Anti-ulcerogenic and proton pump (H\(^+\), K\(^+\) ATPase) inhibitory activity of \textit{Kolaviron} from \textit{Garcinia kola} Heckel in rodents

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Anti-ulcer potential and proton pump inhibitory activity of kolaviron (KV) isolated from \textit{Garcinia kola} Heckel has been evaluated using different ulcer models. Cold-restraint (CRU), aspirin (ASP), alcohol (AL), pyloric ligation (PL) induced gastric ulcer models were used to assess anti-ulcerogenic activity of KV in rats. Effects of KV on gastric juice for free and total acidity, peptic activity and mucin secretion were also evaluated. The H\(^+\), K\(^+\)-ATPase activity was assayed in gastric microsomes, spectrophotometrically. Results of this study showed that KV (200 mg/kg) reduced the incidence of ulcers in CRU (69.0%), PL (67.6%), ASP (68.6%) and AL (51.5%). Reductions were also observed in free acidity (32.6%), total acidity (56.2%) and peptic activity (35.4%) with increase in mucin secretion by 40.1%. KV inhibited the H\(^+\),K\(^+\)-ATPase activity with IC\(_{50}\) of 43.8 µg/ml compared with omeprazole with IC\(_{50}\) of 32.3 µg/ml. KV showed both cytoprotective and anti-secretory potentials against peptic ulcer models, and a proton pump inhibitory activity. KV may emerge as a potent anti-ulcer compound.

Keywords: Anti-secretory, Cyto-protection, \textit{Garcinia kola}, H\(^+\),K\(^+\)-ATPase, Kolaviron, Omeprazole, Peptic ulcer

The gastric mucosal epithelium is continuously exposed to potentially injurious agents such as acid, pepsin, bile acids, food ingredients, bacterial products, and drugs\(^1\) and this exposure leads to the widespread disease states known as gastric ulcers\(^2\). Incidence of gastric ulcer increased due to stress, smoking, nutritional deficiencies and ingestion of non-steroidal anti-inflammatory drugs\(^3\). A positive correlation between oxidative stress and ulcer has been reported clinically, as stress may alter factors that pertain to mucosal integrity, leading to exhausted mucosal defense mechanisms\(^4\). Moreover, treatment of peptic ulcer is generally based on inhibition of gastric acid secretion by H\(_2\) antagonists and proton pump inhibitors such as omeprazole and anti-muscarinics, as well as acid-independent treatment by sucralfate and bismuth\(^5\). The other very promising approach for treating gastric mucosal damage has been to augment defensive factors such as the mucus-bicarbonate layer, prostaglandin (PG), and growth factor. A gastric mucous bicarbonate barrier protects the mucosa from damage caused by acid and pepsin by acting as a blanket over the mucosal layer which protects the epithelium from bacteria by trapping and excreting them in feces.

Efforts are being made by in search for new and suitable therapeutic anti-ulcer agents from natural products of plants and animal origin. Use of bioactive compounds in treating peptic ulcer diseases has become paramount importance. Studies have revealed that medicinal plants contain various classes of bioactive secondary metabolites such as polyphenols, tocopherols, alkaloids and flavonoids\(^6\), and flavonoids particularly are known to exhibit various pharmacological properties like anti-carcinogenic, antimicrobial, anti-inflammatory and anti-proliferative effects\(^7\).

\textit{Garcinia kola} Heckel (Family: Guttiferae) is a largely cultivated forest tree, indigenous to sub-Saharan Africa especially in Nigeria. It is particularly valued in West and Central Africa for its edible nuts\(^8\), and known for its characteristic astringent, bitter and resinous taste. This seed, called “bitter kola” is usually eaten raw by the people with the belief that it promotes longevity\(^9\) while extracts of the plant are used in traditional African medicine for the treatment

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Kolaviron (KV), isolated as a defatted ethanol extract from the seeds of \textit{Garcinia kola}, is known for its numerous medicinal and therapeutic values.\textsuperscript{12} It has been extensively studied for its anti-inflammatory and hepatoprotective properties in various experimental models.\textsuperscript{13-16} Researchers have reported its antioxidant and scavenging properties \textit{in vitro} and \textit{in vivo},\textsuperscript{17,18} and its hypoglycemic effects.\textsuperscript{11,19} KV, which is commonly consumed, plays an important role in ceremony and traditional hospitality in Nigeria. \textit{Garcinia kola} from which kolaviron is obtained is generally non-toxic. However, histological observations in studies involving chronic feeding of \textit{Garcinia kola} seeds in rats indicate that prolonged ingestion of seeds may induce infinitesimally mild changes in liver parenchymal cells and in renal tubular and intestinal epithelium.\textsuperscript{20}

An earlier study justified the anecdotal use of \textit{Garcinia kola} crude extract in ulcer treatment while gastroprotective activity of KV in indomethacin and ethanol/HCl-induced gastric mucosa damage may be linked to its intrinsic antioxidant properties.\textsuperscript{22} In the present study anti-ulcer properties of KV using different ulcer models to understand cyto-protective or/and anti-secretory potentials KV have been reported. The inhibitory potential of KV on proton pump was also investigated to establish its mechanism of action.

Materials and Methods

\textit{Animals}—Sprague Dawley (SD) rats (140-160 g) were obtained from National Animal Laboratory Centre of Central Drug Research Institute, Lucknow. Animals were kept in raised mesh bottom cages to prevent coprophagy and kept in environmentally controlled rooms (25±2°C, 12 h light and dark cycle), with free access to water. Animals were fed with standard Hind Lever diet pellets \textit{ad libitum}. Animals were deprived of food for 24 h before subjecting them to ulcerogens and were randomly allocated to different experimental groups. Experimental protocol and procedure were approved by the Institutional Ethical Committee following guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (34/1999/CPCSEA) with the Approval No. 42/05/Pharma/IAEC. This complies with international norms of INSA (Indian National Science Academy). The “Principle of laboratory animal care” (NIH publication No 85-23) guidelines and procedures were considered in this study.

\textit{Plant material}—\textit{Garcinia kola} seeds were obtained commercially at Ibadan, Nigeria and certified at the herbarium in the Department of Botany, University of Ibadan, Nigeria, where a voucher specimen already exists (UI-00138/01). Peeled seeds (4 kg) was sliced, pulverized with an electric blender and air-dried in laboratory (25-28°C).

\textit{Extraction and isolation of kolaviron (KV)}—Kolaviron (KV) was extracted through the standard method and as previously reported. The powdered seeds (4 kg) were extracted with light petroleum ether (b.pt. 40-60°C) in a soxhlet extractor for 24 h. The defatted, dried marc was re-extracted with acetone. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate (6×250 ml). The concentrated ethyl acetate fraction gave a yellow solid sample known as kolaviron (165 g) with a percentage yield of 4.1% which consist of Garcinia biflavonones GB-1, GB-2 and kolaflavanone. The \textit{Garcinia kola} to kolaviron ratio is approximately 24:1. Kolaviron (180 mg) was initially separated by thin layer chromatography (TLC) using silica gel GF 254 coated plates and solvent mixture chloroform/methanol (80:20). TLC revealed the presence of three compounds GB1, GB2 and kolaflavanone in a ratio 2:2:1, and were identified by their \textit{Rf} values compared with reference compounds, and from the analysis of their spectral data. Also, after analyzing the compounds through Mass Spectra (MS) and Nuclear Magnetic Resonance (NMR), kolaviron (Garcinia biflavonoid) was identified as shown in Fig. 1. The

![Fig. 1—Structure of kolaviron](image-url)
kolaviron extract (50 g) was suspended in 100 mL 0.9% NaCl for oral administration to rats. KV was dissolved in 2–3 drops of tween-80 and diluted to desire concentrations with distilled water to give a water-soluble fraction. The solutions were prepared fresh on the day of experiments.

**Drugs and chemicals**—All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except otherwise stated. These include: aspirin, omeprazole, sucralfate, absolute alcohol, chloral hydrate anaesthesia, NaOH crystals, phenolphthalein, Topfer’s reagent, mannitol, sucrose, EDTA, PIPES, Bradford reagent, KCl, MgSO₄, ATP, EGTA, valinomycin, oligomycin, TCA.

**Anti-ulcer studies**

**Cold restraint stress-induced (CRU) gastric ulcer model in rats**—The rats were subjected to cold-stress paradigm after 45 min of treatment of KV (50, 100 and 200 mg/kg, p.o.) and omeprazole (10 mg/kg, p.o.). All the animals were immobilized in a restraint cages, kept at 4°C in an environmental chamber for 2 h and then sacrificed thereafter. The stomach was cut along the gastric lesion and ulcers were scored with the help of magnascope.

**Alcohol-induced (AL) gastric ulcer model in rats**—Alcohol-induced gastric ulcer was induced in rats by administering absolute alcohol at the dose of 1 ml/200 g, body weight. KV (200 mg/kg, p.o.) and sucralfate (500 mg/kg, p.o.) were administered 45 min before alcohol treatment to the fasting rats. The animals were sacrificed after 1 h and stomach was cut along the greater curvature to observe the gastric lesions which appear as hemorrhagic bands along the mucosal ridges of the stomach. The lesions were analyzed through trinocular stereo-zoom microscope and the lengths of the lesions were measured using biovis image analyzer software and summated to give a total lesion score.

**Aspirin-induced (ASP) gastric ulcer model in rats**—Aspirin at a dose of 150 mg/kg, p.o. was administered to induce ulcer after 45 min of treatment of KV (200 mg/kg, p.o.) and omeprazole (10 mg/kg, p.o.). The animals were sacrificed 5 h after aspirin treatment and the stomach was dissected out, incised along the lesser curvature and the lesion was scored.

**Pyloric ligation-induced (PL) gastric ulcer model in rats**—This method was done by ligating the pyloric end of stomach of rats under chloral hydrate anesthesia (300 mg/kg, i.p.). After 45 min of KV and omeprazole administration, the abdomen was opened below the xiphoid process. The pyloric portion of the stomach was slightly lifted and ligated avoiding any damage to the adjacent blood vessels. Stomach was replaced carefully and the abdomen was stitched. The rats were allowed to recover and stabilize in individual cage during the post-operative period. After 4 h of surgery, rats were sacrificed. The stomach was dissected out and gastric juice was collected for the estimation of free and total acids; mucin and peptic activity.

**Mucin estimation**—The collected supernatant was diluted with methanol and centrifuged at 9000×g for 1 min. After addition of chloroform, centrifuge the sample at 9000 × g for 1 min, then discard the upper phase and add alkaline reagent. The mixture is incubated at 100°C for 30 min. After addition of borate buffer, fluorescence was measure at 383 nm emission and 336 nm excitation wavelength.

**Pepsin estimation**—The gastric juice was added with trichloroacetic acid and incubated at 37°C for 10 min. After addition of HCl, again incubate it for 15 min. Add 0.25N NaOH and folin reagent, mix it by gentle rotation and take the reading at 680 nm.

**Measurement of ulcer index**—Ulcers were scored with the help of magnascope under 5X magnification using the ulcer scoring criteria. The following scoring system was used to grade the incidence and severity of the lesions: (i) shedding of epithelium = 10; (ii) petechial and frank hemorrhages = 20; (iii) one or two ulcers = 30; (iv) more than two ulcers = 40; (v) perforated ulcers = 50. Length of hemorrhagic band is measured in AL model using Biovis Image Analysis Software (BIAS). Percentage protection index is calculated as follows: % protection = (Uᵡ − Uᵢ) × 100 / Uᵡ

where Uᵡ = ulcer index in control group; Uᵢ = ulcer index in treated group.

**Gastric secretion analysis**—The volume of gastric juice obtained in the pyloric ligation model was expressed in terms of ml/100 g of body weight. The free acidity, total acidity, peptic activity and dissolved mucous substances of gastric juice were measured. Free and total acids in the gastric juice were titrated with 0.01N NaOH, using Topfer’s reagent and phenolphthalein as indicators respectively, and were expressed in terms of µeq/ml. Peptic activity was determined by measuring the amount of liberated tyrosine by the action of pepsin on hemoglobin as substrate and expressed in terms of U/ml. Mucin level
in gastric juice was quantified with a fluorometric assay and expressed as µg of mucin/ml of gastric juice.

Preparation of gastric microsomes—The H^+,K^+-ATPase containing gastric microsomes were isolated from non-stimulated rat stomach at 4°C. Gastric mucosal layer was scrapped and homogenized in a buffer containing 125 mM mannitol, 40 mM sucrose, 1 mM EDTA, 5 mM PIPES, pH 6.7 using Teflon pestle. Then, the homogenate was centrifuged at 14,500 g for 10 min. The resulting supernatant was centrifuged at 100,000 × g for 45 min. The microsomal pellets were re-suspended in homogenization buffer and layered on the top of gradients sucrose solutions (33%, 27% and 21%), dissolved in the homogenization buffer. Thereafter, this was ultra-centrifuged at 100,000 × g on a SW 28 swinging rotor (Beckmann) for 2 h. The microsomes fraction present on the top of 21% sucrose layer was harvested. Protein concentration was immediately measured through Bradford method.

Assay of H^+, K^+-ATPase activity—Gastric microsomes, incubated with or without different concentrations of KV as well as standard drug (omeprazole) for 10 min at 37°C, were added to an assay buffer containing 150 mM KCl, 10 mM PIPES, 1 mM MgSO_4, 5 mM Mg-ATP, 1 mM EGTA and 0.1 mM ouabain, at pH 7.2 and 10 µg/ml valinomycin, 2.5 µg/ml oligomycin. The reaction, carried out at 37°C for 20 min was stopped by adding 10% ice-cold trichloroacetic acid. After centrifugation (2000 × g for 1 min), inorganic phosphate release was determined from the resulting supernatant spectrophotometrically at 310 nm wavelength and expressed as µM/hr/mg protein.

Results

Anti-ulcer effects of Kolaviron (KV) against acute gastric ulcer models in rats—The initial biological evaluation of KV was carried out at doses of 100, 150 and 200 mg/kg body wt. The DMHBR exhibited significant anti-ulcer effects against gastric ulcers. From the pilot study conducted using CRU model, graded doses of KV (100, 150 and 200 mg/kg, p.o.) showed percentage protection of 52.1%, 53.5% and 69.0% respectively whereas omeprazole (10 mg/kg, p.o.) showed a protection of 84.6% with reference to the control group. Based on these outcomes, 200 mg/kg was chosen as the effective dose for further studies.

In ASP and PL-induced gastric ulcer model, KV (200 mg/kg, p.o.) showed protection index of 68.6% and 67.6% respectively, whereas omeprazole (10 mg/kg, p.o.) exhibited 84.3% and 69.0% protection respectively. Pre-treatment of rats with KV (200 mg/kg, p.o.) produced 51.5% protection against gastric mucosal damage, induced by absolute alcohol. Sucralfate (500 mg/kg, p.o.), the standard drug, exhibited 76.3% protection under the same condition, when both values were compared with the control group. The results are graphically represented in Fig. 2.

Effect of KV on gastric secretion, in vivo—The effects of KV on different factors such as free acidity, total acidity, peptic activity and defensive factors, mucin, that play a crucial role in the pathogenesis of gastric ulcers, were studied by the analysis of the gastric juice from PL model. KV (200 mg/kg, p.o.) reduced free acidity, total acidity and peptic activity by 32.6%, 56.2% and 35.4% respectively, while omeprazole (10 mg/kg, p.o.) significantly reduced free acidity, total acidity and peptic activity by 61.8%, 69.9% and 46.7% respectively, compared with the control group. Pre-treatment of rats with KV (200 mg/kg, p.o.) produced 51.5% protection against gastric mucosal damage, induced by absolute alcohol. Sucralfate (500 mg/kg, p.o.), the standard drug, exhibited 76.3% protection under the same condition, when both values were compared with the control group. The results are graphically represented in Fig. 2.
of varying concentration of KV (60–100 µg/ml) with the microsomes, inhibited the inorganic phosphate release from proton pump activity proportionately from 17.2% (288.3 µM/h/mg protein) to 74.3% (89.5 µM/h/mg protein) respectively, with IC50 of 43.8 µg/ml. At the same experimental conditions, the proton pump inhibitor omeprazole (10-50 µg/ml) inhibited the enzyme from 12.5% (304.5 µM/h/mg protein) to 87.5% (87.0 µM/h/mg protein) respectively, with IC50 = 32.3 µg/ml.

Discussion

Previous studies have shown that various natural product of plant origin- *Xylocarpus granatum*37, *Tectona grandis*38, *Desmodium gangeticum*39, *Ocimum sanctum*40, *Asparagus racemosus*41 have been reported to possess anti-ulcer activity. Compounds from natural products like plants products are some of the most attractive sources of news drugs, and have shown promising results in the evaluation of anti-ulcer drugs for the treatment of gastric ulcers, induced in the laboratory animals in various experimental models42. In this study, kolaviron (KV), a well established flavonoid compound from *Garcinia kola*, was tested to verify its mode of action in preventing the incidence of gastric ulcer in the laboratory rodents. So, this piece of research work provides a substantial evidence for the anti-ulcer and anti-secretory effects of KV. Pretreatment of gastric mucosal surface epithelium against the attack by physical, chemical or microbiological agents which are involved in multiple pathologies, such as gastritis and peptic ulcer, with plant-originated flavonoid substances could effectively prevent gastric mucosa from the development of erosions and ulcerations43. So, the SD rats were pre-treated with KV.

The graded dose studies carried out on SD rats to identify the effective dose using CRU model showed 200 mg/kg KV to be most effective dose for this study. This is based on the fact that the cold restraint stress-induced ulcer (CRU) has been suggested as the model for rapid massive screening of peptic ulcer44. Moreover, the anti-ulcer properties of KV were significant against CRU, ASP and PL as compared with omeprazole and against AL as compared with sucralfate, with reference to the control group. Generally, gastric ulcers have multiple etiopathogenesis. In CRU model, incidence of ulcers is mainly due to increased acid secretion and generation of free radicals45. Likewise, stress-induced ulcers are due to both physiological and psychological factors, which is crucial for gastrointestinal defense and increased accumulation of acid and pepsin leading to auto-digestion of the gastric mucosa46. Previous investigation has shown the in vivo protective action of kolaviron on antioxidant defense mechanisms and reservation of membrane protein enzyme activities in rats pretreated with potassium bromate18. Hence, the protective effect of KV against CRU model may be due to its antioxidant properties as reported.

<table>
<thead>
<tr>
<th>Group</th>
<th>Free acidity (µeq./ml)</th>
<th>Total acidity (µeq./ml)</th>
<th>Peptic activity (U/ml)</th>
<th>Mucin secretion (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10 ml/kg, p.o.)</td>
<td>74.45±4.56</td>
<td>141.40±5.96</td>
<td>14.92±1.82</td>
<td>102.60±6.90</td>
</tr>
<tr>
<td>Kolaviron (200 mg/kg, p.o.)</td>
<td>50.17±7.30**</td>
<td>61.92±6.16***</td>
<td>9.63±1.02*</td>
<td>171.22±22.03**</td>
</tr>
<tr>
<td>Omeprazole (10 mg/kg, p.o.)</td>
<td>28.43±1.94***</td>
<td>42.63±6.17***</td>
<td>7.95±1.10**</td>
<td>197.20±15.75**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E.M with (n = 6) per group. *P values: *<0.05; ** <0.01; *** <0.001 compared with the control (ANOVA test).
Ulcers formed, in PL model, are due to increased accumulation of acid and pepsin in the stomach. Similarly, the activation of the vagus-vagal reflux by stimulation of pressure receptors in the antral gastric mucosa in the hypersecretion model of pylorus ligation is believed to increase gastric acid secretion. The data from the current experiment clearly showed that KV significantly reduced the gastric acid (both free and total acidity) and pepsin secretion. The gastric mucosal barrier (including mucus), has been suggested to play an important role in protecting gastric mucosa from destructive effects of intraluminal acid. Hence, the significant increase in the mucus secretion by pre-treatment with KV may be due to the stimulation of mucin secretion apart from the maintenance of mucus layer integrity. The cyto-protective and anti-secretory potentials of KV could be evidently considered. Ethanol-induced gastric lesions are thought to arise as a result of direct damage of gastric mucosal cells, resulting in the development of free radicals and hyperoxidation of lipid. Though, pre-treatments with kolaviron significantly inhibited gastric lesions produced by acidified ethanol but the present experiment examined the influence of ethanol alone apart from the endogenous gastric acid released. Also, it has been reported that an active agent that is highly effective in preventing ethanol-induced gastric lesions may possess cyto-protective activity. Hence, this may partially be one of the possible mechanisms by which KV have meliorated ulcers induced by ethanol. So, KV may be cyto-protective in action.

NSAIDs like aspirin induces ulcers due to their effect on cyclo-oxygenase enzyme, leading to reduced prostaglandin production and increase in acid secretion. Aspirin-induced ulcer is mediated through tissue damaging free radicals, which are produced from the conversion of hydroperoxyl to hydroxy fatty acids, which leads to cell destruction. Furthermore, KV has been reported to elicit strong antioxidant activity in both in vivo and in vitro experimental models. Scavenging potentials of KV may not be ruled out in the mechanism of its anti-ulcer properties. Also, polyphenol extracts containing flavonoids are widely employed in peptic ulcer therapy, as food additives and nutritional supplements, mainly because of their strong inhibition of prostaglandin (PG) metabolism and vaso-constrictive leukotriene inhibition. So, KV significantly reduced the incidence of ulcer formation which further supports its cyto-protective potential which may be mediated by prostaglandins.

In the gastric parietal cells, the enzyme H+, K+-ATPase is known to transports the H+ against a concentration gradient. Also, proton pump is the common and final pathway of all stimulation of acid production. Hence, its inhibitor will be a potent anti-secretory agent. The data that emanated from this research work showed that KV inhibited proton pump activities which were comparable with standard proton pump inhibitor, omeprazole. This evidence suggests the interaction of KV with H+, K+-ATPase indicating the proton pump inhibitory potential of KV.

Conclusion
In conclusion, peptic ulcer formation is attributed to the imbalance appearing between aggressive factors e.g. acid and pepsin, and defensive factors e.g. mucosal resistance. The results have shown that KV possesses partial antisecretory and cytoprotective /mucoprotective potential. KV from *Garcinia kola* prevented acid secretion through proton pump inhibition and subsequently intensifying the defensive factors to a significant extent.

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