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Research Article

Protective Role of Green Tea Extract against Cold-Restraint Stress Induced Gastric Ulcerogenesis in Albino Rats

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ABSTRACT
Evaluation of gastric ulcer protective role of green tea *(Camellia sinensis)* extract (GTE) was investigated in cold-restraint stress induced ulcer model in wister strain male albino rats. The experimental animals were divided into control group, cold-restraint stress induced group and green tea extract supplementary pre-treated group which was supplemented with GTE in a dose of 1 ml twice a day per rat for 15 days. Administration of GTE significantly increased the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) by 37.83%, 8.98% and 3.16% respectively as compared with cold stress induced group. The MDA and GSH level were also decreased by 33.32% and 2.19% respectively. The result of histopathological study had indicated that ulcer index was decreased by 76.12 % and a distinct improvement of epithelial lining of gastric mucosa, integration of superficial mucin gel layer and polysaccharide content in gastric tissue were observed in GTE pre-treated group. Thus, the present experimental findings suggest that GTE has protective role against gastric ulcerogenesis and it can enable to restore the normal homeostasis of gastric tissue.

KEYWORDS: Gastric ulcer; green tea extract; antioxidants

INTRODUCTION

Peptic ulcer is a very familiar and one of the major gastro-intestinal disorders of humans in modern days [1]. Basically the word "Peptic" is derived from Greek term "Peptikos" meaning related to digestion. Peptic ulcer is a chronic, non-inflammatory disease which usually occurs in the antrum part of stomach [2]. Several factors like excessive secretion and aggressive action of gastric HCl and pepsin, inappropriate use of non-steroidal anti-inflammatory drugs, infection of *Helicobactor pylori*, breakdown of defensive role of bicarbonate containing mucus, improper functions of prostaglandins and nitric oxide are responsible for initiation of peptic ulcer [3]. Das and Banerjee [4] proposed that generation of reactive oxygen species (ROS) like superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) causes lipid peroxidation as well as pathogenesis of ulceration. Stress also induces the generation of reactive oxygen species (ROS) in gastric tissue level which appears as the major events in ulcer formation [5,6]. The detrimental effects of ROS are reduced by the activity of several anti-oxidant enzymes. SOD, CAT and GPx are the important defensive anti-oxidant enzymes. Among them, SOD catalyses the reaction for

conversion of superoxide radicals into H_2O_2 and O_2 while CAT and GPx help to convert H_2O_2 to H_2O and oxygen molecules. The activities of these enzymes prevent the formation of more harmful hydroxyl radicals.

Though, the hyper secretion of gastric HCl was thought to be the main reason for initiation of ulcer; as a result, histamine receptor 2(H_2) blockers, proton pump inhibitors and use of simple antacids are the choice of treatment regime. It has also been observed that its protection against ulceration is also possible by increasing the production of bicarbonate containing mucus over the gastric epithelial layer [7]. The medicines or drugs which are available for the treatment are mostly synthetic chemicals and have some side effects. Natural products from different plants like *Aloe vera*, *Azadirachta indica*, *Beta vulgaris*, *Curcuma longa*, *Capsicum annuum*, *Piper betel*, *Carica papaya*, *Ocimum sanctum* and *Garcinia indica* are applied as anti-ulcer compounds because those are easily available, very effective and less side effects [8-11].

Peptic ulcers can be induced in laboratory animals by pharmacological, surgical or physiological manipulations. There are several methods for induction of ulcer like cold-water-restraint or cold-restraint stress, non-steroidal anti-inflammatory drugs (NSAIDs: indomethacin, aspirin, and ibuprofen) induced gastric ulcers, cysteamine induced duodenal ulcers, pylorus-ligated-induced peptic ulcers, ethanol-induced gastric ulcers, acetic acid-induced gastric ulcers, histamine-induced gastric ulcers, serotonin-induced gastric ulcers, indomethacin-histamine-induced duodenal ulcers, acetic acid-*Helicobactor. pylori*-induced ulcers etc [12]. Cold-restraint stress induced ulcer model is widely used as experimental model for induction of ulcer which is easy, effective and short time duration procedure to induced gastric ulceration in rats [13]. Green tea (*Camellia sinensis*) is an evergreen shrub or small tree and its leaves contain large number of polyphenols (such as Catechins), vitamins C and vitamin E etc. Previously, it was reported that green tea has anti-inflammatory response, anti-bacterial activity, anti-oxidant capacity, anti-carcinogenic property, anti-obesity effect, anti-ulceration activity [14,15]. Several studies have been carried on to find out the ulcer protective property of green tea extract in different models like ethanol induced gastric ulcer [16], restraint plus water immersion stress-induced ulcer, indomethacin-induced ulcers [17], *Helicobacter pylori*-induced ulcer [18], pyloric ligation-induced ulcer model [19], but the effect of green tea in cold-restraint stress induced ulcer model is not seen properly. Cold exposure is very common for the people of high altitude area, travelers of the high altitude, and military personals working at high altitude. The protective activity of green tea extract as natural phytochemical agents against cold-restraint ulcer model will be beneficial to the society. Thus, this study has focused on the role of green tea extract against histopathological changes in gastric epithelium and oxidative stress in gastric tissue.

MATERIALS AND METHODS

Selection of animals and maintenance

The study was performed on 18 healthy, Wister strain male albino rats having body weight of 100±15 g, supplied by Saha Enterprise, Kolkata (CPSEA, Govt. of India registered farm). They were acclimatized in laboratory condition for period of 2 weeks. CPSEA, Govt. of India approved diet and water ad libitum were given to them. The experimental animals care were provided according to the guidelines of the 'Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)', India and all experimental procedures were approved by Institutional Animal Ethical Committee (Reg No. 1617/GO/Re//S/12/CPCSEA). Experimental animals were housed three rats per cage in a room with temperature 22±2°C with 12–12 h light–dark cycles by the side of humidity of 50±10%. The experimental animals were divided into three groups and each group contains 6 rats. Group-I (control group) received normal diet and water ad libitum, Group-II (Cold-restraint stress exposure group) received normal diet and water ad libitum, Group-III (pretreated with green tea extract,1 ml/100 gm body weight, twice a day for 15 days) also received normal diet and water ad libitum.

Preparation of green tea extract

The dose (1 ml/100 gm body weight) has been selected from the earlier studies of Bakr and Header [20]. Darjeeling green tea was purchased during the month of January to February from

local market of Midnapore town, Paschim Medinipur District, West Bengal, India. To prepare the extract, 20gm of green tea was added to 100ml of warm water (65-70ºC) and was steeped for 15 minutes. The mixture was cooled at room temperature and then filtered.

Cold-restraint stress (CRS) induced ulcer formation

Before exposure to CRS, the animals of Group-II and Group-III were deprived of food for 24 hours but had access to free water. Then the animals were subjected to cold environment (4±1ºC) for 3 hours in a restrained cage. [4].

Sacrifice of animals and macro photography of stomach tissue

Animals were sacrificed and the stomach was cut along the greater curvature. Ulceration point was identified after proper washing with normal saline (NaCl 0.9%) and gently stretching the tissue on a soft paraffinized tray. The macro-photograph was taken by using a digital SLR camera (Nikon D7000, Japan) with macro-lens (Tamron 90 mm, Japan).

Ulcer index determination

The ulcer score (U.S) was calculated according to the method of Allam and El-Gohary [21]: 0 = no damage, 1 = blood at the lumen, 2 = pinpoint erosions, 3 = one to five small erosions < 2 mm, 4 = more than five small erosions < 2 mm, 5 = one to three large erosions >2 mm, 6 = more than three large erosions >2 mm.

Tissue collection and histopathological study

After completion of the ulcer indexing, 5mm/5mm of stomach tissues were cut off from glandular part of each side where the ulcers were observed according to the procedure of Iranloye and Bolarinwa [22]. The Collected tissues were transferred to 10% neutral formaldehyde solution till processed. Later, the tissue was washed in ethanol for dehydration. Then the portion of the tissue was embedded in paraffin wax and 6 µm sections were cut for preparation of histological slides. Eosin and hematoxylin stain were used to observe the histo-architecture of the gastric tissue. The histopathological changes were recorded by the scoring system of Yang et al. [23]. The features of the mucus lining of stomach were identified by using Alcian blue stain. Distribution of polysaccharide was observed after stained with PAS (Periodic acid-Schiff)**.**

Biochemical analysis

Preparation of gastric tissues homogenate

For biochemical analysis of oxidative stress related parameters, tissue homogenate was prepared through the following process; 1.5 g gastric tissue was washed initially in 0.9% normal saline and made homogenate in ice-cold buffer (0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 6000×g for 10 min in 4°C. Then supernatant was separated and stored at -80 ºC for biochemical study [24].

Oxidative stress profile

The assessment of lipid peroxidation in gastric tissue homogenate, thiobarbituric acid reactive substances (TBARS) as malondialdehyde (MDA) content was measured by using the method of Ohkawa et al. [25]. Tissue homogenate (200 µl) was added in 20% TCA (1.5 ml) and 1.34% TBA (1.5 ml) mixture, boiled for 30 minutes and cooled followed by addition of 2.5 ml butanol. Then the whole mixture was centrifuged at 2000×g for 5 minutes. After centrifugation, optical density of supernatants was measured at 535 nm. The amount of malondialdehyde (MDA) content formed as TBARS was expressed as nmol of MDA/mg of protein calculated by using the molar extinction co-efficient $1.43 \times 10^{-3} M^{-1} Cm^{-1}$ [25].

The GSH level in the stomach tissue homogenate was estimated by the principle of Ellman's reaction [26]. The tissue homogenate (100 µl) was mixed with 25% of trichloroacetic acid (200 µl and centrifuged at 2,000×g for 15 minutes. Then the supernatant was separated and diluted to 1 ml of 0.2M sodium phosphate buffer (pH 8.0) followed by addition of 2ml DTNB (0.6mM) and incubated at room temperature for 10 minutes. After formation of yellow-colored complex, optical density was taken at 405 nm. The levels of GSH were expressed as µg of GSH/mg protein [27].

Anti-oxidant enzymes estimation

Superoxide dismutase activity in the stomach tissue homogenate was estimated by the method of Del Mestro and McDonald [28] by using the ability to inhibit auto-oxidation of pyragallol.

The reaction mixture contained 2ml Tris (50mM) and 20µl tissue homogenate followed by addition of 100µl pyragallol (0.2mM) to initiate the reaction and the absorbance was measured at 420nm for 3 minutes .SOD activity was expressed in term of units/mg of protein.

Catalase activity was determined from the tissue homogenate by the method of Luck [29]. Initially, 3% (v/v) H_2O_2 was prepared in 0.05M phosphate buffer (pH 7.0). the reaction was started by addition of 100µl tissue homogenate. The change of absorbance per min was recorded at 240 nm for 3 minutes. The catalase activity was estimated by molar extinction co-effcient of 43.6M^{-1} cm^{-1} for H_2O_2. The level of CAT activity was expressed as units/mg protein.

The activity of GPx was evaluated by method of Paglia and Valentine [30]. A reaction mixture was made with 1.49ml potassium phosphate buffer (50 mM, pH 7.0), 0.1ml EDTA (1mM), 0.1ml sodium azide (1mM), 0.05ml reduced glutathione (1mM) and 0.05 ml glutathione reductase (1 U). Then tissue homogenate (200 µl) was added to the mixture and kept for 5 minutes at 25ºC. Then 0.1 ml of 2.5mM H_2O_2 was added to initiate the reaction. Absorbance was recorded at 340 nm for 5 minutes. Values were expressed as nmol of NADPH converted to NADP by using extinction coefficient of 6.2×103 $M^{-1}cm^{-1}$ at 340 nm. The activity of GPx was given in nmol NADPH consumed/min/mg protein.

Statistical analysis

Statistical analysis was done by using standard software package. The experimental data were presented as mean ±SEM, where n = 6 per group. Student's 't' test was performed to determine the level of significance among the groups.

RESULTS AND DISCUSSION

Observation of ulcers in cold-restraint stress induced stomach

The current study was carried out to evaluate the ulcer protecting effect of green tea extract on cold-restraint stress induced gastric ulceration model in male albino rats. Cold restraint stress is regularly used as experimental model for generation of acute gastric ulcer [31]. The stomach was opened to observe the ulcers in different parts of the tissue. It was observed that no trace of ulcer was present in the tissue of control group (Fig. 1A). Prominent point of hemorrhages, large blood spots, and mucosal damages were present in the tissue of group II animals (Fig. 1B). However few pin point ulcer had been observed in the gastric tissue of green tea extract pretreated group (group III) (Fig. 1C). The protective role of GTE in cold-restraint stress induced ulcer model was reported in Table-1. Ulcer index was decreased by 76.01% in GTE pre-treated group when compared with cold-restraint stress induced treated group (group II). Previously, Panda and Sonkamble [5] reported that *Ipomoea batatas* tuber (sweet potato) exerts maximum 38.48% anti-ulcer activity in CRS induced model. The inhibition was far less than our experiment. Stress-induced ulcer is probably mediated by the release of histamine. It plays various roles for ulceration: increases gastric secretion, causes disturbances of the gastric mucosal microcirculation, creates abnormal motility, and reduces mucus production [32]. The other mechanisms of induction of gastric lesions by CRS are arteriolar vasoconstriction through activation of peripheral sympathetic nerves, excessive production of free radicals, decrease in SOD level and Haber-Weiss reaction [6].

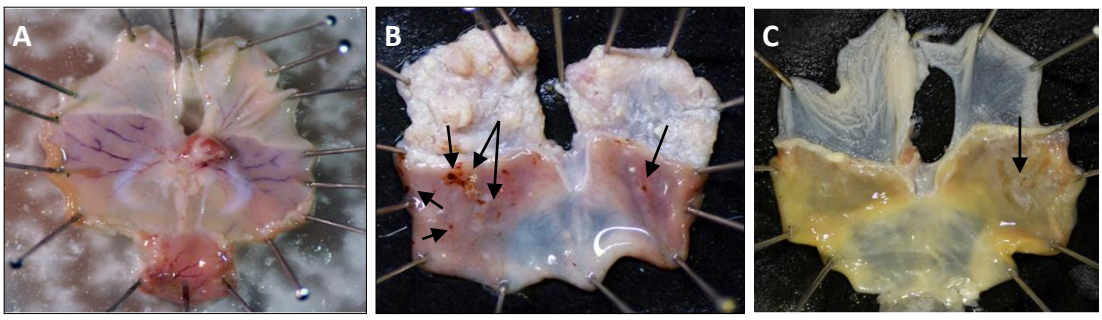

Fig. 1: Macrophotography of open stomach of different groups. A) Normal group: undamaged gastric tissue, B) CRS group: arrow shows the various lesions with extensive necrosis and hemorrhage in gastric mucosa, C) GTE Pre-treated: arrow shows mild damages of gastric mucosa

Table 1: Effect of green tea extract on cold restraint induced gastric ulceration [Values are mean±SEM]

Treatment Groups	Mean Ulcer Score	Mean Ulcer Index (U.I)	Percentage of Inhibition (P.I)
Control group	0	0	-
CRS induced group	13.34±0.92	1334	-
GTE pre-treated group	3.2±0.65	320	76.01%

The ulcer index (U.I) was determined by the following equation: U.I = Mean ulcer score of group × percentage of ulcerated animals of the same group.

The preventive index (P.I) was calculated by the equation:

[(Mean ulcer score in treated group - mean ulcer score in pre-treated group) / mean ulcer score in treated group] × 100

Effect of GTE on oxidative stress

In this study, oxidative stress markers like MDA, reduced glutathione (GSH) were measured to evaluate the effect of GTE on oxidative stress. MDA as the end product of lipid peroxidation was significantly (P<0.001) increased in group-II by 70.73% in comparison to control group; while, GTE significantly (P<0.001) decreased the MDA level in group-III by 33.32% (Fig. 2A). It was established that cold-restraint stress induces the generation of free radicals [33] which starts lipid peroxidation and increases MDA production as end product. Accumulation of MDA alters the redox homeostasis as well as tissue damage [34]. Several phenolic compounds [catechins, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate] of GTE act as anti-ulcer agents by exerting scavenging role against free radicals to prevent gastric ulceration.

Figure 2B had shown the GSH content in the tissue of stomach. It was observed that GSH content significantly (P<0.001) decreased in experimental animals (group-II and group-III) by 3.16% and 2.19% respectively as compared with control group. Reduce glutathione (GSH) non-enzymatic anti-oxidant, acts as electron donor and reduces disulfide bond. During reaction GSH is converted to its oxidized state glutathione disulfide (GSSG) in presence of enzyme GPx. Oxidized glutathione (GSSG) returns back to GSH (reduced form) by glutathione reductase (GR) and maintains the normal cellular GSH level [5,6].

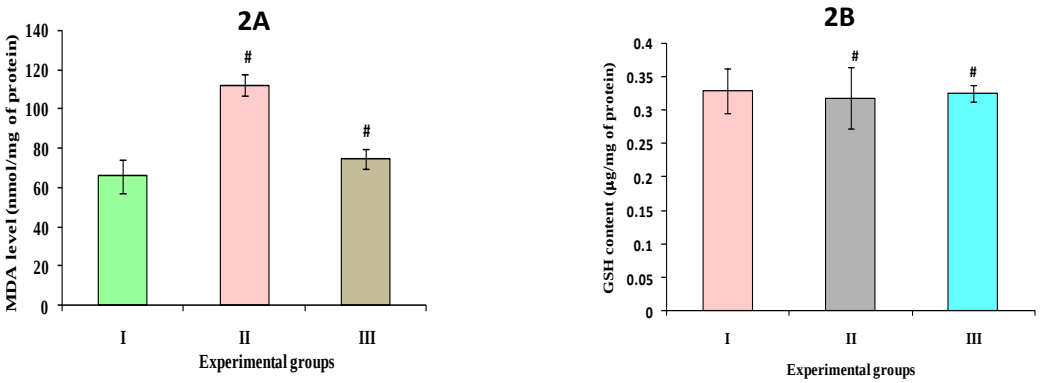

Fig. 2. Effect of GTE on MDA and GSH content in gastric tissue homogenate exposed to cold-restraint stress. All values are expressed as mean ± SEM n=6. # indicates significant difference (P<0.001) in comparison with control group. I- Control group, II- CRS induced group, III- GTE pre-treated group.

Effect of GTE on anti-oxidant enzymes

SOD, first line protective enzyme against ROS had decreased significantly by 36.49% in cold-restraint stress group (P<0.001) as compared with normal group. Although, SOD level had significantly (P<0.001) increased by 37.83% when animals were pre-treated with GTE (Fig.3A). Other enzymes like catalase (CAT) and GPx were also significantly (P<0.001) decreased in group II by 12.3% and 6.68% respectively. Whereas, GTE significantly (P<0.001) increased

the same enzymes by 8.98% and 3.16% respectively (Fig.3B, 3C). Thus, the proposed study had indicated that GTE has the capability to bring back the activity of anti-oxidant enzymes in stress condition.

Cold exposure and immobilization of animals are synergistically responsible for the generation of reactive oxygen species (ROS) [5]. They involve in gastric ulceration and start ischemaia-reoxygenation-induced gastric mucosal injury [6]. Stress induces lipid peroxidation, depletion of GSH, formation of O_2^-, increase level of H_2O_2, •OH radical and inactivation of cyto-protective enzyme prostaglandin synthetase [4]. Stress also causes sympathetic stimulation in the gastrointestinal system which leads to vascular compression and mucosal ischemia [35]. The result is the release of O_2^- from mitochondrial electron transport chain and O_2^- promotes more ROS generation [36]. ROS decreases endogeneous antioxidant status and make the mucosa more prone to oxidative damage. All these effects co-operatively start gastric mucosal lesion and ulceration. Supplementation of anti-oxidant rich GTE improves ulcer protecting activity in group-III. The reduction of ulcer index may be due to the cytoprotective role of GTE or its anti-ulcer activity [1].

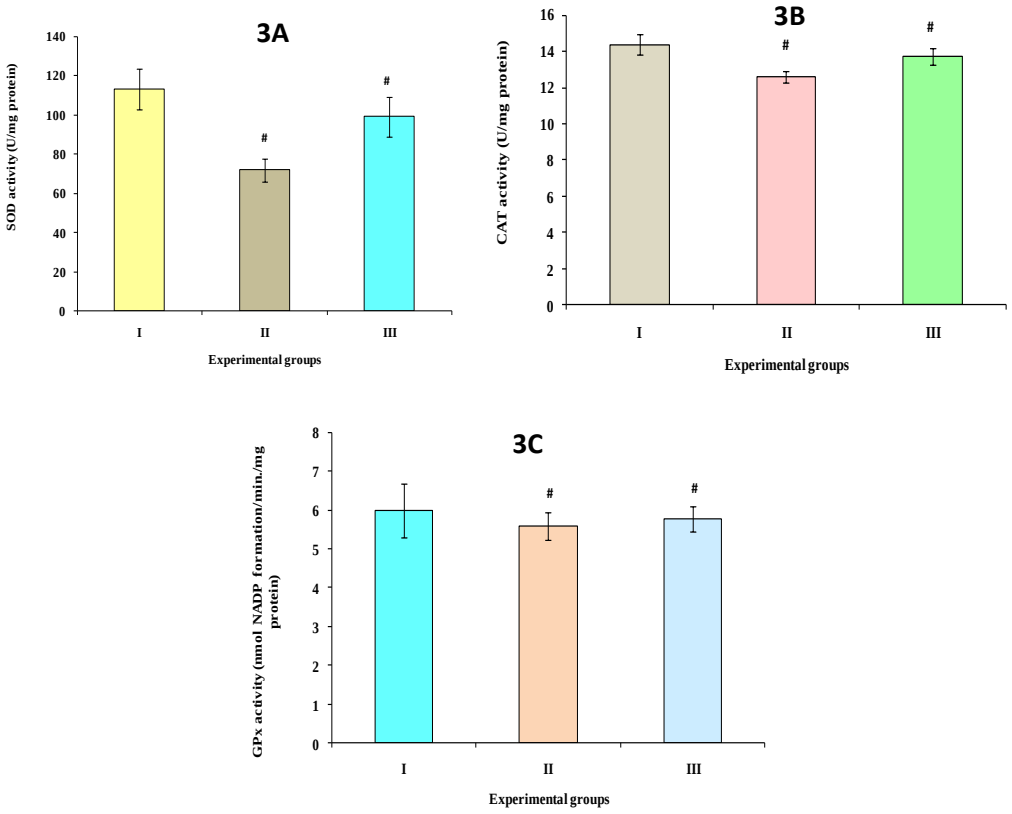

Fig. 3. Effect of GTE on SOD, CAT and GPx activity in gastric tissue homogenate exposed to cold-restraint stress. All values are expressed as mean ± SEM n=6. # indicates significant difference (P<0.001) in comparison with control group. I- Control group, II- CRS induced group, III- GTE pre-treated group.

Histopahological investigations

Histopathological study had revealed the normal mucosal height of the stomach wall, continuous surface epithelial lining, no surface erosion, perfect alignment of glandular tissues in control group (group I, Fig. 4A). Marked changes in the normal pattern of the gastric tissue along with mucosal damages, epithelial cell layer disruption, focal surface erosion, disruption and disarrangement of connective tissue, glandular tissue had been observed in cold-restraint stress treated group (group II, Fig. 4B). A distinct improvement had been seen in pre-treated group (group III) which indicates the preventive action of GTE on gastric ulcer along with recovery of linear alignment of epithelial cell layer, less surface erosion, no vacant spaces and normal arrangement of glandular tissue (Fig. 4C, Table-2). Thus, GTE prevents the gastric

mucosal damage as compared with the control group.

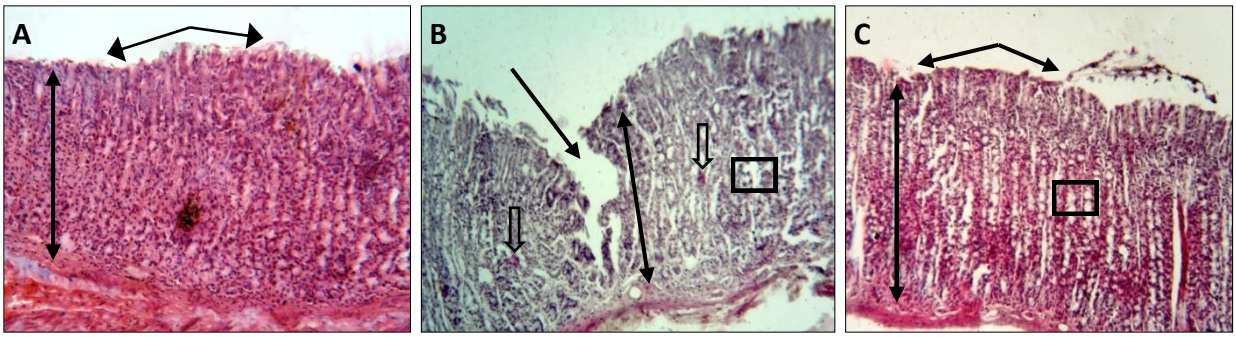

Fig. 4: **Histological characteristics of stomach tissue section in different experimental groups. Hematoxylin-Eosin staning was performed in this purpose. A) Control group: shows intact epithelial layer (arrow), normal muscular arrangement, B) CRS induced group: shows extensive surface disruption, hemorrhage and necrosis in epithelial layer, loss of glandular tissue (indicated by rectangular block), decrease in muscular height, C) GTE Pre-treated group: shows very minimum loss of glandular tissue, apparently normal mucosal height and almost intact epithelia layer.**

Table 2. Histopathological scores after supplementation of green tea extract on cold restraint induced gastric ulceration [Values are mean±SEM]

Treatment Groups	Hyperemia	Bleeding	Epithelial cell degeneration/necrosis	Pathological changes, total score
Control group	0.33±0.21	0.00±0.00	0.00±0.00	0.33
CRS induced group	1.5±0.49	2.83±0.60	4.16±0.87	19.64
GTE pre-treated group	0.83±0.40	1.33±0.33	1.5±0.56	7.99

The total score of pathological changes was calculated by the formula:
Total score = hyperemia points + (bleeding points x2) + (degeneration and necrosis points x3)

Alcian blue was used to identify the mucus containing layer over the tissue and the Figure 5A indicated the normal mucin layer over the gastric epithelium. The damage of the mucin layer was prominently marked in the gastric tissue of group II; whereas harm was found less in GTE pre-treated group (Fig. 5B and 5C). To determine the polysaccharide content, PAS staining was performed. The results showed that the depletion was high in group II in comparison with group I and group III (Fig. 6A-C). Stress induced free radical reacted with polyunsaturated fatty acids of membrane lipids and was bound with their unsaturated units. This reaction converted the membrane lipids (RH) to lipid macro radical (ROO•) in presence of oxygen and ultimately breaks cell membrane which leads to cellular necrosis and erosion of superficial epithelial layer. The mucus is a complex gel consisting of glycoprotein which is one of the most essential first line defense against aggressive action of gastric acid and pepsin [37]. The gastric epithelium secrets a water insoluble mucus layer, known as adherent mucus layer composed of mucin and bicarbonate which act as protective barrier [38,39]. GTE may be cytoprotective in nature which stabilizes the integrity of the gastric mucus or increases mucus secretion.

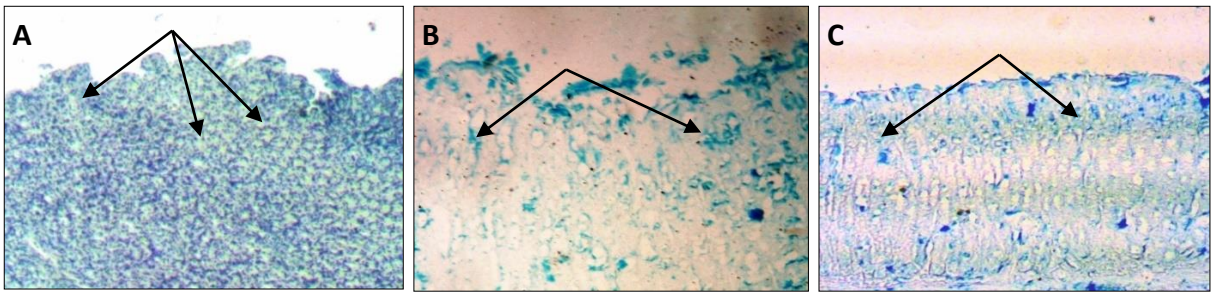

Fig. 5. Histomorphological characteristics gastric sissue of different experimental groups have been observed after Alcian blue staining. A) Control group: arrow represents normal pattern of histological characteristics, B) CRS induced group: shows very less distribution of stain than normal group and C) GTE Pre-treated group: shows higher distribution of stain than CRS group

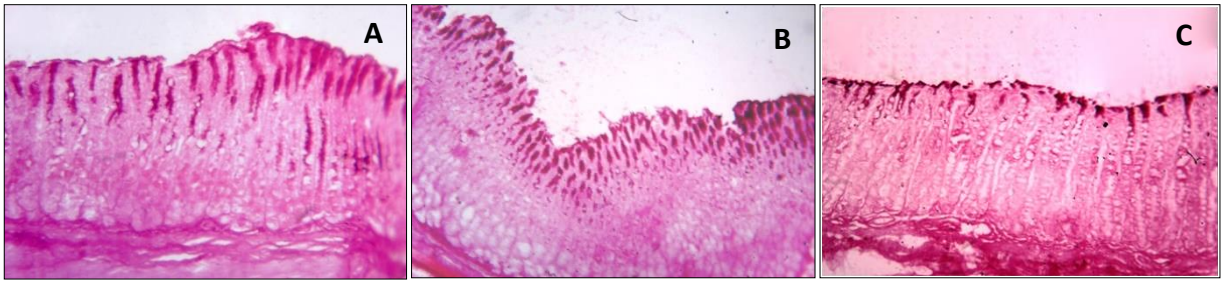

Fig.6: Histochemical study of gastric tissue of different experimental groups by PAS staining. A) Control group: arrow shows normal distribution of polysaccharides, b) CRS induced group: shows depleting the amount of polysaccharides and c) GTE Pre-treated group: shows restoration of mucopolysaccharides than CRS induced group.

CONCLUSION

This study enlightened the ulcer healing effect of GTE which appears to be related to the free radical scavenging property. The significant restoration of SOD, CAT and GPx activities after administration of GTE indicates that it has the ability to reinstate these enzymes along with inhibition of lipid peroxidation and GSH depletion. Furthermore, GTE also ameliorates the gastric mucosal damage by exerting antioxidant mediated cytoprotective activity. Thus, the present study indicates the health benefits of GTE.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest

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