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KOLAVIRON AN EXTRACT FROM GARCINIA KOLA REDUCES PLASMA OXIDATIVE STRESS AND PREVENTS 1,2-DIMETHYLHYDRAZINE-INDUCED ABERRANT CRYPT FOCI DEVELOPMENT IN RAT COLON CARCINOGENESIS

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Abstract
Colon cancer is one of the major causes of cancer mortality worldwide. Several biflavonoids with antioxidant properties have been reported for their chemopreventive properties. In this study, We have evaluated the chemopreventive efficacy of kolaviron on plasma lipid peroxidation, nitrite levels, glutathione and vitamin E, the colonic activity of beta-glucuronidase and beta-glucosidase activities, total number of aberrant crypt foci (ACF), in 1,2 dimethylhydrazine (DMH)-induced colon carcinogenesis using a rat model. Male albino wistar rats were randomly divided into four groups. Group 1 served as control, received 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 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 8 weeks, co-treatment with kolaviron markedly reduced the degree of ACF development and also lowered pro-oxidant markers in plasma and increased the antioxidant levels of GSH and Vit E. Results also showed the decreased activities of colonic beta-glucuronidase and beta-glucosidase activities in the chemopreventive groups of kolaviron. In conclusion, the results of this study suggested that kolaviron has a clear beneficial effect against chemically induced colonic pre-neoplastic progression in rats induced by DMH. Therefore it can serve as a chemopreventive.

Keywords: Antioxidants, Colon carcinogenesis. Aberrant crypt foci, Oxiative stress

INTRODUCTION

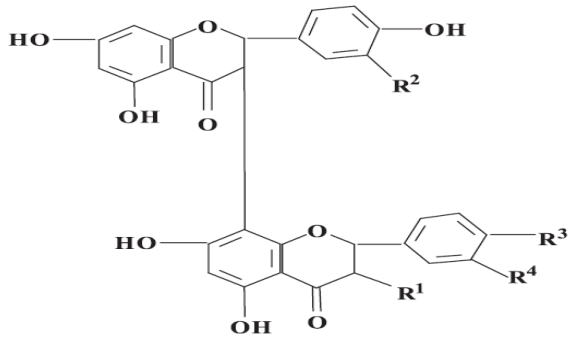
Colorectal cancer is one of the major causes of death in the world. It is the third most common form of malignancy in both men and women (Jemal *et a*l., 2010). Colon carcinogenesis is a multistep process and is thought to arise by the genetic alterations involving a variety of oncogenes and tumor suppressor genes that transform normal colonic epithelium into an invasive carcinoma (Janne and Mayer, 2000). Several environmental factors including lifestyle and perhaps hormones are similarly important in the induction and progression of colon cancer. Recent epidemiological data indicated an increased risk of colorectal cancer through consumption of red or processed meat but not white, whereas dietary fiber is protective (Norat *et al*., 2005).

Colonic crypt cell hyperproliferation has been suggested to play a significant role in the multistep process of colorectal cancer formation and development (Lipkin, 1988). Aberrant crypt foci (ACF) are generally accepted to represent precursor lesions in stepwise colon cancer development. ACF were also found in humans who underwent surgery for colorectal cancer (Newmark *et al*., 1991; Pretlow *et al*., 1991; Bird, 1986).

Dimethylhydrazine (DMH) is a potent carcinogen that has been reported to induce colon cancer in experimental animals. Humans are exposed to DMH and other hydrazines through **the** environment (Fiala *et al*., 1987). DMH is metabolized in the liver, resulting in the production of electrophilic diazonium ion, which is known to elicit oxidative stress. Experimental colon cancer induced by DMH in rats is a prolonged multistage process, bearing many of the same cell kinetics, histopathological and molecular characteristics of carcinogenesis that mimics human colon cancer (Halline *et al*., 1989).

Reactive oxygen species (ROS) are important inducers of both tissue injury and DNA damage. Free radical and non-radical oxidizing species are produced regularly in animals induced with carcinogens, and in human tissues. Accumulating evidence suggests that these free radical and electrophile mediated oxidative stress plays an important role in all stages of chemical carcinogenesis (Sun, 1990). Oxidative stress mediated lipid peroxidation (LPO) plays a significant part in carcinogenesis and is the most studied biologically relevant free radical chain reaction (Banakar *et al*., 2004 and Farombi *et al* 2009). Therefore phytochemicals that possess potent antioxidant properties should constitute effective chemopreventive agents.

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 (Iwu, 1982). Extracts of the plant are employed in African herbal medicine for the treatment of ailments such as laryngitis, liver diseases, cough and hoarseness of voice. (Farombi, 2003). Kolaviron (KV) is a fraction of the defatted ethanol extract of *Garcinia kola*, containing *Garcinia* biflavonoids GB1, GB2 and kolaflavanone (Iwu, 1982). 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 (Abarikwu, et al 2013, Adedara, *et al*., 2013 and Farombi *et al*., 2013). Although the chemopreventive effect of kolaviron has been reported in aflatoxin B1-induced genotoxicity and hepatic oxidative damage (Farombi *et al*., 2005), no study has addressed the effect of Kolaviron against 1,2-dimethylhydrazine induced oxidative stress and lipid peroxidation in the plasma of wistar rats.



	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 (Iwu, 1982)

Materials and methods

Animals

Four-week-old, male wistar rats were obtained from Pharmacology Animal House, Niger Delta University, quarantined for 1 week and allocated randomly to experimental and control groups. Animals were maintained as per the principles and guidelines of Public Health Service (PHS), 1996. The animals were housed ten per cage in a specific pathogen-free animal room under controlled conditions of a 12 h light/12 h dark cycle, with temperature of 22±1 °C and relative humidity of 50±10% till the end of 8 weeks.

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 40 ^{0}C in a drying oven. Powdered seeds were extracted with light n-hexane in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with methanol. The extract was concentrated with chloroform. The concentrated chloroform yielded kolaviron as a golden yellow solid shown in fig 1 (Iwu *et al*., 1990).

Chemicals

1,2-Dimethylhydrazine dihydrochloride was purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Experimental design

Male albino wistar rats were randomly 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 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 (Kanwar *et al*., 2008; Karthikkumar *et al*., 2012). Group 4 rats received DMH injection and kolaviron 100 mg/kg bodyweight.

Aberrant crypt foci determination

The experiment was terminated at the end of 8 weeks and all the animals were sacrificed by cervical dislocation after an overnight fast. The colons of six rats were split open longitudinally and aberrant crypt foci were counted. The colons were removed, flushed with saline, slit open longitudinally from the cecum to anus. For enumerating the ACF, tissues were fixed with 10% buffered formalin for 24 h and stained with 0.2% methylene blue as described by Bird and Good (2000).

The colonic mucosal scrapings were homogenized in phosphate buffer (0.1 M, pH 7.4), centrifuged at 12,000×g for 20 min at 4 °C and the supernatant was used for microbial b-glucuronidase and **b**-galactosidase assays.

Preparation of Plasma

After sacrificing the rats, the blood was collected in 5.0 mL heparinized tubes, and the plasma was separated by centrifugation at 800xg for 5 min at 4 ^{0}C. Plasma was stored for biochemical assays.

Determination of protein in colonic scraping

The protein concentration in all samples was determined by the method of Lowry *et al*., 1951 using BSA as standard.

ß-Glucuronidase activity determination in colonic scraping

ß-Glucuronidase activity was measured by the method described by Freeman (1986). Incubations were performed at 37 °C for 15 min in a water bath. The final concentrations in 1.0 ml of the incubation mixture included 200 µL, 0.01 M phosphate-buffer, pH 7.0; 200 µL, 0.1 mM EDTA, 200 µL, 1mM p-nitrophenyl-b-D-glucuronide; 200 µL, enzyme supernatant (colonic mucosal scraping) and 100 µL distilled water. The reaction was stopped with 4.0 ml of 0.2 M glycine buffer, pH 10.4, in 0.2 M sodium chloride and read at 540 nm. Values are expressed as mg of p-nitrophenol liberated/min/mg protein for mucosal tissue.

ß-Glucosidase activity determination in colonic scraping

b-Glucosidase activity was measured by the method of Freeman (1986). The colonic scraping samples were incubated with the substrate p-nitrophenyl-d-glucopyranoside in a shaking water

bath. The incubation mixture consisted of 200 µL, 0.01 M phosphate-buffer, pH 7.0; 200 µL, 0.1 mM EDTA, 200 µL, 1mM p-nitrophenyl-d-glucopyranoside; 200 µL enzyme supernatant (colonic mucosal scraping) and 100 µL distilled water, 0.2 M Na_2CO_3 solution was added to the reaction mixture. The released p-nitrophenol was measured at 450 nm. The amount of p-nitrophenol released was determined by comparison with a standard nitrophenol curve. Values are expressed as mg of p-nitrophenol liberated/min/mg protein

Nitrite estimation in plasma

Under physiological conditions, NO is readily oxidised to nitrite (NO_2^-) and nitrate (NO_3^-). The Griess reagent reacts with nitrites and nitrates that have been reduced to nitrites.

Nitrite assay was done using Griess reagent by the method of Green *et al*. (1982) with some modifications. In brief, 100 µL of Griess reagent (1:1 solution of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylene diaminedihydrochloride in water) was added to 100 µL of plasma sample incubate for 10 min at room temperature protected from light. Purple/magenta color began to form immediately. Absorbance was measured at 546 nm, nitrite concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were expressed as nmol/ml.

Determination of malondialdehyde (MDA)

The assay for plasma lipid peroxidation was done by the method of Wright *et al*., 1981 with some modifications. The reaction mixture in a total volume of 3.0 ml contained 1.0 ml plasma, 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 3000xg for 10 min. The amount of malondialdehyde (MDA) formed in each of the plasma samples were assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/ml by using a molar extinction coefficient of 1.56 $x10^5$ M^{-1} cm^{-1}.

Determination of reduced glutathione (GSH)

The GSH content in plasma was determined by the method of Jollow *et al*. (1974) in which 1.0 ml of plasma was mixed with 1.0 ml of sulphosalicylic acid (4%). The samples were incubated at 4 ^{0}C for at least 1 h and then subjected to centrifugation at 1200 x g for 15 min at 4 ^{0}C. 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 µmol of DTNB conjugate formed/ml using molar extinction coefficient of 13.6 x 10^3 M^{-1} cm^{-1}.

Determination of Vitamin E

Vitamin E was estimated by the method of Desai (1984). Briefly, 1.0ml of plasma was mixed thoroughly with 1.0ml of ethanol. Then 3.0 ml of petroleum ether was added, shaken rapidly and centrifuged. From this 2.0 ml of supernatant was evaporated to dryness and 0.2 ml of 0.2% bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric

chloride (0.001M) was added followed by 0.2 ml of *O*-phosphoric acid (0.001 M). The total volume was made up to 3.0 ml with ethanol and the colour developed was read at 530 nm. Vitamin E levels were expressed as mg/ml of plasma.

Results

ACFs were distinguished from normal crypts by darker staining and larger size, elliptical shape, thicker epithelial lining, and larger pericryptal zone. Table 1 shows the effect of kolaviron on ACF and the number of crypts/ ACF induced by DMH in rat colon. No incidence of ACF was found in control and kolaviron-treated rats. ACF developed in rats induced with DMH. However, the frequency of ACF per colon and the number of crypts per ACF were significantly lower in kolaviron-treated animals when compared with DMH induced rats.

Table 1 Effect of kolaviron on ACF and number of crypts/ACF induced by DMH in rat colon

			Number of crypts/ACF			
Groups	**Incidence**	**Total ACF**	**1 crypt**	**2 crypts**	**3 crypts**	**≥ 4 crypts**
Control	**0/6**	**0**	**0**	**0**	**0**	**0**
KV	**0/6**	**0**	**0**	**0**	**0**	**0**
DMH	**6/6**	**82.73±1.44[b]**	**22.33±2.37[b]**	**26.45±1.87[b]**	**18.76±1.39[b]**	**15.19±2.54[b]**
DMH+ KV	**6/6**	**62.73±2.19[c]**	**16.91±2.78[c]**	**19.09±1.29[c]**	**9.81 ± 2.67[c]**	**6.92 ± 0.63[c]**

DMH, dimethylhydrazine; ACF, aberrant crypt foci.
Values are given as mean ± SD for groups of six rats each. Values are statistically significant at $P < 0.05$. Values not sharing a common superscript letter (a–c) differ significantly.

Table 2 Effect of kolaviron on GSH, Vit E, MDA and nitrite levels in plasma of DMH induce control and experimental groups in rat

The effect of kolaviron on GSH, Vit E, MDA and nitrite levels were also determined the result on table 2 shows that both GSH and Vit E levels in plasma of DMH challenged rats decreased significantly. This decrease was modulated by the presence of kolaviron at 100 mg/kg bw in the plasma of DMH and KV treated rats significantly as compared with the DMH alone group. The levels of nitrite and MDA in the plasma of DMH treated rats increased significantly as compared with control plasma. The preventive properties of kolaviron also decreased the abnormal high levels of MDA and nitrite in plasma of DMH+KV group as compared to DMH group.

Groups	**GSH**	**Vit E**	**MDA**	**Nitrite**
Control	8.22 ± 0.52[a]	1.26 ± 0.92[a]	2.76 ± 0.56[a]	8.24 ± 1.43[a]
KV	8.24 ± 0.42[a]	1.28 ± 0.73[a]	2.89 ± 0.43[a]	7.44 ± 1.14[a]
DMH	2.18 ± 0.59[b]	0.24 ± 0.05[b]	10.88 ± 0.39[b]	21.67 ± 2.12[b]
DMH+KV	4.26 0.42[c]	1.18 ± 0.41[c]	4.26 ± 0.42[c]	10.67 ± 3.21[c]

GSH glutathione (µmol DTNB/ml)
Vit E (mg/ml)
MDA Malondialdehyde (nmol MDA/ml)
Nitrite (nmol/ml)

Values are mean ± SD for groups of six rats each. Values are statistically significant at $P < 0.05$. Values in a column not sharing a common superscript letter (a–c) differ significantly.

Table 3 Effect of kolaviron on the activities of bacterial b-glucuronidase and b-glucosidase in control and experimental groups in rat

The activities of bacterial b-glucuronidase and b-glucosidase of DMH treated rats increased significantly as compared with control colon. The preventive properties of kolaviron also decreased the abnormal high activities of b-glucuronidase and b-glucosidase in the DMH+KV group as compared to DMH group.

Groups	bacterial b-glucuronidase	bacterial b-glucosidase
Control	**7.65 ± 1.52[a]**	**12.76 ± 2.65[a]**
KV	**9.62 ± 2.79[a]**	**14.23 ± 1.55[a]**
DMH	**22.83 ± 2.96[b]**	**45.21 ± 2.87[b]**
DMH+KV	**11.58 ± 2.58[c]**	**10.82 ± 1.35[c]**

Bacterial b-glucuronidase mg p-nitrophenol liberated/min/mg protein

Bacterial b-glucosidase mg p-nitrophenol liberated/min/mg protein

Values are mean ± SD for groups of six rats each. Values are statistically significant at $P < 0.05$. Values in a column not sharing a common superscript letter (a–c) differ significantly.

Discussion

Oxidative damage to DNA and cellular macromolecules are considered as major events that stimulate the multistage process of cancer development (Norat *et al*., 2005). The use of antioxidants as chemopreventives of carcinogenesis has received much attention. In this study, I have evaluated the possible effects of kolaviron which dampen or modulate excessive ROS in the plasma.

It is recognized that colon carcinogenesis is a multistep process that includes sequential selection and propagation of pre-neoplastic lesions. ACF have been widely used as early markers of colon carcinogenesis in animal studies. ACFs are identified in carcinogen-treated rodent colons as well as in humans at high risk for colon cancer development and in patients with colon cancer. Many different ACF parameters have been measured, including the number of ACF, the number of aberrant crypts (AC) (Bird and Good 2000). This study shows that kolaviron suppresses ACF development which is a useful outcome for evaluating chemopreventive potential in colon carcinogenesis. Kolaviron administration suppressed not only the total number of ACF, but also affected the development of multi crypt foci containing 4 or more AC/ foci in the colon. These results are in line with the recent finding of Aranganathan *et al* (2009) who also reported the chemopreventive properties of hesperitin against DMH induce colon carcinogenesis.

Bacterial enzymes such as glucuronidase and glucosidase enzymes release toxic metabolites in the intestines from non-toxic glycoside conjugates and prolong the lifetime of toxicants in the body through enterohepatic circulation. b-Glucuronidase is responsible for the hydrolysis of conjugated glucuronides in the lumen of the gut (Gorbach, 1986). It may lead to the generation

of toxic and carcinogenic substances, which had been previously detoxified by phase 2 glucuronide conjugation in the liver and subsequently entered the colon via the blood or bile. The increased bacterial b-glucuronidase and b-glucosidase activity observed in this study could be attributed to the altered colonic microflora due to DMH injections. Previous works shows that the products of colonic bacterial metabolism are associated with harmful effects on the host and in particular, may lead to initiation and/or promotion of carcinogenesis (Reddy, 1992). Therefore the present study shows the preventive effects of kolaviron by decreasing the levels of these bacterial enzymes which may be due to elimination of DMH. The results are in line with the results of Karthikkumara *et al* (2012) and Sangeetha *et al* (2009)

Glutathione is a crucial component of the antioxidant defence mechanism and it functions as a direct scavenger of free radical (Romao *et al*., 2006). GSH and GSH dependent enzymes GPx, GST and GR predominantly participate in the detoxification of xenobiotics, carcinogens and free radicals by conjugating toxic substances with GSH, ultimately protecting mammalian cells and organs against carcinogen-induced toxicity and harmful effects. The decreased levels of these enzymes may signify oxidative stress (Gopalan *et al*., 1992). In the present study the levels of GSH decreased significantly in the plasma of DMH treated rats but preventing it with kolaviron increases the levels of this important antioxidant. These findings are in consonant with the recent findings of Sivagam *et al* (2012) who also reported protective effects of p-methoxycinnamic acid.

Lipid peroxidation is one of the important indicators of oxidative damage and high level of lipid peroxidation product (MDA) has been found after treatment with DMH (Sengottuvelan *et al*., 2006). Consistent with previous reports these results also showed remarkable increase in the level of MDA after DMH treatment. Kolaviron treatment significantly attenuated elevated levels of plasma MDA these results are in agreement with recently published report from Sreedharan *et al* (2009) who showed the protective effect of morin on tissue fluids.

Vitamin E is a fat-soluble vitamin, which functions as a free radical quencher and prevents lipid peroxidation of polyunsaturated fatty acids while vitamin C is a water-soluble antioxidant. Ascorbic acid plays a role in the regeneration of α-tocopherol by reducing the α-tocopherol radical at the surface of membranes and also scavenges reactive oxygen metabolites generated during the metabolism of carcinogen, thus protecting genetic material from getting transformed. The present study indicates that levels of plasma Vitamin E decreased significantly in DMH treated rats, while preventing with kolaviron increases these levels significantly in plasma. These findings are also in line with the works of (Sengottuvelan *et al*., 2006).

NO (nitric oxide) is another important mediator in the pathogenesis of inflammatory diseases which has a link in carcinogenesis (Rehman *et al*., 2011). Thus, we also evaluated the effect of kolaviron on DMH induced nitrite levels in plasma. DMH exposure elevated the level of NO significantly though measured indirectly as nitrite as compared with the control plasma values. However, co-treatment with kolaviron prevented DMH induced NO production in plasma. Increase levels of NO are involve in the signaling of colon carcinogenesis. Our results are in agreement with the research findings of (Oday *et al*., 2014) showing that *kolaviron* have preventive effects against plasma NO concentration.

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