNKUN[2], MARTIN OSITA ANAGBOSO[3], JOHN A. UDOBANG[4]

[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo. Uyo 520103, Nigeria. Tel.: +234-8023453678,
♥email: judeefiom@yahoo.com
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Madonna University. Elele Campus, P.M.B 05 Elele, Rivers State, Nigeria
[3]Department of Microbiology, Madonna University. Elele Campus, P.M.B 05 Elele, Rivers State, Nigeria
[4]Department of Clinical Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, University of Uyo. Uyo 520103, Nigeria

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Abstract. *Okokon JE, Onunkun JA, Anagboso MO, Udobang JA. 2024.* Solanum anomalum *leaf extract mitigated doxorubicin-induced kidney toxicity and oxidative stress in male rats. Asian J Nat Prod Biochem 22: 59-66. Solanum anomalum* Thonn. ex Schumach (family Solanaceae) is a shrub whose leaves are used locally to treat various diseases. Evaluation of the antidotal activity of leaf extract of *S. anomalum* was carried out to ascertain its uses in traditional medicine. The leaf extract (70-210 mg/kg) of *S. anomalum* was investigated for nephroprotective activity against doxorubicin-induced kidney toxicity in rats. Kidney function parameters, kidney histology, and kidney oxidative stress markers were used to assess the kidney protective effect of the extract. The leaf extract (70-210 mg/kg) significantly ($p<0.05$-0.01) reduced the levels of creatinine, urea, and electrolytes that were elevated by doxorubicin. Also, the MDA level elevated by doxorubicin was reduced by the extract co-administration, while the levels of GSH, GST, SOD, GPx, and CAT that were decreased by doxorubicin were significantly ($p<0.01$) elevated by the leaf extract. Histology of the kidney sections of extract-treated animals showed reductions in the pathological features compared to the organotoxic-treated animals. The chemical pathological changes were consistent with histopathological observations, suggesting marked nephroprotective potential. The anti-toxic effect of this plant may, in part, be mediated through the plant's chemical constituents. *S. anomalum* possesses anti-toxicant properties that can be exploited in treating doxorubicin-related toxicities.

Keywords: Doxorubicin, oxidative stress, renoprotective, *Solanum anomalum*

INTRODUCTION

Doxorubicin is an anthracycline glycoside antibiotic that possesses a potent and broad-spectrum antitumor activity against various human solid tumors and hematological malignancies (Calabresi and Chamber 1990). However, due to its diverse toxicities, its use in chemotherapy has been limited, including cardiac, hepatic, hematological, and testicular toxicity (Yilmaz et al. 2006). The semiquinone form of doxorubicin is a toxic, short-lived metabolite that initiates a cascade of reactions when it interacts with molecular oxygen, producing Reactive Oxygen Species (ROS). ROS generation, inflammatory processes, and lipid peroxidation have been suggested to be responsible for doxorubicin-induced cardio, hepatic, and nephrotoxicity (Kalender et al. 2005; Injac et al. 2009). It has been proposed that DOX-semiquinone, an unstable metabolite of DOX, reacts with O_2, producing H_2O_2 and O_2^- (superoxide). In addition, DOX enhances the activity of extramitochondrial oxidative enzymes such as NADPH and xanthine oxidases and also interferes with mitochondrial iron export, resulting in ROS formation (Bachur et al. 1979). These free radicals attack the cell's membranes and cause organ dysfunction.

Chronic Kidney Disease (CKD) contributes significantly to the development and progression of kidney failure, cardiovascular disease, and premature death (Levey et al. 2007). Studies have reported that about 1.75 million patients worldwide receive renal replacement therapy through dialysis regularly. This figure is on the increase, and the estimated mortality in patients with Acute Kidney Injury (AKI) was 23.9% in adults and 13.8% in children (Susantitaphong et al. 2013). Previous studies reported that about 10% of the world's population is affected by CKD (Jha et al. 2013) due to increased risk factors such as obesity and diabetes mellitus, among others. Thus, an increased number of patients with CKD has been reported worldwide, and an estimated 843.6 million individuals worldwide were reported to be affected by CKD in 2017 (Jager et al. 2019). The Global Burden of Disease (GBD) reports indicated that CKD has emerged as one of the leading causes of death the world over (GBD 2013 Mortality and Causes of Death Collaborators 2015; Rhee and Kovesdy 2015), although there is a decline in patient mortalities with End-Stage Kidney Disease (ESKD) (Saran et al. 2020). Also, there was an increase of 41.5% in the global all-age mortality rate attributed to CKD between 1990 and 2017 (GBD 2020). About 36.8 million Nigerians (23%) have been estimated to suffer from kidney disease (Ebum 2013), which means that one in seven Nigerians is suffering from some kidney disorder.

Considering the high cost of management of kidney diseases, especially the burden of the high cost of dialysis and kidney transplants in Nigeria and many other

developing countries of the world, the search for an alternative treatment strategy is therefore inevitable. Plants provide a rich reservoir of natural therapeutic compounds that are affordable, readily available, and have less toxic effects. Investigation of plants with kidney protective potentials can lead to the discovery of active compounds that can serve as alternatives to currently available orthodox medicines which are costly and associated with many toxic effects (Okokon et al. 2022a).

Solanum anomalum Thonn. ex Schumach, a plant whose leaves and fruits are used medicinally and nutritionally, is commonly grown in Western and Eastern Africa sub-regions. Its plant parts are utilized locally to treat diabetes, gastrointestinal disorders, infections, inflammation, and pains (Bukenya and Hall 1988; Burkill 2000; Offor and Ubengama 2015). Many studies have been reported on their fruits and leaves hypoglycemic and antidiabetic activities (Offor and Ubengama 2015; Okokon et al. 2022a); in vivo and in vitro antiplasmodial (Okokon et al. 2016, 2017a); anti-edema (Okokon et al. 2017b); antioxidant and antiulcer (Okokon et al. 2019a); anticonvulsant and depressant (Okokon et al. 2019b); analgesic (Okokon et al. 2020) and antidiarrhoeal (Udobang et al. 2022). Moreover, the leaves' phytochemical constituents such as alkaloids, flavonoids, saponins, tannins, diosgenin, a diosgenin glycoside (25(R)-diosgenin-3-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside, uracil, 5-methyluracil, 1-octacosanol, and octacosane have been reported (Okokon et al. 2016, 2022b). In this study, we report the renoprotective activity of the leaf extract against doxorubicin-induced kidney toxicity and oxidative stress.

MATERIALS AND METHODS

Plants collection

Fresh leaves of *S. anomalum* were collected from compounds in the Uruan area, Akwa Ibom State, Nigeria, in August 2022. Next, a taxonomist from the Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria, identified and authenticated the plant's leaves. The herbarium specimen was deposited at the Department of Pharmacognosy and Natural Medicine Herbarium, University of Uyo (UUH.75a).

Extraction

Fresh leaves of *S. anomalum* were washed, cut into smaller pieces, and dried under shade for two weeks. The leaves were further pulverized to powder using an electric grinder. The powdered leaves material (1.5 kg) was macerated in 50% ethanol (7.5 L) for 72 hours at room temperature (28±2°C). Thereafter, the soaked leaves powder was filtered, and the liquid filtrate was concentrated and evaporated to dry in vacuo 40°C using a rotary evaporator (BuchiLab, Switzerland). The extract was stored in a refrigerator at -4°C until used for the proposed experiments (Okokon et al. 2015).

Animals

Albino Wistar rats (120-135 g) of either sex were used for these experiments. They were obtained from the Department of Pharmacology and Toxicology Animal House, Madonna University, Elele, Rivers State. All animals were well acclimatized having been kept in clean standard polypropylene cages with laboratory-grade pine shavings as beddings, contained in well-ventilated house and maintained under standard conditions (temperature: 25±3°C; photoperiod: 12-h natural light and 12-h dark cycle; humidity: 35-60 %) and fed on regular pellets (Guinea feed) and water ad libitum.

Experimental design

This study used a repeated dose model earlier described by Olorundare et al. (2020), which lasted for 14 days. Group I rats, the untreated control, were orally pretreated with distilled water at 10 mL/kg/day. Group 2 rats were given normal saline (10 mL/kg/day) but equally treated with 2.5 mg/kg of doxorubicin hydrochloride (®Celondoxily Injection 50, CELON Laboratories PVT. Limited, India) dissolved in 0.9% normal saline for 14 days on alternate days. Groups 3-5 rats were orally pretreated respectively with 70 mg/kg/day, 140 mg/kg/day, and 210 mg/kg/day of *S. anomalum* leaf extract dissolved in distilled water 2 hours before treatment with 2.5 mg/kg of doxorubicin in 0.9% normal saline administered intraperitoneally on alternate days for 14 days, respectively. Group 6 rats, the positive control group, were equally pretreated with 20 mg/kg/day of Vitamin C two hours before treatment with 2.5 mg/kg of doxorubicin in 0.9% normal saline administered intraperitoneally on alternate days for 14 days.

Blood sample collection

On the last day of the study (day 14), the rats were weighed and fasted overnight but were allowed access to drinking water ad libitum. Rats were sacrificed under light inhaled diethyl ether anesthesia, and whole blood samples were collected directly from the heart by cardiac puncture into plain sample bottles. Blood samples were carefully collected with fine 21G needles and 5 mL syringes without damaging the heart tissues. Finally, the rats' kidneys were identified, harvested, and weighed.

Kidney function test

The following biochemical parameters such as electrolyte levels (Na, K, Cl, and HCO_3), creatinine, and blood urea, were assayed as kidney function markers using diagnostic kits at the Chemical Pathology Department of the University of Port Harcourt Teaching Hospital.

Oxidative stress markers

The antioxidant enzyme assays were performed on kidney homogenates of rats used in this study. These oxidative stress markers assessed the extract's antioxidative stress potentials.

Preparation of renal homogenate

The kidneys were removed in each rat, and one kidney was fixed in 10% formaldehyde for histological processes. At the same time, the other kidney used for oxidative marker assays was dissected free from connective tissue and surrounding fat. The kidneys were longitudinally sectioned, and the renal cortex was separated and kept at -8°C. Subsequently, the renal cortex was homogenized in cold potassium phosphate buffer (0.05M, Ph 7.4). The renal cortical homogenates were centrifuged at 5,000 rpm for 10 min at 4°C; the obtained supernatant determines the superoxide dismutase (SOD) (Marklund and Marklund 1974), catalase (CAT) (Sinha1972), glutathione peroxidase (GPx) (Lawrence and Burk 1976), reduced glutathione (GSH) (Ellman 1959) and malondialdehyde (MDA) content (Esterbauer and Cheeseman 1990).

Histopathological studies

The kidneys of the animals that were surgically removed and fixed in 10% formaldehyde were processed and stained with hematoxylin and eosin (H&E) (Drury and Wallington 1980), according to standard procedures at the Department of Chemical Pathology, University of Port Harcourt Teaching Hospital, Port Harcourt. Morphological changes were observed and recorded in the excised organs of the sacrificed animals. Histologic pictures were taken as micrographs.

Statistical analysis

Data obtained from this work were analyzed statistically using ANOVA (one–way) followed by a post-test (Tukey-Kramer multiple comparison test). Differences between means were considered significant at a 5% significance level, i.e., $p \leq 0.05$.

RESULTS AND DISCUSSION

The effect of leaf extract of *Solanum anomalum* on kidney weights and kidney function parameters of doxorubicin-induced kidney injury in rats

Treatment of rats with doxorubicin (2.5 mg/kg i.p) on alternate days for 14 days caused significant ($p<0.01$-0.001) elevation of serum urea, creatinine, and electrolytes (K^+, Na^+, Cl^- and HCO_3^-) levels when compared to normal control. standard reference values for normal wistar rats are;creatinine (0.2 -0.7 mg/dL), urea (11-25 mg/dl), Na (138-155 mMol/L), K (4.6-6.0 mMol/L), HCO_3^- (22.8-31.1 mEq/L) (Loeb and Quimby 1999). The values of the kidney function parameters of the normal rats in this study were within the standard range, while those of doxorubicin only-treated group were elevated above the standard range. These increased levels of serum urea, creatinine, and electrolytes were significantly ($p<0.05$-0.001) reduced following concomitant treatment of the rats with vitamin C and leaf extract of *S. anomalum* (70-210 mg/kg) with doxorubicin though in a non-dose-dependent fashion (Table 1). Similarly, administration of doxorubicin (2.5 mg/kg i.p) to rats on alternate days for 14 days caused significant ($p<0.05$) reduction of kidney weights of rats when compared to normal control group. Co-administration of leaf extract of *S. anomalum* with doxorubicin reversed these weight losses to near normal (Figure 1)

Effect of leaf extract on kidney oxidative stress markers

Administration of doxorubicin (2.5 mg/kg i.p) on alternate days for 14 days significantly ($p<0.05$-0.001) caused decreases in kidney antioxidant enzymes activities (SOD, GPx, CAT) and GSH levels when compared to control. The MDA level was also elevated by doxorubicin treatment. However, concomitant administration of leaf extract of *S. anomalum* (70-210 mg/kg) with doxorubicin for 14 days caused marked significant ($p<005$-0.01) elevation of the enzymatic and non-enzymatic endogenous antioxidants in the treated rat's groups when compared to the organotoxic groups. The treatment also caused a significant ($p<0.001$) reduction in the level of MDA of the treated rats when compared to organotoxic control (Figures 2,3,4,5,6).

Effect of extract on the histology of kidney of rats

The histological sections of rat kidneys treated with distilled water showed normal glomeruli and tubules. However, sections of the kidneys of rats treated with doxorubicin (2.5 mg/kg) alone showed microvesicles in the tubular lining cells and normal glomeruli. Kidney sections of rats treated with 70 and 140 mg/kg of *S. anomalum* leaf extract and doxorubicin depicted normal glomeruli, and the tubules lined by anucleate epithelial cells with increased eosinophilia that sloughed into the lumen of the tubules. Rats treated with 210 mg/kg of *S. anomalum* leaf extract and doxorubicin had kidney sections with normal glomeruli. At the same time, the kidney sections of rats treated with 40 mg/kg of vitamin C and doxorubicin showed normal glomeruli (Figure 7.A-F).

Table 1. Effect of *Solanum anomalum* leaf extract on renal function parameters of rats with doxorubicin-induced kidney toxicity

Treatment	Dose (mg/kg)	Urea (mMol/L)	Creatinine (µmol/L)	Chloride (mMol/L)	Potassium (mMol/L)	Sodium (mMol/L)	Bicarbonate (mMol/L)
Normal control	-	4.21±1.41	81.66±2.53	60.0±0.06	3.00±0.20	121.3±1.33	22.00±0.81
Doxorubicin	2.5	14.16±0.44^d	263.33±6.67^d	73.33±1.33^d	5.50±0.28^d	158.66±2.96^d	30.66±0.66^d
Vitamin C+DOX	20	4.93±0.43^c	99.0±9.00^c	70.66±2.66	4.13±0.16^b	124.66±4.66^b	22.66±1.66^a
Extract+DOX	70	5.03±0.32^c	104.66±5.04^c	65.33±2.33	3.16±0.08^c	121.33±2.33^b	25.0±1.00
	140	5.73 ±0.39^c	115.0±10.00^c	68.0±1.00	3.73±0.23^b	129.66±1.66^a	24.0±2.00^a
	210	5.00±0.23^c	98.66±5.66^c	79.0±1.04^d	4.36±0.26^a	137.33±7.33	22.33±1.33^a

Note: Data is expressed as MEAN ± SEM, Significant at $^d p<0.001$ compared to normal control; $^a p<0.05$, $^b p< 0.01$, $^c p< 0.001$ when compared to control (n = 6)

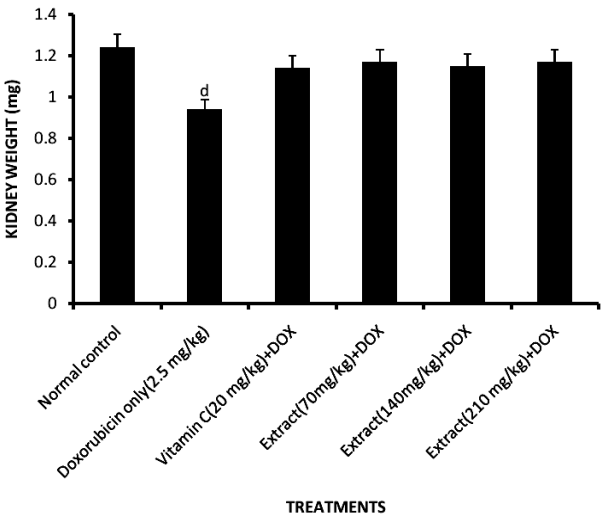

Figure 1. Effect of *S. anomalum* leaf extract on kidney weights of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control when compared to control. (n=6). DOX: Doxorubicin

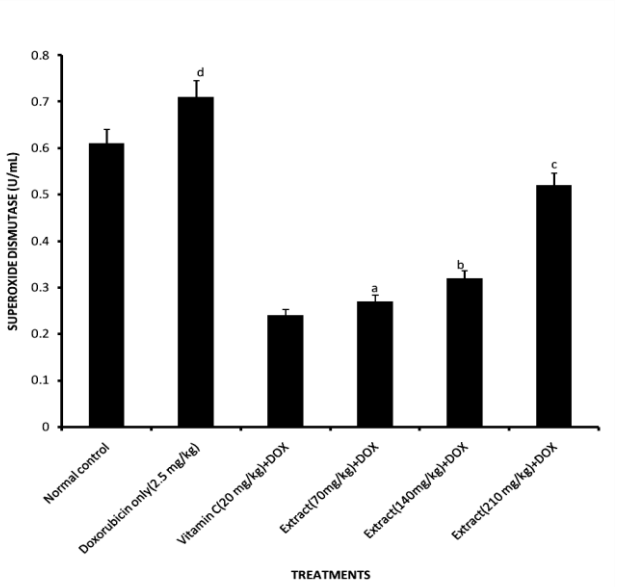

Figure 2. Effect of *S. anomalum* leaf extract on kidney superoxide dismutase levels of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control; $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ when compared to control. (n = 6). DOX: Doxorubicin

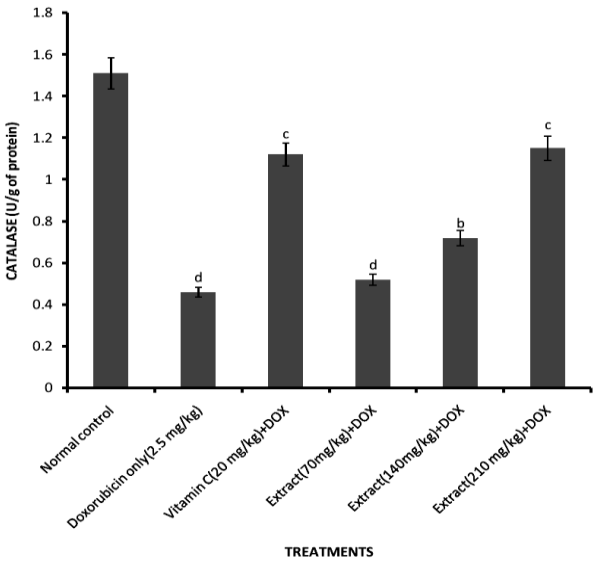

Figure 3. Effect of *S. anomalum* leaf extract on kidney catalase levels of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control; $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ when compared to control. (n=6). DOX: Doxorubicin, N.S: Not significant

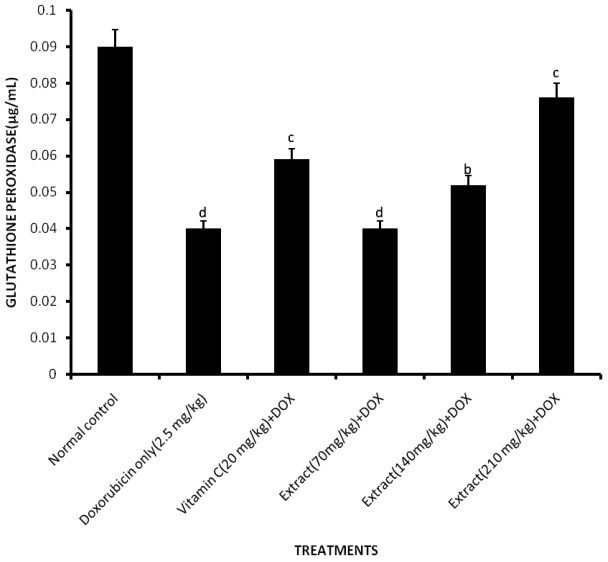

Figure 4. Effect of *S. anomalum* leaf extract on kidney glutathione peroxidase levels of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control; $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ when compared to control. (n = 6). DOX: Doxorubicin

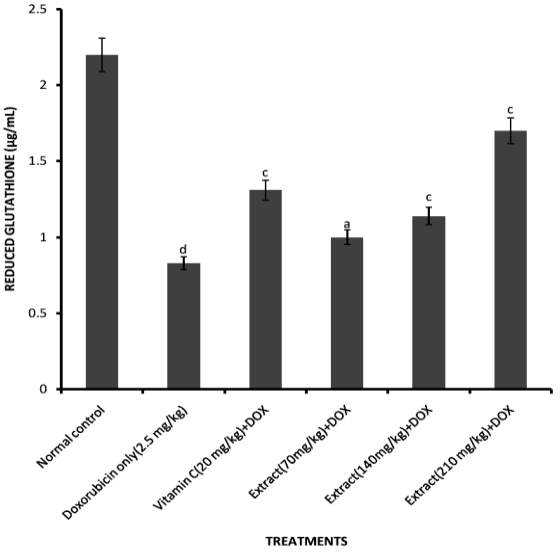

Figure 5. Effect of *S. anomalum* leaf extract on kidney reduced glutathione levels of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control; $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ when compared to control. (n = 6). DOX: Doxorubicin

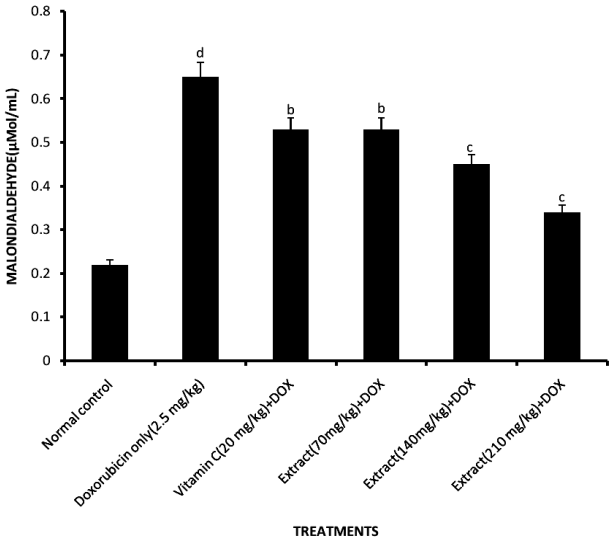

Figure 6. Effect of *S. anomalum* leaf extract on kidney malondialdehyde levesl of rats with doxorubicin-induced toxicity. Data is expressed as MEAN ± SEM, Significant at $^{d}p<0.001$ compared to normal control; $^{a}p<0.05$, $^{b}p< 0.01$, $^{c}p< 0.001$ when compared to control. (n = 6). DOX: Doxorubicin

Discussion

This work was designed to investigate the effect of leaf extract of *S. anomalum* on doxorubicin-induced liver toxicity in rats to confirm the folkloric claim of its antidotal activity. In this study, doxorubicin administration was found to have caused an elevation of serum urea (70.26%), creatinine (68.98%), and electrolytes (K^+, Na^+, Cl^- and HCO_3^-) levels (45.45, 23.54, 18.17 and 28.24% respectively) when compared to normal control. These values were observed to be above the standard reference values ranges for wistar rats; creatinine (0.2-0.7 mg/dL), urea (11-25 mg/dl), Na (138-155 mMol/L), K (4.6-6.0 mMol/L), HCO_3^- (22.8-31.1 mEq/L) (Loeb and Quimby 1999), which is an indication of a serious injury to the kidney. The values of the kidney function parameters of the normal rats in this study as well as those of extract-treated groups were within the standard range. This finding is consistent with an earlier report by Rajasekaran (2019), in which significant elevations were also reported. It is well documented that kidney injury is indicated by an increase in serum levels of creatinine and urea (Lakshmi and Sudhakar 2010) as well as an increase in serum levels of Na, K, Cl, and bicarbonate (James and Mitchel 2006). However, these increases were reduced significantly by the co-administration of leaf extract of *S. anomalum.*

Doxorubicin is reported to cause nephrotoxicity via oxidative stress as free radicals formed caused tubular atrophy and increased glomerular capillary permeability. Nephrotoxicity by doxorubicin can also result from lipid peroxidation and biological macromolecule damage by iron-dependent oxidative damage (Mohan et al. 2010). Degenerative changes in the kidney depend on the cumulative dose and duration of treatment, as doxorubicin metabolites are partly excreted from the kidney. Another mechanism for renal injury is the conversion of DOX to semiquinone free radical by NADPH-cytochrome P-450, which generates hydroxyl radical and superoxide anion, and causes lipid peroxidation (Rashid et al. 2013). The reduction of urea, creatinine, and electrolyte levels by the leaf extract in this study results from the free radical scavenging potentials of the extract, thereby protecting the kidney against oxidative stress by free radicals generated by doxorubicin. This result corroborates previously reported nephroprotective potential of the leaf extract of *S. anomalum* against alloxan-induced kidney injury by Etuk et al. (2023a) and also agrees with Mohan et al. (2010), who reported significant nephroprotection of *Solanum torvum* against doxorubicin-induced kidney toxicity. Phytochemical compounds such as torvanol A, 3, 4-trimethyl triacontane, octacosanyltriacontanoate, 5-hexatriacontanone, triacontanol, 3-tritriacontanone, tetratriacontanoic acid, sitosterol, stigmasterol, campesterol, neochlorogenin 6-O-β-D-quinovopyranoside, neochlorogenin 6-O-β-D-xylopyranosyl-(1→3)-β-D-quinovopyranoside, neochlorogenin 6-O-α-L-rhamnopyranosyl-(1→3)-β-D- quinovopyranoside, solagenin 6-O-β-D-quinovopyranoside, solagenin 6-O-α-L-rhamnopyranosyl-(1→3)-β-D-quinovopyranoside, isoquercetin, rutin, kaempferol and quercetinl have been isolated and identified from the leaves of *S torvum* (Mahmood et al. 1983; Yuan-Yuan et al. 2011). Some of these compounds have also been identified and isolated from *S. anomalum* thereby laying credence to the observed nephroprotective activity in this study.

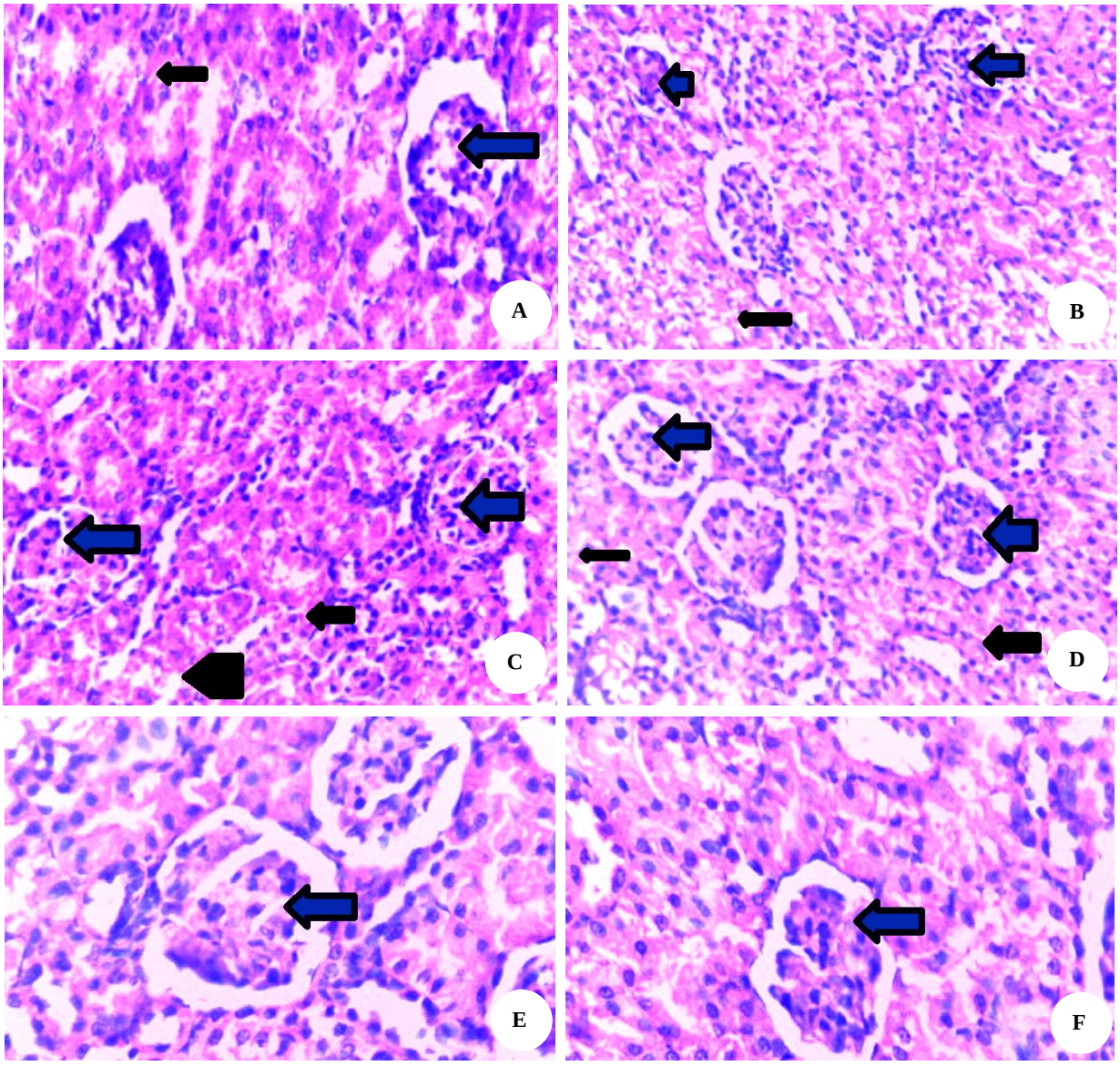

Figure 7. Histological section of rat kidneys. A. Treated with distilled water showing normal glomeruli (*blue*) and normal tubulus (black), B. Treated with doxorubicin (2.5 mg/kg) alone showing microvesicles in the tubular lining cells (*black*) and normal glomeluri (*blue*), C. Treated with 70 kg/kg of *S. anomalum* leaf extract and doxorubicin showing section of kidney with normal glomeruli (*blue*), the tubules (*black*) are lined by anucleate epithelial cells with increased eosinophilia that sloughing into the lumen of the tubulus, D. Treated with 140 mg/kg of *S. anomalum* leaf extract and doxorubicin showing section of kidney with normal glomeruli (*blue*) and the tubulus (*black*) are lined by anucleate epithelial cells with increased eosinophilia that sloughing into the lumen of the tubulus, E. Treated with 210 kg/kg of *S. anomalum* leaf extract and doxorubicin showing section of kidney with normal glomeruli (*blue*), F. Treated with 40 mg/kg of vitamin C and doxorubicin showing section of kidney with normal glomeruli (*blue*)

Moreover, Offor et al. (2021) reported significant protection of the kidney against Lead-induced kidney injury by fruit extract of this plant, further supporting and confirming the nephroprotective activity of *S. anomalum*. The observed nephroprotective activity of the leaf extract against the effect of free radicals generated by doxorubicin is due to antioxidant activities of the phytoconstituents (Okokon et al. 2019a) such as diosgenin (Kanchan et al. 2016), 1-octacosanol and octacosane (Sengupta et al. 2018; Leng et al. 2020; Rhetso et al. 2020), squalene (Gunes 2013; Micera et al. 2020), β-sitosterol (Gupta et al. 2011; Baskar et al. 2012) and phenolic compounds in the leaf extract.

This study further revealed that administration of doxorubicin (2.5 mg/kg, i.p) on alternate days for 14 days to rats caused significant decreases ($p<0.05$) in levels of enzymatic and non-enzymatic endogenous antioxidants (GSH, SOD, CAT, GPX, and GSH) when compared to control, while the MDA level was elevated. Lipid peroxidation is a marker of oxidative stress, and elevations in the amount of malondialdehyde (MDA), a lipid peroxidation product, have been reported following doxorubicin treatment (Rashid et al. 2013; Rehman et al. 2014; Khames et al. 2019); this trend was also observed in this study. Concomitant administration of leaf extract *S.*

anomalum (70-210 mg/kg) with doxorubicin caused significant ($p<0.05$-0.001) non-dose-dependent elevation in the levels of the antioxidant enzymes (SOD, CAT, GPX) when compared to control. Similarly, the GSH level was significantly ($p<0.001$) elevated after treatment with the extract compared to the control. Furthermore, administration of the leaf extract results in significant ($p<0.05$-0.01) reductions in the level of MDA of the extract-treated rats. It has been documented that DOX inhibits the activities of endogenous enzymatic and non-enzymatic antioxidants, as shown in this study. So, an imbalance between ROS generation and neutralization leads to oxidative stress and injury to the kidney (Abushouk et al. 2017, 2019; Abdel-Daim et al. 2017). The reduced MDA level caused by the administration of the leaf extract may have resulted from a reduction in lipid peroxidation and generation of free radicals, which might have been scavenged by the phytoconstituents present in this extract, revealing the antioxidative stress potentials of the leaf extract and hence the protective effect on the kidney as was observed in this study and reported previously by Etuk et al. (2023b).

This study revealed histological findings that the kidneys of rats treated with doxorubicin (2.5 mg/kg) alone showed pathological signs of injury, which were seen as degenerated microvesicles in the tubular lining cells, among others. However, co-administration of *S. anomalum* leaf extract and doxorubicin reduced the toxic effects of the doxorubicin as normal glomeruli devoid of pathological signs were seen in the kidney sections of the extract-treated rats examined. This further confirms the leaf extract's nephroprotective potential, which may have been exerted through its phytochemical constituents' antioxidant and antioxidative stress activities.

In conclusion, the findings of this study show that the leaf extract of *S. anomalum* (70-210 mg/kg) has the potential to counteract the detrimental effect of doxorubicin on the kidney. This activity can be attributed to its phytochemical constituents' antioxidant and antioxidative stress activities. Thus, the leaf can alleviate and/or prevent doxorubicin-induced nephrotoxicity.

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Antibacterial activities of combinations of areca nuts (*Areca catechu*), cardamon seeds (*Amomum compactum*), and green betel leaves (*Piper betle*) ethanol extracts against *Staphylococcus aureus*

AKSAL ANGGARA PRASETYA, NITA ETIKAWATI♥, ARI SUSILOWATI
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: nitaetikawati@staff.uns.ac.id

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Abstract. *Prasetya AA, Etikawati N, Susilowati A. 2024. Antibacterial activities of combinations of areca nuts (*Areca catechu*), cardamon seeds (*Amomum compactum*), and green betel leaves (*Piper betle*) ethanol extracts against* Staphylococcus aureus*. Asian J Nat Prod Biochem 22: 67-73.* Areca nut (*Areca catechu* L.), Javanese cardamom seed (*Amomum compactum* Soland. ex Maton), and betel leaf (*Piper betle* L.) are well-known as the formulas for betel quid chewing in Indonesia. According to tradition, betel quid chewing can prevent tooth decay, gum diseases, and lousy breath. This study investigated the antibacterial activity of the single extract with various concentrations (10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL) and combined extracts with ratios (1:1:1, 2:1:1, 1:2:1, and 1:1:2) against *Staphylococcus aureus* ATCC 25923 using the disc diffusion method based on EUCAST. Data was analyzed on the inhibition zones using one-way ANOVA and Duncan multiple-range tests. The antibacterial activity of each ethanol extract has an optimal concentration of 20 mg/mL against *S. aureus*. The extracts of areca nut, betel leaves, and Javanese cardamom seed obtained inhibitory zone diameters of 8.70±0.570 mm, 7.50±0.395 mm, and 7.35±0.379 mm, respectively. The combinations of three extracts obtained inhibitory zone diameters of 9.60±0.379 mm and 9.90±0.675 mm in 1:1:1 and 2:1:1 ratios, demonstrating a significant effect of the inhibition zone ($p<0.05$) compared to other ratio combinations and single extracts. These combinations of extracts also show a synergistic effect against *S. aureus*.

Keywords: *Amomum compactum*, antimicrobial agents, *Areca catechu*, combinations extract, dental plaque, *Piper betle*

INTRODUCTION

Currently, one potential means of overcoming problems during dental infections is to do it chemically through antibiotics and antimicrobials in the form of mouthwash containing chlorhexidine. According to Cock et al. (2023), the fourth most expensive disease to treat was oral healthcare; some factors, like the limited oral health facilities in rural areas, also lower the investment in oral healthcare. However, people also tend to rely on traditional medicine for problems related to treating dental disease. At least 80% of the world's population uses traditional medicine for primary health care (WHO 2023). Medicinal plants are often considered easy to obtain, cheap, efficient, and rarely accompanied by side effects. The medicinal plants selected for use for thousands of years by society are the most apparent starting point for potential ingredient candidates in developing new and effective antimicrobial agents (Besra and Kumar 2018). Traditional medicine is often an accumulation of various values and knowledge or practices based on theory, beliefs, and culture. Based on regional and cultural factors, conventional medicines are used as single formulations containing part of a plant or as combination formulations with another plant as active ingredients (Che et al. 2013).

The habit of "*nginang*" (betel chew) is an example of an herbal combination in Indonesia; it's usually used for medicinal purposes, social purposes, and religious ceremonies. Betel chewing is often associated with and believed to prevent various complaints related to oral health, such as avoiding bad breath, strengthening teeth, and maintaining healthy gums (Sari et al. 2020). According to Verawati et al. (2017), betel chews also benefit dental health. The standard formulation for betel chew in Indonesia consists of two main ingredients: areca nuts and betel leaves. Areca nuts and other ingredients are wrapped and folded in betel leaves during the preparation procedure. Sanskrit literature recorded several additional ingredients for betel chew in Indonesia, including cloves, nutmeg, and cardamom. According to Portal Informasi Indonesia (2019), the development of other ingredients, such as injet (betel lime) and gambir (*Uncaria gambir* (W.Hunter) Roxb.), has become a common additional ingredient used by people in betel chewing activities.

Staphylococcus aureus is a commensal bacterium and is the primary pathogen of various clinical infections, including abscesses and wound infections. It is found in the skin, skin glands, and mucous membranes, mainly in the nasal passages and oral cavity of humans (Brooks et al. 2013). *Staphylococcus* spp. was reported to colonize 90.4% of the mouths of healthy adults, while *S. aureus* was found to colonize about 24% (Jackson 2000). According to Ohara-Nemoto et al. (2008), *S. aureus* found about 33.9% in dental plaque and 46.4% in the saliva of healthy adults. The *S. aureus* in the oral cavity did not directly cause infection and disease. Furthermore, in advantageous

conditions like an open wound, oral hygiene, and an imbalanced oral microbiome, these oral pathogens may develop into an infection or some dental disorder in the oral cavity of humans (Dewhirst et al. 2010). Huang et al. (2017) reported that *S. aureus* was the most frequently isolated from acute dental abscesses in children and considered an essential microorganism in the etiology of infection in the oral cavity of humans (Ibrahim et al. 2021).

In our comprehensive study, we evaluated the antibacterial activity of each plant and the combined extract of betel chew formulations commonly used in Indonesia. This included areca nuts, cardamom seeds, and betel leaves ethanol extract. We aimed to determine the effect of this combined plant extract against *S. aureus*, a model bacterium, providing a detailed understanding of the potential antibacterial properties of these formulations.

MATERIALS AND METHODS

Material

Areca nut, cardamom seeds, and betel leaves were collected from Ponorogo District, East Java, Indonesia. The plant samples were drained at 50°C for 48 h. The dried samples were then powdered and stored at 4°C in the refrigerator. Chlorohexidine gluconate 2%, distilled water (aquadest), Mueller Hinton Agar (MHA) (Merck), Nutrient Agar (NA) (Merck), and technical ethanol (70%) were provided by the laboratory of the Department of Biology, Universitas Sebelas Maret, Indonesia. The bacteria strain *S. aureus* (ATCC 25923) was obtained from the microbiology laboratory, Faculty of Medical, Universitas Sebelas Maret, and stored at 4°C in the refrigerator.

Procedures

Solvent extraction of areca nuts, cardamon seeds, and betel leaves

Areca Nuts (AN), Betel Leaves (BL), and Cardamom Seeds (CS) were subjected to a rigorous process of drying, powdering, and extraction by maceration using a 70% ethanol solvent. A total of 50 g of each plant sample was mixed with 550 mL of 70% ethanol 1:11 (w/v) in a sample bottle (Zhang et al. 2014). The extraction process was carried out for 3 days, during which the extracts were filtered through Whatman No. 1 every 24 hours and re-macerated three times with 70% ethanol. The ethanol solvent was evaporated using a water bath at 50°C until a thick extract was obtained. After that, the thick extract was stored at 8°C in the refrigerator to maintain the integrity of the samples (Zhang et al. 2014).

Medium preparation

First, the NA medium was prepared by measuring 10 mg of agar and dissolved in 100 mL of distilled water (the manufacturer's protocol for solubility was 20 g/L). The MHA was prepared by measuring 17 g of agar and dissolved in 500 mL of aquadest (the manufacturer's protocol for solubility was 34 g/L). The sterilization process of the medium used an autoclave at 121°C for 15 min. After that, warm agar was dispensed in Petri dishes to attain a depth of 4 mm (EUCAST 2022). Then, a petri dish containing MHA medium was incubated for 24 hours at 37°C. The incubation process is to make the surface of the agar dry before use, with no visible drops of water visible on the surface of the agar. This is to prevent excess moisture, which could cause problems with zone edges and haze within zones (EUCAST 2022).

Preparation of bacterial suspension and inoculation of agar plates

The *S. aureus* ATCC 25923 bacteria strain was prepared after 24 h of incubation on a non-selective medium (NA) with an inoculation loop. One inoculation loop of *S. aureus* culture was added to 0.85% NaCl and visually measured with the 0.5 McFarland turbidity standard. The density of 0.5 McFarland corresponded to approximately 1-2×10^8 CFU/mL of bacteria. Next, a sterile cotton swab is immersed into the bacterial suspension, and the excess fluid is removed by turning the swab inside the tube to avoid over-inoculation. The inoculum was spread over the agar surface with manual swabbing in three directions (EUCAST 2022).

Antibacterial activity assessment of single and combination extracts

The antibacterial activity of a single extract of areca seeds, cardamom seeds, and betel leaves against the Gram-positive bacteria *S. aureus* ATCC 25923 was evaluated using the disk diffusion method by EUCAST (EUCAST 2022). Each plant extract was concentrated at 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL, with aquadest used as the diluted solvent. After that, the plant extract was sterilized with a syringe method with a 0.45 μm filter. The 20 μL of sterilized AN, BL, and CS extracts were put into a 6 mm disk and applied on the agar surface after 15 min of bacteria inoculation. The maximum number of disks applied was six on 90 mm plate dishes (EUCAST 2022). After 15 min of disk application, the plate dish was incubated for 24 hours at 37°C. The positive control used was Chlorhexidine gluconate 2%, and aquadest was used as the negative control.

The antibacterial test of the combinations of *Areca catechu* L. nuts, *Piper betle* L. leaves, and *Amomum compactum* Soland. ex Maton seeds ethanolic extracts were evaluated using a modified antibacterial test of herb combination procedure according to Widiyastuti et al. (2012) and Verawati et al. (2017). The ratio concentration of the combination dilution range of 20 mg/mL was used as the final concentration, which was based on the optimum concentration to inhibit *S. aureus* growth in each single extract, as shown in Table 1.

Measurement of inhibition zone

After 24 hours of incubation, an inhibition zone was determined where no *S. aureus* grew by unaided eye when plates were held about 30 cm from the eyes. The inhibition diameter zones were measured with Vernier calipers (mm) from the back of the plate dish with a dark background.

Table 1. Combination treatment was based on the best concentration of *Staphylococcus aureus* ATCC 25923 inhibitions in single-extract testing

Ratio	Mass (g)			
	***A. catechu* nuts (AN)**	***P. betle* leaves (BL)**	***A. compactum* seeds (CS)**	**Final concentration mg/mL**
1:1:1	6.6	6.6	6.6	20
1:1:2	5	5	10	20
1:2:1	5	10	5	20
2:1:1	10	5	5	20

Data analysis

Each antibacterial test was conducted in quintuplicate. The normality of data was carried out by the Shapiro-Wilk test, and the differences of the inhibitory zone diameter were determined by One-Way Analysis of Variant and Duncan multiple range tests at a significant level of $p<0.05$ using SPSS v. 16.0. The combination extract is called a synergy effect if the diameter of the inhibitory zone is significantly greater than the single extract of each plant at 20 mg/mL.

RESULTS AND DISCUSSION

Antibacterial activity of each plant extract against *S. aureus*

This study showed the susceptibility of *S. aureus* ATCC 25923 to the extracts of *A. catechu* nuts (AN), *P. betle* leaves (BL), and *A. compactum* seeds (CS). Zones of inhibition diameter produced by various concentrations of 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL were measured using the disk diffusion method by EUCAST (2022). Table 2 revealed the antibacterial activity of the AN, BL, and CS extracts; as expected, the results greatly varied for each plant extract. As shown in Table 2, the antibacterial activity of the AN increased with concentrations (Figure 1.A). However, there was no significant difference between the concentration of 20 mg/mL and the highest concentration of 25 mg/mL ($p>0.05$), with the inhibition average diameter zone obtained being 8.70 mm and 9.30 mm, respectively. It was also shown that an increase in the concentrations of BL extract led to an increase in inhibition of *S. aureus* growth, as indicated by the formation of a larger inhibitory zone diameter (Table 2 and Figure 1.B). The BL extract at the highest concentration of 25 mg/mL obtained an average inhibitory zone diameter of 7.90±0.379 mm; nevertheless, these results were not significant ($p>0.05$) compared to 20 mg/mL, which obtained an average of 7.50±0.395 mm. Additionally, compared to 10 mg/mL and 15 mg/mL, the inhibition diameter was significantly larger at a concentration of 20 mg/mL. As shown in Table 2, the antibacterial effect of CS also increased with concentration, as indicated by the larger formation of the inhibitory zone (Figure 1.C). It was shown that CS extracts at 25 mg/mL obtained an average inhibitory zone diameter of 7.75±0.395 mm, and at 20 mg/mL obtained an average of 7.35±0.395 mm; nevertheless, these results were not significant ($p>0.05$). Statistically, a concentration of 20 mg/mL of AN, BL, and CS extracts was obtained as the optimal concentration for inhibiting the growth of *S. aureus*. Furthermore, we found that AN extract was more effective against *S. aureus*, as indicated by the greater inhibition zone diameter than BL and CS extracts at the same concentration. The inhibitory diameter zones produced by the positive control (chlorhexidine gluconate 2%) (23.40 mm) were significantly greater than the plant extracts.

Antibacterial activity of the combined extract against *S. aureus*

The inhibitory zone diameter obtained from the combined extract ranged from 9.00 mm to 9.90 mm against *S. aureus*, as shown in Table 3 and Figure 2. The 2:1:1 ratio (9.90±0.675 mm), dominated by a high concentration of AN extract, exhibited the largest diameter of the inhibitory zone, which was significant at a p level<0.05 compared to the 1:1:2 (9.00±0.586 mm), 1:2:1 (9.40±0.720 mm) ratios, and the three (single) extracts at the same concentration (20 mg/mL). However, the combination ratios 1:1:2 and 1:2:1 also demonstrated good activity when combined, compared to weaker effects when BL and CS extracts were tested independently (Tables 3 and 4). The key finding of this study is the comparison between the combined and single extracts, which is detailed in the Duncan analysis at a p-level of 0.05, shown in Table 3.

Table 2. Antibacterial activity of various concentrations of areca seed, betel leaf, and cardamon seed ethanol extracts against *Staphylococcus aureus* ATCC 25923

Extracts	Concentration (mg/mL)	Inhibitory zone (mm) ± SD
Aquadest (control -)		6.00±0.000[a]
Areca nuts	10	6.70±0.410[a]
Areca nuts	15	7.50±0.353[b]
Areca nuts	20	8.70±0.570[c]
Areca nuts	25	9.30±0.570[c]
Chlorhexidine gluconate 2% (control +)		22.40±0.894[d]
Aquadest (control -)		6.00±0.000[a]
Betel leaves	10	6.45±0.209[a]
Betel leaves	15	6.70±0.209[a]
Betel leaves	20	7.50±0.395[b]
Betel leaves	25	7.90±0.379[b]
Chlorhexidine gluconate 2% (control +)		23.40±1.140[c]
Aquadest (control -)		6.00±0.000[a]
Cardamon seeds	10	6.30±0.273[a]
Cardamon seeds	15	6.60 ±0.285[a]
Cardamon seeds	20	7.35±0.379[b]
Cardamon seeds	25	7.75±0.395[b]
Chlorhexidine gluconate 2% (control +)		23.35±0.335[c]

Note: a, b, c, d: The mean that had a common alphabet in a row are not significantly different at the level of $p<0.05$

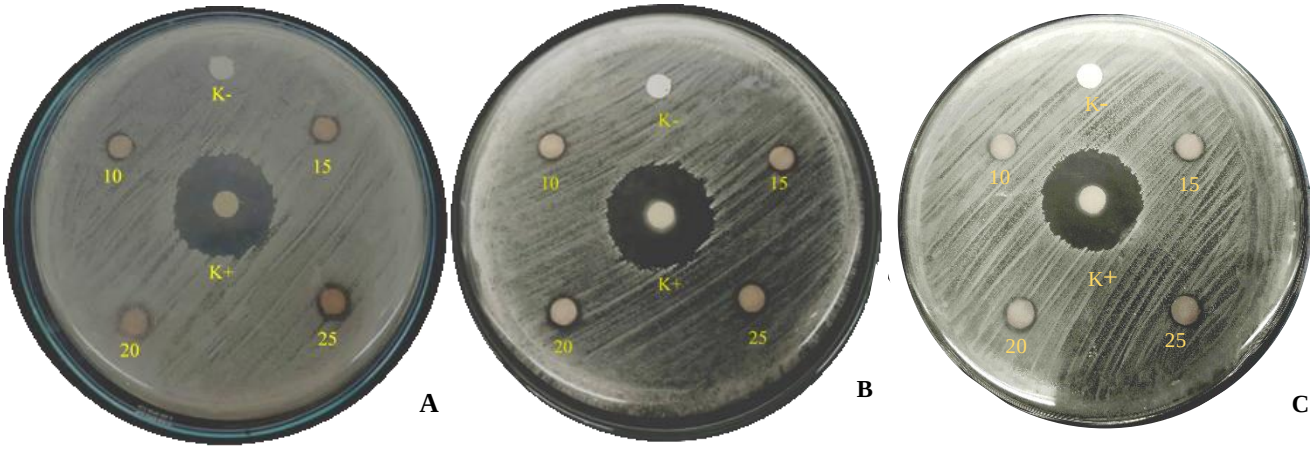

Figure 1. Antibacterial activity of various concentrations of single ethanol extract of A. *Areca catechu*, B. *Piper betle*, and C. *Amomum compactum*. Note: K-: Aquadest; K+: Chlorhexidine gluconate 2%; 10, 15, 20, 25 mg/mL concentration of each extract

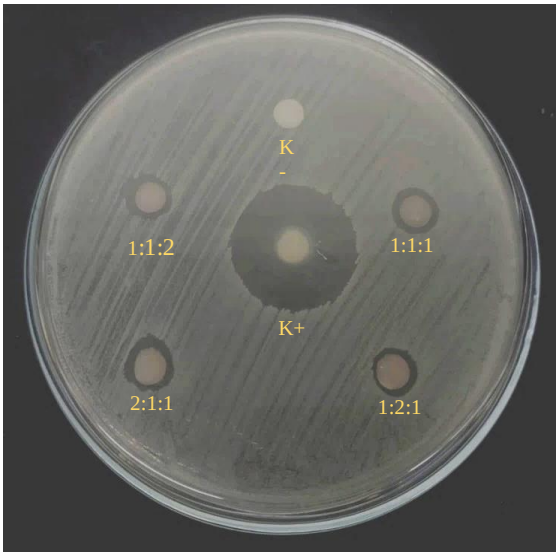

Figure 2. Antibacterial activity of various ratios of combination ethanol extract of *A. catechu*, *P. betle*, and *A. compactum*. Note: K-: Aquadest; K+: Chlorhexidine gluconate 2 %; 1:1:2 (5 mg AN, 5 mg BL, 10 mg CS); 1:1:1 (6.67 mg AN, 6.67 mg BL, 6.67 mg CS); 2:1:1 (10 mg AN, 5 mg BL, 5 mg CS); 1:2:1 (5 mg AN, 10 mg BL, 5 mg CS)

The comparison of inhibitory zone diameters between various ratio combinations can be seen in Figure 2. Based on statistical analysis, it was found that combining the three ethanol extracts at 1:1:1 and 2:1:1 ratios will result in a much larger inhibitory zone diameter than the optimal concentration of each extract, as shown in Table 3. It seems that the combination of AN, BL, and CS had a significant effect on the inhibition zone diameter against *S. aureus* compared to the respective single extract tests, so it can be said that the combination treatment of the three extracts also shows an effect, which is synergistic.

Discussion

The development of a clear inhibition zone in the petri dish assessed the antibacterial activity. The active compounds of three plant extracts, which were used to inhibit the growth of the tested bacteria, are detailed in Table 4. The results of our study show that the inhibition zone of *S. aureus* increased with the concentration of each plant extract. In terms of single extracts, the AN extract demonstrated the highest antibacterial properties against *S. aureus*, outperforming BL and CS (Table 2). The combination of the three extracts, with the AN extract dominating the proportion (2:1:1), exhibited significantly better antibacterial activity ($p<0.05$) against *S. aureus*, indicating a synergistic effect (Table 3; Figure 2).

Table 3. Antibacterial activity of various ratios of combination extract and effective concentration of single extract against *Staphylococcus aureus*

Extracts	Ratio	Concentration (mg/mL)	Inhibitory zone (mm) ± SD
Cardamon seed (CS)	Single extract	20	7.35 ± 0.379^{a}
Betel Leaves (BL)	Single extract	20	7.45 ± 0.480^{a}
Areca Nuts (AN)	Single extract	20	8.70 ± 0.570^{b}
Areca nuts: Betel leaves: Cardamon seeds	1:1:2 (combined)	20	9.00 ± 0.586^{bc}
Areca nuts: Betel leaves: Cardamon seeds	1:2:1 (combined)	20	9.40 ± 0.720^{bc}
Areca nuts: Betel leaves: Cardamon seeds	1:1:1 (combined)	20	9.60 ± 0.379^{cd}
Areca nuts: Betel leaves: Cardamon seeds	2:1:1 (combined)	20	9.90 ± 0.675^{d}

Note: a, b, c, d: The mean that had a common alphabet in a row are not significantly different at the level of $p<0.05$

Table 4. Major phytochemical content of areca nuts, betel leaves, and cardamom seeds with reference

Materials	Major phytochemical content				
	Alkaloid	**Phenolic**	**Tannin**	**Flavonoid**	**Reference**
A. catechu nuts	Arecoline Arecaidine Guvacolin Guvacine		Catechin Epicatechin		Gupta et al. (2020); Sari et al. (2020); Wang et al. (2021); Hugar et al. (2024)
P. betle leaves		Allylpyrocatechol (hydroxychavicol) Eugenol 4-chromanol			Muruganandam et al. (2017); Almasyhuri and Sundari (2019); Nayaka et al. (2021)
A. compactum seeds		Kaempferol 2,2'-metilen bis[6-(1,1-dimetiletil)-4-etil]		Quercetin Hesperetin	Sukandar et al. (2015); Dinata et al. (2021); Cai et al. (2021); Nurcholis et al. (2021, 2022)

Combinations of different medicinal plants in herbal formulations have become common in some traditional medicine systems. According to Ncube et al. (2012), plant extract combinations may offer potential prospects for treating diseases caused by bacteria in conventional medicine. However, Yang et al. (2014) explained that the primary interaction mechanism between plant extract combinations is that the active compounds with different or exact targeting will interact synergistically or antagonistically. The synergistic antibacterial potential is defined as the combinations of plant extracts that strengthen the antibacterial activity of each other, making combined extracts more efficient compared to single extracts (Hussain et al. 2024). In this study, the 2:1:1 ratio's combination of 10 mg AN, 5 mg BL, and 5 mg CS extracts showed a synergistic effect, increasing the diameter of the inhibitory zone against *S. aureus.* While the other ratios of combination 1:2:1 (5 mg AN, 10 mg BL, and 5 mg CS) and 1:1:2 (5 mg AN, 5 mg BL, and 10 mg CS) did not significantly increase compared to 20 mg/mL AN single extract, we suspected that AN ethanolic extract was the most effective plant extract among betel chew formulations against *S. aureus* because the diameter of the inhibitory zone increased when using the high concentration of AN ethanolic extract. Gharbani et al. (2023) also reported that the ethanol extract of AN produced a greater inhibitory zone diameter against *S. aureus* with an increase in concentration. The phytochemical content of AN ethanolic extract may have played the most crucial role in the antibacterial activity against *S. aureus*.

Ethanol extracts of AN, BL, and CS found that all three plants were able to inhibit *S. aureus* bacteria at the lowest test concentration of 10 mg/mL, with areca nut seeds producing a significantly larger average diameter of the inhibition zone compared to BL and CS. According to Hussain et al. (2024), the antibacterial potencies between plant extracts vary based on the phytochemical composition of each plant extract. Previous research by Xin et al. (2021) shows that the areca nut seeds ethanolic extract is potent in inhibiting *S. aureus*. This shows the possibility that the phytochemical content of AN extract is very effective in inhibiting *S. aureus*. The content of active compounds in AN, BL, and CS is briefly summarized in Table 4.

Xin et al. (2021) reported that AN ethanolic extract showed potent antibacterial activity against *S. aureus* strains Methicillin-Sensitive *S. aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA) with minimum Inhibitory Concentration (MIC) at 0.4 mg/mL. Rialita et al. (2019) suggested that the phenolic compound was related to the bacterial activity of the plant extract. Wang et al. (2021) also confirmed that the AN ethanolic extract has a high content of phenolic compounds and showed strong antibacterial activity, especially against Gram-positive bacteria. According to some previous studies, AN ethanolic extract contains alkaloids and catechin at high concentrations, especially in unripe conditions (Sari et al. 2017; Wu et al. 2019; Chen et al. 2021). Another report also showed that catechin and epicatechin were the main compounds in the unripe areca nuts (Sari et al. 2020; Hugar et al. 2024). Catechin was reported to be an effective compound against Gram-positive bacteria such as *Propionibacterium acnes*, *S. epidermidis*, and *S. aureus* (Verawati et al. 2017; Alkufeidy et al. 2024). The mechanism of action of catechin disturbed the cell walls' integrity, increasing cell membrane permeability. In Gram-positive bacteria lacking an outer membrane and thicker cell walls, catechin will mediate the release of lipoteichoic acid from the bacterial wall, weakening the bacterial cell wall (Wu and Brown 2021). Furthermore, arecoline is the primary alkaloid compound isolated from AN, and the concentration of arecoline is estimated to be around 0.30-0.60% in fresh AN (Wu et al. 2019). According to Liu et al. (2016), arecoline has various activities, such as antimicrobial, antiparasitic, and pharmacological effects on the cardiovascular, digestive, and nervous systems. Luo et al. (2010) also confirmed that arecoline had antibacterial activity against *Bacillus proteus* and *B. anthracis* with an MIC value of 0.8 mg/mL. Still, the mechanism of action needs to be better understood.

In the current report by El-Sawy et al. (2024), combinations of ethanolic extracts of cinnamon bark, chamomile flowers, marigold flowers, and sage leaves were more effective against *S. aureus* than each single extract. A previous study by Ncube et al. (2012) also showed that a combination ratio of 1:1 of phenolic and saponin-rich *Hypoxis hemerocallis* corn and leaf extracts

resulted in a synergistic interaction effect against *S. aureus*. Gharbani et al. (2023) also reported that a combination ratio of 1:1 of *A. catechu* and *Punica granatum* L. ethanol extracts had a synergistic effect against *S. aureus*. It has also been reported by Gharbani et al. (2023) that polar and non-polar compounds present in both extracts strengthen each other through a synergistic effect. Jeong et al. (2023) also reported that the combination of *Sanguisorba officinalis* L. and *U. gambir* extracts has shown synergism against MRSA, as well as enhanced antibacterial inhibition with bactericidal effects. According to Hemeg et al. (2020), the potent antibacterial activity of extracts is affected by the bioactive compound, its concentration, and the possibility of interaction with another compound. Combining different extracts can cause different interactions between compounds because of the various compounds contained in the extracts. According to Vaou et al. (2022), in the combination of medicinal plants, there is a unique synergistic interaction between bioactive compounds; for example, phenolic compounds contained in an extract will function to damage the cytoplasmic membrane, causing loss of bacterial cell integrity. This will potentially bring in other compounds that have a mechanism of action by directing the cells, such as targeting DNA replication.

In conclusion, our research demonstrated that the inhibitory zone diameter affected by various ratio combinations of *A. catechu* nuts (AN), *P. betle* leaves (BL), and *A. compactum* seeds (CS) ranged from indifference to synergism compared to each plant extract against *S. aureus* ATCC 25923. Previous studies have explored the antibacterial properties of AN, BL, and CS ethanol extracts and identified active compounds, including alkaloids, phenolics, and tannins. Therefore, the proper antibacterial and toxicity test methods for combinations of extracts of AN, BL, and CS should be optimized in future studies.

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Bioactive compounds of seven seagrass species from the Western Indian Ocean identified by Gas Chromatography-Mass Spectrometry

PHILIPO Y. MAGANGA[1], LUCY D. MBUSI[2], MARIAM I. HAMISI[3], CLARENCE A. MGINA[1], THOMAS J. LYIMO[2,♥]

[1]Department of Chemistry, College of Natural and Applied Science, University of Dar es Salaam. P.O. Box 35179, Dar es Salaam, Tanzania
[2]Department of Molecular Biology and Biotechnology, College of Natural and Applied Science, University of Dar es Salaam. P.O. Box 35179, Dar es Salaam, Tanzania. Tel.: +255-754-375-924, ♥email: tjlyimo@udsm.ac.tz; tlyimo2000@yahoo.com
[3]Department of Crop Science and Horticulture, Sokoine University of Agriculture. P.O. Box 3005, Morogoro, Tanzania

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Abstract. *Maganga PY, Mbusi LD, Hamisi MI, Mgina CA, Lyimo TJ. 2024. Bioactive compounds of seven seagrass species from the Western Indian Ocean identified by Gas Chromatography-Mass Spectrometry. Asian J Nat Prod Biochem 22: 74-88.* Seven seagrass species from the coast of Tanzania, namely *Cymodocea serrulata, Halodule uninervis, Enhalus acoroides, Cymodocea rotundata, Syringodium isoetifolium, Thalassia hemprichii,* and *Thalassodendron ciliatum,* were previously reported to have antibacterial activity against pathogenic microorganisms. This study presents the results of quantitative analysis of the phytocompound composition of hexane extracts of these seagrass species using Gas Chromatography-Mass Spectrometry technique. Overall, 24 known biologically active phytocompounds were revealed, with commonly found compounds being steroids, namely stigmasta-5,22-dien-3-ol acetate and 3*β* sitosterol acetate. On the other hand, 3*β*-cholest-5-en-3-ol tetradecanoate was found only in *C. rotundata* and *T. ciliatum.* Hydrocarbons (1-nonadecene and tetracosane) and a diterpene (phytol acetate) were found only in *H. uninervis* and *T. ciliatum.* In addition, pentadecanal and n-hexadecanoic acid were found in *C. rotundata* and *T. hemprichii,* respectively, while heneicosane and hexadecanoic acid methyl ester were found in *T. ciliatum* only. Moreover, *T. ciliatum* was found to contain more compounds than other seagrass species. This study reports for the first time the occurrence of fourteen compounds from seagrass species. All seven seagrass species are rich sources of phytocompounds with various pharmacological properties, such as antimicrobial, antioxidant, and antiinflammatory activities.

Keywords: Biological activities, Gas Chromatography-Mass Spectrometry, hexane extracts, phytocompounds, seagrass roots and leaves

INTRODUCTION

Seagrasses are plants that live submerged in shallow water areas of temperate, subtropical, and tropical seas **(**Kannan and Thangaradjou 2005; Togashi et al. 2007). They are monocotyledonous plants belonging to four plant families, Posidoniaceae, Zosteraceae, Hydrocharitaceae, and Cymodoceaceae, which have successfully adapted to the marine ecosystems (Ravikumar et al. 2010). There are 60 seagrass species belonging to 13 genera distributed all over the world (Gacia et al. 2003). Seven genera (*Cymodocea*, *Enhalus*, *Halodule*, *Halophila*, *Syringodium*, *Thalassia,* and *Thalassodendron*) are tropical. The remaining six genera (*Amphibolis*, *Heterzostera*, *Phyllospadi*, *Posidonia*, *Pseudalthenia,* and *Zostera*) are largely confined to temperate waters (Ravikumar et al. 2010). The coastal zones of the Western Indian Ocean (WIO) region harbor 14 known species that comprise about 25% of all seagrasses worldwide (Amone-Mabuto et al. 2017). The species occurring in the WIO region are *Enhalus acoroides* (U.) Royle, *Halophila minor* (Zoll.) den Hartog, *H. ovalis* (R. Br.) Hook. f., *H. stipulacee* (Forsk.) Aschers., *Thalassia hemprichii* (Ehrenberg) Asherson, *Zostera capensis* Setchell, *Cymodocea rotundata* Ehrenb. et Hempr. ex Aschers., *C. serrulata* (R. Br.) Aschers. et Magnus, *Halodule uninervis* (Forsk.) Aschers. in Bossier, *H. wrightii* Ascherson, *Syringodium isoetifolium* (Ascherson) Dandy, *Thalassodendron ciliatum* (Forsk.) den Hartog., *T. leptocaule* MC Duarte, Bandeira & Romeiras, and *Ruppia maritina* L. (Gullström et al. 2002; Duarte et al. 2012). The distribution of seagrasses depends on a series of physical factors such as temperature, turbidity, salinity, substrate type, and light availability, where the presence or absence of species depends on their unique adaptation and ability to tolerate those environmental factors (Ha et al. 2019). Twelve seagrass species have been encountered in Tanzania, with the most extensive meadows found on beaches or cliffs and adjacent fringing reefs (Ochieng and Eftemeijer 2003; Hamisi et al. 2004).

Seagrasses are rich sources of secondary metabolites that are believed to be a defense mechanism for these plants, some of which are potential antimicrobial compounds that reduce or control microbial growth (Kannan et al. 2010a; Sangeetha and Asokan 2016; Hamisi et al. 2023). Therefore, recognizing seagrass's potential, several endeavors have been made globally from which various secondary metabolites with pharmacological properties have been extracted and tested. Gono et al. (2022) reported the antiviral, antibacterial, antifungal, antiprotozoal, antifertility, and pharmacological properties of seagrass extracts. Furthermore, seagrass extracts of *H. pinifolia*, *H. ovalis*, and *T. hemprichii* from the southeast coast of India were reported to have antiviral activity (Premanathan et al. 1992). In addition, Kannan et al. (2010a) reported the

antibacterial activity of seagrasses *H. stipulacea*, *C. serrulata*, and *H. pinifolia* from the Mandapam coast, India. Moreover, Saranya et al. (2017) reported that the seagrasses *H. ovalis* and *T. hemprichii* collected from the Keelakarai Coast, Ramnad, Tamil Nadu, have interesting biochemical and bioactive potentials.

In Tanzania, seagrasses have been used as traditional medicine. These include *E. acoroides*, whose roots are popularly used as remedial against stings of rays, muscle pain, wounds, and stomach problems. When burned with other herbs, this species produces smoke (mafusho), which a patient inhales as vapors to cure fever by lowering body temperature. Another species, *Cymodocea* spp., is utilized to combat malaria and cough (De La Torre-Castro and Rönnbäck 2004). *T. ciliatum* is known for its effectiveness in treating various ailments (Abdelhameed et al. 2018). Furthermore, combinations of seagrasses, including *Thalassia* and *Cymodocea*, are recognized for their use in treating fever and skin diseases. In a previous study, Hamisi et al. (2023) reported that extracts from seven common seagrass species—*C. serrulata*, *H. uninervis*, *E. acoroides*, *C. rotundata*, *S. isoetifolium*, *T. hemprichii*, and *T. ciliatum*—exhibited potential antibacterial activities. In this study, we further analyzed the phytocompound composition of these seagrasses using Gas Chromatography-Mass Spectrometry to complement the existing knowledge and provide scientific evidence for their potential in pharmaceutical development.

MATERIALS AND METHODS

Seagrasses collection

Seven seagrass species, namely *C. serrulata*, *H. uninervis*, *E. acoroides*, *C. rotundata*, *S. isoetifolium*, *T. hemprichii*, and *T. ciliatum*, were collected during low tides from the coastal areas of Mjimwema (06°50'S, 39°21'E), 4 km south of the Dar es Salaam harbor, and from Bagamoyo (6°27'32"S, 38°56'E) between the Bagamoyo fish landing site and Kaole ruins (Figure 1). Seagrasses were identified in the field and the laboratory using standard identification guidebooks (Oliveira et al. 2005; Richmond 2011). Sampling was done by uprooting seagrasses using a shovel. The seagrass leaves (phyllosphere) and the below-ground parts (roots/rhizome) were separated using scissors and transported to the University of Dar es Salaam, Department of Molecular Biology and Biotechnology laboratories for processing and analysis.

Preparation of seagrasses for analysis

In the laboratory, the collected seagrass samples were washed twice to three times with fresh water to remove debris. Both leaves and roots were air dried under shade for 10 to 14 days to a constant weight (Figure 2) and then mechanically pulverized to obtain a fine powder using a Thomas Wiley Laboratory mill model 4 (Philadelphia, USA).

Preparation of extracts

Approximately 100 g of the powdered sample of each species was soaked in 500 mL of *n*-hexane at room temperature in a shaker (Edmund Buhler 7400) for 48 hours. The filtrates were then subjected to a rotary evaporator (BUCHI rotary vapor model R-210) to evaporate the solvent and get the crude extract (Kannan et al. 2010b). Table 1 displays the quantity of extracts obtained from each seagrass species following solvent extraction. The extracts were stored at 4°C before analysis using Gas Chromatography-Mass Spectrometry (GC-MS).

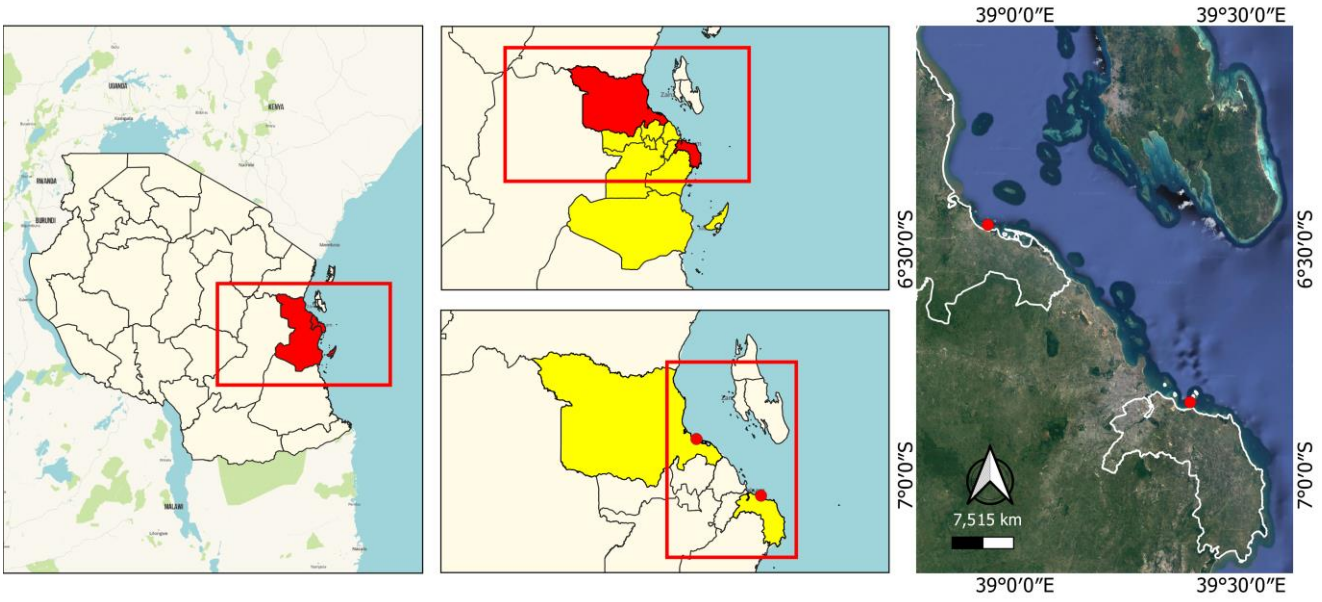


Figure 1. A map showing sampling stations at Mjimwema and Bagamoyo, Tanzania

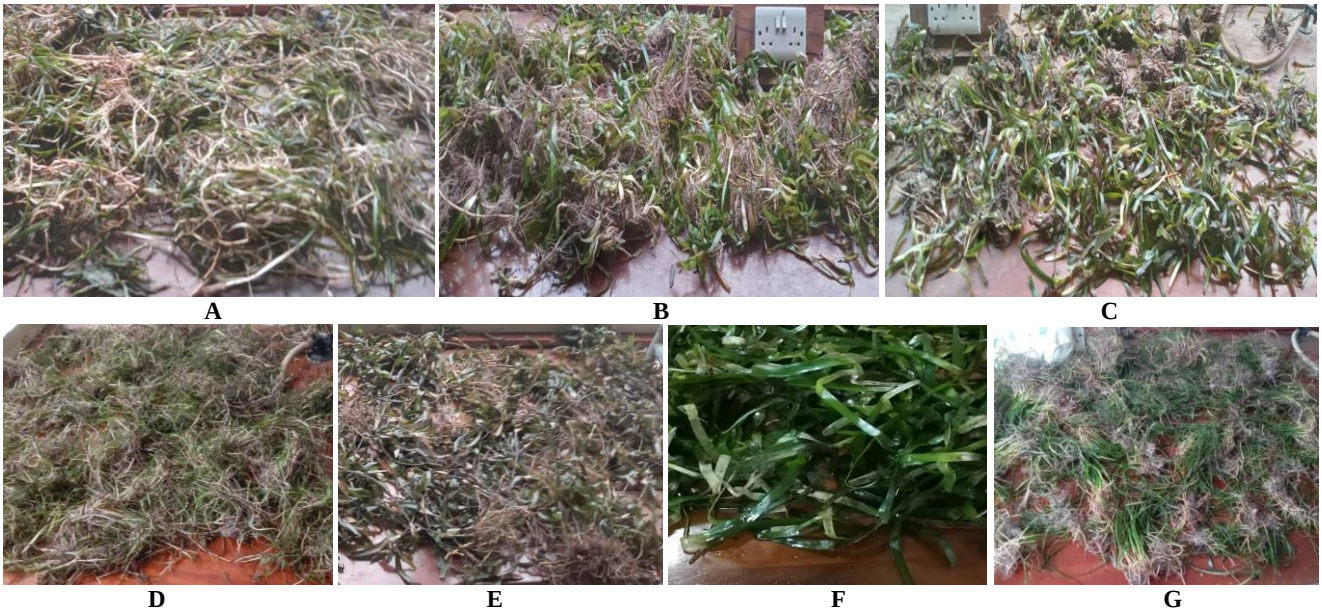


Figure 2. Photographs showing the seven seagrass species on laboratory benches to attain a constant weight: A. *C. rotundata*, B. *C. serrulata*, C. *E. acoroides*, D. *H. uninervis*, E. *T. ciliatum*, F. *T. hemprichii* and, G. *S. isoetifolium*

Table 1. Amount of extracts yield for each seagrass species

Seagrass species	Leaves /roots	Amount of powder (g)	Amount of solvent (ml)	Extracts yield (g)
C. rotundata	Leaves	100	500	0.513
	Roots	100	500	0.274
C. serrulata	Leaves	100	500	0.541
	Roots	100	500	0.523
H. uninervis	Leaves	100	500	0.561
	Roots	NA	NA	NA
T. hemprichii	Leaves	100	500	0.830
	Roots	100	500	0.235
T. ciliatum	Leaves	100	500	0.377
	Roots	100	500	0.226
S. isoetifolium	Leaves	100	500	0.605
	Roots	100	500	0.360
E. acoroides	Leaves	NA	NA	NA
	Roots	100	500	0.1

Note: NA: Not Analysed

Gas Chromatography-Mass Spectrometry analysis

Analysis of the extracts was done by GC-MS, whereby 1 µL of the crude extract in 1 mL of dichloromethane was injected into the instrument. The peaks were recorded in a GCMS-QP 2010 Ultra (Shimadzu instrument) operating in electron ionization (EI) mode (MS) at 70 eV and a flame ionization detector (FID) for GC. A Restek-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The oven temperature program was 90°C to 280°C and was held at 90°C for 2 minutes. The temperature was increased to 280°C for 8 minutes (hold time) at 6°C per minute. The injection temperature was 250°C with split injection mode. Helium was used as carrier gas at a flow rate of 1.21 mL min^{-1}. The ion source temperature and interface temperature in MS were 230°C and 300°C, respectively. The identification of compounds in the sample was performed via the scan method, which involved the use of the Mass Spectral Library & Search Software (NIST 11). The quantification of the compounds in the samples was then performed based on percentage composition using the peak integration method, whereby the ion allowance was 20% (Elkhateeb et al. 2019).

RESULTS AND DISCUSSION

GC-MS revealed biologically active phytocompounds

GC-MS is often referred to as the "gold standard" for analytical substance identification because it is highly effective for specific test analysis (Perez et al. 2016). GC-MS can be used to identify various substances within a test sample, including hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino and nitro compounds, and can be used to identify trace amounts of materials (Saravanan et al. 2014). This study revealed the presence of 24 different biologically active phytocompounds from the hexane extracts of the seven seagrass species, as shown in Table 2 and Supplementary Material Figures S.1-12. Furthermore, the most common phytocompounds observed in all seagrass species, except for *S. isoetifolium*, were stigmasta-5,22-dien-3-ol acetate and 3-β, β-sitosterol acetate. The abundance of stigmasta-5,22-dien-3*β*-ol acetate ranged from 5.01 to 62.4% and 1.08 to 22.3% for the seagrass leaves and root extracts, respectively. The 3*β* sitosterol acetate abundance ranged from 2.69 to 11.6% and 0.88 to 16.7% for the leaf and root extracts, respectively. These results are similar to other findings that have reported the same compounds in various seagrass species, including *Cymodocea serrulata* (Pushpabharathi et al. 2018). Stigmasta-5,22-dien-3*β*-ol acetate and 3*β*-sitosterol acetate

have been reported to possess various bioactive properties, as summarized in Table 3. The presence of these compounds may, therefore, explain and support the local use of the seagrass species in the WIO region for various treatments. Analysis of the results from different seagrass species revealed that they contain different phytocompounds with various compositions, as presented in the following sections.

Phytocompounds from *Cymodocea serrulata* extracts

Eight (8) compounds were identified in both the leaves and roots of seagrass *C. serrulata* extracts. The prevailing compounds in the leaves were stigmasta-5,22-dien-3*β*-ol, acetate (62.4%), 6,10,14-trimethyl-2-pentadecanone (13.0%), and 3*β*-sitosterol acetate (11.6%), whereas cyclic octaatomic sulfur (65.3%), heptadecane (12.0%) and hexadecane (10.2%) were found in the roots (Table 2). Among these compounds, stigmasta-5,22-dien-3*β*-ol, acetate, 2-pentadecanone, and 6,10,14-trimethyl-, have been reported on the same seagrass from India by Pushpabharathi et al. (2018). However, Pushpabharathi et al. (2018) reported five compounds using ethanol extracts while Das et al. (2023) reported the compounds hexadecane and heptadecane among other compounds from the same seagrass that revealed a total of 104 phytocompounds from chloroform, ethanol, and distilled water from *C. serrulata* samples from Palk Bay, India. Moreover, other researchers have detected several compounds from this seagrass species, but none were similar to the compounds extracted from this study. For example, Vijayalingam and Rajesh (2019) revealed nine compounds using ethanol extracts, and Jeevith et al. (2019) detected 26 compounds from the same seagrass using methanol extracts. Thus, to our knowledge, in this study, six compounds from seagrass *C. serrulata* extracts have been identified for the first time.

Phytocompounds from *Cymodocea rotundata* extracts

The extracts of the seagrass *C. rotundata* were found to contain 10 phytocompounds from both the leaves and roots. The main chemical components in the leaves were stigmasta-5,22-dien-3*β*-ol acetate (41.9%), pentadecanal (28.7%), and 6,10,14-trimethyl-2-pentadecanone (16.0%), whereas in the roots, the main chemical compounds were cyclic octaatomic sulfur (56.0%), heptadecane (15.6%), and hexadecane (13.4%). The steroid cholest-5-en-3-ol (3*β*)-, tetradecanoate was uniquely obtained from this seagrass. None of the revealed phytocompounds match the compounds reported by Perez et al. (2018), who used dichloromethane extract on the same seagrass. Hence, the current study has identified ten compounds previously unreported from *C. rotundata*.

Phytocompounds from *Halodule uninervis* extracts

In the seagrass, *H. uninervis* extracts, 13 phytocompounds were found in leaves, as shown in Table 1. The significant phytocompounds detected in *H. uninervis* were tetracosane (33.4%), 1-nonadecene (13.1%), and *β*4-sitosterol-3-one (15.5%). Interestingly, among the studied species, steroid compounds *β*4-sitosterol-3-one and cholestadien-3-one were detected only in *H. uninervis* (Table 2). However, the revealed phytocompounds have never been detected in this seagrass species. Using ethyl acetate, Parthasarathi et al. (2021) reported 23 phytocompounds from the same seagrass *H. uninervis* extracts, but none of the compounds identified matched those releaved in this study.

Phytocompounds from *Enhalus acoroides* extracts

The GC-MS analysis of *E. acoroides* revealed only four compounds. The major phytocompounds identified from this seagrass were cyclic octaatomic sulfur (97.4%), followed by stigmasta-5,22-dien-3*β*-ol acetate (1.08%). Out of the four compounds, cyclic octaatomic sulfur has previously been reported from the same species by Selvam et al. (2022). The study by Amudha et al. (2018) revealed 29 phytocompounds from ethyl acetate crude extracts of this seagrass species from Devipattinam, Ramanathapuram, India. Another study by Vijayalingam and Rajesh (2019) reported 10 phytocompounds from ethanol extracts from the same seagrass species found in India. In both cases, none of the reported compounds were the same as these hereby reported.

Phytocompounds from *Syringodium isoetifolium* extracts

The seagrass *S. isoetifolium* extracts were found to contain six phytocompounds in both the leaves and roots. The major phytocompounds in the leaves were 2,4-di-tert-butylphenol (30.9%), 1-pentadecene (17.1%), and 1-tridecene (16.0%), while in the roots the notable compounds were 2,4-di-tert-butylphenol (30.9%), 1-pentadecene (18.2%), and 1-tridecene (16.6%). All six revealed phytocompounds have not been reported from this species elsewhere. The study by Vijayalingam and Rajesh (2019) revealed eight compounds from ethanol of the same species collected from Seeniyappa Dharka, India, while that conducted by Jeevitha et al. (2019) reported 25 compounds from methanol of the species obtained from the Gulf of Mannar, Tamil Nad. However, none of these previous studies reported the same compounds obtained in this present study.

Phytocompounds from *Thalassia hemprichii* extracts

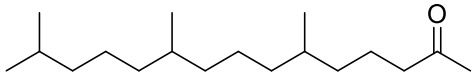
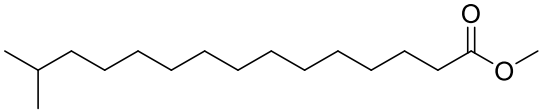
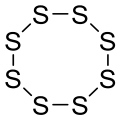
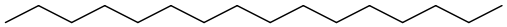
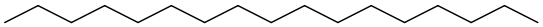
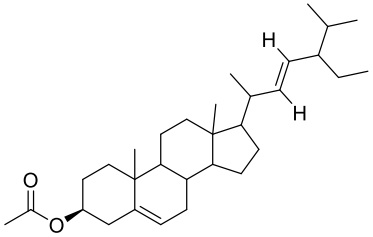
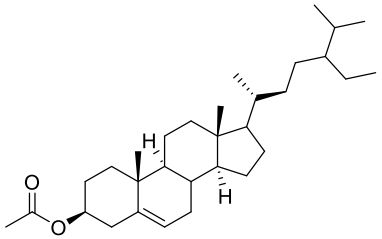
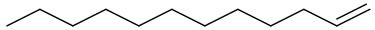
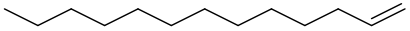
Nine phytocompounds were detected in the leaves and roots of the seagrass *T. hemprichii* hexane extracts. The significant phytocompounds from the leaves were cyclic octaatomic sulfur (54.0%), stigmasta-5,22-dien-3β-ol acetate (10.6%), and *n*-hexadecanoic acid (8.7%). In comparison, in the roots, the significant phytocompounds were cyclic octa atomic sulfur (63.5%), 1-pentadecene (6.86%), and 3*β*-sitosterol acetate (6.19%). Furthermore, the fatty acid (*n*-hexadecanoic acid) was found only in *T. hemprichii*. None of the phytocompounds hereby revealed have been reported from the same seagrass species growing elsewhere. For example, Hassan et al. (2022) analyzed *T. hemprichii* collected from the Red Sea, Egypt and reported the existence of nine phytocompounds from hexane extract, but none of the compounds are similar to those found in this study.

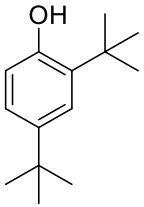
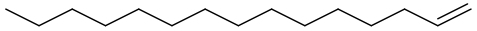
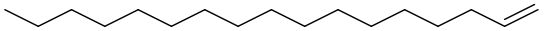
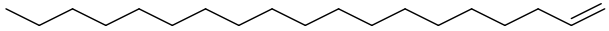
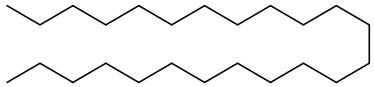
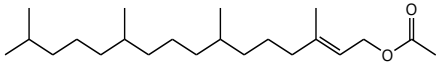
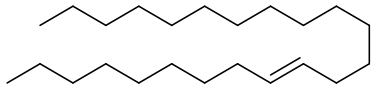
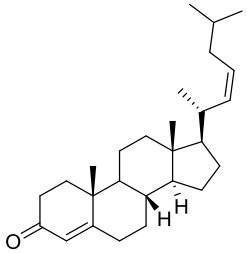
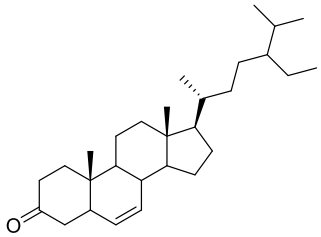
Table 2. Phytocompounds identified in hexane extracts of seven seagrass species and their relative abundance

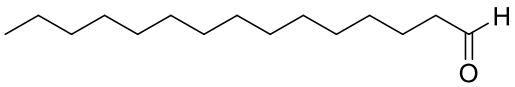
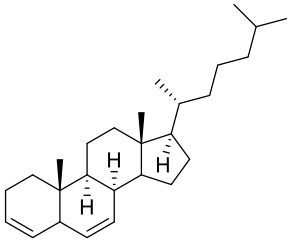
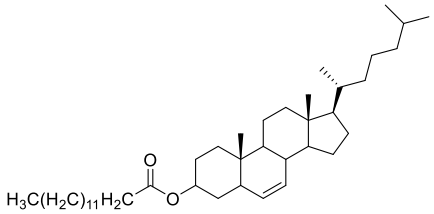
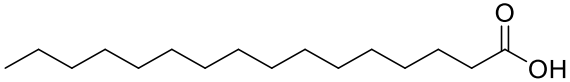
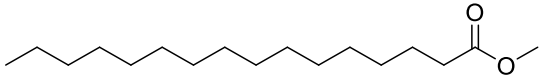
SN	RT	Name of the compound	Formula	MW	Peak Area %													
					CS		HU		EA		CR		SI		TH		TC	
					L	R	L	R	L	R	L	R	L	R	L	R	L	R
1	16.76	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268.0	13.0	0.85	-	-	NA	-	16.0	0.46	-	-	-	-	2.67	-
2	18.12	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.0	6.91	3.16	-	-	-	-	4.76	2.14	-	-	-	-	-	0.33
3	19.52	Cyclic octaatomic sulfur	S_8	256.5	-	65.3	-	-	NA	97.4	-	56.0	-	-	53.97	63.50	-	55.4
4	20.82	Hexadecane	$C_{16}H_{36}$	226.4	-	10.2	-	-	NA	-	-	13.4	-	-	-	-	-	0.74
5	23.69	Heptadecane	$C_{17}H_{36}$	240.5	-	12.0	-	-	NA	0.62	-	15.6	-	-	-	-	-	0.16
6	35.03	Stigmasta-5,22-dien-3β-ol, acetate	$C_{31}H_{50}O_2$	454.0	62.4	6.92	5.01	NA	NA	1.08	41.9	5.75	-	-	10.95	6.19	-	22.3
7	35.79	3β-Sitosterol acetate	$C_{31}H_{52}O_2$	456.7	11.6	1.57	3.18	NA	NA	0.88	7.17	5.81	-	-	2.69	3.88	-	16.7
8	4.58	1-Dodecene	$C_{12}H_{24}$	168.3	-	-	0.69	NA		-	-	-	10.8	10.5	4.44	4.08	2.37	-
9	8.37	1-Tridecene	$C_{13}H_{26}$	162.2	-	-	1.17	NA	-	-	-	-	16.0	16.6	5.43	6.10	3.22	-
10	10.78	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.3	-	-	1.32	NA	-	-	-	-	35.2	30.9	-	-		
11	12.27	1-Pentadecene	$C_{15}H_{30}$	210.4	-	-	1.43	NA	-	-	-	-	17.1	18.2	6.58	6.86	3.57	-
12	15.89	1-Heptadecene	$C_{17}H_{34}$	238.5	-	-	2.12	NA	-	-	-	-	12.2	14.7	5.34	5.66	3.26	-
13	19.18	1-Nonadecene	$C_{19}H_{38}$	266.5	-	-	13.1	-	-	-	-	-	-	-	-	-	2.87	-
14	20.81	Tetracosane	$C_{24}H_5O$	338.7	-	-	33.4	-	-	-	-	-	-	-	-	-	14.9	-
15	21.01	Phytol, acetate	$C_{22}H_{42}O_2$	256.5	-	-	12.0	-	-	-	-	-	-	-	-	-	17.6	-
16	22.19	(*Z*)-9-Tricosene	$C_{23}H_{46}$	322.6	-	-	3.60	-	-	-	-	-	8.72	9.09	-	-	-	-
17	36.88	4,22-Cholestadien-3-one	$C_{27}H_{42}O$	382.6	-	-	6.79	-	-	-	-	-	-	-	-	-	-	-
18	37.85	β 4-Sitosterol-3-one	$C_{29}H_{48}O$	412.7	-	-	15.5	-	-	-	-	-	-	-	-	-	-	-
19	21.01	Pentadecanal-	$C_{15}H_{30}O$	226	-	-	-		-	-	28.7	-	-	-	-	-	-	-
20	33.41	Cholesta-3,5-diene	$C_{27}H_{44}$	368	6.10	-	-		-	-	1.46	-	-	-	1.86	1.00	0.93	-
21	34.62	Cholest-5-en-3-ol (3β)-, tetradecanoate	$C_{41}H_{72}O_2$	386.6	-	-	-	-	-	-	-	0.81	-	-	-	-	-	4.44
22	18.71	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	-	-	-	-	-	-	-	-	-	-	8.71	2.72	-	-
23	17.68	Heneicosane	$C_{21}H_{44}$	296.5	-	-	-	-	-	-	-	-	-	-	-	-	42.1	-
24	18.12	Hexadecanoicacid, methyl ester	$C_{17}H_{34}O_2$	270	-	-	-	-	-	-	-	-	-	-	-	-	6.52	-

Note: -: Absent, NA: Not Determine, RT: Retention Time, MW: Molecular Weight, L: Leaves, R: Roots, CS: *C. serrulata*, HU: *H. uninervis*, EA:*E. acoroides*, CR: *C. rotundata*, SI*: S. isoetifolium*, TH: *T. hemprichii* and TC: *T. ciliatum*

Table 3. Biological activities of reported compounds in the hexane extracts of seagrasses

SN	Compound name	Structure	Nature of compound	Biological activity
1	2-Pentadecanone, 6,10,14-trimethyl-		Essential oil	Antihypertensive activity and vasodilatation affect cerebral and basilar arteries (Pushpabharathi et al. 2018)
2	Pentadecanoic acid, 14-methyl-, methyl ester		Fatty acids	Many odd-length amino acids are derived from pentadecanoic acid. Act as a biological marker for dietary milk intake (Pushpabharathi et al. 2018)
3	Cyclic octa atomic sulfur		Sulfur	Pesticide (Steudel and Chivers 2019)
4	Hexadecane		Alkane hydrocarbon	Antibacterial (Kumaresan et al. 2015)
5	Heptadecane		Straight chain alkane	Antifungal (Abubackerand and Devi 2015)
6	Stigmasta-5,22-dien-3β -ol, acetate		Steroids	Antiosteoarthritic, Antihypercholesterolemi, cytotoxicity, antitumour, hypoglycaemic, antimutagenic, antioxidant, Anti-inflammatory, and CNS effects (Kaur et al. 2011)
7	3β-Sitosterol acetate		Steroids	Anti-inflammatory, antineoplastic, antipyretic, and immunomodulating activity (Amudha et al. 2018)
8	1-Dodecene		Alkene	Antibacterial (Togashi 2007)
9	1-Tridecene		Alkene	Antibacterial activity (Kumar et al. 2011)

10	2,4-Di-tert-butylphenol		Phenol	Antifungal (Sang and Kim 2012), Antimalarial (Kusch et al. 2011)
11	1-Pentadecene		Alkene	Antimicrobial (Dubal et al. 2015)
12	1-Heptadecene		Alkene	Artificial ripening of fruits, metabolism of aromatic compounds in animals antimicrobial (Lakshmi and Bindu 2017)
13	1-Nonadecene		Alkene	Antituberculosis, anticancer, antioxidant, antimicrobial (Amudha et al. 2018)
14	Tetracosane		Straight chain alkane	Cytotoxicity against cancerous cells, Antidiarrheal, Antibacterial, Cardiotonic, laxative, Anthelmintic and removes fatigue, Anti-inflammatory, used in peptic ulcer treatment, Anticorrosive, Antioxidant, and Antitrichomonas (Prabhanna and Jayaraj 2018)
15	Phytol acetate		Diterpene	Antimicrobial activity (Mundt et al. 2003)
16	9-Tricosene		Alkene	Housefly pheromone (Gołębiowskia et al. 2015)
17	4,22-Cholestadien-3-one		Cholesterol	Anticancer (Patil and Singh 2022)
18	β4-Sitosterol-3-one		Steroids	Anticancer (Singariya et al. 2014)

19	Pentadecanal		Long-chain fatty aldehyde	Antimicrobial, a volatile oil component and a plant metabolite (Ogunwande et al. 2013)
20	Cholesta-3,5-diene		Cholesterol	Wound healing property and skin protector activities (Sayik et al. 2017)
21	3β -Cholest-5-en-3-ol, tetradecanoate		Steroids	Antibacterial, antioxidant (Thanigaivel et al. 2015)
22	*n*-Hexadecanoic acid		Palmitic acid	Antioxidant, hypocholesterolemic, nematicide, ant-androgenic hemolytic, and Alpha reductase inhibitor (Vijayalingam and Rajesh 2019)
23	Heneicosane		Straight chain alkane	Antioxidant and antimicrobial (Nimbeshaho et al. 2020)
24	Hexadecanoicacid, methyl ester		Fatty acids	Antibacterial and antifungal activity (Wagh et al. 2006)

Phytocompounds from *Thalassodendron ciliatum* extracts

The seagrass *T. ciliatum* had more phytocompounds compared to other studied seagrass species. Eighteen phytocompounds were found in this seagrass species, of which eleven and seven were found in the leaf and root extracts, respectively. The predominant phyto-compounds from the leaves were heneicosane (42.1%), phytol, acetate (17.6%), and tetracosane (14.9%), while those from the roots were cyclic octaatomic sulfur (55.4%), 3*β*-sitosterol acetate (22.3%), and 1-dodecene (16.7%). Furthermore, hydrocarbon (heneicosane) and fatty acid (hexadecanoic acid, methyl ester) were found only in the leaves of this seagrass. Of the 18 revealed phytocompounds, only one compound, fatty acid methyl ester (14-methyl-pentadecanoic acid methyl ester), was reported from another study conducted in the Red Sea, Egypt, by Goda et al. (2020), identifies 21 compounds from similar seagrass extracted using the same solvent (hexane).

Discussion

As observed in this study, the differences in the composition of the phytocompounds could be attributed to differences in geographical location, the type of seagrass species, and the solvent used. For instance, the variations in phytocompounds in similar seagrass species extracted using similar solvents are most likely due to differences in geographical location (Khanzadi and Tajur 2015). Despite the differences resulting from similar seagrass species, the different solvents used could be attributed to differences in polarity. The solvent used in this study was hexane, which is nonpolar as opposed to the polar solvents used by most of the researchers above. Additionally, the mineral composition, soil type, temperature, light, and water content are frequently reported factors affecting plant total phytochemical contents (Rao and Rao 2007; Hansen et al. 2010).

A comparison of the seven seagrasses from our study revealed that the seagrass *T. ciliatum* had more phytocompounds compared to other studied seagrass species, whereas hydrocarbon (heneicosane) and fatty acid (hexadecanoic acid, methyl ester) were found only in the leaves of this seagrass, and they are known for their antimicrobial and antioxidant properties (Wagh et al. 2006; Nimbeshaho et al. 2020). The seagrass *T. ciliatum* is traditionally popular for the treatment of smallpox and fever (De la Torre-Castro and Rönnbäck 2004); this function corresponds to the functions of 3*β*-sitosterol acetate, 1-dodecene, 1-tridecene, and hexadecane. Additionally, the steroid compounds *β*4-Sitosterol-3-one and 4,22-cholestadien-3-one, which are known for their antineoplastic activities (Singariya et al. 2014; Patil and Singh 2022) were detected only in *H. uninervis*. Furthermore, the steroid cholest-5-en-3-ol (3*β*)-, tetradecanoate was only obtained from *C. rotundata* and is known to have bioactivities (Thanigaivel et al. 2015). Traditionally, *Cymodocea* spp. have been reported to be used as remedies for skin diseases, fever, and cough and are believed to help during pregnancy as tranquilizers for babies (De la Torre-Castro and Rönnbäck 2004). These findings correlate well with the bioactivities of the identified compounds, such as 3*β*-sitosterol acetate, cholesta-3,5-diene, heptadecane, and pentadecanal, which are known for their antipyretic and wound healing properties, skin protector activities, and antifungal and antimicrobial activities (Table 2). The fatty acid (n-hexadecanoic acid), a compound reported to exhibit various bioactive properties (Vijayalingam and Rajesh 2019), was found exclusively in *T. hemprichii*. Additionally, compounds such as heptadecane, stigmasta-5,22-dien-3*β*-ol acetate, and 3*β*sitosterol acetate are recognized for their antimicrobial properties and their ability to reduce fever. Kaur et al. (2011), Abubackerand and Devi (2015), and Amudha et al. (2018) are found in the roots of *E. acoroides*. The anticipated function of the compounds mentioned above correlates well with the traditional use of seagrass. Thus, *E. acoroides* have been widely used against stings of special kinds of rays and scorpions (Kannan et al. 2010b) as well as against fever, muscle pains, wounds, and stomachs (De la Torre-Castro and Rönnbäck 2004).

Generally, seagrasses are rich in antimicrobial, antioxidant, and anti-inflammatory compounds due to the phytocompounds identified. They have therefore been reported to display antibacterial (Hamisi et al. 2023), antifungal, antimalarial (Kim et al. 2021), antioxidantactivities, increased glutathione sulfotransferase (GST) enzyme activity, lipid peroxidation, ferric reducing (Kim et al. 2021), and anti-Inflammatorymuscle aches, wounds, and abdominal pain (Kim et al. 2021).

The present GC-MS analysis revealed multivarious bioactive compounds with diverse chemical structures among which stigmasta-5,22-dien-3*β*-ol acetate and 3*β*-sitosterol acetate were the most common. The structures of the identified compounds and their corresponding known biological activities are presented in Table 3. The compounds 3*β*-Sitosterol acetate and stigmasta-5,22-dien-3*β*-ol acetate are reported to be steroids derived from lanosterol, a tetracyclic triterpenoid that can be used to produce oxy-generated derivatives of cholesterol and lanosterol. This finding demonstrated that oxysterols may be natural regulators of cholesterol biosynthesis in intact cells (Wang et al. 2008). Phytol acetate, a diterpene, has several biological activities such as anti-inflammatory, antimicrobial and antispasmodic activities. Furthermore, diterpenes have been shown to exhibit cardiovascular effects (Tirapelli et al. 2008). In this study, straight-chain alkanes such as heptadecane, were reported from *C. serrulata*, *E. acoroides*, and *C. rotundata* and are known to have antimicrobial properties (Vijayalingam and Rajesh 2019). This alkane was previously reported in *C. serrulata* by Das et al. (2023) from Palk Bay, India.

Generally, the findings of this study revealed the presence of useful chemical compounds such as essential oils, fatty acids, steroids, cholesterol, phenol, diterpenes, straight chain alkanes, and alkenes from seven seagrass species found along the Tanzania coast of the Western Indian Ocean, some of which are known for different bioactivities that reflect the traditional use of these seagrasses. A total of 24 known phytocompounds were identified across the examined species, with notable

common compounds including stigmasta-5,22-dien-3-ol acetate and 3β-sitosterol acetate. Additionally, certain unique compounds, such as 3β-cholest-5-en-3-ol tetradecanoate and phytol acetate were found in specific species. The *T. ciliatum*, in particular, exhibited a greater variety of compounds compared to the other seagrass species studied. This research is the first to report fourteen compounds in these seagrass species, underscoring their potential as sources of pharmacologically significant substances with antimicrobial, antioxidant, and anti-inflammatory properties. The findings contribute to the frontier of knowledge on phytochemicals present in seagrass species growing in the Western Indian Ocean, a scarcity explored ecosystem. We recommend further assessment of the efficacy and safety of these phytocompounds through in vitro and in vivo studies to understand their mechanisms of action and potential therapeutic applications. Additionally, to fully elucidate the spectrum of bioactive compounds present, we suggest exploring other extraction methods and analytical techniques to identify additional compounds.

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SUPPLEMENTARY MATERIAL:
REPRESENTATIVE CHROMATOGRAM OF HEXANE EXTRACTS OF SEVEN SEAGRASSES

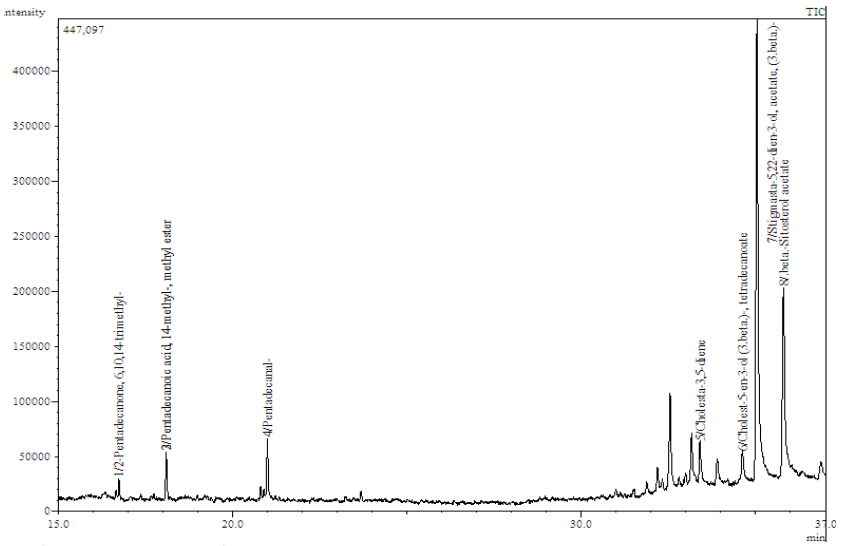

Figure S1. Chromatogram of hexane extracts of *Cymodocea rotundata* leaves

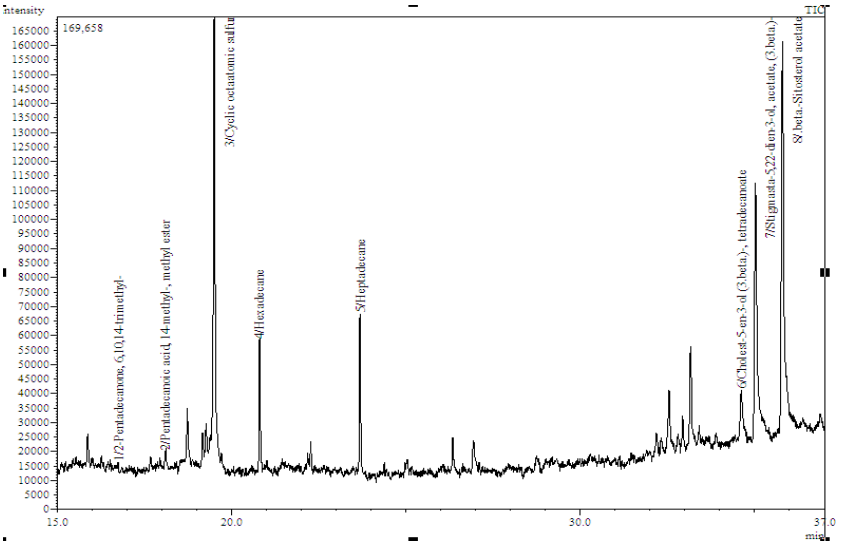

Figure S2. Chromatogram of hexane extracts of *Cymodocea rotundata* roots

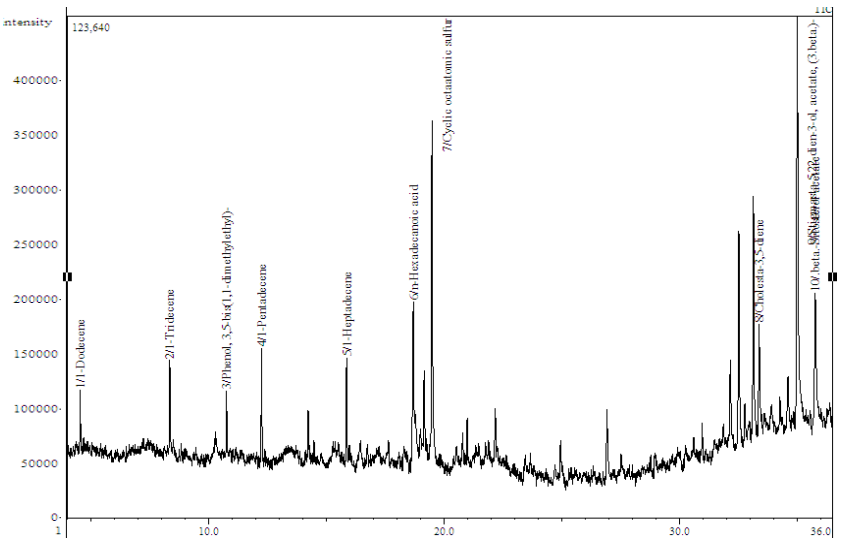

Figure S3. Chromatogram of hexane extracts of *Thalassia hemprichii* leaves

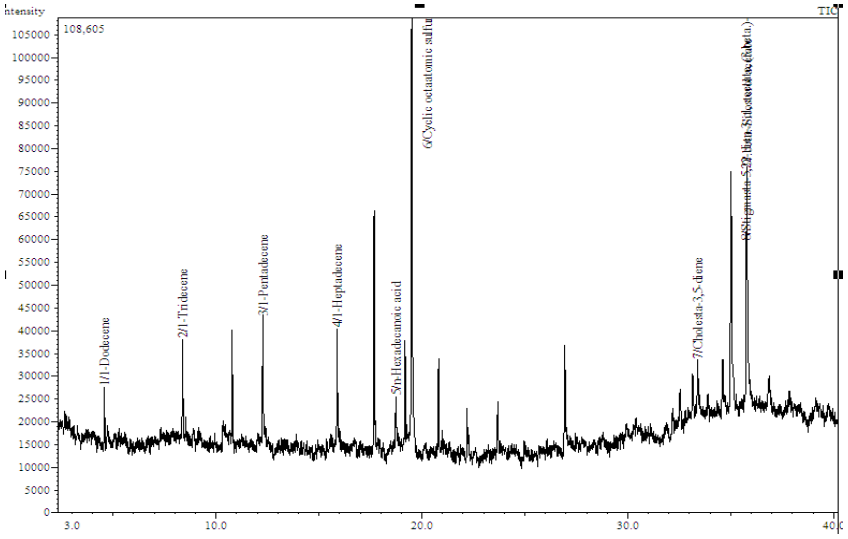

Figure S4. Chromatogram of hexane extracts of *Thalassia hemprichii* roots

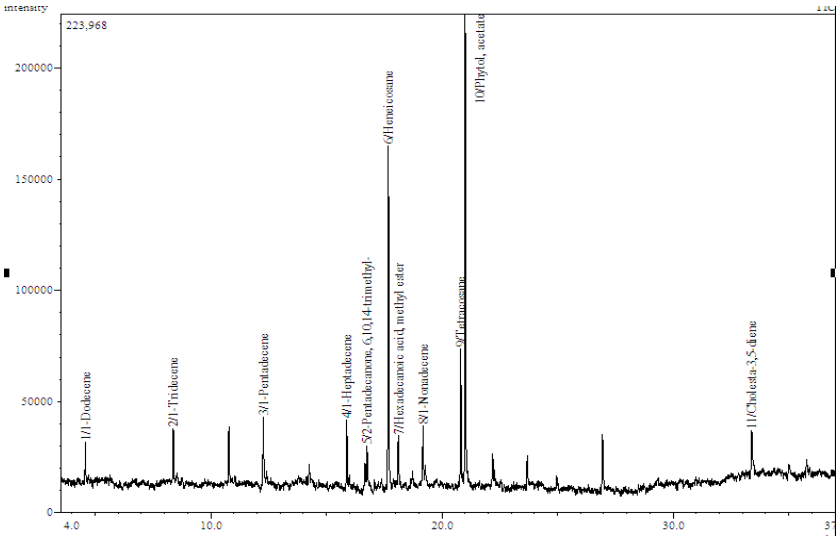

Figure S5. Chromatogram of hexane extracts of *Thalassodendron ciliatum* leaves

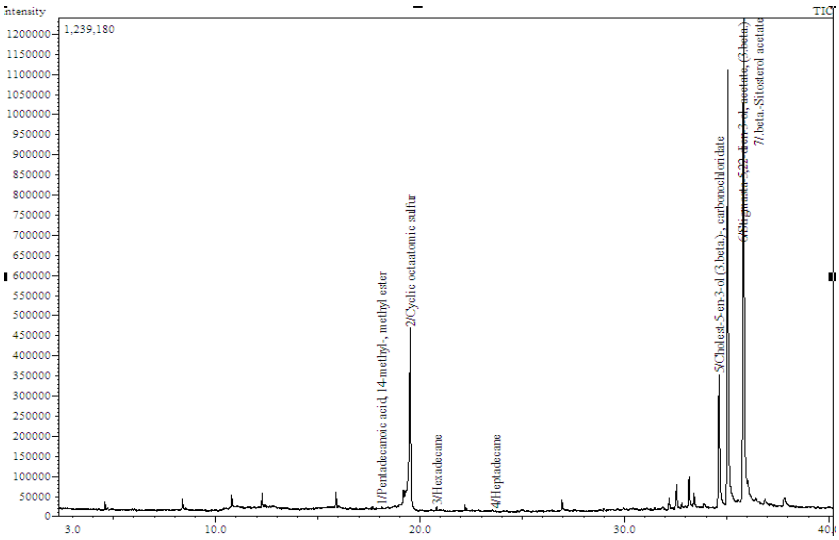

Figure S6. Chromatogram of hexane extracts of *Thalassodendron ciliatum* roots

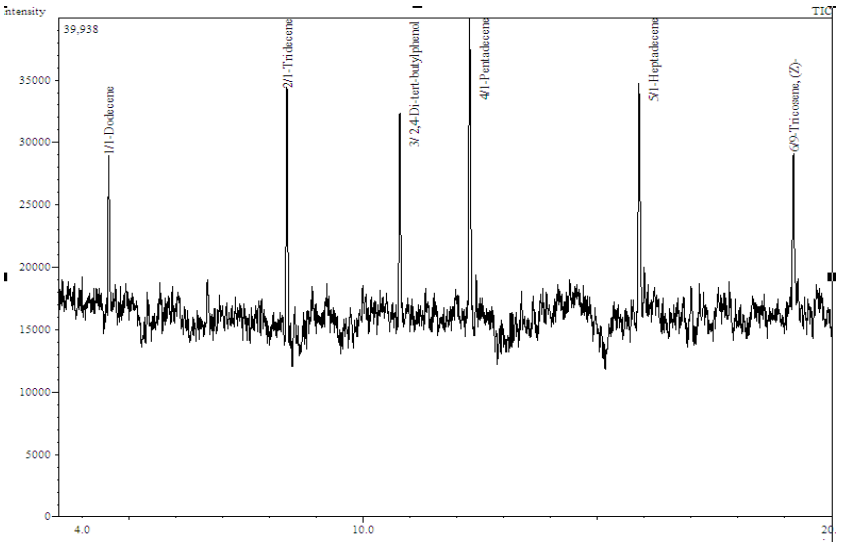

Figure S7. Chromatogram of hexane extracts of *Syringodium isoetifolium* leaves

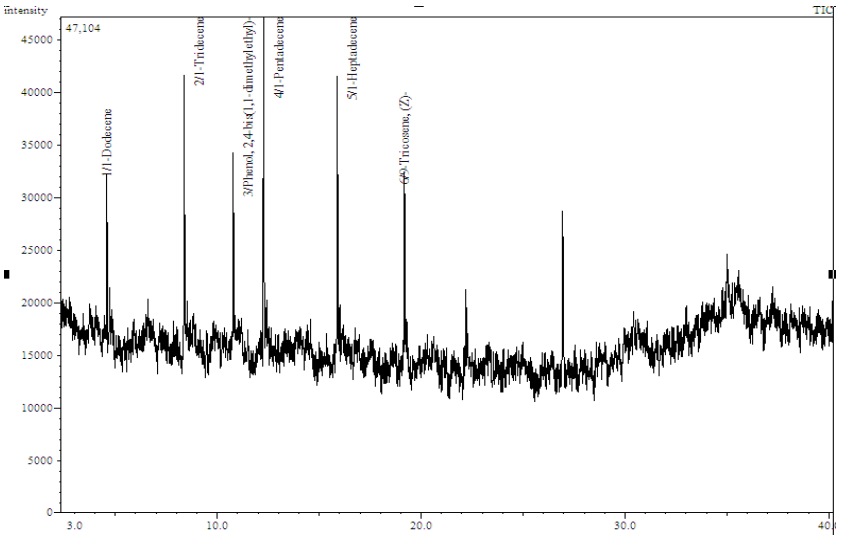

Figure S8. Chromatogram of hexane extracts of *Syringodium isoetifolium* roots

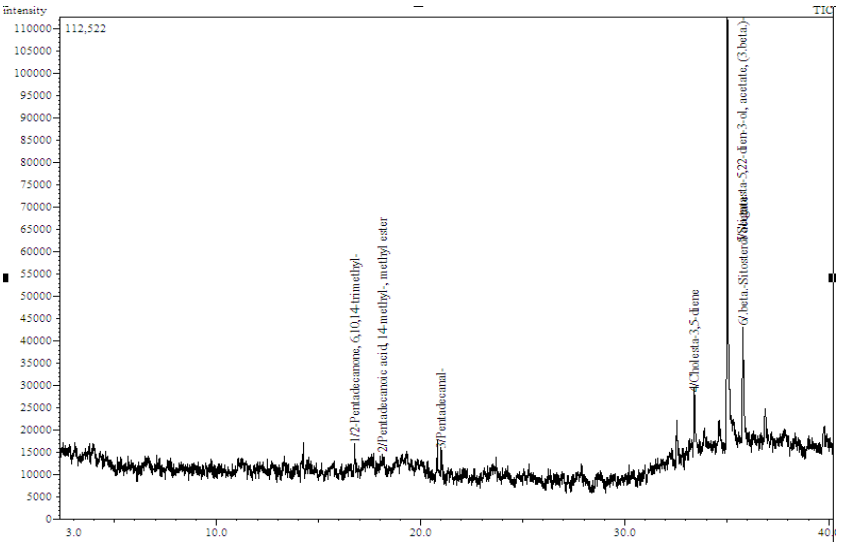

Figure S9. Chromatogram of hexane extracts of *Cymodocea serrulata* leaves

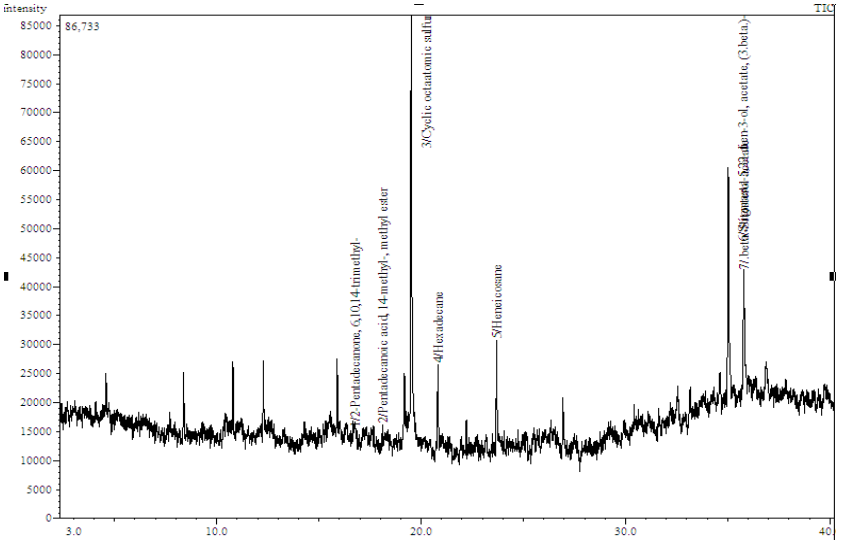

Figure S10. Chromatogram of hexane extracts of *Cymodocea serrulata* roots

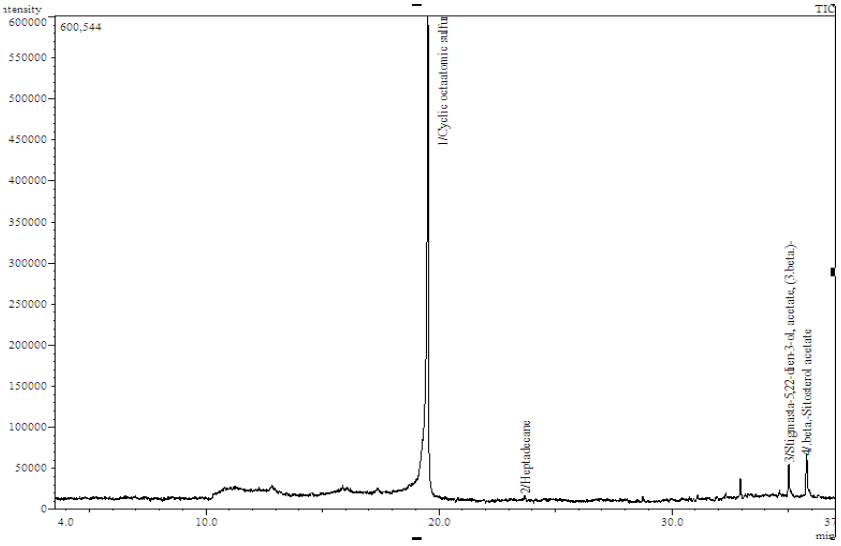

Figure S11. Chromatogram of hexane extracts of *Enhalus acoroides* roots

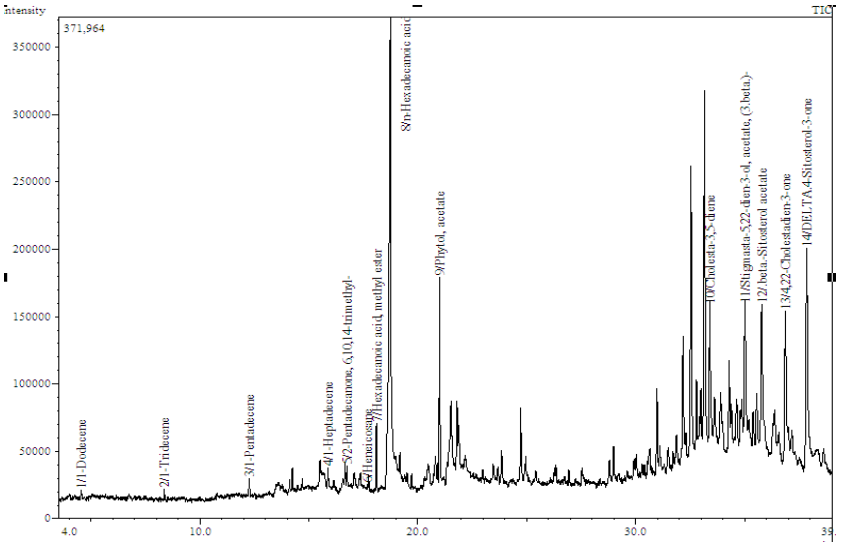

Figure S12. Chromatogram of hexane extracts of *Halodule uninervis* roots

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Investigating the effect of ethanolic extract of *Dioclea reflexa* seeds on antioxidant defense, lipid profile, liver and kidney peroxidation in adult male Wistar rats

BUKUNOLA OLUYEMISI ADEGBESAN[1,♥], ESTHER NKECHI EZIMA[1], ADEFEMI OLUWASEGUN ADEFUYE[1], IFABUNMI ODUYEMI OSONUGA[2]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University. Sagamu Campus, Ogun State, Nigeria. Tel.: +234-805-612-8331, ♥email: adegbesan.bukunola@oouagoiwoye.edu.ng

[2]Department of Physiology, Faculty of Basic Medical Sciences, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University. Sagamu Campus, Ogun State, Nigeria

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Abstract. *Adegbesan BO, Ezima EN, Adefuye AO, Osonuga IO. 2024. Investigating the effect of ethanolic extract of* Dioclea reflexa *seeds on antioxidant defense, lipid profile, liver and kidney peroxidation in adult male Wistar rats. Asian J Nat Prod Biochem 22: 89-97.* Several synthetic agents have been developed to combat oxidative stress associated with degenerative diseases, but factors including high cost, side effects, and lack of availability pose major setbacks in achieving the desired goal. Thus, it is therefore of utmost importance to explore the use of natural products that are less expensive and abundant in many plant sources to circumvent the alarming rates of these diseases. This study assessed the phytochemical content, the antioxidant properties of the ethanolic extract of *Dioclea reflexa* (Hook.f.) C.Wright seeds, and the effects of the extract on the lipid profile, liver, and kidney peroxidative systems of adult male Wistar rats. Therefore, 32 matured male Wistar rats (160-180) g were divided randomly into four treatment groups containing eight (8) rats each. Animals in the normal control group were treated with diluted ethanol, while those in the other three groups received an ethanolic seed extract of *D. reflexa*, 5 mg/kg, 10 mg/kg, and 15 mg/kg, respectively. After two weeks of experimental study, the animals were allowed to fast overnight before sacrifice. After that, their whole blood, kidney, and liver tissues were taken for further analysis. Assays for lipid peroxidation (liver and kidney), antioxidants, and serum lipid profile were evaluated. Our results revealed that *D. reflexa* seeds ethanolic extract contains considerable amounts of phytochemicals; significantly possesses antioxidant activities, repressed LDL and total cholesterol levels with a concomitant increase in HDL levels in Wistar rats; significantly reduced liver and kidney lipid peroxidative damage, especially at 10 and 15 mg/kg doses relative to the control rats. This study suggests that the seeds of *D. reflexa* possess antioxidative, HDL level raising, and non-HDL cholesterol lowering activities and thus may be useful in the management of lipid-associated disorders as well as hepatic and renal malfunctions.

Keywords: Antioxidants, cholesterol, *Dioclea*, lipid peroxidation, lipoprotein

INTRODUCTION

In humans, medicinal plants' therapeutic and beneficial pharmacological effects are very important and cannot be overlooked. Since the medieval age, people have utilized medicinal plants to manage a variety of illnesses and as components for the manufacture of beneficial medications. Natural products are sometimes more useful and efficient than synthetic analogs because they present fewer adverse effects, are economically affordable, and have efficacy in multidrug-resistant cases (Ye et al. 2015). Medicinal plants are now being recognized worldwide as useful tools for researchers in drug discovery, invention, and development (Chopra and Dhingra 2021; Noor et al. 2022).

According to Liguori et al. (2018), oxidative stress is well associated with the pathophysiology of numerous chronic illnesses, including atherosclerosis, cancer, Parkinson's disease, immune system dysfunction, diabetes mellitus, and aging. Concisely, oxidative stress results from an unstable or imbalanced situation between the rate at which free radicals are produced and the rate at which cells eliminate them. This imbalance can lead to lipid peroxidation, cell membranes and lipoproteins damage, and the ultimate development of mutagenic and cytotoxic compounds, such as conjugated diene and malondialdehyde (MDA).

Reduction in the risk of several diseases resulting from antioxidant activities has been attributed to phytochemicals such as flavonoids (present in fruits and vegetables), carotenoids (from carrots), alkyl sulfide (found in onions and garlic), lignans, coumarins, terpenoids, polyphenolics, plant sterols, saponins, phthalides and curcumins (Jimenez-Garcia et al. 2018). Numerous researchers have examined the potential health benefits of medicinal plants, including their hypolipidemic, hypoglycemic, antitumor, and immune-stimulating qualities, which may help to lower the onset of cardiovascular issues and cancer (Ye et al. 2015; Anwar et al. 2016; Shaito et al. 2020). However, despite the use of synthetic drugs, the prevalence of degenerative diseases, including cancer, diabetes, and hypertension, is increasing in both developed and developing nations. Therefore, investigating the usage of natural products is crucial to avoid the problematic rates at which these diseases are occurring.

Dioclea reflexa (Hook.f.) C.Wright is a beneficial species of tropical plant that grows from seeds; other names, including marble vine, sea beans, and horse-eye, also refer to it. *Agba-arin* is the name given to *D. reflexa* by the Yoruba people of South-Western Nigeria. At the same time, it is regarded as *ukpo* and *ebba* by the Igbo people of South-Eastern Nigeria (Ajatta et al. 2019). The *D. reflexa* is found in three different varieties "dark brown, light brown, and black"; it is a member of the family Leguminosae, sub-family Papillionoideae, a dicotyledonous plant and an angiosperm (Akinyede et al. 2017). The plant is a perennial plant; the seeds are in pods, each containing as many as eight seeds (Dutta 2018). The seeds are well known in the central and eastern parts of Nigeria and central African countries and have been used as traditional soup thickeners and as a rheology modifier in processed foods (Iliemene and Atawodi 2014). The importance of two notable sterols found in the seeds of *D. reflexa* has been reported; Stigmasterol has been described as a promising molecule that can be used in the development of drugs against several types of cancers, including breast, gastric, colon, prostate, and ovarian (Bakrim et al. 2022) while Taraxasterol has been described to be an excellent anti-inflammatory, anti-proliferating and antioxidative agent (Jiao et al. 2022; Movahhed et al. 2023). The research done by Balapangu et al. (2021) has also revealed that the acidic eluate of *D. reflexa* seed metabolites exhibits remarkable in vitro anti-proliferative effects on Michigan Cancer Foundation-7 (MCF-7) cells, which are used to model breast cancer.

This current study was designed to examine the phytochemical contents of *D. reflexa* seeds ethanolic extract as well as the extract's dose-dependent effects on oxidative status, lipid profile, and peroxidative status of the liver and kidney in adult male Wistar rats. The findings derived from this current study will provide insight into the antimicrobial properties, free radical scavenging activities, and antioxidant effect of *D. reflexa* seeds, which may suggest its therapeutic effect on liver and kidney health.

MATERIALS AND METHODS

Chemicals and reagents

Every reagent and chemical utilized for this research was pure and analytical grade. Gallic acid, hydrogen peroxide, ascorbic acid, and DPPH were purchased from Sigma–Aldrich, Gillingham, United Kingdom. Commercial assay kits for antioxidant status—GR, GPx activity, and Lipid parameters assays—were procured from Randox Laboratories Limited, Crumlin, United Kingdom.

Collection of plant samples, authentication, and extraction

Viable *D. reflexa* seeds were collected from four locations in Ogun state, Nigeria. The identification of the plant as *D. reflexa* was done at the Plant Science Department on the main campus of Olabisi Onabanjo University, Ago-Iwoye, Nigeria, after which both the plant and the seeds samples were kept in the herbarium. Afterward, the seeds were divided into smaller pieces, cleaned, and air-dried for two weeks at room temperature. After being ground into a coarse powder, 300 g of the dry seeds were soaked in 1000 mL of 99.7% ethanol for five days for sufficient extraction. At the end of the five days, the soaked seed sample was decanted, followed by filtration using a wool funnel to ensure that the filtrate does not contain any impurity that may compromise the usage of pure extract for subsequent analyses. Evaporation of the solvent from the *D. reflexa* seeds sample filtrate was achieved by using RotoVap 110. Further concentration to dryness was obtained on the slurry after evaporation at 40°C through a rotary evaporator. After that, lyophilization of the concentrated product into a powdery substance was done, weighed, and kept in a dry and air-tight container.

Animal care and experimental design

Guidelines for the care and use of laboratory animals were followed in conducting this research, and ethical approval was secured from "The Animal and Human Health Ethics Committee of Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Ago-Iwoye." A week was spent on the acclimatization of thirty-two (32) adult male Wistar rats, weighing between 160 and 180 g, obtained from the central animal house of INRAT "Institute of Advanced Medical Research Training" at UCH "University College Hospital" Ibadan, Nigeria. The rats were then housed in the animal house of the Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Ogun state. Each animal was kept in a metallic cage in a well-ventilated room with a 12:12 hour light and dark cycle every 24 hours while maintaining a temperature of about 25ºC. They were fed regular pelletized rat chow throughout the trial and unlimited water. The rats were randomly divided into four treatment groups, consisting of eight (8) rats each: group CNTL, which received diluted ethanol as control; group DIO-5, which received 5 mg/kg of ethanolic extract of *D. reflexa* seeds; group DIO-10, which received 10 mg/kg of ethanolic extract of *D. reflexa* seeds; and group DIO-15, which received 15 mg/kg of ethanolic extract of *D. reflexa* seeds. Every day for fourteen (14) days, a single dose was administered by oral gavage. Following a twelve-hour fast and diethyl ether anesthesia, rats in each group were sacrificed after fourteen (14) days of treatment. The rats were assessed according to the effects of various extract concentrations after blood samples were drawn from the inferior vena cava of their hearts into plain centrifuge tubes. The serum was then produced by centrifugation. After being removed, the kidney and liver were dried, weighed, and cleaned in an ice-cold potassium chloride (KCl) solution (1.15%). To extract the post-mitochondrial supernatant fraction, liver and kidney samples were homogenized in four volumes of 5 mM phosphate buffer "pH 7.4". The samples were spun at 10,000 x g using a centrifuge to obtain supernatants kept for further analysis at -80°C.

Biochemical assays

Phytochemistry

The qualitative assay for the phytochemical contents of *D. reflexa* seeds ethanolic extract was conducted using different chemical procedures described by Egbuna et al. (2018).

Assessment of the presence of alkaloids

The presence of alkaloids was assessed by combining 1g of the extract with 5 mL of ethanol and 50:50 diluted hydrochloric acid in a test tube. The resultant mixture was then heated to a boiling point for ten minutes. Afterward, the solution was filtered; potassium mercuric iodide, "also known as Mayer's reagent," was added to the filtrate. Alkaloids were present when a yellow-colored precipitate formed.

Assessment of the presence of flavonoids

Exactly 0.5 g of the extract was heated for three minutes in a steam bath with 10 mL of ethyl acetate added. After the mixture was filtered, 1 mL of diluted ammonia solution was mixed with 4 mL of the filtrate. The observation of yellow coloring indicated a positive test result for flavonoids.

Assessment of the presence of tannins

In a test tube, 1 g of the extract was heated with 10 mL of distilled water, and the liquid was subsequently filtered. After adding two to three drops of 5% ferric chloride ($FeCl_3$), the presence of tannins was determined by looking for a brownish-green-black or blue-black hue.

Assessment of the presence of phenols

Exactly 1 g of the extract was dissolved with 5 mL of distilled water, and 2 mL of a 1% gelatin solution comprising 10% sodium chloride was added. The formation of a white precipitate revealed the presence of phenolic compounds.

Assessment of the presence of saponins

Furthermore, 5 mL of distilled water was added to a test tube containing 0.5 g of the extract. After 15 minutes of vigorous shaking, a 1 cm^3 layer of foam was created, indicating the presence of saponins.

Assessment of the presence of steroids

Therefore, 0.6 g of the extract and 5 mL of chloroform were combined, thoroughly agitated, and filtered in a test tube. The test tube was tilted 45 degrees, and 2 mL of concentrated H_2SO_4 was gradually introduced by the side. A color shift was noticed; the acid layer had green fluorescence, indicating the presence of steroids, whereas the chloroform layer displayed a red-to-blue tint.

Assessment of the presence of glycosides

Exactly 2 mL of distilled water was used to dissolve 0.1g of the extract. The extract mixture was supplemented with 3 g of chloroform and 1 mL of a 10% ammonium solution. The development of a pink hue signified the existence of glycoside.

Assessment of the presence of terpenoids

0.5 g of the extract was diluted in 3 mL of distilled water and this was combined with 2 mL of chloroform. After carefully adding 3 mL of concentrated H_2SO_4, a layer of reddish-brown color appeared at the interface, suggesting the presence of terpenoids.

Assays for in vitro antioxidant defense activities

Three distinct chemical methods: the hydroxyl (OH^-) radical scavenging test, FRAP, the ferric reducing antioxidant potential assay, and the DPPH 2,2-Diphenyl-1-picrylhydrazyl free radical elimination potential were followed to estimate the in vitro antioxidant activity of the ethanolic extract of *D. reflexa* seeds. A total phenolic test was also performed to ascertain the extract's capacity for scavenging free radicals. Plant-based phenolic acids are vital human dietary components that exhibit tremendous antioxidant activity, among other health benefits.

Determination of DPPH free radical scavenging activity

Using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), the antioxidant activity of the ethanolic extract of *D. reflexa* seeds was measured for free radical scavenging, as per Burits and Bucar (2000) protocol. A 0.004% DPPH-ethanol solution was added to 50 µL of extracts at varying concentrations (10-50%), and the resulting mixture underwent incubation for 30 minutes at room temperature. The absorbance value was measured to Quercetin at 517 nm using a UV-visible spectrophotometer. Thus, the formula indicated below was used to estimate the inhibition rate (I%) on the DPPH free radical:

Inhibition % (I%) = {(A blank- A sample)÷A blank} × 100

Where:

A blank: Absorbance of the control reaction

A sample: Absorbance of the test compounds, Quercetin: Standard,

Reference (blank)

Determination of hydroxyl radical scavenging activity

The potential effect of *D. reflexa* seeds ethanolic extract on scavenging hydroxyl free radicals was investigated using the method outlined by Kunchandy and Rao (1990), with slight changes. A medium comprising KH_2PO_4-KOH buffer (20 mM, pH 7.4), H_2O_2 (1.0 mM), 2-deoxy-2-ribose (2.8 mM), $FeCl_3$ (100 µM), ascorbic acid (100 µM) and EDTA (100 µM) was combined with plant extract at varying concentrations to make a total volume of 1 mL; succeeded by incubation for 1 hour at 37°C. Next, to develop the color, 1 mL of 2.8% trichloroacetic acid (TCA) and 1 mL of 1% aqueous thiobarbituric acid (TBA) were added to the mixture. Afterward, 0.5 mL of the reaction mixture was heated at 90°C for 15 minutes. Following cooling, the absorbance at 532 nm was measured compared to a suitable blank solution. The following formula was applied for computing the hydroxyl radical scavenging ability (%), and ascorbic acid was used as a benchmark:

[(A absorbance of blank—A absorbance of sample) / A absorbance of blank] ×100

Determination of Ferric Reducing Antioxidant Potential (FRAP)

The method developed by Oboh and Omoregie (2011) was used to calculate the FRAP (Ferric-Reducing Antioxidant Potential) with slight changes. Exactly 250 µL of 1% potassium ferricyanide was added to the mixture of 50 µL of the extract and 450 µL of 200 mM sodium phosphate buffer (pH 6.6). For twenty minutes, the mixture underwent incubation at 50°C. Thereafter, 10% trichloroacetic acid (250 µL) was introduced, after which the mixture was centrifuged at 400 x g for ten minutes. Then 500 µL of 0.1% $FeCl_3$ was combined with 10 µL of the supernatant, and using spectrophotometry, the absorbance value of the mixture was determined at 700 nm. Every test was conducted three times. A rise in the reaction mixture's absorbance suggested that the plant samples' reducing power had increased. The standard was ascorbic acid.

Determination of Total Phenolic Content (TPC)

As per Singleton et al. (1999), the Folin-Ciocalteu assay was estimated to ascertain the phenolic constituent of *D. reflexa* seeds ethanolic extract. This technique provides reducing capability based on electrons, which is measured in terms of phenolic content. The solvent used for extraction affects both the yield and the total phenolic content of *D. reflexa* seeds ethanolic extract. Gallic acid was used for external calibration at 0.00, 0.25, 0.50, 0.75, and 1 mM concentrations. Then, 2.0 mL of solution A (mixture of 0.1 mL of sodium and potassium tartrate, 0.1 mL of $CuSO_4$, 10 mL of 2% Na_2CO_3) and 200 µL of extracts (10 mg/mL) were poured. After 4 minutes, 0.4 mL of 0.5 M sodium hydroxide solution was added. After another 10 minutes, there was an addition of 0.2 mL of the Folin-Ciocalteu reagent (1:1 v/v with water). The solution was left to stand for thirty minutes, after which its absorbance value was taken at 750 nm using a UV-Vis spectrophotometer. Following the adoption of the Gallic acid calibration curve, total phenolic content was estimated as mM Gallic acid equivalent (mM GAE).

Assays for in vivo antioxidant defense activities

Catalase assay

The catalase activity (CAT) in the kidney, liver, and serum was estimated using a slightly modified method attributed to Hadwan (2018). Catalase activity was determined as a function of micromole H_2O_2 broken down per milliliter (mL) of serum or milligrams (mg) of protein (organs) per minute.

Superoxide dismutase assay

The Del Maestro and McDonald (1987) method was adopted to ascertain the activity of superoxide dismutase (SOD) in the serum as well as liver and kidney tissues. The enzyme quantity that suppresses the oxidative conversion of epinephrine by 50%, or 1 U per milligram of protein in the serum or organs, is what is known as the SOD activity. Thus, SOD activity was evaluated as superoxide anion reduced per milliliter (serum) or mg protein per minute.

Glutathione Peroxidase (GP_X) activity assay

Following the usage of commercial kits for GPx assay (Ran-Sel from Randox, UK), the activities of GPx in the serum, liver, and kidney tissues were measured at 340 nm as NADPH oxidized on a Cobas Mira-Plus analyzer of biological fluids, a product of Roche. One micromole of NADPH oxidized per milliliter (serum) or milligram (organs) of protein per minute was the definition of an activity unit.

Glutathione Reductase (GR) activity assay

A commercial kit measured GR enzyme activity in serum, liver, and kidney tissues (Randox, UK). NADPH consumption was used as a spectrophotometric method to evaluate the enzyme activity at 340 nm. One micromole of NADPH oxidized per milliliter (serum) or milligram (organs) of protein per minute was the definition of an activity unit.

Glutathione assay

The method attributed to Tipple and Rogers (2012) was used to conduct the glutathione (GSH) assay in the serum, liver, and kidney. The glutathione concentrations were calculated and expressed as micromoles per milliliter of serum or milligram of protein (organs).

Assessment of lipid parameters

The concentrations of total cholesterol (TC), triglycerides (TG), and high-density lipoprotein (HDL) were determined in the serum using the cholesterol oxidase, glycerol-3-phosphate oxidase, and HDL Cholesterol-Direct Clearance methods, respectively. The serum's low-density lipoprotein (LDL) concentration was extrapolated using the formula in Friedewald et al. (1972). The assays were performed using commercial kits acquired from Randox Laboratories Ltd. (Crumlin, UK) following the instructions provided by the manufacturer.

Liver and kidney lipid peroxidation

The extent of lipid peroxidation in both the kidney and the liver tissue homogenates was determined spectrophotometrically according to the method of Khoubnasabjafari et al. (2015) as regards the measurement of TBARS thiobarbituric acid-reactive substance and the subsequent formation of malondialdehyde (MDA) per mg protein.

Statistical analyses

Data are displayed as mean ± SEM "standard error of means" and shown as bar charts "with SEM as the error bars." Statistical comparisons among the treatment and control groups were determined using one-way or two-way Analysis of Variance (ANOVA) followed by Dunnette's multiple comparison test. Statistical significance was set at values with $p<0.05$. GraphPad Prism software version 9.0.0 GraphPad Software, San Diego, California, USA, was used for the data representation in this study.

RESULTS AND DISCUSSION

Phytochemistry of the ethanolic extract of *D. reflexa* seeds

The qualitative phytochemical screening of *D. reflexa* seeds ethanolic extract was performed and the result is presented in Table 1.

In vitro antioxidant activities and total Phenolic content of ethanolic extract of *D. reflexa* seeds

Table 2 presents the results of the in vitro antioxidant activities and total phenolic content of the ethanolic extract of *D. reflexa* seeds determined in this study.

Effects of ethanolic extract of *D. reflexa* seeds on antioxidant enzyme activities of Wistar rats

Upon administering varying amounts of ethanolic extract of *D. reflexa* seeds, Wistar rats' blood, liver, and kidney showed noticeably elevated antioxidant enzyme activities. The activities of Catalase (Figure 1.A), Superoxide dismutase (Figure 1.B), Glutathione reductase (Figure 1.C), and Glutathione peroxidase (Figure 1.D) were significantly higher in Wistar rats treated with *D. reflexa* relative to the control rats.

Effects of ethanolic extract of *D. reflexa* seeds on reduced glutathione concentration in Wistar rats

Treatment with ethanolic extract of *D. reflexa* seeds significantly elevated GSH levels in male Wistar rats. A marked increase in GSH levels in Wistar rats was observed in the serum, kidney, and liver after administering 10 and 15 mg/kg of *D. reflexa* seed extract via oral route compared to the corresponding controls (Figure 2).

The response of lipid profile status to ethanolic extract of *D. reflexa* seeds in Wistar rats.

The serum total cholesterol level was significantly reduced by 10 and 15 mg/kg ethanolic extract of *D. reflexa* seeds; Low-density lipoprotein level was significantly reduced by 5 and 10 mg/kg ethanolic extract of *D. reflexa* seeds while High-density Lipoprotein level was significantly increased by 15 mg/kg ethanolic extract of *D. reflexa* seeds relative to the corresponding control. The results are presented in mg/dL (Figure 3).

Table 1. The outcome of the qualitative phytochemical screening of ethanolic extract of *D. reflexa* seeds following diverse chemical techniques to detect a range of bioactive compounds spontaneously occurring in medicinal plants

Phytochemicals	*Dioclea reflexa* seeds
Alkaloids	++
Flavonoids	++
Tannins	+
Phenolic compounds	++
Saponins	+
Steroids	++
Glycosides	+
Terpenoids	+

Note: + indicates present, and ++ indicates highly present

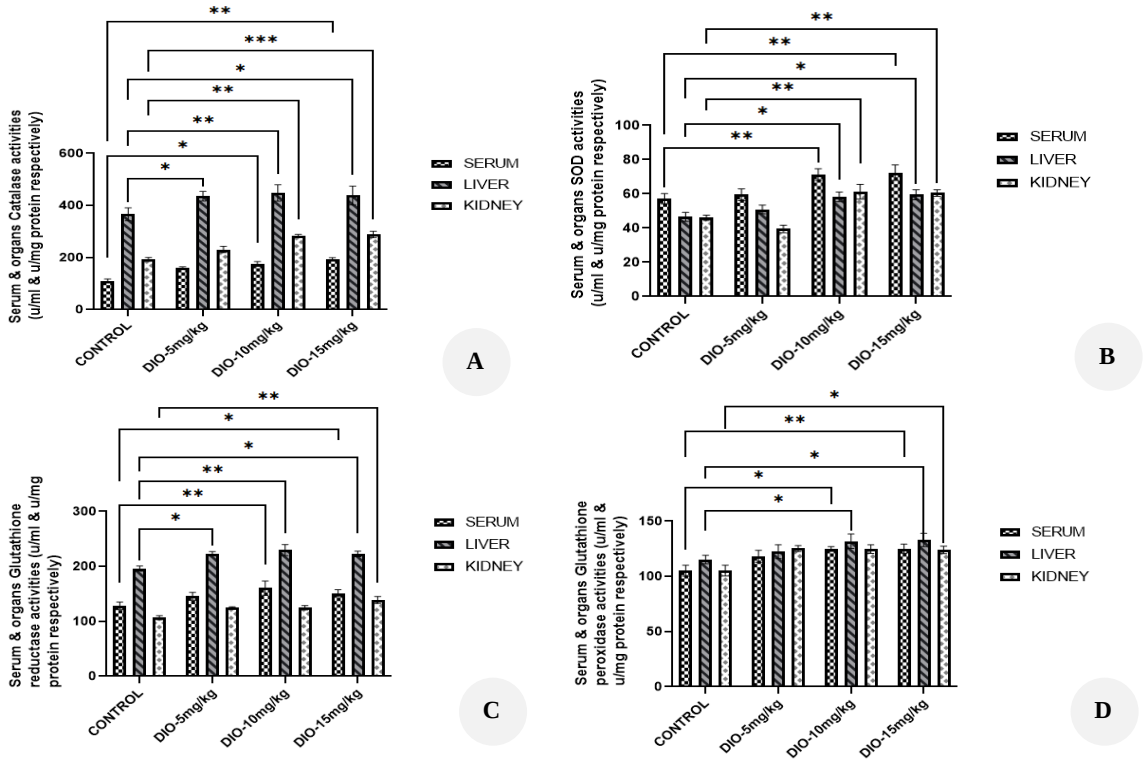

Figure 1. The effects of ethanolic extract of *D. reflexa* seeds on antioxidant enzyme (A. Catalase, B. Superoxide dismutase (SOD), C. Glutathione reductase, D. Glutathione peroxidase) in Wistar rats. Bar charts represent the activities of antioxidant enzyme in control, 5 mg/kg, 10 mg/kg, and 15 mg/kg treated rats' serum, liver, and kidney. Three asterisks indicate $p<0.005$, two indicate $p<0.01$, and one indicates $p<0.05$

Table 2. The 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (OH^-), ferric Reducing Antioxidant Potential (FRAP) free radical scavenging activities are represented as percentages, and the total phenolic contents are expressed as Gallic acid equivalence between the *D. reflexa* seed ethanolic extract and the matching standard for comparison

Test substance	DPPH (%) ± SEM	OH^- (%) ± SEM	FRAP (%) ± SEM	Total phenolic content (mM GAE/g)
Dioclea reflexa seeds ethanolic extract	39.8±0.93[b]	22.5±1.07[b]	1.7±0.03[b]	166.9±5.43[b]
Quercetin	31.7±2/97[a]			
Ascorbic acid		18.9±0.88[a]	0.33±0.01[a]	
Gallic acid				140.3±3.60[a]

Note: Values are presented as mean ± SEM (n = 3). Values in the same column with different superscript letters (a-b) are significantly different ($p < 0.05$)

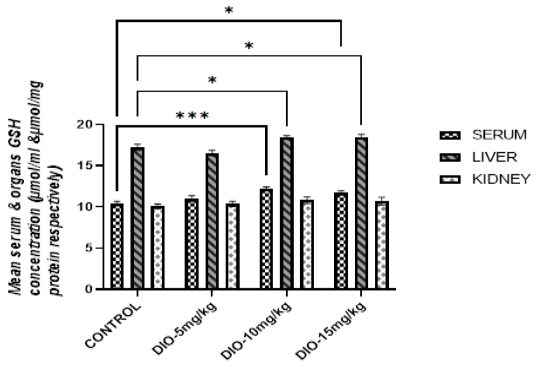

Figure 2. Effects of ethanolic extract of *D. reflexa* seeds on reduced glutathione concentration in Wistar rats. Bar charts represent reduced Glutathione (GSH) levels in the serum, liver, and kidney of Wistar rats. All results are presented as mean ± SEM. One asterisk indicates $p<0.05$, and three indicate $p<0.005$

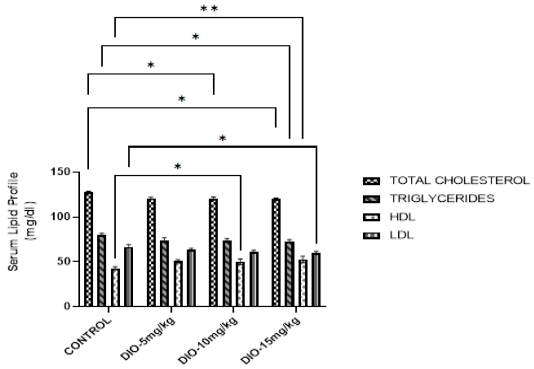

Figure 3. Effects of ethanolic extract of *D. reflexa* seeds on lipid profile status of Wistar rats. Bar charts represent the levels of total cholesterol, Triglycerides, HDL, and LDL in control, 5 mg/kg body weight, 10 mg/kg body weight, and 15 mg/kg treated rats. Two asterisks indicate $p<0.01$, and one indicates $p<0.05$

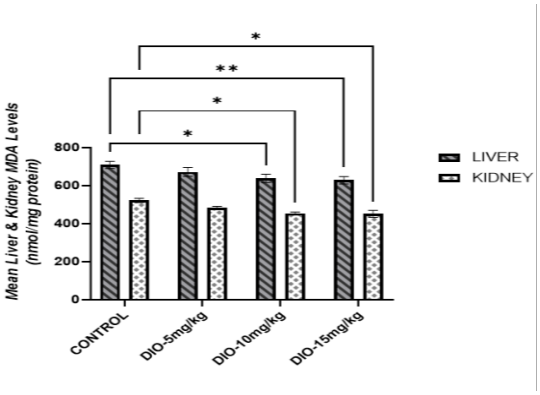

Figure 4. The effects of ethanolic extract of *D. reflexa* seeds on liver and kidney peroxidation in Wistar rats. Bar charts showing the levels of liver MDA and kidney MDA in control, 5 mg/kg bwt, 10 mg/kg, and 15 mg/kg body weight treated rats. Two asterisks indicate $p<0.01$, and one indicates $p<0.05$

The effects of ethanolic extract of *D. reflexa* seeds on kidney and liver lipid peroxidation in Wistar rats

Following the measurement of the synthesized TBARS, the levels of MDA, an index of lipid peroxidation, were significantly reduced in the liver and kidney following treatment with ethanolic extract of *D. reflexa* seeds at 10 and 15 mg/kg body weight relative to the corresponding control groups (Figure 4).

Discussion

It is impossible to overstate the value of medicinal plants as complementary therapies for managing and controlling diseases. In addition to providing food and medicine for human health, plants also aid in managing and controlling diseases (Mbah et al. 2022). Several plants have been studied to learn more about their positive effects on health and medicine by examining how they might enhance certain disease management and disease-prevention processes. In this study, the phytochemistry, in vitro antioxidant status, total phenolic content of the ethanolic extract of *D. reflexa* seeds, and subsequent outcomes of the extract on antioxidant defense, lipid profile, and the peroxidative systems of the liver and kidney investigated provide useful and logical support for using *D. reflexa* seed as an alternative disease control and management effort.

This investigation showed that the ethanolic extract of *D. reflexa* seeds included phytochemicals such as alkaloids, saponin, tannin, flavonoids, phenols, steroids, and glycosides (Table 1). According to a recently conducted research by Sharopov et al. (2018), the presence of alkaloids has been related to their therapeutic relevance in the control of numerous disorders, including Alzheimer's disease, cancer, and malaria. Sharma et al. (2018) state that these phytochemicals found in plants have antibacterial, antiviral, antiallergic, antioxidative, and antispasmodic qualities. It has been proposed that the exceptional physiological potential of tannins, phenolic compounds,

steroids, and saponins is responsible for their antibacterial properties. The use of natural products in managing and treating disease has grown rapidly, especially in underdeveloped countries. Atherosclerosis, diabetes, cancer, and other neurodegenerative diseases are some of the cardiovascular diseases being researched for prevention and treatment through Western or synthetic medicines. However, to provide accessible and affordable treatment for these conditions, developing new treatment pathways involving natural products is essential. Our research thus shows the significant documented potential therapeutic activities of the different phytochemicals present in distinct extracts from medicinal plants. Our results support the research by Oladimeji et al. (2017), who also reported the presence of important bioactive constituents in the *D. reflexa* plant.

Antioxidant properties of natural products have been demonstrated in several in vitro studies on medicinal plants (Ojha and Jain 2021; Adegbesan et al. 2024). This characteristic can help identify significant antioxidant pharmaceutical compounds for managing and treating various health issues. Low hydrogen peroxide (H_2O_2) concentrations exist naturally in the human body, the air, plants, water, food, and microbes. It disintegrates fast into oxygen (O_2) and water (H_2O), and it may also produce hydroxyl radicals (OH^-), which can cause DNA damage and thereafter initiate lipid peroxidation. *D. reflexa* seed ethanolic extract shows notable DPPH radical scavenging activity (39.8±0.93%) and considerable Ferric Reducing Antioxidant Potential (FRAP) (1.7±0.03%); it also successfully scavenges hydroxyl radicals (22.5±1.07%) (Table 2). Compared to Gallic acid, the total phenolic content was significantly higher (166.9±5.43 GAE/g) (Table 2). Our study's in vitro antioxidant results support the outcome of Atawodi and Iliemene (2014) work, who found that when exposed to acute and long-term toxicological challenges, the methanolic extract of *D. reflexa* seeds protects the blood and kidney against oxidative damage and its associated complications related ailments. Phenolic compounds are considered extraordinary and significant antioxidants that occur naturally in several plant species. Research has revealed their relationship with plant defense responses. Our observations regarding the presence and quantification of phenol recorded in this study indicate that *D. reflexa* seed extract has antioxidant potential due to the presence of phenol in the extract.

How the ethanolic extract of *D. reflexa* seeds affected the levels of antioxidant enzymes in the blood, liver, and kidney of Wistar rats, including catalase, superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) were also delved into. After administering varying doses of ethanolic *D. reflexa* seed extract, Wistar rats' blood, liver, and kidney showed noticeably elevated antioxidant enzyme activity. Overall, Wistar rats treated with *D. reflexa* showed considerably higher activities of Catalase (Figure 1.A), SOD (Figure 1.B), GR (Figure 1.C), and GPx (Figure 1.D) than the control rats. The marked increase in antioxidant enzyme activity in rats treated with *D. reflexa* may make this plant a candidate for use as a replacement for potentially toxic synthetic antioxidant analogs. Our findings regarding antioxidant enzyme activity corroborate the research by Iliemene and Atawodi (2014), who reported that the seeds of *D. reflexa* possess the ability to boost the activities of antioxidant enzymes, including catalase and SOD. Bakrim et al. (2022) also reported the antioxidative properties of stigmasterol as well as other properties, including being a molecule with anticancer, anti-osteoarthritis, antidiabetic, antiparasitic, anti-inflammatory, antibacterial, antifungal, and neuroprotective properties. Consequently, it can be used in the development of therapeutic drugs against several cancer types, including colon, breast, gastric, and ovarian. Jiao et al. (2022), also revealed that Taraxasterol found in the seed of *D. reflexa* possesses an outstanding antioxidative, anti-inflammatory, and chemo-preventive property against chemical carcinogenesis. The study's observation of the ethanolic extract of *D. reflexa* seeds' antioxidative action implies that these seeds may have potential pharmaceutical applications in disease management.

In this study, a significant increase ($p<0.05$) in liver glutathione (GSH) levels was observed in rats treated with 10 and 15 mg/kg ethanolic seed extract of *D. reflexa* (Figure 2) compared to the control rats. A similar effect was also observed in the serum of the treated rats following 10 and 15 mg/kg ethanolic seed extract of *D. reflexa*. Increased GSH level indicates that the seeds of *D. reflexa* are a potential antioxidant that may shield the liver and blood from the damaging effects of oxidative stress and other toxicological conditions. These findings are supported by the research of Atawodi and Iliemene, who reported that *D. reflexa* seeds are rich in substances that have the potential to protect the kidneys and blood from oxidative damage and other related diseases (Atawodi and Iliemene 2014; Iliemene and Atawodi 2014, 2023).

Our study examined the impact of ethanolic extract of *D. reflexa* seeds on the lipid profile status of male Wistar rats. The findings (Figure 3) showed that the treated Wistar rats at 10 mg/kg and 15 mg/kg had significantly higher levels of HDL in comparison to the control rats; the treated Wistar rats at 15 mg/kg had significantly lower levels of LDL in comparison to the control rats; both the 10 mg/kg and 15 mg/kg treated Wistar rats had significantly lower levels of total cholesterol in comparison to the control rats; additionally, the level of triglycerides was significantly lower in the 15 mg/kg treated Wistar rats than the control rats. Although ethanolic extract of *D. reflexa* seeds has been shown to have a decreasing effect on the levels of both triglycerides and total cholesterol, high levels of both can pose major health hazards. Individuals with established coronary heart disease have been found to have an all-cause mortality rate that is correlated with high triglycerides (Klempfner et al. 2016); hence, administering *D. reflexa* seed extract to these individuals may lower their death rate. Rats treated with the ethanolic extract likewise showed reduced levels of LDL and a concurrently increased level of HDL. According to a recent study, HDL and LDL are related to wound healing. Chen et al. (2022) showed that treatment with HDL cholesterol restored the substantial connection between lower HDL cholesterol

levels and poorer wound healing. This implies that, given that low HDL cholesterol is a typical hallmark of diabetes, the ethanolic seed extract of *D. reflexa* may be a viable treatment to promote wound healing, particularly in cases of diabetes. Our findings on lipid profile assessment are supported by the research that found phytosterols in the *D. reflexa* seed methanolic extract. Additionally, it has been noted that these phytosterols can decrease cholesterol and Low-Density Lipoprotein (LDL), preventing the buildup of cholesterol within the body. This suggests that the phytosterol constituent of the *D. reflexa* seeds may be linked with the observed modulatory effects of the seeds on the lipid profile status of Wistar rats treated with this extract. These findings on the impact of *D. reflexa* seed ethanolic extract on the lipid profile status of Wistar rats imply that the seeds play a modulatory role in lipid metabolism and subsequently exert a beneficial impact on lipid regulation to enhance human health.

Our findings on the impact of *D. reflexa* seeds ethanolic extract on liver and kidney peroxidation in Wistar rats (Figure 4) showed a noteworthy decrease in liver and kidney MDA levels after administering 10 and 15 mg/kg of the ethanolic seed extract. This implies that the extract may treat renal and liver disorders. The observed outcome in the liver tissues of Wistar rats administered with the seed extract aligns with the findings of Iliemene and Atawodi (2014), who documented the hepatoprotective effect of methanolic *D. reflexa* seed extract in rats after liver damage. Our findings regarding the decreased levels of MDA in the kidney tissues of rats treated with ethanolic seed extract of *D. reflexa* are further supported by a study conducted in 2014 by Atawodi and Iliemene (2014), which found that the methanolic extract of the seeds of this plant protects the blood and the kidney from oxidative damage and other related injuries due to underlying toxicological damage.

In conclusion, our investigation into the phytochemical content of the ethanolic seed extract of *D. reflexa* produced some interesting results, including the presence of substances (such as tannins, saponins, phenolic compounds, and steroids) with possible antibacterial properties. The results of this study also indicate that *D. reflexa* seed has an exceptional ability to scavenge free radicals and reduce oxidative stress; additionally, it positively modulates HDL levels and may facilitate wound healing, particularly in cases of diabetes, due to its modulatory effect on lipid profile status. In addition, due to its antioxidant qualities, *D. reflexa* seeds may help to manage and treat liver and kidney problems. This study offers justification for more investigation into the molecular mechanisms behind the interactions and modifications of metabolic processes by substances found in seed extracts of *D. reflexa*.

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Biochemical evaluation on amelioration of oxidative stress and cardiovascular risk markers in hyperlipidemic rats treated with *Kalanchoe pinnata* aqueous extract

TAMUNO-BOMA ODINGA-ISRAEL[1,♥], BARIZOGE CLETUS LEMII[2], CHRISTINE U. GABRIEL-BRISIBE[3], IYAENEOMI RANSOME DAKA[2], SARAH K. ENEBELI[2], IYINGIALA AUSTIN-ASOMEJI[4], FELICIA UCHEAWAJI[2], CONQUEST C. NODI[1]

[1]Department of Biochemistry, Faculty of Science, Rivers State University. Nkpolu, Oroworukwo P.M.B. 5080 Port Harcourt, Rivers State, Nigeria. Tel.: +234-8037660984, ♥email: tamuno-boma.odinga@ust.edu.ng

[2]Department of Pharmacology and Therapeutics, Faculty of Basic Clinical Sciences, College of Medical Sciences, Rivers State University. Nkpolu, Oroworukwo P.M.B. 5080 Port Harcourt, Rivers State, Nigeria

[3]Department of Medical Biochemistry, Faculty of Basic Medical Sciences, College of Medical Science, Rivers State University. Nkpolu, Oroworukwo P.M.B. 5080 Port Harcourt, Rivers State, Nigeria

[4]Department of Community Medicine, Faculty of Clinical Sciences, College of Medical Sciences, Rivers State University. Nkpolu, Oroworukwo P.M.B. 5080 Port Harcourt, Rivers State, Nigeria

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Abstract. *Odinga-Israel TB, Lemii BC, Gabriel-Brisibe CU, Daka IR, Enebeli SK, Austin-Asomeji I, Ucheawaji F, Nodi CC. 2024. Biochemical evaluation on amelioration of oxidative stress and cardiovascular risk markers in hyperlipidemic rats treated with* Kalanchoe pinnata *aqueous extract. Asian J Nat Prod Biochem 22: 98-105.* The continuous use of plants as a raw material for medicines has gained wide acceptance due to their pharmacological bioactive compounds. Metabolic disorders such as hyperlipidemia could result in cardiovascular diseases. This study evaluated the effect of the aqueous extract of *Kalanchoe pinnata* (Lam.) Pers. on the antioxidant, lipid profile, and clinical indices in hyperlipidemic female albino rats. The female albino rats were induced with hyperlipidemia using a high-fat diet mixture consisting of 68% powdered rat feed (standard rat chow pellet), 20% instant milk powder (peak milk), 6% corn oil (Mazola), and 6% ghee. Thirty female albino rats were grouped into 5, with 6 rats in each group. Group 1 served as normal control, group 2 was positive control, and group 3 was negative control, while groups 4 and 5 were administered 200 mg/kg and 400 mg/kg aqueous *K. pinnata* extract for 21 days. The body weight of the rats was recorded after 21 days. The rats were then sacrificed, and blood samples were collected to determine the antioxidant status, lipid profile, troponin, CK, and myoglobin levels of the rats using standard laboratory procedures. *K. pinnata* extract showed significant antioxidant and antihyperlipidemic activities, with the most at 400 mg/kg, evidenced by a decrease in the TC, TG, and LDLC serum levels with a corresponding increase in the HDLC serum level for anti-hyperlipidemia. An increase in the GSH, GST, GPx, catalase, and SOD levels was observed with a corresponding decrease in MDA serum levels in the experimental rats. Although myoglobin, troponin, and CK are not acutely evaluated for myocardial infarction, they can be used for deduction on biochemical disorders. The serum concentrations of myoglobin, CK, and troponin were decreased on administration of *K. pinnata* when compared to the control groups. The findings of this study suggest that *K. pinnata* could be used as a raw material for the medicinal treatment of cardiovascular diseases and as an antioxidant source.

Keywords: Antioxidant, anti-hyperlipidemia, cardiovascular markers, *Kalanchoe pinnata*, myoglobin, troponin

INTRODUCTION

Biochemical disorders are disruptions or imbalances in the body's normal metabolic processes. These types of disorders are related to the development of various health conditions, including cardiovascular diseases, high cholesterol levels (hyperlipidemia), and diabetes mellitus (Barr 2018). These biochemical disorders increase disease susceptibility. Therefore, proper medical screening and monitoring are necessary to help prevent or manage the onset of these related health conditions. Research has indicated that alterations in antioxidant status and increases in inflammatory markers are often associated with metabolic syndromes and their various components. As a result, those diagnosed with metabolic syndrome are more prone to oxidative stress. It lowers antioxidant levels to compensate for the elevated Reactive Oxygen Species (ROS) levels (Suriyaprom et al. 2019).

The use of natural products from plants has gained attention. It can be attributed to the potency of plants, including their ethnomedicinal value, due to their phytochemical content and biologically active compounds (Odinga et al. 2016). The use of plants for therapeutic, prophylactic, and ameliorative purposes has been encouraged because plants are cost-efficient and readily available (Odinga et al. 2020).

In line with the words of Hippocrates in 400 BC, "Let food be thy medicine and medicine thy food," many plants around our environment serve as food sources and have health benefits such as *Kalanchoe pinnata* (Lam.) Pers.. It is native to Madagascar, an herbaceous perennial, reasonably abundant along the coast, growing in sandy soils. It is used mainly in African, Brazilian, and Indian

traditional medicine for treating several diseases such as diabetes (Nascimento et al. 2023). The crude extracts contained several phytochemical compounds, including tannins (Zawirska-Wojtasiak et al. 2019). It has anthelmintic activity (Muzitano et al. 2006), a protective effect against stomach lesions or ulcers induced by various factors, including aspirin, indomethacin (a type of nonsteroidal anti-inflammatory drug), serotonin, reserpine, stress, ethanol (alcohol) (de Araújo et al. 2018). A study by Richwagen et al. (2019) found that 60% methanolic leaf extract of *K. pinnata* at a concentration of 25 mg/mL inhibited the growth of *Bacillus subtilis, Shigella dysenteriae, Escherichia coli, Proteus vulgaris,* and *Staphylococcus aureus,* but not against, *Klebsiella pneumoniae, Candida albicans,* and *Pseudomonas aeruginosa*.

Hyperlipidemia could arise from an unhealthy diet and lack of exercise and physical activity. This condition can contribute to the buildup of plaque deposits on the inner walls of blood vessels. Over time, the plaque clogs the arteries, leading to high blood pressure and increasing the risk of stroke and cardiovascular diseases such as heart attacks. Therefore, proper diet and regular exercise help prevent and manage hyperlipidemia and its health consequences (Alloubani et al. 2021). Numerous traditional and folkloric plant applications have been documented in peer-reviewed scientific literature. However, further investigation in the Niger Delta region of Nigeria revealed that the local population consumes the plant in a concoction with malt and milk. This traditional preparation is utilized to manage lipid profiles in the blood and enhance overall blood health. Given the absence of prior scientific research on the reported traditional uses of K. pinnata, this study aimed to investigate its effects on antioxidant status, lipid profile, and specific biochemical markers in female albino rats induced hyperlipidemia. The goal was to provide scientific proof of the claimed benefits associated with using K. pinnata for lipid management and related health outcomes.

MATERIALS AND METHODS

Plant collection and preparation of extract

Fresh leaves of *K. pinnata* were harvested in the Emoh community in the Abua/Odual local government area, Rivers State, Nigeria, in September 2022. Healthy and mature leaves were used in this study. The sample was authenticated by Dr. M. Ajuru of the Department of Plant Science and Biotechnology, Rivers State University, Nigeria (voucher number SUK 5279). Two kg of plant sample was ground by a mechanical grinding machine and then macerated with water (3 L) for 24 hours. After filtration and lyophilization, 59.2 g of extract was obtained. The solution (1 g/mL) was prepared by dissolving the extract in distilled water freshly each time before use for administration. The extracts were stored until use at 4°C.

Animals for experiment

Thirty adult female albino rats weighing 250 to 300 g were obtained from the Department of Pharmacology and Therapeutics, Rivers State University, Port Harcourt, Nigeria and were used in this study.

Preparation of standard drug (simvastatin)

Simvastatin was obtained from a commercial Pharmacy in Port Harcourt. Simvastatin (20 mg) was dissolved in 12.5 mL of normal saline in a beaker for a 1.6 mg/mL concentration.

Administration of standard drug (simvastatin)

The standard drug simvastatin was prepared daily and administered using an oral gavage tube to the experimental animals in group 3 as a positive control.

Induction of hyperlipidemia

The female rats with an average body weight of 250 to 300 g were induced hyperlipidemia using a High-Fat Diet (HFD) for 7 days. The composition of the high-fat diet following Kadir et al. (2015) contained 414.0 kcal/100 g, with a composition of 43% carbohydrates, 40% fat, and 17% protein (Table 1). The diets were a mixture of 68% powdered rat feed (standard rat chow pellet), 20% instant milk powder (peak milk), 6% corn oil (Mazola), and 6% ghee (popularly known as *manshanu* in Northern Nigeria). All ingredients for high-fat feed are mixed thoroughly and baked at 65°C in an oven overnight. A standard rat chow diet contains 306.2 kcal/100 g, with 3% fat, 21% protein, and 48.8% carbohydrate. Hyperlipidemia was confirmed by measuring rats' lipoproteins and serum lipids levels.

Experimental design

The animals were weighed before the experiment, observed for physical symptoms, and recorded. The rats were acclimatized for 7 days and were fed ad libitum. The treatment procedure was as follows*:* Thirty (30) albino rats weighing 250-300 g were grouped into 5 groups, with 6 rats in each group. (i) Group 1: Normal Control group (standard feed + water); (ii) Group 2: Negative Control Group (HFD + water); (iii) Group 3: Positive Control Group (HFD + simvastatin (standard drug) + water); (iv) Group 4: 200mg/kg *K. pinnata* extract (HFD + 200 mg/kg *K. pinnata* + water); (v) Group 5: 400mg/kg *K. pinnata* extract (HFD + 400 mg/kg *K. pinnata* + water).

Table 1. Composition of a high-fat diet (Kadir et al. 2015)

High-fat diet	
Nutrients	**%/100 g**
Carbohydrate	43
Protein	17
Fat	40
Ingredients	**g/100 g**
Powdered rat feed	68.0
Maize oil	6.0
Ghee	6.0
Milk powder	20.0
Total energy (kcal/100 g)	414.0

Administration of extracts

Group 4 was treated with *K. pinnata* extract at 200 mg/kg BW and 400 mg/kg BW for group 5 using oral gavage for 21 days.

Body weight gain

The impact of treatments on body weight gain was assessed weekly on each rat using an electronic weighing balance (KERN 440-35 N) throughout the study period. The % mean body weight difference was calculated using the formula as described by Odinga et al. (2023):

$$\% \text{ mean body weight difference} = \frac{Final\ weight - Initial\ weight}{initial\ weight} \times 100$$

Sample collection for biochemical analysis

The experiment lasted for 21 days, after which animals were sacrificed. At the end of the treatment, the animals were fasted for twenty-four hours. The rats were put in a desiccator and allowed to anesthetize slightly following the absence of oxygen, and blood samples were collected by jugular venipuncture. The blood samples (2 mL) were collected into sterile plain sample bottles for each rat, agitated slowly, and appropriately covered. The blood samples were analyzed for antioxidant biomarkers, i.e., Reduced Glutathione (GSH), Glutathione-S-transferase (GST), Glutathione Peroxidase (GPx), Catalase, Superoxide Dismutase (SOD), Malondialdehyde (MDA)), lipid profile (Triglyceride (TG), Total Cholesterol (TC), Low-Density Lipoprotein (LDL), High Density Lipoprotein (HDL)), troponin, creatine kinase, and myoglobin.

Determination of antioxidant biomarkers

The antioxidant activity of *K. pinnata* aqueous leaf extract was evaluated by measuring the antioxidant markers in the serum of the experimental albino rats.

Estimation of malondialdehyde

The malondialdehyde level was calculated using the method of Bahekar and Kale (2016). The malondialdehyde (MDA) level in the plasma samples was measured to indicate lipid peroxidation. MDA is one of the aldehyde products formed during lipid peroxidation. It reacts with thiobarbituric acid (TBA) to produce a colored product. The absorbance of this colored complex was measured spectrophotometrically at 530 nm. 0.5 mL of serum was taken and put in test tubes, added with 3 mL of 10% trichloroacetic acid (TCA), mixed well, and the tubes were left to stand at room temperature for 10 minutes. The tubes were centrifuged for 15 minutes at 5,000 rpm, and 2 sets of test tubes were prepared - one for the blank and one for the test sample. For the test sample, 2 mL of the supernatant was added with 1.5 mL of 0.67% TBA. For the blank, 2 mL of distilled water was added with 2 mL of 0.67% TBA. The tubes were mixed well and then placed in a boiling water bath for 10 minutes; a pale pink color developed after cooling under tap water. The color intensity was measured using a colorimeter at 530 nm. The MDA concentration was calculated by the molar extinction coefficient 1.5×10^5 and expressed as nmol of MDA per 100 mL of serum.

1.5 = 100 µmol/L (here, 100 is conversion from mL to dL)

Then MDA = 100 × OD of unknown/1.5

Where: O.D = Optical density

Estimation of superoxide dismutase

Superoxide dismutase was estimated using the method of Bahekar and Kale (2016). This method utilizes the inhibition of auto-oxidation of pyrogallol by superoxide dismutase (SOD) enzyme. 3 mL mixture consisted of 100 µL each of 0.2 mM pyrogallol, 1 mM EDTA, 1 mM DTPA, and 100 µL of serum in air-equilibrated tris-HCl buffer (50 mM; pH 8.2). The reaction mixture prepared in 3 sets includes standard, test, and control. Pyrogallol was added after all other reagents to start the reaction. The initial 10-second period was considered as the induction period of the enzyme. After 10 seconds, a change in absorbance at 420 nm at 10 s intervals was recorded for 4 min. The average change in the absorbance per minute was calculated. One unit of enzyme SOD was defined as the amount of enzyme to cause 50% inhibition of pyrogallol auto-oxidation.

Estimation of reduced glutathione

Reduced glutathione was estimated following the method of Gabriel-Brisibe et al. (2020). The method is based on developing a yellow color when DTNB (Ellman's Reagent) is added to sulphydryl compounds due to a redox reaction between GSH and DTNB. The developed color was reasonably stable for about 10 min, and temperature variation slightly affected the reaction. The absorbance was measured at 412 nm. GSH in red cells is relatively stable, and venous blood samples anticoagulated with ACD maintain GSH levels for up to 3 weeks at 4°C.

Estimation of catalase

Catalase was estimated using the method of Bahekar and Kale (2016) and Gabriel-Brisibe et al. (2020). This method carefully controls the reduction of dichromate in acetic acid to chromate acetate when heated with H_2O_2, ensuring a predictable reaction. The resulting chromic acetate was measured colorimetrically at 570 nm. Catalase (CAT) enables the separation of H_2O_2. Three sets of tubes were prepared and labeled as blank, test (0 s), and test (60 s) and added with the appropriate reagents to each tube. The tubes were boiled for 10 minutes, cooled to room temperature, and the absorbance was read at 570 nm. The analysis used various H_2O_2 concentrations ranging from 10 to 160 µmoles. One unit of catalase (CAT) activity is defined as the amount of enzyme that decomposes 1 µmole of H_2O_2 per minute.

Evaluation of the lipid profile

After the separation of serum from the whole blood, the various parameters of the lipid profile were estimated using standard laboratory procedures: Total Cholesterol (TC) (Stockbridge et al. 1989), Triglycerides (TG) (Annoni et al. 1982), Low-Density Lipoprotein Cholesterol (LDL) and High-Density Lipoprotein Cholesterol (HDL) (Assmann

1979). Serum LDL was calculated using the following formula by Odinga et al. (2020):

$$\text{LDL (mg/dL)} = \frac{TC-HDL-TG}{5}$$

Evaluation of the clinical markers

Blood was collected in 10 mL heparin-coated tubes and centrifuged without delay. Cells were discarded, and plasma was analyzed using the method described by de Winter et al. (1995) and Fiolet et al. (1977):

Myoglobin

The myoglobin assay (Turbiquant myoglobin, Behringwerke) was used with the Behring Turbitimer analyzer for rapid immunoturbidimetric determination of myoglobin concentrations in plasma. This assay is based on polystyrene particles coated with rabbit anti-human myoglobin antibodies, which form agglutinates with myoglobin present in serum or plasma. The agglutination causes an increase in turbidity, which is measured with a photometer. The measurement range was 50 to 650 ng/mL. The upper reference limit was 90 ng/mL. The turnaround time of the assay was 20 minutes.

Troponin T

Troponin T was measured using an ELISA method (Boehringer Mannheim, product 1289055) on an ES300 analyzer (Boehringer Mannheim). The upper reference limit was 0.1 ng/mL, and the linearity range of this determination was 0 to 15 ng/mL. The turnaround time of the assay was 2.5 hours.

Statistical analysis

IBM SPSS Version 25 was used to analyze the data. The data were analyzed using one-way analysis of variance (ANOVA) and presented as mean ± standard deviation, and Turkey's post hoc test was used for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The results in Table 2 indicated differences in the percentage body weight gain of hyperlipidemic rats treated with different *K. pinnata* extract concentrations. The 400 mg/kg BW treatment had the lowest body weight gain, similar to normal control. The body weight gain of the rats, as shown in their final body weight, could be attributed to their food and water intake *ad libitum* throughout the experimental period with a high-fat diet to induce hyperlipidemia. Odinga et al. (2023) reported that feed with a high content of nutrients and calories increases body weight.

Table 2. % Mean body weight difference of experimental rats

Group	Initial weight (g)	Final body weight (g)	% mean body weight difference
Normal control	159.00±21.49	178.67±8.62	12.37
Negative control	133.67±2.67	171.67±17.17	28.43
Positive control	160.50±2.59	194.00±22.69	19.38
200 mg/kg *K. Pinnata*	153.67±3.98	196.50±31.14	27.87
400 mg/kg *K. pinnata*	146.17±5.49	166.33±16.59	13.79

Note: Values are mean (M) ± Standard Deviation (SD)

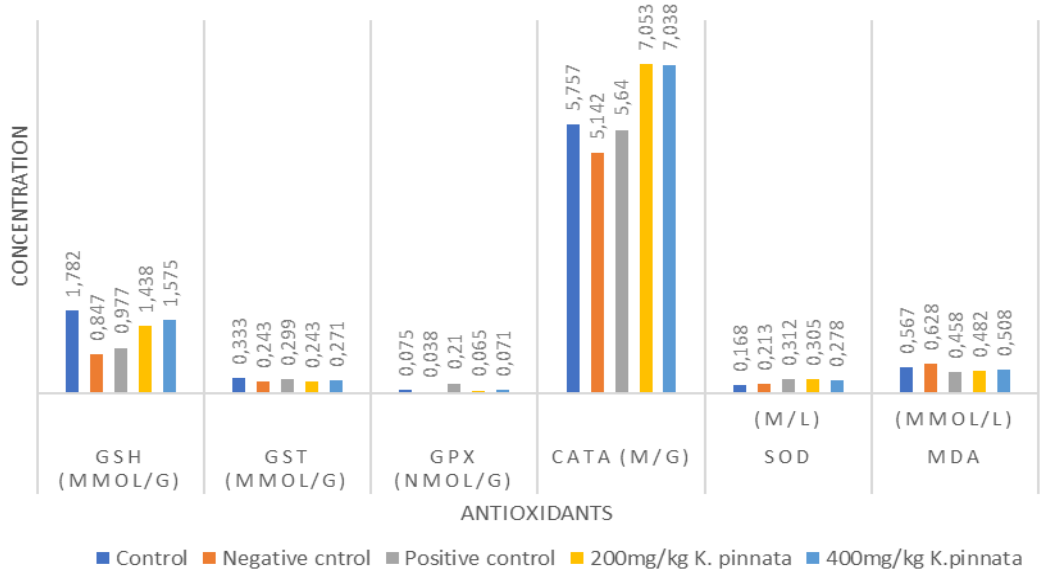

Figure 1. Effect of aqueous extract of *Kalanchoe pinnata* on the antioxidant status of experimental rat model

Table 3. Lipid profile of hyperlipidemic rats treated with aqueous extract of *Kalanchoe pinnata*

Groups	TC (mmol/L)	TG (mmol/L)	HDLC (mmol/L)	LDLC (mmol/L)
Control	3.067±0.493a	1.397±0.228a	1.280±0.232a	2.458±0.391a
Negative control	3.167±0.441a	1.502±0.224a	1.310±0.279a	2.362±0.289ab
Positive control	2.667±0.829a	1.303±0.296a	1.428±0.213ac	1.930±0.839c
200mg/kg *K. pinnata*	2.783±0.412a	1.312±0.227a	1.455±0.283d	1.937±0.383d
400mg/kg *K. pinnata*	2.700±0.693a	1.203±0.355ae	1.487±0.322de	1.935±0.798e

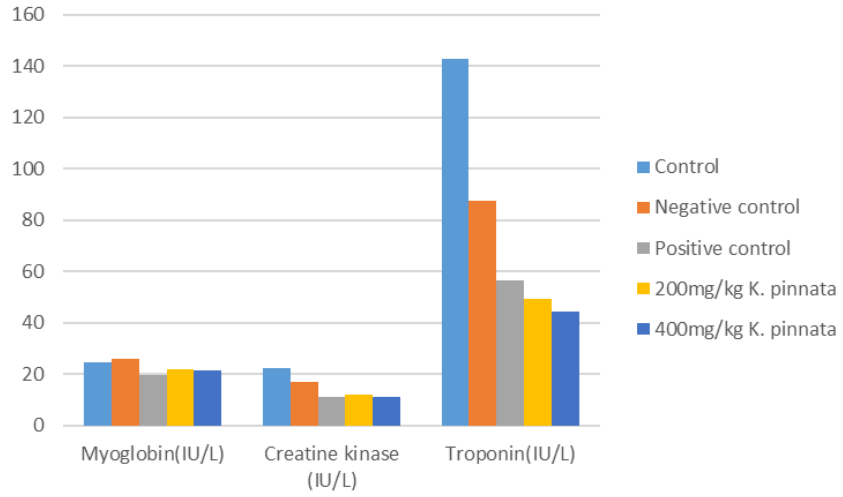

Figure 2. Effect of aqueous extract of *Kalanchoe pinnata* on the myoglobin, creatine kinase, and troponin serum levels of experimental rat model

Figure 1 represents the antioxidant status of experimental hyperlipidemic rats administered with the aqueous extract of *K. pinnata*. The result revealed that the administration of *K. pinnata* extract increased the serum GSH, GST, catalase, and SOD level of the rats. However, the serum MDA and GPx decreased. These findings indicated that the extract contained a significant amount of antioxidants, as evidenced by the increased concentrations of the antioxidant markers GSH, GST, GPx, catalase, and SOD in the serum of rats administered 200 mg/kg and 400 mg/kg of *K. pinnata.* These antioxidant parameters in the treated groups were higher than those in the negative control group, which was not administered with the extract of *K. pinnata* after the inducement of hyperlipidemia. Ramon et al. (2023) reported that *K. pinnata* contained quercetin, kaempferol, apigenin, ECGC, and avicularin, which have antioxidant activity. The findings suggest that the increase in antioxidant markers following the administration of the aqueous extract of *K. pinnata* may be related to its phytochemical contents. It also indicates that the extract of *K. pinnata* has therapeutic potential against diabetes and inflammation.

Glutathione (GSH) is crucial in cellular processes and redox homeostasis. Any deficiency or imbalance in the GSH/GSSG ratio increases cell susceptibility to oxidative stress, inflammation, and tumor development. Conversely, increased GSH levels increase antioxidant capacity and resistance to oxidative stress, which is observed in many types of tumors. The addition of exogenous GSH inhibited the inflammatory response by regulating Reactive Oxygen Species (ROS). However, the role of endogenous GSH in fine-tuning the innate immune response and thus modulating inflammation is also highly significant. GSH acts as an antioxidant, scavenging ROS during oxidative stress, and a signaling molecule that regulates protein function through thiol-disulfide exchange reactions, such as protein glutathionylation. Previous studies showed that GSH can regulate the activity of various oncogenes (e.g., p53, HIF-1, c-jun) through these mechanisms (Kennedy et al. 2020).

Pahwa et al. (2017) reported that increased GST activity in cardiovascular disease patients, especially those with type 2 diabetes, suggests a protective mechanism against increased oxidative stress. Glutathione S-Transferase (GST) is an important enzyme in detoxification and helps lower oxidative stress. This enzyme might be induced under oxidative stress conditions as a protective mechanism.

GPx-1 is a crucial antioxidant enzyme that prevents the harmful accumulation of intracellular hydrogen peroxide. It is present in all cells and is found in cytosolic, mitochondrial, and, in some cells, peroxisomal compartments. It is more effective than catalase at removing intracellular peroxides under many physiological conditions (Savas et al. 2006).

Nandi et al. (2019) noted that catalase is one of the crucial antioxidant enzymes that mitigates oxidative stress by destroying cellular hydrogen peroxide to produce water

and oxygen. Its deficiency or malfunction is related to the pathogenesis of many age-associated degenerative diseases like diabetes mellitus, hypertension, anemia, vitiligo, Alzheimer's disease, Parkinson's disease, bipolar disorder, cancer, and schizophrenia.

SOD is an enzymatic antioxidant that catalyzes the conversion of O_2 to H_2O_2 and helps maintain the redox balance by diffusing the superoxide. Therapeutically, increasing the levels of SOD could be a strategy in oxidative stress-induced pathology (Xue et al. 2021). MDA has been reported as a polyunsaturated fatty acid peroxidation product (Gaweł et al. 2004); thus, it implies the pathogenesis of various disease conditions. Therefore, the MDA level was expected to be decreased by *K. pinnata* administration.

The antioxidant activity in this study may be due to the combined action of the bioflavonoids in *K. pinnata*. Quercetin, a natural flavonoid detected in *K. pinnata*, is therapeutic against type II diabetes by acting as an anti-inflammatory and antioxidant. Hence, *K.pinnata is* a promising source of natural antioxidants.

Table 3 revealed a decrease in the serum TC concentration and Low-Density Lipoprotein Cholesterol (LDLC) of the experimental hyperlipidemic rats administered with 200 mg/kg and 400 mg/kg of *K. pinnata.* The serum HDLC increased in the groups treated with 200 and 400 mg/kg aqueous extracts of *K. pinnata*.

HFD has been used to induce hyperlipidemia in rat models (Pande and Dubey 2009) and cause an elevation in the TC and LDL Cholesterol levels in the serum (Sampathkumar et al. 2011; Odinga et al. 2020). High serum levels of LDL Cholesterol could predispose to most cardiovascular diseases, such as atherosclerosis (Ahmad et al. 2018)

Table 2 showed a decrease in TC, TG, and LDLC concentration in the groups treated with 200 mg/kg and 400 mg/kg aqueous extracts of *K. pinnata* compared to the normal and negative groups; however, the levels of HDLC increased. However, the 400 mg/kg of K. pinnata administration caused the most significant decrease in TC, TG, and LDLC levels. Ahmad et al. (2018) reported that plant extract lowers TC levels by increasing bile acid excretion and preventing reabsorption from the small intestine by disrupting bile acid's micelle formation. Plants' ability to lower TC may be due to the phytochemical composition of plants (Rabizadeh et al. 2022). The increased excretion of bile acid and cholesterol activates cholesterol 7α-hydroxylase and enhances the conversion of liver cholesterol to bile acid, thus reducing cholesterol.

Triglyceride levels in the serum of the experimental rats were significantly reduced after 21 days of treatment with 400 mg/kg of *K. pinnata* (Table 2). Elevated serum TG levels are indicative of pathological conditions related to arterial hardening, which increases the risk of stroke, heart attacks, and heart disease (Jin et al. 2023). Elevated TG levels could arise from complications associated with a high-fat diet, such as obesity (Jin et al. 2023). The reduction in the TG level in the group treated with 200 and 400 mg/kg *K. pinnata* could be attributed to the presence of glycosides in *K. pinnata* (Pavani et al. 2024) that could enhance the lipase enzyme activity in the liver, thereby resulting in the catabolism of lipids. Singh et al. (2013) and Akbarzadeh et al. (2015) also reported that a decrease in TG could also be due to the inhibition of dietary lipid absorption in the intestine by reducing micellar solubilization of cholesterols and by increasing the excretion of TG through feces.

The present study showed increased serum HDLC levels in hyperlipidemic rats treated with *K. pinnata* extract at 200 mg/kg and 400 mg/kg BW. High HDL levels are associated with a lower risk of heart disease. Cho and Jung (2021) showed that the higher the HDL-C level, the better in lowering mortality caused by cardiovascular disease and myocardial infarction risk. Odinga et al. (2020) reported that HDLC helps remove cholesterol from the bloodstream and returns to the liver, where it is catabolized and excreted from the body. It implies that an increase in HDLC serum levels is a good indication. HDLC is commonly known as the good cholesterol.

The LDLC levels in the groups treated with 200 mg/kg and 400 mg/kg BW of *K. pinnata* were lower than the normal and negative control groups. Serum LDLC, also known as bad cholesterol, might be caused by saturated fats and cholesterol in high-fat feeds. LDLC could cause fatty deposits in blood vessels, blocking blood flow in the arteries (Rafieian-Kopaei et al. 2014). The fatty deposits have the potency to form clots, leading to myocardial infarction and stroke (Tanka-Salamon et al. 2016). Ivanova et al. (2021) reported that a plant-based diet effectively lowers LDL cholesterol. The decreased LDLC in the experimental rats treated with *K. pinnata* extract could be due to bioactive compounds with anti-inflammatory and hypolipidemic activities (Odinga et al. 2016). Odinga et al. (2020), in their study on the Antihyperlipidemic effects of *Ricinodendron heudelotii*, reported that LDLC does not facilitate the removal of cholesterol from the body, unlike HDLC. Instead, it deposits cholesterol onto the walls of blood vessels; therefore, elevated levels of LDLC can lead to the accumulation of cholesterol and triglycerides along critical blood vessels. In the long term, it could lead to cardiovascular diseases. Additionally, Zawirska-Wojtasiak et al. (2019) reported the therapeutic use of the leaves of *K. pinnata* for antimicrobial, anti-inflammatory, and antiseptic activities. They also reported that *K. pinnata* is vitamin C-rich.

Myoglobin, CK/CK-MB, and troponin serum levels are crucial because they diagnose Acute Myocardial Infarction (AMI) during the onset of symptoms and emergencies (de Winter et al. 1995). These levels can be affected by various risk factors (Odum and Young 2018). Chiu et al. (1999) suggested combining myoglobin, CK-MB, and troponin parameters could be valuable information in managing Acute Myocardial Infarction (AMI). Figure 2 shows elevated serum myoglobin in the hyperlipidemic rats without any other treatment or negative control. Duan et al. (2018) and Wali et al. (2020) reported that a high-fat diet might affect body metabolism, heart, and muscles. Woo et al. (1995) stated the importance of determining myoglobin level in diagnosing AMI. The experimental rat groups administered standard drugs and *K. pinnata* at various

concentrations had lower serum myoglobin than the control and negative control groups. Detecting cardiac markers such as troponin, myoglobin, and Heart-type Fatty Acid-Binding Protein (H-FABP) could have higher sensitivity and specificity in diagnosing AMI than any single detection. It can provide better data supporting the AMI diagnosis (Sun et al. 2023). Sax et al. (1997) concluded that the ratio of CK-MBm to CK levels reflects, to some extent, the severity of coronary disease and that pre-infarction beta-blockade may lead to lower CK-MB levels.

In conclusion, the administration of *K. pinnata* aqueous extract to induced hyperlipidemic rats reduced the body weight gain, decreased serum concentrations of LDLC, TG, and TC, and increased HDLC serum concentration. It confirms the hypolipidemic activity of *K. pinnata.* The administration of 400 mg/kg body weight of *K.pinnata* aqueous extract had the best results. With the increasing prevalence of hyperlipidemia, primarily driven by risk factors such as dietary habits, *K. pinnata* appears to be a promising natural remedy. This research indicates that *K. pinnata* could serve as a source of natural medicine, and further studies should investigate its potential as a nutritional supplement.

It is recommended that *K. pinnata* be used as an antioxidant and antihyperlipidemic source. This study recommends further elucidation of the effect of the bioactive compounds in *K. pinnata* on health and their possible dose limits.

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