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RESEARCH ARTICLE

Phytochemical Screening, Antioxidant, Antibacterial, and α-Amylase Inhibitory Activity of *Moringa oleifera* Lam. Leaves

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Abstract

Moringa oleifera is a perennial deciduous plant abundant in tropical countries that contains many important bioactive compounds. This study aimed to evaluate phytochemical analysis, antioxidant, antibacterial, and α-amylase inhibitory activities of the methanol and hexane leaf extracts of the plant collected from Nepal. Phytochemical screening of the extracts revealed the presence of a wide spectrum of secondary metabolites such as alkaloids, flavonoids, phenolics, saponins, tannins, terpenoids, etc. Methanol and hexane extracts showed the presence of significant quantities of total phenolics (207.75± 2.75 mg GAE/g, and 137.09 ± 1.1 mg GAE/g) and total flavonoids (94.56 ± 1.88 mg QE/g, and 82.71 ± 1.47 mg QE/g) respectively. The methanol extract exhibited higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The half-maximal concentration causing 50% inhibition of the radical (IC_{50}) of methanol and hexane extracts were 39.19 ± 0.33 and 61.07 ± 1.46 µg/mL respectively which are comparable to that of standard ascorbic acid (28.90 ± 0.24 µg/mL. Methanol extract of *M. oleifera*leavesshowed moderate antibacterial activity against *Staphylococcus aureus* (ATCC25923), *Bacillus cereus,* and *Klebsiella pneumoniae*(ATCC700603). *In-vitro* antidiabetic activity was performed by a starch-iodine method using *α*-amylase enzyme and methanol extract showed significant antidiabetic activity (IC_{50} value 31.78 ± 0.52 µg/mL). The results of this study corroborate the potential application of the plant in traditional medicine and the drug discovery process.

Keywords: Antimicrobial, Antioxidant, α-amylase Inhibition,phytochemical, *Moringaoleifera*

Introduction

Diabetes is a predominant endocrine disorder that arises when the blood glucose level is too high. It cannot be completely cured but it should be kept under tight control. Diabetes mellitus (DM) is one of the most ordinary worldwide diseases. The total number of diabetic patients worldwide has been estimated to increase from 171 million in 2000 to 366 million in 2030[1]. The oxidation reaction is crucial for life but produces free radicals, that lead to oxidative stress and are responsible for the majority of the disease, and impaired antioxidant defense systems progression of DM and its complications[2]. Currently used synthetic drugs cause many side effects therefore, there is expanding interest in the prospective use of medicinal plants as an unconventional treatment for diabetes and even other diseases, as these are commonly cheaper, less toxic, and with fewer side effects[3].

Moringa oleifera (MO) **(Figure 1)** is commonly called drumstick or Horse-radish, not surprisingly; *M. oleifera* is sometimes described as "Mother's Best Friend", "Miracle Tree", "Tree of Life" and "God's Gift to Man"[4]. It is used in traditional medicines due to its various pharmacological properties. It is a perennial, nutritional, flowering plant that is widely cultivated in India,

(a) (b) (c)

Figure 1: *Moringaoleifera plant (a) flowers (b) pods (c) sees*

Pakistan,Africa,and western Asia including Nepal[5]. *Moringa oleifera* belongs to the Magnoliopsida class, Moringaceae family, Moringa genus, and oleifera species[6]. *M. oleifera* grows up to 12 meters and is cultivated in many tropical and subtropical countries of Asia and Africa[7].

Moringa leaves are rich in proteins, calcium, iron, potassium, vitamins (particularly B and C), *β*- carotene. All parts of *Moringa oleifera* are medicinally valuable with overlapping uses in treating myriads of ailments and diseases including body pains and weakness, fever, asthma, cough, blood pressure, arthritis, diabetes, epilepsy, wound, and skin infection. These phytochemicals have been reported to possess antioxidant, hypoglycemic, hypotensive, antidyslipidemic, anticancer, and anti-inflammatory properties[8]. MO plant parts are a good source of phytochemicals such as glucosinolates, flavonoids, phenolic acids, highly bioavailable vitamins, minerals, carotenoids tocopherols, polyunsaturated fatty acids, and folate.In particular, MO plant leaves contain Glycoside niazirin, niazirinin.The Mature MO flowers contain phytochemicals such as *D*-mannose, protein, *D*-glucose, ascorbic acid, and polysaccharides. The MO plant pods contain nitriles, isothiocyanate, thiocarbamates, 0- [2'-hydroxy-3'-(2''-heptenyloxy)] propyl decanoate, and *O*-ethyl-4-[(*α* -1-rhamnosyloxy)- benzyl] carbamate, methyl-p-hydroxybenzoate and *β*-sitosterol. The mature MO seed contains protein, fat, carbohydrate, methionine, cysteine, mono-palmitic, 4-(*α*-L-rhamnopyranosyloxy) - benzylglucosinolate, benzylglucosinolate, moringyne, and di-oleic triglyceride. The seed oil contains essential vitamin A and beta carotene[9], [10]. Moreover, *M. oleifera* leaves have demonstrated better antioxidant, anti-free radicals, and inhibition activities of lipid peroxidation and protein oxidation when compared with standard *α* – tocopherols[7].

The key constituents reported were inositol derivatives, namely the most significant bioactive compounds of plants are alkaloids, flavonoids, tannins, and phenolic compounds[4]. *Moringa* leaves have been investigated as a valuable source of dietary proteins and essential amino acids thereforethe use of the plant as an ingredient in livestock and human nutrition has been encouragedover the last several years[11]**.**

Materials and Methods

Collection of plant material

About 2 kg of fresh leaves of *Moringa oleifera* were selectively collected from the Gulmi district of Nepal. The botanical identity of the plant was taken from the National Herbarium and Plant Laboratories, Godawari. The collected sample was washed with distilled water to remove any traces of dust. The sample was shade dried, and powdered in an electric grinder.

Chemicals

The alpha-amylase enzymewas purchased from Sigma- Aldrich, Mo, USA.chemicals Quercetin, gallic acid, aluminum chloride, ascorbic acid, etc. of analytical grade were provided by the department. Distilled water was used during the entire lab work.

Extraction

The powdered plant materials were extracted successively with methanol and hexane by the cold percolation method. Each plant (~500 g) material was immersed in the respective solvents (500 mL) for 24 hours and the extract was filtered and another fresh solvent was added. The process was repeated 5-6 times until the last filtrate was nearly colorless. The extracts were concentrated by using a rotary evaporatorat reduced pressure to getthe semisolid extract.

Phytochemical analysis

Leaves were dried, powdered, and phytochemicals of leaves were extracted successively with methanol and hexane. Extracts were filtered, dried in Rota-evaporator, and evaluated for the presence of alkaloids, flavonoids, polyphenols, terpenoids, glycosides, and reducing sugar in the different extracts (plant material) were analyzed by reacting with different reagents[12].

Estimation of total phenolic content (TPC)

The total phenolic content in the plant extracts was estimated by using the Folin- Ciocalteu'sreagent (FCR) method by using a standard gallic acid with slight modifications [13], [14].[12]. Briefly, 1 mL of gallic acid of different concentrations or the plant extract (1mg/mL) were added into 5 mL of FCR (10%) and 1 mL of 7% Na_2CO_3 separately. Then, the blue-colored mixture was shaken well and incubated for 30 minutes at 40 ˚ C in the water bath. Finally, the absorbance of these solutions was measured at 760 nm wavelength using UV- spectroscopy against blank.The TPC was calculated from the calibration curve and expressed as mg GAE/g of the dry material.

Estimation of total flavonoid content (TFC)

The total flavonoid content in plant extract was determined using an Aluminumchloride colorimetric assay[15].

Quercetin stock solution 1 mg/mL) was prepared and used to prepare the solutions of various concentrations (100 ppm, 80 ppm, 60 ppm, 40 ppm, 20 ppm, and 10 ppm) were d by serial dilution of the stock solution. First of all, 1 mL of quercetin or the extract (1 mg/mL) in methanol was taken in a test tube containing 4 mL of distilled water. At zero time, 0.3 mL of 5% $NaNO_2$was added to the test tube. After 5 minutes, 0.3 ml of 10% of $AlCl_3$ and after 6 minutes, 2 mL of 1 M NaOH were added to the mixture. Immediately the total volume of the mixture was made up to 10 ml by adding 2.4 mL distilled water and incubated for 30 minutes in the dark. The absorbance of the mixture was measured at 415 nm using a UV-spectrophotometer against the blank solution. The average absorbance values obtained for different concentrations of quercetin were used to plot the calibration curve. The TFC values were calculated from the plot and expressed as mg QE/g of dry material.

Antioxidant assay

Antioxidant activity was determined by DPPH free radical scavenging activity performed[16], [17].The test solutions of ascorbic acid and the extracts of 100, 80, 60, 40, 20, and 10 ppm were prepared from the stock solutions by serial dilution. Each of the test solutions and DPPH (0.2 mM) are added into the test tubes in the ratio of 1:1 by volume and incubated for 30 minutes in dark. Then, the absorbance value was measured at 517 nm by using a UV–Vis spectrophotometer against blank.The capability to scavenge the DPPH radical was calculated by:

$$\text{DPPH scavenged (\%)} = (Ac\text{-}As/Ac) \times 100$$

Where,

Ac = Absorbance of the control

As = absorbance of the sample

α-Amylase inhibition activity

Anti-diabetic activity of the plant was determined by α-amylase inhibition assay using starch iodine method using acarbose as a standard [18]. Briefly, 1 g/mL of the acarbose and the plant extract stock solutions were prepared in dimethyl sulphoxide (DMSO). The stock solutions were used to prepare the test solutions of 100, 80, 40, 20, and 10 ppm by serial dilution. Aliquots of 40 µL of starch were pre-incubated at 40 ˚ C for 3 minutes with 20 µL of acarbose or the plant extract of each concentration and followed by adding 20 µL of 3U/ml α-amylase which

was dissolved in phosphate buffer and incubated at 37 °C for 15 minutes in the dark. Termination of reaction was carried out by adding 80 µL of HCl (0.1M) per incubated mixture having amylase. Then 100 µl of iodine reagent (2.5M) was added to each solution and the final volume was made up to 5 mL with distilled water. Absorbance was measured at 630 nm.

The percentage of inhibition was calculated by using this formula:

Percentage of inhibition =

$$1 - [Ab2 - Ab1 / Ab4 - Ab3] \times 100$$

Where,

Ab1 = absorbance of the incubated mixture of plant sample, starch, and amylase

Ab2 = absorbance of the incubated mixture of sample and starch

Ab3 = absorbance of the incubated mixture of starch and amylase

Ab4 = absorbance of the incubated solution containing starch only

Antimicrobial assay

The antimicrobial properties of the extracts were determined using the agarwell diffusion method [19], [20]. The pure cultures of bacterial strains *Staphylococcus aureus* (ATCC25923), *Bacillus cereus* (ATCC10987) *Klebsiella pneumoniae* (700603), and *Escherichia coli (*ATCC 25922*)* were swabbed onto the Muller Hinton agar in Petri dishes using sterile cotton swabs.Methanol extracts of MO leaf100mg was dissolved in 50 % DMSO and diluted to 100, 50, and 10 mg/mL. The sterile Muller- Hinton agar plates were swabbed carefully all over the surface with the bacterial suspension of 0.5 McFarland's standard ($1.5x10^8$ CFU/mL) and wells of 6 mm diameter were punched with a sterile cork-borer. Then, 10 µL/well of the test solution of the plant extract was loaded into the wells with the help by a micropipette. Chloramphenicol was used as a standard antibiotic in the separate well. The plates were left for an hour with a cover so that the extract diffuse into the mediaandincubated overnight. On the next day, the plates were observed for the zone of inhibition and recorded.

Statistical analysis

The tests were performed in triplicates and the results were expressed as the mean ± standard deviation. The preliminary data of antioxidant and α-amylase inhibition tests were processed using Microsoft Excel. The concentrations inhibiting 50% of the radical were determined by GraphPadPrism9 software.

Results and Discussion

Extraction yield

The extractive values of methanol and hexane solvents were found to be 14 %, and 5% respectively. Methanol is polar to hexane and can extract more secondary metabolites than that hexane.

Phytochemical test

The extracts were evaluated for the presence of 13 phytochemicals by using standard reagents. Methanol extract showed the presence of 12 and hexane showed the presence of 6 phytochemicals. Methanol extract showed the absence of protein only whereas hexane extract indicated the presence of alkaloids, flavonoids, terpenoids, carbohydrates, coumarins, and fatty acids only (Table 1).

Table 1: *Phytochemical screening of the hexane and methanol extract of Moringa oleiferaleaves*

Extract	Test name	Methanol	Hexane
Alkaloid	Wagner's/ Hager's Test	+	+
Flavonoids	Alkaline reagent /Zinc HCL reduction test	+	+
Terpenoids	Copper acetate – diterpenoids	+	+
Polyphenol	Ferric chloride test	+	-
Saponine	Foam test	+	-
Tannine	5% $Fecl_3$/ Gelatin test	+	-
Reducing sugar	Benedicts test	+	-
Carbohydrate	Fehling test	+	+
Glycoside	Molischs test	+	-
Ascorbic acid	Iodine test	+	
Coumarin	Ester test (ferric hydroxamate test)	+	+
Fatty acid	Ninhydrin test	+	+
Protein	Xanthoproteic test	-	-

Note: (++) = abundant, (+) = trace presence, and (-) = absent

Abdulkadiretal.,(2015)reportedthatthephytochemical analysis of different fractions on *Moringa oleifera* leaves showed the presence of tannins, saponins, phenols, alkaloids, and phlobatannins as the major secondary metabolites present in the plant's leaf but saponins wereabsent inthe hexane extractin our study. The phytochemical analysis illustrated that ethanol leaves extract of MO plant had tannins, alkaloids, phytosterols, triterpenoids, flavonoids, saponins, cardiac glycosides, anthraquinones, carbohydrates, fiber, magnesium, vitamin, calcium potassium, amino acids fats, etc. having selective solubility in selected solvents of varying polarities used in succession,therefore, *M. oleifera* is considered to be a good source of nutrients that are necessary for growth and development [22]. But in the present work methanol extract showed alkaloid, polyphenol, flavonoids, terpenoids, carbohydrates, and fatty acids in the case of hexane extract polyphenol, saponins, tannins, reducing sugar and quinine were absent. Protein was absent in both extracts. Many works of literature clearly state that MO is rich in protein but here protein was not detected. The secondary metabolites like alkaloids, flavonoids, phenolics, tannins, steroids, etc. which are abundant in fruits, vegetables, nuts, seeds, leaves, etc. contribute to the protection of plants from disease, and extreme environmental factors, predators, color and flavor. These compounds have significant roles in humans and animals when taken as dietary supplements [23]. The collection of sample, season, maturity of the plant, solvents, extraction method, etc. greatly influence the quality and quantity of the phytoconstituents and no ideal method is adequate for the complete extraction of the phytochemicals [24].

TPC, TFC, and antioxidant activity

The results of TPC and TFC present in methanol and hexane extracts of *M. oleifera* leaf extracts are presented in (Table2). The phenolic content of methanol extract (207.75 ± 2.75 mg GAE/g) is higher than that of hexane extract (137.09 ± 1.1mg GAE/g). Similarly, flavonoids also revealed the same trend with (94.56 ±1.88 mg QE/g) in methanol extract and (82.71 ±1.47 mg QE/g) in hexane extract. Different extracts of *M. oleifera* collected from Southern India were reported to contain the TPC and TFC in the order as EtOAc> Methanol > Water > ethanol > acetone which is comparable to our reports [25].

Table: 2 *List of total phenolic, total flavonoids, and antioxidant activity*

Sample	TPC (mg GAE/g)	TFC(mg QE/g)	IC_{50} (µg/mL)
Ascorbic acid	-	-	28.9 ± 0.24
Methanol extract	207.75 ± 2.75	94.56 ±1.88	39.19 ± 0.33
Hexane extract	137.09 ± 1.1	82.71 ±1.47	61.07± 0.61

Antioxidant activity of leaves extract was expressed in terms of percentage radical scavenging. The half-maximum inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biologicalprocess (or component of a process, i.e. an enzyme, cell, cell receptor, or micro-organism) by half. DPPH radical scavenging activity of leaves of *M. oleifera* was compared with standard ascorbic acid. The dependency of the inhibiting capacity with the concentration of the extracts was shown in a plot of concentration versus % scavenging (figure 2). Both of the extracts showed a linear variation with a concentration comparable to that of ascorbic acid.

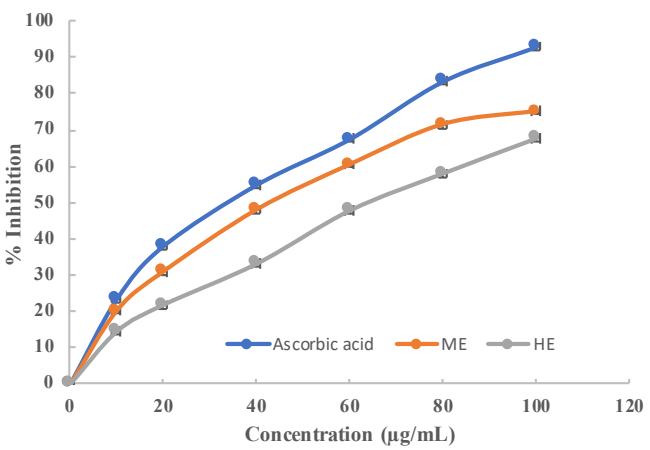

Figure 2:*DPPH radical scavenging activity of extracts and ascorbic acid*

Note: Values are the means of triplicate data with error bars of SD

ME= methanol extract, HE= hexane extract

From the above result, it is revealed that the methanol extract of *M. oleifera*was found to be powerful antioxidant (IC_{50} =39.19 ± 0.33µg/mL) than the hexane extract (IC_{50} = 61.07± 0.61µg/mL). The relatively lower IC_{50} value of 28.9 ± 0.24 µg/mL) indicates the higher antioxidant capacity of ascorbic acid(table3).The antioxidant capacities of the methanol and hexane extracts and standard ascorbic acid are shown in the bar diagram (Figure 3). The antioxidant activity of the extracts is highly correlated with the corresponding TPC and TFC values. Extract with high TPC and TFC showed lower IC_{50} values and vice-versa.

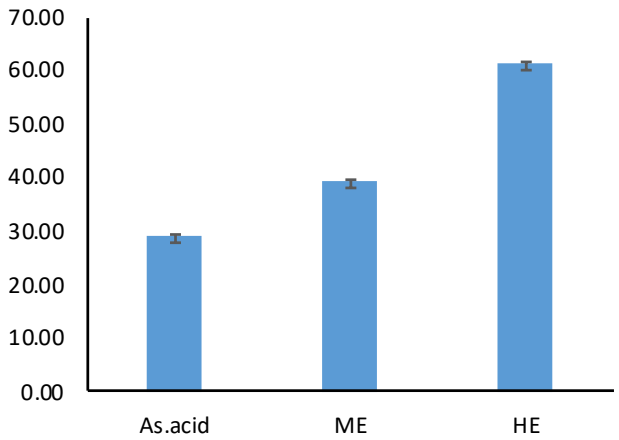

Figure 3: *IC_{50} values for DPPH free radical scavenging of plant extracts along with ascorbic acid (ME= methanol extract, HE= hexane extract)*

The antioxidant capacity expressed in terms of IC_{50} values calculated by linear regression analysis for *M. oleifera* was determined. The methanol showed the highest antioxidant activity (IC_{50} = 49.30 µ/mL) followed by ethyl acetate extract (IC_{50} = 444.10 µg/mL), hexane extract (IC_{50} = 715.57 µg/mL), and the lowest activity was shown by the dichloromethane extract which is analogous to our results [26]. The different extracts of *Beilschmiedia roxburghiana*Nees were evaluated for their antioxidant activity by DPPH method. They reported a higher activity of methanol extract than that of hexane due to the lower levels of TPC and TFC [27]. Similar results were obtained in different studies on the extracts of *M. oleifera* extracts [28], [29].

Antibacterial activity

The results of the disc diffusion method for the test of antibacterial susceptibility of methanol and hexane extracts of *M. oleifera* are presented in (table5). The test was carried out against four bacterial strains.

Table 3: *Result of Antimicrobial screening of Methanol leaves extract of M.oleifera*

Microorganisms	Zones of inhibition (mm)				
	The concentration of the extracts			PC	NC
	100 mg/mL	**50** mg/mL	**10** mg/mL		
S. aureus	10	8	-	37	7
K. pneumoniae	12	9	-	37	7
B. cereus	16	14	-	37	7
E. coli	-	-	-	37	7

Note: PC= positive control (Chloramphenicol), NC= negative control (50% DMSO)

The hexane extract was inert to all of the tested microorganisms but the methanol extract showed moderate antibacterial activity against the gram-positive strains(*S. aureus, B. cereus)* and gram-negative bacterial strain (*K. pneumoniae*). The extract was inactive towards*E. coli* at all of the concentrations. The above result in (table 3)showed thatthemaximum ZOI values were found formethanol extract (100 and 50 mg/ml) against *B. cereus* with 16 mm and 14 mmrespectively. The higher ZOI showed by methanol extract at different concentrations against *K. pneumoniae*are 12, and 9 mm. Similarly, the methanol extract had the ZOI against the *S. aureus* 10, and 8 mm at concentrations of 100, and 50 mg/mL respectively. The plant collected from Africa was reported to exhibit significant antibacterial activities against the *E. coli, S. aureus, Enterococcus faecalis, Pseudomonas aeruginosa,* and antifungal activities against *Candida albicans, Aspergillus fumigatus*, and *Cryptococcus neoformans*[30].The antimicrobial properties of leaf and seed extracts of *M. oleifera*of Iraqi origin were assessed by determining ZOI and minimum inhibitory concentration (MIC). The aqueous and methanol leaf extracts showed significant results against *S. aureus* at the concentration of 200 mg/mL with ZOI of 14.83 and 22.6 mm respectively. They reported MIC values of 16 mg/mL against *S. aureus* and *E. coli,*and 32 mg/

mL against *Bacillus cereus* and *K. pneumoniae* by the methanol extract [31]. The phenolic compounds in the plant extracts sensitize the phospholipid bilayer of the bacterial cell increasing membrane permeability, leaking the cellular material, impairing the enzymatic actions, and inhibiting amino acid decarboxylase in target bacteria [32]. Flavonoids present in the plant extracts exhibit good antibacterial activity which is explained by several mechanisms. These compounds inhibit nucleic acid synthesis, cytoplasmic membrane function, energy metabolism, biofilm formation, membrane permeability, etc. in bacterial cells [33].

Antidiabetic activity

The α-amylase inhibition assay was used to measure the antidiabetic activity of the extract using acarbose as a standard and by the starch-iodine method. The results of the study are presented in the form of a dose-dependent plot of concentration versus % inhibition (figure 4). The extracts show a comparable curve to acarbose where the inhibition of methanol extract is higher than that of the hexane extract.

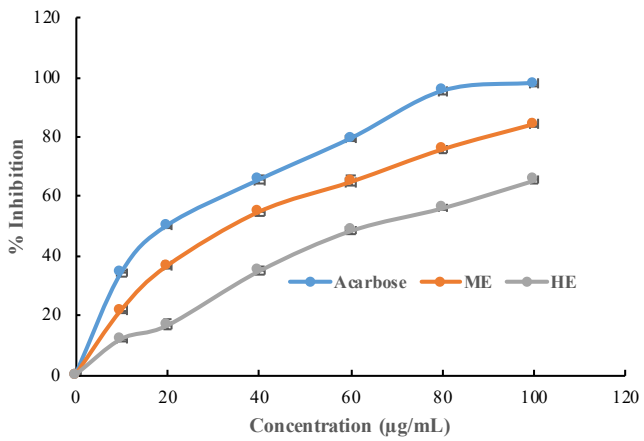

Figure4. ***α****-amylase % inhibition of the extractsof MO and Acarbose in different concentrations.*

The half-maximal inhibitory concentration (IC_{50}) value of the standard and the extracts are shown in (figure 5). A lower IC_{50} value corresponds to higher activity and vice-versa.

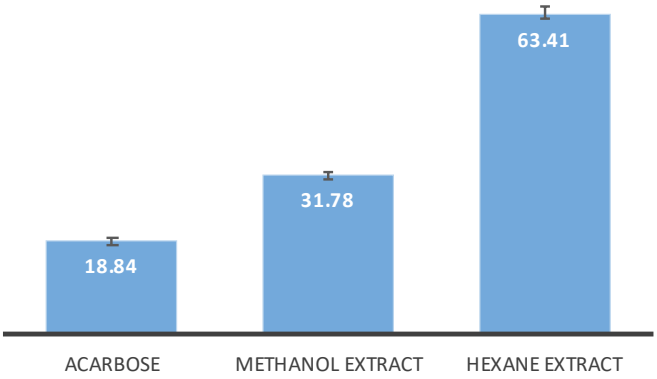

Figure5: *IC_{50}(µg/mL) values for* ***α****-amylase inhibitory activity*

Note: Values are mean ± SD, n=3

Methanol extract of the plant exhibited higher inhibitory activity (IC_{50} = 31.78 ± 0.52 µg/mL) than the hexane extract (IC_{50} = 63.78 ± 1.46 µg/mL). The standard drug acarbose used as a standard exhibited the highest activity with an IC_{50} value of 18.84 ± 0.32 µg/mL. The aqueous leaf extract of *M. Oleifera* collected from India remarkably inhibited the activity of α-amylase and α- glucosidase with maximum inhibitions of 80.5% and 75.65% inhibition was reported at the concentration of 200 µg/mL by the extract for α-amylase and α-glucosidase respectively. The IC_{50} value for α-amylase 52.5 µg/mL is analogous to our result [34]. The α-amylase inhibitory capacity of different solvent extracts of *M. oleifera* seeds was evaluated. At the concentration of 20 mg/mL, ethanol, ethyl acetate, and n-butanol extracts exhibited significant activities with inhibition rates of 61.80%, 52.09%, and 48.34% but the petroleum ether and aqueous extracts had lower inhibitions of 11.68% and 25.26% respectively [35].

Diabetes is a disease caused by the chronic metabolic disorder and escalating rapidly throughout the world. Diabetic can be treated either by administration of insulin (in type I) or inhibition of the enzyme (in type II) like α-amylase, α-glucosidase, dipeptidyl peptidase IV, protein tyrosine phosphate, etc. The quest for alternative drugs of plant origin can help to avoid the side effects of conventional therapies [36]. α-amylase is an important enzyme that plays a crucial role in the hydrolysis of starch and oligosaccharides helping to increase the circulation of glucose in the blood and help the management of hyperglycemia.

The digestion of starch is jammed by the α-amylase inhibitors by blocking the active sites of the molecules. The phenolic compounds in the plants which have significant enzyme inhibitory activity are extensively fascinated for the reduction of postprandial blood glucose [35], [37].

Conclusions

The present study reveals that *Moringa oleifera* leaves are rich in secondary metabolites. This showed substantial quantities of phenolic and flavonoid compounds. Methanol leaves extract of *M. oleifera*showed concentration-dependent inhibition activity against DPPH free radical. Similarly, plant extract exhibited significant α-amylase inhibitory activity and antimicrobial activity against the *S. aureus*, *K. pneumoniae,* and *B. cereus* on the disc diffusion method. The results of the study highlight the potential application of *M. oleifera* for the management of diabetes and microbial infections.

Acknowledgments

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Data availability

All the supportive data of this study can be obtained from the corresponding author upon realistic request.

Conflicts of interest

All the authors have read and declare no conflicts regarding the publication of the manuscript.

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