Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Suppl 2, 2013 ISSN - 0974-2441

Research Article

SAPONINS FROM *SOLANUM ANGUIVI* LAM. FRUIT EXHIBIT *IN VITRO* AND *IN VIVO* ANTIOXIDANT ACTIVITIES IN ALLOXAN-INDUCED OXIDATIVE STRESS

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Received: 11 March 2013, Revised and Accepted: 30 March 2013

ABSTRACT

Objective: To evaluate the antioxidant and radical scavenging activities of saponins from *Solanum anguivi* fruit (SAGU) and its possible effect on oxidative status of rat exposed to alloxan-induced diabetes. **Methods:** Antioxidant activity of SAGU was assayed by using 2,2'-azino-bis3-ethylbenzothiazoline-6-sulphonic acid (ABTS), reducing power, iron chelation and ability to inhibit lipid peroxidation in both liver and brain homogenates of rats. Also, the effect of SAGU on MDA level, an indicator of lipid peroxidation and activity of superoxide dismutase (SOD) and catalase (CAT) were determined in heart and kidney of rat exposed to alloxan (150mg/kg) for 21 days. **Results:** SAGU exhibited potent and concentration dependent free radical-scavenging activity ($IC_{50/ABTS}$ = 290.12 ± 1.34µg/ml). Reductive and iron chelation abilities also increase with increase in SAGU concentration. SAGU also inhibited peroxidation of hepatic and cerebral lipids subjected to iron oxidative assault. In addition, SAGU significantly ($P<0.05$) protected against lipid peroxidation induced by alloxan as well as restored the level of SOD and CAT in both heart and kidney of alloxan-treated rats. **Conclusions:** Taking together, our results suggest that saponins from the fruits of *Solanum anguivi* are potent antioxidant that can be useful not only in pharmaceutical and food industry, but also in the management of diabetes.

Keywords: Saponin, *Solanum anguivi* fruit, Antioxidant activity, Diabetes, MDA, Superoxide dismutase, Catalase, Alloxan, Medicinal plants.

INTRODUCTION

Oxygen is very essential to life due to its utilization by cells to produce energy in form of ATP which is use to drive all cellular processes in the mitochondria. During this process free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as by-products of cell metabolism[1,2]. They play a dual role (toxic and beneficial) in human. For instance, ROS/RNS are beneficially involved in many signaling pathways that control development and maintain cellular homeostasis[3]. However, when present in excess, they can attack biological molecules such as proteins, lipids, enzymes, DNA and RNA and initiate peroxidation of polyunsaturated fatty acids in biological membranes[4,5] leading to cellular injury[6-9]. These events commonly known as oxidative stress, could consequently lead to the occurrence of various free radicals related diseases including diabetes mellitus.

Alloxan, a cytotoxic glucose analogue is extensively used in experimental model of diabetes mellitus. Considerable evidence have shown that chronic hyperglycemia resulting from alloxan-induced diabetes brings about a rise in oxidative stress in a variety of tissues like heart and kidney[10-12], due to overproduction of ROS as a result of glucose-autoxidation and protein glycosylation and decreased antioxidant defenses[13]. In addition, free radicals are also produced during the interaction of alloxan with pancreatic β islets cells[14-15].

Natural antioxidants have been extensively studied for their capacity to protect organisms and cells from damage induced by oxidative stress, the latter being considered a cause of ageing and degenerative diseases[16]. Plants have been a source of medicine in the past centuries and nowadays, scientists and the general public recognize their value as a source of new or complimentary medicinal products in the treatment of diseases like diabetes. Antioxidants have been reported to be involved in the defense mechanisms in alloxan-induced oxidative damage in animals by inhibiting glycation through ROS scavenging, by chelating metals and restoring the level of antioxidant enzymes (SOD, CAT) [17].

Solanum anguivi Lam. is a rare ethanomedicinal herb belonging to the family Solanaceae. The plant is used as therapeutic agent for various diseases. In Nigeria, the fruits are used as sources of food and drugs. It is a nourishing vegetable that is eaten raw or cooked because it is believed to reduce the risk of diabetes and high blood pressure. Recently, we have reported the in vitro antioxidant activity of the fruit of this plant to inhibit lipid peroxidation in rat's brain exposed to both iron and sodium nitropruside insult *in vitro*[18]. *Solanum anguivi* fruit is one of the richest sources of edible saponin in south western and south eastern part of Nigeria. Saponins are natural glycosides of steroid or triterpene which exhibited a variety of biological and pharmacological properties including antioxidant[19,20], hypolipidemic potential[21], and inhibition of erythropoesis in *Rattus novergicus*[22]. However, to the best of our knowledge, there is no information in the literature on the *in vitro* antioxidant as well as effect of saponins from this plant on oxidative status in alloxan induced oxidative stress; hence, this study was aimed at investigating the *in vitro* antioxidant potential of saponins from *Solanum anguivi* fruit and its effect on oxidative status in kidney and heart of rats exposed to alloxan-induced oxidative stress.

MATERIALS AND METHODS

Chemicals

All chemicals used, including solvents, were of analytical grade. Folin Ciocalteu's phenol reagent, malonaldehyde bis-(dimethyl acetal) (MDA), thiobarbituric acid (TBA), sodium dodecyl sulfate, 2,2'-azino-bis3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{+}$), butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (USA). Ferrous sulfate, FeCl3 were obtained from Vetec (Brazil). Alloxan was purchased from Sigma (Sigma-Aldrich, Germany).

Plant materials

The fruits of *S. anguivi* were collected from Adekunle Ajasin University, Akungba Akoko horticultural garden. They were identified and authenticated at the herbarium of plant science and Forestry department, University of Ado Ekiti, Nigeria (voucher specimen number UHAE: 286). The fruits were air dried and

grounded into a powdery fine texture and stored at room temperature in air tight polythene bag prior to use.

Extraction and isolation of saponins from *Solanum anguivi* fruit

Saponins were extracted as described by[23] with slight modification. Hundred grams (100 g) ground sample was extracted with 2000 ml of methanol for 48 hours. The methanolic extract was concentrated using a rotary evaporator and partitioned with hexane and water (1:2, v/v). After a thorough shaking, the mixture was allowed to stand overnight and the water layer was concentrated and partitioned between ethyl acetate and n-butanol (1:3, v/v). The butanol fraction was concentrated to obtain crude saponin fraction. The crude saponin fraction was spotted onto pre-coated silica gel TLC plate (Merck, Kleselgel 60F-254). The plates were developed with n-butanol: acetic acid: water (60:10:30 v/v/v). The spots on the chromatograms which were due to saponins were identified by spraying with Lieberman-Burchard reagent (methanol: sulphuric acid: acetic acid (50:5:5 v/v/v)). Concentrated crude saponin extract was applied to a silica gel column of (60-120 mesh). The impurities were washed with n-hexane through a 2.4 x 50 cm bed of silica gel. The column was eluted with n-butanol: acetic acid: water (1:1:1 v/v/v). The fractions were collected and aliquots applied as a series of spots to a strip of TLC plate, dried, sprayed with Lieberman-Burchard reagent and heated. Positive fractions were pooled together and used for the experiment.

In vitro studies

Animals

Male Wistar rats, weighing 270–320 g and aged from 2 to 3 months, from our own breeding colony (Animal House-holding, UFSM, Brazil) were kept in cages with free access to food and water in a room with controlled temperature (22±3 °C) and in 12 h light/dark cycle. The protocol of this study has been approved by the Brazilian Association for Laboratory animal Science (COBEA).

$ABTS^{\cdot+}$ radical scavenging activity

The antioxidant ability of saponins to trap free radicals was evaluated according to the method described by[24]. The stock solution included 7mM ABTS solution and 2.4mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours at room temperature in the dark. The solution was then diluted by 1ml ABTS solution with 60ml methanol to obtain an absorbance of 0.706±0.001 at 734 nm using the spectrophotometer. Saponins and BHT at various concentrations (50-250µg/ml) were allowed to react with 1ml of ABTS and the absorbance read after 7 mins. BHT was used as a reference standard. The ABTS scavenging capacity was calculated as ABTS scavenging activity (%) = [Absorbance $_{control}$- Absorbance $_{sample}$]/(Absorbance $_{control}$)] x100.

Reducing power

The Fe^{3+} reducing power of the saponins extracted from *Solanum anguvui* fruit was determined by the method of described by[25]. Different concentrations (50 – 500 µg/ml) of saponins (0.5 ml) were mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium ferrycyanate (0.1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 0.5 ml of TCA (10%) was added to terminate the reaction. The upper portion of the solution (1ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. BHT was used as a positive control.

Fe^{2+} chelation Assay

The ferrous ion chelating activity of extract was evaluated by a standard method as described by[26] with minor changes. The reaction was carried out in Tris-HCl buffer (0.1 M, pH 7.5). Briefly, various concentrations (50–500µg/ml) of saponins were added to 100 µM ferrous sulfate solution. The reaction mixture was shaken vigorously and incubated for 30 min at room temperature before the addition of 1,10-Phenanthroline (0.25% w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. Butylated hydroxytoluene (BHT) was used as a positive control.

Thiobarbituric acid reactive substances assay

Thiobarbituric acid reactive substances (TBARS) production was determined as described by[27] with slight modification. Aliquots of the homogenate (100 µl) from tissues (brain or liver) and the pro-oxidant agent (10µM $FeSO_4$) were incubated at 37°C for 1 h in a water bath in the presence or absence of saponins (200–500µg/ml). Color reaction was developed by adding 200 µl of 8.1% SDS (sodium dodecyl sulphate) to the reaction mixture. This was subsequently followed by the addition of 500 µl of acetic acid/ HCl buffer (pH 3.4) and 500 µl of 0.6% thiobarbituric acid (TBA). This mixture was incubated at 100°C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde (MDA).

In vivo studies

Thirty five albino rats of average weight 125±39 g were obtained from Animal unit Achievers University, Ondo State, Nigeria. They were divided into seven groups of five animals each and allowed to acclimatize to experimental condition for two weeks. They were housed in clean cages and maintained under standard laboratory conditions (temperature 25±2°C with dark/light cycle 12/12h). They were fed *ad libitum* on rat pellets (Top Feeds, Nigeria) and water. The principles of Laboratory Animal care (NIH Publication 85-93, revised 1985) were followed throughout the duration of the experiment.

Experimental procedure

Diabetes was induced through a single intraperitoneal injection of a freshly prepared alloxan (Sigma-Aldrich, Germany) solution in normal saline at a dose of 150 mg/kg body weight. Since the injection of alloxan can provoke fatal hypoglycemia due to a reactive massive release of pancreatic insulin, the rats were also orally given 5–10 ml of a 20% glucose solution after 6 h. The animals were then kept with free access to 5% glucose solution for the next 24 h to prevent severe hypoglycemia. Two weeks later, the rats with moderate diabetes having glycosuria and hyperglycemia (i.e. with blood glucose levels of 200-300 mg/dl) were chosen for the experiments. The rats (n=35) were divided equally into 7 groups. Group I served as normal control, and were given 2 ml saline by gavage, group II served as diabetic control, group III-VII were diabetic rats treated with saponin at doses of 20, 40, 60, 80, 100 mg/kg body weight respectively for 21 days. After 21-days of treatment, the rats were sacrificed by decapitation and the tissues (heart and kidney) were removed into 0.25 M ice cold sucrose solution in ratio 1:5 w/v, centrifuged and the supernatant used for determination of lipid peroxidation level, catalase (CAT) and superoxide dismutase (SOD) activities.

Biochemical parameters

Using the supernatant of the centrifuged homogenate of the Heart and kidney tissues, the SOD and CAT levels were determined according to the method described by[28] and[29] respectively. Whereas, the level of lipid peroxidation was determined as described by[30].

Protein determination

Protein content was determined by the method of [31] using bovine serum albumin (BSA) as standard.

Statistical analysis

The experimental results were expressed as mean ± standard error

of mean (SEM) of three replicates and were subjected to one way analysis of variance followed by Duncan's multiple range tests. Significant levels were tested at $p < 0.05$.

RESULTS

In vitro antioxidant activity

ABTS radical scavenging activity

As seen in Fig. 1, saponins as well as the standard antioxidant BHT scavenged the ABTS radical in a concentration dependent-manner. The IC_{50} value (the concentration that inhibited 50% of the radical) of saponins (290.12±1.34 µg/ml) was almost similar to that of BHT (85.03±3.06 µg/ml). However, at the highest concentration tested (500 µg/ml) BHT exerted a more potent activity with 95% ABTS radical inhibition versus 58% for SAGU.

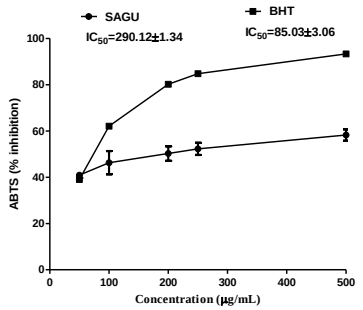


Figure 1: Quenching of ABTS color by SAGU versus BHT. Data represents means ± SEM values averages from 3 to 4 independent experiments performed in triplicate.

Reducing power activity

Reducing power activity is a good indicator of antioxidant activity. Compounds with high reducing power are generally reported to carry high antioxidant potential. Reduction of Fe^{3+} by electron-donating activity of compounds reflects the possible antioxidant mechanism of these compounds. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of test compound. The results for ferric reducing activity of SAGU compared to BHT used as standard are reported in Figure 2. SAGU exhibited dose dependent reducing power potential. However, the efficacy was found to be lower than that of BHT.

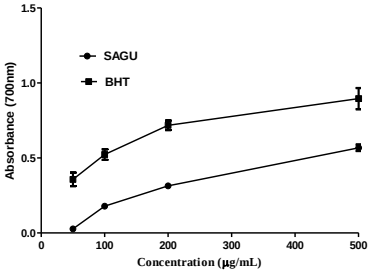


Figure 2: Reducing properties SAGU and standard BHT. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate.

Metal chelating activity

Phenanthroline quantitatively forms complexes with Fe^{2+} and produce red color. In the presence of chelating agents, the formation of this complex is disrupted, thereby impeding the formation of red color. SAGU exhibited dose dependent metal chelating activity (Figure 3). However, the metal chelating activity of BHT was higher than that of SAGU (Figure 3).

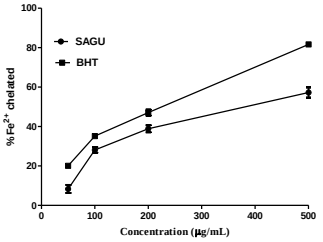


Figure 3: Fe^{2+}- chelating properties of SAGU versus BHT standard. Data show means ± SEM values averages from 3 to 4 independent experiments performed in triplicate.

Lipid peroxidation inhibiting activity

Fe^{2+} caused a significant increase in TBARS production in rat liver and brain homogenates when compared to their respective control ($p < 0.05$, Fig. 4A and B). SAGU caused a significant decrease in Fe^{2+}-induced TBARS production in both rat liver and brain homogenates with increasing concentration ($p < 0.05$, Fig. 4A and B).

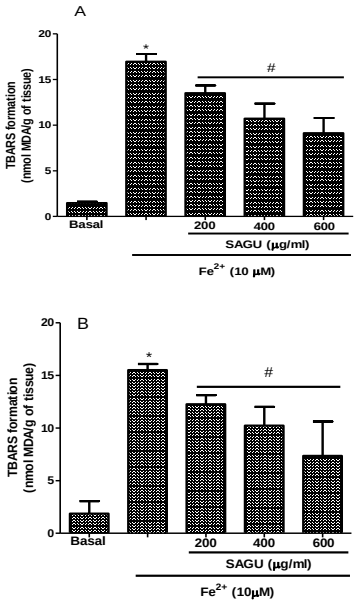
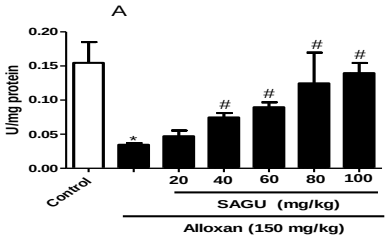


Figure 4: Effects of SAGU on Fe^{2+} (10 µmol L^{-1})-induced TBARS production in liver (A) and brain (B) homogenates. The samples were incubated for 1 h with Fe^{2+} in the presence or absence of SAGU (basal). Mean± SEM, n= 3–4 independent experiments. * represent significant difference from basal, # represent significant difference from Fe^{2+} control at p< 0.05.

In vivo antioxidant activity

Effect of SAGU on the activity of antioxidant enzymes

As shown in Fig. 5 and 6, the activity of CAT and SOD were significantly decreased in alloxan-treated rats in the kidney (Fig. 5A, 6A) and heart (Fig. 5B, 6B) when compared to the control ($p < 0.05$). Treatment with SAGU significantly increased the level of CAT (Fig. 5A and B) and SOD (Fig. 6A and B) in both tissues when compared to diabetic control ($p < 0.05$).



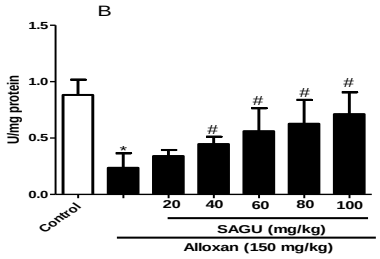


Figure 5: Effect of *SAGU* on Catalase activity in kidney (A) and heart (B) of rats exposed to 150mg/kg alloxan. Results are expressed as means ± S.E.M. (*n* = 5). * Significantly difference from control (p < 0.05); # Significantly different from Alloxan control (*P* < 0.05).

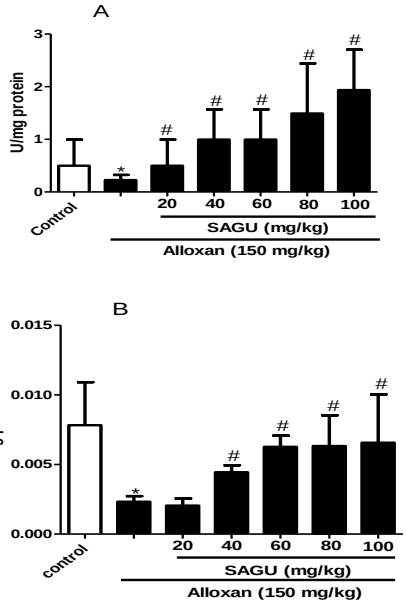
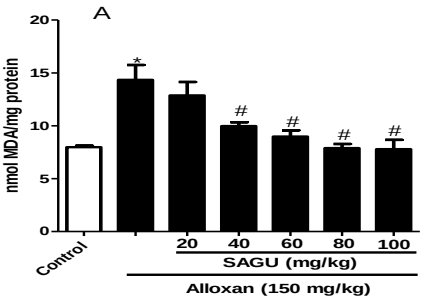


Figure 6: Effect of *SAGU* on SOD activity in kidney (A) and heart (B) of rats exposed to 150mg/kg alloxan. Results are expressed as means ± S.E.M. (*n* = 5). * Significantly different from control (p < 0.05); # Significantly different from Alloxan control (*P* < 0.05).

Effect of SAGU on lipid peroxidation

The effect of SAGU on lipid peroxidation induced by alloxan in heart and kidney of rats is shown in Fig. 7A and B. Alloxan administration significantly increased the level of LPO in kidney and heart, effect that was attenuated by SAGU treatment in a concentration dependent-manner (p < 0.05, Fig. 7A and B).



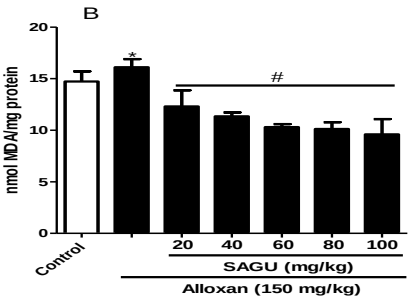


Figure 7: Effect of *SAGU* on MDA level in kidney (A) and heart (B) of rats exposed to 150mg/kg alloxan. * Significantly difference from control (p < 0.05); # Significantly different from Alloxan control (*P* < 0.05).

DISCUSSION

Currently, scientists are focusing on the active compound from the natural sources to develop newer drugs. Several reports have shown that saponins are bioactive compounds in plants responsible for most pharmacological properties such as antidiabetic, antihyperlipidemic, insulin like properties and inhibition of lipid peroxides in vivo and in vitro models[31-35]. In this study, we investigated the antioxidant property *in vitro* as well as the potential protective effect of SAGU on alloxan induced oxidative stress in heart and liver of rats *in vivo*.

The total antioxidant capacity is a summation of different antioxidant mechanisms, including free radical scavenging ability, reducing power and Fe^{2+} chelating ability. ABTS radical scavenging activity is a more drastic radical that is chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids[36]. Its ability in both organic and aqueous media and its stability in a wide pH range raised the interest in the use of $ABTS^{+}$ for the estimation of antioxidant activity[37]. The decolorization of ABTS radical cation reflects the capacity of antioxidants to donate electrons or hydrogen atoms to deactivate these radical species[38,39]. SAGU showed potent antioxidant activity by scavenging ABTS radical as compared to BHT standard used in this study (Fig.1). This result indicates the direct role of SAGU in free radical scavenging by either donating electrons or hydrogen atoms to render the radical species inactive.

The reducing power of SAGU was evaluated by the transformation of Fe^{3+} to Fe^{2+} through electron transfer ability which serves as a significant indicator of its antioxidant capacity. The reductive ability of saponins and BHT increased in a dose dependent manner. The increased reducing power of SAGU observed in this study indicated that saponins from this plant were electron donors that could react with free radicals converting them into a more stable products thereby terminating radical chain reaction[40].

Iron is crucial for the maintenance of cell homeostasis. It is also present in blood pigment hemoglobin which is able to become reversibly bonded by forming coordinate bonds with molecular oxygen thus allowing hemoglobin to transport oxygen to all part of the body and release it where it is needed[41,42]. However, free Fe^{2+} can induce toxicity via stimulation of the Fenton reaction. The ability of antioxidants to chelate and deactivate transition metal like Fe^{2+} prevents such metal from participating in the initiation of lipid peroxidation and oxidative stress through metal-catalyzed reaction[43]. Our result demonstrated that SAGU is not as good as the standard BHT in chelating Fe^{2+}; but the decrease in concentration-dependent colour formation in the presence of saponins indicates that it has iron Fe^{2+} chelating activity.

In this study, we observed that SAGU significantly inhibited Fe^{2+}-induced TBARS formation in rat liver and brain homogenates (Fig. 4A and B). This clearly shows that SAGU has the ability to inhibit lipid peroxidation, a type of oxidative degeneration of polyunsaturated lipids, which has been implicated in a variety of pathogenic processes.

ROS are chemical species which contains one or more unpaired electrons. This makes them highly unstable causing damage to other molecules by extracting electrons from them in order to attain stability. ROS are continuously produced in the human body because they are essential for detoxification, chemical signaling, energy supply and immune function. They are regulated by endogenous antioxidant enzyme system, such as superoxide dismutase (SOD), catalase (CAT) etc. It is possible to reduce the risks of chronic diseases and prevent progression of disease by either enhancing the body's natural antioxidant defense or supplementing with proven antioxidants. Here, we investigated the effect of SAGU on lipid peroxidation and antioxidant enzymes (SOD and CAT) in heart and liver of rat exposed to alloxan-induced oxidative stress. We found that alloxan administration increased both the lipid peroxidation (MDA) and decreased the SOD and CAT activities when compared to control rats ($p < 0.05$). Interestingly, treatment with SAGU in a dose dependent-manner potentially–attenuated the level of LPO and increased SOD and CAT activities in the heart and kidney. The amelioration of the LPO observed in this study may be a consequence of the observed enhancement of the enzymes SOD and CAT. Reports have shown that SOD is one of the most important enzymes in the enzymatic antioxidant defense system in the body since it catalyses the dismutation of superoxide radicals (O_2^{-}.) to produce H_2O_2 and molecular oxygen[44,45]. This diminishes the toxic effects of superoxide radicals or other free radicals derived from secondary reactions[46]. Earlier report by[47] showed that superoxide anion inactivates CAT and this action is involved in the detoxification of hydrogen peroxide[48] . As a result, the increase in SOD activity may indirectly play an important role in the activity of catalase. CAT is a hemeprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals[49]. Reduced activities of SOD and CAT in tissues have been observed in diabetic rat. ROS has been shown to be the cause of complication in diabetes. However, SAGU was able to restore the level of these enzymes in kidney and heart of rat exposed to alloxan.

Our results suggest SAGU as potent inhibitor of ROS both *in vitro* and *in vivo* diabetic models as it is able to re-establish the oxidative status which are reduced in diabetic state. Hence, saponins from *Solanum anguivi* fruits can prevent against of diabetic complications.

CONCLUSION

The results of this study clearly demonstrated that saponins from *Solanum anguivi* fruits exhibit free radical scavenging activities and possess reducing power and iron chelating ability making it an excellent candidate in the treatment of diseases in which ROS has been implicated. SAGU also exhibited antioxidant activities in alloxan-induced diabetes by increasing the level of CAT and SOD activities LPO level both in heart and kidney. Consequently, it has an ability to prevent diabetic complications. Hence, the above findings have given scientific evidence to saponins, a class of phytochemical from *Solanum anguivi* fruit as potent antioxidant that can be employed in the management of diabetes.

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