"Unveiling the Anti-Diabetic Potential of *Lindernia ciliata by using in-vitro methods*: Phytochemical Insights and Mechanistic Pathways"

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Abstract

Objective:
To evaluate the phytochemical screening and anti-diabetic activity of different fractions obtained from the hydroalcoholic extract of *Lindernia ciliata* (Colsm.) Pennell.

Methodology:
The hydroalcoholic extract of *L. ciliata*, a member of the *Linderniaceae* family, was fractionated using petroleum ether, chloroform, methanol, and water. These fractions were analyzed for secondary metabolites through phytochemical screening, and the total phenolic and flavonoid contents were determined. The anti-diabetic activity of the fractions was assessed using multiple assays, including glucose absorption, glucose uptake, insulin secretion by RIN-5F cells, α-amylase inhibition, and DPP-4 inhibition.

Results:
Among the different fractions, the ethanol fraction of the hydroalcoholic extract exhibited significant anti-diabetic potential by enhancing glucose uptake and insulin secretion in RIN-5F cells. Additionally, it demonstrated notable inhibition of DPP-4 and α-amylase, indicating its role in glucose metabolism regulation.

Conclusion:
These findings suggest that *L. ciliata* possesses promising anti-diabetic activity, supporting its potential application in the development of herbal-based pharmaceuticals for diabetes management. Further research is required to elucidate its molecular mechanisms and active phytoconstituents responsible for its therapeutic effects.

Keywords: *L. ciliata;* Diabetes Mellitus; Glucose uptake assay; Insulin secretion; RIN-5F cells

1. Introduction

Diabetes is a prevalent endocrine and metabolic disorder and a major contributor to illness and mortality, particularly in industrialized nations. Its complications are classified into microvascular issues, such as retinopathy, neuropathy, and nephropathy, as well as macrovascular conditions, including myocardial infarction, stroke, and peripheral vascular disease (Jha et al., 2019). This condition significantly affects an individual's physical health, social interactions, and

psychological well-being (Dewanjee et al., 2009). Type 2 diabetes, the most prevalent form, results primarily from insufficient insulin secretion and insulin resistance, leading to elevated blood glucose levels (Jangid et al., 2017). Controlling postprandial hyperglycaemia—spikes in blood sugar following meals—is a crucial strategy in diabetes management, as it contributes to the progression of Type 2 diabetes and its associated complications (Sharma & Sidhu, 2014a).

Indian traditional medicine has long utilized medicinal plants for diabetes management, as they enhance insulin secretion, slow glucose absorption, and regulate insulin-dependent metabolic pathways (Sharma & Sidhu, 2014b). Recognizing the therapeutic potential of natural antidiabetic agents, the World Health Organization (WHO) has recommended further research into plant-based treatments (Pattabiraman & Muthukumaran, 2011). In Assam, India, *Lindernia ciliata* (locally known as Kachidoria) has been traditionally used for diabetes management (Barukial & Sarmah, 2011; (Mondal et al., 2013). This study aims to scientifically evaluate the effects of *L. ciliata* on glucose absorption, cellular uptake, insulin release, alpha-amylase inhibition, and DPP-4 activity in diabetic rats. By integrating traditional knowledge with scientific validation, this research seeks to advance the development of plant-based therapies for diabetes management.

2.Material and methods:

2.1. Collection of Plant Materials, Animals and approval from the animal ethical committee

Whole plants of *L. ciliata* were collected from the fields of Baghmara, District-Bajali, Assam, India (PIN-781328) between January and March 2019. The plant was authenticated by the Botanical Survey of India (BSI), Eastern Regional Centre, Shillong, and identified as *Lindernia Ciliata* (Colsm.) Pennell, belonging to the family *Linderniaceae* (Authentication Letter No. BSI/ERC/Tech/2019/101, dated 02/05/2019).

Animals and approval from the animal ethical committee:

We obtained healthy, nulliparous Wistar albino rats weighing 150-200g from the "College of Pharmaceutical Sciences, Dayananda Sagar University" animal house facility. The tests were carried out after receiving previous approval from Dayananda Sagar University's Institutional Animal Ethical Committee (IAEC) (Approval number: DSU/Ph.D/IAEC/39/2019-20).

2.2. Preparation of Hydroalcoholic Extracts

The collected plant material was shade-dried and ground into a coarse powder before undergoing cold maceration for seven days using 80% aqueous ethanol (v/v) as the extraction solvent. Fractionation was performed by suspending 30 g of *L. ciliata* powder in 50 mL of water, followed by successive partitioning with different solvents in the following order: petroleum ether (LC-Pf), chloroform (LC-Cf), and ethanol (LC-Ef). The remaining aqueous solution was designated as the aqueous fraction (LC-Af). Each fraction was subjected to lyophilization to obtain dry powder, which was subsequently suspended in a 0.3% carboxymethyl cellulose (CMC) solution for use in animal studies. These fractions were further analyzed for their phytochemical composition and potential antidiabetic activity.

2.3. Phytochemical Screening

Phytochemical screening of the hydroalcoholic extract fractions of *L. ciliata* was conducted to identify the presence of various bioactive compounds, including alkaloids, flavonoids, steroids/triterpenoids, carbohydrates, tannins, saponins, and phenolic compounds.

2.3.1. Determination of Total Phenolic Content and Total Flavonoid Content

Total Phenolic Content

The total phenolic content of the hydroalcoholic extract fractions of *L. ciliata* was determined using the Folin-Ciocalteu colorimetric method, as described by Momina et al. (2020). The extract (100–1000 µg/mL) or a standard solution of gallic acid (10–100 µg/mL) was added to a 25 mL volumetric flask containing 9 mL of distilled water. A reagent blank was prepared using distilled water in place of the sample. To this mixture, 1 mL of Folin-Ciocalteu phenol reagent was added and mixed thoroughly. After 5 minutes, 10 mL of 7% aqueous sodium carbonate solution was introduced, and the total volume was adjusted to 25 mL using double-distilled water. The solution was incubated at room temperature for 90 minutes, and the absorbance was measured at 760 nm against the reagent blank. The total phenolic content was quantified using gallic acid as the standard, and results were expressed as gallic acid equivalent (GAE) in milligrams per gram of extract (Momina & Rani, 2020).

Total Flavonoid Content

The total flavonoid content of the hydroalcoholic extract fractions of *L. ciliata* was estimated using the aluminum chloride colorimetric method, following the procedure outlined by Momina et al. (2020). The extract (10–100 µg/mL) or a standard catechin solution (10–100 µg/mL) was added to a 10 mL volumetric flask containing 4 mL of double-distilled water. To this mixture, 0.3 mL of 5% sodium nitrite solution was added, followed by 0.3 mL of 10% aluminum chloride solution after 5 minutes. At the 6th minute, 2 mL of 1M sodium hydroxide solution was introduced. The total volume was adjusted to 10 mL using double-distilled water, and the solution was mixed thoroughly. The absorbance was measured at 510 nm against a reagent blank. The total flavonoid content was calculated and expressed as milligrams of catechin equivalents per gram of extract (mg CE/g extract) (Momina & Rani, 2020).

2.4. Glucose Absorption

Wistar albino rats were euthanized, and their abdominal cavities were opened to extract the jejunum, located approximately 20 cm from the pylorus. The jejunum was inverted, and 5 cm segments were placed in an oxygenated Tyrode solution containing 342 mM NaCl, 6.7 mM KCl, 5.9 mM $CaCl_2 \cdot 2H_2O$, 5.3 mM $MgCl_2$, 59.5 mM $NaHCO_3$, 2.08 mM NaH_2PO_4, and 5.5 mM glucose. Each segment was filled with 0.5 mL of Tyrode solution, sealed at both ends to form sacs, and incubated in 15 mL of Tyrode solution containing the fractions of *L.ciliata*. Incubation was performed at 37°C in an atmosphere of 95% O_2 and 5% CO_2 for 60 minutes. The test substances included fractions of hydroalcoholic extract of *L. ciliata* at concentrations of 0.5, 1, and 2 mg/mL, with acarbose (1.5 and 3 mM) as the positive control and plain Tyrode solution as the negative control. Glucose levels in the Tyrode solution were analyzed before and after incubation.

The amount of glucose transported into the intestinal sacs was calculated using the following formula:

Amount of glucose transported = [(GB – GS) × 198.17] × 100

where GB is the initial glucose concentration, and GS is the glucose concentration outside the sac after incubation (Hassan et al., 2010).

2.5. Glucose Uptake

Wistar albino rats were sacrificed, and the abdominal cavity was opened to expose the muscles. Small muscle sections, weighing 90–150 mg each, were excised from both sides and placed in Kreb's-Ringer bicarbonate (KRB) buffer containing 118 mM NaCl, 5 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$. The buffer was oxygenated with 95% O_2 and 5% CO_2 at 37°C, and the muscle sections were pre-incubated in the buffer with ventilation for 10 minutes. The KRB buffer was then replaced with a solution containing 11.1 mM glucose, and baseline samples were collected for glucose concentration measurement. The test solution contained 1 mg/mL of the hydroalcoholic extract fractions of *L. ciliata* or 1 mg/mL of metformin, either with or without 100 mU/mL insulin. Following pre-aeration for 5 minutes, the tissues were incubated in the treated KRB buffer for 30 minutes at 96 rpm. Post-incubation samples were collected, and glucose content was analyzed using a glucose analyzer. Muscle masses were recorded for data normalization (Gray & Flatt, 1998; (Perez et al., 2000)).

2.6. Insulin Release

RIN-5F cells were cultured in RPMI 1640 medium supplemented with essential nutrients. The cells were regularly passaged and seeded into 24-well Nunclon multiwell plates at a specified density. Insulin secretion was assessed following the method described by Gray and Flatt (1998, 1999). For insulin release measurement, the cells were washed multiple times with KRB buffer containing various components and preincubated at a set temperature for a defined period. Subsequently, the cells were exposed to KRB containing glucose along with different concentrations of hydroalcoholic extract fractions of *L. ciliata* or varying concentrations of glibenclamide. After incubation, small samples were collected from each well and stored at a designated temperature for insulin assay. Insulin levels were quantified using a rat insulin ELISA kit (Crystal Chem, USA), following the methodology outlined by Hassan et al. (2010) (Hassan et al., 2010).

2.7 Alpha-Amylase Inhibition Assay

The alpha-amylase inhibition assay was performed using a modified McCue and Shetty protocol. A stock solution was prepared by dissolving 10 mg of each fraction of hydroalcoholic extract *L. ciliata* in 1 mL of DMSO. A reaction mixture containing 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) with α-amylase solution (0.5 mg/mL) was

added to a tube containing 250 µL of extract (1.25–10 mg/mL). After pre-incubation for 10 minutes at 25°C, 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was introduced at intervals. The mixture was incubated for another 10 minutes at 25°C, and the reaction was terminated by adding 500 µL of Dinitrosalicylic Acid (DNS) reagent. The tubes were then incubated in boiling water for five minutes, followed by cooling to room temperature. The reaction mixture was diluted with 5 mL of distilled water, and absorbance was measured at 540 nm. A control was prepared using the same procedure, substituting the extract with distilled water (McCue & Shetty, 2004).

The percentage of inhibition was calculated using the formula:

% Inhibition = [(Absorbance control - Absorbance extract) / Absorbance control] × 100

2.8.DPP-4 Inhibition

DPP-4 activity was measured using a chromogenic assay with glycine-proline-p-nitroaniline (Gly-Pro-PNA) as the substrate. The reaction mixture included the enzyme, substrate, positive control (Ile-Pro-Ile), and Tris buffer at pH 8.2. Pre-incubation was conducted at room temperature for 30 minutes. In a 96-well microtiter plate, sequential additions of the positive control (0.29 mmol/L) or fractions of hydroalcoholic extract *L. ciliata*, DPP-4 enzyme (10 U/L), Tris buffer (pH 8.2), and substrate (4.68 mmol/L) were performed. The plate was incubated for 2 hours at room temperature, and absorbance was recorded at 405 nm using a Thermo Scientific Multiskan Ascent microplate reader (Geng et al., 2013).

The inhibition activity was calculated by the equation:

Inhibition (%) = [(A Negative control − A control) − (A sample − A sample control)]/ (A Negative control − A control) × 100%

4. Results:

The % yield of different fractions of Hydroalcoholic extracts of *L.ciliata* are presented in figure 1.

Extract	% yeild

Petroleum ether extract (lc-pf)	2.11%
Chloroform extract (lc-cf)	3.24 %
Ethanolic extract (lc-ef)	11.8 %
Aqueous extract (lc-af)	7.24 %

Figure 1: % yield of different fractions of hydroalcoholic extract of *Lindernia ciliata*

The Preliminary phytochemical investigation of the fractions of Hydroalcoholic extracts of *L.ciliata* revealed the presence of different phytochemicals which are been listed in Table 1.

Table 1: Results of preliminary phytochemical investigation

Class of chemical constituents	LC-Pf	LC-Cf	LC-Ef	LC-Af
Alkaloids (Dragendorff's	-	+	+	-
Flavonoids (Shinoda Test)	-	+	+	+
Steroids/triterpenoids (Noller's Test)	-	+	+	-
Carbohydrates (Fehling's Test)	-	-	+	+
Saponins (Foam Test)	+	+	+	+
Phenolic compounds Ferric chloride test.	-	+	+	+
Tannins(Neutral FeCl3)	-	-	+	-

+: Present , -: Absent

LC-Pf: Petroleum ether fraction of hydroalcoholic Extract of *L. Ciliata;* LC-Cf: Chloroform fraction of hydroalcoholic Extract of *L. Ciliata;* LC-Pf: Ethanol fraction of hydroalcoholic Extract of *L. Ciliata;* LC-Af: Aqueous ether fraction of hydroalcoholic Extract of *L. Ciliata*

Total Flavonoid content: Result expressed - mg of catechin equivalents per 100 g of dry weight of extract.

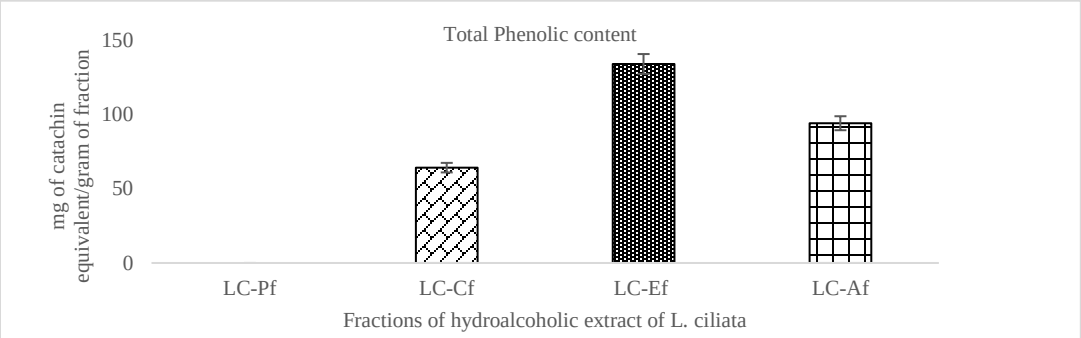

Figure 2: Total Phenolic content of fractions of hydroalcoholic extract of *L.ciliata*

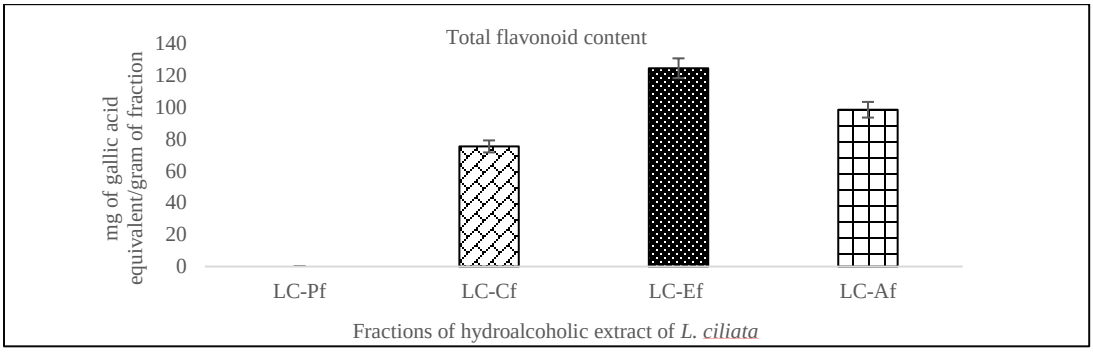

Figure 3: Total Phenolic content of fractions of hydroalcoholic extract of *L.ciliata*

4.1. Glucose absorption:

Figure 4 depicts the effect of acarbose and *L. ciliata* on glucose absorption in everted intestinal sac segments. Acarbose at a concentration of 3 mM significantly reduced glucose absorption, highlighting its inhibitory action compared to the control group. In contrast, treatment with *L. ciliata* fractions showed no noticeable impact on glucose absorption when compared to the control.

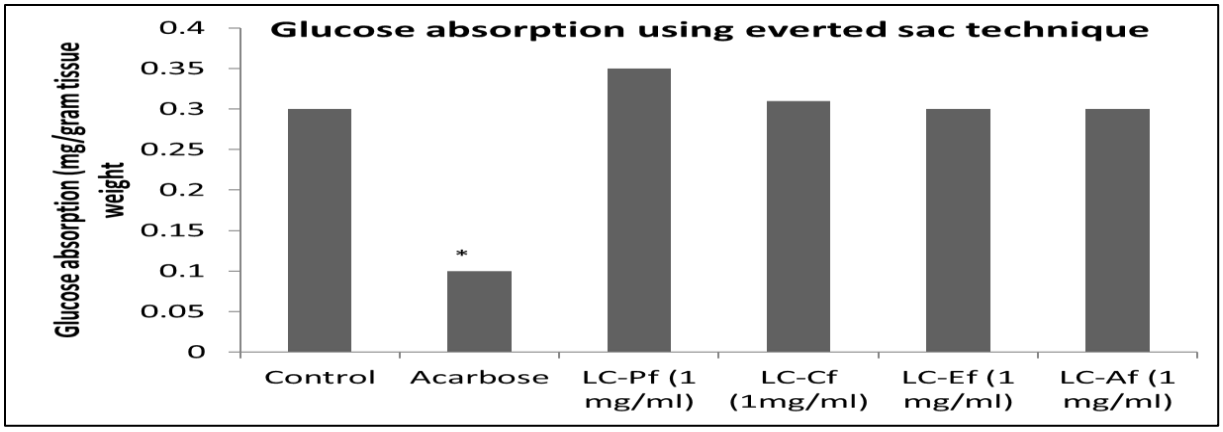

Figure 4: Effect of drugs on glucose absorption**.** Each value represents the mean ±S.E.M, n=6, * indicates significant difference between treated group compared with control group at P <0.05

4.2. Glucose uptake:

Figure 5 illustrates glucose uptake by isolated rat abdominal muscle, showing a significant increase (P < 0.05) in the presence of insulin (100 mU/mL) for LC-Ef (1 mg/mL), LC-Af (1 mg/mL), and metformin (1 mg/mL) compared to the control group. Additionally, glucose uptake with metformin (1 mg/mL) was significantly higher (P < 0.05) when insulin was present (3.06 ± 0.41 mg per g tissue weight) than when absent (1.76 ± 0.35 mg per g tissue weight), relative to the control.

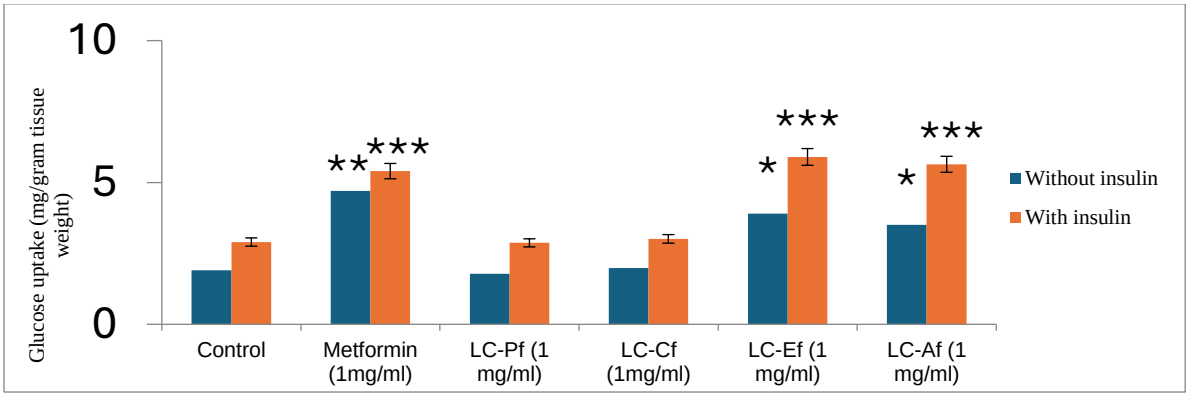

Figure 5: Effect of drugs on Glucose uptake. Each value represents the mean ± SEM, n=6, * indicates significant difference between treated group compared with control group at p <0.05

4.3. Insulin secretion:

Glibenclamide (0.1–10 mM) stimulated insulin secretion from RIN-5F cells in a dose-dependent manner when cultured in 1.1 mM glucose, as shown in Figure 6(a). Figure 6(b) illustrates the effects of various concentrations of *L. ciliata* fractions on RIN-5F cells. Notably, treatment with 10 mM LC-Ef resulted in a significant ($P < 0.05$) increase in insulin levels compared to the control group.

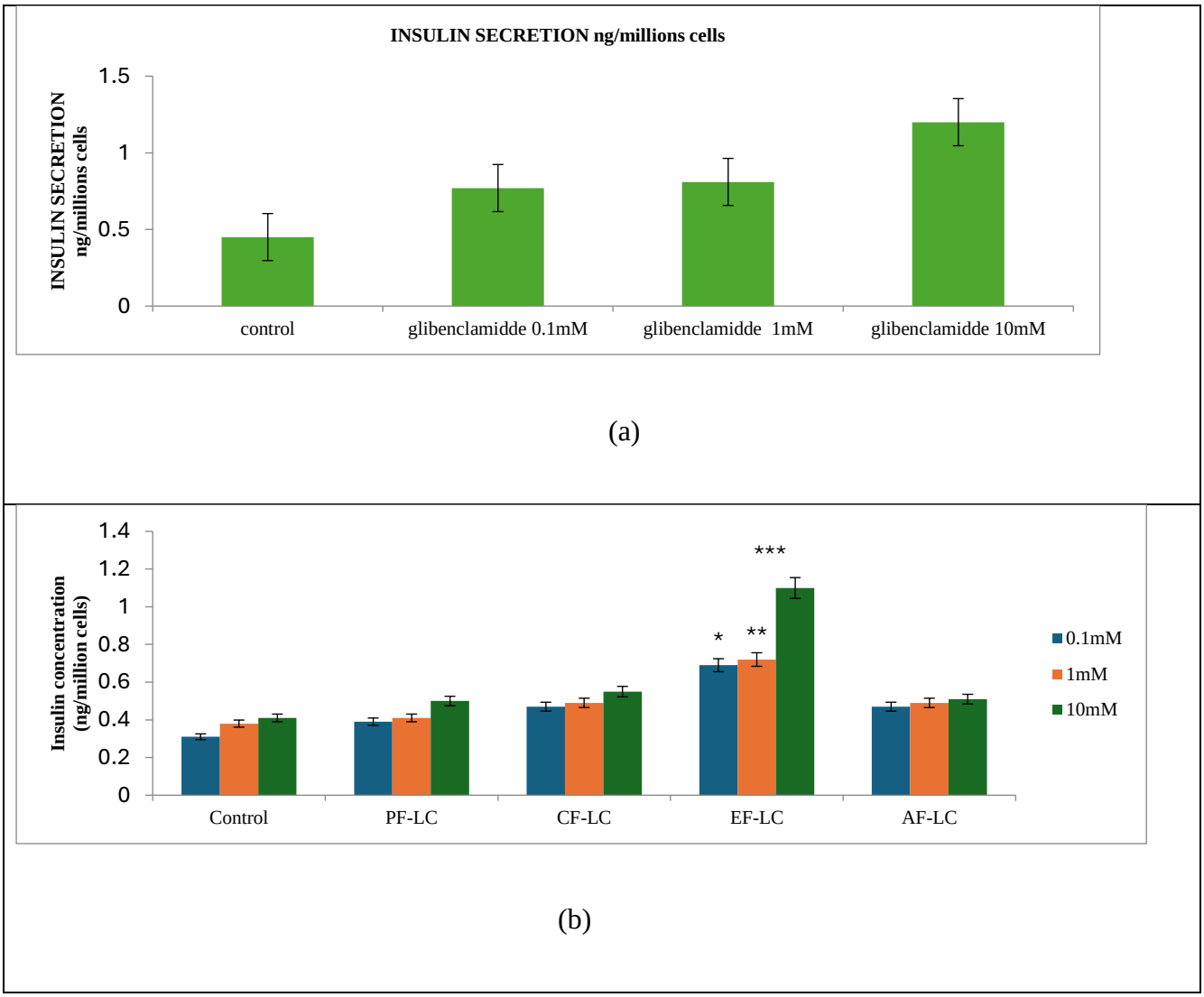

Figure 6: Effect of drugs on insulin secretion. **(a)** Effects of glibenclamide (mM) on insulin secretion. Each value represents the mean ± S.E.M. (n = 6), * Indicates significant difference between treated groups compared with control group without glibenclamide at $P < 0.05$

(b) Effects of LC-Pf: petroleum ether extract of *L. ciliata*; LC-Cf: chloroform extract of *L.ciliata*; LC-Ef - ethanol extract of *L. ciliata*; LC-Af: Aqueous extract of *L. ciliata* on insulin secretion. Each value represents the mean ± S.E.M. (n = 6), * Indicates significant difference between treated groups compared with control group at $P < 0.05$

4.4. Alpha amylase inhibition assay:

The inhibition of α-amylase activity by *L. ciliata* extract was evaluated using acarbose as a positive control. The extract exhibited dose-dependent inhibition. The IC_{50} value for LC-Ef (10 mg/mL) was determined to be 59 µg/mL, showing no significant difference when compared to acarbose, which had an IC_{50} value of 60 µg/mL at the same concentration.

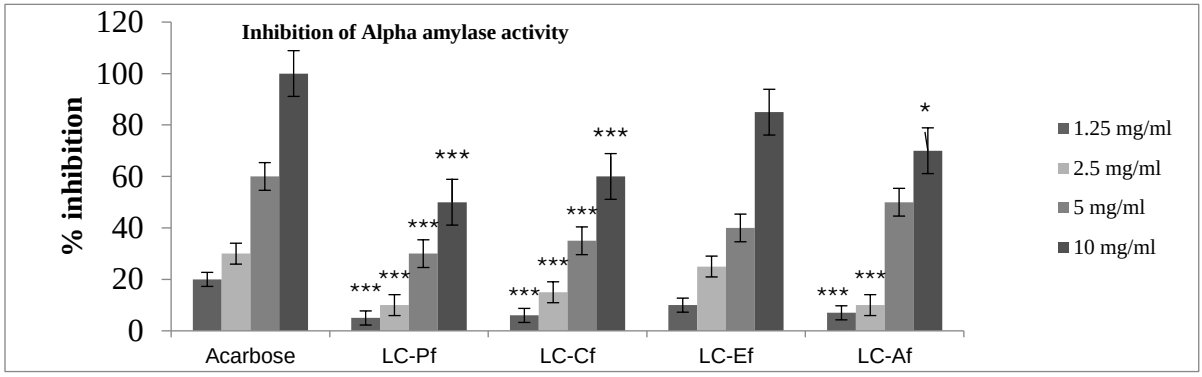

Figure 7: Effect of drugs on α-amylase inhibition. Data expressed as mean ±SEM, (n=6).* Indicates significant difference between treated groups compared with control group at $P < 0.05$

4.5. DPP-4 Inhibition:

Figure 8 illustrate the effect of drugs on DPP-4 inhibition. In the four fractions, LC-Ef showed significantly higher inhibition of DPP-4 than the other fractions. There is no significant difference in inhibition of DPP-4 when compared with the standard drug.

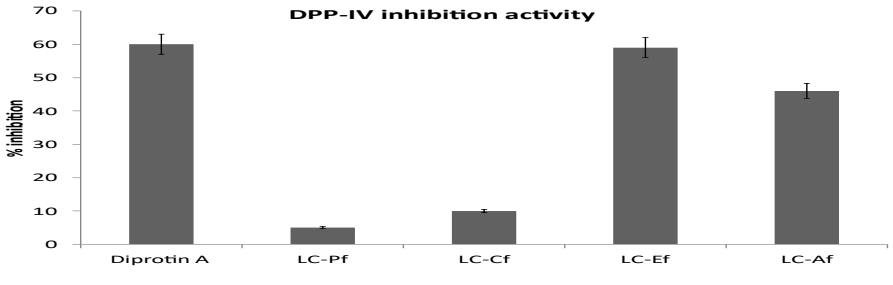

Figure 8: Effect of drugs on DPP-4 inhibition activity. Each value represents the mean ±S.E.M, n=6, * indicates significant difference between treated group compared with control group at P <0.05

5. Discussion:

Limitations associated with insulin and oral hypoglycemic drugs have spurred research efforts toward identifying natural sources with anti-diabetic properties (Soni et al., 2018). As a result, there is a rising claim for safer, more effective herbal-based antidiabetic treatments. A review of existing literature suggests that the antidiabetic potential of *L. ciliata* has yet to undergo widespread scientific evaluation for its probable mechanism (Barukial & Sarmah, 2011; (Mondal et al., 2013). In the present research, in-vitro experiments were performed to explore its anti-diabetic potential. These studies focused on different levels: at the intestinal level, by slowing or inhibiting glucose absorption; at the peripheral level, by enhancing glucose uptake into insulin-sensitive tissues like muscle cells; and at the pancreatic level, by promoting insulin secretion.

Present research findings suggest that LC-Ef inhibits alpha-amylase enzymes, leading to a slower breakdown of carbohydrates and a reduced glucose absorption rate. This mechanism lowers postprandial blood glucose levels, presenting a promising alternative to synthetic drugs and potentially offering fewer side effects (Jain et al., 2023)

Preliminary phytochemical screening of all fractions of the hydroalcoholic extract of *L. ciliata* revealed the presence of alkaloids, steroids, flavonoids, saponins, and phenolic compounds. The phytochemical composition varied among different fractions, with the ethanolic leaf extract exhibiting the strongest alpha-amylase inhibitory activity. This effect may be due to its elevated levels of total phenolic and flavonoid content. Decelerating glucose absorption from the intestine into the bloodstream could mitigate postprandial hyperglycaemia in diabetic patients. Additionally, enhanced dietary fibre has been shown to decrease intestinal glucose uptake and support better glucose regulation (Jain et al., 2023).

LC-Ef promotes plasma insulin levels by enhancing insulin secretion in the RIN-5F cell line, particularly at higher doses. This effect may be attributed to its ability to depolarize pancreatic beta-cell membranes, leading to the opening of voltage-gated calcium channels and a subsequent increase in intracellular calcium levels, which triggers insulin exocytosis (Henquin, 2009). Additionally, LC-Ef may activate the PI3K/Akt signaling pathway, which plays a crucial role in beta-cell survival, proliferation, and glucose metabolism (Saltiel & Kahn, 2001). Similar to glibenclamide, a sulfonylurea that enhances insulin secretion by inhibiting ATP-sensitive potassium (K_ATP) channels, LC-Ef may work through a comparable mechanism, increasing insulin release in a dose-dependent manner (Ashcroft & Rorsman, 2013). These findings align with previous studies indicating that plant-derived bioactive compounds can stimulate insulin secretion, protect pancreatic beta cells from oxidative stress, and promote beta-cell regeneration (Jayasri et al., 2008; (Jung et al., 2006).

Insulin enables glucose uptake into peripheral cells, while metformin continues to be the primary medication used to improve insulin sensitivity. To explore this further, in vitro studies were performed using rat abdominal muscle tissue. Skeletal muscle, which comprises 30-40% of total body weight, is considered one of the most important target tissues for insulin activity and peripheral glucose uptake (DeFronzo et al., 1981). This study found that LC-Ef enhances insulin-stimulated glucose transport across skeletal muscle cell membranes, similar to the action of metformin, even in the absence of insulin. Glucose uptake was significantly higher with LC-Ef when insulin was present than without it (Figure 2). Thus, *L. ciliata* directly promotes glucose uptake at the peripheral level, either independently or as an adjunct to insulin.

Currently, our findings reveal that ethanolic fractions of the hydroalcoholic extract of *L. ciliata* exhibit greater DPP-4 inhibition compared to other fractions when Diprotin is used as the standard drug. DPP-IV inhibitors lowered blood glucose after acute and chronic administration in preclinical studies through mechanisms predominantly dependent on incretin action, leading to potentiation of glucose-stimulated insulin secretion (Ahren et al., 2000; Balkan et al., 1999; Sudre et al., 2002)

This research indicates that *L. ciliata* has an anti-hyperglycemic effect by promoting glucose uptake, enhancing insulin secretion, inhibiting the alpha-amylase enzyme, and suppressing DPP-4 activity. However, further studies at the molecular level, including gene and protein expression analyses, are required to confirm the exact mechanism of action.

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