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Research Article

# AQUEOUS TULSI LEAF (*OCIMUM SANCTUM L.*) EXTRACT PROTECTS AGAINST PIROXICAM-INDUCED GASTRIC ULCERATION IN RATS: INVOLVEMENT OF ANTIOXIDANT MECHANISMS

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**ABSTRACT**

**Objective:** The present studies were aimed at evaluating whether an aqueous extract of Tulsi leaves is capable of ameliorating piroxicam–induced gastric injury in rats.

**Methods:** Male Wister rats were used for the present studies. The aqueous Tulsi leaf extract (TLE) [25, 50, 100 and 200 mg/kg bw] was administered orally to the fasted rats (water *ad libitum*), one hour prior to the piroxicam treatment.

**Results**: The aqueous leaf extract of Tulsi was found to protect the gastric mucosa from becoming ulcerated following piroxicam treatment, in a dose-dependent manner. Pre-treatment of rats with increasing doses of TLE protected the rise in the level of lipid peroxidation, reduced glutathione from being decreased as well as protected the activities of the antioxidant enzymes indicating it may have protected the gastric tissue from piroxicam-induced injury. The results of the biochemical analyses were supported by macroscopic and microscopic studies of gastric tissue.

**Conclusion:** From the results it may be concluded that aqueous leaf extract of Tulsi may be a safe gastro-protective agent and may find extensive pharmaceutical applications especially in situations where the use of NSAIDs is the only choice.

**Keywords:** Antioxidant, gastroprotection, gastric ulceration, piroxicam, tulsi leaf aqueous extract.

## INTRODUCTION

*Ocimum sanctum L.* (Family Lamiaceae) commonly known as Tulsi in India and Holy Basil in Western countries is a small herb seen throughout India. Traditionally different plant parts like leaves, stem, flower, root, seeds and even the whole plant of *Ocimum sanctum L.* have been recommended for the treatment of bronchitis, bronchial asthma, malaria, diarrhoea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite etc. This small herb is known to be used as a therapeutic agent possessing antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, anti-inflammatory, immunomodulatory, adaptogenic and diaphoretic actions [1-3]. Recently, Dharmani et al. [4] have reported a significant anti-gastric ulcer activity in the extract of different plant parts of Tulsi. A hydro-alcoholic extract of the leaves of the Tulsi have been reported to prevent ethanol-induced peptic-ulcers in rats possibly through its antioxidant activities [5,6]. The fixed oil of Tulsi exhibits a significant anti-ulcer activity against alcohol, histamine, reserpine, serotonin, and stress-induced ulceration in experimental models [7]. The present study was performed to evaluate the gastroprotective effect of aqueous leaf extract of Tulsi. To the best of our knowledge, the anti-ulcer effect of the aqueous preparation of the leaves of Tulsi have not yet been studied extensively, despite the widespread use of the aqueous preparation of Tulsi leaf in folk lore medicine to treat different ailment of the body.

Gastro-duodenal ulcer is a very common ailment and presently is of global concern. The gastric lesions develop when the delicate balance between some gastro-protective and aggressive factors is lost. The major aggressive factors are acid, pepsin, *Helicobacter pilori* and bile salts. Defensive factors mainly involve mucus-bicarbonate secretion and prostaglandins [8]. Gastric ulceration is associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [9]. Known additional risk factors include advanced age, previous history of ulceration, stress, smoking, nutritional deficiency, concomitant administration of anticoagulants and coexsisting serious systemic disorders [9].Gastrointestinal bleeding is also related to the type of NSAID and the dosage [10]. Piroxicam, a classic nonselective cyclooxygenase (COX)-1 preferent NSAID, is widely used by patients requiring anti-inflammatory intervention [11]. High ulcerogenic potential of this oxicam anti-inflammatory is due to its ability to decrease the synthesis of prostaglandins through the inhibition of COX-1. This forces clinicians to limit its use. Possible involvement of oxidative stress in piroxicam-induced gastric lesions has also been demonstrated [12].

Although a number of anti-ulcer drugs such as antacids, anticholinergics, proton pump inhibitors, $H_2$ receptor antagonists [13, 14], cytoprotectives and prostaglandin analogues [15] are available for treatment of ulcer, all these drugs have side effects and limitations. Thus development of alternative therapies for the treatment of gastric ulcer is of paramount importance, and in this context, plant extracts are among the most promising source of new treatments for this complaint [16, 17].

The need for safer and effective antisecretory and anti-ulcer drug and the lack of enough scientific data about the antiulcer effect of aqueous leaf extract of Tulsi prompted the present study.

## MATERIALS AND METHODS

### Collection, authentication and preparation of plant material

Fresh leaves of Tulsi [Ocimum sanctum L. (Lamiaceae)] were obtained from the local market and authenticated by the office of the Scientist F Central National Herbarium, Botanical Survey of India, Ministry of Environment and Forests, Government of India (No. CNH / I-I / 40 / 2010 / Tech.II / 231). The leaves were oven dried and pulverized. The powdered leaves were then soaked in double distilled water and kept at room temperature overnight. The supernatant was decanted carefully and centrifuged to remove any unwanted dust particles. The supernatant thus obtained was decanted again and deep frozen and lyophilized (IICB Central Instrumentation Facility). The lyophilized material was then carefully collected and stored at 4ºC for future use. This lyophilized

material was reconstituted in aqua guard clean drinking water (herein-after referred to as TLE) and the rats were fed with this extract at different doses. This method of preparation of the extract was also used by other investigators [18].

**Drugs, reagents and antibodies**

Tthiobarbituric acid (TBA), eosin, NAD+, 2,2-dithiobis-nitro benzoic acid (DTNB), xanthine, xanthine oxidase, cytochrome c, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and piroxicam were obtained from Sigma (St. Louis, MO, USA). Hematoxylin, $H_2O_2$, dimethyl sulfoxide (DMSO) and were obtained from Merck Limited (Delhi, India). Other reagents used were of analytical grade.

**Preliminary phytochemical analysis**

The total phenolic content was measured according to the method adopted by Mitra et al [21] Briefly, to a suitably diluted sample extract, Folin–Ciocalteu reagent and 7.5% aqueous $Na_2CO_3$ solution were added. The mixture was allowed to stand for 30 min at 40°C and then absorbance was measured spectrophotometrically at 765 nm. The amount of total phenolics is expressed as gallic acid equivalents (GAE, mg gallic acid/ml of extract).

The total flavonoid content was measured using 5% $NaNO_2$ solution, a freshly prepared 10% $AlCl_3$ solution, and 1M NaOH solution following the method adopted by Mitra et al [21]. The final volume was adjusted to 2.5 ml with deionised water. The mixture was allowed to stand for 5 min and the absorbance was measured at 510 nm. The amount of total flavonoids is expressed as (+) catechin equivalents [(CE, mg (+) catechin/g sample)]

**Animals**

Male Wister rats, weighing 180-200 g, were obtained from the local animal supplier (CPCSEA registered). The animals were handled and the experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The experimental protocols had the approval of the institutional animal ethics committee (IAEC) [IAEC/PROPOSAL/DB-4/2010], Department of Physiology, University of Calcutta.

**Treatment of rats with piroxicam, and TLE**

Fasted rats (water provided *ad libitum*) were divided into four groups of eight animals each. The rats of the first group served as vehicle treated controls. The rats of the second group were fed orally with different doses of TLE (25, 50, 100 or 200 mg/kg). The rats of the third group were fed orally with piroxicam (30 mg/kg). The fourth group received TLE (100 mg/kg, fed orally) 1 hr before piroxicam administration. The animals were kept at room temperature and sacrificed by cervical dislocation after 4 hr to assess the degree of gastric lesioning. A portion of the collected stomach tissue was kept in suitable fixative for histological studies and the other portion was frozen at -20°C for further biochemical analyses.

**Measurement of mean ulcer Index**

The grade of lesions was scored according to the following scale: 0, no pathology; 1, small 1–2mm ulcers; 2, medium 3– 4 mm ulcers; 4, large 5–6 mm ulcers; 8, ulcers >6 mm. The sum of the total ulcer score in each group of rats was divided by the number of animals was expressed as the mean ulcer index [19].

**Macroscopic and microscopic studies**

Following Sacrifice of rats, the stomach was surgically extirpated from each group and opened through vertical incision along the greater curvature and photographs were taken of the inside surface of the stomach. The stomach tissues were then washed in 0.9% salaine and a portion of it was kept in 10% buffered formalin for histopathological studies. The sections were then stained with hematoxylin and eosin. The tissue sections were examined under an Olympus BX51 (Olympus Corporation, Tokyo, Japan) microscope and images were captured with a digital camera attached to it.

Some of the stomach sections (5 µm thick) were stained with Sirius red (Direct Red 80; Sigma Chemical Co, Louis, MO, USA) according to the method of Ghose Roy et.al.[15] and imaged with laser scanning confocal system (Zeiss LSM 510 META, Germany; University of Calcutta, Central Instrumental Facility, B. C. Guha Center for Biotechnology and Genetic Engineering) and the stacked images through multiple slices were captured. The digitized images were then analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) and the total collagen area fraction of each image was measured and expressed as the % collagen volume.

**Measurement of lipid peroxidation level**

A portion of the fundic stomach was homogenized (5%) in ice-cold 0.9% saline (pH 7.0) with a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 s and lipid peroxides in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Bandyopadhyay et al. [12]. Briefly, the homogenate was mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80°C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 1200 g for 10 min at room temperature was measured at 532 nm using a UV–vis spectrophotometer (Bio-Rad, Smartspec Plus, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. Values were expressed as nmoles of TBARS/mg protein.

**Measurement of reduced glutathione (GSH)**

The level of GSH in the gastric tissue (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsey [20] with some modifications [21]. A portion of the fundic stomach was homogenized (10%) in 2 mM ice-cold ethylene diamine tetra-acetic acid (EDTA). The homogenate was mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer (BIORAD, Smartspec Plus). Values were expressed as nmoles GSH/mg protein.

**Assay of gastric peroxidase (GPO), Cu-Zn superoxide dismutase (SOD), and catalase**

A portion of the fundic stomach was homogenized (10%) in 0.25 M sucrose and 50 mM phosphate buffer (pH 7.2) and the mitochondrial fraction was prepared. The GPO activity in this fraction was measured using iodide as an electron donor. The assay system contained in a final volume of 1 mL: 50 mM sodium acetate buffer (pH 5.2), 1.7 mM KI, a suitable volume of enzyme, and 0.27 mM $H_2O_2$ added last to start the reaction [12]. The enzyme activity was expressed as units/mg protein.

Copper-zinc superoxide dismutase (SOD1) activity was measured by hematoxylin autooxidation method of Martin et al. [22] with some modifications [21]. Briefly, a portion of the fundic stomach was homogenized (10%) in ice-cold 50 mM phosphate buffer containing 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000 g for 15 min and supernatant collected. Inhibition of hematoxylin autooxidation by the cell free supernatant was measured at 560 nm using a UV–VIS spectrophotometer. The enzyme activity was expressed as U / min / mg of tissue protein.

Catalase activity was assayed by the method as adopted by Chattopadhyay et al. [16]. A portion of the fundic stomach was homogenized (5%) in ice-cold 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000 g for 12 min. The supernatant was then collected and incubated with 0.01 mL of absolute ethanol at 4°C for 30 min, after which 10% Triton X-100 was added to have a final concentration of 1%. The sample thus obtained was used to determine catalase activity by measuring the breakdown of $H_2O_2$ spectrophotometrically at 240 nm. The enzyme activity was expressed as µM $H_2O_2$ consumed / min /mg protein.

**Measurement of hydroxyl radical ($^{\cdot}OH$)**

The ·OH generated in the stomach tissue was measured by using DMSO as a specific ·OH scavenger following the method of Bandyopadhyay et al. [12]. Dimethyl sulfoxide (DMSO) forms a stable product (methane sulfonic acid [MSA]) on reaction with ·OH. Accumulation of MSA was measured to estimate the •OH generated *in vitro* after forming a coloured complex with Fast Blue BB salt. Four groups of rats containing four animals in each group were used for each experiment. The animals of the first group were injected i.p. with 0.4ml of 25% DMSO per 100 g body weight 30 min before oral administration of piroxicam (30 mg/kg bw fed orally). The second group was fed orally TLE (100 mg/kg bw fed orally) 15 min after DMSO injection which was followed by oral administration of piroxicam (30 mg/kg bw fed orally) 30 min after the treatment of rats with the extract. The third group of rats was the control group and was treated only with DMSO (i.p. injection). The fourth group of rats were treated with the extract (100 mg/kg bw fed orally) only and received DMSO injection (i.p.) 15 min before the treatment with the extract. The animals of each group were kept at room temperature for 4 hr and were sacrificed through cervical dislocation, abdomen opened and the stomach tissues were collected. The stomach tissue of each animal was processed for MSA which was allowed to react with Fast Blue BB salt to yield a yellow product. This was measured spectrophotometrically at 425 nm using benzenesulfinic acid as the standard. The values obtained were expressed as nm of •OH/g tissue.

**Measurement of xanthine oxidase and xanthine dehydrogenase activity for the indirect assessment of superoxide anion free radical ($O_2^{\cdot-}$) generated in vivo**

Xanthine oxidase (XO) was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler [23] with some modifications [21]. Briefly, the weighed amount of stomach tissues was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.8. The homogenates were centrifuged at 500 g for 10 min. The supernatant, thus obtained, was further centrifuged at 12,000 g for 20 min. The final supernatant was collected and used for spectrophotometric assay at 295 nm using 0.1 mM xanthine in 50 mM phosphate buffer, pH 7.8, as the substrate. The enzyme activity was expressed as milli Units/ mg protein.

Xanthine dehydrogenase (XDH) was assayed by following the reduction of $NAD^+$ to NADH according to the method of Strittmatter [24] with modifications [21]. In brief, a 10% homogenate of stomach tissue was prepared in 50 mM phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were then processed as described earlier. The supernatant, thus obtained was used for enzyme assay at 340 nm with 0.3 mM xanthine as the substrate (in 50 mM phosphate buffer, pH 7.5) and 0.7 mM $NAD^+$ as an electron donor. The enzyme activity was expressed as milli Units/mg protein.

**Measurement of activities of mitochondrial respiratory chain enzymes**

A portion of the gastric tissue was homogenized (10%) in ice-cold 50 mM phosphate buffer, pH 7.4, with a Potter Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s. The homogenate was then centrifuged at 500 g for 10 min and the supernatant was again centrifuged at 12,000 g for 15 min to obtain the mitochondria. The mitochondrial pellet thus obtained was resuspended in the buffer and used for measuring the mitochondrial enzymes.

Pyruvate dehydrogenase activity was measured spectrophotometrically according to the method of Chretien et.al.[25] with some modifications by following the reduction of NAD+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4, 0.5mM sodium pyruvate as substrate and 0.5mM NAD+ in addition to enzyme. Values were expressed as Units/mg protein.

Mitochondrial isocitrate dehydrogenase activity was measured according to the method of Duncan et. al. [26] by measuring the reduction of NAD+ to NADH at 340nm with the help of a UV-VIS spectrophotometer. One ml assay mixture contained 50mM phosphate buffer, pH 7.4, 0.5mM isocitrate, 0.1mM $MnSO_4$, 0.1mM NAD+ and enzyme. The enzyme activity was expressed as Units/mg protein.

Alpha-ketoglutarate dehydrogenase activity was measured spectrophotometrically according to the method of Duncan et. al. [26] by measuring the reduction of 0.35mM NAD+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4, as assay buffer and 0.1mM α-ketoglutarate as the substrate. The enzyme activity was expressed as Units/mg protein.

Mitochondrial succinate dehydrogenase activity was measured spectrophotometrically by following the reduction of potassium ferricyanide ($K_3FeCN_6$) at 420nm according to the method of Veeger et.al [27] with some modifications. One ml assay mixture contained 50mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4mM succinate, 2.5mM $K_3FeCN_6$ and the enzyme. The enzyme activity was expressed as Units/mg protein.

**Estimation of proteins**

Protein was estimated by the method of Lowry et al., (1951) [28] using bovine serum albumin (BSA) as the standard.

**Statistical evaluation**

The values were represented as means ± S.E.M. Significance of mean values of different parameters between the treatment groups were analyzed using one way analysis of variances (ANOVA) followed by Scheffe' multiple comparison test. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

**RESULTS**

**Dose dependent protection of piroxicam-induced gastric injury by TLE**

**Mean ulcer index**

Figure 1 shows that TLE protected the gastric mucosa from piroxicam-induced gastric ulceration in a dose-dependant manner with a maximum protection of gastric ulcer at a dose of 100 mg/kg bw (93%, $p < 0.001$ vs. piroxicam treated animals).

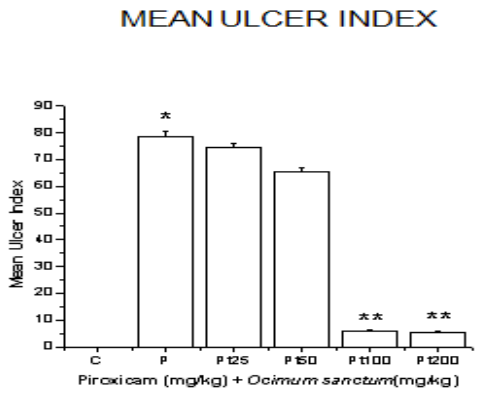


**Fig. 1: (A) Dose-dependent protection by TLE against piroxicam-induced gastric mucosal ulceration.**

Rats were treated with piroxicam (P) (fed orally) and increasing doses of TLE (t) (fed orally). Control (C) animals were treated similarly with vehicle only. Values are mean ± S.E.M of eight rats in each group; *p < 0.001 versus C. **p < 0.001 versus piroxicam-treated animals.

**Macroscopic and microscopic changes in gastric tissue morphology**

Figure 2A and 2B show representative photographs of the rat stomach mucosal surface with the bleeding ulcer spots following treatment of rats with piroxicam at a dose of 30 mg/kg (fed orally). The figures also show a representative photograph of the tissue morphology of the rats of the control, piroxicam treated and TLE protected groups. The results clearly reveal serious bleeding ulcer spots following piroxicam treatment of rats and a gradual decrease of ulcer spots when the rats were pre-treated with increasing doses of TLE with maximum ulcer protection at 100 mg/kg (fed orally). The tissue morphological features also reveal a severe disruption of the gastric mucosa following treatment of rats with piroxicam. However, the extent of mucosal disintegration was found to be decreased when the rats were pre-treated with TLE with maximum protection observed at 100mg/kg bw TLE fed orally (Figure 2B).

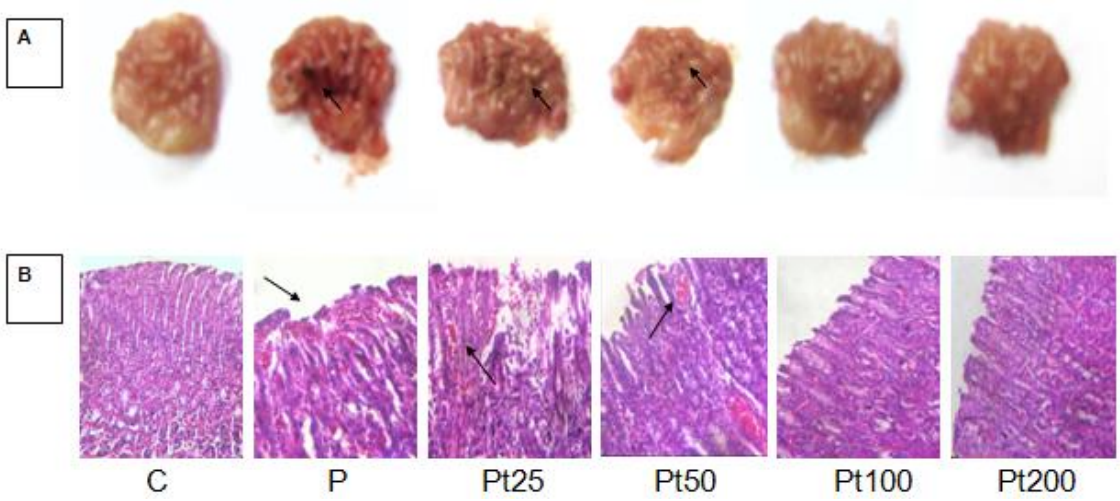


**Fig. 2: Representative image showing dose-dependent morphological changes in rat gastric tissues after treatment of Piroxicam and TLE (t).**

(A) The macroscopic view of mucosal surface of whole stomach showing protective effect of TLE (t) against piroxicam-induced (P) damage of rat gastric tissue. (B) Representative images of hematoxylin-eosin stained sections of stomach tissue (magnification 400X). Arrow heads indicate damaged area of gastric mucosa.

**Lipid peroxidation level (LPO)**

Treatment of rats with piroxicam (30 mg/kg bw.) caused a significant increase (54%, p < 0.001 vs. control) in the level of lipid peroxidation in the gastric tissue which was found to be protected dose-dependently when the rats were pre-treated with increasing doses of TLE (Figure 3A).

**Reduced glutathione (GSH) level**

There occurred a highly significant decrease (63%, p < 0.001 vs. control) in the level of gastric GSH following treatment of rats with piroxicam indicating generation of oxidative stress. The results are presented in Figure 3B shows a dose-dependent increment in the level of gastric GSH when the rats were pre-treated with increasing doses of TLE. Tulsi leaf extract at a dose of 100 mg/kg (fed orally) completely prevented the tissue GSH from getting reduced when compared to the values observed in the gastric tissue of the control rats (Figure 3B).

**Studies with the effective dose of TLE against piroxicam induced gastric injury**

**Mean ulcer index**

In separate experiments, the efficacy of the effective dose of TLE (100 mg/kg BW) against piroxicam-induced gastric ulceration was tested. The Figure 4A illustrates that the effective dose of TLE protected the gastric mucosa from piroxicam-induced gastric ulceration with significant reduction in mean ulcer index (93%, p < 0.001 vs. piroxicam treated animals).

**Macroscopic and microscopic changes in gastric tissue morphology**

Figure 4 illustrates the ulcerated stomach in rats treated with piroxicam and Tulsi extract with a significant injury to gastric mucosa. The figure further reveals that when the rats were pre-treated with the effective dose of TLE (100 mg/ kg), no ulcer spots were detected on the gastric mucosal surface or any injury to tissue morphology.

**Lipid peroxidation level (LPO)**

The data presented in Figure 5A reveals that pretreatment of rats with the effective dose of TLE (100 mg/kg fed orally) reduced the level of LPO in the gastric tissue to the control value when compared to the level measured in piroxicam treated animals (56% reduction vs. piroxicam treated rats; p<0.001).

**Reduced glutathione (GSH) level**

Figure 5B reveals that pretreatment of rats with TLE (100 mg/kg fed orally) prevented the gastric reduced GSH from decreasing (64% increase vs. piroxicam treated rats; p < 0.001).

**Gastric peroxidase (GPO) activity**

There occurred a significant decrease in the gastric peroxidase (GPO) activity (38.79%, p < 0.001 vs. control), an important antioxidant enzyme of rat gastric tissue, following treatment of rats with the present dose of piroxicam. Pre-treatment of rats, however, with the effective dose TLE (100 mg/kg fed orally) completely protected the GPO activity from being decreased (Figure 6A).

**Copper-zinc superoxide dismutase (Cu-Zn SOD) activity**

Piroxicam-induced rise in Cu-Zn SOD activity of gastric tissue was completely restored to the activity observed in the control rats when the animals were pre-treated with TLE (46%, p < 0.001 vs. piroxicam treated rats) (Figure 6B).

**Catalase activity**

Catalase activity of the rat gastric tissue was found to be significantly increased (50.31% vs. control; p < 0.001) following treatment of rats with piroxicam. However, when the rats were pretreated with the

effective dose of TLE, the catalase activity was completely prevented from being increased (Figure 6C).

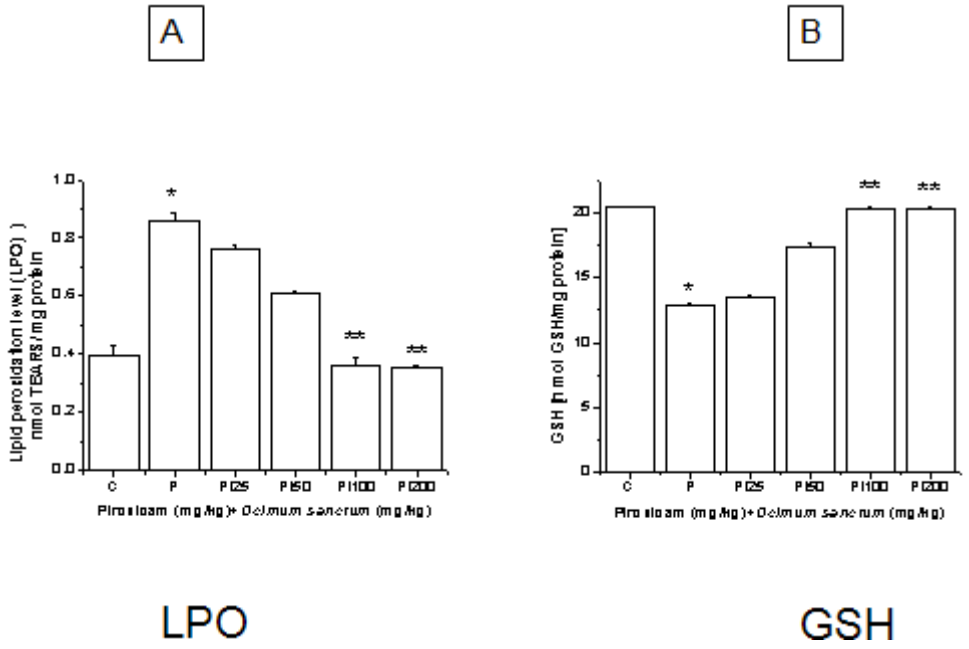


**Fig. 3: (A) Dose-dependent protection by TLE (t) against piroxicam-induced increase in lipid peroxidation.**

Control (C) animals were treated similarly with vehicle only. Values are means ± S.E.M. of eight rats in each group; *p < 0.001 versus control. **p < 0.001 versus piroxicam-treated animals. (B) Dose-dependent protection by TLE (t) against piroxicam-induced decrease in the levels of glutathione of rat gastric tissue. Control (C) animals were treated with vehicle only. Values are mean ± S.E.M. of eight rats in each group; *p < 0.001 versus control. **p < 0.001 versus piroxicam-treated animals.

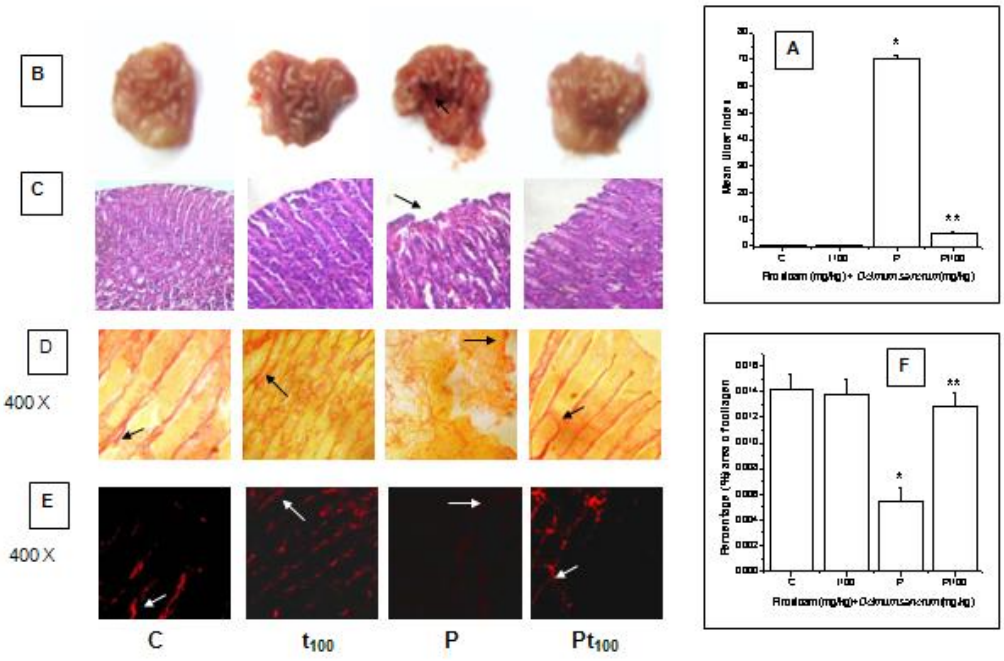


**Fig. 4: (A) Effect of TLE (t) against piroxicam-induced gastric ulceration in rats.**

C= Control, $t_{100}$ = TLE (100mg/kg bw), P= Piroxicam treated (30mg/kg bw), $P_{t100}$ = TLE (100mg/kg bw) + Piroxicam (30mg/kg bw). Values are means ± S.E.M. of eight rats in each group; *p < 0.001 vs control. **p < 0.001 vs piroxicam-treated animals.

**(B) Morphology of rat gastric tissues after treatment with piroxicam (fed orally) and TLE (100mg/kg bw) (fed orally).**

**(C) Hematoxylin-eosin stained sections of stomach tissue (magnification 400X).** Arrow heads indicate damaged area of gastric mucosa.

**(D) Sirius red stained tissue sections of rat stomach of control (400X magnification)**

**(E) Images captured by confocal laser scanning microscope for quantification of fibrosis.** Arrow heads indicate collagen fibers.

**(F) Histogram showing % collagen volume in the gastric tissues.**

Values are mean ± S.E.M. % collagen from 3 images from each of 3 rats of each group; *p < 0.001 versus control; **p < 0.001 versus piroxicam.

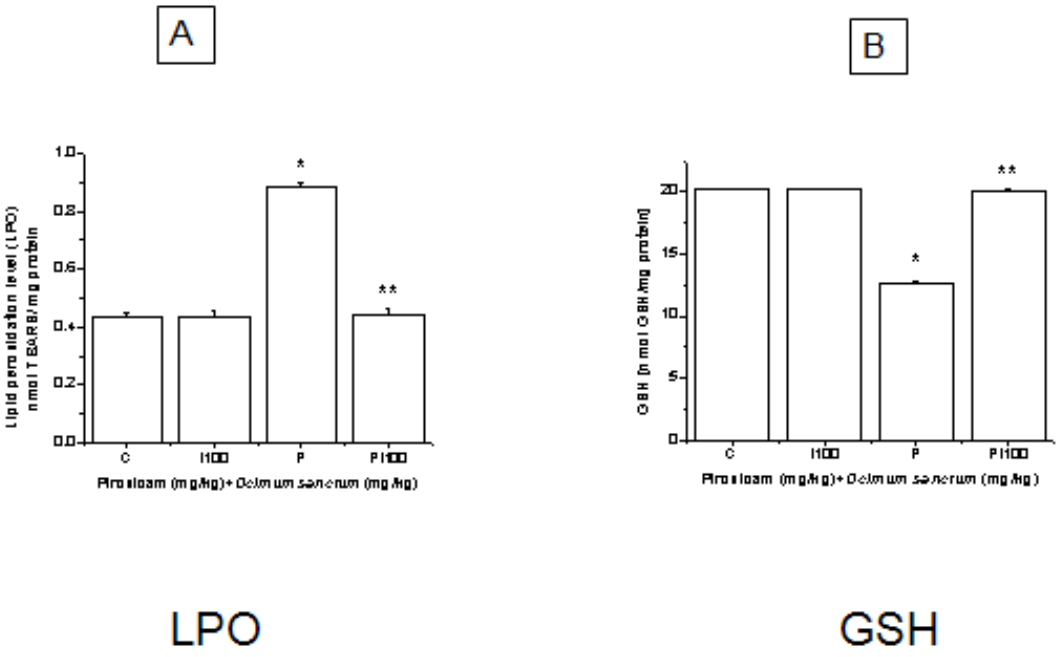


**Fig. 5: (A) Effect of TLE against piroxicam-induced increase in lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS).**

**C=** Control, $\mathbf{t_{100}}$ = TLE (100mg/kg bw), **P**= Piroxicam treated (30mg/kg bw), $\mathbf{P_{t100}}$ = TLE (100mg/kg bw) + Piroxicam (30mg/kg bw) Values are means ± S.E.M. of eight rats in each group; *p < 0.001 vs control. **p < 0.001 vs piroxicam-treated animals.

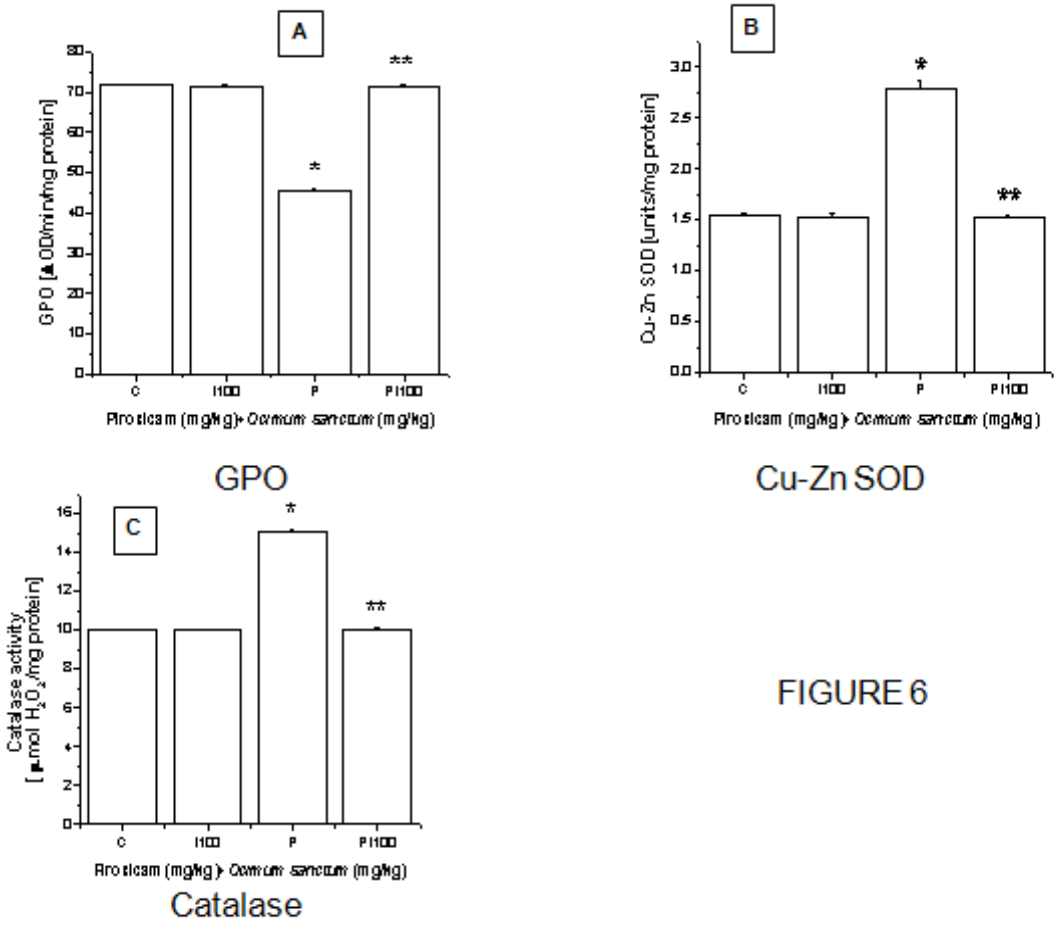


**Fig. 6: (A) Effect of TLE against piroxicam-induced inhibition in gastric peroxidase activity.**

**C=** Control, $\mathbf{t_{100}}$ = TLE (100mg/kg bw), **P**= Piroxicam treated (30mg/kg bw), $\mathbf{P_{t100}}$ = TLE (100mg/kg bw) + Piroxicam (30mg/kg bw) Values are means ± S.E.M. of eight rats in each group; *p < 0.001 vs control. **p < 0.001 vs piroxicam-treated animals.

**Assessment of Superoxide anion radical ($O_2^{\bullet-}$) in vivo**

Whether piroxicam administration to rats has caused the generation of $O_2^{\bullet-}$ was also investigated. The results presented in the Figure 8 clearly indicate that there was an enhancement in the generation of $O_2^{\bullet-}$ *in vivo* following treatment of rats with piroxicam. A 58.46% rise (p< 0.001 vs. control) in xanthine oxidase (XO) activity was observed in the gastric tissue of the piroxicam treated rats while xanthine dehydrogenase (XDH) showed a 40.52% increase vs. control (p< 0.001 vs. control). The total enzyme activity, i.e., XO plus XDH showed a 48.76% rise vs. control while XO-XDH ratio also increased significantly following piroxicam treatment. Pre-treatment of rats, however, with the effective dose of TLE prevented the activities from being increased.

**Generation of hydroxyl radical (•OH) in vivo**

Treatment of rats with the present dose of piroxicam caused more than fourfold increase in the generation of •OH *in vivo* (73.58% vs. control, p < 0.001) when compared to control (Figure 7). Pre-treatment of rats with the effective dose of TLE significantly reduced the generation of •OH compared to piroxicam treated rats (64.59% vs. piroxicam treated rats; p<0.001).

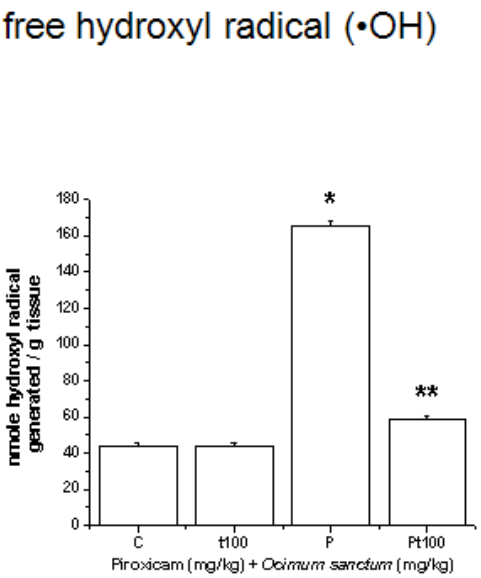


**Fig. 7: Effect of TLE on the scavenging of °OH generated in vivo following treatment of rats with piroxicam.**

**C=** Control, $\mathbf{t_{100}}$ = TLE (100mg/kg bw), **P**= Piroxicam treated (30mg/kg bw), $\mathbf{P_{t100}}$ = TLE (100mg/kg bw) + Piroxicam (30mg/kg bw) Values are means ± S.E.M. of eight rats in each group; *p < 0.001 vs control. **p < 0.001 vs piroxicam-treated animals.

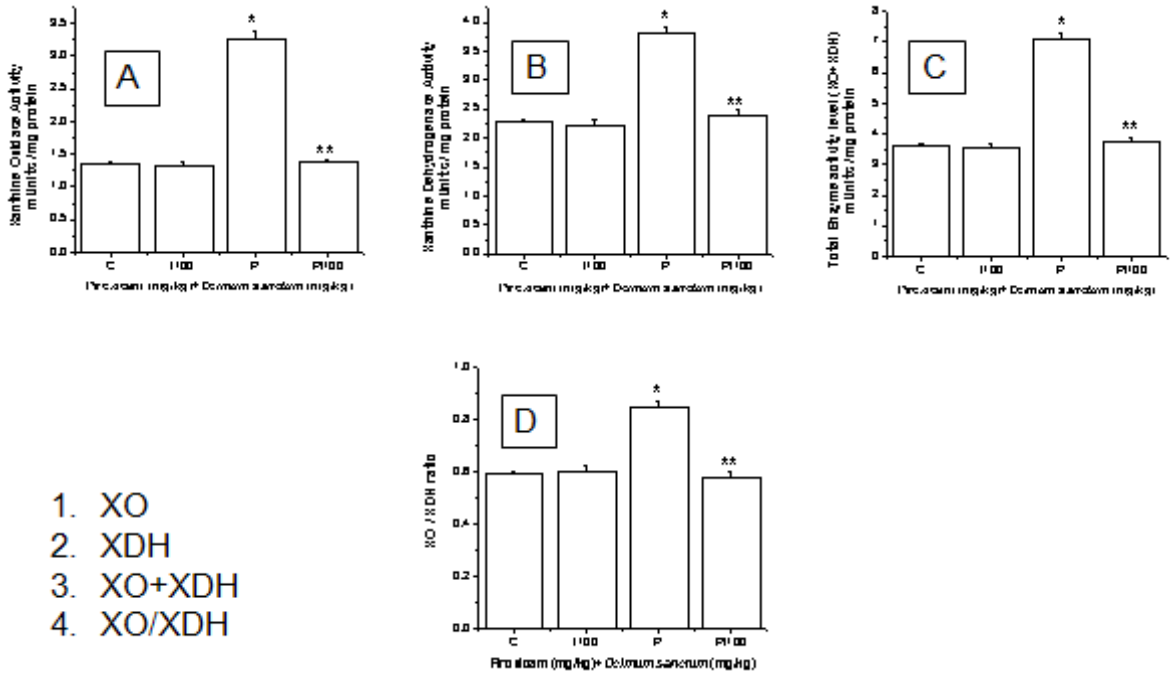

**Fig. 8: Effect of TLE against piroxicam-induced increase in the activities of (A) xanthine oxidase and (B) xanthine dehydrogenase in control (C), TLE (t100) (fed orally), piroxicam-treated (P) (fed orally) and piroxicam + TLE treated (Pt100),rats. Values are means ± S.E.M. of eight rats in each group. *p < 0.001 versus control, **p < 0.001 versus piroxicam-treated animals. (C) Total enzyme activity (XO + XDH), (D) xanthine oxidase/xanthine dehydrogenase (XO/XDH) ratio.**

**Mitochondrial respiratory chain enzyme activity**

Treatment of rats with the present dose of piroxicam caused significant decrease in the activities of the enzymes pyruvate dehydrogenase (82.7%, p < 0.001 vs. control), isocytrate dehydrogenase (51.07%, p < 0.001 vs. control), α-ketoglutarate dehydrogenase (75.94%, p < 0.001 vs. control) and succinate dehydrogenase (79.32%, p < 0.001 vs. control). Pre-treatment of rats, however, with the effective dose of TLE (100 mg/kg bw) prevented the activities from being decreased.

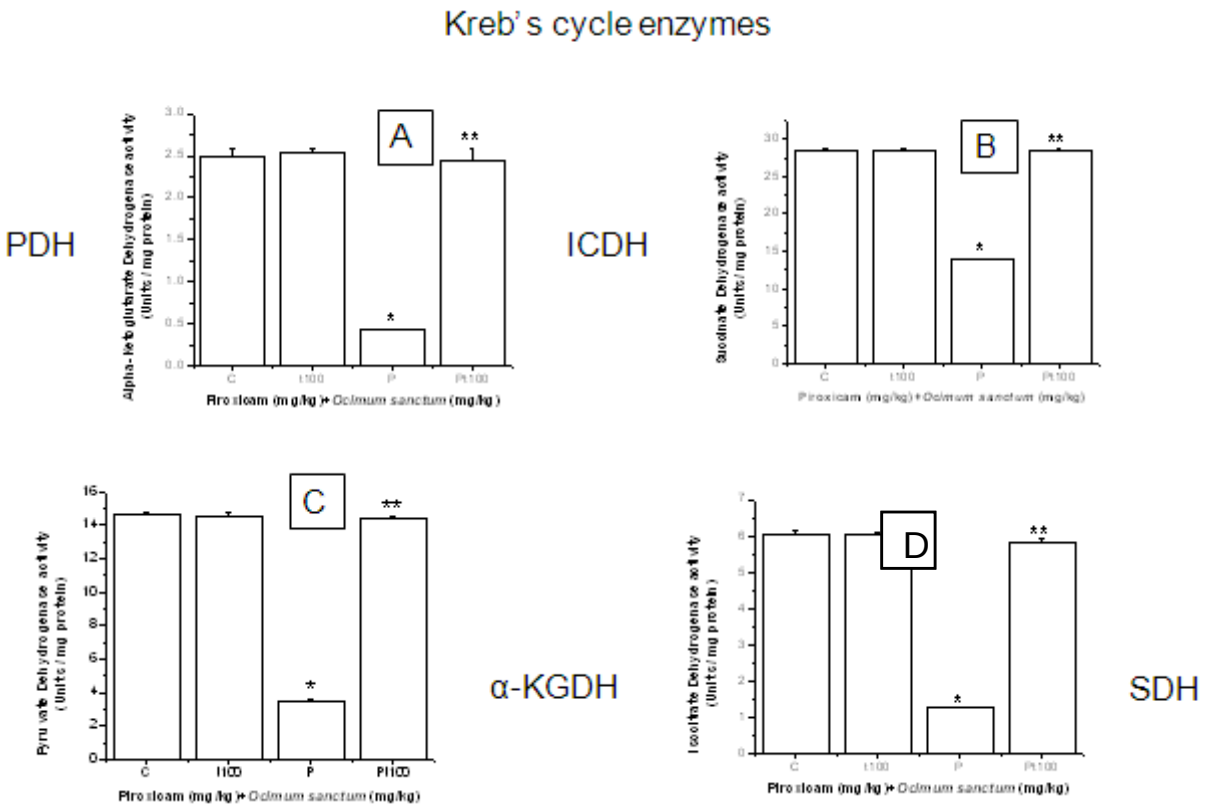


**Fig. 9: Effect of TLE against piroxicam-induced decrease in the activities of (A) pyruvate dehydrogenase, (B) isocitrate dehydrogenase, (C) α-ketoglutarate dehydrogenase and (D) succinate dehydrogenase in control (C), TLE (t100), piroxicam-treated (P) and TLE (Pt100) protected rats. Values are mean ± S.E.M. of eight rats in each group. *p < 0.001 versus control; **p < 0.001 versus piroxicam.**

**Total Phenolics and total flavonoids contant of TLE**

Table 1 reveals that TLE was found to be particularly rich in phenolics and flavonoids.

**Table 1: Estimation of total phenolics and total flavonoids content of TLE**

| Analysed parameters | Values |
|---|---|
| **Total Phenolic Content** [mg gallic acid/ml extract] | **5.237** |
| **Total Flavonoids Content** [mg (+)catechin/gm of leaves] | **6.190** |

**DISCUSSION**

Currently there are several drugs for gastric ulcer. However, the majority of these have side effects and are expensive, limiting their use. Thus, the development of antiulcer agents is focused on the search for cheaper, more effective and less toxic agents. Medicinal plants are among the most attractive source of new drugs. *Ocimum sanctum*, commonly known as Tulsi, is a medicinal plant widely used by the population as a remedy for different problems including gastric problems. However, there are no studies in the literature that

confirm the activity of the aqueous leaf extract of the plant. This study was therefore undertaken to evaluate and contribute to the validation of antiulcer activity of an aqueous extract of leaves of *Ocimum sanctum* (TLE).

The integrity of the gastric mucosa depends on the balance between aggressive (HCl, pepsine) and protective factors (mucus and $HCO_3$ – secretion, prostaglandins, mucosal blood flow, nitric oxide) [29]. Therefore, whether the treatment is effective depends not only on the blockade of acid secretion, but also on the increased production of factors responsible for protecting the gastric mucosa, thus avoiding damage to the epithelium [30].

Inhibition of prostaglandin synthesis is well recognized as the central mechanism by which gastrointestinal injury occurs [31]. This is a result of inhibition of cyclooxygenase enzyme which converts unsaturated fatty acids (which are released during cell injury) such as arachidonic acid to prostaglandins. In the stomach, prostaglandin synthesis is protective as a result of enhanced mucosal blood flow and stimulation of mucous and bicarbonate secretion [32]. In contrast, in arthritis, prostaglandins mediate pain and some components of inflammation. Recognition of two isoforms of cyclooxygenase, with COX1-predominating in the stomach and an inducible COX2 [33] expressed at sites of inflammation offer the prospect of separating the beneficial effects of inhibiting prostaglandin synthesis in joints from the harmful effects of inhibiting it in the stomach [33].

The primary objective of the present investigation was to determine whether TLE provides protection against piroxicam-induced damage to gastric mucosa. Results presented in Figure 1 and Figure 3A and 3B demonstrate clearly that TLE is capable of providing protection to the gastric mucosa against piroxicam-induced gastric injury in a dose-dependent manner. In most of our experiments, the piroxicam-induced gastric ulceration was maximally protected by TLE at the dose of 100mg/kg bw (fed orally). These biochemical observations have been confirmed by the macroscopic and microscopic observations of the gastric mucosa (Figure 2) which indicate gastric tissue damage following piroxicam treatment. This mucosal injury was found to be protected when the rats were pretreated with different doses of TLE. Maximum protection, however was observed at 100 mg/kg bw TLE (fed orally).

Our experiments revealed that pre-treatment of rats with the TLE almost completely inhibited the gastric mucosal ulceration (as measured by mean ulcer index) (Figure 4) indicating the strong potential of the agent.

The results of the present investigation indicate clearly that TLE not only inhibited piroxicam-induced ulceration of the gastric mucosa (Figure 4) but also decreased the piroxicam-induced oxidative stress as evident from the significantly reduced level of LPO and increased level of gastric GSH (Figure 5A and 5B) which indicates that TLE is highly efficient in mitigating the ill effects of piroxicam administration on gastric mucosa. TLE also ameliorates the alterations in the activities of gastric antioxidant enzymes like gastric peroxidise (Figure 6A), Cu-Zn SOD (Figure 6B) and catalase (Figure 6C) indicating that this agent mitigate the changes brought about in the tissue antioxidant system following piroxicam treatment.

It was shown earlier by Bandyopadhyay et al. [8] that piroxicam-induced gastric ulceration results in *in vivo* generation of •OH. Our experiments again confirmed the generation of •OH *in vivo* following treatment of rats with the indicated dose of piroxicam. When the rats were pre-treated with TLE, the formation of •OH was inhibited to near control levels.

Treatment of rats with piroxicam was also found to be involved with the generation of $O_2\bullet^-$, as is evident from the enhanced activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) as well as the increased XO/XDH ratio and the level of total enzyme XO+XDH (Figure 8). These indicate strongly that piroxicam-induced gastric ulceration is the outcome of severe oxidative stress developed within the gastric tissue. Pre-treatment of the rats with the TLE almost completely prevented the activities of these enzymes from getting increased. This indicates that TLE may have the potential to either inhibit the generation of $O_2\bullet^-$ or scavenge this ROS.

Our histological studies further support the biochemical observations as is represented in Figure 4B, 4C, 4D and 4E. This is evident from our macroscopic as well as microscopic studies. However, when TLE were used at effective doses, a complete protection of the tissue morphology with no ulcers was observed, indicating again the effectiveness of this agent against piroxicam-induced gastric ulceration in rats. The rat gastric mucosa was not affected when TLE were administered alone (positive control).

Tulsi (*O. sanctum*), a plant widely used in Ayurveda, possesses anti-inflammatory and antioxidant properties [3]. Flavonoids isolated from Tulsi scavenged free radicals *in vitro* and showed antilipoperoxidant activity *in vivo* at very low concentrations [34]. The free radical scavenging activity of plant flavonoids help in the healing of wounds [34]. Since Tulsi is ubiquitous and abundantly grown, it could be a fairly economical therapeutic agent for wound management as a preventive agent, as well as to control healing.

Given that the number of COX-2 specific NSAIDs is limited [35] and because misoprostol in combination with anti-inflammatory agents is the only recognized form of protection against gastropathy, the results of the present studies seem highly important. With these agents it may be possible to minimize the gastro-toxic effects of piroxicam, when their long-term use is the only choice, especially if TLE is also included in the treatment regimen. Furthermore, these studies establish the versatility in the action of TLE as an antioxidant, i.e., direct scavenging actions as well as indirect antioxidant functions. Besides neutralizing free radicals, a portion of TLE's protective actions may derive from its ability to reduce $O_2^{\bullet-}$ anion free radical generation at the level of the inner mitochondrial membrane by increasing the efficiency of the electron transport chain.

The protection against piroxicam-induced ulcers in our model by TLE which was found to be rich in antioxidants like phenolics and flavonoids, may be used as a gatroprotective agent in gastric ulcers in humans and oxidative stress in general.

## CONCLUSION

The results of this study confirm the use of aqueous leaf extract of Tulsi, i.e., Ocimum sanctum (TLE) in the traditional disease management including gastric ulcer disease. The mode of action of TLE may pave the way for the establishment of a new gastric antisecretory and antiulcer therapy regimen that will not require the use of antacids and antisecretory agents. However, further studies to identify the active moieties and elucidations of the mechanism of action are recommended.

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