Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Issue 2, 2013 ISSN - 0974-2441

Research Article

MELATONIN AUGMENTS THE PROTECTIVE EFFECTS OF AQUEOUS LEAF HOMOGENATE OF TULSI (*Ocimum sanctum L.*) AGAINST PIROXICAM-INDUCED GASTRIC ULCERATION IN RATS

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Received: 14 January 2013, Revised and Accepted: 1 March 2013

ABSTRACT

The effect of different doses of melatonin or aqueous Tulsi (Holy Basil) leaf homogenate (TLH) was studied against piroxicam-induced gastric ulceration in rats. Melatonin or TLH was found to protect the gastric mucosa individually following piroxicam treatment. Pre-treatment of rats with increasing doses of melatonin or TLH decreased the piroxicam-induced oxidative stress in the gastric tissue. Our studies also revealed that both melatonin (20 mg/kg BW i.p.) and TLH (100 mg/kg BW fed orally), individually, could not protect the gastric tissue from developing ulcers. However, when rats were pre-treated with melatonin and TLH in combination, at the doses at which neither was effective individually, the combined therapy completely prevented ulcers following piroxicam treatment. Melatonin and TLH in combination ameliorated the oxidative stress induced in the gastric tissue due to piroxicam by preventing the alterations in the bio-markers of oxidative stress and the activities and levels of antioxidant enzymes. The biochemical analyses were supported by macroscopic and microscopic studies of gastric tissue. Thus, the combination seems to work synergistically to reduce piroxicam-induced gastric damage in rats, strongly indicating that it may be a safe gastro-protective co-therapy especially, in situations where the use of anti-inflammatory drugs is the only choice.

Keywords: Melatonin, Tulsi leaf extract, piroxicam, gastric ulceration, co-therapy.

INTRODUCTION

In traditional systems of medicine, different parts like leaves, stem, flower, root, seeds and even the whole plants of *Ocimum sanctum Linn.* [Holy Basil] (known as Tulsi in India), a small herb seen throughout India, have been recommended for the treatment of bronchitis, bronchial asthma, malaria, diarrhoea, dysentery, skin diseases, arthritis, painful eye diseases, chronic fever, insect bite etc. The *Ocimum sanctum L.*(Holy Basil), apart from its use as a food item in Thailand, Italy and certain parts of India, is also 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. (2004) [4] have reported a significant anti-gastric ulcer activity in the extract of different plant parts of Tulsi. A hydroalcoholic 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 aspirin, alcohol, histamine, reserpine, serotonin, and stress-induced ulceration in experimental models [7].

Melatonin (N-acetyl-5-methoxytryptamine) is a highly evolutionarily conserved molecule present virtually in all organisms, i.e., in both plants and animals. The presence of melatonin in many food items has been reported and a high concentration of this indole has been detected in walnuts [8, 9]. Melatonin has also been detected in different herbs of medicinal importance [10]. Melatonin has several physiological functions in mammals including seasonal reproductive regulation, immune enhancement and regulation of light–dark signal transduction along with the capacity to influence possibly some aspects of aging. Additionally, melatonin and its metabolites have widespread antioxidant effects [11-15]. Moreover, this indoleamine reduces ethanol induced gastric damage and prevents gastric mucosal lesions caused by water-immersion restraint stress, ischemia and aspirin, with these changes being accompanied by an elevation in gastric levels of prostaglandin [16-18], an important gastroprotective agent. Melatonin in these situations also reduced free radical levels in blood as measured by chemiluminescence. Recently, evidence has been provided to show that melatonin protects against restraint-cold stress induced gastric ulceration in rats [19] not only by scavenging hydroxyl radical (◦OH) but also possibly by altering activities of two key gastric antioxidant enzymes, i.e., gastric peroxidase (GPO) and superoxide dismutase (SOD) [20].

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 pylori* and bile salts. Defensive factors mainly involve mucus-bicarbonate secretion and prostaglandins

[18]. Gastric ulceration is associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) [21]. Known additional risk factors include advanced age, previous history of ulceration, stress, smoking, nutritional deficiency, concomitant administration of anticoagulants and coexsisting serious systemic disorders [21, 22].Gastrointestinal bleeding is also related to the type of NSAID and the dosage [23]. Piroxicam, a classic nonselective cyclooxygenase (COX)-1 preferent NSAID, is widely used by patients requiring anti-inflammatory intervention [24]. 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 and ameliorative role of melatonin has also been demonstrated [25]. The present work demonstrates that both melatonin as well as aqueous Tulsi leaf homogenate (TLH), individually, is capable of ameliorating the piroxicam-induced gastric damage in rats dose-dependently. The results further indicate that their combination is highly effective against piroxicam-induced gastropathy at the dose at which neither of them alone demonstrated any efficiency. This raises the possibility of use of a low dose of a crude aqueous preparation of Tulsi and melatonin in combination against piroxicam-induced gastric damage in humans with minimal or no side-effects.

MATERIALS AND METHODS

Animals

Male Wister rats, weighing 180-200 g, were obtained from the CPCSEA approved local animal supplier (Chackraborty Enterprise). 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) [IAEC/PROPOSAL/DB-4/2010], Ministry of Social Justice and Empowerment, Government of India. All the experimental protocols had the approval of Institutional Animal Ethics Committee (IAEC) of Department of Physiology, University of Calcutta. The treatment and care of animals were personally monitored at each step by Prof. P. K. Samanta, M. V. Sc., Ph. D., CPCSEA Nominee to Department of Physiology, University of Calcutta.

Drugs, reagents and antibodies

Melatonin, thiobarbituric acid (TBA), eosin, NAD+, 2,2-dithiobis-nitro benzoic acid (DTNB), xanthine, xanthine oxidase, cytochrome c, fast blue BB salt, nitro blue tetrazolium (NBT), 5- bromo-4-chloro-3-indolyl phosphate (BCIP)and glutathione peroxidase kit were obtained from Sigma (St. Louis, MO, USA). Hematoxylin, H_2O_2, dimethyl sulfoxide (DMSO) and piroxicam were obtained from Merck Limited (Delhi, India). The superoxide dismutase (SOD) 1(C-17), SOD 2(G-20) and actin (I-19) antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal anti-catalase was obtained from Sigma. Other reagents used were of analytical grade.

Preparation of TLH

Fresh leaves of 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 washed in cold water, then weighed and homogenized in a sterilized Teflon glass homogenizer using aqua guard purified cold drinking water for 1-2 minutes (100mg leaf in 1 ml homogenate). The homogeneous suspension thus obtained (herein-after referred to as TLE) was brought to room temperature before administration. This method of TLH preparation was also used by other investigators [26, 27]. A small portion of this preparation was used for the quantitative analyses of some of the constituents and the rest was used for animal treatment.

Treatment of rats with piroxicam, melatonin and TLH

Fasted rats (water provided ad libitum) were divided into seven groups of eight animals each. The rats of the first group served as vehicle treated controls (7 rats). The rats of the second group were fed orally with different doses of TLH (100, 200, 300 or 400 mg/kg; for each dose 7 rats were used). To the third group, different doses of melatonin (20, 40 or 60 mg/kg; 7 rats for each dose) [dissolved in not more than 10% ethanol] was injected (i.p.). The rats of the fourth group were fed orally with piroxicam (30 mg/kg; 7 rats). The fifth group received TLH (100 mg/kg, fed orally; 7 rats) 1 hr before piroxicam administration and the sixth group received melatonin (20 mg/kg, i.p. injection; 7 rats) 30 min before piroxicam administration. To the seventh group, both TLH (100 mg/kg, fed orally; 7 rats) and melatonin (20 mg/kg, i.p. injection; 7 rats) were administered respectively 1 hr or 30 min before piroxicam administration, respectively.

In the first set dose-response experiments were carried out (total number of rats used, Control; 7, piroxicam group; 7, TLH; 28, melatonin; 21, total = 63). In the second set piroxicam, melatonin and TLH combination experiments were carried out (total number of rats used, Control; 7, piroxicam; 7, TLH control 7; melatonin control; 7, piroxicam plus TLH; 7, piroxicam plus melatonin; 7, piroxicam, TLH and melatonin combination; 7, total = 49). The animals were kept at room temperature and were sacrificed by cervical dislocation after 4 hr to assess the degree of gastric lesioning. The collected stomach tissues were stored at -80˚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 divided by the number of animals was expressed as the mean ulcer index [20].

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 Buege and Aust (1978) [28] with some modification as described by Bandyopadhyay et al. (2004) [25]. 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 Sedlac and Lindsey (1968) [29] with some modifications [25]. A portion of the fundic stomach was homogenized (10%) in 2 mm icecold ethylenediaminetetraacetic 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/mg protein.

Assay of gastric peroxidase (GPO), superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)

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 [20]. The activity was expressed as units/mg protein.

Copper-zinc superoxide dismutase (SOD1) activity was measured by hematoxylin autooxidation method of Martin et al. (1987) [30] with some modifications [31]. 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.

Manganese superoxide dismutase (SOD2) activity was measured in the mitochondrial fraction by the xanthine oxidase–cytochrome c method as described by McCord and Fridovich (1969) [32] with some modifications as modified by Bandyopadhyay et al. (2002) [20]. In brief, a portion of the fundic stomach was homogenized (10%) in ice-cold 50 mM phosphate buffer, pH 7.8. 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 mitochondrial fraction. The supernatant was discarded and the pellet was re-suspended in the buffer and used for the enzyme assay using a UV / VIS spectrophotometer at 550 nm with an O_2•- generating system (xanthine / xanthine oxidase) in the presence of cytochrome c. The enzyme activity was expressed as U/mg protein.

Catalase was assayed by the method of Beers and Sizer (1952) [33] with some modifications as adopted by Chattopadhyay et al. (2003) [34]. 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 40C 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. Values were expressed as µM H2O2 consumed / min /mg protein.

A portion of the fundic stomach was homogenized (10%) in ice-cold 50 mM Tris–HCl buffer containing 0.5 mM EDTA, pH 8.0. The homogenate was centrifuged at 3000 g for 10 min and supernatant collected. This supernatant was used for assaying the glutathione peroxidase activity spectrophotometrically at 340 nm using a commercially available GPx kit (Sigma). The enzyme activity was expressed as Units/mg of tissue protein.

Measurement of hydroxyl radical (•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. (2004) [25]. DMSO forms a stable product (methane sulfonic acid [MSA]) on reaction with •OH. Accumulation of MSA was measured to estimate the •OH generated after forming a colored complex with Fast Blue BB salt. Seven 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 per os). The second group was fed orally TLH (100 mg/kg per os) 15 min after DMSO injection which was followed by oral administration of piroxicam (30 mg/kg per os) 30 min after the treatment of rats with the extract. The third group was injected with melatonin (20 mg/kg i.p.) 15 min after DMSO injection which was followed by oral administration of piroxicam (30 mg/kg per os) 30 min after treatment with melatonin. The fourth group was treated with both TLH (100 mg/kg fed orally) and melatonin (20 mg/kg, i.p.) 15 min after DMSO injection which was followed by oral administration of piroxicam (30 mg/kg per os) 30 min after treatment with the TLH and the melatonin respectively. The fifth group of rats was the control group and was treated only with DMSO (i.p. injection). The sixth and the seventh groups of rats were treated with the TLH (100 mg/kg per os) only and melatonin (20 mg/kg, i.p.) only. Both the groups (i.e., sixth and the seventh groups) of rats received DMSO injection (i.p.) 15 min before the treatment with the TLH as well as melatonin. Altogether 28 rats were used for this particular set of experiment. 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 whole stomach tissue of each animal was processed for MSA that 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/Xanthine dehydrogenase activity for the assessment of superoxide anion radical ($O_2^{\bullet-}$) in vivo

Xanthine oxidase (XO) was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler (1964) [35] with some modifications [31]. Briefly, the stomach tissues were 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 (1965) [36] with modifications [31]. In brief, the stomach tissues were homogenized in cold (10%) in 50 mM phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were then processed as in case of xanthine oxidase. 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.

Western blot analysis

Western blot analysis was performed with the stomach homogenates which were prepared as described earlier by Bandyopadhyay et al. (2004) [25] with minor modifications [31]. Briefly, a portion of the fundic stomach was homogenized in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 1 µg/mL each of pepstatin A, leupeptin, and aprotinin. The homogenate was centrifuged at 800 g for 10 min. The supernatant was resolved by 10% SDS–PAGE according to the method of Laemmli (1970) [37] using Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Protein (25 µg) for SOD1 (Cu-Zn SOD) and 35 µg protein for catalase were loaded for immunodetection. After SDS–PAGE, the proteins were transferred to nitrocellulose membranes in an electroblotting apparatus (Mini Trans-Blot, Bio-Rad) at 85 V for 60 min using 193 mM glycine, 25 mM Tris and 20% methanol as transfer buffer. After transfer, the membranes were blocked using 10% nonfat dried milk in Tris-buffered saline containing 0.05% Na-azide (blocking solution, pH 7.6), and incubated at room temperature for 2 hr. The membranes were then rinsed twice with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and then incubated with the respective primary antibody (1:3000 dilutions for all in 5% blocking solution) overnight. After washing thrice with TBS-T, the membranes were incubated with secondary antibody for 2 hr at room temperature, followed by a further washing with TBS-T for 15 min twice. The immunoreactive bands were detected with alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl2, and 100 mM Tris–HCl; pH 9.5) in presence of nitro blue tetrazolium (NBT) and BCIP in the ratio of 2:1. The pixel density of bands obtained through Western blotting was quantified using Image J software (NIH, Bethesda, MD, USA).

Determination of the total phenolics, flavonoids, condensed tannins, chlorophyll content and pH of TLH

The total phenolic content was measured according to the method of Singleton et al. (1999) [38]. Briefly, to a suitably diluted sample TLH, 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% AlCl3 solution, and 1M NaOH solution following the method of Dewanto et al. (2002) [39]. 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)].

The analysis of condensed tannins (procyanidins) was carried out according to the method of Broadhurst and Jones (1978) [40]. To the suitably diluted sample, 4% vanillin and concentrated HCl were added. The mixture was allowed to stand for 15 min in room temperature and the absorption was measured at 500 nm. The amount of total condensed tannins is expressed as (+) catechin equivalents [CE, mg (+) catechin /g sample].

The total chlorophyll content of the leaves was determined according to the method of Arnon (1949) [41]. Fresh leaves were blended and then extracted with 80% acetone and left for 15 minutes for thorough extraction. The liquid portion was decanted into another test-tube and centrifuged at 2,500 rpm for 3 mins. The supernatant was then collected and the absorbance measured spectrophotometrically at 645nm and 663nm to determine the chlorophyll a, chlorophyll b and total chlorophyll content. The pH of the TLH was determined after calibrating pH meter with buffer solution of pH 4 and 9 [42].

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 tissue was then washed in 0.9% saline and a portion of the tissue was kept in 10% buffered formalin for histopathological studies. The tissue sections were then stained with hematoxylin and eosin and observed under microscope for possible histopathological changes like congestion, hemorrhage etc. [43].

Estimation of proteins

Proteins of the different samples were determined by the method of Lowry et al. (1951) [44].

Statistical evaluation

Difference between groups were analysed using one-way analysis of variance (ANOVA) followed by Scheffe' multiple comparison test. All values are presented as means ± S.E.M. Pairwise comparison was done by calculating the least significance. Statistical test were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Dose response studies for TLH and melatonin against piroxicam induced gastric injury.
Mean ulcer index

Table 1 shows that TLH 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 400 mg/kg BW. Similarly, Table 2 exhibits a dose-dependant protection of the gastric mucosa against piroxicam-induced gastric ulceration by melatonin with more than 90% reduction in mean ulcer index at a dose of 60 mg/kg BW. The results presented in Table 1 and 2 indicate further the dose of TLH (i.e., 100mg / kg bw fed orally) or melatonin (i.e., 20 mg / kg bw, i.p.) at which neither of them was able to provide protection against piroxicam-induced gastric ulceration in rats.

Lipid peroxidation level (LPO)

Treatment of rats with piroxicam (30 mg/kg per os.) caused a highly significant increase (95%, $p < 0.001$ 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 TLH (Table 1) indicating that the TLH possess the capability to provide protection to gastric mucosa against piroxicam-induced gastric ulceration possibly by decreasing the level of oxidative stress due to piroxicam. The results presented in Table 2 reveal that the piroxicam-induced increase in the level of lipid peroxidation in the gastric tissue was also protected dose-dependently when the rats were pre-treated with melatonin.

Reduced glutathione (GSH) level

A highly significant decrease (40.33%, $p < 0.001$ vs. control) in the level of gastric GSH following treatment of rats with piroxicam indicated generation of oxidative stress. The results presented in Table 1 shows a dose-dependent protection in the level of gastric GSH when the rats were pre-treated with increasing doses of TLH. Tulsi leaf homogenate at a dose of 400 mg/kg (fed orally) completely protected the tissue GSH from being reduced when compared to the values observed in the gastric tissue of the control rats. Similarly, melatonin at a dose of 60 mg/kg (i.p.) completely protected the tissue GSH from being reduced as is evident from the results presented in Table 2.

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

Table 1 further illustrates the activity of Cu-Zn SOD which was found to be significantly elevated (80%, $p < 0.001$ vs. control) following treatment of rats with piroxicam at a dose of 30 mg/kg (fed orally). Pre-treatment of rats with increasing doses of TLH protected the activity of this antioxidant enzyme from being increased. The enzyme activity was found to be near control values when the rats were pre-treated with 400 mg/kg (fed orally). Table 2 shows that pre-treatment of rats with increasing doses of melatonin protected the activity of this antioxidant enzyme from being increased and melatonin at a dose of 60 mg/kg (i.p.) was found to provide maximum protection to the enzyme activity.

Catalase activity

Treatment of rats with piroxicam caused a significant increase in the activity of catalase of the rat stomach tissue (47.74%, $p < 0.001$ vs. control) when compared to control (Table 1). The table further reveals that pre-treatment of rats with increasing doses of TLH protects the gastric catalase activity from being increased. Similarly, Table 2 reveals that pre-treatment of rats with melatonin at increasing doses protected the gastric catalase activity from being increased following piroxicam treatment.

Macroscopic and microscopic changes in gastric tissue morphology

Figure 1A and 2A show the 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 gastric tissue morphology of the rats of the control, piroxicam treated and TLH protected or melatonin 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 TLH with maximum ulcer protection at 400 mg/kg BW (fed orally). The rats of the melatonin protected group were found to be maximally protected at a dose of 60mg/kg BW (i.p.). 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 TLH or melatonin with maximum protection observed at 400mg/kg BW TLH fed orally (Figure 1B) or 60 mg/kg BW (i. p.) of melatonin (Figure 2B). The figures further documents that melatonin at a dose of 20 mg/kg BW (i.p.) and TLE at a dose of 100 mg/kg BW (fed orally) did not provide protection against piroxicam-induced gastric ulceration.

Studies with the combined doses of TLH and melatonin against piroxicam induced gastric injury

Mean ulcer index

In separate experiments, the efficacy of the combination of melatonin and TLH at their otherwise ineffective doses i.e. the dose at which neither of them singly afforded protection to gastric mucosa against piroxicam-induced gastric ulceration was tested. Table 3A illustrates that the combination of the ineffective doses of both melatonin and TLH protected the gastric mucosa from piroxicam induced gastric ulceration with significant reduction in mean ulcer index.

Lipid peroxidation level (LPO)

The data presented in Table 3A reveals that pre-treatment of rats with the otherwise ineffective doses of melatonin (20 mg/kg BW i.p.) or TLH (100 mg/kg BW fed orally) when administered in combination reduced the level of LPO in the gastric tissue to the control value when compared to the level measured in piroxicam treated animals (51% reduction vs. piroxicam treated rats; $p<0.001$). However, the level of LPO in the rats treated only with the TLH (100 mg/kg BW fed orally) or melatonin (20 mg/kg BW i.p.) did not differ from that observed in the control animals.

Reduced glutathione (GSH) level

Table 3A reveals that pre-treatment of rats with melatonin (20 mg/kg BW i.p.) and TLH (100 mg/kg BW fed orally) in combination protected the gastric GSH from being decreased (68.67% increase vs. piroxicam treated rats; $p < 0.001$), an effect not observed when the two were tested separately for their efficacy, at the doses mentioned above, against piroxicam-induced gastric ulceration. However, the levels of gastric GSH measured in rats treated with the ineffective doses of melatonin or TLH alone did not differ from the GSH levels as measured in the rats of the control group.

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

Piroxicam-induced rise in Cu-Zn SOD activity of rat gastric tissue was completely restored to the activity observed in the control rats when the animals were pre-treated with melatonin and TLH in combination ($p < 0.001$ vs. piroxicam treated rats) [Table 3A]. Pre-treatment of rats with the TLH (100 mg/kg BW fed orally) or melatonin (20 mg/kg BW i.p.) separately, did not reduce the elevated activity of CU-Zn SOD significantly following piroxicam treatment. Also, the TLH (100 mg / kg BW fed orally) or the melatonin (20 mg / kg BW i.p.) alone had no effect on the gastric Cu-Zn SOD activity when compared to control. The expression level of Cu-Zn-SOD protein was also studied via western blot and the results are presented in Figure 3. A 57% increase ($p<0.001$) in the level of Cu-Zn-SOD protein in rats treated with the present dose of piroxicam was observed when compared to the protein level in control animals. Pre-treatment of rats with the ineffective doses of melatonin and TLH in combination, decreased Cu-Zn-SOD protein level to that observed in the control rats.

Manganese superoxide dismutase (Mn SOD) activity

Pre-treatment of rats with the ineffective doses of melatonin (20 mg/kg BW i.p.) and TLH (100 mg/kg BW fed orally) in combination completely protected (63% rise vs. control, $p < 0.001$; 40% reduction vs. piroxicam, $p < 0.001$) the Mn-SOD activity of the gastric tissue from being increased (Table 3A). Pre-treatment of rats, however, with the ineffective dose of melatonin or TLH alone did not significantly alter gastric Mn-SOD activity. The activity of Mn-SOD in rats treated with the ineffective dose of melatonin or TLH alone did not differ from the activity of the enzyme observed in the control rats.

Catalase activity

Catalase activity of the rat gastric tissue was found to be significantly increased (47.74% vs. control; $p < 0.001$) following treatment of rats with piroxicam. However, when the rats were pre-treated with the ineffective doses of melatonin and the TLH in combination, the catalase activity was completely protected from being increased and its activity was similar to that observed in the control rats (Table 3A). The results further revealed that pre-treatment of rats with the ineffective dose of melatonin or the TLH only did not offer any protection to catalase activity. Similarly, catalase activity of rat gastric tissue of melatonin or the TLH alone treated rats did not differ from the level of catalase activity observed in the control rats. Figure 4 reveals a significant increase (71% vs. control; $p<0.001$) in the level of catalase protein in piroxicam-treated rats as determined by western blot analysis. The level of catalase protein was found to be restored to control level when the rats were pre-treated with the otherwise ineffective doses of melatonin and TLH in combination.

Gastric peroxidase (GPO) activity

There occurred a significant decrease in the gastric peroxidase (GPO) activity (39.72%, $p < 0.001$ 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 ineffective doses of melatonin (20 mg/kg BW i.p.) and TLH (100 mg/kg BW fed orally) in combination completely protected the GPO activity from being decreased. Besides, pre-treatment of rats with the ineffective dose of melatonin or the TLH separately failed to protect the GPO activity. Further, the GPO activity in the rats treated with the ineffective dose of melatonin or TLH alone did not differ from the level of activity of the enzyme observed in control rats.

Glutathione peroxidase (GPx) activity

Pre-treatment of rats with the ineffective doses of melatonin (20 mg /kg BW i.p.) and the TLH (100 mg / kg BW fed orally) in combination completely protected the glutathione peroxidase activity from being decreased (28.35% decrease vs. control, $p < 0.001$; 44% increase vs. piroxicam, $p < 0.001$). However, pre-treatment of rats with the ineffective dose of melatonin or TLH alone was found to elevate the activity of the enzyme slightly but this change was not statistically significant.

Generation of hydroxyl radical ($^{\bullet}OH$) in vivo

Treatment of rats with the present dose of piroxicam caused more than four fold increase in the generation of $^{\bullet}OH$ in vivo (74% vs. control, $p < 0.001$) when compared to control (Table 3B). Pre-treatment of rats with the ineffective doses of melatonin (20 mg / kg BW i.p.) and the TLH (100 mg / kg fed orally) in combination highly significantly reduced the generation of $^{\bullet}OH$ compared to piroxicam treated rats (70% vs. piroxicam treated rats; $p<0.001$) and the level measured was near to that measured in the control rats. Besides,,pre-treatment of rats with the ineffective dose of melatonin or TLH separately did not influence $^{\bullet}OH$ formation. Furthermore, the level of $^{\bullet}OH$ in the stomach tissue of rats treated with the ineffective dose of melatonin or TLH alone did not differ from that measured in the control rats.

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

Whether piroxicam administration to rats has caused the generation of $O_2^{\bullet-}$ was also assessed indirectly. The results presented in Table 3B clearly indicate that there was an enhancement in the generation of $O_2^{\bullet-}$ in vivo following treatment of rats with piroxicam. A 56% 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% increase compared to control ($p< 0.001$ vs. control). The total enzyme activity, i.e., XO plus XDH showed a 49% rise compared to control while XO-XDH ratio and XO / XO+XDH ratio also increased significantly following piroxicam treatment. Pre-treatment of rats, however, with the ineffective doses of melatonin (20 mg / kg BW i.p.) and TLH (100 mg / kg BW fed orally) in combination protected the activity of the enzymes from being increased and the activities were similar to that observed in control rats. Moreover, pre-treatment of rats with the ineffective dose of melatonin or TLH failed to protect the enzyme activity to any significant extent. The enzyme activities in the rats treated with the ineffective dose of melatonin or TLH alone did not differ from the activities measured in the control rats.

Macroscopic and microscopic changes in gastric tissue morphology

Figure 5 illustrates the ulcerated stomach in rats treated with piroxicam (30 mg /kg BW fed orally), melatonin (ineffective dose i.e, 20 mg / kg BW i.p.) and TLH (ineffective dose i.e., 100 mg / kg BW fed orally)) with a significant injury to gastric mucosa. The figure further reveals that when the rats were pre-treated with the otherwise ineffective doses of melatonin and TLH in combination, no ulcer spots were detected on the gastric mucosal surface or any injury to tissue morphology. The ineffective dose of melatonin or TLH when administered alone did not cause any damage to the gastric mucosa when compared to tissues of the control rats.

Determination of the content of the total phenolics, total flavonoids, total condensed tannins (procyanidins), chlorophyll and pH of TLH

Table 4 shows that the pH of this preparation was between 6.85-7.04. The table further shows the total content of phenols, flavonoids, procyanidins and chlorophyll in TLH.

DISCUSSION

The NSAIDs are among the most widely prescribed and used drugs for rheumatologic as well as non-rheumatologic conditions which include acute and chronic pain, biliary and ureteric colic, dysmennorhea, fever, closure of patent ductus arteriosus in infants and other applications that derive from the suppression of prostaglandin synthesis [45]. It has been estimated that one in seven Americans is likely to be treated with an NSAID for a chronic rheumatologic disorder [46].

More than 80 million prescriptions are written each year [47], accounting for about 4.5% of all prescriptions written in the United States. A recent report claim that approximately 2,000 patients per annum may die as a result of NSAID-induced ulcer bleeding and perforations in the UK [48]. 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 [49] and are responsible for a high level of dyspepsia [50]. Besides damaging the gastrointestinal tract, NSAIDs can cause fluid retention [51], hypertension [52] and renal impairment. Whether they predispose to or protect against vascular disease is uncertain and recent studies do not establish a clear effect [53].

Inhibition of prostaglandin synthesis is well recognized as the central mechanism by which gastrointestinal injury occurs [54]. 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 [53]. 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 [55] 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 [56].

The primary objective of the present investigation was to determine whether the combination of melatonin and TLH provides protection against piroxicam-induced damage to gastric mucosa at the doses at which neither of them protects the gastric mucosa individually. Results presented in Table 1 and 2 demonstrate clearly that both melatonin and TLH are

capable of providing protection to the gastric mucosa against piroxicam-induced gastric injury in a dose-dependent manner. In our experiments, the piroxicam-induced gastric ulceration was maximally protected by melatonin at 60mg/kg BW (i.p.) and by TLE at the dose of 400mg/kg BW (fed orally), indicating clearly an increased requirement of dose in case of the TLH. These biochemical observations have been confirmed by the macroscopic and microscopic observations of the gastric mucosa which indicate gastric tissue damage following piroxicam treatment. This mucosal injury was found to be protected when the rats were pre-treated with different doses of either melatonin or TLH. Figure 1 and 2 further reveal that melatonin at a dose of 20mg/kg BW (i.p.) and TLH at a dose of 100mg/kg BW (fed orally) do not protect against piroxicam-induced gastric injury.

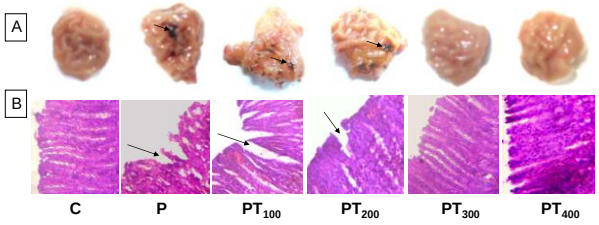

Fig.1. Representative images showing morphology of rat gastric tissues after treatment with piroxicam and TLH.

(A) The macroscopic view of mucosal surface of whole stomach showing protective effect of TLH against piroxicam-induced (P) damage of rat gastric tissue. The rats were treated with piroxicam (P) (fed orally) and increasing doses of TLH (fed orally).The control rats were treated with vehicle only. **(B)** Representative images of hematoxylin-eosin stained sections of stomach tissue (magnification 400X). Arrow heads indicate damaged area of gastric mucosa.

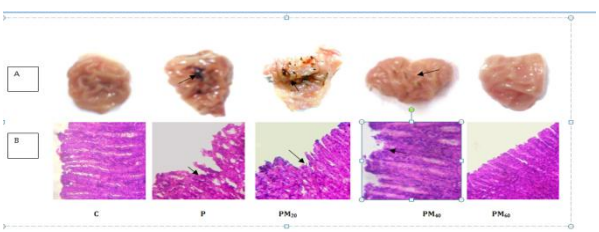

Fig.2. Representative images showing morphology of rat gastric tissues after treatment with piroxicam and melatonin.
(A) The macroscopic view of mucosal surface of whole stomach showing protective effect of melatonin against piroxicam-induced damage of rat gastric tissue. The rats were treated with piroxicam (P) (fed orally) and increasing doses of melatonin (M) (injected i.p.).The control rats were treated with vehicle only. **(B)** Hematoxylin-eosin stained sections of stomach tissue
(magnification 400X). Arrow heads indicate damaged area of gastric mucosa.

This prompted us to investigate whether these two ineffective doses of melatonin and TLH would protect the gastric mucosa when administered in combination. Our experiments revealed that pre-treatment of rats with the combined doses of melatonin and TLH almost completely inhibited the gastric mucosal ulceration (as measured by mean ulcer index) indicating the strong potential of the combination. The individual doses were ineffective. Additionally, when the rats

were treated singly with melatonin (20 mg/kg BW, i.p.) or TLH (100 mg/kg BW, fed orally), they had no effect on the mucosa (positive control). Earlier studies have demonstrated the co-therapeutic effect of melatonin with ranitidine or omeprazole in providing protection to the gastric mucosa against cold-restraint stress-induced gastric ulceration in rats at the doses at which none of them were effective individually [19].

The results of the present investigation indicate clearly that this combination not only inhibited piroxicam-induced ulceration of the gastric mucosa (Table 3A) but also decreased the piroxicam-induced oxidative stress as evident from the significantly reduced level of LPO and increased level of gastric GSH (Table 3A) which indicates that this combination of a pure compound like melatonin and TLH are highly efficient in mitigating the ill effects of piroxicam administration on gastric mucosa. The combination of melatonin and TLH at their otherwise ineffective doses also ameliorates the alterations in the activities of gastric antioxidant enzymes like Cu-Zn SOD, Mn SOD, gastric peroxidase, glutathione peroxidase and catalase indicating that this combination mitigate the changes brought about in the tissue antioxidant system following piroxicam treatment. The alteration in the activities of the antioxidant enzymes Cu-Zn SOD and catalase following piroxicam treatment were further analysed through western blot analysis (Figure 3 and Figure 4). It was revealed that the expressions of these two enzymes were increased significantly by piroxicam treatment. However, pre-treatment of rats with the combined ineffective doses of melatonin and TLH restored the activities as well as the levels of the enzyme proteins to near control values indicating again the superior efficacy of this combination in ameliorating piroxicam-induced alterations in the rat gastric mucosa. However, neither melatonin nor TLH had any effect on the antioxidant levels and the activities of antioxidant enzymes indicating that they do not influence these parameters by themselves (positive control).

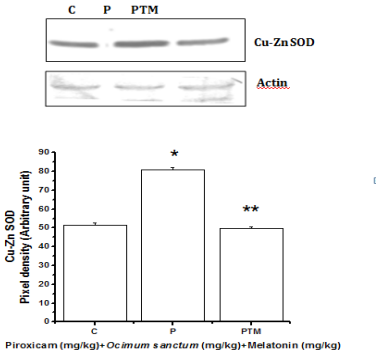

Fig.3. Western blot analysis for determining the level of Cu-Zn SOD. Representative result of Western blot analysis for determining the level of Cu-Zn SOD (lanes from left) of rat gastric tissue in control (C), piroxicam-treated (P) and melatonin and TLH combined dose (PTM) protected.The Western blot analysis was repeated at least

three times with stomach tissue of three different rats. The pixel density of bands obtained through Western blotting was quantified with ImageJ software (NIH, Bethesda, MD, USA) and the values (means ± S.E.M.) are presented below in the form of a bar graph. *P < 0.001 versus control, **P < 0.001 versus piroxicam-treated animals (one-way ANOVA followed by Scheffe' multiple comparison test).

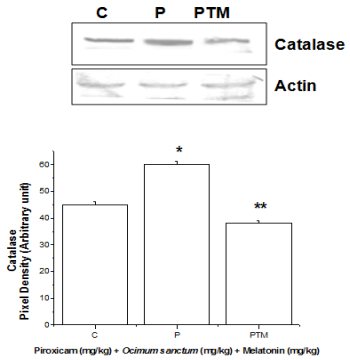

Fig.4. Western blot analysis for determining the level of catalase. Representative result of Western blot analysis for determining the level of catalase (lanes from left) of rat gastric tissue in control (C), piroxicam-treated (P) and melatonin and TLH combined dose (PTM) protected.The Western blot analysis was repeated at least three times with stomach tissue of three different rats. The pixel density of bands obtained through Western blotting was quantified with ImageJ software (NIH, Bethesda, MD, USA) and the values (means ± S.E.M.) were presented in the form of a bar graph. *P < 0.001 versus control, **P < 0.001 versus piroxicam-treated animals (one-way ANOVA followed by Scheffe' multiple comparison test).

It was shown earlier by Bandyopadhyay et al. (2004) [20] 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 the combination of melatonin and TLH at their otherwise ineffective doses, the formation of •OH was inhibited to near control levels. However, melatonin or TLH alone at their individual ineffective dose failed to provide any protection against •OH formation indicating that this combination has strong potential to either inhibit •OH generation *in vivo* or scavenge the •OH generated *in vivo*. 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, the level of total enzyme (XO+XDH) and XO/XO+XDH ratio (Table 3B). 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 combination of melatonin and TLH at their ineffective doses almost completely protected the activities of these enzymes from being increased. This indicates that the combination may have the potential to either inhibit the generation of $O_2^{\bullet-}$ or scavenge this ROS. Our histological studies presented in Figure 5 further support the biochemical observations. The results reveal that melatonin at 20mg/kg BW (i.p.) and TLH at 100mg/kg BW (fed orally), individually, are unable to protect the tissue from being damaged following piroxicam treatment. This is evident from our macroscopic as well as microscopic studies. However, when melatonin and TLH were used in combination at their otherwise ineffective doses, a complete protection of the tissue morphology with no ulcers was observed, indicating again the effectiveness of this combination against piroxicam-induced gastric ulceration in rats. The rat gastric mucosa was not affected when melatonin or TLH were administered alone (positive control).

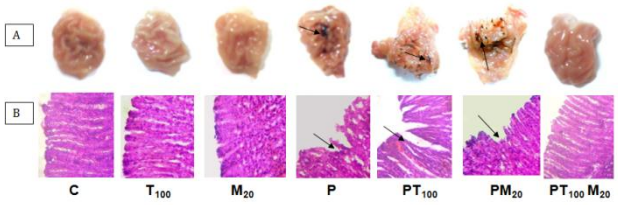

Figure 5

An advantage of melatonin as an antioxidant lies in the fact that it is amphiphilic [57, 58]; thus, it readily reaches all subcellular compartments to scavenge the reactants generated during oxidative stress at their sites of generation. Its potent antioxidant activities, availability in many dietary items and the absence of any demonstrated (short or long-term) toxicity at even pharmacological concentration [59, 60] may predictably make this small indole an important therapeutic molecule in gastroprotection.

Tulsi (*O. sanctum*), a plant widely used in Ayurveda, possesses anti-inflammatory and antioxidant properties [61]. Flavonoids isolated from Tulsi scavenged free radicals *in vitro* and showed anti-lipoperoxidant activity *in vivo* at very low concentrations [61]. The free radical scavenging activity of plant flavonoids help in the healing of wounds [61]. Other plants like *Murraya koenigii* also have been reported to have antioxidant property [62]. 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 [62] and because misoprostol in combination with anti-inflammatory agents is the only recognized form of protection against gastropathy [63], 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 melatonin together with TLH is also included in the treatment regimen. Furthermore, these studies establish the versatility in the action of melatonin and TLH combination as an antioxidant, i.e., direct scavenging actions as well as indirect antioxidant functions. Besides neutralizing free radicals, a portion of this combination'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 [64,65].

CONCLUSION

The present studies reveal that melatonin and TLH both have the ability to provide protection individually to rat gastric mucosa against piroxicam-induced gastric injury dose-dependently. Their combination at the indicated dose exhibited complete protection against piroxicam-induced gastric injury in rats. However, individually neither of them at the minimum dose (i.e., melatonin at 20 mg /kg BW i.p.; TLH at 100 mg / kg BW fed orally) is capable of providing any protection to rat gastric mucosa. The studies further reveal that the combination exerts its gastro-protective effect through antioxidant mechanism. The protection against piroxicam-induced ulcers in our model by melatonin and TLH combination could be used as justification for testing their efficacy in combination for reducing gastric ulcers in humans and oxidative stress in general.

Table 1: Piroxicam induced gastric ulceration and protection by TLH in a dose-dependent manner in rats.

Group of animals	Extent of ulceration	Oxidative Stress Parameters		Antioxidant enzymes	
	Mean Ulcer Index	LPO[nmol TBARS/mg protein]	GSH[nmoles/mg protein]	Cu-Zn SOD[Units/mg protein]	Catalase[µmol H_2O_2 /mg protein]
Control	0 ± 0	0.44 ± 0.01	20.38 ± 0.06	1.45 ± 0.02	10.16 ± 0.05
Piroxicam	70 ± 0.91$^{\alpha}$	0.86 ± 0.04$^{\alpha}$	12.16 ± 0.30$^{\alpha}$	2.61 ± 0.06$^{\alpha}$	15.01 ± 0.26 $^{\alpha}$
P+M_{20}	66 ± 1.08$^{\gamma}$	0.75 ± 0.02$^{\gamma}$	12.91 ± 0.43$^{\gamma}$	2.38 ± 0.01$^{\gamma}$	14.61 ± 0.12 $^{\beta}$
P+M_{40}	42.75 ± 1.89$^{\alpha 1}$	0.61 ± 0.02$^{\alpha 1}$	16.3 ± 0.18$^{\alpha 1}$	1.98 ± 0.03$^{\alpha 1}$	12.88 ± 0.36$^{\alpha 1}$
P+M_{60}	6.75 ± 0.63$^{\alpha 1}$	0.43 ± 0.01$^{\alpha 1}$	20.24 ± 0.11$^{\alpha 1}$	1.5 ± 0.01$^{\alpha 1}$	10.33 ± 0.12$^{\alpha 1}$

Values are expressed as mean ± S.E.M. of 7 rats in each group. Statistical analysis done by one-way ANOVA followed by Scheffe' multiple comparison tests. $^{\alpha}p < 0.001$ vs. control,. $^{\alpha 1}p < 0.001$ vs. piroxicam-treated rats, $^{\beta}$NS vs. piroxicam-treated rats,$^{\gamma}p < 0.05$ vs. piroxicam-treated rats . NS, not significant.

Table 2: Piroxicam induced gastric ulceration and protection by melatonin in a dose-dependent manner in rats.

Group of animals	Extent of ulceration	Oxidative Stress Parameters		Antioxidant enzymes	
	Mean Ulcer Index	LPO [nmol TBARS/mg protein]	GSH[nmoles/ mg protein]	Cu-Zn SOD[Units/ mg protein]	Catalase[µmol H_2O_2 /mg protein]
Control	0 ± 0	0.44 ± 0.01	20.38 ± 0.06	1.45 ± 0.02	10.16 ± 0.05
Piroxicam	70 ± 0.91$^{\alpha}$	0.86 ± 0.04$^{\alpha}$	12.16 ± 0.30$^{\alpha}$	2.61 ± 0.06$^{\alpha}$	15.01 ± 0.26 $^{\alpha}$
P+M_{20}	66 ± 1.08$^{\gamma}$	0.75 ± 0.02$^{\gamma}$	12.91 ± 0.43$^{\gamma}$	2.38 ± 0.01$^{\gamma}$	14.61 ± 0.12 $^{\beta}$
P+M_{40}	42.75 ± 1.89$^{\alpha 1}$	0.61 ± 0.02$^{\alpha 1}$	16.3 ± 0.18$^{\alpha 1}$	1.98 ± 0.03$^{\alpha 1}$	12.88 ± 0.36$^{\alpha 1}$
P+M_{60}	6.75 ± 0.63$^{\alpha 1}$	0.43 ± 0.01$^{\alpha 1}$	20.24 ± 0.11$^{\alpha 1}$	1.5 ± 0.01$^{\alpha 1}$	10.33 ± 0.12$^{\alpha 1}$

Values are expressed as mean ± S.E.M. of 7 rats in each group. Statistical analysis done by one-way ANOVA followed by Scheffe' multiple comparison tests. $^{\alpha}p < 0.001$ vs. control,. $^{\alpha 1}p < 0.001$ vs. piroxicam-treated rats, $^{\beta}$NS vs. piroxicam-treated rats,$^{\gamma}p <$ 0.05 vs. piroxicam-treated rats . NS, not significant.

Table 3A: Piroxicam induced gastric ulceration and protection by the combination of melatonin and TLH in rats.

Group of animals	Extent of Ulceration	Oxidative StressParameters		Antioxidant enzymes				
	Mean Ulcer Index	LPO [nmol TBARS/ mg protein]	GSH [nmoles/ mg protein]	Cu-Zn OD [Units/mg protein]	Mn SOD[Units/mg protein]	Catalase[µmol H_2O_2 /mg protein]	GPO [Δ OD/min /mg protein]	GPx [Units /mg protein]
Control	0 ± 0	0.44 ± 0.01	20.38 ± 0.06	1.45 ± 0.02	43.53 ± 0.47	10.16 ± 0.05	73.33 ± 0.31	1.27 ± 0.01
T_{100}	0 ± 0	0.43 ± 0.01	20.34 ± 0.27	1.46 ± 0.02	43.17 ± 0.77	10.14 ± 0.05	73.03 ± 0.27	1.24 ± 0.01
M_{20}	0 ± 0	0.42 ± 0.01	20.37 ± 0.17	1.44 ± 0.01	42.1 ± 0.50	10.12 ± 0.05	73.08 ± 0.10	1.15 ± 0.03
Piroxicam	70 ± 0.91$^{\alpha}$	0.86 ±0.04$^{\alpha}$	12.16 ± 0.30$^{\alpha}$	2.61 ± 0.06$^{\alpha}$	71.23 ± 0.68$^{\alpha}$	15.01 ± 0.26$^{\alpha}$	44.20 ± 0.15$^{\alpha}$	0.91 ± 0.01$^{\alpha}$
P+T_{100}	67.25 ± 1.38$^{\beta}$	0.77± 0.01$^{\beta}$	14.98 ± 0.06$^{\alpha 1}$	2.34 ± 0.01$^{\alpha 1}$	67.42 ± 0.41$^{\gamma}$	13.34 ± 0.29$^{\beta}$	45.45 ± 0.42$^{\beta}$	1.02 ± 0.02$^{\alpha 1}$
P+M_{20}	66 ± 1.08$^{\gamma}$	0.75 ± 0.02$^{\gamma}$	14.25 ± 0.04$^{\alpha 1}$	2.38 ± 0.01$^{\alpha 1}$	66.84 ± 0.49$^{\gamma}$	14.61 ± 0.12$^{\beta}$	45.72 ± 0.40$^{\beta}$	1.05 ± 0.04$^{\alpha 1}$
P+T_{100}+M_{20}	4.75 ± 0.25$^{\alpha 1}$	0.42 ± 0.01$^{\alpha 1}$	20.53 ± 0.02$^{\alpha 1}$	1.46 ± 0.01$^{\alpha 1}$	42.75 ± 0.97$^{\alpha 1}$	9.87 ± 0.29$^{\alpha 1}$	87.88 ± 0.07$^{\alpha 1}$	1.31 ± .01$^{\alpha 1}$

values are expressed as mean ± S.E.M. of 7 rats in each group. Statistical analysis done by one-way ANOVA followed by Scheffe' multiple comparison tests. $^{\alpha}p < 0.001$ vs. control,. $^{\alpha 1}p < 0.001$ vs. piroxicam-treated rats, $^{\beta}$NS vs. piroxicam-treated rats,$^{\gamma}p < 0.05$ vs. piroxicam-treated rats . NS, not significant.

Table 3B: Piroxicam induced gastric ulceration and protection by the combination of melatonin and TLH in rats.

Group of animals	Parameters for ROS generation				
	OHº generated [nmoles/gm tissue]	XO[milli units /mg protein]	XDH[milli units /mg protein]	XO+XDH[milli units/mg protein]	XO/XDH ratio
Control	42 ± 1.15	1.35 ± 0.01	2.18 ± 0.04	3.54 ± 0.06	0.61 ± 0.01
T_{100}	41.23 ± 1.15	1.35 ± 0.03	2.25 ± 0.06	3.60 ± 0.10	0.60 ± 0.01
M_{20}	40.7 ± 1.13	1.30 ± 0.05	2.14 ± 0.01	3.44 ± 0.06	0.60 ± 0.02
Piroxicam	161.9 ± 1.15$^{\alpha}$	3.07 ± 0.10$^{\alpha}$	3.73 ± 0.03$^{\alpha}$	6.78 ± 0.13$^{\alpha}$	0.83 ± 0.02$^{\alpha}$
P+T_{100}	145.14 ± 1.15$^{\alpha 1}$	2.78 ± 0.05$^{\beta}$	3.58 ± 0.03$^{\beta}$	6.47 ± 0.08$^{\beta}$	0.81 ± 0.01$^{\beta}$
P+M_{20}	144.3 ± 62.11$^{\alpha 1}$	2.79 ± 0.02$^{\gamma}$	3.53 ± 0.04$^{\beta}$	6.32 ± 0.06$^{\beta}$	0.79 ± 0.01$^{\beta}$
P+T_{100}+M_{20}	47.66 ± 1.15$^{\alpha 1}$	1.18 ± 0.04$^{\alpha 1}$	2.24 ± 0.03$^{\alpha 1}$	3.14 ± 0.08$^{\alpha 1}$	0.51 ± 0.02$^{\alpha 1}$

Values are expressed as mean ± S.E.M. of 7 rats in each group. Statistical analysis done by one-way ANOVA followed by Scheffe' multiple comparison tests. $^{\alpha}p < 0.001$ vs. control,. $^{\alpha 1}p < 0.001$ vs. piroxicam-treated rats, $^{\beta}$NS vs. piroxicam-treated rats,$^{\gamma}p < 0.05$ vs. piroxicam-treated rats . NS, not significant.

Table 4: Total phenolics, flavonoids, condensed tannins, chlorophyll content and pH of TLH

Parameters	Values
Total phenolic content (mg gallic acid / ml TLH)	3.135 ± 0.10
Total flavonoids content [mg (+) catechin / gm of leaves]	3.39 ± 0.16
Total condensed tannins [mg (+) catechin / gm of leaves]	2.71 ± 0.16
Chlorophyll content (μM)	
Chlorophyll a	0.0245 ± 0.08
Chlorophyll b	0.0193 ± 0.11
Total Chlorophyll	0.0438 ± 0.04
pH of the TLH	6.85 – 7.04

Values are expressed as mean ± S.E.M.

ACKNOWLEDGEMENT

Anjali Basu is supported from the fund available to Dr. DB from Teachers' Research Grant. DM gratefully acknowledges the receipt of a Senior Research Fellowship (SRF) from CSIR, Govt. of India.. AKG is a SRF under RFSMS Fellowship Program, Govt. of India. EM is a Project Fellow under UGC Major Research Project of Dr. DB. SBF is a University Research Fellow (URF) of UGC, Govt. of India at University of Calcutta. DG is a DST INSPIRE Junior Research Fellow (JRF), Govt. of India. Dr. KJ is supported from grants available to him from Bose Institute, DST Govt. of India, Kolkata. Dr. AC is supported from grants available to her from UGC Minor Research Project, Govt. of India. This work is partially supported by UGC Major Research Project Grant to Dr. DB [(F. No. 37-396 / 2009 (SR)] and also by CSIR Grant to Dr. AB (SIP 007).

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