sciendo

herba polonica
International journal edited by the Institute of Natural Fibres and Medicinal Plants
Vol. 66 No. 1 2020

Received: 2020-01-20
Accepted: 2020-03-20
Available online: 2020-03-31

DOI: 10.2478/hepo-2020-0005

EXPERIMENTAL PAPER

Antioxidant status and hepato-protective role of *Anchomanes difformis* in streptozotocin-induced diabetes in male Wistar rats

TOYIN D. ALABI[1], NICOLE L. BROOKS[2], OLUWAFEMI O. OGUNTIBEJU[*1]

[1]Phytomedicine and Phytochemistry Group
Oxidative Stress Research Centre
Department of Biomedical Sciences
Faculty of Health and Wellness Sciences
Cape Peninsula University of Technology
Bellville 7535, South Africa

[2]Faculty of Health and Wellness Sciences
Cape Peninsula University of Technology
Cape Town, South Africa

*Corresponding author: e-mail: oguntibejuo@cput.ac.za, bejufemi@yahoo.co.uk, phone: +272 19538495

Summary

Introduction: The liver is involved in the metabolism of xenobiotics and their metabolites and it is vulnerable to oxidative damage. Hyperglycaemia is highly implicated in the progression of diabetes mellitus, and adversely affects the liver. Though, conventional hypoglycaemic drugs may be effective in reducing blood glucose, they do not appear to be effective in attenuating the progression of diabetes and its complications.

Objective: This study evaluated the ameliorative effects of *Anchomanes difformis* on hyperglycaemia and hepatic injuries in type 2 diabetes.

Methods: Type 2 diabetes was induced in male Wistar rats with a single intraperitoneal injection of streptozotocin (40 mg/kg BW) after two weeks of fructose (10%) administration. Aqueous extract of *A. difformis* (200 and 400 mg/kg BW) and glibenclamide (5 mg/kg BW) were administered orally for six weeks. Blood glucose concentrations were measured. Serum levels of liver dysfunction markers (ALT, AST, and ALP), total cholesterol, triglycerides, HDL- and LDL-cholesterol were investigated. Total protein, albumin, and globulin were also assessed. Antioxidant parameters: ORAC, GSH, GSSG, SOD, CAT and FRAP were evaluated in the liver while ORAC, FRAP and lipid peroxidation were determined in the serum. Histological examination of the liver tissue was carried out.

Results: Treatment with aqueous extract of *A. difformis* significantly (p<0.05) reduced blood glucose and reversed steatosis in the diabetic-treated rats. The antioxidant status of diabetic-treated rats was significantly (p<0.05) improved. Serum levels of liver dysfunction markers were significantly (p<0.05) reduced in diabetic-treated rats.

Conclusion: The findings in this study revealed that 400 mg/kgBW *Anchomanes difformis* was more effective than 200 mg/kg BW in ameliorating diabetes-induced hepatopathy, however, both doses of *Anchomanes difformis* demonstrated more antidiabetic ability than glibenclamide. *Anchomanes difformis* may be a novel and potential therapeutic agent in the management of diabetes and resulted hepatic injuries.

Key words: *Anchomanes difformis, antioxidants, diabetes, hyperglycaemia, hyperlipidaemia, liver*

Słowa kluczowe: *Anchomanes difformis, antyoksydanty, cukrzyca, hiperglikemia, hiperlipidemia, wątroba*

INTRODUCTION

Sedentary lifestyle and nutritional overload have been directly implicated in the increased prevalence of type 2 diabetes (T2D) [1]. T2D which is shown by constant hyperglycaemia and insulin resistance leads to various organs dysfunction, such as kidneys, liver, heart, blood vessels, reproductive system and eyes [2]. Persistent hyperglycaemia results in an increased production of free radicals and ultimately oxidative stress. Oxidative stress is an important factor in most pathological conditions. Oxidative damage in the structure and functions of cellular biomolecules including lipids, nucleic acids, and proteins, is highly implicative in the progression of diabetes and the development of its complications [3].

The liver is highly essential in glucose and lipid homeostasis [4, 5]. Diabetes mellitus leads to severe damage in liver such as necrosis, cirrhosis, hepatic steatosis, inflammation [6]. This is usually depicted by abnormal levels of serum hepatic enzymes due to leakage from the damaged hepatocytes into the bloodstream. Increase in the serum levels of the hepatic enzymes may lead to reduced concentrations of total protein (TP) and albumin in the serum [7]. Poor glycaemic control during diabetes mellitus causes hyperlipidaemia, which is associated with an increased flux of free fatty acid due to insulin resistance [8, 9].

Human body has its own antioxidant defence mechanisms by which it combats reactive oxygen species (ROS) generated from metabolic and environmental sources. The antioxidant defence mechanisms prevent the initiation of free radical chain reactions [10]. These defence mechanisms may be enzymatic or non-enzymatic [11]. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and they act as free radical scavengers by donating electrons to react with the ROS [3]. Glutathione (GSH) and glutathione disulphide (GSSG) are small non-enzymatic molecules used in fighting oxidants in the body. Other factors used to determine antioxidant status in the body are oxygen radical absorbing capacity (ORAC) and ferric reducing antioxidant power (FRAP). These antioxidant mechanisms that safeguard the body against oxidative damage is compromised in diabetic conditions [3, 12].

Fructose is majorly metabolised in liver and its consumption disturbs glucose metabolism and uptake. This results in a significantly increased rate of *de novo* lipogenesis and triglyceride synthesis, which contributes to decreased insulin sensitivity and hepatic insulin resistance. This appears to be the underlying mechanism of insulin resistance. Furthermore, fructose, is preferentially metabolized to lipids in the liver compared with glucose [13]. Fructose consumption therefore induces insulin resistance, impairs glucose tolerance, causes hyperinsulinemia and hypercholesterolemia in animal models [14]. A study on commonly consumed fruit juices revealed that natural fructose can alter lipid and protein oxidation biomarkers in the blood, and mediate oxidative stress responses *in vivo* [15]. Also, the short-term consumption of fructose was demonstrated to promote insulin resistance in the liver of non-diabetic adults [14]. Administration of 10% fructose for two weeks, followed by a single intraperitoneal or intravenous injection of streptozotocin (40 mg/kg body weight) has been shown to result in a persistent diseased state characterized by hyperglycaemia (high blood glucose) with major clinical signs of T2D such as insulin resistance [16].

Anchomanes difformis (AD), a tropical plant found mostly in African forests, is rich in phytochemicals and has been reported for its biological activities [17]. Folkloric uses of AD include the intake of the its decoction to treat coughs, diabetes, dysentery and throat related

problems [18]. Some of these ethno-medicinal uses of *A. differmis* has been established through scientific evidences [19]. Previous *in vitro* studies carried out in our research group on six different extracts of AD revealed its free radical scavenging property and antioxidant ability, however, the aqueous leaf extract demonstrated the highest antioxidant property and was most abundant in polyphenols [20]. Further investigations were performed on the phytochemical characterization of AD extracts using HPLC and LC-MS: we identified nineteen bioactive compounds from the aqueous extracts of AD (20). Three compounds have been isolated from the rhizome of AD by Nkoh et al. [21]. A study reported only the hypoglycemic effect of ethanolic extract of AD rhizome in alloxan-induced diabetes using Wistar rats [22], however, the hepatoprotective ability and antioxidative properties of AD extracts *in vivo* diabetic model has not been investigated. This study therefore explored the hypoglycaemic property of aqueous leaves extract of AD in fructose-streptozotocin-induced T2D in male Wistar rats, focusing on its role in ameliorating liver injury, hyperlipidaemia and restoring structural liver architecture. This is the first study to evaluate the antioxidant effect of aqueous leaf extract of AD in diabetes and diabetic complications. The potentials of AD were measured in comparison to a standard anti-diabetic drug: glibenclamide.

MATERIALS AND METHODS

Plant preparation

Collection and registration

The leaves of *Anchomanes differmis* (eng. blume) were harvested from a farm in Abeokuta, Ogun state, Nigeria. The authentication was conducted by O.O. Oyebanji at the Herbarium, Department of Botany, University of Lagos, Nigeria (LUH6623), a specimen voucher was deposited at the herbarium. The plant's name has been checked with http://www.theplantlist.org. (ID: kew-8734). Some of local names of *A. differmis* are as follows: *Ògìrìòsákó (Yoruba); Kabaka-kachulu (Lunda); Niamé kwanba (Baule).*

Extraction

Aqueous extraction of AD leaves was done using cold-stirred extraction method. The leaves were dried under shade and blended to increase the surface area. The blended leaves were defatted using n-hexane (10% w/v) for 48 hours. The leaves were further soaked in water for 48 hours in the ratio 1:10 of plant material and solvent at 2–4°C. A vacuum filtration method was used to filter off the debris, the filtrate was lyophilized giving an extraction yield of 25%. The pulverized extract was stored at –20°C for further analysis.

Animals

Male Wistar rats (180±10 g, 8 weeks old) were obtained from the Animal facility, Stellenbosch University, South Africa. This study was carried out at the Primate Unit & Delft Animal Centre (PUDAC), South African Medical Research Council (SAMRC), Cape Town, South Africa. Animal handling, care and other procedures were done in accordance to the standard operating procedure of SAMRC PUDAC (SOP No: 2016-R01) which conforms to the internationally accepted, revised, South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008). The animals were acclimatized for 3 weeks. The rats were housed under controlled, standard, laboratory conditions; humidity between 45% to 55%, and an ambient temperature between 22°C to 26°C. Standard rat chow (SRC) and water was fed to all the rats *ad libitum* and they were exposed to the normal photo period (12 hour dark/12 hour light).

The experimental protocols described in this study was approved by the Faculty of Health and Wellness Sciences, Research Ethics Committee (REC) of Cape Peninsula University of Technology (CPUT), Bellville, South Africa. Ethical approval was granted for this study with REC approval reference number: CPUT/HW-REC 2016/A4. Also, ethical clearance was obtained (REF. 04/17) from the Ethics Committee for Research on Animals from South African Medical Research Council where the animal study from acclimatization to euthanasia was carried out.

Experimental design

Sixty-four male, Wistar rats with weights ranging from 270 to 300 g were used for this study. The rats were randomly grouped into one of seven

(7) groups with a minimum of eight rats in each group (8 rats in normal groups and 10 in diabetic groups), as summarised in figure 1. Water served as a vehicle for fructose and AD administration, while 0.1 M citrate buffer (pH 4.5) was the vehicle for streptozotocin (STZ). Animals in group 1 served as the normal control (NC) and received vehicle only. Animals in group 2 and 3 are normal rats who received only 200 and 400 mg/kg BW of AD aqueous extract, respectively (N+AD 200 and N+AD 400), these served as the treated control. Groups 4 to 7 consisted of animals that were given 10% fructose for 2 weeks followed by streptozotocin (STZ). Group 4 received vehicle only (DC), group 5 and 6 were given 200 and 400 mg/kg BW of AD aqueous extract (D+AD 200 and D+AD 400) respectively while group 7 received 5 mg/kg BW of glibenclamide [23]; an antidiabetic drug (D+G).

Animals were randomly assigned into 7 groups (n≥8). 14 days administration of 10% fructose preceded a single-dose injection of STZ (40 mg/kg). Animals confirmed diabetic after 5 days. Normal rats were administered with the vehicle, water and citrate buffer, accordingly. Treatment commenced immediately for 42 days. Animals were starved for 12 hours prior to anesthesia and euthanized on the 43rd day (red bar) via exsanguination.

Induction of type 2 diabetes

A 10% fructose solution was administered to the rats *ad libitum* (150 ml per day) for 2 weeks followed by a single dose intraperitoneal injection of STZ; 40 mg/kg body weight [16]. Diabetes was confirmed after 5 days of STZ administration, rats with fasting (overnight; 16 hours) blood glucose level higher than 15 mmol/l were considered diabetic. Treatment commenced immediately with aqueous extract of AD which was delivered *via* oral gavage. Stock solutions of AD extracts were freshly prepared, and each rat was administered with the appropriate volume, taken into consideration the stock concentration, the concentration to be given, and their weight.

Measurement of fasting blood glucose and oral glucose tolerance test

Fasting blood glucose (FBG) levels were measured weekly for 10 weeks, this period spans from fructose administration, induction of diabetes, treatment with AD to euthanasia. Rats were fasted overnight (16 hours), blood glucose concentrations were taken using ACCU-CHEK glucometer (Roche, South Africa). Oral glucose tolerance test was done over a period of 2 hours, readings were taken at 0, 30, 60, 90 and 120 minutes immediately after an oral administration of 0.5 g/kg body weight of glucose. Blood samples were collected by tail prick according to the standard operating procedures at PUDAC, SAMRC (SOP 2017-R01).

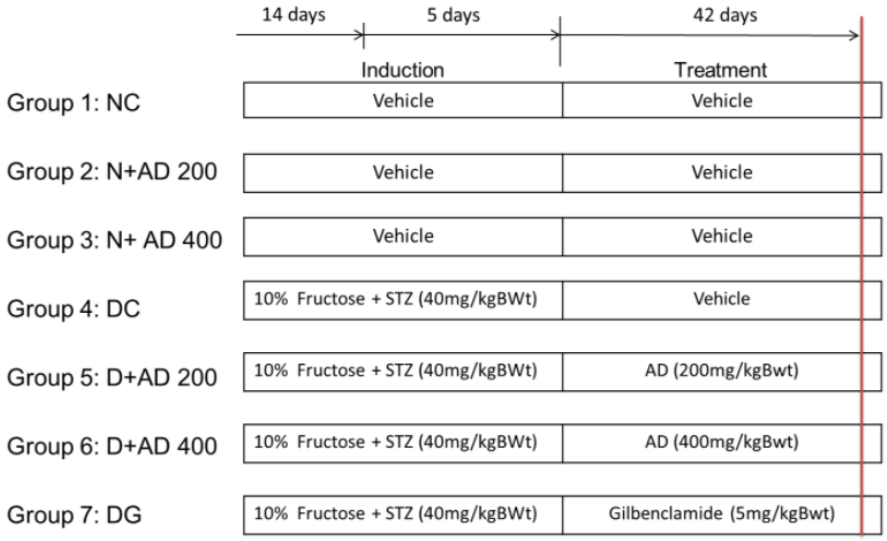

Figure 1

Experimental design

Blood and tissue collection

After the treatment, the rats were weighed and anaesthetized with 2% isoflurane per oxygen (1 l/min flow rate) *via* inhalation and exsanguinated. Blood was collected *via* cardiac puncture into Z-serum clot activator tubes. Blood samples were centrifuged at 4,000 g for 10 min at 4°C. Aliquots of the supernatant were stored at –80°C for biochemical analysis. The liver was excised immediately, washed in ice-cold phosphate buffered saline (PBS), dabbed and weighed. The liver was then frozen using liquid nitrogen and later stored at –80°C for further analysis.

Determination of biomarkers for organ function

In this study, biochemical and histological parameters were assessed for organ integrity. Alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), albumin, total protein and lipid profile; total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were measured in the serum. Transaminases (ALT, AST) and ALP were quantified following a modified method of Thomas [24]. Serum AST and ALT levels were estimated using optimized ultraviolet-test (340 nm) while ALP was measured in the serum at 405 nm by a kinetic photometric procedure [24]. HDL cholesterol was determined using an enzymatic colorimetric test, accelerator selective detergent method [25], while LDL cholesterol was estimated with Friedewald equation [26]. Total cholesterol and triglycerides were measured by an enzymatic photometric assay [27, 28]. The assays in this section were carried out using Horiba kits (Montpellier, France) and performed on an ABX Pentra 400 Chemistry Analyzer (Horiba) according to manufacturer's instructions.

Tissue preparation

A 200 mg sample of the liver was homogenized on ice in 2 ml of 50 mM phosphate buffer with 0.5% triton and centrifuged at 10,000 x gravitational force for 15 minutes at 4°C. The supernatants were aliquoted and stored at –80°C.

Enzymatic and non-enzymatic antioxidant indices; superoxide dismutase (SOD), catalase (CAT), ORAC, FRAP, total glutathione (GSH), oxidized glutathione (GSSG) were measured in the liver homogenates. Lipid peroxidation (TBARS), ORAC and FRAP were measured in the serum to estimate the antioxidant levels of the serum.

Lipid peroxidation

Lipid peroxidation was measured using thiobarbituric acid (TBA) reaction according to the modified methods of Matsunami *et al.* [29] and Wasowicz *et al.* [30]. A 100 μl of sample was pipetted into a 2ml Eppendorf tube followed by the addition of 12.5 μl of 4mM BHT (butylated hydroxy toluene) in ethanol. Approximately 100 μl of 0.2m o-phosphoric acid was added and the solution vortexed for 10 seconds. This was followed by the addition of 12.5 μl of 0.11M TBA reagent and the mixture was then vortexed. The solution was incubated at 90°C for 45 minutes, left on ice for 2 minutes, and further allowed to cool at room temperature for 5 minutes. The thiobarbituric reactive substances (TBARS) were extracted with *n*-butanol, followed by the addition of 100 μl of saturated NaCl for better separation and the samples were then vortexed. The solution was centrifuged at 15000 x g at 4°C for 2 minutes and the solution separated into two layers. The butanol-TBARS solution formed the top layer of which 300 μl was pipetted into the microplate and absorbance read at 532 nm.

ORAC and FRAP

ORAC and FRAP were used to evaluate the antioxidant capacity of the serum and liver using a fluorescence Multiskan Spectrum plate reader (Thermoscan Electron Corporation, USA). The ORAC assay was performed following a modified method of Prior *et al.*, [31] which measured the potential of the antioxidants present in the sample to quench peroxyl radicals in comparison to Trolox; the standard reference. FRAP was assayed according to the method described by Benzie and Strain [32], where the ability of a sample to reduce iron (III) to iron (II) was measured at a wavelength of 593 nm using L-Ascorbic acid as the standard reference.

Superoxide dismutase

The activity of SOD was determined by measuring the auto-oxidation of 6-hydroxy-dopamine (6-HD) and the amount of the enzyme needed to exhibit dismutation of the superoxide radicals (33). This assay quantifies all the three types of SOD (CU/Zn–, Mn–, and Fe-SOD) that could be

present in the sample. A 15 μl sample was placed into wells containing 170 μl of 0.1 mM DETAPAC (diethylenetriaminepentaacetic acid), followed by the addition of 15 μl of 6-HD (1.6 mM) to kick off the reaction. The absorbance was read at 490 nm for 4 minutes in 1-minute intervals. The activity of SOD present in the sample was calculated from the equation obtained from the linear regression of the SOD standard curve.

Catalase

The activity of catalase was assessed by the method of Ellerby and Bredesen [33] where the rate of conversion of hydrogen peroxide to water and oxygen by catalase was measured at 240 nm. A sample of 10 μl was added to 170 μl sodium phosphate buffer (50 mM, pH 7.0) and 75 μl of hydrogen peroxide (30% v/v) was added to initiate the reaction. The absorbance was read over a minute at 15 seconds intervals. The activity of catalase present in the sample was extrapolated from the activity of the standard: the catalase enzyme.

Total glutathione and glutathione disulphide (GSH/GSSG)

A sample of 200 mg of the liver was homogenized in 2 ml of ice-cold, 50 mM sodium phosphate buffer containing 1mM EDTA. For GSSG, liver samples were homogenized in the same phosphate buffer containing 1mM EDTA and 3mM M2VP. Homogenates were then centrifuged at 15,000 x g for 5 minutes, the supernatant was used for the analysis. A sample of 50 μl of the supernatant, 50 μl of GR (0.02 U/μl) and 50 μl of 0.3 mM DNTB were added to each of the wells of the microplate. To this, 50 μl of GSH (3 μM) was added, the reaction was initiated by the addition of 50 μl of 1mM NADPH. A change in absorbance over 5 minutes was measured at 412 nm.

Histological examination of liver tissues

Liver tissues were harvested from all the animals and fixed in 10% buffered formalin solution for 24 hours. Tissues were placed in small cassettes, dehydrated using ethyl alcohol series ranging from 50% to 100% and cleared in xylene. Embedding of tissues in paraffin was done at 56°C and paraffin blocks were sectioned at 5 μm using a rotatory ultra-microtome. Sections were deparaffinised, rehydrated and stained with haematoxylin and eosin (H&E) dyes and mounted. The slides were examined under a light microscope at 10x and 40x magnification. Observations of any changes in the structural architecture, portal or lobular inflammation, sinusoidal dilatation and congestion, oedema, degeneration, necrosis and fatty change were noted, and photomicrographs taken with Motic camera (MOTICAM BTU10) using a Moticonnect Image Plus 2.0 software.

Statistical analysis

Values were expressed as mean ± standard error of mean (SEM). Statistical analysis of results was performed using one-way or two-way analysis of variance (ANOVA) to find differences between groups. Bonferroni test was used for all pair-wise comparisons. Differences (*F* values) were considered statistically significant at *p* values less than 0.05. All statistical calculations were done using GraphPad Prism Version 5.00 for Windows, GraphPad Inc., San Diego, California USA.

ABBREVIATIONS

T2D - type 2 diabetes
TP - total protein
ROS - reactive oxygen species
SOD - superoxide dismutase
CAT - catalase
GSH - glutathione
GSSG - glutathione disulphide/oxidized glutathione
ORAC - oxygen radical absorbing capacity
FRAP - ferric reducing antioxidant power
AD - *Anchomanes difformis*
STZ - streptozotocin
SRC - standard rat chow
FBG - fasting blood glucose
ALT - alanine transaminase
ALP - alkaline phosphatase
AST - aspartate transaminase
TBA - thiobarbaturic acid
TBARS - thiobarbaturic acid reactive substances
MDA - malondialdehyde
FAS - fatty acid synthetase
HDL - high-density lipoprotein
LDL - low-density lipoprotein

RESULTS

Hypoglycaemic effect of AD treatment in diabetic rats

The administration of 10% fructose for two weeks and 40 mg/kg BW of STZ led to a significant (p<0.05) increase in the blood glucose concentration in the diabetic rats when compared to normal rats (fig. 2A). However, treatment with 200mg and 400 mg/kg BW of AD brought about a significant (p<0.05) reduction in the glucose levels of treated diabetic rats when compared to the untreated diabetic rats. Interestingly, both concentrations of AD were able to lower glucose concentrations more than the standard drug; glibenclamide throughout the treatment period. The oral glucose tolerance test (OGTT) is a derived measure of insulin resistance and this was done to confirm constant hyperglycaemia and insulin resistance in the rats administered with 10% fructose and injected with STZ in comparison with normal rats. The result revealed a constant significant (p<0.05) increase in the blood glucose concentration of rats administered fructose and STZ when compared to normal rats. There was no significant (p<0.05) reduction in the glucose concentrations for 2 hours of observation.

Treatment with *A. differmis* abated serum levels of hepatic enzymes

The administration of AD for six (6) weeks significantly (p<0.05) lowered biomarkers of hepatic injury in treated diabetic rats as illustrated in figure 3. Induction of type II diabetes with STZ caused a significant (p<0.05) increase in the serum levels of ALT, AST, and ALP. Treatment with 400 mg/kg BW of AD extract and glibenclamide significantly reduced ALT levels in diabetic rats to normal levels (fig. 3A), while treatment with the 200 mg/kg BW AD extract showed a non-significant decrease (p<0.05). A similar trend was observed in the levels of AST (Figure 3B), however, treatment with both 200 mg/kg BW and 400 mg/kg BW AD extract significantly (p<0.05) lowered AST levels in diabetic treated rats when compared to normal rats. It is interesting to note that AST levels in diabetic rats treated with glibenclamide were significantly reduced (p<0.05) when compared with normal rats. ALP activity declined significantly (p<0.05) in diabetic rats treated with 400 mg/kg BW AD extracts when compared with non-treated diabetic rats, while an observable decrease was observed in the diabetic rats treated with 200 mg/kg BW of AD extract and glibenclamide (fig. 3C).

Regulation/modulation of lipid profile by AD treatment in type II diabetes

The concentrations of total cholesterol, HDL-cholesterol and triglycerides increased significantly (p<0.05) in the serum of diabetic rats (fig. 4 A, C and D). Likewise, the serum concentration of LDL-cholesterol also increased in diabetic rats, but not significantly (fig. 4B). Treatment with 200 mg/kg BW and 400 mg/kg BW of AD extracts significantly (p<0.05) lowered the concentration of total cholesterol and HDL-cholesterol to normal when compared with normal control rats and diabetic rats treated with glibenclamide (fig. 5A, 5C). Level of triglycerides was significantly (p<0.05) reduced only in the diabetic rats treated with 400 mg/kg BW of AD extract as illustrated in figure 4D. Similarly, only diabetic rats treated with glibenclamide (5 mg/kg BW) showed a significant (p<0.05) decrease in serum levels of LDL-cholesterol (fig. 4B).

Impact of AD treatment on protein synthesis in STZ-induced type II diabetes

Figures 5A to D illustrate the concentrations of total proteins, albumin, and globulin in the serum. The figures indicate that total protein, albumin concentrations were affected by induction of diabetes as their concentrations were significantly (p<0.05) lowered. Inversely, albumin levels as illustrated in figure 5B were restored to normalcy in the diabetic rats placed on 400 mg/kg BW of AD extracts and glibenclamide (5 mg/kg BW). Total protein level was increased to normal in diabetic rats treated with glibenclamide only as presented in figure 5A. Treatment with 200 mg/kg BW of AD significantly (p<0.05) reduced globulin levels in the serum of diabetic rats when compared with non-treated diabetic rats as exhibited in figure 5C.

AD intervention ameliorated lipid peroxidation, oxidative stress in type II diabetes

Constant hyperglycaemia significantly (p<0.05) contributes to the increased levels of TBARS in the

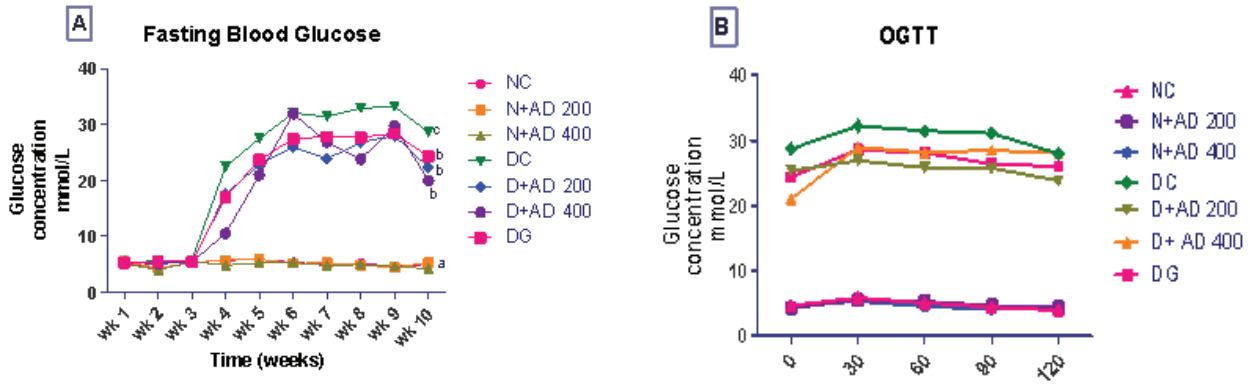

Figure 2

Effect of AD (*Anchomanes differnis)* on (A) weekly blood glucose concentrations and (B) oral glucose tolerance test in normal and diabetic rats. Points are indicative of mean values ±SEM of blood glucose concentrations. Lines marked with different letters (a, b, or c) are significantly (p<0.05) different from each other.

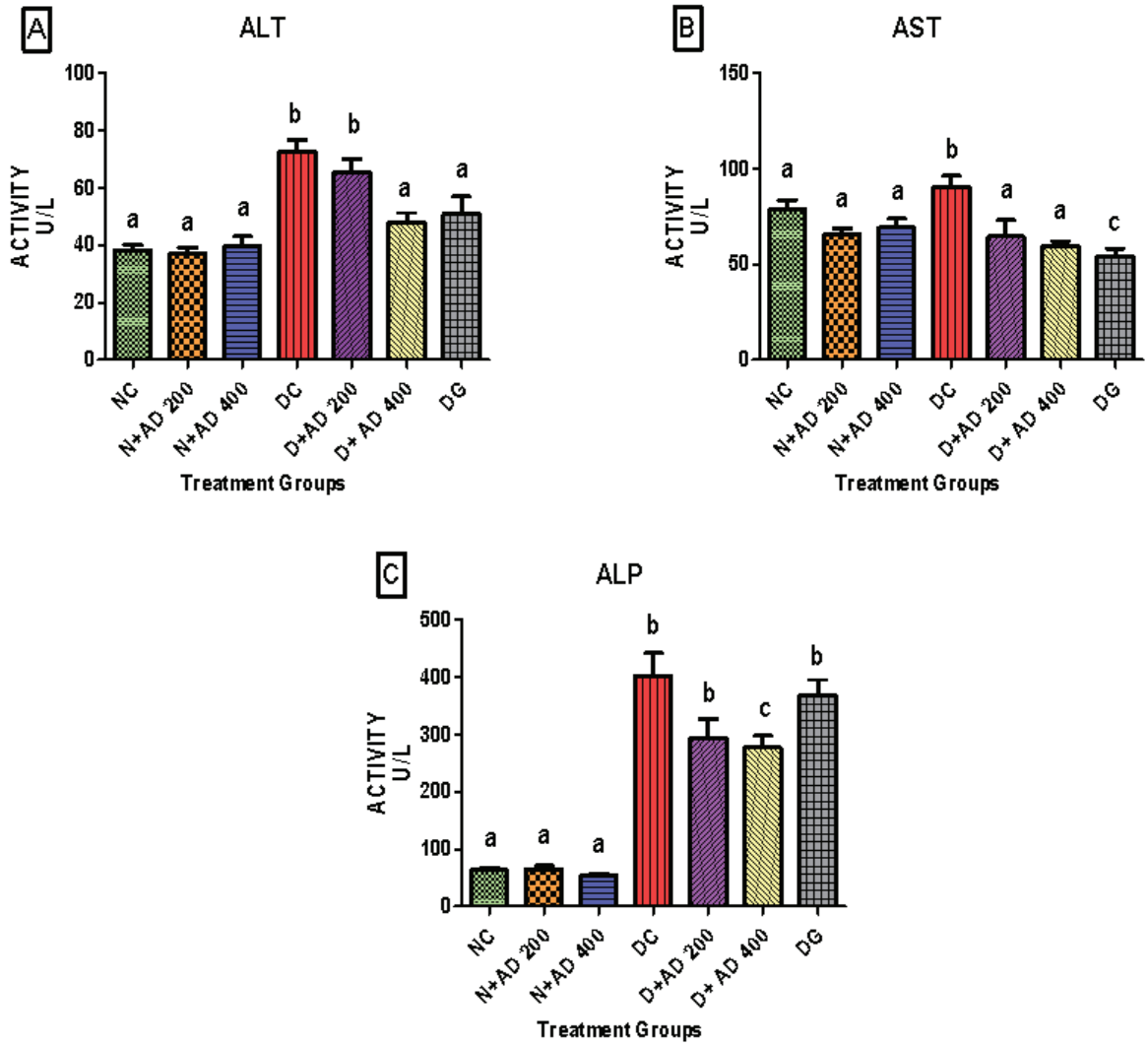

Figure 3

Effect of AD (*Anchomanes differnis)* administration on biomarkers of hepatic injury; (A) ALT, (B) AST and (C) ALP in the serum of normal and diabetic rats. Bars are indicative of mean values ±SEM. Bars marked with different letters (a, b, or c) are significantly (p<0.05) different from each other.

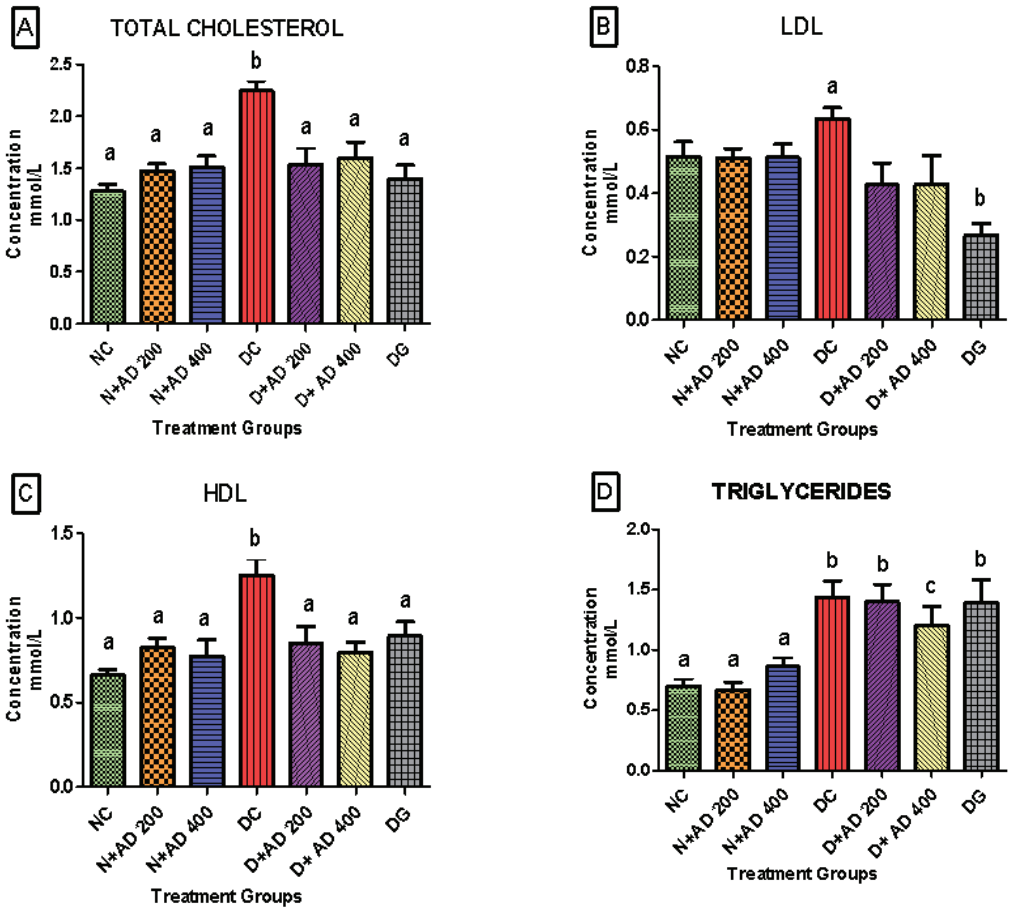

Figure 4

Effect of treatment with AD (*Anchomanes diffornis)* on the lipid profile; (A) Total Cholesterol, (B) LDL-cholesterol, (C) HDL-cholesterol and (D) Triglycerides in the serum of normal and diabetic rats. Bars are indicative of mean values ±SEM. Bars marked with different letters (a, b, or c) are significantly (p<0.05) different from each other.

serum of diabetic rats (fig. 6A). This significantly (p<0.05) declined in diabetic rats that were administered 200 mg/kg BW of AD. Lipid peroxidation decreased (though not significant at p<0.05) in rats treated with 400 mg/kg BW of AD when compared to diabetic untreated. Glibenclamide administration did not reduce lipid peroxidation in diabetic rats. Furthermore, antioxidant capacity measured using FRAP diminished in diabetic rats as illustrated in Figure 6B. Administration of AD extracts and glibenclamide had no significant (p<0.05) influence on the ferric reducing ability in the serum. Diabetes led to a significant reduction in the oxygen radical absorbance capacity of the serum of rats as exhibited in Figure 6C. Furthermore, groups treated with different concentrations of AD was able to restore this capacity compared to normal rats. However, glibenclamide did not have any effect on the ORAC in the serum of diabetic rats.

AD administration enhanced antioxidant status in the liver of diabetic rats

Hyperglycaemia resulted in a significant (p<0.05) reduction in the total glutathione levels (tGSH), glutathione to oxidized glutathione (GSH/GSSG) ratio and ORAC concentration in the liver of diabetic rats (fig. 7A to 7E). Diabetic rats who received 200 mg/kg BW showed a significant (p<0.05) increase in the tGSH by 69.5% and GSH/GSSG by 133%. There was an observed increase in tGSH levels (53.1%) and GSH/GSSG (150.9%) in diabetic rats that were administered 400 mg/kg BW but not significant (p<0.05). Both doses of AD were able to enhance tGSH levels more effectively than diabetic rats given glibenclamide (5 mg/kg BW) which demonstrated increased tGSH and GSH/GSSG ratio by 30.2% and 124%, respectively; this is presented in figure 7A. Figure 7C shows

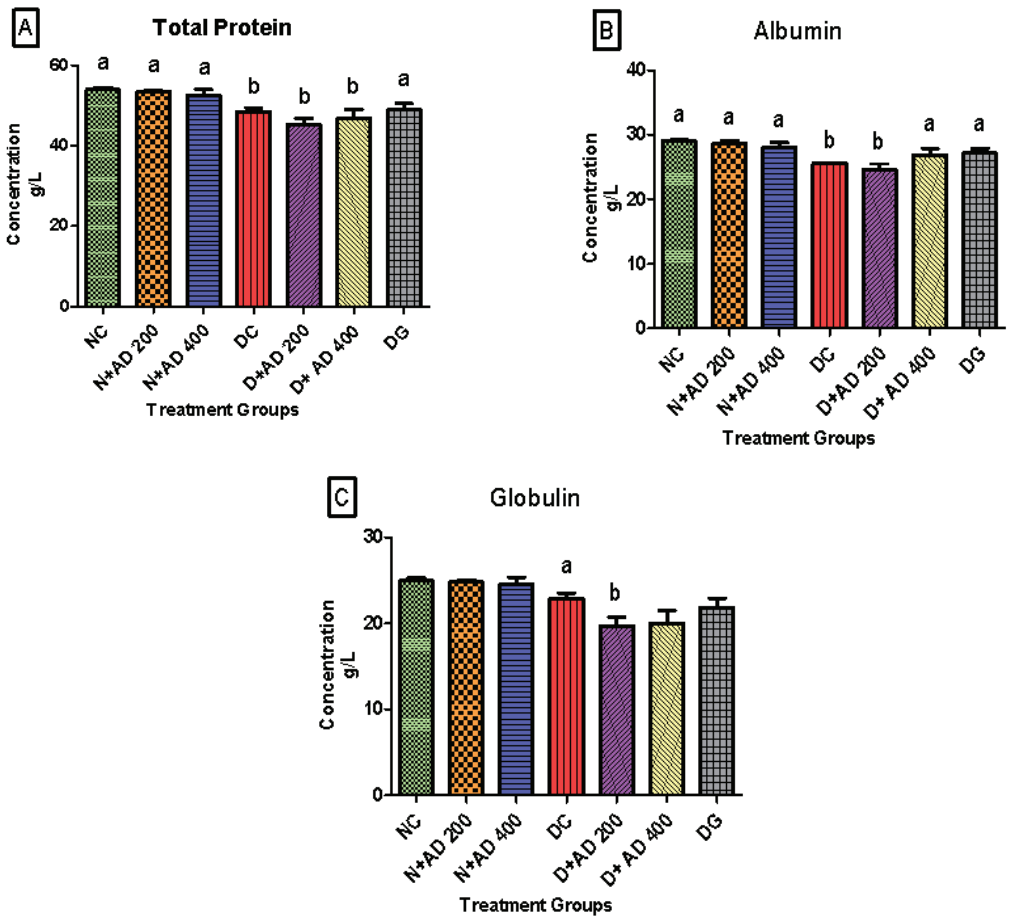

Figure 5

Effect of treatment with AD (*Anchomanes differnis)* on (A) Total protein (B), Albumin and (C) Globulin in the serum of normal and diabetic rats. Bars are indicative of mean values ±SEM. Bars marked with different letters (a or b) are significantly (p<0.05) different from each other.

the comparative levels of GSH and GSSG for all experimental groups, the levels of GSSG were the same in all groups. However, tGSH levels were significantly depleted in diabetic rats indicating increased ROS, treatment with AD increased tGSH levels in normal and diabetic rats. ORAC concentrations were significantly (p<0.05) elevated in treated diabetic rats in response to AD administration as illustrated in Figure 7D. However, treatment with glibenclamide did not have any positive effect on ORAC concentration in diabetic rat liver. There were no significant (p<0.05) changes in the FRAP concentration in normal rats, diabetic rats, and treated rats as exhibited in Figure 7E. Diabetes did not have a significant (p<0.05) effect on SOD levels in the liver as shown in table 1. In addition, SOD activity was not influenced by the administration of AD and glibenclamide. The activity of CAT was enhanced in normal and diabetic rats treated with AD as presented in Table 1. Normal rats treated with 200 mg/kg BW had a non-significant increase (17.32%) in CAT activity when compared with the normal controls (NC), however, treatment with 400 mg/kg BW of AD in normal rats led to a significant (p<0.05) increase in CAT activity. Due to increased oxidative stress, CAT activity was significantly (p<0.05) elevated in diabetic controls when compared with NC. Since AD administration led to significant induction of CAT activity in normal rats (treatment controls), it therefore suggests that the corresponding significantly increased CAT activity in D+AD 200 and D+AD 400 was due to treatment with AD and not increased oxidative stress. Similarly, glibenclamide administration significantly enhanced CAT activity in diabetic rats.

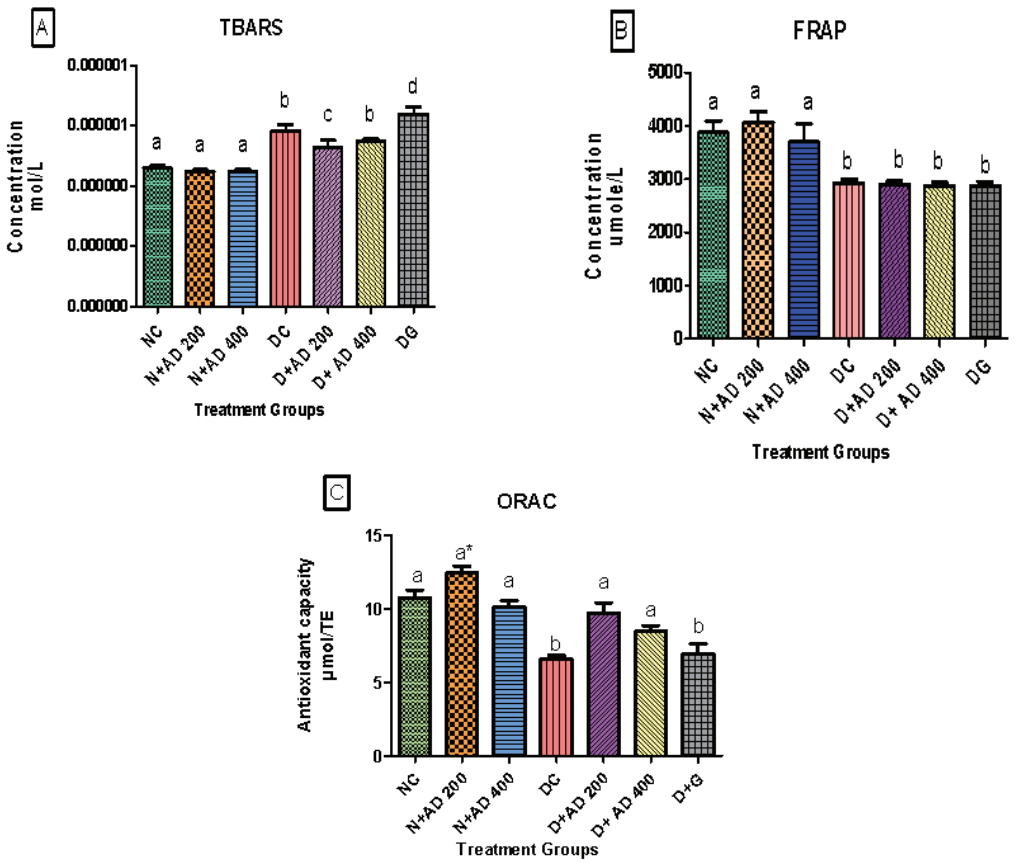

Figure 6

Effect of AD A (*Anchomanes diffornis)* administration on biomarkers of lipid peroxidation and antioxidant activity; (A) TBARS, (B) FRAP and (C) ORAC in the serum of normal and diabetic rats. Bars are indicative of mean values ±SEM. Bars marked with different letters (a, b, or c) are significantly (p<0.05) different from each other. Letters with asterisk are significantly different from the diabetic control at p<0.001.

Table 1.

Effect of treatment on antioxidant enzymes in the liver of diabetic and normal rats

Treatment Groups	SOD [U/mg]	CAT [U/mg]
NC	53.31±5.81	2683±206.2[a]
N+AD 200	50.98±5.61	3147±142.0[a]
N+AD 400	40.85±1.73	6201±401.2[b]
DC	36.53±2.91	6010±694.7[b]
D+AD 200	40.41±1.80	5937±734.7[b]
D+AD 400	36.34±4.33	6202±621.7[b]
D+G	38.46±4.41	6511±772.2[b]

Values with different letters are significantly (p<0.05) different from each other

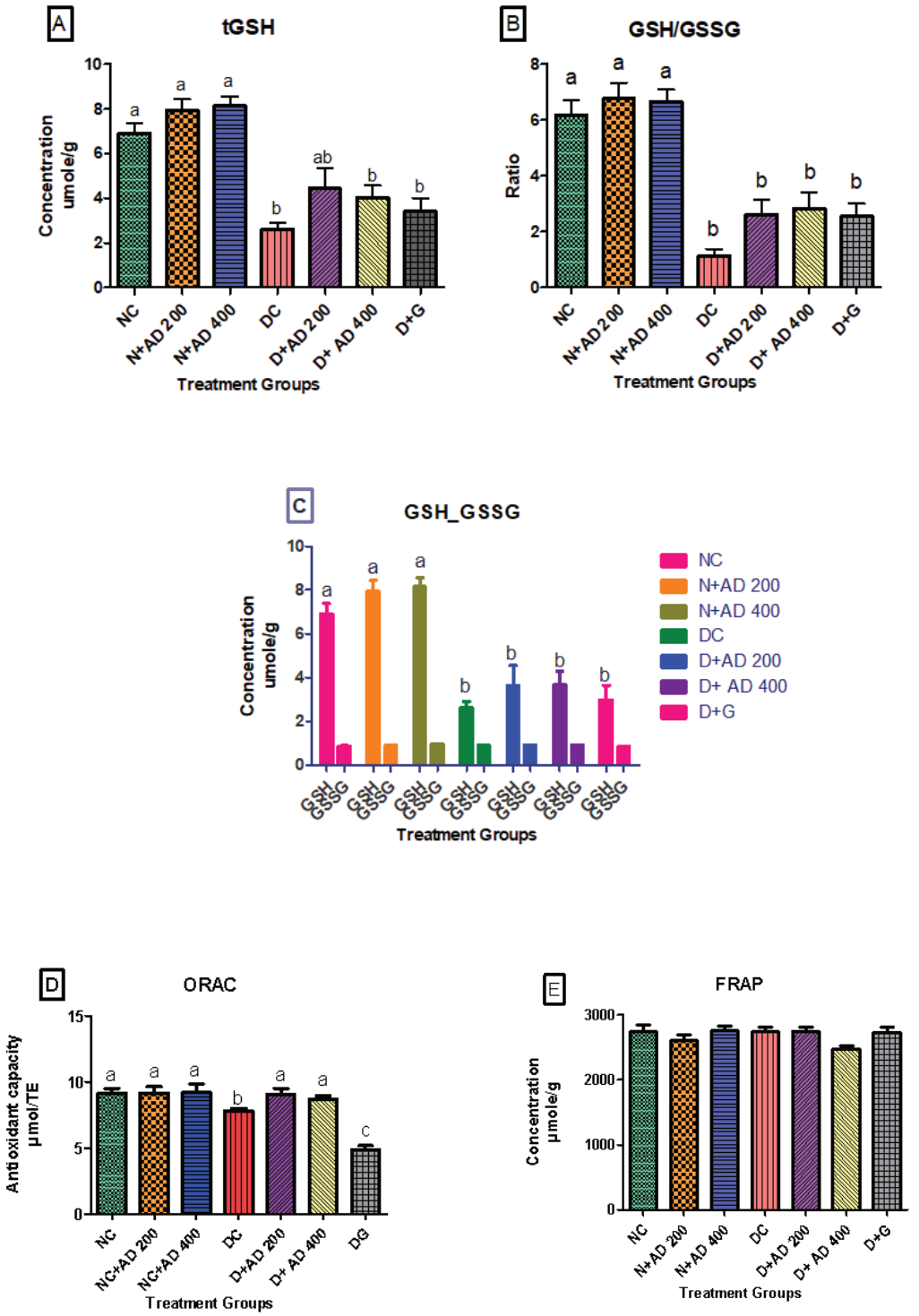

Figure 7

Effect of treatment on non-enzymatic antioxidant indices; (A) total GSH, (B) GSH-GSSG ratio, (C) total GSH and GSSG, (D) ORAC, and (E) FRAP in the liver of diabetic and normal rats. Bars are indicative of mean values ±SEM. Bars marked with different letters (a, b, or c) are significantly (p<0.05) different from each other.

Treatment with AD reversed pathologies and improved the histological structure of the liver in T2D

Findings from the histological examination of the liver from various treatment groups are shown in figure 8. The normal control, non-diabetic rats showed normal histology, sinusoids are well divergent, the nucleus and nucleolus are clearly seen. Diabetic control revealed enlarged sinusoids due to oedema, distorted sinusoidal arrangement, an area of focal necrosis exemplified by nuclear degradation (arrow 'B') and palour in the cytoplasm. Macro-vesicular steatosis can also be seen in the diabetic control rats, this was completely reversed in the diabetic rats treated with AD 200 mg and 400 mg/kg BW compared to normal rats. Mild centrilobular necrosis and widened sinusoids were observed in the liver of diabetic rats treated with 200 mg/kg BW of AD. Rats treated with 400 mg/kg BW of AD showed normal histology in the liver. Steatosis and necrosis can be observed in the rats treated with glibenclamide. The different pathologies that were observed in the hepatic tissues of different treatment groups were scored and graded as displayed in table 2.

DISCUSSION

The present study demonstrates the hypoglycaemic effect of AD aqueous leaves extract in T2D. AD showed a higher potential in lowering blood glucose in diabetic rats than the standard drug; glibenclamide. Ethanolic extracts obtained from the roots of AD has been reported to possess hypoglycaemic effect [22]. Constant hyperglycaemia is associated with distortion in lipid metabolism, this results in hyperlipidaemia and subsequent hepatic steatosis [22]. The data showed that treatment with AD and glibenclamide significantly reduced total cholesterol to normal levels. Significant increase in HDL-cholesterol of diabetic untreated rats may be in response to the increased LDL-cholesterol in the blood, as HDL-cholesterol does reversed-cholesterol transport to get rid of excess cholesterol from the blood [34]. LDL-cholesterol and triglycerides were reduced in diabetic rats with the administration of AD. This result is in agreement with a previous study which reported that root extract of AD was able to reduce plasma cholesterol in alloxan-induce diabetes [22].

Abnormalities in glucose metabolism leads to an overproduction of free radicals. The presence of excess free radicals results in the oxidation of biomolecules such as lipids, leading to breakdown of lipids especially in the cell membrane; which is majorly made up of lipids [35]. Lipid peroxidation plays an important role in the progression of diabetes and diabetic complications such as atherosclerosis which increases the risk of coronary heart disease

Table 2.

Hepatic injury score in the various treatment groups

Histologic feature	NC	N+AD 200	N+AD 400	DC	D+AD 200	D+AD 400	D+G
Portal inflammation	0	0	0	1	1	0	1
Steatosis	0	0	0	2	0	0	2
Sinusoidal dilatation	0	0	0	2	1	0	1
Sinusoidal distortion	0	0	0	2	1	0	1
Centrilobular necrosis	0	0	0	3	1	0	2

The hepatic injury score was graded from 0 to 3, where 0 represents <5% damage and categorised as 'none', 1 represents 5%–33% damage and categorised as 'mild', 2 represents damage between 34%–66% and categorised as 'moderate' and 3 represents damage >66% of the tissue and categorised as 'severe'.

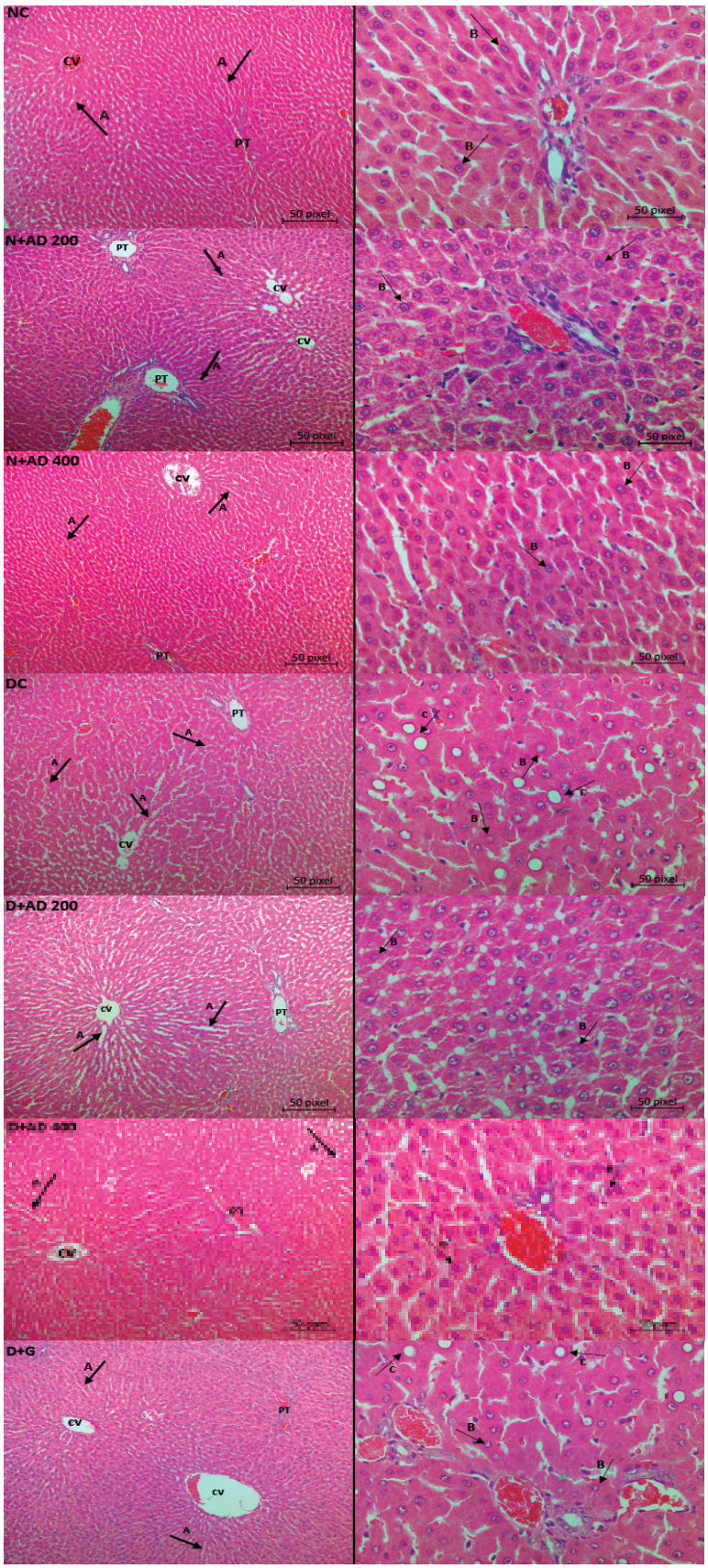

Figure 8

Light micrographs from liver sections of normal and diabetic rats stained with hematoxylin-eosin (x 400). The black thick arrows lettered 'A' points at the sinusoids, arrows lettered 'B' points at the nucleus of the hepatocytes, and arrows lettered 'C' shows fatty droplets in the hepatocytes. Magnification 10 x (left) and 40 x (right). CV- central vein, PT-portal triad.

[36, 37]. Lipid peroxidation significantly increased in diabetic rats which is suggestive of cell membrane damage due to an increased production of free radicals. Treatment with AD significantly lowered lipid peroxidation in the serum of diabetic rats. Previous studies carried out in our research group shows that AD contains bioactive compounds that have antioxidant properties [20].

Loss of cell integrity due to membrane damage results in the leakage of hepatic enzymes in diabetic rats. Hepatic enzymes have been shown to be elevated in diabetic conditions and are associated with liver pathologies [5, 38]. Our findings are consistent with these studies as demonstrated by the significant increase in hepatic enzymes in the serum of diabetic rats. This was however abated by the intervention with AD. Hepatic enzymes have also been used as indices of accumulation of liver fat, this was observed in the diabetic controls (fig. 5, 9). Reports shows that increased hepatic enzymes with liver fat accumulation leads to progression of fibrosis which constitutes a clinically significant risk factor in developing end-stage liver disease [36]. The potency of AD in restoring elevated hepatic enzymes and total cholesterol to normalcy suggests its strong capacity to ameliorate or prevent diabetic complications associated with obesity and insulin resistance.

Proteins play a key role in cell functioning and structure. The amount of protein present in circulation is dependent on the balance between the rate of synthesis and the rate of catabolism or loss [39]. Therefore, the depletion of serum proteins may be due to increased catabolism or decreased synthesis. Abnormalities in serum proteins are observed during pathological conditions, especially diabetes mellitus [40, 41]. STZ-induced diabetes demonstrated a decreased concentration of total proteins and albumin in the serum. The liver is a major site of protein synthesis, therefore pathological conditions that adversely affect the liver can lead to a reduced production of proteins, hence the low concentration of total proteins observed in the diabetic groups. The observed low protein in the diabetic groups may also be as a result of an increased supply of amino acids for gluconeogenesis [22]. The ability of AD to restore protein levels to normalcy in diabetic rats placed on 400 mg/kg BW, is suggestive of its involvement in enhancing protein synthesis. Albumin is one of the most abundant proteins in the serum [42] with numerous functions such as maintaining the colloidal osmotic pressure, acts as a source of amino acids, and in binding and transporting of substances. Low concentration of serum albumin may cause a decline in the antioxidant status of diabetic rats and is a risk factor for the development of diabetic complications [43, 44]. The current study indicated a significant elevation in the albumin concentrations of diabetic rats treated with AD, this may have promoted the antioxidant capacity and delayed the progression of diabetic complications in the treated rats.

The oxygen radical absorbing capacity and ferric reducing antioxidant power of the diabetic rats were significantly ($p<0.05$) reduced. This is associated with the increased production of ROS as depicted by the significantly increased lipid peroxidation. However, AD significantly ($p<0.05$) elevated ORAC levels back to normal, whereas there was no effect on the FRAP levels. Administration of glibenclamide was not able to improve the antioxidant status in the serum of rats. Furthermore, there was an observable increase in the antioxidant indices in normal rats placed on AD in a dose-dependent manner, though not significant. AD increased the ORAC and FRAP levels in the serum and GSH in the liver of normal rats. This indicates the antioxidant ability of AD over glibenclamide.

AD caused a dose-response induction of catalase in normal rats. The increased activity of catalase in the un-supplemented diabetic rats were likely due to the increased hydrogen peroxide and a compensatory response to the oxidative damage. Certain studies have reported a reduction in catalase activity in diabetic conditions [3, 45]. Contrarily, our findings corroborate with those of El Barky and colleagues [46] who observed a significant increase in catalase activity in the liver of STZ-induced diabetic rats. Furthermore, this trend was also reported by Maritim and co-workers [47]. The activity of SOD in the liver of diabetic rats was not affected and administration of AD did not significantly influence SOD levels in normal and diabetic rats. Very similar observations were reported by Turk and his colleagues [10]. Glutathione is an important inhibitor of free radical-mediated lipid peroxidation. Diminished tGSH levels and increased concentrations of TBARS are consistently observed in diabetes [47] and our findings corroborate with these. AD was able to significantly increase tGSH levels in treated rats when compared to normal rats. The decreased tGSH levels in the liver of diabetic rats may be due to an increased utilisation in trapping the oxyradicals produced. AD administration restored antioxidant capacity in the liver of diabetic rats as expressed by the significantly increased ORAC levels in diabetic treated rats when compared to normal levels. This signifies the ability of AD to ameliorate oxidative stress-induced hepatic injuries in T2D.

T2D affected the normal histoarchitecture of the liver as shown by necrosis, macro-vesicular steatosis, dilatated sinusoids due to oedema. Treatment with AD markedly improved the histology of the liver as some of the pathologies such as steatosis were completely reversed. The observations were dose-dependent as 400 mg of AD restored the overall histoarchitecture of the liver in diabetic rats back to normal. Steatosis reflects an impairment of the normal processes of synthesis and elimination of triglycerides. Insulin resistance is a major factor that contributes to hepatic steatosis by inhibiting lipase thereby gaining control over free fatty acid release into the hepatocytes from the adipocytes [48, 49]. This stimulation may be responsible for the observed significant increase in triglyceride levels in the serum of diabetic rats, which was significantly reduced in response to treatment with AD. Also, LDL-cholesterol delivers cholesterol to the cells and this was significantly elevated in the serum of diabetic rats as was the case with total cholesterol levels. These were reversed with administration of AD.

A summary of the likely pathways involved in the antioxidant and hepatoprotective activity of AD is illustrated in figure 9. The administration of fructose and STZ led to the damage of β cells in the pancreas, this resulted into insulin resistance and hyperglycaemia. Insulin resistance brought about disturbed lipid and protein homeostasis which translated to hyperlipidaemia and decreased protein synthesis. Lipids were transported from the blood system to the hepatocytes causing steatosis. Persistent hyperglycaemia generated more ROS and caused increased lipid peroxidation which in turn resulted into oxidative damage of the cell membrane and leakage of hepatic enzymes. AD was able to attenuate these resultants effects of STZ by preventing or reducing oxidative stress, hyperglycaemia, insulin resistance, blocking fat mobilisation, and lipid peroxidation.

CONCLUSION

A. difformis ameliorated hepatic injury in fructose and STZ-induced diabetes in male Wistar rats. *A. difformis* exerts its hepatoprotective effects by demonstrating good glycaemic control, regulating lipid homeostasis, improving the antioxidant status, and restoring hepatic enzymes to normal levels. Treatment with both doses of *A. difformis* displayed more antidiabetic ability than glibenclamide (5 mg/kg BW), however 400 mg/kg BW *A. difformis* was more effective than 200 mg/kg BW in ameliorating diabetes and associated hepatopathy. Administration of *A. difformis* in diabetes may be a potential therapeutic agent in the management of diabetes and resulted hepatic injuries.

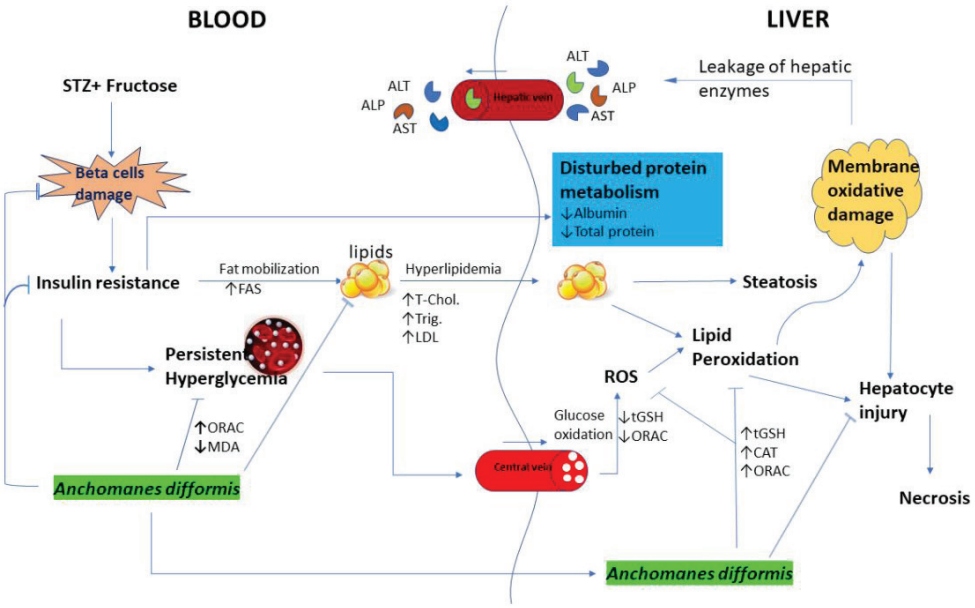

Figure 9

Proposed pathways involved in the antioxidant and hepatoprotective effect of *Anchomanes difformis*

FUTURE PROSPECTS

This study establishes the ability of AD aqueous extract to ameliorate hyperglycaemia, hyperlipidaemia, oxidative stress, liver steatosis and other pathologies in the liver. The findings will be helpful in the management of T2D using AD. However, further molecular studies on the mechanisms by which AD exerts its hypolipidemic, hypoglycaemic and antioxidant potentials in T2D can be explored, this will be a great tool in exploiting the full potentials of AD. Studies investigating the effect of AD on the expression of glucose transporters and other proteins involved in glucose transport and uptake, and insulin signalling is also essential. It is hoped that the current study will encourage further research on the effect of AD in other diabetic complications such as cardiomyopathy, nephropathy, and reproductive dysfunctions.

ACKNOWLEDGMENTS

Thanks to M. Saayman and F. Alexander for their technical assistance.

This research was supported by National Research Foundation, South Africa [NRF 105249] and Cape Peninsula University of Technology, South Africa [CPUT-RJ23 and CPUT-NRF RO22] granted to Professor OO Oguntibeju. The first author also received financial assistance from DST-NRF [107580], this sponsor had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

REFERENCES

1. Anhê FF, Desjardins Y, Pilon G, Dudonné S, Genovese MI, Lajolo FM, et al. Polyphenols and type 2 diabetes: A prospective review. Pharma Nutr 2013 Oct;1(4):105-114. https://linkinghub.elsevier.com/retrieve/pii/S2213434413000406

2. Lüscher TF, Creager MA, Beckman JA, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part II. Circulation 2003; 108(13):1655-1661.

3. Ghanbari E, Nejati V, Khazaei M. Improvement in serum biochemical alterations and oxidative stress of liver and pancreas following use of royal jelly in streptozotocin-induced diabetic rats. Cell J 2016; 18(3):362-370.

4. Sivajothi V, Dey A, Jayakar B, Rajkapoor B. Antihyperglycemic property of *Tragia cannabina* in streptozotocin-induced diabetic rats. J Med Food 2007; 10(2):361-365.

5. Bora K, Borah M, Das D, Chutia H, Ruram A, Nath C. Presence of concurrent derangements of liver function tests in type 2 diabetes and their relationship with glycemic status: A retrospective observational study from Meghalaya. J Lab Physicians 2016; 8(1):30.

6. Guven A, Yavuz O, Cam M, Ercan F, Bukan N, Comunoglu C, et al. Effects of melatonin on streptozotocin-induced liver injury in rats. Acta Histochem 2006; 108(2):85-93.

7. Kaysen GA, Dubin JA, Müller HG, Mitch WE, Rosales LM, Levin NW, et al. Relationships among inflammation nutrition and physiologic mechanisms establishing albumin levels in hemodialysis patients. Kidney Int 2002; 61(6):2240-2249.

8. Sirsikar M, Supriya S, Mohanty S, Pinnelli V. Role of glycated hemoglobin (HBA1c) as a dual marker to predict glycemic status and dyslipidemia in type II diabetes mellitus. Int J Res Med Sci 2016; 4(10):4524-4529. http://www.msjonline.org/index.php/ijrms/article/view/376

9. Biradar SB, Desai AS, Kashinakunti SV, Rangappa M, Kallaganad GS, Devaranavadagi B. Correlation between glycemic control markers and lipid profile in type 2 diabetes mellitus and impaired glucose tolerance. Int J Adv Med 2018 23;5(4):1-6. http://www.ijmedicine.com/index.php/ijam/article/view/1192

10. Turk H, Sevinc A, Camci C, Cigil A, Buyukberber S, Savli H, et al. Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 diabetes mellitus. Acta Diabetol 2002; 39:117-122. http://www.tandfonline.com/doi/full/10.1080/00365519950185823

11. Vertuani S, Angusti A, Manfredini S. The antioxidants and pro-antioxidants network: An Overview. Curr Pharm Des 2005; 10(14):1677-1694.

12. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160(1):1-40.

13. Basciano H, Federico L, Adeli K. Fructose, insulin resistance, and metabolic dyslipidemia. Nutr Metab 2005; 2(5):1-14.

14. Ter Horst KW, Schene MR, Holman R, Romijn JA, Serlie MJ. Effect of fructose consumption on insulin sensitivity in nondiabetic subjects: a systematic review and meta-analysis of diet-intervention trials. Am J Clin Nutr 2016; 104(6):1562-1576.

15. Breinholt VM, Nielsen SE, Knuthsen P, Lauridsen ST, Daneshvar B, Sørensen A. Effects of commonly consumed fruit juices and carbohydrates on redox status and anticancer biomarkers in female rats. Nutr Cancer 2003; 45(1):46-52.

16. Wilson RD, Islam MS. Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes. Pharmacol Reports 2012; 64(1):129-139.

17. Ahmed HA. *Anchomanes difformis*: a multipurpose phytomedicine. IOSR J Pharm Biol Sci 2018; 13(2):62-65.

18. Oyetayo VO. Comparative studies of the phytochemical and antimicrobial properties of the leaf, stem and tuber of *Anchomanes difformis*. J Pharmacol Toxicol. 2007; 2(4):407-410.

19. Udje TD, Brooks NL, Oguntibeju Oluwafemi O. Medicinal activities of *Anchomanes difformis*. and its potential in the treatment of diabetes mellitus and other disease conditions. In: Goyal MR, Ayeleso AO (eds.). Bioactive compounds of medicinal plants: Properties and potential for human health. 1st ed. New York: Apple Academic Press 2018:219-235.

20. Alabi TD, Brooks NL, Oguntibeju OO. Antioxidant capacity, phytochemical analysis and identification of cctive compounds in *Anchomanes difformis*. Nat Prod J 2019; 22:09(1). http://www.eurekaselect.com/171785/article

21. Nkoh NJ, Ngemenya MN, Samje M, Yong JN. Anti-onchocercal and antibacterial activities of crude extracts and secondary metabolites from the rhizome of *Anchomanes difformis* (Araceae). J Cameroon Acad Sci 2015; 12(1):19-30.

22. Adeyemi O, Makinwa TT, Uadia RN. Ethanol extracts of roots of *Anchomanes difformis* roots as an antihyperglycemic agent in diabetic rats. Chem J 2015; 1(3):68-73.

23. Oguntibeju O, Aboua Y, Goboza M. Vindoline – a natural product from *Catharanthus roseus* reduces hyperlipidemia and renal pathophysiology in experimental type 2 diabetes. Biomedicines 2019; 7(3):59. https://www.mdpi.com/2227-9059/7/3/59

24. Thomas L. Alanine aminotransferase (ALT), aspartate aminotransferase (AST). Clin Lab Diagnostics. 1st ed. Frankfurt 1998; 2:55-65.

25. Warnick GR, Wood PD. National cholesterol education program recommendations for measurement of high-density lipoprotein cholesterol: executive summary. The National Cholesterol Education Program Working Group on Lipoprotein Measurement. Clin Chem 1995; 41(10):1427-1433.

26. Friedewald WT, Levy RI, Fredrickson DSF. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18(6): 499-502.

27. Deeg R, Ziegenhorn J. Kinetic enzymic method for automated determination of total cholesterol in serum. Clin Chem 1983; 29(10):1798-1802.

28. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem. 1982;28(10):2077-2080.

29. Matsunami T, Sato Y, Sato T, Yukawa M. Antioxidant status and lipid peroxidation in diabetic rats under hyperbaric oxygen exposure. Physiol Res 2010; 59(1):97-104.

30. Wasowicz W, Neve J, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. Clin Chem 1993; 39(12):2522-2526.

31. Prior RL, Hoang HA, Gu L, Wu X, Bacchiocca

M, Howard L, et al. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. J Agric Food Chem 2003; 51(11):3273-3279.

32. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Methods in Enzymology. Academic Press 1999:15-27.

33. Ellerby LM, Bredesen DE. Measurement of cellular oxidation, reactive oxygen species, and antioxidant enzymes during apoptosis. Methods Enzymol 2000; 322: 413-421.

34. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med 2011; 364(2):127-135.

35. Farombi EO, Abolaji AO, Adedara IA, Maduako I, Omodanisi I. Artemisinin induces hormonal imbalance and oxidative damage in the erythrocytes and uterus but not in the ovary of rats. Hum Exp Toxicol 2015; 34(1):83-92.

36. Ekstedt M, Franzén LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology 2006; 44(4):865-873.

37. Ayepola OR, Brooks NL, Oguntibeju OO. Kolaviron improved resistance to oxidative stress and inflammation in the blood (erythrocyte, serum, and plasma) of streptozotocin-induced diabetic rats. Sci World J 2014; 2:1-8.

38. Chen SC-C, Jhao J-Y, Tsai SP, Tsao CK, Chang L-Y, Jiang W-K. Liver fat, hepatic enzymes, alkaline phosphatase and the risk of incident type 2 diabetes: A prospective study of 132,377 adults. Sci Rep 2017; 7(1):1-9.

39. Hamad AWR, Ibrahim MA, Al-Mohtasib SI, Al-Kobasi KN. Comparative study on saliva proteins in patients of brain tumors and healthy individuals. Trends Med Res 2009; 4(2):16-23.

40. Suzuki H. Protein-protein interactions in the mammalian brain. J Physiol 2006; 575(2):373-377.

41. Hasan HR, Abdulsattar A. Influence of diabetes disease on concentration of total protein, albumin and globulins in saliva and serum: A comparative study. Iraqi Natl J Chem 2015; 15(1):1-11.

42. Wang Y, Wang S, Huang M. Structure and enzymatic activities of human serum albumin. Curr Pharm Des 2015; 21(14):1831-1836.

43. Park KT, Yun CH, Bae CS, Ahn T. Decreased level of albumin in peripheral blood mononuclear cells of streptozotocin-induced diabetic rats. J Vet Med Sci 2014; 76(8):1087-1092.

44. Obia O, Chuemere AN, Chike CPR, Nyeche S. Effect of supplementation of natural honey on serum albumin and total protein of alloxan induced diabetic Wistar rats. Am J Phytomedicine Clin Ther 2017; 5(3):1-3.

45. Erejuwa OO, Sulaiman SA, Wahab MSA, Sirajudeen KNS, Salleh MSM, Gurtu S. Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats. Int J Mol Sci 2010 5;11(5):2056-2066. http://www.mdpi.com/1422-0067/11/5/2056

46. El Barky AR, Hussein SA, Alm-Eldeen AA, Hafez YA, Mohamed TM. Anti-diabetic activity of *Holothuria thomasi* saponin. Biomed Pharmacother 2016; 84:1472-1487.

47. Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: A review. J Biochem Mol Toxicol 2003; 17(1):24-38.

48. Hickman IJ, Clouston AD, Macdonald GA, Purdie DM, Prins JB, Ash S, et al. Effect of weight reduction on liver histology and biochemistry in patients with chronic hepatitis C. Gut 2002; 51(1):89-94.

49. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: Integrating signaling pathways and substrate flux. J Clin Invest 2016; 126(1):12-22. doi: http://dx.doi.org/10.1172/JCI77812