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Free Radical Scavenging and Antioxidant Potential of Ethanolic Leaf Extract of *Diodia sarmentosa* on High Fat Fed Wistar Rats

Ndubuisi Tobias I. Ezejiofor[1], Korie Stephen Chinedumije[1*] and Peter Chukwudi[1]

[1]Department of Biotechnology, Federal University of Technology, Owerri, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ETIN and KSC designed the study and wrote the protocol. Author KSC managed the literature searches, performed the statistical analysis and wrote the first draft of the manuscript. Authors ETIN and KSC managed the analyses of the study. Author PC corrected the final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Diodia sarmentosa (Sw) commonly known as Zimbabwe flora or Tropical button weed is a straggling or procumbent perennial herb which grows in evergreen forest, riverine vegetation and bush land. It has been used traditionally for the treatment of ulcer, diabetes and other ailments. This study investigated the Free radical scavenging and antioxidant potential of ethanolic leaf extract of *Diodia sarmentosa* on high fat fed wistar rats. Thirty (30) male wistar rats (150 g-200 g) were divided into five (5) groups: Negative control rats, Positive control rats, Low dose extract rats treated with 250 mg/kg, High dose extract rats treated with 500mg/kg of the extract, and Standard antihyperlipidaemic drug rats treated with 5 mg/kg of Simvastatin. High fat diet was fed to the rats with Ghee and Coconut oil in the ratio of 3:1 for six (6) weeks, and administration of the treatments started from the 3rd week till the 6th week. The free radical scavenging and antioxidant potentials of *Diodia sarmentosa* was determined by assessing parameters like nitric oxide radical, hydroxyl radical, malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione S-transferase (GST) in the serum of the test rats. *Diodia*

**Corresponding author: E-mail: stephenkorie007@gmail.com;*

sarmentosa scavenged free radicals In vitro by inhibiting Nitric oxide and Hydroxyl radicals in a concentration-dependent manner, showing an inhibitory concentration at 50% (IC_{50}) of 907.17 µg/ml ± 45.36 and 2173.44 µg/ml ± 100.11 respectively. Results obtained showed antioxidant enzymes like SOD, GPx and CAT in the serum of the test rats were significantly increased ($p<0.05$) by both doses of the extract when compared to the PC group and was similar to the standard drug used. GST activity was decreased in the serum by both doses of the extract, this decrease was not significant ($p>0.05$), while Malondialdehyde level was significantly decreased ($p<0.05$) by both doses of the extract in the serum of the tests rats. *Diodia sarmentosa* has antioxidants potentials because of its ability to scavenge free radicals, replenish antioxidant enzymes and reduce lipid peroxidation.

Keywords: High fat diet; Diodia sarmentosa; free radicals; oxidative stress, antioxidant.

1. INTRODUCTION

A free radical is known as any molecular specie that can exist on its own, and contains an unpaired electron in an atomic orbital. The presence of these unpaired electrons in the atomic orbital leads to certain common properties that are shared by most radicals. Most radicals are unstable and highly reactive, they can either be electron donors or electron acceptors thereby making them behave as oxidants or reductants [1]. Radicals are highly reactive species, they include hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radicals. These mentioned radicals are the most important oxygen-containing free radicals, they are involved in various disease state found in the nucleus and membranes of cells causing cell damage and homeostatic disruption to biologically relevant molecules such as DNA, Proteins, Carbohydrates and Lipids [2].

Oxidative stress can be defined as an imbalance or inequality between the generation of reactive species (reactive oxygen or reactive nitrogen species) and the levels of enzymatic antioxidants used as protective or defensive agents [3,4]. Oxidative stress can also be as a result of the rapid production of reactive forms of oxygen than they can be neutralized by antioxidants i.e free radicals exceeding the oxidant system of the body. Hence, increasing RONs and decreasing or lack of antioxidants induces oxidative Stress [3]. Any prolonged imbalance results to oxidative damage to cells, tissues and organs. Generally, oxidative stress are caused by series of activities like alcohol consumption, use of drugs (anti-inflammation, anti-analgesic, anti- cancer and anti-depressants), environmental pollution by pollutants like mercury chloride, lead and other factors like mobile phone/radio frequency radiation exposure, UV radiation, x-Ray exposure, temperature (cold stress), pesticides, high consumption of high fat diet etc [3]. Oxidative stress is said to be the one of the causes of cancer, Alzheimer's diseases, Parkinson's disease, amyotrophic lateral scle rosis, and so many other lifestyle-related disorders such as arterial sclerosis, coronary heart diseases, atherosclerosis, stroke, high blood pressure, myocardial infarction, cerebral apoplexy, dementia, diabetes, cataract, asthma, obesity, lung cancer etc [3,5]. Oxidative stress is one of the major causes of cardiac diseases like congestive heart failure, stroke, hypertension, and atherosclerosis [6,7]. Cardiovascular diseases are characterized with low level of antioxidants enzymes like Superoxide dismutase, Glutathione peroxidase, Glutathione s-transferase, Catalase etc [8]. This is as a result of excess production of free radicals species, inducing oxidative stress which attack many macromolecules like enzymes, membrane lipids, DNA causing a series of chain reaction resulting in cellular damage, lipid peroxidation and many diseases [9]. Various authors have reported feeding with high fat has caused an increase in the production of free radicals and reduced antioxidant enzymes that play vital role in the defense mechanism of the body, leading to diseases such as obesity, hypercholesterolemia, hyperlipidemia, hypertriglyceridemia and cardiovascular diseases due to high fat concentration in the body [10-12].

Antioxidants are substances that inhibit oxidation by balancing/neutralizing out free radicals produced during different metabolic processes, inflammation and injury in the cell [13]. Just like free radicals, antioxidants can also be produced endogenously like superoxide dismutase, glutathione peroxidase, glutathione s-transferase and catalase. They can also be introduced to the biological system exogenously, usually through diet such as Vitamin C, Carotenoids and Vitamin E [14,15].

Diodia sarmentosa (Sw) commonly known as Zimbabwe flora or Tropical button weed is a procumbent perennial herb spreading at irregular intervals. It is from the family of rubiaceae, having length of 1-4m long, its colour can either be green or yellowish-green. It grows in open riverine vegetation, bush land, evergreen forest and also on rocky places near rivers [16]. *Diodia sarmentosa* has been reported to possess anti-ulcer, anti-diabetic, anti-cancer, anti - inflammatory and analgesic potentials [17-19]. The present study aims to determine the free radical scavenging and antioxidant potential of ethanolic leaf extracts of *Diodia sarmentosa* on high fat fed wistar rats.

2. MATERIALS AND METHODS

2.1 Plant Materials

Fresh samples of *Diodia sarmentosa* (Sw) leaves were collected from farm lands and from natural vegetation within Ihiagwa environment. The plant was identified by Prof. I.I Iloegbulam of crop science department in Federal University of Technology, Owerri, Nigeria.

2.2 Chemicals and Reagents

Analytical grade chemicals and reagents were used for this study.

2.3 Experimental Animals

Male Wistar rats weighing between 150-200g were used for this study. The animals were purchased from department of Biochemistry, University of Port Harcourt, Rivers State, Nigeria.

2.4 Preparation of Plant Extract

Fresh leaves of *Diodia sarmentosa* (Sw) were air-dried at room temperature and then ground into fine powder using laboratory mortar and pestle. This leaves now in fine powder were soaked in 80% ethanol for a period of one week then filtered using Whitman filter paper to get the plant extract.

2.5 Experimental Site

The animals were acclimatized in an animal house at Biochemistry department in Federal University of Owerri, under room temperature and relative humidity of 40-65% with a 12h natural light-dark cycle.

2.6 Experimental Design

30 wistar rats (male) used for this experiment were grouped into five (5) groups, six (6) for each group;

Group NC, Normal control: Rats fed normal diet and water.

Group PC, Positive control: Untreated rats fed with high fat diet and water.

Group LDE: High fat diet fed rats treated with 250 mg/kg body weight of ethanolic leaf extract of *Diodia sarmentosa.*

Group HDE: High fat diet fed rats treated with 500 mg/kg body weight of ethanolic leaf extract of *Diodia sarmentosa.*

Group SAD: Standard antihyperlipidaemic drug group treated with 5mg/kg of Simvastatin.

According to the Ezejiofor & Okoroafor (19), the median lethal dose (LD_{50}) of the plant extract was established as 1600 mg/kg, this led to the adoption of safe doses of 250 mg/kg and 500 mg/kg.

2.7 Treatment and Sample Collection Phase

The rats were placed on High-fat diet (Ghee and Coconut oil in the ratio of 3:1) for a period of six (6) weeks adapted from a previous study by Munshi et al. [20] except the negative control group. All animals were allowed access to food and water and body weight of rats were taken weekly.

From the 4^{th} week till the end of the experiment, groups PC, LDE, HDE and SAD continued with high fat diet and their respective doses of extract, while group NC remained on normal rat chew. The appropriate dosages of the *Diodia sarmentosa* (Sw) and Simvastatin were administered to the animals orally once daily by intubation using intravenous cannula tube.

The rats were allowed to fast for Twenty four hours after the last treatment, and then

anesthetized with chloroform vapour and blood samples were collected through cardiac puncture.

2.8 In Vitro Screening of Ethanolic Leaf Extract for Antioxidant Activity

2.8.1 Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of *Diodia sarmentosa* (Sw) was carried out using the Fenton oxidant reaction mixture of Fe^{3+}/ascorbic acid and H_2O_2 method as described by Halliwell et al. [21] as reported by Alisi et al. [22].

2.8.2 Nitric oxide scavenging assay

The Nitric oxide scavenging activity of *Diodia sarmentosa* (Sw) was estimated according to the earlier method described by Marcocci et al. [23] as reported by Lalhminglilui & Jagetia [24].

2.9 In Vivo Screening of Ethanolic Leaf Extract for Antioxidant Activity

2.9.1 Assay of malondialdehyde (Lipid peroxidation)

Lipid peroxidation in the samples was determined spectrophotometrically by assessing the concentration of thiobarbituric acid reactive substances (TBARS) described by Buege & Aust [25] as reported by Nair et al. [26].

2.9.2 Determination of antioxidant enzyme activity

The assay of the antioxidant enzyme superoxide dismutase activity was carried out according to the procedure of Das et al. [27]. The catalase enzyme activity in the samples was assayed following the procedure of Aebi [28] as reported by Hassan et al. [29]. Glutathione-S-transferase (GST) in the samples was measured by the method of Habig et al. [30] as reported by Sasi Bhusana Rao et al. [31]. Glutathione peroxidase (GPx) in the samples was measured by the method of Rotruck et al. [32] as reported by Sajeeth et al. [33].

2.10 Statistical Analysis

Data were analyzed using appropriate software (Microsoft Excel 2013). Results were presented as mean ± Standard deviation of four determinations and statistically analyzed using one-way analysis of variance on statistical computer software program (SPSS 21). The degree of statistical difference was accepted as significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Results of in Vitro Free Radical Scavenging Activities

Nitric oxide is a radical that has the ability to cause damage to the function and structure of many cellular components. The toxic effect of NO. increases when it reacts with superoxide to form peroxynitrite anion (ONOO-), which is a strong oxidant that breaks down to form .OH and NO_2 [34]. The result from Fig. 1. showed the ethanolic extract of *Diodia sarmentosa* scavenged nitric oxide in vitro in a concentration-dependent manner, which could be due to the presence of antioxidants like flavonoids which are phenolic compounds in the plant. The findings of this study is in agreement with Ijonome & Ekpe [18], which showed that *Diodia sarmentosa* possesses flavonoids after the phytochemical analysis of the plant. The IC_{50} of *Diodia sarmentosa* and Quercetin dihydrate was 907.17 µg/ml ± 45.36 and 55.37 µg/ml ± 4.43 respectively. IC_{50} is the measure of the concentration of the extract to give 50% inhibition of radicals. The percentage nitric oxide scavenging ability increased with increase in extract concentration. The ability of *Diodia sarmentosa* to scavenge nitric oxide radicals is similar to the Nitric oxide scavenging ability of *Ocimum gratissimum* extract [34].

Hydroxyl radicals are known as reactive oxygen species that causes oxidation of polyunsaturated fatty acids in food causing cell and tissue damage. Hydroxyl radicals are produced in vivo by the decomposition of superoxide and hydrogen peroxide catalysed by transition metals like iron and copper [34]. The Hydroxyl scavenging ability of *Diodia sarmentosa* was determined by measuring the decrease of Thiobabituric Reactive substance (TBARS), a product of Malondialdehyde (MDA). The Ethanolic extract of *Diodia sarmentosa* scavenged hydroxyl radicals in a concentration dependent manner, an increase in concentration of the extract caused an increase in percentage inhibition of hydroxyl radicals. The result in Fig. 2. showed the IC_{50} of *Diodia sarmentosa* and Quercetin dihydrate was 2173.44µg/ml ± 100.11 and 322.92 µg/ml ± 25.83 respectively. *Diodia sarmentosa* ability to scavenge hydroxyl radicals

is in concurrence with the findings of Lalhminghui & Jagetia [24], which showed *Schima wallichii* has antioxidant properties by scavenging hydroxyl radicals.

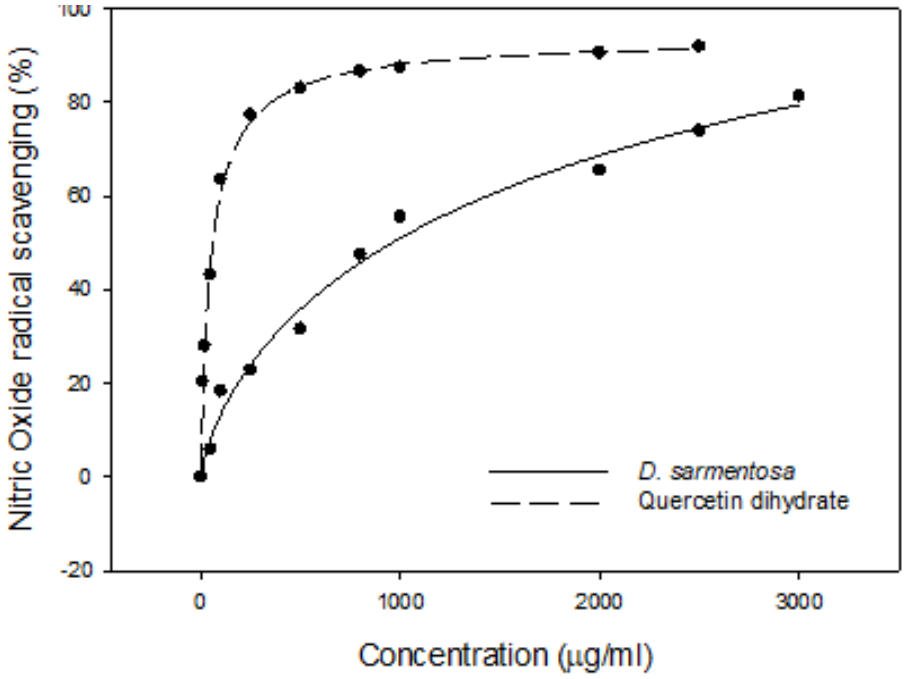

Fig. 1. Result of graded concentration of ethanolic extract of *D. sarmentosa* and Quercetin dihydrate on Scavenging of Nitric Oxide radicals

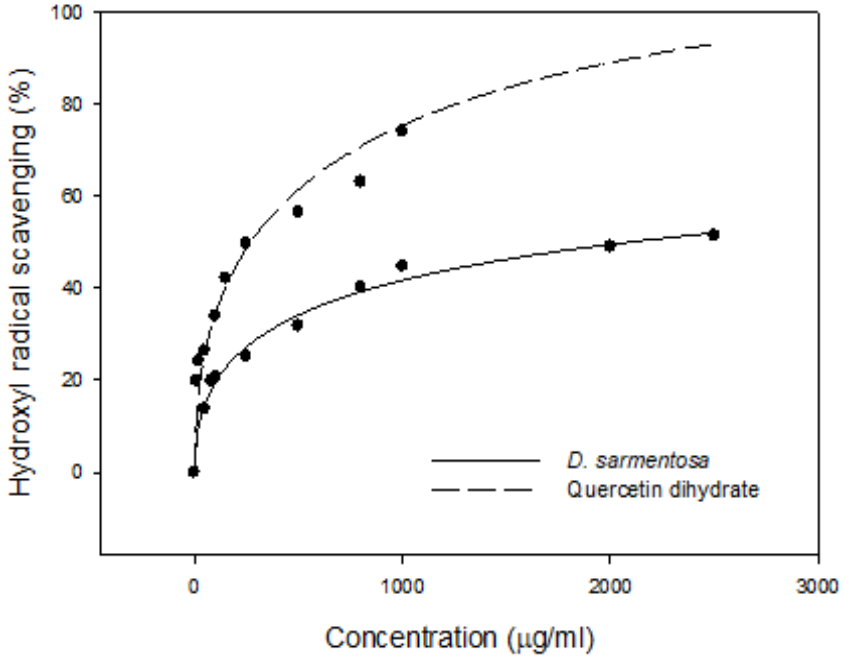

Fig. 2. Result of graded concentration of ethanolic extract of *D. sarmentosa* and Quercetin dihydrate on Scavenging of Hydroxyl radicals

3.2 Results of in Vivo Antioxidant Activities

The In vivo antioxidant potential of *Diodia sarmentosa* was carried out in the serum of the wistar rats, by evaluating the ability of the extract to increase some antioxidant enzymes and decrease lipid peroxidation. The high fat diet caused a high concentration of fat deposition in the rats, this gave rise to oxidative stress by decreasing antioxidant enzymes and increasing lipid peroxidation in the positive control group of the study as shown in Tables 1-5. The result of Superoxide dismutase (SOD) activities in the serum of the different study groups is as presented in Table 1.

Superoxide dismutase (SOD) is an antioxidant enzyme found in aerobic cells that catalyses the breakdown of superoxide anion into oxygen and hydrogen peroxide. The significant reduction ($p<0.05$) of SOD activity in the positive control group shows the overproduction of superoxide radicals by NADPH oxidase in the serum giving rise to oxidative stress. This reduction of SOD activity in the positive control groups makes it difficult for the dismutation of superoxide radical to hydrogen peroxide and oxygen. The range of SOD activities of the study groups is 20.51–28.59 IU/mgProtein, with a significant increase observed in groups LDE, HDE and SAD when compared to the PC group. This increase in the antioxidant enzyme superoxide dismutase, reduced superoxide radicals by the dismutation of superoxide radical to form hydrogen peroxide and oxygen. The efficacy of *Diodia sarmentosa* in the increase of SOD activity can be likened to the efficacy of *Salvadora persica* in the increase of Superoxide dismutase activity in alloxan induced diabetic rats [35].

Glutathione peroxidase (GPx) is an enzyme that is found in almost all tissues though highly found in the liver [1]. Glutathione peroxidase catalyses the oxidation of reduced glutathione into glutathione disulfide, degrading hydrogen peroxide and lipid hydroperoxide into nontoxic forms. It helps in the repair of cellular damage caused by lipid peroxidation. The range of GPx

Table 1. Result of Superoxide dismutase activity in the serum of the various study groups

	Groups	Serum
SOD (IU/mg Protein)x10^-6	NC	28.49 ± 1.05^{c}
	PC	20.51 ± 1.13^{a}
	LDE	$25.73\pm 0.85^{b,c}$
	HDE	28.59 ± 1.86^{c}
	SAD	24.95 ± 1.54^{b}

Each value represents mean ± SD (n=4). Groups with different alphabets are significantly different (p<0.05), while groups with similar alphabets are not significantly different

Table 2. Result of glutathione peroxidase activity in the serum of the various study groups

	Groups	Serum
GPx(mg GSH/min/mgprotein)	NC	3.54 ± 0.27^{c}
	PC	1.52 ± 0.27^{a}
	LDE	2.65 ± 0.28^{b}
	HDE	2.53 ± 0.29^{b}
	SAD	$2.06 \pm 0.27^{a,b}$

Each value represents mean ± SD (n=4). Groups with different alphabets are significantly different (p<0.05), while groups with similar alphabets are not significantly different

Table 3. Result of glutathione s-transferase activity in the serum of the various study groups

	Groups	Serum
GST (µmol GSH-CDNB /min/mgprotein)x10^-6	NC	0.80 ± 0.08^{a}
	PC	1.03 ± 0.14^{a}
	LDE	0.79 ± 0.11^{a}
	HDE	0.78 ± 0.13^{a}
	SAD	0.83 ± 0.15^{a}

Each value represents mean ± SD (n=4). Groups with different alphabets are significantly different (p<0.05), while groups with similar alphabets are not significantly different

Table 4. Result of Catalase activity in the serum of the various study groups

	Groups	Serum
CATALASE (µM H2O2/min/mgProtein)x10^-6	NC	16.02± 0.90[c]
	PC	12.19± 0.85[a]
	LDE	14.12± 0.59[b]
	HDE	15.43± 1.13[b,c]
	SAD	16.35± 0.63[c]

Each value represents mean ± SD (n=4). Groups with different alphabets are significantly different (p<0.05), while groups with similar alphabets are not significantly different

Table 5. Result of malondialdehyde levels in the serum of the various study groups

	Groups	Serum
MDA (nmol/mgprotein)	NC	0.16± 0.01[a]
	PC	0.38± 0.02[c]
	LDE	0.23± 0.02[b]
	HDE	0.23± 0.02[b]
	SAD	0.20± 0.02[b]

Each value represents mean ± SD (n=4). Groups with different alphabets are significantly different (p<0.05), while groups with similar alphabets are not significantly different

activities of the study groups is 1.52–3.54 mg/GSH/min/mgProtein (Table 2). GPx activities were increased in groups LDE, HDE and SAD but only increase by both doses of the extract were significant ($p<0.05$) when compared to the PC group. This increase in GPx activity reduced cellular damage by promoting the oxidation of reduced glutathione and degrading of hydroxyl radicals and lipid hydroperoxide to harmless forms. The efficacy of *Diodia sarmentosa* in the increase of GPx activity is similar to the efficacy of polyherbal formulation in the increase of Glutathione peroxidase activity in streptozotocin induced diabetic male rats [33].

Glutathione S-transferase (GST) is an antioxidant enzyme that is abundant in the liver, it catalyses the conjugation of reduced glutathione to xenobiotics for the purpose of detoxification [36]. Table 3 shows an increase in glutathione s-transferase activities in the serum of the positive control group but was not significantly different ($p>0.05$) compared to the other study groups, thus showing the high fat diet did not have a negative effect on the enzyme GST, rather promotes its production.

Catalase (CAT) is an enzyme whose activity is mostly in the liver and also found in other tissues in the body system. It catalyses the conversion of hydrogen peroxide to water and oxygen [37]. Catalase activity in the study groups ranges from 12.19–16.02 μMH_2O_2/min/mg Protein (Table 4). The significant reduction ($p<0.05$) in catalase activity in the serum of the positive control group maybe be due to the exhaustion of the enzyme catalase, that helps in catalysing the conversion of hydrogen peroxide. This led to the over production of hydrogen peroxide radicals, thus triggering oxidative stress in the test rats. This finding correlates with the decrease in catalase activity in male obese rats [12]. The significant increase ($p<0.05$) in catalase activity in groups LDE, HDE and SAD could be due to the extract has antioxidant potentials to reduce hydrogen peroxide radicals, by increasing the enzyme catalase to catalyse the conversion of hydrogen peroxide to oxygen and water. The effect of this extract is similar to the anti-obesity effect of *Zingiber officinale* extract in male obese rats [12].

Table 5 shows the results of lipid peroxidation expressed as Malondialdehyde levels in the serum ranging from 0.16–0.38 nmol/mgProtein. Lipid peroxidation is oxidative decomposition of lipids, with one of the final products as Malondialdehyde (MDA), which is produced by the overproduction of free radicals in the cell. It is a major marker of oxidative stress [38]. Malondialdehyde activity was significantly increased ($p<0.05$) in the serum of the positive control group, indicating cell injury or damage in the serum caused by production of reactive oxygen species by tumornecrosis factor alpha (cytokine). This result is in concordance with various studies which showed a high activity of lipid peroxidation in high fat fed rats (10,34,39). Groups LDE, HDE and SAD significantly reduced ($p<0.05$) lipid peroxidation expressed as malondialdehyde levels, thus reversing the

cellular damage caused by oxidative stress. The efficacy of *Diodia sarmentosa* is similar to that of *Salvadora persica* in the reduction of lipid peroxidation (MDA activity) [35].

4. CONCLUSIONS

The data of the present study showed that the ethanolic extract of *Diodia sarmentosa* possesses antioxidant potentials by In vitro scavenging of Nitric oxide and Hydroxyl radicals in a similar manner as the standard oxidant. It showed its potentials in reducing lipid peroxidation, and increasing antioxidant enzymes just like the standard antihyperlipidaemic drug.

ETHICAL APPROVAL

The animals were granted free access to water and rats chew in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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