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ASSESSMENT OF ANTIDIABETIC POTENTIAL OF AQUEOUS LEAF EXTRACT OF *MORINGA OLEIFERA* BY *IN VITRO* EXPERIMENTAL METHODS

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Abstract

Diabetes mellitus is a disorder that occurs when there is little or not enough insulin production from the pancreas. The onset and progression of long-term complications in diabetes mellitus appear to be related to the degree of hyperglycemia and the overall metabolic control. The core remedy for managing diabetes is to lower hyperglycaemia and reduce intestinal glucose absorption through the inhibition of carbohydrate metabolizing enzymes *viz.* alpha-amylase and alpha-glucosidase. *Moringa oleifera* is a medicinal plant, and in traditional medicine, it is known as the miracle tree because all the plant parts *viz.* leaves, seeds, bark, roots, and flowers have nutritional and medicinal uses. Thus, we aimed to assess the antidiabetic potential of aqueous (*aq.*) leaf extract of *Moringa oleifera* through *in-vitro* experimental methods such as inhibition of carbohydrate hydrolyzing enzymes namely, alpha-amylase and alpha-glucosidase. Results depicted that *aq.* leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL, and 80µg/mL, shown alpha-amylase inhibition effect of 33.20%, 54.12%, 69.31%, and 79.34% respectively with an IC_{50} value of 61.26 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 69.58 µg/mL. Furthermore, *aq.* leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL, and 80µg/mL, shown inhibition effect of 27.72%, 42.58%, 58.87%, and 71.30% respectively with an IC_{50} value of 51.62 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 64.46 µg/mL. Moreover,

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quantitative estimation of phytochemicals in *aq.* leaf extract of *M. oleifera* revealed total flavonoids quantity was found to be highest (0.21 mg RE/g extract) in aq. leaf extract of *M. oleifera* followed by total phenolics (0.12 mg GAE/g extract) and total tannins (0.06 mg TAE/g extract). In conclusion, results from this study delineated that *aq.* leaf extract of *M. oleifera* possess in-vitro alpha -amylase and alpha-glucosidase activities, and hence it could be presumed from the findings of this study that the crucial mechanisms of anti-diabetic action of leaf parts of *M. oleifera* is via inhibition of carbohydrate inhibiting enzymes such as alpha- amylase and alpha-glucosidase.

Keywords: *Moringa oleifera*, Leaf extract, Diabetes mellitus, Antidiabetic potential, Alpha-amylase, Alpha-glucosidase

Introduction

Diabetes mellitus is a disorder that occurs when there is little or not enough insulin production from the pancreas. It is often regarded as hyperglycaemia *i.e.*, having excessive blood glucose in the blood. According to the International Diabetes Federation (IDF), a total of 387 million people were diagnosed with diabetes worldwide in 2014, with the figure expected to rise to 592 million by 2035.[1] Diabetes mellitus is a lifelong endocrine disease caused by defects in insulin secretion (*i.e.*, deficient or insufficient synthesis of insulin from the pancreas), insulin action (*i.e.*, insulin resistance and hyperinsulinemia), or both,[2,3] leading to hyperglycemia and severe irreversible microvascular and macrovascular complications that affect the eyes (diabetic retinopathy), feet (diabetic foot), nerves (diabetic neuropathy), kidneys (diabetic nephropathy), blood vessels (atherosclerosis), and heart (cardiovascular disease). The management of hyperglycemia is of utmost importance to limit the severe complications of diabetes mellitus.[4]

The onset and progression of long-term complications in diabetes mellitus appear to be related to the degree of hyperglycemia and the overall metabolic control. Although various orthodox medications are available for the control of hyperglycemia but there has been increasing demand for the use of plant products with anti-diabetic activity. The high cost, availability, uncertainly of use during pregnancy and undesirable side effects of synthetic drugs have been some of the factors leading to a strong preference for hypoglycemic drugs of plants origin, which are believed to be suitable for chronic treatments.[5] Till date, numerous medicinal plants have been reported to be effective in the management of diabetes, though further research is required.[6]

The core remedy for managing diabetes is to lower hyperglycaemia and reduce intestinal glucose absorption through the inhibition of carbohydrate metabolizing enzymes *viz.* alpha-amylase and alpha-glucosidase.[7] The toxicity levels and the high cost of pharmaceutical drugs designed for carbohydrate metabolizing enzyme inhibition have been a major concern.[8] Hence, a cost-effective management approach is needed to be adopted to ensure minimal side effects in diabetes treatment. The therapeutic importance of medicinal plants has accelerated researchers' interest in the discovery of the possible activities of plants to prevent and protect against chronic diseases.[9] Phenols, a bioactive compound found in plants were reported to inhibit alpha-amylase and, thereby providing it an excellent approach for type 2 diabetes

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management.[10,11] Furthermore, various prospective studies have shown that some compounds such as catechin, isocatechin, flavonoids, flavones, isoflavone, and anthocyanin have exhibited antidiabetic properties.[12]

Decreasing postprandial hyperglycemia by inhibition of carbohydrate hydrolyzing enzymes such as, alpha-amylase and alpha-glucosidase is one of the therapeutic approaches. Until now, acarbose and voglibose are used either alone or in combination with insulin as an inhibitor of carbohydrate digestive enzymes.[13] However, harmful effects of these compounds, such as liver disorders, flatulence, abdominal fullness and diarrhea, have been reported.[14]

Moringa oleifera is a medicinal plant of the genus *Moringa* of the family Moringaceae (Figure 1).[15,16] In traditional medicine, it is known as the miracle tree because all the plant parts *viz.* leaves, seeds, bark, roots, and flowers have nutritional and medicinal uses.[17,18]

Figure 1A: Showing *M. oleifera* whole plant

Figure 1B: Showing leaves of *M. oleifera*

Several studies have shown that the *aq.* extracts of *M. oleifera* leaves possess a wide range of biological actions including antioxidant, tissue protective, cardioprotective, hepatoprotective, neuroprotective,[19] analgesic,[20] diuretic,[21] antiulcer,[22] anticancer,[23] antidiabetic, anti-inflammatory,[24] antimicrobial,[25] antihypertensive,[26] and immunomodulatory effects.[27] With this background, the present study was designed to assess the antidiabetic potential of leaf parts of *M. oleifera* through *in-vitro* experimental methods such as inhibition of carbohydrate hydrolyzing enzymes namely, alpha-amylase and alpha-glucosidase.

Materials and Methods

Collection of *M. oleifera Leaves*

The leaves of *M. oleifera* plant were collected agricultural fields in and around Bengaluru Rural district, Karnataka, India. The leaves of *M. oleifera* were sprayed with ethanol, and then shade dried at room temperature. The dried leaves were pulverized to obtain a fine powder using electric blender, and stored in airtight containers for further analysis.

Extraction

Approximately 50 g of dried and coarsely powdered leaves of *M. oleifera* was subjected to successive solvent extraction by continuous hot extraction (Soxhlet) with 550 mL of double

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distilled water. All the extracts were concentrated by distilling the solvent in a rotary flash evaporator. The extracts were preserved in airtight containers and stored at room temperature until further use.[28]

Quantitative Estimation of Phytochemicals

Total tannins

0.5 mL (1 mg/mL) of *aq.* leaf extract of *M. oleifera*, double distilled water of 3.75 mL, and 0.25 mL of FCR (1:10 v/v) were mixed together. Lastly, 0.5 mL of 35% sodium carbonate was added to the mixture, and the absorbance was measured. Tannic acid within the concentration range of 0.1–0.00625 mg/mL was used as a reference standard. The total tannin content of extract was calculated using the standard curve and reported as mg tannic acid equivalent per gram extract powder (mg TAE/g extract).[28] The estimation was conducted in triplicate and mean of triplicate was expressed.

Total flavonoids

2 mL of double distilled water was added to 0.15 mL (1 mg/mL) of *aq.* leaf extract of *M. oleifera*, and in addition, 0.15 mL of 5% sodium nitrite was added. The mixture was subjected to incubation at 25°C for 6 minutes. 0.15 mL of 10% aluminium chloride was mixed and further incubated for 5 minutes. 4% of NaOH was added to make up to volume of 5 mL, and then 1.2 mL double distilled water was added. Afterwards, the absorbance of the mixture was measured at 420 nm. The standard calibration curve of rutin (0.02–0.1 mg/mL) was constructed.[29] A flavonoid content in the extract was observed by the presence of pink colouration. The content of flavonoids was calculated and expressed as mg rutin equivalent per gram extract powder (RE/g extract) using the standard calibration curve of rutin. The estimation was conducted in triplicate and mean of triplicate was expressed.

Total phenolics

0.5 mL of *aq.* leaf extract of *M. oleifera* was mixed with 2.5 mL of FCR and incubated for 5 mins at 25°C. Afterwards 2 mL of 7.5% sodium carbonate solution was added. The mixture was measured at 765 nm after incubation at 40°C for 30 minutes. The standard calibration curve of gallic acid (0.02–0.1 mg/mL) was prepared. The total phenolics quantity in *aq.* leaf extract of *M. oleifera* was calculated using standard calibration curve of gallic acid and expressed as mg gallic acid equivalent per gram extract powder (mg GAE/g extract).[28]

Inhibition Assays of Carbohydrate Hydrolyzing Enzymes

Alpha-amylase inhibitory assay

The alpha-amylase inhibition assay was carried out by the method of Miller, (1959).[30] *Aq.* leaf extract of *M. oleifera*/acarbose (20µg/mL, 40µg/mL, 60µg/mL and 80µg/mL) were incubated for 10 minutes at 25°C with 500 µl of 20 mM sodium phosphate buffer (pH 6.8) with 20 µl of amylase (1U/ml). After pre-incubation, each tube was added with 1 ml of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) and incubated for 15 min. One mL DNS was added to arrest the reaction. After that, the tubes were kept in a boiling water bath for 5 min and cooled to room temperature. After that, distilled water (10mL) was added to the reaction mixture, and the absorbance was measured at 540 nm. The test compound was not used in the preparation of the control samples. The following formula was used to determine the percent inhibition of alpha-amylase activity;

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% Inhibition = (Abs control – Abs test) / (Abs control)

Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibition assay was carried out as described by Matsui et al (1996) with slight modifications.[31] The different concentrations of *aq.* leaf extract of *M. oleifera* and standard drug acarbose (20µg/mL, 40µg/mL, 60µg/mL and 80µg/mL) were prepared. Phosphate buffer (1 mL; 100mM, pH 6.8) and 80 µL of test *aq.* leaf extract of *M. oleifera* / and acarbose were added to 20 µL of alpha-glucosidase and incubated at 37°C for 10 minutes. Later, pNPG- 50µl (5mM) was added to the assay mixture to initiate the reaction. Then, the reaction mixture was incubated at room temperature for one hour and arrested the reaction by adding 2.5mL of 0.1 M Na_2CO_3. The absorbance was measured at 400nm to determine the activity of alpha-glucosidase activity. The following formula was used to determine the percent inhibition of alpha-glucosidase activity;

% Inhibition = (Abs control – Abs test) / (Abs control)

Results

Quantitative estimation of phytochemicals

Quantitative estimation of phytochemicals in *aq.* leaf extract of *M. oleifera* was represented in Table 1 and plotted in Figure 2. Results revealed that total flavonoids quantity was found to be highest (0.21 mg RE/g extract) in *aq.* leaf extract of *M. oleifera* followed by total phenolics (0.12 mg GAE/g extract) and total tannins (0.06 mg TAE/g extract).

Table 1: Quantitative analysis of phytochemicals in *aq.* leaf extract of *M. oleifera*

Phytochemicals	***Aq.* Leaf Extract of *M. oleifera***
Total tannins	0.06 mg TAE/g extract
Total flavonoids	0.21 mg RE/g extract
Total phenolics	0.12 mg GAE/g extract

Values are expressed mean; n=3

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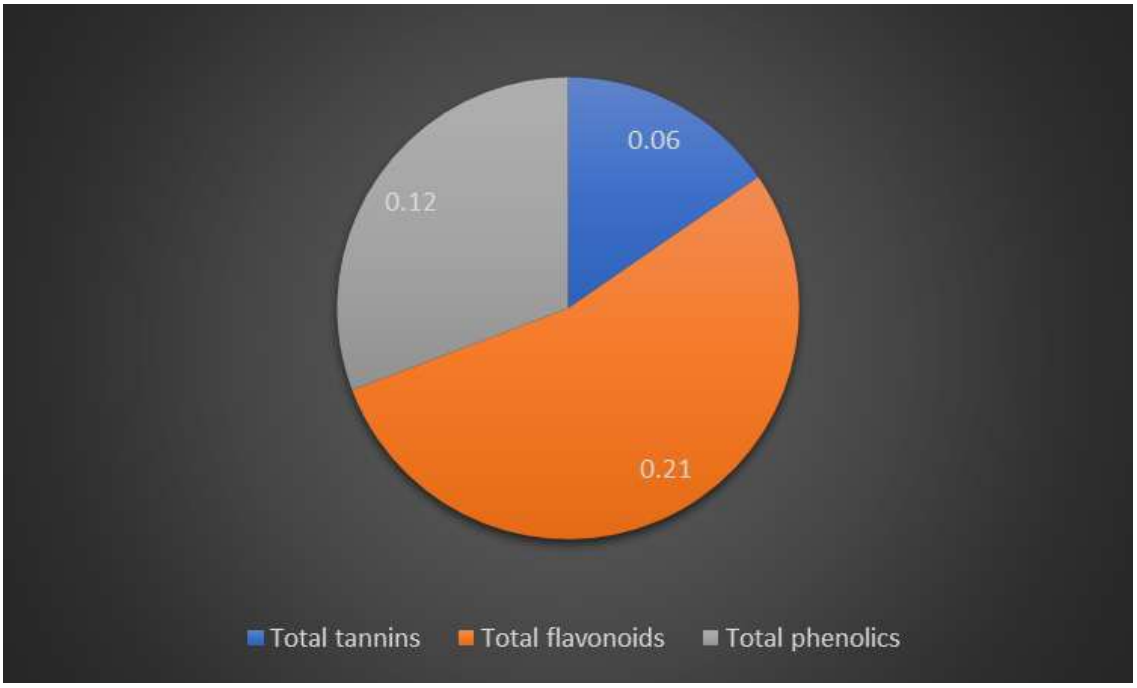

Figure 2: Quantitative analysis of phytochemicals in *aq.* leaf extract of *M. oleifera*

Alpha-amylase inhibitory assay

The results of effect of *aq.* leaf extract of *M. oleifera* on alpha-amylase inhibition activity was represented in Table 2. Results depicted that *aq.* leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL and 80µg/mL, shown inhibition effect of 33.20%, 54.12%, 69.31%, and 79.34% respectively with an IC_{50} value of 61.26 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 69.58 µg/mL.

Table 2: Effect of *aq.* leaf extract of *M. oleifera* on alpha-amylase inhibition activity

Conc. of Aq. Leaf Extract of *M. oleifera* (µg/mL)	**Inhibition (%)**	**Conc. of Acarbose (µg/mL)**	**Inhibition (%)**
20	33.20 ± 0.89	20	46.05 ± 1.11
40	54.12 ± 2.69	40	66.97 ± 2.05
60	69.31 ± 3.24	60	82.16 ± 3.45
80	79.34 ± 4.55	80	92.19 ± 5.62
IC_{50} (ug/mL) = 61.26		IC_{50} (ug/mL) = 69.58	

Values were expressed Mean ± SD; n=3

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Alpha-glucosidase inhibitory assay

The results of effect of *aq.* leaf extract of *M. oleifera* on alpha-glucosidase inhibition activity was represented in Table 3. Results depicted that *aq.* leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL and 80µg/mL, shown inhibition effect of 27.72%, 42.58%, 58.87%, and 71.30% respectively with an IC_{50} value of 51.62 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 64.46 µg/mL.

Table 3: Effect of aq. leaf extract of *M. oleifera* on alpha-glucosidase inhibition activity

Conc. of Aq. Leaf Extract of *M. oleifera* (µg/mL)	**Inhibition (%)**	**Conc. of Acarbose (µg/mL)**	**Inhibition (%)**
20	27.72 ± 2.05	20	40.57 ± 1.83
40	42.58 ± 4.32	40	55.43 ± 3.48
60	58.87 ± 5.68	60	71.72 ± 2.98
80	71.30 ± 3.54	80	84.15 ± 6.34
IC_{50} (ug/mL) = 51.62		IC_{50} (ug/mL) = 64.46	

Values were expressed Mean ± SD; n=3

Discussion

In vitro studies in all fields of biology are aimed at elucidating the mechanisms by which biological substances perform their roles within a cell.[32] The uses of medicinal plants in delivering cost-effective therapy for a variety of ailments are due to the existence of secondary metabolites.[33] Plants contain bioactive compounds, which have been shown in studies to have a variety of therapeutic properties.[34] *M. oleifera* has proven to have various antidiabetic, antiobesity, antioxidant, and anti-inflammatory effects. It was reported to contain a huge amount of proteins, oils, potassium, calcium, carbohydrates, amino acids, and phenolic compounds (such as rutin, kaempferol, p-coumaric acid, and quercetin). The antidiabetic pharmacological properties of *M. oleifera* are a result of its high constituents of flavonoid, glucoside, and glucosinolate.[35] Furthermore, research has shown that many edible wild plants are rich in specific constituents, referred as phytochemicals, which may have health promoting effects. Moreover, alpha-amylase and alpha-glucosidase inhibitors have become a new treatment strategy to combat diabetes mellitus.[36] Thus this research investigation was conducted with the main objectives to assess the antidiabetic potential of *aq.* leaf extract of *M. oleifera* through *in-vitro* experimental methods such as inhibition of carbohydrate hydrolyzing enzymes namely, alpha-amylase and alpha-glucosidase.

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Our study results delineated that *aq.* leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL, and 80µg/mL, shown alpha-amylase inhibition effect of 33.20%, 54.12%, 69.31%, and 79.34% respectively with an IC_{50} value of 61.26 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 69.58 µg/mL. Furthermore, aq. leaf extract of *M. oleifera* at a concentration range of 20µg/mL, 40µg/mL, 60µg/mL, and 80µg/mL, shown inhibition effect of 27.72%, 42.58%, 58.87%, and 71.30% respectively with an IC_{50} value of 51.62 µg/mL in comparison with the standard antidiabetic drug acarbose with an IC_{50} value of 64.46 µg/mL. These findings depicted that *aq.* leaf extract of *M. oleifera* possess carbohydrate hydrolyzing enzymes inhibition activities at par with that of standard acarbose. Furthermore, quantitative estimation of phytochemicals in *aq.* leaf extract of *M. oleifera* revealed total flavonoids quantity was found to be highest (0.21 mg RE/g extract) in *aq.* leaf extract of *M. oleifera* followed by total phenolics (0.12 mg GAE/g extract) and total tannins (0.06 mg TAE/g extract). Hence, in-vitro inhibition of carbohydrate hydrolyzing enzymes potential of *aq.* leaf extract of *M. oleifera* could be ascribed to the presence of secondary metabolites like flavonoids, phenolic compounds, and tannins. These findings were in concurrence with literature findings. Various research investigators demonstrated that herbal plants with secondary metabolites, including alkaloids, polyphenols, flavonoids, saponins, tannins, and terpenoids, have been shown to be responsible for the antihyperglycemic effect.[37-39]

Szkudelski demonstrated in their in-vivo alloxan induced diabetic rat model study that the ethanol extracts of the seed, stem, bark, flowers, leaves and root of *M. oleifera* produced varying degrees of reduction in blood glucose level in diabetic rats in a dose and time dependent manner. The reduction in blood glucose produced by the seed and leaves extract was however more significant than that of the other parts.[40] Moreover, the antidiabetic properties of the leaves have been reported by various other research investigators in the literature.[41,42]

In a controlled study with untreated type 2 diabetes mellitus patients, William et al., reported the anti-hyperglycaemic activity of the *M. oleifera* leaves.[43] Kumari also examined the hypoglycemic effect of *M. oleifera* leaf dietary consumption in type 2 diabetes mellitus patients, and reported that it reduced glucose level significantly after a 40-day period.[44] The therapeutic actions of *M. oleifera* medication has been attributed to the relatively high antioxidant activity of its leaves, flowers, and seeds.[45-48]

Furthermore, the onset of diabetes can be delayed, as revealed in experimental studies with *M. oleifera* leaves extract with high phenolic content.[49,50] The therapeutic potential of phytocompounds in *M. oleifera* has been reported in vitro and in vivo studies for reducing the risk of chronic diseases.[51] Summarily, *M. oleifera* leaf extract has been validated to be an excellent alpha-amylase inhibitor with properties suitable for the prevention and treatment of diabetes mellitus when consumed. The bioactive compounds such as flavonoid, saponin, and tannins present in *M. oleifera* leaf extracts possess antioxidant, antidiabetic, and antiobesity activities, which could be responsible for its potent radical scavenging ability and effective inhibition of carbohydrate hydrolyzing enzymes.[52]

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Conclusion

Conclusively, the results from this study delineated that aqueous leaf extract of *M. oleifera* possess in-vitro alpha -amylase and alpha-glucosidase activities. Furthermore, aqueous leaf extract of *M. oleifera* contains flavonoids as major secondary metabolites along with phenolic compounds and tannins. It could also be presumed from the findings of this study that the crucial mechanisms of anti-diabetic action of leaf parts of *M. oleifera* is via inhibition of carbohydrate hydrolyzing enzymes such as alpha- amylase and alpha-glucosidase.

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