

***Azadirachta indica* Leaf Extract Ameliorates Hyperglycemia and Hepatic Glycogenesis in Streptozotocin-induced Diabetic Wistar Rats**

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Abstract

We studied the effects of ethanolic leaf extract of *Azadirachta indica* (AIE) on hepatic microscopic anatomy and oxidative stress markers in diabetic rats. Seventy-five Wistar rats (8 weeks old) were randomly assigned to five treatment groups: control; diabetic; diabetic+AIE; AIE only; and diabetic+glibenclamide. Hyperglycemia was induced in fasted rats with streptozotocin. AIE was administered orally at 500 mg/kg bw/d and glibenclamide at 600 µg/kg bw/d for 50 days (50 d). Animals were sacrificed on treatment days 7, 21 and 50. The liver was stained with PAS. Hepatic markers of oxidative stress were also estimated. At 50 d, histological study of the liver of diabetic rats showed swollen PAS+ hepatocytes, whose content was confirmed to be glycogen. On the contrary, hepatocytes of AIE-treated diabetic rats lacked glycogen. The major finding in these rats was exacerbated oxidative stress. Our findings in this model showed the beneficial effect of AIE in the amelioration of diabetic hepatic glycogenesis.

Key words: Diabetes, *Azadirachta indica*, hepatic glycogenesis, oxidative stress, liver

Introduction

Diabetes mellitus describes a metabolic disorder of multiple etiologies. It is characterised by chronic hyperglycemia, with perturbations of carbohydrate, lipid and protein metabolism that result from defects in insulin secretion, insulin action or both [1]. Type 1 diabetes is a chronic auto-immune disease characterised by immune-mediated destruction of the insulin-producing β cells of pancreatic islets, resulting in absolute insulin deficiency. More than 90% of diabetics have type 2

form of the disease. This is characterised by insulin resistance in otherwise insulin-sensitive cells [2].

Efforts are ongoing to evaluate botanical drugs for the management of diabetes mellitus [3]. Herbs with potential antidiabetic property include *Azadirachta indica* A. Juss (neem). Currently, neem is included in antidiabetic formulations and food supplements. Such botanical formulations, including pancreatic tonic® and cogent db®, had been subjected to controlled clinical trials in the USA [4] and Malaysia [5], respectively, with promising results.

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However, while botanical drugs are undoubtedly potential sources of new chemical entities in the management of diabetes and its complications, basic and clinical studies directed especially at their efficacy, safety and standardisation are inevitable [6]. Herbal drugs such as *Aristolochia spp* are associated with nephropathy owing to the presence of toxic aristolochic acid [7]. Pennyroyal oil from *Mentha pulegium* could induce liver damage and renal toxicity [8]; while hepatic toxicity is associated with the diterpenoid-containing herbal drug, *Teucrium chamaedrys* [9].

The liver is a central organ in glucose homeostasis; and it is involved in the complications of diabetes mellitus. While non-alcoholic fatty liver disease (NAFLD) may also be associated with type 1 diabetes [10], hepatic histopathology in type 1 diabetic patients could be distinct from type 2. Liver biopsy findings in type 1 diabetics with hepatomegaly are comparable to findings in Mauriac's syndrome, and is characterised by excessive glycogen deposition in hepatocytes [10,11]. Poorly-controlled blood glucose has been associated with this form of glycogenesis.

Moreover, hyperglycemia of diabetes is associated with oxidative stress; and the latter is involved in the etiology of diabetes complications [12]. Excessive glucose delivered to the mitochondria overdrives the electron transport chain, resulting in overproduction of superoxide anion [13]. Autoxidation of glucose in the presence of transition metals, and non-enzymatic glycation of proteins, also generate reactive oxygen species (ROS) in diabetes [14]. Besides, hyperglycemia can lower the activity of antioxidant enzymes such as SOD and glutathione reductase, perhaps by glycation [15]. Glutathione synthesis was also reported to be inhibited under hyperglycemic conditions [16].

However, studies on the structural and biochemical changes in the liver of laboratory animals on AIE therapy have been poorly documented. Thus, the objectives of this work were to study (i) the potential hypoglycemic activity of AIE; and (ii) the histology and oxidative stress markers of the liver of streptozotocin-induced diabetic Wistar rats treated with AIE.

Materials and Methods

Animals

Seventy-five male Wistar rats (Harlan, Milan, Italy) were used. Animals weighed 175.5 g on average and were 8 weeks old. Animals were housed in the animal holdings of the Faculty of Biological and Environmental Sciences and Technology, University of Salento, Italy. They were exposed to 12-hour light, 12-hour dark cycle at a room temperature of 21-23 °C. All animals were maintained on Harlan Global Diet 2018 (Harlan, Milan, Italy). Both feed and water were given freely. Besides, animal handling and experimental procedures conformed to standard principles.

Collection and extraction of *Azadirachta indica* leaves

Mature fresh leaves of *A. indica* (A. Juss) were collected from *A. indica* trees in the premises of the University of Ilorin, Mini Campus, Nigeria, between June and August 2007. A sample of the collection was identified and compared with the voucher specimen at the herbarium of the Botany Department of the same University (Voucher No 542). *A. indica* leaves were air-dried (at room temperature) and extracted by percolation as described by Chattopadhyay [17]. A total of 2.4 kg of the dry leaf powder was extracted at room temperature using 70% ethanol. The initial hydro-alcoholic filtrate was concentrated under vacuum in a Buchi Rotavapor R-114 (Buchi, Switzerland) at 50 °C (bath temperature). The concentrate was dissolved in distilled water and filtered with Whatman No.1 paper. The filtrate was concentrated in the rotavapor at 50 °C bath temperature. The final residue (about 120 g) was stored at 4 °C.

Induction of diabetes mellitus

Hyperglycemia was induced in 45 overnight-fasted, randomly-selected animals, by a single intraperitoneal injection of 70 mg/kg bw streptozotocin (Sigma, MO, USA) in 0.1 M citrate buffer, pH 4.5 [18]. Animals were allowed free access to feed and water after the injection. Hyperglycemia was allowed to develop over a minimum period of 72 hours [19]. Animals with fasting blood glucose ≥ 250 mg/dl were considered hyperglycemic [20]. Non-diabetic control animals (n=9) received a single intraperitoneal injection of 0.1 M citrate buffer (1 ml/kg b w), pH 4.5.

***Azadirachta indica* treatment**

The dose of *A. indica* used was based on the work of Chattopadhyay [17]. Water-soluble, ethanolic extract of *A. indica* (AIE) (500 mg/kg bw/d) was administered by gavage to a group of hyperglycemic rats (n=15) at 9.00-10.00 hour each day for a maximum of 50 d. An antidiabetic drug, glibenclamide (Sigma, MO, USA), was also administered orally at 600 µg/kg bw/d [21] to another group of hyperglycemic rats (n=15), to allow for comparative studies. Furthermore, a separate group of normoglycemic rats (n=15) were administered 500 mg/kg b. w/d of AIE for the same period. Fifteen hyperglycemic rats, which received neither AIE nor glibenclamide, constituted the untreated diabetic group.

Estimation of blood glucose

Blood glucose was estimated in overnight-fasted rats at 9.00-10.00 hour by the glucose oxidase method, using One Touch Ultra 2 Glucometer (Lifescan, CA, USA). Blood was obtained from the dorsal vein of the tail. In the first week of treatment, blood glucose was monitored daily; thereafter, glucose levels were assessed at 72-hour interval.

Body weight

The body weights of the rats were taken prior to the induction of hyperglycemia, at day 0 of AIE and glibenclamide treatment, and at 72-hour interval thereafter.

Termination of treatment

For the purpose of assessing the biochemical and morphological changes occurring in the animals with the progression of treatment, 5 rats were sacrificed in each treatment group at the 7th, 21st, and 50th days of treatment. Animals were anaesthetized with pentobarbital (60 mg/ kg bw). Laparotomy was performed and the liver was excised, rinsed in PBS and fixed in Bouin's fluid for histological studies. Portions of the liver were also stored at -80 °C for analysis of oxidative stress markers.

Tissue processing for light microscopy

After fixation, the liver was rinsed repeatedly in PBS until the yellowish coloration disappeared. Paraffin embedding was done, and 3.5 µm sections were cut on a Reichert-Jung 2050 rotary microtome (Cambridge Instruments, Germany), followed by

Periodic acid-Schiff (PAS) staining. Briefly, sections were de-waxed in xylene, hydrated in descending grades of ethanol and then transferred to 0.8 % periodic for 10 min. Rinsing was done in ordinary water (10 min.), followed by exposure to Schiff reagent (30 min). After rinsing in ordinary water (20 min.), sections were stained in Carazzi's haematoxylin (15 min) and eosin Y (1 min.). Photomicrographs were taken with a Nikon digital camera DXM1200F (Nikon, Japan) coupled to a Nikon Eclipse 80i light microscope (Nikon, Japan).

Oxidative stress markers

The levels of lipid hydroperoxides, aqueous hydroperoxide (hydrogen peroxide), reduced glutathione (GSH), and superoxide dismutase (SOD) were estimated in homogenates of the liver. For each sample, 10% homogenate was prepared with 0.15 M KCl as homogenizing buffer. The homogenate was centrifuged at 1000 g for 10 min at 4 °C [22]; and the supernatant was analysed for total protein and the markers of oxidative stress (except GSH).

Hepatic lipid hydroperoxides

Lipid hydroperoxide levels of each liver sample were estimated with a PeroxiDetect™ kit (Sigma, MO, USA). The assay was based on the oxidation of Fe²⁺ to Fe³⁺ by lipid hydroperoxides in the sample; Fe³⁺ then forms a coloured adduct with xylenol orange. This adduct is detectable by spectrophotometry at 560 nm.

Hepatic aqueous hydroperoxide

Aqueous hydroperoxide levels of each liver sample were estimated using PeroxiDetect™ kit (Sigma, MO, USA). The principle was as described for lipid hydroperoxides. However, colour formation was enhanced by the addition of sorbitol. Sorbitol is converted by H₂O₂ in the samples to a peroxy radical. The latter oxidises Fe²⁺ to Fe³⁺, which then forms a coloured adduct with xylenol orange. Absorbance was read at 560 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Reduced glutathione (GSH)

GSH levels in liver samples were estimated with a glutathione assay kit (Sigma, MO, USA). The samples were homogenised with 5% sulphosalicylic

acid (5% SSA). The homogenates were allowed to stand for 10 min at 4 °C, and then centrifuged at 10,000 x g for 10 minutes. The supernatants were diluted and used in the assay. Absorbance was read at 412 nm in a spectrophotometer.

Protein assay

Protein levels of the samples were estimated by the BioRad Protein Assay Method (BioRad, CA, USA). Bovine serum albumin (100 µg/ml) served as standard, and absorbance was read at 595 nm in an Ultraspec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

Statistical analysis

Data were analysed using SPSS 15.0 (SPSS Inc, Chicago, USA) and Excel 2007 (Microsoft Corporation, USA). Data were expressed as Mean±SEM, while means were compared using one way analysis of variance (ANOVA), and the Bonferroni post-hoc test.

Results

Body weight

At the end of week 1, 1.5% loss in body weight had occurred in the AIE-treated diabetic rats. Similarly, body weight gain in the AIE-treated non-diabetic rats was negligible (Fig. 1). In contrast, relatively higher increases in body weight were obtained in the control, untreated diabetic and glibenclamide-treated diabetic rats by this time (7 d). By 50 d, weight gain in the AIE-treated non-diabetic rats remained the least (19.9%), while the highest weight increase of 49.9% ($P<0.05$) occurred in the glibenclamide-treated rats (Fig. 1).

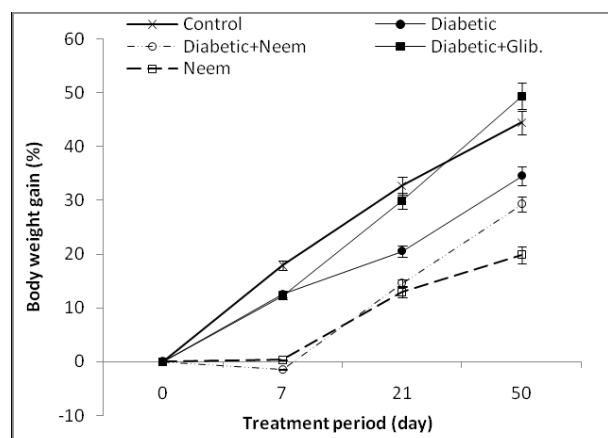


Figure 1: Change (%) in body weights of animals at days 7, 21, and 50 of treatment. Bar represents mean±SEM of 5 animals. * $P<0.05$. (Glib. = Glibenclamide).

Blood glucose

Figure 2 shows details of blood glucose levels. At week 0, all animals in the diabetic, AIE-treated diabetic and glibenclamide-treated diabetic groups were hyperglycemic. By the end of week 1, 87.5% of the hyperglycemic rats treated with 500 mg/kg b w/d of AIE had become normoglycemic, and their blood glucose was therefore not significantly different from control ($P>0.05$). By the end of week 2, normoglycemia had been established in all animals in this group. In hyperglycemic rats treated with glibenclamide (600 µg/kg bw/d), 100% of the animals remained hyperglycemic at 7 d. By week 3, all the rats in this group had become normoglycemic (Fig. 2); and this status was maintained till euthanasia. In contrast, all rats in the untreated diabetic group were hyperglycemic from week 0 to 7 (Fig. 2).

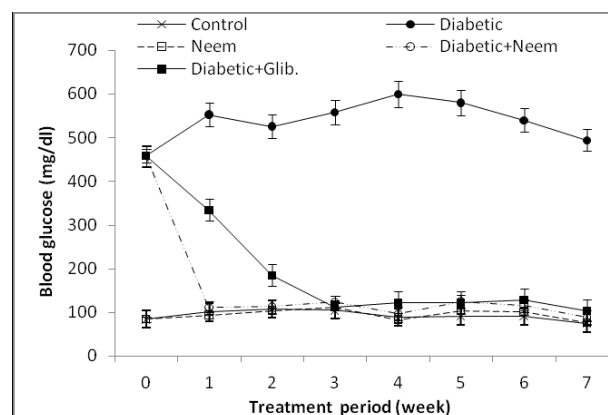


Figure 2: Weekly blood glucose of control and treated animals. Bar represents mean±SEM. * $P<0.05$. (Glib. = Glibenclamide).

Lipid hydroperoxide levels of the liver

Figure 3 A shows changes (%) in lipid hydroperoxide levels of the different treatment groups compared with control. In the untreated diabetic rats, hepatic lipid hydroperoxide levels had increased significantly by 21 d and 50 d ($p<0.05$) (Fig. 3). Treatment of diabetic rats with AIE exacerbated hepatic oxidative stress (by 7 d) compared with control ($P<0.05$). In the latter phase of treatment, hepatic lipid hydroperoxide levels in these diabetic rats remained significantly higher than control ($P<0.05$). (Fig. 3 A).

Furthermore, glibenclamide treatment did not prevent the rise in lipid peroxide levels in the diabetic rats. In these animals, lipid hydroperoxide

levels increased gradually from 31% above control at 7d, to 44% at 50d. Thus, at the latter phase of treatment, hepatic lipid hydroperoxide levels were significantly higher than control in all the groups except AIE-treated normoglycemic rats (Fig. 3).

Aqueous hydroperoxide levels of the liver

Hepatic aqueous hydroperoxide levels are shown in figure 3 B. In the control rats, aqueous hydroperoxide levels remained nearly constant throughout the study period. In the untreated diabetic group, aqueous hydroperoxide levels were similar to control at 7 d. At 50 d, significant increases in hepatic aqueous hydroperoxide levels of these animals had occurred (P<0.05).

Similarly, hepatic aqueous hydroperoxide levels in the AIE-treated diabetic rats did not differ significantly from control at 7 d. However, by 50 d, the levels had increased, and was nearly 60% above control (P<0.05). Moreover, glibenclamide treatment could not prevent the rise in aqueous hydroperoxide levels. In these animals, aqueous hydroperoxide levels rose from as low as 15% above control at 7 d, to nearly 50% at 50 d (Fig. 3 B).

levels of the liver. Bars represent Mean±SEM of 5 animals; *P<0.05.

Superoxide dismutase (SOD) activity of the liver

Hepatic SOD activities are shown in figure 4 A. By 7 d, liver SOD activity had decreased significantly by 42% in the diabetic rats compared with control (P<0.05). SOD activities in hyperglycemic rats treated with AIE were however not significantly different from control (P<0.05). At 50 d, SOD activities in all the treatment groups did not differ statistically from control, except for the AIE-treated normoglycemic rats, where a significant decrease had occurred (Fig. 4 A).

Hepatic reduced glutathione (GSH)

Hepatic GSH levels are shown in figure 4 B. Cellular GSH levels decreased progressively in the untreated diabetic rats from a 3-fold increase above control at 7 d (P<0.05), to a level 5% below control at 50 d. Similarly, GSH levels in AIE-treated diabetic rats decreased from a level 58% above control at 7 d (P>0.05) to a level 20% below control at 50 d (Fig. 4 B).

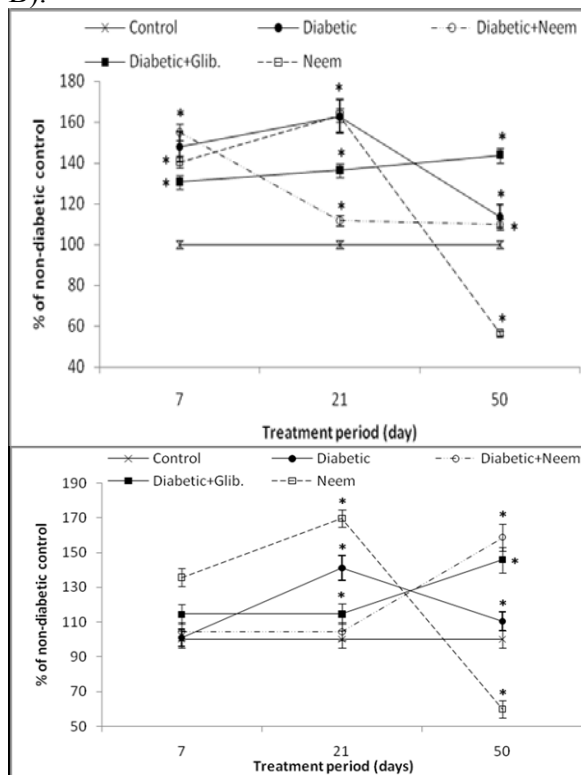


Figure 3: (A) Lipid hydroperoxide levels of the liver. Bars represent Mean±SEM of 5 animals. (B) Aqueous hydroperoxide

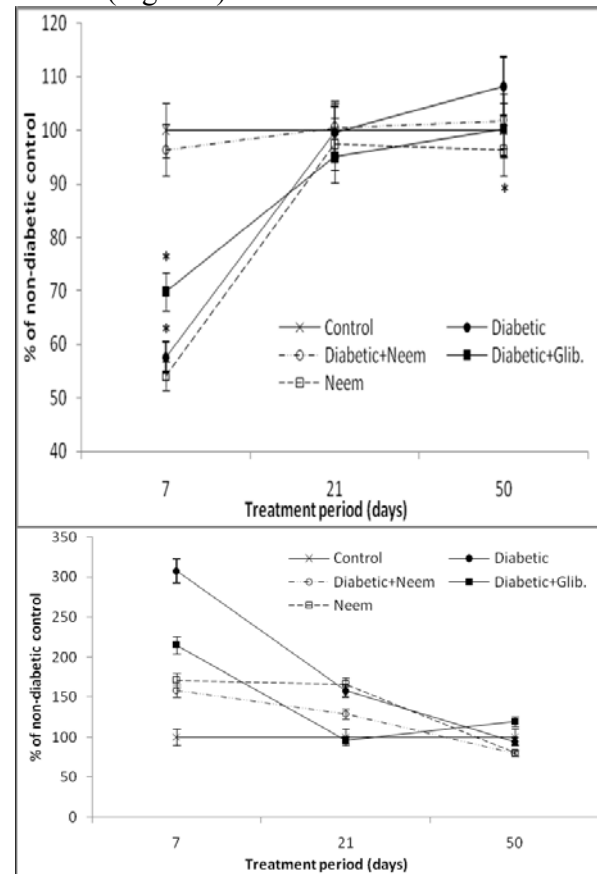
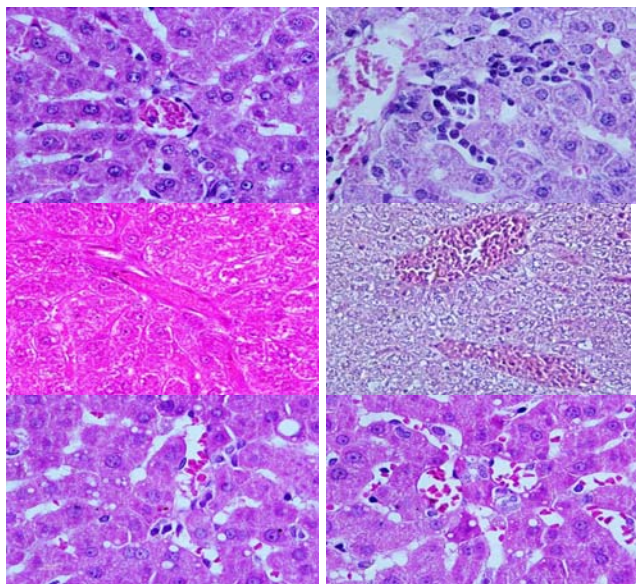


Figure 4: (A) SOD activity of the liver. Bars represent Mean \pm SEM of 5 animals. (B) GSH levels of the liver. Bars represent Mean \pm SEM of 5 animals; *P<0.05.

Hepatic histopathology

At 50 d of treatment, liver sections of untreated diabetic rats showed changes characteristic of hepatic glycogenosis (Fig. 5 C). Hepatocytes were swollen and strongly PAS-positive. This PAS positive-staining was observed in the cytoplasm of hepatocytes, and this finding suggests glycogen accumulation. Diastase digestion of the sections however resulted in loss of the PAS-positive staining (Fig. 5 D), thereby confirming the presence of glycogen in hepatocytes. Besides, the central veins of these untreated diabetic animals were compressed and congested, while sinusoids were either occluded or compressed and congested. Portal areas were rarely discernible, and so were Kupffer cells. In contrast, such histological findings were absent in AIE-treated diabetic rats. The major finding in these animals was mild lobular inflammation (Fig. 5 B). Apart from this, hepatic morphology in this group was comparable to control (Fig. 5 A). In the glibenclamide-treated diabetic group, hepatocytes were characterised by the presence of multivesicular intracytoplasmic vacuoles (Fig. 5 E and F).



28 Figure 5 (A-H): Hepatic histopathology at 50d. (A) Control group. Cords of hepatocytes appear distinct and sinusoids (S) remain patent; P, portal area. Hepatocytes are PAS-. (B) Neem-treated diabetic group; aggregates of leucocytes (arrows) are observable. (C&D) Diabetic group. C shows swollen and strongly PAS+ hepatocytes. Sinusoids are either occluded or compressed and congested. (D) The same tissue as in C, after diastase digestion, showing PAS- staining that confirms the presence of

glycogen in section C. (E&F) Glibenclamide-treated diabetic group. Multivesicular intracytoplasmic vacuoles are seen in hepatocytes. Periodic acid-Schiff, x60; Bar is 10 μ .

Discussion

In this study, ethanolic extract of the leaves of *Azadirachta indica* (AIE), administered at 500 mg/kg bw/d to hyperglycemic rats, produced normoglycemia in the larger percentage of the animals by the end of week 1 of treatment (Fig. 2). This hypoglycemic effect of AIE corroborates previous findings in dogs [23], rabbits [24], and rats [17]. Reported mechanisms of hypoglycemic effect of AIE include inhibition of adrenalin-induced glycogenolysis [25], and an insulinotropic effect [17]. However, in the present study, we observed a modulatory effect of AIE on body weight in diabetic and non-diabetic rats. By 50 d, gain in body weight was least in the AIE-treated rats (Fig. 1). This suggests a modulatory effect of AIE on leptin production and/or action, or some effect on body adiposity. The findings of Szayna *et al* [26] reported such a mechanism for the antidiabetic activity of exendin-4 from the salivary gland of Gila monster lizard. Further study is therefore required in this direction with respect to the hypoglycaemic activity of AIE.

A plethora of phytochemicals is derivable from neem leaf [27]. Among these, quercetin, myricetin, kaemferol, and their glycosides, have been implicated as contributing to the antidiabetic effect of neem leaf [17]. However, Sanders *et al* [28] and Dias *et al* [29] reported negative effect of quercetin on blood glucose in STZ-induced hyperglycemic rats treated with 10 mg/kg bw/d and 150 μ mol/kg bw/d of quercetin respectively. This suggests that phytochemicals other than flavonoids may be responsible for, or contribute to the hypoglycemic property of neem. Besides, it is possible that the negative findings of Sanders *et al* [28] and Dias *et al* [29] might arise from the use of an isolated flavonoid (quercetin). Synergic activity of all bioflavonoids in neem may account for its hypoglycemic effect.

In this work, we report hepatic glycogenosis (glycogenic hepatopathy) in diabetic rats induced to hyperglycemia with STZ (70 mg/kg bw). At euthanasia (50 d after attainment of hyperglycemia), hepatocytes were glycogen-laden,

as shown by their strong PAS-positive staining (Fig. 5 C). Hepatic glycogenosis had been reported as a common complication in type 1 diabetics whose blood glucose is poorly-controlled with insulin [10,11]. Up to 80% of type 1 diabetes patients could be affected [30]. In such patients, marked accumulation of glycogen occurs in hepatocytes when insulin treatment leads to increased uptake of glucose by hepatocytes, followed by rapid conversion of glucose to glycogen, and subsequently, trapping of the latter [31]. Apart from diabetes, hepatic glycogenosis could also occur in Mauriac's syndrome [32], glycogen storage disease [33], and following short-term high dose steroid therapy [34].

In this work, accumulation of glycogen in hepatocytes of diabetic rats might arise from upregulation of glycogen synthase. This enzyme had been shown to be upregulated in alloxan- and STZ-induced diabetes [35,36]. In the latter model, total glycogen synthase was reported to be significantly higher than control 40 d after induction of diabetes. Besides, the work of Ferrannini *et al* [37] showed that longstanding insulin deficiency might actually enhance glycogen synthase activity.

Clinical management of diabetic hepatic glycogenosis include administration of short- and intermediate-acting insulin [38], psychological support and dietary control [10], and pancreas transplantation [39]. Our finding in this study suggests that AIE could ameliorate hepatic glycogenosis when used as an antidiabetic therapy. The marked glycogen accumulation observed in the hepatocytes of untreated diabetic rats at 50 d was absent in those diabetic rats treated with AIE. This beneficial effect might arise from effective control of blood glucose by this botanical extract.

However, though hepatocytes of AIE-treated diabetic rats were devoid of glycogen, one characteristic histological finding in these rats (at 50 d) was the presence of aggregates of leucocytes in hepatic sinusoids (Fig. 5 B). This suggests mild lobular inflammation of the liver in these animals, and the development might be associated with hepatic oxidative stress observed throughout the study (Fig. 3A and 3B). The high levels of hepatic lipid peroxidation products in the untreated diabetic rats treated with AIE suggest increased formation

of reactive oxygen species (ROS) in hepatocytes, and perhaps in other hepatic cells, such as sinusoidal endothelial and Kupffer cells. The work of Vischer *et al* [40] showed that high levels of ROS in vascular endothelial cells could stimulate exocytosis of Weibel-Palade bodies, and thus, expression of selectin platelet (SELP) on the surface of these cells. High ROS levels in sinusoidal endothelial cells could also enhance the transcription of SELP and other adhesion molecules via the activation of the redox-sensitive NF κ B. Expression of SELP on hepatic sinusoidal endothelial cell surface could mediate the tethering and adherence of leucocytes to vessel surface, leading to the aggregation of these cells [41,42]. This phenomenon could explain the aggregation of leucocytes in hepatic sinusoids of AIE-treated diabetic rats. A similar finding was reported by Chosay *et al* [43] in endotoxin-induced liver injury. As shown in figures 3 A and 3 B, significant increases in hepatic lipid hydroperoxide and H₂O₂ levels occurred in the untreated diabetic and AIE-treated diabetic rats throughout the 50-day treatment period. In the untreated diabetic rats, hepatic oxidative stress could be a result of chronic hyperglycemia, as explained elsewhere [13,14,16]. However, the high hepatic lipid hydroperoxide and aqueous hydroperoxide levels obtained in the AIE-treated diabetic and AIE-treated non-diabetic rats, largely in the absence of hyperglycemia, may not be readily explainable. A probable source of high ROS in these animals could be hepatic metabolism of AIE by the cytochrome P450 enzymes. The P450 families play active roles in the metabolism of drugs and xenobiotics [44]. Their induction and activity have been associated with enhanced ROS production [45]; and this suggests that certain phytochemicals in neem could induce some of these enzymes. Such induction may result in increased generation of ROS. Previous findings had reported such an association between hepatic metabolism of drugs/xenobiotics by cytochrome P450, and ROS generation. For example, the toxic effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin was reported to arise from ROS generation via induction of the P450 enzymes [46]. Ethanol could also induce certain families of P450 to produce free radicals [47]. The work of Dasgupta *et al* [48] showed that 80% ethanolic leaf extract of neem, administered to mice

at a dose of 200 mg and 500 mg/kg bw/d, could induce the phase II enzymes, which are involved in the metabolism of xenobiotics. Thus, generation of reactive oxygen species, which may arise from such cellular activities, could account for the high hepatic lipid peroxidation obtained in our model.

However, in non-diabetic rats treated with AIE, increases in hepatic lipid peroxidation products were limited to the early periods of exposure (Fig. 3A and 3B). Levels of these products were significantly lower than control at 50 d, in contrast to AIE-treated diabetic rats, where ROS remained significantly high throughout exposure period. This implies that AIE-associated ROS generation was limited to the early phase of AIE treatment. Thus, the significant reduction in hepatic lipid hydroperoxide levels in AIE-treated non-diabetic rats (at 50 d) suggests that certain mechanisms were induced in the liver to suppress ROS generation (or scavenge these molecules) with continued exposure to AIE; and that such mechanisms were either inhibited or overwhelmed in the AIE-treated diabetic rats. One mechanism that could be involved in the suppression of ROS generation (or accumulation) in AIE-treated non-diabetic rats was upregulation of the antioxidant system. However, our findings in these animals showed that SOD and GSH levels were significantly lower than control (at 50 d) (Fig. 4 A & B). This suggests that the reduced ROS levels in these animals might arise from reduced generation of ROS, rather than enhanced scavenging activity by antioxidants. Down regulation of the cytochrome P450 enzymes in the latter phase of neem exposure might thus be responsible, but this is subject to further studies.

Furthermore, the significantly high hepatic lipid hydroperoxide levels in AIE-treated diabetic rats (at 50 d) could arise from ROS generation by leucocytes seen in higher number in the liver at this time. Leucocytes generate free radicals as part of their defensive mechanisms [49]. But they have been reported to produce significantly high amounts of ROS in experimental hepatitis [50] and pancreatitis [51]. In the present study, AIE could not prevent the generation of such high levels of hepatic ROS in these cells.

Most previous studies of the antioxidant activity of neem reported positive findings based on the high flavonoids content of this plant; and

phytochemicals such as quercetin and rutin have been mostly implicated [52]. However, reports from *in vivo* studies of the antioxidant effect of quercetin in STZ diabetic rats had been conflicting. Suppression of hepatic oxidative stress was reported by Dias *et al* [29], while Sanders *et al* [28] did not observe this. In the present study, the persistently high hepatic lipid hydroperoxide levels and the influx of leucocytes into the liver of diabetic rats treated with AIE suggest that the herb may not possess antioxidant activity in the liver; and that long-term treatment with AIE could be associated with hepatic injury.

Furthermore, hepatocellular ballooning, characterised by the presence of multivesicular intracytoplasmic vacuoles, were seen at 50 d in all zones of hepatic acini in diabetic rats treated with glibenclamide. However, such vacuoles were not observed in the other treatment groups. In diabetic patients, gross liver enlargement and ballooning of hepatocytes are generally interpreted as steatosis or steatohepatitis, arising from impaired hepatic lipid metabolism. The development of a steatotic liver in diabetes is associated with decreased plasma insulin levels, and thus, increased lipolysis [53]. Such metabolic perturbations could imply increased delivery of exogenous fatty acids to hepatocytes; increased endogenous hepatic production of fatty acids; decreased hepatic disposal of fatty acids; or impaired export of VLDL from the liver. These can thus lead to hepatic accumulation of lipids, especially in type 2 diabetic patients [54]. In the present study, Sudan black staining of the ballooned hepatocytes did not confirm the presence of lipid in the vacuoles; and this suggests absence of steatosis. This could be expected as the administered drug (glibenclamide) is an insulin secretagogue. Thus, improved levels of insulin in these animals not only restored normoglycemia but also suppressed lipolysis.

Moreover, vacuolation and ballooning of hepatocytes had also been associated with anoxia that may occur at euthanasia [55]. Trowell [56] observed hepatic vacuolation from 5-15 minutes after death of animals from anoxia or asphyxia; while Sykes *et al* [57] reported marked hepatocyte ballooning as early as 30 seconds post-euthanasia with nitrogen gas. Besides, the work of Li *et al* [58] showed that hepatocyte vacuolation occurred in rats

immediately after cessation of respiration, and that vacuolation progressed with increasing exposure to anoxic conditions. In our work, animals were sacrificed under pentobarbital anaesthesia in a well-ventilated environment. Thus, ballooning of hepatocytes in this instance could not be a result of anoxia. Moreover, hepatocytes vacuoles were absent in other treatment groups euthanized in the same manner. In the study of Li *et al* [58], histochemical staining of ballooned hepatocytes for albumin, adipophilin, and β -catenin was only positive for albumin. This showed that the vacuoles contained plasma, not lipid. Furthermore, congestion of hepatic sinusoids was reported in the affected animals. In the present study, though staining of the vacuoles for albumin (or other markers of the plasma) was not done, hepatic sinusoids appeared dilated (Fig. 5 E & F). This suggests sinusoidal congestion, a development that might account for the influx of plasma into, and thus, vacuolation of hepatocytes [58]. Thus, in our study, hepatocellular vacuoles observed in liver sections of glibenclamide-treated diabetic rats at 50 d might contain plasma. The reports of Trowell [56] also support such a proposition. However, the mechanisms that could account for sinusoidal congestion (and thus raised intrasinusoidal pressure and vacuolation of hepatocytes) in the present work are unclear.

In conclusion, our findings in this work showed the beneficial effect of AIE in the amelioration of hyperglycaemia and hepatic glycogenosis in STZ-induced diabetic Wistar rats. However, the finding of mild lobular inflammation in the liver of AIE-treated diabetic rats, coupled with raised lipid peroxidation products in this organ, suggest the association of a degree of liver injury with chronic AIE treatment.

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