

## PAPER

# Microscopic and biochemical changes on liver and kidney of Wistar rats on combination antiretroviral therapy: the impact of naringenin and quercetin

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## Abstract

Combination antiretroviral therapy (cART), which is a lifelong therapy for people living with human immunodeficiency virus, has been associated with nephrotoxicity and hepatotoxicity leading to its discontinuation. This study aimed at investigating the ameliorative potential of naringenin and quercetin on cART-induced hepatotoxicity and nephrotoxicity. Seventy male Wistar rats (225–260 g) were divided into seven groups as control, cART, naringenin, quercetin, dimethyl sulfoxide (DMSO), naringenin/cART (CN) and quercetin/cART (CQ). cART (24 mg/kg), naringenin (50 mg/kg) and quercetin (50 mg/kg) were dissolved in 1% v/v DMSO and administered orally for 56 days. Combination of cART and bioflavonoids had significant increase in superoxide dismutase ( $P < 0.05$ ), catalase ( $P < 0.01$ ), reduced glutathione ( $P < 0.001$ ) and decreased malondialdehyde ( $P < 0.001$ ) compared to cART only. Tumor necrosis factor Alpha (TNF $\alpha$ ) level increased significantly in cART and CQ ( $P < 0.01$ ) groups, while others showed no significant changes compared to control. TNF $\alpha$  also significantly decreased in CQ level compared to cART ( $P < 0.001$ ). In addition, significant increase in creatinine level in cART only indicated progressive renal toxicity. Also, progressive pathological changes including congested blood vessels and hepatocellular necrosis were found in the liver, while the kidney had glomerular atrophy, and tubular distortion in cART-only group. Control, naringenin- and quercetin-treated groups showed normal renal and hepatic cytoarchitecture. These findings elucidate that progressive renal and hepatic toxicity is associated with the continuous use of cART; however, a combination of quercetin and naringenin with cART showed possible potential of ameliorating the damages posed by cART.

**Key words:** cART; quercetin; naringenin; liver; kidney

## Introduction

The advent of combination antiretroviral therapy (cART), made up of two nucleos(t)ide reverse transcriptase inhibitors (NRTIs)

combined with either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (NNRTI) has been very instrumental to the well-being of human immunodeficiency virus (HIV)-infected patients. It has prolonged their lifespan and reduced

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susceptibility to opportunistic infections [1]. In spite of these benefits, cART has been associated with several adverse events including gonadotoxicity [2], neurotoxicity [3, 4], hepatotoxicity [5] and nephrotoxicity [6], which unfortunately has led to a third of the therapy discontinuation reported [7]. Another startling drawback of cART in the treatment of HIV-infected persons is that it is a lifelong therapy. Consequently, people living with HIV/acquired immunodeficiency syndrome (AIDS) (PLWHAs) suffer the drug's adverse effects for the rest of their lives. There are reports on nephrotoxicity associated with chronic use of tenofovir disoproxil fumarate (TDF), a nucleoside reverse transcriptase inhibitor [8, 9], likewise an irreversible renal failure due to an acute overdose of cART [10]. Similarly, hepatotoxicity has been reported in both experimental [11, 12] and clinical [13] applications of antiretrovirals. It is therefore important to understand the underpinning mechanisms triggered by cART to induce hepatotoxicity and nephrotoxicity in order to proffer possible remedies. Hence the search for alternatives that can significantly minimize these adverse effects is gathering great momentum.

Naringenin and quercetin are nutraceuticals that have taken the center stage and have been hypothesized as lead compounds that may play significant roles in the management of liver and kidney injuries [14], hence the use of these bioflavonoids in the present study.

Naringin is a flavanone-7-O-glycoside naturally found in grape fruits. In humans it is metabolized to the aglycone—naringenin by naringinase present in the gut [15]. It has been reported to display strong antioxidant and anti-inflammatory activity [15]. Likewise, quercetin a plant phenolic glycoside characterized as a flavonol is metabolized by the small intestine, liver, kidney and large intestine, and commonly found in fruits (especially apples) and vegetables including onions, kale, French beans, broccoli, lettuce and tomatoes [16]. It is reported to have strong antioxidant property due to the presence of two antioxidant pharmacophores within its molecule configuring it for free radical scavenging [17]. In light of this, the present study thrives on the hypothesis that naringenin and quercetin may mitigate the adverse effects of the long-term therapy of cART on the liver and kidney.

## Materials and Methods

### Animals

Seventy (70) adult male Wistar rats weighing between 225 and 260 grams were allowed to acclimatize for 3 weeks in the Animal House, Department of Anatomy, College of Medicine of the University of Lagos under standard animal housing condition of  $24 \pm 2^\circ\text{C}$  and 12/12-hour light/dark cycle. All the rats were housed in plastic cages (5 rats/cage) with soft wood shavings 30 cm long, 20 cm wide and 13 cm high employed as bedding in the cages. The rats had access to standard rat chow and water *ad libitum*.

All procedures were carried out in accordance with the principles of laboratory animal care of the National Medical Research Council and the Guide for the Care and Use of Laboratory Animals [18]. This research was approved by the Health Research Ethics Committee of the College of Medicine of the University of Lagos with protocol number CMUL/HREC/03/17/113.

### Chemicals, reagents and drugs

Quercetin (Cat No: Q4951-100G), naringenin (Cat No: W530098-500G) and dimethyl sulfoxide (DMSO) (Cat No: 317275-500ML)

were purchased from Sigma-Aldrich South Africa while cART, a combination of efavirenz 600 mg, lamivudine 300 mg and tenofovir disoproxil fumarate 300 mg, manufactured in India by Macleods Pharmaceuticals Limited was provided by the AIDS Prevention Initiative in Nigeria (APIN) center at Lagos University Teaching Hospital (LUTH).

### Experimental design

The animals were randomly divided into seven (7) groups of 10 each viz control group: 1 ml of distilled water, cART group: 24 mg/kg combination antiretroviral therapy (300 mg tenofovir, 300 mg lamivudine, 600 mg efavirenz) [19], DMSO group: 1% v/v dimethyl sulfoxide, N group: 50 mg/kg naringenin, which was dissolved in 1% v/v DMSO, Q group: 50 mg/kg quercetin, which was dissolved in 1% v/v DMSO, CN group: 24 mg/kg cART +50 mg/kg naringenin, CQ group: 24 mg/kg cART +50 mg/kg quercetin. All treatments were applied daily by oro-gastric gavage and at the end of 56 days all animals were euthanized with excess urethane 24 hours after the last treatment.

### Assessments of superoxide dismutase, catalase, reduced Glutathione, and tumor necrosis factor Alpha Analysis

For the preparation of oxidative stress markers and  $\text{TNF}\alpha$ , Using tissue homogenizer, 0.5 g of the liver was homogenized in 4.5 ml of 0.4 M sodium phosphate buffer (pH 7.0), centrifuged at 3500 rpm for 10 min and the supernatant removed for the assay. Oxidative stress markers including catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA) were determined by modified methods by Sinha, Kakkar et al., Moron et al., Niehaus and Samuelsson, respectively [20–23]. Rat  $\text{TNF}\alpha$  assay was done on liver homogenate using Elabscience ELISA kit with a catalog No: E-EL-R0019 in accordance with manufacturer's instructions.

### Kidney function tests

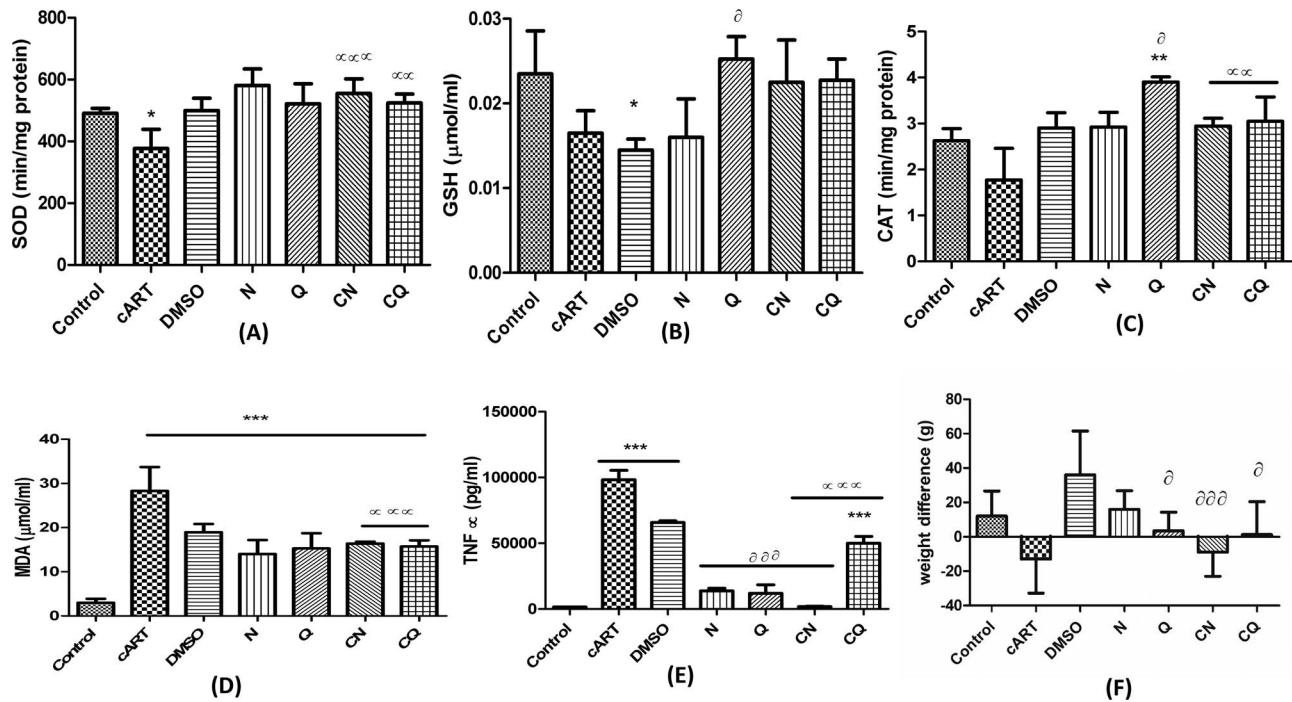
The blood sample was collected via cardiac puncture into lithium heparin bottles and centrifuged at 3000 rpm for 10 min at  $4^\circ\text{C}$  in a refrigerated centrifuge. The resulting supernatant (plasma) was decanted into Eppendorf tubes and prepared for biochemical analyses of blood urea and creatinine (CRT) were determined by the method reported in Pandya et al. [24].

### Mean weight difference

This was determined by subtracting the final body weight of each the animals at the end of the experiment from the initial body weights.

### Histomorphological studies

The tissue samples of liver and kidney were fixed in 10% neutral buffered formalin and processed for hematoxylin and eosin (H&E) via paraffin embedding after which they were sectioned at  $4 \mu\text{m}$  using a Thermo Scientific HM 325 rotatory microtome (CE Bright Company Ltd. Huntington England), cleared in xylene and hydrated in decreasing alcohols, stained with hematoxylin and eosin (H&E) and mounted with DPX [25]. The histological specimen was captured and examined using the Leica Slidepath Gateway LAN software.



**Figure 1:** Showing oxidative stress markers (A-D), tumor necrosis factor alpha (TNF $\alpha$ ) (E) and weight difference (F). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to control;  $\alpha P < 0.05$ ,  $\alpha\alpha P < 0.01$ ,  $\alpha\alpha\alpha P < 0.001$  compared to cART.  $\delta P < 0.05$ ,  $\delta\delta P < 0.01$ ,  $\delta\delta\delta P < 0.001$  compared to DMSO.

## Statistical analysis

The values for the results were expressed as mean  $\pm$  standard deviation (SD). Statistical level of significance was determined using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test to compare difference between groups. The mean difference was significant at 95% confidence interval ( $P < 0.05$ ). Graphpad prism 5 (Graphpad software) was used.

## Results

### Oxidative stress markers and TNF $\alpha$ assessment

Animals that received cART had significantly decreased ( $P < 0.05$ ) SOD level compared to control (Fig. 1). Animals that received a combination of cART and bioflavonoids (CN and CQ) had significant increase ( $P < 0.01$ ) in SOD and CAT compared to those that received cART only. The animals that received only quercetin had a significant increase in CAT compared to both DMSO ( $P < 0.05$ ) and control ( $P < 0.01$ ), while GSH level increased ( $P < 0.05$ ) compared to DMSO. Whereas, those that received only DMSO had significantly decreased ( $P < 0.05$ ) GSH compared to control (Fig. 1).

There was a significant increase ( $P < 0.001$ ) in MDA across the entire groups compared to control. There was also a concomitant decrease ( $P < 0.001$ ) in MDA level in animals that received a combination of both cART and bioflavonoids (CN and CQ) compared to cART-only group. TNF $\alpha$  increased significantly in cART, DMSO and CQ groups compared to control. Whereas, those that received a combination of cART and bioflavonoids (CN and CQ) had significantly decreased ( $P < 0.001$ ) TNF $\alpha$  levels compared to cART-only group. Animals in groups N, Q and CN also had significantly decreased ( $P < 0.001$ ) in TNF $\alpha$  levels compared to DMSO (Fig. 1).

### Kidney function tests

There was no statistically significant difference in urea level across the groups compared to the control group. Creatinine level was statistically significantly increased in cART ( $P < 0.01$ ) compared to control group. Combination of the individual bioflavonoid and cART (CN and CQ) showed a statistically significant decrease ( $P < 0.05$ ) in creatinine level when compared to cART-treated group (Fig. 2).

### Mean weight difference

There was no significant difference between the treated groups and control but there was marked decrease in body weights of animals that received cART and cART and naringenin in comparison with control. Those that received quercetin, cART combined with naringenin, and cART combined with quercetin had significantly decreased weights ( $P < 0.05$ , 0.001 and 0.05 respectively) compared with DMSO (Fig. 1F).

### Histomorphological changes on the liver

The histology of the liver for animals that received distilled water and those that received a combination of cART and naringenin (CN) showed normal cellular architecture of sinusoids (s), hepatocytes (H) and central vein (CV). On the contrary, animals that received cART, DMSO, N, Q and CQ showed different levels of hepatocellular necrosis including dilated sinusoids, congestion of blood vessels and presence of inflammatory cells. Groups treated with N and Q showed the least while those treated with cART presented the most severity of the hepatic cytoarchitecture (Table 1) (Fig. 3).

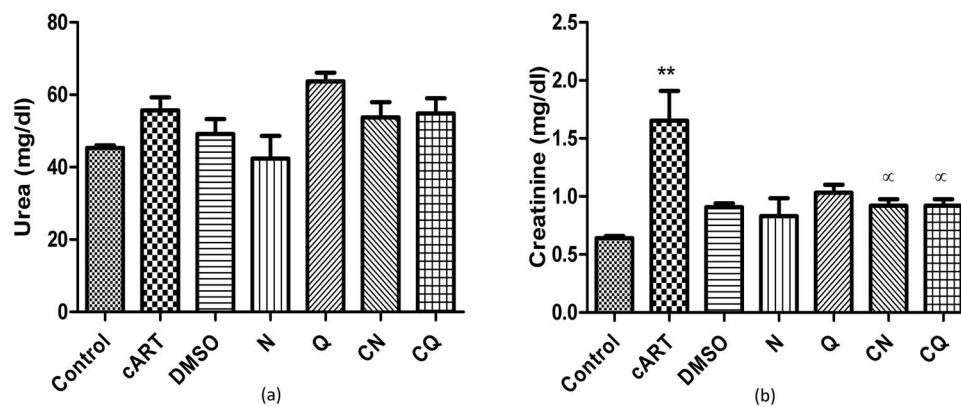


Figure 2: Showing kidney function tests. \*\*P < 0.01 compared to control;  $\alpha$ P < 0.05 compared to cART.

Table 1: Hepatic histopathological incidence and severity scores under light microscopy assessment (+: mild severity, ++: moderate severity, +++: high severity)

Groups	Dilated sinusoid	Damaged blood vessel wall	Inflammatory cells	Congestion of blood vessel
Control				+++
Naringenin	+		+	+
Quercetin				+
DMSO	++	++	+	+
cART	+++	++	+++	++
H/N	+		+	
H/Q		+		++

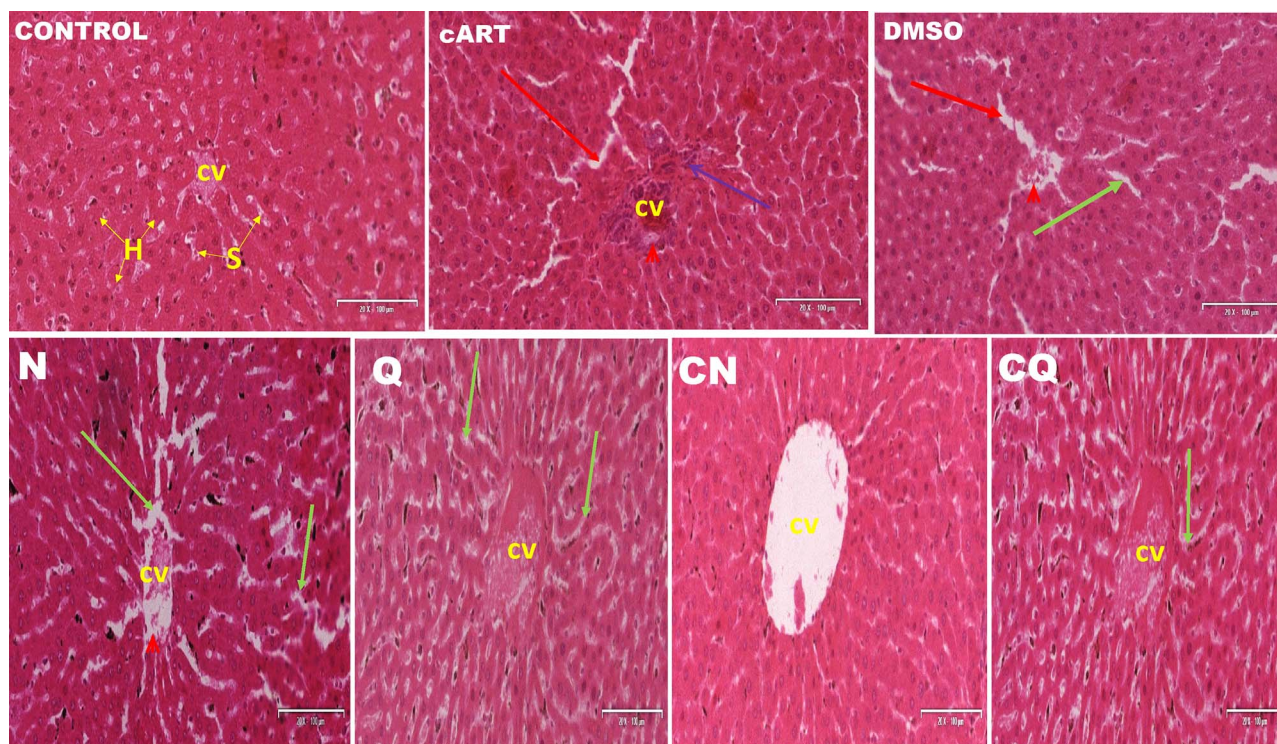
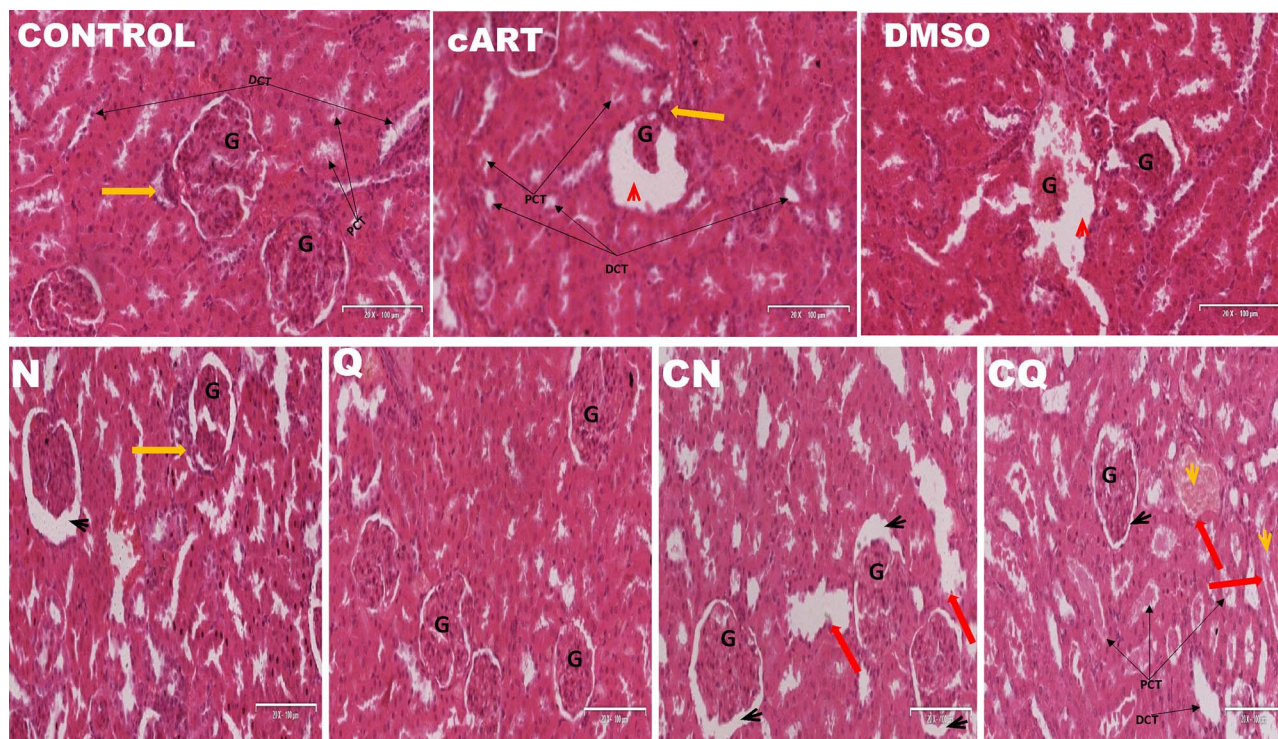


Figure 3: Photomicrograph of transverse section of liver (H&E 20x). CV: central vein, green arrow: dilated sinusoid, S: sinusoid, purple arrow: inflammatory cells, H: hepatocytes, red arrow: degenerating hepatocytes, red head: congested central vein.



**Figure 4:** Photomicrograph of the cytoarchitecture of the kidney (H&E 20x). Yellow arrow: vascular pole, G: glomerulus, red head: widened Bowman's capsule with degenerating wall and glomerulus, PCT: proximal convoluted tubule, DCT: distal convoluted tubule, red arrow: dilated renal tubule with flattened tubular cells, yellow head: intratubular cast formation black head: normal renal corpuscle.

**Table 2:** Nephritic pathological incidence and severity scores under light microscopy assessment (+: mild severity, ++: moderate severity, +++: high severity)

	Atrophied glomerulus	Damaged basement membrane	Dilated urinary space	Fluid filled space
<b>Control</b>				+++
<b>Naringenin</b>	+		+	
<b>Quercetin</b>			+	+
<b>DMSO</b>	+	+	++	
<b>cART</b>	++	++	+++	
<b>H/N</b>	+		+	
<b>H/Q</b>			+	++

### Histomorphological changes on the kidney

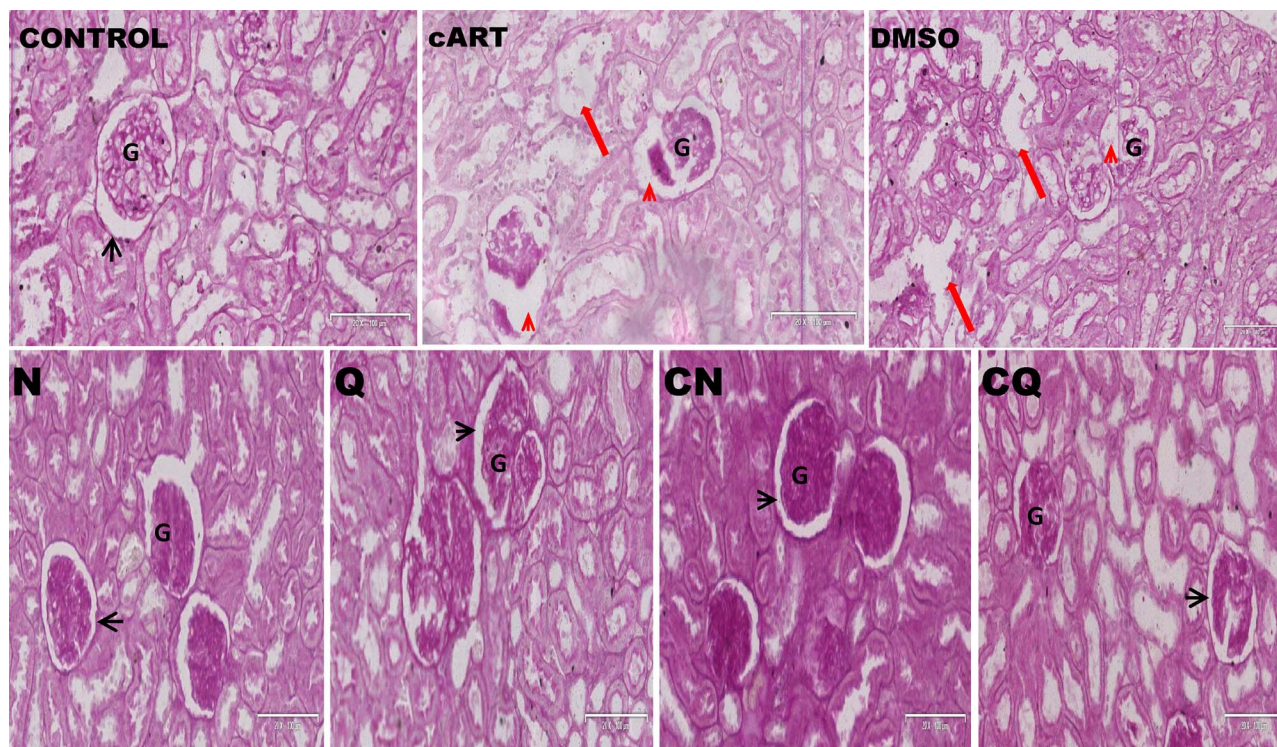
Normal renal glomerular and tubular morphology was observed in control and Q group. There was loss of brush borders in proximal convoluted tubules and macula densa of the DCT, congestion of blood vessels and excessive infiltration of mesangial cells in the Bowman's capsule of cART (Fig. 4). N, Q and CQ showed improved nephritic cytoarchitecture, while persistent mesangial cells, loss of macula densa and congestion of blood vessels persisted in DMSO and CN (Table 2) (Fig. 4).

Control group tested positive to PAS reagent. Well-alienated basement membrane and glomerular were observed. Loss of tubular cast depositions of glycogen and atrophied glomerulus expanding the urinary space occurred in cART. Extensive tubular cast, mineralization, alienated basement membrane and normal glomerulus were observed in CQ, CN, N and Q, while, damaged basement membrane and mild mineralization were observed in DMSO (Fig. 5).

### Discussion

The kidneys are known to be major target sites for drugs interaction (including antiretroviral drugs) due to metabolic detoxification and excretion of metabolite function [26–29]. Therefore, drugs capable of significantly impairing its functions are often considered to be less effective as continuous usage of these drugs could lead to various kidney diseases. Constituents of cART (Tenofovir, efavirenz and lamivudine) have been linked with kidney perturbations and liver damages [29] leading to debates on their removal from first-line [29] treatment and management of HIV.

Non-enzymatic and enzymatic antioxidant activity level has been documented to contribute effectively to normal functioning of the liver and kidney. Antioxidant activity levels from this study showed that the use of cART significantly increased lipid peroxidation level and altered both enzymatic (SOD) and non-enzymatic antioxidant (GSH) activity levels. Reduction in GSH and SOD levels in this study, which normally protect the cells



**Figure 5:** Photomicrograph of the histomorphology of the kidney (PAS 20x). G: glomerulus, red head: widened Bowman's capsule with degenerating wall and glomerulus, red arrow: dilated renal tubule with flattened tubular cells, black head: normal renal corpuscle.

against several ROS generated by converting hydrogen peroxide ( $H_2O_2$ ) to water, was insufficient to neutralize  $H_2O_2$  resulting in the increased lipid peroxidation observed. This correlates with reports that the use of cART has a detrimental effect on the antioxidant level of various organs [4, 30–32]. This mechanism of action might be the basis of major complications including liver damages and kidney perturbation. On the contrary, the use of **quercetin** and **naringenin** as adjuvants significantly improved the reduced enzymatic antioxidant level induced by cART activity. This improvement might be due to the potency of these antioxidants in inhibiting the functioning of ROS-forming enzymes (NADPH-oxidase and myeloperoxidase) and enhancing ROS scavengers through its ability to produce sufficient hydroxyl ( $-OH$ ) substitute [33]. This toxicity of cART is implicated in the weight loss observed. Antiretroviral drugs involving a combination with efavirenz have been reported to cause lipoatrophy on HIV-infected persons [34].

Incidence of oxidative stress and elevations in creatinine and urea level have been strongly linked with onset of renal pathology. Increase in creatinine level in cART is due to alterations to the macula densa of the kidney, impairing the contribution of the cells in the regulation of glomerular filtration, hence, encouraging retention of creatinine in the blood. This is in consonance with the report by Soto et al., that the use of cART relatively altered plasma creatinine levels. Progressive deteriorating kidney function test of cART-treated group can be due to drug-drug interaction among the constituents of cART [35].

Efavirenz has been discussed to be a potent inhibitor of organic cation transporters and toxin extrusion proteins transporters. This might be the mechanism behind it preventing the excretion of lamivudine, tenofovir and other toxins from the kidney, contributing to increased renal toxicity [35]. Improvement in the kidney function test especially in creatinine excretion by **quercetin** and **naringenin** is due to their ability

to enhance podocytes and macula densa functioning and possibly modulation of ionic gate channels responsible for excretion of toxins. Antioxidants, which are present in both bioflavonoids, have also been discussed to have effectively depressed  $Ca^{2+}$ -mediated priming and activation of NADPH oxidase enhancing renal excretion of toxins [36]. In addition, the hydroxy benzoic nature of these bioflavonoid similar to Egyptian Propolis, which have been reported to improve the digestive utilization of calcium, phosphorus and magnesium [37, 38], might have contributed to the functioning of the Bowman's capsule, absorption and re-absorption activities at the proximal and distal convoluted tubules through its interactions and effective utilization of calcium ions at the ion channels of the renal system in this study.

Tumor necrosis factor-alpha ( $TNF\alpha$ ) released by macrophages (Kupffer cells) has been named as one of the main regulators of the inflammatory process. Hence, therapeutic restraints of these pro-inflammatory cytokines enhance kidney, liver and cardiovascular health [39, 40].  $TNF\alpha$  assessment in the present study elucidated that cART enhances the release of pro-inflammatory cytokines by the Mesangial cells and Kupffer cells of the kidney and liver respectively. This is possibly due to its ability to stimulate increased ROS release, mitochondrial autophagic stress triggering inflammatory pathway and the release of pro-inflammatory cytokines. [7]. This will eventually enhance lipid peroxidation within the tissue, hence, lead to necrosis and alteration in cytoarchitecture of the organs [40, 41]. This hypothesis is in tandem with several studies that the use of cART leads to a marked increase in MDA level [4, 31, 42]. Flavonoid (quercetin and naringenin) metabolites have been reported to be more active than their precursor structures (naringin—its glycone precursor) through targeting of the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells ( $NF-\kappa B$ ) [39].

Metabolites of these bioflavonoids released to the kidney and liver might have accounted for the reduction in the release of pro-inflammatory cytokines (TNF $\alpha$ ) by their ability to suppress inflammatory processes.

Pro-inflammatory cytokines levels including TNF $\alpha$  regulate cellular reactions, apoptotic and necrotic cascade. Increase in this level triggers the onset of several pathological changes on the cytoarchitecture. Histomorphological findings of this study agreed with TNF $\alpha$  analysis. cART-treated groups showed progressive hepatocellular necrosis in the liver and tubular distortion, necrosis and glomerular atrophy in the kidney. This agrees with findings of Truter et al., that the use of cART containing both efavirenz and tenofovir triggered similar pathological changes in the kidney as observed in this study [43].

Alterations on the podocytes, brush borders, convoluted tubules of the kidney and the hepatocytes, sinusoids and blood vessels of the liver involved with the detoxification and excretion of toxins might have significantly contributed to the detrimental effects of cART on various biochemical analysis carried out in this study. Naringenin-treated groups showed similar pathological features as cART while quercetin-treated groups showed improved cytoarchitecture. Activities of quercetin on oxidative stress and pro-inflammatory cytokines have contributed to the improved well-being of the hepatic and nephritic cytoarchitecture involved with removal of toxins induced by the use of cART, hence, ameliorating the severity of pathological effects posed by the use of cART.

Similarly, PAS analysis demonstrated severe tubular vacuolization with glycogen retention and severe atrophy of the glomerulus in cART-treated group. This correlates with findings by Offor et al., that the use of highly active antiretroviral therapy containing lamivudine induces tubular vacuolization with high proportion of glycogen [44]; by Adjene et al., that chronic administration of efavirenz altered the microanatomy arrangement of the kidney [45] and by Elias et al., that the use of 600mg/kg of tenofovir for 2–8 weeks induced glomeruli damage and tubular necrosis [46]. On the contrary, the use of quercetin as an adjuvant showed improvement of the nephritic cytoarchitecture. However, the naringenin-treated group showed mild glycogen retention in the basement membrane of the kidney. The mechanism behind the hindrance to the antioxidant activities of these bioflavonoids on the cytoarchitecture of the kidney and liver is yet unknown. Although, the use of 1% v/v DMSO as a solvent as opposed to the use of distilled water in our previous study for dissolving the bioflavonoids (especially naringenin) [4] might have reduced optimal antioxidant functioning of naringenin in this study.

## Conclusion

Continuous use of cART containing efavirenz, lamivudine and tenofovir has the potential of inducing progressive hepatotoxicity and nephrotoxicity due to oxidative damages on liver and kidney respectively. However, its combination with bioflavonoids (quercetin or naringenin) inhibited oxidative stress and inflammatory process, hence, improving the cytoarchitecture and biochemical activities of the organs.

## Recommendation

The authors recommend that further analysis be carried out on ameliorative activities of naringenin and quercetin on liver and kidney upon longer administration periods.

## Conflict of interest

The authors declared no conflict of interest.

## Acknowledgement

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