

Original Research Article

Inhibitory Activity of Ethanolic Extract of Horseradish (*Moringa oleifera*) (Lam) on Oxidative Stress parameters in some organs of Dexamethazone Induced Hypertensive Wistar Rats.

ABSTRACT

The use of herbal remedies in various local communities throughout the world to assuage the scourge of indigenous diseases has gained tremendous popularity. One of the many plants species of herbal remedies is *Moringa oleifera* (horseradish) which has been widely used in West Africa and Nigeria in particular for treating numerous human ailments such as malnutrition, cardiovascular, hepatotoxicity and many others. Regardless of its wide use by communities around the world, there is inadequate scientific information available on the actual pharmacological effects of ethanolic extract of *Moringa oleifera* leaves on the heart and kidney dysfunction. Hence this work aimed at determining the inhibitory activity of ethanolic extract of horseradish on oxidative stress in the heart and kidney of dexamethazone induced hypertensive wistar albino rats. The air-dried leaves of horseradish were pulverized and crude ethanolic extract was prepared. The animals were grouped into four groups of five animals each. Group A animals served as control and fed with water and feed while Groups B and C animals were administered with 0.5mL of 40 % w/v ethanolic extract of *Moringa oleifera* leaf and 0.5ml of 1.67 mg/kg body weight dexamethasone respectively. Group D animals were treated with 0.5ml of 1.67 mg/kg body weight dexamethasone and 0.5mL of 40 % ethanolic extract of *Moringa oleifera* leaf intermittently for 21days. The results obtained from the study showed that the ethanolic extract of *Moringa oleifera* leaf possessed the ability to inhibit and reverse the dexamethasone mediated tissues oxidative damage of both organs as seen in cholesterol, reduced glutathione and malondialdehyde concentrations. The same protective trend was also observed in activities of alkaline phosphatase, aspartate transaminase, alanine transaminase and catalase enzymes in both organs of the hypertensive rats. Hence, ethanolic extract of *Moringa oleifera* leaf reduced the extent of antioxidant loss and restoration of organ dysfunction caused by dexamethasone in the rats.

Keyword: *Moringa oleifera*, oxidative stress, dexamethazone, chemoprevention, antioxidant

Introduction

Moringa oleifera is one of the most useful tropical trees. The relative ease with which it propagates through both sexual and asexual means and its low demand for soil nutrients and water after being planted makes its production and management easy. Introduction of this plant into a farm which has a biodiverse environment can be beneficial for both the owner of the farm

and the surrounding eco-system. *Moringa oleifera* tolerates a wide range of environmental conditions; it will tolerate extremely high temperatures in the shade and can survive a light frost. The drought-tolerant tree grows well in areas that receive annual rainfall amounts ranging between 250 and 1500 mm, it prefers a well-drained sandy loam or loam soil, but tolerates clay. *Moringa oleifera* is planted either by direct seedling, transplanting, or using hard stem cuttings. Nigeria is rich in nutritionally and medicinally important flora and there are series of plants for bioprospecting, *Moringa oleifera* is one of these important plants, it is commonly called horseradish tree or the miracle tree and locally known as ‘Zogale-gandi’ in Hausa, ‘Eweigbale’ in Yoruba. It is mostly grown and widely cultivated in the northern part of Nigeria and many countries in tropical Africa. *M. oleifera* can be grown in a variety of soil conditions preferring well-drained sandy or loamy soil that is slightly alkaline (Abdul, 2007). Almost every part of *M. oleifera* can be used for food and as forage for livestock (Ram, 1994). The leaves can be eaten fresh cooked or stored as dried powder for several months the pods, when young can be cooked; eaten like beans (National Research Council, 2006). Its oil and micronutrients have been reported to contain antitumour, antiepileptic, antidiuretic, anti-inflammatory and venomous bite characters (Hsu, *et al.*, 2006). Virtually every part of the tree is beneficial in some way and both rural and urban people depend on it for their livelihood. Depending on the purpose and quantity, moringa is grown in nurseries, as a community project, or on a small scale at the family level. It can function as windbreaks, for land erosion control, live fences, as an ornamental, or intercropped to provide semi-shade to species requiring less direct sunlight. The useful products obtained from plants directly or indirectly, demonstrate their importance to man, Plants serve as a source of food (Saka and Msonthi, 1994; Katsayal *et al.*, 2004; Kawo, 2007), medicinal product (Adoum *et al.*, 1997; Ezeamuzie *et al.*, 1996; Caceres *et al.*, 1992), energy (Oladele and Yisa, 1989). Hence this study aims at harnessing the potentials of *Moringa oleifera* ethanolic extract to treat and prevent oxidative stress as a result of hypertension induced drugs.

Materials and Methods

Collection and preparation of samples for analysis

Freshly obtained leaves of *Moringa oleifera* were obtained from the Federal University of Technology satellite Estate in Akure, Ondo State, Nigeria. The identification of leaf was done at Plant Science department of, Ekiti State University, Ado Ekiti, Nigeria by plant taxonomist.

Leaves of *Moringa oleifera* were collected and air dried under shade and ground into powder with a Marlex Excella laboratory blender. An ethanolic extract of *Moringa oleifera* leaves was made. 200g of the powder was weighed and mixed in 500mL of ethanol in a corked bottle, shaken and allowed to stand overnight with constant shaking on mechanical shaker.

Experimental protocol

The study was performed on 20 male wistar albino rats housed in ventilated cages at the Biochemistry Department animal house, Ekiti State University, Ado-Ekiti, Nigeria. They were acclimatized for two weeks before administration of the drugs. Animals were divided into four groups of five rats each. Group A served as the positive control and received distilled water and rat feed. Group B animal were administered with 0.5mL of 40 % ethanolic extract of *Moringa oleifera*. Group C animal served as the negative control and were orally administered with 0.5mL single dose of dexamethasone (1.67 mg/kg body weight) while Group D animals were orally given 0.5mL dexamethasone (1.67mg/kg body weight) followed by 0.5mL of 40% ethanolic extract of *Moringa oleifera*. Animals were kept at optimum temperature with a 12hour light/dark cycle and given rat feed and water. The period of induction and treatment lasted for three weeks.

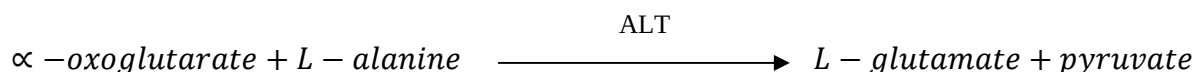
Preparation of organ homogenate and plasma collection

After three weeks of induction and treatment of the rats, they were sacrificed anesthetically using chloroform. Blood was collected using syringes and needles from the heart into anti-coagulant bottles. The organs (kidney and heart) of interest were also collected which were homogenized in 6.7mM potassium phosphate buffer and the supernatants were collected after spinning at 10,000 rpm in a centrifuge.

Biochemical Assay

Standard Randox kits were used to determine Cholesterol, Total protein, Alkaline Phosphatase (ALP), Aspartate Transaminase (AST) and Alanine Transaminase (ALT).

ALT



Alanine Aminotransferase was measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4-dinitrophenylhydrazine.

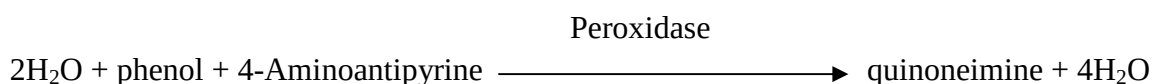
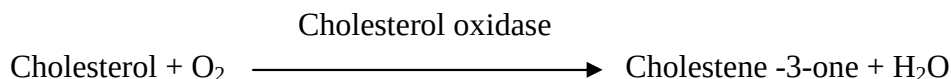
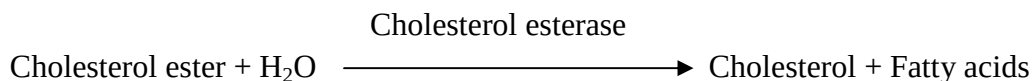
AST



AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2, 4-dinitrophenylhydrazine.

CHOLESTEROL

The Randox laboratory cholesterol kit was used to determine cholesterol on the basis of enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase using the following enzymatic reactions.



Determination of plasma Malondialdehyde (MDA)

The method of Varshney and Kale (1990) was used to determine lipid peroxidation by measuring the formation of thiobarbituric acid reactive substances (TBARS). An aliquot of 0.4mL of the plasma or other organ homogenates was mixed with 1.6mL of Tris-KCl buffer to which 0.5mL of 30% trichloroacetic acid (TCA) was added. Then 0.5mL of 0.75% TBA was added and placed in a water bath for 45 minutes at 80°C. This was then cooled on ice and centrifuged at 3000g. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532nm. The MDA level was calculated according to the method of Adam-Vizi and Seregi (1982). Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

Calculation

$$\text{MDA (units/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532\text{nm}} \times \text{volume of sample} \times \text{mg protein}}$$

Determination of reduced glutathione

The reduced glutathione (GSH) was determined following the method of Jollow *et al.* (1974). 0.2mL of sample was added to 1.8mL of distilled water and 3mL of the precipitating solution was mixed with sample. The mixture was then allowed to stand for approximately 10 minutes and then centrifuged at 3000g for 5 minutes. 0.5mL of the supernatant was added to 4mL of 0.1M phosphate buffer. Finally 0.5mL of the Ellman's reagent was added. The

absorbance of the reaction mixture was read within 30 minutes of colour development at 412nm against a reagent blank.

Determination of catalase activity

This experiment was carried out using the method described by Sinha (1972). 0.2ml of sample was mixed with 0.8ml distilled H₂O to give 1 in 5 dilution of the sample. The assay mixture contained 2mL of solution (800μmol) and 2.5mL of phosphate buffer in a 10mL flat bottom flask. 0.5mL of properly diluted enzyme preparation was rapidly mixed with the reaction mixture by a gentle swirling motion. The reaction was run at room temperature. A 1mL portion of the reaction mixture was withdrawn and blown into 1mL dichromate/acetic acid reagent at 60 seconds intervals. The hydrogen peroxide content of the withdrawn sample was determined by the method described above. The mononuclear velocity constant, K, for the decomposition of H₂O₂ by catalase was determined by using the equation for a first-order reaction: $K = 1/t \log S_0/S$, where S₀ is the initial concentration of H₂O₂ and S is the concentration of the peroxide at t min. The values of the K are plotted against time in minutes and the velocity constant of catalase K₍₀₎ at 0 min determined by extrapolation. The catalase contents of the enzyme preparation were expressed in terms of Katalase feiahigkei or 'Katf' according to von Euler and Josephson (1927).

$$\text{Kat. f} = \frac{K_{(0)}}{\text{mg protein/ml}}$$

Determination of Superoxide Dismutase (SOD)

The level of SOD activity was determined by the method of Misra and Fridovich (1972). 1mL of sample was diluted in 9mL of distilled water to make a 1 in 10 dilution. An aliquot of the diluted sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3ml of freshly prepared 0.3mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5ml buffer, 0.3ml of substrate (adrenaline) and 0.2ml of water. The increase in absorbance at 480nm was monitored every 30 seconds for 150 seconds.

Calculation

$$\text{Increase in absorbance per minute} = \frac{A_3 - A_0}{2.5}$$

where A₀ = absorbance at 0 second

A₃ = absorbance at 150 seconds

$$\% \text{ inhibition} = \frac{\text{increase in absorbance for substrate}}{\text{increase in absorbance of blank}} \times 100$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute.

Statistical Analysis: The experimental results of the analyses were obtained in triplicates with means and standard deviations. Various formulae were used to calculate the individual parameter and enzyme activities. The means and standard deviations of the triplicates results were determined using Microsoft Office Excel 2007

RESULTS AND DISCUSSIONS

Table 1: Specific activity of alkaline phosphatase (ALP) in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaf.

Specific activity (IU/mg protein)				
Groups	Heart	Kidney	Plasma	
A	41.40 ±1.06	5.52 ±0.42	16.56 ±2.10	
B	39.78 ±0.56	11.04 ±0.22	19.32 ±1.64	
C	13.80 ±0.43	30.36 ±0.12	32.76 ±0.06	
D	25.52 ±0.23	8.28 ±0.76	11.04 ±1.26	

Mean of individual triplicate results ±STDEV

Table 1 showed the activity of alkaline phosphatase in the heart, kidney and serum of hypertensive induced rats treated with ethanolic extract of *Moringa oleifera* leaves. We observed about 67% decrease in ALP activity in the heart of the rats treated with dexamethazone when compared with the control (group A) while the ethanolic extract caused about 28% reversal in the activity of ALP in the heart when compared with control. There was a contrary observation in the kidney as the drug showed increased ALP activity when compared with the control though treatment with the extract caused a reversal to the control as seen in group A. These observations showed the toxic effect of the drug and the ability of the ethanolic extract of the plant to reverse the toxic effect on both organs of the animals. The activity of the enzyme was also monitored in the plasma. Normally, enzymes will not always be found in the serum except when there is damage to one or more organs of the body. Therefore, enzymes from diseased organs may become manifested in the serum resulting in increased activity since they must have leaked from the diseased organ. The results obtained showed that the ethanolic extract of the plant in groups B

and D in both organs and plasma caused a reversal in the ALP activity, adjusting their values toward those recorded in the respective controls. The observations in this work showed a resemblance to trends observed in the effect of aqueous extract of nutmeg in bromate induced renal toxicity in rats (Oseni *et al.*, 2015). Increase in plasma level of ALP is due to increased synthesis, in presence of increasing organ dysfunction, as measurement of ALP activity is of clinical and toxicological importance as changes in its activity is indicative of kidney damage by toxicants or in diseased conditions (Singh *et al.*, 2001).

Table 2: Specific activity of aspartate transaminase (AST) in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaf.

Specific activity (IU/mg protein)				
Groups	Heart	Kidney	Plasma	
A	163.00 ± 5.98	82.00 ± 3.39	29.50 ± 2.88	
B	161.00 ± 6.34	76.00 ± 7.23	24.50 ± 7.89	
C	130.00 ± 4.86	115.50 ± 8.80	123.00 ± 3.25	
D	140.00 ± 2.86	68.50 ± 5.54	67.00 ± 2.78	

Mean of individual triplicate results ± STDEV

Tables 2 and 3 showed the AST and ALT activities in the tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaves, the results obtained showed that the extract reversed the effects of the drug in both organs. These tissues participate in a variety of metabolic activities and contain a host of enzymes. The increased plasma AST and ALP activities of the hypertension induced animals showed that the drug resulted in membrane disruption, cellular leakage and loss of functional integrity of cell membrane the organs of the animals in the group C, while the extract performed corrective roles as observed in the treated animals in group D, while the results of animals in group B showed that the extract maintained the organs integrity.

Table 3: Specific activity of alanine transaminase (ALT) in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaves.

Specific activity (IU/mg protein)				
Groups	Heart	Kidney	Plasma	
A	5.40 ±1.65	3.00 ±1.21	67.00 ±2.67	
B	6.25 ±0.80	5.60 ±1.10	69.58 ±2.43	
C	168.20 ±5.34	20.80 ±3.07	125.00 ±2.30	
D	6.80 ±0.75	7.60 ±1.98	57.40 ±1.64	

Mean of individual triplicate results ±STDEV

The elevated activities of AST and ALT in plasma are indicative of cellular leakage and loss of the functional integrity of cell membranes (Drotman and Lawhorn, 1978). Administration of dexamethasone significantly raises the plasma level of AST and ALT activities in rats as observed in our results, which indicated that apart from the hypertensive effect of the drug, it can also cause damage to other organs of the body, measurement AST and ALT activities in rats as observed in our results is of clinical and toxicological importance as changes in their activities are indicative of tissues damage by toxicants or in diseased conditions (Singh *et al.*, 2001). The high level of activities of AST, ALT and ALP in the kidney and heart tissues of the rats and their corresponding decreased activities in the plasma is indicative of non toxicity of the ethanolic extract in these organs.

AST in plasma was increased in dexamethasone-induced rats. A marked elevation in the concentration of the enzyme was also recorded in the kidney and the heart after the injection of the drug. This observation was supported by Fleischer and Schwartz (1971) who reported that any damage done to the cell membrane may lead to leakage of AST and ALP, which is a marker enzyme in the plasma membrane, into extracellular fluid. The study is also corroborated by the work Rajesh and Latha, (2004) that recorded the same trend in CCl₄-intoxicated rats treated with Kamilari. Oral administration of ethanolic extract of *Moringa oleifera* leaves caused a decrease in the activity of the above enzymes, which may be a consequence of the stabilization of plasma membrane as well as repair of damage caused to the organ by dexamethasone. This indicates that the ethanolic extract displayed high degree of protection in the organs and plasma.

Table 4: Reduced GSH in selected tissues of hypertensive induced rats treated with ethanolic extract of *Moringa oleifera* leaf.

(µg/mL)			
Groups	Heart	Kidney	Plasma
A	0.769 ±0.43	0.114 ±0.04	0.641 ±0.42
B	0.841 ±0.03	0.126 ± 0.02	0.763 ±0.04
C	0.280 ±0.04	0.053 ± 0.10	0. 201 ±0.06
D	0.652 ±0.08	0.123 ± 0.03	0. 428 ±0.05

Mean of individual triplicate results ±STDEV

Glutathione is a small tripeptide protein synthesized in the liver (Kaplowitz, *et al.*, 1985). It is a potent antioxidant with high redox potential and it also serves as a co-factor for several oxidative stress detoxifying enzymes (glutathione peroxidase and glutathione transferase) (Valko *et al.*, 2007; Parris, 1997). Glutathione also helps in the regeneration of some important antioxidant vitamins such as C and E. Depletion of GSH has been reported in apoptosis and many degenerative conditions (Parris, 1997). The results of the present study showed that the level of glutathione (GSH) was significantly decreased in the plasma, heart and kidney homogenates of the animals fed with dexamethazone when compared with the control group A animals. This depletion in blood and organs glutathione is attributable primarily to the dexamethasone, a xenobiotic and an inducer of hypertension. The effects of ethanolic extract of horseradish leaf treatments on the organs and plasma concentration of reduced Glutathione (GSH) showed a significant reversal to the control levels in the tissues and plasma as seen in Table 4. The observations in this study were however similar to what was obtained by Ologundudu *et al.*, (2010) in their work on the effect of *Hibiscus sabdariffa* anthocyanins on 2, 4-dinitrophenylhydrazine-induced tissue damage in rabbits. The reduction in glutathione to the level that was observed in this study could lead to a significantly decrease in the total antioxidant status of the animals because glutathione helps in recycling cellular antioxidants, inhibits free radical damage and plays a key role in the detoxification of harmful compounds (Robert *et al.*, 2000). This agrees with earlier works carried out by Unt *et al.*, (2008) and Dominquez *et al.*, (1998) who reported reduced total plasma antioxidant capacity in uncontrolled hypertension. However, the plant extract intake by the hypertensive rats increased their glutathione status to near the control level. This is remarkable as it implies that *Moringa* ethanolic extract could have an ameliorating effect on the altered antioxidant status of the hypertensive rats. It has been

reported that decreased hepatic and serumGSH has been consistent with exposure to toxic and oxidative agents which may also lead to more free reactive oxygen species (ROS) leading to excessive oxidative damage (Vijayaet *al.*, 2011; Ahmed *et al.*, 2008; Videla *et al.*, 1991). The results obtained in this work revealed that dexamethazone treated group showed reduced glutathione in heart, kidney and plasma when compared with the control which however corroborate the results of other previous investigators.

Table5 revealed the effects of ethanolic extracts of horseradish (*Moringa oleifera*) leaf treatments on the catalase activity of dexamethasone induced hypertensive rat. Catalase is an enzyme which functions in the detoxification of hydrogen perioxide. The reduction in catalase activity after injection with dexamethasone in the heart, kidney and plasma respectively is another significant finding in this study. This decreased concentration in catalase activityof organs and plasma is attributable in part to the reduced synthesis of this antioxidant enzyme whose concentrations would have fallen as a result of the dexamethasone that was injected into the animals. This study is in agreement with earlier reports by (Erukainure *et al.*, (2012), Udoh *et al.*, (2007) and Tagami *et al.*, (1992) who reported a decreased red blood cell catalase activity in diabetic conditions, while treatment with aqueous extracts of *Moringa oleifera* leaves caused a slight increase in catalase activity.

Table 5: Specific activity catalase in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaf.

Groups	(µg/ml)		
	Heart	Kidney	Plasma
A	18.40±1.20	10.3±0.20	11.42±0.20
B	22.56±2.50	9.62± 0.01	10.72±0.04
C	4.25±0.20	1.66± 0.01	3.42 ±0.01
D	16.50±0.75	9.18± 0.01	8.97 ±0.01

Mean of individual triplicate results ±STDEV

Table 6 revealed the total cholesterol concentration in heart, kidney and plasma of hypertension induced rats that were treated with ethanolic extract of *Moringa oleifera* leaf. The observations in this study revealed that dexamethazone caused hypercholesterolemiawhich was subsequently reversed by the ethanolic extract of *Moringa oleifera*.This was however contrary to observation by Oseni *et al.*, (2015) where total cholesterol concentration was depleted as a result of rat fed

with bromate. However, the reverse of the total cholesterol concentration to almost control level was observed by the ethanolic extract of *Moringa oleifera*. The ethanolic extract also showed its protective ability against hypercholesterolemia on control animals fed with the extract as seen in Table 6 group B animals

Table 6: Total cholesterol concentration in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaves.

mMol/dL Groups	Heart	Kidney	Plasma
A	21.419± 2.09	11.990 ± 3.11	60.477± 6.34
B	19.138 ± 3.23	8.349 ± 1.87	53.547± 4.23
C	77.486 ± 5.76	16.379 ± 2.98	101.430±6.98
D	26.930 ± 3.87	13.859 ±5.87	67.107±3.47

Mean of individual triplicate results ±STDEV

Table 7 showed the effects of ethanolic extract of horseradish leaf treatment on the malondialdehyde concentration in dexamethasone-induced hypertensive rats. The concentration of plasma Malondialdehyde (MDA) was shown to be significantly increased in the heart, kidney and plasma of hypertensive rats when compared with the control. However, the hypertensive rats, when placed on *Moringa oleifera* ethanolic extracts had a remarkable decrease in their cardiac, renal and plasma malondialdehyde levels when compared with the control, thus indicating the extract ability to protect against cellular lipid peroxidation caused by the drug and free radical scavenging ability of the ethanolic extract of *Moringa oleifera* leaves in hypertensive rats. The ethanolic extract also showed its protective ability against cellular lipid peroxidation on the normal animals fed with the extract as seen in Table 7 group B animals.

The ability of the ethanolic extract to inhibit the process of lipid peroxidation *in vivo* may be due to the free radical scavenging activities of its phytochemical components. The observations in this study however allude to previous work (Oseni *et al.*, 2015) on effects of aqueous extract of nutmeg on potassium bromated induced renal toxicity and on protective effect of *Tulbaghia violacea* rhizome extract against hypercholesterolemia-induced oxidative stress in Wistar rats (Olorunnisola *et al.*, 2012).

Table 7: Malondialdehyde concentration in selected tissues of hypertension induced rats treated with ethanolic extract of *Moringa oleifera* leaf.

(Units/ mg)			
Groups	Heart	Kidney	Plasma
A	0.22 ± 0.01	0.12±0.002	0.50±0.002
B	0.12±0.001	0.08±0.003	0.32±0.004
C	0.31±0.001	0.35±0.010	1.10±0.030
D	0.15±0.001	0.29±0.001	0.30±0.002

Mean of individual triplicate results ±STDEV

CONCLUSSION AND RECOMMENDATION

Moringa oleifera leaf extract has chemopreventive and protective benefits on Dexamethasone mediated tissues oxidative damage in rat as it significantly reduced the extent of antioxidant loss and restoration of organ dysfunction caused by Dexamethasone in rat.

The results of this study show preventive and protective effect on tissues functions that might be due to the presence of some bioactive compounds in plant ethanolic extract. On the basis of our study however, it can be concluded that *Moringa oleifera* leaf ethanolic extract has plasma, cardiac and renal antitoxicity and protective effects as well as antioxidant abilities. Further investigations may therefore be necessary to elucidate the active components of *Moringa oleifera* leaf and establish their chemical nature.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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