A combination of aqueous curry (Murraya koenigii) leaf extract and melatonin protects against piroxicam induced gastric ulcer in male albino rats: Involvement of antioxidant mechanism(s)

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ABSTRACT

Melatonin (M) and aqueous curry leaf extract (CuLE) protected gastric mucosa from piroxicam induced damage in a dose dependent manner. In two different sets of experiments, pre-treatment of rats with melatonin at a dose of 20 mg/kg bw and CuLE at a dose of 50 mg/kg bw, individually, failed to render complete protection against piroxicam-induced gastro-toxicity though mean ulcer index showed some decrease on oral administration of both at the above mentioned dose. Combination of the above mentioned two doses, however, provided complete protection as indicated by the results from a third set of experiment. Gastro-toxic side effects of piroxicam depend on oxidative stress built up in vivo. Potential antioxidant activities of both melatonin and CuLE possibly provided protection against gastric oxidative stress. Biochemical studies of biomarkers of gastro- mucosal damage and oxidative stress parameters along with supporting histopathology reveal that the combination has the potential to provide protection against piroxicam-induced ulceration of the gastric mucosa experimental rats. Thus, the combination (CuLE⁵₀+M₂₀) seems to be an effective and a safe gastro-protective co-therapy in patients where piroxicam can be a useful drug of choice.

KEYWORDS: Antioxidant, aqueous curry leaf extract, co-therapy, gastric ulcer, melatonin, piroxicam, oxidative stress.

INTRODUCTION

Gastric ulcer is the major side effect that has restricted the use of oxicam class of non-steroidal anti-inflammatory drugs (NSAIDs)¹. Piroxicam belonging to enolic subclass of oxicam family has been reported to induce gastric ulcer through in vivo free hydroxyl radical generation². Alternative drugs to piroxicam have serious detrimental effects on long term use in patients. Therefore, our present study involves the search of an effective co-therapy that can reduce the detrimental effect of piroxicam to minimum. Piroxicam, an otherwise safe drug can be widely prescribed, if a co-therapeutic drug introduced protects against gastric oxidative stress and gastric mucosal damage on piroxicam administration.

South Indian culinary spice herb (Murraya koenigii), popularly known as curry plant, has leaves rich in antioxidants. Thus, curry leaves have been found to be effective in many oxidative stress related disease conditions. Curry leaves are useful in the treatment or prevention of diabetes, cancer and cardiovascular disease³. Ingestion of curry leaves improved the plasma lipid profile in the rat feeding model. In obese mouse model, curry leaves exhibited both hypocholesterolemic effects and improved glycemic status⁴. Anti-oxidative and anti-lipid per-oxidative actions of curry leaves are also reported⁵. Thus, the leaves of the curry plant have the potential to provide protection against oxidative stress.

Melatonin, a highly evolutionarily conserved indoleamine has been found to be present in mammals. It regulates seasonal reproduction, immune responses, light-dark signal transduction and aging⁶. Melatonin is also present in other plants and animals and has been synthesized chemically for human supplementation to promote sleep and other health benefits⁷. Melatonin in low pharmacologic doses provides protection against stress and drug-induced gastric ulcer⁸. This
has been currently investigated by a number of researchers and is considered to be an emerging area. It has been shown that melatonin is highly effective against cold-restraint stress and drug-induced gastric ulceration and it possibly acts through its antioxidant mechanism(s). Very recently, it has also been shown that melatonin shows synergism with some natural antioxidants or herbal extracts in mitigating oxidative stress in a number of models, even at a much lower dose.

Considering the wide range of antioxidant effects of CuLE as well as melatonin, in our present study, we used a combination of both to investigate its protective action against piroxicam induced oxidative stress and gastric ulcer. The effects of the combination in protecting oxidative stress, gastric adherent mucus and tissue collagen on pre-treatment open an area of extensive research in co-therapeutic approach where piroxicam is a useful drug of choice. In the present study, we ascertained the protective actions of the combination through histo-pathological examination and biochemical analysis of a variety of oxidative stress related parameters, at a dose, at which neither of them can provide significant protection against piroxicam-induced oxidative stress and gastric ulcer, when administered singly.

**MATERIALS & METHODS**

**Chemicals**

Piroxicam sold under the trade name Dolonex DT was purchased from the local chemist shop. Melatonin and other chemicals and solvents used in the present study were of analytical grade and procured from Sisco Research Laboratories (SRL), Mumbai, India; Qualigens (India/Germany); SD Fine chemicals (India) and Merck Limited, Delhi, India.

**Preparation of aqueous curry leaf extract (CuLE)**

Fresh green curry leaves were collected and confirmed by Mr. P. Venu, Scientist ‘F’, the Botanical Survey of India, Central National Herbarium (Government of India, Ministry of Environment and Forests), Botanic Garden, Howrah 711 103, West Bengal, India. The Herbarium (Government of India, Ministry of Environment and Forests) of the plant was deposited in the BSI against voucher specimen No. CNH/I-I/42/2010/Tech.II/233. An aqueous extract was prepared following the procedure adopted by our laboratory. Lyophilised leaf material was used to prepare the different doses of Cu LE to confirm its efficacy in ulcer protection.

**Animals:** Male Wistar rats of body weight 160-180 g were used throughout the experiments. The animals were handled as per the guidelines of institutional animal ethics committee (IAEC) of Department of Physiology, University of Calcutta in accordance with the committee for the purpose of control and supervision of experiment on animals (CPCSEA), Ministry of Environment and Forest, Government of India. All the experimental protocols had the approval (approved under proposal No. IAEC/ PROPOSAL/ DB-5/ 2010 dated 05/05/2010, approval date: 16/11/2011) of Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., Professor and Veterinary Surgeon and CPCSEA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling.

**Protection against gastric ulcer**

For our present study, the animals were housed in galvanized wire cages, in well ventilated, air conditioned rooms of our animal house with 12 hours light/dark cycle, at about 18°C room temperature for 7 days to get adapted to laboratory condition. All rats had been given a standard diet containing 18% protein, 71% carbohydrate and vitamins which are considered to be an adequate (normal) dietary protein level.

The animals were released from quarantine and immediately they were kept on fasting condition in specially designed cages for the following 40 hours. After that, treatment of rats was carried out as per the schedule mentioned below. The animals were divided into six groups in two different sets of experiments for the dose response studies using melatonin and CuLE respectively.

The first set of experiment used rats divided into six groups - C or control rats (supplied only drinking water), Px or piroxicam treated rats (piroxicam administered orally at 30 mg/kg bw dose), M20 rats (pre-treated with 20 mg/kg bw melatonin orally one hour before piroxicam administration). M40 rats (pre-treated with 40 mg/kg bw melatonin orally one hour before piroxicam administration) and M60 rats (pre-treated with 40 mg/kg bw melatonin orally one hour before piroxicam administration). The second set of experiment tested the gastro-protective action of CuLE. Here also, the animals were divided into six groups. The groups included C or control rats (allowed to drink water supplied ad libitum), Px or piroxicam treated rats (piroxicam administered orally at 30 mg/kg bw dose), CuLE20 rats (pre-treated with 50 mg/kg bw melatonin orally one hour before piroxicam administration), CuLE100 rats (pre-treated with 100 mg/kg bw melatonin orally one hour before piroxicam administration) and CuLE200 rats (pre-treated with 200 mg/kg bw melatonin orally one hour before piroxicam administration).

In another separate set of experiment, animals were divided into the following four groups to ascertain the mechanism underlying Cu LE60+M20 mediated protection against piroxicam induced gastric ulcer. The groups included: C or control rats (rats were allowed to drink water supplied ad libitum), Cu LE60+M20 rats (rats were orally administered with CuLE at 50 mg/kg bw dose followed by melatonin at 20 mg/kg bw dose), Px or piroxicam treated rats (piroxicam administered orally at 30 mg/kg bw dose) and Cu LE60+M20+Px rats (rats were orally administered with CuLE at 50 mg/kg bw dose followed by melatonin at 20 mg/kg bw dose exactly one hour before oral administration piroxicam at 30 mg/kg bw dose).
Each group of animals used in different sets of experiments comprised of six rats. At the end of treatment, all the animals were allowed to drink water and kept undisturbed for four hours. The animals were sacrificed at the end of four hours by cervical dislocation following light ether anesthesia. The abdomen of each rat was surgically opened to collect the stomach for macroscopic observations, histological studies and biochemical estimations. The stomach tissue was kept in sterile plastic vial at -20°C until further biochemical analysis. For histological studies, an appropriate portion of the fundic part of the stomach was placed immediately in formalin fixative. Each set of experiment was repeated at least three times. Another separate set of experiment was carried out to determine the degree of inhibition of free hydroxyl radical generation in vivo with oral administration of CuLE35+M30+ combination.

**Determination of ulcer index**

Stomach was flushed with saline and lesions in glandular portion were then exposed and examined under a magnifying glass. The grade of lesions was scored according to the following scale: 0, no pathology; 1, small 1–2 mm ulcers; 2, medium 3–4-mm ulcers; 4, large 5–6-mm ulcers; 8, ulcers greater than 6 mm. The sum of the total ulcer scores in each group of rats was divided by the number of animals in the group to give the mean ulcer index for that group.

**Determination of oxidative stress biomarkers**

A portion of the fundic stomach was homogenized (5%) in the ice-cold 0.9% saline (pH 7.0) with a Potter–Elvehjem glass homogenizer for 30 s. The homogenate was centrifuged at 800g for 10 min and the supernatant was again centrifuged at 12,000g for 15 min to obtain the mitochondrial fraction. The mitochondrial pellet was suspended in buffer and used for the enzyme assay using a UV/VIS spectrophotometer at 550 nm with an O2− generating system (xanthine/xanthine oxidase) in the presence of cytochrome c. For Cu–Zn SOD activity, a portion from the fundic stomach was homogenized (10%) in ice-cold 50mM phosphate buffer containing 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000g for 15 min and supernatant collected. Inhibition of haematoxylin auto-oxidation by the cell free supernatant was measured at 560 nm using a UV-VIS spectrophotometer.

To determine catalase activity, a weighed amount of gastric tissue was homogenized (5%) in ice cold 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000g for 12 min. The supernatant, thus obtained, was then collected and incubated with 0.01mL of absolute ethanol at 4°C for 30 minutes, after which 10% Triton X-100 was added so as to have a final concentration of 1%. The sample, thus obtained, was used to determine catalase activity by measuring the breakdown of H2O2 spectrophotometrically at 240 nm.

**Determination of activities of pro-oxidant enzymes**

Xanthine oxidase (XO activity) was determined from gastric tissues homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.8. The homogenates were centrifuged at 500g for 10 min. The resulting supernatant was further centrifuged at 12,000g for 20 min in cold. The supernatant, thus obtained, was collected and used for spectrophotometric assay of the enzyme at 295 nm using 0.1 mM xanthine in 50 mM phosphate buffer, pH 7.8, as the substrate. The activity of Xanthine dehydrogenase (XDH) was measured by following the reduction of NAD+ to NADH. In brief, the weighed amount of rat gastric tissue was homogenized in cold (10%) in 50 mM phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were centrifuged in cold at 500g for 10 min. The supernatant, thus obtained, was further centrifuged in cold at 12,000g for 20 min. The final supernatant was used as the source of the enzyme, and the activity of the enzyme was measured spectrophotometrically 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 activities of both the enzymes measured were expressed as milli units/min/mg tissue protein.
Determination of tissue level of endogenous free hydroxyl (‘OH) radical generation
The ‘OH generated in the stomach were measured using DMSO as ‘OH scavenger22, DMSO forms a stable product [methanesulfonic acid (MSA)] on reaction with fast blue BB salt. The stomach of each animal was processed for MSA, which was allowed to react with the fast blue BB salt to yield a yellow product. This was measured spectrophotometrically at 425 nm using benzenesulfonic acid as standard. Values obtained were expressed as nmoles of ‘OH generated per g of stomach.

Estimation of tissue protein
The protein content of different samples was determined by a spectrophotometric method using Folin Ciocalteau reagent23.

Histological studies
A portion from the fundic part of rat stomach was spread out on a wooden block, attached and fixed in formalin. Later an ulcerated part was separated out with the help of a surgical blade. The part of the stomach dissected out was embedded in paraffin following routine procedure and 5µm thick sections were stained24 separately with haematoxylin-eosin, per-iodo-acid Schiff (PAS) reagent, alcian blue dye and sirius red (Direct red 80; Sigma chemical Co., St. Louis, MO, USA) respectively. Quantification of collagen in each of the sirius red stained sections was carried out by analysis of confocal microscope captured digitized images using Image J software (NIH, Bethesda, MI)25.

Determination of adherent gastric mucus
The gastric tissue scrapings were weighed and incubated with 1% alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate , pH 5.8) for 2h. The tubes were then centrifuged at 1450g for 10 min and the absorbance of the supernatant was measured at 489 nm to determine the total adherent mucopolysaccharide content26.

Statistical analysis
Data are represented by Mean ± S.E.M. Significance of mean values of different parameters between the treated groups were analysed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Paired comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin 7.0 for Windows. Each experiment was repeated at least three times with different rats.

RESULTS

Dose response study
Experiments were carried out to determine dose dependent effects of melatonin and CuLE on piroxicam induced gastric ulcer. The mean ulcer index and three biomarkers of oxidative stress namely lipid peroxidation level, protein carbonyl content and reduced glutathione content were studied and the values thus obtained are represented in Table 1 and Table 2. Melatonin at 20 mg/kg bw dose and CuLE at 50 mg/kg bw dose did not provide significant protection against piroxicam induced gastric oxidative stress and ulcer (P<0.001 was considered to be the level of significance for protection). Reduction in mean ulcer index values by 46.7% and 37.5% on melatonin (20 mg/kg bw) and (CuLE 50 mg/kg bw) pre-treatments respectively, indicate induction of gastro-protective actions on piroxicam toxicity. These doses however do not protect maximally. Thus, the two minimum effective doses were combined (CuLE_{50}+M_{20}) and gastro-protective actions of that combination are elaborated in the later results.

Table 1. Dose response study on gastro-protective effects of melatonin

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>C</th>
<th>Animal groups (Value= Mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>Mean ulcer index</td>
<td>0±0.00</td>
<td>60±1.54</td>
</tr>
<tr>
<td>LPO (nmoles TBARS/mg protein)</td>
<td>0.62±0.02</td>
<td>1.54±0.06</td>
</tr>
<tr>
<td>PCO (nmoles carbonyl/mg protein)</td>
<td>2.54±0.26</td>
<td>12.6±0.32</td>
</tr>
<tr>
<td>GSH (nmoles/mg protein)</td>
<td>25.6±0.74</td>
<td>9.52±0.52</td>
</tr>
</tbody>
</table>

*P>0.001, NS compared to piroxicam treated values, values are expressed as Mean±SE and each value represents the mean value of the data obtained from six rats. Statistics were done using one way ANOVA followed by Scheffe’ multiple comparison test in Microcal Origin 7 version

Table 2. Dose response study on gastro-protective effects of CuLE

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>C</th>
<th>Animal groups (Value= Mean±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cu LE_{50}</td>
</tr>
<tr>
<td>Mean ulcer index</td>
<td>0±0.00</td>
<td>64±1.62</td>
</tr>
<tr>
<td>LPO (nmoles TBARS/mg protein)</td>
<td>0.59±0.01</td>
<td>1.42±0.07</td>
</tr>
<tr>
<td>PCO (nmoles carbonyl/mg protein)</td>
<td>2.52±0.28</td>
<td>14.76±0.42</td>
</tr>
<tr>
<td>GSH (nmoles/mg protein)</td>
<td>23.6±0.72</td>
<td>9.64±0.48</td>
</tr>
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</table>

*P>0.001, NS compared to piroxicam treated values, values are expressed as Mean±SE and each value represents the mean value of the data obtained from six rats. Statistics were done using one way ANOVA followed by Scheffe’ multiple comparison test in Microcal Origin 7 version for Windows. NS : not significant.
Antiulcer, antioxidant and gastro-protective actions of CuLE$_{50}$+M$_{20}$

Rats pre-treated with CuLE$_{50}$+M$_{20}$ showed significant decrease in mean ulcer index (*P*≤0.001 compared to piroxicam treated group). Macro-scopic view (Figure 1A) revealed absence of ulcerative spots in animals pre-administered with the combination before piroxicam treatment.

Figure 1A. Digitized images of ulcerative damages viewed and captured in a canon SX 110 IS of 10X optical zoom. C represents stomach of control rat, CuLE$_{50}$+M$_{20}$ represents stomach of positive control group rat, Px represents ulcerated stomach of piroxicam fed rat and CuLE50+M20+Px represents the protected stomach of the combination group rat.

Gastro-protective actions were further studied through histopathological study of haematoxylin-eosin (H & E), alcain blue dye and per-iodo-acid Schiff (PAS) stained sections of rat stomach. H&E stained section of piroxicam treated rats shows interrupted mucosal border, ruptured blood vessel and infiltrated macrophages and neutrophils. Photomicrograph of PAS stained gastric tissue section of piroxicam treated rats also shows a distinct discontinuous pink gastro-mucosal border. This indicates depletion of muco-polysaccharide and damage of the gastric mucus coat. Alcian blue dye stained sections of piroxicam fed animals reveal concentration of blue stained glands. This is indicative of increased acidic mucus secretion on piroxicam treatment. The tissue sections of the other three groups had similar histo-morphology and no pathological changes were observed in all of these sections. These further confirm the gastro-protective actions of the combination. Representative images of microscopy done on gastric tissue sections of different animal groups are shown in Figure 1B.

Table 3 contains the values of mean ulcer index and biomarkers of oxidative stress to further confirm the gastro-protective actions of the combination on piroxicam treatment. Mean ulcer index determined showed absence of any ulcerative damage. Lipid peroxidation level, protein carbonyl content and reduced glutathione titre are near control values (Table 3) in combination pre-treated group.

Table 3. Gastro-protective effects of CuLE50+M20

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>Animal groups (Value= Mean±SE)</th>
<th>CuLE$<em>{50}$+M$</em>{20}$</th>
<th>Px</th>
<th>CuLE$<em>{50}$+M$</em>{20}$+Px</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ulcer index</td>
<td>C</td>
<td>0±0.0</td>
<td>0±0.0</td>
<td>72±2.56*</td>
</tr>
<tr>
<td>LPO (nmoles TBARS/mg protein)</td>
<td>0.61±0.02</td>
<td>0.59±0.01</td>
<td>1.39±0.08*</td>
<td>0.62±0.03**</td>
</tr>
<tr>
<td>PCO (nmoles carbonyl/mg protein)</td>
<td>2.52±0.28</td>
<td>2.46±0.44</td>
<td>14.5±0.24*</td>
<td>2.72±0.26**</td>
</tr>
<tr>
<td>GSH (nmoles/mg protein)</td>
<td>25.6±0.92</td>
<td>26.4±0.72</td>
<td>9.26±0.42*</td>
<td>25.2±0.74**</td>
</tr>
</tbody>
</table>

*P*≤0.001, compared to control values; **P*≤0.001 values, compared to piroxicam treated values. All values are expressed as Mean± SE and each value represents the mean value of the data obtained from six rats. Statistic were done using one way ANOVA followed by Scheffe’ multiple comparison test.
CuLE<sub>50</sub> + M<sub>20</sub> protects against piroxicam-induced alterations in adherent gastric mucus in rats

Adherent gastric mucus depletion is a marker for gastro-mucosal damage. Mucus content significantly depleted in piroxicam fed rats (28.5% decrease compared to control, *P<0.001). Pre-treatment of rats with the combination showed 10.01% increase in mucus content compared to control. This increased mucus protected the pre-treated rats from piroxicam induced mucus depletion (Figure 2).

Figure 2. Bar graph showing changes in gastric mucus content. C represents stomach of control rat, CuLE<sub>50</sub>+M<sub>20</sub> represents stomach of positive control group rat, Px represents ulcerated stomach of piroxicam fed rat and CuLE<sub>50</sub>+M<sub>20</sub>+Px represents the protected stomach of the combination group rat. The values are expressed as Mean ± SE. *P<0.001 compared to control rats and **P<0.001 compared to piroxicam treated rats.

CuLE<sub>50</sub> + M<sub>20</sub> protects against the piroxicam-induced alterations in the activities of gastric antioxidant and pro-oxidant enzymes

Oxidative stress mediates alterations in the activities of tissue antioxidant and pro-oxidant enzymes. Key gastric tissue antioxidant enzymes like gastric peroxidase, superoxide dismutases (both Cu-Zn and Mn-types), catalase and pro-oxidant enzymes like xanthine oxidase and xanthine dehydrogenase showed marked alterations in their activities on piroxicam treatment. There were significant changes in the activities of the enzymes studied in piroxicam treated rats when compared to control rats (*P<0.001). Gastric peroxidase (GPO) activity was inhibited by 53.6% in piroxicam treated rats. Cu-Zn SOD activity and Mn SOD activity are increased by 2.25 folds and 2.28 folds, respectively, on piroxicam treatment. Catalase and the pro-oxidant enzymes also showed increased activities in piroxicam treated group when compared to control [Table 4].

Table 4. Gastro-protective effects of CuLE<sub>50</sub> + M<sub>20</sub> on activities of antioxidant and pro-oxidant enzyme.

<table>
<thead>
<tr>
<th>Parameters studied</th>
<th>C</th>
<th>CuLE&lt;sub&gt;50&lt;/sub&gt;+M&lt;sub&gt;20&lt;/sub&gt;</th>
<th>Px</th>
<th>CuLE&lt;sub&gt;50&lt;/sub&gt;+M&lt;sub&gt;20&lt;/sub&gt;+Px</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPO activity (Units/min/mg tissue protein)</td>
<td>70.2±1.72</td>
<td>73.4±1.33</td>
<td>32.6±1.12*</td>
<td>70±1.12**</td>
</tr>
<tr>
<td>Cu-Zn SOD activity (Units/min/mg tissue protein)</td>
<td>2.54±0.32</td>
<td>2.52±0.23</td>
<td>5.72±0.41*</td>
<td>2.62±0.27**</td>
</tr>
<tr>
<td>Mn SOD activity (Units/min/mg tissue protein)</td>
<td>1.42±0.26</td>
<td>1.39±0.21</td>
<td>3.24±0.22*</td>
<td>1.41±0.24**</td>
</tr>
<tr>
<td>Catalase activity (µmol H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/mg tissue protein)</td>
<td>10.72±0.64</td>
<td>10.52±0.47</td>
<td>19.2±0.52*</td>
<td>10.8±0.64**</td>
</tr>
<tr>
<td>XO activity (milli units/min/mg tissue protein)</td>
<td>25.6±0.86</td>
<td>25.4±0.92</td>
<td>59.2±0.89*</td>
<td>26.2±0.98**</td>
</tr>
<tr>
<td>XDH activity (milli units/min/mg tissue protein)</td>
<td>0.52±0.02</td>
<td>0.48±0.03</td>
<td>0.82±0.02*</td>
<td>0.54±0.03**</td>
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</table>

*P<0.001, compared to control values; **P<0.001 values, compared to piroxicam treated values. All values are expressed as Mean±SE and each value represents the mean value of the data obtained from six rats. Statistic were done using one way ANOVA followed by Scheffe’s multiple comparison test.

CuLE<sub>50</sub> + M<sub>20</sub> protects against piroxicam-induced in vivo free hydroxyl radical accumulation

Significant increase in gastric tissue level free hydroxyl radical was observed in piroxicam treated rats (*P<0.001 compared to control rats). There was 3.77 folds increase in in vivo free hydroxyl radical on piroxicam treatment. This indicated a marked increase in tissue free hydroxyl radical generation in the course of piroxicam action. Combination pre-treatment protected against increase in free hydroxyl radical titre in vivo (Figure 3).

Figure 3. Bar graph showing changes in in vivo free hydroxyl radical titre. C represents stomach of control rat, CuLE<sub>50</sub>+M<sub>20</sub> represents stomach of positive control group rat, Px represents ulcerated stomach of piroxicam fed rat and CuLE<sub>50</sub>+M<sub>20</sub>+Px represents the protected stomach of the combination group rat. The values are expressed as Mean ± SE, *P<0.001 compared to control rats and **P<0.001 compared to piroxicam treated rats.

CuLE<sub>50</sub> + M<sub>20</sub> protects against piroxicam-induced gastric collagen depletion

Gastric tissue collagen depleted significantly on piroxicam treated group (*P<0.001 compared to control group). Photomicrographs of acid Sirius stained tissue sections captured in an inverted phase contrast microscope reveal minimum collagen content in tissue sections of piroxicam treated rats. Confocal microscopy was done on identical areas in each of the picro-sirius stained gastric tissue sections and collagen content quantified using Image J software indicated maximum protection on pre-treatment of rats with the combination.

*Syed Benazir Firdaus et al. / Journal of Pharmacy Research 2014,8(3),428-436*
Collagen quantified using Image J analysis software revealed a significant decrease on piroxicam treatment (P<0.01 compared to control) but combination pre-treatment protected tissue collagen against damage. Figure 4B represents the bar graph showing the changes and protective action against changes in the tissue collagen content in rats.

Figure 4B. Bar graph showing changes in gastric tissue collagen content. C represents stomach of control rat, CuLE50+M20 represents stomach of positive control group rat, Px represents ulcerated stomach of piroxicam fed rat and CuLE50+M20+Px represents the protected stomach of the combination group rat. The values are expressed as Mean ±SE, *P<0.001 compared to control rats and **P<0.001 compared to piroxicam treated rats.

DISCUSSION

Worldwide, more than 30 million or even more people are exposed to the risk of gastric ulcer due to consumption of NSAIDs. NSAID treatment becomes indispensable in the management of arthritis and pain. Although there are a great number of alternatives to piroxicam, those NSAIDs also have fatal side effects. Many co-therapeutic approaches have been associated with NSAID treatment. The co-therapeutic drugs in many cases fail to provide complete protection and sometimes bear harmful consequences on prolonged use. Considering these problems, we searched for a co-therapy that can fully protect against side effects of piroxicam and by itself has with no side effects.

Melatonin has been tested to be effective in piroxicam induced ulcer model in some studies. Melatonin and its health benefits are widely present in scientific literatures. Anioxidant activity of melatonin in lead induced toxicity is reported in a study by our group. Similarly, CuLE has been well tested for its protective potential in metal toxicity induced oxidative stress. Hydroxyl radical, the main causative factor in stress needs to be scavenged to prevent building up of piroxicam-induced oxidative stress. Both melatonin and CuLE when used singly require high doses (60mg/kg bw for melatonin & 200 mg/kg bw dose for CuLE) [refer, Table 1 and Table 2] to provide protection against gastric oxidative stress and subsequent ulceration. Requirements in such high amounts, when used individually, impose difficulty in oral administration and also cannot restore all the altered parameters to control levels. Thus, we investigated the potency of a combination of melatonin and CuLE for complete protection in piroxicam mediated oxidative stress and gastric ulcer in rats.

Mean ulcer index determined from macroscopic study of rat stomach
hydroxyl radicals [refer, Figure 3], indicating that the combination has complete protective effect on piroxicam induced gastric ulcer. Biomarkers of oxidative stress, viz., lipid peroxidation level, protein carbonyl content and reduced glutathione levels were similar in the pre-treated group when compared to control. Such findings, strengthens the result obtained in mean ulcer index determination. The combination shielded the alterations in biomarkers of oxidative stress, thereby resulted in absence of oxidative stress built up in the course of piroxicam action. The effects of pre-treatment of the combination on altered activities of antioxidant and pro-oxidant enzymes represented in Table 4 reveal complete protective action.

Histo-pathological examination reveals interrupted and discontinuous gastro-mucosal border in piroxicam treated rats. Combination co-therapy maintained a continuous border as observed in H & E stained and PAS stained sections indicating there was no damage to the mucosa [Figure 1B]. The change in the nature of secreted mucus as observed in ACB stained piroxicam fed rat’s tissue section was left unaltered when combination pre-treatment was done. This clearly indicated that the combination preserves the nature of mucus which is likely to turn acidic on piroxicam treatment. Gastric adherent mucus, a protective coat on gastric epithelium gets significantly reduced on free radical attack26. This was well combated by the policing of the combination on free radicals. Moreover, the combination also promoted neutral mucus secretion in rat stomach when administered alone [refer, Figure 2]. The free radical scavenging activity of the combination was confirmed by determination of gastric tissue free hydroxyl radicals [refer, Figure 3], indicating that the combination has the potential to serve the role of a direct antioxidant.

Light microscopy and confocal microscopy of the areas selected in light microscopy was carried out in picro-sirius stained gastric tissue sections. Collagen volume determined from the confocal images using Image J analysis software is a measure of tissue integrity. Significant collagen depletion marks tissue disintegration and breakdown13. In the present study, the bar graph of collagen content [refer, Figure 4B] reveals there was no extracellular matrix disintegration or collagen breakdown on pre-treatment of combination in piroxicam fed rats. This well support that potential scavenging of free hydroxyl radical and subsequent blocking of oxidative stress pathways protected rat stomach from piroxicam toxicity in combination co-therapy.

Our results and findings are subjected to future human trials for introduction of the combination as a co-therapy in piroxicam treated patients. Prolonged treatment of such patients will enable them to get relief from pain without any adverse heath effect.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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