Evaluation of the *in vivo* antioxidant, toxicological and chromatographical profiling of leaf extract and fractions of *Crescentia cujete* Linn. (Bignoniaceae)

Amarachukwu Ukamaka Anwuchaepe[1], Felix Ahamefule Onyegbule[1, 2]*, Daniel Lotanna Ajaghaku[3], Felix Ifeanyi Nwafor[4], Festus Basden C. Okoye[2]

[1]*Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria*
[2]*Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria*
[3]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Agbani, Enugu State, Nigeria*
[4]*Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka, Enugu State, Nigeria*

ABSTRACT

Oxidative stress has been reported to be involved in many diseases pathogenesis. This study evaluated the antioxidant potentials of *Crescentia cujete* leaf. The leaves were extracted in methanol by cold maceration and fractionated into n-hexane (NHF), ethyl acetate and butanol fractions. The *in vitro* antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazil (DPPH) and ferric reducing antioxidant power (FRAP) tests while Catalase (CAT), Superoxide dismutase (SOD) and Lipid peroxidation (LPO) assays were used to evaluate *in vivo* antioxidant potentials. The constituents of the extract and fractions where analyzed using high performance liquid chromatography coupled to diode array detector (HPLC-DAD). The median lethal dose (LD$_{50}$) of CME and serum liver marker (SLM) enzymes were evaluated using standard protocols. The crude methanol extract (CME) and the fractions showed concentration dependent *in vitro* antioxidant activity (EC$_{50}$ values of 15.22 to 569.22 μg/ml for DPPH and 54.69 to 581.40 μg/ml for FRAP) with ethyl acetate fraction (EAF) showing the highest activity. The CME at 400 mg/kg and EAF at 200 mg/kg showed more than 3-fold increase in CAT and SOD activities compared with the vehicle treated group. EAF (400 mg/kg) significantly (p< 0.05) inhibited LPO. HPLC-DAD analysis of CME and EAF revealed the presence of phenolic compounds (chlorogenic acid, protocatechuic acid and several quercetin derivatives). The LD$_{50}$ of CME was above 5000 mg/kg. The CME and EAF showed significant (p< 0.05) reduction of SLM enzymes. The study validated the antioxidant activity of *C. Cujete,* which may be connected to high abundance of the detected phenolic compounds.

Key words: *Crescentia cujete*, oxidative stress, FRAP, DPPH, LD$_{50}$, liver enzymes.

Introduction

Free radicals are constantly produced in all living

---

*Correspondence
Dr. Felix Ahamefule Onyegbule
 Department of Pharmaceutical and Medicinal Chemistry, Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
E Mail: af.onyegbule@unizik.edu.ng

organisms with damaging effects that result to cell injury or death [1].
These free radicals can be generated by both external and biological processes. When the generation of free radicals overwhelms the antioxidant capacity of the biological defence system; it gives rise to oxidative stress [2].
Oxidative damage to proteins, lipid membranes and genetic molecules play a significant role in disease pathogenesis [3] such as cancer [4,5],

neurodegenerative diseases [6, 7], metabolic [8, 9] and cardiovascular disorders [10]. Therefore, antioxidants are highly important in maintaining optimal well-being. Plants are abundant sources of antioxidants. Plants often owe their medicinal properties to their antioxidant activities [11- 14].

Crescentia cujete **Linn (Bignoniaceae)** is commonly known as calabash tree. It is widely distributed in the Caribbean region, Mexico, Northern and Southern American and later introduced to tropical Africa from Senegal to Cameroon then to other parts of Africa. In Nigeria, it is called osisi mkpo/oba in Igbo tribe, igi igba in Yoruba tribe while the Bini and Fufulde tribes call it uko and gumbusi mboro respectively [15]. Traditionally, *C. cujete* have been reported to be used among many tribes and different culture around the world to treat different aliments [16]. The fruit mesocarp is used as a febrifuge, purgative and diuretic while the fruit decoction is used for treating hypertension, stomach ache, cold and coughs [17]. The fresh leaves are used topically for wound healing while the powdered leaves are used for headaches, and internally as a diuretic and in the treatment of hematomas and tumors. Since oxidative stress has been implicated in the pathogenesis of many diseases [3], we hypothesized that the use of *C. cujete* in ethnomedicine for the treatment of the mentioned above aliments could be attributed to its antioxidant activity. The antimicrobial activity of the leaf extracts of *C. cujete* [19] has been reported. Due to the complexity of the biological system, direct correlation of *in vitro* pharmacological activities with *in vivo* applications is not always guaranteed. Depending on the reaction environment, some antioxidants can behave as pro-oxidants; thus, the need for bioassay guided evaluation of *in vitro* and subsequent validation of the *in vitro* results with *in vivo* experiments. Inspired by the ethnomedicinal uses of *C. cujete* leaf, this study was designed to evaluate the *in vitro* and *in vivo* antioxidant potentials of *C. cujete* leaf extracts and fractions with emphasis on validation of the *in vitro* results with its *in vivo* antioxidant activity assays; using multiple assay models.

Materials and methods

Plant material

The leaves of *C. cujete* were sourced from Nanka in Anambra State, Nigeria. The plant collected was authenticated by a trained taxonomist, Mr. Felix Nwafor of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka. A voucher specimen (PCG 474/A/035) of the plant was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka for future reference. The plant material was subsequently cleaned, air-dried under room temperature for 14 days and pulverized with a mechanical grinding machine.

Animals

Swiss Albino mice (25 – 30 g) were used for this study. The animals were obtained from the animal house of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Awka. The animals were sheltered in standard laboratory conditions of 12 h light, room temperature, 40-60% relative humidity and fed with commercial rodent feed (Guinea Feeds Nigeria Ltd). They were allowed access to food and water *ad libitum*. All the animal experiments were conducted in compliance with National Institute of health (NIH) guide for care and use of laboratory animals [20].

Extraction and fractionation

The pulverized plant (2kg) was cold macerated in 5 L of methanol for 72 h at room temperature. The resulting solution was filtered, the filtrate concentrated to dryness *in vacuo* using rotary evaporator (RE300 Model, United Kingdom) at 40°C to obtain the crude methanol extract (CME). A 79.23 g of the CME was subjected to liquid-liquid partition with n-hexane, ethyl acetate and butanol in increasing order of polarity. The fractions were concentrated *in vacuo* using rotary evaporator at 40°C to obtain the n-hexane (NHF), ethyl acetate (EAF) and butanol (BF) soluble fractions. The CME and all the fractions were stored in refrigerator between 0-4°C until they were used.

Quantification of the total phenolic content

The total phenolic content was quantified by folin-Ciocalteu's method as described by [21].

High performance liquid chromatography (HPLC) analysis

HPLC analysis of the CME and fractions were done using Dionex P580 HPLC system coupled to photodiode array detector (UVD340S, Dionex Softtron GmbH, Germany). Detection was at 235, 254 and 340 nm.

In vitro antioxidant screening

The *in vitro* free radical scavenging activities of the CME and fractions were evaluated using DPPH test [22] and ferric reducing antioxidant power (FRAP) assay [23]. Serial dilutions (500 – 15.625 ug/ml) of the CME and fractions were tested and the plot of percentage inhibitions against concentrations were used to derive the half maximum effective concentration (EC_{50}) of the CME and fractions. The fraction with the highest *in vitro* antioxidant activity was selected for further *in vivo* analysis along with the CME.

Acute toxicity test

Acute toxicity test was performed using Organization of Economic Co-operation and Development (OECD) guidelines for the testing of chemicals [24]. Six Swiss albino mice of either sex selected at random were employed in this study. The animals were fasted over night with free access to water. The CME at 2000 and 5000 mg/kg were used for determining the acute limit dose. Obvious toxicity and mortality were monitored for 14 days.

***In vivo* antioxidant screening**

Forty-two Swiss albino mice were divided into seven groups of six mice each and treated with the CME and fraction for 10 days. Groups 1 and 2 were treated with 200 and 400 mg/kg of CME respectively; while groups 3 and 4 were treated with 200 mg/kg and 400 mg/kg of EAF respectively; group 5 served as the naïve (un-induced control); group 6 received 100 mg/kg of ascorbic acid (reference) while group 7 was treated with 10 ml/kg of the vehicle (5% Tween 80). Six hours after the last day of the treatment oxidative stress was induced with carbon tetrachloride (CCl_4) (2 ml/kg) intraperitoneal in the animals in all the groups except the naive group. Eighteen hours later, blood samples were collected from all the animals through retro-orbital puncture and the liver harvested through surgical dissection. The blood samples collected were centrifuged at 3000 rpm using a centrifuge (Model TGL-20M, China) for 10 min and the supernatant was decanted to get the serum. The harvested liver was rinsed in phosphate buffer saline, homogenized and centrifuged at 12,000 rpm for 20 min at 4°C; the supernatant was decanted and stored at 4°C. The serum was used for the estimation of serum liver marker enzymes - ALP, AST and ALT- while the supernatant of the liver homogenate was used for the determination of liver antioxidant enzymes [catalase and superoxide dismutase (SOD)] and lipid peroxidation. Histological examination of an animal from each group was done using H&E staining procedure according to [25].

Evaluation of liver antioxidant enzyme activity and lipid peroxidation

Catalase activity was evaluated by the ability of the enzyme to degrade hydrogen peroxide as described by Sinha [26] while SOD enzyme activity was determined by its ability to inhibit the auto-oxidation of adrenaline as described by Olatosin *et al* [27]. Lipid peroxidation was assessed through maolondialdehyde (MDA) - an aldehyde product of lipid peroxidation using the method of Gutteridge and Wilkins [28].

Biochemical assay of serum liver marker enzymes

The enzyme activities were determined using commercial reagent kits (Randox Laboratories Limited, United Kingdom). Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) were determined according to the method of Reitman and Frankel [29] while the alkaline phosphatase (ALP) was determined according to the method of King and Amstrong [30].

Statistical analysis

The results were analyzed using SPSS version 16 and presented as Mean ± standard error of mean (SEM). Significance between control and CME and fraction treated groups were determined using student t-test and one way ANOVA. Differences between means were considered statistically significant at $P < 0.05$.

Results

Extraction yield of the CME and Fractions of *C. cujete* leaves

The extraction yield of CME and fractions of *C. Cujete* leaves are shown in Table 1. Two kilogram of *C. Cujete* leaves yielded 97.93 g of CME. BF (41.13 g) gave the highest yield while NHF (8.1 g) gave the lowest yield.

Table 1: Extraction yield of the CME and Fractions of *C. cujete* leaves

Extracts/Fractions	Yield (g)	Yield (%w/w)
CME	97.93	4.90[a]
NHF	8.1	10.22[b]
EAF	30	37.86[b]
BF	41.13	51.91[b]

[a] = Yield calculated from 2000 g of powdered leaves; [b] = Yield calculated from 79.23 g of methanol extract; CME = Crude methanol extract; EAF = Ethyl acetate fraction; BF = Butanol fraction; NHF = n-hexane fraction.

DPPH scavenging activity

The DPPH scavenging activity of CME and various fractions is shown in Figure 1 using AA as reference. CME, EAF and BF greatly diminished the deep purple colour of DPPH which is the evidence of their antioxidant activity. EAF showed the best antioxidant activity with EC_{50} (Table 2) of 15. 22 µg/ml close to

that of AA (14.48 µg/ml) while the NHF showed the least antioxidant activity with EC_{50} 569.22 µg/ml

Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) of the CME and fractions of *C. cujete* with their EC_{50}'s are shown in Figure 2 and Table 2 respectively. The order of the FRAP activity of CME and its various fractions is as follows; EAF> BF>CME>NHF. The EAF showed the best antioxidant activity with EC_{50} 54.69µg/ml not very close to AA (31. 25 µg/ml). The CME and fractions showed lower FRAP activity compared with their DPPH activity. The weak antioxidant activity exhibited by the FRAP assay may be as a result of the fact that FRAP cannot detect species that act by radical quenching (H transfer), particularly sulfhydryl group containing antioxidants like thiols, such as glutathione

and proteins [31, 32]. Also, some phenolic compounds have been reported to react more slowly in FRAP assay [31].

Total phenolic content

The total phenolic content (TPC) of the CME and fractions are presented in Table 2. The DPPH results are in agreement with the phenolic contents obtained for the CME and fractions. The CME and its various fractions contained considerable amount of phenolic compounds. The highest amount of phenolics was found in EAF (384 mgGAE/g) while the lowest amount of phenolics was found in NHF (192 mgGAE/g). Based on the results of the DPPH, FRAP and TPC assay, further *in vivo* antioxidant analysis was carried out on the EAF along with the CME.
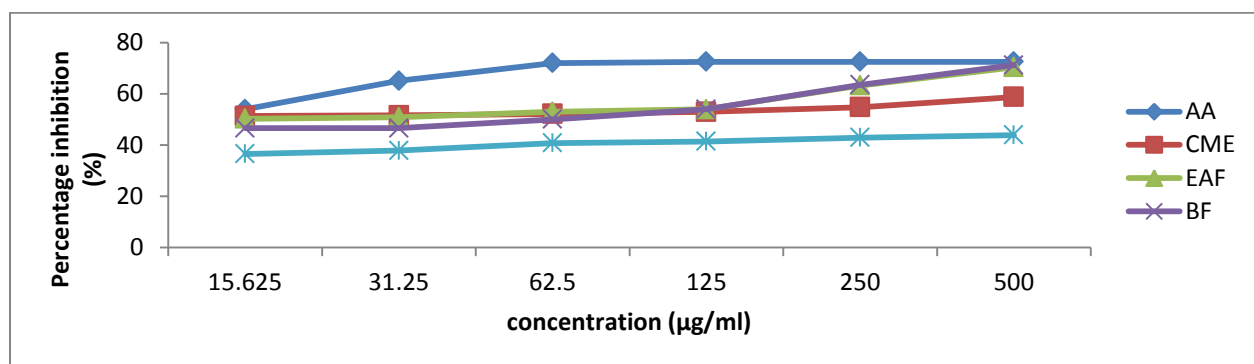

Fig 1: Graph of DPPH scavenging activity of the CME and fractions

AA = ascorbic acid; CME = crude methanol extract; EAF = ethyl acetate fraction; BF = butanol fraction; NHF = n-hexane fraction
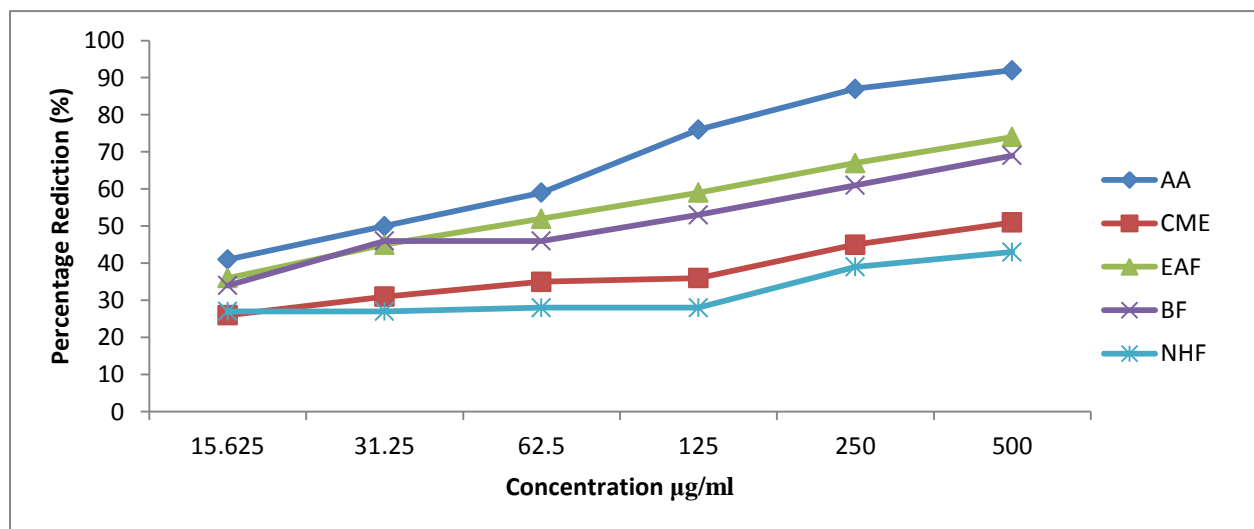

Fig 2: Graph of ferric reducing antioxidant power of the CME and fractions

AA = ascorbic acid; CME = crude methanol extract; EAF = ethyl acetate fraction; BF = butanol fraction; NHF = n-hexane fraction.

Table 2: Half maximum effective concentration (EC$_{50}$) and total phenolic content of the CME and fractions

Extract/Fractions	DPPH EC$_{50}$(μg/ml)	FRAP EC$_{50}$(μg/ml)	Total phenolic content (mgGAE/g)
CME	15.54	458.34	304 ± 0.15
EAF	15.22	54.69	384± 0.02
BF	62.50	93.75	240± 0.01
NHF	569.22	581.40	192± 0.06
AA	14.48	31.25	-

AA = ascorbic acid; CME = crude methanol extract; EAF = ethyl acetate fraction; BF = butanol fraction; NHF = n-hexane fraction

In vivo antioxidant activity
Catalase and SOD enzyme activity
The effects of CME and EAF on the activities of catalase and SOD enzymes are presented in Table 3. The CME and EAF at 200 and 400 mg/kg produced a dose dependent activity in catalase SOD and LPO analysis. The ability of CCl$_4$ to induce oxidative stress in the experimental animals was evident in the decreased level of catalase and SOD activity in the CCl$_4$ treated groups compared to the naïve group. However, pre-treatment with CME and EAF at a dose of 200 and 400 mg/kg for 10 days showed significant ($P < 0.05$) restoration of the antioxidant enzymes near to normal values. The 5% tween 80 control group showed the lowest level of antioxidant enzyme activity.

Lipid peroxidation
In this investigation (Table 3), increased MDA level was observed in the liver of CCl$_4$ treated groups suggesting enhanced lipid peroxidation leading to failure of antioxidant defence mechanism to prevent formation of excessive free radicals. Pre-treatment with 400 mg/kg CME and EAF significantly ($P < 0.05$) decreased the MDA level compared with the 5% tween 80 control group. At 400 mg/kg, CME and EAF showed comparable MDA level with the naïve and AA control group. This finding reveals that the CME and EAF at higher concentration (400 mg/kg) significantly reduced MDA level in mice.

Table 3: Effect of CME and EAF on liver antioxidant enzymes and lipid peroxidation

Treatment	Dose (mg/kg)	Catalase (μmol)	SOD(U/ml)	MDA(nmol/ml)
CME	200	*[a]21.88 ± 11.74	*12.00 ± 1.14	[ab]2.31 ± 0.51
CME	400	*34.95 ± 7.27	*13.60 ± 0.93	*2.04 ± 0.51
EAF	200	*30.25 ± 9.30	*12.00 ± 1.14	[ab]2.32 ± 0.09
EAF	400	*36.69 ± 14.98	*13.80 ± 1.20	*1.42 ± 0.24
AA (Reference)	100	*33.56 ± 12.73	*14.00 ± 1.96	*1.47 ± 0.02
Naïve (CCl$_4$ un-induced)	-	*44.56 ± 1.50	*16.00 ± 1.69	*1.37 ± 0.02
5% Tween 80	10 ml/kg	[ab]7.43 ± 0.33	[ab]3.66 ± 0.97	[ab]3.08 ± 0.02

a = $P< 0.05$ compared with naïve group; b = $P< 0.05$ compared with ascorbic acid; * = $P< 0.05$ compared with 5% tween 80; AA = ascorbic acid; CME = crude methanol extract; EAF = ethyl acetate fraction; BF = butanol fraction; NHF = n-hexane fraction

HPLC analysis of the CME and fractions
The chromatograms of the CME, EAF, BF and NHF with their retention times are presented in Figure 3. The chromatograms revealed the presence of many compounds most of which are phenolic. The identification of these compounds was through the comparison of the UV scan of each of these compounds and that of the in-built library. Further identification was done by comparing these UV scans with reported literature for these compounds. CME revealed Isoprunetin 8C gluco, chlorogenic acid, hyperoside, bastadin, sulochrin, methylasteric acid and paclitaxel. EAF revealed p-Hydrozybenzoic acid, protocatechuic acid (PCA), chlorogenic acid and quercetin 3-O (6-O acetyl) galactopyranose. BF revealed Isochavicine, Triterpene acetate, Indol-3-amin, Chlorogenic acid, Isoflavone, 5-Hydroxy-3-hydromethyl-2-methyl-7-methoxychromone and

Isorhamnetin-3O-glucosid while the NHF revealed only three compounds Helenalin, Fettsaure and Aureonitol of which only Helenalin has been reported to show antioxidant activity [33].

Acute toxicity

Result of oral acute toxicity study in mice revealed no lethality or toxic reactions at any of the selected doses, showing that the LD_{50} of CME is above 5000 mg/kg.

Serum liver marker enzymes and histopathology examination

Following this investigation (Table 4), these enzymes were significantly ($P < 0.05$) elevated in the serum of the CCl_4 treated groups however, pre-treatment with the CME and EAF showed varying degrees of hepatic protection against the CCl_4 toxicant. This finding was further supported with histophathological studies (Figure 4). The histophathological examination clearly reveals that the hepatocytes are almost normal in the 200 and 400 mg/kg CME and EAF treated groups compared to the 5% tween 80 control group.
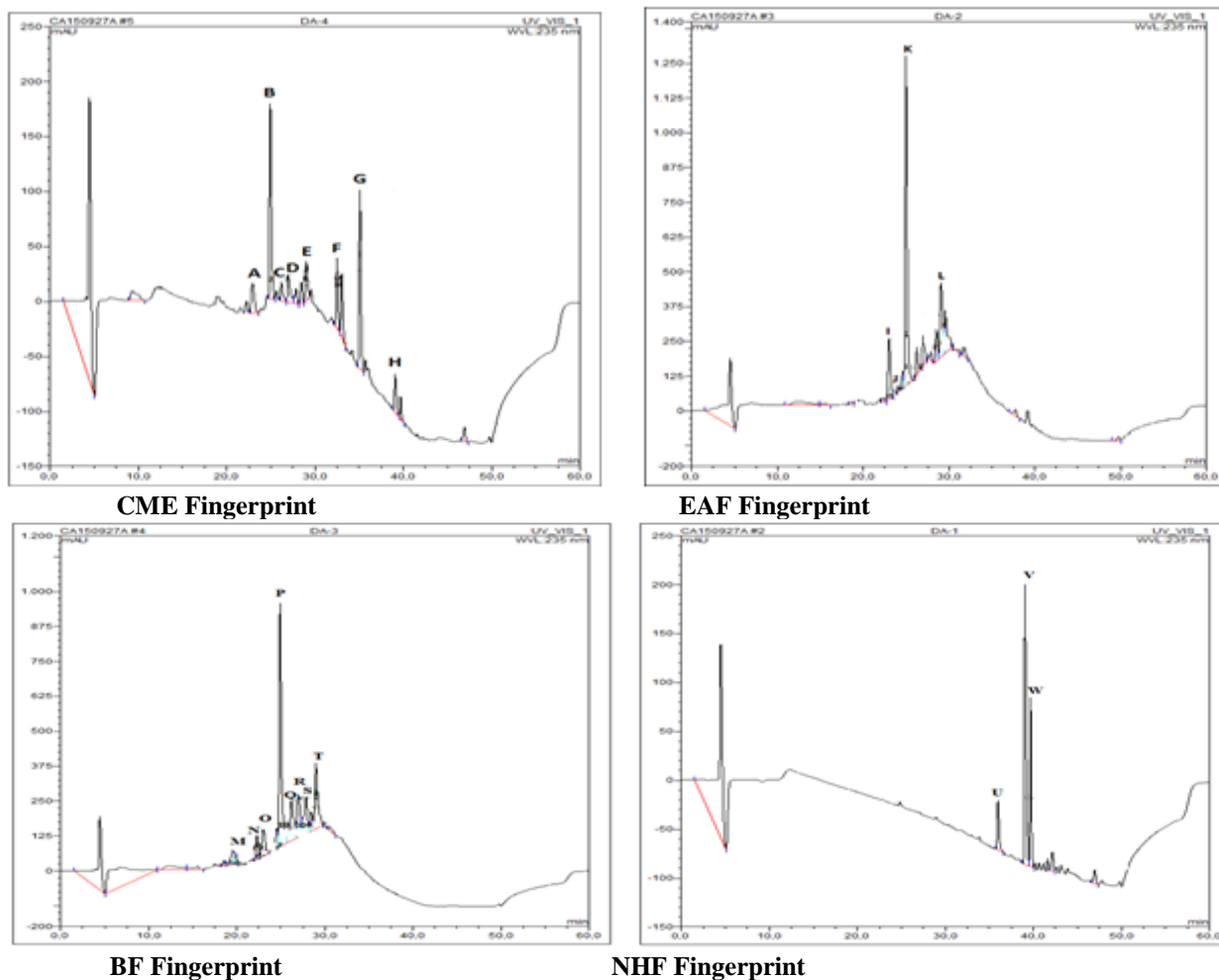

CME Fingerprint   **EAF Fingerprint**

BF Fingerprint   **NHF Fingerprint**

Fig 3: HPLC fingerprinting of the CME and fractions

A = Isoprunetin 8C gluco (R_t = 22,50); **B** = Chlorogenic acid (R_t = 24,92); **C** = Chlorogenic acid (R_t = 26,97); **D** = (Hyperoside) Quercetin-3-galactoside (R_t = 28,99); **E** = Bastadin 3 (R_t = 32,49);**F** = Sulochrin (R_t = 33,04); **G** = Methylasteric acid (R_t = 35,08); **H** = Paclitaxel (R_t = 39,68);   **I =** p-Hydrozybenzoicacid (R_t = 23,02)**; J =** Protocatechuic acid(R_t = 24,00)**; K =** Chlorogenic acid (R_t = 24,97)**; L =** Quercetin 3-O (6-O acetyl)galactopyranose (R_t = 29,07); **M** = Isochavicine (R_t = 19,57);   **N** = Triterpene acetate (R_t = 22,27); **O** = Indol-3-amin (R_t = 23,03); **P** = Chlorogenic acid (R_t = 24,91); **Q** = Isoflavone (R_t= 26,19); **R** = Chlorogenic acid (R_t=26,96); **S** = 5-Hydroxy-3-hydromethyl-2-methyl-7-methoxychromone (R_t = 27,85); **T** = Isorhamnetin-3O-glucosid (R_t = 28,98);   **U** = Helenalin (R_t = 35,97); **V** = Fettsaure (R_t = 39,04); **W** = Aureonitol (R_t = 39,66). Where   **Rt** = Retention time.

Table 4: Effect of the CME and EAF on serum liver marker enzymes

Treatment(mg/kg)	Dose (mg/kg)	Mean serum liver marker enzymes (U/L)		
		ALT	AST	ALP
CME	200	*[ab]63.67 ± 0.33	*[ab]130.00 ± 3.54	31.25 ± 2.35
CME	400	*[ab]63.67 ± 0.61	*[ab]129.17 ± 3.52	*29.90 ± 2.62
EAF	200	*[ab]64.00 ± 0.00	*[a]119.17 ± 5.07	[ab]34.19 ± 2.64
EAF	400	*[a]62.67 ± 1.61	*[a]117.00 ± 3.39	*27.94 ± 2.11
AA (Reference)	100	*[a]58.4 ± 2.22	*[a]112.00 ± 7.16	*29.12 ± 1.57
Naïve (CCl₄ un-induced)	-	*[b]10.67 ± 2.81	*[b]31.67 ± 5.87	*26.72 ± 0.25
5% tween 80	10 ml/kg	[ab]79.71 ± 8.75	[ab]197.86 ± 19.05	[ab]35.6 ± 3.51

a = $P < 0.05$ compared with naïve group; b = $P < 0.05$ compared with ascorbic acid while   * = $P < 0.05$
compared with 5% tween 80; AA = ascorbic acid; CME = crude methanol extract;   EAF = ethyl acetate
fraction; BF = butanol fraction; NHF = n-hexane fraction.

Discussion

This study validated the reported *in vitro* antioxidant activity and further evaluated the *in vivo* antioxidant potentials of *C. cujete* leaf extract and fractions which could be linked to their phenolic content. Thus, free radical scavenging and induction of antioxidant enzymes could be the possible mechanism of action of *C. cujete* leaves. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen atom [34]. Antioxidant compounds neutralize the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH, thereby inhibiting it by changing the colour from deep purple to the yellow coloured stable diamagnetic molecule – diphenylpicrylhydrazine [35]. High free radical scavenging activity was recorded by EAF in this study. FRAP accesses antioxidant activity based on reduction principle. The good DPPH scavenging activity and ferric reducing ability shown by EAF, CME, and BF suggests that they contain compounds that are capable of donating hydrogen atom and/or electron to free radicals/oxidants thus inhibiting/reducing them as the case may be. The antioxidant activity exhibited by CME and its fractions could be due to the phenolic compounds detected them. Phenolic compounds have been demonstrated to possess high antioxidant capacity due to the presence of redox active groups in their structures [36]. The low phenolic content recorded by the NHF could be responsible for the weak antioxidant activity exhibited by this fraction. The high phenolic contents observed in the CME, EAF and BF may be responsible for their good antioxidant activity. Catalase and SOD are powerful antioxidant enzymes found in almost all living organism. SOD catalyzes the dismutase of superoxide radical into oxygen and hydrogen peroxide while catalase catalyzes the breakdown of hydrogen peroxide to harmless products

[37]. The effect of CME and EAF on catalase and SOD activities was comparable to that of AA. The significant catalase and SOD activity recorded in the CME and the EAF treated groups is an indicator of their antioxidant potentials which may be attributed to the phenolic compounds detected in them.

Lipid peroxidation is the oxidative degradation of lipids. Cell membranes contain polyunsaturated fatty acid which is highly prone to free radical attack (38). Reaction of free radicals with polyunsaturated fatty acid leads to lipid peroxidation with their associated end products like malondialdehyde (MDA) [39]. In this study, the level of MDA was used to measure LPO. The lower level of MDA recorded in the CME and EAF treated groups is a supportive indicator of their antioxidant potentials.

The bulk of the plant constituents of the CME are most soluble in butanol than the other fractionation solvents as seen in the HPLC chromatograms, the BF revealed more compounds than the other fractions. Thus the plant constituents could be more polar in nature. HPLC is a powerful tool in analysis. This tool revealed strong antioxidant compounds in *C. cujete* leaves which include; chlorogenic acid (CGA), a polyphenol and the most abundant compound (highest peak) in the CME, EAF and BF suggesting that this could be one of the major active compounds of this plant. Several researches have reported the antioxidant activity of chlorogenic acid [40], [41]. Chlorogenic acid and its metabolite (caffeic acid) have been reported to show protective effect against oxidative injury [40]. Suppression of superoxide generation has also been reported as one of the mechanisms of action of chlorogenic acid [41]. Isoprunetin has been reported to exhibit antioxidant activity [42]. Hyperoside (Quercetin-3-galactoside) is a flavonol glycoside with potent antioxidant properties [43]. Studies have shown that hyperoside possess cytoprotective properties

against oxidative stress by scavenging intracellular reactive oxygen species and enhancing antioxidant enzyme activity [43]. Sulochrin is a benzophenone and possess antioxidant activity [44]. Bastadin is a bromophenol and most bromophenols have been reported to display potent antioxidant activity than synthetic antioxidants like BHT (butylated hydroxy toluene) [45]. Paclitaxel, a chemotheraphy drug, it has been reported to decrease lipid peroxidation with concomitant increase in the activities of enzymatic antioxidants (SOD, catalase and glutathione peroxidase) and non-enzymatic antioxidants (glutathione and vitamin C and E) in animals induced with breast cancer [46]. p-Hydrozybenzoic acid is a phenolic derivative of benzoic acid. Merkl *et al.,* [47] reported that phenolic acids and its esters (of which p-hydroxybenzoic acid is an example) possess good antioxidant effect as reveratol and trolox. Protochatechuic acid (dihydroxy benzoic acid) is also phenolic acid and has been reported to show much more antioxidant activity *in vitro* compared to trolox by chelating metal transition ions as well as scavenging

free radicals via donation of hydrogen atom (H^+) or electron (e) [48], [49]. Quercetin 3-O (6-O acetyl) galactopyranose has been reported to show good antioxidant activity [50]. Isochavicine is a photo-isomer of piperine found in *Piper guineense,* it has been reported to show antioxidant activity [51, 52]. Helenalin which is a sesquiterpene lactone has been reported to exhibit antioxidant activity [31].Result of oral acute toxicity study in mice revealed no lethality or toxic reactions at any of the selected doses, with LD_{50} of CME above 5000 mg/kg. This means that the leave extract of the plant is relatively non- toxic. This is the first study to report the acute toxicity of *C. cujete* leaves. This plant part may be safe for human consumption as revealed in the acute toxicity. However, the evaluation of the effect of its long-term administration is recommended. Elevated level of serum liver marker enzymes (ALT, AST and ALP) is an indicator of liver rupture. The significant decrease in serum liver marker enzymes recorded in the CME and EAF treated groups is an indicator of their protective effect against oxidative stress induced hepatotoxicity.

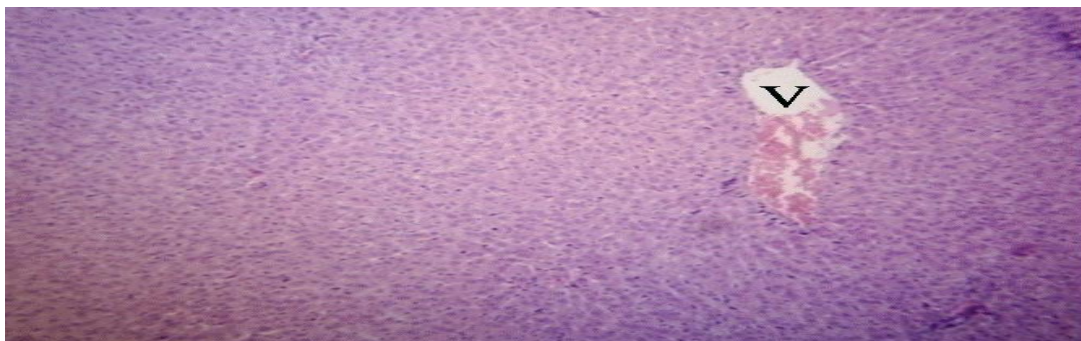**The photomicrograph of liver sections at different doses (Figures 4- 10)**

Fig 4: Photomicrograph of liver for naïve (un-induced control) (H&E X100) showing a normal liver histology with the central vein (V) intact
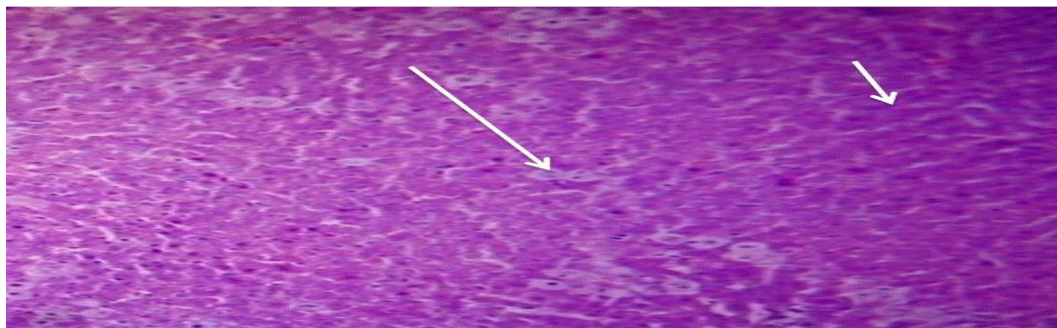

Fig 5: Photomicrograph of liver at dose 100 mg/kg AA (standard) showing mild fatty change (short arrow) and mixed necrosis (long arrow) (H&E X400)
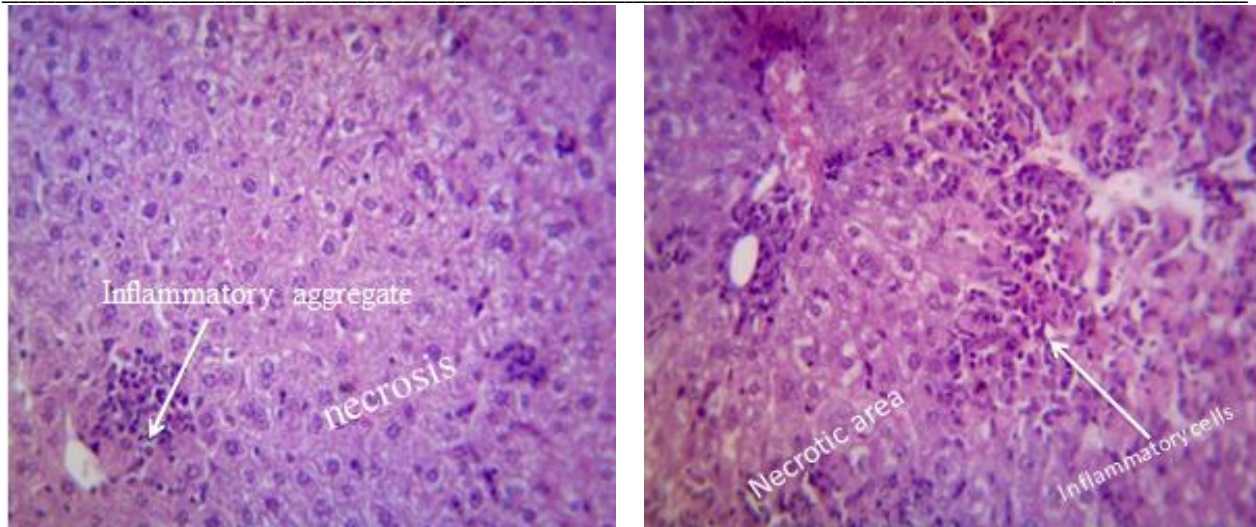
Fig 6: Photomicrograph of the liver for 5% tween 80 group showing severe necrosis and numerous inflammatory cells (CCl$_4$ treated) (H&E X400)
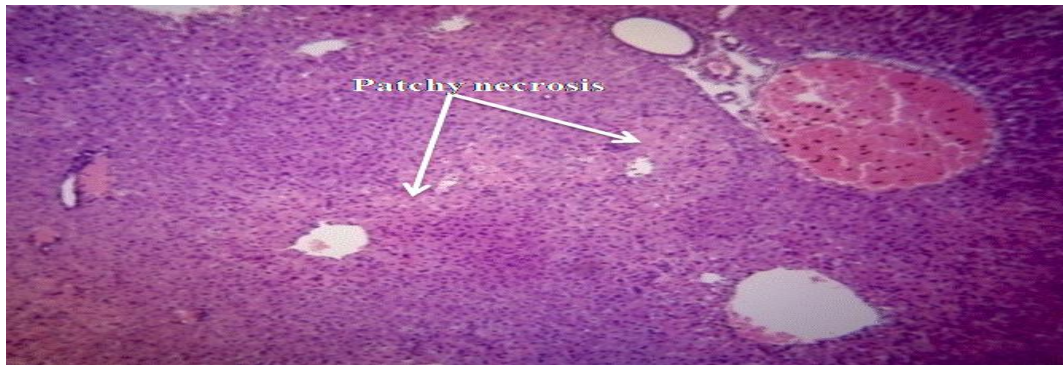

Fig 7: Photomicrograph of the liver at dose 400 mg/kg CME showing few patchy necrosis (double arrow) and mild congestion (H&E X100)
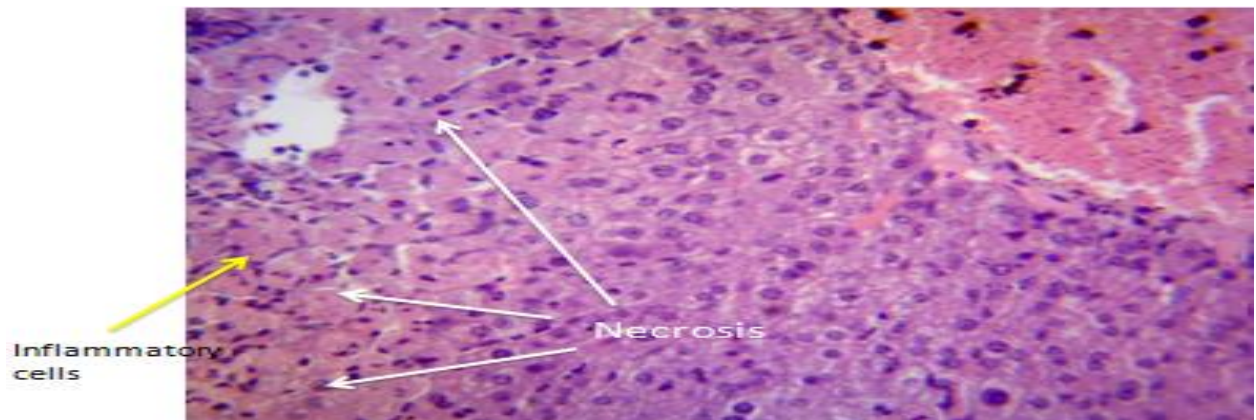

Fig 8: Photomicrograph of the liver at dose 200 mg/kg CME showing inflammatory cells and some necrotic regions (H&E X400)
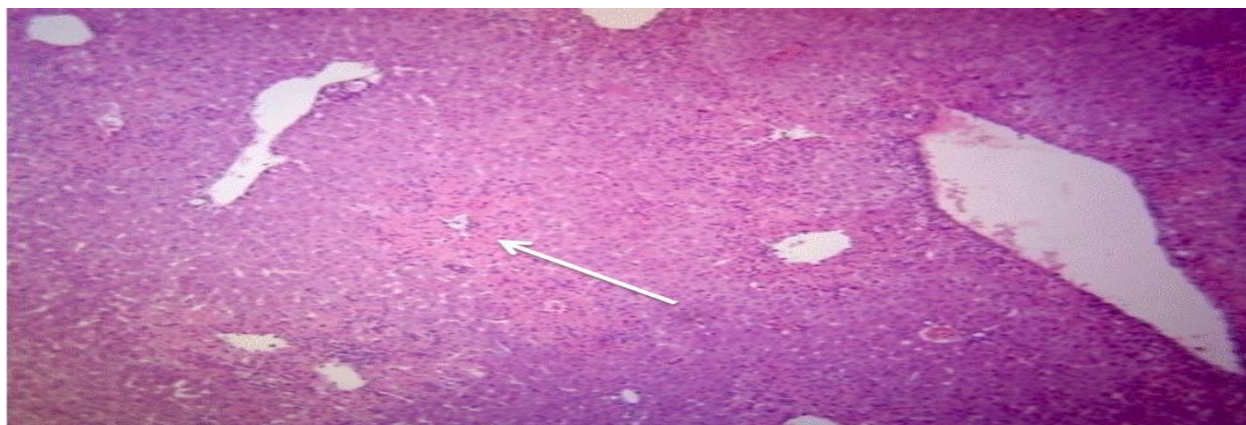
Fig 9: Photomicrograph of the liver at dose 400 mg/kg EAF showing mild necrosis (arrow) (H&E X100)
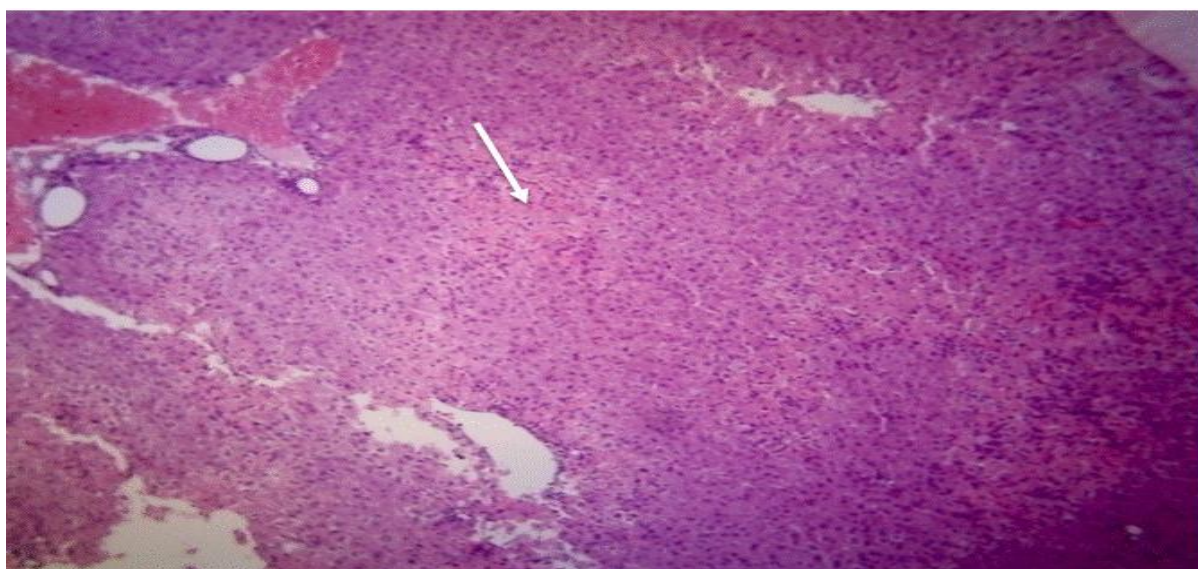

Fig 10: Photomicrograph of the liver at dose 200 mg/kg EAF showing Mild necrosis (arrow) with little inflammation (H&E X100)

Conclusion

Findings from this study revealed the good antioxidant potential of *C. cujete* which may explain its role in eliminating oxidative stress and its traditional usefulness in the treatment and management of many aliments. The results established the antioxidant activity of the leaf extract of *C. cujete* by demonstrating both the *in vitro* and *in vivo* antioxidant activities; and further established its toxicological profiles. These antioxidant potentials may be connected to its phenolic compounds.

References

1. Faiza P, Satheeshan B, Kumar M, Adarsh AK, Shilpa R, Roshni P, *et al*. Evaluation of Glutathione, Albumin and Ascorbic Acid Levels in Breast Cancer Patients. Int J Clin Med. 2013; 4: 405-408
2. Zima TS, Fialova L, Mestek O, Janebova M, Crkovska J, Malbohan I, *et al*. Oxidative stress

metabolism of ethanol and alcohol-related diseases. J. Biomed. Sci. 2001; 8: 59-70

3. Zengin G, Aktumsek A, Guler GO, Cakmak YS, Yildiztugay E. Antioxidant properties of methanolic extract and fatty acid composition of *Centaurea urvillei. Hayekiana wagenitz.* Rec. Nat. Prod. 2011; 5(2): 123–132

4. Devasagayam TP, Tilak JC, Boloor KK. Review: Free radical and antioxidants in human health J. Assoc Physicians India. 2004; 53(1): 794-804.

5. Halliwell B. Biochemistry of oxidative stress. Biochem Soc Trans. 2007; 35: 1147-1150

6. Valko M, Leibfritz D, Moncol J, Cronin M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007; 39(1): 44-84

7. Pohanka M. Alzheimer's disease and oxidative stress: a review. Curr Med Chem. 2013; 21(3): 356-364

8. Kikkawa RD, Koya, Haneda M. Progress of diabetic neuropathy. Am J Kidney Dis. 2003; 41: 19-21

9. Udoh EA, Iya N, Okon E, Mary N. Red cell catalase activity in diabetes. Pak J Nutr. 2007; 6(1): 511-515.

10. Singh N, Dhalla AK, Seneviratne C, Singal PK. Oxidative stress and heart failure. Mol Cell Biochem. 1995; 147 (1): 77-81

11. Aruoma, O.I. Free radicals, oxidative stress and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* 1998;75:199–212.

12. Ashokkumar, D.; Mazumder, U.K.; Gupta, M.; Senthilkumar, G.P.; Selvan, V.T. Evaluation of antioxidant and free radical scavenging activities of *Oxystelma esculentum* in various *in vitro* models. *J. Compliment. Integr. Med.* 2008, 5, 1553–3840.

13. Veerapur, V.P.; Prabhakar, K.R.; Parihar, V.P.; Kandadi, M.R.; Ramakrishana, S.; Mishra, B.; Satish Rao, B.S.; Srinivasan, K.K.; Priyadarsini, K.I.; Unnikrishnan, M.K. *Ficus racemosa* stem bark extract: A potent antioxidant and a probable natural radioprotector. *Evid. Based Complement. Alternat Med.* 2009, 6, 317–324.

14. Barku VY, Opoku-Boahen Y, Owusu-Ansah E, Mensah F. Antioxidant activity and the estimation of total phenolic and flavonoid contents of the root extract of *Amaranthus spinosus*. Asian J Plant Sci Res. 2013; 3(1): 69-74

15. Nigerian Natural Medicine development Agency. 1st ed. Lagos: Medicinal plants of Nigeria, South-West Nigeria. Nigeria Natural Development Agency; 2009. 240 p

16. Stuartxchange. Philippine medicinal plants. www.stuartxchange.com/cujete.hmtl [Accessed 02 September 2015]

17. Pravin MS, Das N, Jahan N, Akhter MA, Nahar L, Islam ME. Evaluation of *in vitro* anti-inflammatory and antibacterial potential of *Crescentia cujete* leaves and stem bark. BMC Res Notes. 2015; 8(1): 412. /doi: 10.1186/s13104-015-1384-5

18. Agarwal M, Chauhan S. Anti-mycobacterial potential of *Crescentia cujete* (Bignoniaceae). Int J Adv Res Bot. 2015; 1(1): 1-9.

19. National Institute of health. Institutional administrator's manual for laboratory animal care and use (Revised). 8th ed. Maryland: National Institute of health; 2011. 82 p

20. Ajaghaku DL, Obasi O, Umeokoli BO, Ogbuatu P, Nworu CS, Ilodigwe EE, *et al*. *In vitro* and *in vivo* antioxidant potentials of *Alchornea floribunda* leaf extract, fractions and isolated bioactive compounds. Avicenna J Phytomed, 2016; Epub ahead of print

21. Obiagwu MO, Ihekwereme CP, Ajaghaku DL and Okoye FBC. The useful medicinal properties of the root bark extract of *Alstoniaboonei* (Apocynaceae) may be connected to antioxidant activity. ISRN Pharmacol. 2014; http://dx.doi.org/10.1155/2014/741478

22. Okoye FBC, Odimegwu DC, Anyasor CN, Ajaghaku DL, Gugu TH, Osadebe PO, *et al*. New antioxidant and antimicrobial compound isolated from *Alchornea cordifolia* leaves. AJOPRED. 2015; 7(2): 87-94

23. OECD Guidelines for the testing of chemicals (No 423). Acute toxic class method. 3rd ed. France: Organization of Economic Co-operation and Development (OECD); 2011; 14 p

24. Bancroft, J. D. and Stevens, A. 3rd ed. Edinburgh: Theory and practice of histopathological techniques. Churchhill Livingstone; 1990. 740 p

25. Sinha AK. Colorimeteric assay of catalase. Anal Biochem.1972; 47: 389-394

26. Olatosin TM, Akinduko DS, Uche CZ.Antioxidant capacity of *Moringa oleifera* seed oil against CCl_4-induced hepatocellular lipid peroxidation in wistar albino rats. Eur J Exp Biol. 2014; 4(1): 514-518.

27. Gutteridge JM, Wilkins S. Copper dependent hydroxyl radical damage to ascorbic acid; formation of thiobarbituric acid reactive products. Fed Eur Biochem Soc J. 1982; 137: 327-330

28. Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am J ClinPathol.1957; 28: 56-63

_____

29. King EJ, Armstrong AR. A convenient method for determining serum and bile phosphatase activity. J ClinPathol.1954; 7(4): 322-326

30. Prior RL, Wu X, Schaich K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. J Agric Food Chem. 2005; 53: 4290-4302

31. Huang DB, Prior RL. The chemistry behind antioxidants capacity assays, J Agric food chem. 2005; 53(6): 1841–1856

32. Jodynis-Liebet J, Murias M, Bloszyk E. Effect of sesquiterpene lactones on antioxidant enzymes and some drug-metabolizing enzymes in rat liver and kidney. Planta Med. 2000; 66(3): 199-205

33. Prior RL, Cao G. Analysis of botanicals and dietary supplements for antioxidant capacity; a review. J. AOAC Int. 2000; 83(4): 950–955

34. Naik GH, Priyadarsini KL, Satav JG. Comparative antioxidant activity of individual herbal components used in ayurvedic medicine. Phytochem. 2003; 63(1): 97-104

35. Pisoschi AM, Negulescu GP. Methods for total antioxidant activity determination: a review. Biochem Anal Biochem. 2011; 1, 1–10

36. National Center for Biotechnology Information. 2016. CAT (catalase) [*Homo sapiens* (Human)]. [ONLINE] Available at: http://www.ncbi.nlm.nih.gov/gene/847 [Accessed 27 January 2016].

37. Olakunle S, Onyechi O, James O. Toxicity, anti-lipid peroxidation, *in vitro* and *in vivo* evaluation of antioxidant activity of *Annona muricata* ethanol stem bark extract. Am J Life *Sci*.2014; 2(5): 271-277.

38. Bakirel T, Bakirel, U, Keles OU, Ulgen SG, Yardibi H. *In vivo* assessment of antidiabetic and antioxidant activities of rosemary (Rosmarinus officinalis) in alloxan-diabetic rabbits. J Ethnopharmacol. 2008; 116: 64-73

39. Sato Y, Itaqaki S, Kurokawa T, Oqura J, Kobayashi M, Hirano T, *et al*. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. Int J Pharm. 2011; 403(1-2): 136-138

40. Mee-Hyang K, Han-Joon H, Ha-Chin S. Identification and antioxidant activity of novel chlorogenic acid derivatives from Bamboo (*Phllostachys edulis*). J Agric Food Chem. 2001; 49(10): 4646-4655.

41. Mahsa SJ, Movahedin M, Mazaheri Z, Amanlu M, Mowla SJ, Batooli H. Evaluating the effects of *Escanbil* (Calligonum) extract on the expression level of catspergene variants and sperm mortality in aging mice. Iran J Reprod Med. 2014; 12(7): 459-466

42. Piao MJ, Kang KA, Zhang R, Ko D, Wang ZH, You HJ, *et al*. Hyperoside prevents oxidative stress induced by hydrogen peroxide in lung fibroblast cells via antioxidant effect. Biochim Biophys Acta. 2008; 1780(12): 1448-1457

43. Inamori Y, Kato Y, Kubo M, Kamiki T, Takemoto T, Nomoto K. Studies on metabolites produced by *Aspergillus terreus* var. *aureus*: chemical structures and antimicrobial activities of metabolites isolated from culture broth. Chem Pharm Bull. 1984; 31(12): 4543-4548.

44. Gribble GW.Biological activity of recently discovered halogenated marine natural products. Mar Drugs. 2015; 13(7): 4044-4136

45. Padmavathi R, Senthilnathan P, Chodon D, Sakthisekaran D. Therapeutic effects of paclitaxel and propolis on lipid peroxidation and antioxidant system in dimethyl benz(a)anthracene-induced breast cancer in female Sprague Dawley rats. Life Sci. 2006; 78(24): 2820-2825.

46. Merkl R, Hradkova I, Filip V, Smidrkal J. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. Czech J Food Sci. 2010; 28, 275-279

47. Li X, Wang X, Chen D, Chen S. Antioxidant activity and mechanism of protocatechuic acid *in vitro*. Funct Food Health Dis. 2011; 7: 232-244

48. Kakkar S, Bais S. A review on protocatechuic acid and its pharmacological potentials. ISRN: Pharmacology. 2014; http://dx.doi.org/10.1155/

49. Kicel A, Wolbis M. Study on the phenolic constituents of the flowers and leaves of *Trifolium repens* L. Nat Prod Res. 2012; 26(21): 2050-2054.

50. Anand R, Saundane VK, Viajinath AV, Walmik P. Antimicrobial and antioxidant activity of some new indole derivatives containing pyridopyrimidine and pyrazolopyridine moieties. Med Chem Res. 2015; 22(2): 806-817

51. Saxena R, Venkaiah K, Anitha P, Venu L, Raghunath M. Antioxidant activity of commonly consumed plant foods of India: contribution of their phenolic content. Int J Food Sci Nutr. 2007; 58(4): 250-260.

_____