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Harpephyllum caffrum stimulates glucose uptake, abates redox imbalance and modulates purinergic and glucogenic enzyme activities in oxidative hepatic injury

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ABSTRACT

Objective: To investigate the antioxidative and antidiabetic effects of *Harpephyllum caffrum* bark infusion as well as its effects on glucogenic and nucleotide hydrolyzing enzyme activities in $FeSO_4$-induced oxidative stress in rat hepatic tissue.

Methods: *Harpephyllum caffrum* infusion was prepared from dried plant materials (40 g) infused in boiling water (400 mL) for 20 min at room temperature. The antioxidative and inhibitory activities against carbohydrate digestive enzymes of the infusion were determined using established protocols. The liver tissues of rats were used for glucose uptake assay and to evaluate the infusion's effect on endogenous antioxidant, glucogenic, and nucleotide hydrolyzing enzyme activities in $FeSO_4$-induced hepatic injury.

Results: The *Harpephyllum caffrum* infusion significantly reduced ferric iron (FRAP) and free radicals ($OH^{\bullet}$ and DPPH) in a dose-dependent manner. It inhibited α-amylase and α-glucosidase activities and increased glucose uptake in hepatic tissues. $FeSO_4$ significantly decreased glutathione concentration, catalase, and superoxide dismutase activities while increasing malondialdehyde level, glycogen phosphorylase, fructose-1,6-bisphosphatase, and adenosine triphosphatase activities. However, treatment with *Harpephyllum caffrum* infusion reversed $FeSO_4$-induced changes. Characterization of the infusion revealed the presence of catechol, *O*-pyrocatechuic acid, mequinol, maltol, and glycoside derivatives.

Conclusions: The *Harpephyllum caffrum* infusion demonstrates antidiabetic and antioxidative potentials in *in vitro* models of type 2 diabetes as depicted by its ability to inhibit carbohydrate digestive enzymes, mitigate oxidative imbalance, and regulate glucogenic and nucleotide hydrolyzing enzyme activities in oxidative hepatic injury.

KEYWORDS: *Harpephyllum caffrum*; Antioxidant; Oxidative injury; Carbohydrate dysmetabolism

1. Introduction

Diabetes is one of the diseases of global public health significance, with a continuous annual increase in global prevalence. Epidemiological data released by the International Diabetes Federation in 2019 revealed that nearly 463 million individuals worldwide are affected by diabetes. This value represents an 11.56% increase of 415 million earlier reported in 2015[1]. Moreover, it was projected that by 2030, an estimated 578 million people constituting 5.01% Africans, will suffer from this

Significance

Harpephyllum caffrum is an underutilized wild food plant that is indigenous to South Africa. The plant is valued traditionally due to the nutritional and medicinal properties associated with its parts. More importantly, an aqueous extract of the bark is utilized in diabetes management but without a detailed scientific basis to support the reported activity. The ability of the bark infusion to stimulate glucose uptake and ameliorate oxidative-induced biochemical dysfunction in liver tissues may indicate its protective role in type 2 diabetes. *Harpephyllum caffrum* presents a source of natural chemical agents with hypoglycemic and antioxidant efficacies in managing diabetes comorbid complications.

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pathological condition. Amongst the various types of diabetes, type 2 diabetes (T2D) accounts for over 90% of all reported cases contributing to the global health burden[1].

In T2D, diminished pancreatic β-cells insulin production or cellular insensitivity to its signal causes chronic hyperglycemia, leading to cardiopathy, nephropathy, retinopathy, and neuropathy[2]. Nevertheless, T2D comorbid conditions have been significantly linked to oxidative stress, emanating from free radicals mediated suppression of the body's innate antioxidant defence system[3].

The liver is a vital body organ that performs unique roles in energy metabolism. One way it carries out this crucial cellular function is by regulating blood glucose homeostasis. In the fed state, the liver takes up blood glucose for storage as glycogen *via* glycogenesis, while under fasting conditions, the organ produces more sugar into the general circulation through gluconeogenesis and glycogenolysis[4]. Liver energy metabolism function is coordinated by insulin and other hormones signals[4]. However, in T2D pathology, insulin resistance and excessive free radical production act as synergistic mediators of pathological liver conditions such as hepatic ischemia, hepatic cirrhosis, non-alcoholic fatty liver disease, obstructive cholestasis, and hepatocellular carcinoma[5]. Hence, chemical agents that are capable of regulating various pathways exacerbating hyperglycemia may present valuable therapeutic targets in managing T2D disorders.

Currently, there are many medications for regulating high blood glucose levels in diabetic patients. These chemical interventions utilize different mechanisms in lowering blood glucose. One of these drugs is metformin which exerts its hypoglycemic effect by inhibiting liver gluconeogenesis and enhancing glucose uptake in peripheral tissues[6]. This drug brings about its physiological response by promoting GLUT-4 expression in skeletal and adipose tissues while inhibiting fatty acid oxidation and reducing circulating triacylglycerol levels[7]. Despite the therapeutic potentials of metformin and other synthetic antidiabetic drugs, studies have documented their side effects such as gastrointestinal disturbance, weight gain, and β-cell functional suppression[8]. Therefore, the search for alternative regimens in diabetes treatment with little or no adverse effects has led to the exploration of medicinal plants[9].

Harpephyllum caffrum (*H. caffrum*) is a deciduous evergreen plant that is endemic and widely distributed within Southern Africa vegetations. The tree is a member of the 4th largest tree family (Anacardiaceae) in South Africa[10]. In English, the plant is commonly called "wild plums" or "Bush mango", whereas it is known as "umgwenya" in the isiZulu native language. The fruits of *H. caffrum* are consumed as snacks, sweet preserves, and in the production of alcoholic and non-alcoholic beverages[11]. In addition, the bark extract is utilized in local medicine to treat headaches, pains, epilepsy, and convulsion in children[12]. Pharmacological evidence also reported that *H. caffrum* has antidiabetic, antioxidant, anti-inflammatory, hypotensive, and analgesic properties[13]. However, there is scarce information about the possible protective effect of *H. caffrum* bark on the oxidative hepatic injury.

Therefore, this study was undertaken to investigate the effect of *H. caffrum* infusion on Fe^{2+} induced oxidative imbalance in hepatic tissue, glucogenic and purinergic enzyme activities, and liver glucose uptake *ex vivo*. Its effect on carbohydrate digestive enzyme activities and glucose uptake in yeast cells were also evaluated. Bioactive compounds in the infusion were characterized, and their molecular binding affinities with some of the studied enzymes were determined *in silico*.

2. Materials and methods

2.1. Plant material and infusion preparation

Specimen of *H. caffrum* stem bark was collected from the University of KwaZulu-Natal, South Africa. The plant was authenticated at the school ward herbarium, and a voucher specimen (K. Olofinsan & F. Olawale 4) of the plant sample was deposited. Then the fresh plant material was washed with water and air-dried to constant weight. About 60 g of dried plant sample was transferred into a beaker containing 600 mL boiling water (100 ℃). Infusion of the plant sample was carried out for 20 min at room temperature. After sieving with Whatman filter paper (No. 1), the filtrate obtained from the infusion was concentrated on a boiling water bath.

2.2. Estimation of total phenolic contents

The total phenolics content of *H. caffrum* infusion was estimated using the protocol of McDonald *et al*.[14] with some modifications. Briefly, 800 µL of 700 mM Na_2CO_3 and 900 µL of Folin Ciocalteau reagent (prepared in distilled water 1:10 *v/v*) were added to 0.2 mL of 320 µg/mL solution of the infusion. The resulting solution was incubated at 25 ℃ for 30 min, and absorbance was measured at 765 nm. The concentration of phenolics in the infusion was extrapolated from a standard curve with 0-700 µg/mL gallic acid.

2.3. In vitro antioxidant activities

2.3.1. Ferric reducing antioxidant power (FRAP)

The infusion's ferric reducing power was evaluated using the method of Oyaizu[15] with minor modifications. Briefly, 500 µL of the infusion was incubated with 250 µL of 200 mM phosphate buffer (pH 6.6) and 125 µL potassium ferricyanide (1%) for 20 min at 50 ℃. Then 250 µL of 10% trichloroacetic acid was added, followed by 250 µL of distilled water and 50 µL $FeCl_3$ (0.1%). The absorbance of the resulting solution was read at 700 nm with a Shimadzu spectrophotometer (UV min 1240, Shimadzu Corporation, Japan). FRAP of the aqueous infusion was expressed as % equivalent of 320 µg/mL Trolox.

2.3.2. Hydroxyl ($OH^{\bullet}$) radical scavenging activity

The ability of the infusion to scavenge hydroxyl radical ($OH^{\bullet}$) was determined using Halliwell and Gutteridge[16] with some modifications. Briefly, 100 μL of different concentrations of the aqueous infusion, 150 μL deoxyribose (20 mM), 250 μL phosphate-buffered saline, 100 μL Fe_2SO_4 (500 μM) and 100 μL H_2O_2 (1%) were added together in sequence. The mixture was incubated for 30 min at 37 ℃ before 200 μL of 10% TCA, and 600 μL of 0.25% thiobarbituric acid were added. The resulting solution was boiled for 20 min and then cooled to room temperature. Absorbance was measured at 532 nm, and % $OH^{\bullet}$ scavenging activity was calculated as described previously by Olofinsan *et al*.[17].

2.3.3. 2,2–Diphenyl–1–picrylhydrazyl (DPPH) scavenging activity

The ability of the infusion to scavenge DPPH free radical activity was evaluated by the method of Ak and Gülçin[18], with minor modifications. A total of 100 μL of 0.3 mM DPPH (prepared in methanol) was added to 100 μL infusion or Trolox (20-320 μg/mL) in 96-well microplates. The mixture was kept in the dark for 30 min, and absorbance was measured at 517 nm.

2.4. In vitro enzyme assays

2.4.1. α–Amylase inhibition assay

The α-amylase enzyme inhibitory activity of *H. caffrum* infusion was determined with a modified protocol described by Ibitoye *et al*.[19]. Briefly, 200 μL of infusion at various concentrations (20-320 μg/mL) were added to 300 μL solution of 0.5 mg/mL porcine pancreatic α-amylase prepared in 20 mM sodium phosphate buffer (pH 6.9). The mixture was incubated for 10 min at 37 ℃, followed by the addition of 500 μL starch solution (1%). After further incubation at the same temperature for 20 min, 300 μL of DNSA reagent was added, and the solution was boiled for 10 min. Then, 1 mL distilled water was added to the cooled reaction mixture before absorbance was taken at 540 nm.

2.4.2. α–Glucosidase inhibition assay

The α-glucosidase inhibitory activity of the infusion was evaluated with the method outlined by Ademiluyi and Oboh[20] with slight modifications. Briefly, 200 μL of acarbose or infusion at different concentrations (20-320 μg/mL) was transferred into tubes containing 400 μL of 1 U/mL yeast α-glucosidase, prepared in 100 mM phosphate buffer (pH 6.8). The mixture was incubated for 10 min at 37 ℃, and then 200 μL *p*-nitrophenol glucopyranoside (5 mM), prepared in the same buffer. The resulting solution was incubated for 20 min, and the absorbance was subsequently measured at 405 nm.

2.4.3. Pancreatic lipase inhibition assay

The lipase inhibitory activity of infusion was assayed with the method of Kim *et al*.[21], using *p*-nitrophenyl butyrate as substrate. Briefly, porcine pancreatic lipase (2 mg/mL) was prepared in buffer containing 10 mM 3-(*N*-morpholino) propane sulfonic acid and 1 mM EDTA, pH 6.8. Twenty μL of enzyme solution was incubated with 170 μL of Tris buffer (100 mM Tris–HC1 and 5 mM $CaCl_2$, pH 7.0) and 20 μL of various concentrations of the infusion or orlistat for 30 min at 37 ℃. Then, 5 μL of *p*-nitrophenyl butyrate (20 mM dimethylformamide) was added to the mixtures in a 96-well microplate, and the reaction proceeded at 37 ℃ for 5 min. The absorbance of 2, 4-dinitrophenol released from the reaction was read at 405 nm.

2.5. Glucose uptake in yeast cells

The activity of the infusion to enhance glucose transport into yeast cells was determined by measuring the decrease in glucose concentration in a solution containing yeast cell suspension and the infusion as described by Nirupama *et al*.[22] with modifications. Briefly, 500 μL of infusion (20-320 μg/mL) or distilled water (control) was incubated with 500 μL glucose (25 mM) at 37 ℃ for 10 min. Then, 100 μL yeast suspension (1%) was added, followed by further incubation at 37 ℃ for 1 h. Final glucose concentrations in the reaction mixtures were determined with DNSA reagent and their values were extrapolated from a glucose (0-50 mM) standard curve.

2.6. Animals

Six male rats of Sprague-Dawley strain (190-220 g) were procured from the Biomedical Resource Unit of the University of KwaZulu-Natal, South Africa. The animals were transferred to a holding room in the same building at temperatures between (21± 2) ℃ and under a daily 12 hours light and 12 hours dark standard photoperiods. After 12 h of overnight fasting, the rats were euthanized in isofor, gas chamber before their liver was excised. The handling and processing of the tissues specimen were done according to the procedures outlined by the Animal Research Ethics Committee of the University of KwaZulu-Natal.

2.7. Glucose uptake in isolated rat liver

The effect of the infusion on glucose uptake in freshly excised liver tissue was evaluated according to the protocol of Chukwuma and Islam[23]. Briefly, 0.5 g of whole rat liver was incubated in an 8 mL solution containing varying concentrations of the infusion (20-320 μg/mL) and metformin (320 μg/mL) dissolved in Krebs's buffer and 11.1 mM glucose. An aliquot of 2 mL solution was removed from each sample tubes before they were maintained for 2 h in an incubator at 37 ℃ and 5% CO_2. Then, 2 mL of incubated solution in each tube was taken for the measurement of glucose concentration. The liver glucose uptake was calculated as:

Liver glucose uptake = (GC_1 – GC_2)/(0.5 g of liver tissue)

Where GC_1 = glucose concentration in the sample tubes before 2 h incubation; GC_2 = glucose concentration in the sample tubes after 2 h incubation.

2.8. Preparation of liver homogenate

One gram of liver sample was homogenized in a buffer solution containing a mixture of 10% Triton X-100 and sodium phosphate buffer (50 mM, pH 7.5). The homogenate was centrifuged for 10 min at 15 000 rpm in a cold centrifuge (4 ℃). Then the supernatant was decanted and then kept at −80 ℃ for biochemical assays.

2.9. Induction of oxidative hepatic injury by $FeSO_4$

The induction of oxidative injury in liver homogenate was done using $FeSO_4$ solution as described by Oboh *et al*.[24]. Briefly, a mixture containing 200 μL tissue homogenate, 60 μL $FeSO_4$, and 20-320 μg/mL infusion or Trolox were incubated together at 37 ℃ for 30 min. The normal sample had the liver homogenate only while the untreated sample contained the homogenate and the pro-oxidant. The test sample had the liver homogenate, pro-oxidant, and infusion or Trolox.

2.10. Determination of reduced glutathione (GSH)

The concentration of GSH in the hepatic homogenate was estimated, according to the method of Ellman[25]. Briefly, 300 μL of 10% trichloroacetic acid was added to 100 μL of sample in Eppendorf tubes. The mixture was centrifuged at 3 500 rpm for 5 min, and 100 μL of the supernatant was transferred into 96-well microplates. Then 40 μL DTNB (0.5 mM) in 0.2 M sodium phosphate buffer (pH 7.8) was added, followed by gentle tapping of the plate to mix the content. The plate was incubated at 25 ℃ for 15 min, and the absorbance was read at 415 nm.

2.11. Determination of antioxidant enzyme activities

2.11.1. Superoxide dismutase (SOD) assay

The SOD activity in the hepatic homogenate was determined according to the protocol of Kakkar *et al*.[26] with minor modifications. Briefly, 25 μL of each sample was added to 170 μL of 0.1 mM diethylenetriaminepentaacetic acid in a 96-well plate. After incubation at room temperature for 20 min, 20 μL of 1.6 mM 6-hydroxydopamine was added, and immediately, absorbance was read at 492 nm every 1 min for three times.

2.11.2. Catalase (CAT) enzyme assay

The CAT enzyme activity in the sample homogenate was estimated *via* the spectrophotometric protocol described by Hadwan and Abed[27]. Briefly, 100 μL of homogenate was incubated with 1 mL of 65 μM H_2O_2 (prepared in 6 mM sodium phosphate buffer, pH 7.4) for 2 min at 37 ℃. The reaction was terminated by adding 5 μL of 32.4 mM ammonium molybdate, and the colour of the molybdate/H_2O_2 complex formed was measured at 347 nm. The blank tube contained H_2O_2 only, while the standard had all the reagents without the sample, and the control had all the reagents except H_2O_2.

2.12. Determination of lipid peroxidation

The concentration of lipid peroxidation product in the hepatic homogenate was estimated as malonaldehyde (MDA) equivalent by employing the method of Fraga *et al*.[28] with modifications. Briefly, 850 μL MilliQ water, 200 μL of 8.1% SDS solution, 2 mL of 0.25% thiobarbituric acid, and 750 μL of 20% acetic acid were added to 100 μL of the tissue homogenate. The resulting solution was kept in a boiling water bath for 1 h and then cooled to room temperature. The absorbance of 150 μL aliquot of each sample was measured at 532 nm.

2.13. Determination of nitric oxide (NO) concentration

The NO concentration in the liver homogenates was determined with the Griess method of Erukainure *et al*.[29] and Tsikas[30]. Briefly, 200 μL of liver supernatant or blank (distilled water) was incubated with 300 μL of Griess reagent for 30 min at 25 ℃ in a dark chamber. Then, the absorbance of the reaction medium was read at 548 nm in 96-well microplates.

2.14. Determination of glucogenic enzyme activities

2.14.1. Fructose-1,6-bisphosphatase (FBPase) activity

FBPase assay was carried out using the method outlined by Balogun and Ashafa[31] with modifications. Briefly, 200 μL of the tissue homogenate was added to 250 μL EDTA (1 mM), 250 μL of KCl (0.1 M), 100 μL $MgCl_2$ (0.1 M), 100 μL of 0.05 M fructose, and 1 200 μL 0.1 M Tris-HCl buffer (pH 7.0). The resulting solution was incubated for 15 min at 37 ℃ before adding 100 μL TCA (10%). Then the mixture was centrifuged at 5 000 ×*g* for 10 min, and the supernatant was pipetted in separate tubes containing freshly prepared 9% ascorbic acid. After further incubation at 37 ℃ for 30 min, the absorbance of the experimental mixture was measured at 680 nm, and FBPase activity was calculated.

2.14.2. Glycogen phosphorylase (GP) enzyme assay

GP activity was determined with the modified method described by Balogun and Ashafa[31]. Briefly, a mixture containing 100 μL of glycogen (4%), 100 μL glucose-1-phosphate (64 mM) and 200 μL of the tissue homogenate was incubated together at 30 ℃ for

10 min. This step was followed by the addition of 2.5 mL of 20% ammonium molybdate. After further incubation at 30 ℃ for 45 min, Elon reducer and distilled water were added. The absorbance of the resulting solution was read at 600 nm, and GP activity was calculated.

2.15. Adenosine triphosphatase (ATPase) enzyme assay

ATPase assay was carried out using the protocol of Erukainure *et al*.[2]. A mixture containing 200 µL of tissue, 200 µL of 5 mM KCl, 1 300 µL of 0.1 M Tris-HCl buffer, and 40 µL of 50 mM ATP was incubated together for 30 min at 37 ℃ on an automatic shaker. The reaction was terminated with 1 000 µL of ammonium molybdate solution. Then 1 000 µL of freshly prepared solution of 9% ascorbic acid was added to the mixture before incubation at 25 ℃ for 30 min. Absorbance was read at 660 nm, and ATPase enzyme activity was calculated subsequently.

2.16. Gas chromatography–mass spectrometric (GC–MS) analysis

A mixture of 2 M trifluoroacetic acid and 40 mg dried *H. caffrum* infusion was incubated at 100 ℃ for 2 h. After cooling, the resulting solution was air-dried for GC-MS analysis. Then, 50 µL solution of pyridine, hexamethyldisilazane, and trimethylchlorosilane in ratio 9:3:1 *v/v/v* was added for each gram of the sample to produce a silylated derivative. The derivatized compound was analyzed with a Shimadzu gas chromatograph (series AOC-20i) coupled Mass Spectrophotometer (GCMS-QP2010 SE). The carrier gas used contained ultra-pure helium flowing at 1.03 mL/min and a linear velocity of 37 cm/s. The injector temperature was kept at 250 ℃ while the oven temperature was programmed to operate until 280 ℃ maximum at the rate of 10 ℃/min and 3 min hold time. Then, 1 µL of the samples were injected in a splitless mode of 20:1 split ratio. The mass spectrometer was operated in an electron ionization mode of 70 eV and electron multiplier voltage of 1 859 V. Additionally, ion source temperature was 230 ℃, and the quadrupole temperature was 150 ℃. The solvent cut time was maintained at 3 min, and the scan range was between 50-700 amu. The compound identification was carried out *via* comparison of relative mass spectral data with those on the NIST database.

2.17. In silico molecular docking

In silico screening of the compounds identified in the infusion was conducted to determine their binding affinity with α-amylase, α-glucosidase, SOD, and CAT enzymes. The 3D structures of human α-amylase (1B2Y), α-glucosidase (3CTT), CAT (1F4J), and SOD (2C9V) were retrieved from the NCBI protein data bank. The proteins were prepared for protein-ligand docking using the Dock prep tool of UCFS Chimera software V. 1.14 (Pettersen *et al*., 2004). The three-dimensional structure (SDF format) of the compounds was also downloaded from the PubChem database. The compounds were optimized to their maximum global structure with Avogadro V1.2. The optimal binding pocket of the proteins was obtained using the CASTp online server before molecular docking with AutodockVina. Calculated values for the best binding pose of each compound in the proteins' active site were recorded, followed by virtual inspection with BIOVIA Discovery Studio.

2.18. Statistical analysis

Experiments were carried out in triplicate (n=3), and the data were expressed as mean ± SD. Significance level between the experimental groups at P<0.05 was calculated using the one-way analysis of variance (ANOVA) tool of GraphPad Prism V5 (GraphPad Software, USA), followed by the HSD *post hoc* test to compare the values between the experimental groups.

2.19. Ethical statement

This study was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa with protocol number: AREC/00002325/2021 on 10th August 2021.

3. Results

3.1. Polyphenol concentration of H. caffrum

The estimated concentration of total phenolics present in *H. caffrum* bark infusion was 408 mg GAE/g.

3.2. Antioxidant activities of H. caffrum

In Supplementary Figure 1A, *H. caffrum* infusion showed significantly higher Fe^{3+} reducing activities than Trolox at all the tested concentrations (20-320 µg/mL) (P<0.05). The infusion also significantly scavenged hydroxyl and DPPH free radicals in a dose-dependent manner (P<0.05), as depicted in Supplementary Figure 1B and 1C, respectively. The lower IC_{50} values (32.3 µg/mL for $OH^{\bullet}$ scavenging assay and 45.2 µg/mL for DPPH scavenging assay) of the infusion indicate its better antioxidant property over Trolox (Supplementary Table 1).

3.3. Carbohydrate digestive enzyme inhibitory activities of H. caffrum

As depicted in Supplementary Figure 2, *H. caffrum* infusion

inhibited α-amylase enzyme. At 160 and 320 μg/mL, it displayed significantly higher activities than the standard drug, acarbose (IC_{50}= 397.5 μg/mL) with a lower IC_{50} value (298.2 μg/mL). The infusion also showed remarkable dose-dependent inhibition of α-glucosidase, which was significantly higher than acarbose (P<0.05).

3.4. Lipase inhibitory activity of *H. caffrum*

As depicted in Supplementary Figure 3, *H. caffrum* infusion showed an inhibitory effect on pancreatic lipase. Although the infusion at 160 and 320 μg/mL had more pronounced activities than orlistat (P<0.05), its lower IC_{50} (88.8 μg/mL) in comparison with orlistat (40.9 μg/mL) suggested its weaker inhibition of the enzyme (Supplementary Table 1).

3.5. *H. caffrum* glucose uptake properties

The infusion dose-dependently increased glucose uptake in yeast cells (Figure 1A). Similarly, in isolated liver tissue (Figure 1B), the infusion at 40-320 μg/mL displayed remarkable glucose uptake activities (P<0.05).

3.6. Effect of *H. caffrum* on endogenous antioxidant capacity in oxidative hepatic injury

As displayed in Figure 2A-2C, $FeSO_4$ significantly decreased GSH content, SOD, and CAT activities in untreated liver

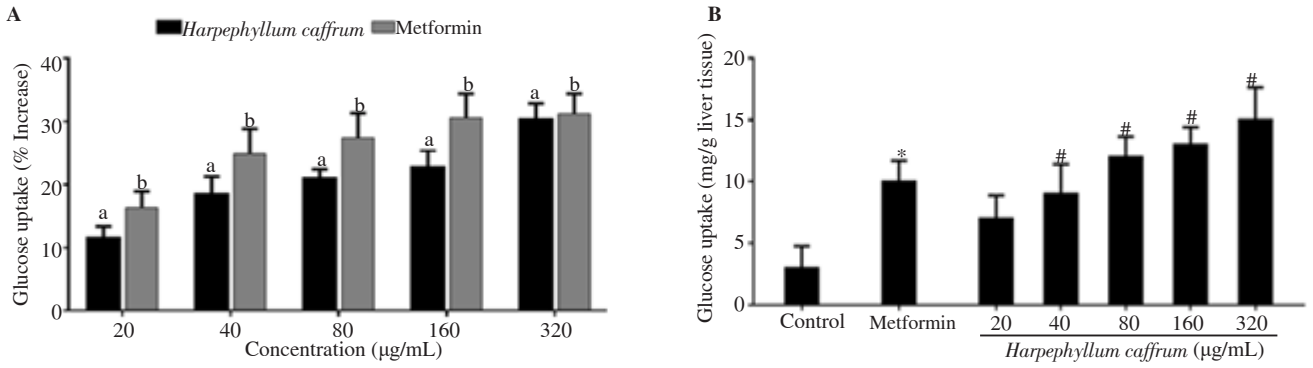

Figure 1. Effect of *Harpephyllum caffrum* infusion on glucose uptake in (A) yeast cell suspension and (B) isolated rat liver. Values are presented as mean ± SD of triplicate determinations. * and # indicate significant difference from the control and metformin groups, respectively at P<0.05. Different letters mean significant difference, P<0.05.

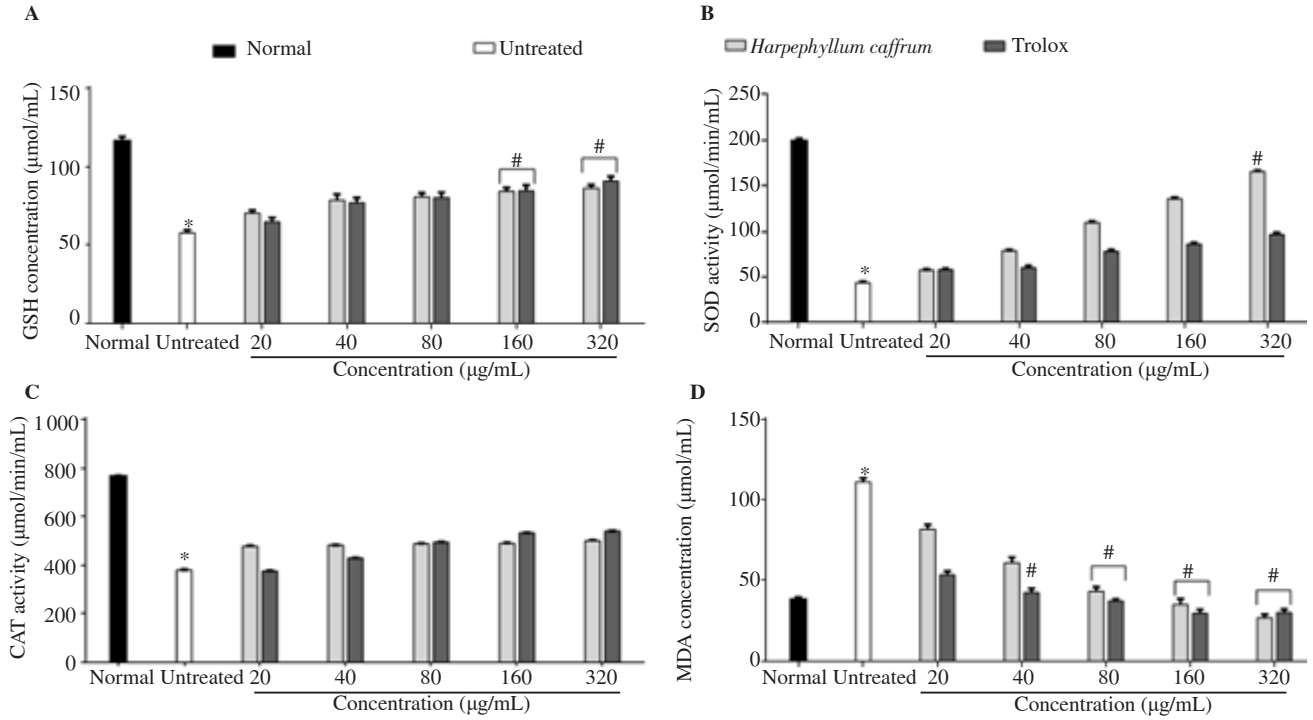

Figure 2. Effect of *Harpephyllum caffrum* infusion on (A) reduced glutathione (GSH) level, (B) superoxide dismutase (SOD) activity, (C) catalase (CAT) activity, and (D) malonaldehyde (MDA) in oxidative hepatic injury. Values are presented as mean ± SD of triplicate determinations. Bars with # and * are significantly (P<0.05) different from the untreated and normal groups, respectively.

homogenate (P<0.05), accompanied by an increase in MDA concentration (Figure 2D). Treatment with the infusion reversed the $FeSO_4$-induced changes, and it significantly raised SOD activity dose-dependently while reducing MDA (80-320 µg/mL) to a similar level to the normal control (P<0.05).

3.7. Effect of H. caffrum on NO level in oxidative hepatic injury

In Figure 3, $FeSO_4$ significantly elevated NO content in untreated liver homogenate (P<0.05). However, the infusion lowered NO level in the treatment groups.

3.8. Effect of H. caffrum on gluconeogenic enzyme activities in oxidative hepatic injury

$FeSO_4$ elevated FBPase and GP activities in untreated liver homogenate. The plant infusion at 40-320 µg/mL significantly lowered FBPase in a dose-dependent manner (P<0.05) (Figure 4A). Similarly, the infusion significantly decreased GP activities in the treatment groups at 160-320 µg/mL (P<0.05) (Figure 4B).

3.9. Effect of H. caffrum on ATPase activity in oxidative hepatic injury

ATPase activity was increased significantly by $FeSO_4$ (P<0.05). Treatment with the infusion reduced the enzyme activity to the levels significantly different from those in the untreated tissue sample (P<0.05) (Figure 5).

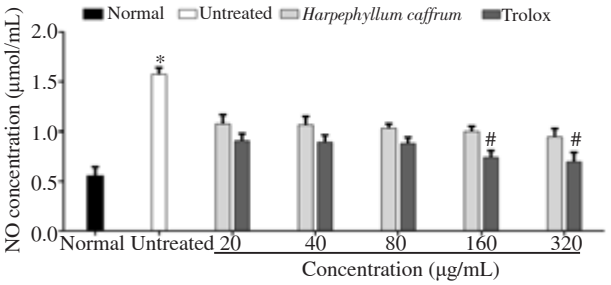

Figure 3. Effect of *Harpephyllum caffrum* infusion on nitric oxide (NO) level in oxidative hepatic injury. Values are presented as mean ± SD of triplicate determinations. Bars with # and * are significantly different from the untreated and normal groups, respectively at P<0.05.

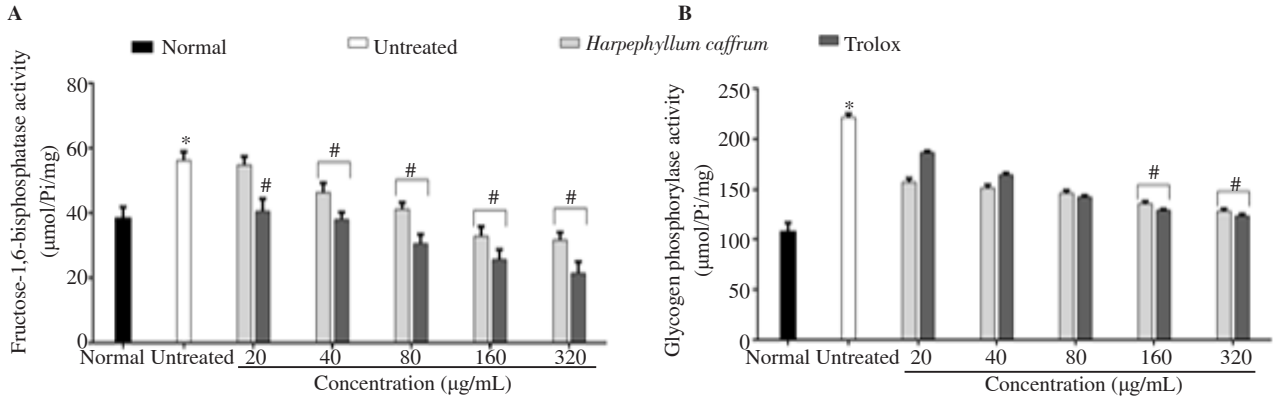

Figure 4. Effect of *Harpephyllum caffrum* infusion on (A) fructose-1,6-bisphosphatase, (B) glycogen phosphorylase activities in oxidative hepatic injury. Values are presented as mean ± SD of triplicate determinations. Bars with # and * are significantly different from the untreated and normal groups, respectively at P<0.05.

Table 1. Bioactive compounds identified in *Harpephyllum caffrum* infusion.

Compounds	Retention time (Minute)	Relative abundance (%)
Maltol	6.917	0.47
Mequinol	8.401	0.95
Catechol	8.560	1.76
2-Methoxyhydroquinone	8.946	1.23
Hydroquinone	9.515	2.58
Hydroquinone, acetate	9.665	1.38
Protocatechuic acid	9.825	0.38
Pyrogallol 1-methyl ether	10.860	0.63
10-Undecenyl hexofuranoside	13.967	0.82
Phytol, acetate	15.688	0.79
-(+)-Ascorbic acid 2,6-dihexadecanoate	17.044	2.84
24,25-Dihydroxyvitamin D	22.540	0.17
Hydrocortisone acetate	22.915	0.75
Retinal	23.075	0.77
Strophanthidol	23.570	0.46

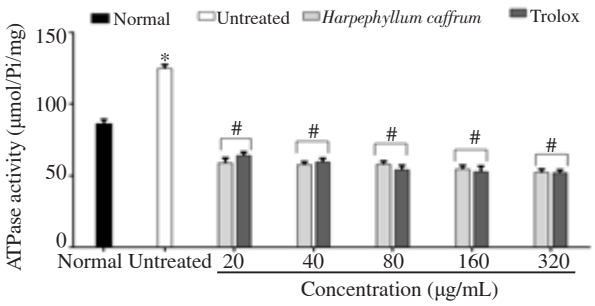

Figure 5. Effect of *Harpephyllum caffrum* on ATPase activity in oxidative hepatic injury. Values are presented as mean ± SD of triplicate determinations. Bars with # and * are significantly different from the untreated and normal groups, respectively at $P<0.05$. ATPase: adenosine triphosphatase.

3.10. H. caffrum GC–MS analysis

GC-MS data in Table 1 displays chemical compounds identified in *H. caffrum*. The analysis revealed the presence of phenols, namely catechol, maltol, protocatechuic acid, and mequinol. Additionally, (+)-ascorbic acid 2,6-dihexadecanoate, 10-undecenyl hexofuranoside, hydrocortisone acetate, phytol acetate, and strophanthidol were also found in the infusion. The binding energies of some of these phytochemicals with antidiabetic (α-amylase; α-glucosidase) and antioxidant (CAT; SOD) protein targets are presented in Table 2.

3.11. H. caffrum bioactive compounds–protein interaction

Figure 6 gives 2D images of active site amino acids interaction of strophanthidol and α-amylase (Figure 6A), hydrocortisone acetate and α-glucosidase (Figure 6B), as well as 24,25-dihydroxyvitamin D and SOD and CAT (Figure 6C-D). The chemical compounds interacted with the proteins *via* molecular forces, including van da Waal forces, hydrogen bonds, carbon-hydrogen bonds, *etc*. Strophanthidol shared two strong hydrogen bonds with α-amylase. In contrast, hydrocortisone acetate had a single hydrogen bond with α-glucosidase, while 24,25-dihydroxyvitamin D formed a single and double bond with CAT and SOD, respectively.

Table 2. Calculated binding energies of *Harpephyllum caffrum* phytochemical constituents with antioxidant and carbohydrate metabolizing enzymes (kcal/mol).

Compounds	α-Amylase	α-Glucosidase	SOD	CAT
Catechol	−5.0	−5.4	−4.0	−5.6
Protocatechuic acid	−5.6	−6.1	−4.4	−7.0
Phytol, acetate	−6.2	−5.3	−4.8	−8.3
-(+)-Ascorbic acid 2,6-dihexadecanoate	−5.9	−5.8	−4.3	−8.1
24,25-Dihydroxy vitamin D	−9.0	−6.0	−6.4	−8.9
Hydrocortisone acetate	−8.8	−7.0	−5.9	−7.8
Strophanthidol	−9.5	−6.4	−6.3	−5.7

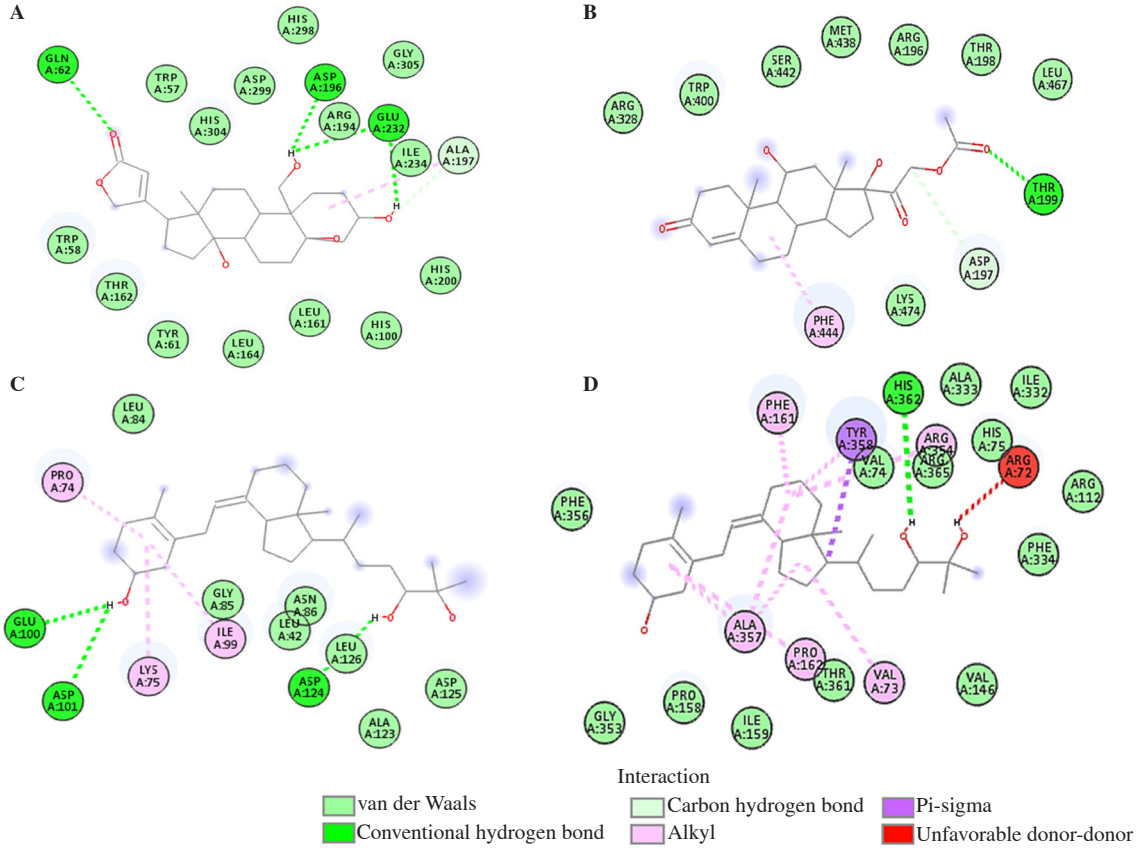

Figure 6. 2D images showing chemical interactions between *Harpephyllum caffrum* bioactive compounds and amino acid residues involved in the catalytic site of key antidiabetic and endogenous antioxidant enzymes. (A) Strophanthidol and α-amylase; (B) Hydrocortisone acetate and α-glucosidase; (C) 24,25-Dihydroxyvitamin D and SOD; (D) 24,25-Dihydroxyvitamin D and CAT.

4. Discussion

The use of medicinal plants in the traditional management of T2D diabetes has been reviewed extensively in previous articles[29]. Evidence from experimental studies has ascribed the antidiabetic pharmacological properties of these plants to their inherent phytochemical constituents[9]. However, detailed reports elucidating the mechanism through which the plants exert their interesting biological properties are scanty. *H. caffrum* bark aqueous extract has been reported to show antidiabetic activity in rats[32]. Since the liver plays a critical role in blood glucose regulation, this study investigated the possible mechanism underlying the antioxidant and antidiabetic activities of *H. caffrum* bark infusion using *in vitro* models.

Reactive oxygen species (ROS) constitute the free radical and non-radical species produced in various physiological processes in normal cells[33]. Although non-radical ROS such as NO may function in metabolic signaling, other free radicals like $OH^{\bullet}$ and $O_2^{\bullet-}$ can damage cellular macromolecules. Endogenous antioxidant molecules protect body tissues by donating electrons to these free radicals and limiting their harmful effects. The increasing ferric reducing power of the infusion coupled with its high DPPH and $OH^{\bullet}$ radical scavenging activities indicates its potent antioxidant properties, which could be attributed to the total phenol content of this plant. Interestingly, GC-MS bioactive phytochemicals such as catechol, protocatechuic acid, and dihydroxyvitamin D identified in *H. caffrum* infusion have been reported to have potent antioxidant properties[34]. These findings are similar to the study of Moodley *et al*.[10] where catechin and different alkyl *p*-coumaric acid esters (cardanols) isolated from *H. caffrum* bark extract possessed impressive free radical scavenging activities.

Reducing plasma blood glucose *via* inhibiting intestinal carbohydrate catabolic enzymes has been explored as a target in diabetes management[29]. In this regard, plant-derived products have shown promising antidiabetic potency due to their ability to inhibit α-amylase and α-glucosidase enzymes, thus delaying glucose absorption[9]. The infusion's ability to repress the carbohydrate hydrolyzing enzymes showed its antidiabetic potency, which may be associated with its bioactive compounds. The negative binding energies of these compounds and, more importantly, the formation of strong hydrogen bonds with active site amino acid moieties of α-amylase and α-glucosidase enzymes may suggest excellent modulatory activities. The infusion's inhibitory effect on the intestinal carbohydrate degrading enzymes may explain the observed *H. caffrum* bark hypoglycemic property documented in the previous findings[32].

Excessive breakdown of triacylglycerol with concomitant elevation of plasma-free fatty acid has been reported in T2D-related obesity with insulin resistance[35]. This condition that is attributed to elevated lipase activity increases lipid availability for absorption through the gastrointestinal tracts. Interestingly, the evidence suggests that lipase inhibitors present explorable therapeutic options for diabetic obesity[36]. The infusion demonstrated pancreatic lipase inhibitory properties that might have resulted from its chemical constituents. The anti-lipase activities of protocatechuic acid and other phenolic compounds have been well documented[36]. Lambrechts *et al*.[37] indicates the topical application of *H. caffrum* bark powder in local acne treatment. Since lipase produced by *Propionibacterium acnes* is linked with skin acne[38], the inhibitory effect of the infusion on this enzyme as observed in this study may further give credence to its previously reported medicinal use[37].

Under physiological conditions, insulin triggers postprandial hypoglycemia by signaling events leading to glucose uptake in body cells. However, in T2D, insulin insensitivity impedes this process, thus resulting in chronic hyperglycemia. Therefore, chemical agents capable of improving cellular glucose uptake are considered as another potential target for diabetes therapy. Interestingly, the infusion enhanced glucose uptake in yeast cells and the liver tissue. This observation may be indicative of its promotion of hepatic glucose transport. Insulin stimulates glucose transporter 2 to increase glucose uptake in the liver depending on glucose concentration in the bloodstream[39]. This study is similar to the finding by Ho *et al*.[40], where berry extracts enhance glucose uptake in human hepatic cell lines.

In normal cells, endogenous antioxidants such as CAT, SOD, and GSH mitigate the damaging effects of free radicals on macromolecules. During the respiratory process in the mitochondria, SOD converts superoxide radical ($O_2^{\bullet-}$) from singlet oxygen reduction into hydrogen peroxide (H_2O_2). In the Fenton reaction, H_2O_2 produces hydroxyl radical ($OH^{\bullet}$) in the presence of Fe^{2+}. CAT, in turn, lowers the cellular concentration of H_2O_2 by hydrolyzing it to water and molecular oxygen. The depressed SOD and CAT activities with GSH reduction after the induction of oxidative hepatic injury may indicate suppressed intrinsic antioxidant capacities. The high MDA level in the untreated liver homogenate may further suggest redox imbalance. However, the infusion's ability to improve the enzyme capacities with a simultaneous reduction in MDA concentration may show its antioxidant activity similar to those observed previously. Moreover, the formation of strong hydrogen bonds and other interaction forces between the phytochemicals of the plant infusion and the active site amino residues of CAT and SOD may suggest a modulatory effect. While oxidative stress is involved in ageing, Chen *et al*.[41] reported that 1,25-dihydroxyvitamin D, a compound identified in *H. caffrum*, stalled this process in mice by activating pathways that ultimately increase the cellular antioxidant system.

Inducible nitrogen synthase found in hepatic Kupffer cells, cholangiocytes, and stellate cells produce high NO levels in different liver pathologies[42]. When NO reacts with $O_2^{\bullet-}$ radicals under suppressed SOD activities, it produces peroxynitrite (ONOO-). Thus, ONOO- further aggravates oxidative stress and thus causes lethal damage to protein, lipid, and DNA molecules. Elevated NO level in untreated liver homogenate may suggest inflammation resulting from oxidative hepatic injury. Nevertheless, the reduction in NO after treatment with the infusion demonstrates

anti-inflammatory properties. *In vitro* findings from previous experiments have shown that extract from *H. caffrum* plant possesses cyclooxygenase inhibitory activities[43].

The liver utilizes gluconeogenesis and glycogenesis pathways to produce glucose to release into the bloodstream during the fasting state. However, in the fed state, insulin signals deactivate the processes. Hepatocyte inability to detect insulin signal in T2D exacerbates hyperglycemia by mobilizing more glucose from non-carbohydrate molecules. This latter process exacerbates hyperglycemia and its associated complications. The elevated FBPase and GP activities in the untreated liver homogenate may cause the release of more glucose molecules from the liver tissue. The ability of the infusion to lower these enzyme activities to a normal level may indicate its hypoglycemic effect. This study corroborates the findings documented by Ojewole[32] where *H. caffrum* bark aqueous extract lowered fasting blood glucose in diabetic rats.

Alterations in hepatic ATP levels have been implicated as one of the mechanisms of drug-induced hepatoxicity[44]. This is depicted in the present study by the increased ATPase level following the induction of oxidative hepatic damage, which insinuates a reduction in the cellular level of ATP. The reduced ATPase activity following treatment with *H. caffrum* suggests a restorative effect on hepatic ATP levels, indicating the ability of the infusion to protect against alteration in hepatic ATP concentration resulting from oxidative attack.

In conclusion, *H. caffrum* is an underutilized tropical food plant with reported hypoglycemic activities. This study demonstrates that the aqueous infusion of the plant enhances hepatic glucose uptake while modulating key enzymes of type 2 diabetes. The infusion's ability to attenuate redox imbalance in oxidative hepatic injury may give further credence to its previously reported antioxidant activity. The plant contained several active chemical agents that may account for its pharmacological activities; therefore, more studies are required to determine specific compounds responsible for its antidiabetic properties. Moreover, detailed *in vivo* studies are necessary to understand further the effect of this plant on the expression of protein targets identified in type 2 diabetes management.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

KAO conceptualized the study, collected data, and wrote the original draft. KAO and BKB performed data validation and statistical analysis. OLE reviewed and edited the manuscript while MSI was responsible for resources acquisition and supervision of the project.

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