

Antioxidant and Antidiabetic Potential of Saponin Fraction Isolated from *Moringa oleifera* Leaves

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Abstract. To assess total phenolic contents, total flavonoid contents (TPC, TFC), DPPH scavenging and antidiabetic (antiglycation and alpha amylase hindrance) activities of *Moringa oleifera* (MO) leaves, bio-assay guided fractionation was used with thin layer chromatography profile to isolate saponin fraction (SF). The TPC and TFC were 9.7-12.8 GAE and 5.3-9.3 CE (g/100 g dry weight), respectively and aqueous and methanol fractions were more effective. DPPH scavenging activity ranged 69.5-83.1%. SF displayed noteworthy antioxidant ability ($p < 0.046$) and it was leading inhibitor of glycation ($p < 0.041$) after MeOH with 48.1% restriction. Enzyme inhibition by MO fractions was negligible (3.4-9.2%) and it was less impressive by SF as compared to MeOH extract. Percent reduction in enzyme activity by SF was non-significant ($p > 0.198$), nullifying its concentration dependent impact.

Keywords: *Moringa oleifera*, antioxidant activity, antidiabetic activity, saponin fraction

Introduction

Pharmacological exploitation of natural compounds is promising at betterment of treatment of a variety of diseases. Medicinal plants such as *Moringa oleifera* attract increasing interest in the beneficial management of diabetes mellitus owing to remarkable antioxidant properties. *M. oleifera* Lam. has been an important plant since ancient times, both for its nutritional and medicinal uses. Recently, several therapeutic potentials of *M. oleifera* such as antioxidant, antimicrobial, anti-inflammatory, anticancer and antidiabetic have been studied. Apart from these effects, the potential therapeutic implications in treatment of endocrine, circulatory, neural, respiratory and digestive disorders are well reported (Jung, 2014; Sahoo *et al.*, 2014).

M. oleifera leaves have been used as an important source to treat downstream complications related with elevated serum creatinine (Sahoo *et al.*, 2014). Protecting effect of *M. oleifera* against carcinogenesis, cataract, inflammation and diabetes mellitus are clearly indicated (Kurmi *et al.*, 2014; Waterman *et al.*, 2014; Yassa and Tohamy, 2014; Bharali *et al.*, 2003). *M. oleifera* seeds hydro-alcoholic extract and its chloroform fraction were effective to treat experimental colitis (Minaiyan *et al.*, 2014). Chemopreventive, anti-leukemic and antipyretic activities of *M. oleifera* strongly supports its ethnopharmacological uses (Ahmad *et al.*, 2014; Akanni *et al.*, 2014).

Oxidative stress is mainly involved in the development of various degenerative ailments, such as cardiovascular disease and diabetes mellitus. *M. oleifera* could be beneficial by reducing oxidative stress and especially hyperglycemia that leads to formation of advanced glycation endproducts (AGE). Moreover, *M. oleifera* may decrease lipid synthesis and cholesterol and can act as antileishmanial agent (Kaur *et al.*, 2014; Ngamukote *et al.*, 2014; Sangkitikomol *et al.*, 2014).

Saponins, the natural glycosides of steroid or triterpene are pharmaceutical and nutraceutical agents mostly present in plant leaves and roots. These possess hypo-cholesterolemic, antidiabetic, anticoagulant, hepatoprotective, anticarcinogenic, hypoglycemic, neuroprotective, immunomodulatory, anti-inflammatory, antimicrobial and antioxidant activity, leading to significant concentration on their benefit to be used as vaccine adjuvants (Koneri *et al.*, 2014; Thao *et al.*, 2014; Astuti *et al.*, 2011; Sun *et al.*, 2009; Rao and Gurfinkel, 2000). Estimation of total saponins in MO is a routine practice that needs to be more elaborated in terms of antioxidant and antidiabetic efficacy of isolated saponins. Phytochemicals are isolated by extraction with numerous polarity based solvents and these constituents exhibit variation in their chemistry and ultimately solubility in specific solvent (Sultana *et al.*, 2009). Despite the natural abundance and widespread use, data on biological activities of MO leaves in different solvents and the isolated saponins is limited. The purpose of the present

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study was to investigate *M. oleifera* leaves solvent fractions and isolated saponins fraction for antioxidant and antidiabetic activities.

Materials and Methods

Collection, identification and preparation of plant material. Samples collected from local Botanical garden were authenticated by a botanist. Leaves were washed, shade dried and ground to coarse powder. Powdered material was initially extracted with methanol at 25 °C for 4 days. Methanolic extracts were evaporated and resulting viscous greenish residues were partitioned into fractions by separating funnel. Fractionation was done into *n*-hexane (120 g), *n*-butanol (40 g), ethyl acetate (55 g), chloroform (60 g) and aqueous (85 g) fractions.

Phytochemical analysis. Phytochemical screening was accomplished for the detection of active components like saponins, tannins, alkaloids, terpenoids and flavonoids (Krishnaiah *et al.*, 2009).

Chromatographic isolation of saponin. The *n*-butanol fraction with optimal saponin concentration was further processed by means of thin layer chromatography (TLC) using silica gel G60 to validate and isolate saponins as described previously (Wagner and Bladt, 1996). Three spots that matched the reference R_f values of saponins were scratched from TLC plates, centrifuged and supernatant was dried to obtain pure saponin fraction.

Antioxidant components. Total phenolic contents (TPC). The amount of phenol contents was measured by Folin-Ciocalteu method, explained previously (Chahardehi *et al.*, 2009). Briefly, solvent fractions and saponin fraction (SF) were incorporated in 1 mL DMSO (dimethyl sulfoxide) and 1 mL Folin-Ciocalteu reagent (10%). 3 min later, 3 mL Na_2CO_3 (1% w/v) was added and incubated at room temperature for 2 h. The results were reported as gallic acid equivalent (g gallic acid/100 g dry weight) after measuring absorbance (760 nm).

Total flavonoid contents (TFC). Spectrophotometric method (Siddique *et al.*, 2010) was used for this estimation. 0.5 mL of extracts (1:10 g/mL) was mixed with respective solvent (1.5 mL), 10% AlCl_3 (0.1 mL), 2.8 mL distilled water and 0.1 mL of potassium acetate (1 M). Contents were articulated as g catechin equivalents/100 g dry weight) by taking absorbance at 510 nm.

Antioxidant activity (DPPH scavenging). The fractions were analyzed for their abilities to scavenge DPPH (2, 2-diphenyl 1-1-picrylhydrazyl) as described by Souri *et al.* (2010). 50 μL aliquot of some concentrations of each extract was mixed with 5 mL DPPH methanolic solution of 0.004%. After incubating for 30 min at room temperature, absorbance was taken at 517 nm. Inhibition percentage was calculated: Radical scavenging ($\text{IC}_{50}\%$) = $100 \times (\text{Absorbance blank} - \text{Absorbance test sample} / \text{Absorbance blank})$. IC_{50} denote the sample concentration required to scavenge 50% of DPPH radicals. The experiment was done in triplicates. BHT (Butylated hydroxy toluene) was the standard control.

Antioxidant components and activity were assessed in each sample (extract/fraction/synthetic), which was collected in triplicate and for each collection, three measurements were made. That concludes the use of sixty three samples each for TPC, TFC contents and DPPH activity assays and statistical analysis.

Antidiabetic evaluation. Antiglycation assay. Antiglycation assay was performed as described before (Ayatollahi *et al.*, 2010) and results were compared to those of the synthetic inhibitor aminoguanidine. Solvent fractions and SF were reconstituted in dimethylsulphoxide (DMSO). Bovine serum albumin (BSA, 10 mg/mL) and anhydrous glucose (50 mg/mL) were made in sodium phosphate buffer (67 mM, pH 7). Sample mixture contained equal amounts (100 μL) of fraction, BSA and glucose solution. Glycated (positive) control had sodium phosphate buffer instead of plant extract, while blank (negative control) contained BSA and sodium phosphate buffer only. After incubation at 37 °C for 7 days, 100 μL of 100% trichloroacetic acid was added and centrifuged for 4 min at 4 °C. Pellets containing advanced glycation end product were dissolved in phosphate buffer saline (pH 10). Absorbance was noted at 440 nm and inhibition percentage was calculated, % inhibition = $100 - [(\text{OD}(\text{test}) / \text{OD}(\text{blank}) \times 100)]$. Same procedure was repeated after 3 and 5 weeks.

Antiglycation activity was assessed in each sample (extract/fraction/synthetic), which was collected in triplicate for 1st, 3rd and 5th weeks. For each collection, three measurements were made. That concludes the use of one hundred and eighty nine samples for the antiglycation tests and statistical analysis.

Enzyme inhibition assay. Assay of alpha amylase inhibition was performed according to Apostolidis *et al.* (2006). Porcine pancreatic α -amylase (EC 3.2.1.1)

was bought from Sigma Chemical Co. Extracts, SF (500 μ L) and 500 μ L amylase solution (0.5 mg/mL) in buffer (0.02 M; pH 6.9) were incubated at 25 °C. After 10 min, 500 μ L starch solution (1%) was added at fixed time intervals and incubated. The reaction was arrested by adding 1 mL of dinitrosalicylic acid (DNS) reagent, heated in a boiling water bath for 5 min, cooled to room temperature and diluted with 10 mL distilled water. Absorbance was noted against blank at 540 nm. IC_{50} (%) was calculated as: $[(Ac - As)/Ac] \times 100$. Where, Ac = the absorbance of control and As = the absorbance of test samples. For control (negative), buffer was used and positive control was acarbose drug.

Enzyme inhibition was assessed with 0.1, 0.3, 0.5 mL extracts/fractions/synthetic inhibitor volume, which was collected in triplicate and for each collection, three measurements were made. That concludes the use of one hundred and eighty nine samples in enzyme inhibitory and statistical analysis.

Statistical analysis. All the results were stated as values of means \pm SD (standard deviation) and analysis of variance (ANOVA) was used to analyze data by SPSS software (SPSS Inc. Chicago, IL, USA; version 15.0) at level of significance $p < 0.05$. To assess within group differences due to week wise treatments, repeated measure ANOVA (tukey test) and for multiple comparisons, LSD test (post hoc test) were used at 5% level of significance (Statistical Software SAS 9.0).

Results and Discussion

Antioxidant contents and activity. Phytochemicals play an important pharmacological role (Kasolo *et al.*, 2010). This study screened *M. oleifera* leaves fractions especially saponins for antioxidant and antidiabetic activities. Phytochemical screening indicated the

availability of imperative chemicals such as tannins, saponins, terpenoids, alkaloids and flavonoids in MO leaves. SF that appeared on chromatograms had three R_f values (0.89, 0.90, 0.91) with green coloured spots. These SF were pooled together into one fraction for the estimation of antioxidant and antidiabetic potentials. TPC and TFC of MO leaf fractions in ranges 9.7-12.8 GAE and 5.3-9.3 CE (g/100 g dry weight) are summarized in Table 1. TPC was significantly higher in methanol than *n*-hexane fraction ($p < 0.034$). Analysis revealed variations in TPC and TFC of different solvents. Aqueous and methanol were more effective in extracting the highest TPC and TFC, followed by ethyl acetate, chloroform, *n*-butanol and *n*-hexane.

All the fractions had free radical scavenging abilities but to significantly different degrees ($p < 0.05$), in the range 69.5-83.1% (Table 1). MO methanol extract exhibited highest scavenging activity ($p < 0.037$). Although SF displayed noteworthy antioxidant ability ($p < 0.046$) as compared to standard BHT, its efficiency was trivial than most of the other extracts. Regarding the solvent partitions, DPPH scavenging activity in descending order was ethyl acetate > chloroform > *n*-butanol > SF > *n*-hexane. In terms of IC_{50} , all the extracts showed considerable activity as compared to BHT ($p < 0.05$).

Phenolic compounds in plants have antioxidant activity. Intensive studies on the phenolic and flavonoid constituents in *Moringa oleifera* have been conducted. Present study is the first to find antioxidant and antidiabetic role of saponin isolated from MO leaves. Reference to preliminary phytochemical investigation, important chemicals like alkaloids, tannins, flavonoids, saponins and terpenoids were prevalent in MO samples and these findings are agreeing with previously reports (Kasolo *et al.*, 2010; Krishnaiah *et al.*, 2009).

Table 1. Antioxidant profile

Antioxidant components	ME	SF	HF	BF	CF	EF	BHT
TPC (GAE g/100 g DW)	12.8 \pm 2.1	-	9.7 \pm 1.4	10.0 \pm 1.2	10.4 \pm 3.1	11.6 \pm 2.4	-
TFC (CE g/100 g DW)	9.3 \pm 2.5	-	5.3 \pm 1.0	6.5 \pm 1.07	7.0 \pm 2.3	7.8 \pm 1.9	-
DPPH, IC_{50} (μ g/mL)	83.1 \pm 1.3	70.4 \pm 2.1	69.5 \pm 1.56	72.4 \pm 1.6	76.5 \pm 1.78	78.1 \pm 2.34	21.5 \pm 0.27

Data (Mean \pm SD) are average of three samples of each extract/fraction, analyzed individually in triplicate ($n = 1 \times 3 \times 3$).

DW = dry weight; TPC = total phenolic contents expressed g gallic acid equivalent /100 g dry weight; TFC = total flavonoid contents expressed as g catechin equivalents/100 g dry weight; DPPH = 2, 2-diphenyl 1-1-picrylhydrazyl, ME = methanol extract; SF = saponins fraction; HF = *n*-hexane fraction; BF = *n*-butanol fraction, CF = chloroform fraction; EF = ethyl acetate fraction; BHT = Butylated hydroxy toluene.

Data regarding TPC and TFC was in accordance with that of the Sultana *et al.* (2009). They reported 9.72-12.2 GAE (g/100 g dry weight) TPC and 5.3-8.66 CE (g/100 g dry weight) TFC in MO. Solubility of phytoconstituents although variable, plays a vital role in their activities. This can be due to the higher ability of polar solvents such as methanol and aqueous extract phenolics oftenly in higher amounts (Sultana *et al.*, 2009). Another study (Kasolo *et al.*, 2010), mentioned 45.21 mg GAE/g extract and 15.39 mg CE/g extract in MO leaves.

A very familiar trend often observed in the study of phytoconstituents is the parallel relation between total phenols and free radical scavenging power. Higher the TFC, higher will be the antioxidant efficacy observable in DPPH methodology. With free radical scavenging power, antioxidants offer defense against numerous diseases. A simple and precise approach to investigate antioxidant potential is DPPH stable free radical method (Choi *et al.*, 2002; Koleva *et al.*, 2002). Likewise, DPPH activity measured in present results were analogous to DPPH activity (69.5-83.1%S) stated previously (Sultana *et al.*, 2009). Although saponins do have antioxidant potential (DPPH scavenging by SF was not quite impressive as compared to other fractions) in fact it was lower than most of them, as it lacked the major radical scavenging components.

Antidiabetic activities. Data on antiglycation potential is summarized in Fig. 1. SF was leading inhibitor ($p < 0.041$) after MeOH throughout the studied weeks, attaining up to 48.1% glycation restriction. Three fractions *viz.* *n*-hexane, *n*-butanol, chloroform demonstrated almost same though noticeable ($p < 0.05$) inhibition patterns (W1: 12.86-25.71%, W3: 34.78-42%, W5: 44.93-47.83%). Comparatively, there was almost 4-fold difference between the least and most potent glycation inhibitors (MeOH *vs.* ethyl acetate) in the first week of incubation. Nonetheless, this difference was lessened in the last study week. Aminoguanidine was most potent ($p < 0.023$) against glycation process (W1: 44%, W3: 62%, W5: 68%). Contrary to that, ethyl acetate fraction was least effective, as glycation inhibition was enhanced by 8.57% in 1st week to 37.68% in the 5th week. Significant differences ($p < 0.011$) in glycation inhibition means were evident. Follow up examinations after two and three weeks revealed that corresponding response activities for each group were positive. Diabetes mellitus and its manifestations originate from persistent hyperglycemia. Central theme in therapeutic options against such complications is to restrict high blood glucose

through the mediation of natural products. *Moringa oleifera* is one of the renowned medicinal plants proved to have the promising effects in the prevention and treatment of diabetes through different mechanisms (Adisakwattana and Chanathong, 2011). MO leaves extract, fraction were assessed for their antiglycation ability, and competence was compared with synthetic glycation inhibitor aminoguanidine. MeOH repressed glycation up to 20-57% after 5 weeks. Similar to our results, Awasthi (2013) questioned the antiglycation activity of MO leaves on the basis of formation of AGEs and amadori products. The amadori and AGEs inhibitory activity was about 70% and was persistent with the incubation time. Regarding SF, antiglycation ability was higher among most of the other samples. This data can be justified by the fact that antioxidant and antiglycation activities of many medicinal plants correlate with corresponding saponin content (Bi *et al.*, 2012; Xi *et al.*, 2008). It was further revealed that glycation inhibition was associated with the prolonged incubation period. Similar to present inferences, Ngamukote *et al.* (2014) determined hindering effect of extract of *M. oleifera* leaf on fructose-induced glycation for 7,

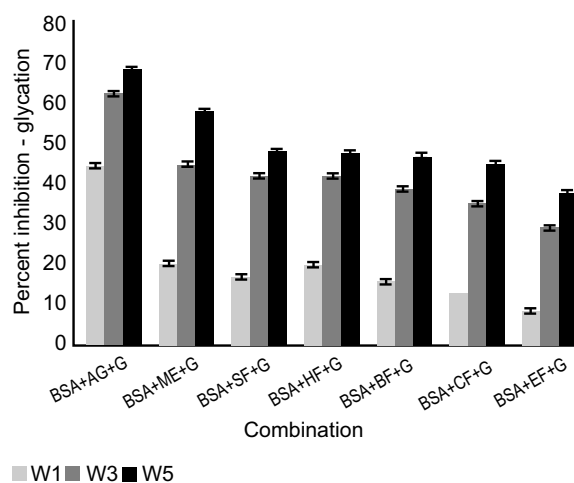


Fig. 1. Antiglycation activity.

Data (mean \pm SD) are average of three samples of each extract/fraction, analyzed individually in triplicate ($n = 1 \times 3 \times 3$). BSA = Bovine serum albumin; AG = Aminoguanidine; G = Glucose; ME = Methanol extract; SF = saponins fraction; HF = *n*-hexane fraction; BF = *n*-butanol fraction; CF = chloroform fraction; EF = ethyl acetate fraction. Inhibition was assessed after 1st, 3rd and 5th weeks (W1, W3, W5) incubations.

14, 21 and 28 days. A decline in formation of AGEs was observed with prolonged incubations.

Starch hydrolyzing enzymes inhibitors may exert some of their benefits in the therapy of diabetes mellitus. It was investigated whether alpha amylase activity could be minimized by plant extracts (Fig. 2). Negligible enzyme inhibition (3.4-9.2%) was reflected by MO fractions, though methanol extract attained greatest (5.5-9.2%) amylase inhibitory potential. Enzyme inhibition by SF was less impressive as compared to that of MeOH extract. Nearly equivalent yet non-significant ($p > 0.198$) percent reduction in enzyme activity by 0.1 mL, 0.3 mL and 0.5 mL SF was evident, nullifying its concentration dependent impact. Concentration dependent effect of methanol extracts was more apparent ($p < 0.043$) as increasing extract levels from 0.1 mL to 0.5 mL lowered the amylase activity by 3.7%. Least enzyme inhibition was shown by *n*-butanol (0.5 mL). Significant differences ($p < 0.01$) in enzyme inhibition means were evident. Throughout the treatment period, there was a significant decrease in enzyme activities ($p < 0.001$) with the magnitude of the reduction increasing with the inhibitor concentrations.

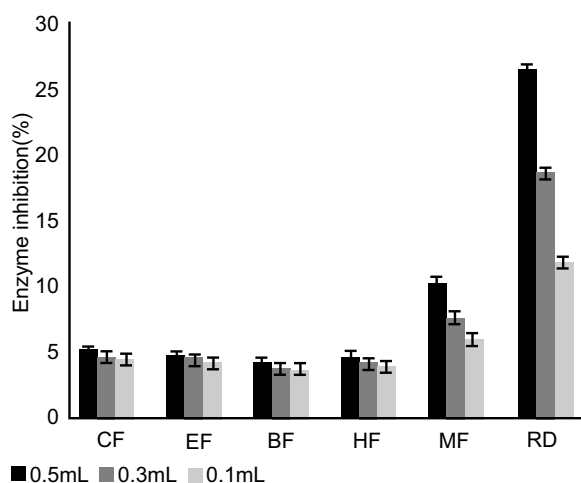


Fig. 2. Alpha amylase inhibition by plant extract, fractions and standard drug.

Data (mean \pm SD) are average of three samples of each extract/fraction, analyzed individually in triplicate ($n = 1 \times 3 \times 3$). RD = reference drug (acarbose); ME = Methanol extract; SF = saponins fraction; HF = *n*-hexane fraction; BF = *n*-butanol fraction; EF = ethyl acetate fraction; CF = chloroform fraction. Inhibitions were assessed with 0.1, 0.3, 0.5 mL extracts/fractions/synthetic inhibitor volume.

All the other extracts improved their inhibition potential up to 0.6% for the same increase in corresponding concentrations. Maximum enzyme inhibitory potential was exhibited by synthetic inhibitor acarbose (23.7%, $p < 0.025$).

Using the inhibitors of carbohydrate digesting enzymes obtained from natural resources with less adverse effects than synthetic drugs can be advantageous to block the absorption of dietary carbohydrate (Etxeberria *et al.*, 2012). Plants are important source of chemical compounds with ability of α -amylase inhibition (Sales *et al.*, 2012).

Enzyme inhibition by MO in present study was lesser than that calculated for other medicinal plants. Present results can be justified by the fact that an association exists between phenolic, flavonoids and tannins and the alpha amylase inhibitory activities. Studies on MO for enzyme inhibition phenomenon are scarce. Adisakwattana and Chanathong (2011) documented slight inhibitory potential of pancreatic alpha amylase by MO leaves extracts. Amongst the phytoconstituents investigated, flavonoids demonstrated maximum inhibitory potential related to the number of hydroxyl groups present in molecules of the compound (Sales *et al.*, 2012). SF showed negligible amylase inhibition being devoid of flavonoids. Triterpenoids represent a favourable and expanding natural source of biologically active constituents. Despite their wide distribution in plants, investigations on inhibition of activities of alpha amylase and alpha glucosidase are limited. A few reports available to date dearth the mechanism by which this activity occurs (Sales *et al.*, 2012; Luo *et al.*, 2008). Multiple comparisons of means and inter-dependence of weekly analysis showed significant associations for glycation assay. Similarly, enzyme inhibition improved after increasing inhibitor concentrations. It is assumed that saponins existing in MO leaves hold antioxidant, antiglycation and enzyme inhibitory activities that can lead to the development of innovative therapeutic adjuvants. However, despite some promising results, more research is needed to validate current inferences.

Conflict of interest. The authors declare no conflict of interest.

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