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In-Vitro antidiabetic activity of leaf juice of *Mespilus Germanica L*

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Abstract

Diabetes mellitus, a modern civilization disease, is marked by chronic hyperglycemia and severe complications if untreated. Type II DM, a multifactorial form, accounts for 90% of global cases. Controlling postprandial glucose is vital for preventing complications, as elevated plasma glucose is influenced by α-amylase and α-glucosidases. Current enzyme inhibitors like Acarbose and voglibose cause gastrointestinal side effects. This study aimed to find natural source inhibitors with fewer adverse effects using in-vitro antidiabetic assays. Mespilus germanica L. leaf juice (20-100 µg/ml) was tested against fungal α-amylase and α-glucosidases from albino rat intestines. The enzymes showed dose-dependent inhibition comparable to Acarbose. LJMG and Acarbose had IC50 values of 83.35 and 52.15 µg/ml for fungal α-amylase, and 92.44 and 54.84 µg/ml for α-glucosidase, respectively. The leaf juice had a protein content of 10.6 mg/ml and increased glucose uptake by yeast similarly to Metronidazole. Results suggest that polyphenolic compounds in the leaf juice mimic insulin in glucose utilization and inhibit α-amylase and α-glucosidase. Further pre-clinical assessments are needed to confirm the antidiabetic potential of this herb.

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INTRODUCTION

Being healthy goes beyond being a basic human necessity and is a common human ambition. The degree of protection against disadvantage resulting from illness, the fairness with which health is spread throughout the socioeconomic spectrum, and the general state of health can all be used to assess the growth of any community, affluent or poor. The WHO Charter and other international treaties guarantee everyone the right to the best possible level of health, and no nation or area should have to tolerate levels of illness that cannot be prevented [1].

The World Health Organization (WHO) defined "Health" in its broader sense as "a state of complete physical, mental, and social well-being (referred to as the *health triangle*) and not merely the absence of disease or infirmity." The Ottawa Charter claimed that health is not just a state but also "a resource for everyday life, not the objective of living.

According to Theodore Schwann and Matthias Jakob Schleiden's "Cell theory," a cell is the structural and functional building block of all living things. Many intricate and diverse chemical processes carry out every function, including cell division, differentiation, growth, reproduction, senescence, and others. The cascade of molecular changes known as biochemical pathways with strict, intrinsic control begins with one type of biomolecule interacting with another, which then influences another, and so on.

These pathways are sometimes over- or under-driven as a result of both genetic and non-genomic factors that cause the disorder or disease in a variety of intricate and diverse ways. An error in one process could prevent the synthesis of a crucial protein, cause an excess of it, cause it to have an abnormal structure, cause it to misfold, or result in chronic disorders ranging in severity from moderate to fatal [2].

METHODOLOGY

Preparation of *Mespilus germanica L* leaf juice: Freshly collected leaves of *Plectranthus amboinicus*are washed thoroughly to remove adherent impurities, ground to paste using mortar and pestle, and squeezed through muslin cloth to obtain juice. The juice was centrifuged at 5000 rpm for 15 minutes at room temperature to obtain the clear supernatant. To determine the percentage yield, the volume of the obtained juice was measured using a measuring cylinder [3].

$$\%\text{Yield of juice} = \frac{\text{Quantity of juice obtained (ml)}}{\text{Weight of leaves taken (gm)}} \text{X}100$$

Random determination of weight/volume of leaf juice of *Mespilus germanica L*: A clean specific gravity bottle was filled with 1 ml of leaf juice, its weight recorded as W1, and it was heated to a temperature between 40 and 450 degrees Celsius in a hot air oven until a semisolid consistency was achieved. W2 was the weight specified for it. The weight/volume of the juice is given by the difference in weights or W1-W2. The resultant value is used to prepare appropriate juice dilutions for additional parameter measurements [4].

The preparation of LJMG concentrations of 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, and 100µg/ml for an in-vitro anti-diabetic test

The desired sample dilutions were made using the formula based on the weight/milliliter value of LJMG, which was determined using the previously described method.

$$C_1V_1 = C_2V_2$$

Chemicals

LJMG's total protein content was calculated using the Bradford technique.

Principle: This assay's foundation is the shift in absorbance maxima of Coomassie Brilliant Blue G-250 in an acidic solution from 465 nm to 595 nm upon protein binding [5].

Reagents:

1. Bradford reagent: 50 milliliters of 95% ethanol were used to dissolve 200 mg of Coomassie Blue G250 dye. This mixture was combined with 100 milliliters of 85% concentrated phosphoric acid, and distilled water was added to bring the total amount to one liter. Whatman No. 1 filter paper filters the reagent, and the filtrate is placed in an amber-colored bottle and kept at room temperature (250C).

2. **Bovine serum albumin (Protein standard**): A freshly manufactured protein stock solution with a 1 mg/ml concentration was obtained by dissolving a precisely weighed quantity of 5 mg of bovine serum albumin in 5 ml of distilled water.

Procedure:

The experiment was conducted using a range of dilutions (1, 1:10, 1:100, and 1:1000), and 100 µl of each dilution was used because the content of Mespilus germanica L leaf juice is unknown and can be anything. Test tubes were labeled from 1 to 15, and glass tubes labeled 1 through 10 and tube 11 as blank were pipetted with ten µl, 20 µl, 30 µl, and 100 µl BSA standards. To make the final volume in each tube, including the blank, 100µl was added with distilled water. The tubes labeled 12 through 15 were filled with 100µl of every unknown leaf juice dilution that contained the protein. Bradford reagent (5 ml) was added to each tube and thoroughly mixed by inversion. For tubes 1 -10 and 12 -15, the absorbance was measured at 595 nm in the quartz/glass cuvette compared to the reagent blank (tube 11) in five to sixty minutes [6].

Standard: Bovine serum albumin (1mg/ml)

Table 1: Observations table for Bradford assay

Tube No.	Volume (μl)	Mass (μg)	Distilled water (μl)	Bradford reagent (ml)
Standard DNA				
1	10	10	90	5
2	20	20	80	5
3	30	30	70	5
4	40	40	60	5
5	50	50	50	5
6	60	60	40	5
7	70	70	30	5
8	80	80	20	5
9	90	90	10	5
10	100	100	0	5
11	Blank (0)	0	100	5
Unknown sample				
12	100 (1:1000 dil.)	Unknown	0	5
13	100 (1:100 dil.)	Unknown	0	5
14	100 (1:10 dil.)	Unknown	0	5
15	100 (Undiluted)	Unknown	0	5

The following formula can be used to calculate the concentration of the unknown sample directly: Protein concentration equals absorbance × dilution factor/slope (1/µg) × volume of the assay's diluted protein (ml).

α-Amylase inhibition assay by DNSA method

There are various steps involved in the digestion of starch in humans. Polymeric substrates are first broken down into shorter oligomers by partial digestion by salivary amylase. These are further degraded into maltose, maltotriose, and tiny malto-oligosaccharides in the stomach by pancreatic α-amylases later on. The hydrolysis of dietary starch (maltose) into glucose before absorption is carried out by the digestive enzyme α-amylase. When α-amylase is inhibited, postprandial hyperglycemia in diabetic patients can be reduced [7].

The quantification of the reducing sugar (maltose equivalent) released during the hydrolysis of starch under assay conditions was used to determine the inhibition of α-Amylase. The result was expressed as a decrease in units of maltose freed. Measuring alpha-amylase activity in vitro involves hydrolyzing starch when α-amylase is present. The decreased intensity of the orange-yellow color denotes the suppression of starch's breakdown into monosaccharides by enzymes. Stated differently, the α-amylase inhibitory activity of a test sample is directly correlated with the intensity of its orange-yellow color [8].

Enzyme: (Fungal α-amylase, 0.5128 U/ml) kept between 2 and 8 0C. Phosphate buffer (0.02 M) with 0.006 M sodium chloride and 100 ml of sodium phosphate buffer (pH 6.9) contained 3.246 mg of α-amylase.

Sodium phosphate buffer (0.02 M), pH 6.9, in the presence of 0.006 M NaCl. The three solutions that followed were each produced independently. 0.002 M of Na2HPO4 preparation: 1.582g of Di Sodium hydrogen phosphate was mixed with 1.2 ml of distilled water.

How to prepare 0.002 M NaH2PO4? To 1.062g of NaH2PO4, 2.2 ml of distilled water was added.

Setting up 0.006 M NaCl.

All three solutions were thoroughly combined to achieve a pH of 6.9, and 400 milliliters of distilled water were added. If the pH is not 6.9, Na2HPO4 as base or NaH2PO4 as acid was added to bring the pH back to 6.9. In the end, the solution was raised in the volumetric flask to its ultimate volume of 1000 ml. The produced buffer was utilized within two weeks and kept at 25°C [9].

Substrate:

1% starch solution: One gram of soluble starch (100 ml) was dissolved in sodium phosphate buffer. The starch in the buffer dissolved more easily with constant stirring at 90°C. After cooling, the starch solution was kept in storage at 4°C. Before the experiment, the starch solution was incubated for five minutes at 25°C [10].

Positive Control: Acarbose (Glucobay, India: Bayer Pharma)

Stock Solution of Acarbose: 50 cc of 0.02 M phosphate buffer containing 50 mg of Acarbose

1% Starch solution: Soluble starch (1g) was dissolved in 100 ml sodium phosphate buffer [11].

Dinitro salicylic acid (DNSA) reagent: After dissolving 1 gram of Dinitro salicylic acid in 50 milliliters of distilled water, 28.2 grams of Rochelle salt are added. After adding 20 ml of 2N NaOH, 100 ml of distilled water was added, and the mixture was kept in a light-resistant container. Before the assay, the reagent was newly produced [12].

α –amylase inhibition assay procedure:

500 µL of Plectranthus amboinicus/Acarbose leaf juice sample (positive control), Using 0.006 M NaCl and 0.02 M sodium phosphate buffer (pH 6.9), 200 µL of fungal α-amylase was produced. Pre-incubation for roughly ten minutes at 37 °C. Using 0.006 M NaCl and 0.02 M sodium phosphate buffer (pH 6.9), 500 µL of 1% starch solution was prepared. Re-incubation for roughly ten minutes at 37 °C. Reaction termination with the addition of 500 µL of DNSA reagent. Five minutes of incubation in a boiling water bath, Cooled to Room Temperature, diluted with 5 ml of distilled water, Measurement of OD at 540 nm. (The appropriate inhibitor and reagent blank controls were carried out concurrently and subtracted). Every experiment was conducted three times.

% Inhibition = (Control Absorbance - Test Absorbance) x 100 / Control Absorbance.

α-Glucosidase inhibition assay

The in vitro measurement of α-glucosidase activity involves identifying the reducing sugar, or glucose, that results from the enzyme's breakdown of sucrose [13].

Materials

Substrate: Sucrose

Positive control: Acarbose (Glucobay, Bayer Pharma, India)

Glucose reagent- Span Diagnostics, India

Lyophilized powder of ∝-Glucosidase from saccharomyces *cerevisiae* (Sigma-Aldrich. (10 units/mg of protein).

Preparation of 0.1M potassium phosphate buffer (pH 6.9) :

To achieve the desired pH of 6.90, 9.11 g of K2HPO4 and 6.49 g of K2HPO4 were dissolved in 200 ml of distilled H2O and 200 ml of distilled H2O, respectively. The two solutions were then well mixed. If the pH fell short of 6.9, K2HPO4 was added. Ultimately, the solution was increased in the volumetric flask to a final volume of 1000 ml. The produced buffer was utilized and stored within two weeks at 25°C [14].

Substrate (37 mM): A solution of 316 mg sucrose in 25 ml of 0.1M phosphate buffer (pH 6.9)

Positive control: Acarbose 50 mg in 50 ml of 0.1M phosphate buffer (pH 6.9) is in stock.

Procedure for α-glucosidase inhibition assay:

500 µL of fresh Plectranthus amboinicus/Acarbose leaf juice sample (positive control), Alpha-glucosidase was produced in 100 µL of 0.1M sodium phosphate buffer (pH 7). Pre-incubation for roughly ten minutes at 37°C, 500 µL of Sucrose solution at 37 Mm, Re-incubation for roughly ten minutes at 37°C. I kept it in a boiling water bath for 2 minutes and cooled it to RT. A 250 µL addition of glucose reagent. Measure the absorbance at 510 nm after 10 minutes of incubation at Rt. (The appropriate inhibitor and reagent blank controls were carried out concurrently and subtracted) Every experiment was conducted three times. The inhibitory activity of ∝-glucosidase was expressed as an inhibition percentage [15].

$$\text{Inhibition percentage} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Glucose uptake studies by Yeast cells

Materials:

Substrate: Glucose 25mM

Positive control: Metronidazole (Flagyl, Pfizer)

DNSA reagent

Yeast suspension

For the investigation of glucose uptake in response to Mespilus germanica L leaf juice and the commonly used drug metronidazole, commercial baker's yeast was obtained from a nearby bakery. The yeast was rinsed by centrifuging it in distilled water for five minutes at 3000 rpm until the supernatant fluids were clear. A yeast suspension was made in distilled water at a ratio of 10% (v/v). LJMG in different concentrations (20, 40, 60, 80, and 100 µg/mL) was combined with 1 mL of a 25 mM glucose solution and incubated for 10 minutes at 37 °C. To initiate the reaction, 100 µL of yeast suspension was added, and then it was vortexed and incubated for an additional 60 minutes at 37 °C [16]. The tubes were centrifuged (2,500 × g, 5 min) after 60 min, and the amount of glucose in the supernatant was determined spectrophotometrically (540 nm) using the DNSA method. Metronidazole was the prescribed medication. Every experiment was run in three duplicates. Using the following formula, the

percentage increase in glucose absorption by yeast cells was determined:

$$\text{Increase in glucose uptake (\%)} = \frac{\text{absorbance (Sample)} - \text{Absorbance(Control)}}{\text{Absorbance (Sample)}} \times 100$$

Calculation of IC_{50} for enzyme inhibitory activity

The efficiency of a substance in blocking a specific biological or biochemical function is measured by the half-maximum inhibitory concentration (IC50). This metric represents the amount of a particular drug or other material (inhibitor) required to slash the amount of a biological process or its component (such as an enzyme, cell, cell receptor, or microbe) in half. In pharmacological studies, it is frequently used to gauge antagonist medication potency. According to the FDA, the drug's IC50 value is the concentration needed to achieve 50% inhibition in vitro [17].

The formula was used to compute the IC50 using the linear interpolation method.

$$IC_{50} = \frac{50 - A}{B - A} \times (D\text{-}C) + C$$

Statistical analysis

The statistical program Graph Pad Instat 7.0 (GraphPad Software, Inc. La Jolla, CA USA) was used to analyze the experimental data [18].

RESULTS AND DISCUSSION

The Percentage yield of fresh leaf juice of *Mespilus germanica L.*

The herb *Mespilus germanica L.*, belonging to the Lamiaceae family, has been extensively reported for its myriad and versatile therapeutic potential in traditional systems of medicine like antibacterial, antimicrobial, insecticidal, free radical scavenging and radio-protective activities and appetizing potential, etc. Hence, we have selected the herb for our–*in vitro* antidiabetic study.

The prime objective of employing fresh leaf juice of *Mespilus germanica L.* for our investigation instead of extractive form is that crude consumption of juice of certain herbs is the widely followed and prescribed practice from ancient times and is cost-effective and affordable relative to processed one as eating tomato is cheaper than oral administration of its antioxidant phytochemical *lycopene* as the latter is unaffordable for large masses. This concept also supports the universal claim that "Prevention is better than cure," as regular consumption might have preventive and curative effects.

The plant produced a modest juice production from its succulent, meaty leaves, which is evident given that the amount of juice does not necessarily indicate the presence of proteins or other phytochemicals.

Table 2: Percentage yield of fresh leaf juice of *Mespilus germanica L.*

S. No.	Name of the herb	% Yield (ml/100 gm)
1.	Fresh leaf juice of *Mespilus germanica L*	42.1 ± 2.14

A mean ± standard error of three observations (n = 3) was expressed.

Random determination of weight/milliliter of leaf juice of *Mespilus germanica L.*

Fresh leaf juice of *Mespilus germanica L* was subjected to random weight/volume determination, and the value obtained was the average of a triplicate of readings. This parameter i.e. ,wt./mL enabled us to prepare fresh juice dilutions over specific concentrations of 20, 40, 60, 80, and 100 µg/ml for *in-vitro* α-amylase, α- α-glucosidase inhibitory and glucose uptake assay using baker's yeast.

Table 3: Weight/milliliter of fresh leaf juice of *Mespilus germanica L.*

S. No.	Name of the Herb	wt/ml
1.	Fresh leaf juice of *Mespilus germanica L*	200 mg/mL of juice

The value obtained was the average of a triplicate of readings.

Total protein concentration in fresh leaf juice of *Mespilus germanica L.*

The total protein concentration of LJMG assayed by the Bradford method was approximately 10.65mg/ml. This value confirms the presence of

proteins, and we can infer that some of these proteins might interfere with the function of enzymes through various unknown protein-protein interactions, either occupying the orthosteric site (complementary structure) or allosteric modulation.

(Bradford method)

Table 4: Absorbance at 585 nm readings of varied amounts of Bovine serum albumin (Standard)

S. No	Amount of Bovine serum albumin (µg)	Absorbance (585 nm)
1.	10	0.02
2.	20	0.072
3.	30	0.118
4.	40	0.152
5.	50	0.212
6.	60	0.232
7.	70	0.264
8.	80	0.314
9.	90	0.359
10.	100	0.412

Absorbance at 585 nm plotted against the albumin (µg) quantity in bovine serum.

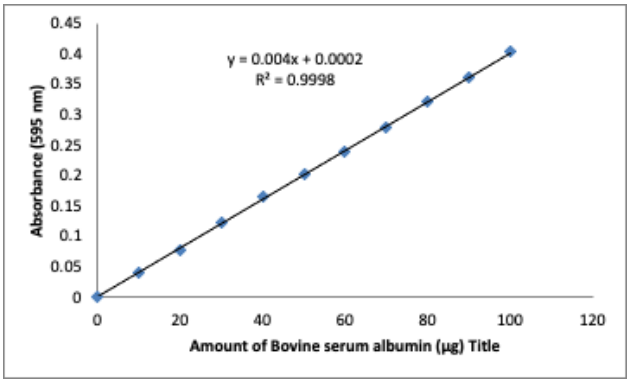

Figure 1: Absorbance at 585 nm plotted against the quantity of albumin (µg) in bovine serum

Absorbance (585nm) of different dilutions of LJMG samples

Table: Absorbance (585nm) of different folds of dilutions of LJMG

The absorbance of LJMG at various dilutions can be used to calculate the total protein concentration because it closely matches the conventional BSA absorbance values. As a result, we will determine the concentration for the sample diluted ten times and had an absorbance of 0.426. The regression line's equation is Y = 0.004X + 0.002, where 0.004 represents the slope since this much protein was found in 100 µl of the protein sample diluted ten times.

Table 5: Volume and Absorbance of Leaf Juice of Mespilus germanica L(µl)

S. No.	Volume of Leaf Juice of Mespilus germanica L(µl)	Absorbance(585 nm)
1	100 (1:1000 dilutions)	0.008
2	100 (1:100 dilutions)	0.032
3	100 (1:10 dilutions)	0.431
4	100 (undiluted)	1.282

Protein concentration is calculated as follows: absorbance × dilution factor/Slope(1/µg) × volume of the assay's diluted protein (ml)

Protein concentration of LJMG= 0.426×10/ 0.004×0.1 = 10650µg/ml=10.65 mg/ml

***In-vitro* assay of α-amylase inhibition by LJMG (3,5-Di nitro salicylic acid method)**

The LJMG has shown a dose-dependent inhibition of α- amylase with 22.68%, 32.44%, 38.74%, 48.18%, and 57.69% at the concentration ranges of 20, 40, 60, 80 and 100 µg/ ml respectively. Concentration-dependent inhibition of α-amylase activity by leaf juice of *Mespilus germanica L* was observed, and the probable mechanism for the inhibition might be a blockade of the active site or allosteric inhibition, etc.

The $IC_{50,}$ i.e., half maximal inhibitory concentration value of LJMG on α- amylase was found to be 82.35µg/ml, which is more than that of standard employed, i.e., Acarbose and hence LJMG was inferior in inhibiting the enzyme α- amylase.

Table 6: Percentage inhibition of α-amylase activity by LJMG

S. No.	Concentration of LJMG (µg/ml)	% α-amylase inhibition
1	20	22.68±0.262
2	40	32.44±0.126
3	60	38.74±0.172
4	80	48.18±0.212
5	100	57.69±0.034

The values expressed were the Mean ± SEM of 3 observations (n=3)

Graphical representation of α-amylase inhibitory activity by LJMG

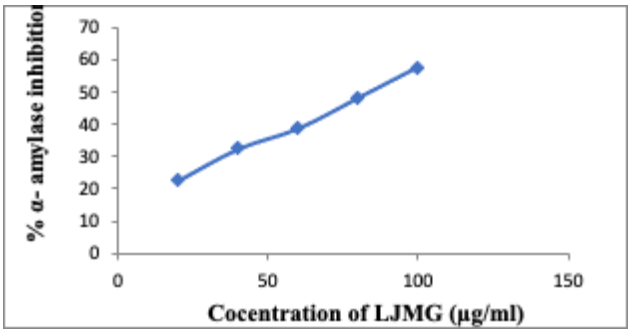

Figure 2: Percentage inhibition of α-amylase activity by LJMG

IC_{50} value of LJMG on α- amylase inhibition

Plotting the log (inhibitor) vs. normalized response-variable slope (GRAPH PAD PRISM VERSION 7) allowed for the determination of the half maximum inhibitory concentration (IC50) of LJMG on α-amylase using non-linear regression analysis. The result was 83.35µg/ml.

***In-vitro* assay of α-amylase inhibition by Acarbose (3,5-Dinitro salicylic acid method)**

The standard drug acarbose has also shown dose-dependent inhibition of α- amylase enzyme with 31.43%, 39.27%, 49.18%, 54.12%, and 59.75% at the concentration ranges of 20, 40,60, 80, and 100 µg/ml, respectively.

We used Acarbose as the standard reference drug in this in-vitro antidiabetic investigation. It effectively reduces postprandial hyperglycemia by competitively blocking alpha-glucosidase and alpha-amylase in the gastrointestinal tract. An oligosaccharide analog called Acarbose can sparingly bind α-glucosidase 1000 times more strongly than genuine carbs, saving insulin.

Plotting log(inhibitor) vs. normalized response-variable slope allowed researchers to determine Acarbose's half maximum inhibitory concentration (IC50) on α-amylase. The result was 52.15µg/ml.

Graphical representation of α-amylase inhibitory activity by Acarbose (Reference Standard drug)

The LJMG has shown a dose-dependent inhibition of α- amylase with 22.68%, 32.44%, 38.74%, 48.18%, and 57.69% at the concentration ranges of 20, 40, 60, 80 and 100 µg/ ml respectively.

The percentage α-amylase inhibitory values for the selected positive control, i.e., Acarbose, were 32.44%, 38.26%, 46.22%, 51.15%, and 58.79% at the concentration ranges of 20, 40,60, 80, and 100 µg/ ml respectively and the IC_{50} value was defined at 52.15µg/ml.

The leaf juice has significant α-amylase inhibitory activity, which may help reduce the conversion of dietary carbohydrates into other oligosaccharides like maltose and maltotriose. The graphical comparison of the % α-amylase inhibition by LJMG versus Acarbosea and the IC50values of LJMG versus Acarbose demonstrated this.

Table 7: Percentage inhibition of α-amylase activity by Acarbose.

S. No	Concentration of Acarbose (µg/ml)	% α-amylase inhibition
1	20	32.44±0.12
2	40	38.26±0.63
3	60	46.22±0.28
4	80	51.15±0.29
5	100	58.79±0.08

The data displayed were the Mean ± SEM of three observations (n = 3)

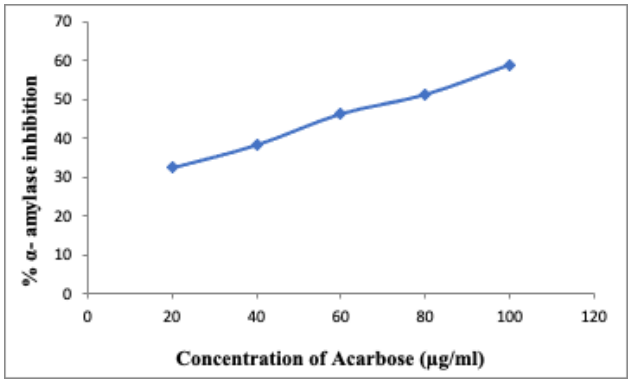

Figure 3: Percentage inhibition of α-amylase activity by Acarbose

IC_{50} value of Acarbose on α- -amylase inhibition

Plotting log (inhibitor) vs. normalized response-variable slope (GRAPH PAD PRISM VERSION 7) allowed for the determination of the half maximum inhibitory concentration (IC50) of

Acarbose on α-amylase. The result was 52.15µg/ml.

Graphical comparison of % α-amylase inhibition by LJMG *vs* Acarbose

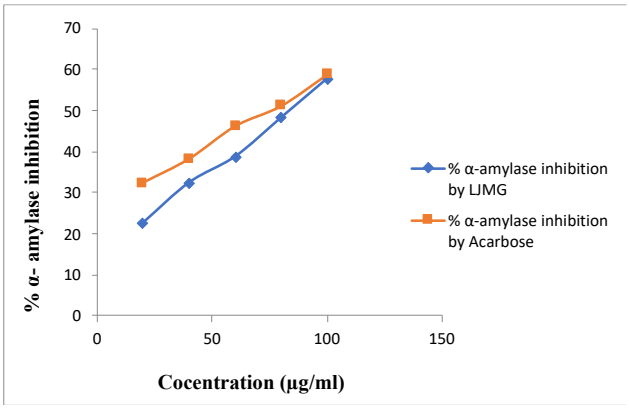

Figure 4: Comparison of % α-amylase inhibition by LJMG *vs* Acarbose

***In-vitro* assay of α-glucosidase inhibition by LJMG**

Leaf juice of *Mespilus germanica L* has shown a moderate dose-dependent inhibition of α-glucosidase inhibition of 14.26%, 22.59%, 35.14%, 41.77%, and 56.15% at the concentration ranges of 20, 40, 60, 80 and 100 µg/ ml respectively.

The *in-vitro* α-glucosidase assay results indicate a linear relationship between the concentration of LJMG and % inhibition of enzyme activity. However, LJMG has slightly higher α-amylase inhibitory activity relative to α-glucosidase inhibition and has to be further investigated to know the reason for the subtle difference.

The IC_{50}i.e., half maximal inhibitory concentration value of LJMG on α-glucosidase was found to be 93.44µg/ml, much larger than the standard Acarbose drug.

Table 8: Percentage inhibition of *in-vitro* α-glucosidase activity by LJMG

S. No.	Concentration of LJMG (µg/ml)	% α-glucosidase inhibition
1	20	14.26±0.032
2	40	22.59±0.027
3	60	35.14±0.092
4	80	41.77±0.077
5	100	56.15±0.14

The data displayed were the Mean ± SEM of three observations (n = 3).

Graphical representation of α-glucosidase inhibitory activity by LJMG

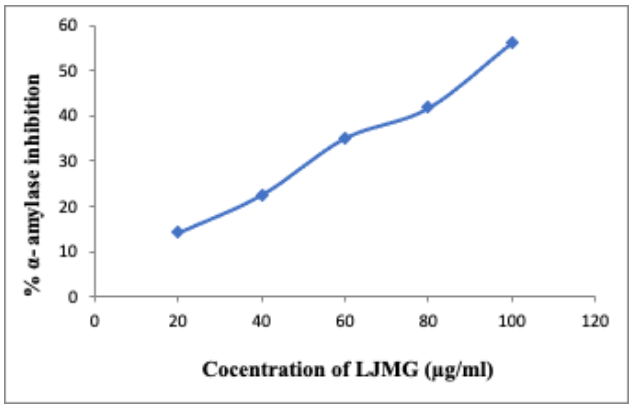

Figure 5: Percentage inhibition of α-glucosidase activity by LJMG

IC_{50} value of LJMG on α- glucosidase inhibition:

Plotting log (inhibitor) vs. normalized response-variable slope (GRAPH PAD PRISM VERSION 7) allowed for the determination of the half maximum inhibitory concentration (IC50) of LJMG on α-glucosidase. The result was 92.44µg/ml.

***In-vitro* assay of α-Glucosidase inhibition by Acarbose**

The reference standard drug Acarbose has shown dose-dependent inhibition of α- glucosidase with 18.79%, 32.56%, 52.84%, 68.22% and 82.29% at the concentration ranges of 20, 40, 60, 80, and 100 µg/ml, respectively, and the IC_{50}i.e., half maximal inhibitory concentration value of Acarbose on α-glucosidase was found to be 54.84 µg/ml.

The *in-vitro* α-glucosidase inhibition assay results indicated a linear relationship between the concentration of acarbose and % inhibition of enzyme activity. However, Acarbose has little more α-glucosidase inhibitory activity relative to α-amylase inhibition, which might be the reason for establishing Acarbose as the prototype α-glucosidase inhibitor. The $IC_{50,}$ i.e., half maximal inhibitory concentration value of Acarbose on α-glucosidase, was found to be 54.84 µg/ml.

The graphical comparison of %α-glucosidase inhibitory values of LJMG versus Acarbose indicated that leaf juice has appreciable α-glucosidase inhibitory activity but much less than that of Acarbose and hence may contribute to a

reduction in the conversion of dietary carbohydrates into further oligosaccharides like maltose and maltotriose and thus, can reduce postprandial hyperglycemia. This paves the way for the passage of nutritional carbohydrates without being converted and absorbed. The enzyme source for α- glucosidase was obtained by appropriate processing without compromising the denaturation of the enzyme.

The IC 50 values, which indicate how well a drug inhibits a particular biological or biochemical process, were found using non-linear regression analysis. This quantitative measurement shows the amount of a specific drug or other chemical (inhibitor) required to halve the activity of a biological process or one of its constituent parts (such as an enzyme, cell, cell receptor, or microbe). It is frequently employed in pharmacological studies as a gauge for antagonist medication strength. The FDA states that IC50 is the drug concentration needed to achieve 50% inhibition in vitro.

Table 9: Percentage inhibition of *in-vitro* α-Glucosidase activity by Acarbose

S. No.	Concentration of Acarbose (µg/ml)	% α-glucosidase inhibition
1	20	18.79±0.24
2	40	32.56±0.392
3	60	52.84±0.214
4	80	68.22±0.124
5	100	82.29±0.12

The data displayed were the Mean ± SEM of three observations (n = 3).

Graphical representation of α-Glucosidase inhibitory activity by Acarbose.

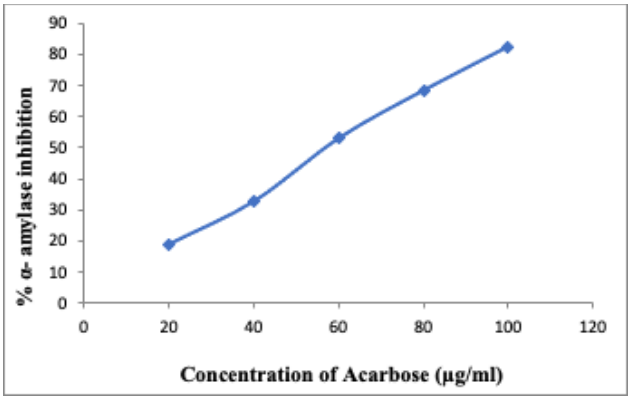

Figure 6: The percentage that Acarbose inhibits the action of α-glucosidase

IC_{50} value of Acarbose on α- glucosidase inhibition

Plotting log (inhibitor) vs. normalized response-variable slope (GRAPH PAD PRISM VERSION 7) allowed for the determination of the half maximum inhibitory concentration (IC50) of Acarbose on α-glucosidase. The result was 54.84 µg/ml.

Graphical comparison of % α-glucosidase inhibition by LJMG *vs* Acarbose

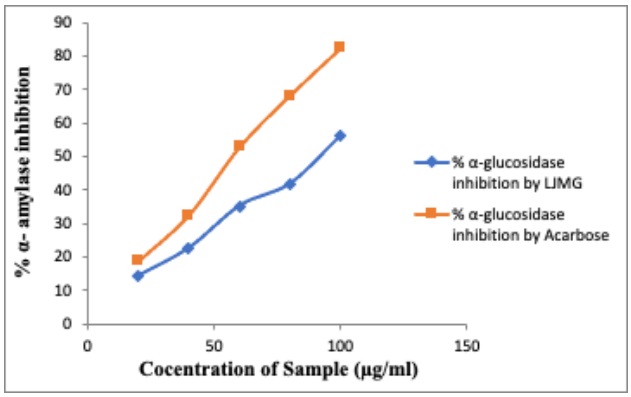

Figure 7: Comparison of LJMG and Acarbose's percentage inhibition of α-glucosidase

Effect of LPJA treatment on glucose uptake in yeast

Most cells use glucose as their primary energy source, and the first rate-limiting step in glucose metabolism is the tightly controlled absorption of glucose into cells. A significant in-vitro screening method for assessing the hypoglycaemic effects of various medicinal plants, aside from the impact on glycosylation of the hemoglobin and inhibition of alpha-glucosidase and alpha-amylase enzymes, is glucose uptake study. This information is crucial for understanding glucose metabolism and its regulation in normal and disease development. The glucose transport mechanism across the yeast cell membrane has gained significance as a perfect in-vitro antidiabetic model for evaluating glucose uptake and utilization. Hence, we have selected this model in our study.

Glucose transport in yeast (Saccharomyces cerevisiae) is achieved by multiple hexose transporters, or members of the GLUT family, whose expression and activity are controlled by various mechanisms. Glucose transport only happens when intracellular glucose is efficiently reduced or utilized.

In this study, we have employed various concentrations of LJMG, i.e., 20,40,60,80 and 100 (µg/ml), and it was observed that the uptake was dose-dependent with 14%.22%, 35%, 41%, and 56%, respectively. The influence of LJMG treatment on glucose uptake was compared with the reference standard drug Metronidazole. The increase in glucose uptake is 18%, 32%, 52%, 68%, and 82% at the concentration of 20,40,60,80 and 100 µg/ml respectively. The results suggest that the leaf juice of Mespilus germanica L can effectively enhance glucose uptake in a dose-dependent manner but at a slightly lower rate than the standard drug metronidazole. We can infer that LJMG can enhance peripheral tissues' effective glucose utilization, thereby controlling blood glucose levels. The probable mechanism of increased uptake might be the translocation of GLUTs to the cell's surface or decreasing insulin resistance.

Many plant-derived secondary metabolites associated with plant defense need thorough dredging for their pharmacological activities. Gut enzyme inhibitors, which block the action of digestive α-amylases and α-glucosidases, which are essential for the breakdown of starch, are among them. The literature reports that *Mespilus germanica L* contains myriad, pharmacologically active phytochemical constituents which, alone or in synergy with one another, might interfere with various molecular pathways. The scientific basis for this inhibitory activity might be that the mixture of compounds present in LJMG imparts efficacies by stimulating multiple therapeutic targets. This study also verified the antidiabetic activity by promoting glucose transport across the cell membrane, as revealed by the simple *in-vitro* model of yeast cells. In conclusion, the *in-vitro* hypoglycemic effect exhibited by Mespilus germanica L leaf juice at the cellular level must be confirmed by employing different *in-vivo* models and clinical trials.

Hence, the present study substantiates the folklore claim of *Mespilus germanica L* as an antidiabetic herb. Future studies were essential to confirm the *in-vitro* findings and isolate the component responsible for the evaluated activities by a battery of pharmacological, phytochemical, and bioanalytical studies.

Table 10: % increase in yeast cells' absorption of glucose after treatment with LJMG

S. No.	Concentration of LJMG (µg/ml)	% increase in glucose uptake by yeast cells
1	20	14.53±0.462
2	40	23.44±0.124
3	60	32.71±0.372
4	80	38.15±0.411
5	100	43.69±0.21

The data displayed were the Mean ± SEM of three observations (n = 3).

A representation of how LPJA treatment affects yeast's ability to absorb glucose

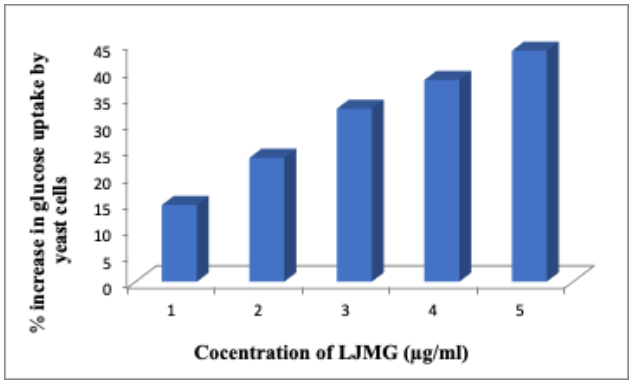

Figure 8: Effect of LPJA treatment on glucose uptake in yeast

The effect of metronidazole therapy on yeast's absorption of glucose

Table 11: % increase in yeast cells' absorption of glucose after metronidazole treatment

S. No.	Concentration of Metronidazole(µg/ml)	% increase glucose uptake by Yeast cells
1	20	15.66±0.24
2	40	32.64±0.525
3	60	48.74±0.236
4	80	53.18±0.811
5	100	55.72±0.028

The data displayed were the Mean ± SEM of three observations (n = 3)

A graphical representation of how metronidazole treatment affects yeast's ability to uptake glucose

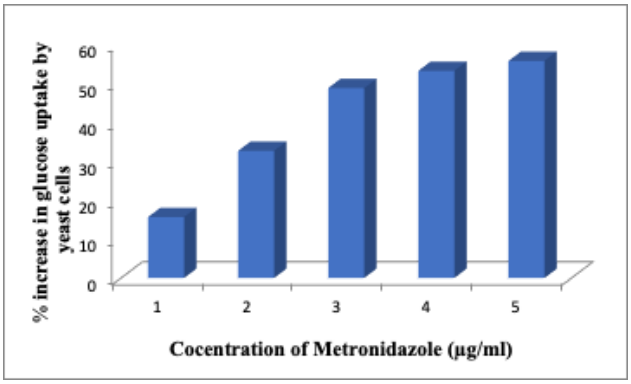

Figure 9: Effect of Metronidazole treatment on glucose uptake in yeast

Graphical comparison of % increase in glucose uptake by Metronidazole *vs* LJMG

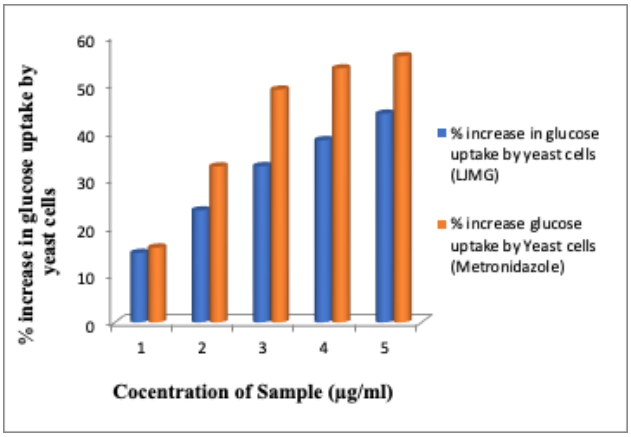

Figure 10: Comparison of % increase in glucose uptake by Metronidazole *vs.* LJMG

Statistical analysis:

Graph Pad Prism, San Diego version (Prism graph pad version 7) trail version was used to analyze the trial data statistically.

SUMMARY & CONCLUSION

Diabetes, the disease of modern civilization, characterized by chronic hyperglycemia, may lead to multi-organ dysfunction syndrome if not diagnosed and intervened appropriately and is met with large-scale economic and disease burdens in the current century. Elevated postprandial blood glucose due to the activity of two principal carbohydrate metabolizing enzymes, viz. α-amylase and α- α-glucosidase, were intricately associated with the pathogenesis of Type 2 Diabetes mellitus. Acarbose, miglitol, voglibose, etc. are the promising inhibitors of the above enzymes and were quite successful in maintaining glucose homeostasis but are met with severe gastrointestinal side effects; hence, active research is highly essential to screen for novel inhibitors of these enzymes, especially from natural sources because they are "natural" and fit into the image of a gentle and, therefore, harmless alternative to conventional medicine.

According to folk medicine, Mespilus germanica L. treats many diseases. Because of its spicy and aromatic flavor, it is also a common ingredient in green leafy vegetables. It has been reported that these vegetables contain bioactive secondary metabolites in pharmacologically active concentrations, which, when taken in sufficient amounts, can have drug-like effects by controlling critical molecular pathways involved in some diseases, incredibly infectious and long-term types. In this view, we wished to subject the fresh leaf juice of *Mespilus germanica L* to α-amylase and α- α-glucosidase inhibitory activities by *in-vitro* assays and glucose uptake assay using yeast model. In addition to the fact that enzymes are necessary for catalysis, small molecular weight, drug-like compounds can highly effectively block enzymes by nature, which makes them appealing targets for drugs.

Thus, dietary ingredients may be ethically investigated by traditional drug development methods to bring the pipeline dream of dietary disease management with plant-derived formulas into virtual reality, further abiding by the ICH charter of 3Rs, i.e., Reduction, Refinement, and Replacement of experimental animals.

The determination of wt/mL of LJMG served to prepare the appropriate concentrations necessary for intended assays. The total protein assay revealed the presence of proteins that can be molecules of interest for the inhibitory assays and serve as a source of nutrients.

Experimental findings indicate that LJMG has comparable α-amylase and α- αglucosidase inhibitory activities and enhancement of glucose uptake as evidenced by *in-vitro* assays using yeast as a model organism due to the presence of hexose transporters that share ordinary functional homology with human. Hence consumption of leaf juice regularly can inhibit the conversion of dietary polysaccharides into monosaccharides leading to reduced absorption and maintenance of normoglycemia; therefore, the progression of the

disease can be retarded. In susceptible individuals, this treatment may prevent the development of the pre-diabetic stage. *Mespilus germanica L,* the traditional culinary herb, has diverse phytochemicals that, through the unelucidated mechanism of action, have significant *in-vitro* antidiabetic activity. The IC_{50} values of LJMG are significantly comparable with those of positive controls. We can infer that the LJMG under investigation can be developed as a functional food incorporated into the dietary armory to combat Type II Diabetes effectively.

CONCLUSION

According to historical reports, several plant foods have high enough concentrations of pharmacologically active chemicals to serve as drugs in moderation. Promising results were found in the in-vitro antidiabetic assays conducted on fresh leaf juice of Mespilus germanica L. However, these studies are insufficient to make a claim. Therefore, a rigorous battery of pharmacological, photochemical, and bio-analytical studies, followed by observational studies in humans, must be conducted to support Hippocrates' (the Father of Medicine) purported statement, "Let food be your medicine and medicine be your food," and to assign this herb, specifically Mespilus germanica L.

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