

Antioxidative Potential of Aqueous Extract of *Ocimum americanum* L. Leaves: An *in vitro* and *in vivo* Evaluation

B. T. Aluko, O. I. Oloyede

Abstract—*Ocimum americanum* L. (*Lamiaceae*) is an annual herb that is native to tropical Africa. The *in vitro* and *in vivo* antioxidant activity of its aqueous extract was carefully investigated by assessing the DPPH radical scavenging activity, ABTS radical scavenging activity and hydrogen peroxide radical scavenging activity. The reducing power, total phenol, total flavonoids and flavonols content of the extract were also evaluated. The data obtained revealed that the extract is rich in polyphenolic compounds and scavenged the radicals in a concentration dependent manner. This was done in comparison with the standard antioxidants such as BHT and Vitamin C. Also, the induction of oxidative damage with paracetamol (2000 mg/kg) resulted in the elevation of lipid peroxides and significant ($P < 0.05$) decrease in activities of superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase in the liver and kidney of rats. However, the pretreatment of rats with aqueous extract of *O. americanum* leaves (200 and 400 mg/kg) and silymarin (100 mg/kg) caused a significant ($P < 0.05$) reduction in the values of lipid peroxides and restored the levels of antioxidant parameters in these organs. These findings suggest that the leaves of *O. americanum* have potent antioxidant properties which may be responsible for its acclaimed folkloric uses.

Keywords—Antioxidants, free radicals, *Ocimum americanum*, scavenging activity.

I. INTRODUCTION

ANTIOXIDANTS are synthetic or plant derived agents which protect the cell from harmful effects of reactive oxygen species [1]. Reactive oxygen species (ROS) such as hydroxyl radical (OH \cdot), hydrogen peroxide (H $_2$ O $_2$), organic hydroperoxide (ROOH) and peroxynitrite (ONOO \cdot) are produced in the mitochondrial and functions majorly as intracellular messengers. However, the accumulation of ROS is known to be associated with various kinds of degenerative disorders [2], [3]. Although the body is endowed with endogenous antioxidants to quench the reactions of ROS, in some pathological conditions, these antioxidant molecules may be insufficient to neutralize all the radicals [4]. This has necessitated the search for antioxidant compounds of plant origin [5]. Plants are generally rich in phenolics compounds and have been reported to possess anti-inflammatory, anti-cancer and anti-aging activities attributable to their antioxidant potentials [6], [7].

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Ocimum americanum L. (Family *Lamiaceae*) locally known as "Efinrin elewe dudu" in south-western Nigeria, is a wild herb that grows in tropical Africa [8]. The leaf sap is widely used in traditional folk medicine in Ghana for the management of diabetes [9]. In Nigeria, the aqueous leaf extract is administered orally for the treatment of constipation, diarrhoea, piles and dysentery. It is also used as insect repellent. The leaf contains essential oils of therapeutic importance and is widely used for the preparation of local delicacy by the Yorubas' in Nigeria because of its aromatic properties [10]. Oboh [11] reported the free radical inhibitory effect of acetone extract of this plant on some neurotoxins induced brain damage in rats.

This present study is aimed at establishing the *in vitro* and *in vivo* antioxidant potentials of aqueous extract of *O. americanum* leaves.

II. MATERIALS AND METHODS

A. Collection and Identification of Plant Material

The leaves of *O. americanum* L. were collected in May, 2011 from a local farmland near Orin Ekiti, Nigeria. The plant was identified and authenticated by Mr Omotayo (herbarium curator) at the Department of Plant Science, University of Ado Ekiti, Nigeria. A voucher specimen (U.H.A.E 09/01) was deposited in the herbarium of the Department. The leaves were air dried for 10 days and pulverized into fine powder using an electric blender. The powdered sample was stored in sealed glass bottles and kept refrigerated until required.

B. Preparation of Plant Extract

The aqueous extract was prepared by extracting fifty gram of the powdered sample in 1 liter of cold sterile distilled water maintained on a mechanical shaker (Stuart Scientific Orbital Shaker SO1, Essex, UK) for 24 h. The extract was filtered using a Buchner funnel with Whatman's No 1 filter paper. The filtrate was frozen at -40°C and dried for 72 h using a freeze drier (Savant Refrigerated vapor Trap, RVT 41404, USA) to give a percentage yield of 14.2 w/w. The extract was preserved in the refrigerator and later reconstituted to give the doses of 200 and 400 mg/kg body weight respectively.

C. Experimental Animals

Wistar rats (*Rattus norvegicus*) weighing 150 – 190 g were obtained from the animal house of the laboratory of School of Biological Sciences, University of Fort Hare Alice 5700 South Africa. They were kept in clean cages placed in a well

ventilated house condition (temperature: $22 \pm 2^\circ\text{C}$; photoperiod: 12 h light and 12 h dark cycle; humidity: 40 – 45%). The animals were fed *ad libitum* with rat pellets and tap water freed of contaminants. The study was carried out in line with the guidelines of Ethics Committee on the use and care of Experimental Animals of the University of Fort Hare, South Africa.

D. Determination of in vitro Antioxidant Activities of Extract of O. americanum Leaves

1. Determination of Total Phenolics

The method described by [12] was used to determine the total phenolic contents of aqueous extract *O. americanum* leaves. The reaction mixture contained 2.5 ml of 10 % (v/v) Folin-Ciocalteu reagent, 2 ml of 7.5 % (w/v) of sodium carbonate and 0.5 ml (1 mg/ml) of the extract. The mixture was vortexed and incubated at 40°C for 30 min after which the absorbance was measured at 765 nm. The total phenolic content was calculated from the equation obtained from the calibration curve of Tannic acid ($Y = 0.1216x$, $R^2 = 0.936512$) and expressed as mg/g tannic acid equivalent where x is the absorbance and Y is the tannic acid equivalent.

2. Determination of Total Flavonoids

The modified method of [13] was adopted to estimate total flavonoids content of *O. americanum* leaves. The extract (1 ml in a final concentration of 0.1 mg/ml) was mixed with 1 ml of 2 % (w/v) aluminium chloride prepared in ethanol and left in the dark at room temperature for 1 h. A yellow colour was observed which was measured spectrophotometrically at 420 nm. The total flavonoid content was calculated as mg/g quercetin equivalent from $Y = 0.0255x$, $R^2 = 0.9812$ obtained from the calibration curve where x is the absorbance and Y is the quercetin equivalent.

3. Determination of Total Flavonols

The determination of the total flavonols content of the extracts was done according to the modified method of [14]. Two millilitres (2 ml) of the extract was mixed with 1 ml of 2 % aluminium chloride in ethanol and 1.5 ml of 5 % sodium acetate solution and allowed to stand for 2.5 h at room temperature and later, measured the absorbance at 440 nm.

The flavonols content was expressed as mg/g quercetin equivalent using the calibration equation ($Y = 0.0255x$, $R^2 = 0.9812$) from standard quercetin where x is the absorbance and Y is the quercetin equivalent.

4. DPPH Scavenging Assay

The radical scavenging activity of the extract against DPPH was performed as described by [15] with some modifications. Briefly, 1 ml of the extract or standard at different concentrations (50-300 $\mu\text{g/ml}$) was added to 2 ml DPPH (10 mg/l in methanol). After a 30-min reaction, the absorbance was measured at 517 nm. The scavenging ability of the extract was calculated as:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100$$

where Abs control is the absorbance of DPPH radicals + methanol; Abs sample is the absorbance of DPPH radical + sample or standard.

5. ABTS Scavenging Assay

The ABTS scavenging ability of the extract was assayed according to the method of [16] with slight modifications. The stock solution consisting of 7 mM ABTS solution and 2.4 mM potassium persulfate (1:1) was allowed to react in the dark fume hood for twelve hours at room temperature. The radical generated was mixed with methanol to obtain an absorbance of 0.702 ± 0.001 unit at 734 nm. Two millilitres (2ml) of the resulting solution was added to 2 ml of the extract or vitamin C (50-300 $\mu\text{g/ml}$) and the absorbance was measured at 734 nm after 7 min. The percentage scavenging ability was calculated from:

$$\text{ABTS radical scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100$$

where Abs control is the absorbance of ABTS radicals + methanol; Abs sample is the absorbance of ABTS radical + sample or standard.

6. Hydrogen Peroxide Scavenging Assay

The slightly modified method of [12] was employed to assess the hydrogen peroxide scavenging potential of the aqueous extract of *O. americanum* leaves. The stock solution was 4 mM hydrogen peroxide prepared in 0.1 M phosphate buffer (pH 7.4). The solution (0.8 ml) was added to 2.5 ml of the extract and standard (50 – 300 $\mu\text{g/ml}$) and incubated for 15 min at room temperature. The absorbance was read at 230 nm and the percentage inhibition of hydrogen peroxide was calculated as:

$$\text{H}_2\text{O}_2 \text{ scavenging activity (\%)} = [(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100$$

where Abs control is the absorbance of H_2O_2 radicals; Abs sample is the absorbance of H_2O_2 radical + sample or standard.

7. Determination of Reducing Power

The ferric reducing power assay was done as previously described by [17]. The extract (1 ml) at different concentrations (50 – 300 $\mu\text{g/ml}$) was first mixed with 0.2 M phosphate buffer (pH 6.6) (2.5 ml), and 1% $\text{K}_3\text{Fe}(\text{CN})_6$ (w/v) (2.5 ml). After incubation at 50°C for 20 min, trichloroacetic acid (TCA, 10% w/v) (2.5 ml) were added to the mixture and centrifuged at 3000 rpm for 10 min to stop the reaction. 2.5 ml of the supernatant of the mixture was mixed with 2.5 ml distilled water and 0.5 ml ferric chloride (FeCl_3 , 0.1% w/v), and the absorbance of the resulting solution was read at 700 nm against a blank. BHT was used as standard.

E. In vivo Antioxidant Studies in Wistar Rats

To study the antioxidant effects of the extract, rats were equally divided into five groups ($n = 4$) and treated for a period of seven days as follows:

- Group 1 (normal control) received 1ml of distilled water p.o. for 7 days
- Group 2 (toxic group) received 1ml of distilled water p.o. for 7 days and paracetamol (2 g/kg b.w., p.o.) on the 4th and 5th day.
- Groups 3 and 4 (test groups) received aqueous extract (200 and 400 mg/kg b.w. p.o.) for 7 days and paracetamol (2 g/kg b.w., p.o.) on the 4th and 5th day, 30 min after extract administration.
- Group 5 (standard group) received silymarin (100 mg/kg b.w. p.o.) for 7 days and paracetamol (2 g/kg b.w., p.o.) on the 4th and 5th day 30 min after the administration of silymarin.

On the 8th day, the animals were sacrificed under light ether anesthesia 24 h after their respective doses. The liver and kidney were quickly removed into ice cold 0.25 M sucrose solution. Thereafter, they were blotted with clean tissue paper and homogenized in Tris HCl buffer (0.1 M; pH 7.4; 1: 10 w/v). The homogenates were kept frozen overnight before being used for various antioxidant enzyme assays.

1. Estimation of Lipid Peroxides (LPO)

Lipid peroxidation in the tissues were estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modified method of [18]. A volume of 0.1 ml of tissue homogenate (10% w/v) was treated with 2 ml of (1:1:1 ratio) TBA -TCA- HCl reagent. All the tubes were placed in a boiling water bath for 30 min and cooled. After cooling the mixture was centrifuged at 3000 rpm for 10 min. The lipid peroxides formed in each of the samples was assessed by measuring the absorbance of clear supernatant at 535 nm. A standard curve was generated using 1, 1, 3, 3-tetramethoxypropane. The results were expressed as μM 1, 1, 3, 3-tetraethoxypropane equiv/g of wet tissue.

2. Determination of Superoxide Dismutase Activity

The mixed substrate solution (340 μL) and samples (10 μL) were added to microplate wells. Reaction was initiated by adding 50 μL of diluted xanthine oxidase to the mixture followed by shaking for a few seconds to mix. The plate was then incubated on a shaker for 5 minutes at room temperature. The initial absorbance (A_1) was read after 30 minutes while the final absorbance (A_2) of the sample was read after 3 minutes at 505nm using a microtitre plate reader. A series of standard was prepared and assayed the same way to generate a standard curve.

3. Determination of Glutathione Peroxidase Activity

The method of [19] was adopted for assay of glutathione peroxidase (GPX). A volume of 500 μL of the reconstituted reagent (Glutathione 4mmol/l, Glutathione reductase 0.5 U/I, NADPH 0.34mmol/l) and samples (10 μL) were added to microplate wells. Reaction was initiated by adding 20 μL of Cumene hydroperoxide to the mixture followed by shaking for a few seconds to mix. The well that contained the blank had the sample replaced with 10 μL of distilled water. The initial absorbance of the sample and blank was read at 340 nm using a microtitre plate reader. Thereafter, absorbance was also

measured again after 1 and 2 minutes. The absorbance of the reagent blank was subtracted from that of the sample. Glutathione peroxidase concentration was calculated as:

$$U/I = 8412 \times \text{change in absorbance at 340 nm/ minute} \times \text{dilution factor}$$

4. Determination of Glutathione Reductase Activity

The method of [20] was used for the determination of glutathione reductase (GRD) in the tissue homogenates. The tissue homogenates (20 μL) was mixed with 500 μL of the substrate. The reaction was stopped by the addition of 100 μL of NADPH. The initial absorbance of the sample was read at 340 nm using a microtitre plate reader. Thereafter, absorbance was also measured again after 1, 2, 3, 4 and 5 minutes. Glutathione reductase concentration was calculated as:

$$U/I = 4983 \times \text{change in absorbance at 340 nm/ minute} \times \text{dilution factor}$$

5. Estimation of Catalase Activity

The catalase was colorimetrically assayed as described by [21]. The reaction mixture (1.5 ml) contained 1.0 ml of phosphate buffer (10 mM, pH 7.0), 0.1 ml of liver homogenate and the reaction was started by addition of 0.4 ml of H_2O_2 (2000 mM). The reaction mixture was incubated for 3 min at room temperature. The reaction was stopped by addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio), was incubated at 100 °C for 2 min. The absorbance was measured at 620 nm. The concentration of the standard (H_2O_2) was plotted against absorbance. Catalase activity is expressed as nM H_2O_2 consumed / min / mg protein.

F. Statistical Analysis

Results were expressed as mean \pm SD and analyzed by one – way analysis of variance (ANOVA) followed by Duncan multiple range test. The results obtained from both the extract and standard drug were compared that of the control group. P values < 0.05 were considered significant.

III. RESULTS AND DISCUSSIONS

A. Phenolic Content

Phenolic compounds are plant derived antioxidative agents which are important for maintaining health [22]. The major mechanism of action of these polyphenols has been their ability to neutralize free radicals, chelate metals and inhibit the activity of oxidizing enzymes in biological systems [23], [12]. The data obtained from this study revealed high concentrations of total phenolics (240.16 mgTE/g), flavonoids (32.43 mgQE/g) and appreciable amounts of flavonols (23.55 mgQE/g) in the extract of *O. americanum* leaves (Table I). Scientific research has shown that these phenolic compounds possess the ability to inhibit oxidative stress by antioxidant mechanisms [24]. The folklore use of this plant may be attributed to the presence of these polyphenols.

TABLE I
POLYPHENOLIC CONTENTS OF AQUEOUS EXTRACT OF *O. AMERICANUM*
LEAVES

Polyphenol	Concentration
Total phenolics (mg TE/g)	240.16 ± 0.17
Total flavonoids (mg QE/g)	32.43 ± 0.84
Total flavonols (mg QE/g)	23.55 ± 0.04

Data expressed as means ± SD (n=3). TE, QE and CE are tannic acid, quercetin and CE catechin equivalent respectively.

B. DPPH Radical Scavenging Assay

DPPH radical scavenging activity of plant extracts is widely used to assess their antioxidant potentials. The aqueous extract of *O. americanum* leaves exhibited the inhibition of DPPH radical in a concentration related manner (Fig. 1). Although, the inhibitory effect displayed by the standard drug was significantly higher than the extract, the observed trend can be attributed to the electron donating potentials of the extract [7].

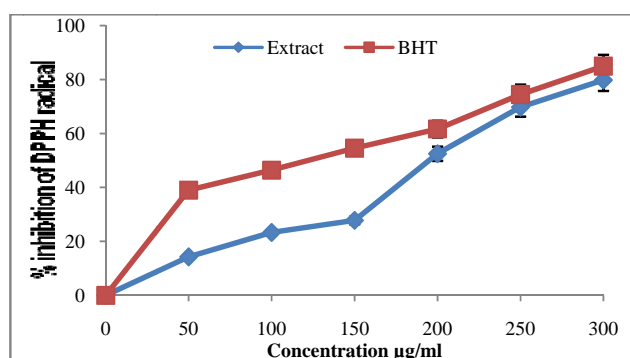


Fig. 1 Scavenging effect of aqueous extract of *O. americanum* leaves on DPPH radical. The results are means ± SD (n=3)

C. ABTS Radical Scavenging Assay

ABTS radical has a characteristic absorbance maxima at 734 nm and mostly reactive toward phenolics, thiols and other antioxidants [25]. ABTS radical scavenging activity of aqueous extract of the leaf of *O. americanum* was concentration dependent (Fig. 2). This observed effect can be attributed to the presence of phenolic compounds in the extract.

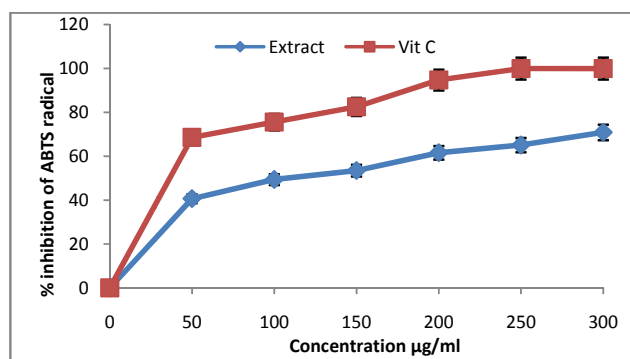


Fig. 2 Scavenging effect of aqueous extract of *O. americanum* leaves on ABTS radical. The results are means ± SD (n=3)

D. Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide is a powerful oxidizing agent which is reduced to hydroxyl radical through the catalytic actions of glutathione peroxidase and catalase in the presence of iron or copper. Hydroxyl radical the product of this reaction, is a highly reactive radical capable of reacting with sugars, nucleotides and lipids in the body thereby causing their damage [26]. The hydrogen peroxide inhibition increased with increasing concentration of the extract and the standard (Fig 3). The inhibition of H₂O₂ by the extract can be attributed to the proton donating abilities of its phytoconstituents.

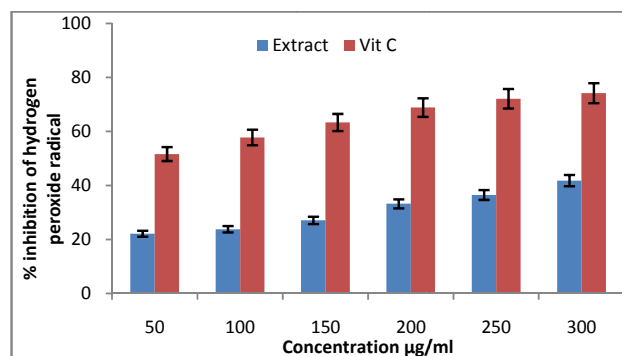


Fig. 3 Scavenging effect of aqueous extract of *O. americanum* leaves on hydrogen peroxide radical. The results are means ± SD (n=3)

E. Ferric Reducing Ability

Fig 4 shows the ferric reducing power of the extract in comparison with BHT. The presence of polyphenols in the extract of *O. americanum* reduced ferric ions to its ferrous form. The complex formed with Perl's Prussian blue was monitored spectrophotometrically at 700 nm. The reducing ability of the extract was significantly lower than that of BHT. Nevertheless, the result revealed that *O. americanum* contain components capable of electron transfer which can react with free radicals to convert them to stable products quench the reactions that precipitates oxidative stress.

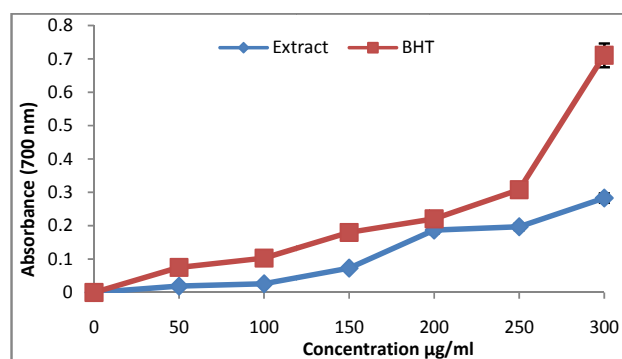


Fig. 4 The ferric reducing ability of aqueous extract of *O. americanum* leaves. The results are means ± SD (n=3)

F. In vivo Antioxidant Effects of Aqueous Extract of *O. americanum* Leaves in Rats

Paracetamol is an analgesic and antipyretic drug which is primarily metabolized by the liver and excreted by the

kidneys. The overdose of this drug leads the accumulation of a toxic metabolite known as N – acetyl – p – benzo quinoneimine (NAPQI). Increased levels of NAPQI precipitates oxidative damage, and thus enhance hepato renal damage [27]. Although the occurrence of nephrotoxicity is less common than that of hepatotoxicity, a number of studies have reported acetaminophen induced renal tubular damage [28], [29].

The administration of paracetamol (2000 mg/kg b.w.) induced a marked significant ($P < 0.05$) increase the level of lipid peroxides in the liver and kidney of induced rats as compared to that of normal rats. Pre treatment of rats with the extract and reference drugs resulted in a significant ($P < 0.05$) reduction in the level of lipid peroxides in these organs (Fig 5). Also, the activities of glutathione peroxidase (GPX), glutathione reductase (GRD) and antiperoxidative enzymes [catalase (CAT) and super oxide dismutase (SOD)] were significantly ($P < 0.05$) lower in the liver and kidney of rats as compared to that of control animals (Figs. 6-9). However, the prior oral administration of extract and silymarin significantly ($P < 0.05$) attenuated paracetamol – induced hepatorenal alterations in the activities of glutathione peroxidase enzymes (GPX) and antiperoxidative enzymes.

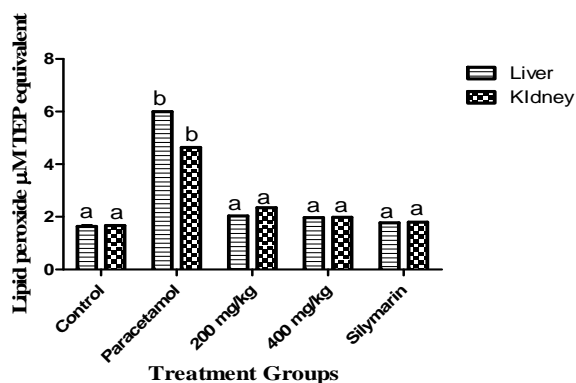


Fig. 5 Effect of *O. americanum* leaf extract on lipid peroxidation in the liver and kidney of normal and paracetamol intoxicated rats

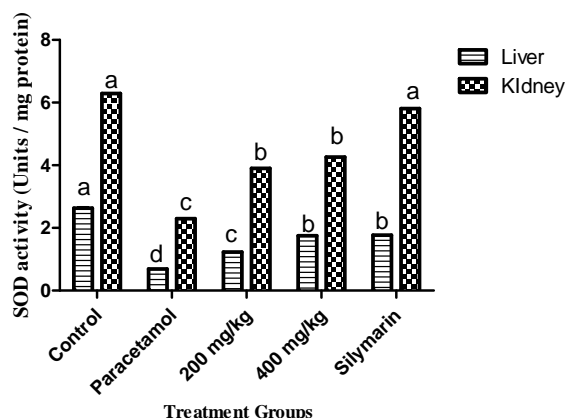


Fig. 6 Effect of *O. americanum* leaf extract on the activities of SOD in the liver and kidney of normal and paracetamol intoxicated rats

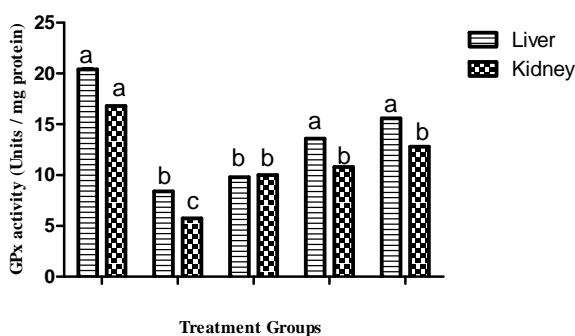


Fig. 7 Effect of *O. americanum* leaf extract on the activities of GPX in the liver and kidney of normal and paracetamol intoxicated rats.

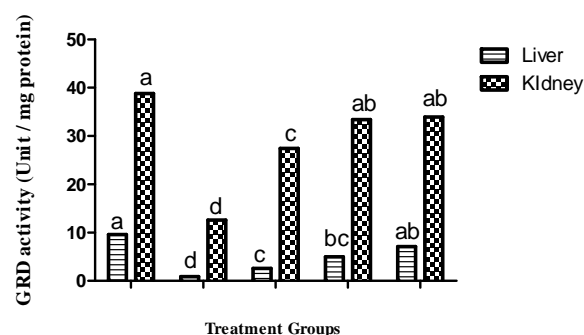


Fig. 8 Effect of *O. americanum* leaf extract on the activities of GRD in the liver and kidney of normal and paracetamol intoxicated rats.

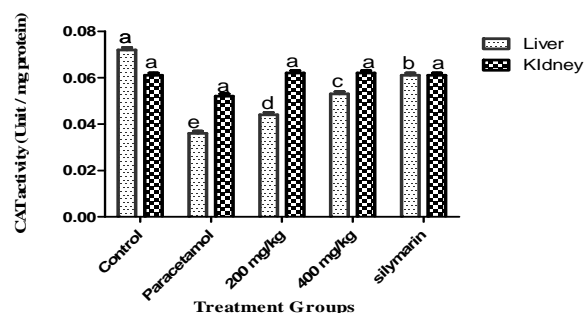


Fig. 9 Effect of *O. americanum* leaf extract on the activities of CAT in the liver and kidney of normal and paracetamol intoxicated rats.

In the present study, elevated level of TBARS in paracetamol treated rats indicates excessive formation of free radicals and activation of lipid peroxidation system resulting in hepatic and renal damage. TBARS is produced as by product of lipid peroxidation that occurs in hydrophobic core of biomembranes [30]. The significant decline in the concentration of these constituents in the liver and kidney of silymarin and extract pretreated rats indicates antilipid peroxidative effect of *O. americanum*. SOD, GRD and GPX constitute a mutually supportive team of defense against ROS. SOD is a metalloprotein and is the first enzyme involved in the antioxidant defense by lowering the steady-state level of O_2^- . GPX is a selenoenzyme two third of which (in liver) is present in the cytosol and one third in the mitochondria. It

catalyses the reaction of hydroperoxides with reduced glutathione to form glutathione disulphide [7]. In this study, there was a decline in the activities of these enzymes in Paracetamol intoxicated animals. However, the observed increase in the activities of these enzymes in extract pretreated rats revealed that lipid peroxidation and oxidative stress elicited by Paracetamol intoxication have been nullified due to the effect of *O. americanum* aqueous extract. GRD is concerned with the maintenance of cellular level of GSH (especially in the reduced state) by effecting fast reduction of oxidized glutathione to reduced form [31]. In our study, the activity of GRD was significantly decreased in the liver and kidney tissues of Paracetamol administered rats, compared with control group. However, pretreatment with extract of *O. americanum* significantly increased the activity of this enzyme, thus unearthing the antioxidant effect of *O. americanum*.

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