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Subchronic oral toxicity evaluation of ethanolic whole plant extract of *Eleucine indica* on haematological and biochemical indices in Wistar albino rats

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ABSTRACT

Objective: To evaluate the effect of ingestion of ethanolic whole plant extract of *Eleucine indica* on haematological and biochemical parameters of Wistar albino rats.
Methods: Subchronic toxicity study was carried out by oral administration of different doses (200, 400 and 600 mg/kg body weight) of the extract on alternate-day basis to different groups of rats for 28 days. The animals were subsequently sacrificed and blood samples were collected by cardiac puncture for haematological and biochemical analyses.
Results: Haematological indices were preserved and the extract showed significant ($P < 0.01$–0.001) haemostatic potentials. There was significant reduction ($P < 0.05$–0.001) in total bilirubin, aspartate aminotransferase ($P < 0.001$), alanine transaminase ($P < 0.05$), alkaline phosphatase ($P < 0.001$) and blood glucose ($P < 0.001$) compared to control. The level of total protein increased significantly ($P < 0.05$–0.001). Kidney functions were, however, intact.
Conclusions: The results obtained indicated that ingestion of *Eleucine indica* whole plant extract for a long period of time reduces both bleeding and clotting times, reduces blood sugar and shows no apparent toxic effect on liver and kidneys. The results of this study may be useful as a basis for clinical trials in humans.

1. Introduction

The plant *Eleucine indica* (*E. indica*) is generally considered as an adventitious species native to the tropics and subtropical regions[1]. It has a broad tolerance to a wide range of environmental conditions, but its vegetative growth is significantly reduced during dry season[2]. It is an annual growing to 0.45 m. It is in flower from July to August and the seeds ripen from August to October. The flowers are monoecious and are pollinated by wind. The plant prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well drained soil. The plant prefers acid, neutral and basic (alkaline) soil. It cannot grow in the shade. It requires much soil mostly cultivated beds for habitats and possible locations[3]. It is commonly called crowsfoot grass, goose grass, Indian goose grass, wiregrass, and silver crabgrass. The Ibibios and the Ekid people of Akwa Ibom State of Nigeria call it "Nkimenang", the Hausas "Ciyawar Tuji", the Nupe "Chinchere" and the Yorubas "Ese-Kannakanna" or "Gbegi".

This plant is used for the treatment of malaria among the Ibibios of Southern Nigeria. The whole plant, especially the root, is depurative, diuretic, febrifuge and laxative, and hence is used for the treatment of influenza, hypertension, oliguria and urinary retention[4]. It is also used for kidney problems in Trinidad and Tobago[5]. The seed is sometimes used as a famine food as well as in the treatment of liver complaints[6].

Two main flavonoids have been isolated: schaftoside (6-C-β-glucopyranosyl-8-C-α-arabinopyranosylapigenin) and vitexin (8-C-β-glucopyranosylapigenin) based on ^{1}H and ^{13}C NMR spectra and found to have strong anti-inflammatory activities[7]. *E. indica* has been reported to have phytochemical content of sterol glucoside forms[8] and C-glycosyl flavone possessing anti-inflammatory activities[9]. *E. indica* leaves are reported to have good bactericidal activity towards methicillin-resistant *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis*, antioxidant and non-cytotoxic properties[6]. Phytochemical screening has indicated the presence of alkaloids, tannins, flavonoids, cardiac glycosides, terpenes and simple sugar. The LD_{50} was determined to be (2 090.00 ± 0.01) mg/kg. The

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extract has antiplasmodial[10], analgesic, anti-inflammatory[11], antipyretic and antioxidant activities[12]. Howevr, There is, up till date, no scientific report in literature about its subchronic toxicological profile. This study is aimed at evaluating the subchronic effects of this plant on haematological and biochemical parameters and thereby laying the framework for further beneficial exploitation of its medicinal potentials.

2. Materials and methods

2.1. Collection and identification of plant material

The plant material (*E. indica*) was collected in April, 2009 from Uyo, Akwa Ibom State, Nigeria. The plant was identified and authenticated by Dr. (Mrs.) Margaret Bassey (a plant taxonomist) in the Department of Botany and Ecological Studies, University of Uyo, where a voucher specimen (UUH 1409) was deposited.

2.2. Preparation of extracts

The plant material was air-dried and then oven-dried at reduced temperature (28 ± 2) ˚C. It was thereafter ground into powder using a mixer grinder and 1.5 kg of powder-dried plant was cold-macerated in 12 L of 70% ethanol at room temperature for 72 h, and filtered. The filtrate was dried *in vacuo* using the rotary evaporator to obtain 100 g of dried extract. The extract was stored in a refrigerator at -4 ˚C until required for use.

2.3. Animal stock

Adult albino rats were obtained from the Animal House of the University of Jos, Jos, Plateau State and were maintained in the University of Uyo Animal House and fed with growers pellet feed (Bendel Feeds and Flour Mills Ltd., Edo State, Nigeria) with water given *ad libitum*. Animal Ethics Committee, Faculty of Pharmacy, University of Uyo, granted approval for animal use.

2.4. Evaluation of subchronic toxicological effect of extract in rats

Adult albino rats (140–200 g) of both sexes were weighed and randomized into five groups of six animals per group. Group 1 received 10 mL/kg of distilled water orally, and served as control. Groups 2–4 received the extract at 200–600 mg/kg orally respectively. Group 5 animals received 100 mg/kg of silymarin orally. Drugs were administered on alternate days for 28 days at 09.00 a.m. On Day 29, after an overnight fast, the animals were anaesthetized with light chloroform and blood samples were collected by cardiac puncture for haematological and biochemical analyses. Blood samples were collected into tubes with or without ethylene diamine tetraacetic acid respectively. Haemoglobin (Hb) (g/dL), haematocrit (HCT) (%), red blood cell (RBC) count (µL), white blood cell (WBC) count (µL), mean corpuscular haemoglobin concentration (MCHC) (g/dL), mean corpuscular haemoglobin (MCH) (ph), mean corpuscular volume (MCV) (fL) and platelet count (µL) were determined using automatic counter sysmex (K 21, Tokyo, Japan).

The biochemical parameters were determined in serum obtained after centrifugation of total blood without anticoagulant, at 2 500 r/min for 15 min. Standardized diagnostic kits and spectrophotometer were used for spectrophotometric determination of the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Others were creatinine, urea, total protein, albumin, Na^+, K^+, Cl^- and HCO_3^-, total and direct (conjugated) bilirubins, total cholesterol, high density lipoprotein (HDL)-cholesterol, and triglyceride levels. The blood glucose was measured with a glucometer[13,14].

2.5. Haemostatic effects of extract

2.5.1. Effect of extract on bleeding time

The tail bases of rats were sterilized by cleaning with 70% ethanol. The sterilized areas were punctured with disposable lancet and simultaneously timed using stop clock. The blood from punctured site was gently blotted out every 15 s using filter paper until the bleeding stopped. The interval between the time the skin was punctured and the time the skin stopped bleeding was considered as the bleeding time[15,16].

2.5.2. Determination of effect of extract on clotting time

As previously described, the clotting time of the rats was determined by cutting the tip of the tail of the animal each and about 2–3 drops of blood was placed on a glass slide. The tip of an office pin was used to pick the blood at about 15 s interval until a clot was observed. The time taken from the dropping of the blood to the formation of a streak blood, indicating clotting formation, was recorded as the clothing time[15,16].

2.6. Statistical analysis

Results were expressed as multiple comparisons of mean ± SEM. Significance was determined using One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison post test. A probability level of less than 5% was considered significant.

3. Results

3.1. Haematological indices

Administration of ethanolic extract of *E. indica* at 200–600 mg/kg body weight over 28 days had no significant effects on RBCs, Hb, MCH, basophils, eosinophils and moneutrophilsnocytes, neutrophils and lymphocytes. There was significant ($P < 0.001$) but none dose- dependent reduction in MCV and significant ($P < 0.001$) dose-dependent reduction in HCT when compared to control. However, there was significant increase in MCHC ($P < 0.001$), WBC count ($P < 0.001$) and platelets ($P < 0.05$) when compared to control as shown in Table 1.

Table 1

Effect of *E. indica* extract on haemetological indices in rats

Group		WBC ($\times10^3$/μL)	RBC ($\times10^3$/μL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (ph)	MCHC (g/dL)	Platelet ($\times10^3$/μL)	LYM (%)	NEUT (%)	MXD (%)
Control		8.80 ± 0.43	7.79 ± 0.41	13.70 ± 0.63	45.82 ± 0.12	60.15 ± 0.03	17.95 ± 0.34	29.85 ± 0.14	937.75 ± 10.11	75.92 ± 5.77	20.30 ± 5.04	3.77 ± 0.77
Extract (mg/kg)	200	11.40 ± 0.22[b]	7.65 ± 0.42	13.62 ± 0.02	43.50 ± 0.21[b]	56.12 ± 0.12[b]	17.65 ± 0.94	31.37 ± 0.14[b]	938.50 ± 10.12	76.00 ± 2.26	19.00 ± 1.64	3.60 ± 0.73
	400	11.55 ± 0.14[b]	7.38 ± 0.41	13.30 ± 0.69	42.42 ± 0.13[b]	56.47 ± 0.07[b]	17.55 ± 0.54	31.17 ± 0.21[b]	971.00 ± 12.02	77.67 ± 4.62	19.15 ± 3.97	3.17 ± 0.68
	600	11.67 ± 0.30[b]	7.33 ± 0.56	12.77 ± 0.66	40.95 ± 0.30[b]	56.45 ± 0.06[b]	17.22 ± 0.19	31.40 ± 0.14[b]	1008.25 ± 11.07[a]	78.07 ± 3.26	19.30 ± 3.13	2.63 ± 0.17
Silymarin (mg/kg)	100	9.77 ± 0.11	6.40 ± 0.05	11.80 ± 0.04	36.42 ± 0.16[b]	56.52 ± 0.21[b]	18.35 ± 0.06	32.45 ± 0.13[b]	630.00 ± 11.04[b]	77.40 ± 0.44	19.92 ± 0.05	2.95 ± 0.61

Values are represented as mean ± SEM (n = 6). Significance relative to control [a]: $P < 0.05$; [b]: $P < 0.001$. LYM: Lymphocytes; NEUT: Neutrophils; MXD: Basophils, eosinophils and moneutrophilsnocytes.

The extract showed significant haemostatic potentials by reducing both bleeding ($P < 0.001$) and clotting ($P < 0.01$) times as shown in Table 2.

Table 2

Haemostatic effects of *Eleucine indica* extract in rats

Dose (mg/kg)	Bleeding Time (s)	Clotting Time (s)
Control	65.00 ± 0.90	88.33 ± 2.50
200	30.00 ± 0.00[a]	77.50 ± 1.05[a]
400	45.00 ± 0.00[a]	70.50 ± 0.14[a]
600	45.00 ± 0.00[a]	56.75 ± 0.50[a]
Silymarin 100	42.50 ± 0.44[a]	57.00 ± 1.03[a]

Values are represented as mean ± SEM (n = 6). Significance relative to control [a]: $P < 0.001$.

3.2. Effect of E. indica extract on biochemical parameters in rats

The extract showed no significant effects on serum albumin, total cholesterol, trigyceride, HDL and conjugated bilirubin. However, there was significant ($P < 0.05$–0.001) reduction in total bilirubin, AST ($P < 0.001$), ALT ($P < 0.05$), ALP ($P < 0.001$) and blood glucose ($P < 0.001$) by the extract when compared with control. The level of total protein increased significantly ($P < 0.05$–0.001) especially with the low and middle doses of the extract when compared to control (Table 3).

3.3. Effect of E. indica extract on renal functions of rats

The result showed a dose-dependent decrease in creatinine which was statistically significant ($P < 0.001$) at the highest dose. Similarly, potassium exhibited a non dose-related decrease relative to control. There was a non dose-dependent increase in urea, Na^+ and Cl^-. However, the increase in sodium and chloride were statistically significant. HCO_3^- was not affected by the extract administration (Table 4).

4. Discussion

There was no significant effect on RBCs and Hb in extract-treated animals which indicates that the extract did not cause anaemia[17], the observed significant decrease in HCT notwithstanding as this decrease is within normal ranges[18]. Blood is an important index of physiological and pathological status of man and animal, and the parameters measured are packed cell volume, Hb, WBC count and platelets count[19]. The significant increase in WBCs in extract-treated animals suggests a good potential in boosting immune system[20]. The WBCs and their differentials are useful indicators of the ability of an organism to fight infections which suggests also an intact immune system. The increase in platelets suggests stimulatory effect of extract on platelet production possibly by enhancing thrombopoietin secretion[21]. Bleeding and clotting times are parameters used to measure blood coagulation. The bleeding time measures the vascular and platelet responses associated with haemostasis while the clotting time measures the intrinsic pathway clotting factors. Therefore, deficiency in

Table 3

Subchronic toxicity effect of *E. indica* extract on biochemical parameters in rats

Groups		TB	CB	AST	ALT	ALP	TC	TG	HDL	TP	ALB	GLU
Control		16.67 ± 0.13	1.00 ± 0.07	131.67 ± 4.59	35.00 ± 2.19	409.00 ± 5.21	2.00 ± 0.11	0.55 ± 0.05	1.20 ± 0.06	61.00 ± 1.67	34.33 ± 1.12	95.83 ± 0.32
Extract (mg/kg)	200	16.23 ± 0.06[a]	1.20 ± 0.04	115.00 ± 0.73[c]	33.67 ± 1.12	397.67 ± 4.40	2.00 ± 0.15	0.54 ± 0.06	1.27 ± 0.04	68.00 ± 0.21[c]	36.00 ± 0.13	77.50 ± 1.28[c]
	400	16.17 ± 0.11[b]	1.10 ± 0.04	115.00 ± 2.76[c]	32.67 ± 0.15	310.33 ± 3.57[c]	1.97 ± 0.11	0.53 ± 0.56	1.03 ± 0.02	65.00 ± 0.20[a]	35.67 ± 0.21	62.33 ± 0.89[c]
	600	16.03 ± 0.08[c]	1.07 ± 0.04	110.33 ± 2.59[c]	29.67 ± 0.14[b]	265.33 ± 4.55[c]	1.87 ± 0.10	0.46 ± 0.11	1.00 ± 0.09	60.67 ± 1.32	33.67 ± 0.17	60.00 ± 0.27[c]
Silymarin (mg/kg)	100	16.00 ± 0.10[c]	1.10 ± 0.06	95.67 ± 1.52[c]	30.67 ± 0.20	374.33 ± 4.15[c]	2.00 ± 0.06	0.54 ± 0.03	1.10 ± 0.06	58.00 ± 0.34	32.00 ± 0.16	98.00 ± 1.55

Values are represented as mean ± SEM (n = 6). Significance relative to control [a]: $P < 0.05$; [b]: $P < 0.01$; [c]: $P < 0.001$. TB: Total bilirubin; CB: Conjugated bilirubin; TC: Total cholesterol; TG: Total glyceride; TP: Total protein; ALB: Albumin, GLU: Glucose.

Table 4

Effect of *E. indica* extract on kidney function in rats.

Dose (mg/kg)	Urea	Creatinine	K^+	Na^+	Cl^-	HCO_3^-
Control	4.40 ± 0.19	121.00 ± 1.70	8.87 ± 0.20	136.33 ± 1.38	92.67 ± 0.76	21.00 ± 0.36
200	4.20 ± 0.26	118.67 ± 1.90	6.67 ± 0.26[c]	141.00 ± 0.63[a]	95.00 ± 0.36[b]	21.00 ± 0.00
400	5.13 ± 0.10	116.67 ± 1.90	6.90 ± 0.17[c]	140.67 ± 0.76[a]	95.00 ± 0.36[b]	21.00 ± 0.63
600	5.77 ± 0.03[c]	110.33 ± 1.50[c]	5.80 ± 0.18[c]	141.33 ± 0.56[b]	96.33 ± 0.21[c]	22.00 ± 0.36
Silymarin 100	5.50 ± 0.22[c]	74.67 ± 0.16[c]	5.93 ± 0.27[c]	142.33 ± 0.21[c]	94.67 ± 0.21[a]	24.00 ± 0.36[a]

Values are represented as mean ± SEM (n = 6). Significance relative to control [a]: $P < 0.05$; [b]: $P < 0.01$, [c]: $P < 0.001$.

the factors of the intrinsic pathway (I, II, V, VIII, IX, X, XI, and XII) will affect the result. From the results obtained, there was significant decrease in clotting and bleeding times. This reflects the fact that there was an increase in one or more of the clotting factors involved in the intrinsic pathway[22]. Okoli *et al*.[23] had earlier reported similar results on the haemostatic activities of the leaf extract of *Aspilia africana* which arrested bleeding from fresh wounds by reducing both bleeding and clotting times. *E. indica* extract is reported to contain tannins. Tannins have been implicated in the haemostatic activity of plants where they arrest bleeding from damaged or injured vessels by precipitating proteins to form vascular plugs[23,24].

Biochemical indices are useful markers in evaluating the safety or toxic potentials of plant extracts in living cells. Increased serum activity of enzymes such as ALP, AST and ALT could suggest damage to the plasma membrane leading to compromise of membrane integrity[25]. Such alterations are capable of causing leakage from hepatocytes and damage resulting from change in membrane permeability[26]. ALT is more specific to the liver. AST is also found in cardiac and skeletal muscle and RBCs. During liver damage, ALT is released into serum causing raised levels that may remain high for weeks or months. Because AST is found in the liver, RBC, cardiac and skeletal muscle, kidney and brain tissue, damage to any of these areas can result in an increased level on test result. ALP is a marker enzyme that can be used to detect whether or not the plasmamembrane structure has been damaged or disrupted[27,28]. *E. indica* extract caused a significant reduction in the activity of ALP which suggests that there was no damage of the plasma membrane. The significant reduction in ALP levels following the administration of the extract also shows that no possible cholestasis occurred at the dose levels tested since a rise in plasma ALP level is usually a characteristic finding in cholestatic liver disease[29]. Excessive as well as insufficient liver enzymes indicate dysfunction of the liver. The *E. indica* extract did not produce any significant effect on ALT activity in low and middle doses but significant decrease was observed in the high dose. This suggests that the extract is not hepatotoxic since it did not cause any significant alteration in ALT in low doses, ALT being a specific liver enzyme.

Alteration in the concentration of major lipids such as cholesterol, HDL, low density lipoprotein and triacylglycerol gives useful information on the lipid metabolism and predisposition of the heart to atherosclerosis as well as coronary heart disease[30]. The reduction in HDL, total glyceride and total cholesterol parameters, even though not statistically significant, suggests the lack of predisposition of the extract-treated animals to cardiovascular risk.

The serum glucose level was significantly lowered in animals pretreated with the extract which suggests that the extract possesses hypoglycaemic activity.

The levels of serum albumin were not significantly altered by the extract which shows that the protein synthetic capacity of the liver was intact. This is also reflected in the significant increase in total protein especially in low and middle doses of the extract. Serum albumin is frequently used as an index of the ability of the hepatocyte to carry out synthetic function. Serum albumin does not change in mild liver injury but readily declines in the face of sub-massive liver necrosis[31].

Bilirubin is a breakdown product of heme. About 70%–80% of bilirubin produced each day is derived from the breakdown of Hb in senescent RBCs. The remainder comes from prematurely destroyed erythroid cells in bone marrow and from the turnover of haemoproteins such as myoglobin and cytochromes found in tissues throughout the body[32]. The formation of bilirubin occurs in reticuloendothelial cells, primarily in the spleen and liver. This unconjugated bilirubin is bound to albumin in the blood and transported to the liver where it undergoes conjugation with glucuronic acid to conjugated bilirubin and mostly excreted in bile. A small fraction is excreted in urine[32]. Bilirubin is a conventional indicator of liver diseases and its elevation in the serum has been associated with hepatocellular damage and hepatic biliary tract obstruction[31]. The extract showed a significantly low total bilirubin in the extract-treated animals compared to control. Conjugated bilirubin was not affected. The observed decrease in total bilirubin and the lack of alteration in the level of conjugated bilirubin suggested that the hepatic capacity to excrete bilirubin was not impaired.

The kidney is the chief regulator of all body fluids and is primarily responsible for maintaining homeostasis, or equilibrium of fluid and electrolytes in the body. The main functions of the kidney are urine formation, regulation of acid-base balance, excretion of waste products of protein metabolism, protein conservation and hormonal function[33]. Nephrons are lost via toxic, anoxic, or immunological injury that may initially injure the glomerulus, tubule or both together. Glomerular damage can involve endothelial, epithelial, or mesangial cells and/or basement membrane[34]. Sodium is the most abundant cation in the extracellular fluid, constituting 90% of all extracellular fluid cations, where it helps to regulate the amount of water in the body. Potassium is primarily intracellular. A small but vital amount of potassium is found in the plasma. Monitoring potassium is important. Small changes in the plasma K^+ level can affect the heart's rhythm and ability to contract. Chloride moves in and out of the cells to help maintain electrical neutrality, and its level usually mirrors that of sodium. The primary role of bicarbonate, which is excreted and reabsorbed by the kidneys, is to help maintain a stable pH level (acid-base balance) and, secondarily, to help maintain electrical neutrality[35]. The balance of these chemicals is an indication of the functional well-being of several

basic body functions, including those performed by the kidneys and heart.

It was observed earlier that the extract may possess hypoglycaemic effect which suggests its ability to have antidiabetic potential. Potassium facilitates the function of insulin in the delivery of glucose to cells; when insulin binds to its receptors on the cell membrane, it causes potassium to flow into the cells. As levels of insulin increase in the blood, more potassium is driven into cells. In fact soon after insulin treatment is started, there is likely to be a precipitous fall in the plasma potassium due to dilution of extracellular potassium by administration of intravenous fluids, the movement of potassium into cells induced by insulin, and the continuing renal loss of potassium, causing hypokalaemia. Therefore, the reduced potassium may not be unconnected with the hypoglycaemic effect of the extract[36]. Bicarbonate was not affected except for an insignificant rise which is to be expected since regeneration of H^+ to aid reabsorption of HCO_3^- is a renal tubular function. The potassium depletion equally forces renal tubular regeneration of HCO_3^-, hence the observed increase in HCO_3^-[37]. There was significant increase in the levels of Na^+ and Cl^- by the extract. Increased Na^+ retention can be associated directly with Cl^- since most sodium ion reabsorption is coupled with chloride ion reabsorption. The excretion of chloride ion would naturally follow the excretion of sodium ions. Chloride reabsorption is associated with excretion of HCO_3^-, as plasma chloride ion decreases, the bicarbonate ion increases to keep the total concentration constant[37]. Na^+ and K^+ work and operate inversely as a result of the activity of sodium-potassium pump. Increased excretion of sodium ion usually leads to reabsorption of K^+ from the renal tubules[38].

Most diseases that affect the kidneys or liver can affect the amount of urea present in the blood. If increased amounts of urea are produced by the liver or decreased amounts are removed by the kidneys, then blood urea concentrations will rise. If significant liver damage or disease reduces the production of urea, then urea concentrations may fall. Generally, decreased renal function causes an increase in plasma urea concentration. The extract showed a non dose dependent increase in urea. This increase in urea was reflected in the increase in total protein, the liver capacity to produce the metabolite and also the preservation of the liver integrity of extract-treated rats[29]. Almost all creatinine is removed from the body by the kidneys, so levels in the blood are a good indication of how well the filtering units in the kidneys, called glomeruli, are functioning. The amount of creatinine removed from the blood depends on the filtering ability of the glomeruli in the kidneys and the rate at which blood is carried to the kidneys. The laboratory marker that has long served as the mainstay for detecting impaired kidney function is serum creatinine[35,39]. Measurement of serum creatinine is the most commonly used indicator of renal function. A rise in blood creatinine level is observed only with marked damage to functioning nephrons. In general, increased urea levels are associated with nephritis, renal ischemia and urinary tract obstruction. Low blood levels of creatinine are not common, but they are also not usually a cause for concern[31]. Low doses of the extract caused a decrease in the level of creatinine with significant decrease in the highest dose of the extract. The reduction in creatinine level observed in low doses of extract-treated groups indicated that extract did not exert deleterious effect on renal function in low doses.

This study has demonstrated that subchronic administration of ethanolic whole plant extract of *E. indica* causes a reduction in both bleeding and clotting times, reduces blood glucose but is apparently non-toxic to the liver and the kidneys. However, further histopathological work is advocated in order to fully assess its toxicity profile and safety.

Conflict of interest statement

We declare that we have no conflict of interest.

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