Sub-acute effect of $N^G$-nitro-l-arginine methyl-ester (L-NAME) on biochemical indices in rats: Protective effects of Kolaviron and extract of Curcuma longa L

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ABSTRACT

Background: Kolaviron (KV) (biflavonoid from Garcinia kola) and extract of Curcuma longa (CL) are frequently used in folk medicine for treatment of hypertension. One of their mechanisms of action is to enhance antioxidant properties in animals. $N^G$-nitro-l-arginine methyl-ester (L-NAME) is L-arginine analogue, which by binding to Nitric Oxide Synthase (NOS) may induce hypertension partly due to increase in tissues oxidative stress. Objectives: To investigate the effect of L-NAME on some biochemical indices and the possible protective effect of KV or CL. Materials and Methods: Four groups consisting of 6 rats each were used. One group served as control, second group received L-NAME (40 mg/kg/day). Third and fourth groups were treated with KV and CL, respectively and also received L-NAME. KV and CL were given at a dose of 200 mg/kg/day. Results: L-NAME caused a significant ($P<0.05$) increase in the levels of serum urea, creatine kinase and alanine aminotransferase relative to controls. L-NAME treated rats had markedly decreased hepatic catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and reduced glutathione (GSH) levels. Precisely, L-NAME decreased CAT, SOD, GST and GSH by 48, 52, 76 and 40%, respectively. L-NAME intoxication significantly decreased ($P<0.05$) renal GSH and SOD levels. Also, L-NAME caused a significant ($P<0.05$) induction of lipid peroxidation (LPO) in the animals. Administration of KV or CL with L-NAME caused significant ($P<0.05$) inhibition of LPO and augments tissue antioxidant indices. Conclusion: These results confirm the adverse effect of L-NAME on biochemical indices and the ability of kolaviron or Curcuma longa to ameliorate the alterations.

Key words: Antioxidant, biochemical indices, Curcuma longa, NG-nitro-l-arginine methyl ester, kolaviron

INTRODUCTION

$N^G$-nitro-l-arginine methyl ester (L-NAME) is one of L-arginine analogues, which by binding competitively to NOS, has been shown to attenuate both production and metabolic pathway of NO. NO is known to mediate vasodilation, inhibit platelet aggregation, and prevent leukocyte adhesion to endothelial cells. One of the effects of NO on biochemical indices in relation to cardiovascular system is its action on ion channels. For instance, NO has been shown to inhibit Na⁺–K⁺ ATPase or Na⁺/K⁺ exchangers, both playing pivotal roles in tubular fluid and sodium reabsorption in renal tubular epithelial cells thereby regulating blood pressure. The critical feature of rat model lacking NO synthesis is persistent activation of the rennin-angiotensin system and regional inflammatory changes, such as monocyte chemo-attractant protein-1 expression, synthesis of growth factors in the endothelium and increase in neutrophil infiltration, which finally leads to myocardial remodeling (hypertrophy and fibrosis) and hypertension. On the other hand, it has been reported that reduced plasma NO level is linearly correlated with severity of hypertension. It is known that chronic inhibition of NOS by L-NAME can alter some biochemical indices. For example, L-NAME administration has been reported to cause a decrease of urinary sodium and potassium excretions along with a decrease of diuresis. However, the effect of L-NAME on biochemical indices of liver...
and kidney function tests as well as markers of oxidative stress still remains unknown.

Nowadays, medicinal plants are being used as an alternative therapy for treatment of several illnesses including hypertension.[9-12] A special attention is paid to the bioactive elements extracted from plants in order to discover new drugs toward the treatment of several pathologies.

Kolaviron (KV), the predominant constituent in *Garcinia kola* seed is a biflavonoid complex. KV has been reported to prevent hepatotoxicity mediated by several toxins.[13] KV is known to exhibit hypoglycemic effects in normal, alloxan and streptozotocin- diabetic animals.[14] Also, KV has been reported to elicit strong antioxidant activity in both *in vivo* and *in vitro* experimental models.[15] In a preliminary study, we demonstrated that KV elicited hypocholesterolemic effects and reduced the relative weight of heart in cholesterol fed animals.[10] Also, we have shown that the vasorelaxant effects of KV in smooth muscle is mediated by mechanism that involves extracellular Ca$^{2+}$ influx blockade, inhibition of intracellular Ca$^{2+}$ release and the opening of K$^+$ channels.[17]

*Curcuma longa* L. (CL) (Family: Zingiberaceae) is a perennial herb that grows predominantly in the tropical regions of Asia and Africa. CL has been used by traditional Medicine Practitioners to treat several ailments, such as cough, fever, liver and urinary diseases, inflammation, palpitation, eczema, itching, measles, chicken pox, vascular disorders and hypertension.[18] CL has been studied for its biological activities, such as; anti-microbial,[19] hepatoprotective,[20] hypoglycemic,[21] anti-ulcer,[22] neuroprotective,[23] anti-hemolytic[24] and anti-oxidant.[25] The vasorelaxant effect of CL in vascular smooth muscles has been reported by many authors.[26] From the aforementioned, both KV and CL elicit strong biological activities including antioxidant functions in animal model. Therefore, this study was designed to investigate the effect of L-NAME on some biochemical indices and, the possible protective effects of KV or CL during co-administration with L-NAME in Wistar rats.

**MATERIALS AND METHODS**

**Animals**

Inbred 8 to 9 weeks old male Wistar albino rats weighing 220 – 230 g were purchased from the Animal House of the Physiology Department, University of Ibadan, Nigeria. The animals were kept in well-ventilated cages at room temperature (28- 30°C). They were maintained on normal laboratory chow (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*. All animal experiments conform to the guidelines of National Institute of Health (NIH publication 85- 23, 1985) for laboratory animal care and use.

**Chemicals**

Thiobarbituric acid, Tris, bovine serum albumin, 5,5'-dithio-bis(2- nitrobenzoic acid) (DTNB), reduced GSH and 1- chloro- 2,4- dinitrobenzene (CDNB) were obtained from Sigma Chemical Company, St. Louis, USA. N$^6$- nitro- l- arginine methyl- ester (L-NAME) was purchased from BDH Chemicals Limited, Poole, Dorset, UK. Urea, creatinine, protein, alanine and aspartate aminotransferases kits were purchased from Randox chemical company, UK. Other reagents were of analytical grade and the purest quality available.

**Preparation of plant materials**

*Garcinia kola* seeds were obtained commercially in Ibadan, Nigeria and certified at the herbarium in the Department of Botany, University of Ibadan, Nigeria, where a voucher specimen already exists (UI- 00530). Three kilogram of peeled seeds was sliced and pulverized with an electric blender and air-dried in the laboratory (25 - 28°C). Extraction of KV was achieved by the method of Iwu *et al.* Briefly, powdered seeds were extracted with light petroleum ether (b.p. 40 to 60°C) in a soxhlet extractor. The defatted, dried marc was repacked and then extracted with methanol. 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 known as kolaviron (KV) with a percentage yield of 6%.

Fresh samples of CL (rhizome) were obtained from a local market in Ibadan, Nigeria. Their botanical identification and authentication were confirmed at the department of botany, University of Ibadan, Nigeria (The voucher specimen number is UI- 02577). The rhizome was sliced into pieces and air-dried at room temperature and then powdered. The powdered samples (1 kg) were de-fatted with n-hexane and extracted with methanol overnight in a soxhlet extractor. The methanolic extract of CL was concentrated and evaporated to dryness at 50°C with a rotary evaporator under reduced pressure. The yield of the preparation was 7.9%. Prior to the experiments, KV and methanolic extract of CL were dissolved in corn oil overnight and administered at a dose of 200 mg/kg body weight by oral gavage to the animals.

**Study design**

Twenty four male Wistar rats were randomly divided into four groups of six rats each and were given a period of two weeks for acclimatization before the commencement of the experiment. Group A received the drug vehicle (Corn oil) and served as control, while group B received L-NAME...
alone, group C was given L-NAME and KV, while group D received L-NAME and CL. Groups C and D were pre-treated with KV and CL, respectively, for 7 consecutive days before treatment with L-NAME. L-NAME was administered at a dose of 40 mg/kg/day,[27] while KV and CL were given at a dose of 200 mg/kg/day.[16,20] All drugs were given by oral gavages, 5 times in a week for a period of 3 weeks. The animals were fasted overnight and sacrificed by cervical decapitation under light ether anesthesia 24 h after the last dose of drugs. Blood samples were obtained directly from the heart of the animals. After dissection, liver, kidney and heart samples were obtained and processed to post mitochondrial fraction (PMF).

Preparation of post-mitochondrial fraction
Tissues (liver, kidney and heart) from the animals were quickly removed and washed in ice-cold 1.15% KCl solution, dried and weighed. The samples were homogenized in 4 volumes of isotonic phosphate buffer, pH 7.4 and then centrifuged at 10,000g for 20 min to obtain the post-mitochondrial supernatant fraction. All procedures were carried out at temperature 0-4°C.

Preparation of serum
Blood samples were collected from inferior vena cava of the heart into clean centrifuge tubes and allowed to stand for 1 h. Serum was prepared by centrifugation at 3,000g for 15 min in an MSC bench centrifuge.

Biochemical assays
Protein determination: Protein contents of serum and PMF were determined according to Lowry et al,[28] using bovine serum albumin (BSA) as a standard.

Alanine and Aspartate aminotransferases (ALT and AST) determination: Serum ALT and AST activities were determined using Randox kits. The kits used the combined methods of Mohun and Cook[29] and Reitman and Frankel.[30]

Creatine kinase, creatinine and urea determination:
Serum creatinine and urea levels were estimated using Randox kits, involving the methods of Jaffe[31] and Talke and Schubert,[32] respectively. Creatine kinase was measured in the presence of an antibody to CK-M monomer utilizing a kit purchased from United Diagnostics Industry (Riyadh, SA).

Glutathione S-transferase (GST) determination: The PMF GST level was determined spectrophotometrically at 37°C by the method of Habig et al.[33]

Reduced glutathione (GSH) determination: PMF GSH level was estimated as total non-protein sulphydryl group by the method of Moron et al.[34]

Superoxide dismutase and catalase determination: Superoxide dismutase activity (SOD) was measured by the nitro blue tetrazolium (NBT) reduction method of McCord and Fridovich.[35] Catalase (CAT) activity was assayed by measuring the rate of decomposition of hydrogen peroxide at 240 nm as described by Aebi.[36]

Lipid peroxidation (LPO) determination: LPO in the PMF and serum were assayed spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method, as described by Buege and Aust.[37]

Statistical analysis
All values were expressed as the mean ± S.D. (n = 6 in all the groups). Data were analyzed using one-way ANOVA followed by the post-hoc Duncan multiple range test for analysis of biochemical data using SPSS version 11 (SPSS Inc Chicago, Illinois). Values were considered statistically significant at P < 0.05.

RESULTS
In Table 1, administration of L-NAME for 3 consecutive weeks to rats caused a significant increase (P <0.05) in the levels of serum urea, alanine aminotransferase (ALT) and lipid peroxidation (LPO) when compared to controls.

Table 1: Effect of kolaviron (a biflavonoid complex from Garcinia kola seeds) and methanolic extract of Curcuma longa L on the levels of serum protein, lipid peroxidation and biochemical parameters of liver and kidney functions in L-NAME intoxicated rats

| Grouping | Protein (mg/ ml) | Urea (µmol/L) | Creatinine (mmol/L) | LPO (µmol/ mg protein) | ALT (U/L) | AST (U/L) | CK (U/L) |
|----------|-----------------|--------------|---------------------|------------------------|-----------|-----------|---------|
| Control  | 1.81 ± 0.5      | 1.05 ± 0.2   | 0.56 ± 0.2          | 4.7 ± 1.0              | 10.15 ± 1.4 | 42.5 ± 3.6 | 85.8 ± 9.6 |
| L-NAME   | 1.65 ± 0.4      | 2.49 ± 0.4*  | 0.61 ± 0.1          | 10.3 ± 2.1*            | 19.04 ± 2.1* | 44.3 ± 2.6 | 127.3 ± 10.4* |
| L-NAME + KV | 1.86 ± 0.5    | 1.13 ± 0.2   | 0.59 ± 0.2          | 5.8 ± 1.3              | 11.38 ± 1.5 | 40.3 ± 3.9 | 79.6 ± 9.1  |
| L-NAME + CL | 1.73 ± 0.3    | 1.23 ± 0.2   | 0.57 ± 0.1          | 6.5 ± 1.2              | 10.72 ± 1.7 | 43.0 ± 2.9 | 72.9 ± 8.4  |

Values are the means ± S.D. of six rats in each group. *Significantly different from the control (P < 0.05). L-NAME = N⁶- nitro- l- arginine methyl ester, KV= Kolaviron, CL= Curcuma longa L, LPO= Lipid peroxidation, ALT= Alanine aminotransferase, AST= Aspartate aminotransferase, CK= Creatine Kinase
Specifically, serum urea, ALT and LPO increased by 137, 88 and 118%, respectively, in L-NAME intoxicated rats. However, simultaneous treatment of rats with KV or CL attenuated the L-NAME- mediated increase in the levels of these biochemical parameters. Furthermore, L-NAME intoxication produced insignificant effect ($P > 0.05$) on the levels of serum protein, creatinine and aspartate aminotransferase in the rats. In addition, L-NAME intoxication caused a significant ($P < 0.05$) elevation in the activities of serum creatine kinase by 50% relative to the control, whereas supplementation with KV or CL significantly ($P < 0.05$) decreased the activities of creatine kinase relative to L-NAME alone [Table 1].

Figures 1 and 2 depict the effect of KV or CL on the levels of reduced glutathione (GSH) and glutathione-S-transferase (GST) in liver, kidney and heart of L-NAME intoxicated rats. Administration of L-NAME for 3 weeks significantly ($P < 0.05$) decreased the levels of hepatic GSH and GST as well as renal GSH of the animals relative to controls. Supplementation with KV or CL augmented the L-NAME induced decrease in hepatic and renal GSH and GST. However, CL failed to attenuate the observed decrease in the levels of renal GSH in L-NAME treated rats. Also, L-NAME produced no effect ($P > 0.05$) on the levels of cardiac GSH and GST in these animals when compared to the control. Figures 3 and 4 show that the L-NAME intoxication- caused marked decrease in the activities of hepatic catalase (CAT), superoxide dismutase (SOD) and renal SOD of the animals. Precisely, the activities of...
hepatic CAT, SOD and renal SOD were decreased by 48, 52 and 31%, respectively relative to controls. Simultaneous treatment of rats with L-NAME and KV or CL prevented the adverse effect of L-NAME on hepatic CAT, SOD and renal SOD. In addition, treatment with KV or CL restored the activities of these antioxidant enzymes to values that were statistically ($P > 0.05$) similar to the control.

Furthermore, L-NAME increased the levels of lipid peroxidation (LPO) products in the liver, kidney and heart of the animals [Figure 5] when compared to controls. Specifically, hepatic, renal and cardiac LPO were increased by 72, 87 and 101%, respectively, in L-NAME treated rats relative to the control. However, treatment with either KV or CL significantly ($P < 0.05$) ameliorated the increased serum creatine kinase and lipid peroxidation in tissues of L-NAME treated rats.

**DISCUSSION**

L-NAME can competitively bind NO synthase (NOS), which may impair metabolic pathway of NO and causes tissue injuries, especially cardiac remodelling, impairment of endothelial dependent relaxation and renal dysfunction. In addition to vascular endothelial dysfunction and activation of the renin–angiotensin system, oxidative stress appears to play a prominent role in L-NAME induced hypertension. In NO deficient hypertension induced by L-NAME, alteration in biochemical indices should therefore be expected. The present study, shows the ability of natural antioxidants (KV or CL) to prevent the L-NAME induced biochemical alterations in rats by activating other biochemical processes that may serve as alternatives to metabolic pathway of NO inhibited by L-NAME. The major findings of our study are that L-NAME intoxication increased the levels of serum biochemical parameters such as lipid peroxidation, urea, alanine aminotransferase and creatine kinase, and also decreased the hepatic levels of catalase, superoxide dismutase, reduced glutathione and glutathione-S-transferase in the rats. The present study provides evidence that the natural antioxidants, kolaviron (biflavonoid complex from *Garcinia kola*) and extract of *Curcuma longa* L are effective at reversing the early consequences of biochemical alterations in rats with NO deficiency.

Several investigators have proposed that oxidative stress contributes to the generation or maintenance of hypertension through inactivation of NO, inhibiting its vasodilator and natriuretic actions, and through non-enzymatic generation of vasoconstrictor isoprostanes from arachidonic acid peroxidation. The hypertension caused by NOS inhibition is thus associated with increased oxidative stress, and was confirmed in this study. Duarte et al. showed that chronic intake of oral quercetin (a flavonoid with antioxidant properties) has a protective effect in rats with hypertension from L-NAME, which further confirmed the role of oxidative stress in this model of hypertension. The kidney plays a central role in the regulation of body salt and water balance, and that any disorder in the regulation of renal functions could alter this balance in pathophysiological states including hypertension. In the present study, chronic inhibition of NOS by L-NAME caused an increase in serum urea level. Serum urea is a sensitive and reliable biochemical index for evaluation of renal function in animal model. The increased serum urea indicates impairment to the kidney function. Both extracts (KV and CL) normalize the serum urea levels of the treated rats. It may be suggested that both KV and CL offer protection against L-NAME induced renal dysfunction in these animals. These observations are consistent with the studies of Chidrawar et al. and Bhalodi et al. in which extracts of *Hemidesmus indicus* and *Benincasa cerifera* attenuate the serum urea levels of rats with cardiovascular disorder, respectively.

![Figure 5: Effect of kolaviron (flavonoid of *Garcinia kola*) and *Curcuma longa* L on the levels of lipid peroxidation (LPO) in L-NAME intoxicated rats. *Significantly different from others ($P < 0.05$); L-NAME= NG-nitro-L-arginine methyl ester, KV= Kolaviron, CL= *Curcuma longa* L.](image-url)
protection against ischemic reperfusion injury in rat heart via reduction of cardiac infarct size, and amelioration of plasma levels of lactate dehydrogenase and CK.

A major outcome of the present study is the evidence provided that L-NAME intoxication caused a significant increase in various oxidative stress parameters in the blood, kidney, liver and heart of the animals. In the present study, L-NAME treated rats exhibited marked depletion of renal and hepatic reduced glutathione (GSH). Reduced GSH is an endogenous antioxidant that acts among the first line of defence against pro-oxidant status.[49] Decreased hepatic GSH contents result in increased susceptibility to injuries via induction of lipid peroxidation and TNF-α.[44] Furthermore, significant decreases in the activities of glutathione-S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) were observed in the liver and kidney of L-NAME treated rats. On the other hand, L-NAME intoxication increased the levels of lipid peroxidation products in the blood, kidney, liver and heart of the animals. Interestingly, treatment with KV or CL offered remarkable protection against oxidative stress induced by L-NAME by normalizing the values of biochemical markers of oxidative stress. Several lines of evidence previously reported support the superb cardiac, renal and hepatic protective capabilities of KV and CL.[16,17,26]

In conclusion, our results support potent tissues protective roles for KV and CL by reversing the altered biochemical indices observed during L-NAME administration in rats. The protection may be primarily attributed to the antioxidant effectiveness of these natural products, and suggests that pretreatment with KV or CL may contribute in developing novel strategies in prevention and treatment of the cardio toxic agents that are capable of inducing free radicals. Further studies are needed to determine whether the beneficial effect of KV or CL on biochemical indices can be extended to hemodynamic parameters in L-NAME treated rats.

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