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Variation in Anti-Glycation and Cross-Link Breaking Effects of Crude Extracts of *Moringa oleifera* Leaf, Seed and Root

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Study and comparing the anti-glycation and cross-link breaking effects of *Moringa oleifera* leaf, seed and root extracts with aminoguanidine, a known inhibitor of advanced glycation end-products (AGEs). Their phytochemical composition and extraction yields were also assessed. All seed and root extracts demonstrated significantly higher anti-glycation effect than aminoguanidine against total immunogenic AGEs ($p < 0.05$). Both seed and root polar (methanol and water) extracts exhibited significantly higher anti-glycation effect against *N'*-(carboxymethyl)lysine than aminoguanidine ($p < 0.05$). Polar extracts of all *M. oleifera* tested parts demonstrated higher anti-glycation activity against fluorescent AGEs than aminoguanidine ($p < 0.05$). With exception to the aqueous root extracts, all leaf, seed and root extracts of *M. oleifera* demonstrated an ability to break AGE-protein cross-links. The highest cross-link breaking effect was exhibited by ethyl acetate extract of leaves.The methanol extract of seeds of *M. oleifera* showed the presence of all tested secondary metabolites.

Keywords: *Moringa oleifera*, Anti-glycation, Cross-link, Crude extracts.

INTRODUCTION

Advanced glycation end-products (AGEs) are implicated in the pathogenesis of diabetes, age-related and neurodegenerative diseases [1,2]. AGEs are a complex group of compounds which result from the non-enzymatic covalent reactions that occur between the carbonyl group of reducing sugars (*e.g.* glucose) and the free amino groups of proteins, lipoproteins and nucleic acids [2-4]. These AGEs are classified based on their cross-linking and/or fluorescence properties [5] and often detected with the use of immunogenic assays and with spectrofluorometry based on their characteristic fluorescence spectrum [6,7]. An example of AGEs is *N'*-(carboxymethyl)lysine (CML), a non-cross-linking non-fluorescent AGE [4].

When AGEs accumulate in living systems, as shown by numerous scientific evidences, they lead to the development of age-related diseases and diabetes and its associated complications [2,8,9]. Therefore, it is considered that preventing the formation of AGEs as well as uncoupling AGE-protein cross-links may prevent the development and progression of diabetic complications [9]. A number of synthetic agents, which have the capacity to prevent the formation of AGEs or break cross-links formed by AGEs are documented in literature [10,11]. However, most of these agents, for example aminoguanidine, have been found to have undesirable side effects [12]. Also, most of the AGE-protein cross-linking breakers do not act on glucosepane, the most abundant glucose derived AGE-protein cross-link found in human tissues [13].

In light of the problems encountered with these synthetic drugs, there is a need to search for new and safer anti-glycating agents, especially from natural products such as medicinal plants [14,15]. The aim of the current study was to screen crude extracts of the leaf, seed and root parts of *Moringa oleifera* for their anti-glycation and AGE-protein cross-link breaking activities and also profile their phytochemical components. The knowledge of the anti-glycation effect and cross-link breaking ability of the leaf, seed and root extracts of *M. oleifera* on different types of AGEs may lead to the discovery and isolation of active anti-glycation agents that can be used to prevent late onset of diabetic complications.

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EXPERIMENTAL

M. oleifera leaf powder was purchased from Winterveldt Organic Agricultural primary co-operative (Gauteng, South Africa) as finely grounded leaf powder. The seed and root of *M. oleifera* were obtained from Danice Farms (Ilorin, Kwara State, Nigeria). A voucher specimen (PRE 0984450-0) was deposited at the National Herbarium, South Africa National Biodiversity Institute, Pretoria, South Africa. The seeds and roots were dried and pulverized using a laboratory mill (Polymix PX-MFC 90D, Kinematica, Thermo-Fisher Scientific, Sweden). The different fine powders of *M. oleifera* were extracted sequentially with hexane, ethyl acetate, methanol and water according to the procedure as described by Mogale *et al*. [16]. For each part of the plant materials, the mixture was left shaking for 24 h (for 3 days per solvent) at 120 rpm on an orbital shaker at room temperature. The resulting mixtures were filtered using Whatman No. 1 filter paper. The filtrates from hexane, ethyl acetate and methanol extraction were concentrated using Eyela SB-1100 rotary evaporator under reduced pressure, then transferred into pre-weighed beakers and evaporated to dryness at room temperature. The water filtrates were lyophilized with Advantage Plus SP freeze dryer (Scientific, Sweden). The extracts were kept in the dark at low temperature for further usage. For the investigation, both the hexane and ethyl acetate dry extracts were dissolved in DMSO, while methanol and water dry extracts were reconstituted with water.

General procedure for anti-glycation activity: The *in vitro* glycated test of *M. oleifera* parts (leaf, seed and root) was carried out as prescribed by Ho & Chang [17] with modification. Briefly, bovine serum albumin (BSA) was dissolved in 76 mM sodium phosphate buffer (pH 7.4) containing 0.02% sodium azide. The resultant solution (10 mg/mL BSA) was incubated with glucose (50 mg/mL) at 37 ºC for 40 days with and without *M. oleifera* extracts (as test samples) or aminoguanidine (used as positive control). The amounts of immunogenic AGEs (total immunogenic AGEs (TIAGEs) and CML) and fluorescent AGEs (FAGEs) were determined. The measurement of immunogenic AGEs for TIAGES and CML was obtained from commercially available enzyme linked immunosorbent assay (ELISA) kits (STA-317 and STA-316 OxiselectTM) purchased from Cell Biolabs (San Diego, USA), using a primary antibody against different AGEs or specific primary antibody against CML. The procedure was carried out according to manufacturer's instruction. Briefly, 100 µL of BSA-glucose mixture was incubated in the presence and absence of the extracts (1 mg/mL) or aminoguanidine (1 mg/mL) and added to a 96-well protein binding plate, then incubated overnight at 4 °C. Phosphate buffer saline (PBS) was used to wash wells twice and 200 µL of assay diluent was added to each well and incubated for 2 h at room temperature. The primary antibody (polyclonal anti-AGE or anti-CML) were added to all wells and incubated for a further 1 h at 25 ºC. After the incubation, the wells were washed thrice. Then 100 µL of diluted secondary antibody-HRP conjugate was added to each well and incubated for 1 h at room temperature. The antibodies were washed off in 5 washes and 100 µL of substrate solution was added. The enzyme reaction was terminated with 100 µL of the stop solution. The absorbance of each well was measured using Tecan Spectra microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm. The method of Münch *et al*. [18] was used to measure the effect of *M. oleifera* extracts on the formation of fluorescent AGEs. This was performed in triplicate at room temperature by means of Promega GloMax multi detection plate reader (Wisconsin, USA) at excitation of 370 nm and an emission at 445 nm. The amounts of FAGEs were determined using a concentration of 1 mg/mL for each plant extract and aminoguanidine. Results were expressed in arbitrary units as percentage anti-glycation. Percentage anti-glycation activity of the extracts were calculated using the following equation:

$$\text{Anti-glycation activity (\%)} = \left(\frac{[\text{AGEs}]_{\text{no extract}} - [\text{AGEs}]_{\text{extract}}}{[\text{AGEs}]_{\text{no extract}}} \right) \times 100$$

where $[AGES]_{no\ extract}$ and $[AGEs]_{extract}$ is absorbance of BSA + glucose only and absorbance of BSA + glucose + test samples/aminoguanidine, respectively.

Cross-link breaking activity: The cross-link breaking activities of the different crude plant extracts on preformed AGE-protein cross-links were assessed *in vitro* according to the procedure described by Cheng *et al*. [19]. An aliquot of glucose was incubated with equal volume of BSA at 37 ºC for 80 days to form glucose-derived AGE-BSA. After the incubation period, aliquots of this resultant mixture was added into each well of a 96-well collagen coated plate (Gibco®, Maryland, USA). This mixture in the 96-well collagen coated plate was incubated further (4 h) at 37 ºC to allow for the formation of collagen-AGE-BSA cross-links. The AGE-BSA unbound to collagen in wells were removed by washing the wells with phosphate buffered saline solution containing 0.05% Tween-20 (PBST), product of Amresco Inc. (Ohio, USA). The collagen-AGE-BSA complexes formed in the wells were incubated for 24 h in the presence and absence of plant extracts (1 mg/mL) or aminoguanidine (1 mg/mL). Aminoguanidine was used as positive control. Collagen-AGE-BSA mixtures without aminoguanidine or plant extracts served as negative control. After incubation (24 h), the amount of BSA released was quantified with E11-113 bovine albumin ELISA kit (Bethyl Laboratories, Texas, USA). Procedure was carried out using an anti-bovine albumin detection antibody and a streptavidin-conjugated horseradish peroxidase as secondary antibody, according to manufacturer's instruction. A chromogenic substrate, 0.045 M TMB (3,3′,5,5′-tetramethylbenzidine) was added to each well and the reaction terminated with the stop solution (0.18 M H_2SO_4). The absorbance was measured using a Tecan Spectra microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at a primary wavelength of 450 nm. The percentage of breaking activity of each plant extract and aminoguanidine was calculated using the following equation:

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

where $A_{control}$ and $A_{extract}$ is absorbance of collagen + glucose + BSA only and absorbance of collagen + glucose + BSA + extracts/aminoguanidine, respectively.

The qualitative phytochemical analysis gives information about the presence or absence of different classes of secondary metabolites in plants. Various phytochemical tests were carried out on extracts of the different *M. oleifera* parts under investigation (leaf, seed and root). The detection of the several compounds *viz*. phenols, flavonoids, quinones, cardiac glycosides, saponins, steroids, terpenoids and coumarins was conducted according to the reported methods described elsewhere [20-23]. All the experiments were carried out in triplicates and results obtained for the anti-glycation and AGE-protein cross-link breaking effects are presented as mean ± standard deviation. Comparisons between groups were made by means of one-way analysis of variance (ANOVA). The differences between groups were considered significant at $p < 0.05$. The data obtained were analyzed using version 24 of the IBM SPSS® Statistical package.

RESULTS AND DISCUSSION

Studies have highlighted the benefits of using medicinal plants with combined anti-glycation and antioxidant properties in diabetic patients [24]. The antioxidant, antidiabetic and anti-inflammatory activities of *M. oleifera* plant parts have been reported previously [25-27]. There are however, limited reports on the anti-glycation and AGE-protein cross-link breaking effect of *M. oleifera* plant [8,28-30]. The result of this study indicates that the crude extracts of *M. oleifera* leaf, seed and root give different percentage yields when extracted with solvents of varying polarities. These extracts contain different secondary metabolites, can prevent the formation of different types of AGEs and also reverse AGE-protein cross-links.

Percentage yields of the extracts of the selected parts of *M. oleifera* (leaf, seed and root) subjected to sequential solvent extraction are presented in Table-1. The highest percentage yield for the *M. oleifera* leaf was obtained with methanol (15.9%). With the seed part, the highest extraction yield was obtained from the hexane extract (17.2%). For *M. oleifera* root, water gave the highest percentage extraction yield (4.35%). The lowest extraction yields for all three plant parts were obtained with ethyl acetate solvent (leaf, 1.5%; seed, 3.68%; root, 0.25%).

The result obtained from the study shows the richness of the leaf and root in polar substances and the seed in non-polar content (Table-1). This result is at variance with that obtained by Akinyeye *et al.* [31] when they evaluated the phytochemical and antimicrobial activities of *M. oleifera* leaf and seed extracts where they reported higher non-polar content for leaf (obtained with hexane). Also, the outcome of the investigation by Akinyeye *et al*. [31] showed maximum extraction yield or the methanol extract of *Moringa oleifera* seed therefore alluding to the seed having higher polar content than the leaf. However, it was identified that the different soil types and season of harvesting the materials could have effect on phytochemical composition [32,33]. This suggests that the outcomes obtained in this study, especially with the percentage yields and phytochemical composition, could be related to those differences, as such variation in results obtained in this study and those of Akinyeye *et al*. [31] could be as a result of geographical location where each plant material was sourced based on the soil types, weather patterns as well as the time of the year and season each material was obtained. Furthermore, variations between the secondary metabolite composition among different parts of *M. oleifera* has also been documented [34].

TABLE-1
PERCENTAGE YIELD OF *M. oleifera* LEAF, SEED AND ROOT EXTRACTS

Extracts	*M. oleifera*		
	Leaf	Seed	Root
Hexane (%)	3.28	17.20	0.30
Ethyl acetate (%)	1.50	3.68	0.25
Methanol (%)	15.90	4.88	3.55
Water (%)	10.80	5.92	4.35

The results of the anti-glycation effects of the different parts of *M. oleifera* selected for use in this study are expressed as percentage anti-glycation. For the investigation on the anti-glycation effect of *M. oleifera* leaf, seed and root crude extracts and aminoguanidine on TIAGEs, the results are presented in Table-2. All leaf, seed and root extracts of *M. oleifera* and aminoguanidine were able to exert anti-glycation effect against the formation of BSA-glucose derived TIAGEs. The highest anti-glycation effect against TIAGEs was observed with the ethyl acetate extract of *M. oleifera* seed (98.1%). The seed and root extracts were more effective against TIAGEs formation, exerting statistically higher anti-glycation effect than aminoguanidine ($p < 0.05$). All the root extracts, in addition to their significantly higher anti-glycation effect than aminoguanidine, showed comparable anti-glycation effect among themselves. Statistically, there was no significant difference between the anti-glycation effects of the seed hexane, ethyl acetate and water extracts and the entire extracts of *M. oleifera* root. For the leaf extracts, the methanol extract alone demonstrated a comparable effect with aminoguaindine.

For CML, the results as presented in Table-3, showed that all polar (methanol and water) extracts of *M. oleifera* leaf, seed

TABLE-2
PERCENTAGE ANTI-GLYCATION EFFECT OF *M. oleifera* LEAF, SEED AND ROOT EXTRACTS AND AMINOGUANIDINE ON BSA-GLUCOSE DERIVED TIAGEs

Plant part	Standard inhibitor/Extracts				
	Aminoguanidine	Hexane	Ethyl acetate	Methanol	Water
Leaf	$90.3^{a} \pm 1.87$	$83.5^{b} \pm 1.31$	$79.1^{c} \pm 2.60$	$88.9^{a} \pm 0.71$	$83.5^{b} \pm 2.24$
Seed	$90.3^{c} \pm 1.87$	$97.8^{a} \pm 4.46$	$98.1^{a} \pm 3.33$	$93.8^{b} \pm 0.21$	$97.3^{a} \pm 0.62$
Root	$90.3^{b} \pm 1.87$	$97.2^{a} \pm 1.62$	$96.9^{a} \pm 2.18$	$97.6^{a} \pm 0.97$	$97.7^{a} \pm 4.77$

Values with same letter within the same row are not significantly different ($p > 0.05$) while values with different letter are significantly different ($p < 0.05$).

TABLE-3
PERCENTAGE ANTI-GLYCATION EFFECT OF *M. oleifera* LEAF, SEED AND ROOT EXTRACTS AND AMINOGUANIDINE ON BSA-GLUCOSE DERIVED CML

Plant part	Standard inhibitor/Extracts				
	Aminoguanidine	Hexane	Ethyl acetate	Methanol	Water
Leaf	$95.3^{a} \pm 0.11$	$40.6^{b} \pm 0.20$	$3.0^{c} \pm 0.16$	$97.7^{a} \pm 0.77$	$97.3^{a} \pm 1.33$
Seed	$95.3^{b} \pm 0.11$	$62.4^{d} \pm 1.06$	$82.2^{c} \pm 1.38$	$97.9^{a} \pm 0.31$	$98.1^{a} \pm 2.75$
Root	$95.3^{b} \pm 0.11$	$72.8^{d} \pm 0.19$	$74.5^{c} \pm 2.50$	$97.6^{a} \pm 1.23$	$98.3^{a} \pm 4.53$

Values with same letter within the same row are not significantly different ($p > 0.05$) while values with different letter are significantly different ($p < 0.05$).

TABLE-4
PERCENTAGE ANTI-GLYCATION EFFECT OF *M. oleifera* LEAF, SEED AND ROOT EXTRACTS AND AMINOGUANIDINE ON FAGEs

Plant part	Standard inhibitor/Extracts				
	Aminoguanidine	Hexane	Ethyl acetate	Methanol	Water
Leaf	$75.7^{b} \pm 0.28$	$10.3^{d} \pm 2.57$	$45.1^{c} \pm 4.71$	$100^{a} \pm 3.59$	$100^{a} \pm 4.84$
Seed	$75.7^{b} \pm 0.28$	0	$21.4^{d} \pm 2.87$	$58.1^{c} \pm 4.06$	$97.4^{a} \pm 4.91$
Root	$75.7^{a} \pm 0.28$	$14.3^{d} \pm 3.36$	0	$70.1^{b} \pm 2.88$	$50.7^{c} \pm 3.67$

Values with same letter within the same row are not significantly different ($p > 0.05$) while values with different letter are significantly different ($p < 0.05$).

and root demonstrated higher anti-glycation effect than aminoguanidine against CML formation. The seed and root polar extracts were found to have statistically higher anti-glycation effect than aminoguanidine. The results of the anti-glycation effect of *M. oleifera* leaf, seed and root extracts compare with aminoguanidine on BSA-glucose derived FAGEs are presented in Table-4. The highest anti-glycation effects against FAGEs were observed with the leaf polar extracts. This was followed by the seed water extract. The anti-glycation effects of these three extracts (methanol and water extracts of *M. oleifera* leaf and water extract of *M. oleifera* seed) were found to be significantly higher than that of aminoguanidine ($p < 0.05$).

The results of the AGE-protein cross-link breaking ability of *M. oleifera* leaf, seed and root extracts and aminoguanidine are expressed as percentage cross-link breaking ability (Table-5). From the investigation, the result showed that aminoguanidine did not exert any cross-link breaking effect against the BSA-glucose derived AGE-protein cross-links. All leaf extracts showed considerably high cross-link breaking ability on AGE-protein cross-link derived from BSA-glucose. The leaf ethyl acetate extract demonstrated the highest cross-link breaking ability (98.7%) followed by the leaf water extract (96.9%). The AGE-protein cross-link breaking ability of these two extracts were not significantly different from each other. The *M. oleifera* seed extracts and *M. oleifera* root hexane and ethyl acetate extracts were found to exhibit less than 50 % cross-link breaking ability. No cross-link breaking ability was demonstrated by the root water extract. The methanol extract of root demonstrated above 50% AGE-protein cross-link breaking effect.

TABLE-5
PERCENTAGE CROSS-LINK BREAKING ABILITY OF *M. oleifera* LEAF, SEED AND ROOT EXTRACTS AND AMINOGUANIDINE ON BSA-GLUCOSE DERIVED AGES-PROTEIN CROSS-LINKS

Standard inhibitor/extracts	Plant parts		
	Leaf	Seed	Root
Aminoguanidine	0	0	0
Hexane	$86.9^{b} \pm 0.39$	$40.1^{a} \pm 1.07$	$30.5^{b} \pm 3.37$
Ethyl acetate	$98.7^{a} \pm 4.67$	$33.1^{b} \pm 4.76$	$35.1^{b} \pm 2.72$
Methanol	$86.3^{b} \pm 1.55$	$45.5^{a} \pm 4.27$	$57.0^{a} \pm 3.30$
Water	$96.9^{a} \pm 1.46$	$24.5^{c} \pm 0.76$	0

Values with same letter within the same column are not significantly different ($p > 0.05$) while values with different letters are significantly different ($p < 0.05$).

Phytochemical screening of different *M. oleifera* selected parts used in this study is shown in Table-6. Eight secondary metabolites were tested in this study, namely coumarins, phenols, flavonoids, quinones, cardiac glycosides, saponins, steroids and terpenoids. In this study, *M. oleifera leaf* extracts revealed the presence of various phytochemical composition, anti-glycation effect and cross-link breaking activities in various degrees. Of the secondary metabolites tested, the leaf hexane extract was found to contain only cardiac glycosides and coumarins yet was able to exert significantly higher anti-glycation effect against the polygenic AGEs, TIAGEs than aminoguanidine. However, the anti-glycation effect it showed on the more specific AGE, CML was less than 50 % and also low against FAGEs. Contrary to expectations, hexane extract of *M. oleifera* leaves exerted high cross-link breaking effect on AGE-protein cross-links. These results suggested that the hexane extract of the leaf contains phytochemicals which are effective against a wide range of immunogenic AGEs and these secondary metabolites have the capacity to reverse cross-links formed between AGEs and proteins.

Ethyl acetate extract of *M. oleifera* leaves showed the least anti-glycation effect against TIAGEs and CML but slightly higher anti-glycation effect than the hexane extract against FAGEs, yet demonstrated the highest cross-link breaking effect of all the *M. oleifera* parts crude extracts. Phytochemical screening test revealed only the presence of coumarins in the leaf ethyl acetate extract. This suggests that the ethyl acetate extract of *M. oleifera* leaves contains compounds that have high cross-link breaking ability. The leaf polar extracts demonstrated high anti-glycation effects against all the tested AGEs as well as the ability to reverse AGE-protein cross-links. The results from the study revealed that the leaf methanol extract had statistically

TABLE-6
PHYTOCHEMICAL COMPOSITION OF EXTRACTS FROM *M. oleifera* LEAF, SEED AND ROOT

Phytochemicals	Leaf				Seed				Root			
	HX	EA	MT	WT	HX	EA	MT	WT	HX	EA	MT	WT
Phenol	–	–	+	+	–	–	+	–	–	–	+	+
Flavonoids	–	–	+	–	–	–	+	–	–	–	–	+
Quinones	–	–	–	–	–	–	+	+	–	–	+	+
Cardiac glycosides	+	–	+	+	–	+	+	–	+	+	+	–
Saponins	–	–	+	+	–	–	+	+	–	–	+	+
Steroids	–	–	–	+	–	–	+	+	–	–	–	+
Terpenoids	–	–	+	+	–	–	+	–	–	–	+	–
Coumarins	+	+	+	+	–	+	+	+	+	–	+	–

+ Indicates presence; – Indicates absence; HX = Hexane extract; EA = Ethyl acetate extracts; MT = Methanol extract; WT = Water extract

higher anti-glycation effect against FAGEs than aminoguanidine ($p < 0.05$). The methanol extract of leaves anti-glycation effect against TIAGEs and CML were similar to aminoguanidine. Although its cross-link breaking effect (86.3 ± 1.55%) was the least among the leaf extracts, the methanol leaf extract showed strong AGE-protein cross-link breaking effect. Phytochemical screening showed that methanol extract of leaves contained flavonoids, phenols, cardiac glycosides, saponins, terpenoids and coumarins. The phytochemicals detected in the methanol extract of leaves were similar to that of methanol extract of leaves except for the absence of flavonoids and the presence of steroids. The anti-glycation activity assay revealed that the leaf water extract was effective in preventing the formation of TIAGEs and CML and highly effective against fluorescent AGEs and as cross-link breakers. This suggests that *M. oleifera* leaf water extract, in addition to possessing compounds capable of preventing the formation of various types of AGEs, contains components, which are highly effective against FAGEs and also have the capacity to reverse AGE-protein cross-links.

For the seed extracts, hexane extract had over 60% anti-glycation effect against CML. It also displayed a 97.8% anti-glycation effect against TIAGEs, which was significantly higher than that of aminoguanidine and a 40 % cross-link breaking effect. This shows that *M. oleifera* seed hexane extract contains components, which might not have been screened for in this study that are capable of exerting anti-glycation effect on the formation of these types of AGEs as well as cleave AGE-protein cross-links. On the other hand, seed ethyl acetate extract revealed the presence of cardiac glycosides and coumarins only. However, the anti-glycation test showed that the seed ethyl acetate extract contained components that can exert strong inhibitory effect against the formation of CML and TIAGEs. *M. oleifera* seed ethyl acetate crude extract showed 33 % cross-link breaking effect.

The methanol extract of seed gave indication of the presence of phenols, flavonoids, quinones, cardiac glycosides, saponins, steroids, terpenoids and coumarins. The anti-glycation effect displayed by the methanol extract of seed spanned across the different types of AGEs tested for. The seed methanol extracts showed a much higher inhibitory effect against the immunogenic AGEs (TIAGEs and CML) than aminoguanidine ($p < 0.05$). In case of the water extract of the seeds, it revealed the presence of quinones, saponins, steroids and coumarins and also anti-glycation effect against TIAGEs, CML and FAGEs. The anti-glycation effect of water crude extract of seeds of *M. oleifera* was significantly higher than that of aminoguanidine against all tested AGEs. Mild AGE-protein cross-link breaking effect was observed with the water extract of seed. This suggests that *M. oleifera* crude seed water extract contains compounds that are highly effective as anti-glycation agents mostly.

The hexane extract of root showed the presence of cardiac glycosides and coumarins and was found to have significantly higher anti-glycation effect than aminoguanidine against BSA-glucose derived TIAGEs, good anti-glycation effect against CML, poor anti-glycation effect against FAGEs and mild cross-link breaking effect (30.5 ± 3.37 %). In ethyl acetate extract of root, only the presence of cardiac glycosides was detected. The root ethyl acetate extract exhibited a significantly higher anti-glycation effect than aminoguanidine against TIAGEs ($p < 0.05$) and moderate anti-glycation effect against CML.

The methanol extract of roots of of *M. oleifera* was found to contain phenols, coumarins, quinones, cardiac glycosides, saponins and terpenoids. The anti-glycation effects of root methanol extract were significantly higher than aminoguanidine against both BSA-glucose derived TIAGEs and CML. This suggests the presence of components that are effective against immunogenic AGEs present in the root methanol crude extract. Since several compounds present in crude extracts usually work through a combination as synergetic and additive effects [35], the root methanol extract may not be a good target for isolating strong compounds capable of acting as anti-glycation agents against AGEs of fluorescent nature and as cross-link breaking agents. For the root water extract, the most outstanding effects were observed against TIAGEs (97.7 ± 4.77 %) and CML (98. 3 ± 4.53 %), significantly higher than that of aminoguanidine ($p < 0.05$). These anti-glycation effect by the root water extract were highly effective and significantly more effective than aminoguaunidine against these immunogenic AGEs.

Previous studies [4,36] indicated that the extracts from various parts of the same plant could display different anti-glycation effects. For instance, extracts from various parts of *Alpina zerumbet* (Zingiberaceae) were found to have the highest and least inhibitory effects against formation of α-dicarbonyl compounds in the flowers and leaves, respectively [36]. Further support of reports of the differences in anti-glycation effects obtained with different plant parts were provided by the anti-glycation activities of alcoholic extracts of different parts of Calophyllaceae and Clusiaceae species, which showed

significant differences between various parts of the plants [4,37]. This may account for the variation in anti-glycation effect exerted by the leaf, seed and root extracts of *M. oleifera*, as documented in this study.

In this study, it could be seen that all the extracts of seed showed increasing anti-glycation effect against CML and FAGEs (Tables 3 and 4) with the increasing polarity of the extracting solvents (hexane < ethyl acetate < methanol < water). In a previous study carried out for a reduced incubation period of 20 days, a zero anti-glycation effect on FAGEs by the hexane and ethyl acetate extracts of *M. oleifera* leaf, seed and root was reported [30]. In this study, these extracts displayed some degree of anti-glycation effect against FAGEs. Thus suggesting a possibility that the compounds extracted by both hexane and ethyl acetate in *M. oleifera* investigated parts may be more active with pro-longed incubation duration. In present study, carried out for 40 days at physiological temperature (37 ºC) showed an improvement in anti-glycation effects of the three parts with prolonged incubation duration. The study previously reported was carried out by incubating BSA, glucose and *M. oleifera* extract test samples (and aminoguanidine) for 20 days at 37 ºC [30].

In addition, present findings indicate that *M. oleifera* leaf extracts (especially the polar ones) can effectively inhibit FAGEs derived from glucose. The seed and root, on the one hand can be seen to be highly effective in targeting the inhibition of TIAGEs. In addition, the result shows that all the extracts of the seed and root demonstrated over 90% effectiveness against TIAGEs formation and as such could be possible sources of anti-glycation agents considering that their effectiveness was found to be statistically higher than that of aminoguanidine. Also, this study revealed that all the leaf extracts have high potency for breaking cross-link bonds formed between collagen-AGE-BSA derived from glucose (Table-5). As such, there is a possibility that agents that break these bonds, thereby easing the elimination of the now smaller peptide bonds resulting, by the kidney from the body could be isolated from both the non-polar, mildly polar and polar extracts of the leaf of *M. oleifera* plant. However, it was not surprising that aminoguanidine had zero cross-link breaking effect on BSA-glucose derived AGE-protein cross-links. Evidence in several studies abounds of the α-dicarbonyl scavenging properties of aminoguanidine and its affinity for preventing AGEs formation in the initial stage of glycation by trapping reactive carbonyl intermediates rather than as a cross-link breaker [3,9,38].

Conclusion

Crude extracts of *Moringa oleifera* plant in different solvents have the potential to prevent the formation of different types of AGEs and also break pre-existing AGE cross-links formed from glucose-induced protein cross-links. All the methanol and water extracts of leaf, seed and root of *M. oleifera* plant showed strong anti-glycation effect on TIAGEs as well as CML. As such if studied further, potent agents with the ability to inhibit the formation of CML (the most abundant AGEs in the body) and other immunogenic AGEs could be isolated and developed from *M. oleifera* leaf, seed and root. The leaf polar extracts promise to be good candidates for further investigations for isolation of bioactive anti-glycation compounds with effect on fluorescent AGEs as well as cross-link breaking. To the best of our knowledge, this is the first study to report on the AGE-protein cross-link breaking ability of *M. oleifera* parts.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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