AOSIS

Mitigation of free radicals and carbohydrate-linked enzymes by extracts and partitioned fractions of *Elephantorrhiza elephantina* (Burch.) Skeels root

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Background: *Elephantorrhiza elephantina* (Burch.) Skeels is a medicinal plant used in folkloric medicine for the management of several metabolic and infectious diseases.

Aim: This aim of this research study was to investigate the antioxidant and antidiabetic effects of extracts and partitioned fractions in order to validate its folkloric use.

Setting: The plant material purchased from herb sellers in Qwaqwa township, authenticated at Department of Plant Science Qwaqwa herbarium, was evaluated in the same unit of the University of the Free State.

Methods: The antioxidative and antidiabetic activities of extracts and fractions were assessed with 1,1-diphenyl-2-picrylhydrazyl, 2,2-azino-bis(3-ethylbenzothiazoline-6)-sulphonic acid, hydroxyl radicals, metal chelating agents, and α-amylase, as well as α-glucosidase inhibitions based on standard methods. The subfractions with considerable yields from the partitioned n-hexane fraction of the crude extract were subjected to gas chromatography-mass spectrometry analysis or profiling for possible compound identification.

Results: The aqueous extract showed the most effective 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radical and metal chelating activities judging by half-maximal inhibitory concentrations (IC_{50}: 0.573, 0.059 and 1.937 mg/mL, respectively), whilst the ethanol extract revealed maximum activity (0.017 mg/mL) against 2,2-azino-bis(3-ethylbenzothiazoline-6)-sulphonic acid. However, the ethanol extract displayed the most potent alpha-amylase (0.346 mg/mL) inhibition, whilst the aqueous extract (0.363 mg/mL) was best against alpha-glucosidase. The modes of enzymes inhibition revealed that the aqueous extract displayed near-competitive inhibition against alpha-amylase and uncompetitive inhibition against alpha-glucosidase. Additionally, good antioxidative and antihyperglycaemic effects were established by the n-hexane fraction when compared with standards (gallic acid and acarbose). The GC-MS chromatogram of subfractions (4 and 9) from the n-hexane fraction afforded compounds, such as 2,4-bis (1, 1-dimethylethyl)-phenol, 9-octadecenoic acid (Z)-, methyl ester, dodecanoic acid and 1-methylethyl ester already established in the literature with potential pharmacological activities (antioxidant, anti-inflammatory, etc.).

Conclusion: The research study provides evidence on the folkloric use and insights on the prospect of the plant as natural antioxidative and antidiabetic agents.

Keywords: antioxidants; diabetes mellitus; *Elephantorrhiza elephantine*; free radicals; antihyperglycaemia.

Introduction

Elephantorrhiza elephantina (Burch.) Skeels is one of the prominent medicinal plants used in the treatment of several degenerative and infectious diseases (Maroyi 2017). The plant, belonging to the subfamily Mimosoideae of the family Fabaceae is a subshrub commonly known as elephant's root or eland's wattle. The perennial shrub plant is endowed with a stem that grows up to 0.9 m above the ground originating from a thick, woody rhizome. The leaves that appear dull-green in colour consist of two pinnate compounds of 2–17 oppositely arranged pinnae. The flowers, golden yellow or pale yellow or white, are arranged in axillary or solitary manner. Fruits are dark or reddish brown, with squeezed pods consisting of valves distinguishing them from continuous margins upon opening. Seeds are dark brown and ellipsoidal in shape (Grobler 2010; Olaokun et al. 2020).

Elephantorrhiza elephantina is used by South Africans as a food source in medicine and other fields (e.g. the rhizome being used as a source of dye and tanning materials). Interestingly, because of its numerous medicinal properties, it is found mostly in *muthi* markets and across several provinces, such as Free State, Eastern Cape, Limpopo, Gauteng and Northern Cape, within South Africa. Traditionally, it is used for treating syphilis, internal disorders and to stop bleeding; these indigenous uses have been attributed to a variety of pharmacological and biological activities, such as antioxidant, antifungal, anthelmintic, antibacterial, antinociceptive, anti-inflammatory and antiplasmodial (Maroyi 2017). It is worth mentioning that despite the use of the plant in Basotho traditional medicine for the management of diabetes mellitus (DM; Balogun, Tshabalala & Ashafa 2016), there is a dearth of scientific information on its antidiabetic potential in the literature. Intriguingly, information gathered from the ethnobotanical survey on the use of these plants (Tshabalala & Ashafa 2011), subsequently reviewed in the work of Balogun et al. (2016), suggested that only the root (rhizome) part of *E. elephantina* is used in the management of diabetes by the tribe.

Free radicals (nitrogen or oxygen) are cellular modulators produced within the body system (He & Zou 2015). An increased free radical production results in a state of oxidative stress, leading to the development of many degenerative diseases (Liguori et al. 2018), including diabetes and its complications (Pieme et al. 2017). They attack cells or tissues resulting in the loss of body function. It must be noted that oxidative stress causes glucotoxicity by reducing insulin production from pancreatic beta cells and also disrupting the absorption of glucose into muscle cells (Chatterjee, Khunti & Davies 2017).

Diabetes mellitus is a chronic derangement whose global prevalence has continued to increase, thus greatly affecting nations around the world (undeveloped, developing and developed) (Larijani & Forozandeh 2003). It is a metabolic disorder characterised by hyperglycaemia, resulting in elevated glucose levels in the blood or urine arising from the inability of the pancreas to produce insulin (glucose-regulating hormone), which, in turn, leads to the abnormality in carbohydrate, protein and fat metabolism (Maiti et al. 2004). During the development of the disease, pathogenic processes come into play from the destruction of the pancreatic -cells to insulin resistance. These pathogenic processes have been established to be the primary cause of hyperglycaemia (high level of glucose in the blood) (Narkhede et al. 2011).

According to the International Diabetes Federation (IDF 2015), approximately 415 million of adult population around the world have diabetes, meaning that one in every 11 adults suffer from this ailment. In Africa, South Africa followed by Nigeria (in terms of prevalence per million populations) accounts for the highest incidence of diabetes, with about 6% of the entire population having diabetes (Balogun et al. 2016).

Additionally, an estimated 5 million people in South Africa have pre-diabetes (a condition where blood glucose levels are found to be higher than normal probably because of insulin resistance although not high enough to be classified as type 2 diabetes). The primary goal for the management of diabetes (pharmacological or non-pharmacological) is to control the blood glucose levels whilst preventing further complications of the disease. The pharmacological management involves the use of drugs or oral hypoglycaemic agents (OHAs), such as insulin, glibenclamide (sulphonylurea), metformin (biguanides) and acarbose, whilst the non-pharmacological activities include exercise regimen, dietary modification and healthy lifestyles. However, the use of these OHAs, particularly the alpha-glucosidase inhibitors, such as acarbose and voglibose, in the management of diabetes has been reported to be accompanied with debilitating gastrointestinal side effects, such as bloating and diarrhoea (Yin et al. 2014). There is a need to search for potent natural products with less toxic effects. Thus, this study is, therefore, aimed at evaluating the *in vitro* antioxidant and antidiabetic effects of the root of *E. elephantina* in order to search for suitable antidiabetic agents because of the adverse effects of synthetic ones.

Materials and methods
Chemicals

Porcine pancreatic alpha-amylase, rat intestinal alpha-glucosidase, 1, -diphenyl-2 picrylhydrazyl (DPPH), ascorbic acid, acarbose and para-nitrophenyl glucopyranoside (pNPG) were products obtained from Sigma-Aldrich Co. (St Louis, USA), whilst potato starch (extra pure) was obtained from J. T. Baker Inc. (Phillipsburg, USA). Other chemicals and reagents used were of analytical grade, and glass distilled water was used for the study.

Collection, preparation and extraction of the plant materials

The whole plant of *E. elephantina* was obtained from the herb sellers around the Free State Province. The authentication of the plant was performed by Professor AOT Ashafa from the University of Free State, Free State, South Africa. The *E. elephantina* sample was separated and washed under a running tap to remove all debris and chopped into small pieces before being dried in an oven for 9 days at 45 °C. The dried root material was then pulverised into fine powder using a Waring (instrument) Laboratory Blender. In the study involving the use of extracts, about 30 g each of the dried powdered material was exhaustively extracted in 900 mL each of distilled water (DW), ethanol and aqueous ethanol (ratio 1:1), respectively. The mixtures were then shaken constantly on a platform shaker (Labcon) at 110 revolutions per minute (rpm) for 72 h, filtered using Whatman No 1 filter paper and concentrated the organic solvents under low pressure using a rotary evaporator (Cole-Palmer, model SB-1100 Shanghai, China) at 40 °C, whilst the aqueous extract was lyophilised (VirTis BenchTop, SP Scientific, USA). The obtained extracts (1 g)

were dissolved in dimethyl sulphoxide (DMSO) to obtain stock solutions of 1.0 mg/mL, and different concentrations (0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL and 1.000 mg/mL) of the extracts (working concentrations) were prepared by serial dilution with DW. All extracts were stored at 4°C prior to analysis.

The preparation for obtaining the fractions was carried out in the following way: approximately 500 g of the dried powdered material was exhaustively extracted with 1500 mL of DW (the choice of the solvent was based on the pharmacological study outcomes from the crude extract evaluation). The mixture was then shaken constantly on a platform shaker (Labcon) at 110 rpm for 72 h, filtered using Whatman No 1 filter paper and the extract was lyophilised (VirTis BenchTop, SP Scientific, USA) to obtain the crude extract, which was later dissolved in 550 mL of DW. The solution was partitioned in the order of increasing polarity with hexane (1230 mL), chloroform (1430 mL), ethyl acetate (1420 mL), n-butanol (1585 mL) and leftover (water); all designated as hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), butanol fraction (BF) and aqueous fraction (AF). The fractions were concentrated accordingly and evaluated for antioxidant and antidiabetic activities using the standard selected assays with the concentration range of 0.062–1.000 mg/mL.

In vitro antioxidant assays

1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity
1, 1-Diphenyl-2-picrylhydrazyl radical scavenging activity of the samples (crude extracts and fractions) was evaluated based on the method of Saha et al. (2008) where the scavenging ability of the extracts was evaluated against DPPH (crystalline powder containing free radical molecules). In brief, 150 µL of the various concentrations of the samples and/or standard (ascorbic acid) with 150 µL of methanolic solution (95%) DPPH radicals (0.0004 mol/L) were mixed and left in the dark room for 30 min. This was followed by measuring the absorbance at 515 nm using WPA BIOWAVE II (Bichrom, England) spectrophotometer. The whole procedure was similarly repeated for the control sample using DW to replace the extract.

Hydroxyl radical scavenging activity
The hydroxyl radical scavenging activity of the crude extract was analysed using the deoxyribose method based on Sindhu and Emilia's (2005) protocol. One hundred and twenty microlitres (µL) 0.2 M of deoxyribose, 40 µL 0.2 M H_2O_2, 400 µL 0.1 M phosphate buffer and 40 µL 0.0005 M $FeSO_4$ were mixed in 2 mL Eppendorf tubes, followed by the addition of 100 µL DW, and the reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped with 500 µL of 2.8% trichloroacetic acid (TCA) and 400 µL of 0.6% thiobarbituric acid (TBA). About 300 µL from the mixture was pipetted and emptied into a 96-well microtitre plate, and the absorbance was measured at 532 nm using a microtitre plate reader (BIO-RAD, model 950).

2, 2-Azino-bis(3-ethylbenzothiazoline-6-)sulphonic acid assay
Adopting the method of Re, Pellegrini and Proteggente (1999), the scavenging ability of the samples against the azino-bis(3-ethylbenzothiazoline-6-)sulphonic acid (ABTS) radical was determined. Briefly, 7 mM aq. ABTS and 2.45 mM potassium persulphate (50 mL each) were prepared and allowed to react in the dark for 16 h. The reaction mixture was then adjusted with ethanol to 0.7 pH at 734 nm using a spectrophotometer. Twenty microlitres of the sample were reacted with 200 µL of ABTS–potassium persulphate mixture, and the absorbance was measured at 734 nm.

Metal chelating activity
According to the method of Dinis, Madeira and Almeida (1994), the chelation of ferrous ions was estimated by the crude extracts. About 40 µL of the extracts were readily mixed with 200 µL of 2 mM $FeCl_2$ solution in a 96-well microtiter plate; the reaction was started by adding 80 µL of 5 mM ferrozine, then shaken vigorously and the resulting mixture was left standing at room temperature for 10 min. The absorbance of the reaction mixture was then assessed at 562 nm using a microplate reader (BIO-RAD, model 680, Japan).

In vitro antidiabetic assays

α-Glucosidase enzyme inhibition
The varying concentrations of the samples (50 µL) were mixed with 100 µL of 0.1 mol/L phosphate buffer (pH 6.9) containing α-glucosidase (1.0 mol/L), and the mixture was incubated at 25 °C for 10 min. Following this, 50 µL of 0.1 mol/L phosphate buffer having 5 mM p-nitrophenyl-α-D glucopyranoside (p-NPG) was added to the solution at different time intervals, and the reaction mixture was incubated for 5 min at 25 °C. The absorbance was measured at 405 nm, and the values were then compared with the standard (acarbose) obtained following the same steps above, although DW was used in place of the sample for the control or blank (Elsnoussi et al. 2012).

α-amylase enzyme inhibition
About 100 µL of 0.5 mg/mL α-amylase prepared in 0.02 M sodium phosphate buffer (pH6.9) was mixed with 100 µL of different concentrations of the samples in test tubes, and the reaction mixture was incubated at 25 °C for 10 min. Thereafter, 100 µL of 1% starch solution prepared with 0.02 M sodium phosphate (buffer) was added to each tube at different time intervals to initiate the reaction. Following this, the mixture was again incubated at 25 °C for 10 min, and subsequently, 10 µL of dinitrosalicylic acid (DNS) colour reagent was added to stop the reaction, and the reaction mixture was suspended in a hot (100 °C) water bath for 5 min and was cooled to room temperature. The absorbance was measured at 504 nm following the addition of 5 mL DW into the mixture. Using the same concentrations as the samples, acarbose (prepared in DW) was utilised as a standard (Elsnoussi et al. 2012).

Mode of α-glucosidase inhibition

The kinetics of the inhibition of α-glucosidase by an aqueous root extract of *E. elephantina* was determined using a modified method of Dnyaneshwar and Archana (2013) with a slight modification. Briefly, 5 mg/mL of the extract (25 µL) was pre-incubated with 50 µL of α-glucosidase solution for 10 min at 25 °C in one set of test tubes. In another set of tubes, α-glucosidase was pre-incubated with 25 µL of phosphate buffer (pH 6.9). Twenty-five microlitres of pNPG at increasing concentrations (0.25–2.00 mg/mL) were added to both sets of reaction mixtures to start the reaction. Whilst the mixture was incubated for 10 min at 25 °C, 170 µL of Na_2CO_3 was added to stop the reaction. The amount of reducing sugars released was determined colourimetrically using a *p*-nitrophenol standard curve. Reaction rates or velocity (V) was calculated, and the double reciprocal plots of enzyme kinetics were constructed according to the Lineweaver and Burk method in order to study the nature of inhibition. K_m and V_{max} values were also calculated from the Lineweaver–Burk plot (1/V vs. 1/[S]).

Mode of α-amylase inhibition

The kinetics of alpha-amylase inhibition was determined using a modified method of Ali, Houghton and Soumyanath (2006). Briefly, 250 µL of the extract (5 mg/mL) was pre-incubated with 250 µL of α-amylase solution for 10 min at 25 °C in one set of tubes, whilst α-amylase (0.5 mg/mL) was pre-incubated with 250 µL of phosphate buffer (pH 6.9) in another set of tubes. The reaction of the two sets of the mixtures was initiated by adding 250 µL of the starch solution at increasing concentrations (0.31–5.00 mg/mL). The mixture was then incubated for 10 min at 25 °C, followed by the addition of DNS (500 µL), boiled for 5 min and then 2.5 mL DW was finally added to dilute the mixture. The amount of reducing sugars released was determined spectrophotometrically using a maltose standard curve and converted to reaction velocities. A double reciprocal (Lineweaver–Burk) plot (1/V vs. 1/[S]), where V is the reaction velocity and [S] is substrate concentration, was plotted to determine the mode of inhibition.

Percentage inhibitory determinations

The inhibitory activity of the samples was calculated by adopting the following expression: $Ab_0 - Ab_1 / Ab_0 \times 100$, where Ab_0 is the absorbance of the control and Ab_1 is the absorbance of the sample or standard. The half-maximal inhibitory concentration (IC_{50}) value was calculated from the linear regression equation using y = mx + c, where y is the percentage activity and equals to 50, m connotes the slope, c signifies the intercept and x represents the IC_{50} value (Balogun & Ashafa 2016).

Chromatographic analysis

The n-hexane fraction being the most active was subjected to chromatographic separation using the silica gel (100–200 mesh size) column and the mixture of chloroform and ethanol in varying ratios as the mobile phase. Briefly, silica gel (150 g) was mixed with about 300 mL of 100% chloroform to form a homogenous suspension or slurry, and the mixture was stirred using a glass-stirring rod to remove bubbles. The slurry was poured time to time into the column (closed at the tail end with cotton wool) very carefully to form a uniform packing (to prevent cracking of silica), and this was allowed to stand for 24 h so as to equilibrate. Then, a slurry of the n-hexane fraction (5.03 g) with a small amount of silica gel prepared was applied carefully on the top layer of the packed column and successfully eluted with solvent or solvent system chosen (Barbara 2014; Natarajan 2015). The ratio of solvent system (chloroform: ethanol) prepared (250 mL) was 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9. Eluents (134 fractions) were collected in numbered conical flasks (20 mL), concentrated by air-drying and bulked using TLC profiles of each fraction to obtain 15 subfractions.

Preparative Thin-Layer Chromatography

A streak of subfractions 4 and 9 (chosen based on the yield) were loaded onto a TLC plate (20 cm × 10 cm diameter and thickness of 1.5 mm), dried using a hair hand-dryer and then developed in a chromatographic glass chamber containing the mobile phases, such as chloroform–ethanol–acetic acid (ration 4:1:3 drops) for subfraction 4 and chloroform–ethanol (3:2) for subfraction 9. For each set of the fractions, four plates (20 × 10) were used and a plate each (out of the 4) was sprayed with anisaldehyde–sulphuric acid spraying reagent (followed by exposure to heat at 110°C for few minutes) to identify bands where the scrapping on the remaining three plates were carried out. The scrapped samples were extracted with methanol and chloroform (30 mL each), filtered, concentrated (by reducing the volume of the extracting solvents) and later sent for gas chromatography-mass spectroscopy (GCMS) analysis following a second round of pTLC processes (involving re-spotting, development and visualisation) in order to confirm the purity of the identified compound.

Gas chromatography-mass spectroscopy

All the samples by GC-MS were analysed using Perkin Emler® gas chromatography (Clarus® 580) functioning with a mass selective detector (MSD) mass spectrophotometer (Clarus® SQ8S) instrument that has a built-in auto-sampler. The samples were analysed on an Elite-5 ms (30 m × 0.25 mm internal diameter × 0.25 µm) column. Oven temperature was programmed to progress from 37 °C to 320 °C at a rate of 18 °C/min – 25 °C/min and delayed for 0.5 and 1.85 min at 18 °C and 320 °C, respectively. The temperature of the injector was 250 °C with the MS ion source temperature being 280 °C, with a full scan and solvent delay of 0.00–2.30 min. The MS scan range was m/z 35–500 in 0.10 sec. One microlitre of each sample was injected at a split flow rate of 20 mL/min in helium carrier gas.

Statistical analysis

Data analyses were performed by one-way analysis of variance (ANOVA), followed by Bonferroni's multiple

comparison test, and the results were expressed as mean ± standard error of mean (SEM) using Graph Pad Prism, version 3.0 for Windows, Graph Pad Software, San Diego, California, USA. A value of $p < 0.05$ was considered to be statistically significant.

Results

Sample yield

Table 1 reveals the yields and percentage yields of the extracts and fractions. Aqueous ethanol and hexane were found to produce the highest yields amongst the crude extract (9.18 g) and fractions (20.03), respectively.

Antioxidant assays

The IC_{50} values for the free radical scavenging abilities of the root extracts of *E. elephantina* are presented in Table 2. The aqueous extract displayed the best radical (DPPH) scavenging and metal-chelating effects, judging by its lowest IC_{50} values (0.573 mg/mL and 1.937 mg/mL, respectively), which was insignificantly lower ($p > 0.05$) compared with other extracts, except for ascorbic acid, having ($p < 0.05$) IC_{50} values of 1.439 mg/mL and 0.282 mg/mL respectively.

TABLE 1: Yield of crude extracts and partitioned fractions of *E. elephantina* root.

Extracts/Fractions	Yield (g)	Yield (%)
Crude extracts		
Aqueous	4.86	16.2
Ethanol	7.08	23.6
Aqueous-ethanol	9.18	30.6
Partitioned fractions		
Hexane	20.03	21.5
Chloroform	15.25	16.4
Ethyl acetate	14.00	15.1
Butanol	13.59	14.6
Aqueous	13.05	14.0

Note: The yield of crude extracts from aqueous ethanol was found to give the highest with 9.18 g followed by ethanol (7.08 g) and finally aqueous (4.86 g) corresponding to percentage yields of 30.6, 23.6 and 16.2, respectively (Table 1). Additionally, a total yield of 93.04 g corresponding to a percentage yield of 18.61 obtained for the partitioning study.

TABLE 2: Free radical scavenging and metal chelating abilities of different extracts and fractions of *E. elephantina* root.

Variable	Antidiabetic assays (IC_{50} [mg/mL])			
	DPPH	Hydroxyl radical	ABTS	Metal chelating
Extracts†				
Ethanol	0.855 ± 0.111[a]	0.641 ± 0.048[a]	0.017 ± 0.243[a]	7.261 ± 5.567[a]
Aqueous ethanol	0.834 ± 0.072[a]	0.596 ± 0.052[a]	9.468 ± 8.089[b]	3.209 ± 5.567[b]
Aqueous	0.573 ± 0.001[b]	0.059 ± 0.026[b]	2.126 ± 2.605[c]	1.937 ± 0.411[c]
Ascorbic acid	1.439 ± 2.827[c]	1.419 ± 3.803[c]	35.52 ± 24.27[d]	0.282 ± 0.039[d]
Fractions†				
Hexane	0.171 ± 0.46[a]	ND	0.170 ± 0.55[a]	ND
Chloroform	0.226 ± 0.15[b]	ND	0.236 ± 0.55[b]	ND
Ethyl acetate	0.583 ± 0.58[c]	ND	1.189 ± 0.02[c]	ND
Butanol	0.445 ± 0.22[d]	ND	0.397 ± 0.58[d]	ND
Aqueous	0.154 ± 0.51[a]	ND	0.216 ± 0.34[b]	ND
Gallic acid	0.497 ± 0.51[e]	ND	0.434 ± 0.58[e]	ND

Note: The values are expressed as mean ± standard error of mean (SEM) of triplicate determinations.

ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; DPPH, 1.1-diphenyl-2-picrylhydrazyl; ND, not determined; IC_{50}, inhibitory concentrations.

†, Values with different superscript letters along the same rows for each parameter are significantly different from each other at $p < 0.05$.

Similarly for hydroxyl radical assay, the aqueous extract displayed the lowest IC_{50} value (0.059 mg/mL), which is significantly lower ($p < 0.05$) than the other extracts and the standard, indicating a better activity. The ethanol extract exhibited the lowest IC_{50} value (0.017 mg/mL) compared with other extracts and ascorbic acid, signifying the most potent effect against the ABTS radical. Overall, the aqueous extract showed the best free radical scavenging effect by inhibiting the activities of these radicals in three of the four evaluated assays.

As shown in Table 2, HF, however, revealed the best radical scavenging effect against DPPH and ABTS. This was observed by its lower IC_{50} value (0.171 mg/mL) for DPPH and the lowest ABTS (0.170 mg/mL) IC_{50} values. The activity was followed by AF, with IC_{50} values of 0.154 mg/mL and 0.216 mg/mL, respectively, whilst the least inhibition was observed in EF, with the IC_{50} values of 0.583 mg/mL and 1.189 mg/mL, respectively.

Antidiabetic assays

Root extracts of *E. elephantina* showed good inhibitory activities against the carbohydrate-hydrolysing enzymes, such as α-amylase and α-glucosidase, as shown in Table 3. The aqueous extract of *E. elephantina* exhibited the highest inhibition of the activity of α-glucosidase indicated by the lowest IC_{50} value (0.363 mg/mL), which was significantly lower ($p < 0.05$) than other extracts and the standard. However, for alpha-amylase, the ethanol extract revealed the lowest IC_{50} value (0.346 mg/mL), suggesting its ability to strongly inhibit the enzyme. The inhibition is followed by aqueous ethanol (0.391 mg/mL) and finally aqueous (0.453 mg/mL). The modes of inhibitions of α-amylase and α-glucosidase, as shown in Figures 1 and 2, respectively, by the aqueous root extract of *E. elephantina* showed a near-competitive inhibition towards α-amylase, signifying a close V_{max} value (0.16 mM/min) between the control and the extract with a decrease in K_m values from 0.182 (extract) to 0.156 (control) mM^{-1} (Figure 1). For

TABLE 3: *E. elephantina* root extenuates the activities of carbohydrate-hydrolysing enzymes.

Variable	Antidiabetic assays (IC_{50} [mg/mL])	
	α-glucosidase	α-amylase
Extracts		
Ethanol	0.739 ± 0.091[a]	0.346 ± 0.107[a]
Aqueous ethanol	0.536 ± 0.032[b]	0.391 ± 0.079[a]
Aqueous	0.363 ± 0.038[c]	0.453 ± 0.018[b]
Acarbose	45.77 ± 51.24[d]	4.178 ± 1.327[c]
Fractions		
Hexane	0.894 ± 0.52[a]	4.373 ± 0.35[a]
Chloroform	0.813 ± 0.32[a]	1.793 ± 0.20[b]
Ethyl acetate	0.361 ± 0.53[b]	4.101 ± 0.03[c]
Butanol	1.031 ± 0.25[c]	2.212 ± 0.02[d]
Aqueous	2.115 ± 0.79[d]	5.591 ± 0.59[e]
Acarbose	2.621 ± 0.02[d]	1.697 ± 0.18[b]

Note: Values are expressed as mean ± standard error of mean (SEM) of triplicate determinations. Values bearing different superscript along the same row for each parameter are significantly different from each other at $p < 0.05$.

α, alpha; IC_{50}, inhibitory concentrations.

α-glucosidase, it was uncompetitively inhibited with a decrease in Vmax values from 0.005 (extract) to 0.0005 (control) mM/min and Km values from 0.038 (extract) to 0.004 (control) mM^{-1} between the extract and the control (Figure 2).
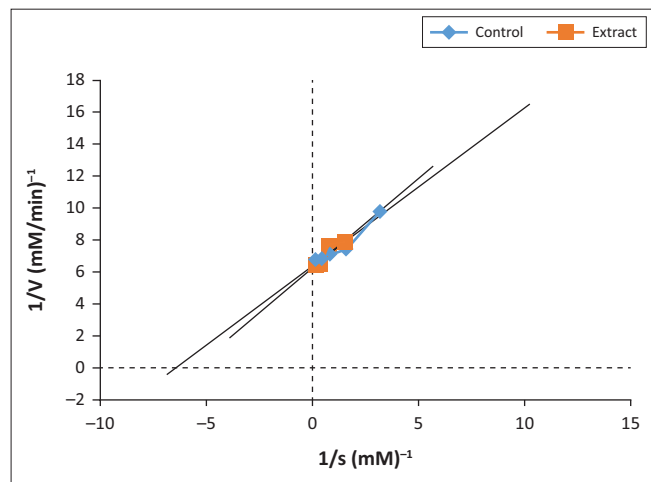

FIGURE 1: Mode of inhibition of α-amylase by aqueous root extract of *E. elephantina* showing a near-competitive inhibition.
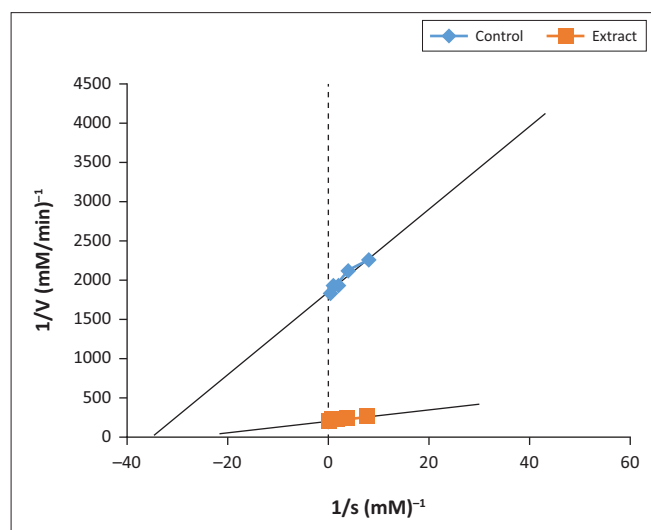

FIGURE 2: Mode of inhibition of α-glucosidase by aqueous extract of *E. elephantina* root revealing an uncompetitive inhibition.

The fractions of *E. elephantina* showed mild-to-moderate inhibitory activities against the carbohydrate-hydrolysing enzymes, such as α-glucosidase and α-amylase, as shown in Table 3. Ethyl acetate (0.361 mg/mL), chloroform (0.813 mg/mL) and hexane (0.894 mg/mL) fractions going by IC$_{50}$ reported the lowest values against α-glucosidase, revealing better activities than the standard, acarbose (2.621 mg/mL). Acarbose (1.697 mg/mL), however, followed by chloroform (1.793 mg/mL) and butanol (2.212 mg/mL) displayed the lowest IC$_{50}$ values in the inhibition of α-amylase activity.

Chromatographic reports

The column chromatography of HFs produced 134 fractions, and based on the chromatographic features of each fraction on TLC, they were pooled together to afford 15 subfractions. The subfractions 4 and 9 were further analysed using chloroform: ethanol: acetic acid (4:1:3 drops) (for subfraction 4) and chloroform: ethanol (3:2) (subfraction 9) solvent systems, where a prospective spot was obtained for further analysis. The GCMS chromatogram for subfraction 4 revealed a number of phytoconstituents, such as triphenylphosphine oxide, with 46.86% abundance for subfraction 4 (Table 4), whilst subfraction 9 depicted 1-Tetracosanol (20.65%) (Table 5).

Discussion

Free radicals could be toxic and, at the same time, beneficial to the human body; this is because at low levels, they can be helpful for maintaining cellular responses and immune function. However, a state of oxidative stress may be induced as a result of its excessive generation in the human body, leading to the destruction of body biomolecules, such as proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA) (Lobo et al. 2010; Young & Woodside 2001). Intriguingly, many of the human degenerative diseases, such as DM and cancer, (Szalay 2016), are reported to be triggered by excessive production of free radicals (Satlisha, Lingaraju & Sham Prasad 2011). Hence, prompt intervention of an antioxidant, particularly those arising from natural products, may come handy in neutralising the ravishing effects of

TABLE 4: Depicted phytoconstituents from gas chromatography-mass spectroscopy chromatogram of subfraction 4.

Peak number	Retention times	Area (%)	Compound names	Pharmacological activities	References
1	3.665	0.60	Toluene	INA	INA
2	11.535	0.24	Cetene	INA	INA
3	12.827	5.64	2, 4-bis (1, 1-dimethylethyl)-Phenol	Anti-inflammatory, antibacterial, antioxidant	Amaral et al. 2014; Chandrasekar et al. 2015; Kumar et al. 2011; Yogeswari et al. 2012
4	13.715	3.11	E-15-Heptadecenal	Antibacterial	Afifah et al. 2012
5	13.948	0.41	Hexadecane	Antibacterial	Gideon 2015; Slantchev et al. 2002; Yayli et al. 2006
6	14.905	0.27	Dodecanoic acid, 1-methylethyl ester	Antibacterial, antioxidant, hypercholesterolemic, candidicidal	Gideon 2015; Mitova et al. 2011
7	15.099	4.93	Triphenylphosphine oxide	INA	INA
8	15.215	12.34	Triphenylphosphine oxide	INA	INA
9	15.383	9.14	Triphenylphosphine oxide	INA	INA
10	15.541	20.45	Triphenylphosphine oxide	INA	INA

INA, Information not available.

Note: Please see the full reference list of the article, Moloi, L.D, Balogun, F.O. & Ashafa, A.O.T., 2021, 'Mitigation of free radicals and carbohydrate-linked enzymes by extracts and partitioned fractions of *Elephantorrhiza elephantina* (Burch.) Skeels root', *Journal of Medicinal Plants for Economic Development* 5(1), a109. https://doi.org/10.4102/jomped.v5i1.109, for more information.

TABLE 5: Identified phytoconstituents in the chromatogram of subfraction 9.

Peak number	Retention times	Area (%)	Compound names	Pharmacological activities	References
1	3.701	0.84	Toluene	INA	INA
2	11.536	0.64	1-Tetradecene	INA	INA
3	12.829	20.56	2,4-Bis (1,1-dimethylethyl)-phenol	Anti-inflammatory, antibacterial, antioxidant	Amaral et al. 2014; Chandrasekar et al. 2015; Kumar et al. 2011; Yogeswari et al. 2012
4	13.645	5.66	Tetradecyl trifluoroacetate	INA	INA
5	13.715	0.92	Hexadecane	Antibacterial	Gideon 2015; Slantchev et al. 2002; Yayli et al. 2006
6	13.948	0.65	Dodecanoic acid, 1-methylethyl ester	Antibacterial, antioxidant, hypercholesterolemic, candidicidal	Gideon 2015; Mitova et al. 2011
7	14.624	2.24	2-Propenoic acid, pentadecyl ester	INA	INA
8	15.768	17.40	Trifluoroacetic acid, pentadecyl ester	INA	INA
9	15.847	3.38	Heptadecane	INA	INA
10	17.869	3.73	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	INA	INA
11	19.161	20.65	n-Tetracosanol-1	Antibacterial	Kuppuswamy et al. 2013
12	19.291	3.24	Heneicosane	INA	INA
13	21.750	1.43	Methyl 10-trans,12-cis-octadecadienoate	INA	INA
14	21.974	1.67	9-Octadecenoic acid (Z)-, methyl ester	Antimicrobial, anti-inflammatory, cancer preventative	Gideon 2015; Simin et al. 2000; Wagh et al. 2007
15	22.644	5.65	[1,1'-Biphenyl]-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethylethyl)-	INA	INA

INA, information not available.

Note: Please see the full reference list of the article, Moloi, L.D, Balogun, F.O. & Ashafa, A.O.T., 2021, 'Mitigation of free radicals and carbohydrate-linked enzymes by extracts and partitioned fractions of *Elephantorrhiza elephantina* (Burch.) Skeels root', *Journal of Medicinal Plants for Economic Development* 5(1), a109. https://doi.org/10.4102/jomped.v5i1.109, for more information.

these free radicals. This study evaluated the antioxidant and antidiabetic potentials of different extracts and fractions of *E. elephantina* roots. The results of this investigation revealed that the aqueous extract of *E. elephantina* exhibited the best activity in scavenging free radicals (DPPH and hydroxyl radical) and also chelate metal ions. Thus, indicating its excellent antioxidant and disease-control capacities since it has been reported that hydroxyl radical is one of the major oxygen-containing free radicals resulting in the ontogenesis of many diseases (Lobo et al. 2010). Moreover, the ability of the aqueous extract to wipe the DPPH and OH⁻ radical partly suggests the tendency of the extract to decolorise DPPH with the potential ability to scavenge DPPH radicals. Aside the fact that the result corroborated the findings of Mpofu et al. (2014) on the scavenging ability of DPPH radical by the aqueous root extract, it may be suggested that the antioxidative potential might be attributed to phenolic compounds in the extract of the plant (Olaokun et al. 2020), which might be irrespective of the plant parts as the hot leaf extract similarly in a recent study by Olaokun et al. (2020) inhibited DPPH and ABTS radicals. Additionally, the ethanol extract reported the lowest IC_{50} value for ABTS, indicating that the extract also has the ability to scavenge the free radical and prevent oxidative stress. Judging by the revelation in this study and other reports (Mpofu et al. 2014; Olaokun et al. 2020), it could be deduced that the use of polar solvents (aqueous, ethanol and methanol) maximally extracts antioxidative polar compounds. This is best felt or established with the aqueous extract when compared with the other polar solvent (ethanol) in this study; however, the reverse is the case with the study of Mpofu et al. (2014) where the methanol extract exhibited a superior radical scavenging effect compared with the aqueous extract. The variation might be attributed to differences in methods. In line with the aforementioned results, the observed activity of HF (from the crude aqueous extract) against ABTS and DPPH (although AF

highly inhibited DPPH) followed by CF may be suggestive of the antioxidant potential of the plant across solvent ranges (polar and non-polar).

Alpha-glucosidase is a carbohydrate-hydrolysing enzyme found in the brush border of the small intestine. It functions by breaking down disaccharides into glucose. The control of postprandial hyperglycaemia is very crucial in the treatment of diabetes and its associated complications. Hence, there is an absolute need for regulating enzymes, such as α-glucosidase and α-amylase, involved in the constant metabolism of carbohydrates to glucose (Kwon et al. 2007). In this study, the aqueous extract of *E. elephantina* displayed the strongest inhibition of α-glucosidase. This was exhibited by its lowest IC_{50} value for the enzyme, which is lower than that for acarbose (a known antihyperglycaemic agent). A strong inhibition of α-glucosidase is one of the features of an antidiabetic agent (Kwon et al. 2007). However, the findings of this study contradict the report of Olaokun et al. (2020) where ethanol and aqueous (cold) leaf extracts are the weakest inhibitors of the enzyme. Notwithstanding the aforementioned findings, the result of this study is in consonance with the report of Kazeem and Ashafa (2015) where the aqueous extract of *Dianthus basuticus* reported a strong inhibition against α-glucosidase, which consequently may be attributed to the antioxidant activity of the aqueous extract of the plant. Alpha-amylase is found in the saliva and pancreas, and it catalyses the conversion of polysaccharides to disaccharides (Manohar et al. 2002). Thus, a good inhibitor of this enzyme would go a long way in slowing down its activity, and for this to happen, the extract or the inhibitor should have the capacity to inhibit the enzyme mildly to be considered a good antidiabetic agent. Mild inhibition is depicted by an extract with a higher IC_{50} value for α-amylase. Although it is worth mentioning that the ethanol extract

was the strongest inhibitor of this enzyme in this study as similarly reflected in the work of Olaokun et al. (2020), however, the aqueous extract displayed mildest inhibition depicted by its highest IC_{50} value of 0.453 mg/mL; this makes it suitable for use as a potent hypoglycaemic agent better than the synthetic agent. Generally, a good antidiabetic agent with the possibility of being effective above OHAs (such as acarbose and miglitol) without revealing any adverse side effects, such as constipation and bloating, should be a mild inhibitor of alpha-amylase and a strong inhibitor of alpha-glucosidase. The findings of the study regarding the crude extract (aqueous) were also corroborated or consolidated by HF, which depicted strong alpha-glucosidase and mild alpha-amylase inhibitions. Thus, this was in line with the aforementioned antioxidant potential of the fraction and the effectiveness of varying solvent ranges (polar and non-polar alike). The antidiabetic activity of medicinal plants (such as *Cucumis prophetarum*) partitioned-non-polar solvents (chloroform, hexane, etc.) has been reported (Gawli & Lakshimidevi 2015).

The Lineweaver–Burk plot otherwise known as a double reciprocal plot is a graphical representation described by Hans Lineweaver and Dean Burk to reflect and or analyse the (Michaelis–Menten) equation for understanding the kinetics of enzyme inhibition, which can be competitive, uncompetitive and non-competitive. In fact, the plot helps in determining important parameters, such as maximum reaction velocity (V_{max}) and K_m, which is the Michaelis–Menten constant. In this study, the possible kinetics of inhibition of α-amylase by the aqueous root extract of *E. elephantina* depicts a near-competitive inhibition, suggesting that the active components of the extract may bind reversibly to the active site of the enzyme. This shows that the extract has a potential to slow down the breakdown of oligosaccharides to disaccharides. Moreover, the extract displayed an uncompetitive inhibition against α-glucosidase, implying that the active components of the extract binds to the other sites of the enzyme–substrate complex, resulting in the slowing down of disaccharide synthesis to glucose (Cornish-Bowden 2013).

The GC-MS chromatograms of *E. elephantina* (subfractions 4 & 9) revealed the presence of seven major phytochemicals, including triphenylphosphine oxide, 2,4-bis (1,1-dimethylethyl)-phenol, E-15-heptadecenal, hexadecane, 1-methylethyl ester dodecanoic acid, cetene, toluene (subfraction 4) and 2,4-bis (1,1-dimethylethyl)-phenol, tetradecyl trifluoroacetate, 1-tetradecene, hexadecane, 1-methylethyl ester dodecanoic acid, pentadecyl ester 2-propenoic acid, heptadecane, methyl ester benzenepropanoic acid, n-tetracosanol, heneicosane, methyl 10-trans,12-*cis*-octadecadienoate and (Z)-, methyl ester 9-octadecenoic acid. A number of these chemical components have established pharmacological potentials. Typically, 2,4-bis (1,1-dimethylethyl)-phenol found in both the subfractions is reported to possess anti-inflammatory (Amaral et al. 2014; Chandrasekar et al. 2015; Kumar, Bhatnagar & Srivastava 2011; Yogeswari et al. 2012), neuronal and antioxidant

(Yang et al. 2016) effects. The 2,4-di-*tert*-butylphenol plays a significant role as an antioxidant and marine metabolite (NLM 2020). Z-Methyl 9-octadecenoic acid was found to act as anti-inflammatory and anticancer agents (Gideon 2015), whilst 1-methyllethyl ester dodecanoic acid possessed antioxidant and hypercholesterolaemic activities (Gideon 2015; Mitova et al. 2011), which is suggestive of antihyperglycaemic effect of the plant as hypercholesterolemia is one of the risk factors or complications of diabetes.

Conclusion

The results of this investigation revealed that the extracts and fractions of *E. elephantina* possessed antioxidant and antidiabetic effects *in vitro*. A possible mechanism of antidiabetic effect of this plant could be through the inhibition of diabetes-related enzymes. Therefore, it could be inferred that the study supports the traditional use of *E. elephantina* in the management of diabetes in the eastern Free State Province of South Africa.

Furthermore, with this submission, research work on pharmacological potentials (especially against diabetes and safety profile) of the plant in animal models is on-going, which to our understanding would go a long way in efforts towards developing a drug moiety against diabetes in order to fully elucidate the potentials of the plant. Additionally, as GC-MS may not be the only technique required for the identification of the active compounds responsible for the elicited effects; hence, isolation and characterisation of the plant involving additional techniques, such as mass spectrophotometry and nuclear magnetic resonance (carbon 1 and 13), are recommended.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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