EFFECT OF n-BUTANOL FRACTION OF *NIGELLA SATIVA* (L.) SEED EXTRACT ON NSAID-INDUCED GASTRIC MUCOSAL INJURY ON GASTRIC HORMONES IN RATS

SALEH, M.I.A.[1*], ISA, A.I.[1], MABROUK, M.A.[1]., MUSA, K.Y.[2], MOHAMMED, A.[1], ALHASSAN, A.[1] AND HEBA, D.[3]

[1]Department of Human Physiology, [2]Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Nigeria.

[3]Animal Science Unit, El-Kasr el-Ain, Cairo University, Cairo, Egypt.

ABSTRACT

This study investigated the gastroprotective effect of n-butanol fraction of *Nigella sativa* L. (family: Ranunculaceae) seed extract on gastric hormonal changes of prostaglandins, somatostatin and gastrin in rats using ELISA kits. The rats were grouped into 9 groups with 5 rats per group and treated with extract fractions at 50, 100 and 200 mg/kg subcutaneously, followed with indomethacin and cimetidine used as standard drugs at various doses. Preliminary phytochemical screening of the fraction revealed the presence of flavonoids, saponins, alkaloids, glucocinolates, tannins and steroids, while the LD_{50} of the fraction was found to be above 5000 mg/kg subcutaneously in rats according to Lorke's method. There was a significant increase ($p<0.05$) in the prostaglandin and somatostatin levels in a dose-dependent manner with highest increase at 200 mg/kg with consequent decrease in the level of gastrin compared to the control. The results obtained suggest a safe and gastroprotective role for *Nigella sativa* n-butanol fraction at the doses used in this study.

Keywords: Hormones, NSAID's, *Nigella sativa*, gastroprotection

***Correspondence**: alhajisaleh@yahoo.com

INTRODUCTION

Gastric and duodenal ulcers are illnesses that affect a considerable number of people in the world. It is estimated that about 10% of the world's population will have an ulcer at some point during their lives [1]. Factors such as stress, cigarette smoking, nutritional deficiencies, inadequate dietary habits, hereditary predisposition and frequent ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) increase the gastric ulcer incidences [2]. The mucosal barrier's ability to resist gastric injury requires the integrity of cytoprotective factors such as the pre-epithelial mucus bicarbonate layer, the intercellular tight junctions connecting the epithelial cells, the submucosal acid sensors, the presence of prostaglandins, cytokines, enteric nerves and blood flow [3]. Cyclooxygenase (COX) is constitutively expressed in the gastrointestinal tract in large amounts and has been reported to maintain mucosal integrity through continuous generation of prostaglandins. The COX-produced prostaglandins enhance gastric mucosal protection by stimulating mucus and bicarbonate secretion, epithelial cell proliferation and increasing mucosal blood flow [4]. The gastric ulcer production by indomethacin is due to the fact that this compound inhibits the synthesis of cytoprotective prostaglandins, synthesized by COX-1 and COX-2 in the stomach tissue [5]. 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. For example, in West Africa, 80% of children may be infected with the organism by age five. Many natural products and modern synthetic drugs have been used to treat the peptic ulcer disease, but so far a complete cure has not been achieved or discovered, and exploration of new anti-ulcer drugs has remained a field of active research [6]. Although there are many products in the market for the management of gastric ulcers, including antacids, proton-pump inhibitors, anti-cholinergics and H_2-receptor antagonists, most of these drugs produce several adverse reactions, such as hypersensitivity reactions, arrhythmias, impotence, gynaecomastia, nephrotoxicity, and haemopoetic changes [7]. Development of tolerance and incidence of relapses and side-effects on clinical evaluation make their efficacy arguable, further promoting non-drug compliance to therapy. In addition, most of these medications are expensive, which further restricts their use [8].

This has been the basis for the development of new antiulcer drugs, which include herbal drugs [9]. Herbs are used in many domains including medicine, nutrition, flavouring, beverages, dyeing, repellants, fragrances and cosmetics [10]. The plant *Nigella sativa* has been used for medicinal purposes for centuries, both as a herb and when pressed into oil in Asia, Middle East and Africa. It has been traditionally used for a variety of conditions and treatments related to respiratory health, stomach, intestinal, kidney, liver, circulatory and immune system support, and for general well-being. The seeds are used as carminative, aromatic, stimulant, diuretic, antihelmintic, galactagogue and to increase sweat. They are used as a condiment in curries. A tincture prepared from the seeds is useful in indigestion, loss of appetite, diarrhoea, dropsy, amenorrhoea, dysmenorrhoea and in the treatment of worms and skin eruptions. Externally, the oil is used as an antiseptic. To arrest vomiting, the seeds are roasted and given internally [11].

MATERIALS AND METHODS

Plant material

Nigella sativa dried seeds were obtained during the month of July, 2011 from Sabon-Gari market in Zaria, Nigeria. Botanical identification and authentication was carried out by Mr. U.A Gallah at the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University, Zaria. A voucher specimen (No: 101201) was deposited at the herbarium for future references.

Extraction of the plant material

Nigella sativa seeds weighing 2 kg 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, suspended in water and partitioned with n-butanol to obtain the n-butanol fraction. The fraction obtained was further concentrated *in-vacuo* and the residue collected. The fraction yielded about 80% of the residue.

Phytochemical screening of the fractions

The n-butanol fraction was analysed for the presence of flavonoids, alkaloids, saponins, steroids, anthraquinones, resins and reducing sugars using standard procedures for analysis [12].

Acute toxicity studies

Median lethal Dose (LD_{50}) determination was conducted using the method of Lorke [13]. In the initial phase, male rats (indicate weight/age of rats were divided into three groups of 3 each, making a total of 9 rats. The rats were treated with the n-butanol fraction of the extract at doses of 10, 100 and 1000 mg/kg subcutaneously. Animals were observed for the first 4 hr and occasionally for 24 hr and the number of death(s) or those that showed neurological signs were recorded. In the second phase, four animals were grouped into 3 groups of one rat each and treated with the fraction at 1600, 2900 and 5000 mg/kg subcutaneously. The rats were observed for 24 hr 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

The following drugs and reagents were used for the study. Cimetidine (Lek Pharma, Slovenia), Indomethacin (Liomethacin$^{(R)}$) (Cheisi, Egypt), Thiopental Sodium (Abbott Laboratories, UK), Phosphate Buffered Saline (PBS) solution at pH 7.4 (Life Technologies, USA), Heparin (Pfizer Pharmaceuticals, USA), Ethylene Diamine Tetraacetic Acid (EDTA) (DOW Chemical Company, USA). All other drugs and chemicals used were of analytical grade.

Experimental animals and design

A total of fifty eight (58) adult male albino Wistar rats weighing 180-240 g. were used in this study. The animals were obtained from the Animal House, Faculty of Medicine, Cairo University, Egypt. They were maintained under similar conditions of humidity, temperature and light/dark cycle respectively and each of the animal was kept in a single 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 Principles of Laboratory Animal Care. At the time of the experiment, all treatments were conducted between 9:00 and 10:00 (GMT+1) hr to minimize variations in animal response due to circadian rhythm after which blood samples were collected. The animals were divided into the following groups:-

Group 1: Normal saline: Five rats received normal saline 1 ml/kg/rat subcutaneously (SC).
Group 2: Indomethacin treatment: Five rats received indomethacin 20 mg/kg SC.
Group 3: Cimetidine treatment: Five rats received cimetidine 50 mg/kg SC.
Group 4: Cimetidine treatment: Five rats received cimetidine 100 mg/kg SC.
Group 5: Cimeticine/indomethacin treatment: Five rats received cimetidine 50 mg/kg SC 30 min prior to indomethacin administration.
Group 6: Cimeticine/indomethacin treatment: Five rats received cimetidine 100 mg/kg SC 30 min prior to indomethacin administration.
Group 7: *Nigella sativa*/indomethacin treatment: Five rats received the n-butanol fraction of *Nigella sativa* at 50 mg/kg SC 30 min prior to indomethacin administration.
Group 8: *Nigella sativa*/indomethacin treatment: Five rats received n-butanol fraction of *Nigella sativa* at 100 mg/kg SC 30 min prior to indomethacin administration.
Group 9: *Nigella sativa*/indomethacin treatment: Five rats received n-butanol fraction of *Nigella sativa* at 200 mg/kg SC 30 min prior to indomethacin administration.

Drug treatments

After 48 hr of starvation, the animals were weighed and maintained in their individual cages. Then, indomethacin 20 mg/kg was injected subcutaneously and the animals were then deprived of both food and water for 7 hr [13]. Pretreatments were administered as earlier enumerated in each of the groups above.

Collection of blood samples

Blood samples were collected using heparinized capillary tubes from the retro-orbital plexuses of each rat, and kept in EDTA bottles. The blood was immediately centrifuged at 1,006xg at 6^{o}C for 10 min. Plasma was obtained and preserved at -20^{o}C until used for the hormonal assays.

Prostaglandin assay

The animals were later sacrificed by decapitation. Their stomachs were opened along the greater curvature, rinsed slowly with water. In the already dissected rats, samples of the corpus (full thickness) were excised, weighed and suspended in 1 ml of 10 mM sodium phosphate buffer at

pH 7.4. The tissue was minced finely with scissors, and then incubated at 37^{o}C for 20-25 min [14].
An enzyme immunoassay kit Biotrack^{TM} (RPN 222) was used to measure the prostaglandin level in the buffer. 10 µL Cell lysis reagent 1 was added to the homogenate or plasma sample, incubated for 5 min followed by dissociation of PGE_2 from soluble receptors and interfering binding proteins. The antisera and PGE_2 peroxidase conjugate were reconstituted in lysis reagents 2 and added to the homogenate or plasma sample. The reagent 1 key component was then sequestered in 10 µL lysis reagent 2. The peroxidase labeled ligand bound to the antibody was immobilized to the wells of a microtitre plate, coated with second antibody. Any unbound ligand was removed by washing and the amount of peroxidase labeled PGE_2 bound to the antibody was determined by the addition of tetramethylbenzidine (TMB) substrate, incubated for 30 min and the optical density measured at 405nm [15].

Gastrin assay (merge)
It uses a polyclonal antibody to gastrin 1 to bind in a competitive manner the gastrin 1 in the sample or an alkaline phosphatase molecule, which has gastrin I covalently attached to it. The gastrin hormonal level was measured in the serum of the rats through enzyme-immunoassay kit to gastrin (Bio-ENZOlife). 50 µL and 25 µL of samples and assay buffer were pipetted into appropriate wells. The plate was incubated at room temperature on a plate shaker for 2 hr at 500 rpm covered with a plate sealer. The contents were emptied into the wells and washed by adding 400µL of wash solution to every well. The wells were aspirated or emptied, and any remaining wash buffer was removed. Five (5) µL was added to the conjugate 1:10 dilution to the TA wells afterwhich 200 µL of the pNpp substrate solution added to each well. The plate was sealed and incubated at 37^{o}C for 3hr. The reaction was stopped with Stop Solution 50 µL added to every well and was read at optical density of 405 nm [1].

Somatostatin assay
The level of somatostatin hormone was measured in the serum through enzyme-immunoassay kit to somatostatin PHOENIX Inc (EK-060-03). Added 50 µL/well of standard, sample or positive control, 25 µL primary antibody and 25 µL biotinylated peptide was incubated at room temperature (20-25^{o}C) for 2 h. Immunoplate 4x was washed with 350 µL/well of 1x assay buffer. Then, 100 µL/well of SA-HRP solution was added and incubated for 1 h at room temperature (20-25^{o}C), then 100 µL/well of TMB substrate solution was added. It was then incubated for 1hr at room temperature, reaction was terminated with 100 µL/well of 2N HCl and absorbance at optical density of 450 nm was read (1).

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 constituents

Table 1: Phytochemical constituents of n-butanol fraction of *Nigella sativa*

Phytochemical tests	n-butanol
Test for flavonoids	Present
Test for alkaloids	Present
Test for tannins	Present
Test for saponins	Present
Test for steroids	Absent
Test for anthraquinones	Absent
Test for reducing sugars	Absent
Test for resins	Present

Acute toxicity studies
The toxicity studies of the n-butanol seed extract of *Nigella sativa* 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 5000 mg/kg body weight, hence, they were considered safe for further pharmacological screening. The LD_{50} is therefore reported to be above 5000 mg/kg.

Effect of n-butanol fraction on gastric hormonal levels
As shown in Table 2, the gastric mucosal hormonal assays of prostaglandin, somatostatin and gastrin were carried out. In the indomethacin 20 mg/kg group, the gastric mucosal prostaglandin was significantly suppressed as compared to the normal saline control group ($p<0.05$). It was decreased from 64.46±2.16 to 31.40±2.84 pg/mg. somatostatin was significantly elevated compared to the normal saline control. Gastrin showed a significant reduction compared to the normal saline from 302.92±4.61 µU/mL to 94.24±6.46 µU/mL. For cimetidine at 50 and 100mg/kg pre-treatments, the mean value of gastrin measured were 305.63±7.74 and 312.41±13.48µU/mL respectively, both significantly increased above the indomethacin 20mg/kg treated group.

Table 2: The Hormonal levels of gastrin (µU/ml), prostaglandin (pg/mg) and somatostatin (µU/ml) for the various treatments.

Group (n=5)	Gastrin (µU/ml) Mean ± SEM	Prostaglandin (pg/mg) Mean ± SEM	Somatostatin (µU/ml) Mean ± SEM
Normal Saline (1ml/kg/rat)	302.92 ± 4.61	64.46 ± 2.16	21.20 ± 1.40
Indomethacin (20mg/kg)	94.24 ± 6.46*	31.40 ± 2.84*	81.06 ± 3.55*
NBut (50mg/kg) + Indo (20mg/kg)	49.19 ± 4.67*†	75.74 ± 1.61†	38.95 ± 3.63†
NBut (100mg/kg) + Indo (20mg/kg)	56.69 ± 3.37*	87.50 ± 4.71*†	47.83 ± 2.27*†
NBut (200mg/kg) + Indo (20mg/kg)	86.67 ± 2.65*	104.05 ± 2.28*†	84.68 ± 4.64*
Cimetidine (50mg/kg)	305.63 ± 7.74†	67.20 ± 1.80†	25.91 ± 2.26†
Cimetidine (100mg/kg)	312.41 ± 13.48†	67.88 ± 1.85†	24.49 ± 1.55†
Cimetidine (50mg/kg) + Indo (20mg/kg)	218.98 ± 7.38*†	62.92 ± 1.97†	25.70 ± 2.67†
Cimetidine (100mg/kg) + Indo (20mg/kg)	275.58 ± 8.92†	64.70 ± 0.80†	18.78 ± 1.74†

* P<0.05 compared to normal saline, † P<0.05 compared to indomethacin, Indo=Indomethacin, NBut=n-nutanol

Prostaglandin values were 67.20±1.80 and 67.88±1.85pg/mg, while somatostatin values obtained were 25.91±2.26 and 24.49±1.55 µU/mL respectively. Administration of cimetidine 50 mg/kg plus indomethacin 20mg/kg, the gastrin, prostaglandin and somatostatin values obtained were 218.98±7.38 µU/mL, 62.92±1.97 pg/ml and 25.70 ±2.67 µU/mL respectively.

Both gastrin and prostaglandin in the 100 mg/kg cimetidine plus indomethacin groups were significantly higher than the indomethacin control, but lower than the normal saline control group for gastrin and higher in prostaglandin group, though both insignificant. Somatostatin value of 18.78±74 µU/mL was lower than both normal saline and indomethacin control groups.

In the n-butanol 50, 100 and 200 mg/kg plus indomethacin 20 mg/kg groups, level of somatostatin recorded at the 200 mg/kg significantly decreased below the normal saline group. Prostaglandin was also markedly elevated to 104.05±2.28 pg/kg, while gastrin significantly (p>0.05) decreased compared to normal saline group.

DISCUSSION AND CONCLUSION

This work evaluated the effect(s) of n-butanol fraction on some key gastric hormones namely, prostaglandin, gastrin and somatostatin due to the fact that they all play a crucial role on mucosal integrity and defence [1].

In the indomethacin 20 mg/kg group, the two hormones gastrin and prostaglandin assayed decreased significantly (p<0.05) compared to control. Indomethacin is a non-selective inhibitor of COX and it does not inhibit the lipo-oxygenase pathway of arachidonic acid, while it strongly inhibits the COX pathway, thereby potentiating the secretion response elicited by histamine due to inhibition of Prostaglandin E_2 ($PGE_{2)}$ synthesis. Therefore, induction of gastric ulcers and erosions was possible due to progressive decrease in the level of prostaglandin, gastrin and somatostatin. Amongst the three hormones evaluated, prostaglandin plays a key role that stimulates the complex array of ulcer healing mechanisms, gets synthesized in the mucosal cells by COX enzymes. It stimulates the secretion of mucus and bicarbonate, maintains mucosal blood flow, and regulates mucosal turn over and repair [1]. The suppression of prostaglandin (PG) synthesis by indomethacin results in increased susceptibility of the gastroduodenal mucosa to inflammation and ulcerative injury [1]. Indomethacin significantly reduced gastric mucosal PG level compared to control.

The n-butanol at 50, 100 and 200 mg/kg, level of gastrin significantly reduced in dose-dependent manner, whereas PG level increased in dose-dependent manner, similar to that of somatostatin. These findings are in agreement with the results of Fabio-Cruz *et al.* [16], who used extracts of *Alchornea casterneafolia* and *Mouriri elliptica* to assay prostaglandin synthesis in the gastric mucosa, respectively. This corroborated with the findings of Borelli and Izzo [17] that flavonoids have the ability to increase PG content in the mucosa and decrease mast cell-mediated histamine production with inhibition of *Helicobacter pylori* growth, subsequently protecting the mucosa against aggressive factors. The results of cimetidine treated groups are insignificant compared to the indomethacin control. The multiple cytoprotective role that included suppression of gastrin production were reported by several authors [18], which corroborates with our findings. Incidentally, our work showed a similar pattern with the n-butanol fraction upregulating PG and somatostatin levels.

This work further showed the possible gastroprotective potentials possessed by this plant. It has been documented that PGs, which are up-regulated in this study, play a vital role via several mechanisms to confer gastric mucosal viability and defence. Some of these mechanisms were prevention of the increase in the permeability of the gastric mucosa to ions, increased mucosal blood flow, restoration of active sodium transport, stimulation of mucus production/secretion amongst others [19, 20, 21].

The reported results have validated the folkloric use of *N. sativa* in the therapy of peptic ulcer disease. *N. sativa* offers protection against NSAIDs-induced gastric ulceration, down-regulate basal acid secretions, modulate oxidative stress markers and gastric hormones to promote mucosal cytoprotection. The presence of phytoconstituents in this medicinal plant might be responsible for those pharmacological actions. In this context, extracts and active

principles from plants could serve as leads for the development of new drugs. Therefore, this plant species have a great potential to be used as a gastroprotective agent in combination with others or alone.

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