RESEARCH PAPER

BIOASSAY GUIDED FRACTIONATION OF METHANOLIC LEAF EXTRACT OF *MORUS INDICA* FOR GLUCOSE LOWERING ACTION

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Abstract

The present study investigated the antidiabetic potential of methanolic leaf extract of *Morus indica* through bioassay-guided fractionation. Phytochemical screening revealed the presence of alkaloids, saponins, cardiac glycosides, phenolics, tannins, flavonoids, terpenoids, and proteins. Quantitative analysis confirmed high levels of phenolics (145.58 mg GAE/g) and flavonoids (173.39 mg QE/g). Column chromatography yielded four pooled fractions (F1–F4), which were evaluated for glucose-lowering activity using α-amylase and α-glucosidase inhibition assays. Among these, fraction F2 exhibited the strongest inhibitory activity, achieving 59.66% α - amylase inhibition and 68.90% α-glucosidase inhibition, values approaching those of the reference drug acarbose. The dual enzyme inhibition suggests that F2 is enriched with bioactive phytoconstituents capable of modulating carbohydrate metabolism and reducing postprandial hyperglycemia. These findings highlight *Morus indica* as a promising natural source of antidiabetic agents, with fraction F2 emerging as a potential candidate for nutraceutical or phytopharmaceutical development. Further in vivo and clinical studies are warranted to validate efficacy, bioavailability, and safety.

Keywords

Morus indica, bioassay-guided fractionation, antidiabetic, α-glucosidase, α-amylase

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Introduction

Diabetes represents a spectrum of metabolic disorders, which has become a major health challenge worldwide[1]. By the year 2030, over 85 percent of the world's diabetic patients will be in developing countries. In India alone, the prevalence of diabetes is expected to increase from 31. million in 2000 to 79.4 million in 2030[2].

For thousands of years plants and their derivatives are being used for the treatment of various diseases. A large number of studies have demonstrated the anti-hyperglycemic property of many of these plants.[3] In addition, clinical trials have shown some plants as useful anti-diabetic agents, but the pure chemical compounds isolated from the crude extracts of these plants neither bear structural resemblance to the anti-diabetic drugs in current clinical use nor have similar mechanisms of action. But still the search for a novel anti diabetic drug advocates the utilization of plants as a potential source and can be achieved by application of modern scientific technology and recent knowledge on the physiological changes in case of diabetes. [4]

Morus indica is a fruit bearing plant that has been associated with constituents like anthocyanins, flavonoids, phenolics and sterols.[5] Phytochemicals from Moraceae family across the globe have been widely reported for its multi-faceted biological propensities viz. antioxidant, cardio protective, anti-obesity, and anti-diabetic.[6-8] Along with a rich pool of phenolics and flavonoids that contribute majorly to its bioactivities, 1-deoxynojirimycin (1-DNJ), a vital phytochemical in mulberry leaves, has gained visible interest owing to its strong α-glucosidase inhibitory ability, anti-obesity, and antiviral activities.[9]

The objective of this work was to obtain extract of leaves of *Morus indica* using methanol as the solvent; identify the class of secondary metabolites present in the extract; determine the total phenolics and flavonoids in the extract; fractionate extract using column chromatography and evaluate the glucose lowering property of the fractions using α-amylase and α-glucosidase inhibition.

Material and Method

Extraction and phytochemical screening

The leaves of *Morus indica* have been collected from locally grown trees in the month of December. The authenticated plant material was shade dried and ground to coarse powder using a slow speed motorized blender. The powder was passed through sieve no. 20 to obtain uniform size material for extraction. The powdered plant material was accurately weighed and filled in a cellulose thimble. The thimble was placed in the extractor of soxhlet extraction apparatus for carrying out the extraction. Weighed quantity of powdered leaf was accurately weighed and filled in a cellulose thimble and placed in the extractor of soxhlet extraction apparatus and the assembly was set up. The powdered material was defatted using petroleum ether followed by extraction with methanol as the solvent.[10,11]

Mayer's test

The testing for presence of alkaloids was done by dissolving the extracts in 5 mL of 1% HCl solution. To a few ml of this plant sample extract, two drops of Mayer's reagent was added along the sides of test tube. The formation of a cream-colored precipitate was taken as indicator of a positive response to the test.[12]

Froth test

1 ml solution of the extract was boiled in distilled water and filtered. To the filtrate was added 3 ml distilled water, shaken vigorously and heated. The samples were observed for the persistence appearance of foam lasting for at least 15 min was taken as confirmation for the presence of saponins.[12]

Keller-Kiliani test

The extract was *re-e*xtracted with chloroform and evaporated to dryness. To this residue was added 0.4 ml of glacial acetic acid containing a trace amount of ferric chloride. The solution was transferred to a test tube and 0.5 ml of concentrated sulphuric acid is added along the wall of the test tube. Brown ring formed at the interface may indicate the presence of deoxysugar cardenolides. A violet ring may appear just below the brown ring, while in the acetic acid layer, a greenish ring may also form gradually throughout the layer, if the cardiac glycosides are present.[12]

Salkowski test

The extract was dissolved in chloroform and a few drops of concentrated sulphuric acid were added to it. The mixture was shaken well and allowed to stand for some time to separate into layers. The formation of greyish colour indicates the presence of terpenes/terpenoids.[12]

Ferric chloride test

To the extract is added a freshly prepared solution of ferric chloride. Development of blue-green color is taken as indication for the presence of tannins and phenolics.[12]

Shinoda test

To the test solution of the extract, few fragments of magnesium ribbon were added and concentrated hydrochloric acid was mixed drop wise to it. Colour variation of the solution from pink scarlet to blue color indicates the presence of flavonoids in the sample.[12]

Alkaline reagent test

To the test solution a few drops of sodium hydroxide solution was added. Later if intense yellow colour appeared, a few drops of concentrated hydrochloric acid were added to it. The disappearance of the yellow colour was marked as confirmation for the presence of flavonoids in the sample.[12]

Ninhydrin test

The solution of extract was boiled with 0.2% solution of ninhydrin. Development of violet colour in the solutions is an indicator for the presence of amino acids and proteins in the sample.[12]

Molisch reagent test

A few drops of Molischs reagent was added to 2 mL of aqueous solution of the extract, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple colour as indicative of positive for carbohydrates.[12]

Total Phenolic content determination

In order to determine the total phenolic content, 200 µL of the extracted sample (50 µg/mL) was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent. After 3 min, 300 µL of 20%Na_2CO_3 aqueous solution was added and the mixture was allowed to stand for 2 h.[13,14] The absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100 ppm) were similarly treated to plot the calibration curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

Total Flavonoid content determination

Determination of total flavonoids content was based on aluminium chloride method. 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 25- 150µg/ml were prepared in methanol. 0.1 g of dried extract was extracted with 10 ml methanol, filtered, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoid. 1 ml of 5% $NaNO_2$ solution was added to 1 ml of extract or standard and allowed to stand for 5 min at room temperature. To this was added 1 ml of 10% $AlCl_3$ solution and allowed to stand for 6 min. To this was added 5 ml of 1M NaOH solution and kept aside for 15 min. The absorbance was measured at 485 nm.[15]

Fractionation of extract

The extract was subjected to column chromatography for fractionation of components and each fraction was subjected to testing for

glucose lowering potential. Wet packing method was adopted for packing the gla ss column. The methanolic extract was dissolved in small volume of methanol by sonication and the solution was used to load on the column for fractionation. The extract

was loaded on a silica gel column (100-200 mesh) and subjected to column chromatography with ethylacetate-hexane (2:8) as the elution solvent. A total of 13 fractions of 10 mL each were collected. The TLC profiles of these fractions were obtained and the fractions with similar profiles were pooled together to obtain three major fractions (F1-F4) and these fractions were evaporated to dryness and were screened for their α-amylase and α-glucosidase inhibition potential using the method reported. [16]

***In vitro* Glucose lowering potential**

In vitro testing using α-glucosidase and α-amylase inhibition assay was used for testing the glucose lowering potential of the fractions.

α-amylase inhibition assay

Accurately measured 100 µL of the test substance (prepared by dissolving the fraction in DMSO to obtain 100 µg/mL) was reacted with 200 µl of α-amylase and 100 µl of 2 mM of phosphate buffer (pH 6.9) of 0.02 M sodium phosphate (pH 6.9). Subsequent to incubation for 20 min, 100 µL of 1% starch substrate was added. Blank controls where the enzyme was replaced with the buffer were also prepared alongside. Following incubation of the reaction mixture for 5 min, 500 µl of dinitrosalicylic acid reagent was mixed with the control and the test. These were kept in a water bath at a boiling temperature for 5 min.[17] The absorbance readings were taken at 540 nm, and the percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

α-glucosidase inhibition assay

An accurately measured 50 µL of the test substance (prepared by dissolving the extract in DMSO to obtain 100 µg/mL) was 125 µL of α-glucosidase (0.5 U/mL in phosphate buffer solution, pH 6.9), and 700 µL of phosphate buffer was preincubated at 37 °C for 15 min. 125 µL of 5 mM *p*-nitrophenyl glucopyranoside was added, and the mixture was held for 30 min at 37 °C. The reaction was stopped by adding 1000 µL of 0.2 M Na_2CO_3, and the absorbance was read at 405 nm (Yellow color). Blank controls where the enzyme was replaced with the buffer were also prepared alongside.[18] The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{A_{control} - A_t}{A_{control}} \times 100$$

Results and Discussion

The extraction yield of the leaves of *Morus indica* in petroleum ether and methanol was found to be 5.18% w/w and 16.66% w/w respectively. The methanolic extract was resinous and dark brown in color. The findings of the phytochemical analysis suggest the presence of alkaloids, saponins, cardiac glycosides, phenolics, tannins, flavonoids, terpenoids, and protein in the extract (Table 1).

Table 1. Observations of phytochemical screening tests

Chemical Tests	Observation	Inference
Mayer's reagent	Cream colour precipitate	**Positive** (alkaloids present)
Froth test	Frothing is seen	**Positive** (saponins present)
Keller-Kiliani	Violet color in acetic acid layer	**Positive** (cardiac glycosides detected)
Ferric chloride	Blue green color	**Positive** (phenols & tannins present)
Shinoda test	Red color	**Positive** (flavonoids present)

Alkaline reagent test	Yellow color that turns red on acidification	**Positive** (flavonoids present)
Ninhydrin Test	Voilet color	**Positive** (proteins & amino acids present)
Salkowski Test	Yellow color in lower layer	**Positive** (terpenoids/steroids present)
Molisch's Test	Purple color produced	**Negative** (reducing sugars absent)

Total Phenolic content

The methanolic extract of *Morus indica* leaves was evaluated for quantifying the total phenolic content by Folin-Ciocalteu method. The absorbance of the extract sample was found to be 0.867 and the total phenolic content of methanolic extract of *Morus indica* was calculated to be 145.58 GAE mg/g.

Total Phenolic content

The methanolic extract of *Morus indica* leaves was evaluated for quantifying the total flavoniod content by Aluminium chloride method. The absorbance of the extract sample was found to be 0.744 and the total flavonoid content of aqueous extract of *Morus indica* was calculated to be 173.39 QE mg/g.

Fractionation of extract

Ethyl acetate-hexane (2:8) was selected as the solvent to carry out fractionation and fractionation of bioactives from the aqueous extract.

Silica gel column chromatography of the aqueous extract resulted in collection of 13 fractions of 10 mL each. The TLC of all these fractions was performed to identify fractions having similar compounds (Table 2). This resulted in pooling of fractions 1, 2 & 13 (F1), 4-7 (F2), 8-10 (F3) and 11-12 (F4).

Table 2. R_f values from TLC of chromatographic fractions

Fraction	Color	Rf value
1	Colorless	-
2	Colorless	-
3	Dark Yellow	0.39, 0.47, 0.62
4	Dark Yellow	0.39, 0.47, 0.62
5	Dark Yellow	0.39, 0.47, 0.62
6	Dark Yellow	0.39, 0.47, 0.62
7	Dark Yellow	0.39, 0.47, 0.62
8	Yellow	0.37, 0.43
9	Yellow	0.37, 0.43
10	Yellow	0.37, 0.43
11	Pale Yellow	0.26, 0.34
12	Pale Yellow	0.26, 0.34
13	Colorless	-

α-amylase inhibition assay

The α-amylase inhibition assay is a standard *in vitro* method used to evaluate antidiabetic potential of plant extracts or compounds by measuring their ability to block starch breakdown into sugars. It relies on colorimetric detection of reducing sugars, typically using the DNS (3,5-dinitrosalicylic acid) method. The inhibitors of α-amylase reduce this activity, slowing carbohydrate digestion and lowering postprandial blood glucose.

The α-amylase inhibition potential was studied using in vitro method and the results are presented in Figure 1.

Figure 1. Comparative α-amylase inhibition by fractions and standard

The highest α-amylase inhibition was exhibited by F2 (59.66 ± 0.300 %) and was considered to be the most potent fraction for antidiabetic activity.

α-glucosidase inhibition assay

One of the enzymes that the body uses is alpha-glucosidase, which is responsible for converting carbs into glucose. To reduce blood glucose levels, medications called alpha-glucosidase inhibitors (AGIs) delay the intestinal digestion of carbs. Type 2 diabetes is treated with AGIs.
The alpha glucosidase enzyme controls the rate of carbohydrate digestion in the intestines, which in turn regulates blood sugar levels. Therefore,

inhibiting this enzyme can be a crucial strategy for managing postprandial hyperglycemia (high blood sugar after meals) in individuals. An alpha glucosidase inhibition test evaluates the ability of a compound or drug to block the activity of this enzyme.
The α-glucosidase inhibition potential was studied using in vitro method and the results are presented in Figure 2.

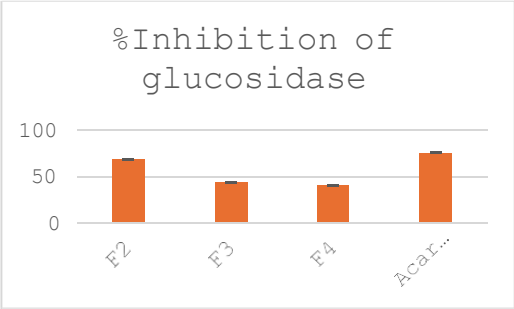

Figure 2. Comparative α-glucosidase inhibition by fractions and standard

Fraction F2 exhibited the strongest inhibitory activity against both carbohydrate-hydrolyzing enzymes. In the **α-amylase assay**, F2 achieved 59.66 ± 0.300% inhibition, which, although lower than acarbose (71.43 ± 0.244%), still represents substantial suppression of starch hydrolysis. Fractions F1 and F3 showed comparatively weaker activity, indicating that bioactive phytoconstituents are unevenly distributed, with F2 enriched in compounds possessing higher affinity for α-amylase.

In the **α-glucosidase assay**, F2 again demonstrated the highest inhibition (68.90 ± 0.357%), followed by F3 (43.70 ± 0.507%) and F4 (40.96 ± 0.440%). The inhibitory effect of F2 approached that of acarbose (75.47 ± 0.634%), underscoring its potential as a natural alternative to synthetic α-glucosidase inhibitors. The dual inhibitory action of F2 suggests comprehensive modulation of carbohydrate digestion, which could translate into improved postprandial glycemic control.

The observed enzyme inhibition is likely attributable to phytochemicals such as flavonoids, phenolics, saponins, and alkaloids. These compounds are known to interact with digestive enzymes through hydrogen bonding, hydrophobic interactions, and conformational modifications. Similar findings have been reported in flavonoid-rich plant extracts, reinforcing the therapeutic relevance of natural products in diabetes management.

Notably, the inhibition levels of F2 were close to those of acarbose, positioning it as a promising candidate for nutraceutical or phytopharmaceutical development. Nevertheless, while in vitro assays provide valuable preliminary insights, *in vivo* validation is essential to establish bioavailability, efficacy, and safety. Future work should focus on isolating and characterizing the active constituents of F2, elucidating their mechanisms of enzyme inhibition, and evaluating their antidiabetic potential in animal models and clinical studies.

Conclusion

The present study demonstrated that the methanolic leaf extract of *Morus indica* is rich in diverse bioactive phytochemicals, including alkaloids, saponins, cardiac glycosides, phenolics, tannins, flavonoids, terpenoids, and proteins. Quantitative analysis confirmed notably high levels of phenolics and flavonoids, compounds widely recognized for their antioxidant and enzyme inhibitory properties. Bioassay-guided fractionation identified fraction F2 as the most active, showing strong inhibitory effects against both α-amylase and α-glucosidase, with activity approaching that of the reference drug acarbose. This dual inhibition suggests that F2 contains potent phytoconstituents capable of modulating carbohydrate metabolism and reducing postprandial glucose levels. These findings highlight *Morus indica* as a promising natural source of antidiabetic agents. In particular, fraction F2 emerges as a potential candidate for development into nutraceuticals or phytopharmaceuticals for type 2 diabetes management. However, while the in vitro results are encouraging, further *in vivo* studies and clinical validation are essential to establish bioavailability, safety, and therapeutic efficacy.

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