ORIGINAL ARTICLE

Preliminary Phytochemical Screening and *in vitro* Antidiabetic Activity of Multi-Solvent Extracts of *Marchantia paleacea*

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Abstract

Introduction: Increased blood sugar levels are a hallmark of diabetes mellitus, a metabolic disease caused by insulin resistance and decreased pancreatic insulin secretion. *Marchantia paleacea*, a species of liverwort, has demonstrated potential antidiabetic effects in both *in vitro* and *in vivo* studies. This study aims to conduct phytochemical screening and evaluate the *in vitro* antidiabetic activity of thallus extracts of *M. paleacea* using multiple solvents. **Materials and Methods:** Approximately 150 g of dried thallus powder was separately extracted using methanol, ethyl acetate, and chloroform through Soxhlet extraction. The resulting extracts underwent phytochemical analysis and were tested for *in vitro* antidiabetic activities, including α-amylase inhibition (AAI), α-glucosidase inhibition (AGI), and glucose uptake assay using 3T3-L1 adipocytes and L-6 myotubes. GraphPad Prism version 4 was employed to analyze the information, and one-way analysis of variance and Dunnett's post-test were used. *P*-values below 0.05 were regarded as statistically significant. **Results:** All extracts contained flavonoids, phenolics, and terpenoids, which may be responsible for their antidiabetic effects, according to phytochemical screening. The strongest inhibitory action was shown by the methanol extract. With half-maximal inhibitory concentration values of 24.17 ± 0.002 µg/mL for AAI and 30.07 ± 0.016 µg/mL for AGI. In addition, the percentage of glucose uptake in 3T3-L1 and L-6 cell lines (449.72 ± 0.032 and 229.69 ± 0.20, respectively) was comparable to that of acarbose. **Conclusion:** The methanol extract was one of the examined extracts that demonstrated the strongest *in vitro* antidiabetic activity, likely because of the existence of bioactive phytoconstituents. These findings support the traditional medicinal use of *M. paleacea* for managing diabetes.

Key words: 3T3L-1 and L-6 cells, glucose uptake activity, *Marchantia paleacea,* α-amylase, α-glucosidase.

INTRODUCTION

Diabetes is a major global public health concern that has several underlying causes and is classified as a metabolic condition. It is marked by impaired glucose regulation and disruptions in the metabolism of carbohydrates, fats, and proteins due to abnormalities in insulin secretion and/or function.[1] According to the International Diabetes Federation (IDF), elevated blood glucose ranks as the third most important risk factor for early death worldwide, surpassed only by high blood pressure and tobacco use.[2] *Marchantia paleacea*, a member of the Marchantiaceae family, is a type of liverwort. Liverworts are non-vascular plants typically found in moist or damp habitats, where they contribute to ecological processes such as soil development and nutrient recycling.[3]

M. paleacea thrives in damp, shaded environments such as riverbanks, moist soils, or near waterfalls. It is frequently found in tropical and subtropical regions, often colonizing bare or disturbed soils, which helps stabilize them.[4] The plant has a flattened, ribbon-like thallus that is green to yellow-green. It exhibits dichotomous branching and lacks true roots, stems, or leaves. It reproduces both sexually and asexually. Sexual reproduction involves the production of gametes through specialized structures called archegoniophores

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(female) and antheridiophores (male). Asexual reproduction is achieved through the formation of gemmae within gemma cups located on the upper surface of the thallus [Figure 1].[5]

Based on the above research, the present study aimed to evaluate the *in vitro* antidiabetic activity and the preliminary phytochemical screening of *M. paleacea* thallus.

MATERIALS AND METHODS

Plant materials and authentication

The Nagadevi Organic Zone, located near Thiruvanmiyur, Chennai, India, is where the *M. paleacea* thallus was gathered. On June 07, 2024, the Botanical Survey of India, Deccan Regional Centre, Hyderabad, verified the plant substance using identification reference number BSI/DRC/2024-25/Tech./identification/189. For future use, there is a voucher specimen which is placed at the same location reference.

Preparation extracts

The *M. paleacea* thallus was ground into a fine powder after being shade-dried. Methanol, ethyl acetate, and chloroform were utilized in independent extractions using around 150 g of this powdered material. For the methanol extract, 500 mL of 100% methanol was added to 150 g of the powder, and for 6 h, the mixture was constantly mixed at 30°C. Following filtration and reduced-pressure concentration, the resultant extract was allowed to air dry. The same procedures were used to get the ethyl acetate extract: 500 mL of 100% ethyl acetate was extracted from 150 g of thallus powder using a Soxhlet extraction at 30°C for 6 h while stirring continuously. In addition, this extract was air-dried, condensed, and filtered. Similarly, under the same circumstances, 150 g of powder was extracted using 500 mL of 100% chloroform to make the chloroform extract. After that, the chloroform extract was filtered, concentrated, and then allowed to air dry. For later use, all dried extracts were kept at −20°C in sealed containers.[6-10] Before analysis, each extract was reconstituted using the appropriate solvent[11] to a final concentration of 5% (w/v).

Preliminary qualitative analysis

The analysis was carried out by utilizing standard protocols.[12,13]

Quantitative analysis

Total flavonoid content

The overall amount of flavonoids in the powder extracts of *M. paleacea* thallus in methanol, ethyl acetate, and chloroform was ascertained using the aluminium chloride colorimetric method. In this assay, in a test tube, 4 mL of distilled water was combined with 1 mL of each extract (at a concentration of 5 mg/mL). A 5% sodium nitrite ($NaNO_2$) solution (0.3 mL) was then added. After 5 min of incubation, 0.3 mL of a 10% aluminium chloride ($AlCl_3$) solution was added.

After the liquid stood for 6 min, 2 mL of 1 M sodium hydroxide (NaOH) was added. After adding distilled water until the level reached 10 mL, everything was thoroughly mixed. Absorbance was determined by ultraviolet (UV)-visible spectrophotometer at 510 nm. With quercetin serving as the reference, the results were expressed in milligrams of quercetin equivalents per gram of extract (mg QE/g extract). Each sample underwent three examinations.

Total phenolic content

The total phenolic content of the extracts was determined by the Folin–Ciocalteu colorimetric method. For this procedure, 1 mL of each extract (at a concentration of 5 mg/mL) was combined with 1 mL of diluted Folin–Ciocalteu reagent (1:10 dilution). Two millilitres of a 7.5% sodium carbonate ($NaCO_3$) solution were added to the mixture after it had been allowed to settle at room temperature for 5 min. The samples were then incubated in the dark at 30°C for 30 min. Absorbance was determined using a UV-Visible spectrophotometer at 765 nm. Gallic acid was used as the reference standard, and the results were reported in milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract). Each sample underwent three examinations.

Total terpenoid content

The total terpenoid concentration was ascertained using the vanillin-sulfuric acid colorimetric technique. In this test, 2 mL of chloroform and 1 mL of each extract (at a concentration of 5 mg/mL) were mixed together in a test tube. 1.5 mL of concentrated sulphuric acid (H_2SO_4) and 1.5 mL of a 5% vanillin solution made in glacial acetic acid were added to this. After giving the mixture a vigorous vortex, it was incubated for 20 min at 30°C. At 548 nm, absorbance was measured with a UV-Visible spectrophotometer. The results were reported in milligrams of linalool equivalents per gram of extract (mg LE/g extract), with linalool acting as the reference standard. All measurements were taken 3 times.

Antidiabetic screening[14-17]

α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of the methanol, ethyl acetate, and chloroform extracts from the thallus of *M. paleacea* was evaluated using an *in vitro* enzymatic assay conducted in a 96-well microplate format. After dissolving each extract in dimethyl sulfoxide (DMSO), 10 µL of each concentration (10, 25, 50, 100, 200, and 500 µg/mL) was applied to each well for testing. 50 µL of the α-glucosidase enzyme was added to each well, and the wells were left to

Figure 1: Thallus of *Marchantia paleacea*

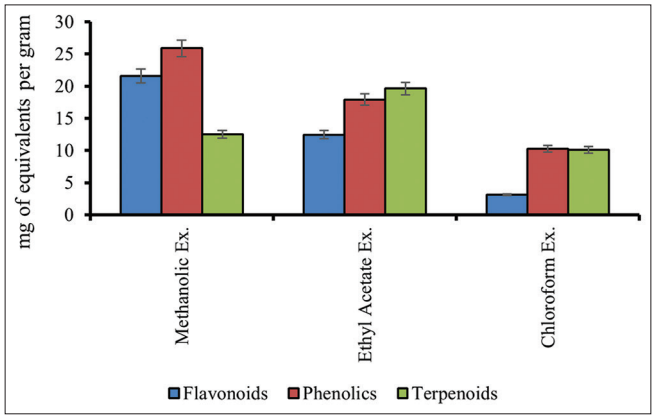

Figure 2: Quantitative analysis of methanolic, ethyl acetate, and chloroform extracts of *M. paleacea* thallus powder

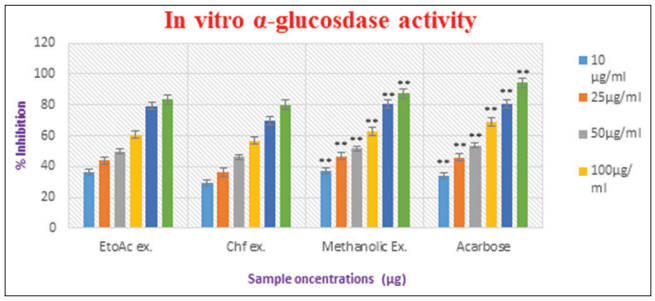

Figure 3: Percentage inhibition of α-glucosidase enzyme activity at different concentrations of acarbose, plant extracts from the *Marchantia paleacea* thallus. The values are expressed as mean ± standard deviation (n = 3)

incubate. The enzymatic reaction was initiated by adding 50 µL of the substrate p-nitrophenyl-α-D-glucopyranoside at a final concentration of 5 mM. The mixture was incubated at 37°C for 30 min.

To stop the reaction, 100 µL of 0.2 M sodium carbonate was added to each well. The absorbance at 405 nm, which represents the quantity of p-nitrophenol generated (a measure of α-glucosidase activity), was measured using a microplate reader. The control wells included both a blank (without

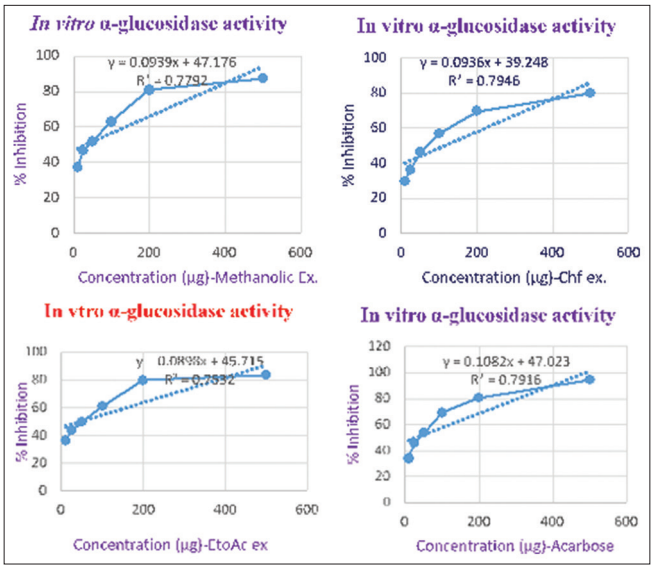

Figure 4: Linear graphs of different extracts of *Marchantia paleacea* thallus and standard acarbose: α-glucosidaseinhibitory activity

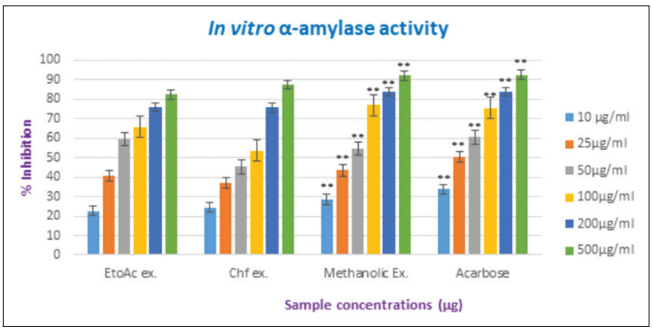

Figure 5: Percentage of *Marchantia paleacea* thallus plant extracts that inhibit α-amylase enzyme activity at varying acarbose doses. The values are expressed as mean ± standard deviation (n = 3)

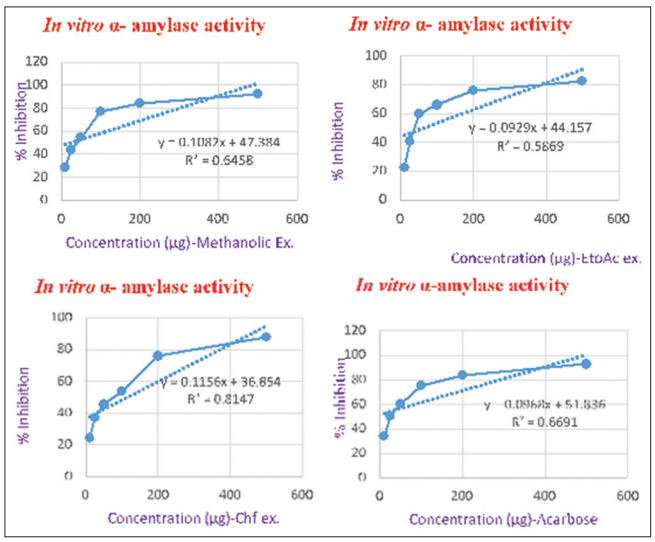

Figure 6: Linear graphs of different extracts of *Marchantia paleacea thallus and standard* acarbos*e:* α-amylase inhibitory activity

enzyme) and a control (containing enzyme, substrate but no extract). Acarbose, a well-known α-glucosidase inhibitor, was used as a positive control to gauge the extracts' effectiveness. The percentage of α-glucosidase activity inhibition was calculated using the following formula:

$$\%\text{Inhibition} = [(\text{Abs of control} - \text{Abs of test})/\text{Abs of control}] \times 100$$

The half-maximal inhibitory concentration value, representing the concentration of extract needed to inhibit 50% of α-glucosidase activity, was determined through non-linear regression analysis. All experiments were conducted in triplicate to ensure the reliability and consistency of the results.

α-amylase inhibitory activity

The inhibitory effect of methanol, ethyl acetate, and chloroform extracts of *M. paleacea* thallus on α-amylase was evaluated using an *in vitro* enzymatic assay in a 96-well microplate format. Each extract was dissolved in DMSO and tested at different concentrations (10, 25, 50, 100, 200, and 500 µg/mL), with 10 µL added per well. The extracts were incubated with 50 µL of α-amylase enzyme at 37°C for 10 min. 50 µL of 1% starch solution was added as the substrate to start the reaction, and it was then incubated at 37°C for 30 min. Each well received 100 µL of the dinitrosalicylic acid (DNS) reagent to stop the reaction. After 10 min of colour development at 90°C in a water bath, the plate was allowed to cool to room temperature. Using a microplate reader, the absorbance – a measure of α-amylase activity – was taken at 540 nm. There were two control groups: One with enzyme and substrate but no extract, and one blank (without enzyme). The classic α-amylase inhibitor, acarbose, was employed to assess and contrast the extracts' inhibitory activity. Using the following formula, the percentage inhibition of α-amylase activity was determined:

$$\%\text{Inhibition} = [(\text{Abs of control} - \text{Abs of test})/\text{Abs of control}] \times 100$$

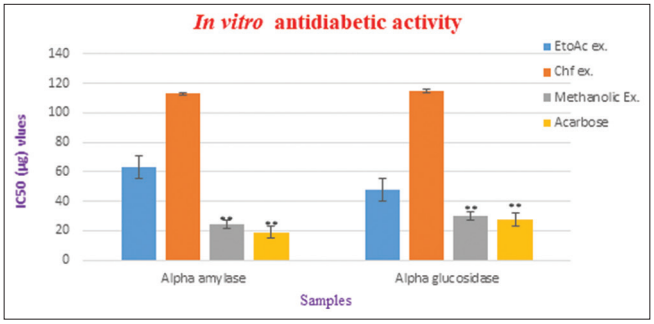

Figure 7: IC_{50} values (inµg/mL) of α-glucosidase and α-amylase inhibitory effects of acarbose (standard), methanolic, ethyl acetate, and chloroform extracts of *Marchantia paleacea* thallus. Values were expressed as mean ± standard deviation ($n = 3$); $P < 0.05$ significant difference as compared to standard

Cell culture

L6 myotubes (muscle cells) and 3T3-L1 adipocytes (fat cells) were cultivated in Dulbecco's modified Eagle medium (DMEM), which was enhanced with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). The cultures were kept at 37°C with 5% CO_2 in a humidified incubator. Every 48 h, the medium was changed to promote the best possible cell growth. The cells were harvested using 0.25% trypsin-EDTA and subcultured into fresh flasks once they had reached 80–90% confluence. 3T3-L1 pre-adipocytes and L6 myoblasts were planted at a density of 1×10^4 cells/cm^2 onto six-well plates or other appropriate culture vessels to stimulate differentiation into myotubes. Every 48 h, the media was changed for the cells, which were kept in DMEM with 2% FBS. Cells showed an elongated, multinucleated myotube shape by the 6th day, confirming effective differentiation, which was monitored every day. Following differentiation, these 3T3-L1 and L6 cells were employed for subsequent tests, such as glucose absorption measurement.

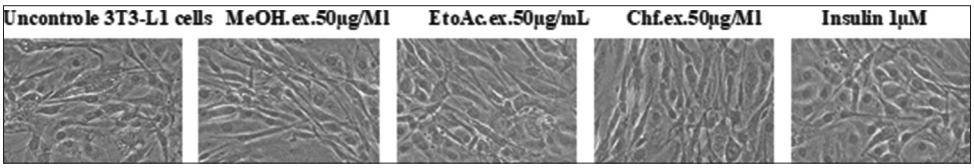

Figure 8: Microscopic images of 3T3-L1 adipocytes treated with control (untreated), Ethyl acetate and methanolic extracts, chloroform extract of *Marchantia paleacea*, and insulin (1 µM)

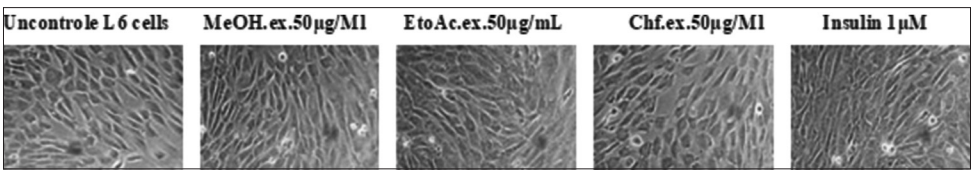

Figure 9: Microscopic images of L6 myotubes treated with control (untreated), extracts of methanol, ethyl acetate, and chloroform of *Marchantia paleacea*, and insulin (1 µM)

Glucose uptake assay using 3T3-L1 adipocytes and L6 myotubes

The purpose of this test was to examine how *M. paleacea* thallus extracts in methanol, ethyl acetate, and chloroform affected the uptake of glucose by 3T3-L1 adipocytes and L6 myotubes. 3T3-L1 and L6 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under standard growth conditions. Cells were cultured for 6 days after confluence in DMEM supplemented with 2% FBS, with medium changes occurring every 48 h. Differentiated cells were plated at a density of 1×10^4 cells per well in 96-well plates for the glucose absorption test. Cells were serum-starved for 12 h before treatment. Ten microliters of each plant extract were added to the proper well after it had been diluted in DMSO to a final concentration of 50 µg/mL. Untreated wells were utilized as negative controls, and insulin (1 µM) was employed as a positive control to enhance glucose absorption. Background controls were cell-free wells. After adding 10 µL of 2-deoxy-D-glucose (2-DG) at a final concentration of 10 mM to initiate the uptake of glucose, the plate was incubated for 30 min at 37°C in a 5% CO_2 environment. To stop the reaction after incubation, the wells were rinsed 3 times with ice-cold PBS.

The cells were subsequently broken down using 50 µL of a lysis buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100. Glucose uptake was assessed by measuring the levels of 2-DG-6-phosphate, an intracellular indicator, using a colorimetric assay kit. Absorbance was recorded at 540 nm with the help of a microplate reader. The percentage of glucose uptake was calculated relative to the untreated control group. All experiments were repeated 3 times to ensure consistency, and statistical analysis was performed to evaluate any significant differences between treated and control samples [Figure 2].

Statistical analysis

There were three duplicates of each experiment, and the mean ± S.E. was used to express the results [Table 2]. GraphPad Prism version 4 was used to do the statistical analysis. Dunnett's test for statistical analysis of the data, post-test, and one-way analysis of variance were employed. $P < 0.05$* was considered statistically significant [Figure 3].

RESULTS

Preliminary qualitative analysis of *M. paleacea* thallus powder [Figure 4]

Preliminary phytochemical analysis of the different extracts shows the presence of various classes of compounds as represented in Table 1 and Figure 5.

Table 1: Preliminary phytochemical screening of *Marchantia paleacea*

Chemical class	Methanolic Ex.	Ethyl acetate Ex.	Chloroform Ex.
Carbohydrates	+	-	-
Proteins and amino acids	-	-	-
Alkaloids	+	+	+
Saponins	-	+	+
Terpenoids	+	++	+
Fats	-	-	-
Flavonoids	+++	++	+
Tannins	+	+	+
Glycosides	-	-	-
Phenols	+++	++	+

+++: Most Abundant; ++: Found in good quantities; +: Slightly abundant; -: Absent

Table 2: IC_{50} values (in µg/mL) for α-glucosidase and α-amylase inhibitory activities of methanolic, ethyl acetate, and chloroform extracts of Marchantia paleacea thallus compared to the standard inhibitor acarbose

	IC_{50} (µg/mL)	
Samples	**Alpha amylase**	**Alpha glucosidase**
EtoAc ex.	62.89±0.324	47.71±0.285
Chf ex.	113±0.462	114.87±0.121
Methanolic Ex.	24.17±0.002**	30.07±0.016**
Acarbose	18.96±0.001**	27.51±0.002**

**: P<0.001. Data are mean (n=3)±SD.

Quantitative analysis

From the results, methanolic extract showed the highest content of flavonoids and phenolic contents, whereas ethyl acetate extract exhibited the highest terpenoidal content.

In vitro antidiabetic activity

α-glucosidase activity

The activity was tested at varying concentrations (10–500 µg/mL). Significantly more inhibitory action was shown by the methanolic extract.

($P < 0.05$) compared to chloroform and ethyl acetate extracts across most concentrations, with activity approaching that of acarbose at higher concentrations.

α-Amylase activity

The activity was tested across varying concentrations (10–500 µg/mL). The methanolic extract's inhibitory efficacy was significantly greater.

Table 3: Effect of *Marchantia paleacea thallus* extracts, control, and insulin on the uptake of glucose by 3T3-L1 cells

Samples	Glucose uptake (µg/mL)	% Glucose uptake
Control	10.72±2.43	100±23.52
EtoAc ex.	20.31±3.02	189.45±2.90
Chf ex.	25.43±2.43	237.22±5.56
Methanolic Ex.	48.21±2.07	449.72±0.032**
Insuline (1µM)	52±0.162	484.07±0.015**

**: *P*<0.001. Data are mean (n=3)±SD.

Table 4: Effect of *Marchantia paleacea thallus* extracts, control, and insulin on the uptake of glucose by L-6 cells

Samples	Glucose uptake (µg/mL)	% Glucose uptake
Control	10.98±3.04	100±3.13
EtoAc ex.	18.03±5.32	164.20±1.76
Chf ex.	12.32±4.01	112.20±4.23
Methanolic Ex.	25.22±1.032	229.69±0.20**
Insuline (1 µM)	30.43±0.034	277.14±0.12**

**: *P*<0.001. Data are mean (n=3)±SD.

($P < 0.05$) than that of the ethyl acetate and chloroform extracts, and at higher doses, it was almost identical to that of acarbose [Figure 6].

Glucose uptake by3T3-L1adipocytes and L6 myotubes [Tables 3 and 4]

M. paleacea methanolic extract showed glucose uptake comparable to insulin ($P < 0.05$). The chloroform extract had weak efficacy whereas insulin was somewhat more active than ethyl acetate extract ($P < 0.05$) [Figures 8 and 9].

DISCUSSION

In the present in vitro study, *M. paleacea* thallus extracts were used to evaluate antidiabetic activity [Figure 7]. Preliminary phytochemical screening of the extracts showed the presence of alkaloids, flavonoids, phenolics, and terpenoids. The methanol extract reported inhibition of α-amylase at an IC_{50} value of 24.17 ± 0.002 µg/mL, and standard at an IC_{50} value of 18.96 ± 0.001 µg/mL; α-glucosidase IC_{50} value of 30.07 ± 0.016 µg/mL, and the standard at an IC_{50} value of 27.51 ± 0.002 µg/m. Whereas the % glucose uptake of 3T3-L1 cells was 449.72 ± 0.032 and the standard was 484.07 ± 0.015. The % glucose uptake methanol extract reported for L-6 cells at 229.69 ± 0.20 and standard at 277.14 ± 0.12. There was a notable antidiabetic benefit from the methanol extract. Pharmacologically, it could help create a new, powerful herbal diabetic medication. These possible methanolic extract effects are utilized to treat diabetes and its associated problems.[18] The treatment of insulin resistance and metabolic diseases that accompany diabetes or obesity may benefit from the use of extract h1a3. Consequently, the extract exhibited α-amylase inhibition (AAI), α-glucosidase inhibition (AGI), and GUI inhibitory effects because it included strong bioactive components.[19,20]

CONCLUSION

This study evaluated *M. paleacea* thallus extracts' initial phytochemical screening as well as their *in vitro* AGI, AAI, and GUI activities. The methanol extracts demonstrated strong inhibitory action against the alpha-glucosidase and alpha-amylase enzymes in the test, in addition to effectively enhancing the glucose absorption enzyme activity by 3T3-L1 and L-6 cell lines. Extracts from *M. paleacea* thallus were discovered to have specific bioactive chemicals that controlled blood glucose levels by inhibiting the activity of the enzymes α-amylase, α-glucosidase, and glucose uptake. This information may help develop new medications with fewer side effects.

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