Journal of Global Biosciences ISSN 2320-1355
Vol. 2(1), 2013, pp. 1-9 http://mutagens.co.in/jgb.html

EFFECTS OF EXTRACTS OF *TRIPLOCHITON SCLEROXYLON* (K. SCHUM) ON SOME PLASMA NON-ENZYME ANTI-OXIDANT CONCENTRATIONS IN DIABETIC RATS

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Abstract

In the aim to ascertain some of the protection offered by extracts of *Triplochiton scleroxylon* used in some parts of Nigeria to treat diabetes mellitus, some plasma antioxidant concentrations were determined in streptozotocin-induced diabetic rats treated with the extracts for 28 days. Plasma glucose concentration was also determined. Blood was collected from the tails of rats (Wistar strain) and analysed spectrophotometrically. Streptozotocin-induced diabetes mellitus increased plasma glucose but caused a decrease in reduced glutathione, vitamins C and E concentrations in experimental rats. However, treatment with aqueous and 50% ethanol extracts at 200 mg/kg body weight, increased plasma reduced glutathione, vitamins C and E concentrations ($P<0.05$), when compared to diabetic controls, respectively. Aqueous extract exerted a greater decrease in plasma glucose concentration ($P<0.05$) while 50% ethanol extract recorded a greater increase in plasma vitamins C and E in treated streptozotocin-induced diabetic rats. Aqueous extract did not cause any adverse histological changes in the tissues investigated whereas 50% ethanol extract precipitated tubular necrosis and mild fatty changes in the kidneys and liver of rats, respectively.

Anti-diabetic potential of extracts of *T. scleroxylon* is a justification for the use of this plant to treat diabetes mellitus in some areas of Nigeria. Extracts also possess appreciable anti-oxidant property essential in guiding against free radical destruction and complications associated with oxidative stress.

Key words: plasma anti-oxidants, aqueous and ethanol extracts, *Triplochiton scleroxylon*, diabetic rats.

INTRODUCTION

Antioxidants are generally touted as anti-aging, anti-cancer, anti-disease substances that are believed could improve health, increase longevity, and fight certain types of diseases like cancer [1]. Antioxidative vitamins have a number of biological activities such as immune stimulation, inhibition of nitrosamine formation and an alteration of metabolic activation of carcinogens. They can prevent genetic changes by inhibiting DNA damage induced ROS. They protect the somatic cell from free radicals that are thought to be responsible for wide range of diseases [2]. Some plasma anti-oxidant vitamins (e.g. C and E) play excellent roles in protecting the cells from oxidative stress or damage [3]. Most of the natural products used in traditional medical systems for treating diabetes contain a wide scale of antioxidants with a potent scavenging activity for reactive oxygen species (ROS) [4, 5]. Aqueous bark extract of *Triplochiton scleroxylon* is *commonly* used to treat diabetes mellitus in some of the southern and western parts of Nigeria [6, 7, 8]. It is found in the humid ever green semi-deciduous forest along water ways in the tropical West Africa [9, 10]. *T. scleroxylon* belongs to the family of tropical medicinal plants [9] whose active ingredients are believed to be at the stem bark. *T. scleroxylon* has been classified as follows: kingdom: plantae, division: magnoliophyta, class: magnoliopsida, order: malvales, family: sterculiaceae (APG: Malvaceae), genus: *triplochiton* and species: *T. scleroxylon*, K. Schum [9, 10].

This study examined the possible relationship between the use of these extracts to treat diabetes mellitus in rats and the activities of non-enzyme antioxidants in the plasma; as any compromise would cause oxidative stress and other health risks.

MATERIALS AND METHODS

All the experimental protocols were according to our Institutional Animal Ethics Committee guidelines as well as internationally accepted practices for use and care of laboratory animals as contained in US guidelines [11].

Experimental Animals

Male albino rats (Wistar strain) obtained from the animal house of the College of Medicine, Ambrose Alli University, Ekpoma, Edo State, Nigeria, were used in this study. The rats weighed between 130 to 186 g and were housed

in clean cages under standard laboratory conditions of temperature, humidity and light. All the albino rats were allowed free access to standard laboratory diet supplied by Ewu feeds Ltd. Ewu, Edo State, Nigeria and distilled water *ad libitum* for a period of 2 weeks to acclimatize to the new environment. All animals were handled with humane care [12].

Medicinal plant

The barks of *Triplochiton scleroxylon* were obtained from the forest of Uokha, Owan - East local government area, Edo State, Nigeria. They were then identified by experts in the Department of Botany, University of Ibadan, Ibadan, Oyo State, Nigeria, as *Triplochiton scleroxylon* K. Schum where a voucher specimen (UIH – 22329) had been deposited [12].

Extraction and Preparation of Plant Extracts

The barks of *T. scleroxylon* were washed with clean water, dried and cut into tiny strands. They were pulverized into powder and 1000 g of powdered bark of this plant was then extracted separately in 7000 ml of aqueous (distilled water) and 50 % ethanol in cold percolation by maceration technique under room temperature. This was followed by periodic stirring. The macerated samples were filtered with sintered glass funnel under suction to eliminate particles after 72 hours. The filtrates collected were then concentrated on a reduced pressure using the rotary evaporator to yield thick brown viscous pastes which were further dried under vacuum with the aid of a freeze dryer [13]. The freeze dried samples were then kept in the freezer at -21 ^{o}C until used. The yields were 14.22% (w/w) and 12.81% (w/w) for aqueous and 50% ethanol extracts, respectively [12].

Blood collection

The tail of the restrained rat was cleansed with a ball of cotton wool soaked in methylated spirit. A little vaseline was then smeared on the tail to reduce friction while massaging to redness. Gentle massage towards the tip of the tail continued until the tip became red; sign of blood accumulation. The red tip of the tail was then slightly incised with new and sterilized blade and further massaged gently as the blood tickled into immobilized sample tubes containing lithium heparin (for anti-oxidant assays) and fluoride oxalate (for glucose assay). Cotton wool soaked in methylated spirit was again used to cleanse the incised area of the tail. Blood samples collected were subjected to centrifugation for 10 minutes at 3,000 G to obtain the plasma for all biochemical analyses. Analyses were carried out immediately after centrifugation [12].

Streptozotocin injection

Streptozotocin (100 mg), dissolved in commercial saline (5 ml) was administered to overnight fasted rats (65 mg/kg body weight) by intra-peritoneal route. Rats with blood glucose level two or three times the basal values, seven days after injection were selected for the experimental study [14, 15, 16, 17].

Experimental procedure

Male albino rats of Wistar strain after acclimatization for a period of two weeks were fasted overnight and randomly divided into four groups of four rats each. Rats in groups 2, 3 and 4 were injected streptozotocin (i.p., 65 mg/kg body weight) and having being certified diabetic seven days after this injection were treated simultaneously with rats in group 1 as follows:

Group 1: served as normal control and received distilled water *ad libitum*

Group 2: served as diabetic control and received distilled water *ad libitum*

Group 3: served as diabetic rats treated with aqueous extract of *T. scleroxylon* (200 mg/kg body weight)

Group 4: served as diabetic rats treated with 50% ethanol extract of *T. scleroxylon* (200 mg/kg body weight)

Administration of extracts

Aqueous and 50% ethanol extracts of *T. scleroxylon* were administered to experimental rats orally (p. o.) with the aid of the gavage.

Biochemical assays

Plasma glucose was determined by the procedure described in the kit supplied by Randox Laboratories Ltd. United Kingdom.

Estimation of plasma reduced glutathione [18].

1ml of supernatant (0.5ml of plasma precipitated in 2ml of 5% TCA) was measured into a test tube, 0.5ml of Ellman's reagent (0.0198% DTNB in 1% sodium citrate) and 3ml of 0.1M phosphate buffer (pH 8.0) were added and colour which developed was read at 412nm as a function of reduced glutathione concentration.

Estimation of vitamin C concentrations [19].

To 0.5 ml of plasma, 1.5 ml of 6 % Trichloroacetic acid (TCA) was added and centrifuged at 3,500 g for 20 minutes. To 0.5 ml of supernatant, 0.5 ml of dinitrophenylhydrazine (DNPH) reagent, prepared by dissolving 2% DNPH and 4% thiourea in 40 ml of 9 N sulphuric acid (H_2SO_4) and made up to 100 ml

with 9N H_2SO_4, was added and incubated for 3 hours at room temperature. 2.5 ml of 85% H_2SO_4 was added after incubation and the colour developed read at 530 nm in a spectrophotometer, after 30 minutes. Concentration of vitamin C (µmole/ml) was calculated from absorbance of sample/absorbance of standard x concentration of standard.

Estimation of vitamin E concentrations [20]
Vitamin E was extracted from plasma by adding 1.6 ml absolute ethanol and 2.0 ml petroleum ether to 0.5 ml of plasma. This was centrifuged for 5 minutes at 1,500 g. The supernatant was separated and evaporated. To the residue, 0.2 ml of 0.2 % 2, 2-dipyridyl and 0.2 ml of 0.5% ferric chloride were added and the reaction mixture kept in the dark for 5 minutes. A red coloured layer which developed on addition of 4 ml butanol, was read in the spectrophotometer at 540 nm. Concentrations (µmole/ml) of vitamin E in the plasma were extrapolated from the standard curve.

Statistical analysis
Data were expressed as mean ± S. E. M. of three separate determinations. The statistical significance was evaluated by one-way ANOVA using SPSS (statistical package for social sciences) version 16.0, followed by post –hoc LSD and Turkey tests for individual comparisons. Values lower than 0.05 probabilities were accepted as statistically significant.

RESULTS

Results have been presented in Tables (1 to 4), Figures (1 to 4) and further elaborated in histological photomicrographs (Plates 1 to 12). Extracts have anti-diabetic potentials (Table 1 and Fig. 1) but caused significant increase in the plasma concentrations of reduced glutathione (Table 2 and Fig. 2), vitamins C (Table 3 and Fig. 3) and E (Table 4 and Fig. 4) in the treated streptozotocin-induced diabetic rats when compared to diabetic control. Aqueous extract was safer in treating diabetes mellitus in rats as its use did not cause adverse histological changes in the tissues examined, in contrast with 50% ethanol extract of *Triplochiton scleroxylon*.

Table 1: Mean plasma glucose concentrations (mg/dl) of controls and treated streptozotocin-induced diabetic rats.

S/N.	Treatments	0days	1days	6days	12days	18days	24days	28days
1.	NC	88.92±1.52^a	89.95±1.88^a	91.94±1.35^a	93.28±1.13^a	93.44±1.18^a	92.38±2.25^a	93.24±1.28^a
2.	DC	81.00±1.58^a	206.94±1.73^b	206.93±1.94^b	208.36±0.97^b	210.00±2.21^b	214.43±8.05^b	203.93±2.11^b
3.	ATD	77.25±2.29^a	211.38±3.59^b	186.18±4.12^c	140.92±4.04^c	126.57±2.36^c	107.39±2.80^c	96.39±2.02^a
4.	ETD	81.00±2.04^a	212.77±2.00^b	199.89±0.64^b	168.23±2.41^c	146.17±2.36^d	113.72±2.69^c	108.29±1.97^c

Data are mean ± S.E.M. (n = 4). Mean in the same column with different superscript letters are significantly different, P<0.05(one way ANOVA followed by post-hoc LSD) when compared to diabetic control. NC: normal control; DC: diabetic control; ATD: aqueous treated diabetes; ETD: ethanol treated diabetes.

Table 2: Mean plasma reduced glutathione concentrations (mg/dl) of controls and treated streptozotocin-induced diabetic rats.

S/N.	Treatments.	0days	1days	6days	12days	18days	24days	28days
1.	NC	0.45±0.00^a	0.44±0.00^a	0.44±0.00^a	0.44±0.00^a	0.44±0.00^a	0.44±0.01^a	0.45±0.01^a
2.	DC	0.44±0.00^a	0.29±0.00^b	0.28±0.01^b	0.27±0.01^b	0.26±0.01^b	0.24±0.00^b	0.21±0.01^b
3.	ATD	0.44±0.00^a	0.29±0.00^b	0.29±0.00^b	0.29±0.00^c	0.30±0.00^c	0.34±0.00^c	0.36±0.01^c
4.	ETD	0.44±0.00^a	0.30±0.00^b	0.30±0.00^c	0.30±0.00^c	0.29±0.01^c	0.30±0.01^d	0.31±0.00^d

Data are mean ± S.E.M. (n = 4). Mean in the same column with different superscript letters are significantly different, P<0.05(one way ANOVA followed by post-hoc LSD). NC: normal control; DC: diabetic control; ATD: aqueous treated diabetes; ETD: ethanol treated diabetes.

Table 3: Mean plasma vitamin C concentrations (mg/dl) of controls and treated streptozotocin-induced diabetic rats.

S/N.	Treatments	0days	1days	6days	12days	18days	24days	28days
1.	NC	0.19±0.00^a	0.19±0.00^a	0.19±0.00^a	0.19±0.00^a	0.20±0.00^a	0.20±0.01^a	0.20±0.00^a
2.	DC	0.20±0.01^a	0.17±0.01^a	0.14±0.01^b	0.14±0.01^b	0.13±0.01^b	0.11±0.01^b	0.08±0.01^b
3.	ATD	0.19±0.00^a	0.15±0.00^a	0.15±0.00^b	0.17±0.01^c	0.22±0.01^a	0.23±0.01^c	0.23±0.01^c
4.	ETD	0.20±0.01^a	0.15±0.01^a	0.16±0.01^b	0.19±0.00^a	0.22±0.01^a	0.24±0.01^c	0.24±0.01^c

Data are mean ± S.E.M. (n = 4). Mean in the same column with different superscript letters are significantly different, P<0.05(one way ANOVA followed by post-hoc LSD) when compared to diabetic control. NC: normal control; DC: diabetic control; ATD: aqueous treated diabetes; ETD: ethanol treated diabetes.

Table 4: Mean plasma vitamin E concentrations (mg/dl) of controls and treated streptozotocin-induced diabetic rats.

S/N.	Treatments	0days	1days	6days	12days	18days	24days	28days
1.	NC	46.52±1.04^{a}	45.21±1.04^{a}	45.70±1.09^{a}	44.83±1.73^{a}	45.22±1.12^{a}	46.27±1.62^{a}	47.26±1.09^{a}
2.	DC	45.54±1.47^{a}	31.20±1.10^{b}	26.24±1.54^{b}	20.50±1.28^{b}	15.43±1.18^{b}	10.83±0.76^{b}	8.00±1.02^{b}
3.	ATD	45.73±2.34^{a}	33.53±2.56^{b}	31.61±2.33^{c}	35.15±3.21^{c}	39.09±3.02^{c}	42.22±2.48^{a}	48.39±1.95^{a}
4.	ETD	47.20±1.36^{a}	35.47±0.83^{b}	43.40±1.66^{a}	48.54±1.27^{a}	49.12±0.88^{a}	50.46±0.88^{c}	54.73±1.02^{c}

Data are mean ± S.E.M. (n = 4). Mean in the same column with different superscript letters are significantly different, P<0.05(one way ANOVA followed by post-hoc LSD) when compared to diabetic control. NC: normal control; DC: diabetic control; ATD: aqueous treated diabetes; ETD: ethanol treated diabetes.

DISCUSSION

The blood glucose concentrations of diabetic rats (Table 1) obtained in this study were indicative of the diabetic status of experimental rats. The values of plasma glucose following streptozotocin injection agreed with values reported by Venkatesh et al., [21] in alloxan induced diabetic rats and Bhaskar et al., [22] in streptozotocin diabetic rats. Aqueous and ethanol (50%) extracts of the plant resulted in significant decrease (P<0.05) in plasma glucose concentrations in treated streptozotocin-induced diabetic rats when compared to diabetic control. The anti-diabetic potentials of aqueous and ethanol (50%) extracts of *T. scleroxylon* could be attributable to the presence of some phytochemicals viz:, flavonoids, tannins, steroids, phlobatannins and saponins [23] which are common in plants with known hypoglycemic effect [24]. Anti-diabetic and hypoglycaemic properties of aqueous extract of *T. scleroxylon* in streptozotocin-induced diabetic and normal rabbits have been documented [16, 17]. Substances with known hypoglycaemic and anti-diabetic properties would be effective in the management of diabetes mellitus [25].

Non–enzyme antioxidants such as reduced glutathione (GSH), vitamin C and vitamin E play excellent roles in protecting the cells from oxidative stress or damage. It is well established that GSH in blood keeps up the cellular levels of the active forms of vitamin C and vitamin E by neutralizing the free radicals. Whenever there is a reduction in plasma GSH the cellular levels of vitamin C is also lowered, indicating that GSH, vitamin C and vitamin E are interlinked [3]. Vitamin E is one of the most important free radicals scavenging chain-breaking antioxidant in the cell membrane [26] and it is a lipid soluble vitamin which occurs in the plasma as a variety of tocopherols, most important being α-tocopherols. It scavenges peroxy radical intermediates in lipid peroxidation and is responsible for protecting polyunsaturated fatty acid (PUFA) present in cell membranes and low density lipoprotein against lipid peroxidation. It is probably the most efficient antioxidant in the lipid phase. The sole physiological role of vitamin E is to quench free radical reaction as a chain – breaking antioxidant [27, 28]. Vitamin C is an important water soluble antioxidant in biological fluids and an essential micronutrient required for normal metabolic functions of the body. As an antioxidant, vitamin C helps in cardiovascular disease by protecting the linings of arteries from oxidative damage. It neutralizes reactive oxygen species (ROS) and reduces oxidative DNA damage and genetic mutations. It reacts with superoxide, hydroxyl ion and also with hydroperoxides to form dehydroascorbate. Ascorbate also reacts with α-tocopheroxyl radical to generate the reduced form of vitamin E (thereby functioning as a chain breaking antioxidant) [29, 2]. Reduced glutathione by its free radical scavenging activity protects tissues against damage.

In diabetes, disturbances in ascorbic acid metabolism might have a great role in the pathogenesis of diabetic complications [30]. Reduced glutathione, vitamins C and E significantly increased (P<0.05) (Tables 2, 3, 4 and Figs. 2, 3, 4) in streptozotocin-induced diabetic rats treated with aqueous and ethanol extracts of *T. scleroxylon* when compared to diabetic controls, respectively. The increased concentrations of vitamins C, E and GSH may have resulted from the scavenging activities of these antioxidants of the radicals that could have been generated by streptozotocin–induced diabetes mellitus [31] and in part by flavonoid content of extracts of *T. scleroxylon* known to have antioxidant activities [32, 26]. High levels of reduced GSH protects the cells from oxidative stress and hence from damage [33]. Prohp and Onoagbe [34] have reported an increase in the concentrations of plasma anti-oxidants in non-diabetic rats following administration of aqueous and 50% ethanol extracts for 28 days. The organs investigated showed no adverse histopathological changes in streptozotocin induced diabetic rats treated with aqueous extract (200 mg/kg body weight) of *T. scleroxylon* for 28 days (Plates 7, 8, 9) relative to diabetic controls (Plates 4, 5, 6). The organs

of normal (non-diabetic) rats showed normal histology (Plates 1, 2, 3). The hearts of rats tolerated both extracts of *T. scleroxylon* throughout the experiment (Plates 4, 7, 10). Vacuolation of tubular epithelium with necrosis in the kidneys (Plate 11) were observed in diabetic rats treated with 50% ethanol extract (200 mg/kg body weight). However the heart and liver showed normal histology (Plates 10 & 12). The result of this study was a justification for the intuitive use of aqueous extract of *T. scleroxylon* in some parts of Nigeria to treat diabetes mellitus.

CONCLUSION

Aqueous extract exhibited a greater and safer anti-diabetic property and would be more useful in the treatment of diabetes mellitus. Extracts also demonstrated ability to protect against oxidative stress.

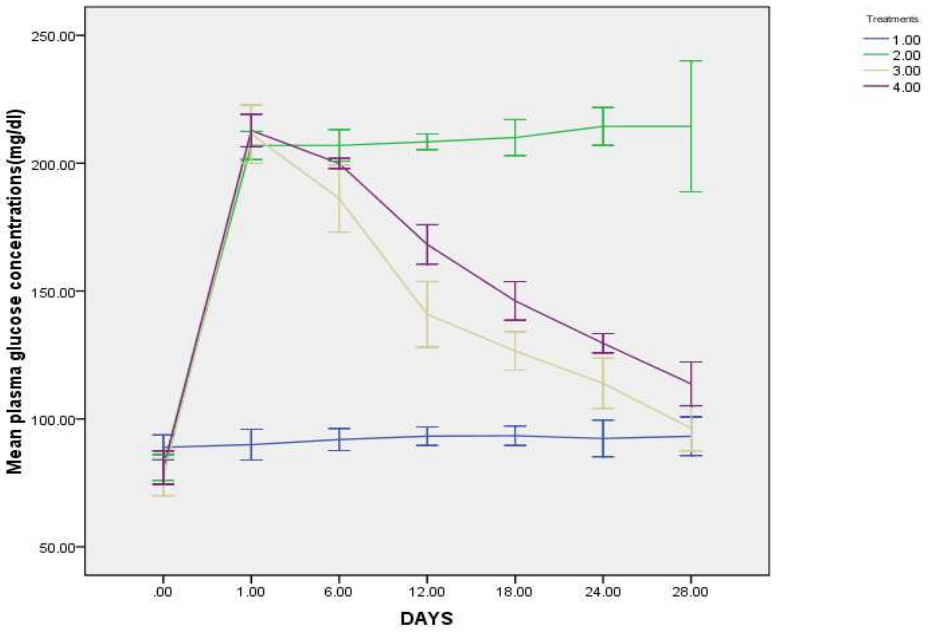

Fig.1:Mean plasma glucose concentrations(mg/dl) of controls and treated streptozotocin-induced diabetic rats.1.00:Normal Control; 2.00:Diabetic Control;3.00:Aqueous extract;4.00:50% Ethanol extract.

Error bars: 95% CI

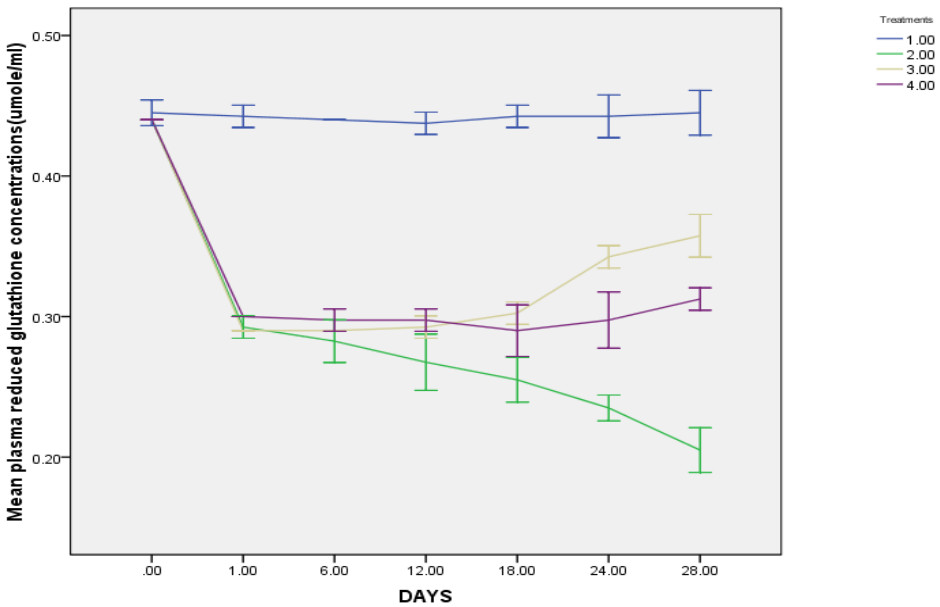

Fig. 2:Mean plasma glutathione concentrations(umole/ml) of controls and treated streptozotocin-induced diabetic rats.1.00:Normal Control;2.00:Diabetic Control;3.00:Aqueous extract;4.00:50% Ethanol extract.

Error bars: 95% CI

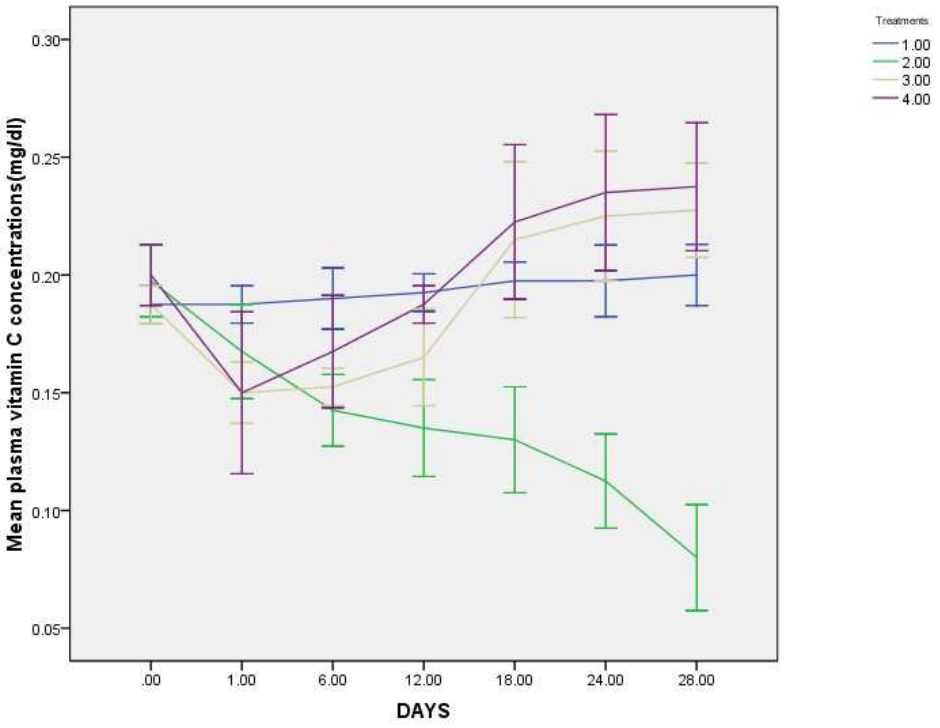

Fig.3:Mean plasma vitamin C concentrations(mg/dl) of controls and treated streptozotocin-induced diabetic rats.1.00:Normal Control;2.00:Diabetic Control;3.00:Aqueous extract,4.00:50% Ethanol extract.

Error bars: 95% CI

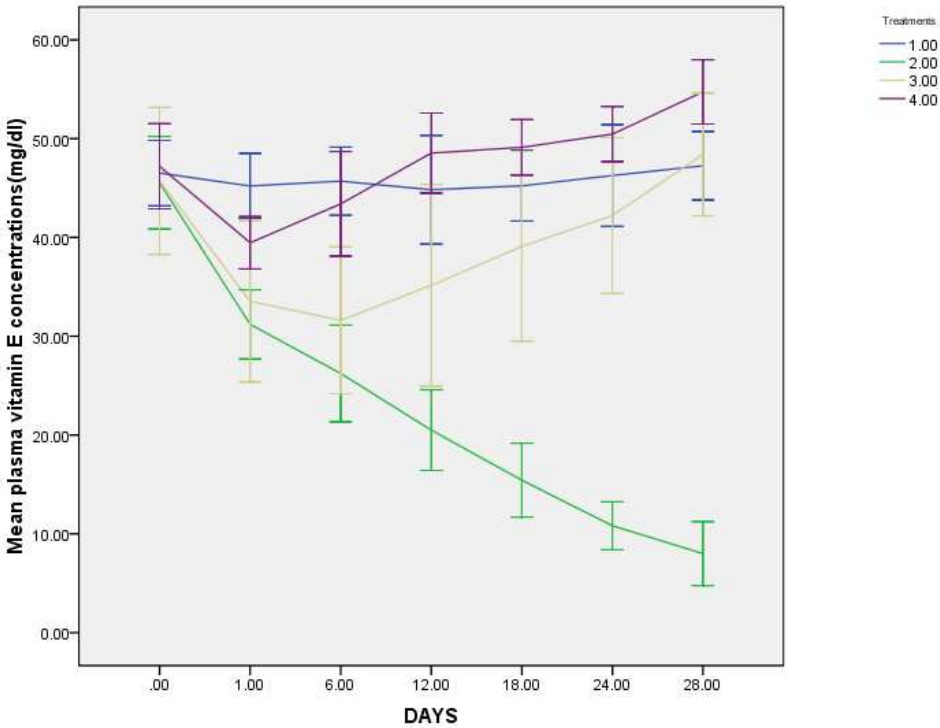

Fig.4:Mean plasma vitamin E concentrations(mg/dl) of controls and treated streptozotocin-induced diabetic rats.1.00:Normal Control;2.00:Diabetic Control;3.00:Aqueous extract;4.00:50% Ethanol extract.

Error bars: 95% CI

Plate 1: A photomicrograph showing a section of the heart of a normal rat given distilled water for 28 days. Section shows normal histology. (Control group). H & E. X 100.

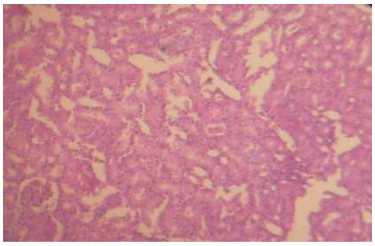

Plate 2: A photomicrograph showing a section of the kidney of a normal rat given distilled water for 28 days. Section shows normal histology. (Control group). H & E Stain, X 100

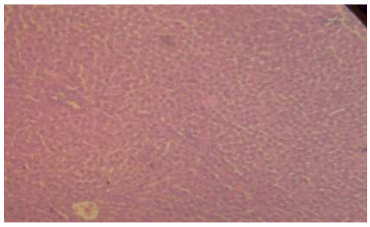

Plate 3: A photomicrograph showing a section of the liver of a normal rat given distilled water for 28 days. Section shows normal histology. (Control group). H & E Stain, X 100.

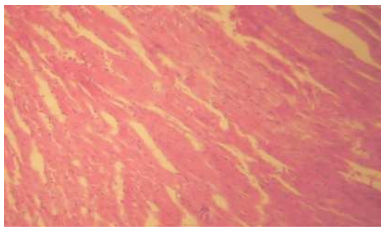

Plate 4: A photomicrograph showing a section of the heart of streptozotocin induced (65 mg/kg body weight) *i. p.* diabetic rat given distilled water for 28 days. Section shows normal histology. (Diabetic control). H & E. X 100.

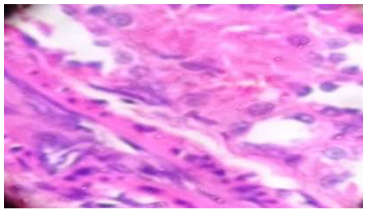

Plate 5: A photomicrograph showing a section of kidney of streptozotocin induced (65 mg/kg body weight) *i. p.* diabetic rat given distilled water for 28 days. Section shows tubular necrosis with infiltrations of inflammatory cells. (Diabetic control). H & E. X 100.

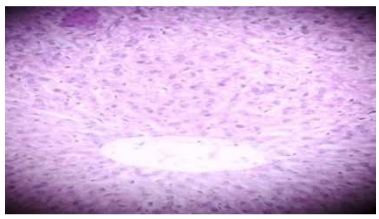

Plate 6: A photomicrograph showing a section of liver of streptozotocin induced (65 mg/kg body weight) *i. p.* diabetic rat given distilled water for 28 days. Section shows fatty changes (fatty liver). (Diabetic control). H & E. X 100.

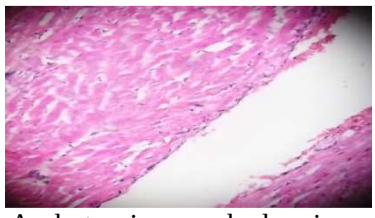

Plate 7: A photomicrograph showing a section of the heart of streptozotocin induced (65 mg/kg body weight) *i. p.* diabetic rat treated with aqueous extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows normal histology. (Aqueous extract treated diabetes). H & E. X 100.

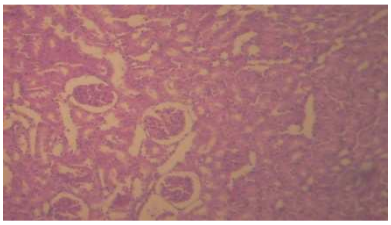

Plate 8: A photomicrograph showing a section of the kidney of streptozotocin induced (65 mg/kg body weight) *i. p.* diabetic rat treated with aqueous extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows normal glomerular architecture with mild infiltrations. (Aqueous extract treated diabetes). H & E. X 100.

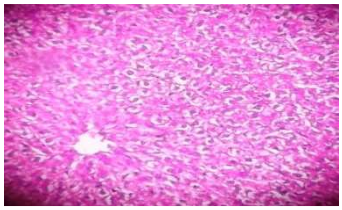

Plate 9: A photomicrograph showing a section of the liver of streptozotocin induced (65 mg/kg body weight) *i. p*. diabetic rat treated with aqueous extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows normal histology. (Aqueous extract treated diabetes). H & E. X 100.

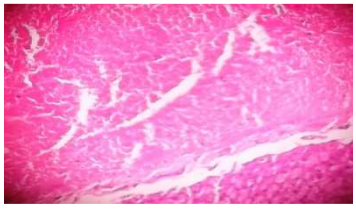

Plate 10: A photomicrograph showing a section of the heart of streptozotocin induced (65 mg/kg body weight) *i. p*. diabetic rat treated with ethanol extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows normal histology. (Ethanol extract treated diabetes). H & E. X 100.

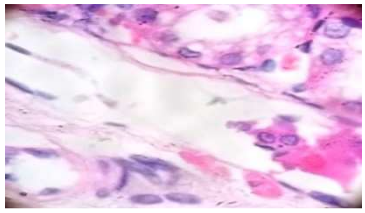

Plate 11: A photomicrograph showing a section of the kidney of streptozotocin induced (65 mg/kg body weight) *i. p*. diabetic rat treated with ethanol extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows vacuolation of tubular epithelium with area of necrosis. (Ethanol extracts treated diabetes). H & E. X 100.

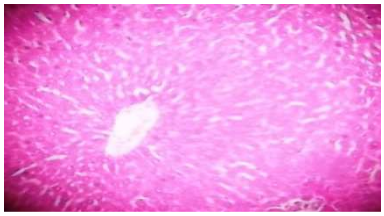

Plate 12: A photomicrograph showing a section of the liver of streptozotocin induced (65 mg/kg body weight) *i. p*. diabetic rat treated with ethanol extract of *Triplochiton scleroxylon* (200 mg/kg body weight) *p. o*. for 28 days. Section shows normal histology. (Ethanol extract treated diabetes). H & E. X 100.

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