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Impact of *Garcinia kola* and *Kigelia africana* Polyherbal Extract on Endothelial Dysfunction and Angiogenesis in Type-2 Diabetic Wistar rats: Regulation of Angiopoeitin-1 and Heme Oxygenase-1

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ABSTRACT

Diabetes mellitus, particularly type-2 diabetes is associated with complications such as increased gluconeogenesis, ketogenesis, advanced glycation end product formation (AGEs), and free radicals that promote the inflammatory process, thereby affecting vascular integrity that eventually leads to endothelial dysfunction. This study aimed to investigate the impact of *Garcinia kola* and *Kigelia africana* polyherbal extract on endothelial dysfunction and angiogenesis in Type-2 Diabetic Wistar rats.

Forty-two male Wistar rats were divided into six groups of seven rats each, namely; [Diabetic control (DMC)]; normal control [PC], extracts only (EC) [250 mg/kg], synthetic drugs only (SDM) [5 mg/kg glibenclamide + 100 mg/kg metformin], extract low dose (ELD) [250 mg/kg] and extract high dose (EHD) [500 mg/kg]. Diabetes was induced by intraperitoneal injection of 110 mg/kg nicotinamide (NA) followed by 65 mg/kg of streptozocin (STZ). The extracts were administered once daily for six weeks. After the treatment period, blood samples, mesenteric arteries, and kidney tissues were collected and used for biochemical and immunohistochemical analysis, including antioxidant activity, liver function [aspartate aminotransferase (AST) and alanine transaminase (ALT)], renal function (uric acid, urea, and creatinine), nitric oxide (NO), angiopoeitin-1, heme oxygenase-1 (HO-1), as well as indirect bilirubin assays using commercial assay kits following the manufacturers' protocols. Results showed that administration of polyherbal extract of *G. kola* and *K. africana* significantly increased antioxidant enzymes activities, angiopoeitin-1, NO, HO-1, and indirect bilirubin levels. These findings suggest that the polyherbal extract of *G. kola* and *K. africana* has the potential to ameliorates endothelia dysfunction in STZ-nicotinamide-induced diabetic Wistar rats.

Keywords: Diabetes, Angiopoeitin-1, Nitric Oxide (NO), Heme Oxygenase-1 (HO-1).

Introduction

Type 2 diabetes mellitus (DM) is an inflammation-driven metabolic disorder of heterogeneous origin, characterized by persistent hyperglycemia resulting from impaired insulin secretion, action, or both,[1] leading to the generation of free radicals. The generated free radicals exacerbate the disease progression due to the overactivity that overwhelms the endogenous antioxidant system. It has been reported that the free radicals generated undergo auto-oxidation with glucose, impairing metabolism and altering the oxidant/antioxidant balance in diabetic conditions.[2,3] Improved heme oxygenase-1 (HO-1) level protects tissue vasculature against oxidative stress through several mechanisms, including mitochondrial biogenesis.[4] Furthermore, angiopoietin-1 protects vascular tissue by preventing endothelial cell death.[5]

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Decoctions from various parts and whole plants of *Kigelia africana* and *Garcinia kola* have been effectively used for several ailments, including type-2 diabetes mellitus.[6,7] In our previous study, we reported the composition, synergistic effect, and beneficial effects of polyherbal extract of *G. kola* and *K. africana*.[8] Consequently, this present study was designed to investigate the possible ameliorative effect of the polyherbal extract of *G. kola* and *K. africana* on endothelial dysfunction in type-2 diabetic rats.

Materials and Methods

Plant collection and identification

Fresh stems of *Kigelia africana* were harvested in November 2018 from Afe Babalola University farm, while *Garcinia kola* seeds were purchased from King's market, Ado-Ekiti, Ekiti State. The plants were identified and authenticated in the Department of Plant Science, Ekiti State University, Ado-Ekiti, where voucher numbers UHAE2018074 for *Kigelia africana* and UHAE2018075 for *Garcinia kola* were assigned.

Extract preparation

The plant materials were air-dried under ambient temperature for 4 weeks. The dried plant materials were ground into fine powder. Equal amount (1 kg each) of powdered *Kigelia africana* stem and *Garcinia kola* were thoroughly homogenized and macerated in 2 L of distilled water at room temperature for 48 hours with occasional agitation to facilitate optimal extraction of phytoconstituents.[8]

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Animals
Forty-two (42) male Wistar rats were obtained from the animal house of Afe Babalola University, Ado-Ekiti, Nigeria. The rats were acclimatized to the laboratory conditions for one week. The animals were placed on standard rodent chow and water *ad libitum* throughout the experimental period.

Ethical approval
The study was approved by the Health Research Ethics Committee of Afe Babalola University, Ado-Ekiti, with approval number PHS/2023/2024-17. All procedures were conducted in accordance with established ethical standards.

Induction of type-2 diabetes
Type 2 diabetes mellitus was induced in the experimental animals following a previously described protocol. Briefly, nicotinamide (110 mg/kg body weight), dissolved in distilled water, was administered intraperitoneally. After a 15-minute interval, streptozotocin (65 mg/kg), freshly prepared in 0.1 M citrate buffer (pH 4.5), was also administered intraperitoneally to complete the induction process. Diabetes mellitus was confirmed by measuring fasting blood glucose levels using a OneTouch Ultra 2 glucometer. Rats exhibiting glucose concentrations ≥140 mg/dL were classified as diabetic.[8]

Animal grouping and extract administration
The Wistar rats were evenly divided into six groups of seven rats each, namely; Diabetic control (DMC), Normal control (PC), extracts only (EC) [250 mg/kg], Synthetid drug (SDM) [5 mg/kg Glibenclamide + 100 mg/kg Metformin], Extract low dose (ELD) [250 mg/kg] and Extract high dose (EHD) [500 mg/kg] groups.
The polyherbal extract was administered by oral gavage once daily for 6 weeks.

Animal sacrifice and collection of blood samples
After the treatment period, the animals were humanely sacrificed under ketamine anesthesia. The rats were anesthetized by intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Blood samples were withdrawn through cardiac puncture into heparinized tubes, and centrifuged at 3000 rpm for 15 minutes at room temperature. The plasma samples obtained were frozen until needed for biochemical assay.

Isolation of mesenteric arteries
The mesenteric arteries were detached from the stomach wall and introduce into a dissecting dish containing ice-cold homogenization buffer (50 mM Tris HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 250 mM sucrose and 10% glycerol). The fats were cautiously removed from the vessel under a microscope. The defatted arteries were transferred into a tube containing homogenization buffer and snap-frozen in liquid nitrogen. The arterial tissues were separated within 5 minutes of excision and homogenized in the homogenization buffer using a mechanical homogenizer. The resulting homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C to fractionate the sample into a cytosolic supernatant and a coarse particulate pellet.[9]

Kidney tissue homogenization
Kidney tissues were dissected, weighed, and rinsed three times with cold phosphate-buffered saline (PBS) to eliminate blood and surface contaminants. Tissues were subsequently transferred into bead lysis tubes pre-loaded with zirconium oxide beads. Homogenization was performed using a Bullet Blender (Next Advance Inc., USA) in radioimmunoprecipitation assay (RIPA) buffer (or an appropriate buffer based on the intended downstream assay) at a ratio of 2:1 (buffer volume to tissue weight). The samples were homogenized at 10,000 rpm for 4 minutes followed by an additional 3 minutes cycle at 10,000 rpm to ensure complete tissue disruption. Following homogenization, tubes were centrifuged at 15,000 rpm for 5 minutes at 4°C. Supernatants were collected and stored at –80°C until further biochemical analysis.[10]

Biochemical analysis
The activities of aspartate aminotransferase (AST) and alanine transaminase (ALT), as well as levels of uric acid, urea, and creatinine, were determined using commercial assay kits (Fortress Diagnostics Limited, United Kingdom) following standard enzymatic and colorimetric protocols as previously described.[11,12] Oxidative stress markers, including malondialdehyde (MDA), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and total protein were also quantified using spectrophotometric methods in accordance with established procedures.[13-15]

Determination of nitric oxide concentration
Nitric oxide (NO) level in the mesenteric artery homogenate was quantified using a colorimetric assay kit supplied by Oxford Biomedical Research Inc., USA.[16] The assay protocol was based on the Griess reaction, which detects NO indirectly by measuring its stable metabolic end-products: nitrate (NO_3^-) and nitrite (NO_2^-). Nitrate reductase was used to enzymatically convert nitrate to nitrite, followed by reaction with sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (Griess reagent). This yielded a chromophoric azo compound measurable at 540 nm using a microplate spectrophotometer. Total NO concentration was determined by comparison with a standard curve prepared from known concentrations of sodium nitrite.

Determination of angiopoietin-1 concentration
The plasma and homogenized supernatant of mesenteric arteries as described above were used for the determination of angiopoietin-1 using the enzyme-linked immunosorbent assay (ELISA) (ER0007) kits following the manufacturer's instructions.[17]

Determination of heme oxygenase-1 plasma concentration
Plasma samples were analyzed for heme oxygenase-1 (HO-1) concentration using a sandwich ELISA kit (Cat. No. ER1041; Oxford Biomedical Research Inc., USA) according to the manufacturer's instructions. The assay involves the binding of HO-1 to a pre-coated capture antibody, followed by detection with a biotin-conjugated antibody and HRP-streptavidin complex. Colorimetric detection was performed using TMB substrate, and absorbance was read at 450 nm. HO-1 concentration was calculated from a standard curve generated using known concentrations of rat HO-1 standard.[18]
Determination of indirect bilirubin plasma concentration
The plasma was used for the determination of indirect bilirubin. The assay kits used were products of Fortress Diagnostics UK, and standard procedures were followed.[19]

Statistical analysis
Graph Pad Prism 5 (Version 6.1; Graph pad Software Inc., San Diego, CA, USA) was used for data analysis. Data were presented as mean ± standard deviation (SD), n = 7. Differences between means were analyzed by One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Statistically significant difference was set at $P < 0.05$.

Results and Discussion

Effect of the polyherbal extract on oxidative stress marker and antioxidant enzymes
Oxidative stress marker and several antioxidant activities were assessed post-treatment. The results as presented in Figure 1A showed that malondialdehyde (MDA) levels increased significantly ($P < 0.05$) in the diabetic group compared to the other groups. There were significant ($P < 0.05$) increases in the levels of all the antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase) and **t**otal protein in the polyherbal extract treated groups compared to the diabetic control group.
Malondialdehyde (MDA), a product of lipid peroxidation that frequently increases in diabetes mellitus, was reduced on the administration of polyherbal extract of *G. kola* and *K. africana*. The increase in MDA level is usually accompanied by an increase in the concentration of free fatty acids (FFAs) in animals with diabetes compared to non-diabetic or diabetic-treated animals. The increased FFAs level in diabetic animals may be due to the decrease in plasma

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levels of insulin, which increases the action of fatty acyl coenzyme A oxidase, and subsequent initiation of beta-oxidation of fatty acids and lipid peroxidation.[3] This finding could be attributed to both monounsaturated and polyunsaturated fatty acids.[8]

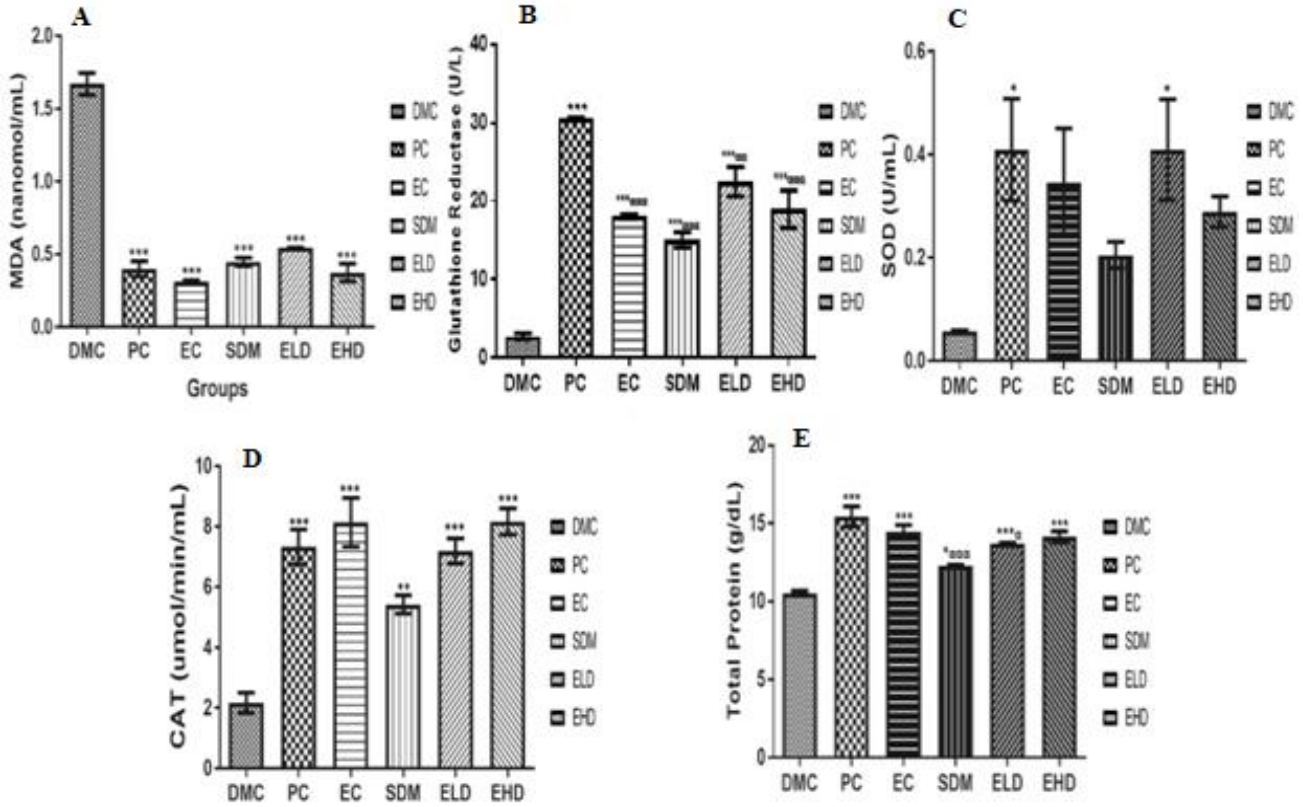

Figure 1: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Malondialdehyde (MDA) **(B)** Plasma glutathione reductase, **(C)** Super oxide dismutase (SOD), **(D)** Catalase (CAT) and **(E)** Total protein in diabetic rats.
$^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

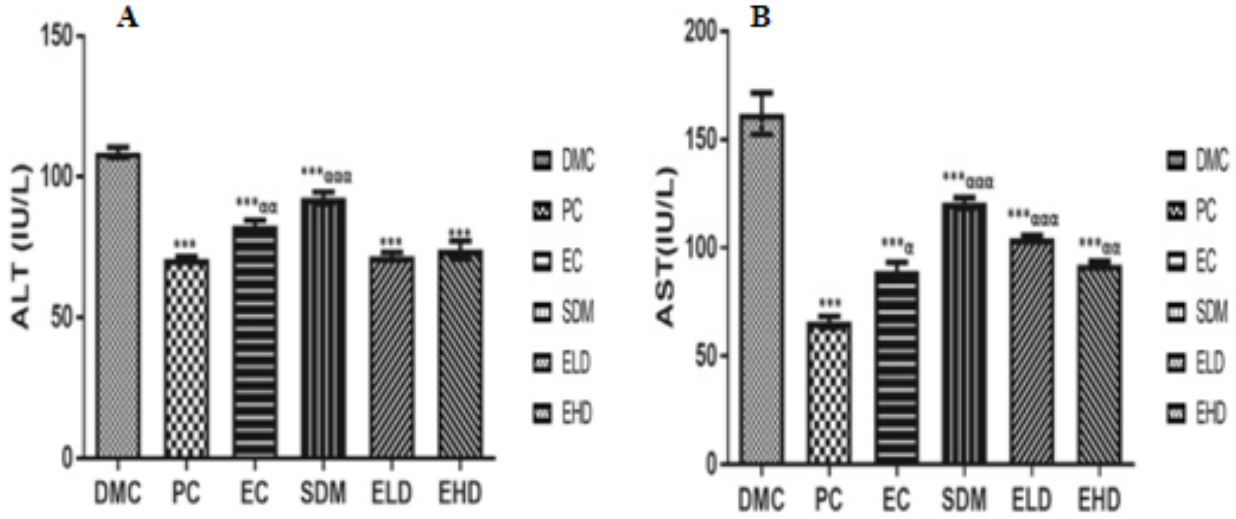

Figure 2: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Alanine aminotransferase (ALT) and **(B)** Aspartate aminotransferase (AST) in diabetic rats.
$^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

On the other hand, the significant increase in antioxidant enzymes in the polyherbal extract-treated group leads to reduced free radicals. Untreated hyperglycemia increases the generation of free radicals, leading to several diabetic associated complications.[20,21] The results obtained from this study have shown the potential of *G. kola* and *K. africana* polyherbal extract in treating diabetes and its complications. However, there are inconsistent reports on the antioxidant status in diabetic patients, with some studies documenting increased antioxidant enzymes activities, while others report decreased antioxidant enzymes levels. The findings from the present study

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reinforce the hypothesis that monounsaturated fatty acids, particularly oleic acid, exhibit resistance to lipid peroxidation and mitigate ROS-induced cellular damage. This was demonstrated by a significant improvement in antioxidant status, characterized by elevated levels of

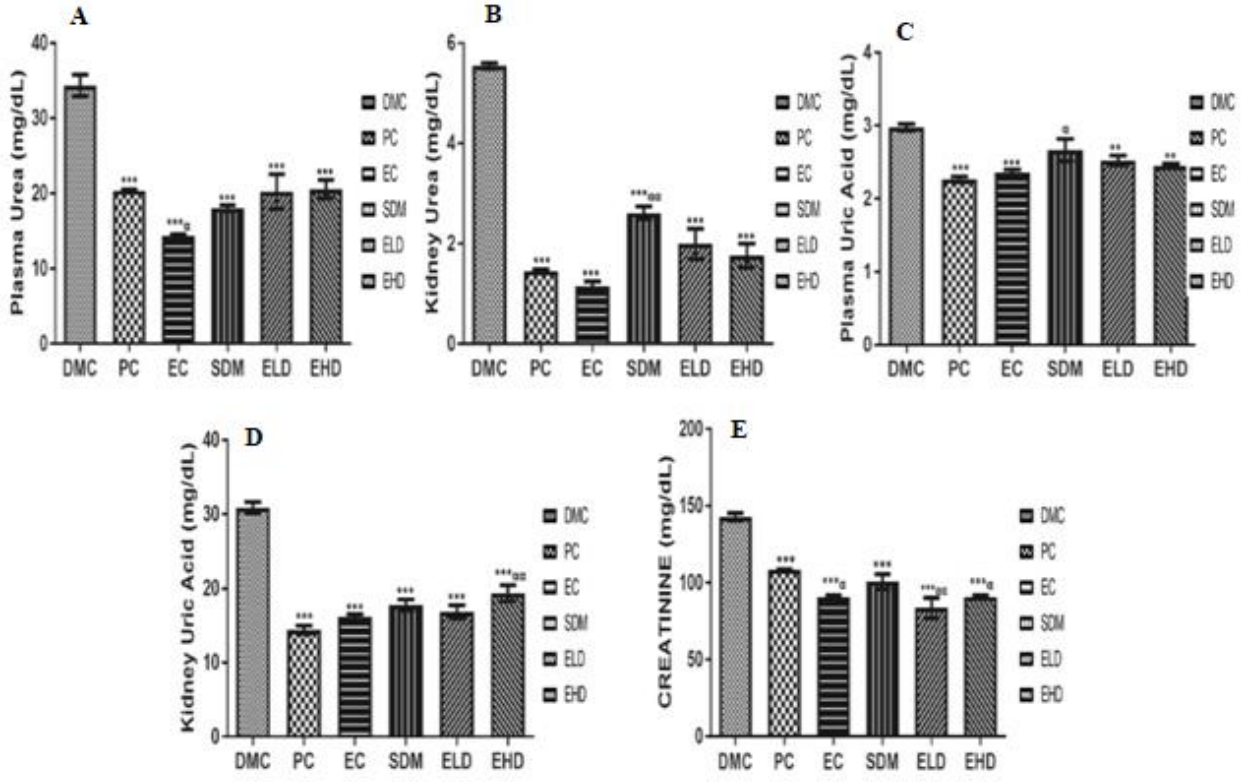

Figure 3: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Plasma urea, **(B)**Kidney urea, **(C)** Plasma uric acid **(D)** Kidney uric acid and **(E)** Creatinine in diabetic rats. $^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

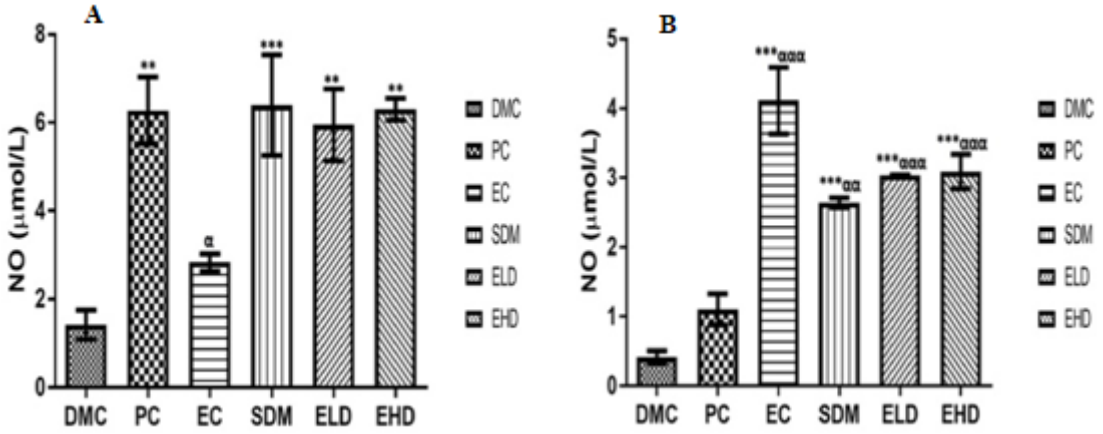

Figure 4: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Plasma nitric oxide (NO) and **(B)** Mesenteric artery homogenate nitric oxide (NO) in diabetic rats. $^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione reductase (Figure 1B – D), alongside reduced concentrations of oxidative stress markers like malondialdehyde (MDA). These results align with the results of our previous study, which reported that oleic acid identified in the polyherbal extract of *Garcinia kola* and *Kigelia Africana* contributed to decreased lipid peroxidation and enhanced redox homeostasis in diabetic rat models.[3,22,23] The decrease in total plasma proteins in the diabetic control group could be associated with the generated free radicals, which have been shown to have negative impact on plasma proteins in diabetic conditions due to oxidative stress.[24] Hence, the increase in total plasma proteins in the polyherbal extract-treated groups (Figure 1E) could be attributed to the extract's ability to inhibit free radicals.

Effect of the polyherbal extract on liver enzymes

In this study, plasma levels of liver enzymes including aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased in untreated diabetic rats, indicating diabetes-induced damage to the hepatocytes. Alanine amino transferase (ALT) is largely found in the liver, and its high level in the blood stream is a specific marker of liver dysfunction.[25,26] In contrast, aspartate amino transferase (AST) does not represent a highly specific biomarker of liver injury because its elevation can also be due to injuries to other

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tissues.[3] Treatment of the diabetic rats with the polyherbal extract of *G. kola* and *K. africana* resulted in a significant reduction in ALT and AST levels (Figure 2A-B), indicating an improved metabolic activities following treatment with the polyherbal extract.

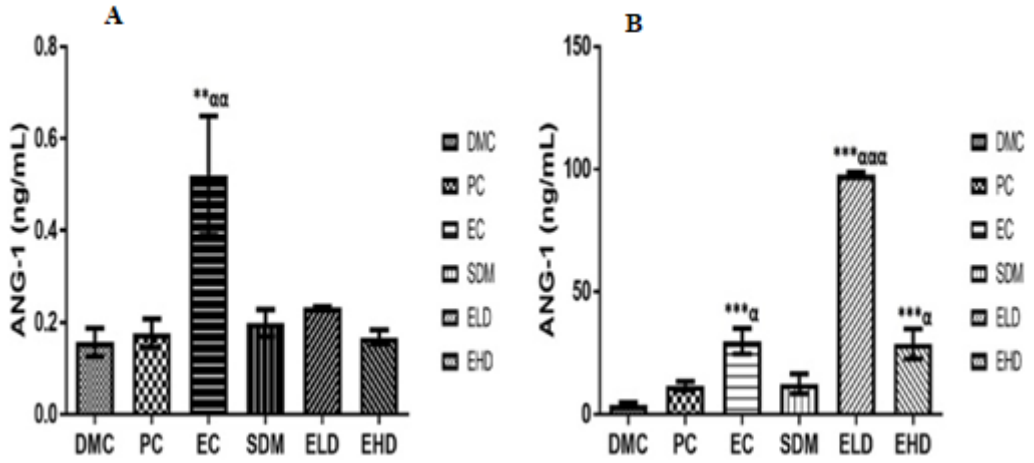

Figure 5: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Plasma angiopoeitin-1 and **(B)** Mesenteric artery homogenate angiopoeitin-1 in diabetic rats.
$^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

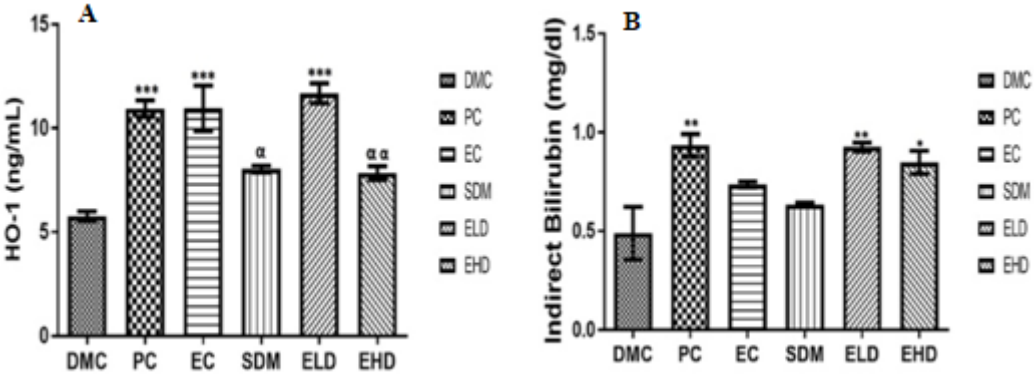

Figure 6: Effect of *Garcinia kola* and *Kigelia africana* polyherbal extract on **(A)** Plasma heme oxygenase-1(HO-1) and **(B)** Indirect bilirubin in diabetic rats.
$^{\alpha}p<0.05$, $^{\alpha\alpha}p<0.001$, ***p<0.0001

Effect of the extracts on uric acid, urea, and creatinine levels

The results as presented in Figure 3A-E showed significant increases in urea, uric acid and creatinine levels both in the plasma and kidney homogenate of the diabetic group when compared to the other groups ($p < 0.05$).

Uric acid, urea, and creatinine are consistent markers of renal function.[27] An increase in uric acid levels has a direct correlation with hyperinsulemia, insulin resistance, and diabetes.[28,29] Hyperuricemia causes diabetic complications, in particular those related to the renal and cardiovascular systems.[30] Hyperuricemia in diabetes is correlated with situations like insulin resistance and impaired glucose tolerance, followed by nephropathy. Uric acid reacts with free radicals to increase lipid oxidation and induce various effects in vascular smooth muscle cells.[31] High level of uric acid decreases the production of endothelial nitric oxide, leading to endothelial dysfunction and insulin resistance.[32,33] Therefore, the polyherbal extract-induced reduction in uric acid level is suggested to attenuate hyperinsulinemia, and improved renal function, which is consistent with report from a previous study.[34]

Urea, a terminal product of protein metabolism, reflects both dietary protein intake and the body's catabolic rate. Elevated serum urea levels are commonly associated with impaired renal clearance and may signal progressive kidney dysfunction.[35] In the present study, treatment with the polyherbal extract of *G. kola* and *K. africana* significantly attenuated urea concentrations in diabetic rats, suggesting a potential nephroprotective effect. Creatinine, a breakdown product of creatine phosphate in skeletal muscle, serves as a more sensitive and specific indicator of glomerular filtration rate (GFR) and overall kidney function.[36] Diabetic rats treated with the polyherbal extract exhibited reduced serum creatinine levels, indicating improved renal performance. These findings are consistent with previous reports, further supporting the renoprotective potential of *Garcinia kola* and *Kigelia africana* extracts.[36]

Effect of the polyherbal extract on nitric oxide level

Figure 4A-B shows the effect of *G. kola* and *K. africana* polyherbal extract on nitric oxide levels in the plasma and mesenteric arteries. The results revealed a significant ($p < 0.05$) decrease in plasma and mesenteric arteries nitric oxide (NO) levels in the diabetic rats compared to the control. However, treatment with *G. kola* and *K. africana* polyherbal extract restored plasma nitric oxide in diabetic rats to normal level, while resulting in a significantly ($P < 0.05$) higher nitric oxide concentration in the mesenteric arteries of treated animals compared to the control. This result is supported by the findings from the work of Perona *et al.* (2006)[37], which reported the positive effects of olive oil (contains majorly oleic acid) on endothelial nitric oxide synthase activity.

Effect of polyherbal extract on angiopoeitin-1 level

The administration of polyherbal extract of *G. kola* and *K. africana* produced beneficial effects on endothelial dysfunction resulting from type-2 diabetes by up-regulating angiopoeitin-1 levels in both plasma and mesenteric artery homogenate.

The results as presented in Figure 5A and 5B showed a significant ($p < 0.05$) increase in plasma and mesenteric artery angiopoietin-1 levels in the polyherbal extract treated groups when compared to the diabetic control group.

Vascular protection is dependent on several factors, including angiopoeitin-1 and heme oxygenase-1 expression to support angiogenesis and vasculogenesis, and there is a positive correlation between angiopoeitin-1 and hemeoxygenase-1 levels.[38] Angiopoietins are the angiopoietin isomers of the Tie signaling system with a twofold pathway. Angiopoeitin-2 is linked to inflammation and/or cancer in which endothelial cell integrity is compromised.[39] Diabetic rats treated with the polyherbal extract of *G. kola* and *K. africana* up-regulated angiopoeitin-1 levels in the mesenteric artery homogenate. Angiopoeitin-1 is known to exhibit powerful vascular protection by suppressing plasma leakage, restricting vascular inflammation, and barring endothelial cell death (apoptosis).[5] Although, there is no clarity on the possible mechanism of action of the polyherbal extracts of *G. kola* and *K. africana* on angiopoeitin-1, it action could be attributed to the oleic and linoleic acids contents. Oleic acid protects against cardiovascular insulin resistance, ameliorates endothelial dysfunction and inflammation, reduces the rise in apoptosis in vascular smooth muscle cells, and may contribute to the inhibition of the atherosclerotic process and vascular strength.[40] Linoleic acid on the other hand is known for its role in wound healing in diabetic rats.[41,42]

The endothelium is a monolayer of cells that line the vascular arrangement, segregating circulating blood in the lumen from the rest of the vessel wall and controls many key processes such as the formation of new blood vessels (angiogenesis) and inflammation.[43] The endothelium releases a variety of vasoactive substances such as prostacyclin (PGI2) and prostaglandins, endothelial-derived hyperpolarizing factor (EDHF), platelet-activating factor, endothelin-1, ROS, and NO that are involved in vessel relaxation, inhibition of platelet and leukocyte adhesion, proliferation and migration of vascular smooth muscle cells, maintenance of endothelial function, and prevention of endothelial dysfunction.[44]

Effects of the polyherbal extract on heme oxygenase-1 and indirect bilirubin levels

The effects of *G. kola* and *K. africana* polyherbal extract on heme oxygenase-1 and indirect bilirubin levels are shown in Figure 6A and 6B, respectively. The result showed that heme oxygenase-1 (HO-1) and indirect bilirubin were significantly decreased in the untreated diabetic rats compared to the control. However, treatment with *G. kola* and *K. africana* polyherbal extract restored plasma HO-1 and indirect bilirubin to levels comparable to that of the control, and significantly ($p < 0.05$) higher than that of the synthetic drugs group.

Heme oxygenase with two major types; the inducible and non-inducible (HO-1 and HO-2, respectively), act as a rate-limiting factor in the heme degradation to biliverdin, iron and carbon monoxide.[45] The generated biliverdin is quickly changed to bilirubin by biliverdin reductase, which has a positive effect on a number of biological functions. The correlation between HO-1 activity and diabetes have been well documented. HO-1 demonstrates an extensive action on the endothelial tissue, including improved vasodilation and amplification of endothelial progenitor cell numbers while reducing vasoconstriction and inflammation. Upregulation of HO-1 leads to increased production of bilirubin and carbon monoxide. Bilirubin acts as a potent antioxidant, while carbon monoxide exerts anti-apoptotic and anti-inflammatory effects, partly by modulating oxidative stress through enzymes such as superoxide dismutase and catalase.[45] Diabetic rats treated with the polyherbal extract of *G. kola* and *K. africana* significantly elevated HO-1 levels, demonstrating a protective function against oxidative stress. Heme oxygenase-1 breaks down heme into bilirubin and carbon monoxide, both of which exert antioxidant and cytoprotective effects that counteract the pathogenesis of diabetes.[46] The increased HO-1 levels make this pathway a significant adaptive mechanism for lowering the severity of vascular dysfunction and a potential therapeutic target.[46] Similar to the findings from this study, is the report that increased HO-1 expression attenuates the detrimental effects of diabetes complications.[47]

Bilirubin, a product of heme degradation, offers cytoprotective mechanisms against damage caused by generated radicals.[37] The treatment of diabetic rats with the polyherbal extract of *G. kola* and *K. africana* increased bilirubin levels, demonstrating an effective protective function against oxidative stress. Diabetes accompanied by a slight elevation of bilirubin level shows a lower predominance of vascular reactivities, thereby preventing oxidative stress and improving venous relaxation. In the present study, treatment of diabetic rats with *G. kola* and *K. africana* polyherbal extract resulted in an improved level of indirect bilirubin, which is in agreement with the study of Inoguchi *et al*. (2016)[48], which reported bilirubin as a key modulator of oxidative stress and systemic inflammation in diabetes. Bilirubin effectively protects lipids from oxidation and supports the bioavailability of nitric oxide, thereby contributing to improved endothelial function.[49,50]

Conclusion

The findings from this study showed that the polyherbal extract of *Garcinia kola* and *Kigelia africana* improved the antioxidant status of type-2 diabetic Wistar rats and attenuated the resulting endothelial dysfunction by upregulating angiopoietin-1 and heme-oxyenase-1 levels.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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