A BIOCHEMICAL ESTIMATION ON GASTROPROTECTIVE EFFECT OF PHYTOEXTRACTS IN GASTRIC ULCER INDUCED RODENT MODEL

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Abstract

The primary objective of the study was to evaluate the gastroprotective properties of *Callicarpa macrophylla, Grewia abutifolia, and Ipomoea cairica* leaves in preventing ulcers in rats. A dose-responsive investigation involved pre-treating rats for 15 days with these leaves before inducing ulcers via pyloric ligation. The assessment of ulcer-preventive efficiency included measuring free acidity, pH, total acidity, stomach oxidative stress, and enzymatic antioxidant activity in gastric tissue. This comprehensive approach aimed to elucidate the potential gastroprotective effects of these plant extracts In the rat model, a significant reduction in ulcers was observed after administering test medications, leading to favourable increases in pH and decreases in overall acidity. Animals with ulcers exhibited heightened stomach tissue activity, but those treated with *Grewia abutifolia, Ipomoea cairica, and Callicarpa macrophylla* extracts displayed a notable decrease in enzyme activity. Pre-treatment with these extracts preserved antioxidants and mucin levels in the stomach tissue of ulcer-afflicted rats. No harmful effects were observed in healthy rats. The study suggests that the antiacid secretory, cytoprotective, and antioxidant properties of these plant extracts contribute to their ulcer-preventing capabilities.

Keywords: *Grewia abutifolia, Ipomoea cairica, and Callicarpa macrophylla*, gastric ulcer, H^+-K^+ ATPase, lipid peroxides, mucin.

1. INTRODUCTION

Peptic ulcer is an acid-induced lesion of the digestive tract that is usually located in the stomach or proximal duodenum, and is characterized by denuded mucosa with the defect extending into the submucosa or muscularis propria.[1,2] The estimated prevalence of peptic ulcer disease in the general population is 5–10%, but recent epidemiological studies have shown a decrease in the incidence, rates of hospital admissions, and mortality associated with peptic ulcer.[3,4] This is most likely secondary to the introduction of new therapies and improved hygiene, which resulted in a decline in *Helicobacter pylori (H. pylori)* infections.[5]

Traditionally, mucosal disruption in patients with the acid peptic disease is considered to be a result of a hypersecretory acidic environment together with dietary factors or stress. Risk factors for developing peptic ulcer include *H. pylori* infection, alcohol and tobacco consumption, non-steroidal anti inflammatory drugs (NSAIDs) use, and Zollinger–Ellison syndrome. The main risk factors for both gastric and duodenal ulcers are *H. pylori* infection and NSAID use.[6,7] However, only a small proportion of people affected with *H. pylori* or using NSAIDs develop peptic ulcer disease, meaning that individual susceptibility is important in the beginning of mucosal damage. Functional polymorphisms in different cytokine genes are associated with peptic ulcers. For example, polymorphisms of interleukin 1 beta (*IL1B)* affect mucosal interleukin 1β production, causing *H. pylori*-associated gastroduodenal diseases.[8,9]

On the other hand, the risk of complications of peptic ulcer is increased four times in NSAID users, and two times in aspirin users. The concomitant use of NSAIDs or aspirin with anticoagulants, corticosteroids, and selective serotonin reuptake inhibitors increase the risk of upper gastrointestinal bleeding. Although many people who use NSAIDs or aspirin have concurrent *H. pylori* infection, their interaction in the pathogenesis of peptic ulcer disease remains controversial. A meta-analysis of observational studies resulted in a conclusion that NSAIDs, aspirin use, and *H. pylori* infection increase the risk of peptic ulcer disease independently.[10,11]

H. pylori-negative, NSAID-negative, and aspirin-negative peptic ulcer disease, which is classified as an idiopathic ulcer, can be diagnosed in about one-fifth of cases. It is caused by the imbalance between factors that contribute to mucosal integrity and aggressive insults, but the pathogenic mechanisms behind the development of idiopathic peptic ulcer are still unknown.[12,13] A Danish study showed that

psychological stress could increase the incidence of peptic ulcer. Other etiologies include ischemia, drugs and radiotherapy, viruses, histamine, eosinophilic infiltration, gastric bypass surgery, and metabolic disturbances.[14,15]

Grewia abutilifolia, a deciduous shrub or tree, holds significance in various cultures for its therapeutic properties. The plant contains mucilage in its leaves, stems, and roots, known for its calming and healing qualities. Internally, it is commonly utilized as a remedy for dysentery and diarrhea, showcasing the plant's traditional medicinal applications rooted in its mucilage content. Ipomoea cairica, a perennial climbing evergreen herb, features slender stems from a tuberous root stock, reaching up to 5 meters. The entire plant is employed in traditional medicine for treating external ailments, ulcers, and eye conditions.[16,17]

Callicarpa macrophylla, a small tree or evergreen shrub, attains a height of 2.5 meters. Harvested in the wild, it serves local purposes as fuel, food, and medicine. The leaves are utilized to address gastrointestinal bleeding, diarrhea, and dysentery. A juice, prepared by combining the leaves with Oxalis corniculata and Drymaria diandra, is employed for treating stomach issues. This plant plays a multifaceted role in local communities, serving both practical and medicinal needs.[18,19]

2. MATERIAL AND METHODS

2.1 Collection of plants

Fresh disease-free leaves and seeds of the plant were collected from Safia science college, dept. of botany. A voucher specimen and seed of the plant has been deposited in the herbarium of Botany, Bhopal, India. The National Identity number of *Grewia abutifolia, Ipomoea cairica, and Callicarpa macrophylla leaves* 172/SAIF/SCI/CLG/BPL. The leaves were washed thoroughly several times with running water and once with sterile distilled water. The leaf material was then air-dried on a sterile blotter under shade.

2.2 Animals used

Wistar Albino rats of either sex (130–160 g) were used in the study. Animals were housed individually in polypropylene cages in a ventilated room under ambient temperature of 22 ± 2 °C and 45–65% relative humidity, with a 12 h light followed by 12 h dark. All the animals were acclimatized for at least 7 days to the laboratory conditions prior to experimentation. Tap water and food pellets were provided ad libitum. Food pellets was with-held overnight prior to dosing. All rats were handled and maintained strictly as per guidelines.

2.3 Biochemical Parameter

2.3.1 Gastric mucosal studies of free radicals and antioxidants in animals

Protein (mg/g wet tissue), free radicals, lipid peroxidation (LPO, MDA nmol/mg protein) and nitric oxide (NO, nmol/mg protein) and antioxidants, catalase (CAT, mU/mg protein) superoxide dismutase (SOD), U/mg protein) and reduced glutathione (GSH, nmol/mg protein) were estimated in the rat gastric mucosal of CMC (negative control) and Pylorus ligation alone or treated with oral CMC (control)/GA (200 mg/kg, test drug)/positive controls, IC (200 mg/kg)/CM (200 mg/kg).

2.3.2 Gastric ulcer and Inflammatory Markers Studies

The dose of GA, IC and CM and the standard anti-ulcer drugs, ranitidine (20 mg/kg), was selected. GA, IC, CM and ranitidine were suspended in 0.5% carboxymethyl cellulose (CMC). The animals received the drugs orally with the help of an orogastric tube in the volume of 10 ml/kg body weight and were given orally once daily for 7 days. The experiments were conducted on day 7, one hour after the last dose of GA, IC and CM/standard drugs to 18 h fasted rats while control rats received CMC only. The effects of GA, IC and CM were studied on gastric ulceration and gastric mucosal MPO in PL-induced GU rats while gastric ulceration and cytokines, TNF-α and IL-1β, and VEGF were studied in EtOH-induced GU.

2.3.3 Determination of Gastric Tissue Level of Nf-kβ Activity

The NF- kβ driven transcription, cells were plated in 24-well plates (30000 cells/well); after 48 hours, cells were transfected by calcium-phosphate method with a plasmid containing the luciferase reporter gene under the control of NF- kβ promoter.

After 16 hours, cells were placed in a medium deprived of FCS, and stimulated with TNF-α and IL-1β at 10 ng/ml. ETs were tested at 1–10 mg/ml; individual compounds at 0.5–10 mM. After 6 hours cells were harvested and luciferase assays were performed using Britelite TM Plus reagent according to manufacturer's instructions. Data were expressed considering 100% the luciferase activity related to the cytokine-induced NF-kβ driven transcription.

Preliminary time-course experiments were performed to set the best conditions for further experiments. AGS were treated with TNF-a, IL-6, IL-21 and IL-8 and IL-1b 10 ng/ml, for 3, 6, 24, and 30 hrs. TNF-α and IL-1β only stimulated the NF-kB driven transcription. The maximal effect was observed at 6 hrs, and decreased at later times.

For the evaluation of the time-course of NF-kβ (p65) translocation, AGS were treated with TNF-α and IL-1β 10 ng/ ml, for 1,2,3, and 6 hrs. The maximal effect of nuclear

translocation was observed at 1hr and decreased at later times. These conditions were used for testing ETs (0.5– 2 mg/ml) and individual compounds (0.5–10 mM). Ranitidine at 5 mM was used as reference inhibitor of NF- kβ translocation. Results are the mean ± sd of three experiments in triplicate.

For the NF- kβ (p65) nuclear translocation assay, AGS cells were plated at the concentration of 1.5 6106 cells/ml in 60-mm plates. After 48 hours, cells were treated for 1 hour with the inflammatory mediators and the extracts/compounds under study. Nuclear extracts were prepared using Nuclear Extraction Kit and stored at 28^0C until assayed. The same quantity of total nuclear proteins, measured by the method of Bradford, was used to assess NF-kβ nuclear translocation using the NF- kβ (p65) transcription factor assay kit followed by spectroscopy. Data were expressed considering 100% the absorbance related to the cytokine-induced NF- kβ nuclear translocation.

2.3.4 Determination of Gastric Nf-kβ Protein Expression Using Western Blotting

Western blot analysis. Whole tissue lysates were prepared from gastric tissue specimens. Standard western blotting was performed using anti-IL-6 and anti-NF-κB, anti-VEGF antibodies. Simultaneous determination of the expression level of β-actin was carried out as an internal control. Proteins were detected using the enhanced chemiluminescence system in accordance with the manufacturer's instructions. Separate analyses were performed for each sample and the experiment was repeated three times.

2.3.5 Determination of Genes Expression Using Real Time Polymerase Chain Reaction (PCR)

Samples (including gastric tissue and the tissue of corresponding normal areas) were treated with the TRIzol reagent for total-RNA extraction. The potentially contaminated genomic DNA was removed by treating 10 mg of the RNA sample at 37˚C for 30 min with 1 ml of TURBO DNase followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1). Real-time PCR analysis was carried out on the ABI PrismH 7300 Sequence Detection System. Expression of IL-6, NF-κB and VEGF were analyzed using the PCR Reagents kit. The TaqMan probe and primers for IL-6, NF-κB and VEGF designed using the Primer Express 2.0 version were: NF-κB forward 5'-gaaccacacccctgcatatag-3', reverse 5'-gcattttcccaagagtcatcc-3' and probe 5'-agaggcta aagttctccaccagg-3'; IL-6 forward 5'-ccactcacctcttcagaacg-3', reverse 5'-catctttggaaggttcaggttg-3' and probe 5'-aaattcggta catcctcgacggcatc-3'; VEGF forward 5'-agtccaacatcaccatgcag-3',reverse

5'-ttccctttcctcgaactgattt-3' and probe 5'-tcaccaaggccag cacataggag-3'. The cDNA was synthesized from 500 ng of RNA using the TaqMan RT Reagents kit (Applied Biosystems). The optimized concentrations for real-time PCR were 0.4 µM for both primers, 0.2 µM for the probe and 5 ng cDNA in a 20 µl reaction volume. Actin primers (forward 5'-tgcagaaag agatcaccgc-3', reverse 5'-ccgatccacaccgagtatttg-3') were used as an internal control. Each sample was tested in triplicate. Cycle threshold (Ct) values were obtained graphically for IL-6, NF-κB, VEGF and actin. The difference in Ct values between actin and IL-6, NF-κB, VEGF are presented as ΔCt values. The ΔΔCt values were obtained by subtracting the ΔCt values of the control samples from those of the treated samples. Relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$

2.3.6 Gastric Level of Nitric Oxide

In present study, we used male Sprague-Dawley rats (weighing 155-180 g). The animals were fed with a conventional pellet diet. Three rats were housed per cage and kept at room temperature (22 ± 1^0C) with humidity of 65-70%. The rats were randomly assigned to tap water control group and test group. In the test group, the animals drank a solution tap water containing test solution (GA (200mg/ml), IC (200mg/ml) and CM (200mg/ml)). For 25 days. NO donor was given to some test group treated rats twice at 1 hour and at 10 min before experimentation. In the second experiment, rats drank the test solution for 25 days and then the solution was replaced by tap water. NO donor was administered to some test solution -treated rats for an additional 12 days. Solid food was withdrawn 48 hours before the start of experimentation. The animals were kept in fasting cages and allowed to drink a solution containing 8% sucrose in 0.2% NaCl (w/v). In the test-treated group, rats were given the 8% sucrose/0.2% NaCl solution containing the same concentration.

2.3.7 Gastric secretion/mucosal studies in pylorus ligated rats

After 4 h of pylorus ligated, the stomachs were dissected out. The gastric juice was collected into a centrifuge tube through a nick along the greater curvature of the stomach and centrifuged for 5 min at 2000 rpm. The volume of the supernatant was expressed as ml/100g body weight. Free and total acidity and peptic activity were studied in the gastric juice while, muco-substances like total hexoses, hexosamine, fucose, sialic acid and protein were estimated in the 90% ethanol precipitate of gastric juice using standard procedures and were compared with respect to the control CMC group. The mucosal scrapings were also taken from the glandular portion of the stomach and were homogenized in phosphate buffered saline for the estimation

of various fractions of glycoproteins, for muco-substances.

2.3.8 Determination of Gastric Mucosal Prostaglandin E2

The stomach was removed 30 min after the administration of OPC-12759, and was opened along the greater curvature, followed by rinsing with ice-cold Tris-HCl buffer (pH 7.4) containing indomethacin 50µg/ml to inhibit biosynthesis of PGs. The corpus mucosa was carefully stripped off from the muscle layer on a filter paper. Approximately 100 mg of the tissue was homogenized in 1 ml of Tris buffer containing indomethacin (50 µg/ml). After centrifugation at 15000RPM for 2 min, the supernatant was stored at -80^{o}C until assayed.

PGE2-1ike activity was analysed in rat stomach strips and colon by a sensitive super fusion method. These preparations were suspended with a tension of 1g in an organ bath surrounded by a 37°C water jacket. The organ bath was filled with liquid paraffin and was super fused with Krebs solution maintained at 37°C and oxygenated with 95% O_2 and 5% CO_2 onto the upper tissue at a rate of 1.5 ml/min. Contractions of these tissues were determined with an isotonic transducer and recorded on a recorder. Indomethacin (1µg/ml) was also added to prevent the biosynthesis of endogenous PGs.

3. RESULTS

3.1 Protein, free radicals and antioxidants

Protein (mg/g wet tissue), free radicals, LPO (MDA nmol/mg protein) and NO (nmol/mg protein), and antioxidants, CAT (mU/mg protein), SOD (U/mg protein) and GSH (nmol/mg protein) were estimated in the gastric mucosal homogenate of CMC (negative control)-treated and Pylorus ligation (PL, control) rats or rats treated with GA, IC, CM+PL (test extract) and ranitidine+ GA, IC, CM (positive controls).

Parameters	Normal control	PL Control	PL + GA (200)	IC (200) +PL	CM (200) +PL	Ranitidine (20) +PL
PROTEIN (PR) PR *(mg/g wet tissue)* FREE RADICALS	0.00±0.00	11.03±0.163	59.9±1.45	61.2±4.25	59.6±3.65	58.3±3.65
LPO (nmol/mg protein)	0.00±0.00	12.26±0.157	2.65±1.62	4.56±1.36	4.25±1.25	4.25±1.25
NO (nmol/mg protein) ANTIOXIDANTS	0.00±0.00	13.58±0.159	3.65±1.63	7.65±1.52	5.65±1.68	5.32±1.65
SOD (U/mg protein)	0.00±0.00	12.58±0.185	2.65±0.36	2.85±.0.03	2.33±1.85	1.65±1.98

CAT (mU/mg protein)	0.00±0.00	13.65±0.187	6.35±0.64	4.25±0.26	6.35±1.98	4.25±1.57
GSH (nmol/mg protein)	0.00±0.00	12.65±0.136	41.2±0.78	24.6±1.47	40.2±3.65	35.6±0.35

Table 1. Effect of GA, IC, CM and ranitidine on wet gastric mucosal protein, free radicals, LPO and NO and antioxidants, CAT, SOD and GSH in PL induced GU rats

3.2 Gastric Tissue Levels of Inflammatory Markers

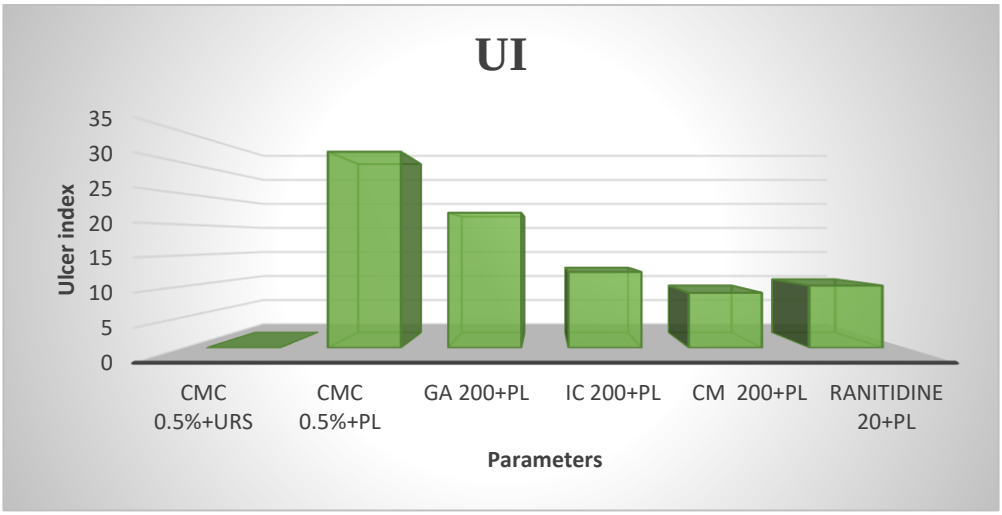

Figure 1. Effects of GA, IC, CM and ranitidine on UI in PL-GU rat

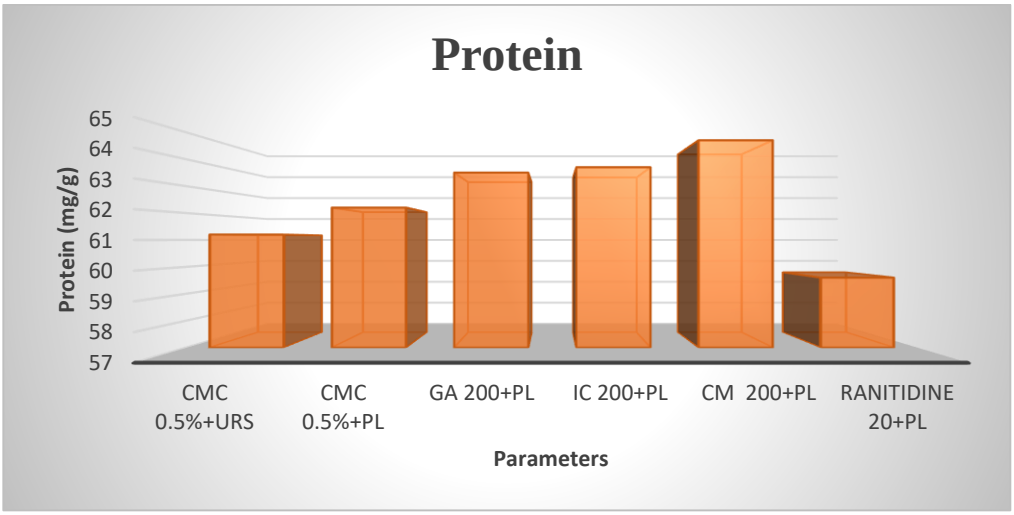

Figure 2. Effects of GA, IC, CM and ranitidine on Protein in PL-GU rat

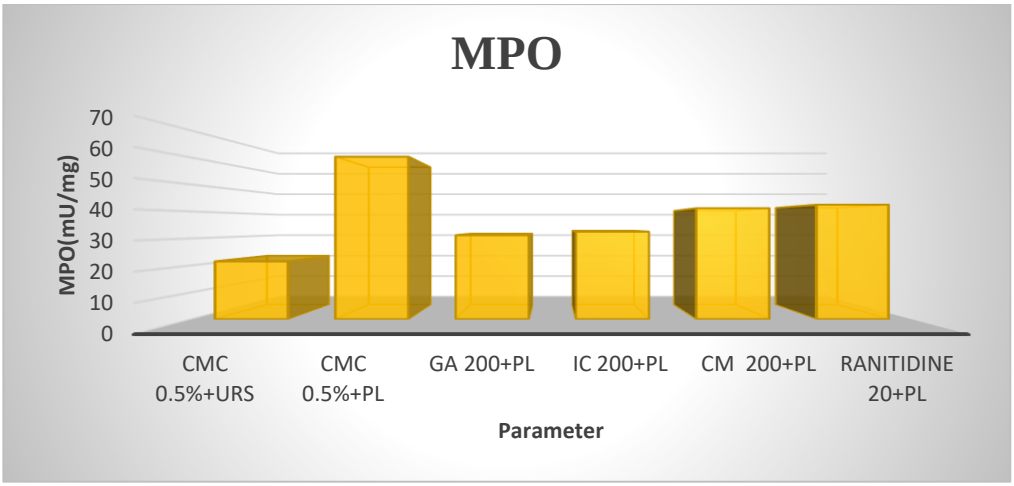

Figure 3. Effects of GA, IC, CM and ranitidine on MPO in PL-GU rat

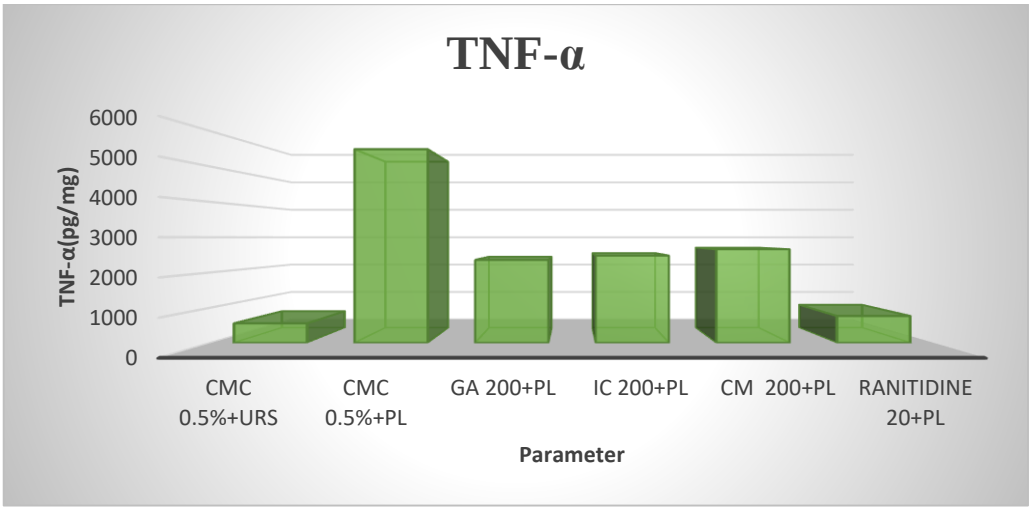

Figure 4. Effects of GA, IC, CM and ranitidine on TNF-α in PL-GU rat

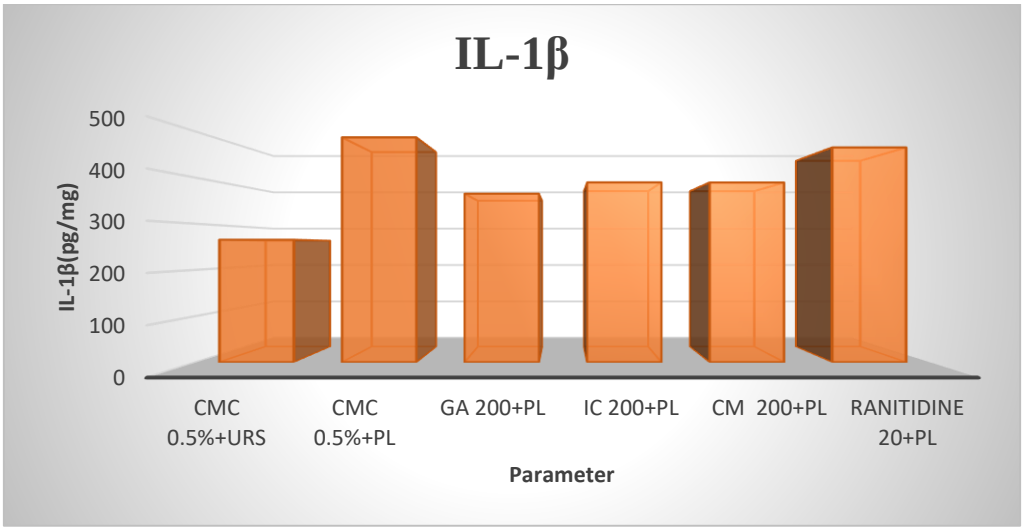

Figure 5. Effects of GA, IC, CM and ranitidine on IL-1β in PL-GU rat

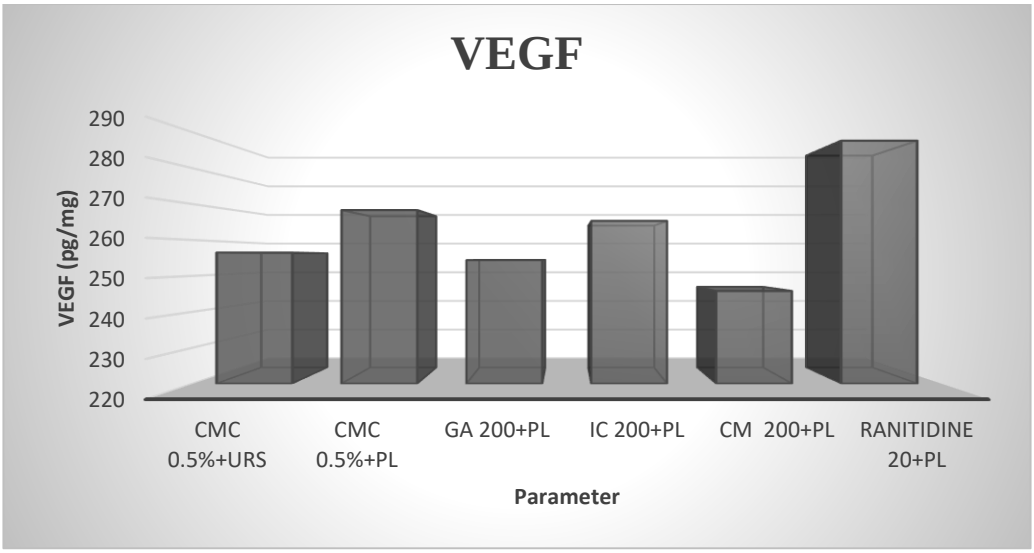

Figure 6. Effects of GA, IC, CM and ranitidine on VEGF in PL-GU rat

3.3 Gastric Tissue Level of Nf-Kβ Activity

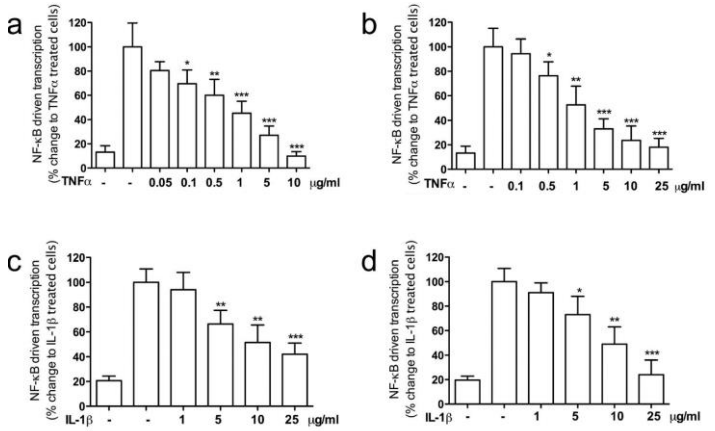

Figure 7. Effect of GA, IC, CM and ranitidine on NF-kB driven transcription induced by TNF-α and IL-1β.

3.4 Gastric Nf-Kβ Protein Expression Using Western Blotting

The production of IL-6, NF-κB and VEGF in gastric tissue and adjacent normal mucosa were all examined using immunohistochemical staining. The findings of the immunohistochemical staining confirmed a weak expression of NF-κB, IL-6 and VEGF in adjacent normal mucosa but a strong expression in gastric tissue. The overexpression of IL-6 was directly associated with NF-κB activation. Overexpression of VEGF was also directly associated with NF-κB activation according to further correlation analysis. Moreover, an association of increased IL-6, VEGF and NF-κB expression. mRNA levels are significantly increased in gastric tissue. We investigated the mRNA levels of IL-6, NF-κB and VEGF in gastric tissue according to RT-PCR. As we expected, mRNA levels of IL-6, NF-κB and VEGF in human gastric

tissue were all significantly increased compared to those in adjacent normal mucosa tissue samples, suggesting that high NF-κB mRNA levels might be positively correlated with IL-6 mRNA levels.

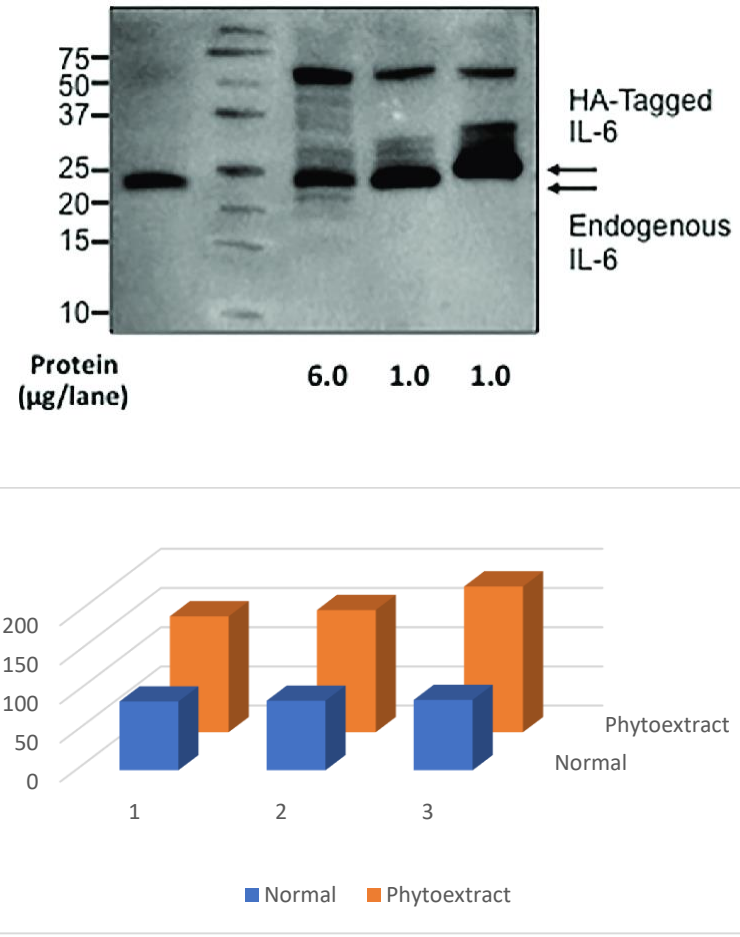

Figure 8. The protein levels of IL-6, NF-κB, and VEGF increased significantly in gastric tissue compared with those in the adjacent normal tissue

3.5 Genes Expression Using Real Time Polymerase Chain Reaction (PCR)

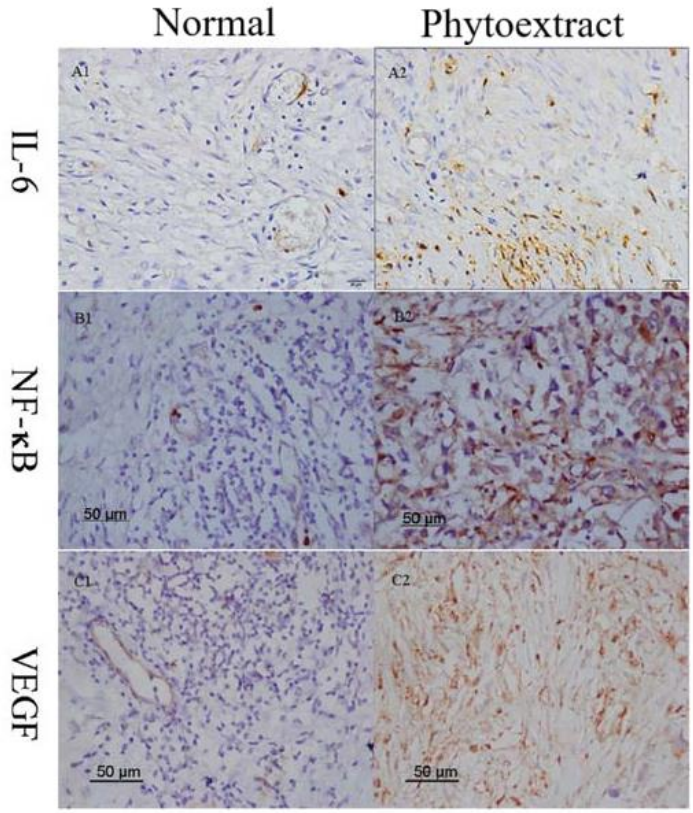

Figure 9. Immunostaining showed that NF-κB activity is directly associated with protein overexpression in gastric tissue

3.6 Gastric Level of Nitric Oxide

Only petechiae were found in the stomach of animals in control group and the mean ulcer index were unaffected by tap water, test solution or acute NO donor treatments. Pylorus ligation induced gastric ulcers in the glandular mucosa with a 100% incidence as indicated by the mean ulcer index in all stressed animals, whether given tap water, test solution with or without NO donor. Treatment with test solution for 25 days intensified Pylorus ligation-induced gastric ulceration significantly but had no effect on non-stressed group. Acute NO administration twice shortly before Pylorus ligation-restraint in test solution-treated animals failed to reduce the ulcerogenic effect. Interestingly, test solution treatment alone in the normal group significantly reduced the gastric adhesion mucus content as compared to tap water control group, while acute NO treatment failed to reverse the effect of test solution. When animals were subjected to Pylorus ligation, the adhesion mucus content was reduced to 50% of that of normal group. Test solution treatment further reduced the adhesion mucus content in stressed animals and acute NO treatment did not reverse the effects.

Experimental group	No. of rats	Ulcer index(mm)	Adhesion mucus content (µg/g)
A. Pylorus ligation-induced gastric ulceration (unrestrained at 22^0C for 2 hours)			
Tap water	2	0.05±0.04	382±38
GA+NO (acute)	3	0.06±0.03	283±27
IC+ NO (acute)	2	0.07±0.06	276±28
CM+ NO (acute)	2	0.08±0.08	230±23
B. Normal (restrained at 4^0C for 2 hours)			
Tap water	8	7.5±0.8	221±12
GA+NO (acute)	8	20.9±1.8	167±15
IC+ NO (acute)	7	21.7±1.9	151±16
CM+ NO (acute)	7	22.8±1.2	145±19

Table 2. Effects of GA, IC, CM and acute NO treatment on Pylorus ligation induced gastric ulceration

3.7 Gastric secretion/mucosal studies in pylorus ligated rats

Parameters (mg/kg)	CMC (0.5%)	GA (200)	IC (200)	CM (200)
Gastric Mucosa glycoproteins (µg/100 mg wet tissue)				
Total hexoses	1923±159	2489±201	1956±132	2682±219
Hexosamine	1236±89.6	1632±65.3	1125±8.65	1457±95.8
Fucose	165.3±20.6	165.3±18.6	185.3±26.3	265.4±19.6
Sialic acid	89.6±8.65	132.7±12.3	98.5±8.65	140.3±12.5
Total carbohydrates (TC)	4562±236	5632±156	4578±285	4568±319
Protein (P)	6593±365	6478±356	5687±198	5785±315
TC: P ratio	0.74±0.96	0.85±0.03	0.48±0.06	0.89±0.08

Table 3. Effect of GA, IC and CM on gastric acid, pepsin and mucin secretion and mucosal glycoproteins in PL-induced GU in rats

3.8 Gastric Mucosal Prostaglandin E2

Effect of OPC-12759 on the generation of PGE2-like activity in gastric mucosa OPC-12759 given i.p. increased the generation of PGE2-1ike activity in the corpus mucosa above that in the control for 30 min in a dose-dependent manner. The generation of PGE2-1ike material was markedly suppressed by pretreatment with indomethacin 50mg/kg s.c. OPC-12759 did not modify the suppressed PGE 2 levels in indomethacin-treated animals.

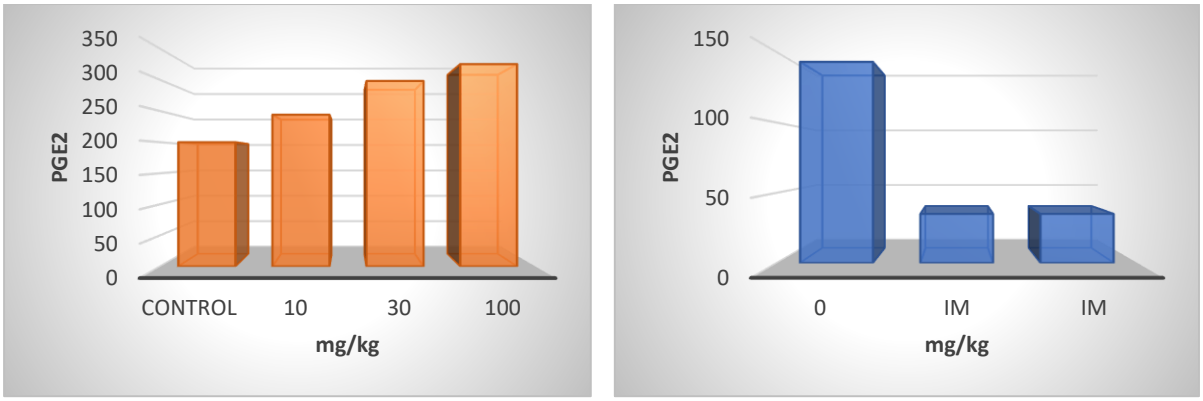

Figure 10. Effect of OPC-12759 on the generation of PGE2-1ike activity in gastric mucosa with and without indomethacin pretreatment.

4. CONCLUSION

The results indicate that *Grewia abutifolia, Ipomoea cairica, and Callicarpa macrophylla* has potent antiulcer property by decreasing the gastric acid secretion by H+-K+ ATPase, decreasing the free radical formation and also by protecting the gastric mucin from acid and free radical induced damage. The test drug has been found to be nontoxic when tested with normal rats. The present study revealed that *Grewia abutifolia, Ipomoea cairica, and Callicarpa macrophylla* can be claimed for the gastroprotective effect the traditional medicinal plant used for gastric complications.

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