Gastric 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 pylori* and bile salts. Defensive factors mainly involve mucus-bicarbonate secretion and prostaglandins [33]. Gastric ulceration is associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [17] like Piroxicam. Known additional risk factors include advanced age, previous history of ulceration, stress, smoking, nutritional deficiency, concomitant administration of anticoagulants and co-existing serious systemic disorders [17, 132]. Possible involvement of oxidative stress in piroxicam-induced gastric lesions has also been demonstrated [60].

In the first chapter of our study, we demonstrate that piroxicam, when fed orally, once at different doses induced damage to the gastric mucosa as is evident from the mean ulcer index. Our studies further indicate the involvement of oxidative stress in the piroxicam-induced gastric damage as evident from the changes observed in the levels of biomarkers of oxidative stress and antioxidant enzymes.

Use of antioxidant therapy against piroxicam induced gastric damage is an area of extensive research. Many drugs, like antacids, H$_2$ receptor blockers, proton pump blockers, anticholinergics, antigastrins etc., are currently used to treat gastric ulceration but all of them exhibit side effects. Therefore, the demand of the time is to look for a safer antioxidant or a preparation with antioxidant activity with anti-secretory and anti-inflammatory properties, and in this context, plant extracts are among the most promising agents [81-83].

*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. [85, 86]. 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, anti-secretory, immunomodulatory, adaptogenic and diaphoretic actions [133-135]. Recently, Dharmani et al. [15] 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 [13, 14]. The fixed oil of Tulsi exhibits a significant anti-ulcer activity against alcohol, histamine, reserpine, serotonin, and stress-induced ulceration in experimental models [16]. These studies though interesting and important did not deal with the detailed mechanism(s) which are responsible for gastro-protection by Tulsi. Furthermore, detailed studies have not been carried out yet with an aqueous extract of Tulsi leaf.

In the first chapter, we provide evidence that a crude aqueous leaf extract (fresh leaf homogenate) of Tulsi, fed orally, 1 hr prior to piroxicam treatment dose-dependently ameliorated the piroxicam-induced gastric ulceration in rat stomach. Additionally, the lyophilized material obtained from the aqueous Tulsi leaf extract (TLEL) was found to be more effective than crude aqueous Tulsi leaf extract (TLE) [i.e., fresh leaf homogenate] in protecting against piroxicam-induced ulceration of the rat gastric mucosa in our experimental conditions. Our studies further reveal that both the fresh Tulsi leaf homogenate (TLE) and the lyophilized material (TLEL) provided gastro-protection against piroxicam in rats through direct as well as indirect antioxidant mechanism(s).
Results

Section-A

(I) Dose-response studies for NSAID of choice i.e., Piroxicam

Induction of gastric ulceration by piroxicam:
Figure 1 reveals that oral administration of piroxicam induced ulceration of the rat gastric mucosa in a dose-dependent manner, with a maximum lesion observed at 30 mg/kg bw (*p < 0.001 vs. control).

![Figure 1: Dose-dependent piroxicam-induced increase in gastric mucosal ulceration (expressed as mean ulcer index). Values are mean ± S.E.M of 7 animals in each group. *p < 0.001 versus control group (i.e., P0).]

Induction of oxidative stress by piroxicam:
To examine whether administration of piroxicam induces oxidative stress, we have measured two important biomarkers of oxidative stress, viz., lipid peroxidation and reduced glutathione (GSH) content of rat stomach tissue. Treatment of rats with different doses of piroxicam elicited a dose-dependent increase in the level of lipid peroxidation (LPO) measured as TBARS in the gastric tissue [Fig 2 A] (*p < 0.001 vs. control group (i.e., P0) at the dose 30 mg / kg bw, fed orally]. Treatment of rats with piroxicam also caused a dose-dependent highly significant decrease in the reduced glutathione (GSH) content of the rat stomach tissue [Fig 2 B].
Figure 2 (A): Effect of piroxicam on lipid peroxidation level measured as thiobarbituric acid reactive substances (TBARS).

Figure 2 (B): Effect of piroxicam on reduced glutathione level of rat stomach tissue. Rats were treated with increasing doses of piroxicam (P). PO i.e. control rats were treated with vehicle only.

Values are mean ± SEM of 7 rats in each group; *p < 0.001 vs. PO.

**Effect of piroxicam on gastric antioxidant enzymes:**

To determine the effect of piroxicam on the activities of the rat gastric antioxidant enzymes, we measured the activities of Cu-Zn-SOD and catalase. The results presented in figure 3 A reveals that piroxicam at the doses of 5.0, 7.5, 10.0, 20.0 and 30.0 mg / kg bw, (fed orally) significantly increased dose-dependently the activity of Cu-Zn SOD in gastric tissue.

Figure 3 B demonstrate that piroxicam also increases the catalase activity, another important antioxidant enzyme, in a dose-dependent manner with the maximum increment of activity observed at 30 mg / kg bw, (fed orally). (*p < 0.001 vs. control group (i.e., PO).
The results presented above clearly reveals that piroxicam at the dose of 30 mg / kg bw was most effective in ulcer formation in rat stomach with no mortality of animals during the period of experiment. Now, we have made an attempt to examine whether a crude homogenate of Tulsi leaves at increasing doses can protect against piroxicam-induced gastric ulceration in rats and the results are presented in the following section.

(II) Dose-dependent studies for Tulsi (*Ocimum sanctum*) [crude homogenate (TLE)]

**Induction of gastric ulceration by piroxicam and protection by TLE:**

Figure 4 documents that pre-treatment of rats with TLE dose-dependently protected the gastric mucosa from being ulcerated following piroxicam treatment at a dose of 30 mg / kg bw, fed orally.

Our macroscopic [figure 5A] as well as microscopic (histopathological) [figure 5B] studies revealed that the changes brought about to the rat gastric mucosa due to piroxicam treatment at a dose of 30 mg/kg bw, fed orally, were found to be dose-dependently protected by TLE, the best protection being afforded at the doses of 300 and 400 mg/kg bw, also fed orally.
**Induction of oxidative stress by piroxicam and protection by TLE:**

Figure 6 A reveals that pre-treatment of rats with TLE dose-dependently protected the level of LPO from being increased in the rat gastric tissue ($p < 0.001$ vs. control). A dose-dependent restoration of the GSH content of the gastric tissue by pre-treatment of rats with increasing doses of TLE is also clearly evident from the data presented in the figure 6 B.
Effect of piroxicam on the activity of gastric antioxidant enzymes and protection by TLE:

Figure 7 A reveals that treatment of rats with piroxicam at a dose of 30 mg / kg bw (fed orally) caused enhancement in Cu-Zn SOD activity of the gastric tissue (44 % increase over control, p < 0.001 versus control). However, when the rats were pre-treated with increasing doses of TLE, the enzyme activity was protected from being increased dose-dependently. Figure 7 B shows a highly significant increase of catalase activity of rat gastric tissue following treatment of the animals with piroxicam at a dose of 30 mg /kg bw, fed orally. The enzyme activity was found to be dose-dependently protected from being increased when rats were pre-treated with increasing doses of TLE (also fed orally).

![Graph A: Protective effect of TLE against piroxicam-induced increase in Cu-Zn SOD activity of rat stomach tissue.](image)

**Figure 7 (A): Protective effect of TLE against piroxicam-induced increase in Cu-Zn SOD activity of rat stomach tissue.**

**Figure 7 (B): Protective effect of TLE against piroxicam-induced increase in catalase activity of rat stomach tissue.** Rats were pre-treated with increasing doses of TLE (T). Control (C) animals were treated with vehicle only.

Values are mean ± SEM of 7 rats in each group; *p < 0.001 vs. C. **p<0.001 vs. P.

From these dose-dependent studies, the dose of 300 mg/kg bw was found to be the best effective dose of TLE at which it was able to almost completely ameliorate the piroxicam-induced alterations to both the tissue morphology and the biochemical parameters studied of the gastric tissue of rats.

Hence, in our subsequent experiments piroxicam was used at a dose of 30 mg / kg bw (fed orally) to induce gastric ulceration in rats and the protective effects of TLE on
other parameters were examined at a dose of 300 mg/kg bw (also fed orally) and the results are presented in the following section.

(III) Studies with the best effective doses of piroxicam and TLE

Induction of gastric mucosal ulceration by piroxicam and protection by TLE:
The data presented in figure 8 demonstrate that piroxicam, at a dose of 30 mg/kg bw fed orally, induced ulceration of the rat gastric mucosa as evident from the mean ulcer index (*p < 0.001 versus control). Pre-treatment of rats with TLE at the dose of 300 mg/kg bw also fed orally, was found to almost completely protect the gastric mucosa from being ulcerated (**p < 0.001 versus P).

![Figure 8: Protective effect of TLE against piroxicam-induced gastric ulceration as evident from the mean ulcer index. The rats were treated with piroxicam (P) at a dose of 30 mg/kg bw, fed orally. TLE protected rats were treated with 300 mg/kg bw (also fed orally) 1 hr before piroxicam treatment (PT300). The rats of the positive control group (T300) were fed with only TLE at the same time. The control (C) rats were treated with vehicle only. Positive control rats were treated with TLE (T300) only. Values are means ± S.E.M. of seven rats in each group. *p < 0.001 versus C; **p < 0.001 versus P.]

Protective effect of TLE against piroxicam-induced changes in the gastric mucosal tissue morphology: Treatment of rats with piroxicam at a dose of 30 mg/kg bw (fed orally) resulted in the ulceration of the gastric mucosa and the results are presented in figure 9 (A and B).
Figure 9 (A): Photograph of rat gastric mucosal surface.

Figure 9 (B): Representative images (400X magnification) of haematoxylin-eosin stained gastric tissue sections of rat stomach of Control (C), TLE (T\textsubscript{300}), piroxicam (P) treated and TLE protected (PT\textsubscript{300}) rats.

Figure 9A shows the photographs of rat gastric mucosal surface (cavity side) and 9B shows the H-E stained rat gastric tissue sections at 400X magnification. A significant damage to the gastric mucosa is evident in the piroxicam treated tissue sections. Pre-treatment of rats, however, with TLE at a dose of 300mg/kg bw (also fed orally), 1 hr before piroxicam treatment, protects the gastric mucosa from being ulcerated due to piroxicam treatment.

**Quantification of gastric tissue collagen through confocal microscopy:**

The damage to the rat gastric tissue morphology due to piroxicam treatment was further confirmed by the observation that there was a loss of tissue collagen from the intercellular space compared to the gastric tissue of

![Image of confocal microscopy results](image)

Figure 10: (A) Representative images (400X magnification) of Sirius red stained sections of rat stomach tissue of control (C), TLE (T\textsubscript{300}), piroxicam (P) treated and TLE (PT\textsubscript{300}) protected rats. Red colour stretches show the areas containing collagen.

Figure 10: (B) The similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibers.

Figure 10: (C) Histogram showing % collagen volume in the gastric tissues. Values are means ± S.E.M. % collagen from 3 images from each of 3 rats of each group; *p < 0.001 versus C; **p < 0.001 versus P.
the control rats and the results are presented in the figure 10[A [Syrus red staining] and B [representative images through Laser-Confocal Microscopy]].

This loss of collagen due to piroxicam treatment was found to be partially but significantly prevented when the animals were pre-treated with TLE at the dose of 300 mg /kg bw (fed orally). The results strongly indicate that TLE has the ability to provide protection to the gastric tissue against piroxicam-induced damage. However, in respect of tissue collagen content, the indicated dose of TLE may be insufficient to completely protect the stomach tissue from piroxicam induced damage or the complete protection of the tissue collagen content may require some more time.

**Piroxicam-induced oxidative stress and protection by TLE:** Figure 11A shows that treatment of rats with piroxicam caused elevation in lipid peroxidation (LPO) level (48.84% increase versus control, *p < 0.001) of the rats gastric tissue. This elevation in the level of LPO was significantly lowered on pre-treatment of rats with TLE (**p < 0.001 versus piroxicam). The results presented in the figure 11B indicate a significant decrease in the gastric reduced glutathione levels due to piroxicam treatment (40.33% versus control, *p < 0.001). The GSH level of the gastric tissue was found to be significantly ameliorated when the rats were pre-treated with 300 mg /kg bw (fed orally) of TLE (**p < 0.001 versus piroxicam).

![Graph A](image)

**Graph A:** Protective effect of TLE against piroxicam-induced increase in lipid peroxidation (LPO) level of rat stomach tissue. The rats were treated with piroxicam (P) at a dose of 30 mg /kg bw (fed orally). TLE protected rats were treated with 300 mg/kg bw (also fed orally) 1 hr before piroxicam treatment (PT300). The control (C) rats were treated with vehicle only. Positive control rats were treated with 300 mg/kg bw piroxicam.

![Graph B](image)

**Graph B:** Glutathione (GSH) levels in rat stomach tissue. The results indicate a significant decrease in the GSH level of the gastric tissue due to piroxicam treatment (40.33% versus control, *p < 0.001). The GSH level of the gastric tissue was found to be significantly ameliorated when the rats were pre-treated with 300 mg/kg bw TLE (**p < 0.001 versus piroxicam).
treated with TLE (T300) only. Values are means ± S.E.M. of seven rats in each group; *p < 0.001 versus C; **p < 0.001 versus P.

Figure 11 (B): Protective effect of TLE against piroxicam-induced decrease in reduced glutathione (GSH) level of rat stomach tissue.

Values are mean ± S.E.M. of seven rats in each group; *p < 0.001 versus C; **p < 0.001 versus P.

The data presented in the figure 12A and 12B demonstrate that the activity and the level of Cu-Zn SOD, an important gastric antioxidant enzyme, were found to be elevated following treatment of rats with piroxicam at the indicated dose. The activity and the protein level of the enzyme were found to be almost completely protected from being altered when the rats were pretreated with TLE at a dose of 300 mg/kg bw (fed orally).

Figure 12 (A): Protective effect of TLE against piroxicam-induced increase in Cu-Zn SOD activity of rat gastric tissue in control (C), TLE only (T300), piroxicam-treated (P) and TLE protected (PT300) rats. Values are mean ± SEM of 7 rats in each group; *p < 0.001 vs. C. **p<0.001 vs. P.

Figure 12 (B): Representative result of Western blot analysis for determining the level of Cu-Zn SOD (lanes from left) of gastric tissue in control (C), TLE only (T300), piroxicam-treated (P) and TLE (PT300) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control.

The pixel density of bands obtained through Western blotting was quantified with imageJ software (NIH, USA) and the values (mean ± SEM) were presented in the form of a bar graph. *p < 0.001 vs.C; **p < 0.001 vs. P.

Figure 13 reveals a highly significant increase in the activity of Mn-SOD, a key mitochondrial antioxidant enzyme, in the rats treated with the same
dose of piroxicam. The activity of this enzyme was found to be almost completely protected from being altered when the rats were pre-treated with TLE.

Figure 13 Protective effect of TLE against piroxicam induced increase in Mn-SOD activity of rat stomach tissue. The rats were treated with piroxicam (P) at a dose of 30 mg/kg bw body weight (oral). TLE protected rats were treated with 300 mg/kg bw body weight (oral) TLE, 1 hr before piroxicam treatment (PT300). The control (C) rats were treated with vehicle only. Rats of the positive control group were treated with TLE (T300) only. Values are means ± S.E.M. of seven rats in each group; *p < 0.001 versus C; *p < 0.001 versus P.

Figure 14 shows a significant decrease in gastric peroxidase (GPO) activity of the rats treated with 30mg /kg bw (fed orally) of piroxicam. The activity of GPO was protected completely from being decreased when the rats were pre-treated with TLE at the dose of 300 mg / kg be (fed orally).

Figure 14: Protective effect of TLE against piroxicam-induced decrease in GPO activity of rat stomach tissue. The rats were treated with piroxicam (P) at a dose of 30 mg/kg bw (fed orally). TLE protected rats were treated with 300 mg/kg bw (also fed orally), 1 hr before piroxicam treatment (PT300). The control (C) rats were treated with vehicle only. Positive control rats were treated with TLE (T300) only. Values are mean ± S.E.M. of seven rats in each group; *p < 0.001 versus C; *p < 0.001 versus P.

Figure 15 A demonstrates piroxicam (30 mg /kg bw, fed orally) induced increase in catalase activity, another important antioxidant enzyme of the rat gastric tissue. The activity of this enzyme was found to be completely protected from being increased when the rats were pre-treated with TLE (300 mg/kg bw, also fed orally). This enhanced activity of catalase following piroxicam treatment of rats is supported by an increased level of the enzyme Mn-SOD [units/mg protein].

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protein as is evident from our Western-blot analysis. The level of the enzyme protein was also found to be almost completely protected from being increased when the rats were pre-treated with the TLE at the indicated dose. (Fig. 15 B).

Figure 15 (A): Protective effect of TLE against piroxicam-induced increase in catalase activity of rat gastric tissue. The rats were treated with piroxicam (P) only; and piroxicam and TLE (PT300). The control rats were treated with vehicle only. Positive control rats were treated with TLE only. Values are mean ± SEM of 7 rats in each group; *p < 0.001 vs. C. **p < 0.001 vs. P.

Figure 15 (B): Representative result of Western blot analysis for determining the level of catalase (lanes from left) of stomach tissue in control (C), TLE only (T300), piroxicam-treated (P) and TLE (PT300) protected rats. The Western blot analysis was repeated at least three times. Actin served as loading control. The pixel density of bands obtained through Western blotting was quantified with imageJ software. (NIH, USA) and the values (mean ± SEM) were presented below in the form of a bar graph. *p <0.001 versus C; **p < 0.001 versus P.

Figure 16 shows that treatment of rats with piroxicam, at the present dose of 30 mg/kg bw (fed orally), significantly decreased the activity of gastric peroxidase (GPx) in the stomach tissue of rats (28% versus control, *p < 0.001). However, GPx activity was found to be completely protected from being decreased when the rats were pre-treated with TLE at the dose of 300 mg / kg bw fed orally.
Piroxicam-induced generation of reactive oxygen species (ROS) and protection by TLE: We have indirectly assessed whether piroxicam administration to rats have caused the endogenous generation of ROS. The results presented in the figure 17 A-E clearly indicate that there was an enhancement in the generation of $O_2^\cdot$ in vivo following treatment of rats with piroxicam. The activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH), the total enzyme activity, i.e., XO plus XDH, XO-XDH ratio and XO/XO+XDH ratio were found to be significantly increased following treatment of rats with piroxicam. All these parameters were found to be protected from being increased when the rats were pre-treated with TLE indicating TLE’s ability to neutralize oxygen free radicals in vivo.

However, in TLE only treated rats (positive control), the activities of XO and XDH, the total enzyme activity as well as their ratios exhibited no significant change when compared to that of the values observed in the control rats.
Figure 17: Protective effect of TLE against piroxicam-induced increase in the activities of (A) xanthine oxidase and (B) xanthine dehydrogenase in control (C), TLE only (T300), piroxicam-treated (P) and TLE protected (PT300) rats. Values are Mean ± SEM of 7 rats in each group. *p < 0.001 vs. C, **p < 0.001 vs. P. (C) Total enzyme activity (XO + XDH), (D) xanthine oxidase / xanthine dehydrogenase (XO/XDH) ratio, (E) xanthine oxidase / xanthine oxidase + xanthine dehydrogenase (XO/XO+XDH) ratio.
Figure 18 demonstrates that treatment of rats with piroxicam caused nearly a four-fold increase of endogenous generation of $^\cdot$OH compared to control. Pre-treatment of rats with TLE at the indicated dose decreased the piroxicam-induced $^\cdot$OH formation to near control level (i.e., basal value).

**Protective effects of TLE against piroxicam-induced alterations in the activities of pyruvate dehydrogenase (PDH), mitochondrial tricarboxylic acid cycle (TCA) cycle and respiratory chain enzymes:**

(a) Pyruvate dehydrogenase (PDH) activity:

Figure 19A depicts a significant decrease in the activity of pyruvate dehydrogenase (PDH) (*p < 0.001 versus control), the enzyme that couples glycolysis to TCA cycle following treatment of rats with piroxicam. Pre-treatment of rats with TLE at a dose of 300 mg /kg bw (fed orally) almost completely protected the PDH activity from being decreased (**p < 0.001 versus P).

(b) Mitochondrial TCA cycle enzymes:

Since mitochondria are the major site of cellular oxidative stress, we studied the activities of some of the enzymes of the gastric mitochondrial tricarboxylic acid (TCA) cycle as well as mitochondrial respiratory chain enzymes in control, piroxicam treated and TLE protected rats.
Figures 19B, 19C and 19D show the piroxicam-induced decrease (*p < 0.001 versus control) in the activities of some of the TCA cycle enzymes like isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) respectively. The activities of all the three enzymes were found to be significantly protected from being decreased when the rats were pre-treated with TLE at the indicated dose (**p < 0.001 versus P for ICDH, α-KGDH and SDH). It was further noted that the activities of the enzymes in the TLE protected rats were close to the activities measured in the control rats.

Figure 19: Protective 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 (T300), piroxicam-treated (P) and TLE (PT300) protected rats. Values are mean ± S.E.M. of seven rats in each group. *p < 0.001 versus C; **p < 0.001 versus P.
Estimation of the total phenolics, flavonoids, condensed tannins, chlorophyll content and pH of TLE

Table 1 represents the total phenolics, flavonoids, condensed tannins, chlorophyll content and pH of TLE.

Table 1. Estimation of the total phenolics, flavonoids, condensed tannins, chlorophyll content and pH of TLE

<table>
<thead>
<tr>
<th>Analysed parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phenolic Content</td>
<td>3.135 ± 0.10</td>
</tr>
<tr>
<td>[mg gallic acid/ml extract]</td>
<td></td>
</tr>
<tr>
<td>Total Flavonoids Content</td>
<td>3.39 ± 0.16</td>
</tr>
<tr>
<td>[mg (+)catechin/gm of leaves]</td>
<td></td>
</tr>
<tr>
<td>Total condensed tannins (procyanidins)</td>
<td>2.71 ± 0.16</td>
</tr>
<tr>
<td>[mg (+)catechin/gm of leaves]</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll Content [μM]: Chlorophyll a</td>
<td>0.0245 ± 0.08</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>0.0193 ± 0.11</td>
</tr>
<tr>
<td>Total Chlorophyll</td>
<td>0.0438 ± 0.04</td>
</tr>
<tr>
<td>pH of the leaves</td>
<td>6.85 – 7.04</td>
</tr>
</tbody>
</table>

Each value is a mean ± S.E.M.
Section-B

The studies of this section were carried out by using the lyophilized material obtained from aqueous extract of Tulsi leaves to find out whether this preparation is more effective than the crude aqueous homogenate of Tulsi leaves in ameliorating the piroxicam induced gastric ulceration in rats. To obtain the lyophilized material, Tulsi leaves were oven dried and pulverized. The powdered leaves were then dissolved in distilled water and kept at room temperature overnight. A supernatant was obtained by centrifuging the decanted material at a low speed in cold to remove any unwanted dust particles. The supernatant, thus obtained, was collected, deep frozen in lyophilizer bottles and lyophilized (IICB, Kolkata, Central Instrumentation Facility). The lyophilized material was then carefully collected and stored at 4°C for future use. This lyophilized material was reconstituted in aqua guard clean drinking water and the rats were fed with this extract at different doses [115]. The results obtained with this lyophilized preparation are presented in the following sections.

(I) Dose-dependent studies for aqueous Tulsi leaf extract

[Lyophilized material: TLE\textsuperscript{L}]

Induction of gastric ulceration by piroxicam and protection by TLE\textsuperscript{L}:

Figure 20 documents that pre-treatment of rats with TLE\textsuperscript{L} dose-dependently protected the gastric mucosa from being ulcerated following treatment with piroxicam at a dose of 30 mg / kg bw, fed orally. The TLE\textsuperscript{L} was found to be maximally effective at the dose of 100 mg / kg bw fed orally.
Protective effect of TLE against piroxicam-induced changes in the gastric tissue morphology: Treatment of rats with piroxicam at a dose of 30 mg/kg bw (fed orally) resulted in ulcer formation on the mucosal surface in the rat stomach, as is evident from the results presented in the Fig. 21 A that shows the photographs of rat stomach (inner mucosal surface). Figure 21 B shows the H-E stained rat gastric tissue sections at 400X magnification. A significant damage to the rat gastric mucosa was observed in the piroxicam-treated tissue sections. There is marked congestion in the mucosal blood vessels along with interstitial and luminal hemorrhage. However, pre-treatment of rats with TLE (fed orally at the indicated doses) 1 hr before piroxicam treatment dose-dependently protected the rat gastric mucosa from being ulcerated.
Induction of oxidative stress by piroxicam and protection by TLE:

Figure 22 A reveals that pre-treatment of rats with TLE-dose-dependently protected the gastric tissue from being peroxidized due to treatment with piroxicam (*p < 0.001 vs. control). Furthermore, when the rats were pre-treated with TLE, a dose-dependent protection of the tissue GSH content was also observed indicating that the extract is capable of providing protection against piroxicam-induced oxidative stress in rat gastric mucosa (figure 22 B).

Protective effect of TLE against piroxicam-induced alterations in gastric antioxidant enzymes:

Treatment of rats with piroxicam at a dose of 30 mg / kg bw (fed orally) caused significant elevation in gastric Cu-Zn SOD activity. The results presented in figure 23A reveal that pre-treatment of rats with increasing doses of TLE-dose-dependently protected the activity of this gastric antioxidant enzyme Figure 23B shows a highly significant increase of
catalase activity of rat gastric tissue following treatment of the animals with piroxicam at a dose of 30 mg /kg bw, fed orally. This elevation of catalase activity was found to be completely protected when the rats were pre-treated with increasing doses of TLEL. At the dose of 100 mg /kg bw fed orally, the activity of catalase was found to be completely protected. Treatment of rats with the indicated dose of piroxicam caused a significant decrease [41.60% versus control, p < 0.001 versus control] in rat gastric peroxidase (GPO) activity, an important gastric antioxidant enzyme capable of scavenging hydrogen peroxide (H₂O₂). The activity of GPO was found to be dose-dependently protected from being decreased when the rats were pre-treated with increasing doses of TLEL. The results indicate that at the dose of 100 mg / kg bw, TLEL almost completely protected the GPO activity from being decreased.

Figure 23 (A): Protective effect of TLEL against piroxicam-induced increase in Cu-Zn SOD activity in rat stomach.
Figure 23 (B): Protective effect of TLEL against piroxicam-induced increase in catalase activity of rat stomach.
Figure 23 (C): Protective effect of TLEL against piroxicam-induced decrease in gastric peroxidase activity of rat stomach.
Rats were treated with piroxicam (P) and increasing doses of TLEL (t). Control (C) animals were treated with vehicle only.
Values are mean ± SEM of 7 rats in each group; *p < 0.001 vs. C. **p<0.001 vs. P.
From the dose-dependent studies, the dose of 100 mg/kg bw (fed orally) was found to be the effective dose of TLE against piroxicam-induced ulceration at a dose of 30 mg/kg bw as evident from the mean ulcer index (*p < 0.001 versus control). Pre-treatment of rats with TLE at the dose of 100 mg/kg bw, fed orally, was found to be able to significantly protect the gastric mucosa from being ulcerated (**p < 0.001 versus P).

(II) Studies with the best effective dose of piroxicam and TLE

Induction of gastric mucosal ulceration by piroxicam and protection by TLE: Figure 24 demonstrates piroxicam-induced gastric ulceration at a dose of 30 mg/kg bw as evident from the mean ulcer index (*p < 0.001 versus control). Pre-treatment of rats with TLE at the dose of 100 mg/kg bw, fed orally, was found to be able to significantly protect the gastric mucosa from being ulcerated (**p < 0.001 versus P).

Protective effects of TLE against piroxicam-induced changes in the rat gastric tissue morphology: Treatment of rats with piroxicam at the present dose [30 mg/kg bw (fed orally)] resulted in ulcer formation in the rat gastric mucosa which is evident from the results presented in figure 25 A and B. The figure 25 A shows the photographs of rat gastric mucosal surface (cavity side) which shows a number of bleeding ulcer spots following
treatment with piroxicam, and Figure 25 B shows considerable damage to rat gastric mucosa as evident from the H-E stained rat gastric tissue sections at 400X magnification. Pre-treatment of rats with TLE\textsuperscript{i}, however, at a dose of 100mg/kg bw, also fed orally, 1 hr before piroxicam treatment protected the gastric mucosa from being ulcerated due to piroxicam treatment.

Figure 25 (A): Representative photographs of rat stomach (gastric mucosal surface; cavity side).
Figure 25 (B): Representative images (400X magnification) of haematoxylin-eosin stained rat gastric tissue sections of Control (C), TLE\textsuperscript{i} (t\textsubscript{100}), piroxicam (P) treated and TLE\textsuperscript{i} protected (Pt\textsubscript{100}) rats.

The damage to the gastric tissue morphology due to piroxicam treatment was further confirmed by the observation that there was a loss of tissue collagen from the intercellular space compared to control and the results are presented in the figure 26(A and B). This loss of collagen was found to be partially protected when the animals were pre-treated with TLE\textsuperscript{i} at the dose of 100 mg kg bw (fed orally). The results further indicate that TLE\textsuperscript{i} has the ability to provide protection to the gastric tissue against piroxicam-induced damage.
Figure 26 (A): Representative images (400X magnification) of Sirius red stained tissue sections of rat stomach of control (C), TLE\textsuperscript{l} (T\textsubscript{100}), piroxicam (P) treated and TLE\textsuperscript{l} (PT\textsubscript{100}) protected rats. Red colour stretches are collagen depositions.

Figure 26 (B): The similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibers.

Figure 26 (C): 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 C; **p < 0.001 versus P.

**Protective effects of TLE\textsuperscript{l} against piroxicam-induced oxidative stress in rat gastric tissue:** Figure 27A shows piroxicam-induced increase in lipid peroxidation level [LPO] (50.56% increase versus control, *p < 0.001) of the gastric tissue. Treatment of rats with TLE\textsuperscript{l} at a dose of 100 mg / kg bw (fed orally) completely protected the tissue LPO level from being increased (**p < 0.001 versus piroxicam). The results presented in figure 27B indicate a significant decrease in the gastric reduced glutathione levels due to piroxicam treatment (37.67% versus control, *p < 0.001). The level of tissue GSH was found to be completely protected from being decreased when the rats were pre-treated with 100 mg / kg bw (fed orally) of TLE\textsuperscript{l} (**p < 0.001 versus piroxicam).
Figure 27 (A): Protective effect of TLE against piroxicam-induced increase in lipid peroxidation (LPO) level of rat stomach tissue. The rats were treated with piroxicam (P) at a dose of 30 mg/kg bw (fed orally). TLE-protected rats were treated with 100 mg/kg bw (also fed orally), 1 hr before piroxicam treatment (Pt100). The control (C) rats were treated with vehicle only. Positive control rats were treated with TLE (tl00) only. Values are mean ± S.E.M. of seven rats in each group; *p < 0.001 versus C; *p < 0.001 versus P.

Figure 27 (B): Protective effect of TLE against piroxicam-induced decrease in reduced glutathione (GSH) level of rat stomach tissue. Values are means ± S.E.M. of seven rats in each group; *p < 0.001 versus C; **p < 0.001 versus P.

Treatment of rats with the present dose of piroxicam caused elevation the activities of Cu-Zn SOD and catalase of the rat stomach tissue with a concomitant decrease in the activity of gastric peroxidase. However, pre-treatment of rats with 100 mg / kg bw (fed orally) of TLE completely protected the activity of these enzymes from being altered, indicating clearly that TLE is effective at a low dose compared to crude homogenate.
The protective effects of TLE against piroxicam-induced generation of reactive oxygen species (ROS) in rat gastric tissues: The results presented in the figure 29A-E clearly indicate that the activities of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) as well as the total enzyme activity, i.e., XO plus XDH, XO-XDH ratio and XO / XO+XDH ratio all were found to be significantly increased following piroxicam treatment of rats. All these parameters studied were found to be protected from being altered when the rats were pre-treated with TLE indicating its ability to neutralize oxygen free radicals in vivo. The XO and XDH activities in the rats treated with TLE only (positive control) did not show any change compared to that of the control rats.
Figure 29: Protective effect of TLE against piroxicam-induced increase in the activities of (A) xanthine oxidase and (B) xanthine dehydrogenase in control (C), TLE-only (t100), piroxicam-treated (P) and TLE protected (P100) rats. Values are Mean ± SEM of 7 rats in each group. *p < 0.001 vs. C, **p < 0.001 vs. P. (C) The other parameters like total enzyme activity (XO + XDH), (D) xanthine oxidase / xanthine dehydrogenase (XO/XDH) ratio, (E) xanthine oxidase / xanthine oxidase + xanthine dehydrogenase (XO/XO+XDH) ratio were calculated from the activity values of the XO and XDH.
Protective effects of TLB against piroxicam-induced alterations in the activities of pyruvate dehydrogenase (PDH), mitochondrial tricarboxylic acid cycle (TCA) cycle and respiratory chain enzymes:

Figure 30A depicts a significant decrease in the activity of pyruvate dehydrogenase (PDH) (*p < 0.001 versus control), the enzyme that couples glycolysis to TCA cycle. Pre-treatment of the rats with TLB significantly ameliorated the piroxicam-induced effects (**p < 0.001 versus P). Figures 30B, 30C and 30D show the piroxicam-induced decrease (*p < 0.001 versus control) in the activity of the TCA cycle enzymes isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) respectively. The activities of all the three enzymes were found to be almost completely protected from being decreased when the rats were pre-treated with the indicated dose of TLB (** p< 0.001 versus P for ICDH, α-KGDH and SDH).
Figure 30: Protective 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 (T300), piroxicam-treated (P) and TLE (PT300) protected rats. Values are mean ± S.E.M. of seven rats in each group. *p < 0.001 versus C; **p < 0.001 versus P.

(III) Estimation of total phenolics and total flavonoids content of TLE

Table 2 shows the total phenolics and total flavonoids contents of TLE.

**Table 2. Estimation of total phenolics and total flavonoids content of TLE.**

<table>
<thead>
<tr>
<th>Analysed parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phenolic Content [mg gallic acid/ml extract]</td>
<td>5.237 ± 0.09</td>
</tr>
<tr>
<td>Total Flavonoids Content [mg (+)catechin/gm of leaves]</td>
<td>6.019 ± 0.08</td>
</tr>
<tr>
<td>pH of the leaves</td>
<td>6.86 - 7.00</td>
</tr>
</tbody>
</table>

Each value is a mean ± S.E.M.
Discussion

The NSAIDs are among the most widely prescribed and used drugs for rheumatologic as well as non-rheumatologic conditions, because of their suppression of prostaglandin synthesis [2]. A recent report claimed that approximately 2000 patients per annum may die as a result of NSAID-induced ulcer bleeding and perforation in the UK [5]. Worldwide, more than 30 million or even more people consume NSAIDs daily and of these 40% of the patients are more than 60 years of age. NSAIDs have been reported to affect the entire gastrointestinal tract [6].

The current studies revealed an increase in rat gastric mucosal lesions (as expressed by ulcer index) following oral administration of piroxicam (30 mg piroxicam/kg bw). The crude aqueous Tulsi leaf extract (TLE) [crude homogenate], was found to attenuate piroxicam-induced gastric mucosal lesions in this model.

Administration of piroxicam to rats caused increased lipid peroxidation and depletion of antioxidants of rat gastric tissue, indicating production of ROS and generation of oxidative stress during gastric ulceration. However, all these parameters tested were found to be protected from being altered when the rats were pre-treated with TLE. This documents TLE's ability to provide gastro-protection when piroxicam is used as an anti-inflammatory intervention. Treatment of rats with piroxicam at the indicated dose (30 mg/kg bw) increases the activity of gastric SOD and inhibits GPO activity which favors accumulation of H$_2$O$_2$. Hydrogen peroxide (H$_2$O$_2$) is generated at an enhanced rate as suggested by an increased Cu-Zn-SOD activity. Hydroxyl radicals (·OH) are also probably produced at a higher rate from O$_2^-$ and H$_2$O$_2$ which would account for a major portion of the oxidative damage in piroxicam-induced gastric ulceration. Our studies indicate that one of the TLE's actions in protecting against piroxicam-induced gastric ulcers in vivo may be the result of directly scavenging the ·OH generated following oral piroxicam administration in rats. The present work shows that pre-treatment of the rats with TLE restores the activities of GPO, a key
antioxidant enzyme in the rat stomach. Thus, TLE may also protect against piroxicam-induced gastric ulceration by modulating the activities of the gastric antioxidant enzymes.

The lyophilized material (TLE\textsuperscript{L}) obtained from the aqueous Tulsi leaf extract was found to be more effective than TLE in protecting the gastric mucosa from being ulcerated due to piroxicam in our experimental conditions.

The current studies indicate that it may be possible to minimize the gastro-toxic effects of piroxicam, when their long-term use is the only choice, if the aqueous leaf extract of Tulsi or the lyophilized material obtained there from is also included in the treatment regimen. Additionally, it was also found that this lyophilized material is more rich in total phenolic and total flavonoid contents than the crude aqueous material [table 1 & 2] and also stable with time (shelf-life) [results not shown]. Therefore, protection against piroxicam-induced ulcers in our model by aqueous Tulsi leaf extract [crude homogenate] (TLE) as well as the lyophilized material (TLE\textsuperscript{L}) obtained from the aqueous leaf extract could be used as a justification for testing their efficacy in reducing gastric ulcers in humans and oxidative stress in general.