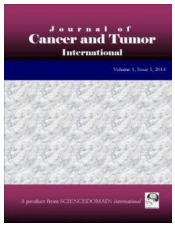

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Kolaviron an Active Biflavonoid of *Garcinia kola* Extract Prevent 1,2-dimethylhydrazine Induced Oxidative Stress and Lipid Peroxidation in the Initiation Phase of Colon Carcinogenesis in Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author ASE designed the study, wrote the protocol, author DE wrote the first draft of the manuscript. Authors LCC and AAU managed the literature searches, analyses of the study and performed the spectroscopy analysis and Author ASE also managed the experimental process and author DE also identified the species of plant. All authors read and approved the final manuscript.

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ABSTRACT

Colon cancer is steadily increasing in Africa with high mortality and it is a pathological consequence of persistent oxidative stress. Kolaviron an active biflavonoid, has been shown to possess antioxidant, anti-lipid peroxidation and chemopreventive properties. The present study was performed to evaluate the protective efficacy of Kolaviron against 1,2-dimethylhydrazine (DMH) induced oxidative stress and lipid peroxidation. Male wistar rats were divided into four groups. Group 1 served as control, animals have access to water and the rodent feeds for 8 weeks plus 1mM EDTA-saline injection subcutaneous (s.c) once a week for 4 weeks. Group 2 rats served

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as kolaviron (KV) control received 100 mg/kg bodyweight of kolaviron per oral (p.o.) every day. Group 3 served as carcinogen control, received pellet diet and 30 mg/kg bodyweight of 1,2-dimethylhydrazine (DMH) subcutaneous injection once a week for 4 weeks to induce colon carcinogenesis. Group 4 rats received DMH injection and kolaviron 100 mg/kg bodyweight. All rats were sacrifice at the end of 8 weeks (56 days) by cervical dislocation. Protective effects of kolaviron were assessed by using tissue lipid peroxidation (LPO) and antioxidant status as end point markers. Prophylactic treatment with kolaviron 100 mg/kg b.w significantly ameliorates DMH induced oxidative damage by diminishing the tissue LPO accompanied by increase antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST) and non-enzymatic antioxidants reduced glutathione (GSH) antioxidant status. The results revealed that supplementation with kolaviron significantly reduced the formation of ACF in DMH treated rats. The data of the present study suggest that kolaviron effectively suppressed DMH induced colonic carcinogenesis by ameliorating ACF multiplicity, oxidative stress and lipid peroxidation.

Keywords: Kolaviron; DMH; free radicals; ACF; carcinogenesis.

1. INTRODUCTION

It is estimated that cancers of the large and small intestine are major contributors to worldwide health hazard and its prevention is of great challenge in the modern medicine to conquer its morbidity and mortality [1]. Colon carcinogenesis is a multistep process and is thought to arise by the accretion of genetic alterations involving a variety of oncogenes and tumor suppressor genes that transform normal colonic epithelium into an invasive carcinoma [2]. Colon cancer is frequently a pathological consequence of persistent oxidative stress and inflammation [3]. Oxidative stress is a state which occurs when the balance between the productions of reactive oxygen species (ROS) overcomes the endogenous antioxidant defense system and inflammation is a complex biological response of tissues to pathogens and damaged cells [4].

1, 2-dimethylhydrazine (DMH) is a toxic environmental pro-carcinogen that is metabolically activated to the active carcinogen with selectivity for colon and can produce colon cancer in experimental models. Animal studies showed that experimental colonic tumors induced by DMH were closely parallel to the human colon carcinoma in terms of histology, morphology, anatomy of human colonic mucosa, microscopic pathology and immune-biology. This pro-carcinogen could thus provide an excellent experimental model to study the pathogenesis of colon cancer in humans [5].

Several epidemiological studies suggest that diet is considered as one of the major factor associated with increased risk for colon cancer incidence and mortality. Many experimental animal models have supported the idea that high fat diet augments the incidence of colon carcinogenesis whereas low fat and high fiber (present in fruits and vegetables) diet, decreases the risk of colon cancer. Many natural products present in the high fiber diets have been reported to possess chemopreventive properties against cancer. Therefore, chemoprevention is a logical and current strategy to reduce the mortality from colon cancer because numerous chemopreventive agents are present in the diet [6,7,8].

Bitter kola (*Garcinia kola*) belongs to the family of plants called Guttiferae and the genus *Garcinia*. The seed, commonly known, as 'bitter kola' is eaten by many and it is culturally acceptable in Nigeria. Extracts of the plant have been employed in the African herbalmedicine for the treatment of ailments such as laryngitis, liver diseases, cough and hoarseness of voice. *Garcinia kola* seeds have been shown to contain a complex mixture of polyphenolic compounds, biflavonoids, prenylated benzophenones and xanthones which account for the majority of its effects [9]. Kolaviron (KV) is a fraction of the defatted ethanol extract, containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone [10, 11]. A number of studies have confirmed the antioxidative and anti-inflammatory effects of kolaviron in chemically-induced toxicity, animal models of diseases and in cell culture [12,13,14]. Although the chemopreventive effect of kolaviron has been reported in aflatoxin B1-induced genotoxicity and hepatic oxidative damage and 2-acetylaminofluorene-induced hepatotoxicity and lipid peroxidation in animal models [15,16],

no study has addressed the effect of Kolaviron against1,2-dimethylhydrazine induced oxidative stress and lipid peroxidation in the colon of Wistar rats.

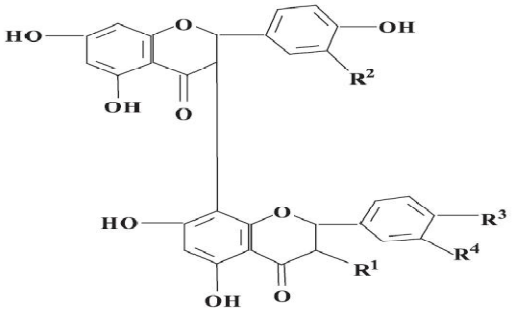
2. MATERIALS AND METHODS

2.1 Chemicals

DMH was purchased from Sigma Chemical Co. (USA). All other chemicals and reagents used were of analytical grade.

2.2 Extraction of Kolaviron

Garcinia kola seeds purchased from a local market in Yenagoa, Nigeria, were certified at the department of Botany, Niger Delta University, Nigeria. Peeled seeds were sliced, pulverized with an electric blender and dried at 40ºC in a drying oven. Powdered seeds were extracted with light petroleum ether (boiling point 40–60ºC) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6 x 300 mL). The concentrated ethylacetate yielded kolaviron as a golden yellow solid shown in Fig. 1 [17].



	R1	R2	R3	R4
GB 1	OH	H	OH	H
GB 2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Fig. 1. Structure of kolaviron

2.3 Animals

Three to four-weeks-old, male albino rats (120–150 g) of Wistar strain were obtained from Central Animal House of Niger Delta University, Bayelsa State, Nigeria. All procedures for using experimental animals were checked and permitted by the University Animal Ethical Committee, Bayelsa State, Nigeria. They were housed in aluminum cages in groups of 10 rats per cage and were kept in a room maintained at 25±2ºC with a 12 h light/dark cycle. They were allowed to acclimatize for 1 week before the experiments and were given free access to standard laboratory animal diet and water *ad libitum*.

2.4 Induction of Colon Carcinogenesis

DMH was dissolved in 1 mM EDTA just prior to use and the pH adjusted to 6.5 with 1 mMNaOH to ensure the stability of the carcinogen. The rats were given subcutaneous injections of DMH for 4consecutive weeks at a dose of 30 mg/kg body weight [18].

2.5 Experimental Design

Group 1 served as control, animals have access to water and the rodent feeds for 8 weeks plus 1mM EDTA-saline injection subcutaneous (s.c) once a week for 4 weeks. Group 2 rats served as kolaviron (KV) control received 100 mg/kg bodyweight of kolaviron per oral (p.o.) every day. Group 3 served as carcinogen control, received pellet diet and 30 mg/kg bodyweight of 1,2-dimethylhydrazine (DMH) subcutaneous injection once a week for 4 weeks to induce colon carcinogenesis. Group 4 rats received DMH injection and kolaviron 100 mg/kg bodyweight. At the end of 56 days (8 weeks) rats were sacrifice by cervical dislocation after an overnight fasting. The body weight and growth rate were determined.

2.6 Determination of Aberrant Crypt Foci

The detached colons of five rats were washed thoroughly with 0.9% NaCl, opened longitudinally from caecum to anus and fixed flat between two pieces of filter paper. Microscopic slides were placed on top of the filter paper to ensure that the tissue remained flat during fixation. After 24 h in buffered formalin, the colon was stained with 0.2% methylene blue as described by Bird and Good [19]. It was then placed mucosal side up, on a microscopic slide and observed under a light microscope. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from laminae to basal surface of cells, and the easily discernible pericryptal zone. Crypt multiplicity was determined as the number of

crypts in each focus, and was categorized as containing 1, 2, 3, 4 or more aberrant crypts/focus. For topographical assessment of the colon mucosal ACF was counted using a light microscope.

2.7 Post-mitochondrial Supernatant (PMS) Preparation and Estimation of Different Parameters

Colons were removed quickly, cleaned free of irrelevant material and immediately perfused with ice-cold saline (0.85% sodium chloride). The colons (10% w/v) were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjen homogenizer. The homogenate were centrifuged at 3000 rpm for 10 min at 4ºC by Eltek Refrigerated Centrifuge (Model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12,000 rpm for 20 min at 4ºC to obtain PMS, which was used as a source of various enzymes.

2.8 Determination of Protein

The protein concentration in all samples was determined by the method of Lowry et al. [20] using BSA as standard.

2.9 Determination of Reduced Glutathione (GSH)

The GSH content in colon was determined by the method of Jollow et al. [21] in which 1.0 ml of PMS fraction was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4ºC for at least 1 h and then subjected to centrifugation at 1200 x g for 15 min at 4ºC. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1 M, pH 7.4) and 0.4 ml DTNB (10 mM) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on spectrophotometer. The GSH content was calculated as nmol of DTNB conjugate formed/mg protein using molar extinction coefficient of 13.6 x 10^3 M^{-1} cm^{-1}.

2.10 Determination of Glutathione Peroxidase (GPx)

The GPx activity was measured spectrophotometrically [22]. The reaction mixture consisted of 50 mM potassium-phosphate buffer (pH 7.0) containing 1 mM EDTA, 1.125 M NaN_3, 0.2 mM NADPH, 0.3 mM GSH, 12 mM cumene hydroperoxide and an appropriate amount of the cytosol sample in a total volume of 1.0 ml. The reaction was started by adding NADPH. The change in absorbance of system at 340 nm was monitored. One unit of enzyme activity is expressed as nmoles NADPH consumed/ min/mg protein related to an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

2.11 Determination of Malondialdehyde (MDA)

The assay for membrane lipid peroxidation was done by the method of Wright et al. [23] with some modifications. The reaction mixture in a total volume of 3.0 ml contained 1.0 ml tissue homogenate, 1.0 ml of TCA (10%), and 1.0 ml TBA (0.67%). All the test tubes were placed in a boiling water bath for a period of 45 min. The tubes were shifted to ice bath and then centrifuged at 2500 x g for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmolMDA formed/mg protein by using a molar extinction coefficient of 1.56 x10^5 M^{-1} cm^{-1}.

2.12 Determination of Glutathione-S-transferase (GST) Activity

The GST activity was measured by the method of Habig et al. [24]. The reaction mixture consisted of 2.4 ml phosphate buffer (0.1 M, pH 6.5), 0.2 ml reduced glutathione (1.0 mM), 0.2 ml CDNB (1.0 mM) and 0.2 ml of cytosolic fraction in a total volume of 3.0 ml. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol CDNB conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 x10^3 M^{-1} cm^{-1}.

2.13 Determination of Catalase Activity

The activity of catalase was assayed by the method described by Sinha [25]. The reaction was started by the addition of 0.4 mL of H_2O_2 to the reaction mixture containing 1 mL of phosphate buffer and 0.1 mL of enzyme solution. The reaction was stopped at 30 s by the addition of 2 mL dichromate acetic acid reagent. The tubes were kept in a boiling water bath for 10 min and cooled. The utilization of H_2O_2 by the enzyme was read at 620 nm. Values are

expressed in micromoles of H_2O_2 utilized per minute per milligram protein.

2.14 Determination of Superoxide Dismutase (SOD) Activity

The SOD activity was measured by the method of Marklund et al. [26] The reaction mixture consisted of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), pyrogallol (24 mM in 10 mMHCl) and 100 µL PMS in a total volume of 3 ml. The enzyme activity was measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

2.15 Statistical Analysis

Results are expressed as mean±SD and all statistical comparisons were made by means of one-way ANOVA test followed by Turkey's post hoc analysis and p-values less than or equal to 0.05 were considered significant.

3. RESULTS

3.1 General Observations

All the rats in the experimental groups tolerated subcutaneous injections of DMH as well as kolaviron feeding. Normal animal behavior, improved body weight gain and absence of mortality in kolaviron treated rats emphasize the safety of kolaviron at 100 mg/kg b.w. Effect of DMH and kolaviron on change in body weight and growth rate of control and experimental animals are shown in Table 1. Body weight of the animals in all the groups increased gradually during the 8 week experimental period. The growth rate of rats in DMH alone treated group was not significantly ($p>0.05$) lower than control rats. There was a significant ($p\leq0.05$) increase in the growth rate on kolaviron supplementation to DMH treated rats as compared to the DMH alone treated rats.

3.2 Effect of Kolaviron on ACF Formation

ACF analysis was carried out at the end of the experimental period. Effect of kolaviron and DMH on ACF and total ACF, are shown in Table 2. Control rats and kolaviron alone treated rats showed nil ACF. DMH treated rats alone show increase number of crypts and ACF. A statistically significant ($p\leq0.05$) reduction in aberrant crypts and total ACF was observed the group supplemented with kolaviron.

3.3 Effect of DMH and Kolavironon GSH and Glutathione Dependent Enzymes

The level of GSH and activities of GSH dependent enzymes such as GST and GPx were significantly decreased ($p\leq0.05$) in colon tissues

Table 1. Effect of kolaviron on DMH induce body weight gain and growth rate of experimental and control rats

Groups	Initial weight (g)	Final weight (g)	Weight gain (g)	Growth rate (g/day)
Control	127.80±13.31	182.2±11.34[a]	54.40±9.04[a]	0.97±0.16[a]
KV control	146.40±18.90	193.0±14.40[a]	46.60±15.26[a]	0.83±0.27[a]
DMH control	136.6±12.66	176.6±8.14[b]	40.00±5.61[b]	0.73±0.12[a]
DMH + KV	139.0±8.39	201.8±8.31[c]	62.80±15.42[c]	1.12±0.28[c]

Values are mean ±SD from 5 rats. Values not sharing a common superscript letter (a–c) in a column differ significantly at P ≤0.05

Table 2. Effect of kolaviron on DMH induce aberrant crypt foci in experimental and control rats

Groups	Aberrant crypt foci containing				
	1 crypt	2 crypt	3 crypt	>4 crypt	Total
Control	Nil	Nil	Nil	Nil	Nil
KV control	Nil	Nil	Nil	Nil	Nil
DMH control	22.33±4.04[b]	13.79±4.01[b]	7.33±2.52[b]	5.00±2.65[b]	47.67±1.97[b]
DMH+ KV	13.33±4.16[c]	6.77±1.53[c]	3.67±1.53[c]	2.67±1.58[c]	23.00±1.73[c]

Values are mean ±SD from 5 rats. Values not sharing a common superscript letter (a–c) in a column differ significantly at P≤0.05

of DMH treated rats as compared to the control results as shown in Table 3. Administration of kolaviron (100 mg/ kg body weight) to the experimental group of animals markedly (p≤0.05) increased the reduced glutathione level as well as glutathione dependent enzymes activities, as compared to rats treated alone with DMH.

3.4 Effect of DMH and Kolaviron on MDA, Catalase and Superoxide Dismutase

The level of MDA in colon of DMH treated rats increased significantly, but chemoprevention with kolaviron decreased the levels of MDA. The activities of catalase and superoxide dismutase were significantly decreased (p≤0.05) in colon tissues of DMH treated rats as compared to the control results as shown in Table 4. Prevention with kolaviron (100 mg/ kg body weight) to the experimental group of animals markedly (p≤0.05) elevated the activities of catalase and superoxide dismutase as compared to rats treated alone with DMH.

4. DISCUSSION

The decreased (p≤0.05) growth rate observed in DMH challenged rats may be due to the occurrence of tumours in the colonic tract. However, the elevated growth rate of kolaviron supplemented rats obviously shows its role as a chemopreventive agent. It is reported that colon cancer is often associated with an abdominal mass, weight loss, decreased appetite and blood in the stool [27]. Thus the body weight gain upon kolaviron administration, observed in our study, emphasizes its preventive potential against DMH induced colon cancer.

The earliest recognizable morphological biomarkers of colorectal carcinoma are the ACF. These are considered to be the useful biomarkers to assess the chemopreventive potential of natural products against colon carcinogenesis [28]. In this study, the inhibitory effects of kolaviron on the occurrence of ACF were observed during colorectal carcinogenesis. Larger ACF (four or more aberrant crypts per focus) are considered more likely to progress into tumors [29] and in our study, kolaviron treatment had a significant inverse influence on larger ACF formation in the colon. Significant reduction in the occurrence of ACF in DMH treated rats supplemented with kolaviron denotes that it has remarkable potential in suppressing the occurrence of pre-neoplastic changes and the formation and progression of preneoplasia to malignant neoplasia. This result is in line with the work of Ansil et al. [30] who also reported the chemopreventive effect of *Amorphophallus campanulatus* against aberrant crypt foci.

DMH treatment generates free radicals in colonic tissue and their level is controlled by antioxidants [31]. Elimination of free radicals in biological systems is achieved through enzymatic (GST and GPx) and non-enzymatic (GSH) antioxidants, which act as major defense systems against free radicals [32]. Low level of GSH, GST and GPx activity in the colon tissue promotes the growth of cancer and its infiltration into the surrounding tissues, which is important for invasion and metastasis [33]. Our study also demonstrated the decreased levels of colonic GSH, GST and GPx activity in rats treated alone with DMH. However the supplementation of kolaviron significantly (p≤0.05) elevated the GST and GPx activity and GSH levels and could be important in inhibiting the carcinogenic changes induced by DMH.

Results of the present investigation correlate with previous studies that the level of lipid peroxidation in colonic tissues of rats increases on DMH exposure [34]. DMH treated rats alone shows an increase in the level of MDA. In this study, kolaviron supplementation to DMH treated rats resulted in the decrease of colonic MDA levels. It clearly suggests that kolaviron can protect cells from loss of their oxidative capacity due to the administration of the pro-carcinogen DMH.

Table 3. Effect of kolaviron on DMH induce glutathione (nmol DTNB conjugated/mg protein) level, GPx (nmolNADPH consumed/min/mg protein) and GST (nmol CDNB conjugated/min/mg protein) activity of experimental and control rats

	Control	KV control	DMH control	DMH + KV
GSH	10.75±0.39[a]	11.87±0.36[a]	3.11±0.26[b]	8.99±0.82[c]
GPx	12.14±1.83[a]	12.56±2.35[a]	6.53±1.34[b]	12.41±2.75[c]
GST	14.27±0.97[a]	14.43±2.2[a]	9.75±0.90[b]	13.81±1.81[c]

Values are mean ±SD from 5 rats. Values not sharing a common superscript letter (a–d) in a row differ significantly at P ≤0.05

Table 4. Effect of kolaviron on DMH induce MDA (nmol MDA/mg protein) level, catalase (µmol H_2O_2 consumed/min/mg protein) and SOD (U/mg protein) activity in experimental and control rats

	Control	KV control	DMH control	DMH + KV
MDA	4.51±0.95[a]	5.04±0.78[a]	11.97±0.83[b]	5.38±1.44[c]
CAT	60.75±6.11[a]	56.90±3.72[a]	33.25±5.34[b]	60.20±4.82[c]
SOD	4.35±0.97[a]	5.33±0.94[a]	1.30±0.47[b]	3.96±0.83[c]

Values are mean ±SD from 5 rats. Values not sharing a common superscript letter (a–c) in a row differ significantly at P≤0.05

The activities of other antioxidant enzymes like SOD and catalase were also reduced by DMH treatment. Kolaviron co-treatment significantly enhanced the activities of all these antioxidant enzymes in colonic tissue. The results of the present study are in accordance of the previous findings which has been shown that the activities of these antioxidant enzymes decreased in colonic tissue after DMH treatment [35]. H_2O_2 content formed in the colonic tissue is associated with oxidative DNA damage and it may lead to or play a role in cancer development [36], due to the decreased activities of catalase.

5. CONCLUSION

In conclusion kolaviron might have practical applications as a chemopreventive agent; however, further studies are required before kolaviron can be claimed as a therapeutic agent against colon cancer.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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