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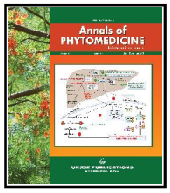

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In vitro antidiabetic and antioxidant assessment of *Hydrocharis laevigata* (Hump. & Bonl. ex Willd.) Byng & Christenh.

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Abstract

Seaweeds serve as remarkable reservoirs of biologically active components. Numerous nations across Asia boast a rich heritage of incorporating various types of seaweeds into traditional herbal medicine formulations. These marine plants hold considerable promise as abundant sources of highly bioactive secondary metabolites, show casing a diverse array of therapeutic effects such as antioxidative, anticancer, antidiabetic, and anti-inflammatory properties. In the ongoing investigation, a total of 15 compounds were successfully extracted from the ethanolic fractions of *Hydrocharis laevigata* (Hump.& Bonl. ex Willd.) Byng & Christenh. (*H. laevigata*) through the implementation of column chromatography. These compounds were subsequently subjected to a meticulous process of structural elucidation. Validation of their structures was achieved using advanced techniques such as 1H-NMR, 13C-NMR, and mass spectrometry. From the isolated compounds, two compounds of particular interest; namely, perillic acid and strychnine, were singled out for focused analysis. Rigorous *in vitro* evaluations encompassing antioxidant and antidiabetic properties were conducted, involving various assessments such as MTT assay, reactive oxygen species analysis, alpha amylase inhibitory activity, alpha glucosidase inhibitory activity, as well as apoptosis assays. The outcomes of these tests are anticipated to reveal substantial antioxidant and antidiabetic potentials inherent in the tested compounds.

1. Introduction

Diabetes has emerged as a significant and escalating health concern, posing a considerable global burden. Characterized by abnormal blood glucose levels, diabetes encompasses a spectrum of disorders, primarily type 1 and type 2 diabetes, with distinct etiologies and implications (Gautam *et al*., 2021; Ibrahim *et al*., 2021). The prevalence of diabetes has witnessed a relentless increase, attributed to factors such as sedentary lifestyles, unhealthy diets, and genetic predisposition. This growing epidemic has profound socio-economic consequences, straining healthcare systems and economies. Insulin stands as the primary regulatory hormone governing intermediatory metabolism. The pancreas' islets of Langerhans, specifically the β-cells, are responsible for secreting insulin. Inadequate insulin function leading to the mismanagement of blood glucose levels is the underlying cause of diabetes mellitus. Among individuals afflicted with diabetes, elevated blood glucose levels give rise to heightened sensations of thirst, hunger, and urine production. However, prolonged and uncontrolled elevation of blood glucose levels in chronic cases contributes to severe and consequential health complications.

The implications of diabetes are far-reaching, extending beyond elevated blood sugar levels. It is intricately linked to various complications, including cardiovascular diseases, neuropathy, retinopathy, and kidney dysfunction, thereby impairing both the quality and duration of life. The multifaceted nature of diabetes underscores the urgency for holistic strategies that encompass prevention, early detection, and comprehensive management (Ansari *et al*., 2020; Mehrotra, 2020). Public health initiatives focusing on awareness, lifestyle modifications, and improved access to healthcare are pivotal in curbing the relentless rise of diabetes and its associated complications, ultimately fostering a healthier global population (Insaf *et al*., 2022; Khan *et al*., 2021).

Alongside conventional treatments, the role of medicinal plants and natural products has garnered significant attention in the battle against diabetes (Mehrotra, 2020; Rana *et al*., 2021; Salar *et al*., 2022). These natural resources obtained from plants contain a wealth of bioactive components. They hold significant potential in controlling the way glucose is processed in the body, enhancing the body's responsiveness to insulin, and reducing oxidative stress. These factors play a crucial role in the successful treatment of diabetes. Compounds such as berberine sourced from bitter melon, resveratrol extracted from grapes, and curcumin derived from turmeric have displayed encouraging antidiabetic properties within various research investigations. Type 1 diabetes mellitus comprises a minority, accounting for 5-10% of all diabetes cases, but its global incidence is on the rise. On the other hand, type 2 diabetes mellitus, characterized by compromised insulin secretion from pancreatic â cells, stands as the most prevalent form. The hereditary predisposition for type 2 diabetes can be attributed to the human leukocyte antigen complex (Gaurav, 2022; Gaurav *et al*., 2023, 2022).

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Furthermore, it has been determined as the major source of the energy for the cell is glucose. Maintaining a stable glucose level is essential, as cells require a continuous supply of energy regardless of sporadic food consumption and fluctuating activity levels. The historical identification of diabetes dates back to approximately 1500 BC when ancient Egyptians characterized it as a rare condition marked by excessive urination and weight loss. The Greek physician Aretaeus is credited with coining the term "diabetes" at a later point in time. The phrase "diabetes mellitus" originates from two Greek words: "diabetes," signifying the occurrence of excessive urine flow, and "mellitus," emphasizing its sweet nature (Perez *et al*.,1998). Metabolic disorder refers to a condition where there is an elevation in blood glucose levels due to disruptions in both insulin secretion and action. Diabetes mellitus is marked by this particular anomaly. This ailment manifests in two main variations: type 1 and type 2. Type 1 diabetes mellitus stems from an autoimmune assault directed at the pancreatic β-cells responsible for producing insulin, consequently reducing the supply of insulin available for metabolic functions. This form of diabetes tends to manifest at an early age, typically before the age of 40, although onset can occur at any stage of life (Ekbbal *et al*., 2023; Gaurav *et al*., 2020; Gautam, 2022).

A successful therapeutic approach for controlling blood glucose levels revolves around managing the functions of carbohydrate-hydrolysing enzymes; namely, alpha amylase and alpha glucosidase. Alpha amylase is central to the initial phase of starch degradation, where it breaks down intricate polysaccharides (starch) into three primary components: maltose, maltriose, and limit dextrins. Conversely, α-glucosidase aids in the concluding phase of starch and disaccharide digestion. As a result, substances that impede the function of α-amylase cause a slowdown in the digestion of carbohydrates in the small intestine. This ultimately leads to a decrease in the levels of postprandial blood glucose in individuals suffering from type 2 diabetes (T2D) (Meenakshi *et al*., 2018; Venkatachalam *et al*., 2021). Carbohydrate-hydrolysing enzyme inhibitors employed for the clinical management of type 2 diabetes encompass acarbose, miglitol, and voglibose. These inhibitors are associated with adverse effects like flatulence, diarrhoea, and hepatic complications. Additionally, a majority of these inhibitors incorporate sugar components, necessitating intricate and time-consuming synthetic pathways. As a result, there is a demand for inhibitors derived from non-sugar origins, characterized by reduced side effects (Rana *et al*., 2021; Sinha and Haque, 2022).

Oxidation plays a crucial role in the functioning of living organisms. The process of oxidation gives rise to reactive oxygen species (ROS) (Gaurav *et al*., 2023). When ROS production becomes overly excessive, it can contribute to the emergence of numerous diseases, such as cellular ageing, mutagenesis, carcinogenesis, diabetes, and neurodegeneration. To counteract these negative outcomes, the internal antioxidant defense mechanisms of organisms play a significant role in mitigating the detrimental effects of ROS. Natural antioxidants have received considered attention due to safety and effective properties (Khan *et al*., 2022).

Sea weeds are recognized for their potential as a valuable reservoir of bioactive compounds, particularly those with antioxidant and α-glycosidase inhibitor properties. Among these marine resources, *H. laevigaetum*, commonly referred to as the South American frog bit, has been investigated for its potential antioxidant content. The extracted perillic acid and strychnine from this source have demonstrated notable antioxidant and antidiabetic properties. The bounty of bioactive compounds present in sea weeds offers a promising avenue for the discovery of diverse metabolites that could find applications in pharmaceuticals, cosmetics, as well as food and feed supplements. Drawing from these considerations, this research aimed to evaluate the potential of *H. laevigata* in terms of its antidiabetic properties through *in vitro* methodologies.

2. Materials and Methods

2.1 Requirements

A variety of drugs and chemicals were procured in the analytical category. These included substances like *H. laevigata,* glucose, 2,7 dichloro dihydro fluorescein diacetate (DCFH-DA), potassium phosphate, sodium carbonate, as well as solvents such as petroleum ether, chloroform, ethanol, and ethyl acetate.

2.2 Sea weed collection and processing

The seaweed *H. laevigata* was sourced from Sree Padma Aqua Flora located in Aluva, Kerala, India. Authentication of the specimen was carried out by Dr. Madhav Shetty, a Botanist associated with S V University in Tirupati, Andhra Pradesh, India. The assigned voucher number for this specimen is 1047. The collected seaweed underwent a meticulous cleansing process to eliminate salt, surface impurities, sand particles, and epiphytes adhering to its surface. Following the cleansing procedure, excess water was drained, and the samples were finely chopped before being subjected to natural sun drying. The ensuing dried seaweed was then ground into a powdered form, preparing it for the subsequent extraction process. The extraction process involved utilizing various solvents, namely petroleum ether, chloroform, ethyl acetate and ethanol, in a Soxhlet apparatus. Each of these extracts underwent an initial phytochemical screening, aiming to discern the array of biological constituents present. Among the extracts, the ethanolic extract exhibited the highest concentration of phytochemical compounds. As a result of this outcome, the ethanolic extract was chosen for further investigation and analysis (Ekbbal *et al*., 2022).

2.3 Characterization of ethanolic extract of the plant

The composition of the ethanolic extract was analyzed using LCMS, resulting in the identification of 34 distinct compounds. Further, through the application of column chromatography, 15 of these compounds were successfully isolated. Based on the yield of the isolated compounds, 2 compounds were selected for the study. The 2 isolated compounds were structure elucidated by using FTIR, ^{13}C and ^{1}H NMR and mass spectroscopic techniques, respectively (Ekbbal *et al*., 2022).

2.4 *In vitro* antioxidant activity

2.4.1 MTT assay

The cytotoxicity nature of plant extract and isolated compound were studied against C_2C_{12} cell lines through MTT assay with slight modification of minimal essential medium supplemented with 50 µl of 8 mm glucose solution. About 5000 cells/well were seeded on 96 well plate (triplicate) and incubated for 1 day at 37ºC. then the cells are rinsed with 100 µl of serum-free medium and kept starved at 37ºC for 1hr, then the different concentrations of test sample (15.625, 31.15, 62.50, 125, 250 µg/ml) were treated with starved C_2C_{12} cells and incubated at 37ºC for 24 h. Next, the liquid in the container was

removed and replaced with MTT solution (0.05 mg/ml). The setup was then placed in a CO_2 incubator at 37°C for a duration of 4 h. Subsequently, the liquid was discarded, and the cells were carefully washed using phosphate buffer saline (PBS). Following this, 100 µl of DMSO mixed with formazan stain was added to dissolve the cells. The mixture was thoroughly blended to ensure uniformity. To measure the outcomes, colorimetric method is employed for analyzing cell content. The 96-well plate was examined at a wavelength of 570 nm. In order to compare results, the antidiabetic agent metformin was utilized as a reference. Metformin, reconstituted in a mixture of water, ethanol, and propylene glycol (35:5:60 v/v), was diluted using the complete culture medium and introduced into the wells (Khan *et al*., 2023; Khan *et al*., 2022).

The percentage of cell viability was calculated using the following formula:

Percentage of cell viability = Absorbance of sample / Absorbance of control × 100

2.4.2 Reactive oxygen species assay

The influence of the botanical extract on reactive oxygen species was evaluated employing the procedure described in the work by Davis *et al* (2020) In this study, cell cultures were exposed to varying concentrations of the plant extract for around 24 h. Subsequently, the cells underwent a PBS wash and were then treated with 20 µM of the 2,7-dichloro dihydro fluorescein diacetate (DCFH-DA) dye, a fluorescent probe, for a duration of 30 min in a lightless environment at 37°C. Following this, the cells were examined using a fluorescence microscope (Khan *et al*., 2022).

2.5 *In vitro* antidiabetic assay

2.5.1 α-amylase inhibitory assay

The standard procedure with a slight modification was employed to assess the α-amylase inhibitory activity of the plant extract. Different concentrations of the plant extract and the isolated compound (20, 49, 60, 80, 100 µg/ml) were prepared from a 1 mg/ml standard phosphate buffer solution. Each sample (250 µl) was incubated with 250 µl of α-amylase solution for a duration of 10 min at a temperature of 28°C. Following this, 250 µl of starch solution was introduced and incubated for an additional 10 min. To halt the reaction, a color reagent consisting of dinitro salicylic acid (0.5 ml) was introduced, after which the mixture was subjected to a 10 min heating in a boiling water bath. Following the heating process, the solution was permitted to undergo a cooling phase. Subsequently, it was thinned by introducing 5 ml of distilled water. In preparation for every test sample concentration, a blank solution was readied by replacing the enzyme with a buffer. Alongside, a control was up held where in the sample addition was omitted; this control stood to depict the enzyme activity at 100%. Measurement of absorbance for the solution's color was carried out at a wavelength of 540 nm. Notably, the positive control for this investigation involved the use of acarbose (Gaurav *et al*., 2020).

% α-amylase inhibition = Control (Abs) – Sample (Abs)/Control (Abs) × 100

2.5.2 α-glucosidase inhibitory activity

The plant extract was found to contain perillic acid and strychnine, both of which demonstrated α-glycosidase inhibitory properties. To assess this inhibition, the extract was tested at various concentrations (20, 40, 60, and 80 µg/ml). A volume of 0.2 ml of each concentration was combined with an assay mixture consisting of 0.3 ml of 10 mM p-nitrophenyl α-D-glucopyranoside, 1.0 ml of 0.1 M potassium phosphate buffer (pH: 6.8), and 0.2 ml of enzyme solution. The mixture was then incubated for 30 min at a temperature of 28°C. To complete the reaction, 2.0 ml of 100 mM sodium carbonate was added. The resulting reaction was analyzed by measuring the absorbance at 400 nm using a spectrophotometer. For comparative purposes, a positive control, acarbose, was introduced. This control allowed the assessment of inhibitory activity of the samples. The inhibitory activity of the plant extract was quantified by calculating the percentage of inhibition. This provided a measure of the α-glucosidase inhibitory potential of the extract, with acarbose serving as a reference for comparison (Gaurav *et al*., 2020).

α-glucosidase inhibition % = [Control (Absorbance at 540 nm) – Sample (Absorbance at 540 nm) / Control × 100

2.6 Real time polymerase reaction for gene expression analysis

2.6.1 Matrix metalloproteinase 2

The RT-PCR procedure was carried out using a standard protocol with minor adjustments. In the initial steps, RNA extraction and cDNA synthesis were performed utilizing the Transgene Biotech ER 105-02 kit. To begin, 1 µg of RNA from various concentrations of plant extract (15.625, 31.25, and 62.5 µg/ml) that had been exposed to C2C12 cell lines, was introduced into an RNAse-free tube. Subsequently, 1 µl of DNase I along with 1 µl of enzyme buffer was added, and the mixture was incubated at 37°C for a duration of 30 min. Following this, 1 µl of 50 mM EDTA was introduced to deactivate the enzyme, and the mixture was then incubated at 65°C for 10 min.

For evaluation of gene expression, the SYBR green was applied using prime script TM RT reagent kit. The reference gene was GADPH (F: 5⁻- GAAGGTGAAGGTCGGAGTC- 3⁻ and R: 5⁻ - GAAGATGGT GATGGGATTTC-3⁻). The RTPCR conditions included are 1µl DNA (50 ng), 27 µl primer (200 nm), 10 µl SYBR green master mix and 7 µl deionised water (Artika *et al*., 2022; Caballero-Solares *et al*., 2022).

2.6.2 Apoptosis analysis by RT- PCR and ETBr/acridine orange staining

The apoptosis process on C2C12 cells that are exposed to plant extract at different concentrations for 24 h (15.62, 31.25, 62.5 and 125 µg/ml). The cycling conditions used for gene amplication are heat denaturing step at 95°C for 10 min, 45 cycles each at 95°C for 20 s, annealing at 58°C for 20 s and elongation period at 72°C for 20 s.

The primers used for gene amplification were Bax F- 5Œ§ TGCTTCAGGGTTTCATCCAG 3- R-52 GGCGGCAATCATC CTCTG32, p53 F-52 CCCAGCCAAAGAAGAAACCA32 , R-52 TTCCAA GGCCT CATT CAGCT32 , Caspase-3 F-52 ACAT GGCGTGTCATAAAATACC32 R-52 CACAAAGCGACTG GATGAAC3-. These collective genes were controlled to normalise the expression levels of p55, caspase and Bax. Partially, the cell samples subjected to RT-PCR analysis were then subjected to standard ETBr/ acridin orange staining protocol to observe the apoptotic cells morphological changes (Ahagh *et al*., 2019; Mahdavi *et al*., 2015).

3. Results

In this study, after getting isolation of the compounds their characterization, the process was performed to evaluate the *in vitro*

cell viability assay and the antioxidant activity. Thereafter, RT-PCR studies were conformed to determine biomolecular changes in the cells against the diabetic pathophysiological changes.

3.1 MTT assay

The efficacy of the drugs in providing cytoprotection was evaluated through an MTT assay. This analysis involved exposing cells to various concentrations of the samples. The findings revealed that the drugs conferred a safe guarding effect against the harmful impacts of the toxic substance, shielding approximately 94.32% of the cells from its deleterious effects. Furthermore, the results of the study have been summarised on the Table 1 while morphological analysis, cell viability, cytotoxicity and IC_{50} of the cells has been depicted in the Figures 1-4.

Table 1: Antioxidant activity by MTT assay

S.No.	Sample	Treated conc. µg/ml	Absorbance at 570 nm (ELISA reader)	Percentage of C2C12 cell viability	Percentage of cytotoxicity
1.	Plant extract	15.62	1.051 ± 0.27	94.32	5.68
		31.25	0.975 ± 0.29	73.45	26.55
		62.50	0.768 ± 0.30	40.35	59.65
		125	0.652 ± 0.52	20.31	79.69
		250	0.427 ± 0.35	16.25	83.75
2.	Metformin (control)	100 (ng/ml) (35:5:60, v/v)	0.105 ± 0.32	99.14	0.86

The provided values represent the average and standard error (± SE) from three separate measurements.

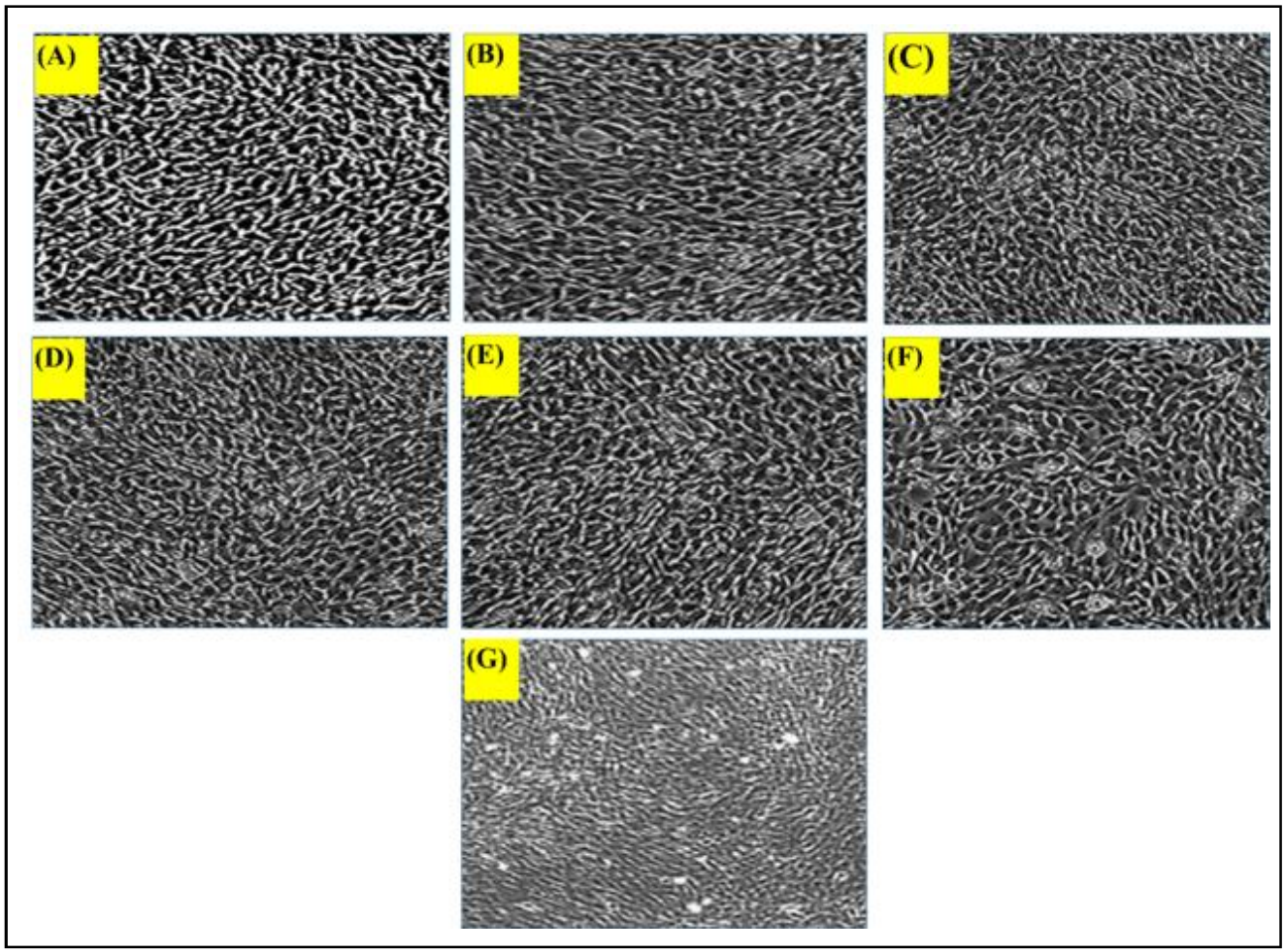

Figure 1: The morphological appearance of C2C12 cells, which exposed to different concentrations of plant extract and compared with control metformin through MTT assay. (A): Control (untreated) C2C12 cells (B): 15.625 µg ml^{-1} (C): 31.25 µg ml^{-1} (D): 62.50 µg ml^{-1} (E): 125 µg ml^{-1} (F): 250 µg ml^{-1} (G): metformin.

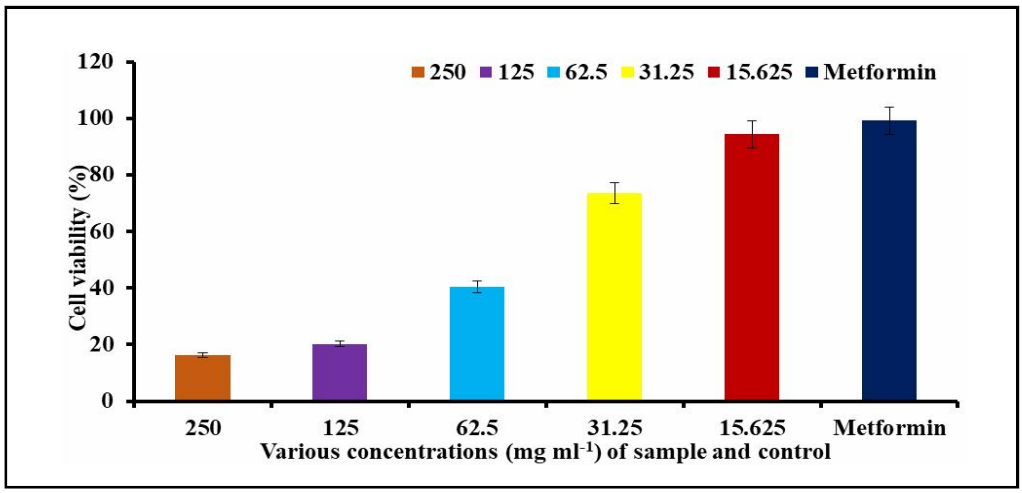

Figure 2: The cell viability of C2C12 cells was assessed after exposure to various concentrations of plant extract as well as a control group. The provided values represent the average along with the standard error (± SE) and were obtained from three separate replicates.

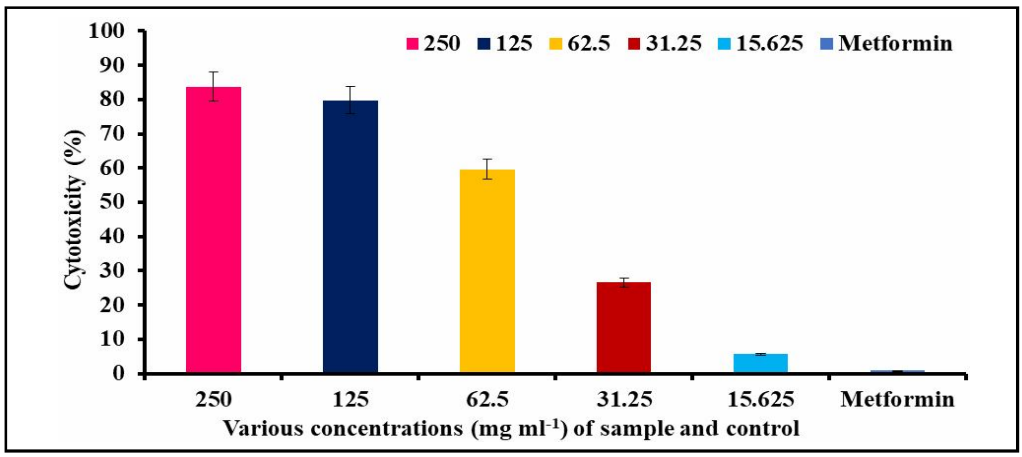

Figure 3: The cytotoxicity percentage of C2C12 cells was evaluated upon exposure to varying concentrations of a plant extract, along with a control group. The provided values represent the average, as well as the standard error (± SE), based on three independent replicates.

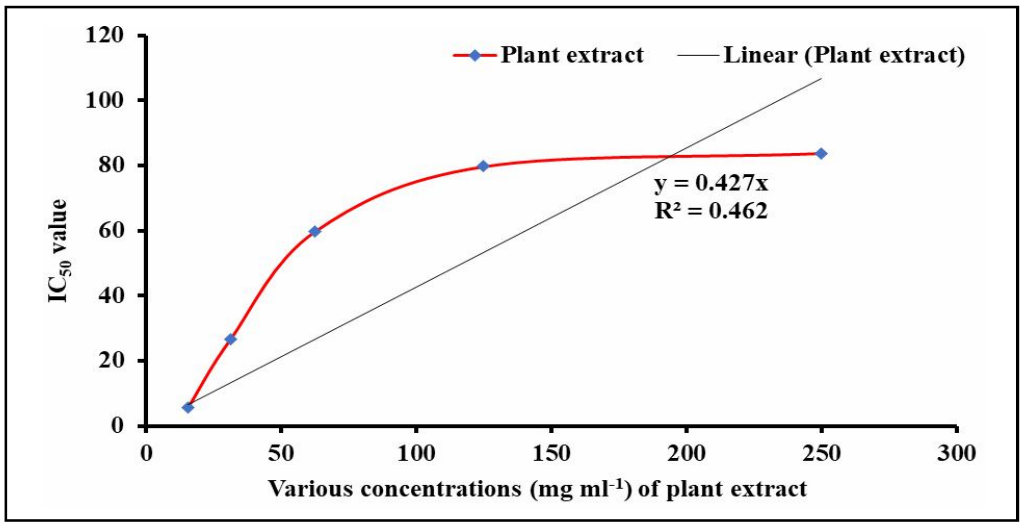

Figure 4: IC_{50} values of the botanical extract were assessed using the MTT assay in conjunction with C2C12 cell lines.

Furthermore, it is determined that various concentrations (15.625, 331.25,62.50, 125 and 250 µg/ml) of plant extract showed low cytotoxic C2C12 cells at 250 µg/ml and showed as 83.75 ± 2.5 of cytotoxicity and the OD was in the range of 1.051 ± 0.27 to 0.427 ± 0.35 at 15.625 to 250 µg/ml concentrations, respectively. The lower number of dead cells due to membrane blabbing and a greater number of viable cells were noted in the microscopic images (Figure 7) (Gaurav *et al*., 2020). There was considerable viability recorded at the concentration of 250 mg/ml of plant extract (Figure 4). Correspondingly, the Figure 4 represents the cytotoxicity percentage of C2C12 cells. The IC_{50} value of the plant extract was calculated as 108.22 mg/ml. The microphotograph showed that the increased cytotoxicity on C2C12 cell line with increased concentration of plant extract (250 µg/ml).

3.2 Reactive oxygen species assay

Furthermore, it has been demonstrated from Table 2 and Figure 5, The ROS level was not considerably increased in C2C12 cells exposed different concentrations of plant extract, and slight ROS was formed at a concentration of 125 µg/ml as evident from the images as shown above. It was correlated with the values as represented in the graph as percentage of ROS formation in C2C12 cells. Effect of drugs against reactive oxygen species has been summarised in Table 2, while Figures 5 and 6 revealed the graphical and morphological representation of cells.

Table 2: Effect of drugs against reactive oxygen species

S.No.	Concentration in µg/ml	ROS production in C2C12 Cell lines
1	Untreated cells	103.89 ± 0.614
2	Plant extract -15.625	118.5 ± 0.65
3	Plant extract- 31.25	113 ± 0.49
4	Plant extract- 62.5	107.1 ± 0.86
5	Plant extract- 125	26.5 ± 0.65
6	Metformin -100 ng/ml	21.34 ± 0.151

Mean ± SD The average ± standard deviation (SD) of reactive oxygen species (ROS) generation was measured in C2C12 cells following exposure to different concentrations of plant extract and metformin.

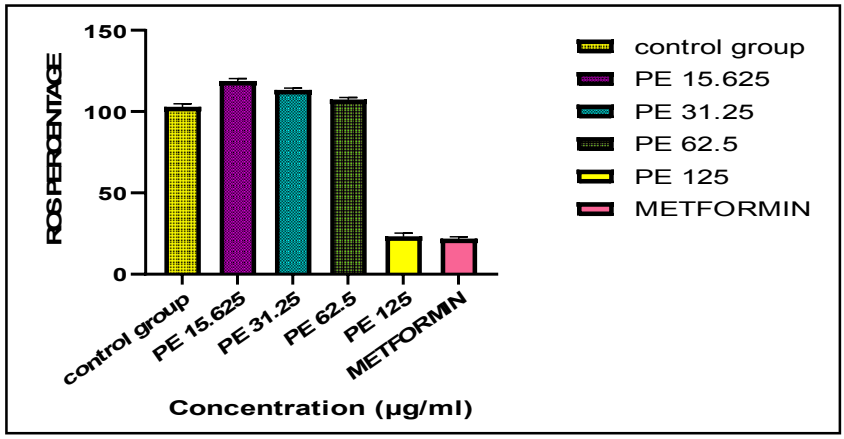

Figure 5: The generation of reactive oxygen species (ROS) was examined in C2C12 cell lines following exposure to different concentrations of plant extract and metformin.

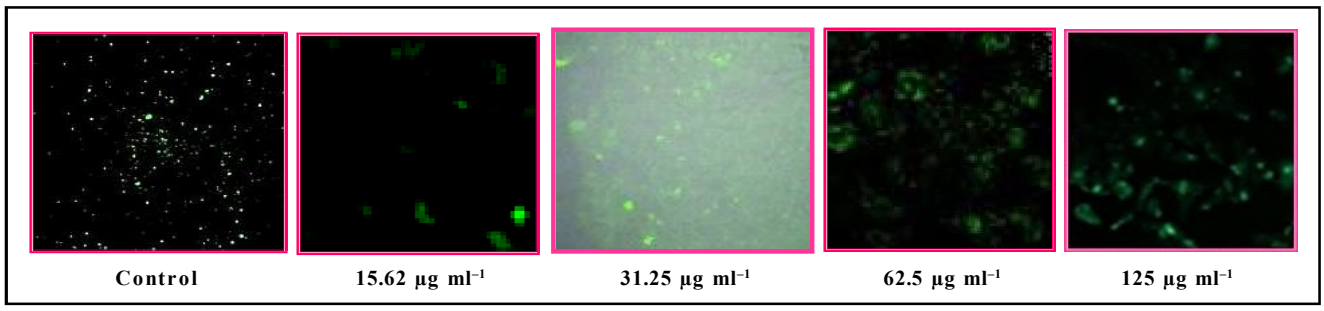

Figure 6: ROS Production in C2C12 cell lines was assessed following exposure to various concentrations of plant extract and metformin. The presence of a green hue in the C2C12 cells indicated the generation of ROS after exposure to distinct plant extract concentrations. When the plant extract was administered at a higher concentration (125 µg/ml), there was observed a minor occurrence of ROS generation independently (Gautham *et al*., 2021).

3.2.1 α-amylase inhibitory assay

Alpha amylase, functioning as a calcium metalloenzyme, plays a vital role in digestion by catalysing the breakdown of complex polysaccharides into simpler sugars like glucose and maltose (Ibrahim *et al*., 2021, Insaf *et al*., 2022). Notably, it contributes to the occurrence of postprandial hyperglycaemia, leading to elevated blood glucose levels. Consequently, targeting alpha amylase has emerged as a recognized approach for managing postprandial blood glucose levels. Several inhibitors, including acarbose, have demonstrated effectiveness in modulating the activity of this enzyme (Khan *et al*., 2021). Various concentrations of plant extract, perillic acid, strychnine and acarbose (20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml) were exhibits potent alpha amylase inhibitory activity. The IC_{50} values for plant extract, perillic acid and strychnine were found be similar like that of the IC_{50} values of acarbose. By the alpha amylase, the plant extract and the isolated compounds perillic acid and strychnine were exhibits potent antidiabetic activity similar to that of acarbose. α-amylase, inhibitory activity of isolated compounds of plant extract and acarbose has been summarised in the Table 3 while α-amylase inhibitory activity of isolated compounds of plant extract and acarbose has been depicted in Figure 7.

Table 3: Efficacy in managing diabetes through inhibition of α-amylase

S.No.	Concentration (µg/ml)	α-amylase inhibitory activity (% of inhibition)			
		Plant extract	Perillic acid	Strychnine	Acarbose
1.	20	21.01	22.06	24.08	52.85
2.	40	24.35	26.35	29.87	60.27
3.	60	31.24	33.45	34.57	68.95
4.	80	43.51	45.67	48.86	79.24
5.	100	49.12	52.23	55.45	92.34

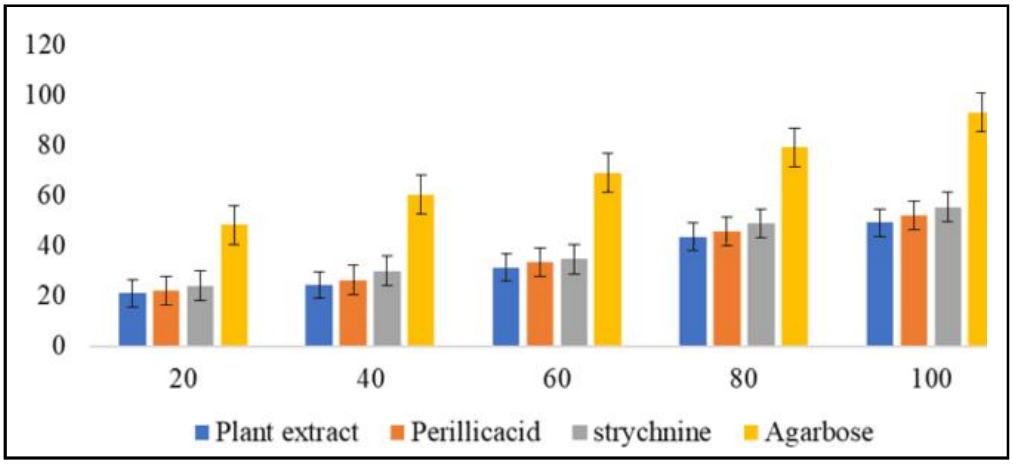

Figure 7: α-amylase inhibitory activity of isolated compounds of plant extract and acarbose.

3.2.2 α-glucosidase inhibitory activity

α-glucosidase is an enzyme which is responsible for causing the type 2 diabetes mellitus. It causes diabetes by converting the polysaccharides like starch into the monosaccharides like glucose and dextrose. Alpha glucosidase inhibitors such as acarbose and voglibose function by diminishing the absorption of glucose (Khan *et al*., 2022). Researchers evaluated the impact of individual compounds from a plant extract on inhibiting alpha glucosidase enzyme activity. The study demonstrated a dose-dependent effect, with perillic acid, strychnine, and the plant extracts exhibiting noteworthy antidiabetic activity similar to acarbose at concentrations of 100 µg/ml. The study also detailed the á-glucosidase inhibitory potential of both the isolated compounds from the plant extract and acarbose itself in Table 4 and Figure 8.

Table 4: The effectiveness of individual components from a plant extract and acarbose in inhibiting α-glucosidase activity was evaluated

S. No.	Concentration (µg/ml)	α-glucosidase inhibitory activity (% of inhibition)			
		Plant extract	Perillic acid	Strychnine	Acarbose
1	20	20.04	34.06	42.08	55.25
2	40	25.37	38.35	53.87	63.18
3	60	28.65	45.45	64.57	69.34
4	80	33.52	52.67	72.86	74.28
5	100	41.29	63.23	82.45	87.95

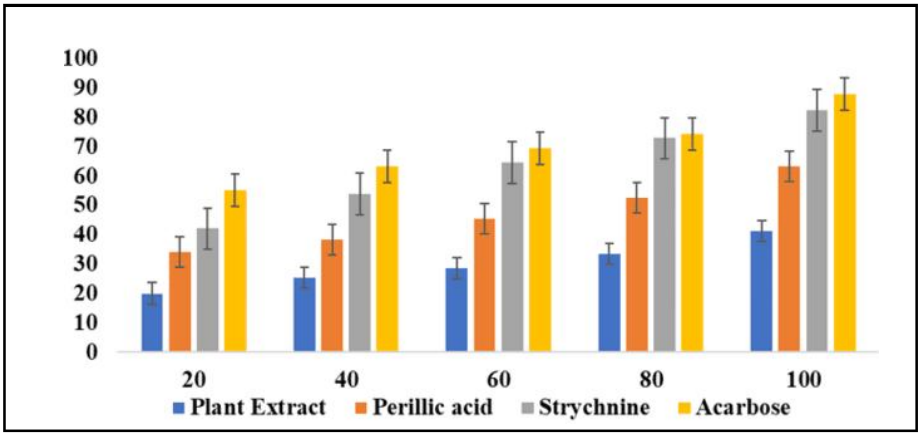

Figure 8: The study focused on evaluating the ability of individual compounds from a plant extract and acarbose to inhibit α-glucosidase activity RT PCR for gene expression.

3.2.3 Matrix metalloproteinase 2

Matrix metalloproteinases (MMPs) constitute a group of proteases that necessitate zinc to operate effectively. These enzymes have the ability to degrade various constituents of the extracellular matrix. The precise functioning of these proteases is meticulously regulated through the influence of molecules recognized as tissue inhibitors of metalloproteinases. Table 5 represents the changes in the folds of the gene expression based on the concentrations of the plant extracts. At the concentration of 62 µg/ml of plant extract shows the significant change similar to that of the antidiabetic drug metformin. C2C12 cells are the mouse myeloblast which are used to study the insulin resistance. The changes in the folds of gene expression by MMP2 on C2C12 cells has been summarised in Table 5, Figures 9 and 10.

Table 5: Alterations in the patterns of gene expression induced by MMP2 in C2C12 cells

S. No	Concentration (µg/ml) of the sample	MMP2 (changes in fold)
1	Plant extract- 15.625	0.624 ± 0.019
2	Plant extract- 31.25	0.872 ± 0.037
3	Plant extract- 62.50	1.054 ± 0.064
4	Control (metformin 100 ng/ml)	1.184 ± 0.052
5	Untreated cell	0.486 ± 0.056

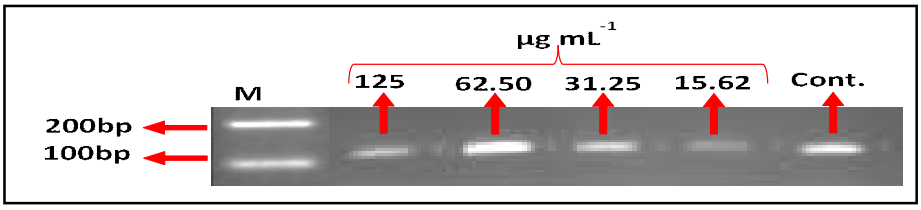

Figure 9: Gene expression of MMP-2 in C2C12 cells when exposed to varying concentrations of plant extract.

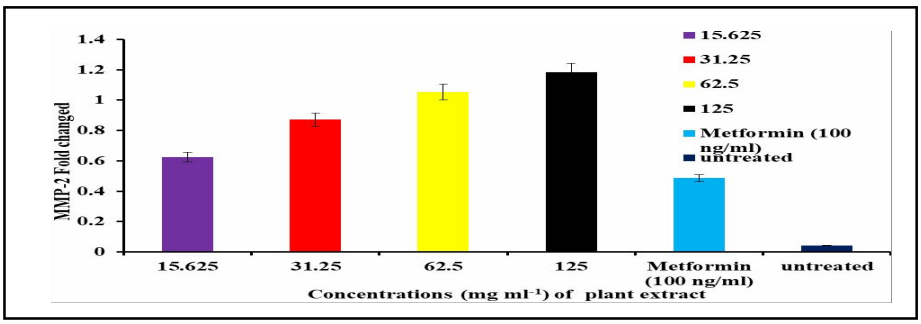

Figure 10: Alterations in the MMP-2 gene expression were observed within C2C12 cells subsequent to exposure to varying concentrations of plant extract.

3.2.4 Apoptosis analysis by RT- PCR and ETBr/acridine orange staining

Apoptosis of the beta cells will lead to the diabetes mellitus. In the context of type 1 diabetes (T1DM), immune-related mechanisms lead to the destruction of β-cells, whereas in type 2 diabetes (T2DM), metabolic irregularities play a role in the decline of β-cell function, eventually resulting in apoptosis (Meenakshi *et al*., 2018). Irrespective of the specific diabetes type, common cellular pathways responsible for apoptosis initiation are activated. In an upcoming review, the authors will elucidate the intricate molecular factors governing β-cell apoptosis and the internal pathways that become operational in this process (Mehrotra *et al*., 2020). The apoptotic gene markers like caspase -3 P53 and Bax were studied in the present study. The plant extract at the concentration of 125µg/ml shows the significant changes in the biomarkers of apoptosis. Thus, shows the significant antidiabetic activity. The fluorescence microscopic image and quantitative analysis of mRNA level fold-changes of apoptotic genes such as p53, bax, and casp3 on C2C12 cells exposed to different concentrations of plant extract have been depicted in Figures 11 and 12.

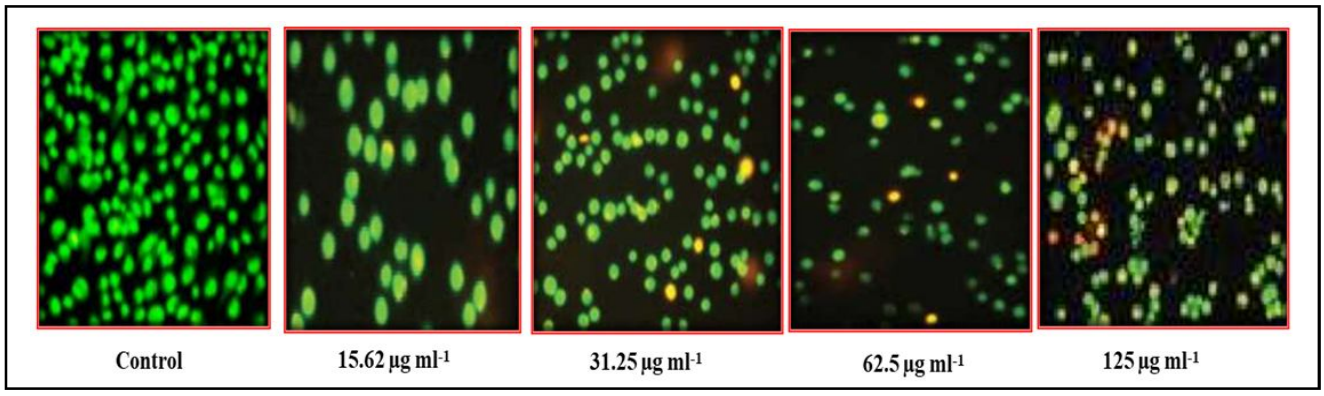

Figure 11: The fluorescence microscopy picture illustrates cellular contraction and the emergence of bulges on the cell membrane, both linked to apoptosis as detected through acridine orange and ethidium bromide staining techniques.

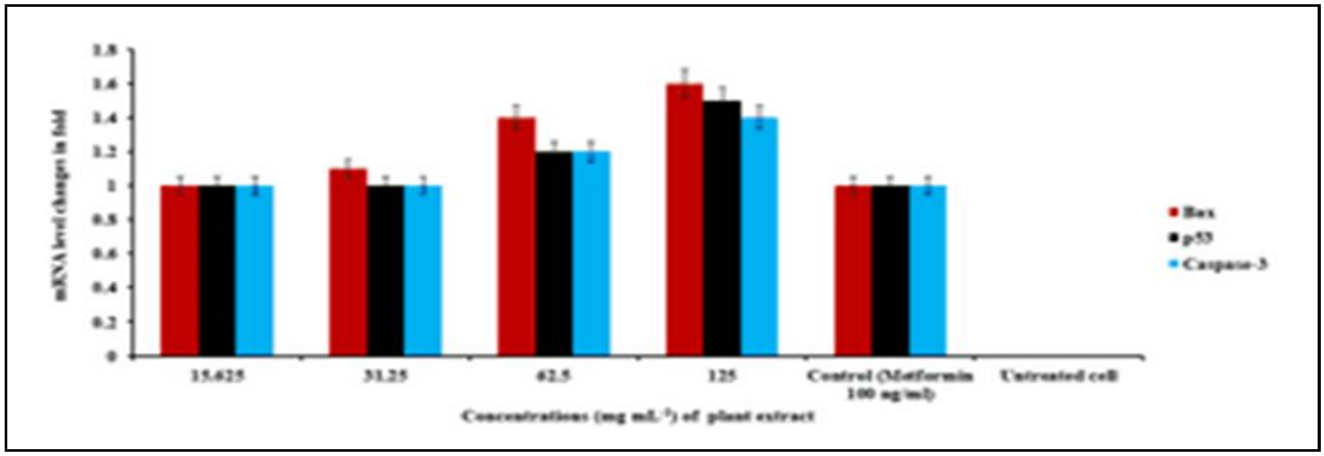

Figure 12: Quantitative analysis of mRNA level fold-changes of apoptotic genes such as p53, bax, and casp3 on C2C12 cells exposed to different concentrations of plant extract.

Table 6: Quantitative analysis of mRNA level fold-changes of apoptotic genes such as p53, bax, and casp3 on C2C12 cells exposed to different concentrations of plant extract. Mentioned values are mean and standard error of triplicates

Concentration of samples (µg/ml)	Apoptosis marker genes expression (mRNA level fold changed)		
	Bax	p53	Caspase-3
Plant extract- 15.625	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Plant extract-31.25	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.0
Plant extract- 62.5	1.4 ± 0.6	1.2 ± 0.3	1.2 ± 0.3
Plant extract- 125	1.6 ± 0.3	1.5 ± 0.14	1.4 ± 0.2
Control (metformin 100 ng/ml)	1± 0.35	1 ± 0.21	1 ± 0.21
Untreated cell	0.40 ± 0.02	0.37 ± 0.01	0.55 ± 0.05

4. Discussion

Medicinal plants exhibit a significant role in alleviation of various acute and chronic ailments and thus contributing an immense role in health care system (Mehrotra, 2020; Venkatachalam *et al*., 2021). The findings from this study highlight the remarkable potential of the ethanolic extract of *H. laevigata* as well as the isolated compounds perillic acid and strychnine, in exhibiting potent antioxidant and antidiabetic activities. These results shed light on the valuable role that natural compounds derived from plants can play in addressing the global health challenges posed by diabetes and oxidative stress-related disorders. The discovery of potent antioxidant activity within the ethanolic extract of *H. laevigata* is particularly noteworthy. Oxidative stress is a significant contributor to the development and progression of various chronic diseases, including diabetes (Amarnath Satheesh and Pari, 2004; Singh and Chaudhuri, 2018). The ability of this plant extract to combat oxidative stress may offer a novel therapeutic avenue for preventing and managing diabetes and its associated complications. Moreover, the isolation of perillic acid and strychnine as key bioactive components further supports the potential health benefits of *H. laevigata*. MTT assay showed that 94.32% cells were protected against the deleterious effect of the toxicant.

In the present study, C2C12 myoblast cell lines isolated from mouse skeletal muscle were used. C2C12 cell lines can facilitate the glucose metabolism, insulin signaling mechanism, insulin resistance, oxidative stress and glucose transporters at cellular and molecular levels. Plasma insulin levels are increased in the normal individual in response to blood sugar level after meals. Adipose tissue and the skeletal muscle will play a major role in the metabolism of the glucose. Glut-4 protein (glucose transporter) is highly expressed in C2C12 cell lines which is used for investigations of insulin resistance and the glucose uptake (Chun. Y. Wong *et al*., 2020)

The observed antidiabetic activity of perillic acid, strychnine, and the plant extracts presents a promising prospect for addressing the multifaceted nature of diabetes. The fact that these natural compounds demonstrate comparable or even superior activity to the established alpha amylase and alpha glucosidase inhibitor, acarbose, is particularly encouraging. Alpha amylase and alpha glucosidase inhibitors are widely used in diabetes management to control postprandial hyperglycemia. The discovery of alternative compounds from natural sources that exhibit similar or better inhibitory effects suggests a potential expansion of therapeutic options.

Furthermore, it is determined that various concentrations (15.625, 331.25,62.50, 125 and 250 µg/ml) of plant extract showed low cytotoxic C2C12 cells at 250 mg/ml and showed as 83.75 ± 2.5 of cytotoxicity and the OD was in the range of 1.051 ± 0.27 to 0.427 ± 0.35 at 15.625 to 250 µg/ml concentrations, respectively. The ROS level was not considerably increased in C2C12 cells exposed different concentrations of plant extract, and slight ROS was formed at a concentration of 125 mg/ml.

Alpha amylase stands out as a recognized focal point in therapeutic endeavours aimed at managing and stabilizing blood glucose levels after meals. A range of inhibitors, including acarbose, have demonstrated their efficacy in selectively addressing this particular enzyme (Khan *et al*., 2021). Various concentrations of plant extract, perillic acid, strychnine and acarbose (20 µg/ml, 40 µg/ml, 80 µg/ml, 100 µg/ml) were exhibits potent alpha amylase inhibitory activity. The IC_{50} values for plant extract, perillic acid and strychnine were found be similar like that of the IC_{50} values of acarbose. By the alpha amylase, the plant extract and the isolated compounds perillic acid and strychnine were exhibits potent antidiabetic activity similar to that of acarbose (Mukhtar *et al*., 2018).

The destruction of beta cells through apoptosis is a critical factor in the development of diabetes mellitus. In the case of type 1 diabetes (T1DM), immune-related mechanisms result in the elimination of β-cells, while type 2 diabetes (T2DM) involves metabolic irregularities that lead to β-cell dysfunction and eventual apoptosis (Meenakshi *et al*., 2018). Despite the distinction between diabetes types, the process of apoptosis involves consistent intracellular pathways. A comprehensive overview of these molecular agents responsible for β-cell apoptosis and the subsequent activation of intracellular pathways will be presented (Mehrotra *et al*., 2020). The apoptotic gene markers like caspase -3 P53 and Bax were studied in the present study. The plant extract at the concentration of 125 µg/ml shows the significant changes in the biomarkers of apoptosis and thus shows the significant antidiabetic activity.

However, the translation of these findings into clinical applications warrants careful consideration and further investigation. While the results provide a strong foundation for the potential benefits of *H. laevigata* and its isolated compounds, the study's experimental context and methodology must be critically evaluated. Factors such as bioavailability, dosage, safety profile, and potential side effects need to be rigorously assessed before these compounds can be recommended for human use. Additionally, studies involving animal models and clinical trials are crucial to ascertain the efficacy and safety of these compounds in a real-world setting (Abeysinghe *et al*., 2021).

Furthermore, the mechanistic insights into how perillic acid and strychnine exert their antidiabetic effects remain an area of interest. Understanding the underlying molecular pathways and cellular mechanisms can provide valuable knowledge for drug development and therapeutic strategies (Mukhtar *et al*., 2018). It is also essential to explore potential synergistic interactions between these compounds and conventional antidiabetic drugs to determine if they can enhance the overall efficacy of diabetes management.

5. Conclusion

The study findings indicate that the ethanolic extract derived from H. *laevigata*, along with its individual components perillic acid and strychnine, demonstrate strong antioxidant and notable antidiabetic properties. Notably, perillic acid, strychnine, and the plant extracts displayed significant antidiabetic effects, outperforming the alpha amylase and alpha glucosidase inhibitor acarbose in the study.

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Conflict of interest

The authors declare no conflict of interest relevant to this article.

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