

Role of *Moringa oleifera* on enterochromaffin cell count and serotonin content of experimental ulcer model

Siddhartha Debnath & Debjani Guha*

S N Pradhan Centre for Neurosciences, University of Calcutta, 244 B A.J.C. Bose Road, Kolkata 700 020

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The present study has been undertaken to observe the effect of aqueous extract of *M. oleifera* (MO) leaf (300mg/kg body weight) on mean ulcer index, enterochromaffin (EC) cells and serotonin (5-hydroxytryptamine; 5-HT) content of ulcerated gastric tissue. Ulceration was induced by using aspirin (500 mg/kg, po), cerebellar nodular lesion and applying cold stress. In all cases increased mean ulcer index in gastric tissue along with decreased EC cell count was observed with concomitant decrease of 5-HT content. Pretreatment with MO for 14 days decreased mean ulcer index, increased both EC cell count and 5-HT content in all ulcerated group, but treatment with ondansetron, a 5-HT₃ receptor antagonist, along with MO pretreatment increased mean ulcer index, decreased 5-HT content without any alteration in EC cell count. The results suggest that the protective effect of MO on ulceration is mediated by increased EC cell count and 5-HT levels which may act via 5-HT₃ receptors on gastric tissue.

Keywords: Enterochromaffin cell, *Moringa oleifera*, Ondansetron, Serotonin, Ulceration

Gastric ulcer usually develops due to a break in the tissue lining of the stomach. The term peptic ulcer refers to those ulcers which occur in either the stomach or first part of small intestine that leads out of stomach, called duodenum¹. Formation of gastric ulcer is a complex mechanism. Several factors are responsible for pathogenesis of gastric ulcer². A variety of factors produce damage of gastro-duodenal mucosa including systemic events such as application of stress or local administration of various irritants like aspirin, NSAIDs that are commonly breakers of mucosal barrier³. The mucosal barrier is composed of mucous secreting epithelial cells with tight junctions⁴, which secrete bicarbonate along with mucous for maintenance of normal lining of layers for defence. Good regional blood supply is necessary for metabolic activity to maintain the process¹. Studies with different animal models demonstrate that severity of stress ulceration (cold, endotoxin etc) is associated with stress-induced decreased blood flow in gastro-duodenal tissue¹. Cerebellar nodulation is also responsible for pathogenesis of gastro-duodenal damage by decreasing the mucous secretion⁵⁻⁸. Chemicals like neurotransmitters are also involved in

ulcer formation in human and animals^{7, 9,10}. 5-HT or serotonin is a neurotransmitter located in the EC cells of the gastric mucosa^{7, 11-13}. It is suggested that 5-HT is the specific substance in the EC cells¹⁴⁻¹⁸. EC cells are distributed throughout the alimentary canal^{18, 19}, mainly in gastric mucosa. Shape of EC cells varies considerably in different parts of mucosa^{18, 19}. Average size of the EC cell is 21 - 24 μm in length and 8 - 12 μm in breadth even with a variation in different animals¹⁸. Coarse cytoplasmic granules are generally filled with 5-HT¹⁸. In rat the 5-HT content per EC cell is $1.2 \times 10^{-6} \mu\text{g}$ ¹⁸. The EC cell count and 5-HT concentration greatly depend on anatomical and environmental conditions¹⁸. There is a high significant correlation between EC cell count and 5-HT content in duodenum in all adult mammals¹⁸. In preliminary investigations aqueous extract of MO leaf at a dose of 300 mg/kg body weight exhibited maximum cytoprotection²⁰ and MO may exert its protective effect by modulating 5-HT secretion. In gastro intestinal tract 5-HT acts through 5-HT₃ receptors²¹. Ondansetron, a commonly used antagonist of 5-HT₃ receptors, along with other 5-HT₃ receptor antagonist for chemotherapy induced emesis²¹ is the prototypical drug which has been in use since early 1990s. Other agents in these classes which are now also available include granisetron, dolasetron and tropisetron, which are closely related to their chemical structures, and

* Correspondent author

Phone: 91-033-24796471/8674(Res)

Fax: 91-033-22413222

E-mail: debjaniguha@rediffmail.com

differ only in 5-HT₃ receptor affinities and pharmacokinetic properties²¹. The present study has been undertaken to observe the role of aqueous extract of *Moringa oleifera* (MO) (300mg/kg body weight) on mean ulcer index, 5-HT content and EC cell count of gastric mucosa of rat in three different rat ulcer models using ondansetron a 5-HT₃ receptor antagonist.

Materials and Methods

Chemicals— Aspirin (SRL, India) and ondansetron, a 5-HT₃ receptor antagonist (Sandoz Ltd., Switzerland) were used. All other chemicals used were of analytical grade.

Preparation of extract— Green young, healthy leaves of *M. oleifera* (MO; 2 kg) were collected locally. The leaves were identified and authenticated by the Botanical Survey of India, Howrah and kept in the S. N. Pradhan Centre for Neurosciences, Calcutta University. A voucher specimen of leaves has been deposited at S. N. Pradhan Centre for Neurosciences, Calcutta University. The leaves were then shade dried, ground in an electrical grinder and spread over tray with shifting of materials everyday to avoid growth of fungus. The powder thus obtained was soaked in distilled water for 24 hr at room temperature. The solution was filtered with Whatman No. 1 filter paper and vacuum dried at 40°–50°C to get a dry powder. The final yield was 12%. The extract was dissolved in double distilled water for final use²².

Treatment with MO and ondansetron— Aqueous extract of MO leaf at a dose of 300 mg/kg body weight was administered orally using orogastric cannula for 14 days. After completion of 14 days pretreatment with MO, ondansetron was given subcutaneously on the 15th day at a dose of 2 mg/kg body weight²³.

Animals and experimental design— Adult Holtzman strain albino rats of either sex (weight 150–200 g) were used. The rats were housed in stainless steel cages maintained in an ambient temperature of 25±1°C and 45.5% RH, with a 12: 12 hr photoperiod cycle with food and water *ad libitum*. Experiments were carried out after the approval of Animal Ethical Committee of the institute.

In schedule I of the experiment, 72 rats were divided into 4 groups (control, MO treated, ulcerated and MO pretreated ulcerated) for three different ulcer models (aspirin, cerebellar nodular lesioned and cold

stress induced); each group having 6 animals. The rats of the control group received saline (5 ml/kg body weight, po) whereas the rats of the MO pretreated and the MO pretreated ulcerated groups were treated with MO leaf extract of 300 mg/kg body weight for 14 days. On the 15th day all the rats were sacrificed by cervical dislocation (between 1100–1200 hrs) and the stomach were taken for histological study (mean ulcer index and EC cell count) and biochemical estimation of 5-HT.

In another experiment (schedule II) 36 rats were divided into 2 groups (ondansetron treated ulcerated and MO pretreated ondansetron treated ulcerated) for three different ulcer models (aspirin, cerebellar nodular lesioned and cold stress induced). The rats of the MO pretreated ulcerated and MO pretreated ondansetron treated ulcerated groups were treated with MO leaf extract of 300 mg/kg body weight, po for 14 days. On the 15th day all the rats were sacrificed by cervical dislocation (between 1100–1200 hrs) and the stomach were taken for histological study (mean ulcer index and EC cell count) and biochemical estimation of 5-HT.

Preparation of aspirin induced ulcer model— Rats were fasted for overnight and only water was allowed *ad libitum*. A single dose of aspirin (500mg/kg body weight) was given orally using orogastric cannula. After 4 hr, animals were sacrificed by cervical dislocation. Stomach was cut along the greater curvature and dissected longitudinally, stretched on paraffin bed, washed with normal saline and distributions of haemorrhagic spots were counted²⁴. Different doses of aspirin (50, 100, 200, 400 and 500 mg/kg body weight) were used for ulceration, but 100% ulceration was observed in 500 mg/kg dose. Hence this dose was selected for further experiments.

Electrolytic lesion of cerebellum— Prior to surgery all the animals were fasted overnight but had free access to water. Animals were anaesthetized with sodium pentobarbitone (40 mg/kg ip, Abbott India Ltd.). Each animal was placed in an Inco stereotaxic instrument equipped with ear bar that prevents damage to the tympanic membrane. Surgery was performed by a midline incision at the back of the head, the scalp was incisioned posteriorly in the midline and the adjacent pericranial muscles were retracted laterally. A burr hole of 1–2 mm diameter was made on the posterior aspect of the scalp stereotaxically following the coordinates of rat brain [AP= 12.8 mm, D (depth)= 6.8 mm, L= 0.4 mm]²⁵ so

that the bipolar electrode could penetrate the required area of the cerebellum. Then electrolytic lesion of cerebellar nodule was produced by passing electrical current of 0.2 mA for 20 sec (using Grass lesion maker) stereotaxically following the coordinates of rat brain²⁵. After lesions were done, the hole overlying the skull surface was closed with aseptic bone wax. Particular cares were taken for feeding and the animals were kept under observations till they recovered from surgical stress. Routine antibiotic injections (penicillin 100,00 IU) were given through intramuscular route to all the animals as an antibiotic measure to avoid any type of infection²⁶.

Cold stress induced ulcer model— For application of cold stress, rats kept in perplexed glass made restraining cages were subjected to the refrigerator compartment at 4°C for three hours daily and repeated consecutively for seven days^{6, 27, 28}.

Determination of mean ulcer index— The mean ulcer index was calculated following the method of Szabo *et al*²⁹. Briefly, stomach was taken from the sacrificed animal and cut along the length of greater curvature. The tissues were then rinsed in normal saline and spread over a paraffin tray. The number of bleeding spots was examined using a magnifying glass. The lengths of bleeding spots (in mm) were also measured using a scale. The scoring of ulcer index was done by the following grade for a single animal.

0 = normal i.e. no ulceration; 1 = one small ulcer (bleeding spot with 1-2mm in length); 2 = medium ulcer (bleeding spot with 3-4mm in length); 4 = a large ulcer (bleeding spot with 5-6mm in length); and 8 = a large ulcer (bleeding spot with >6mm in length)

The sum total scores were measured for all animals in a group and mean ulcer index were calculated by dividing the sum total scores by the number of animals. Then some tissue parts of the glandular mucosa was taken for staining of EC cells and estimation of 5-HT.

Histomorphological evaluation and staining of enterochromaffin cells— Stomach tissues were collected, rinsed in 0.9% saline and fixed in Bouin's fluid. After dehydration, all tissues were sectioned at 4-5 μ m thickness and stained to study EC cells following the method of Singh³⁰. EC cell density was determined by counting the total number of EC cells in each section with visible nuclei in the sections (objective $\times 10$, magnification $\times 200$). The values thus obtained were referred to as cell density.

For staining of EC cells, 10% aqueous silver nitrate solution was prepared and concentrated ammonia solution was added to it drop by drop until it formed a clear solution. To this solution, 10% aqueous silver nitrate solution was added drop by drop carefully until a faint opalescence was seen. It was kept at 60°C in an incubator. Rehydrated sections were placed in that preheated silver solution for 30 min until light brown colour was seen. The sections were removed, washed well in distilled water and immersed in 1% aqueous sodium thiosulphate for 1 min. Sections were then washed well in distilled water, counterstained using 0.5% aqueous neutral red, dehydrated and mounted³⁰.

Estimation of 5-HT content— Stomach tissues were weighed and 5-HT levels were estimated following the methods of Ray *et al*³¹. Stomach tissues were dissected out, washed in ice cold saline and homogenized in 10 ml acidified butanol. Homogenate (4 ml) was mixed with 10 ml 10% heptane and 5 ml 0.003 N HCl and then shaken for 5 min and centrifuged at 2000 rpm for 10 min. Acid layer (4.5 ml) was eluted and mixed with 200 mg alumina and 1 ml of 2 M sodium acetate. The mixture was shaken for 5 min and centrifuged at 2000 rpm for 10 min. Supernatant was taken for estimation of 5-HT. Supernatant was mixed with 3 volume of 10% isobutanol, shaken twice with equal volume of salt saturated buffer at pH 10. Then 2 volumes of 10% heptane was added to the butanol phase and 5 ml of 0.1 N HCl was added and shaken well and then the 1 ml of 0.3 N HCL was added finally to the mixture. This was taken for estimation of 5-HT. The fluorescence of 5-HT was measured in the Perkin-Elmer MPF 44B Fluorescence spectrophotometer with activation and emission wavelength set at 295 and 550 nm.

Statistical analysis— The results were analyzed statistically using one-way ANOVA followed by multiple comparison t-test.

Results

Mean ulcer index— Significant increase in the extent of ulcerations was observed in three different ulcer models as evidenced by increased mean ulcer index (Table 1). But pretreatment with MO for 14 days reduced the severity of ulcerations in gastric tissues in three different ulcer models (Table 1). When MO pretreated groups were subjected to ondansetron treatment prior to ulceration by aspirin, cerebellar lesioned and cold stress MO did not protect

ulcer formation as evidenced by increased mean ulcer index (Table 1).

Enterochromaffin cells count— EC cell density of gastric tissue was also decreased in three models of ulcers (Table 1). But MO pretreatment caused an increase in EC cell density as compared to ulcerated groups. But ondansetron treatment did not affect the EC cell count (Table 1).

5-HT content— In ulcerated conditions, 5-HT content of gastric tissues decreased significantly in three different ulcer models (Table 1). MO pretreatment for 14 days increased 5-HT content (Table 1). But in MO pretreated and ondansetron treated ulcerated groups (aspirin, cerebellar lesioned, cold stress) 5-HT content decreased significantly as compared to MO pretreated group (Table 1).

Discussion

From the present results it is evident that severity of ulcerations i.e., mean ulcer index were increased along with decrease in the amount of 5-HT as well as

EC cell density of gastric tissue of three different ulcer models (aspirin induced, cerebellar nodular lesioned and cold stressed). However pretreatment with MO showed protective effect against three different ulcerations. 5-HT is a neurotransmitter in the central nervous system and gastrointestinal tract^{14, 15}. The presence of 5-HT in the gastroduodenal tract has been demonstrated immunohistochemically in EC cells^{14, 15}. 5-HT is generally considered to be involved in the modulation of motor and sensory functions of the gastrointestinal tract¹⁹. Apart from motor and sensory functions, the role of 5-HT in gastric acid secretion has been evaluated. 5-HT inhibits gastric acidity by increasing the gastric mucous secretion⁷. It was also observed that 5-HT stimulates prostaglandins synthesis (mainly PGE₂, PGH₂) by enhanced activity of the cyclooxygenase pathway, the major biochemical pathway for prostaglandin synthesis from arachidonic acid^{7,32}. Prostaglandins stimulate mucosal blood flow and help in the secretion of mucous along with bicarbonate³². Aspirin

Table 1—Effect of *M. oleifera* (MO) on mean ulcer index, EC cell and 5-HT of stomach tissues of different ulcer models

[Values are in mean ± SE from 6 animals in each group]

Experimental condition: Aspirin ulcer model

Parameters	Control	MO	ASP	MO+ASP	ASP+OND	OND	MO+OND+ASP
Mean ulcer index	1.17± 0.48	0.33± 0.21	41.83± 1.0*	9.50± 0.76 [#]	52.33± 1.82 [#]	1.18± 0.32	24.17± 1.42 ^a
ECcell (cells/mm ³)	2649.17± 37.39	2691.67± 43.10	1342± 47.05*	2633.33± 23.76 [#]	1338.33± 47.52	2630± 43.83	2640.33± 22.68
5-HT (µg/100g)	1.58± 0.10	1.80± 0.09	0.90± 0.09*	1.32± 0.08 [#]	0.68± 0.04 [#]	1.56± 0.09	0.89± 0.04 ^a

Experimental condition: Cerebellar nodular lesion ulcer model

Parameters	Control	MO	CNL	MO+CNL	CNL+OND	OND	MO+OND+CNL
Mean ulcer index	1.33± 0.21	0.50± 0.34	38.67± 1.65*	8.83± 0.95 [#]	52.17± 1.70 [#]	1.26± 0.33	19.17± 0.70 ^a
ECcell (cells/mm ³)	2638.50± 64.20	2731.17± 67.48	1449.17± 28.25*	2384.83± 83.70 [#]	1451.83± 31.51	2669.67± 71.18	2392.50± 76.78
5-HT (µg/100g)	1.54± 0.11	1.81± 0.08	0.97± 0.10*	1.59± 0.15 [#]	0.56± 0.04 [#]	1.59± 0.10	0.86± 0.04 ^a

Experimental condition: Cold stress ulcer model

Parameters	Control	MO	CS	MO+CS	CS+OND	OND	MO+OND+CS
Mean ulcer index	1.83± 0.60	0.67± 0.33	24.17± 2.99*	9.33± 0.67 [#]	33.67± 1.02 [#]	1.88± 0.50	19.33± 1.23 ^a
ECcell (cells/mm ³)	2635± 67.72	2640.17± 66.83	1485.67± 104.87*	2517.83± 121.67 [#]	1464.67± 102.10	2610.63± 59.83	2501.67± 121.20
5-HT (µg/100g)	1.60± 0.10	1.82± 0.09	0.92± 0.09*	1.29± 0.07 [#]	0.64± 0.03 [#]	1.62± 0.09	0.71± 0.02 ^a

MO = *Moringa oleifera*; ASP = Aspirin; OND = Ondansetron; CNL = Cerebellar nodular lesion; CS = Cold stress.

Statistical analysis were done using one way ANOVA followed by multiple comparison t-test

P values: <0.001; when compared to *control; [#]group in which ulceration was produced; and ^a mo+ ulcerated group

and related NSAIDs have been found to prevent the synthesis of prostaglandin and hence the mucous secretion is hampered^{7, 32}. Mucosal cells are rapidly replaced in the crypts by proliferation and migrate to the damaged surface¹. Prostaglandins play an important role in this defence mechanism, which requires good regional gastroduodenal blood supply¹. As cold stress causes the damage of the mucosa by decreasing the blood flow, involvement of prostaglandin synthesis pathway is also responsible for the mechanism¹. Since 5-HT is a neurotransmitter in the enteric nervous system, release of 5-HT from EC cells acts as chemical and mechanical transducers for the initiation of local reflexes¹⁹. Although EC cells count and 5-HT concentration greatly depend upon anatomical and environmental conditions¹⁸, in all adult mammals there is a high significant correlation between the EC cell count and 5-HT content in gastro-duodenal tissue¹⁸. In the EC cells, 5-HT is synthesized from tryptophan by tryptophan hydroxylase (a mixed function oxidase) and stored with other autocooids, such as vasoconstrictor peptide, substance P and other kinins²¹. 5-HT released by the mechanical or vagal stimulation acts locally to regulate gastrointestinal function. Serotonergic terminals located in the gastro duodenal mucosa are responsible for liberation of 5-HT from EC cells under response to acetylcholine, noradrenergic stimulation and vagal influence²¹.

Several 5-HT receptor subtypes have been identified in the gastroduodenal tract. These are located in the nerves or on smooth muscle cells where they mediate a number of different actions^{33, 34}. In the EC cells 5-HT acts mainly via 5-HT₃ receptors²¹. These receptors are unique; becoming the only monoamine neurotransmitter receptors those are known to function as a ligand operated ion channel²¹. Activation of 5-HT₃ receptor elicits the release of 5-HT from EC cells²¹. Abundant 5-HT₃ receptors on Vagal and other afferent neurons on enterochromaffin cells play a vital role in the emission process of 5-HT²¹. MO pretreatment exhibited an enhancement of EC cell density as well as an increase in 5-HT content in gastric tissues of different ulcerated group with concomitant decrease in mean ulcer index showing the protective effect. From the present results it is evident that aspirin, cold stress and cerebellar nodular lesion caused gastric tissue damage by decreasing 5-HT content. Thus from these findings, the proposed mechanism of MO can be explained as

follow: MO possibly triggers the healing of gastric damage through the release of 5-HT from EC cells of gastric mucosa via 5-HT₃ receptors as is evident from the study using 5-HT₃ receptor antagonist ondansetron. The use of this antagonist decreased the amount of 5-HT without any change in EC cell count resulting in gastric tissue damage thus reducing the protective effect of MO. MO may also be stimulatory for formation of EC cells in gastric mucosa. Thus from the present investigation it can be concluded that MO protects gastric ulceration by modulating 5-HT and EC cell.

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