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Phytochemical profile, anti-glycation effect, and advanced glycation end-products protein cross-link breaking ability of *Sclerocarya birrea* stem-bark crude extracts

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ABSTRACT

Introduction: *Sclerocarya birrea* stem-bark is widely used for the treatment of many medical conditions. Advanced glycation end-products (AGEs) are implicated in the pathogenesis of vascular complications of diabetes mellitus. The study, other than phytochemical composition, evaluated the anti-glycation and AGEs-protein cross-link breaking effects of *S. birrea* stem-bark extracts.

Methods: Different *S. birrea* extracts and aminoguanidine (used as control) were incubated with bovine serum albumin (BSA) and glucose/fructose at 37°C for 40 days. Amounts of fluorescent AGEs (FAGEs) and immunogenic AGEs formed were determined. Anti-glycation activity percentage of each extract and aminoguanidine was calculated. Their AGEs-protein cross-link breaking abilities were also assessed. Standard techniques were employed for phytochemical screening. Volatile compounds were identified by means of gas chromatography mass spectrometry (GC-MS).

Results: *S. birrea* stem-bark *n*-hexane extract was statistically more effective than aminoguanidine against the formation of total immunogenic AGEs ($P<0.05$). For FAGEs, ethyl acetate, methanol, and water extracts exerted significantly higher anti-glycation effects than aminoguanidine ($P<0.001$). Methanol extract exhibited the highest anti-glycation effect with an average IC_{50} value of 0.142 mg/mL against FAGEs. All extracts were effective in releasing BSA from the preformed collagen-AGEs-BSA cross-links. GC-MS enabled the identification of many biologically important compounds, including campesterol, stigmasterol, and 1-heptatricontanol.

Conclusion: *S. birrea* stem-bark has a potential for usage in the management of complications in uncontrolled glucose metabolism.

Implication for health policy/practice/research/medical education:
The results of this study may lead to the isolation of bioactive phytochemicals that might be used for the prevention of vascular complications of diabetes and other age-related or neurodegenerative diseases.

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Introduction

A large body of evidence has implicated advanced glycation end-products (AGEs) in the development of microvascular and macrovascular complications of diabetes and other age-related diseases (1-4). AGEs are known to promote the development of vascular complications of diabetes through two main mechanisms, i.e., the cross-linking of proteins in the extracellular matrix of the blood vessels trapping and oxidizing the low-density lipoprotein cholesterol (LDL-C) (5) and receptor binding on vascular endothelial cells (6). The latter mechanism activates a signal transduction pathway, which results in vascular endothelial dysfunction, an established mediator of atherosclerotic vascular diseases (7).

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In light of the involvement of AGEs in the pathogenesis of vascular complications of diabetes and age-related diseases, investigations seeking to find agents that can prevent the formation of AGEs or break down existing AGEs-protein cross-links are increasingly important (8). In this regard, several chemical agents, such as aminoguanidine, benfotiamine, alagebrium, and *N*-phenacylthiazolium bromide (PTB) with the capacity to either prevent the formation of AGEs or break the existing AGEs-protein cross-links have been discovered (9). However, most of these substances, for example, aminoguanidine, have failed clinical trials due to their toxicities (8). Furthermore, most of the AGEs-protein cross-linking breakers do not act on glucosepane, the most abundant glucose-derived AGEs-protein cross-link found in human tissues (10). Thus, there is a need for the discovery of new effective and non-toxic anti-glycation agents.

Sclerocarya birrea [(A. Rich.) Hochst.] subspecies *caffra* (Sond.) Kokwaro, commonly known as 'Cider tree' or 'Morula', belongs to the family Anacardiaceae and is indigenous to sub-Saharan Africa (11-13). Its fruit is popularly known as marula, which is a term used to refer to the fruit in the Sotho language of the Republic of South Africa. The stem bark is widely used across the African continent to manage a plethora of ailments such as malaria, diarrhoea, dysentery, ulcers, and diabetes mellitus (14,15). Crude extracts of the stem bark of *S. birrea* have been shown to have blood glucose lowering effects in both animal and human studies (16,17). The anti-diabetic, anti-oxidant, anti-inflammatory, and anti-cancer activities of *S. birrea* stem-bark extracts have also been documented (13,16,18). Furthermore, the anti-aging potential of *S. birrea* extracts have been reported (19). In addition to its blood glucose lowering effect, antioxidant and anti-inflammatory effects of *S. birrea* could inhibit the formation of AGEs and/or break the cross-links formed with proteins.

Sclerocarya birrea stem-bark was selected on the basis of its known and scientifically proven blood glucose lowering property, as well as its anti-inflammatory and antioxidant activities. To our knowledge, no study has reported the anti-glycation effect of *S. birrea* or its ability to release bovine serum albumin (BSA) from collagen-AGEs-BSA cross-links. This study, other than phytochemical composition, evaluated the anti-glycation and AGEs-protein cross-link breaking effects of *S. birrea* stem-bark extracts.

Materials and Methods

Plant material collection and identification

Sclerocarya birrea [(A. Rich.) Hochst.] is listed in the plant list (http://www.theplantlist.org/). The stem-bark was obtained locally at the Sefako Makgatho Health Sciences University (25°37'06.2"S, 28°01'26.7"E) in October 2012. The sample of *S. birrea* was sent to the National herbarium, Pretoria, South Africa National Biodiversity Institute (SANBI) for identification and was confirmed to be that of *S. birrea* (Genspec 4558000).

Preparation of plant extracts

The collected plant material was dried and grounded into fine powder. Dry powdered stem-bark of *S. birrea* was extracted sequentially (ratio 1:10 w/v) with *n*-hexane, ethyl acetate, methanol, and water according to a previously reported procedure (Figure 1) (20). The *n*-hexane and ethyl acetate extracts were dissolved in dimethyl sulfoxide (DMSO) because of their solubility and used in the experiment. Similarly, the methanol and water extracts were reconstituted in water for use in the experiment because of their solubility.

Gas chromatography-mass spectrometry (GC-MS) analysis

Separation of hydrocarbons and other volatile compounds present in the *n*-hexane and ethyl acetate stem-bark extracts of *S. birrea* were determined with a SHIMADZU GC coupled to a QP2010 SE mass detector. A Zebron capillary column (ZB-$^{Tm-1}$), 30 mm long with an internal diameter of 0.25 mm ID and film thickness of 0.25 µm, was used. The electron ionization system (ionizing energy of 70 eV) was used for the analysis. The GC oven temperature was initially held at 50°C/1 min and gradually increased to 180°C, 240°C, and 280°C at a rate of 20°C until the final temperature of 300°C was reached for 10 min. Injector and detector temperature were maintained at 290°C. The carrier gas used, Helium (5.0) was utilized at a flow rate of 2.21 mL/min, with an injection volume of 5 µL. For the mass detector, the operation was carried out at 230°C with a scan range of 0.30 scan/second from 50 to 700 m/z. The solvent delay time was 6 minutes with

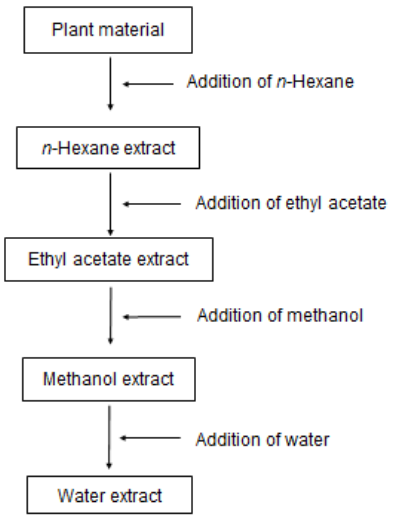

Figure 1. Sequential solvent extraction adapted from a previously reported procedure (20).

total sample runtime of 33.5 minutes. Software used was GCMSsolution version 2.6.

Determination of the anti-glycation effect of plant extracts

The extracts were screened for their anti-glycation effects according to the reported procedure (21) with some slight modifications. In brief, an aliquot (500 µL) of 10 mg/mL BSA solution (dissolved in 76 mM sodium phosphate buffer, pH 7.4 containing 0.02% sodium azide) was incubated with an aliquot (500 µL) of a glucose or fructose solution (50 mg/mL) at 37°C for 40 days with and without the plant extract test samples. An aliquot (500 µL) of aminoguanidine solution was used as a positive control. At the end of the incubation period, the amounts of fluorescent AGEs (FAGEs) were determined at various concentrations (0.25. 0.5. 1.0, 2.0, and 4.0 mg/mL) according to a known procedure (22). In brief, 100 µL of the reaction mixture was measured spectrofluorometrically (excitation at 370 nm and emission at 445 nm) on a Promega GloMax multi-detection system (Wisconsin, USA), and the results were expressed in arbitrary units (ratio of emission and excitation light intensities). The amount of total immunogenic AGEs (TIAGEs), N^{ϵ}-(carboxymethyl)lysine (CML) and N^{ϵ}-(carboxyethyl) lysine (CEL) were determined using enzyme immunoassay kits (STA-317, STA-316 and STA-300 OxiSelect™, respectively) purchased from Cell Biolabs inc. (San Diego, USA) according to the manufacturer's instructions. A final concentration of 1 mg/mL for the test samples and aminoguanidine was used for the immunogenic AGEs investigation. The percentage of anti-glycation activity was calculated using the following formula:

$$\%Anti-glycation\ activity = \left(\frac{[AGEs]_{control} - [AGEs]_{extract}}{[AGEs]_{control}}\right) \times 100$$

Where $[AGES]_{control}$ and $[AGEs]_{extract}$ are the absorbance of BSA + glucose/fructose only and absorbance of BSA + glucose/fructose + test samples/aminoguanidine, respectively.

Ability to breakdown AGEs-protein cross-links

The ability of crude plant extracts to breakdown AGE-protein cross-links was assessed according to a previously reported procedure (23). In brief, aliquots (500 µL) of BSA were incubated with an aliquot (500 µL) of glucose or fructose at 37°C for 80 days, followed by the addition of 50 µL of this reaction mixture into each well of a 96-well collagen-coated plate (Gibco®, Maryland, USA). The mixture was further incubated for 4 hours at 37°C to allow for the formation of collagen-AGEs-BSA cross-links. The wells were then washed three times with phosphate-buffered saline solution (containing 0.05% Tween-20) (PBST), a product of Amresco Inc. (Ohio, USA) to remove collagen un-bound AGE-BSA. Thereafter, 50 µL of each plant extract in triplicate was added to the mixtures in the plate. Aminoguanine solution (50 µL) served as a positive control, whereas collagen-AGEs-BSA mixtures without plant extracts served as negative controls. The wells were then washed four times with PBST, followed by the addition of an anti-BSA primary antibody (E11-113 Bovine Albumin ELISA kit, Bethyl Laboratories, Texas, USA). Next, it was incubated for 1 hour at 25°C, washed, and further incubated with streptavidin-conjugated horseradish peroxidase secondary antibody (100 µL). One hundred microliters (100 µL) of 3,3',5,5'-tetramethylbenzidine substrate was added to each well, incubated for further 30 minutes, and the enzymatic reaction was terminated with the addition of 100 µL stop solution (0.18 M H_2SO_4).

Absorbance measurements were recorded at 450 nm using a Tecan Spectra Microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The percentage of AGE-protein cross-linking breaking activity of each plant extract and aminoguanidine was calculated according to the following formula:

$$\%\ \text{AGE cross} - \text{link breaking activity} = \left(\frac{A_{450}\ \text{control} - A_{450}\ \text{extract}}{A_{450}\ \text{control}}\right) \times 100$$

Where A_{450} control and A_{450} extract are the absorbance of collagen + glucose/fructose + BSA only and the absorbance of collagen + glucose/fructose + BSA + extracts/aminoguanidine, respectively.

Statistical analysis

Data were expressed in tables and figures as mean ± standard error of mean (SEM) for continuous variables and as percentages for categorical variables. Comparisons between/among different groups were performed using either the students' *t* test (two groups) or analysis of variance (ANOVA) (three or more groups) for continuous variables and the chi-square test for categorical variables. Differences between groups were considered to be significant when the *P* value was less than 0.05. Statistical analysis was performed using the IBM SPSS® Statistical package (version 24).

Results

Yields of *Sclerocarya birrea* stem-bark extracts

Seventy-four grams of dry powdered stem-bark of *S. birrea* was extracted sequentially with 740 mL each of *n*-hexane, ethyl acetate, methanol, and water. The highest percentage yield of 11.96% was obtained with the polar solvent, methanol (8.85 g), compared to other solvents. The higher yield for methanol was obtained because, during the sequential extraction, most of the polar components were extracted by this solvent, and the remaining was then extracted by water. Water gave a percentage yield of 3.45% (2.55 g). With ethyl acetate, a percentage yield of 1.16% (0.86 g) was obtained. The lowest percentage yield was obtained with the *n*-hexane solvent (0.24%, equal to 0.18 g).

Phytochemical composition of *Sclerocarya birrea* stem-bark extracts

Identification of the phytoconstituents of *S. birrea* stem-bark was achieved by gas chromatography-mass spectrometry (GC-MS) analysis of the less polar extracts. The result revealed the presence of various volatile compounds (Table 1). Identified compounds included hydrocarbons, fatty acids, esters, alcoholic compounds, aldehyde, and ketone-containing compounds, halogen-containing compounds, and sulfur-containing compounds.

Anti-glycation against FAGEs

The percentage anti-glycation effects of *S. birrea* stem-bark extracts and aminoguanidine against both BSA-glucose and BSA-fructose derived FAGEs are shown in Figure 2 (A and B). This revealed that significantly higher anti-glycation activities were demonstrated by the ethyl acetate, methanol, and water extracts than aminoguanidine ($P < 0.001$) for both sugar-derived FAGEs.

The results of anti-glycation activities of *S. birrea* stem-bark extracts and standard inhibitor (aminoguanidine) at various concentrations against BSA-glucose and BSA-fructose derived FAGEs are presented in Figure 3. The *n*-hexane, ethyl acetate, and water extracts of *S. birrea* stem-bark and aminoguanidine exhibited increasing anti-glycation activities with increasing concentration against BSA-glucose and BSA-fructose derived FAGEs. In contrast, methanol stem-bark extract of *S. birrea* showed a decline in activity with increased concentration.

The result shown in Table 2 revealed that methanol and water stem-bark extracts of *S. birrea* were able to exert 50% inhibitory effect against formation of both BSA-glucose and BSA-fructose derived FAGEs at concentrations lower than that of aminoguanidine.

The methanol and water extracts of *S. birrea* stem-bark exhibited lower inhibitory activities of 0.145 mg/mL and 0.158 mg/mL, respectively, compared to aminoguanidine (with an IC_{50} of 0.16 mg/mL) against BSA-glucose derived FAGEs. Similarly, these two extracts had good anti-glycation effects of 0.139 mg/mL (for methanol) and 0.141 mg/mL (for water) compared to aminoguanidine (0.171

Table 1. Some chemical constituents detected by gas chromatography-mass spectrometry (GC-MS) from the *n*-hexane and ethyl acetate stem-bark extracts of *S. birrea*

Compound name	Class of compound	*n*-Hexane	Ethyl acetate
Heptadecane	Hydrocarbon	+	-
Heneicosane	Hydrocarbon	+	-
l-(+)-Ascorbic acid 2,6-dihexadecanoate	Fatty acid	+	+
6-Octadecenoic acid (Z)	Fatty acid	+	+
Tetratetracontane	Hydrocarbon	+	-
1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	Ester	+	-
1-Hentetracontanol	Alcohol	+	+
Octatriacontyl pentafluoropropionate	Fatty acid	+	
1,2-Benzenedicarboxylic acid, ditridecyl ester (phthalic acid, ditridecyl ester)	Ester	+	+
Triacontane, 1-bromo	Halogen-containing	+	-
3 beta-Acetoxystigmasta-4,6,22-triene	Aldehyde	+	-
Cholesta-4,6-dien-3-ol, (3 beta)	Alcohol	+	-
Cholest-7-en-3-ol, 14-methyl-, (3 beta)	Alcohol	-	+
Nonahexacontanoic acid	Fatty acid	-	+
Pentadecanoic acid	Fatty acid	-	+
Stigmasterol	Alcohol	-	+
Gamma-Sitosterol	Alcohol	+	+
Campesterol *[Ergost-5-en-3-ol, (3 beta,24R] * Ergost-5-en-3-ol, (2 beta) *[beta-Methylcholesterol]	Alcohol	+	+
9-Octadecenoic acid (Z)-, phenylmethyl ester *[Benzyl oleate]	Ester	-	+
Octatriacontyl pentafluoropropionate	Halogen-containing	+	-
Stigmasta-5,22-diene, 3-methoxy-, (3 beta, 22E)	Aldehyde	+	-
Stigmastane-3,6-dione, (5, alpha)	Ketone	+	-
Sulfurous acid, octadecyl 2-propyl ester	Sulphur-containing ester	+	-
Triarachine *[Eicosanoic acid, 1,2,3-propanetriyl ester]	Ester	-	+
1-Heptatricontanol	Alcohol	+	-

+ indicates presence; - indicates absence; *[ ] indicates alternative name.

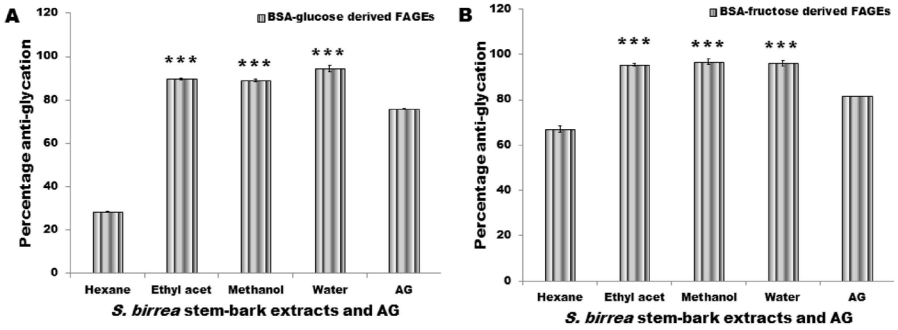

Figure 2. Percentage anti-glycation effects of *S. birrea* stem-bark extracts and aminoguanidine on bovine serum albumin (BSA)-glucose and BSA-fructose derived fluorescent AGEs. *** Significant at $P < 0.001$ in relation to positive standard (AG); AG, aminoguanidine; AGEs, advanced glycation end-products.

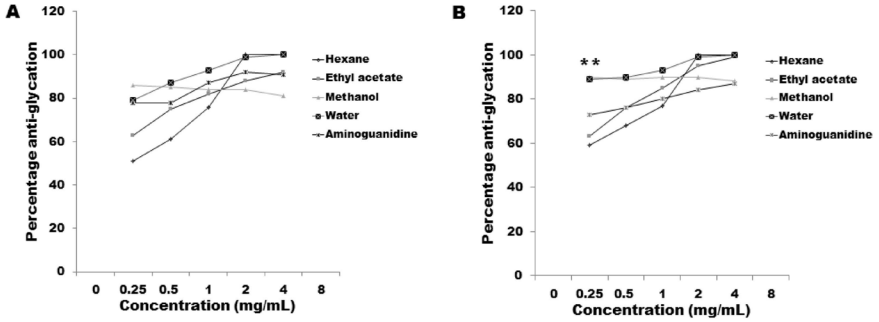

Figure 3. Anti-glycation activities of *S. birrea* stem-bark extracts and standard inhibitor (aminoguanidine) against the formation of (A) bovine serum albumin (BSA)-glucose derived and (B) BSA-fructose derived fluorescent AGEs at various concentrations. ** Significance at $P < 0.01$ for higher inhibitory effect by methanol and water with a minimum concentration of 0.25 mg/mL used compared with aminoguanidine; AGEs, advanced glycation end-products.

mg/mL) against BSA-fructose derived FAGEs. On average, the inhibitory effects of *S. birrea* stem-bark methanol and water extracts against the formation of FAGEs were significantly more effective than that of aminoguanidine ($P<0.05$). The average IC_{50} values recorded for the methanol and water extracts were 0.142 mg/mL and 0.149 mg/mL, respectively.

Anti-glycation effect on immunogenic AGEs

Anti-glycation against total immunogenic AGEs (TIAGEs)

The percentage anti-glycation effects of the different *S. birrea* stem-bark extracts and standard inhibitor, aminoguanidine, on BSA-glucose and BSA-fructose derived TIAGEs are presented in Figure 4 (A and B). All extracts and aminoguanidine exerted anti-glycation effects against both types of sugar-derived TIAGEs. A significantly higher percentage anti-glycation effect compared with aminoguanidine against BSA-glucose and BSA-fructose-derived TIAGEs ($P<0.05$) was observed for the *n*-hexane extract only.

Anti-glycation against N^{ε}-(carboxymethyl)lysine (CML)

All extracts exhibited anti-glycation effects against both BSA-glucose and BSA-fructose derived CML as shown in Figure 5 (A and B). The water stem-bark extract was able to exert a significantly higher anti-glycation effect, even more than the positive control (aminoguanidine) ($P<0.05$), as seen in the BSA-glucose model.

Anti-glycation against N^{ε}-(carboxyethyl)lysine (CEL)

Figure 6 (A and B) illustrates the calculated percentage anti-glycation effects of *S. birrea* stem-bark extracts

Table 2. Inhibitory concentrations of *S. birrea* stem-bark extracts and standard inhibitor aminoguanidine at 50%

Solvent	Glucose IC_{50} (mg/mL)	Fructose IC_{50} (mg/mL)	IC_{50} average values (mg/mL)
n-Hexane	0.245	0.212	0.229^{e}
Ethyl acetate	0.198	0.198	0.198^{d}
Methanol	0.145	0.139	0.142^{a}
Water	0.158	0.141	0.149^{b}
Aminoguanidine	0.160	0.171	0.166^{c}

Values in the same column with different letters are significantly different ($P < 0.05$).

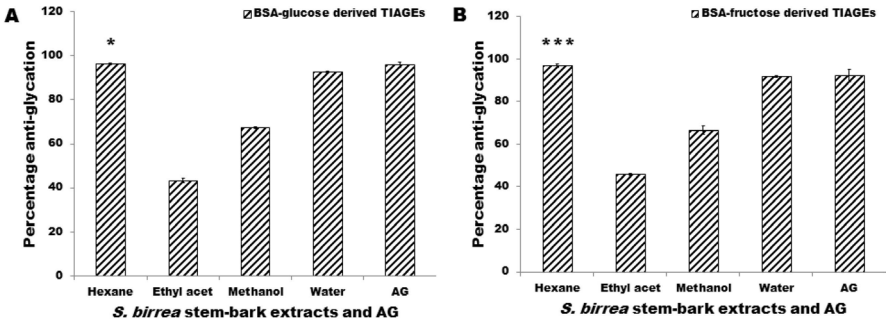

Figure 4. Percentage anti-glycation effects of *S. birrea* stem-bark extracts and aminoguanidine on bovine serum albumin (BSA)-glucose and BSA-fructose derived total immunogenic AGEs. * Significant at $P < 0.05$; *** Significant at $P < 0.001$ compared to the positive standard (AG); AG, aminoguanidine; AGEs, advanced glycation end-products.

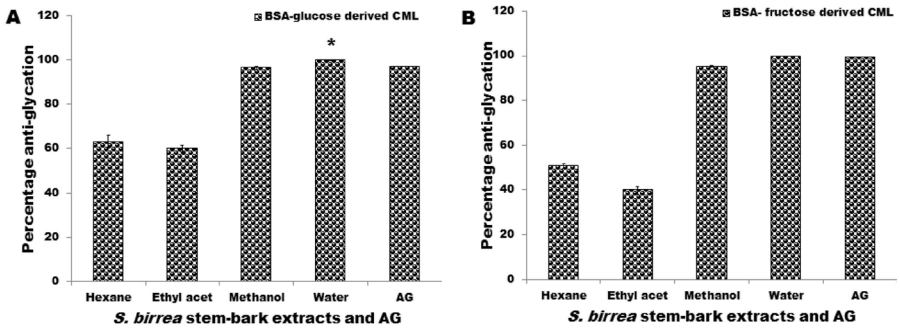

Figure 5. Percentage anti-glycation effects of *S. birrea* stem-bark extracts and aminoguanidine on bovine serum albumin (BSA)-glucose and BSA-fructose derived N^{ε}-(carboxymethyl)lysine. * Significant at $P < 0.05$; AG, aminoguanidine; CML, N^{ε}-(carboxymethyl)lysine.

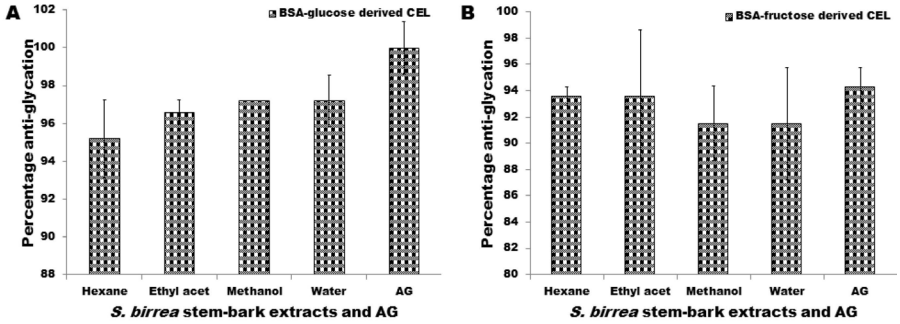

Figure 6. Percentage anti-glycation effects of *S. birrea* stem-bark extracts and aminoguanidine on bovine serum albumin (BSA)-glucose and BSA-fructose derived N^{ε}-(carboxyethyl)lysine). AG, aminoguanidine; CEL, N^{ε}-(carboxyethyl)lysine.

and aminoguanidine on BSA-glucose and BSA-fructose derived CEL. The extracts were not as effective as aminoguanidine against both BSA-glucose and BSA-fructose-derived CEL.

Ability to break AGEs-protein cross-links

The results obtained in the investigation on the ability of the different *S. birrea* stem-bark extracts and aminoguanidine to break AGEs-protein cross-links formed from either BSA-glucose or BSA-fructose derived AGEs are presented in Figure 7 (A and B).

At a test concentration of 1 mg/mL, aminoguanidine did not show any cross-link breaking ability against BSA-glucose-derived protein cross-links. However, at the same concentration, all stem-bark extracts of *S. birrea* showed appreciable AGE-protein cross-link breaking ability ranging from 59.1 to 90.4%. The highest cross-link breaking ability was observed with the methanol extract (92.6 ± 1.36 %). The cross-link breaking ability was found to be significantly different between all the extracts

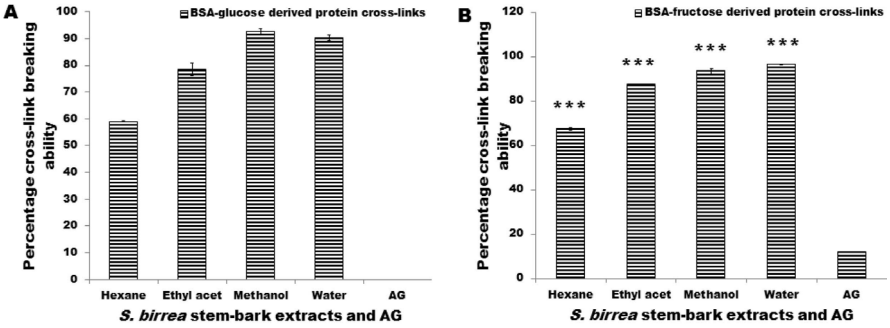

Figure 7. Percentage cross-link breaking ability of *S. birrea* stem-bark extracts and aminoguanidine against both bovine serum albumin (BSA)-glucose and BSA-fructose derived protein cross-links. *** Significant at $P < 0.001$ compared to the positive standard (AG); AG, aminoguanidine.

($P < 0.001$). Also, a significant difference was observed between the cross-link breaking ability of the methanol and water extracts ($P < 0.001$) and between those of *n*-hexane and ethyl acetate ($P < 0.001$).

In the case of BSA-fructose-derived AGEs-protein cross-links, all *S. birrea* stem-bark extracts and aminoguanidine were able to exert some degree of cross-link breaking abilities. Compared with the cross-link breaking ability of aminoguanidine against BSA-fructose derived AGEs-protein cross-links, all *S. birrea* stem-bark extracts demonstrated significantly higher cross-link breaking abilities ($P < 0.001$). The highest cross-link breaking ability was displayed by the water extract (96.4 ± 3.3%).

Discussion

The results of the present study suggest that all extracts of *S. birrea* stem-bark have the ability to prevent the formation of major types of AGEs as well as the ability to break AGEs-protein cross-links, albeit to varying degrees. These effects are against AGEs and their cross-links derived from glucose or fructose. While glucose and fructose are both common sugars frequently consumed globally, evidence abounds that fructose is a more potent glycating agent than glucose (24,25). Hence, they were both used in this study. To our knowledge, there has been no previous scientific report on the anti-glycation and/or cross-link breaking effects of extracts of *S. birrea* stem-bark, neither in BSA-glucose nor BSA-fructose models.

The comparison of the anti-glycation activities of the *S. birrea* stem-bark extracts and aminoguanidine, an established inhibitor of AGEs revealed that the mildly polar extract (ethyl acetate) and polar extracts (methanol and water) consisted of components that were effective against fluorescent AGEs formation (Figure 1). The *S. birrea* stem-bark ethyl acetate extract showed effectiveness with increasing concentration against FAGEs formation as well as the ability to break AGEs-protein cross-links. However, the ethyl acetate extract did not show an outstanding anti-glycation effect against immunogenic AGEs.

In this study, sequential serial extraction was carried out to extract secondary metabolites based on polarity and identify solvents extracting the most active ingredients (19). After the initial extraction with *n*-hexane and subsequent extraction with ethyl acetate, there was a higher concentration of polar compounds. Methanol is a polar solvent, and it demonstrated a highly potent anti-glycation effect against FAGEs (Figure 1). Of the plant extracts, methanol had the lowest IC_{50} (Table 2). As seen in Figure 3, for methanol extract, an increase in concentration did not result in a significant increase in the anti-glycation effect on FAGEs. For the other extracts (*n*-hexane, ethyl acetate, and water) and aminoguanidine, increasing concentration led to an increase in the anti-glycation effect against FAGEs. With the methanol extract, the percentage of anti-glycation activity against FAGEs decreased slightly with increasing concentration, while with other extracts, the effect increased with increasing concentration (Figure 3). The methanol extract of *S. birrea* stem-bark showed better anti-glycation at a lower concentration of 0.25 mg/mL than higher concentrations of 0.5, 1, 2, and 4 mg/mL (Figure 3). Usually, the chemical components in mixtures work through a combination of synergetic and additive effects (19). Although the methanol extract showed the lowest concentration at which fluorescent AGEs formation could be inhibited, an increase in concentration did not lead to an increase in effectiveness. This suggests that the active ingredient might not work effectively in synergy with other compounds. Thus, the result obtained with increasing concentration indicates that other compounds capable of suppressing the active compound(s) may be present in the methanol extract.

The inhibition of the fluorescent AGEs by *S. birrea* stem-bark ethyl acetate, methanol, and water extracts is an indication that there are compounds present in these extracts that are effective as antiglycative agents against both glucose- and fructose-derived fluorescent AGEs. Moreover, the result showed that the methanol and water extracts of *S. birrea* stem-bark have the capacity to inhibit the formation of FAGEs by 50% at much lower concentrations than aminoguanidine. Ethanolic extract of *S. birrea* is reported to have similar mechanism of action as metformin against hyperglycaemia (17). In addition, metformin is reported to have anti-glycation effect and is able to inhibit AGEs formation by exerting its action in

the post-Amadori stage (26).

Many articles have shown that polar extracts, especially those containing phenols and flavonoids, are more potent anti-glycating agents (27-29). The current results indicating higher antiglycation effect of the *n*-hexane extract was thus unprecedented. The result obtained suggests that *n*-hexane can extract active ingredients responsible for preventing the formation of various types of AGEs, especially polygenic AGEs. For total immunogenic AGEs (TIAGEs), *n*-hexane displayed the best ability to extract the most active ingredients (Figure 4). The *n*-hexane extract of *S. birrea* stem-bark was significantly more effective against TIAGEs than aminoguanidine at the concentration of 1 mg/mL. The higher inhibition of TIAGEs formation than aminoguanidine and other extracts of *S. birrea* stem-bark by the *n*-hexane extract suggests that the non-polar component of the stem-bark of *S. birrea* contains compounds that can selectively prevent the formation of polyclonal immunogenic AGEs derived from both glucose and fructose. As further observed, increasing the concentration of the *n*-hexane extract showed a consistent rise in activity against the formation of AGEs of fluorescent nature (Figure 3). The anti-glycation effect of *S. birrea* stem-bark *n*-hexane extract was significantly higher than the other extracts. Taken together, this suggests that there are compounds extracted by *n*-hexane, which at higher concentrations would be effective against FAGEs and highly effective against TIAGEs.

GC-MS analysis enabled the identification of 1-heptatricontanol as a volatile compound in the *n*-hexane stem-bark of *S. birrea*. 1-Heptatricontanol is credited with antidiabetic effect (30). Campesterol, which is noted to have antioxidant and hypocholesterolemic properties, was identified in both the *n*-hexane and ethyl acetate stem-bark of *S. birrea* used in this study (31). L-(+)-ascorbic acid, 2,6-dihexadecanoate, a fat-soluble form of ascorbic acid reported to have antioxidant and anti-inflammatory properties, was identified in the *n*-hexane and ethyl acetate stem-bark extracts (32,33). Stigmasterol, which is credited with hypoglycemic, hyperlipidemic, and antioxidant properties (38) was identified in the ethyl acetate stem-bark extract of *S. birrea*. These compounds, with their attributed effects, may contribute to the anti-glycation effect observed with the non-polar extracts, especially the *n*-hexane stem-bark extract of the plant.

It is known that different sugars produce different AGEs (34). A wide range of AGEs exists; some have been well characterized such as CML. However, the structures of the majority of the fructose-derived AGEs have not been elucidated (35). Compared with aminoguanidine, the extracts of *S. birrea* stem-bark while showing good inhibitory effects against the formation of the two specific AGEs, CML, and CEL (Figures 5 and 6) were not as effective as aminoguanidine against their formation. A number of studies have focused on the hypoglcaemic and antioxidant effects of *S. birrea* (12,16). It is considered that agents with both antiglycation and antioxidant properties may slow down the progression of AGEs formation Such agents can delay complications associated with diabetes and other age-related diseases (36). Therefore, inhibitors of AGEs that have been observed to exhibit antioxidant activity have great potential as preventive agents against diabetic complications (37,38).

The antioxidant properties observed in *S. birrea* are documented to be predominantly polar (12). Antioxidant activities in the stem-bark and other parts of *S. birrea,* such as leaf, fruit, and root, are attributed to the high flavonoids and polyphenolic content found in the studied methanol extracts (39,40). Evidence from the work of Mncwangi et al (18) showed that *S. birrea* bark ethanolic extract has a strong inhibitory effect on nicotinamide adenine dinucleotide phosphate hydrogen oxidase- 4 (Nox-4). Nox-4 is a member of the nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) family of enzymes associated with the generation of reactive oxygen species (41).

The investigation into the phytochemical composition of *S. birrea* by Russo et al (12) confirmed the presence of galloylated tannins and procyanidins such as epicatechin-3-*O*-gallate (ECG), predominantly in *S. birrea* stem-bark. Others include catechin, epicatechin, galloylepicatechin-epigallocatechin-3-*O*-gallate, epigallocatechin 3-*O*-gallate, trigalloylated procyanidin trimer (P3G3), and digalloylated procyanidin dimer B (P2G2) (13). Also, common phytochemicals reported presently in various parts of *S. birrea* (leaf, stem-bark and root) are gallic acid, epicatechin-3-*O*-gallate (P1G1), and procyanidin dimer B (P2G1). In addition, Mncwangi et al (18) reported the isolation and identification of (-)4'-O-methylepigallocatechin-3-O-gallate in the ethanolic extract of *S. birrea* bark. It is also documented that stem bark from *S. birrea* contains a significant amount of high molecular weight tannins and traces of alkaloids (12, 15). Catechin and epicatechin procyanidin B2 inhibit AGEs formation by trapping the dicarbonyl compound methyl glyoxal (42). Russo et al (12) also reported the presence of galloylated tannins predominantly in *S. birrea* stem bark as well as procyanidins such as epicatechin-3-*O*-gallate (ECG).

Extracts of *S. birrea* stem-bark have been able to prove both their ability to prevent the formation of different AGEs and also reverse cross-links formed between these adducts and other proteins. Our findings suggest that *S. birrea* stem-bark may have high potential as cross-link breakers, especially the polar extracts. The component responsible for this might be present only in the more polar extracts and not the non-polar extracts. The increasing polarity of the extracts showed an increasing cross-link breaking effect by extracts. Aminoguanidine, though identified as an inhibitor, was not able to show any remarkable effect on cross-links formed. The inability of aminoguanidine to effectively reverse the formation of collagen-AGEs-BSA is

not surprising. Aminoguanidine is identified as a strong inhibitor of AGEs and is documented to prevent AGEs formation by trapping reactive intermediates (43).

The extracts of *S. birrea* have exhibited anti-elastase and anti-collagenase activities (19). This implies that *S. birrea* extracts (especially the polar ethanolic extracts) have the ability to prevent the breakdown of elastin and collagen, as such promoting the continued strength and elasticity of the skin. Shoko et al (19) concluded in their investigation that epicatechin gallate and epigallocatechin gallate present in the tested extracts play a role in the anti-aging activity of *S. birrea*.

While the above-mentioned studies by various independent investigators have not demonstrated the anti-glycation and AGEs-protein cross-link breaking abilities of the medicinal plant of interest, they have given some insights to the possibility of exploiting the plant for antiglycation and reversal of AGEs cross-links, which are deleterious to health. These previous studies support findings from our research, which suggest that *S. birrea* stem-bark should be exploited as a source of active components to prevent the rapid degeneration associated with aging as well as the vascular complications of diabetes mellitus. Furthermore, the result of this study may lead to the isolation of bio-active compounds that may be used for the prevention of vascular complications of diabetes. Further work is required to determine the compounds responsible for the anti-glycation and cross-link breaking ability of *S. birrea* stem-bark.

Conclusion and Recommendations

Polar stem-bark extracts of *Sclerocarya birrea* may have high potential as cross-link breakers. The findings of this study suggest that *S. birrea* stem-bark extracts have antiglycation effects against AGEs and also the ability to break AGEs-protein cross-links. However, it remains to be seen how the compounds identified in *S. birrea* stem-bark will do in their pure form with regard to anti-glycation and AGEs-protein cross-link breaking activities. A full-scale purification/isolation of bioactive compounds inherent within the stem-bark of *S. birrea* is, therefore, recommended in order to compare their anti-glycation activity strengths with those of the crude extracts. In addition, *in vivo* studies are required to demonstrate the effects of these extracts on living cells and animals and to better understand the benefits of the plant extracts. This may proceed to further clinical studies to shed light on how human health can be enhanced.

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Authors' contributions

Conceptualization of the research idea was made by MAM and LJS; The study was performed, analysed, and results interpreted by OIA. The original draft preparation was done by OIA and AMM. AMM, LSS, SSG, and LJS reviewed and edited the manuscript. The project was keenly supervised by SSG and LJS. The software used was made available by LSS. All authors have read and agreed to the published version of the manuscript.

Conflicts of interests

The authors declare no conflict of interest.

Ethical considerations

This research was approved by the Sefako Makgatho Health Sciences University Ethical Committee with approval number MREC/P/245/2012:PG. All authors have inspected for ethical issues of plagiarism, misconduct, data fabrication, falsification, double publication or redundancy related to the manuscript and adhered to the guidelines given for publication in this journal.

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