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Original Article

Antioxidant activity and protective effects of cocoa and kola nut mistletoe (*Globimetula cupulata*) against ischemia/reperfusion injury in Langendorff-perfused rat hearts



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ABSTRACT

Protection against cardiomyocyte damage following ischemia/reperfusion (I/R) injury is highly desirable in patients with ischemic heart disease. Hydromethanol extracts of *Globimetula cupulata* (mistletoe) growing on cocoa (CGCE) and kola nut (KGCE) trees were assessed for antioxidant content and cardioprotective potential against I/R. Graded concentrations (1–50 µg/mL) of CGCE or KGCE were tested on Langendorff-perfused rat hearts to evaluate the effects on the flow rate, heart rate, and force of cardiac contraction, while another set of hearts were subjected to biochemical analyses. Both extracts showed good antioxidant content and activity, but KGCE (EC₅₀: 24.8±1.8 µg/mL) showed higher hydroxyl radical scavenging activity than CGCE (70.2±4.5 µg/mL). Both extracts at 3 µg/mL reversed ($p < 0.001$) membrane peroxidation and the significant decrease in nitrite level, coronary flow rate, and superoxide dismutase and catalase activity caused by the I/R cycle. It is concluded that *G. cupulata* protects against ischemia–reperfusion injury in rat hearts via augmenting endogenous antioxidants and significant restoration of altered hemodynamic parameters.

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1.0. Introduction

Ischemic heart disease (IHD), which arises from a deficiency or lack of blood supply to cardiac myocytes due to the partial or

total obstruction of coronary arteries, is the major precursor of myocardial infarction (MI). MI, which has been described as the most lethal manifestation of cardiovascular disease, has been the object of intense investigation by clinicians and basic

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medical scientists [1]. Prevention of tissue damage in patients with IHD can be successfully accomplished only through reperfusion of the ischemic myocardium [2]. The process of reperfusion, however, is accompanied by the development of oxidative stress and the generation of free radicals, which play a major role in tissue damage arising from the ischemia/reperfusion (I/R) episode [1]. This very nature of I/R has raised speculations as to the possible therapeutic efficacy of antioxidants as antidotes to the voracious electron appetite and damaging effect of free radicals. Age-long safety concerns about synthetic antioxidants [3,4] have led to increased interest in those from natural sources [5]. Plants remain the reservoir of natural antioxidants, and their free-radical scavenging or antioxidant activity has been established in numerous studies [4,6,7]. Therefore, plant products with antioxidant activity would be good natural substances for protection against IHD.

Globimetula cupulata (a mistletoe) is a parasitic shrub belonging to the Loranthaceae family of plants and widespread in some tropical African countries, including Nigeria [8,9]. The plant grows on some dicotyledonous trees, like cocoa, coffee, and kola nut, and attaches itself to the host by modified roots. *G. cupulata* has popular ethnomedicinal use in the management of high blood pressure and diabetes mellitus [10]. Aqueous extract of the plant has been shown to possess hypoglycemic activity [11]. Methanolic extract of the plant was reported to contain steroids, terpenoids, cardiac glycosides, alkanoids, saponins, tannins, and flavonoids and showed the highest hydroxyl radical scavenging activity among 10 plants in one study [12].

Currently, there is paucity of data on the cardioprotective potential of cocoa and kola nut mistletoe leaf extracts. Furthermore, there is increasing awareness that medicinal plants might influence the course of heart disease and its treatment by providing an integrated structure of therapeutic substances which aid in restoring and maintaining a balanced system.

The present work has therefore been designed to evaluate the antioxidant activity of the hydro-alcoholic leaf extracts of cocoa and kola nut *G. cupulata* and to ascertain whether they can protect against heart ischemia–reperfusion episodes using the Langendorff heart preparation model.

2.0. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), malonaldehyde bis-(dimethyl acetal), epinephrine, and hydrogen peroxide were purchased from Sigma Chem., Co. (London, UK). All other chemicals were of analytical grade and were obtained from British Drug Houses (Poole, UK). The water used was glass-distilled.

2.2. Plant material

G. cupulata (mistletoe) plants growing on cocoa and kola nut trees were obtained from farmlands in Akure, Nigeria, in March 2013. Botanical identification and authentication were carried out at the herbarium of the Forestry Research Institute

of Nigeria, Ibadan, Nigeria. The leaves were stripped from the plants and air-dried. The dried leaves were then pulverized and stored in air-tight containers.

2.3. Extraction and fractionation

A 1 kg sample of the powdered cocoa or kola nut mistletoe was macerated in 2400 mL of a mixture of methanol and water (4:1) for 72 h [13]. This was filtered and concentrated to a small volume to remove all of the methanol using a rotary evaporator. The small volume was later freeze-dried to obtain a crude extract. The extracts were kept at 4°C until needed for use.

2.4. In vitro antioxidant tests

2.4.1. Determination of total phenolic content

The total phenolic content of the hydromethanolic extracts of cocoa and kola mistletoe was determined using the Folin–Ciocalteu method as modified by Singleton et al [14].

2.4.2. Determination of total flavonoid content

The total flavonoid content was determined using a colorimetric method described by Dewanto et al [15].

2.4.3. Determination of total proanthocyanidins

Determination of the total proanthocyanidins was based on the procedure reported by Sun et al [16]. A volume of 0.5 mL of 100 µg/mL of the extract was mixed with 3 mL of a 4% vanillin–methanol solution and 1.5 mL hydrochloric acid. The mixture was allowed to stand for 15 minutes. Absorbance was measured at 500 nm. The total amount of proanthocyanidins was expressed as catechin equivalents (CE, mg catechin/g sample) through the calibration curve of catechin.

2.4.4. Assay for total antioxidant activity

The total antioxidant capacity of the extracts was determined with phosphomolybdenum using ascorbic acid as the standard. The assay was based on the reduction of Mo (vi) to Mo (v) by the extracts and the subsequent formation of a green phosphomolybdate (v) complex at acidic pH. Exactly 0.1 mL of each extract (100 µg/mL) solution was combined with 3 mL of reagent (0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The mixtures were incubated in a boiling water bath at 95°C for 90 minutes. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was read at 695 nm against the appropriate blank in a spectrophotometer. The antioxidant capacity was expressed as the equivalent of that of ascorbic acid [17].

2.4.5. Assay for nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside was measured by the Griess reagent [18]. Various concentrations of cocoa and kola mistletoe extracts were mixed with sodium nitroprusside (1mM in phosphate buffer saline) and incubated at 25°C for 150 minutes. Half a milliliter of this solution was mixed with an equal volume of Griess reagent (1% sulfanilamide, 2% orthophosphoric acid, and 0.1%

naphthalene ethylenediamine dihydrochloride, NED) and allowed to react for 30 minutes. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with NED was read at 546 nm. The percentage inhibition was calculated [19].

2.4.6. Assay for hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the samples was determined according to the method of Halliwell and Gutteridge [20]. The assay mixture consisted of 100 mL of pre-mixed ferric chloride (100mM), 250 mL of 2.8mM 2-deoxyribose in 50mM phosphate buffer (pH 7.4), 100mM EDTA solution (1:1; v/v), and 200mM H₂O₂ (50 mL) without or with the 50 mL extract/fraction solutions (25–100 µg/mL final concentrations). Reaction was triggered by adding 50 mL of ascorbate (300mM) and heating for 60 minutes at 37°C. A solution of 1% TBA in 500 mL of NaOH (50mM) and 500 mL of 2.8% trichloroacetic acid (TCA) was added. Then the mixture was placed in a boiling water bath for 15 minutes. After cooling, the absorbance was taken at 532 nm.

Hydroxyl radical scavenging was calculated according to the following equation:

$$\text{Percentage inhibition activity} = \frac{[(\text{absorbance of control}) - (\text{absorbance of sample})/(\text{absorbance of control})] \times 100}{\text{absorbance of control}}$$

2.5. Animals

Adult male rats (Wistar strain) weighing 120–150 g, obtained from the Animal House of the College of Medicine, University of Lagos, Nigeria, were used for this study. The animals were kept in wire mesh cages under a controlled light cycle (12 h light/12 h dark), fed with commercial rat chow (Vital Feeds Nigeria Limited) ad libitum, and liberally supplied with water. All animal experiments were conducted according to the guidelines of European Commission Directive 86/609/EEC for laboratory animal care and use.

2.6. Isolated rat heart preparation

Perfusion of hearts with Ringer–Locke's solution in a Langendorff apparatus proceeded as described in previous works [21,22]. The animals were killed by cervical dislocation, with heparin premedication as an anticoagulant (0.2 mL/100 g animal; i.p.). After thoracotomy was performed and the heart rapidly excised into a basin of ice-cold Ringer–Locke's solution [NaCl, 9 g; KCl, 0.24 g; NaHCO₃, 0.5 g; CaCl₂, 0.42 g; glucose, 1 g (for 1 L)], blood was gently squeezed from the aorta, which was detached from the pulmonary artery and cannulated on a shortened and blunted 21-gauge needle. Perfusion was initiated at a constant pressure of 60 mmHg through a tube leading from a reservoir of Ringer–Locke's solution. The reservoir was in turn connected to an oxygen tank so that the pressure closed the semilunar valves and forced the perfusion fluid through the coronary vessels. From the coronary vessels the fluid flowed out over the entire heart through the pulmonary artery and the cut end of the superior vena cava. During the process of perfusion, it was ensured that no bubbles entered

the aorta; effluent from the heart was then collected in a measuring cylinder, transferred into a universal bottle, immediately kept in a cooler containing lumps of ice blocks, and then transferred to a freezer until needed for biochemical analysis.

The amplitude of the ventricular contractions was recorded on a graph sheet by the lever of a Ugol Basil Bio-recorder connected to a force transducer, which in turn was attached to the apex of the left ventricle of the heart by a thread and hook. The equipment was calibrated by attaching a 1 g tension to the force transducer and noting the height of deflection on the graph sheet. The heart rate and force of contraction were determined as written on the recording equipment. The outflow from the heart was the outflow of fluid which had passed through the coronary vessels.

2.7. Experimental design

2.7.1. Preliminary toxicological study

Twelve hearts divided into two groups ($n = 6$) were used for physiopharmacological assays of the hydromethanolic extracts of cocoa (CGCE) and kola (KGCE) *G. cupulata*. The heart perfusion was set up as described in Section 2.6, and a 30-minute stabilization period was observed. Thereafter, every 15 minutes, 1 mL of 1, 3, 5, 10, 30, and 50 µg/mL of CGCE or KGCE was introduced successively into the perfusion fluid in order to study the chronotropic (heart rate) and inotropic (force of contraction) effects of the extracts [23]. Based on a preliminary study, the duration of perfusion for each concentration was 15 minutes, during which the total volume (as well as the effect) of the previous extract/drug would have been completely lost with the perfusion fluid. The successive administration of both extracts was continued until the heart collapsed, in order to determine the effective and lethal concentrations.

2.7.2. Biochemical assay

Rat hearts divided into four groups ($n = 6$) were used for this study and treated as follows:

Group 1 (Control)	No ischemia
Group 2	Ischemia, no treatment
Group 3	Ischemia + 3 µg/mL CGCE
Group 4	Ischemia + 3 µg/mL KGCE

Hearts were prepared as earlier described (Section 2.6). Ischemia was induced by turning off the tap on the tube leading to the heart, thereby stopping the flow of the perfusion fluid to the heart. Reperfusion was established by reopening the tap after 20 minutes. One mL of extract (CGCE or KGCE) was introduced into the perfusion fluid just before entrance into the heart at time (minutes) 0, 15, 30, and 45. Effluents were collected at intervals of 5 minutes for 50 minutes concurrently with extract treatment, and the protein concentrations of the effluents were determined by the method of Bradford (BioRad) using bovine serum albumin (BSA) as the standard. The protein concentrations ranged from 25 to 44 µg/ml.

The effluents were later subjected to biochemical assays.

2.8. Antioxidant status determination

2.8.1. Assay for superoxide dismutase activity

Superoxide dismutase activity was determined by the ability to inhibit the auto-oxidation of epinephrine by monitoring the increase in absorbance at 480 nm as described by Sun and Zigma [24]. A mixture (3 mL) containing 2.95 mL 0.05M sodium carbonate buffer (pH 10.2), 0.02 mL of the effluent, and 0.03 mL of epinephrine in 0.005N HCl was used to initiate reaction. The reference cuvette contained 2.95 mL buffer, 0.03 mL of substrate (epinephrine), and 0.02 mL of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 minutes.

2.8.2. Assay for catalase activity

Catalase activity was determined by measuring the decrease in absorbance at 240 nm due to the decomposition of H₂O₂ in a UV recording spectrophotometer as described by Aebi [25]. The reaction mixture (3 mL) contained 0.1 mL of the effluent in phosphate buffer (50mM, pH 7.0) and 2.9 mL of 30mM H₂O₂ in phosphate buffer pH 7.0. An extinction coefficient for H₂O₂ at 240 nm of 40.0 M⁻¹cm⁻¹ was used for the calculation. The specific activity of catalase was expressed as moles of H₂O₂ reduced per minute per mg protein.

2.8.3. Assay for membrane lipid peroxidation

Malondialdehyde (MDA), an index of lipid peroxidation, was determined using the method of Buege and Aust [26]. One milliliter of the effluent was added to 2 mL of TBA–HCl–TCA reagent (0.37% TBA, 0.24N HCl, and 15% TCA in a 1:1:1 ratio) and boiled for 15 minutes. After cooling on ice, flocculent materials were removed by centrifuging at 3000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 532 nm against a distilled-water blank. MDA was calculated using the molar extinction coefficient for the MDA–TBA–complex of 1.56 × 10⁵ M⁻¹cm⁻¹.

2.9. Protein estimation

The protein concentrations of the effluents were measured by the method described by Lowry et al [27] using BSA as the standard.

2.10. Statistical analysis

Experiments conducted in replicates were expressed as mean ± standard deviation. Data generated from time-course measurements were analyzed using two-way analysis of variance followed by Bonferroni posttest to account for the two variables of time and treatment. Other data were analyzed using either Student t test or one-way analysis of variance followed by the Newman–Keuls post hoc test.

3.0. Results

3.1. Antioxidant indices and radical scavenging activity

The antioxidant indices and radical scavenging activity of CGCE and KGCE are shown in Table 1 and Fig. 1. The total

Table 1 – Antioxidant indices of CGCE and KGCE.

Property	Level ^{e,f}	
	CGCE	KGCE
Total phenolics ^a	21.06±2.3	17.56±3.3
Total flavonoids ^b	0.12±0.001	0.14±0.002**
Total proanthocyanidins ^c	0.20±0.02	0.26±0.01*
Total antioxidant activity ^d	0.037±0.005	0.031±0.008*

* *p* < 0.01, compared with the corresponding property of CGCE.
 ** *p* < 0.001, compared with the corresponding property of CGCE.
 CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees.
^a mg tannic acid equivalent/g extract.
^b mg rutin equivalent/g extract.
^c mg catechin equivalent/g extract.
^d mg ascorbic acid equivalent/g extract.
^e Results are presented as the mean ± standard deviation of replicate measurements (*n* = 3).
^f Data analysis was done using Student t test.

phenolic content (mg tannic acid equivalent/g) was higher for CGCE (21.06±2.3) than for KGCE (17.56±3.3). The total flavonoid content (mg rutin equivalent/g extract) and total proanthocyanidin content (mg catechin equivalent/g extract) were however found to be significantly (*p* < 0.001 and *p* < 0.01) higher in KGCE (0.14±0.002 and 0.26±0.01) than in CGCE (0.12±0.0006 and 0.20±0.02). The total antioxidant activity (mg ascorbic acid equivalent/g extract) was higher in CGCE (0.037±0.005) than in KGCE (0.031±0.008). Both CGCE and KGCE showed considerable nitric oxide scavenging activity *in vitro*. Hydroxyl radical scavenging potency was higher for KGCE (EC₅₀: 24.8±1.8 µg/mL) than for CGCE (EC₅₀: 70.2±4.5 µg/mL). For both CGCE and KGCE, the nitric oxide and hydroxyl radical scavenging activity were dose-dependent and significant (*p* < 0.001) at the evaluated concentrations (25, 50, 75, and 100 µg/mL).

3.2. Effect on hemodynamic parameters

CGCE and KGCE exhibited a profound effect on the cardiac function of rats (Table 2). In the preliminary study (data not shown), CGCE and KGCE, at concentrations as low as 1 µg/mL, lowered the amplitude of contraction from 2.3±0.4 cm to 1.73±0.15 cm and from 2.98±0.6 cm to 2.05±0.5 cm, respectively. The highest concentration tolerated for both plant extracts was 30 µg/mL. The lethality to rat hearts at higher doses (>30 µg/mL) was manifested by the observed collapse of the hearts. As shown in Table 2, CGCE and KGCE caused a significant dose-dependent decrease (*p* < 0.001) in the flow rate, working rate, and force of contraction of the hearts at the 3–30 µg/mL concentrations when compared to the control (0 µg/mL CGCE or KGCE). At the highest effective concentration (30 µg/mL), the respective reductions (%) by CGCE and KGCE of the flow rate (78.2 and 81.7), heart rate (30.4 and 46.2), and force of contraction (79.7 and 87.6) were significant (*p* < 0.01).

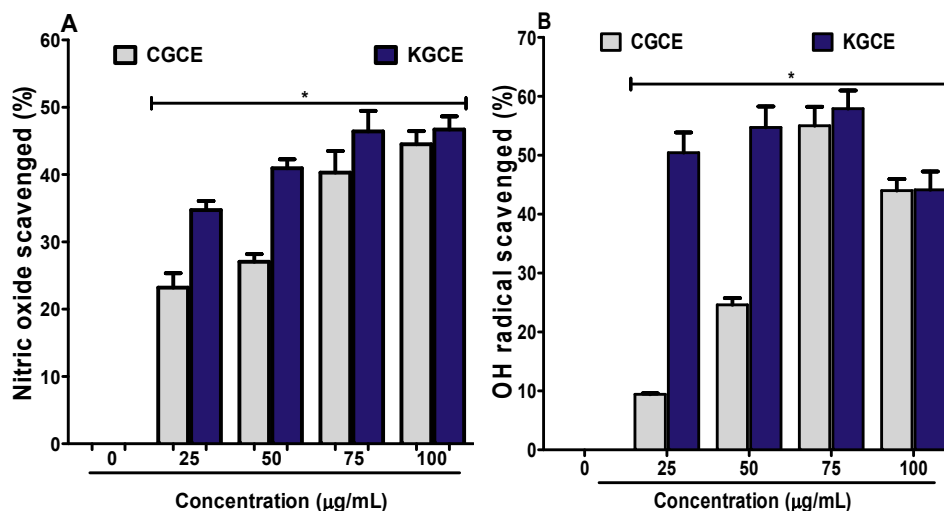


Fig. 1 – (A) Nitric oxide and (B) hydroxyl radical scavenging activity of CGCE and KGCE. ^aThe results shown are the mean ± standard deviation of replicate measurements (n = 3). ^bData analysis was done by one-way analysis of variance followed by the Newman–Keuls post hoc test (p < 0.05). *p < 0.001, compared with 0 µg/mL CGCE/KGCE. CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees; OH = hydroxyl.

3.3. Effect on biochemical and antioxidant indices

The reperfusion of the ischemic hearts produced a significant (p < 0.001) decrease in the nitrite content of the effluents compared with the level in the hearts that were not subjected to ischemia. CGCE and KGCE administered with the reperfusion fluid reversed the trend in a significant manner. A significant decrease (p < 0.001) in the coronary flow rate during the reperfusion of the ischemic hearts was observed when compared to that of the non-ischemic hearts. The administration of extracts during the reperfusion of the hearts also ameliorated the effects of ischemia on flow rate. The ameliorative effect of both CGCE and KGCE on the nitrite level and coronary flow rate was significant throughout the course of reperfusion. At 30–40 minutes of reperfusion, the flow rate of the KGCE-treated hearts was not different from

that obtained in the normal, non-ischemic hearts (p > 0.05) (Fig. 2).

A significant increase (p < 0.001) in MDA levels in the perfusates was observed during the reperfusion of the ischemic hearts (Fig. 3). The MDA level was however reduced in the effluents of the hearts administered with CGCE and KGCE. The extent of cardiac membrane peroxidation was not statistically different from that in the control in both the CGCE- and KGCE-treated hearts at 5–25 minutes of reperfusion. The activity of superoxide dismutase (SOD) and catalase in the perfusates of the ischemia untreated rats was significantly reduced (p < 0.001) during the entire 50 minutes and 25–40 minutes, respectively, of reperfusion (Fig. 4). The reversal of the effects of ischemia on the activity of the antioxidant enzymes was significant (p < 0.001) in the perfusates of the CGCE- and KGCE-treated hearts.

Table 2 – Effect of CGCE and KGCE on hemodynamic parameters in rats.

Concentration (µg/mL)	Flow rate ^{a,b} (mL/min)		Heart rate ^{a,b} (beats/min)		Force of contraction ^{a,b} (N)	
	CGCE	KGCE	CGCE	KGCE	CGCE	KGCE
0	2.43±0.02	2.90±0.06	224±22	195±27	0.0069±0.0012	0.0089±0.0018
1	1.15±0.13***	1.25±0.19**	207±19*	172±21*	0.0052±0.0005**	0.0061±0.0015**
3	0.90±0.08***	0.92±0.13**	173±20**	158±10**	0.0041±0.0005**	0.0044±0.0009**
5	0.70±0.08***	0.81±0.10**	174±09**	141±31**	0.0038±0.0008**	0.0037±0.0004**
10	0.63±0.09***	0.65±0.09**	161±22**	121±22**	0.0034±0.0004**	0.0022±0.0007**
30	0.53±0.05***	0.53±0.09**	156±25**	105±21**	0.0014±0.0004**	0.0011±0.0002**
50	0	0	0	0	0	0

*p < 0.05, compared with 0 µg/mL CGCE/KGCE.

**p < 0.01, compared with 0 µg/mL CGCE/KGCE.

***p < 0.001, compared with 0 µg/mL CGCE/KGCE.

CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees.

^a Results are presented as mean ± standard deviation (n = 12).

^b Data analysis was done by one-way analysis of variance followed by the Newman–Keuls post hoc test (p < 0.05).

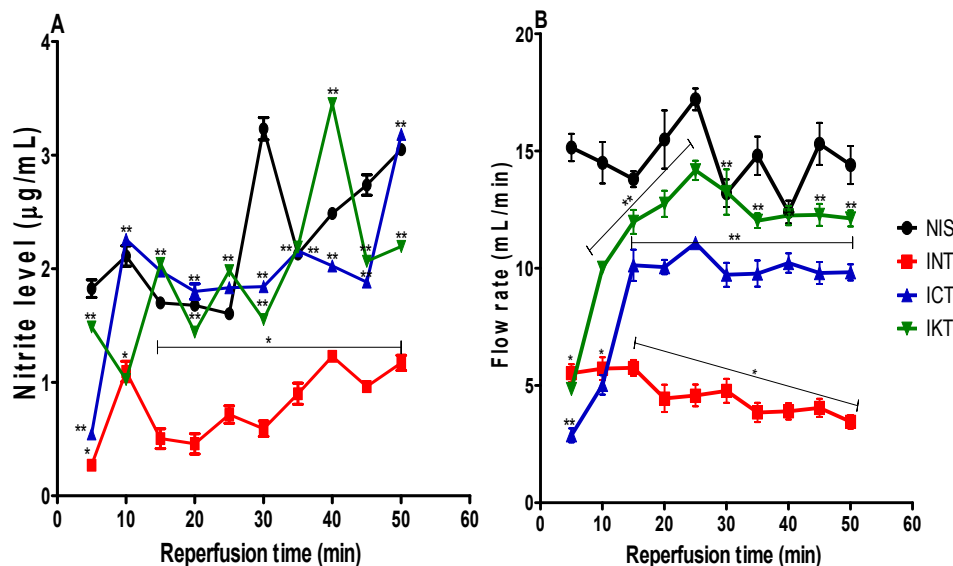


Fig. 2 – Effects of CGCE and KGCE on (A) nitrite level and (B) time-course of coronary flow during reperfusion of hearts subjected to ischemia/reperfusion cycle. ^aThe vertical bars represent the standard deviations ($n = 6$). ^bData analysis was done by two-way ANOVA, followed by Bonferroni posttests ($p < 0.05$). * $p < 0.001$, compared with NIS group (control). ** $p < 0.001$, compared with INT group. CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; ICT = ischemia, CGCE-treated; IKT = ischemia, KGCE-treated; INT = ischemia, not treated; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees; NIS = no ischemia.

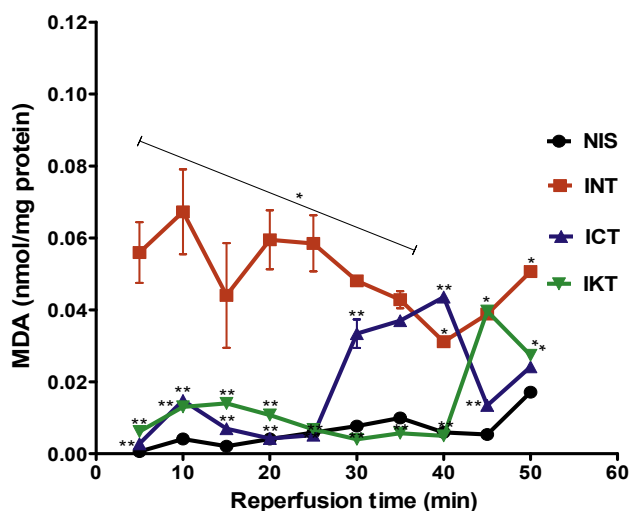


Fig. 3 – Effect of CGCE and KGCE on MDA level in perfusates of hearts subjected to ischemia/reperfusion cycle. ^aThe vertical bars represent the standard deviations ($n = 6$). ^bData analysis was done by two-way ANOVA, followed by Bonferroni posttests ($p < 0.05$). * $p < 0.001$, compared with NIS group (control). ** $p < 0.001$, compared with INT group. CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; ICT = ischemia, CGCE-treated; IKT = ischemia, KGCE-treated; INT = ischemia, not treated; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees; MDA = malondialdehyde; NIS = no ischemia.

4.0. Discussion

In spite of the pharmacological efficacy of cardioprotective drugs, new products inhibiting ischemic organ damage are needed [28], and special attention is being paid to the discovery of new pharmacological agents from plants [29]. The antioxidant content of medicinal plants may contribute to the protection they offer against disease [30]. Phenolic compounds, which are ubiquitous in plants, are the major antioxidant phytochemicals. When plants are consumed as foods, these phytochemicals contribute to the intake of natural antioxidants in the diets of humans as well as animals.

In line with several studies which reported a strong relationship between total phenolic content and antioxidant activity in plants [31,32], the higher total phenolic content of CGCE was commensurate with the total antioxidant activity. The total antioxidant activity was measured spectrophotometrically through the phosphomolybdenum method and based on the reduction of Mo (VI) to Mo (V) by the test samples. Polyphenolics contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [33]. Flavonoids represent a major subset of phenolics, and they are highly effective scavengers of most oxidizing molecules and free radicals implicated in several diseases [34]. Subclasses of flavonoids include, but are not limited to, flavones (chrysin, apigenin, luteolin), flavonols (rutin, quercetin, myricetin, kaempferol, quercitrin), and flavan-3-ols (catechin, epicatechin) [35]. Proanthocyanidins could be oligomeric or polymeric flavan-3-ols. Those consisting exclusively of (epi) catechin units (i.e., procyanidins) are the most abundant type of proanthocyanidins in plants.

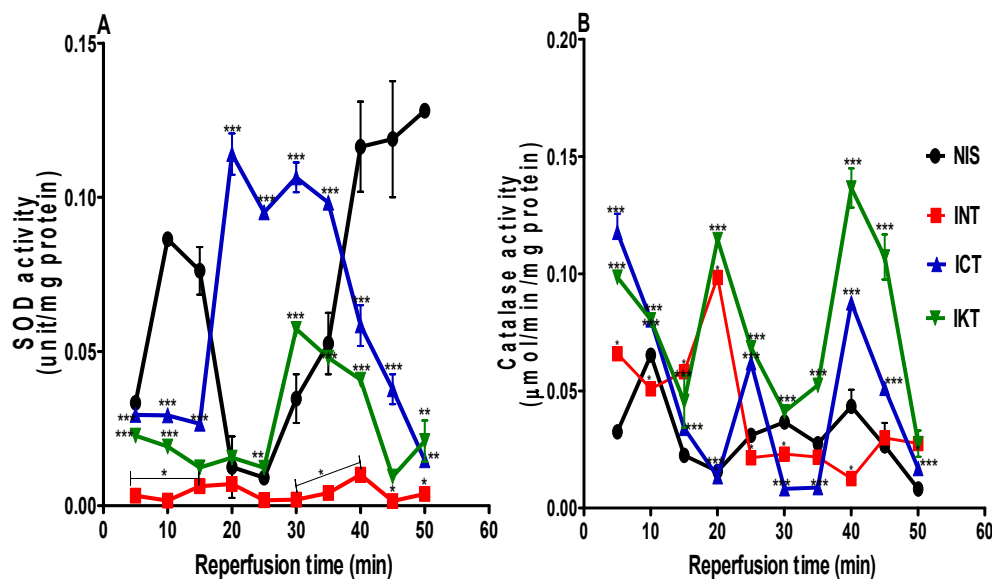


Fig. 4 – Effects of CGCE and KGCE on activity of (A) SOD and (B) catalase in perfusates of hearts subjected to ischemia/reperfusion cycle. ^aThe vertical bars represent the standard deviations ($n = 6$). ^bData analysis was done by two-way ANOVA, followed by Bonferroni posttests ($p < 0.05$). * $p < 0.001$, compared with NIS group (control). ** $p < 0.01$, compared with INT group. *** $p < 0.001$, compared with INT group. CGCE = hydromethanolic extract of *Globimetula cupulata* from cocoa trees; ICT = ischemia, CGCE-treated; IKT = ischemia, KGCE-treated; INT = ischemia, not treated; KGCE = hydromethanolic extract of *G. cupulata* from kola nut trees; NIS = no ischemia; SOD = superoxide dismutase.

The extracts of cocoa and kola *G. cupulata* contained considerable concentrations of proanthocyanidins. Proanthocyanidins are polymeric flavan-3-ols whose antioxidant property hinges on their ability to serve as electron donors to terminate the radical chain reaction [36]. They are capable of preventing experimentally induced myocardial ischemic injury in rats [37]. In the present study, KGCE possessed higher total flavonoid and proanthocyanidin contents than CGCE. Accordingly, the scavenging potency of KGCE against nitric oxide and hydroxyl radicals was superior to that of CGCE. The hydroxyl radical, one of the most potent reactive oxygen species in living systems, damages cells by causing a membrane peroxidation attack on cellular macromolecules [38]. Protection of biological systems is thus afforded by antioxidants that can remove the radical. The hydroxyl radical scavenging capacity of an extract is known to have direct proportionality to its antioxidant activity [39]. With EC_{50} values of $24.8 \pm 1.8 \mu\text{g/mL}$ and $70.2 \pm 4.5 \mu\text{g/mL}$ for KGCE and CGCE, respectively, the extracts exhibited good hydroxyl radical scavenging activity [40]. There is little doubt phenolic acids and flavonoids are the major contributors to the antioxidant activity of the extracts in the present investigation, based on findings in the literature on other extracts of plant products [41].

Langendorff heart preparation is one of the best experimental models usually employed in the evaluation of the cardioprotective effects of drugs and drug candidates [42]. The model allows the administration of drugs (via the perfusate) and observation of their effect on parameters, such as the heart rate and contractile strength, without the complications involved in *in vivo* experimentation, such as neuronal and hormonal interferences [42,43]. In the present

study, we sought to know the effects of varying concentrations of both CGCE and KGCE on vital heart parameters using the Langendorff heart preparation method. An assessment of the role of the extracts revealed their effects on the heart rate and the hearts' force of contraction and flow rate. Both extracts in the present study possessed negative inotropic (force-of-contraction-lowering) and negative chronotropic (heart-rate-lowering) effects. These results are consistent with a previous study that showed that *G. cupulata* exhibits an antihypertensive effect [10], although the exact mechanism was not described. The superior potency of kola mistletoe over cocoa mistletoe extract, however, is noteworthy in the present investigation and may be due to kola mistletoe's higher content of flavonoids and proanthocyanidins.

As observed in the increased concentration of nitrite in the effluents of the treated hearts, both CGCE and KGCE could increase the nitrite level in perfused hearts following I/R. There have been claims of cardioprotection by nitrite therapy in animal models of myocardial I/R injury [44,45]. The cardioprotective potential of nitrite donors or boosters in the setting of I/R could be related to two general mechanisms. First, nitrite could be reduced to the potent vasodilator and anti-inflammatory agent nitric oxide and thus serve as a nitric oxide synthase (NOS)-independent source of the molecule when NOS is inactive due to low oxygen tension. Second, nitrite reacts with critical thiols to form nitrosothiols, which act as antioxidants that prevent the irreversible oxidation of proteins and lipids during the early oxidative burst of reperfusion [45,46]. In the present study, the coronary flow rate of the hearts subjected to the I/R cycle indicated a positive improvement by the administration of cocoa and kola

mistletoe extracts during reperfusion. Taken together, the results on the nitrite level and flow rate suggest that *G. cupulata* may have a beneficial influence on coronary vascular tone and might exert cardioprotective effects through its vasodilatory anti-ischemic activity. The therapeutic effects of cardioprotective plants have been ascribed to the antioxidative tendency of the constituent antioxidant phytochemicals (mostly phenolics) to neutralize nitric-oxide-destroying oxygen radicals, of which superoxide radicals (O_2^-) is a principal member [47]. Such effects could increase nitric oxide bioavailability, causing overall improvement in vascular tone, and is one of the major mechanisms underlying the improvement of endothelium-dependent vasodilatation by antioxidants [48]. Thus, the increased levels of nitrite and, by extension, flow rate in the groups treated with CGCE and KGCE may be attributed to their antioxidant properties. In contrast, the reduced levels of nitrite as well as the decrease in flow rate in the hearts subjected to the I/R cycle without extract treatment may be indicative of the effect of superoxide anions on nitric oxide production. This result is consistent with a study in which nordihydroguaiacetic acid, a lipooxygenase inhibitor, was thought to have increased endothelial nitric oxide synthase gene expression in a culture of endothelial cells as mediated by the antioxidant properties of its phenolic groups [49]. The observed collapse of the hearts at 50 $\mu\text{g/mL}$ concentrations of the extracts is an indication of toxicity at that dose; thus, pharmacology would definitely transcend to toxicology over a particular threshold dose. Under cellular conditions, lower concentrations (low- μM -to-mM range) of antioxidant phytochemicals are usually employed to elicit therapeutic responses [50]. Even with this, the bioavailability and hence effective concentrations of dietary polyphenolics *in vivo* rarely exceed the nM range [50].

During I/R damage to the myocardium, reactive oxygen species, such as superoxide and hydrogen peroxide, are produced in enormous amounts. Hence, the decreased activity of endogenous antioxidant enzymes, such as SOD and catalase, in perfusates, as observed in the present study, is normally associated with I/R injury [51]. SOD dismutates superoxide into hydrogen peroxide, which is in turn decomposed to water by catalase. The excessive generation of superoxide in the presence of nitric oxide has been described as the primary source of oxidative stress, and perturbation in the production and/or metabolism of either molecule can have pathologic consequences [51]. There is substantial evidence that the associated contractile and rhythmic disturbances observed following the I/R cycle in the present study might involve a contribution from reactive oxygen species [52]. In the present investigation, the reduced levels of these antioxidant enzymes observed in the ischemic/reperfused untreated hearts were significantly reversed by treatment with the cocoa and kola mistletoe extracts. This might suggest that the intervention assisted the inherent cardiac antioxidant system in combating the superoxide and hydrogen peroxide generated by the I/R episode. The enormous amount of H_2O_2 resulting from the IR cycle could trigger the production of the highly reactive hydroxyl radicals via Fenton reaction involving transition metal ions, like Fe^{2+} , which are considerably present in the heart [53]. Such hydroxyl radicals and other resulting ROS

are capable of degrading polyunsaturated lipids, thus forming MDA. The relatively high degree of membrane peroxidation (increased MDA level) observed for the ischemic/reperfused untreated hearts when compared with that of the non-ischemic and CGCE- and KGCE-treated hearts might be indicative of the protection afforded by the treatment.

In conclusion, the present study suggests that extracts and fractions of cocoa and kola mistletoe exhibit *in vitro* radical scavenging activity and offer protection against I/R injury in rat hearts via augmenting endogenous antioxidants and preventing decline in cardiac function. The study plants could thus serve as sources of useful agents to combat complications associated with myocardial I/R injury.

Conflicts of interest

The authors declare no conflicts of interest.

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