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Purified Extract of *Costus pictus* D. Don containing 25 kDa protein induces antidiabetic activity by down-regulating gsk3β gene expression in pancreatic cell lines *in vitro*

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Costus pictus is found to have antidiabetic activity, but the real mechanism of action and the potent bioactive molecule responsible is still unknown. This work aims to identify the potential mechanism of action of the partially purified antidiabetic extract of *C. pictus* extract. The purified methanol extract of *C. pictus* was prepared and the presence and isolation of protein were done by ammonium sulfate precipitation. The carbohydrate enzyme, α-amylase, and α-glucosidase inhibition activity of the crude, protein, and nonprotein part of the extract were investigated *in vitro*. Glucose-induced insulin secretion assay and calcium levels in the cytoplasm were also evaluated using pancreatic cell lines. The effect of GSK3β gene expression was studied in RIN5MF cells treated with crude extract of *C. pictus*. The crude methanol extract showed the presence of several classes of compounds, with significant levels of polyphenols and flavonoids. A protein of 25 kDa molecular weight was found in the crude extract. Protein and crude extract showed enzyme inhibitory activity, glucose uptake, and increased calcium uptake, and increased insulin secretion. The effect was more in crude extract than protein and nonprotein parts. Moreover, the crude extract reduced the gene expression of GSK3β, which may be one of the possible mechanisms by which the antidiabetic property of *C. pictus* relies upon. The results showed that the antidiabetic and insulin secretagogue property of *C. pictus* involves multiple mechanisms and requires the involvement of both phytochemicals as well as the protein present in it.

Keywords: Antioxidant, Insulin, Protein, RIN5MF cells

Diabetes, a chronic metabolic disease is associated with elevated fasting blood glucose levels and impaired insulin production[1] or the body cannot utilize the insulin. This deadly disease makes millions of people suffer worldwide and type 2 diabetes mellitus (T2DM) is the most prominent form accounting for more than 80% of the total cases. Insulin, a peptide hormone identified and isolated in 1921 is still being used as an effective medication for combating diabetes mellitus[2]. Insulin stimulates different cells to uptake glucose from the circulatory system. The underutilization of insulin leads to insulin resistance, which is characterized by the inability of cells to respond to the circulating insulin[3], leading to diabetes.

Plants have been widely used since ancient times for the treatment of diabetes. Insulin-like substances/ proteins isolated from plant tissues, yeast, and fungi are found to be capable of producing a profound beneficial effect on carbohydrate metabolism[4]. The occurrence of such protein in plants did not evoke much interest among the scientific community until the presence of insulin-like peptides was confirmed from the fruits of *Momordica charanti* which is rich in nutrients and phytochemicals[5]. From the developing fruits of cowpea, insulin-like protein, involved in carbohydrate transport with similarity to the amino acid sequence of bovine insulin was identified[6]. A similar protein was also isolated from the antidiabetic plant *Bauhinia variegate,* which was found to have significant involvement in carbohydrate metabolism[7]. Reports indicate that insulin-like protein was purified from *Costus igneus*, which showed cross-reactivity with murine anti-insulin antibodies, and was purified by affinity chromatography. The protein showed hypoglycemic activity and a significant decrease in blood glucose levels. *C. pictus* (insulin plant), a native of Mexico is utilized for the treatment of diabetes, cancer, and inflammation[8,9]. The rhizomes of *C. pictus* are eaten by the people cooked, roots form a major constituent in tonic and anthelmintic medicines, and leaves are consumed by the patients for curing diabetes[10]. The first scientific study on the

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hypoglycemic activity of *C. pictus* leaves was undertaken by Arjuna Natural Extracts LTD, Kerala, India, and found that the extract obtained from the plant had considerable hypoglycaemic activity[10]. Though *C. pictus* is used for reducing blood sugar, the exact molecular mechanism by which the effect is exerted is largely unknown.

In this, study the presence of any kind of protein in the partially purified methanolic extract of *C. pictus* is evaluated. The effect of the extract and protein on carbohydrate metabolic enzyme inhibition was investigated with α-amylase and α-glucosidase. A comparative study on glucose uptake was done with crude methanolic extract, protein, and nonprotein part using intestinal epithelial cell lines (HT 29). Glucose-induced insulin secretion assay, calcium levels in the cytoplasm, have been investigated for the antidiabetic properties of the extracts.

Materials and Methods

Chemicals

DMSO, McCOY's 5A medium, RPMI-1640, Fetal Bovine Serum (FBS), antibiotics were purchased from Hi-Media, India. 2-[N-(-7-Nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2NBDG) was purchased from Sigma chemicals, USA. Fura-2AM for calcium staining was procured from Invitrogen, USA. RIN5F cells, HT 29 cells, Miapaca cells were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Previously A composition for treating diabetes and dyslipidemia obtained from the extract of *C. pictus* plant and a method of preparing the same was patented (Merina Benny Antony., Inventor-. US Patent (No. US 9981000, dated May 29, 2018).

Extraction of *C.pictus*

C. pictus was collected from the nursery of Arjuna Naturals Extracts Ltd, Alwaye, Kerala, India. *C. pictus* was extracted with 90% methanol by refluxing. The residue obtained was cooled, filtered, and concentrated. The extract was evaluated for the presence of acids, carboxylic, and oxalic acid using HPLC[11]. Oxalic acid, which contributes towards the sour taste of the plant was removed to obtain a purified methanol extract of *C. pictus* (ME_{CP}), which was used for the evaluation of antidiabetic activity *in vitro*.

Preliminary phytochemical analysis

The purified methanol extract, ME_{CP} was found to be brown with a moisture content of 4.8%. The extract was found to be soluble in both methanol and water. The extract was found to contain proteins (49%), Carbohydrates (33%), and fats (2.5%), respectively[11]. The extract was also analyzed qualitatively for the presence of phenolic compounds (Lead acetate test), flavonoids (Alkaline reagent test), tannins (Ferric chloride test), steroids, triterpenoids (Salkowski's test), saponins (Froth test), cardiac glycosides (Keller Killiani test) and alkaloids (Wagner's test) using standard procedures[12].

Determination of polyphenols and flavonoids in ME_{CP} and NP_{CP}

The total phenol content was determined by the Folin-Ciocalteu method. To the different concentration of the samples (adjusted using methanol) 80 µL of Folin-Ciocalteu reagent and 200 µL of Na_2CO_3 was added. The mixture was mixed well and absorbance was determined after 90 min at 725 nm against standard Gallic acid. The total phenolic content of the extract was expressed as mg gallic acid equivalence/g of the extract. For total flavonoid estimation, the extract in DMSO was mixed with 1.5 mL of 95% alcohol, 0.1 mL of 10% aluminum chloride hexahydrate ($AlCl_3$), 0.1 mL of 1 M CH_3COOK, and 2.8 mL of distilled water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm on a spectrophotometer (Shimadzu UV-vis-Spectrophotometer, model 2600). The total flavonoid content of the extract was expressed mg quercetin equivalence/g of the extract[13].

Isolation of proteins from ME_{CP}

Methanolic extract of *C. pictus* was mixed with 10 volumes of cold 10% TCA in acetone. The mixture was vortexed and incubated for 3 h at −200°C or overnight. The same volume of cold acetone was added, vortexed, and stored at −20°C for 10 min and centrifuged at 15000 g for 5 min. The supernatant was removed and the pelleted protein (P_{CP}) obtained was air-dried. The concentration of protein was estimated using the Bradford assay[14]. The protein obtained by precipitation was subjected to SDS–PAGE by the method of Laemmli, 1970[15].

α amylase inhibition assay

The α-amylase repressing activity of ME_{CP}, P_{CP}, and NP_{CP} was evaluated by the strategy represented earlier with slight modification[16]. The reaction mixture containing 50 µL phosphate buffer (50 mM, pH= 6.8), 10 µL α-amylase (10 U/mL) and 20 µL of varied concentrations of extracts were pre-incubated at 37°C for 10 min. Then 20 µL soluble starch (0.05%) was added as a substrate and incubated

further at 37°C for fifteen min. The reaction was stopped by adding 20 µL 1N HCl, followed by the addition of 100 µL iodine. The absorbance was read at 620 nm using a Multimode Reader. Each experiment was performed in triplicates and the inhibitory effect was calculated as:

$$\text{Inhibition }(\%) = \frac{Ab_{Test} - Ab_{Negative\ control}}{Ab_{Test}} \times 100$$

where, A is absorbance.

α-Glucosidase inhibition assay

The reaction mixture containing 50 µL phosphate buffer (50 mM, pH-6.8), 10 µL α-glucosidase (1 U/mL), and 20 µL of varying concentrations of the extracts were pre-incubated at 37°C for fifteen min. The substrate, p-nitrophenyl-α-D-Glucopyranoside (PNPG; 1 mM; 20 µL) was added and incubated at 37°C for thirty min. The reaction was stopped by adding sodium carbonate (50 µL; 0.1 M). The absorbance was measured at 405 nm. Acarbose at numerous concentrations (200-1000 µg/mL) was used as the standard. The result was expressed as percentage inhibition, which was calculated as, Inhibition

$$(\%) = \frac{Ab_{Test} - Ab_{Negative\ control}}{Ab_{Test}} \times 100$$

where, A is absorbance[17].

Glucose uptake studies on ME_{CP}, $P_{CP,}$ and NP_{CP} on HT 29 cells using 2NBDG

The level of glucose uptake in the presence of ME_{CP}, $P_{CP,}$ and NP_{CP} was carried out using a fluorescent structural analogue of glucose. Briefly, McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics in 5% CO_2 at 37°C served as the medium for maintenance of Intestinal epithelial cell lines, HT29. After attaining 70% confluency, cells were seeded in 96 well plates with BSA (1 mg/mL) and 80 µM fluorescent analog, 2NBDG (Invitrogen) in presence of different concentrations of ME_{CP}, $P_{CP,}$ and NP_{CP} for 60 min. For stimulation experiments, 100 µL insulin was added along with extracts. The cells were washed and plates and the absorbance was were read at an ex/em wavelength of 485 nm and 35 nm, respectively[18].

Glucose-induced insulin secretion in the presence of ME_{CP}, $P_{CP,}$ and NP_{CP} in RIN-m5Fcells

RIN-m5F cells were cultured at 37°C in a humidified atmosphere of 5% CO_{2-} in the air in RPMI-1640 containing 10% FBS and antibiotics. Cells were seeded in a 48 well plate and allowed to attach 48 h before the acute tests. Wells were washed three times with KRB; 115 mM NaCl, 4.7 mM KCl; 1.3 mM $CaCl_2$. 1.2 mMKH_2PO_4, 1.2 mM $MgSO_4$ 24 mM $NaHCO_3$, 10 mM HEPES, 1 g/mL BSA. A 1 mM glucose; pH 7.4. Cells were and pre-incubated for 1 h at 37°C followed by incubation with KRB with 4.5 mM glucose and ME_{CP}, $P_{CP,}$ and NP_{CP} for 1 h. Aliquots were removed, centrifuged at 2000 rpm for 5 min at 4°C, and assayed for insulin levels using radio immune assay[19].

Estimation of Intracellular calcium levels in the presence of ME_{CP}, $P_{CP,}$ and NP_{CP} in Miapaca cells

The intracellular calcium concentration, $[Ca^{2+}]$, was measured using Fura-2AM (Molecular Probes, Invitrogen). Islets were incubated in calcium-free HBSS with 5 µM FURA-2AM at 37°C for 30 min[20]. After washing (2x) with calcium-free HBSS, islets were suspended in complete HBSS and treated with various concentrations of ME_{CP}, $P_{CP,}$ and NP_{CP} for 60 min. fluorescence was measured in the multiplate reader at an emission wavelength of 500 nm for dual excitation wavelength at 340 and 380 nm. The Ca^{2+} level was expressed as nmol/50 islet equivalents[21].

Expression AKT, GSK3β, IRS 1 mRNA in ME_{CP}, P_{CP} and NP_{CP} treated RIN-m5F cells

Total RNA was isolated from control and treated cells by Trizol reagent (Himedia) as described by the manufacturer's instructions. cDNA was synthesized by adding 1 ng of RNA to the 5X cDNA synthesis buffer (4 µL), dNTP Mix (2 µL) RNA primer (1 µL), RT Enhancer (1 µL) Verso Enzyme Mix (1 µL) and nuclease-free water (20 µL) using Thermo Scientific Verso cDNA Synthesis Kit. PCR was done by using GoTaq Green Master Mix in thermal cycler using the following primer sequences GSK3β (Forward 5′–GGAACTCCAACAAGGGAGCA-3′ Backward 5′–TTCGGGGTCGGAAGACCTTA-3′)., AKT (5′-TCA CCTCTGAGACCGACACC TCACCTCTGAGACC GACACC-3′, 5′-ACTGGCTGAGTAGGAGAACTGG-3′), Insulin receptor1 (5′-AGTGGCCATGGCT CCACA-3′, 5′-TTGCCACCCATGCAGAT-3′ GAPDH was used as an endogenous control. The finished PCR product was run on 1.5% agarose and analyzed using a bio rad gel documentation system.

Results

Preliminary phytochemical analysis

Preliminary phytochemical screening was done to identify the different classes of components present in

the *C. pictus* methanolic extract. Results showed that the ME_{CP} contains different classes of compounds *viz* polyphenols, flavonoids, proteins, tannins, steroids, triterpenoids, saponins, and cardiac glycosides, *etc*. Figure 1 indicates that ME_{CP} contains a higher amount of polyphenols than flavonoids (120 and 56 µg/mg sample), while after removal of proteins showed a total phenol content of 129.3 µg/mg and flavonoid content of 39.9 µg/mg sample, respectively. Cold TCA treatment of 5g ME_{CP} yielded 1.2 g of the precipitate. Bradford assay showed that the protein concentration was 0.46±0.21 mg/mg TCA precipitate. Electrophoresis of the precipitate showed the presence of a single band of protein with an approximate molecular weight 25 kDa. These results suggest that the ME_{CP} contains components conjugated to proteins probably in the form of glycoproteins as well as many biologically active secondary metabolites that contribute towards its antidiabetic activity.

α amylase and α glucosidase Enzyme inhibitory activity

The crude methanol extract of *C.pictus*, ME_{CP} showed a significant α amylase inhibitory effect with an IC_{50} value of 33.53 µg/mL. The amylase inhibitory activity of protein isolated from *C. pictus,* P_{CP} also showed profound amylase inhibition ranged from 29.66 to 53.64%, but at mg concentration (Fig. 2A). P_{CP} showed maximum inhibition of 53.64 % at 2 mg/mL. The methanol extract obtained after protein isolation, NP_{CP} inhibited α amylase activity at higher concentrations, with an IC_{50} value of 1.14 mg/mL. The *in vitro* α glucosidase inhibition studies demonstrated that ME_{CP} inhibited the enzyme in *in vitro* conditions. 6 mg/mL of the extract showed inhibition of about 80%. P_{CP} had profound concentration-dependent α glucosidase inhibitory activity, with the highest concentration of 2 µg showed maximum inhibition of nearly 61.18% (Fig. 2B). The percentage inhibition varied from 35.8-61.18% from the lowest concentration to the highest concentration. NP_{CP} showed higher inhibition (IC_{50} value of 95.36 µg/mL) than P_{CP}. The inhibitory effect of the extract was comparable with that obtained for standard acarbose. It has been shown that NP_{CP} had higher activity than acarbose.

Increased glucose uptake by HT 29 cells in the presence of ME_{CP}, P_{CP}, and NP_{CP}

Differentiated HT29 cells were incubated with or without insulin along with various fractions. ME_{CP} and P_{CP} showed a dose-dependent increment in glucose uptake, which was significantly greater than NP_{CP} part. However, higher concentrations of P_{CP} showed maximal glucose uptake compared to both ME_{CP} and NP_{CP}. In the presence of insulin, ME_{CP} and P_{CP} showed a dose-dependent increment in glucose uptake as evidenced by higher fluorescence (Fig. 3).

Effect on GLUT 2 translocation in a colon cell line, HT-29cells treated with ME_{CP}, NP_{CP}, and P_{CP}

Tissues whose function is regulated by glucose also possess particular glucose transporters, GLUT 4 in adipocytes and GLUT 2 in tissues secreting glucose into the blood (liver, small intestine, and proximal tubular cells of the kidney) as well as GLUT 2 in the β cells of the islets of Langerhans. We used HT 29, intestinal epithelial for the translocation study. HT29 cells were treated with an increasing concentration of ME_{CP}, NP_{CP}, and P_{CP}. Cells were treated with GLUT-2 primary antibody and then with Alexa fluor conjugated secondary antibody which gives

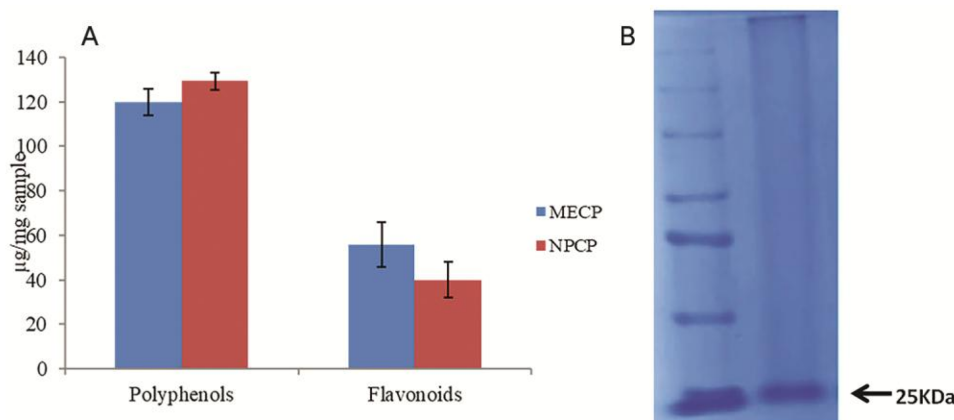

Fig. 1 — Total polyphenols, flavonoids in ME_{CP} , and $NP_{CP,}$ and the electrophoresis of protein isolated from ME_{CP}. A: Total polyphenols and flavonoids, B: Protein isolated from the crude extract. Results are expressed as mean ± SEM of three independent experiments. ME_{CP}: Methanol extract of *C.pictus*, NP_{CP}: Non-protein part after protein removal

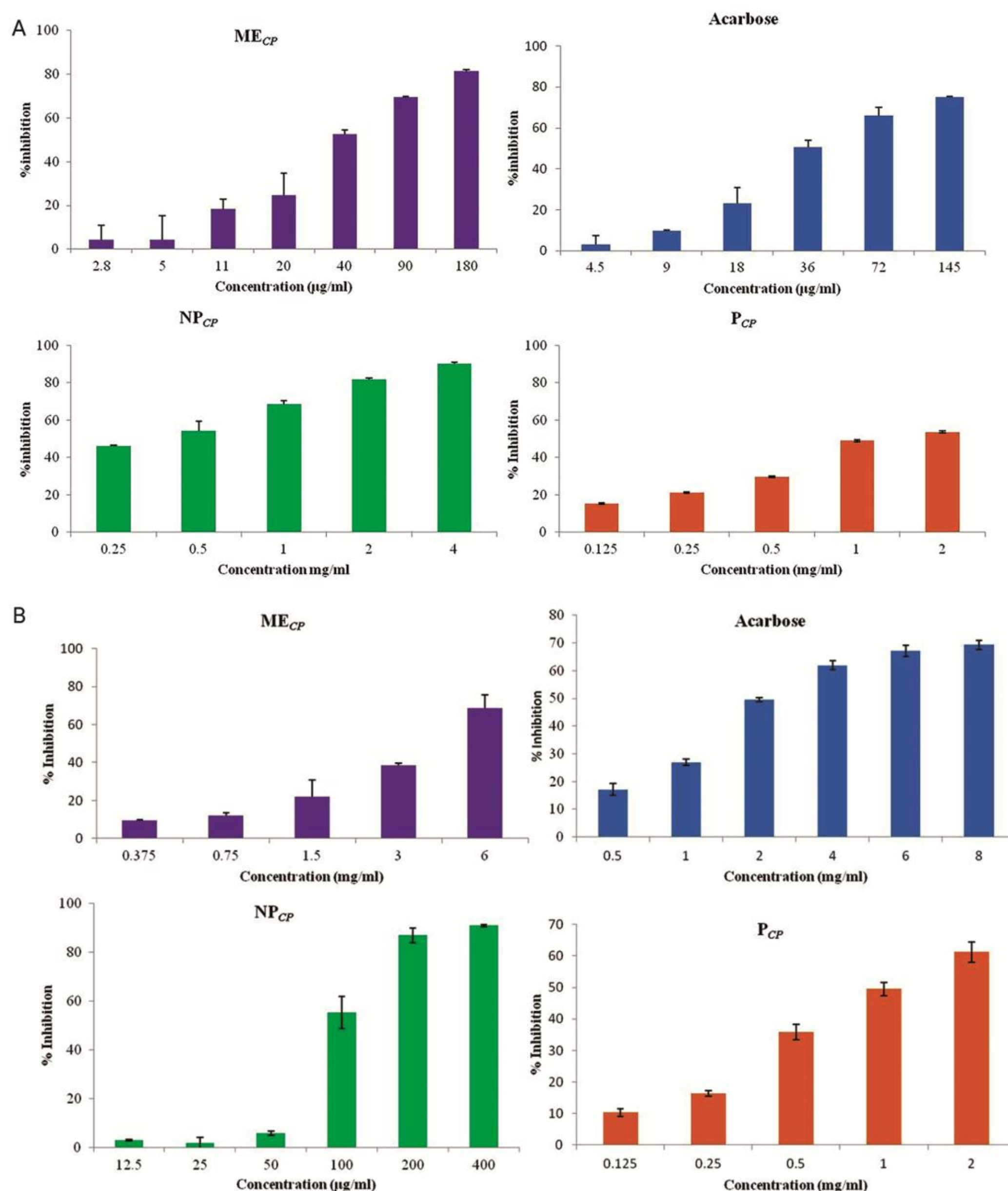

Fig. 2 — *In vitro* α (A) amylase; (B) glucosidase inhibitory activity of ME_{CP}, NP_{CP}, and P_{CP}. Results are expressed as mean ± SEM of three independent experiments. ME_{CP}: Methanol extract of *C.pictus*, NP_{CP}: Non-protein part after protein removal, P_{CP}: Protein isolated from *C. pictus*

fluorescence. The intensity of the fluorescence was found to be increased in a dose-dependent manner when treated with ME_{CP} and NP_{CP} parts. While P_{CP} showed lower translocation of GLUT 2 in HT 29 cells.

ME_{CP} induces insulin secretion from RIN5MF cells

ME_{CP} showed a significant dose-dependent increment in insulin secretion from mouse pancreatic cell lines, RIN5MF cells under-stimulated glucose concentration (Fig. 4). P_{CP}, at 20 µg/mL showed increased insulin secretion, comparable to that of NP_{CP}, but significantly lower than that of ME_{CP}. Glibenclamide was used as standard and found to have a lower effect on insulin secretion from RIN5MF cells compared to that of ME_{CP}.

P_{CP} increased intracellular calcium in the pancreatic cell, MIA PaCa-2 than ME_{CP} and NP_{CP}

There was an increase in calcium levels when the extracellular glucose concentration was increased up to 16.7 mM. Under the stimulating concentration of glucose, a dose-dependent increment in intracellular calcium levels was noticed in the presence of P_{CP} in a dose-dependent manner (Fig. 5). ME_{CP} exhibited narrow variation in calcium uptake and NP_{CP} part showed very less calcium uptake.

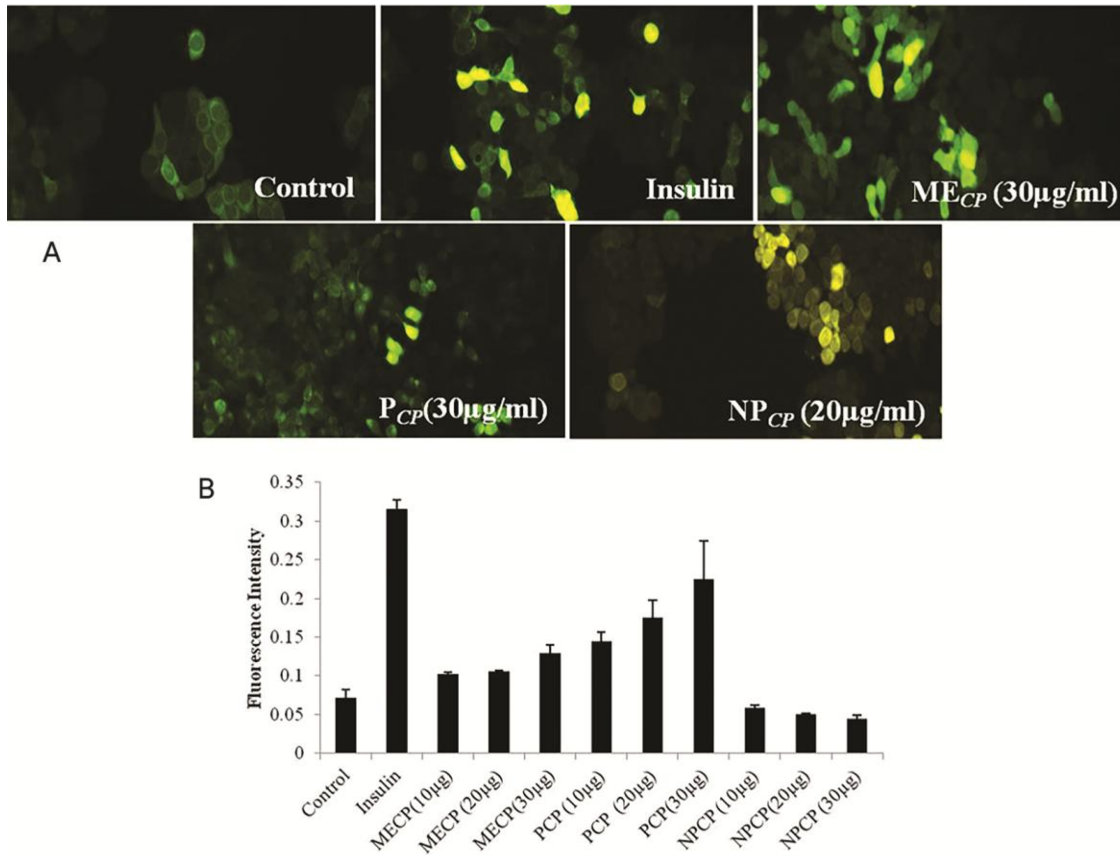

Fig. 3 — Effect of ME$_{CP}$, NP$_{CP}$, and P$_{CP}$ on glucose uptake in HT 29 cells. Pictures are the representation of three separate experiments (magnification 20x). Results are expressed as mean ± SEM of three independent experiments. ME$_{CP}$: Methanol extract of *C.pictus*, NP$_{CP}$: Non-protein part after protein removal, P$_{CP}$: Protein isolated from *C. pictus*

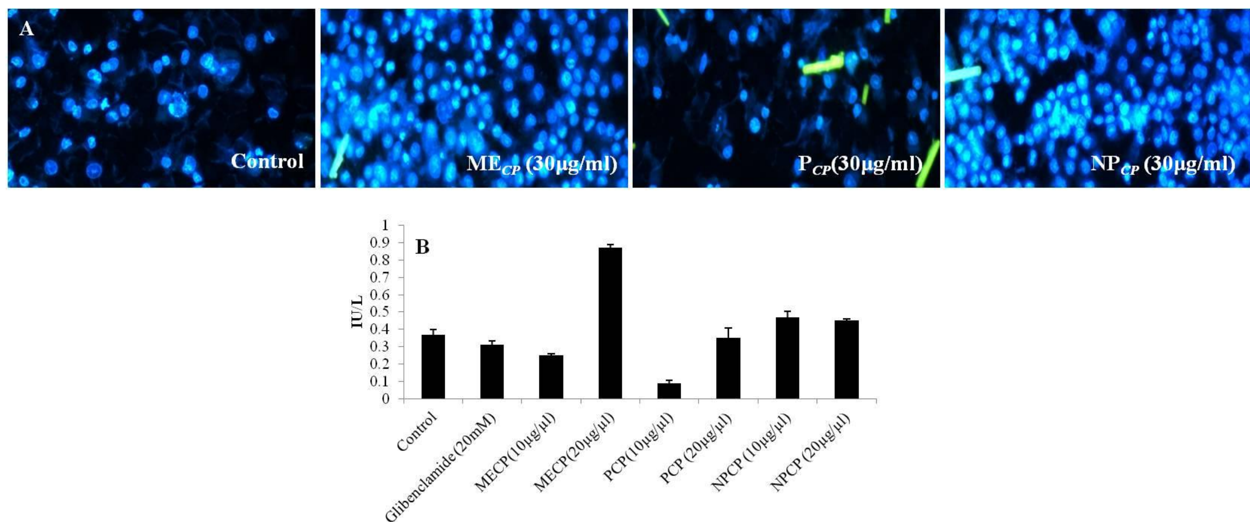

Fig. 4 — Effect of ME$_{CP}$, NP$_{CP}$, and P$_{CP}$ on glucose-stimulated insulin secretion in RIN5F cells and intracellular calcium levels in Miapaca cells. Pictures are the representation of three separate experiments (magnification 20x). Results are expressed as mean ± SEM of three independent experiments. A: Intracellular Calcium levels; B: Insulin secretion. ME$_{CP}$: Methanol extract of *C.pictus*, NP$_{CP}$: Non-protein part after protein removal, P$_{CP}$: Protein isolated from *C. pictus*

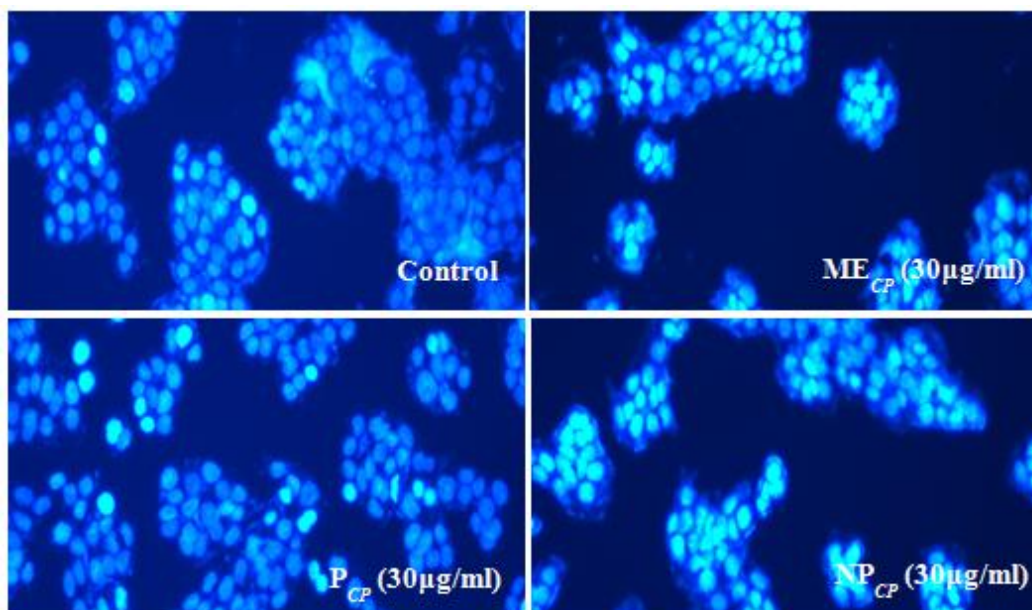

Fig. 5 — Effect of ME_{CP}, NP_{CP}, and P_{CP} on glucose-stimulated intracellular calcium levels in MiaPaca cells. Pictures are the representation of three separate experiments (magnification 20x). ME_{CP}: Methanol extract of *C.pictus*, NP_{CP}: Non-protein part after protein removal, P_{CP}: Protein isolated from *C.pictus*

Discussion

Diabetes is a major health problem for millions of people worldwide, which becomes chronic due to the deficiency of insulin/resistance or both. Presently this condition is treated by insulin injection or by other synthetic chemicals which often leads to hypoglycemia. A natural drug that could prevent diabetes without creating hypoglycemia is quite interesting. India has a well-defined indigenous medical system that utilizes plant products or derived components for curing various diseases. *C. pictus* is one such plant used by many patients to cure diabetes. Previous reports indicate that *C. pictus* extract lowered toxicity in rats treated with 200 mg/kg body wt. for 15 days with no mortality without hyperglycemic effect[22]. According to the survey conducted by Arjuna Natural Extracts, Kerala, India, it was found that that *C. pictus* was widely used by the population of Kerala as an antidiabetic medicine without being prescribed or without any proper scientific authentication of the species consumed. It was also known that many people used *C. speciosus* without knowing its adverse toxicity and other inflammatory reactions. About 97.8% of people had a decrease in fasting blood sugar and more than 70% people found a decrease in postprandial sugar level, while 77.8% of patients did not notice any complications after consuming the plant, but a few people complained about gastric discomfort or acidity, which may be due to higher concentration of plant material[23]. Though the *C. pictus* extract shows significant antidiabetic activity, the molecular mechanism by which it acts remains unclear. This study was designed to evaluate the presence of proteins in *C. pictus* extract, and its effect on the insulin release and inhibition of carbohydrate metabolism enzymes as well as the preliminary molecular mechanism of antidiabetic activity in pancreatic cell lines. The results showed that the crude methanol extract of *C. pictus* (ME_{CP}) contains several classes of compounds, *viz*. protein, saponins, tannins, steroids, triterpenoids, polyphenols, and flavonoids. Quantitative analysis showed the polyphenol levels were much higher than flavonoids. Ammonium sulfate precipitation showed the presence of a protein with a molecular weight of 25 kDa. Previous studies showed that *C. pictus* leaves contain the presence of fibers, steroids α-tocopherol, ergastanol, terpenoid lupeol, and stigmasterol[24]. These observations are comparable with the results obtained from our studies. Research across the world has proved that secondary metabolites isolated and purified from plants have antidiabetic effects in various experimental animal models[25-27].

Experiments were carried out to evaluate the effect of Crude (ME_{CP}), Protein (P_{CP}), and the extract

obtained after the removal of protein (NP_{CP}) portion of *C. pictus*. α-amylase enzyme activity was significantly reduced by ME_{CP} at µg concentration range and the effect was comparable with that obtained for acarbose. Protein isolated from *C. pictus* also showed an inhibitory effect, but a higher concentration was required for even 60% inhibition, while NP_{CP} showed a maximum effect of 80% inhibition at a concentration of 4 mg/mL which is much higher than that of ME_{CP}. α-glucosidase inhibitory studies showed that NP_{CP} with profound effects at µg concentration, while ME_{CP}, $P_{CP,}$ and acarbose showed an inhibitory effect at higher concentrations. This varied effect indicates that both protein and other phytochemicals are working in coordination in the antidiabetic activity of *C.pictus*.

Glucose uptake studies using human colon cancer cell lines, HT-29 cells showed that P_{CP} increased the glucose uptake by the cells in a concentration-dependent manner, but found to be lesser than insulin which showed a very high effect. Glucose uptake in the presence of ME_{CP} was lesser, while the presence of NP_{CP} showed a significantly lower uptake. This result showed that the protein present in *C. pictus* may have some insulin-like activity which contributes to the glucose uptake effect of ME_{CP}.

Insulin secretion from mouse pancreatic cells, RIN-m5F was increased by the treatment with NP_{CP} and ME_{CP} compared to the P_{CP}. The P_{CP} influences the intracellular calcium dynamics in pancreatic cells, MIA PaCa-2 compared to NP_{CP}. The Non protein part showed a significant effect in the translocation of GLUT-2 in HT-29 cells, an effect more profound than P_{CP}. Gene expression analysis showed that levels of AKT were found to be stable in control and treated groups, while the expression of GSK3β gene was found to be decreased in ME*CP* treated groups in a dose-dependent manner. GSK3β, a modulator of glycogen metabolism is also involved in multiple roles in regulating protein synthesis and cell differentiation[28]. GSK3β is a negative regulator of the insulin signaling pathway and is involved in insulin resistance, and this gene is now considered to be a potent target for the intervention and control of type 2 diabetes[29]. GSK-3b is the most potent inhibitor of Glycogen Synthase and subsequent glycogenesis and is therefore considered as a promising target for diabetes treatment[30]. Studies report that insulin activates p13K/Akt and reduces glycogen synthase activity mediated through GSK3β[31]. IRS-1 gene

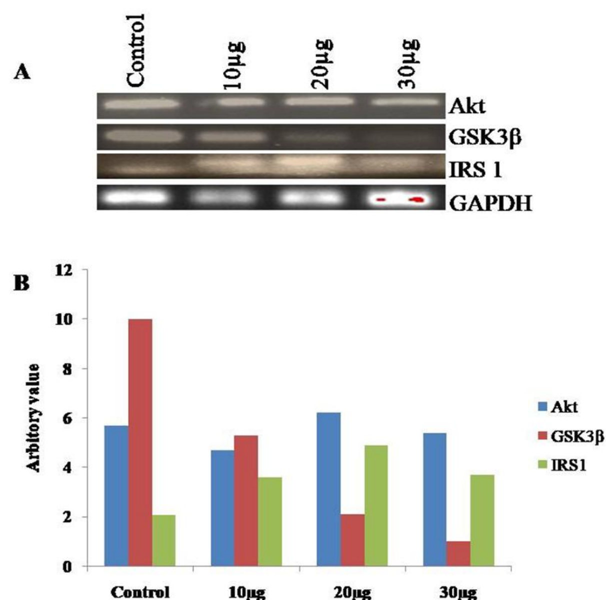

Fig. 6 — Expression levels of Akt, GSK3β, IRS1 in RIN 5 cells treated with ME_{CP}. Pictures are the representation of three separate experiments

expression was found to be increased in cells treated with varying concentrations of *C. pictus* extract (Fig. 6). Earlier studies showed that the methanol extract of *C. pictus* elevated the GLUT4 mRNA expression compared to the non-treated controls. Experiments also prove that when cells were treated with the extract, modulated IRb, and PI3K protein expressions equivalent to that of positive controls[32]. Apart from protein, extract from ME_{CP} showed the presence of polyphenolic components and flavonoids, which are capable of scavenging free radicals and chelate metals. Phytochemicals in *C.pictus*, especially the polyphenols and flavonoids can reduce the complications of diabetes by modulating multiple molecular pathways by maintaining blood glucose levels, reduce glucose uptake, and enhance insulin secretion as well as modulating the overall immune function[33].

Conclusion

The outcome of the present study showed that *C. pictus* methanol extract can reduce diabetic conditions. Both protein and the nonprotein part, containing several bioactive secondary metabolites contribute toward this activity. *C. pictus* extract was found to induces antidiabetic activity through different mechanisms which include the inhibition of carbohydrate digesting enzymes, enhance insulin

secretion from islets, modulation of glucose uptake by the cells, and inhibition of GSK3β gene expression.

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Conflict of interest

All authors declare no conflict of interest.

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