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Full Length Research Paper

Acute oral toxicity of *Euphorbia heterophylla* Linn. ethanolic extract in albino mice

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This study was carried out to determine acute oral toxicity and histopathological effects associated with consumption of *Euphorbia heterophylla* ethanolic extract using 9 to 10 weeks old Albino mice randomized in six groups. The five groups were orally administered with single graded doses of plant extract at 1500, 2000, 2500, 3500 and 4000 mg/kg body weight while the sixth group was administered 1 ml of physiological saline and the animals were observed for toxicity signs and death. V**iscera organs** were obtained after **cervical dislocation for histopathological assessment. The graded extracts induced dose-dependent toxicity signs with major clinical manifestation prior to death including: polyurea, circling, paralysis, thirst, loss of appetite and gait, tachypnea, dehydration and stupor. The major dose-dependent histopathological lesions included: Hemorrhages, congestion, peri-vascular degeneration and necrosis in viscera organs in the groups that received 2000 to 4000 mg/kg body weight. The 24 h median lethal dose was 2831 mg/kg body weight and the 95% confidence interval of median lethal dose was 2490 to 3218 mg/kg body weight and R^2 is 0.96 indicating *E. heterophylla* is of low toxicity. The study demonstrated the toxicity potential associated with uncontrolled use of this plant by the communities. Toxicological studies of sub chronic and chronic toxicity, as well as *in vitro* mutagenicity and genotoxicity need to be conducted considering the well claimed prolonged use of the plant extract to assess the effect prolonged use on animals.**

Key words: Acute oral toxicity, histopathological lessions, *Euphorbia heterophylla*.

INTRODUCTION

Medicinal plants play a significant role in the livelihoods of rural communities of developing countries especially where health and veterinary services are limited. About 80% of the world population depends partially or whole on medicinal plants for primary healthcare (WHO, 2010). The medicinal plants are used in humans and livestock to control a multiplicity of diseases and conditions. For instance, *Euphorbia heterophylla Linn* belong to the family Euphorbiaceae, has been used for treatment of several diseases and conditions of man and livestock.

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Although most members of Euphorbiaceae family are reportedly poisonous, some are of economic and medicinal value (Burkill, 1994). The leaves and stem of Euphorbiaceae species exude a milky sap when injured and contain latex in all parts of the plant (Parsons and Cuthbertson, 1992).

In Uganda, the *E. heterophylla* is used to treat a number of diseases and conditions like helminthes and constipation in man and livestock in addition to feed the plant to animals like pigs and rabbits (Nalule et al., 2011). In Africa and India, *E. heterophylla* is used to treat constipation, bacterial and inflammatory disease conditions such, arthritis and rheumatism, migraine and wart cures (Falodun et al., 2008; Anilkumar, 2010; Karimi et al., 2010). The plant lattices are used as fish poison, repulsive, insecticide and ordeal poisons (Rodriguez et al., 1976; Falodun et al., 2003) and medically for fungal infection and gonorrheal treatment (Moshi et al., 2007). The plant is also reported to have diuretic and purgative action in addition to treating asthma by causing bronchial relaxation (Johnson et al., 1999, Edeoga and Okafor, 2005). In 2010, Oluduro and Olumide demonstrated anti-typhoid activity of aqueous and methanolic leaf extracts. According to Edeoga et al. (2005), the plant vegetable and latex are used in insect bites, treatment of erysipelas, cough, bronchial paroxymal asthma by causing bronchial relaxation hay fever and catarrh. *E. heterophylla* was reported useful in rheumatism, dropsy, gout, neuropathy, deafness and cough (Kirtikar and Basu, 1975). A study by Okoli et al. (2009) revealed alcoholic extract of *E. heterophylla*, was effective against Streptococcus sp. In Nigeria, the plant extract is used to treat ear pain, and induces milk flow in addition to increasing sperm quality (Okoli et al., 2009). A study of anthelmintic activity of *E. heterophylla* used by Ugandan pastoralists revealed the plant is very effective and potent (Nalule et al., 2013). In the same study, phytochemical screening revealed presence of tannins, alkaloid, saponins, flavonoids, steroids glycosides, triterpenes, coumarin derivatives, anthocyanocides, anthracenocides and reducing sugars whose intensity varied with solvent used for extraction.

However, despite the several uses of medicinal plants, herbal medicine popularity has raised concerns over the quality and safety with adverse effects resulting from taking herbal medicines having been reported (Chan, 2003; Fennell et al., 2004). For instance, toxicity has been reported in most members of genus Euphorbia where individuals sensitive to latex show strong reactions, including dermatitis and anaphylaxis to the sap exuded by this plant (Wilken and Schempp, 2005). Milkweed has been recorded as toxic to stock (Parsons and Cuthbertson, 1992) despite reports that root bark is used as medicines. There has been a tendency of generalized reports of euphorbia species latex toxicity to animals and humans (Burkill, 1935; Wattman and Breyer-Brandwijk, 1962). Considering the medicinal importance of this plant, the current study was undertaken to determine acute oral toxicity and histopathological effects of single graded doses of ethanolic extract of whole plant over a twenty four hour period.

MATERIALS AND METHODS

Plant collection and preparation of plant extract

The plant material was collected from Nakasongola district and identified in Makerere University herbarium where a sample specimen was deposited. The whole aerial plant part material was oven dried and milled into fine powder. Two hundred fifty grams (250 g) was macerated in 2 L of 70% ethanol for 72 h in a dark room with intermittent shaking. Filtration through cotton wool was done to remove coarse particles (residues) and finely through filter paper 12.5 mm (Whatman®, England). This was followed by concentration on Rota-vapor type Buchi-R, Switzerland under reduced pressure at 50°C. The extracts was then put into an oven to dry completely at 50°C and finally packed into universal bottles and kept at 4°C till needed for toxicity studies.

Experimental animals

Thirty six 9-10 week old mice of either sex, of an average body weight of 19.4 g were randomly divided into six animals per group and put in a cage for easy observation. The animals were kept at 24 to 28°C and at a relative humidity of 60 to 70% for one week to acclimatize to experimental laboratory conditions. During this period, the mice were fed on a standard diet and given ordinary tap water *ad libitum*. After one week of acclimatization, the animals were starved for 18 h but water provided *ad libtum* prior to administration of extracts. The individual body weights were taken shortly before drug administration to guide dose and drug volume for each animal.

Acute oral toxicity and determination of median lethal dose (LD_{50})

The studies were carried out according to Good Laboratory Practice (GLP) Regulations of Organization for Economic Cooperation and Development (OECD) (UNDP/World Bank/WHO, 2001). Acute oral toxicity of ethanolic *Euphorbia heterophylla* extract was determined orally in mice following the procedure used by Ghosh (1984) and Toma et al. (2009). The five animal groups were orally administered with single graded doses of plant ethanolic extract at 1500, 2000, 2500, 3500, and 4000 mg/kg body weight as treatments 1 to 5. The mice in the control group (group 6) were each orally administered 1ml of physiological saline. After applying the treatments, the animals were observed for six hours continuously and thereafter at six hour intervals up to 24 h. The animals were observed for signs of toxicity: motor activity, tremors, convulsions, posture and spasticity, ataxia, righting reflex, lacrymation, salivation, urination, urine output, diarrhoea, sensation, respiratory rates and mortality. The LD_{50} of the ethanolic extract was determined using Graph pad prism version 5.01 computer programme and also calculated using the arithmetic method of Karber as described by Ghosh (1984) using the formula;

$$LD_{50} = \frac{X - \sum(D \times \overline{D})}{N}$$

Where, LD_{50}, is the median effective dose; X, is the least dose that killed all the animals; D, is the dose difference; $\overline{D}$, is the mean dose and N, is the number of animals in a group.

Histopathological examination

Collection of organs samples

After the experimental regimen (24 h) post oral administration of the extract, the animals were sacrificed by cervical dislocation under mild chloroform anesthesia **and** their viscera organs including intestine, liver, brain, lung and kidneys were collected and preserved in 10% formalin immediately after removal from the animal.

Tissue processing for histopathology

The tissue processing was conducted in an automatic processor model Leica TP 1020 in the college of veterinary medicine animal resources and Bio security, Pathology laboratory. The procedures described by Drury and Wallington (1980) and Prophet et al. (1994) were followed with slight modification. **T**he tissues were placed in 10% formalin (10 parts of formalin and 90 parts of distilled water) for 1 h to rectify shrinkage due to high concentration of formalin. The tissues were dehydrated by ascending grades of isopropyl alcohol by immersing in a series of isopropyl alcohol including 70 and 80% for an hour each, then through 90 and 96% for 1.5 h each to avoid shrinkage of cells then in three 100% each for 1½ h each to ensure no traces of water in the cells. The dehydrated tissues were cleared in two changes of xylene for 1½ h each. The tissues were impregnated in two changes of molten paraffin wax for two hours each to provide an internal support. The wax impregnated tissues were
embedded in paraffin wax using the same grade wax. The paraffin wax were mounted and cut with rotary microtome machine model Leica RM 2235 at 5µ thickness. The sections were floated on a tissue floatation water bath model Leica HI 1210 at 44°C and taken on plain glass slides. The cut sections were then transferred to an incubator at 53°C for overnight, and thereafter cooled and ready for staining.

Tissue staining

The staining procedures described by Prophet et al. (1994) were followed. The sections were deparaffinised by immersing in two changes of xylene for 2 min each in horizontal staining jar. The deparaffinised sections were washed in two changes of 100% isopropyl alcohol, then in 95% alcohol thereafter dipped in cold water and stained in Meyers hematoxylin for 8 to 12 min in horizontal staining jar. After staining in Meyer's hematoxylin, the sections were blued in tap water for 10 to 15 min to wash out excess hematoxylin in others structures and make the nuclei distinct. The sections were counter stained with eosin for 5 min (the cytoplasm and other organelles stain varying shades of pink to red and nucleus stain blue). The sections were counter stained in 1% aqueous eosin (1 g in 100 ml tap water) for 5 min and the excess stain was washed in tap water. Complete dehydration of stained sections was ensured by placing the sections in graded alcohols including 95, 100 and 100% for 5 min each. The sections were then mounted in DPX having the optical index of glass (the sections were wetted in xylene and inverted on to the mount and placed on the cover slip). The slides were observed at low power objective under microscope. The cell injury and over aspects were observed under high power dry objective (Dunn, 1974). After mounting the slides were left on table to air dry for microscopic observation and evaluation. The slide imaging was carried out using Carl Zeiss photographic microscope. Model: German-176045 and Canon camera PC 1200. The haematoxylin and Eosin stained slides images were read and the pathological lesions described at magnification x5.

Data analysis

The Graph pad prism computer programme version 5.01, software was used to transform the dataand determinate LD_{50} and the 95% confidence interval of median lethal dose and the slope.

RESULTS

Toxicity signs in mice dosed with whole plant ethanolic *Euphorbia heterophylla* crude extract

The animals to which the different doses of ethanolic crude extract of *E. heterophylla* were administered displayed graded clinical signs whose severity increased with increasing dose before death (Table 1).

Median lethal dose (LD_{50})

The survival and mortality results of mice dosed with ethanolic extract of *E. heterophylla* are given in Table 2. The 24 h post treatment LD_{50} by graph pad prism computer programme was determined to be 2831 mg/kg body weight and the 95% confidence interval of median lethal dose (LD_{50}) was 2490 to 3218 mg/kg body weight and R^2 was 0.96 indicating the plant is of low toxicity. Table 2 also provides data used in calculation of median lethal dose using arithmetic method of Karber for comparison. The LD_{50} of *E. heterophylla* was 2958 mg/kg when the Karber arithmetic method (Ghosh, 1984) was used. The calculated LD_{50} still indicate the plant is relatively of low toxicity.

Gross and histopathological findings

The histopathological examination was conducted to determine the damages caused by ethanolic extract to the tissues**.** At gross examination, the animals in groups treated with 4000 mg/kg of extracts looked dehydrated and weak. There were hemorrhages on the brain meninges and peritoneum, while their liver looked congested. However, the kidneys apparently appeared to be of normal colour and consistence.

The organs from the both groups administered 2500 and 4000 mg/kg body weight, showed histopathological lesions or alterations in histology including congestion of blood vessels, hemorrhages, degenerative changes and cell damage (Table 3). The effects obtained from both doses were the same although varied in magnitude with 4000 mg/kg causing severe injuries compared with 2500 mg/kg. However, the kidney, liver, brain, intestine and lungs of mice from the control groups showed normal architecture and normal cells. The stomach and intestines from treated groups revealed severe inflammation of the walls of the stomach and intestine, ulcers, erosive necrosis and sloughing off of mucosa though the basement remained intact. The histopathological representations of light micrograms of intestine, liver, kidney, brain and lungs lesions are illustrated in Figures 1 to 5 respectively.

Table 1. Toxicity clinical signs in mice treated with a single dose of ethanolic crude extract of *E. heterophylla* Linn.

Dose (mg/kg of body weight)	Noticeable toxicity signs post *E. heterophylla* ethanol extract administration(N = 6)													
	IR	CV	ATX	IUR	DHD	THT	LOA	DEP	IST	MLT	STP	CHN	DTH	TTD
Normal saline (1 ml)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1500	-	-	-	+ (2)	-	-	+ (2)	-	-	-	-	-	-	-
2000	+ (2)	+ (1)	+ (1)	+ (3)	+ (3)	+ (3)	+ (3)	+ (2)	+(1)	+(1)	+(3)	+ (1)	+ (1)	22
2500	+ (3)	+ (2)	+ (3)	+ (3)	+ (2)	+ (2)	+ (3)	+ (2)	+ (2)	+ (2)	+ (3)	+ (1)	+ (2)	22
3500	+ (4)	+ (4)	+ (5)	+ (6)	+ (6)	+ (6)	+ (6)	+(6)	+ (4)	+ (6)	+ (6)	+ (4)	+ (4)	18
4000	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	+ (6)	8

+, Present; -, absent; (), number of animals showing the signs; IR, increased respiration; CV, convulsions; ATX, Ataxia; IUR, increased urination rate; DHD, Dehydration; THT, thirst; LoA, loss of appetite; Dep, depression; IST, insensitivity; MLT, misuse of limbs and tail; STP, stupor; CHN, circling and head nodding; DTH, death; TTD, time to death (h).

Table 2. Twenty four hour dose -response relationship and Median Lethal dose (LD_{50}) of mice dosed with ethanolic crude extract of *Euphorbia heterophylla.*

Group	Dose (mg/kg)	Dose difference (a)	Log dose (mg/kg)	No. of dead animals	Mean death (b)	% of dead animals	Dose difference × mean death (a × b)
1	0.0	-	0.0	0	0	0	0
2	1500	-	3.176	0	0	0	0
3	2000	500	3.301	1	0.5	16.67	250
4	2500	500	3.398	2	1	33.33	500
5	3500	1000	3.544	4	3	66.67	3000
6	4000	500	3.602	6	5	100.00	2500
Total							6250

N= 6 mice; LD_{50} = 4000 - (6250/6) mg/kg body weight. Therefore, LD_{50} = 2958 mg/kg.

Table 3. Histopathological lesions of mice treated with ethanolic crude extract of *E. heterophylla.*

Organ	Histopathological Lesions		Microscopic pathological diagnosis*
	Control (Physiological saline)	Animals treated with 2500 and 4000 mg/kg body weight[1]	
Liver	Normal cell architecture	Haemorrhages, distension of hepatic sinosides, congestion of vessels, perivascular necrosis	Haemorrhagic necrotizing hepatitis
Intestine	Intact mucosa and basement	a) Sloughing off of mucosa and mucosa membrane b) Haemorrhages, ulcers, erosion of mucosa, glands not affected	Erosive haemorrhagic enteritis
Brain	Normal cells	a) Renal cortical haemorrhages, degeneration of neurons, b) Haemorrhages	Haemorrhagic encephalitis
Kidney	Normal architecture	Haemorrhages on renal cortex, congestion and perivascular necrosis (nuclear pycknosis, and tubule degeneration)	Haemorrhagic necrotizing nephritis
Lung	Normal architecture	Necrosis of lung tissue, haemorrhages, Emphysema, and congestion of vessels	Haemorrhagic pneumonia and emphysema

[1]The two doses displayed similar lesions but varied in severity; *overall pathological diagnosis: Severe vascular degeneration leading to haemorrhagic shock that caused death of the animals

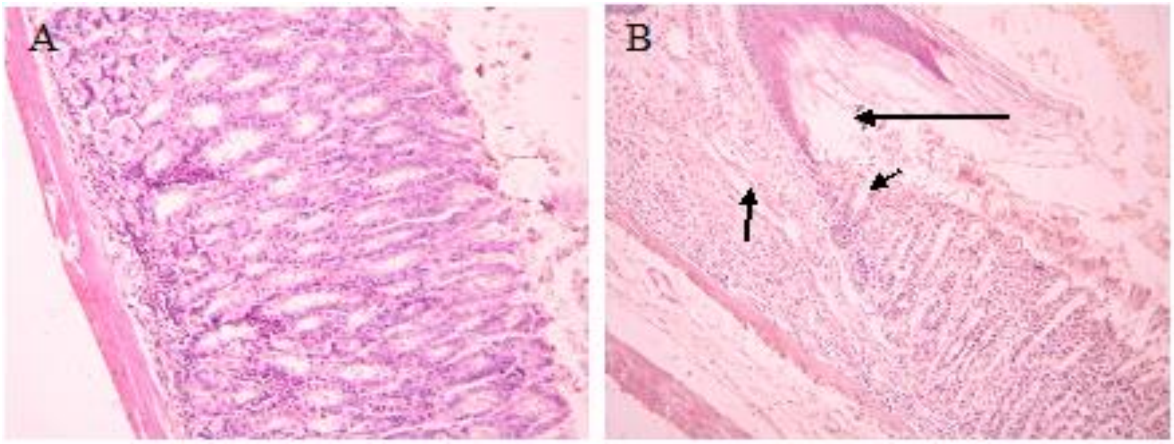

Figure 1. Histopathological presentation of the intestine from mice orally administered with 4000 mg/kg of ethanol crude extracts of *E. heterophylla* (B) at 24 h post exposure, magnification x10. The architecture of organs were severely affected indicated by sloughing off of intestinal mucosa and ulcerative lesions compared with intact mucosa of untreated control (A). Note the loss of lamina propria and lamina muscularis mucosa, basement membrane and damage of epithelial cells (B).

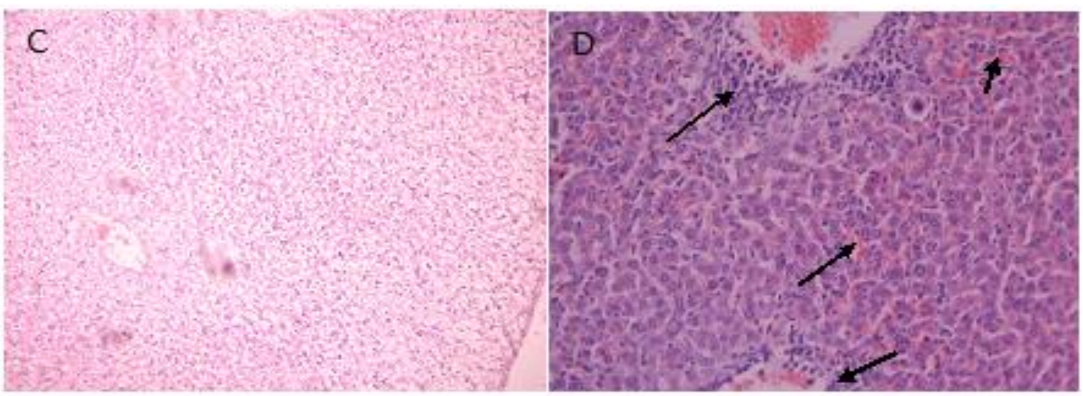

Figure 2. Histopathological presentation of the liver from albino mice orally administered with 4000 mg/kg of ethanol crude extracts of *E. heterophylla* (D) at 24 h post exposure, magnification x 10. The architecture of liver from treated mice shows hemorrhages, distension of hepatic sinosides and cell degeneration around blood vessel compared with untreated control (C). The arrows point to haemorrhages and cell degeneration.

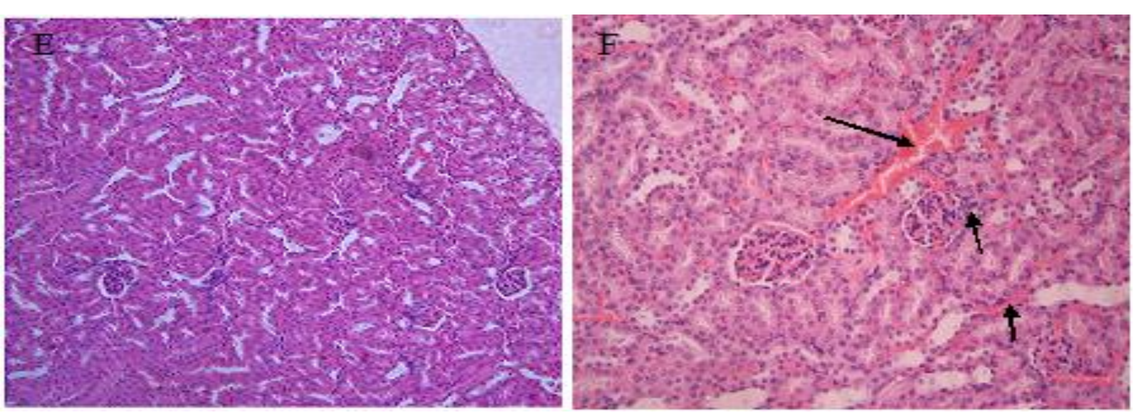

Figure 3. Histopathological presentation of the kidney from albino mice orally administered 4000 mg/kg of ethanol crude extracts of *E. heterophylla* (F) at 24 h post exposure, magnification x 10. The kidney cortex from treated mice shows hemorrhages, renal corpuscles with cellular infiltration, blood between ducts of nephron cells and cell degeneration compared with untreated control (E).

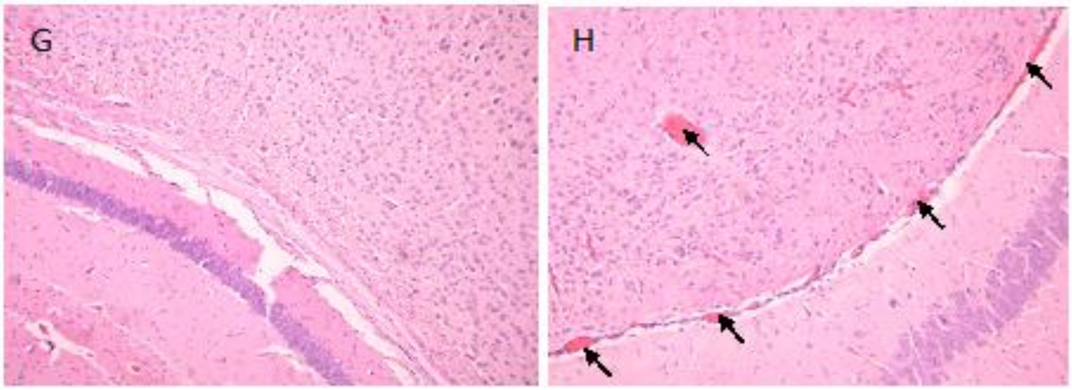

Figure 4. Histopathological presentation of the brain from mice orally administered 4000 mg/kg of ethanol crude extracts of *E. heterophylla* (H) at 24 h post exposure, magnification x 10 compared with untreated control (G). The architecture of brain from treated mice shows exposures to *E.heterophylla* produce hemorrhages in different parts of the brain indicated by arrows.

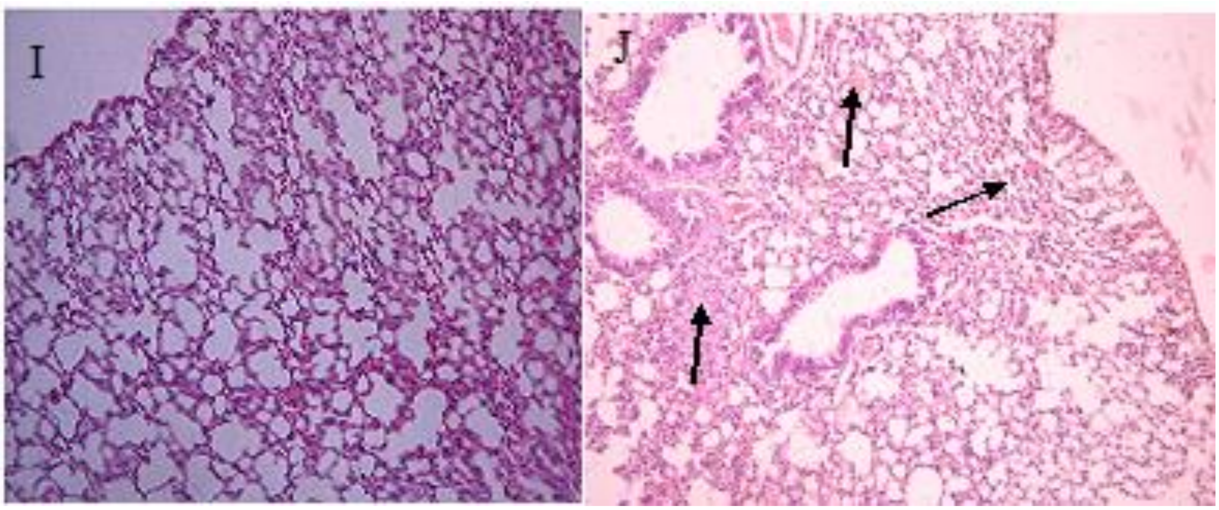

Figure 5. Histopathological pictures of the lung from albino mice orally administered 4000 mg/kg of ethanol crude extracts of *E. heterophylla* (J) at 24 h post exposure compared with untreated control (I), magnification x 10. The architecture of lung from treated mice shows exposures to *E.heterophylla* cause hemorrhages, congestion and edema reducing the alveoli space in the lungs.

DISCUSSION

This study has established that the ethanolic extract of *E. heterophylla* is slightly toxic with LD_{50} of 2831 mg/kg according to Matsumura (1975) and Ghosh (1984) classification of toxicity. This is in agreement with the report by Moshi et al. (2007) who also established an LC_{50} of 80.2 µg/ml using brine shrimp model; and a study by Ologe and Sogbesan (2007) that established an LC_{50} of 1.8 g/L of the aqueous extract on *Barbus occidentalis* fingerlings. The difference in the LC_{50} could be attributed to the source of the plant, maturity, species of the animals on which it is tested and solvent of extraction. Kuppusamy and Murugan (2008) reported an ovicidal and pupicidal activity of ethanolic extract of *E. heterophylla* on mosquito development stages with LC_{50} ranging 14.98 to 25.72 ppm. Adedapo et al. (2004) reported macrocytic normochromic anaemia and a total white blood cell count resulting from 14 day's administration of aqueous fresh extract at 1 g/100 g in rats.

The study further indicated that the ethanolic extract has potential to cause severe clinical signs in the mice (Table 1) and damage to viscera organs exhibiting as severe haemorrhgaes, cell degeneration, ulcerations and necrosis (Figures 1 to 5). The severe haemorrhages observed indicate that animals feeding on the plant or unregulated use of the plant may cause anaemia and reduced immunity. These results agree with the findings of Adedapo et al. (2004) who reported anaemia and reduction in total white cell count. The haemorrhage could be due to a plant lectin, phytohaemagglutinins reported by Nsimba-Lubaki et al. (1983). The observed damages in the viscera organs of animals is agreement with earlier observations that euphorbia species induces inflammation on mucous membranes in animals (Karimi et al., 2010). The clinical manifestations observed in the mice may be linked to presence of polyphenols such as tannins and triterpenes such as saponins present in the plant (Nalule et al., 2013). Damage to the brain and kidney could have caused excessive urination probably due to the effect on the anti-diuretic hormone (ADH). Alldredge (1993) and Kongvongxay et al. (2011) attributed reduced feed intake in animals to tannin containing diets that have strong astringent property and cause induction of internal malaise in mammals, which may contribute to reduce feed intake.

Haemorrhages, cell infiltration and cell degeneration present in all organs assess could be attributed to presence saponins and tannins which damage cells. A plant lectin (phyto-haemagglutinins) has been isolated from this plant (Nsimba-Lubaki et al., 1983). The authors reported that *E. heterophylla* agglutinins agglutinate the human and animals erythrocytes in a non blood group specific. The observed mucosal erosion and ulceration (Figure 1) in the stomach and intestine of the test mice administered high doses are consistent with a website report that a post-mortem examination of people killed by *Euphorbia* latex have severe inflammation of the walls of the stomach and intestine while in some patients, the wall of the stomach are perforated (http://www.the amateur.digest.com?epoisons.htm, accessed April, 2011).

The excitement and increased urination observed in the mice could be due to the damage caused to the brain and kidney that were characterized by severe haemorrhages and cell degeneration. Thirst was observed in all animals and it could also be associated with the excessive urination that directs to the diuretic effect of the plant extract on the anti-diuretic hormone. The diuretic activity observed in this study agrees with report by Johnson et al. (1999) who reported that euphorbia species have diuretic activity. Similar observations were reported by community who said that humans taking the aqueous plant extract need to drink a lot of water due to the excessive thirst the extract cause to the patient (Nalule et al., 2011).

The histopathological lesions in the viscera organs suggest the level of toxicity and the affected body systems targeted by the *E. heterophylla* extract. Beside these perilous effects, Euphorbia species have numerous therapeutic beneficial effects including anti-inflammatory and purgative, antioxidant and hepato-protective activity (Jyothi et al., 2008). These effects may be attributed to the rich phytochemical composition present in this plant which include but not limited to alkalloids saponins, glycocides, flavonoids, tannins (Falodun et al., 2006; Karimi et al., 2010). The many secondary metabolites are said to have a positive or negative effect on animals and as parasites/disease causing agent.

Tannins and anthraquinones are said to have both proxidant and antioxidant effects on the animal. While antioxidant act as an anti-inflammatory (Ogueke et al., 2007; Anilkumar, 2010; Karimi et al., 2010) and protect body, the proxidants cause damage to animal tissues and organs. The tannins and saponins are responsible for the antibacterial activity of the plant extracts (Gloor, 1997). Saponins have been used in veterinary vaccines such foot-and-mouth disease vaccines as adjuvant and helping to enhance immune response. The presence of anthraquinones in the extracts could be contributing to the damage observed. Huang et al. (1992) reported that anthraquinones produce free radicals and hydrogen peroxide during its oxidation to semiquinone in the body that damages the body cells. The terpen ester composition in the plant determines how caustic and irritating to body tissues such as the skin and mucous membranes (Karimi et al., 2010). Karimi et al. (2010) reported the sap from euphorbia species cause keratoconjuctivities and that the latex (milky sap) of spurges act as deterrent for the herbiovores as well as wound healer. A study by Adedapo et al. (2004) showed slight reduction in packed cell volume (PCV) level of the test mice but a significant decrease in the levels of red blood cells (RBC) and haemoglobin concentration, indicating that the leaves of this plant could also cause anaemia in animals.

Flavonoids are well known for ant-oxidant activity and in this study they may have acted to reduce some of the inflammatory reactions in the animal. Flavonoids have also been reported to show anti-inflammatory, anti-allergic, antimicrobical and anticancer activity (Balch and Balch, 2000). The nitrogen containing alkaloids are physiologically active with sedative and analgesic properties and are reported to relieve pains, anxiety and depression (Jisika et al., 1992). However, alkaloids are toxic due to their stimulatory effects that lead to excitation of cells and neurological dysfunction (Ekam and Ebong, 2007; Malu et al., 2009).

The excitatory behavour is consistent with previous findings of Adedapo et al. (2004) who reported that the aqueous crude extracts of *E. heterophylla,* caused an excitatory effect followed by dullness in addition to anaemia and leucocytosis. These effects could be explained by the severe haemorrhage that was observed in all internal organs in the current study. Adedapo et al (2004) study also reported increase in the level of albumin, ALT and AST. Although the *E. heterophylla* lower doses, caused no observable effects, the hemorrhages caused indicate that prolonged consumption of this plant could also cause anaemia in animals that feed on it like the pigs and rabbits. Gross and histopathological findings, which included, increased urine output, hemorrhages, depression, cellular degenerative loss of appetite among others, were indicative of poisoning by this plant that were also consistent with findings of Adedapo et al. (2004).

The significant change in the architecture of the viscera organs observed in this study is a clear indication of toxicity potential associated with this plant. Adedapo et al. (2004) reported increase in the levels of aspartate aminotransferase (AST) and alanine associated with the liver damage observed in this study. It therefore evident that with continuous administration of this plant extracts to animal, the principal function of vital organs would be compromised with consequent failures of a number of body systems. The toxicity of the plant, especially of the root and latex, was also recognized in East Africa (Burkill, 1985).

CONCLUSIONS AND RECOMMENDATIONS

It should be concluded that although *E. heterophylla* possess medicinal benefits to humans and their livestock, this study has shown that using the extract at a high dose can cause fatal vital organs' damage. It is imperative to note that continuous exposure of livestock to these plants may lead to morbidity and/or mortality leading to reduced livestock productivity. Caution should therefore be exercised in the use of *E. heterophylla* for medicinal purposes and feed. Patients, whether human or animal taking the extract should equally be availed with enough fluids due to severe loss of fluids. Understanding the toxic principle and its mechanism of action would greatly guide on the protection of the patients. Toxicological studies of sub chronic and chronic toxicity, as well as in vitro mutagenicity and genotoxicity need to conduct considering the well claimed prolonged use of the plant extract to assess the effect prolonged use on animals.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Ethical approval

Authors hereby declared that the experimental protocol was reviewed and approved by the competent members of graduate research and ethics of Faculty of Veterinary medicine and re-reviewed by the University graduate research and training committee of Makerere University. All ethical consideration for using animal subjects were considered. Experimental protocol was followed according to OECD Guidelines 2000. All rules were followed as well as specific national laws where applicable.

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Full Length Research Paper

Anti-ulcerogenic evaluation of the fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *Dacryodes edulis*

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In traditional medicine, the leaves of *Dacryodes edulis* are utilised in the treatment of gastric ulcer and hence, the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* were evaluated for their anti-oxidant vitamin contents and proximate compositions. Based on preliminary investigations, the more promising layer (ethanol layer) was further fractionated and the effects of the fractions on ulcer index and curative ratio of diclofenac-ulcerated Wistar rats were determined using standard methods. The anti-oxidant vitamin contents of the chloroform and ethanol layers showed the following: vitamins A (1.83 ± 0.08 and 1.79 ± 0.08 µg/100 g), C (0.62 ± 0.05 and 0.79 ± 0.03 mg/100 g) and E (1.17 ± 0.07 and 1.04 ± 0.05 mg/100 g) respectively. The proximate compositions of the chloroform and ethanol layers were: moisture (3.15 ± 0.11 and 4.08 ± 0.18%), crude fibre (5.41 ± 0.29 and 5.29 ± 0.27%), ash (5.09 ± 0.27 and 4.12 ± 0.18%), crude fats (4.43 ± 0.13 and 3.98 ± 0.12%), crude proteins (17.24 ± 0.43 and 17.80 ± 0.29%) and carbohydrates (64.68 ± 1.40 and 64.73 ± 1.35%) respectively. Each of the chloroform and ethanol layers of the chloroform-ethanol extract was found to be safe at a dose as high as 5000 mg/kg body weight (b.w). At the two doses (100 and 200 mg/kg b.w), the n-hexane, chloroform, ethyl acetate and methanol fractions of the ethanol layer dose-dependently decreased and increased the ulcer indices and curative ratios respectively of the rats in the test groups when compared with those of the rats in the ulcer-untreated group albeit these effects were not significant (p>0.05) except those of the methanol fraction at the dose of 200 mg/kg b.w. Results of the methanol fraction (200 mg/kg b.w only) were comparable to those of the standard anti-ulcer drug, ranitidine at the dose of 150 mg/kg b.w. The data of this study indicate that the methanol fraction of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* possess remarkable anti-ulcerogenic effect and might serve as source of novel drugs for ulcers in future.

Key words: *Dacryodes edulis*, anti-oxidant vitamins, proximate composition, ulcer index and curative ratio.

INTRODUCTION

A peptic ulcer is a sore on the lining of the stomach or duodenum, the beginning of the small intestine. Less commonly, a peptic ulcer may develop just above the stomach in the oesophagus, the tube that connects the mouth to the stomach (Verma et al., 2011). Peptic ulcer affects a large proportion of the world's population and is

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Figure 1. A young *D. edulis* tree showing the stem, the leaves and the fruits.

induced by several factors including stress, smoking, nutritional deficiencies and ingestion of non-steroidal anti-inflammatory drugs (Devi et al., 2011). A number of drugs are available for the treatment of peptic ulcers in current medicine as histamine II (H_2) receptor antagonist, proton pump inhibitors or cytoprotective agents such as sucralfate inter alia but clinical evaluation of these drugs indicates high incidences of relapse, side effects (arrhythmias, gynaecomastia, impotence, arthralgia, hypergastrinaemia and haemopoeitic changes) and drug interactions (Ahmad et al., 2013). Several studies have shown the beneficial effects of medicinal plants on ulcer (Wandale et al., 2012; Ahmad et al., 2013). Many natural products and modern synthetic drugs have been used to treat gastric ulcer but so far, a complete cure has not been discovered. The exploration of new anti-ulcer drugs has therefore, continued to be a field of active research.

Dacryodes edulis (Figure 1) is of the family, Burseraceae. It is a versatile plant in African ethnomedicine as its various parts are utilised in the treatment of array of ailments. Its bark has long been used to cicatrise wound in Gabon (Walker and Silans, 1961). In this case, the bark is pulped and then applied directly to the wound. In Democratic Republic of Congo, the plant is employed in the treatment of diverse diseases. The decoction of the bark is taken orally to treat leprosy. It is also used as a gargle and mouth-wash to treat tonsillitis. The bark is comminuted with meleguetta pepper to cure dysentery, anaemia, spitting blood and as an emmenagogue; when mixed with palm oil, it is applied topically to relieve pains, debility, stiffness and skin diseases (Bouquet, 1969). The leaves are chewed with kolanut as an anti-emetic. The leaf sap is used as an ear drop to treat ear trouble while the leaf decoction is prepared to produce vapour that treats fever and headache (Bouet, 1980). In Congo Brazzaville, the leaves are boiled with those of *Lanata camara*, *Cymbopogon citratus* and *Persea americana* in water to form a decoction for treating malaria. A steam bath can also be taken from the decoction to treat the same ailment. Boiling the leaves with those of *P. americana* alone can be used to treat headache, antalgic and cephalgy (Diafouka, 1997). Recently, Jiofack et al. (2010) reported that the leaves are made into plaster to treat snakebite in southwest Cameroon. The bark resin is used in Nigeria to treat parasitic skin diseases and jiggers (Dalziel, 1937; Hutchinson et al., 1963). When applied in lotions and creams, the resin smoothens and protects the skin (Ekpa, 1993). The aroma of the resin when liberated through burning is believed to ward off evil spirit in Nigeria (Sofowora, 2008). The leaves are often crushed and the juice released to treat generalised skin diseases such as scabies, ringworm, rashes and wound while the stem or stem twigs are employed as chewing sticks for oral hygiene (Igoli et al., 2005; Ajibesin et al., 2008). The leaves boiled singly or with lemon grass or pulp oil are employed in the treatment of peptic ulcer (Omonhinmin, 2014). In this study, the anti-ulcerogenic properties of the fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* are reported.

MATERIALS AND METHODS

Fresh leaves of *D. edulis* were plucked from their tree at Government Reserved Area (GRA), Nsukka Local Government Area of Enugu State, Nigeria. The leaves were identified by Mr. Alfred Ozioko of Bioresource Development and Conservation Programme (BDCP) Research Centre, Nsukka.

Extraction procedure for the chloroform-ethanol extract

A known weight (1200 g) of the pulverised *D. edulis* leaves was macerated in 5 volumes (w/v) of chloroform-ethanol (2:1) at room temperature for 24 h. The mixture was filtered with Whatman No 1 filter paper. The filtrate of the macerate was vigorously shaken with 20% distilled water to obtain two layers. The upper layer (ethanol layer) was separated from the lower layer (chloroform layer). The ethanol and the chloroform layers were concentrated in a rotary evaporator, air-dried, weighed and stored in the refrigerator.

Vacuum liquid chromatography (VLC) of the ethanol layer

Twenty-five grams (25 g) of the ethanol layer was purified by vacuum liquid chromatography using silica gel (230-400 mesh, 3.0 x 30 cm, 500 g) as the stationary phase and eluted with n-hexane (1500 ml), chloroform (3500 ml), ethyl acetate (2500 ml) and methanol (2500 ml) to obtain the n-hexane, chloroform, ethyl acetate and methanol fractions respectively.

Anti-oxidant vitamin analyses

The concentrations of vitamins A, C and E were determined for the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* using the methods described by Pearson (1976).

Proximate analyses

Percentage composition of moisture, crude fibre, ash, crude fats, crude proteins and carbohydrates were determined for the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* using the methods described by AOAC (1990).

Animals

Adult male Wistar rats of between 8 and 12 weeks old with an average weight of 125 ± 25 g and albino mice weighing 30 ± 5 g were obtained from the Animal house of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. The rats were acclimatised for one week under a standard environmental condition with a 12 h- light and dark cycle and maintained on a regular feed and water *ad libitum*. The Principles of Laboratory Animal Care were adhered to. The University Animal Research Ethical Committee approved the experimental protocol.

Chemicals and reagents

The chemicals used for this study were of analytical grade. They included the following: diclofenac (Evans Medical Plc., Nigeria), ranitidine (Evans Medical Plc., Nigeria), absolute ethanol, chloroform, acetone, diethyl ether, chloroform-acetic anhydride, TCA-chloroform, oxalic acid, boric acid, methyl red, ferric chloride, α-α-dipyridine, concentrated and dilute H_2SO_4, loctanol, sodium sulphate, KOH, NaOH and Indolephenol reagent (Sigma-Aldrich, Inc., St. Louis, USA).

Acute toxicity study

The acute toxicity and lethality (LD_{50}) of the chloroform and ethanol layers of the chloroform-ethanol extract were determined using mice according to slightly modified method of Lorke (1983).

Gastric ulcer index and curative ratio

The methods described by Rezq and Elmallh (2010) were employed in these studies.

Statistical analysis

The data obtained were subjected to one-way analysis of variance (ANOVA). Significant differences are observed at $p<0.05$. The results are expressed as means ± standard errors of means (SEM). The analysis was done using the computer software known as Statistical Product and Service Solutions (SPSS), version 18.

RESULTS

Anti-oxidant vitamin contents of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis*

Table 1 shows that the ethanol layer contained the higher concentration of vitamin C (0.79 ± 0.03 mg/100 g) while the chloroform layer contained the higher concentrations of vitamins A (1.83 ± 0.08 μg/g) and E (1.17 ± 0.07 mg/100 g).

Table 1. Anti-oxidant vitamin contents of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis.*

Anti-oxidant vitamins	Chloroform layer	Ethanol layer
A (µg/100 g)	1.83 ± 0.08	1.79 ± 0.08
C (mg/100 g)	0.62 ± 0.05	0.79 ± 0.03
E (mg/100 g)	1.17 ± 0.07	1.04 ± 0.05

Values are expressed as means of three determinations ± SEM.

Table 2. Proximate compositions of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis.*

Proximate constituents	Compositions (%)	
	Chloroform layer	Ethanol layer
Moisture	3.15 ± 0.11	4.08 ± 0.18
Crude fibre	5.41 ± 0.29	5.29 ± 0.27
Ash	5.09 ± 0.27	4.12 ± 0.18
Crude fats	4.43 ± 0.13	3.98 ± 0.12
Crude proteins	17.24 ± 0.43	17.80 ± 0.29
Carbohydrates	64.68 ± 1.40	64.73 ± 1.35

Values are expressed as means of three determinations ± SEM

Proximate compositions of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis*

The ethanol layer contained higher percentage of crude proteins (17.80 ± 0.29%) while the chloroform layer contained higher percentages of crude fibre (5.41 ± 0.29%), ash (5.09 ± 0.27%) and crude fats (4.43 ± 0.13%) as shown in Table 2.

The acute toxicity and lethality (LD_{50}) of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis*

There was no lethality or any sign of toxicity in the four groups of mice that received 10, 100 and 1000 mg/kg body weight of the chloroform and ethanol layers and 5 ml/kg body weight of the vehicle respectively at the end of the first phase of the study. At the end of the second phase of the study, no death was recorded in the groups of mice that received 1600, 2900 and 5000 mg/kg body weight of the chloroform and ethanol layers within 24 h of administration.

Effects of the graded doses of the n-hexane, chloroform, ethyl acetate and methanol fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* on ulcer index

The ulcer index of the rats in the ulcer-untreated group (group 2) was significantly ($p<0.05$) higher (6.02 ± 0.31 mm) when compared to the value (0.00 ± 0.00 mm) obtained for the rats in the normal control group (group 1) which received 5 ml/kg body weight of normal saline only. The ulcer indices of the rats in groups 3 (1.02 ± 0.06 mm) and 11 (2.89 ± 0.15 mm) were significantly ($p<0.05$) lower than that of the rats in group 2 (6.02 ± 0.31 mm). There were however, no significant ($p>0.05$) differences between the ulcer indices of the rats in groups 4 (5.91 ± 0.30 mm), 5 (5.68 ± 0.29 mm), 6 (5.22 ± 0.30 mm), 7 (4.01 ± 0.30 mm), 8 (5.79 ± 0.29 mm), 9 (5.33 ± 0.31 mm) and 10 (4.15 ± 0.32 mm) and the value obtained for the rats in group 2 (6.02 ± 0.31 mm) as shown in Figure 2.

Effects of the graded doses of the n-hexane, chloroform, ethyl acetate and methanol fractions of the ethanol layer of chloroform-ethanol extract of the leaves of *D. edulis* on curative ratio

Table 3 shows that the curative ratios of the rats in groups 3 (83.06 ± 7.05%) and 11 (51.99 ± 5.38%) were significantly ($p<0.05$) higher than that of the rats in group 2 (0.00 ± 0.00%). There were however, no significant ($p>0.05$) differences between the curative ratios of the rats in groups 4 (1.83 ± 0.22%), 5 (5.65 ± 0.54%), 6 (13.29 ± 1.37%), 7 (33.39 ± 3.03%), 8 (3.82 ± 0.39%) 9 (11.46 ± 1.28%) and 10 (30.40 ± 2.95%) and that of the rats in group 2 (0.00 ± 0.00%).

DISCUSSION

The amounts of the anti-oxidant vitamins in the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* were: vitamins A (1.83 ± 0.08 and 1.79 ± 0.08 µg/100 g), C (0.62 ± 0.05 and 0.79 ± 0.03 mg/100 g) and E (1.17 ± 0.07 and 1.04 ± 0.05 mg/100 g) respectively. This means that the vitamins A and E present in the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* were more in the chloroform layer than in the ethanol layer whereas vitamin C was more in the ethanol layer than in the chloroform layer. The anti-ulcerogenic properties of the fractions as seen in the present study might be, in part, due to the amount of the anti-oxidant vitamins in the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis.* While the optimal nutrient intake to promote wound healing is unknown, increased needs for carbohydrates, proteins, zinc and vitamins A, C and E have been documented (Scholl and Langkamp, 2001). Vitamin C aids in wound healing by increasing collagen synthesis, neutrophil function and angiogenesis. Collagen production also serves to produce a barrier to pathogens (Scholl and Langkamp, 2001).

Proximate compositions of the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* were: moisture (3.15 ± 0.11 and 4.08 ± 0.18%),

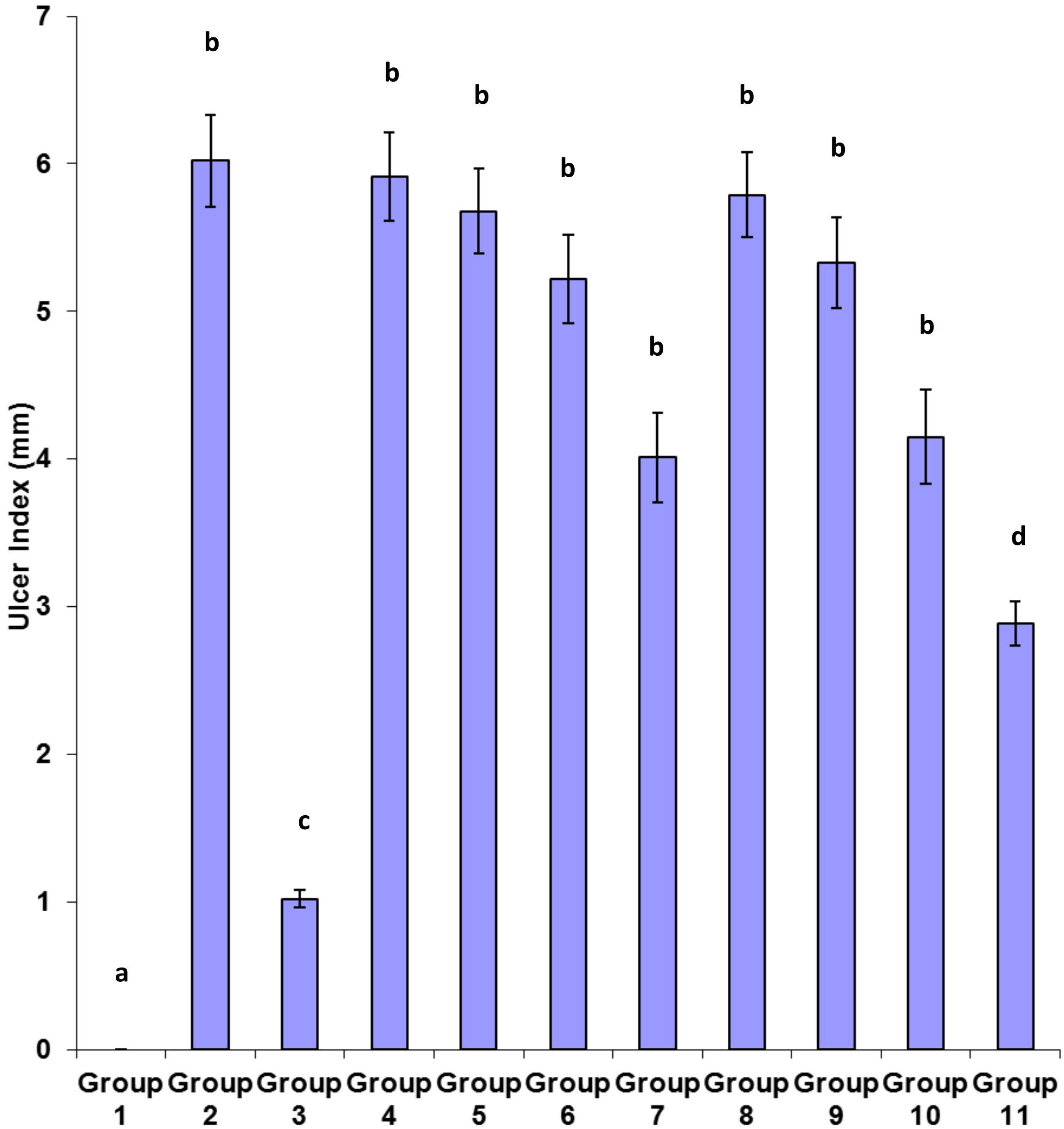

Figure 2. Effects of the n-hexane, chloroform, ethyl acetate and methanol fractions of the chloroform-ethanol extract of the leaves of *D. edulis* on ulcer index [values for groups with different alphabets are significantly ($p<0.05$) different]. Group 1: 5 ml/kg body weight (b.w) of normal saline only (Normal control); Group 2: 5 ml/kg b.w of normal saline + 200 mg/kg b.w of diclofenac (Positive control); Group 3: 150 mg/kg b.w of ranitidine + 200 mg/kg b.w of diclofenac (Standard control); Group 4: 100 mg/kg b.w of the n-hexane fraction + 200 mg/kg b.w of diclofenac; Group 5: 200 mg/kg b.w of the n-hexane fraction + 200 mg/kg b.w of diclofenac; Group 6: 100 mg/kg b.w of the chloroform fraction + 200 mg/kg b.w of diclofenac; Group 7: 200 mg/kg b.w of the chloroform fraction + 200 mg/kg b.w of diclofenac; Group 8: 100 mg/kg b.w of the ethyl acetate fraction + 200 mg/kg b.w of diclofenac; Group 9: 200 mg/kg b.w of the ethyl acetate fraction + 200 mg/kg b.w of diclofenac; Group 10: 100 mg/kg b.w of the methanol fraction + 200 mg/kg b.w of diclofenac; Group 11: 200 mg/kg b.w of the methanol fraction + 200 mg/kg b.w of diclofenac.

crude fibre (5.41 ± 0.29 and 5.29 ± 0.27%), ash (5.09 ± 0.27 and 4.12 ± 0.18%), crude fats (4.43 ± 0.13 and 3.98 ± 0.12%), crude proteins (17.24 ± 0.43 and 17.80 ± 0.29%) and carbohydrates (64.68 ± 1.40 and 64.73 ± 1.35%), respectively. This implies that the crude proteins and carbohydrates present in the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* were more in the ethanol layer than in the chloroform layer. The anti-ulcer properties of the fractions as evidenced in this study could also be, in part, due to the presence and/or amounts of some of the proximate constituents in the ethanol layer of the chloroform-ethanol

Table 3. Effects of the graded doses of the n-hexane, chloroform, ethyl acetate and methanol fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* on curative ratio.

Groups	Curative ratios (%)
1	100.00 ± 0.00[a]
2	0.00 ± 0.00[b]
3	83.06 ± 7.05[c]
4	1.83 ± 0.22[b]
5	5.65 ± 0.54[b]
6	13.29 ± 1.37[b]
7	33.39 ± 3.03[b]
8	3.82 ± 0.39[b]
9	11.46 ± 1.28[b]
10	30.40 ± 2.95[b]
11	51.99 ± 5.38[d]

Values for groups with different superscript letters are significantly ($p<0.05$) different.

extract of the leaves of *D. edulis*. Carbohydrates and proteins are important in the wound healing process or recovery from ulcers (Crowe and Brockbank, 2009). While proteins are required for all stages of the wound healing process including fibroblast proliferation, collagen synthesis, angiogenesis and immune function, adequate intake of carbohydrates prevents the utilisation of the proteins as energy substrates (Crowe and Brockbank, 2009).

Acute toxicity test on the chloroform and ethanol layers of the chloroform-ethanol extract of the leaves of *D. edulis* using mice showed that each of the layers administered to the mice at a dose as high as 5000 mg/kg body weight by oral route within twenty-four hours of observation, had no fatal effect on the mice which indicates that the chloroform and ethanol layers have low toxicity at high doses when administered by oral route.

Evaluation of the effects of the fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* on gastric ulcer experimentally induced with diclofenac in rats showed that, they dose-dependently decreased and increased the ulcer indices and curative ratios respectively of the treated rats with the effects of the 200 mg/kg body weight of the methanol fraction being the most remarkable. These indicate that the leaves of *D. edulis* contain anti-ulcerogenic agents. The observations that the fractions of the ethanol layer decreased and increased the ulcer indices and curative ratios respectively of the treated rats might be attributed to the anti-oxidant vitamin content and proximate composition of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* as shown by the results of their analyses. Also, the finding that diclofenac induced ulcer in all the treated rats is in accord with the reports of Verma et al. (2011), Santos et al. (2012), Wandale et al. (2012) and Khan and Khan (2013). The mechanism by which diclofenac and other NSAIDs cause injury to the gastric mucosa is mainly due to the inhibition of cyclooxygenase and suppression of prostaglandin-mediated effects on mucosal protection. Besides, it has been proposed that neutrophil and oxygen radical-dependent microvascular injuries may be important processes that lead to mucosal damage in response to NSAID administration (Wallace, 2000). These agents cause the activation of neutrophils and their adherence to the vascular endothelium, hence, blocking capillaries and reducing local gastric blood flow. NSAIDs inhibit COX and thereby reduce the intrinsic ability of the mucosa to resist injury induced by endogenous and exogenous aggressors (Wandale et al., 2012). Administration of exogenous prostaglandins has also been shown to decrease or prevent gastric damage induced by NSAIDs (Wallace, 2001). Therefore, the decreases and increases in the ulcer indices and curative ratios respectively of the rats treated with the fractions of the ethanol layer of the chloroform-ethanol extract of the leaves of *D. edulis* in this study are in parts, indications of the anti-ulcerogenic properties of the leaves of *D. edulis*.

In conclusion, the findings of this study lend credence to the use of the leaves of *D. edulis* in traditional medicine for the treatment of gastric ulcer.

Conflict of Interests

The authors have not declared any conflict of interests.

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