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Anti-Gastric Ulcer Effect of Betulinic Acid in Male Albino Rats

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Summary: Betulinic acid (BA) is a lupane-type triterpene that has been identified and isolated from various plant species used in ethnomedicine in various cultures across the world. This study was undertaken to elucidate the mechanisms underlying the anti-ulcer effect of Betulinic acid. The effect of BA on indomethacin-induced ulcer, gastric mucus secretion, gastric mucus cells count, basal and histamine-induced gastric acid secretion and levels of malondialdehyde formation were studied using dose of 0.5, 1.5, and 3.0 mg/kg. The results showed that BA reduced indomethacin-induced ulceration significantly and significantly increased ($p < 0.05$) gastric mucus secretion in the 1.5 mg/kg and 3.0 mg/kg BA treated rats compared to the control rats. There was a significant increase ($p < 0.05$) in the mucus cells count in all the treated groups which is in a dose- dependent manner compared to the control group. There was significant decrease ($p < 0.05$) in gastric acid secretion in each of the BA treated groups compared to the control. Malondialdehyde concentration significantly decrease ($P < 0.05$) in all the treated groups compared to the control. The anti-gastric ulcer effect of BA may be mediated via decreasing gastric acid secretion, increasing gastric mucus secretions, increasing the number of gastric mucus cells and also by reducing the level of MDA concentration.

Keywords*:* Betulinic acid, Gastric acid, Mucus secretion, Malondialdehyde.

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INTRODUCTION

Natural products have been used for combating human diseases for thousands of years since they exhibit a wide range of biological properties that can be exploited for medical application such as pentacyclic triterpenes that have been investigated for anti-inflammatory properties (Safayhi and Sailer, 1997). Among these are the lupane-type triterpenes betulinic acid and betulin tested in a number of in vitro and in vivo model systems. Betulinic acid has been isolated from various parts of plants species used in ethnomedicine (Takeoka *et al*, 2000) and in particular, those reported to have anti-secretory and anti-ulcer properties (Adesanwo *et al*, 2003; Pisha *et al*,1995). Animal studies reveal that BA lack toxicity even at high concentrations (Pisha *et al*, 1995). Other reported biological effects of betulinic acid include anti-spasmodic, anti-retroviral (Mayaux *et al*, 1994; Evers *et al*, 1996), anti-tumor (Pisha *et al*, 1995; Schmidt *et al*, 2007) and antimicrobial (Adekunle *et al*, 2003; Setzer *et al*, 2000). The imbalance between the gastroduodenal mucosal defensive factors such as bicarbonate, mucus and aggressive factors such as acid, pepsin (Sostres and Lanas, 2011) has been associated with gastric ulcer and many of the anti-ulcer drugs in use have been found to have adverse effects and recurrent infection after a few weeks (Chan and Leung, 2002). There is dearth of information on the mechanisms of the anti-ulcer effect of betulinic acid. In this study, the effect of betulinic acid on indomethacin-induced ulcer, gastric mucus secretion, gastric mucus cell count, basal and histamine stimulated gastric acid secretion and malondialdehyde concentration were evaluated as means of elucidating the mechanisms of the anti-ulcer effects.

MATERIALS AND METHODS

Animals

Adult male albino rats of Wistar strains weighting 210-240 grams and divided into five studies groups of thirty-two animals each were used for this study. The animals were purchased from the Central Animal House, College of Medicine, University of Ibadan, Nigeria and were kept four per cage in a clean, well-ventilated room maintained under standard condition (12 hours light and 12 hours darkness). The animals were allowed to acclimatize before the studies commences. They were fed with commercial rat chow obtained from Ladokun Livestock Feeds Limited, Ibadan Oyo State Nigeria and water was provided *ad libitum*. The studies were conducted in accordance with the Organization for Economic Development (OECD) guidelines on good laboratory practice (OECD, 2001).

Chemicals

Betulinic acid (Aldrich Sigma) was dissolved in Dimethyl sulphuroxide (DMSO), Sodium acetate (May & Baker, England), Indomethacin (Merck, Sharp & Dohme, Canada) was dissolved in distilled water

with a pinch of sodium carbonate (Na2CO3) added. All other chemicals and reagents were of analytical grade and were products of Aldrich Sigma Chemical limited, Poole, England or Sigma Chemical Company, St Louis, MO, USA.

Experimental Design

The animals for the studies were further divided into five groups of eight animals each. Group 1 is the control and was treated with DMSO in normal saline. Groups 2, 3, 4 and 5 were treated with 0.5mg/kg, 1.5mg/kg, and 3.0mg/kg of BA dissolved with DMSO respectively for seven (7) days. The studies were on effects of betulinic acid on indomethacin-induced ulcer, gastric mucus 4 secretion, gastric mucus cell count, basal and histamine-stimulated gastric acid secretion and possible anti-oxidant effect of betulinic acid.

Effect of betulinic acid on indomethacin induced gastric ulceration

A total of thirty-two (32) rats were used for this study divided into four groups, each with eight rats. Group 1 was the control, treated with DMSO in normal saline. Groups 2, 3 and 4 were treated with BA doses of 0.5mg/kg, 1.5 mg/kg and 3.0 mg/kg orally respectively (Durst *et al*, 2002). The animals were fasted for 24 hours only but allowed free access to water. The method of indomethacin-induced gastric ulceration adopted was that described in previous works (Njar *et al*, 1995; Oluwole *et al*, 2008). One hour after the administration of betulinic acid and DMSO in normal saline, indomethacin at 40mg/kg BW (Merck, Sharp & Dohme, Canada) was administered subcutaneously to all the animals in all the groups. After 4 hours, the animals were sacrificed by cervical dislocation. Their stomachs were removed, opened by cutting along the whole length of the greater curvature, turned inside out and then pinned to a cork mat. This was moistened with normal saline to prevent autolysis. The method used for assessment of the degree of gastric ulceration was that of Alphin and Ward (1967) as modified by Elegbe and Bamgbose (1976). Macroscopic examinations of the washed stomachs were carried out with a magnifying hand lens.

$$Mean\ Ulcer\ Score = \frac{\text{Total Ulcer Score}}{n}$$

Where n = number of rats

Effect of Betulinic acid on Gastric Mucus Secretion

Each glandular portion of the stomach in sacrificed rats was opened along the lesser curvature, everted and soaked for two hours in 0.1% Alcian blue dissolved in 0.16M sucrose buffered with 5 0.05M sodium acetate, adjusted to pH 5.8 with hydrochloric acid. Uncomplexed dye was removed with two successive washes at 15 and 45 minutes in 0.25M sucrose. Dye complexed with mucus was diluted by immersion in 10ml aliquots of 0.5M Magnesium Chloride for 2 hours. The resulting blue solutions were shaken briefly with equal volume of diethyl ether and absorbance of the aqueous phase was measured at 605nm using spectrophotometer (Corney *et al*., 1974). The absorbance of each solution was used to calculate the various concentrations of dye. The weight of dye (expressed in mg) was deduced using a standard curve. The weight of the dye was expressed over the weight of the stomach to give the weight of the mucus.

Thus, gastric mucus secretion (mg/g tissue)

$$= \frac{\text{Weight of dye (mg)}}{\text{Weight of stomach (g)}}$$

Effect of Betulinic acid on Gastric Mucus Cell Count

The rats were sacrificed by cervical dislocation, the stomachs removed and weighed. The glandular portion of each stomach was opened along the lesser curvature and histological slides prepared using Haematoxylin and Eosin as stain. Gastric mucus cell count was done by counting the number of gastric mucus cells that stain with Haematoxylin and Eosin under calibrated light microscope. These are indicated as blue patches. The gastric mucus cells were counted in five randomly selected area of the gastric mucosal tissue. Five cubic boxes each with an area of $1mm^2$ were assessed. This method is an improvement over the earlier described approach for counting by Li *et al* (2002).

Effect of Betulinic acid on basal and histamine-stimulated gastric acid secretion.

Basal Secretion

Each rat was anaesthetized with urethane according to their body weights (0.6 ml/100g body weight), abdomen dissected open and stomach exposed. The femoral vein was exposed by blunt dissection and later cannulated for intravenous administration of histamine. Esophageal cannula was passed down into the stomach. This was used to perfuse the stomach with normal saline. The perfusion was regulated at a rate of 1.0 ml/min using a modified Langardoff apparatus and 10ml of gastric effluent was collected from the stomach cannula. This perfusion preparation is the modified method of Ghosh and Schild (1958).

Histamine-stimulated Secretion

A dose of 0.1mg/g body weight histamine was injected intravenously (i.v) through the femoral vein into the rats. Four samples of gastric contents were collected at 10 minutes intervals. The total acidity of the gastric contents was determined by using 0.0025N NaOH with an initial drop of 1% phenolphthalein added and titrated to end-point.

Effect of Betulinic acid on malonialdehyde (MDA) concentration.

Lipid peroxidation which is also a marker of oxidation was assessed by measuring Thiobarbituric acid

reactive substances (TBARS) produced according to the method of Gutteridge and Wilkins (1982). This method is based on the reaction between 2-thiobarbituric acid (TBA) and malonialdehyde (MDA) which is an end-product of lipid peroxides during lipid peroxidation. On heating in acidic solution, a pink coloured complex was produced that absorbs maximally at 532 nm on the spectrophotometer. 0.1ml of the test sample was mixed with 0.5ml of 10% TCA and 0.5ml of 75% TBA was then added. The mixture was placed in water bath at 80°C for 45 minutes. The absorbance of the resulting pink colour solution was measured against a reference blank of distilled water at 532nm. The test sample was calibrated using the MDA as standard and the result was expressed as the amount of free MDA produced or MDA quantified by using the molar extinction coefficient, C of 1.56×10^5 $M^{-1}cm^{-1}$ according to the expression of Adam Vizi and Seregi (1982).

$$\text{MDA (units/g tissue)} = \frac{\text{Absorbance of sample}}{\text{Molar extinction coefficient,C}}$$

Statistical Analysis

Data are expressed as the mean ± Standard Error of Mean (SEM). Data were analysed using one-way Analysis of Variance (ANOVA) and Graph Pad prism 4. The differences in mean were considered significant at $p \leq 0.05$.

RESULTS

Effect of betulinic acid (BA) on indomethacin-induced gastric ulceration

The mean ulcer score decreases with increasing doses of BA when compared with the control (Table 1). Each administered dose of 1.5 mg/kg BA (0.8± 0.10) and 3.0 mg/kg BA (0.3± 0.09) compared to the control rats (7.0± 0.27) showed significant reduction in mean ulcer score ($p < 0.05$). However, the difference in mean ulcer score for 0.5 mg/kg dose of BA (6.3 ± 0.42) and the control (7.0± 0.27) was not significant ($p > 0.05$). Also it was noticed that, as the dose of betulinic acid increases in the treated animals, percentage inhibition tends toward 100%. These showed a lower incidence of ulceration. The inhibition of gastric ulceration by betulinic acid is therefore dose-dependent.

Gastric Mucus Secretion

The results in figure 1 showed the gastric mucus secretion in the 1.5 mg/kg (4.9 ± 0.22) and 3.0 mg/kg BA treated rats (5.2 ± 0.09) being significantly different ($p < 0.05$) compared to the control rats (4.4± 0.20). Whereas, no significant difference ($p>0.05$) was observed in mucus secretion in the 0.5 mg/kg BA treated rats (4.6 ± 0.16) compared to the control rats (4.4 ± 0.20).

Table 1: The effect of betulinic acid (BA) on indomethacin induced gastric ulceration

Treatment	Mean Ulcer score [a]	Inhibition of ulceration (%)[b]
Control	7.0 ± 0.27	-
BA (0.5mgkg^{-1})	6.3 ± 0.42	10.7
BA (1.5mgkg^{-1})	0.8 ± 0.10*	89.3*
BA (3.0mgkg^{-1})	0.3 ± 0.09*	96.4*

[a]Values are Mean ± SEM for 8 animals per group. [b]Percentage inhibition as described by Raji et al, 2000. *$p < 0.05$ significantly lower compared with control.

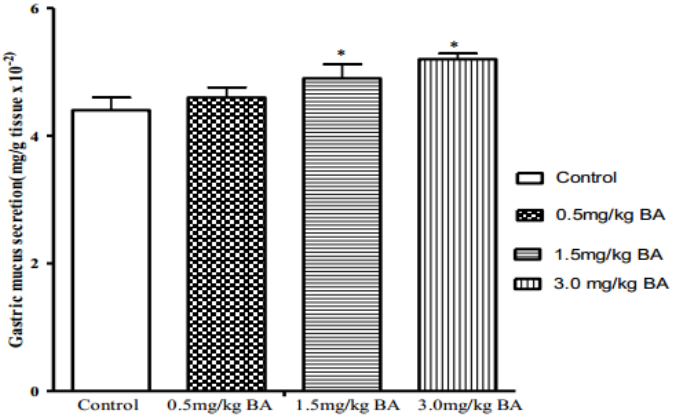

Figure 1: Effect of Betulinic Acid on Gastric Mucus Secretion

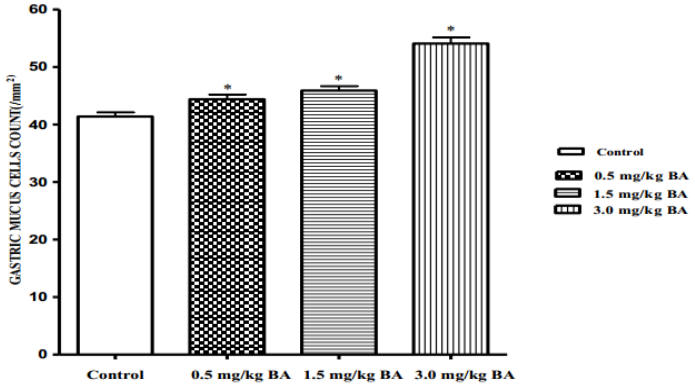

Figure 2. Effect of Betulinic Acid on Gastric Mucus cells count (mm^2)

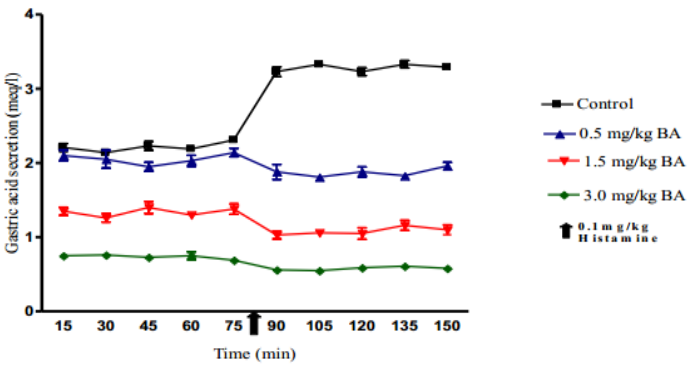

Figure 3: Effect of Betulinic acid on Gastric acid secretion

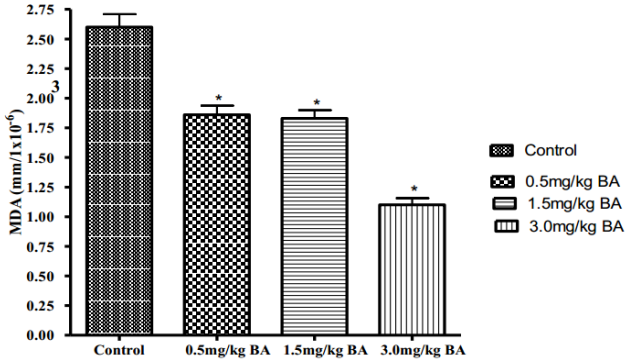

Figure 4: Effect of Betulinic acid on Malonialdehyde (MDA) Concentration

Gastric acid secretion

From fig. 3, there were decreases in basal gastric acid secretion between 0-50 minutes in all the treated rat groups. After the 50th minutes, histamine a known secretagogue was administered intravenously through the femoral vein. After 10 minutes there was a sharp increase in gastric acid secretion in the control rats, while the BA treated groups showed a fall in gastric acid secretion that were all significantly different ($P < 0.05$), compared to those of the control group. Analysis of the 50th - 70th minutes interval also showed a significant decrease in the pretreated rats compared to the control rats.

Malonialdehyde (MDA) Concentration

The results obtained shows that there is a significant decrease ($P < 0.05$) in MDA concentration between the treated rats groups; 0.5 mg/kg BA (1.86 ± 0.076), 1.5 mg/kg BA (1.83 ± 0.069) and 3.0 mg/kg BA (1.10 ± 0.056) compared to the controls group (2.60 ± 0.110).

DISCUSSION

The results obtained showed that BA has anti-ulcer effect (Table 1) and a stimulatory effect on gastric mucus secretion (Fig. 1) and gastric mucus cells (Fig. 2). These effects are similar to that of known drugs such as sucralfate and misoprostol reported to increase gastric mucus production in vivo through increasing inositol triphosphate (IP_3) content by activating phospholipase C (Slomiany et al, 1991). The resulting IP_3 elicited Ca^{2+} mobilization is then involved in the stimulatory effect of sucralfate.

BA in the present study significantly suppressed gastric acid secretion when compared with the control group ($p < 0.05$). The result (Fig. 3) clearly suggests that there is a good relationship between reduction of gastric acid secretion by BA and its anti-ulcer effect. Several workers have reported that prostaglandins of the A, E and F types are potent anti-secretory agents. BA being a triterpene exhibits similar effect to that of related triterpenoid, sodium carbenoxolone which has been reported to protect gastric mucosa from acid effect by selectively inhibiting prostaglandin F_2 (Aguwa and Okunji, 1986). Also BA had been reported to inhibit prostaglandin synthesis in vitro (Carter, 1980). BA shares the same five membered ring structures of H2 receptor antagonists with Cimetidine, Ranitidine and Famotidine that are known to relieve or heal peptic ulcers (Yamada, 1996). In this regard, BA may be acting as antihistaminic agent while the stimulation of gastric acid secretion might be inhibited competitively by selective H_2 -receptor antagonists (Hirschowitz and Molina, 1983).

The pretreatment of the animals with BA significantly decrease ($p < 0.05$) the MDA concentration compared to the control (Fig. 4). This reduction of MDA concentration may be due to the ability of BA to increase anti-oxidant activity. This supports other studies that had demonstrated a reduction in lipid peroxidation of the gastric mucosa (DelaLastra and Motilva, 1999). Other studies have also shown that the protective activity of gastric mucus is due to the anti-oxidant activity conferred on it by its rich glycoprotein content (Oluwole and Saka, 2001). The results of these studies showed that the anti-gastric ulcer effect of BA may be mediated via decreasing gastric acid secretion, increasing gastric mucus secretions, increasing the number of gastric mucus cells and also by reducing the level of MDA concentration. Thus BA could be used as a promising drug against peptic ulcer. More studies are needed to shed more light in all these areas.

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