

Effects of combined leaf extract of *Vernonia amygdalina* and *Azadirachta indica* on hepatic morphology and hepatotoxicity markers in streptozotocin-induced diabetic rats

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Objectives: In this work, we studied liver morphology, markers of hepatic oxidative stress and some liver enzymes in diabetic rats treated with the combined leaf extract (CLE) of *Vernonia amygdalina* (bitter leaf) and *Azadirachta indica* (neem).

Methods: Diabetes was induced in fasted male Wistar rats with intraperitoneal injection of streptozotocin (STZ). Oral CLE (500 mg/kg body weight) and metformin (150 mg/kg body weight) were administered to different groups of diabetic rats for eight weeks. Blood glucose and change in body weight were estimated weekly. All animals were sacrificed under anaesthesia after eight weeks. Hepatic sections were stained with periodic acid-Schiff. Liver samples were homogenized and assayed for contents of malondialdehyde (MDA) and glutathione peroxidase (GPx), while the plasma was assayed for contents of alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Results: Metformin and CLE treatment produced normoglycaemia in the diabetic rats in the course of the treatment period. Significant increases in body weight were observed in the treatment groups compared with the diabetic control rats ($P < 0.05$). In the control and treatment groups, light microscopic study showed intact hepatic histology. Plasma ALT and AST were not significantly different from the control values in the CLE-treated rats. In addition, from week four onwards, blood glucose concentrations in the CLE-treated rats were not different from the normal control ($P > 0.05$). Besides, hepatic MDA ($P < 0.05$) significantly decreased in the CLE-treated rats compared with the normal control.

Conclusion: These findings suggest that CLE ameliorates hyperglycemia and hepatic oxidative stress when administered to diabetic rats as a chronic regimen, and there was no morphologic or biochemical evidence of liver damage at the dose tested.

Keywords: diabetes mellitus, experimental; *Vernonia*; *Azadirachta*; plant extracts; hepatotoxicity; oxidative stress; rats

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In diabetes mellitus, insulin deficiency and insulin resistance result in chronic hyperglycemia, with the risk of microvascular and macrovascular complications that are associated with long-term poorly controlled blood glucose. These diabetes-induced complications include atherosclerosis, cardiopathy, nephropathy and retinopathy.

The liver plays crucial roles in glucose and drug metabolism. Its cells (hepatocytes, Kupffer cells, etc.) are constantly exposed to numerous chemical insults via the gastrointestinal route, with the risk of hepatic toxicity. Because many complementary and alternative medicines (CAMs) are taken orally, their thorough laboratory and clinical screening is vital to patients' safety. Savvidou *et al*^[1] reported hepatic toxicity in patients taking an hypoglycaemic preparation from *Teucrium polium*. The toxicity led to the discontinuation of the clinical use of this herbal preparation. Moreover, in animal studies, Freire *et al*^[2] reported liver toxicity in mice treated with an antiparasitic preparation from the extract of *Ruta graveolens*.

Similarly, antidiabetic drugs such as phenformin and troglitazone were withdrawn from the American markets in 1977 and 2000, respectively. This was due to their toxic effects on the liver^[3]. In 2010, rosiglitazone (Avandia) was also withdrawn from the American markets because of reported myocardial infarction in treated patients. These findings underscore the benefits of standardised screening of existing and potential conventional and herbal antidiabetic medications prior to clinical use.

Presently, data on antidiabetic medicinal plants are accumulating, and CAMs are receiving unprecedented attention as veritable means of disease management. Previous animal studies in our laboratory reported the efficacy of *Vernonia amygdalina* (bitter leaf)^[4] and *Azadirachta indica* (neem)^[5] as antihyperglycaemic therapies. Similarly, Ebong *et al*^[6] reported the hypoglycaemic activity of the combined leaf extract (CLE) of neem and bitter leaf in alloxan-induced diabetic rats. Thus, owing to the prospect of producing potent antidiabetic

remedy from the combined leaf extract of neem and bitter leaf, additional work on the biosafety of the CLE is needed.

In the present study, we reported the effects of the CLE of neem and bitter leaf on hepatic histology. We also studied the effects of the extract on some liver enzymes and oxidative stress markers in a streptozotocin (STZ) model of diabetic rats.

1 Materials and methods

1.1 Chemicals All chemicals used were of analytical grades. STZ, ethanol, citric acid, sodium citrate, disodium hydrogen phosphate and monosodium phosphate were procured from Sigma (MO, USA). Xylene was procured from Carlo Erba (Milan, Italy), and paraffin wax was from Bio-Optica (Milan, Italy). Periodic acid and Schiff reagent were from Sigma (MO, USA). Reagents for Bouin's fluid (picric acid, formaldehyde and glacial acetic acid) were from Carlo Erba (Milan, Italy). All other chemicals used were either supplied with the assay kits or procured locally, and they were of analytical grades.

1.2 Plant materials Mature fresh leaves of neem (*Azadirachta indica*) were collected in the premises of the University of Ilorin (Mini Campus), Nigeria. Mature fresh leaves of bitter leaf (*Vernonia amygdalina*) were obtained from a botanical garden in Ilorin, Nigeria. Both plants were collected in October 2009, and were authenticated at the herbarium of the University of Ilorin, where they were compared to the herbarium specimens (voucher numbers: neem (542); bitter leaf (10)).

The leaves were separately shade-dried and pulverized. Ethanolic extraction was done for each dry leaf material using 70% ethanol (in a percolator) at room temperature (23 °C). The extracts were evaporated to dryness in a water bath at 45 °C. A compound herbal extract was thereafter prepared at a weight ratio of 5 : 4 (neem : bitter leaf). This ratio of the combined neem and bitter leaf extract was selected because it is within the range reported to produce normo-



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glycaemia in experimental diabetic Wistar rats^[6].

1.3 Experimental animals Male Wistar rats (eight weeks old) were randomly assigned to one of the following treatment groups: normal control group, STZ diabetic control group, STZ plus CLE group, STZ plus metformin group, and CLE group. Each group consisted of eight animals. Fasted rats were induced to hyperglycemia with intraperitoneal injection of STZ (70 mg/kg body weight), in sodium citrate buffer (0.1 mol/L, pH 4.5). Animals with fasting blood glucose 250 mg/dL or more (96 h after STZ induction) were included in the study. CLE was administered orally at 500 mg/kg body weight per day^[6], while oral metformin was administered at 150 mg/kg body weight per day^[7]. All rats were treated for eight weeks. Animals were exposed to a 12-hour light/dark photocycle. They were maintained on pelletized rat feed (Bendel Feed, Nigeria). Food and water were served *ad libitum*.

1.4 Estimation of blood glucose and body weight

Starting at day 0, blood glucose was estimated weekly by the glucose oxidase method using the One-Touch glucometer (Lifescan, CA, USA). Besides, in each group, change in body weight of the rats was estimated as follows: change in body weight = (final body weight — initial body weight) × 100% / (initial body weight).

1.5 Termination of treatment After the last dose of CLE or metformin, all animals were fasted for 12 h and then placed under anaesthesia (diethyl ether, Sigma, USA). Laparotomy was performed and blood was collected from the inferior vena cava into heparinised tubes. Blood was centrifuged at 8 000 × g.

Liver samples were fixed in chilled Bouin's fluid, embedded in paraffin wax, and sectioned at 4 μm using a rotary microtome (Cambridge Instruments, Germany). Liver sections were then stained by the periodic acid-Schiff (PAS) method, as described by Bancroft and Stevens^[8]. Moreover, 10% liver homogenate was prepared from fresh liver tissue samples using phosphate buffer (0.1 mol/L, pH 7.4) as the homogenizing buffer.

1.6 Estimation of plasma alanine aminotransferase and aspartate aminotransferase Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated by colourimetry. ALT and AST levels were estimated by the method of Reitman and Franke^[9], using kits from Fortress Diagnostics (Antrim, UK). Assessment of enzyme levels was done as indicated in the kit manuals.

1.7 Estimation of malondialdehyde and glutathione peroxidase Hepatic malondialdehyde (MDA) was estimated by the thiobarbituric acid test^[10]. Hepatic glutathione peroxidase (GPx) was estimated by the method of Paglia and Valentine^[11] using a kit from Randox Laboratories (Antrim, UK). Total liver protein was determined by the biuret method^[12] using a kit from Randox Laboratories

(Antrim, UK).

1.8 Statistical analysis Data were presented as mean ± standard error of mean and analysed by one-way analysis of variance, followed by the Waller-Duncan post-hoc test. Statistical significance was accepted at $P < 0.05$.

2 Results

2.1 Blood glucose and body weights Figure 1 shows the mean blood glucose concentrations of each group of rats. Blood glucose in the untreated diabetic group remained significantly higher than the normal control group ($P < 0.05$). Meanwhile, metformin and CLE treatment produced normoglycaemia in the diabetic rats in the course of the treatment period. The difference in body weight of the rats was estimated and expressed as the percentage change in body weight, as shown in Figure 2. Significant increases were observed in the treatment groups compared with the diabetic control rats.

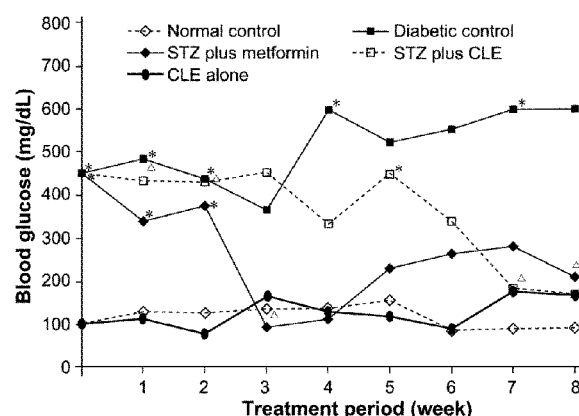


Figure 1 Blood glucose concentrations of control and treatment groups over a period of eight weeks

Data are presented as mean ± SEM, $n = 8$, * $P < 0.05$, vs normal control group; $\triangle P < 0.05$, vs diabetic control group. SEM: standard error of mean; STZ: streptozotocin; CLE: combined leaf extract of *Vernonia amygdalina* and *Azadirachta indica*.

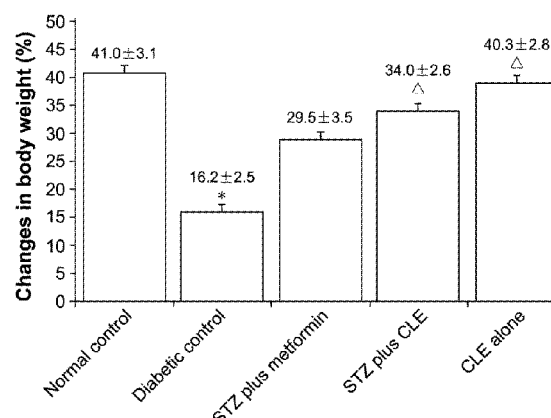


Figure 2 Changes in body weights of the rats after eight weeks of treatment

Data are presented as mean ± SEM, $n = 8$, * $P < 0.05$, vs normal control group; $\triangle P < 0.05$, vs diabetic control group. SEM: standard error of mean; STZ: streptozotocin; CLE: combined leaf extract of *Vernonia amygdalina* and *Azadirachta indica*.

2.2 Plasma ALT and AST and hepatic MDA and GPx levels As shown in Table 1, after 8 weeks, metformin-treated diabetic rats had the higher levels of plasma ALT and AST than the normal control ($P<0.05$); hepatic MDA was not significantly different from the normal control values in all the treatment groups except the CLE-treated rats, where significant decreases occurred ($P<0.05$); hepatic GPx activity was higher in the metformin-treated rats than that in the normal control ($P<0.05$).

2.3 Hepatic histology Figure 3 shows the histologic findings of PAS-stained liver sections. Hepatic sections of the control and treatment groups were PAS-negative. There were no striking differences in the hepatic morphology of these groups after 8 weeks of treatment. Liver sections did not show any necrotic changes, and inflammatory cells were not observable. Hepatocytes retained their cord-like organization, and adjoining cords of hepatocytes were separated by intact sinusoids.

Table 1 Effects of treatment with CLE and metformin on liver enzymes and oxidative stress markers in STZ diabetic rats (mean $\pm$ SEM)

Group	n	Plasma ALT (IU/L)	Plasma AST (IU/L)	Hepatic MDA (μ mol/mg protein)	Hepatic GPx (U/mg protein)
Normal control	8	36.3 $\pm$ 5.1	68.1 $\pm$ 6.2	1 650 $\pm$ 150	1.20 $\pm$ 0.15
Diabetic control	8	37.0 $\pm$ 3.2	88.3 $\pm$ 7.1*	1 410 $\pm$ 132	1.22 $\pm$ 0.30
STZ plus metformin	8	45.2 $\pm$ 4.8*	89.8 $\pm$ 6.5*	1 305 $\pm$ 98	1.60 $\pm$ 0.41*
STZ plus CLE	8	28.6 $\pm$ 3.1	30.1 $\pm$ 2.8 Δ	903 $\pm$ 69* Δ	0.62 $\pm$ 0.05
CLE control	8	25.1 $\pm$ 5.5	30.1 $\pm$ 3.1 Δ	1 906 $\pm$ 161 Δ	1.50 $\pm$ 0.35*

* $P<0.05$, vs normal control group; $\Delta P<0.05$, vs diabetic control group. SEM: standard error of mean; STZ: streptozotocin; CLE: combined leaf extract of *Vernonia amygdalina* and *Azadirachta indica*; ALT: alanine aminotransferase; AST: aspartate aminotransferase; MDA: malondialdehyde; GPx: glutathione peroxidase.

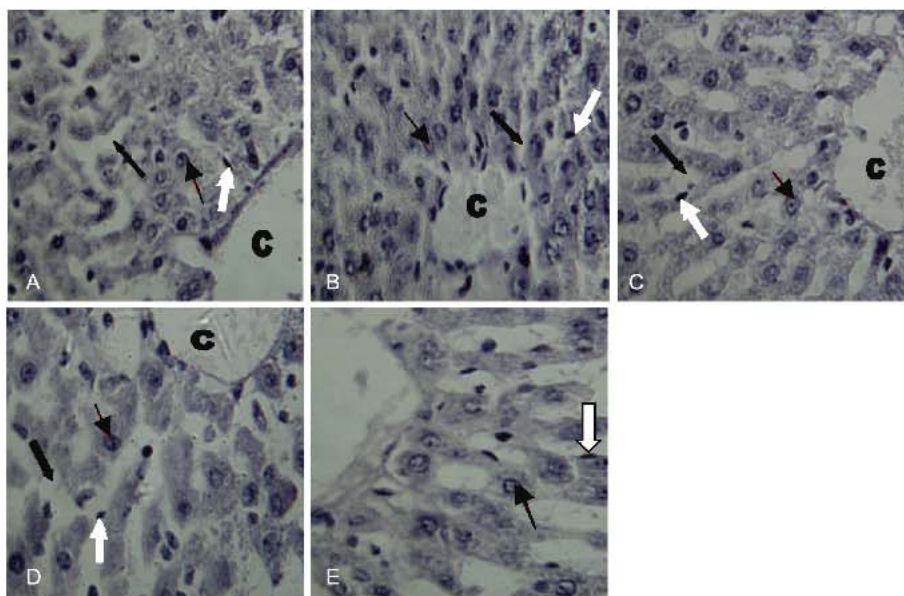


Figure 3 Hepatic histology after eight weeks of treatment (Light microscopy, $\times 400$)

A: Normal control; B: Diabetic control; C: STZ plus CLE; D: STZ plus metformin; E: CLE alone. In each of the groups, hepatic sections are periodic acid-Schiff-negative; besides, no notable differences exist in hepatic morphology of the control and treated rats. Thick white arrows point to the Kupffer cells; thick black arrows point to the sinusoid; thin arrows point to the hepatocyte nucleus; C means the central vein. STZ: streptozotocin; CLE: combined leaf extract of *Vernonia amygdalina* and *Azadirachta indica*.

3 Discussion

In the present work, we studied the effects of the CLE of neem and bitter leaf on blood glucose, hepatic histology, liver enzymes and oxidative stress markers in STZ-induced diabetic Wistar rats. CLE ameliorated STZ-induced hyperglycemia in the treated diabetic rats beginning at week 4 of treatment, when blood glucose levels of the CLE-treated rats were not significantly different from the control values. The work of Ebong *et al*^[6] showed that oral treatment of alloxan-induced

hyperglycemic rats with CLE of neem and bitter leaf (500 mg/kg body weight per day) resulted in significant reduction in blood glucose at the end of the 24-day treatment period. Meanwhile, in the present study, hyperglycemia persisted in the CLE-treated rats until the 4th week (28 d), when normoglycaemia was established. This is also in spite of the higher dose of CLE used in the present study (500 mg/kg body weight). Earlier studies in our laboratory showed that oral ethanolic leaf extract of neem produced normoglycaemia as early as week 1 of treatment when administered

to STZ-induced diabetic rats at 500 mg/kg body weight^[5]. Similarly, the work of Akinola *et al*^[4] showed that oral treatment of STZ-induced hyperglycaemic rats with ethanolic leaf extract of bitter leaf (400 mg/kg body weight) ameliorated hyperglycemia after 21 d of treatment. Moreover, Osinubi *et al*^[13] reported amelioration of hyperglycemia in an acute study of alloxan-induced hyperglycemia in Sprague-Dawley rats treated orally with aqueous leaf extract of bitter leaf (500 mg/kg body weight). Normoglycaemia was established in this treated group 12 h after bitter leaf dosing. These studies showed that the leaf extracts of neem and bitter leaf possess antidiabetic property when used separately. However, their combined regimen as used in the present study produced normoglycaemia in the treated rats later than the separate leaf extracts. This suggests that CLE does not have a remarkable advantage on hyperglycemia in STZ-induced diabetic rats, when compared with the individual extract of neem and bitter leaf respectively.

Moreover, in the control and treatment groups, PAS-stained paraffin sections of the liver showed normal morphology. This finding suggests that CLE and metformin did not produce any morphologic damage to the liver during the 8-week treatment period. Although many herbal medications are safe and are not hepatotoxic, a few animal and human studies reported hepatic toxicity associated with the ingestion of certain phytomedicines. A study by Fu *et al*^[14] in rats showed that *Piper methysticum* Forst (kava) produced severe vascular and endothelial damage in the liver of the exposed rats. In human studies, isolated cases of phytotherapy-induced liver injury have been reported. Hepatotoxicity of hydroxycut and herbalife, which are weight-loss herbal supplements, was reported by Chen *et al*^[15]. Similarly, elevated liver enzymes and hepatobiliary injury were reported in a patient on a weight-loss medication containing materials from *Lycopodium serratum* and *Chelidonium majus*^[16]. These animal and human reports validate the need for thorough screening of herbal medications, to forestall hepatic and other organ injuries. In addition, several conventional drugs are also known hepatotoxins. A recent review by Chau^[17] explicitly summarized these conventional hepatotoxic drugs.

In the control and CLE-treated diabetic rats, serological analysis showed that plasma AST and ALT were not significantly different. In contrast, plasma levels of these liver enzymes (ALT and AST) were significantly higher in the metformin-treated rats compared with the control. These suggest deleterious effect of oral metformin in these rats at a dose of 150 mg/kg body weight. This relatively high dose of metformin is within the range reported to be the effective dose for reversing hyperglycemia in diabetic rats^[18]. Meanwhile, this dose (150 mg/kg) is much higher

than the human metformin dose range for type 2 diabetes and does not suggest toxicity of this drug at the human dose range.

However, a few studies reported the adverse effects of oral metformin in diabetic patients. Olivera-González *et al*^[19] reported severe liver injury in a diabetic patient on oral metformin therapy. Besides, one of the most commonly reported side effects of oral metformin is lactic acidosis^[20]. This suggests that oral metformin does have potential adverse effects on the metabolic functions of the liver. Metformin reportedly precipitates lactic acidosis possibly by impairing gluconeogenesis^[21]. Moreover, biguanide therapy decreases the activity of pyruvate dehydrogenase and the transport of mitochondrial reducing agents; and these properties enhance anaerobic metabolism^[22]. Thus, increased conversion of pyruvate to lactate is associated with biguanide use, resulting in increased lactic acid production, and lactic acidosis. Results from the present and previous studies therefore suggest that metformin doses should be as low as possible.

We also studied certain markers of oxidative stress in the control and treated rats. Oxidative stress is a confounding factor in diabetes, and it may account for the pathogenesis of some diabetic complications^[23]. In the CLE-treated diabetic group, hepatic MDA levels were significantly lower than those of the normal control group ($P < 0.05$), but there was no significant difference in the hepatic levels of GPx between the CLE group and the normal control group. These findings suggest the absence of hepatic oxidative stress in the CLE-treated rats. Earlier work in our laboratory showed that oral treatment of diabetic rats with the leaf extract of neem alone was associated with exacerbation of hepatic oxidative stress^[5]. Thus, with respect to its effects on hepatic oxidative stress, CLE had better antioxidant protection on the liver compared with the use of neem leaf extract alone.

Furthermore, body weights of the treatment groups were not significantly different from the control group at the end of the treatment period. In the untreated diabetic rats, significant reduction ($P < 0.05$) in body weight occurred compared with the normal control rats. The lack of significant difference between the body weights of rats in the CLE-treated group and the normal control group suggests improved disease (diabetes) management in the treated animals.

The present study shows that CLE ameliorates hyperglycemia and hepatic oxidative stress when administered to diabetic rats as a chronic regimen, and there is no morphologic or biochemical evidence of liver damage at the dose tested.

4 Competing interests

The authors declare that they have no competing interests.

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扁桃斑鸠菊及非洲印楝叶的提取物对链脲佐菌素致糖尿病大鼠肝脏形态学及肝毒性标志物的影响

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目的: 研究扁桃斑鸠菊及非洲印楝叶的提取物对链脲佐菌素致糖尿病大鼠肝脏形态学、肝脏氧化性应激标志物及部分肝脏酶类的影响。

方法: 大鼠腹腔注射链脲佐菌素致糖尿病。不同治疗组大鼠分别口服扁桃斑鸠菊及非洲印楝叶的提取物(500 mg/kg)或二甲双胍(150 mg/kg), 疗程 8 周, 每周测量大鼠的血糖水平及体质量变化。8 周后麻醉处死大鼠。取肝组织制成切片, 希夫染色法染色, 并测量肝匀浆中丙二醛和谷胱甘肽过氧化物酶的含量; 下腔静脉采血, 分离血浆, 检测血浆中丙氨酸氨基转移酶和天冬氨酸氨基转移酶的活性。

结果: 二甲双胍及植物提取物均能显著改善糖尿病大鼠的血糖水平, 且与糖尿病模型组相比, 治疗组的体质量明显增加($P < 0.05$)。光学显微镜下, 各组大鼠的肝脏形态学无明显差别。植物提取物治疗组大鼠的血浆丙氨酸氨基转移酶及天冬氨酸氨基转移酶的活性与正常对照组相比无明显变化($P > 0.05$), 而丙二醛含量明显下降($P < 0.05$)。

结论: 扁桃斑鸠菊及非洲印楝叶的提取物对糖尿病大鼠有明显的降糖作用, 而肝脏形态学及肝毒性标志物没有明显变化。

关键词: 糖尿病, 实验性; 斑鸠菊属; 印楝属; 植物提取物; 肝脏毒性; 氧化性应激; 大鼠