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Full Length Research Paper

Effect of Hydro-Methanolic Leaf Extract of *Indigofera pulchra* on Gastric Mucosal Damage and Acid Secretion in Rats

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ABSTRACT

Indigofera pulchra (family: *fabaceae*) is found throughout the tropical and subtropical regions of the world. It has been used for medicinal purposes for centuries from Sudan to the dry deciduous parts of Madagascar for variety of conditions and ailments related to stomach, intestinal, liver, circulatory and immune system support .This study was aimed at investigating the effect of hydro-methanolic extract of this plant as it affects gastric mucosal integrity and basal gastric secretions using the ethanol-induced ulcer model. Phytochemical screening of the plant revealed the presence of flavonoids, alkaloids, tannins and cardiac glycosides amongst others, while acute toxicity studies (Lorke's method, 1983) revealed a median lethal dose above 5000mg/kg indicating that the plant is less toxic. A total of 60 rats were used for the two studies, with 30 rats for the mucosal integrity study and 30 rats for the basal acid secretion studies. The first group was subdivided into four(A,B,C,D) with subgroup A,B, and C containing five rats each, and subgroup D containing fifteen rats. Subgroup A served as normal control and was given normal saline while subgroup B represented negative control and was administered 70% ethanol, subgroup C was given cimetidine and served as positive control. Subgroup D was further subdivided into three experimental groups which contained five rats each and were administered *Indigofera pulchra* extract at a dose of 100mg/kg, 200mg/kg, and 400mg/kg respectively 30 minutes prior to the administration of 70% ethanol to induce gastric mucosal damage followed by pyloric ligation. The same procedure was followed in major group II to study basal gastric output. Ulcer and preventive indices were assessed for mucosal integrity while volume of gastric juice, titratable acidity and acid output were assessed for basal gastric secretions. The result showed a dose –dependent decrease in both ulcer and preventive indices. It also showed a significant ($p<0.05$) decrease in volume of gastric juice, titratable acidity and acid output in dose-dependent manner with the three experimental doses administered with the highest reduction at the 400mg/kg dose. The results obtained suggest that this plant extract down-regulated all those parameters which might be attributed to the presence of the phytoconstituents present in the fraction. Therefore, this plant possesses gastro protective and antisecretory effects further explaining the folkloric use of this plant in the therapy of peptic ulcer disease.

Keywords; Basal Secretions, Ethanol, Gastroprotection, *Indigofera pulchra*,

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INTRODUCTION

Peptic ulcers are breakages or discontinuities that can occur in the mucosal epithelial lining of either the stomach, small intestine, large intestine or the Merkel's diverticulum along the gastrointestinal tract (Falase and Akinkugbe, 2010). They are known to occur worldwide (Cemek et al., 2010) and are major causes of morbidity and mortality (Chaturvedi et al., 2007). The pathophysiology of peptic ulcers has been centralized on an imbalance between aggressive and protective factors. Factors such as stress, cigarette smoking, nutritional deficiencies, inadequate dietary habits, hereditary predisposition and frequent ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) all are known to increase the gastric ulcer incidences (Klein et al.,2010). In developing countries, usually 50-90% of

the populations are infected with Helicobacter pylori, which is the main organism responsible for majority of peptic ulcer cases, and children acquire the infection soon after being weaned. Many natural products and modern synthetic drugs have been used to treat peptic ulcer disease, but so far a complete cure has not been achieved, and exploration of new anti-ulcer drugs has remained a field of active research (Bandyopadhyay et al.,2001).Although there are many products in the market for the treatment of gastric ulcers, including antacids, proton-pump inhibitors, anticholinergics and H_2-receptor antagonists, most of these drugs produce several adverse reactions such as hypersensitivity reactions, arrythmias, impotence, gynaecomastia, nephrotoxicity, and haemopoetic changes(Chang and Leung.,2002; Scholl et al.,2005).Development of tolerance and incidence of relapses and side-effects on clinical evaluation make their efficacy arguable, promoting non-drug compliance to therapy (Santin et al., 2011). In addition, most of these medications are expensive, which further compound financial burden for their usage (Santin et al., 2010). This has been the basis for the development of new antiulcer drugs, which include herbal drugs (Altinkaynak et al., 2003).

Herbs are used in many domains including medicine, nutrition, flavouring, beverages, dyeing, repellants, fragrances and cosmetics (Djeridane et al., 2006). The plant Indigofera pulchra has been used for medicinal purposes for centuries, both as herb and as well as when pressed into oil in Asia, Middle East and Africa. Traditionally, this plant has been used for a variety of conditions and ailment related to respiratory health, stomach, intestinal, kidney, liver, circulatory and immune system support, and for general well-being. The aim of the present study was to evaluate the effectiveness of the traditional claim, using Hydro-Methanolic extract the plant leaves on gastric mucosal damage and secretions.

MATERIALS AND METHODS

Plant material: *Indigofera pulchra* plant leaves were obtained at the Botanical Garden of Department of Biological Sciences, Ahmadu Bello University, Zaria during the month of July, 2008. Botanical identification and authentication was done at the Herbarium section with a voucher specimen (No:6558) deposited for future references.

Extraction of the plant material: The air dried leaves weighing about 3kg were crushed and pounded with pestle and mortar. The powder was extracted with aqueous ethanol (70%) in a Soxhlet Extractor, concentrated using rotary evaporator at reduced pressure and suspended in methanol. The extract was further concentrated in-vacuo and the concentrate obtained that yielded about 80% of the residue.

Phytochemical screening of the fractions: The preliminary analysis of the extract was conducted for the presence phytoconstituents using standard procedures for analysis (Evans, 2002 and Harborne, 2007).

Acute toxicity studies: Lethal Dose (LD_{50}) determination was conducted using Lorke's Method, 1983. In the initial phase, male rats were divided into three groups of 3 rats each, making a total of 9 rats. The rats were treated with the fraction of the extract at doses of 10, 100 and 1000 mg/kg subcutaneously. Animals were observed for 24 hours and the number of death(s) or those that showed neurological signs were recorded. In the second phase, the animals were grouped into 4 groups of one rat each and treated with the fraction at appropriate doses subcutaneously. The rats were observed for 4 h for deaths or neurological signs, and the final LD_{50} was calculated as the square root of the highest non-lethal dose in which the animal survived multiplied by the lowest lethal dose in which the animal died.

Drugs and chemicals/reagents: Cimetidine (Lek Pharma, Slovenia), Chloroform (Abbott Laboratories, UK), Phenolphthalein indicator (BDH Poole, England), Sodium Hydroxide (NaOH) (BDH Poole, England) for the preparation of 0.01N NaOH solution, Phosphate Buffered Saline (PBS), Hydrochloric Acid (HCl) 0.1N (Sigma-Aldrich, USA), Ethanol (Riel-de-Haen, Germany). All other chemicals and reagents were analytical grade.

Experimental animals: A total of sixty (60) adult male albino Wistar rats were used in this study. They were obtained from the Animal House, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. Their weights ranged from 180 – 240g. They were maintained under similar conditions of humidity, temperature and light/dark cycle respectively and each animal was kept in a single individual cage, with wide-meshed galvanized wire bottoms to decrease coprophagy as much as possible. The rats were given access to food and water ad libitum for two weeks to acclimatize, prior to the commencement of the experiment. The rats were treated in accordance to the internationally accepted principles of laboratory animal use and care. At the time of the experiment, all treatments were conducted between 9:00 and 10:00 (GMT+1) h to minimize variations in animal response due to circadian rhythm. The animals were divided into the following groups and subgroups for gastric mucosal damage and gastric secretion studies respectively.

Experimental design: The Experiment was divided into two major groups namely group I and II

Group I: Study of gastric mucosal damage

Group IA: Five rats were administered normal saline (1ml/kg orally) which served as normal control.

Group IB: Five rats were administered 70% Ethanol (1 ml orally) to induce gastric mucosal damage and served as negative control.

Group IC: Contained five rats and were administered with only Cimetidine 100mg/kg subcutaneously and served as positive control group.

Group ID: Consisted of three subgroup with five rats each (n=5) to Study the effect of Indigofera pulchra extract. The extract was administered 30 minutes prior to the administration of 70% Ethanol at the doses of 100mg/kg, 200mg/kg and 400mg/kg.

Group II: Study of basal gastric secretion

Group IIA; Contained five rats administered normal saline (1ml/kg orally) and served as control for this group.

Group IIB: Contained five rats administered 70% Ethanol (1 ml orally)for the study of basal gastric secretions and served as negative control.

Group IIC: Contained five rats and were administered Cimetidine 100mg/kg alone subcutaneously and served as positive control group.

Group IID: Fifteen rats were used for the study of the effect of Indigofera pulchra extract on basal acid secretion. The rats were divided into three subgroups; each subgroup received the extract at doses of 100mg/kg, 200mg/kg and 400mg/kg orally respectively, all doses were given 30 minutes prior to the administration of 70% Ethanol after which basal gastric acid secretions were evaluated.

Induction of gastric ulceration

After 48 hours of starvation, the animals were weighed and maintained in their individual cages. Then, 70% ethanol was administered orally followed by the respective graded doses of the extract and the animals were then deprived of both food and water for 7 h (Urushidani et al., 1979). The animals were later sacrificed by decapitation (Satoh et al., 1983). Their stomachs were opened along the greater curvature, rinsed slowly with running water, stretched out as much as possible by the use of metal pins on No.1 Whatman's filter paper on a ceiling board.

Quantitative assessment of mucosal damage

The ulcerated areas in each stomach were measured with a transparent (mm) ruler scale, the result of each group were expressed as ulcer index (U.I) in mm of mean ulcer ± standard error of mean (Scepovic and Radmanovic, 1984) and was calculated by multiplying ulcer score by 100 (Robert et al., 1968). Ulcer score for each group was calculated by dividing the total number of ulcers in each group by the total number of rats in that group (Robert et al., 1968).The percentage preventive index was calculated according to the method of Hano et al (1976), which is expressed as:

$$\text{Preventive Index (\%)} = \frac{\text{(U.I. Ethanol} - \text{U.I Extract/drug) Ethanol}}{\text{U.I. Ethanol}} \times 100$$

Collection of gastric secretion

The gastric juice was collected according to the technique of Shay et al. (1954) as modified by Levine (1965), where oesophageal ligation was avoided. The animals were briefly fasted for 48 hrs to ensure complete emptying of the stomach, but allowed water ad libitum. Each animal was weighed at the end of the fasting period. Light chloroform anaesthesia was used (Juliane et al., 2009), abdomen of each rat was opened via a midline incision and the stomach exteriorated. A pyloric ligature was made using a thread with care to avoid damage to the blood vessels or traction to the stomach. The abdomen was then closed by suture, cleaned thoroughly with distilled water or saline, and the animal was allowed to recover.

After 3 hrs, the rats were sacrificed by decapitation, abdomen of each of the animals were opened. The oesophagus was ligated, and the stomachs removed and washed with distilled water or saline. An opening along the greater curvature was made (Nwafor et al., 2000) and the gastric content drained into a graduated centrifuge tube, and the volume measured.

Analysis of the gastric juice

The volume of 3 hours gastric secretion was measured after being subjected to centrifugation at 1,006 x g for 15 mins. A given volume of the gastric juice (1ml) was titrated against 0.01N NaOH. An end point of pH 7.0 as determined colorimetrically at 280nm by phenolphthalein indicator was used (Grossman, 1973; Davenport, 1977).The values were calculated as micro-equivalents per litre (Meq/L), which is equal to the number of millitres (ml) of 0.01N NaOH required to neutralize 1ml of gastric juice.

Titritable Acidity = Volume of 0.01 N NaOH (mol) which neutralized 1ml of gastric juice

10Acid Output was calculated by multiplying the volume (ml) of the gastric juice of each animal by the titratable acidity in that animal.

Statistical analysis

All data were expressed as Mean ± S.E.M (standard error of the mean) using SPSS Version 20. Statistical evaluation was done by analysis of variance (ANOVA) followed by post-hoc analysis by Duncan and Scheffe. Values of $P<0.05$ were considered significant (Microcal Software Inc., Northampton, USA)

RESULTS

Phytochemical Analysis

It revealed the presence of carbohydrates, saponins, tannins, flavonoids, resins, alkaloids and cardiac glycosides

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Acute Toxicity Studies

The toxicity studies of the extract in the first phase after being observed for 24hr, the rats did not show any signs and symptoms of toxicity or death. In the second phase, none of the rats produced any toxic symptoms or mortality up to the dose level of 5000mg/kg body weight, hence, they were considered safe for further pharmacological screening.

Effect of on ulcer and preventive indices

As shown in tables I, pre-treatment with 1ml/kg/rat normal saline produced an ulcer index of 7.30±0.45mm, with a preventive index of 47%. Administration of 70% ethanol significantly elevated ($p<0.05$) the ulcer index at 10.10 ± 0.35mm. It has the highest ulcer index compared to all the groups with a zero preventive index.

Hydromethanolic portion of the extract at 100, 200 and 400mg/kg administered, followed by 70% ethanol produced ulcer indices of 1.56 ± 0.59mm, 0.94 ± 0.56mm and 0.72 ± 0.26mm with preventive indices of 76, 89 and 94% respectively. The highest dose of 400mg/kg of the extract significantly ($p<0.05$) reduced the ulcer index compared to the control group, and the standard drug cimetidine at a dose of 100mg/kg.

Table 1:

Ulcer Index in Millimetre (mm) and Preventive Index in Percent (%) for Normal Saline, 70% Ethanol, Cimetidine and Hydromethanolic Extract - Treated Portions of *Indigofera pulchra*

Groups (n=5)	Ulcer Index (mm) Mean ± SEM	Preventive Index (%) Mean ± SEM
Normal Saline (1ml/kg/rat)	7.30 ± 0.45	47.03 ± 0.01 (47%)
Ethanol (70%) (1ml)	10.10 ± 0.35*	0.00 ± 0.00* (0%)
I.pulchra (100mg/kg) + 70% Ethanol	1.56± 0.59*	76.24 ± 0.95* (76%)
I.pulchra (200mg/kg)+ 70% Ethanol	0.94± 0.56*	89.11 ± 0.63* (89%)
I.pulchra (400mg/kg) + 70% Ethanol	0.72± 0.26*	94.06 ± 0.63* (94%)
Cimetidine (100mg/kg)	2.10 ± 0.35*	79.21 ± 0.32*s (79%)

* P<0.05 compared to Normal Saline

Table 2:
Comparism Of the Effect of Different Doses of *Indigofera Pulchra* On the Volumes of Gastric Juice, Titrable Acidity and Acid Output as Compared to Normal Control, Ethanol and Cimetidine

Groups (n=5)	Volume of Gastric Juice (ml/3hr) Mean ± SEM	Titratable Acidity (mEq/L) Mean ± SEM	Acid Output (µEq/hr) Mean ± SEM
Normal Saline (1ml/kg/rat)	3.68 ± 0.19	42.40 ± 1.78	52.34 ± 4.49
Ethanol (70%) (1ml)	5.38 ± 0.18	27.40 ± 4.56	22.18 ± 4.43*
I.pulchra (100mg/kg) + 70% Ethanol	3.58 ± 0.29	47.00 ± 3.88	25.08 ± 8.20
I.pulchra (200mg/kg)+ 70% Ethanol	2.42 ± 0.22*	45.80 ± 2.58	18.04 ± 3.58
I.pulchra (400mg/kg) + 70% Ethanol	1.06 ± 0.07*	42.20 ± 4.01	13.94 ± 3.00
Cimetidine (100mg/kg)	1.72 ± 0.15*	38.40 ± 4.71	21.62 ± 2.56*

Effect on Basal Gastric Secretions

Table 2 indicate the effect of *Indigofera pulchra* extract on gastric juice analysis, mainly on the major parameters of gastric secretion viz; volume of gastric juice, titratable acidity and acid output. It shows that ethanol administration caused a significant ($p<0.05$) increase in the volume of gastric juice compared to the normal saline control from 3.68 ± 0.19mls/3hr to 5.38 ± 0.18mls/3hr. Titratable acidity and acid output were significantly reduced. Co-administration of 100mg/kg *I. pulchra* extract with 70% ethanol recorded a gastric juice volume of 3.58 ± 0.29mls/3hr among the three different doses of the extract used, though slightly lower than the control group. The extract at 200 and 400mg/kg plus ethanol recorded mean values of gastric juice 2.42 ± 0.22mls/3hr and 1.06 ± 0.07ml/3hr respectively, both showing a significant decrease compared to normal saline and ethanol control groups. The titratable acidities obtained were 41.80 ± 4.02 and 39.51 ± 4.02mEq/L, while acid outputs obtained were 17.58 ± 2.81 and 2.73mEq/hr, with the 400mg/kg of the extract group significantly ($p<0.05$) decreased compared to control.

For cimetidine 100mg/kg on basal secretions, the volume of gastric juice significantly ($p<0.05$) decreased compared to the normal saline control, and was found to be 1.72 ±0.15ml/ 3hr. Acid output was 21.62 ± 2.56 mEq/hr which equally showed a reduction compared to control. However, the titratable acidity in that group was insignificant.

DISCUSSION

We evaluated the gastro-protective effect of the hydromethanolic portion of *I. pulchra* using the model of acute ulcer induced by a non-steroidal anti-inflammatory compound indomethacin. it was observed that the group that received 100mg/kg of the standard drug cimetidine alone, showed a significant decrease in gastric lesions ($p<0.05$) compared to the ethanol-treated group, this corroborated with the studies of Marivane *et al*. (2011) who reported a decrease in lesion index on the gastric mucosa of rats treated with the H_2-receptor antagonist cimetidine compared to ethanol.

Equally, it was found that rats treated with different doses of *Indigofera pulchra* treatment (100mg/kg, 200mg/kg and 400mg/kg) significantly reduced the lesion index compared to the standard control group ($P<0.05$) in a dose-dependent manner in this ethanol-induced model. The highest dose of 400mg/kg produced the highest preventive index of 94% above that of the standard drug cimetidine. A similar finding was reported by Dashputre*et al*. (2011) that a significant reduction in lesion index, total injured area and the percentage gastric mucosal injured area when extract of *Abutilon indicum* was used on indomethacin ulcer model.

Both *Abutilon indicum* and *Indigofera pulchra* are found to contain some flavonoids, especially quecertin and kacempferol mainly in glycosodic form and these are secondary metabolites that are widely distributed in nature with several biological activities including gastroprotective potentials (Martin *et al*., 1998). *Croton urucurana* with high flavonoid content also exhibited same mucosal cytoprotective potentials (Alves *et al*., 2008). The flavonoid content of this extract had prevented and exerted a protective effect possibly by its inherent ability to scavenge free radicals, inhibit lipid peroxidation, increase mucous and prostaglandin contents of the gastric mucosa (Alanko *et al*., 1999).

Through phytochemical analysis of this plant, apart from flavonoids, the presence of terpenoids, tannins, cardiac glycosides, steroids, saponins were detected amongst others. To further support and corroborate the possible factors for the mucosal cytoprotection exhibited by this portion of *I.pulchra* containing flavonoids are the studies by Alcaraz and Hoult, 1985 that flavonoids increase mucosal prostaglandin content, inhibit histidine decarboxylase thereby decreasing histamine secretion (Bromer and Landry, 1985).

The presence of saponins in this extract of *I.pulchra* to improve on mucosal integrity has been reported in several other studies where plants containing saponins have been shown to possess antiulcer activity in several experimental ulcer models. Among these, saponins isolated from the leaves of *Maytenus robusta* and the fruit of *Kochia scoparia* (which contain approximately 20% of saponins) have been demonstrated to possess gastroprotective properties (Matsuda *et al*., 2003; Sergio *et al*., 2007) in conformity with this study. The protective activities of all these saponins are not due to inhibition of gastric acid secretion, but probably due to activation of mucous membrane protective factors (Borreli and Izzo, 2000). Moreover, several plants containing high amount of saponins have been shown to possess antiulcer activity in several experimental bioassays, probably acting as an activator of mucus membrane stabilizing factors (Morikawa *et al*., 2006; Lima *et al*., 2006). Similarly, presence of tannins, terpenoids in this extract further validate the cytoprotective property in the gastric mucosa observed in our study as reported by Al-Rehaily *et al*. (2002), where several saponins, tannins, terpenoids were found to possess gastroprotective properties. Additionally, Terpenoids are a widespread class of secondary compounds with several pharmacological activities, including anti-inflammatory and antiulcer activities (Arrietta *et al*., 2003).

Furthermore, the interference of the extract using same model was also evaluated on parameters of basal gastric secretion. This method is an important procedure that reveals the possible changes of gastric secretory physiological parameters relating to volume of gastric secretion, titratable acidity, acid output relating to pH. Our findings suggest that the fraction interfered with these major basal secretory indices of gastric juice.

Considering that the volume of gastric juice which is mainly acidic encompasses mucus, hydrochloric acid, pepsinogen, bicarbonates, intrinsic factor of Castle and protein plays a vital role in the aetiopathogenesis of gastric mucosal integrity, it is plausible to consider the two fractions as putative cytoprotective agents. This assumption was made based on the observation that the different quantities or volume of gastric juices obtained in this study showed a general inhibitory pattern with regard to its production in the stomach.

All the *I.pulchra*-treated groups of 100, 200 and 400mg/kg administered showed a reduction that was significant in terms of volume of gastric juice, with the 400mg/kg significantly decreasing compared to both ethanol and normal saline controls. A similar finding was reported by Halter *et al*.,(1988) and Hatazawa *et al*.,(2006).

With regard to the titratable acidity, the extract at 100, 200 and 400mg/kg administered showed a reduction that was significant ($P<0.05$).This decrease in stomach acidity facilitated the healing of gastric ulcers, because exposing the mucosa to high concentrations of acid favours mucosal epithelial damage (Laine *et al*., 2008). Contact between stomach acid and the mast cells of the submucosa and lamina propria causes mast cell degranulation and the release of histamine, which stimulates hydrochloric acid secretion by parietal cells and produces inflammation and acute oedema at the site of contact (Rodrigues *et al*., 2008). Overall, concentration of hydrogen ions in the gastric juice decreases reflective of high pH, further aggravating the aggressive factors (Lullmann *et al*., 2000). Consistently, hyperacidity is known to result due to uncontrolled hypersecretion of hydrochloric acid from parietal cells of gastric mucosa through the proton pump H^+-K^+ ATPase (Kishor *et al*., 2007). These results suggest a direct protective effect of this extract on gastric mucosal barrier.

In conclusion, the reported results have validated the folkloric use of *I.pulchra* in the therapy of peptic ulcer disease. It protects against the ethanol-induced gastric ulceration and down-regulated the basal acid secretory parameters to promote mucosal cytoprotection. The presence of phytoconstituents in this medicinal plant might be responsible for those pharmacological actions observed. In this context extracts and active principles from plants could serve as leads for the development of new drugs. Therefore, this plant specie(s) have a great potential to be used as a gastroprotective agent either alone or in combination with others.

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