International Journal of Advanced Science and Research
ISSN: 2455-4227, Impact Factor: RJIF 5.12
www.newresearchjournal.com/science
Volume 1; Issue 4; April 2016; Page No. 1-6

Phytoconstituents, acute toxicity study and protective effect of ethanol extract of *Dennettia Tripetala* seed against aspirin-induced ulcer in rats

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Abstract
In this study, the phytoconstituents, acute toxicity and protective effects of ethanol extract of *Dennettia tripetala* (pepper fruit) seed against aspirin-induced ulcers in rats were evaluated using classical methods. For the antiulcer study, 20 albino wistar rats weighing between 120-250 g were acclimatized for 14 days and randomly assigned into 5 groups of 4 rats each. The rats were treated orally as follows: Group 1 (3 ml/kg 3% Tween 80), Groups 2, 3 and 4 (100, 200 and 400 mg/kg b.w. of ethanol extract of *D. tripetala* seed respectively) and Group 5 (100 mg/kg b.w. Omeprazole). Ulcerogenic lesions were induced using aspirin, and mean ulcer index, percentage inhibition of ulcer lesions and histological profiles were determined. Phytochemical analysis of *D. tripetala* seeds showed the presence of alkaloids, reducing sugars, cardiac glycosides, carbohydrates, flavonoids, tannins, oil and steroids in high concentration. Similarly, moderate amounts of saponins and resins, and low amounts of glycosides, acid and phlobatannins were detected. There was neither clinical sign nor mortality after 24 hours post-treatment observation in the rats, up to 5000 mg/kg b.w. of the extract in the acute toxicity study. Oral administration of ethanol extract of *Dennettia tripetala* seed at doses of 100, 200 and 400 mg/kg b.w. inhibited gastric ulceration in rats in a dose-dependent manner when compared to normal control. There was a strong positive correlation between % ulcer inhibition and concentration of extract administered ($R^2 = 0.997$). Histological examination of the rat gastric tissues indicated that rats treated with extract and standard drug showed reduction in the ulcerations when compared with the control group. The results showed that ethanol extract of *D. tripetala* seed has potent and dose-dependent anti-ulcer effect against aspirin-induced ulcer which could be attributed to its flavonoid content.

Keywords: *Dennettia tripetala*, Ulcer index, rats, phytochemicals, acute toxicity

1. Introduction

Plants synthesize a wide variety of chemical compounds that perform various biological functions such as defence against physicochemical and biological attacks. Herbal practitioners harness this observation and make use of medicinal plants and their extracts in the management of ailments such as those caused by fungi, bacteria as well as viruses [1].

Dennettia tripetala (pepperfruit) is an indigenous fruit tree of the family Annonaceae [2]. The fruits (Fig. 1) appear red when ripe and green when unripe. The mature fruits constitute the main edible portion. The leaves, fruit, bark and root of the plants possess strong pepperish and pungent spicy taste with a characteristic aroma and fragrance. The young leaves and fruits have instinctive spicy taste [3]. The fruits are chewed in different forms (fresh green, fresh ripened red, black dry fruit and dry seed). It is widely grown in the rain forest zones of Nigeria and some parts of West Africa. It is commonly consumed for its spicy taste and used to welcome visitors in eastern part of Nigeria [4]. It is also used in traditional medicine as a remedy for cough, fever, toothache, diarrhea, diabetes, and nausea in pregnant women [5].

Fig 1: Ripe (red) and unripe (green) seeds of *D. tripetala*

Preliminary studies showed that various parts of the plant have bioactivities. The essential oil from the fruits has analgesic and anti-inflammatory effects [6]. Extract from the leaf has antioxidant and antimicrobial activities [7, 8]. Ethanol extract of the fruit is haematotoxic [9]. The present study was aimed at evaluating the phytoconstituents, acute toxicity and antiulcer potential of ethanol extract of the plant seed extract.

2.0 Materials and Methods

2.1 Materials

2.1.1 Plant Material

Fresh mature fruits of *D. tripetala* (pepper fruit) were purchased from Obollo market, Obollo-Afor Town, Udenu Local Government Area of Enugu State, Nigeria and were authenticated at the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria. Voucher specimen was deposited in the herbarium of the Department. Seeds of *D. tripetala* fruits were air-dried and crushed into coarse powders using a mechanical grinder for extraction.

2.1.2 Experimental Animals

Twenty (20) wistar albino rats of both sex with body weight between 120-250 g and eighteen (18) wistar albino mice of both sexes and body weight between 30-50 g obtained from the Department of Pharmacology, Faculty of Pharmacy, University of Nigeria, Nsukka were used for the study. The animals were acclimatized to laboratory environment for 14 days under standard laboratory conditions and fed with animal feeds (Ladokun feeds Ibadan, Nigeria). The animals had free access to water throughout the period of experiment. The rats were used for the antiulcer study while the mice were used for the acute toxicity study. They received human care throughout the experimental period in accordance with the ethical rules and recommendations of the University of Nigeria committee on the care and use of laboratory animals and the revised National Institute of Health Guide for Care and Use of Laboratory Animal (Pub No.85-23, revised 1985).

2.1.3 Chemicals and Reagents

All reagents and chemicals used for this study were of analytical grades and they include: ethanol, sodium chloride, chloroform, formaldehyde, Tween 80, acetic acid, acetic anhydride, conc. sulphuric acid, 2% hydrochloric acid (HCl), ethanol, 1% aluminum chloride, acetone and ferric chloride (BDH Chemicals Ltd., Poole, England), Aspirin (Dispirin®) (Reckitt and Colman, UK), Omeprazole (Vinco), ammonia and Wagner's reagent, Meyer's reagent, Fehling's solution, distilled water and normal saline.

2.2 Methods

2.2.1 Extract Preparation

A quantity (2000 g) of the coarse powdered *D. tripetala* seeds was suspended in 4000 ml of ethanol in an air tight container and kept at room temperature for 24 hr. The suspension was filtered with a chess cloth, followed by Whatman No. 1 filter paper and the filtrate was concentrated using rotary evaporator at 50 ^{o}C. The concentrate was further evaporated to dryness to give an oily crude extract of percentage yield of 1.325%.

2.2.2 Qualitative Phytochemical Analysis

The phytochemical analysis of *D. tripetala* seed was done using the methods described by Harborne [10], and Trease and Evans [11].

2.2.3 Acute Toxicity

Acute toxicity (LD_{50}) study of the ethanol extract of *D. tripetala* seed was carried out using the method of Lorke [12]. Eighteen (18) albino mice (both sexes) were used, and the acute toxicity test consisted of two phases: phase I and phase II.

In the phase I, nine (9) mice were randomly assigned into three groups of three mice each. Groups 1, 2 and 3 were administered orally with ethanol extract of *D. tripetala* seed at the doses of 100, 250 and 500 mg/kg b.w. respectively. Based on the observation of the phase I study, the procedure was repeated using another set of nine (9) mice which were randomly assigned into three groups of three mice each. Groups 4, 5 and 6 of phase two were administered orally with the ethanol extract of *D. tripetala* seed at the doses of 1000, 3000 and 5000 mg/kg b.w. respectively. The mice in phase I and II were observed for general behavioral, neurological and autonomic profile for 24 hours.

Table 1: Experimental Design for Aspirin-Induced Gastric Ulcer in Rats

Groups	Treatment (administered orally (p.o))
Group 1 (normal control)	3 ml/kg of 3% Tween 80 + 200 mg/kg b.w. of Aspirin
Group 2 (low dose of extract)	100 mg/kg b.w. of extract + 200 mg/kg b.w. of Aspirin
Group 3 (mid-dose of extract)	200 mg/kg b.w. of extract + 200 mg/kg b.w. of Aspirin
Group 4 (high dose of extract)	400 mg/kg b.w. of extract + 200 mg/kg bwt of Aspirin
Group 5 (Standard control)	100 mg/kg b.w. of Omeprazole + 200 mg/kg b.w. of Aspirin

2.2.4 Aspirin-Induced Gastric Ulcer in Rats

Twenty wistar albino rats were randomly assigned into five groups of four rats each. After acclimatization, the animals were starved for 18 hours but had free access to water. Thereafter, the rats were treated with the extract, 3% Tween 80 and Omeprazole orally as described in Table 1 below. Thirty (30) min after treatment, 200 mg/kg b.w. of Aspirin was administered orally (p.o) to all the rats. After 8 hours, rats from each group were sacrificed using chloroform anesthesia and dissected, the stomach removed and opened along the greater curvature, rinsed with copious volume of normal saline and pinned on a flat PVC board. Erosions formed on the glandular portions of the stomach were counted and the ulcer index calculated as described by Singh and Majumdar [13].

2.2.5 Gross Lesion Evaluation

Ulcer indices were examined using magnifying lens (x10) and scored. Ulcers found in the gastric mucosa became visible as elongated bands of hemorrhagic lesions parallel to the long axis of the stomach. The ulcers observed in the gastric mucosa were assessed and scored using the method described by Rao *et al*. [14] as follows: 0 = no ulcer, 1 = superficial ulcer, 2 = deep ulcer, 3 = perforations. Total ulcer score (summation of all the ulcers/lesions, in all the animals for each group) was used to compute the ulcer index. The ulcer inhibition expressed in percent (%) was calculated relative to control, by the formula:

$$\%\ \text{Ulcer inhibition}\ (\%\ \text{U. I}) = \frac{1 - \text{Ulcer Index of the treated group}}{\text{Ulcer Index of the control grou}} \times 100$$

2.2.6 Histological Lesion Evaluation

The histological examination of the gastric wall specimens of wistar albino rats was done using the method described by Wang *et al*. [15]. Five rats, one from each of the 5 groups of rats were randomly selected for this study. The slides prepared from the gastric wall specimens were mounted on photomicroscope, one after the other and viewed at different magnification power of the microscope. Photograph of each of the slides was taken.

2.3 Statistical Analysis

The primary data were analyzed using one way analysis of variance (ANOVA) in IBM Statistical Product and Service Solution (SPSS) software, Version 16. The results were expressed as mean ± standard deviation (SD). The Fischer LSD post hoc test was used to test the difference between means of treated and control groups. Differences between means at $p < 0.05$ were regarded as statistically significant.

3.0 Results and Discussion

3.1 Results

3.1.1 Results of the phytochemical screening of *Dennettia tripetala* seeds

The results of the phytochemical screening of the *D. tripetala* seeds showed the presence of alkaloids, reducing sugars, cardiac glycosides, carbohydrates, flavonoids, tannins, oil and steroids in high quantity. Similarly, moderate amount of saponins and resins, and low amounts of glycosides, acid and Phlobatannins were detected (Table 2).

Table 2: Results of the phytochemical screening of *Dennettia tripetala* seeds

Phytochemicals	Bioavailability
Alkaloids	++
Glycosides	+
Reducing sugar	+++
Cardiac glycosides	+++
Carbohydrates	+++
Flavonoids	+++
Tannins	+++
Saponin	++
Resins	+
Acid	+
Oil	+++
Steroids	+++
Phlobatannins	+

Keys: + + + = detected in high quantity
+ + = detected in moderate quantity
+ = detected in low quantity.

3.1.2 Result of the Acute Toxicity Test

At the all the doses of ethanol extract of *D. tripetala* seed administered orally to the mice for acute toxicity study, there were neither clinical signs nor mortality after 24 hours post-treatment observation. Therefore, the median lethal dose (LD_{50}) value of the extract was estimated to be above 5000 mg/kg body weight (Table 3).

Table 3: Results of Phase I and Phase II of the acute toxicity test of the ethanol extract of *Dennettia tripetala* seed in mice

PHASE I		
Groups	**Dose of extract administered (mg/kg b.w.)**	**Number of deaths**
Group 1	100	0/3
Group 2	250	0/3
Group 3	500	0/3
PHASE II		
Group 4	1000	0/3
Group 5	3000	0/3
Group 6	5000	0/3

n= 3

3.1.3 Effects of Ethanol Extract of *Dennettia tripetala* on Aspirin-Induced Ulcer in Rats

The crude ethanol extract of *D. tripetala* seeds at doses of 100, 200 and 400 mg/kg b.w. (p.o) inhibited gastric ulceration in rats in a dose-dependent manner when compared to normal control (treated with 3 ml/kg b.w. of 3% Tween 80). However, the percentage ulcer inhibitions by the extracts were lower compared to that of the standard drug (Omeprazole) used (Table 4).

Table 4: Percentage inhibition and mean ulcer index of the experimented groups

Groups/Treatment	Mean Ulcer Index ±SEM	%inhibition
Group 1 (3 ml 3% Tween 80)	1.63±0.49	-
Group 2 (100 mg/kg b.w. extract)	1.17±0.32	28.22
Group 3 (100 mg/kg b.w. extract)	1.00±0.21	38.65
Group 4 (100 mg/kg b.w. extract)	0.57±0.18	65.03
Group 5 (100 mg/kg Omeprazole)	0.33±0.15	79.75

Data are mean ± SD (n = 4)

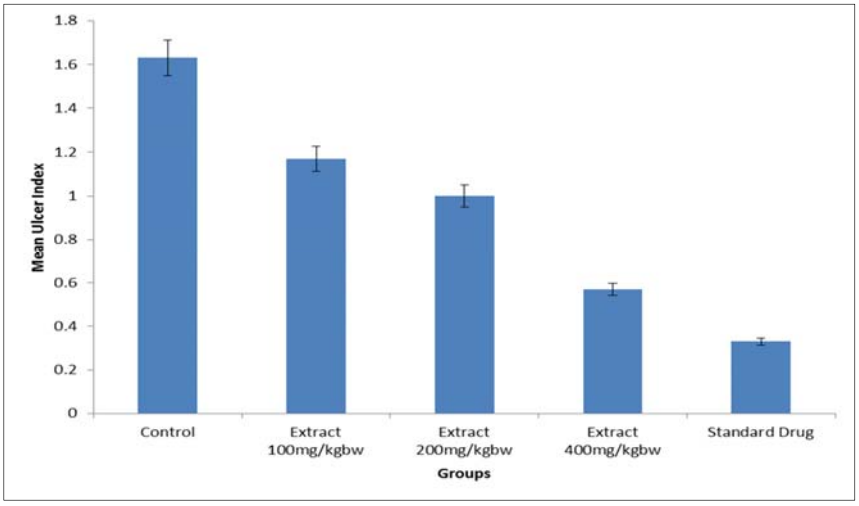

Fig 2: Effect of Ethanol Extract of *Dennettia tripetala* on Aspirin-Induced Ulcer in Rats

As shown in Fig. 2, the mean ulcer index in animals in groups 2, 3 and 4 (treated with 100, 200 and 400 mg/kg b.w. extracts) and group 5 (100 mg/kg of Omeprazole) were significantly ($p < 0.05$) higher compared to that treated with the vehicle only (3 ml/kg of 3% Tween 80). However, the mean ulcer index in animals treated with standard drug (group 5) was significantly ($p < 0.05$) higher than those treated with the extracts (Groups 2, 3 and 4).

There was a strong positive correlation between % ulcer inhibition and concentration of extract ($R^2 = 0.997$). This implies that the % ulcer inhibition effect of the plant extract was dose-dependent (Fig. 3).

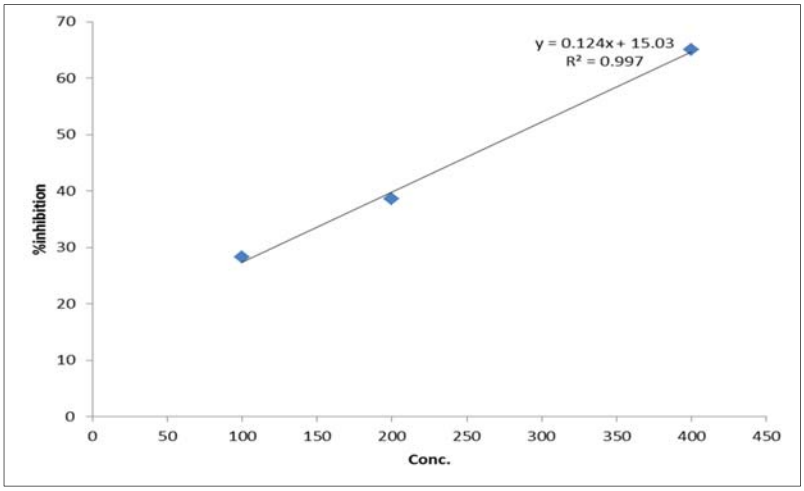

Fig 3: A correlation curve of % ulcer inhibition vs concentration of the extracts

3.4 Results of Histological Studies on Rats Treated with Extracts and Standard drug

Figs. 4, 5, 6, 7 and 8 show the photomicrographs (X 400) of the histological section of the gastric mucosa of rats treated with 3 ml/kg b.w of 3% Tween 80, extract (100, 200 and 400 mg/kg b.w.) and standard drug (100 mg/kg b.w Omeprazole). The extract-treated groups (Figs. 5, 6 and 7) showed reduction in the ulcerations when compared with the control group (Fig. 4). Numerous inflammations marked by disorientation of the gastric epithelium were observed in the photomicrographs of control rats (Fig. 4). Result in Fig. 7 shows that the extract when administered at high dose (400 mg/kg b.w.) protected the mucosa surface.

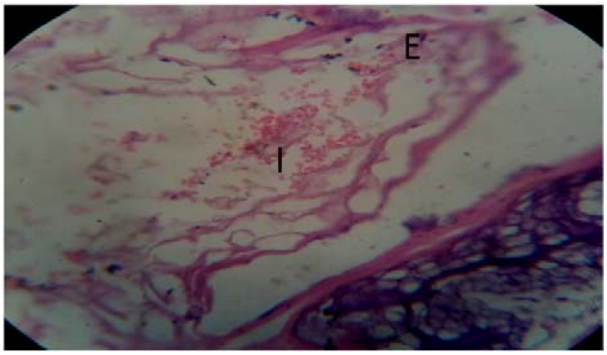

Fig 4: Histological section of the stomach from rat treated with 3 ml of 3% Tween 80, showing numerous inflammatory cells (I) and severe erosions (E). H and E. X400.

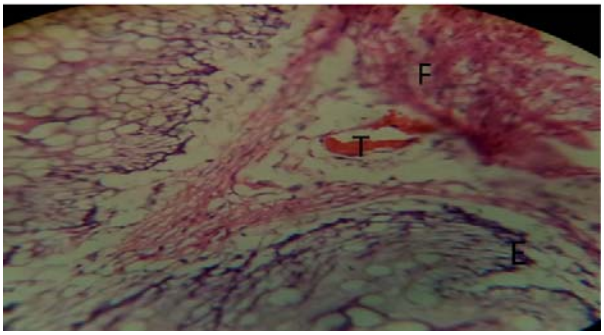

Fig 5: Histological section of the stomach from rat treated with 100 mg/kg b.w. of the extract, showing fibrinoid necrosis (F), thrombosis (T) and erosion (E). H and E. X400.

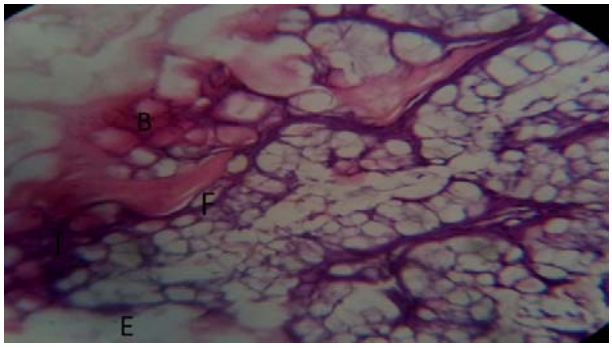

Fig 6: Histological section of the stomach from rat treated with 200 mg/kg b.w. of the extract, showing excessive bleeding (B), fibrinoid necrosis (F), inflammatory cells (I) and erosion (E). H and E. X400.

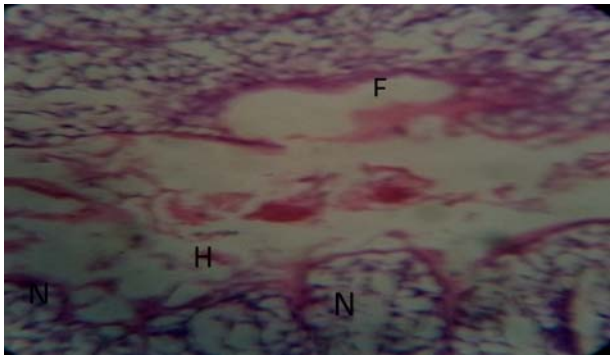

Fig 7: Histological section of the stomach from rat treated with 400 mg/kg b.w. of the extract, showing normal gastric mucosa (N), mild erosion (E), and possible hemorrhaging (H). H and E. X400.

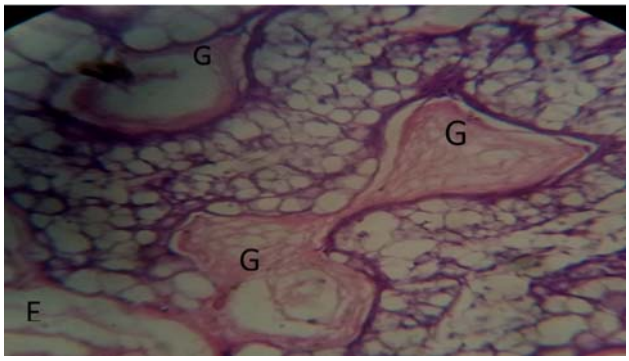

Fig 8: Histological section of the stomach from rat treated with 100 mg/kg b.w. of the standard drug (Omeprazole), showing re-epithelialization of mucosal surface (i.e. granulation tissue; G) and mild erosion (E). H and E. X400.

3.2 Discussion

Many plants are known to have a long history of use for soothing inflamed and injured mucous membranes in the digestive tract. In the present study, the phytoconstituents, the acute toxicity and antiulcer potential of ethanol extract of *D. tripetala* seed in rats were evaluated. Aspirin was used to induce ulcerogenic lesion because it has the ability to cause gastroduodenal ulceration and this effect is related to the ability of aspirin to suppress prostaglandin synthesis [16, 17]. In the stomach, prostaglandins play a vital protective role, stimulating the secretion of bicarbonate and mucus, maintaining mucosal blood flow and regulating mucosal cell turnover and repair [18]. Thus, the suppression of prostaglandin synthesis by NSAIDs result in increased susceptibility to mucosal injury and gastroduodenal ulceration [19].

Another mechanism by which aspirin damages the gastric mucosa is the increased production of nitric oxide (NO) due to the over expression of intestinal nitric oxide synthase (iNOS) [20]. NO is a mediator not only of gastrointestinal mucosal defense [21], but also causes its damage in high concentration [22]. It has been shown that different concentrations of NO have completely opposite effects in same tissue [23]. In general, the mucosal and epithelial iNOS isoforms produce low amounts of NO. However, the high quantity of NO produced by iNOS damages the epithelium [24]. The excessive release of NO from gastric epithelial cells induced by aspirin has been reported to exert detrimental effects [25]. In this study, exposure of the animals to aspirin may have caused severe ulcerogenic effects, as aspirin is known to increase gastric acid secretion which is involved in the formation of aspirin-induced mucosal lesions. Hence, inhibition of aspirin-induced increase in iNOS expression in gastric mucosa leads to a reduction in gastric mucosal damage [20].

The results of the present study showed that ethanol extract of *D. tripetala* seed has potent and dose-dependent anti-ulcerogenic activity against aspirin-induced ulcer. Treatment with 400 mg/kg b.w. gave a percentage ulcerogenic inhibition which is comparable to that of the standard drug, Omeprazole. Osuagwu and Eme [8] showed that the extract of *D. tripetala* leaf is rich in phytochemicals. The present study showed that *D. tripetala* seed is rich in phytochemicals with antiulcer properties such as flavonoids and alkaloids [26]. Flavonoids present in the extract could be responsible for the protective effect on ulcer. This could be by reducing iNOS activity and inhibiting the production of gastric ulcers, even in the presence of aspirin. However, further studies is needed to fully elucidate the mechanisms of antiulcer action of ethanol extract of *D. tripetala* seed and more detailed pharmacological actions could be explored for potential development of potent and effective antiulcer agents. Efforts are in progress to formulate drugs from the plant extract and investigate the precise mode of action [27].

The histological findings of the gastric mucosa obtained from the control (Group 1) rats showed distorted gastric glands, a damaged mucosal epithelium, inflammatory exudates and cellular debris after aspirin treatment (Fig. 4). The protection against these Histopathological changes induced by the varied doses of the extract and standard drug to rats resulted in the maintenance of glandular organization and the structure of muscular is mucosa (Figs. 5, 6, 7, and 8). The correlation analysis showed a strong positive correlation between ulcerogenic inhibition and concentration of extract, which

implies that the antiulcer effect is dose-dependent. The result of the acute toxicity study showed that the plant extract could be safe up to 5000 mg/kg b.w. which implies that the extract is of low toxicity.

3.3 Conclusion

D. tripetala seed is rich in phytochemicals and ethanol extract of the seed has low toxicity. The study also showed that methanol extract of *D. tripetala* seeds possess dose-dependent antiulcer effect on aspirin-induced ulcer in albino wistar rats and may be a promising material for treatment of ulcer genesis and gastric mucosal injury. Studies are suggested to elucidate the biochemical mechanism of gastro protective effects of *D. tripetala.* The active constituents of the plant extract needs to be isolated and screened for bioactivity for drug formulation.

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