Evaluation of the Inhibition of Carbohydrate Hydrolysing Enzymes and Antioxidant Activity of Hydroalcoholic Extract of Leaves of Moringa Oleifera

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Abstract

Background: Medicinal plants constitute an important source of potential therapeutic agents for diabetes and hyperlipidemia. One of the most important strategies for prevention of postprandial hyperglycemia and hyperlipidemia is inhibition of carbohydrate digestive enzymes (α-glucosidase and α-amylase), inhibition of fat digestion and absorption.

Aim: The purpose of present study was to investigate the effect of Hydro alcoholic extract (HEMO) of *Moringa oleifera* on inhibition of alpha-glucosidase and pancreatic alpha-amylase related to diabetes mellitus.

Method: *In-vitro* antioxidant activity and anti- diabetic studies.

Results: The IC_{50} values of HEMO for sucrase inhibitory activity and α-amylase inhibitory activity had been 248.26 ± 0.31μg/ml and 71.35 ± 0.52μg/ml respectively. The study's findings indicated that HEMO shown robust activity almost dose-dependently, indicating that it is a potent -glucosidase inhibitor that is comparable to the gold standard, acarbose. But compared to nojirimycin, it has also been found to exhibit -amylase inhibitory action.

Conclusion: These outcomes verify that HEMO exhibits α-glucosidase and α-amylase inhibitory properties. The results provide evidence that *Moringa oleifera* leaf extract may have the potential to treat diabetes mellitus.

Keywords: *Moringa oleifera*, inhibitory activity, diabetes and hyperlipidemia.

1. Introduction

Diabetes mellitus is a serious chronic metabolic ailment originated by innate and /or pancreatic deficit in insulin making, or by a decrease in insulin production[1]. DM is symbolized by hyperglycemia, diabetic specific micro vascular complications within the eye, kidney and peripheral neurons and macro vascular complications affecting arteries that provide the heart, brain and other organs[2]. A category of metabolic disorders known as hyperlipidemia are brought on by elevated triglyceride and/or cholesterol levels in the blood. The progression of micro- and macro-vascular problems, such as microangiopathy, cardiovascular, cerebrovascular, and metabolic syndrome disorders, is significantly

influenced by long-term hyperglycemia and hyperlipidemia.. Due to a modern lifestyle and an increase in the intake of high-carbohydrate and high-fat diets, the prevalence of hyperlipidemic and diabetic patients has sharply grown worldwide[3-5]. One of the most important strategies is Prevention of postprandial hyperglycemia and hyperlipidemia consists of inhibition of carbohydrate-degrading enzymes (α-glucosidase, α-amylase) and inhibition of fat digestion and absorption. [6]. The Global Ethnobotanical Information reports that many herbal medicines derived from plants and vegetables are used to control hyperglycemia and hyperlipidemia7. Many herbal medicines offer possibilities and opportunities to develop new treatments of antihyperglycemic and antihyperlipidemic active ingredients from natural sources[8-10]. *Moringa oleifera (Ma-rum)* is the most commonly cultivated member of the single *genus Moringaceae,* containing 13 species of trees and shrubs distributed in India, Sri Lanka, Pakistan, Bangladesh, Northeast and Southwest Africa, Arabia, India and Thailand. It is a seed.

Moringa oleifera has shown various beneficial pharmacological effects in prevention or treatment of a variety of diseases such as anti-diabetic [11], anti-microbial [12], anti-inflammatory [13], anti-oxidant properties [14]. According to a recent study, Moringa oleifera leaf extract lowers blood glucose levels in both healthy and diabetic rats generated by streptozotocin (STZ). Additionally, giving rats fed a high-fat diet the crude leaf extract of Moringa oleifera lowers their blood cholesterol levels in the liver and kidney[15]. However, it is unknown whether Moringa oleifera leaf extract has anti-diabetic and anti-hyperlipidemic properties through inhibiting enzymes that break down carbohydrates as well as enzymes that break down and absorb lipids. The current study's objective was to ascertain the impact of Moringa oleifera leaf extract on the inhibition of -glucosidase and pancreatic -amylase activities. Additionally, the extract's in vitro antioxidant activity was assessed.

2. Materials and Methods

Chemicals

Nicotinamide adenine dinucleotide (NADH), 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), and phenazine methosulphate (PMT) were purchased from Sigma Chemicals Co. in St. Louis, Missouri. Methanol, dimethyl sulphoxide, and sodium nitroprusside were purchased from Ranbaxy Fine Chemicals Ltd. in Mohali, India. The supplier of sulphanilic acid was Himedia Laboratories Ltd. in Mumbai, India. It was possible to buy naphthyl ethylene diamine dihydrochloride from LobaChemie in Mumbai, India. NBT, which is nitro blue tetrazolium, was purchased from Merk in Germany. The supplier of streptozotocin was SRL Private Ltd. in Mumbai, India. Rutin and ascorbic acid were purchased from S.D. Fine Chem. in Biosar, India. The other compounds employed were all of an analytical calibre.

Preparation of Extract

Following plant harvest and shade drying, Moringa oleifera leaves were ground into a fine powder and utilised for the hydroalcoholic extraction with Reflux. Reflux was used in a 1:4 ratio for 5–6 hours to extract 80% hydroalcoholic extract (HEMO). The extracted material was concentrated at a regulated temperature of 40 to 50 C while under reduced pressure.

Phytochemical Analysis

All of the extracts were subjected to routine phytochemical analyses. Coumarin, quinones, flavonoids, tannins (iron chloride), saponin, and terpenoids (Liebermann-Burchard reaction), reducing substances (Dragendorff and Mayer reagent), alkaloids (Fehling reagent), and coumarin, quinones, flavonoids. [16,17].

1. **Invitro Antioxidant activity**

By deciding on the following actions, HEMO's anti-oxidant activity was assessed. 1, 1-Diphenyl-2-pycrylhydrazyl (DPPH) free radical scavenger activity, Superoxide anion radical scavenger activity, and nitric oxide radical inhibitor assay.

a. **1,1-Diphenyl-2-pycrylhydrazy l(DPPH) free radical scavenging activity**[18]
b. 500 l of the methanolic DPPH solution (0.2 mM) was added to the same volume of ethanolic extract at various concentrations (5–40 g/ml) or to 500 l of methanol, which served as the control. At 517 nm, the absorbance was measured after 30 minutes.At various quantities, ascorbic acid and rutin were used as positive controls. DPPH free radical scavenging activity was calculated using the following formula.

DPPH scavenging effect (%) = $[(A_x - A_y)/ A_x] \times 100$

A_x =Absorbance of the control reaction

A_y = Absorbance in the presence of extracts or standards.

The amount of the samples needed to inhibit 50% of the radical (IC_{50}) had been then calculated

c. **Superoxide anion radical scavenging activity**[19]

Different amounts of ethanolic extract (25-200 g per ml) or 150 l of water (as the control) were added to a solution mixture containing 150 l of NADH (156 mM) and 150 l of NBT (630 mM) in 400 l of 0.1 M phosphate buffer pH of 7.4. As a reference solution, NBT (630 M) of 150 l in 700 l of 0.1 mole phosphate buffer (pH 7.4) was utilised. After adding 150 l of PMS (30 M) to all of the combinations to start the reaction, the absorbance at 560 nm was measured after 5 minutes. This process involves dissolving oxygen to create superoxide anions, which then diminish NBT. Antioxidants prevent the synthesis of blue NBT, as shown by the decline in 560 nm absorbance.The superoxide anion ***radical scavenging activity*** was calculated as percentage using the following formula

Inhibition (%) = $[(A_x - A_y)/ A_x] \times 100$

A_x =Absorbance of the control reaction

A_y =Absorbance in the presence of extracts or standards.

For each extract IC_{50} values ± S.E.M (IC_{50} value: Sample concentration needed to reduce 50 percent of radical) had been determined.

d. **Nitric oxide radical inhibition assay**

At normal physiological pH, an aqueous solution containing sodium nitroprusside will generate nitric oxide. Nitric oxide interacts with oxygen to create nitrite ions, and this can be

predicted by the usage of Griess Illosvoy reaction. Nitric oxide scavengers compete with oxygen and decreases the output of nitric oxide.

Sodium nitroprusside (10mM) of 2ml was added to 0.5ml of phosphate buffer saline. This mixture was then added to 0.5ml of extracts or 0.5 ml of standard solution and at 25^0C it was incubated for 150min. Then reaction mixture of 0.5ml had been pipette away and blended with 1ml of sulphanilic acid reagent (0.33 percent in 20 percent of glacial acetic acid) and kept apart 5min for diazotization reaction.

N-(1-*Naphthyl*) *ethylenediamine dihydrochloride*(1%) of 1ml was mixed to it and allowed to stand for 30min.The resultant index was formation of a pink colour chromophore in incandescent light. Finally, all the solutions were measured for absorbance at 540 nm. Gallic acid and rutin were taken as standard reference. The IC_{50} value has been measured for all the solutions.

2. In vitro Anti- diabetic studies

2.1. Inhibition of carbohydrate digesting enzymes

a. α-glucosidase inhibitory assay

This assay had been carried off to analyze the in vitro inhibitory effect that is inhibitory of on α-glucosidase like sucrase and maltase. The outcomes did maybe perhaps not constantly concur with those acquired in animals although α-glucosidase separated from yeast is extensively utilized as an assessment material for α-glucosidase inhibitors. Consequently, we used a small intestine homogenate of a rat as α-glucosidase solution so it would better imitate the in vivo state because we guess.

The inhibitory effect has been calculated by fairly altering the technique employed by "Dahlqvist" [10]. After 20 hours of fasting, part of the animal small intestine below the duodenum and instantly above the caecum had been cut, rinsed with ice-cold saline, and homogenized with 12 ml of maleate buffer (100 milli moles, pH 6). The homogenate has been used as α-glucosidase solution. The assay mixture contains 100 mM maleate buffer of pH 6, 2% (w/v) of each glucose substrate solution (100 µl), and the sample extract (50-500 µg/ml). The mixture was pre-incubated for 5 minutes at 37^0C, and the reaction ended up being initiated with the addition of α-glucosidase (50 µl) solution, accompanied by incubating the mixture once more for 10 minutes at 37^0C. The amount of glucose released in this effect was determined by a commercially available glucose estimation kit (Span Diagnostic Ltd., Mumbai, India). The carbohydrate decomposition rate was determined as a percentage ratio to the quantity of glucose accomplished by the completion of digestion. The inhibition rate was calculated by the formula that is following

Inhibition rate (%) = [{(amount of glucose produced by the control that is positive - (amount of glucose produced by the addition of HEMO) - (glucose production value in blank)/ (amount of glucose produced by the positive control)}] x 100.

α-amylase inhibitory assay

Both test samples and nojirimycin (20-120 g/ml) were added to 500 l of 0.02 moles of sodium phosphate buffer, pH 6.9, 0.006 M sodium chloride, and 0.5 g/ml porcine pancreatic -amylase solution. The mixture was then incubated at 25 °C for 10 minutes. Following the pre-incubation, 500 l of a 1% starch solution in a pH 6.9 buffer solution of sodium phosphate and 0.006 M salt chloride were systematically added to each tube. The reaction mixtures were then incubated for a further ten minutes at 25 °C. By adding 1 ml of 3, 5-dinitrosalicylic acid colour reagent, the response was stopped. The test tubes were then heated for five minutes in a boiling water bath and cooled to room temperature. The reaction mixture was then diluted by the addition of 10 ml of distilled water and the absorbance was measured at 540 nm[18].

$$\% \text{ inhibition} = A_C - A_E / A_C$$

A_C : Absorbance of control at 540 nm

A_E : Absorbance of extract at 540 nm

b. Glucose uptake

After 28 days of therapy, the rats were beheaded and their hemidiaphragms were separated from a few of them. Hemidiaphragms were placed in culture tubes with 2 ml of a tyrode solution containing 2 g of glucose and incubated for 30 min at 37 °C in a 95% oxygen, 5% carbon dioxide environment while being shaken. Similar studies had been conducted in six other models, where

Group-I - diabetic control
Group-II - reference standard insulin (0.25 IU/ml)
Group-III - HEMO (200 mg/ml)
Group-IV - HEMO (400 mg/ml)
Group-V - Insulin (0.25 IU/ml) + HEMO (200mg/ml)
Group-VI - insulin (0.25 IU/ml) + HEMO (400 mg/ml) respectively.

After incubation, the hemi diaphragms had been removed and weighed. The incubated medium's glucose content had been calculated. The difference between the starting and end glucose contents of the incubation medium had been identified as glucose absorption.

Statistical Analysis:

The IC 50 values were computed using the Sigma Plot 10.0 programme (Systat Software Inc., San Jose, IL, USA) using plots of log inhibitor concentration against percentage inhibition curves.For n = 3, values were presented as mean standard error (SE).

3. Results

Extraction of plant material

The powdered whole plant material was subjected to soxhlet extraction using solvent ethanol. The color, consistency and yield of ethanolic extract is depicted in **Table 1**.

Table 1. Color, consistency and yield of the extract

Extract	Color	Consistency	%Yield (W/W)
Hydroalcoholic extract	Greenish brown	Sticky	42

Preliminary phytochemical screening

The data corresponding to **Table 2** describes the preliminary phytochemical investigation report of HEMO. Phenols, flavonoids. saponins, phytosterols, steroids and terpenoids are present in HEMO.

Table No. 2: Phyto-chemical screening of leaves of Hydroalcoholic extract of *Moringa oleifera*

S No.	Constituent	Hydroalcoholic extract
1	Alkaloids	-ve
2	Glycosides	-ve
3	Saponin glycosides	-ve
4	Flavonoids	+ve
5	Tannins	+ve
6	Steroids	+ve
7	Terpenoids	+ve
9	Phenols	+ve
10	Proteins	+ve
11	Carbohydrates	-ve

+ve sign indicates presence; - ve sign indicates absence;

In vitro antioxidant activity

1-diphenyl-2-picryl hydrazyl [DPPH], superoxide [O_2•]), Superoxide anion radical scavenging activity and Nitric oxide radical inhibition assay.

In vitro tests were done to determine HEMO's capacity to scavenge DPPH, superoxide, and nitric oxide. In **Table 3**, the samples' respective IC50 values are listed. DPPH levels were dramatically lowered with HEMO. As evidenced by their low IC50 values, superoxide and nitric oxide scavenging activity for HEMO was also discovered to be high.

Table 3: Invitro antioxidant activity of HEMO

Test material	IC_{50} (µg/ml) ± SEM[a]		
	DPPH	Super oxide	Nitric oxide
HEMO	8.97±0.73	29.23±3.19	149.28±2.16
Ascorbic acid	3.45±0.16	-	-
Rutin	4.03±0.74	-	83.41±3.07

[a] Average of 3 determinations.

Invitro studies on HEMO for its antidiabetic activity

α- glucosidase and α-amylase inhibitory activities.

The IC50 value of HEMO's sucrase inhibitory activity was 248.26±0.31 µg/ml. The results in **Table 4, Figure 1** showed that HEMO exhibited potent activity in an almost dose-dependent manner and is therefore considered to be a potent α-glucosidase inhibitor. Moreover, HEMO inhibited the activity of α-amylase with a His IC50 value of 71.35±0.52 µg/ml. Results are shown in **Table 5** and FIG. In this study, HEMO showed potent inhibitory activity against α-glucosidase similar to standard. H. Acarbose. However, unlike acarbose and nojirimycin, HEMO exhibited moderate α-glucosidase and α-amylase inhibitory activity. These results confirm that HEMO has α-glucosidase and α-amylase inhibitory properties.

Table 4: α-glucosidase (sucrase) inhibitory activity of HEMO

Concentration (µg/ml)	Percentage inhibition (%) of sucrase by		IC $_{50}$ (µg/ml)	
	HEMO	Acarbose	HEMO	Acarbose
50	10.32 ± 0.06	18.97 ± 0.59	248.26 ± 0.31	141.02± 0.06
100	15.53 ± 0.69	36.42 ± 0.26		
150	32.05 ± 0.48	53.52 ± 0.35		
200	35.74 ± 0.57	72.94 ± 0.10		
250	49.12 ± 0.43	84.75 ± 0.04		
300	60.02 ± 0.20	94.83 ± 0.05		

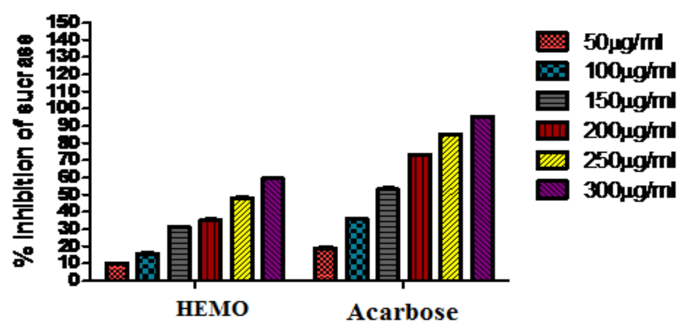

Fig 1. α-glucosidase (sucrase) inhibitory activity of HEMO

Table 5: α-amylase inhibitory activity of HEMO

Concentration (µg/ml)	Percentage inhibition (%) of α-amylase by		IC$_{50}$ (µg/ml)	
	HEMO	Nojirimycin	HEMO	Nojirimycin
20	12.37 ± 0.75	22.71 ± 0.12	71.35 ± 0.52	43.95 ± 0.67
40	31.04 ± 0.42	46.02 ± 0.40		
60	41.63 ± 0.71	61.13 ± 0.58		
80	62.94 ± 1.12	75.62 ± 0.65		
100	70.02 ± 0.10	86.59 ± 0.92		
120	87.11 ± 1.07	98.01 ± 0.73		

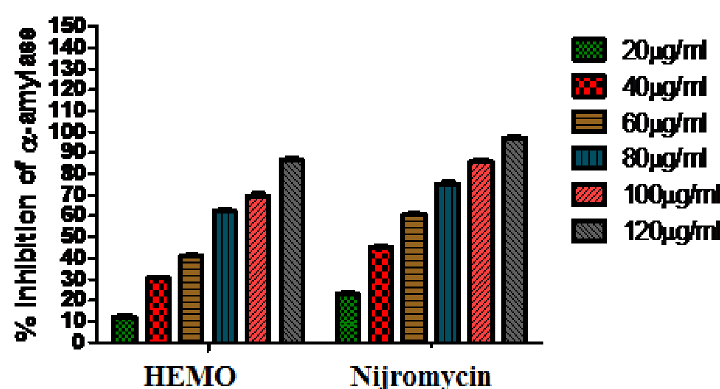

Fig 2. α-amylase inhibitory activity of HEMO

Glucose uptake

The data in **Table 6** and **Fig 3** illustrates the study outcome of glucose uptake by isolated rat hemi diaphragm. It reveals that HEMO at 200 mg/ml and 400 mg/ml concentration exhibited the glucose uptake of 5.41±0.52 and 6.84±0.12 milligram/gram/30 minutes with statistical significance of $p<0.05$ and $p<0.001$ correspondingly, by the isolated rat hemi diaphragm, when compared with diseased group. Simultaneously, the group treated with only insulin, showed 6.29±0.91 mg/gm/30min of the glucose uptake with a statistical significance of $p<0.001$. But, insulin and HEMO (200 mg/ml and 400mg/ml) combination respond to significant ($p<0.001$) increase of 7.19±0.42 and 8.53±0.75 mg/g uptake of glucose at the same instant in comparison to that of diseased group

Table 6: Effect of HEMO on glucose uptake

Groups & Treatment	**Glucose uptake (mg/gm/30 min)**
I. Tyrode solution in glucose(2g%) +Diabetic control	4.13±0.29
II. Tyrode solution in glucose(2g%) +Insulin (0.25 IU/ml)	6.29±0.91***
III. Tyrode solution in glucose (2g%) + HEMO (200mg/ml)	5.41±0.52*
IV. Tyrode solution in glucose(2g%) + HEMO (400mg/ml)	6.84±0.12***
V. Tyrode solution in glucose(2g%) +Insulin (0.25 IU/ml) + HEMO (200mg/ml)	7.19±0.42***
VI. Tyrode solution in glucose(2g%) +Insulin (0.25 IU/ml) + HEMO (400mg/ml)	8.53±0.75***

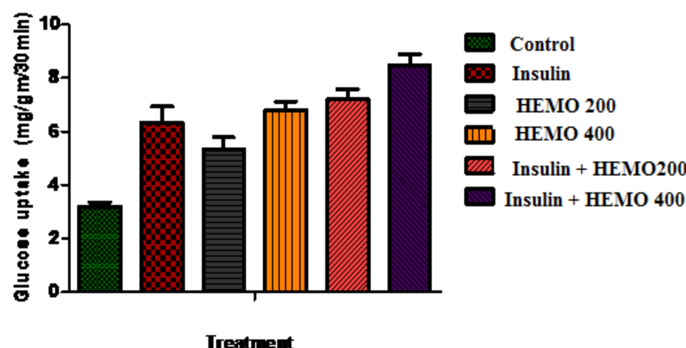

Fig 3. Effect of HEMO on glucose uptake

4. Discussion

Diabetes mellitus (DM) is a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism. As the disease progresses tissue or vascular damage ensues leading to severe diabetic complications such as retinopathy, neuropathy, nephropathy, cardiovascular complications and ulceration. Thus, diabetes covers a wide range of heterogeneous diseases.

Currently available drug regimens for management of diabetes have certain drawbacks and therefore, there is a need for safer and more effective anti-diabetic drugs. The plant kingdom is a wide field to search for natural and effective oral hypoglycemic agents that have no side effects. The increase in number of diabetic patients has motivated the scientists to find new methods to cure diabetes. In spite of the presence of known antidiabetic medicine in the pharmaceutical market, remedies from medicinal plants are used with success to treat this disease.

Moringa oleifera was a plant enriched with antioxidant constituents it may be used for relieving free radical induced pathogenesis. As there is no much pharmacological activity work recorded on this medicinally potent plant, the present work was undertaken to put down the standards which could be useful for establishing its authenticity.

Hence the present study was aimed to evaluate antidiabetic activity of HEMO by preliminary Phyto-chemical screening, preliminary assessment of the anti-diabetic activity and antioxidant activities in *invitro* model. Literature survey states that presence of alkaloids,flavonoids,terpenes,steroids,polysaccharides,phenols,coumarins and proteins in the plant extract contribute to pharmacological activities such as antidiabetic, hypoglycemic, antihyperlipidemic and antioxidant properties.

The information obtained from the preliminary Phyto-chemical screening will reveal the useful findings about the chemical nature of the drug. Preliminary Phyto-chemical screenings ascertain the presence of Glycosides, Flavonoids, Triterpenoids, Steroids, Phenols and Coumarin etc., in whole plant extract. Hence, keeping all this in view, research work was focused on the above-mentioned constituents in ethanolic extract, for the invitro evaluation of antidiabetic and antioxidant potential. Invitro studies using DPPH method, superoxide radical and nitric oxide inhibition assays showed strong antioxidant nature of the ethanolic extract. The IC_{50} values were found to be equal to that of standards ascorbic acid and rutin. The results clearly indicated that HEMO was found to be more effective in scavenging the DPPH free radical when compared to the superoxide radical and nitric radical, since IC_{50} values obtained were found to be low in DPPH method.

Based on the in vitro studies, it was assumed that HEMO provides better protection against oxidative damage. Free radical scavengers like enzymes such as SOD and CAT system protect biological system against the harmful impacts of activated species.

Certainly, one of the therapies approaches for diabetes is to decrease the hyperglycemia that is post-prandial delaying the utilization of glucose through the inhibition of carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase. α-glucosidases are enzymes that increase the absorption of digested glucose from nutritional polysaccharides in the small

intestine. The α -glucosidase inhibition of HEMO was indeed examined by determining the α-glucosidase inhibitory activity by the use of 4-Nitrophenyl-β-D-glucopyranosiduronic acid (pNPG) as the response substrate. But, it is confusing perhaps the mode of inhibition of a-amylase and α-glucosidase by HEMO is due to competitive and noncompetitive techniques. The theory that α-amylase and α- glucosidase showed different inhibition that is unquestionable because of structural variations pertaining to the origins of the enzymes. The inhibition rate for α-glucosidase ended up being close to that of acarbose. This suggested that HEMO had been an effective inhibitor of α-glucosidase.

5. Conclusion

The present work was undertaken with a positive approach to put down standards which could be useful to detect the potency and authenticity of *Moringa oleifera* for their invitro antioxidant and antidiabetic activity.

Moringa oleifera claims to treat various disease ailments like neurological disorders and inflammation etc. In Indian indigenous system of medicine was as anti-diabetic, however the scientific studies have not been done. Therefore, the invitro anti diabetic activity of Hydroalcoholic extract of *Moringa oleifera* (HEMO) was evaluated.

Obesity and the onset of diabetes are two closely liked medical complications prevalent globally. Postprandial hyperglycemia is one of the earliest abnormalities of glucose homeostasis associated with type 2 diabetes. Postprandial glucose levels can be regulated through α-glucosidase inhibition. Medicinal plants constitute an important source of potential therapeutic agents for Type 2 Diabetes Mellitus. One vital therapeutic approach is the use of agents that can decrease postprandial hyperglycemia by inhibiting carbohydrate digesting enzymes resulting in a delay of carbohydrate digestion to absorbable monosaccharide. In this preliminary work, we attended to evaluate the α-amylase and α-glucosidase inhibitory activities of some extracts of *Moringa oleifera* to clarify its traditional use as antidiabetic treatment. Obtained results of the both enzyme inhibition activity, found in a dose-dependent manner constitute the first report for this plant. Further, in vitro and in vivo researches are required to confirm the present results, isolate and determine active substances and phenolic components contained in the extract of this plant that may be responsible for improvements in health conditions by regulating digestives enzymes inhibitory activities. In vivo studies are necessary to recognize a potential substance for clinical use in the therapy of diabetes and related disorders, so it is desirable to optimize secondary metabolite production and purification of compounds for the pharmaceutical applications.

Furthermore, other studies in vitro and in vivo are needed to confirm these findings and characterize and determine bioactive components responsible of this effect. The present study confirms the traditional use of *Moringa oleifera* to treat diabetes mellitus.

Hence, *Moringa oleifera* may have great potential as an alternative to the therapeutic agents currently available for treatment of diabetes.

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Conflict of Interest:

The authors declare that there is no conflict of interest.

Abbreviations:

HEMO: Hydro alcoholic extract of *Moringa oleifera*; **IC50:** Half Maximal Inhibitory Concentration; **µg:** Microgram; **DPPH**: 1, 1-diphenyl-2-picrylhydrazyl.

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