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Rhus longipes (Engl.) infusions improve glucose metabolism and mitigate oxidative biomarkers in ferrous sulfate-induced renal injury

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ABSTRACT

Objective: To explore the antioxidant and antidiabetic activities of *Rhus longipes* (*R. longipes*) leaf and stem bark aqueous infusions.

Methods: *R. longipes* leaf and stem bark infusions were characterized *via* gas-chromatography mass-spectroscopy (GC-MS) analysis. *In vitro* antioxidant and carbohydrate and lipid digestive enzyme inhibitory activities of *R. longipes* infusions were determined. Additionally, the modulatory effects of *R. longipes* infusions on intestinal glucose absorption, muscle glucose uptake, and biomarkers of renal oxidative injury were evaluated. Molecular docking was performed to determine the binding affinities of the identified compounds from the leaf and stem bark infusions on carbohydrate and lipid digestive enzymes.

Results: GC-MS analysis revealed the presence of several phytocompounds, including palmitoleic acid, octadecanamide, 24,25-dihydroxyvitamin *D* and *L*-ascorbic acid. The bark infusion had significantly higher total phenolic contents compared with the leaf infusion, with better DPPH scavenging [IC_{50}: (10.50±1.03) μg/mL] and ferric reducing [IC_{50}: (9.85±0.32) μg/mL] activities (P<0.05). Both *R. longipes* infusions at their highest concentrations significantly increased glucose uptake in yeast suspension and rat psoas muscle with marked suppression of glucose absorption in the rat jejunum (P<0.05). With no cytotoxicity on Vero cells, the infusions lowered lipid peroxidation, increased cellular reduced glutathione concentration, and the activities of superoxide dismutase and catalase in renal homogenate treated with $FeSO_4$.

Conclusions: *R. longipes* shows antioxidant and antidiabetic activities and could be a potential therapeutic candidate for diabetes.

KEYWORDS: Antioxidant; Oxidative biomarkers; Enzyme inhibition; Antidiabetic; *Rhus longipes*; *Searsia longipes*

1. Introduction

Diabetes mellitus (DM) is an enervating metabolic disorder characterized by chronic hyperglycemia, with type 1, type 2, and gestational diabetes being the most prevalent forms of diabetes[1,2]. The risk factors of DM include genetic predisposition, age, ethnicity, and unhealthy lifestyle[1]. Similarly, high-calorie diets and lack of physical activity, which progressively develop obesity, have also been implicated as major risk factors and comorbidity associated with diabetes pathology[2], when obesity is one of the major risk factors behind the development of type 2 diabetes.

In obese individuals, the elevated amounts of pro-inflammatory factors, non-esterified fatty acids, and cytokines lead to an increased cellular concentration of reactive free radicals, which ultimately

Significance

Some previous studies reported the antioxidative, antimicrobial and toxicological effects of *Rhus longipes* with some identified bioactive phytochemicals. However, there is scarce information on its antidiabetic activity. The present study shows the antioxidant and antidiabetic activities of the leaf and stem bark aqueous infusions of this plant in *in vitro* and *ex vivo* experimental models.

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suppress the activities of the body's intrinsic antioxidant enzymes, such as superoxide dismutase (SOD) and catalase[3]. Then, redox imbalance or oxidative stress that emerges allows the excess free radicals to react with DNA, proteins, and lipid biomolecules, which gradually lose their biological structure and function[4].

Another critical biochemical process associated with diabetes is insulin resistance. The development and progression of this condition at the cellular level occur when there is a systemic loss of function of cell surface receptors on insulin-responsive tissues. Besides the insulin resistance in diabetes, there is also the impairment of pancreatic β-cells function, resulting in reduced insulin production. These two situations synergistically lead to aberrant glucose metabolism, which is proceeded by hyperglycemia.

Chronic hyperglycemia has been implicated as an underlying cause of increased morbidity and premature mortality in diabetes-associated complications[5]. These complications, which include cardiovascular and cerebrovascular diseases, retinopathy, nephropathy, and neuropathy, have been well-documented in previous studies[6]. As part of the complications, findings have indicated that diabetes patients have higher odds of developing diabetic kidney disease than those without diabetes[7]. Interestingly, hyperglycemia-mediated oxidative damage to renal blood vessels has been linked to dysfunctional glomerular hemodynamics in diabetic nephropathy[8]. While there are many commercially available drugs for diabetes and its complications, available reports have shown that their utilization, especially by people living in low to middle-income countries, is limited by undesirable side effects and higher costs[9]. In South Africa, up to 80% of people rely on or consult with traditional medicine practitioners for their primary healthcare needs[10]. Consequently, the efficacies of these traditional medicines need to be scrutinized since there is no documented evidence of their standardized mode of action[9].

Rhus longipes (*R. longipes*), also referred to as *Searsia longipes*, is a small tree with long drooping stems and branches of the family Anacardiaceae that bears small fleshy raisin-tasting fruits[11]. It is commonly known as Inhlokotshiyane (Zulu), large-leaved rhus (English), or Mufokosiana (Shona). *R. longipes* roots are traditionally used to treat infertility in women, while Zimbabweans in Mashonaland used leaves to alleviate diabetes-related symptoms[12]. Moreover, in Southwest Nigeria, the plant has been reported to manage asthma and malaria[13]. In this study, Olorunnisola *et al*.[13] reported the protective effect of *R. longipes* leaf extract in paracetamol-induced oxidative stress in Wistar rats. Another study reported the antioxidant and antimicrobial activities as well as identified the bioactive compounds in *R. longipes* extracts[14].

However, to the best of our knowledge, the antidiabetic potentials of *R. longipes* remain poorly investigated. Although *R. longipes* is used in managing diabetes and its complications in traditional medicine in many African countries, its proper scientific validation in this regard has not been done.

Therefore, this study was conducted to evaluate the antidiabetic activity of *R. longipes*, as well as antioxidative potentials in oxidative renal injury using different experimental models.

2. Materials and methods

2.1. Plant extract preparation

2.1.1. Plant collection and verification

The leaves and stem bark of *R. longipes* were collected from the Westville area in KwaZulu Natal province, South Africa. The plant was selected based on its folkloric use as an antidiabetic agent by traditional healers and village elders in KwaZulu Natal Province. The plant was sampled from the same soil strata within a 2 km radius. The plant identity and authentication were done by Dr Ramdhani and Mr Khathi (curators) at Ward Herbarium, School of Life Sciences, University of KwaZulu-Natal (Specimen voucher number BB-003-WV10/19).

2.1.2. Plant infusion preparation

Air-dried leaves and bark of plant materials were ground into fine powder using a domestic warring blender. The plant material was then defatted overnight with *n*-hexane and dried. Dry powdered plant material (100 g) was then infused in boiling distilled water (1 000 mL) and allowed to stand overnight. The supernatants were filtered using a Whatman No.1 filter paper into pre-weighed glass vials and concentrated in a water bath at 50 ℃. The plant infusions were then stored in amber air-tight 10 mL glass vials to be protected from light until further analysis. Stock solutions (1 mg/mL) of dry plant infusions were prepared by reconstituting them in distilled water.

2.2. Phytochemical characterization and quantification

2.2.1. Total phenolic content

The total phenolic content of the leaf and bark infusions was determined spectrophotometrically using the Folin-Ciocalteu's phenol reagent method[15,16]. Briefly, 240 μg/mL of the different infusions (20 μL) were added to 10% Folin-Ciocalteu reagent (100 μL) and left to stand for 5 min in the dark at 25 ℃. Thereafter, 0.7 M sodium carbonate (80 μL) solution was added. The mixture was allowed to stand for 30 min in the dark at 25 ℃. The absorbance of independent triplicates was measured at 765 nm using a multimode microtiter plate reader (Synergy HTX, BioTek Instruments, Sata Clara, CA, USA). The total phenolic content was determined by linear regression from a gallic acid calibration standard curve. Results were expressed as gallic acid equivalents (GAE) in milligrams per gram of dry weight.

2.2.2. Total flavonoid content

The aluminum chloride colorimetric method was used for the determination of total flavonoids[15,16]. Briefly, 240 μg/mL of the different infusions were prepared. Each of the infusions (10 μL) was mixed with 10% aluminum chloride (10 μL), 1 M potassium acetate (10 μL), and distilled water (200 μL). The mixture was left to stand at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in triplicates. The total flavonoid content was determined by linear regression from a quercetin calibration standard curve. Results were expressed as quercetin equivalent (QE) in milligrams per gram of dry weight.

2.2.3. Gas chromatography–mass spectroscopy (GC–MS) analysis

R. longipes leaf and bark infusions were subjected to GC-MS analysis using Agilent technology 6890 series GC coupled with (an Agilent) 5973 Mass Selective Detector, driven by Agilent Chemstation software. An HP-5MS capillary column (30 m×0.25 mm ID, 0.25 μm film thickness, 5% phenylmethyl siloxane) was used for the analysis with ultra-pure helium as the carrier gas and at a flow rate of 1.0 mL/min and a linear velocity of 37 cm/s. The injector temperature was set at 250℃. The oven temperature was programmed to 280℃ from 60℃ at the rate of 10℃/min with a hold time of 3 min. One microliter (1 μL) injection of each sample was made in split mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV, while the electron multiplier voltage at 1 859 V. The ion source temperature was 230℃, and the quadrupole temperature 150℃. The solvent delay was set at 4 min, with a scan range of 50-70 amu. The direct comparison of the retention times and mass spectral data in the NIST library was used for identifying the compounds.

2.3. In vitro antioxidant analysis

2.3.1. Quantitative DPPH radical scavenging activity assay

The antioxidant activity of the different infusions was quantitatively determined spectrophotometrically using a slightly modified DPPH free radical scavenging assay[17]. Equal volumes of 0.3 mM DPPH in methanol and different concentrations (0-240 μg/mL) of the infusions were incubated in the dark at room temperature for 30 min. The DPPH in methanol solution was used as the experimental control, while *L*-ascorbic acid (vitamin C) as the reference standard. The degree of discoloration and decrease in absorbance was measured against a blank solution at 517 nm, and the percentage scavenging activity was calculated.

2.3.2. Ferric reducing antioxidant power (FRAP) assay

The ferric ion-reducing capacities of the different infusions were determined by FRAP assay[18]. Varying concentrations (15-240 μg/mL) of the infusions in deionized water (100 μL) were prepared. A blank was prepared without infusion, while *L*-ascorbic acid (vitamin C) was used as the reference standard. They were then mixed with 250 μL of phosphate buffer (0.2 M, pH 7.4) and potassium ferricyanide (250 μL) before incubation at 50℃ for 20 min. Then aliquots of trichloroacetic acid (250 μL) were added to the mixture and centrifuged at 3 000 rpm for 10 min. The supernatant (250 μL) was mixed with distilled water (250 μL) and freshly prepared ferric chloride solution (50 μL). The absorbance of the samples was measured at 700 nm, and the percentage reducing power was calculated.

2.3.3. Nitric oxide scavenging assay

The nitric oxide scavenging assay was carried out as previously described[19]. The leaf and bark infusion samples (50 μL) at increasing concentrations (15-240 μg/mL) were incubated with equal volumes of sodium nitroprusside (10 mM) in phosphate buffer (pH 7.4) at 37℃ for 2 h. Thereafter, Griess reagent (50 μL) was added to the reaction mixture and mixed gently by tapping the side of the plate. The resultant chromophores' absorbance was read at 546 nm, and the percentage inhibition of nitric oxide generated was calculated.

2.4. In vitro enzyme inhibition analysis

2.4.1. α–Glucosidase inhibition assay

The ability of the infusions to inhibit α-glucosidase *in vitro* was carried out according to the method previously described[20,21]. Plant infusion samples (50 μL) at increasing concentrations (15-240 μg/mL) were incubated with an equal volume of α-glucosidase (1.0 U/mL) in phosphate buffer (100 mM, pH 6.8) at 37℃ for 15 min. Thereafter, 100 μL of 5 mM *p*-nitrophenyl-α-*D*-glucopyranoside solution in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture, which was further incubated for 20 min at 37℃. Acarbose was used as the reference standard. The absorbance of liberated *p*-nitrophenol was measured at 405 nm, and the inhibitory activity was expressed as a percentage of the experimental control lacking inhibitors/infusion.

2.4.2. α–Amylase inhibition assay

The ability of *R. longipes* infusions to inhibit α-amylase *in vitro* was carried out according to the method previously described[22]. Plant infusion samples (50 μL) at increasing concentrations (15-240 μg/mL) were incubated with equal volumes of porcine pancreatic amylase (2 U/mL) in phosphate buffer (100 mM, pH 6.8) for 10 min at 37℃. An aliquot of 1% starch solution (50 μL) in phosphate buffer (100 mM, pH 6.8) was added to the reaction mixture and incubated at 37℃ for 10 min. Dinitrosalicylic acid color reagent (100 μL) was then added to the mixture and boiled for 10 min. Acarbose was used as a positive control (standard drug). The absorbance was then read at 540 nm, and the inhibitory activity was

expressed as a percentage of control without inhibitors.

2.4.3. Porcine pancreatic lipase inhibition assay

The ability of *R. longipes* infusions to inhibit pancreatic lipase, a lipid digestive enzyme *in vitro*, was carried out according to the previously described method[23]. The leaf and bark infusion samples (100 µL) at increasing concentrations (15-240 µg/mL) were incubated with 20 µL of porcine pancreatic lipase (2.5 mg/mL) buffered solution. The buffer system contained 10 mM 3-(*N*-morpholino)propane sulfonic acid and 1 mM EDTA at pH 6.8. After 15 min incubation at 37℃, 169 µL of Tris buffer (100 mM Tris-HCl and 5 mM $CaCl_2$, pH 7.0) was added. Orlistat was used as the reference standard, while the experimental control had enzyme only. The substrate solution of 5 µL of 10 mM *p*-nitrophenyl butyrate (*p*-NPB) in dimethyl formamide was then added to initiate the reaction. After gentle mixing, the solution was further incubated for 30 min at 37℃. The lipase activity was determined by measuring the hydrolysis of *p*-NPB to *p*-nitrophenol at 405 nm. The inhibitory activity was expressed as a percentage of the experimental control lacking inhibitors/infusion.

2.4.4. Mode of α-amylase inhibition assay

The mode of inhibition of α-amylase enzyme by leaf and bark infusions was investigated using their IC_{50} value as determined previously from the inhibition assay through linear regression[24]. The leaf or bark infusions (250 µL) were preincubated with α-amylase (250 µL) (2 U/mL) solution at 37℃ for 10 min in one set of tubes. In another set of tubes, α-amylase was preincubated with phosphate buffer (250 µL) (100 mM, pH 6.8). After that, starch (250 µL) solution at increasing concentrations (0.0-8.0 mM) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated at 37℃ for 10 min. Dinitrosalicylic acid (100 µL) was then added to the mixture to stop the reaction and boiled for 10 min. The amount of reducing sugars released was determined spectrophotometrically at 540 nm using a maltose standard curve and converted to reaction velocities.

2.4.5. Mode of α-glucosidase inhibition assay

The mode of inhibition of α-glucosidase enzyme by leaf and bark infusions was investigated using their IC_{50} value determined previously from the inhibition assay through linear regression as described[24]. The leaf or bark infusions (50 µL) were preincubated with aliquots of 1.0 U/mL α-glucosidase (100 µL) solution at 37℃ for 15 min in one set of tubes. In another set of tubes, α-glucosidase was preincubated with phosphate buffer (50 µL) (100 mM, pH 6.8). *p*-Nitrophenyl-α-*D*-glucopyranoside (50 µL) at increasing concentrations (0.0-10.0 mM) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated at 37℃ for 15 min before 500 µL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically using a para-nitrophenol standard curve and converted to reaction velocities.

2.4.6. Mode of pancreatic lipase inhibition

The mode of inhibition of porcine pancreatic lipase by the leaf and bark infusions was investigated using their IC_{50} value determined previously from the inhibition assay through linear regression[25]. The leaf or bark infusions (100 µL) were preincubated with aliquots of 2.5 mg/mL porcine pancreatic lipase (20 µL) solution at 37℃ for 15 min. Porcine pancreatic lipase preincubated with the buffer was used as a control. A 5 µL of substrate solution (10 mM *p*-NPB) was then added to initiate the reaction (0.0-10.0 mM). After gentle mixing, the solution was further incubated for 30 min at 37℃. The absorbance was measured at 405 nm, and the amount of *p*-nitrophenol released from *p*-NPB hydrolysis was calculated from a *p*-nitrophenol standard curve before conversion to reaction velocities.

2.4.7. Mode of inhibition analysis

The modes of inhibition of α-amylase, α-glucosidase, and pancreatic lipase were analyzed by constructing Lineweaver-Burk plots (double reciprocal plots) from the reaction velocities (V_0) and substrate concentrations (S). From this plot, the Michaelis constant (K_m) and the maximum rate of the enzymatic reaction (V_{max}) were obtained using the expression below:

$$1/V_0=K_m/V_{max}[S]+1/V_{max}$$

2.5. Cell lines and cytotoxicity assay

Vero monkey kidney epithelial cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured at 5% CO_2, and 37℃ in a Dulbecco's Modified Eagle Medium (Sigma, South Africa) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The culture medium was replaced frequently with a fresh medium until the cells reached a 70%-80% confluency. The cultured cells were also constantly observed under a light microscope (Nikon TS100, Germany) for their attachment, viability, and morphological changes during this period.

The *in vitro* screening of the cytotoxicity of leaf and bark infusions in Vero cells was carried out *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier[26]. The cells were resuspended in fresh media to a seeding concentration of 2 500 cells/mL. Then, they were seeded into 96 well microplates for 24 h and incubated at 37℃ in a 5% CO_2 incubator, and allowed to re-attach before exposure to increasing concentrations of infusions (15-240 µg/mL) for a further 24 h. After treatment, 20 µL of MTT reagent (5 mg/mL in PBS) was added to each well, and the plates were incubated for 4 h. The spent media was carefully aspirated, and intracellular purple formazan crystals formed were resolubilized by

the addition of acidified isopropanol (100 μL). The absorbance was measured at 570 nm in a multiplate reader, and the percentage of cell viability was calculated.

2.6. Glucose uptake/transport by yeast cells

The effect of the *R. longipes* leaf and bark infusions on glucose uptake/transport by yeast cells was carried out according to a previously established protocol[27]. The dry *R. longipes* leaf and bark infusions were reconstituted in 1 mL of distilled water containing 25 mM glucose to desired concentrations of 15 μg/mL-240 μg/mL. The resulting solutions were equilibrated for 10 min at 37℃. Thereafter, 100 μL of 1% yeast [Gold star instant yeast, Rymco (Pty) Ltd., Johannesburg, South Africa] suspension was added, vortexed, and incubated for a further 60 min at 37℃. Metformin was used as a reference standard drug. The glucose concentration of the solution was determined with dinitrosalicylic acid method, and glucose uptake (%) was calculated using the formula:

$$\% \text{ Glucose uptake} = \frac{(A_{540nm} \text{ of sample} - A_{540nm} \text{ of control}) \times 100}{A_{540nm} \text{ of control}}$$

2.7. Ex vivo study

2.7.1. Experimental animals

Male Sprague-Dawley rats weighing between 180-200 g were obtained from the Biomedical Research Unit, University of KwaZulu-Natal, Durban, South Africa. They were acclimatized for a week and supplied with standard pellet rat chow and water *ad libitum*. During this period, a standard laboratory condition of a 12 h light-dark cycle was maintained. After the animals fasted for 12 h overnight, they were subjected to euthanasia before being sacrificed. The muscles, intestines, and kidneys were harvested immediately for use in *ex vivo* studies.

2.7.2. Glucose uptake in excised rat psoas muscles

The effects of plant infusions on glucose uptake in excised rat psoas muscles were determined according to a previously described procedure[28]. Psoas muscles collected from the rats were sectioned into 0.5 g pieces. The pieces were then incubated in tubes containing 8 mL each of Glucose-Krebs solution (GKS) only (control), GKS solution was added with different concentrations of plant infusions or with 100 U/mL insulin solution (positive control) for 1 h at 37℃ in an incubator with 5% CO_2 and 95% oxygen. Then, aliquot solutions (1 mL) were collected from each incubation tube before and after the incubation with muscle tissue. The glucose concentrations were determined using a glucose kit (Thermo Scientific) in a Labmax Plenno Chemistry Analyzer (Labtest Inc., Costa Brava, Brazil), and the extent of glucose uptake was calculated using the following formula:

Muscle glucose uptake=(GC1–GC2)/0.5 g of muscle tissue

Where "GC1" and "GC2" are glucose concentrations (mg/dL) before and after incubation, respectively.

2.7.3. Intestinal glucose absorption

The effects of the *R. longipes* leaf and bark infusions on glucose absorption were evaluated in isolated rat intestinal mucosa using a previously described technique[28]. The jejunum of rats' gastrointestinal tract (GIT) was sectioned into equal portions (5 cm in length). The partitioned sections were rinsed by injecting 2 mL of Kreb's buffer through the jejunal lumen with a sterile syringe before their inversion to expose their inner wall and villi. The inverted sections were then incubated in tubes containing 8 mL each of GKS solution only (control), GKS solution with different concentrations of plant infusions and GKS solution with 3 mM acarbose solution (positive control) for 2 h at 37℃ under 5% CO_2 and 95% oxygen in an incubator. Aliquots (1 mL) were collected from each incubation tube before and after the incubation with intestinal tissue. The glucose concentrations were determined using a glucose kit (Thermo Scientific) in a Labmax Plenno Chemistry Analyzer (Labtest Inc., Costa Brava, Brazil), and the extent of glucose absorption was calculated using the following formula:

Intestinal glucose absorption=(GC1–GC2)/5 cm of jejunum

Where "GC1" and "GC2" are glucose concentrations (mg/dL) before and after incubation, respectively.

2.7.4. Preparation of kidney homogenates and induction of oxidative stress ex vivo

The harvested kidneys were homogenized in a phosphate buffer (0.2 mM, pH 6.9) and centrifuged at 3 500 ×*g* for 10 min (4℃). The supernatant was transferred to sample tubes and stored at −20℃ for biochemical analysis.

Aliquots of the kidney homogenates (100 μL) were incubated with an equal volume of the plant infusion and the pro-oxidant, 0.1 M $FeSO_4$ (30 μL), for 30 min at 37℃. The reaction containing no infusion (untreated) served as a negative control, while ascorbic acid served as a positive control. After incubation, the modulatory effects of plant infusions on the activities and concentrations of selected oxidative biomarkers were analyzed.

2.7.5. Studies on the biomarkers of oxidative stress

2.7.5.1. Determination of lipid peroxidation levels

Lipid peroxidation levels of the samples were determined by measuring thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalent[29]. Samples (100 μL) were mixed with an equal volume of 8.1% SDS solution, 375 μL of 20% acetic acid, 1 mL of 0.25% thiobarbituric acid, and 425 μL of distilled water. The reaction mixture was boiled at 95℃ for 1 h in a water bath. After cooling, the boiled mixture (200 μL) was then pipetted into a 96-well plate, and the absorbance was read at 532 nm. The TBARS concentration of the samples was obtained by

extrapolation from an MDA standard curve.

2.7.5.2. Determination of reduced glutathione (GSH) levels

GSH levels in the kidney homogenates were determined according to a previously described method[30]. Homogenates (200 μL) were mixed with an equal volume of 10% TCA to precipitate proteins and then gently mixed and centrifuged at 3 500 rpm for 5 min (25 ℃). After that, aliquots of the supernatant (200 μL) were pipetted into a 96-well plate. Ellman reagent (50 μL) was then added, and the plate was allowed to stand for 5 min for the color to develop. After the absorbance was read at 415 nm, the GSH level was then extrapolated from the standard curve of plotted GSH concentrations.

2.7.5.3. Determination of SOD activities

SOD activity in the kidney homogenates was determined using a previously described method[31]. Briefly, 0.170 mL diethylenetriaminepentaacetic acid (0.1 mM) was added to the samples (15 μL) in a 96-well plate. Thereafter, 1.6 mM 6-hydroxydopamine (15 μL) was added and mixed by gently tapping all four sides of the plates. The absorbance of the chromophore generated from the oxidation of 6-hydroxydopamine by H_2O_2 was immediately measured at 492 nm for 5 min at 1 min intervals.

2.7.5.4. Determination of catalase activities

The catalase enzyme activity in kidney homogenate was determined according to a previously described method[32]. The homogenates (100 μL) were incubated with 65 μM H_2O_2 (1 mL) prepared in 6 mM sodium phosphate buffer (pH 7.4) for 2 min at 37 ℃. The reaction was terminated by the addition of 32.4 mM ammonium molybdate (5 μL). Then, the absorbance of the resulting chromophore (molybdate/H_2O_2 complex) was measured at 347 nm.

2.8. Molecular docking screening

This virtual screening was done using PyRx V 0.8 software which uses Autodock Vina simulation engine[33]. The grid center and box sizes for each protein docking were set with maximized blind coordinates. X-ray crystal structures of α-amylase (PDB ID: 1B2Y, resolution: 3.20 Å), α-glucosidase (PDB ID: 3TOP, resolution: 2.88 Å), and pancreatic lipase (PDB ID: 1LPB, resolution: 2.46 Å) proteins responsible for carbohydrate and lipid digestion were downloaded from the Protein Data Bank website (https://www.rcsb.org/). The structures were prepared by removing all heteroatom coordinates and water molecules. This step was followed by the addition of hydrogens atoms, Kollman charges, and missing C-terminal oxygen. However, the plant compounds were prepared by minimizing their structural energy coefficients with Open Babel software after their 3D structures were retrieved from the PubChem database. The binding energy for the best poses of the protein-ligand complex was tabulated, and their 2D interaction details were visualized using Biovia Discovery Studio 2017R2 Client software.

2.9. Statistical analysis

All data are presented as mean±SD of 3 independent experiments. One-way analysis of variance (ANOVA) was used to determine statistical significance at $P<0.05$. The experimental statistical analysis was conducted using IBM SPSS for Windows, version 25.0 (SPSS Inc., Chicago, IL, USA).

2.10. Ethical statement

All animals used were maintained and processed under the guidelines and approval of the animal ethics committee of the University of KwaZulu-Natal, Durban, South Africa (protocol approval number: AREC/038/019D).

3. Results

3.1. Total phenolic and flavonoid contents of R. longipes

Both infusions contained significantly higher amounts of phenolics than flavonoids ($P<0.05$) (Supplementary Figure 1). The total polyphenol (60.16 mg/g GAE) of the bark infusion, however, was significantly higher than that of the leaf infusion ($P<0.05$).

3.2. GC–MS analysis of R. longipes

As shown in Table 1, GC-MS analysis of *R. longipes* leaf and bark aqueous infusion revealed the presence of *n*-pentadecanol, 2-ethyl-1-dodecanol, l-(+)-ascorbic acid 2,6-dihexadecanoate, palmitoleic acid, octadecanamide, 24,25-dihydroxyvitamin *D*, *cis*-11-eicosenamide, beta-*D*-mannofuranoside, 1-*O*-(10-undecenyl)-, 2-amino-1,3-propanediol, 1-heneicosanol, phytol, acetate, 5,15-dimethylnonadecane, *n*-nonadecanol-1, *cis*-vaccenic acid, *L*-ascorbic acid, 6-octadecanoic. Palmitoleic acid had the highest % relative abundance (1.04%) in the leaf infusion, while 2-amino-1,3-propanediol was the most abundant (7.35%) in the bark infusion. The compounds in the bark infusion had a higher percentage relative abundance than those in the leaf infusion of *R. longipes*. Although the two parts contained different phytocompounds, some compounds such as l-(+)-ascorbic acid 2,6-dihexadecanoate, octadecanamide, and *cis*-11-eicosenamide were present in both parts.

3.3. In vitro antioxidant properties of R. longipes

The infusions' DPPH scavenging and electron donating capabilities were dose-dependent (Supplementary Figures 2A and 2B). At lower concentrations (15-30 μg/mL), there was no significant difference

in radical scavenging activities of both leaf and bark infusions (Supplementary Figure 2A). The bark infusion, however, had significantly higher activity at concentrations of 60-240 µg/mL with a lower IC_{50} value [(10.50±1.03) µg/mL], suggesting better antioxidant potency compared with the leaf infusion [IC_{50}=(12.86±0.22) µg/mL] as depicted Table 2. Although the electron donating capacities of bark infusion [IC_{50}=(9.85±0.32) µg/mL] were lower than that of vitamin C [IC_{50}=(6.67±0.88) µg/mL], its activity was indeed better than that of the leaf infusion [IC_{50}= (11.00±0.10) µg/mL]. Moreover, the leaf and bark infusions had significantly higher nitric oxide scavenging activities than the positive control (vitamin C) at all test concentrations (P<0.05), and the activities of both infusions were not significantly different from each other at 60-240 µg/mL (Supplementary Figure 2C).

3.4. Inhibitory effects of R. longipes on carbohydrate and lipid digesting enzymes

The leaf infusion significantly outperformed the bark infusion in inhibiting α-amylase at all concentrations with an IC_{50} value of

Table 1. Phytocompounds in *Rhus longipes* leaf and bark infusions.

Compounds	Retention time (min)	Molecular formula	Molecular weight (g/mol)	Relative abundance (%)
Leaf infusions				
n-Pentadecanol	13.050	$C_{15}H_{32}O$	228.41	0.63
2-Ethyl-1-dodecanol	16.300	$C_{14}H_{30}O$	214.39	0.59
l-(+)-Ascorbic acid 2,6-dihexadecanoate	17.170	$C_{38}H_{68}O_8$	652.90	0.86
Palmitoleic acid	18.910	$C_{16}H_{30}O_2$	254.41	1.04
Octadecanamide	19.220	$C_{18}H_{37}NO$	283.50	0.88
24,25-Dihydroxyvitamin *D*	20.480	$C_{27}H_{44}O_3$	416.60	0.59
cis-11-Eicosenamide	20.830	$C_{20}H_{39}NO$	309.50	0.51
β-*D*-Mannofuranoside, 1-*O*-(10-undecenyl)-	22.400	$C_{17}H_{32}O_6$	332.40	0.68
Bark infusions				
2-Amino-1,3-propanediol	4.070	$C_3H_9NO_2$	91.11	7.35
1-Heneicosanol	15.267	$C_{21}H_{44}O$	312.60	1.24
Phytol, acetate	15.721	$C_{22}H_{42}O_2$	338.60	1.93
5,15-Dimethylnonadecane	16.899	$C_{21}H_{44}$	296.60	1.00
l-(+)-Ascorbic acid 2,6-dihexadecanoate	17.092	$C_{38}H_{68}O_8$	652.90	5.49
n-Nonadecanol-1	18.252	$C_{19}H_{40}O$	284.50	4.91
cis-Vaccenic acid	18.871	$C_{18}H_{34}O_2$	282.50	3.91
L-Ascorbic acid, 6-octadecanoate	19.042	$C_{24}H_{42}O_7$	442.60	1.82
Octadecanamide	19.185	$C_{18}H_{37}NO$	283.50	1.47
cis-11-Eicosenamide	20.885	$C_{20}H_{39}NO$	309.50	1.56

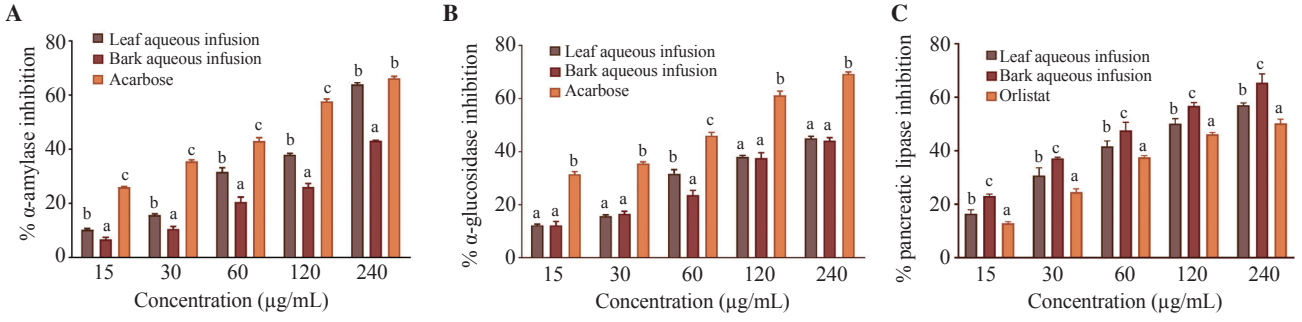

Figure 1. Inhibitory effects of *Rhus longipes* leaf and bark infusions on (A) α-amylase, (B) α-glucosidase, and (C) pancreatic lipase. [a-c]Bars with different letters for a given concentration are statistically different from each other (P<0.05).

Table 2. IC_{50} values of *in vitro* biological activities of *Rhus longipes* leaf and bark aqueous infusions (µg/mL).

Activities	Leaf infusion	Bark infusion	Vitamin C	Acarbose	Orlistat
DPPH	12.86 ± 0.22	10.50 ± 1.03	9.21 ± 0.12	-	-
FRAP	11.00 ± 0.10	9.85 ± 0.32	6.67 ± 0.88	-	-
NO	485.47 ± 0.12	472.01 ± 0.36	552.22 ± 0.41	-	-
α-Glucosidase	238.67 ± 0.45	242.32 ± 0.73	-	100.68 ± 0.09	-
α-Amylase	135.62 ± 0.01	311.38 ± 0.02	-	118.57 ± 0.97	-
Pancreatic lipase	62.52 ± 0.31	48.37 ± 0.27		-	123.55 ± 0.81

DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: Ferric reducing antioxidant power, NO: nitric oxide.

(135.62±0.01) μg/mL (P<0.05) (Figure 1A and Table 2). At 240 μg/mL, there was no significant difference in the inhibitory activity of the leaf infusion and the positive control (acarbose). Besides, no significant difference was found in the α-glucosidase inhibitory activities between the leaf and the bark infusions of *R. longipes* at the test concentrations except at 60 μg/mL (Figure 1B). Moreover, the bark infusion had significantly higher pancreatic lipase inhibitory activities with a lower IC_{50} value [(48.37±0.27) μg/mL] (P<0.05) than the leaf infusion [IC_{50}=(62.52±0.31) μg/mL] and the orlistat [IC_{50}=(123.55±0.81) μg/mL] (Table 2 and Figure 1C).

3.5. Mode of inhibition of carbohydrate and lipid digesting enzymes by R. longipes

The leaf infusion showed competitive inhibition for α-amylase with a K_m value of 2.075 mM and a V_{max} value of 0.109 μM/min, while the bark infusion showed mixed inhibition for α-amylase with a K_m value of 0.991 mM and a V_{max} value of 0.098 μM/min. The same trend was observed for α-glucosidase inhibition (leaf infusion: K_m value 1.838 mM and V_{max} value 0.379 μM/min; bark infusion: K_m value 4.053 mM and V_{max} value 0.530 μM/min). Both leaf (K_m value 6.982 mM and V_{max} value 1.651 μM/min) and bark infusions (K_m value 8.750 mM and V_{max} value 1.651 μM/min) of *R. longipes* showed competitive inhibition of pancreatic lipase.

3.6. R. longipes cytotoxicity in Vero kidney cells

As depicted in Figure 2, there was no significant difference between the % cell viability between leaf and bark infusions at 15-120 μg/mL. Moreover, no significant difference was found between the % cell viability of the normal Vero cells and those treated with the leaf and bark infusions. Treatment with H_2O_2, however, significantly reduced the cell viability to 57.27% in relation to the normal control (P<0.05).

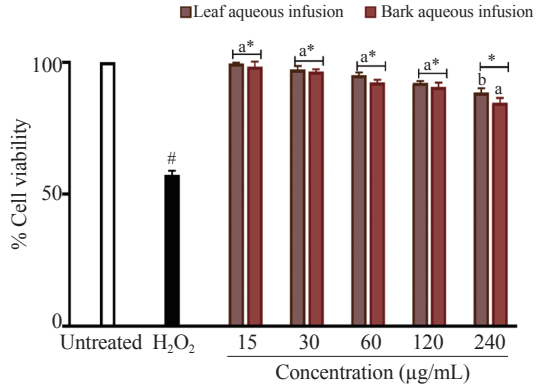

Figure 2. Cytotoxic activities of *Rhus longipies* leaf and bark infusions against Vero cells. [a-b]Different letters for a given concentration show a statistical difference between the bark and leaf infusions of *Rhus longipies* (P<0.05) while those with * and # indicate a significant difference from the H_2O_2 group and the normal control group, respectively.

Table 3. Free binding energy of *Rhus longipes* leaf and bark infusions secondary metabolites with carbohydrate and lipid digestive enzymes (kcal/mol).

Ligands	α-Glucosidase	α-Amylase	Pancreatic lipase
Acarbose	−7.5	−7.8	-
Orlistat	-	-	−6.7
n-Pentadecanol	−4.6	−5.1	−5.8
2-Ethyl-1-dodecanol	−5.7	−5.0	−5.4
l-(+)-Ascorbic acid 2,6-dihexadecanoate	−7.0	−5.8	−6.6
Palmitoleic acid	−5.3	−5.0	−5.1
Octadecanamide	−5.9	−5.4	−6.2
24,25-Dihydroxyvitamin *D*	−8.6	−9.4	−9.0
cis-11-Eicosenamide	−5.6	−5.0	−6.4
β-*D*-Mannofuranoside, 1-*O*-(10-undecenyl)-	−6.7	−5.9	−6.5
2-Amino-1,3-propanediol	−3.8	−3.9	−3.7
1-Heneicosanol	−5.4	−5.0	−5.7
Phytol, acetate	−5.9	−6.0	−6.9
5,15-Dimethylnonadecane	−5.9	−5.6	−5.8
n-Nonadecanol-1	−5.8	−4.9	−5.0
cis-Vaccenic acid	−5.2	−5.3	−6.2
L-Ascorbic acid, 6-octadecanoate	−7.0	−6.0	−6.4

3.7. Effects of R. longipes on glucose uptake and absorption in rat tissues

As depicted in Figure 3A, there was a dose-dependent increase in glucose uptake in yeast cells incubated with the plant infusions. At the assayed concentrations, the bark infusion significantly increased glucose uptake to an extent better than the leaf infusion (P<0.05). Similarly, in Figure 3B, there was an upward trend in the amount of glucose taken up by the rat psoas muscle tissue with increasing concentrations of the leaf and bark infusions. However, the bark infusion only at 240 μg/mL had significantly higher glucose uptake activity than positive control treatment (insulin). Both infusions also suppressed intestinal glucose absorption dose-dependently. At the higher concentrations (120-240 μg/mL), both infusions significantly outperformed acarbose (P<0.05) (Figure 3C).

3.8. Effects of R. longipes on kidney oxidative stress biomarkers

As shown in Figures 4A-4D, $FeSO_4$ induction led to significantly increased MDA levels while reducing GSH levels as well as SOD and catalase activities (P<0.05). Treatment with the leaf and bark aqueous infusions of *R. longipes* resulted in a dose-dependent reduction in MDA to nearly normal levels. Also, both infusions markedly increased GSH, SOD, and catalase activities (P<0.05).

3.9. Molecular docking results

The molecular docking scores indicate that 24,25-dihydroxyvitamin *D* had the lowest free binding energy with the digestive enzymes (Table

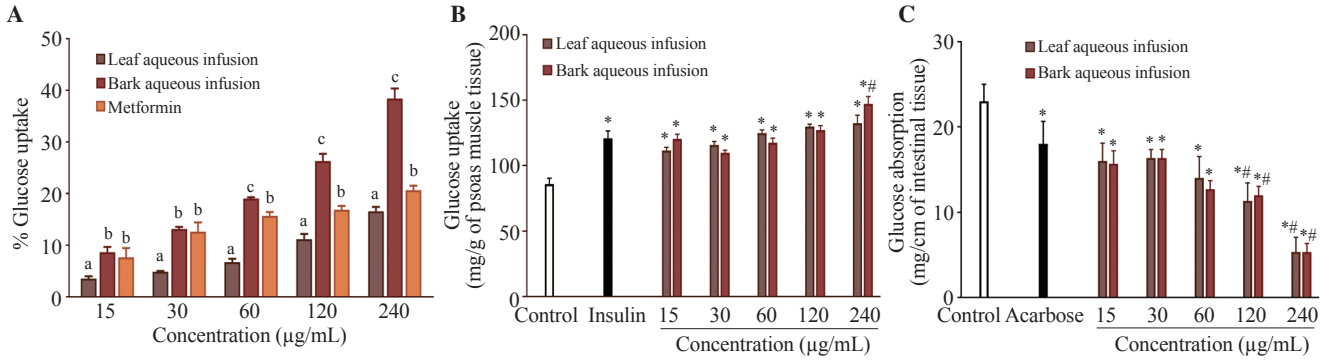

Figure 3. Effects of *Rhus longipes* leaf and bark infusion on glucose uptake in (A) yeast suspension and (B) excised rat psoas muscle as well as (C) glucose absorption in rat intestinal jejunum. [a-b]Bars with different letters for a given concentration are statistically different from each other (P<0.05) whereas those with * and # are statistically different from the control group and the insulin or acarbose group, respectively.

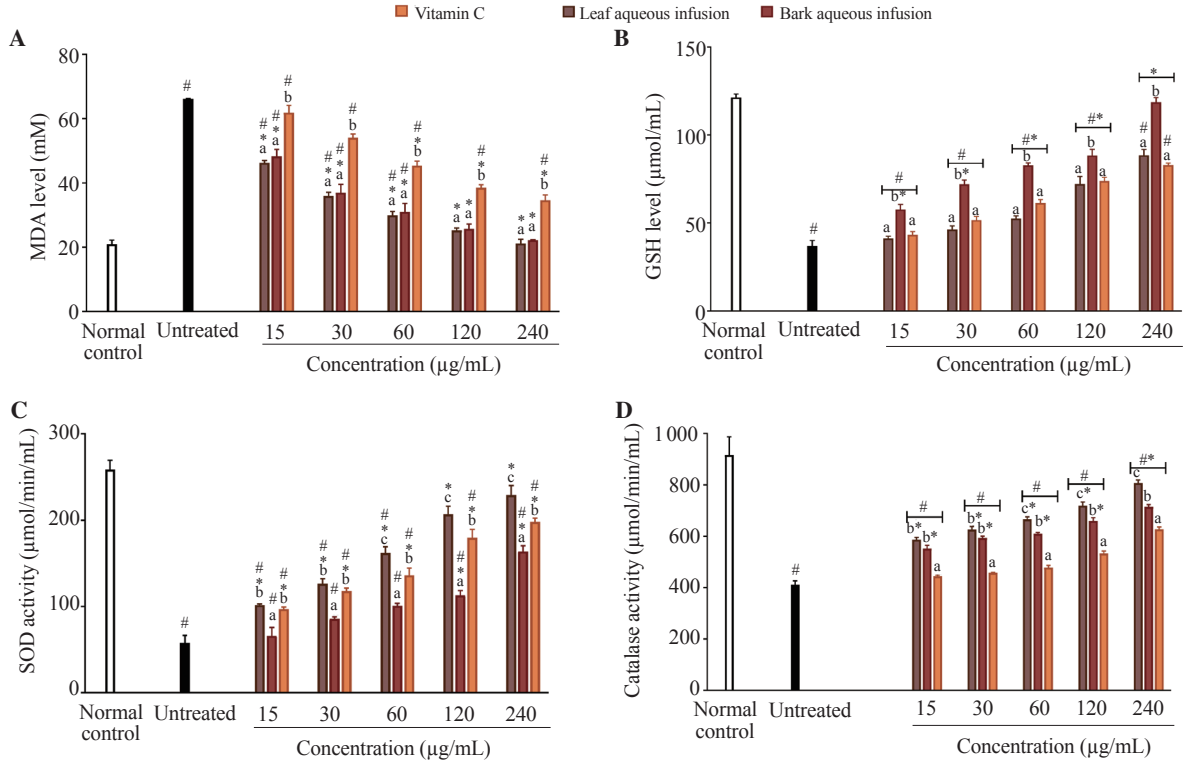

Figure 4. Effects of *Rhus longipes* leaf and bark infusion on (A) malondialdehyde (MDA), (B) reduced glutathione (GSH), (C) superoxide dismutase (SOD) and (D) catalase on $FeSO_4$-induced kidney injury. [a-b]Bars with different letters for a given concentration are statistically different from each other (P<0.05) whereas those with * and # are different from the untreated and normal control treatment, respectively.

3). The 24,25-dihydroxyvitamin *D* best 2D ligand-receptor interactions posed with α-glucosidase, α-amylase, and pancreatic lipase are shown in Figures 5A-5C, respectively. The results indicate that the compound formed multiple interactions with the pocket protein residues of enzymes, including a conventional H-bond with HIS 1584 and Pi-Alkyl-bonds with TRP1369, TRP1355, and PHE1427 amino residues of α-glucosidase (Figure 5A). While forming only Pi-Sigma Pi-Alkyl with α-amylase active site residues, the compound was able to interact *via* stronger hydrogen bonds amongst other forces with amino acids found at the pancreatic lipase catalytic pocket (Figure 5C).

4. Discussion

The global upsurge in the prevalence of diabetes in the last decade has prompted the immediate need for novel intervention strategies[34]. Plants produce various secondary metabolites, constituting a reservoir of beneficial pharmacological agents. Reports have indicated that about 70% to 80% of the rural African population still employs traditional medicine for their primary healthcare needs[10,34]. The current study investigates the cytotoxicity, antidiabetic and antioxidant activities of the leaf and bark infusions

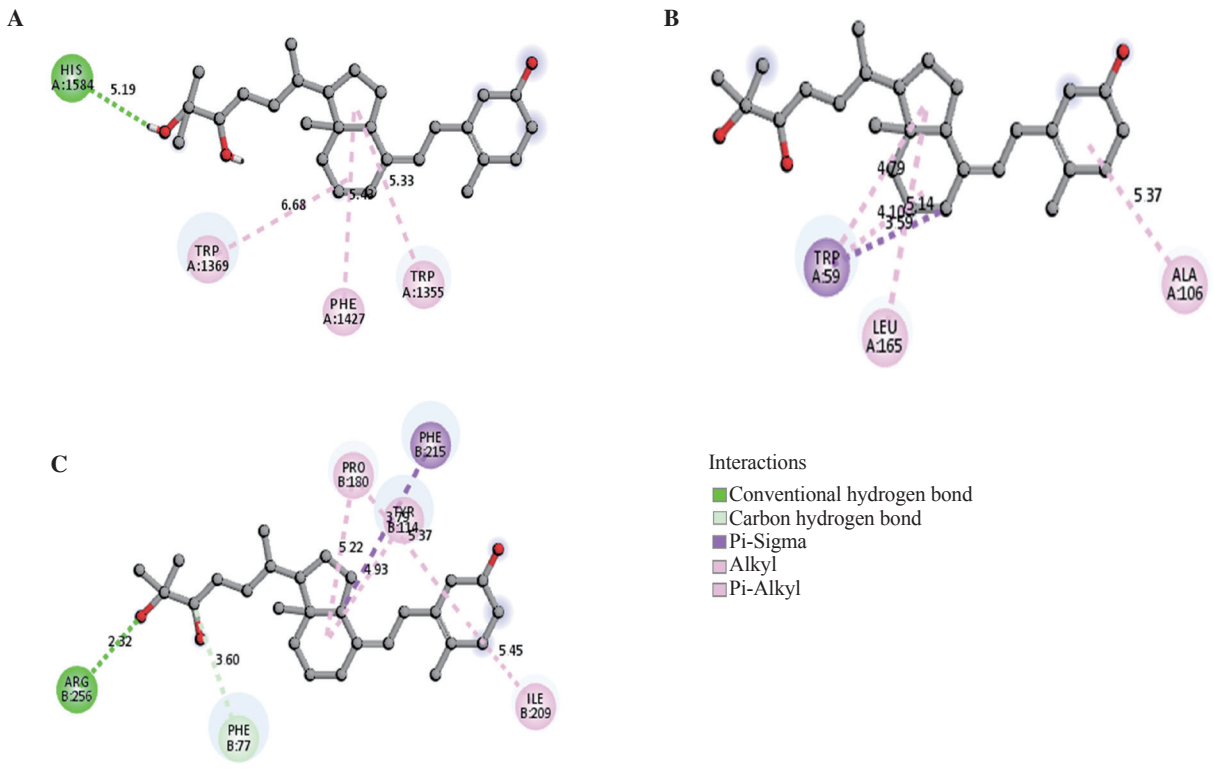

Figure 5. The 2D images of the molecular interactions between 24,25-dihydroxyvitamin *D* and active site amino acid residues of (A) α-glucosidase, (B) α-amylase and (C) pancreatic lipase.

of the folkloric medicinal plant *R. longipes*.

Innate and dietary acquired exogenous antioxidants counteract the harmful effects of excessive reactive species produced during the normal metabolic activities of the body[35]. When the protective capacities of these antioxidant systems become insufficient in the body, it causes oxidative stress. This biochemical process has been implicated in the etiology of many degenerative conditions, including diabetes[36]. Indeed, studies have described chronic hyperglycemia-induced oxidative stress as a factor promoting the development and progression of nephropathy. While representing one of the most common symptoms of end-stage diabetes complications, this renal tissues-associated pathology is characterized by severe impairment of kidney functions. The abilities of the *R. longipes* stem bark and leaf infusions to donate free electrons (FRAP) and scavenge free radicals (DPPH and NO)could evidence their potent antioxidant pharmacological properties. These observations are further supported by reduced MDA and enhanced antioxidant activities in kidney tissues treated with $FeSO_4$ and *R. longipes* infusions. Plants contain a wide variety of phytoconstituents, including polyphenols, flavonoids, carotenoids, and tannins with excellent antioxidant properties[37]. Findings have shown that the aromatic ring on phenolic compounds is endowed with hydrogen and electron-donating abilities in addition to transition metal ion chelating capabilities[38]. Therefore, the observed antioxidative effects of these infusions may be attributed to their total phytochemical composition and the presence of several phytocompounds in the plant, as revealed in the GC-MS analysis. This observation is consistent with previous studies, which show a positive correlation between high total phenolic and flavonoid content and antioxidant capacity[38]. However, further study should be carried out to examine the antioxidative and antidiabetic effects of compounds identified in the plant parts.

When complex dietary carbohydrates are ingested, they pass through the digestive tract and are broken down into simple sugars, which are subsequently absorbed by the small intestines. Consequently, one of the modes of action employed by some antidiabetic agents in restoring homeostatic blood glucose levels in a diabetic state is retarding the absorption of glucose in the small intestine[39]. These pharmacological agents perform these functions by competing with substrate molecules at the active site of carbohydrate digestive enzymes such as α-amylase and α-glucosidase. Thus, these inhibitors stall carbohydrate polymer hydrolysis into monosaccharide sugar units and decrease the latter biomolecule's absorption into the bloodstream[39]. In the current study, the potency of the plant infusions, especially those of the leaf infusion in inhibiting α-amylase and α-glucosidase enzymes, may suggest the plant is a viable source of alternative antidiabetic therapy. Interestingly, these findings are also supported by kinetic inhibitory studies, which indicated the competitive inhibition of the enzymes by leaf infusion. Additionally, the infusion's ability to reduce glucose absorption in intestinal tissue could be further evidence of

the antidiabetic properties of *R. longipes*. However, future studies in experimental animals are required to confirm these effects of extracts from the different parts of *R. longipes*.

Studies have shown that increased dietary lipid intake and assimilation lead to obesity, a significant risk factor, and comorbidity of diabetes. In this regard, compounds from plants with pancreatic lipase inhibitory activities are described as better alternatives to synthetic chemicals[40]. Indeed, *R. longipes* leaf and bark infusions displayed lipase inhibitory properties that exceed the effect of the orlistat. Moreover, the result of the enzyme mode of inhibition studies suggests that phytocompounds in the infusion may compete with lipid substrate at the active site of the digestive enzyme. Among the compounds in the infusions that could be attributed to this anti-lipase bioactivity is 24,25-dihydroxyvitamin *D*, which had the lowest binding affinity with the enzymes. In addition, the ability of the infusions to competitively inhibit lipase may be associated with the strong hydrogen bond and other molecular interactions formed between the compound and the enzyme's active site amino residues. Previous investigations have shown that dihydroxyvitamin D and some of the other compounds found in this plant exert their inhibitory activity by acting as competitive, mixed, non-competitive, or un-competitive inhibitors[41]. The effects of extracts on other enzymes related to glucose metabolism could explore the additional mechanism of action, which will be conducted in our future studies.

Cytotoxicity studies, in which the toxic effects of plant-derived compounds are tested on normal cells, are an important first step in determining the success of a pharmaceutical therapeutic agent's development. In this regard, plant extracts with minimal or no significant adverse effects on the viability of normal cell lines are sought after in the development of oral pharmacological agents because of their low toxicological effects[42]. In a previous study, *R. longipes* was shown to be non-toxic to female Swiss albino mice at concentrations of up to 2 000 mg/kg body weight[43]. Plant extracts with low toxicity additionally have reduced undesirable side effects as they work harmoniously with the body's natural biochemical pathways[44]. The reduced cytotoxic effects of *R. longipes* infusions demonstrated in this study at tested doses may suggest its use in traditional diabetes management. However, it could be better to conduct the cytotoxicity study on more than one cell line which could not be done due to lack of cell lines in our laboratory.

Another mechanism employed by antidiabetic agents in restoring the normoglycemic state under diabetic conditions is by promoting glucose uptake in peripheral tissues[45,46]. Under normal physiological conditions, insulin signals facilitate glucose transport in skeletal muscle tissues *via* activating their cell surface receptors of glucose transporter 4. However, in diabetes, this glucose metabolism signaling pathway is impaired due to insulin resistance. The ability of *R. longipes* infusions to increase glucose uptake in yeast cells and, more importantly, psoas muscle tissues may justify the antidiabetic efficacies of plants in traditional diabetic management. In support of this study, Woldemariam and Van Winkle[47] demonstrated that plant extracts that increase glucose uptake in yeast models may encourage increased glucose utilization by peripheral tissues in diabetic patients.

In conclusion, the results of this study demonstrate that *R. longipes* leaf and bark infusions have antioxidant potential, as evidenced by scavenged free radicals and enhanced endogenous antioxidant systems in oxidative renal injury. The effects of the infusions on suppressing intestinal glucose absorption and increasing the glucose uptake in isolated rat psoas muscle also indicate its anti-diabetic properties. Although the plant did not show much cytotoxicity, further *in vivo* studies in the animal model are warranted to ascertain the results of this study.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

BKB: Conceptualization, formal analysis, methodology, and writing original draft. KAO: Formatting, writing review and proof editing. VFS: Writing review and proof editing. OLE: Writing-review and proof editing. MSI: Supervision, project administration, writing review, and proof editing.

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