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Potentilla fulgens Wall. Ex sims. Upregulates insulin receptor substrate 1 and Akt in alloxan-induced diabetic mice

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Abstract

Background *Potentilla fulgens* Wall. ex Sims. is a medicinal plant used by the locals of Meghalaya. However, its mechanism of action has not been well elucidated. Hence, this study investigated the effect of *P. fulgens* on IRS1 and Akt. The interaction of the various polyphenols present in *P. fulgens* with the IR tyrosine kinase and IRS1 PTB domain was studied using auto dock. Changes in expression of antioxidant enzymes, IRS-1, Akt and behavior of normal, diabetic, and diabetic mice treated mice were assessed after 14 days of treatment. Morphological changes in the liver tissue were determined by Transmission Electron Microscopy.

Results The effect of *P. fulgens* on blood glucose was time and dose dependent. Treatment with *P. fulgens*, Cat, E, CE, CEP and metformin improved the activity of catalase, glutathione peroxidase, glycogen, IRS-1 and Akt. The Forced Swimming test showed an altered behavior in diabetic mice. The altered mobility was reverted back to near normal on treatment with *P.fulgens*, Cat, E, CE, CEP and metformin. The morphological aberrations seen in diabetic animals considerably improved in the treated diabetic group.

Conclusion *P. fulgens* and its phytochemicals-catechin and epicatechin are potent sources of antidiabetic drugs, possibly mediating their effects through upregulation of insulin IRS-1 and Akt.

Keywords Akt, Catechin, Diabetes, IRS-1, *Potentilla fulgens*

Background

According to the International Diabetes Federation report, Diabetes Mellitus (DM) currently affects more than 537 million people worldwide, and this number is expected to rise to 783 million by 2045 [1]. DM is a complex metabolic disorder characterized by hyperglycemic condition that results from a dysfunction in insulin secretion, action, or a combination of both [2]. Studies have shown that diabetes results when there is impairment at the level of receptor or post-receptor events [3]. Insulin signal transduction occurs when insulin binds to its receptor and activates the Insulin receptor (IR) tyrosine kinase, leading to autophosphorylation of tyrosine residues in several regions of the intracellular beta subunit [4, 5]. This autophosphorylation further enhances the tyrosine kinase activity of the receptor towards other protein substrates, the first of which is the Insulin receptor substrate 1(IRS1). Phosphorylated IRS1 activates the phosphatidylinositol-3-kinase (PI3K) pathway that stimulates AKT and ultimately promotes glucose transport and glycogen synthesis [5–8]. Most patients with diabetes mellitus have impaired insulin signaling including impaired activation of the IR tyrosine kinase [9]. Therefore, any natural product that interacts with the insulin

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receptor tyrosine kinase and augments it would be considered useful in the treatment of DM [10].

Potentilla fulgens Wall. ex Hook.(Rosaceae) is a medicinal plant traditionally used for treatment of cough, cold, gum and tooth problems, stomach disorders, cancer, diabetes [11–14]. Pharmacologically, it has been reported to have strong antioxidant, antihyperglycemic, hypoglycemic, anti-hyperlipidemic, anti-tumor, anti-inflammatory, and anthelminthic properties [15–18]. This plant has also been reported to contain flavans, including oligomeric flavanols, as major constituents followed by triterpene acids [19]. Reports from our previous study [20] indicate that this plant includes polyphenols like catechin, epicatechin, afzelechin, epiafzelechin, and rutin. Catechins are a class of polyphenols, also reported with anti-diabetic activity, and have shown to exert insulin-like activities thereby improving insulin sensitivity [21, 22]. However, the underlying molecular mechanisms of action of this plant and its active components have not been thoroughly studied.

Accumulating evidence suggests that combining different isolated compounds can further improve the potency of each other. Curcumin for one is a potential phytochemical but its therapeutic potential is limited by its poor bioavailability. However, it was demonstrated that combining curcumin with piperine increased the bioavailability of curcumin [23]. In another study it was shown that combination of curcumin, piperine and quercetin improved the hepatoprotective activity of curcumin [24]. Epicatechin is another potential polyphenol with a variety of therapeutic effects in cancer, inflammation, diabetes and neurodegeneration. However, it has been reported that the bioavailability of native (-)-epicatechin is smaller than for vitamins C and E, with about 1/200 and 1/150 bioavailability, respectively [25]. In another study, it was shown that piperine enhanced the antihyperglycemic effects of metformin (Met) [26]. No reports are available till date to check whether piperine can improve the potency of catechin (Cat) and (-)-epicatechin (E). Therefore, combining catechin and (-)-epicatechin (CE) with piperine is a rationale in this particular study. Thus, modulation of insulin signaling by catechin hydrate, (-)-epicatechin, their combination with piperine (CEP) or without piperine (CE), and *P. fulgens* methanolic extract (PF), was investigated by analyzing the modification of tyrosine and threonine phosphorylation states of the key signaling protein IRS1 in the liver tissues of normal, diabetic and diabetic treated mice. Given their presence in this plant, it is likely that these compounds are key mediators of the medicinal effects of PF. Therefore, the present study focuses on the effect of these compounds on the phosphorylation states of IRS1 and to draw correlation from the study aimed at understanding the mechanistic aspect of this plant. Further, in this study, the compounds found to be present in PF were docked with insulin receptor tyrosine kinase and IRS1 PTB domain to gain more insights to the interactions.

Materials and methods

Chemicals and reagent

All chemicals and reagents used were of analytical grade purchased from Bio-Rad Laboratories Inc., Germany, Sigma Aldrich Co. (St. Louis, USA), Sisco Research Laboratories (SRL), India, Thermo Scientific India Pvt. Ltd. The antibodies were purchased from Santa Cruz Biotechnology, Inc. USA. The purity of piperine, catechin and epicatechin procured from Sigma Aldrich Co. (St. Louis, USA) was ≥ 97% (HPLC) and 98% (HPLC) respectively.

Plant collection and extraction

500 g of *P. fulgens* (voucher no. 464) collected from Shillong Peak, East Khasi Hills Shillong, Meghalaya, India and identified by Dr. P.B. Gurung, herbarium curator, Department of Botany, North-Eastern Hill University was extracted in 10 volumes of aqueous-methanol (1:4). The mixture was filtered and filtrate was dried under vacuum in a rotary evaporator to obtain the crude extract. On November 4, 2023, the plant name was verified using http://www.worldfloraonline.org.

Experimental animals and induction of diabetes

Healthy Balb/c strain Swiss albino mice weighing 25–30 gm procured from Pasteur Institute, Shillong, Meghalaya were housed at 12 h dark and light condition with temperature set to 25°C.

Prior the experiments, mice were fasted overnight but provided with *ad libitum.* 80 mg/kg b.w. of alloxan monohydrate (ALX) in 0.15 M acetate buffer (pH4.5) was intravenously administered to the fasting mice. Mice with a blood glucose level ≥ 200 mg/dL after 48 h were considered diabetic and used for the study [27].

Antihyperglycemic study

Alloxan-induced diabetic mice were intraperitoneally administered with catechin (Cat), epicatechin (E), PF, and metformin (Met). The fasting blood glucose levels were assessed at various time intervals for a period of 24 h. In this study, mice were divided into eleven groups -.

Group 1 - Normal control (NC), received 2% of ethanol.

Group 2 - Diabetic control (DC), received 2% of ethanol.

Groups 3, 4 and 5 - Diabetic mice treated with 5, 10 and 20 mg/kg b.w Cat.

Groups 6, 7 and 8 - Diabetic mice treated with 5, 10 and 20 mg/kg b.w E.

Group 9 - Diabetic mice treated with a combination of Cat and E (CE) (10 mg/kg b.w dose in a 1:1 ratio).

Group 10 - Diabetic mice treated with a combination of Cat, E and piperine (P) (CEP) (10 mg/kg b.w dose in a 1:1:1 ratio).

Group 11- Diabetic mice treated with PF (250 mg/kg b.w).

Group 12- Diabetic mice treated with Met (500 mg/kg b.w).

Intraperitoneal glucose tolerance test (IPGTT)

Alloxan-diabetic mice, fasted overnight and provided with water *ad libitum*, were administered the test samples intraperitoneally 1 h prior to the glucose load of 2 g/kg b.w. Glucose concentration was measured before administration and subsequently at 30, 60, 120, 180 and 1440 min after the glucose load. The control groups received only the glucose load and Met was used as the reference standard. In this study, mice were divided into eight groups, each group comprising of six mice –.

Group a- Normal control (NC), received 2% of vehicle.

Group b- Diabetic control (DC), received 2% of vehicle.

Group c- Diabetic mice treated with the effective optimum dose of Cat (10 mg/kg b.w dose).

Group d- Diabetic mice treated with the effective optimum dose of E (10 mg/kg b.w dose).

Group e-Diabetic mice treated with a combination of CE (10 mg/kg b.w in a 1:1 ratio).

Group f- Diabetic mice treated with CEP (10 mg/kg b.w in a 1:1:1 ratio).

Group g- Diabetic mice treated with PF (250 mg/kg b.w).

Group h- Diabetic mice treated with Met (500 mg/kg b.w).

Antioxidant enzyme studies

Catalase (CAT)

The tissue homogenate was reacted with 0.07 M potassium phosphate buffer and 50 mM H_20_2 and the absorbance was read at λ240 nm [28].

Glutathione peroxidase (GPX)

To 0.1 ml of tissue homogenate 0.5 M sodium phosphate buffer, 10 mM sodium azide, 4 mM reduced glutathione, distilled water and 2.5 M H_20_2 was added and incubated for 30 min at 37 °C. The reaction was stopped by adding 10% TCA and centrifuged at 1000 xg for 10 min. The supernatant obtained was reacted with 0.3% NaH_2PO_4 and DNTB and the absorbance was read at λ412 nm [29].

Estimation of glycogen

Tissue homogenate was centrifuged at 3000 xg for 10 min at 4 °C. 45% ethanol was added to the supernatant obtained and incubated at 4 °C overnight. The precipitated glycogen was collected after centrifugation and quantified using Anthrone method [30].

Protein extraction and western blot of IRS-1 and Akt

IRS-1 and Akt from the liver was extracted using a lysis buffer containing 20 mM Tris HCL (pH 7.6), 150 mM NaCl, 1 mM CaCl2, 10% glycerol, 2 mM EDTA, 1% NP-40, 2 mM Na_3VO_4, and protease inhibitor cocktail. The tissue homogenates was centrifuged at 14,000 xg for 60 min at 40 °C [31].

IRS-1 and Akt from the skeletal muscle was extracted using a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 10 mM NaF, 10 mM Na_3VO_4, 1 mM PMSF, 1 g/ml leupeptin, 1 g/ml aprotinin and protease inhibitor cocktail. The tissue homogenates were centrifuged at 14000xg for 60 min at 40 °C [32].

100 µg of tissue homogenate was separated by SDS-PAGE and transferred to a NC membrane using the Mini Trans-blot Turbo Transfer System. The membrane was block with skim milk, washed with TTBS, and incubated with primary and secondary antibody respectively. Following incubation, the membrane was washed with TTBS, incubated with ECL substrate for 5 min in the dark and the blot was analyzed with a ChemiDoc imaging system.

RNA isolation and quantification of IRS-1 and akt gene

100 mg of liver and skeletal muscle tissue was homogenized using TRI reagent and the RNA obtained was screened for its purity and concentration using a NanoDrop-1000 spectrophotometer. The ratio of absorbance at λ260 nm to λ280 nm was used to assed the purity. RNA samples were converted to cDNA using an iScript cDNA synthesis kit. IRS- and Akt genes were then quantified using a CFX real-Time PCR system. The relative mRNA expression was assessed and calculated using ddCt [33]. The data was normalized to GAPDH. The primers (Table 1) used were designed using the Primer Blast from National Centre for Biotechnology Information (NCBI).

Animal behavioral test: force swimming test

Normal, diabetic and diabetic treated mice were screened for their behavioral changes. The test involved placing mice individually in a borosil cylindrical tank (40×15 cm, 25 cm deep) filled with clean water at 25 °C for 6 min.

Table 1 List of designed primer sequences

	Forward primer	Reverse Primer
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′	3′-TGTAGACCATGTAGTTGAGGTCA-5′
t-IRS1	5′-TTAGGCAGCAATGAGGGCAA-3′	3′-TCTTCATTCTGCTGTGATGTCCA-5′
t-Akt	5′-GGTTCCGTGGCAGACTCTTTA-3′	3′-CTTGGTCCTGCTCCTAGGCT-5′

The immobility of the mice was recorded for 5 min and the first min was taken as adaption period [34].

Transmission electron microscopy study

Liver Tissues were excised, washed, cut into mm sections, and fixed in Karnovsky's fixative and 1% OsO_4. Fixed tissue blocks were dehydrated in 0.2 M cacodylate buffer, cleansed in propylene oxide and embedded in BEEM capsules. The blocks were sectioned, stained with uranyl acetate, and viewed using TEM at 2000x magnification.

Docking studies

The structure of phytochemicals such as quercetin, kaempferol, gallic acid, ursolic acid, ellagic acid, tormentic acid, epicatechin, epiafzelechin, and catechin that were previously reported to be present in PF in our investigations [20] and Choudhary et al. [19] were extracted from PubChem. The 3-D structure of IRS tyrosine kinase and PTB domain was retrieved from the Protein Databank.

Statistical analysis

The results were expressed as Mean ± SEM and data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test.

Results

Antihyperglycemic study

The anti-hyperglycemic effect of PF, Cat, E, CE, CEP and Met was dose and time-dependent. Maximum reduction was observed at 6 h following test sample administration for all the doses used. At 6 h, the reduction was 57.8% ($p < 0.001$), 68.3% ($p < 0.001$) and 68.9% ($p < 0.001$) for 5 mg/kg b.w, 10 mg/kg b.w and 20 mg/kg b.w of Cat, 53% ($p < 0.001$), 70.7% ($p < 0.001$) and 72.6% ($p < 0.001$) for 5 mg/kg b.w, 10 mg/kg b.w and 20 mg/kg b.w of E. It was

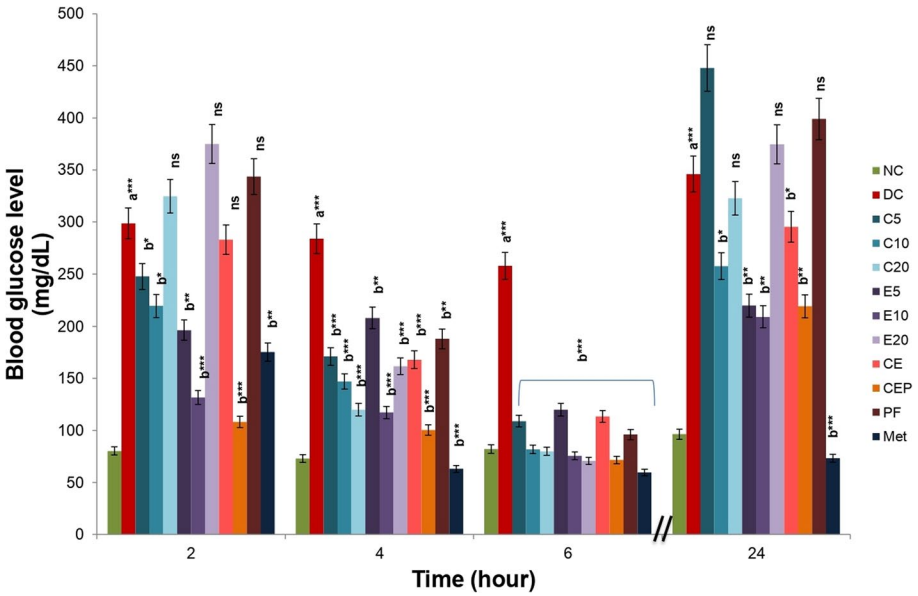

Fig. 1 Effects of NC, DC C5, C10, C20, E5, E10, E20, CE, CEP, PF, and Met on blood glucose levels of alloxan-induced diabetic mice. Values are expressed as mean ± SEM. SEM: a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control. SEM: Standard error of mean; ns: non significant; NC; normal control; DC: diabetic control; C5: catechin hydrate at a dose of 5 mg/kg b.w; C10: catechin hydrate at a dose of 10 mg/kg b.w; C20: catechin hydrate at a dose of 20 mg/kg b.w; E5: (-)-epicatechin at a dose of 5 mg/kg b.w; E10: epicatechin at a dose of 10 mg/kg b.w; E20: (-)-epicatechin at a dose of 20 mg/kg b.w; CE: catechin hydrate and epicatechin at a dose of 20 mg/kg b.w; CEP: catechin hydrate, epicatechin and piperine at a dose of 20 mg/kg b.w; PF: methanolic extract of *Potentilla fulgens*; Met: metformin

56% ($p < 0.01$) for E and 72% ($p < 0.001$) for CE respectively, when compared to DC. For PF, the reduction was found to be 61.5% ($p < 0.001$) respectively (Fig. 1). 10 mg of Cat and E were found to be more effective than 5 and 20 mg.

Intraperitoneal glucose tolerance test (IPGTT)

Results of the IPGTT showed that both diabetic and treated groups could significantly suppress the glucose peak after glucose load (Fig. 2A). The effect of CE and CEP on the glucose peak was comparable. After 60 min of glucose load the effect was of Cat, E, CE, CEP was very prominent (Fig. 2A). The effect of Cat, E, CE, CEP, PF, and met was very significant. The effect of the plant extract was rather slow in comparison to the compounds. However, after 24 h the suppression of the glucose peak by PF was very significant in comparison to Cat, E, CE, CEP, and met. Overall when compared to the diabetic group, the AUC-G for met treated group was the lowest followed CEP, CE, PF, E, and C (Fig. 2B). The effect of PF

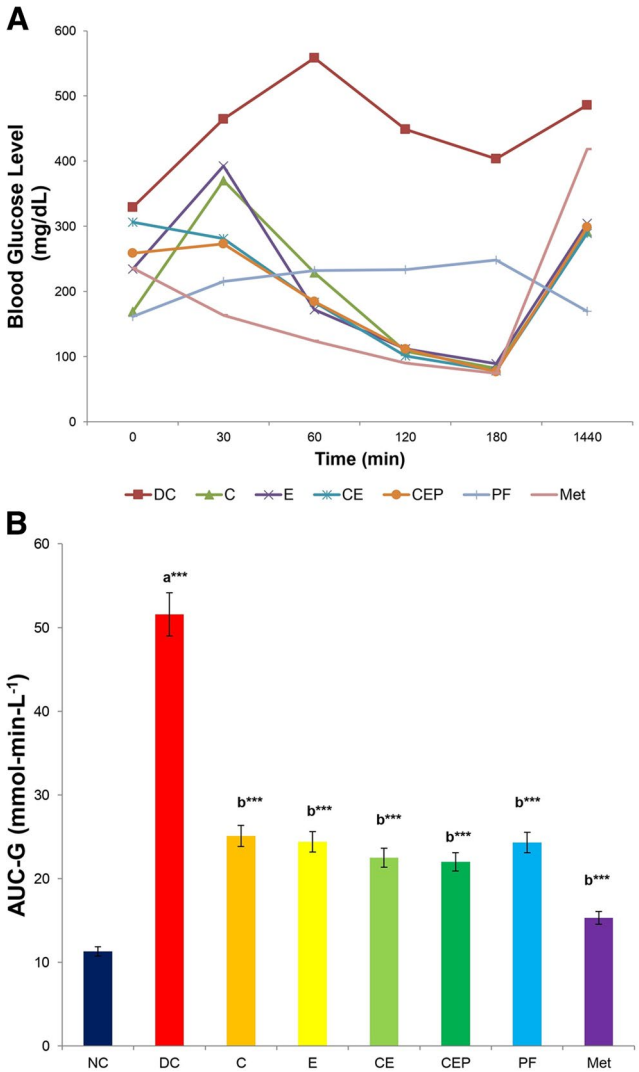

Fig. 2 **A** Intraperitoneal Glucose tolerance in NC, DC, C, E, CE, CEP, PF and Met. Results are expressed as mean ± SEM. SEM: Standard error of mean; NC; normal control; DC: diabetic control; C: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin. **B** Area under the curve of the intraperitoneal glucose tolerance in NC, DC, C, E, CE, CEP, PF and Met. Results are expressed as mean ± SEM. a*** represents the level of significance at 0.001 against the normal control; b**, and b*** represents the level of significance at 0.01, and 0.001 respectively against the diabetic control. SEM: Standard error of mean; NC: normal control; DC: diabetic control; C: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

may not be as in the case of Cat, E, CE, CEP, and Met but in the context of diabetes, PF has a long-term effect.

Antioxidant enzyme studies

Catalase (CAT)

Significant decrease in CAT activity was observed in the liver tissues of untreated diabetic group compared to the normal levels (3.6 ± 0.0002). CAT activity liver was reduced by 78% ($p < 0.001$) as compared to the normal group. Administration of *P. fulgens* extract increased enzyme activity by 172% ($p < 0.01$) from the diabetic control group. Treatment with Cat and CE increased CAT activity by 175% ($p < 0.05$) and 178% ($p < 0.01$) respectively. Although the results were significant only in Cat, CE, PF and Met group, E and CEP, treated group also decreased the activity of CAT (Table 2).

Glutathione peroxidase (GPx)

Significant decrease in GPx activity was observed in the liver tissues of untreated diabetic group compared to the normal levels (10.4 ± 0.013). GPx activity liver was reduced by 72% ($p < 0.01$) as compared to the normal group. Administration of P. fulgens extract increased enzyme activity by 44.3% ($p < 0.001$) from the diabetic control group. Treatment with Cat, EC, Cat/EC and Cat/ECPincreased enzyme activity by 36% ($p < 0.05$), 51.3% ($p < 0.001$), 61.2% ($p < 0.001$), and 33.5% ($p < 0.05$) respectively. Thus, all the treated groups with exception to Met significantly increased the activity of GPx (Table 2).

Table 2 Showing the Mean ± SEM activity of glutathione peroxidase (GPx) and catalase (CAT) in liver tissue of normoglycemic (NC), diabetic untreated (DC), Catechin treated diabetic mice (C), epicatechin treated diabetic mice (E), catechin and epicatechin treated diabetic mice (CE), catechin, epicatechin, and piperine treated diabetic mice (CEP), Potentilla fulgens treated diabetic mice (PF), and metformin treated diabetic mice (Met), as well as the percentage increase in their activities compared to their respective controls

GROUP	LIVER			
	CAT activity (U/mg protein) X 10^3	% increase/ decrease	GPx activity (U/mg protein) X 10	% increase/ decrease
NC	16(±0.001)	-	36.9(±0.27)	-
DC	3.6 ^{a***}(±0.0002)	-78	10.4 ^{a***} (±0.013)	-72
C	9.887 b*(±0.0018)	175	36.0^{b*} (±0.39)	246
E	9.9^{ns}(±0.002)	175	51.3^{b***} (±0.42)	393
CE	10 ^{b**} (±0.0017)	178	61.2^{b***} (±0.28)	488
CEP	13^{ns} (±0.0023)	261	33.5^{b*}(±0.21)	222
PF	9.8^{b**} (±0.0009)	172	44.3^{b***} (±0.38)	325
Met	5^{b***} (±0.0017)	39	19.5^{ns} (±0.21)	88

Values are expressed as mean ± SEM of three separate experiments

a** represents level of significance at p < 0.01 compared against NC

a*** represents level of significance at p < 0.001 compared against NC

b* represents level of significance at p < 0.05 compared against DC

b** represents level of significance at p < 0.01 compared against DC

b*** represents level of significance at p < 0.001 compared against DC

Ns Non significant

Estimation of glycogen

The hepatic glycogen which decreased significantly by 73% ($p < 0.001$) inthe DC group got significantly elevated in all the treated groups. The increase inhepatic glycogen content was 97.1% ($P < 0.05$), 134.5% ($P < 0.05$), 200.8%($p < 0.001$), 163.2% ($P < 0.001$), 215.7% ($P < 0.05$), 248.8% ($P < 0.001$) for Cat, EC, Cat/ EC, Cat/EC/P, PF and Met respectively. Cat/EC displayed a similarpattern of increasing the hepatic glycogen content as the plant extract. (Table 3).

Protein expression of IRS-1 and Akt

The total IRS1 and phosphorylated IRS1 protein in the liver and skeletal muscle significantly decreased in the DC ($p < 0.0001$) (Figs. 3 and 4). The treated groups exhibit an increased in the expression of t-IRS1by 67% and 41%, p-IRS1 by 66% and 80% in skeletal muscle, respectively when compared to the diabetic control groups. However, the effect of E was not significant when compared to DC.

When compared to NC, t-Akt and p-Akt protein level in the liver and skeletal muscle of the DC significantly decreased ($p < 0.0001$) (Figs. 5 and 6). When compared to the DC however, the treated groups showed increased

Table 3 Glycogen content in hepatic tissue of normoglycemic (NC), diabetic untreated (DC), Catechin treated diabetic mice (C), epicatechin treated diabetic mice (E), catechin and epicatechin treated diabetic mice (CE), catechin, epicatechin, and piperine treated diabetic mice (CEP), Potentilla fulgens treated diabetic mice (PF), and metformin treated diabetic mice (Met), as well as the percentage increase in their activities compared to their respective controls

Group	M±SEM (mg/g tissue)	% increase/decrease
NC	5±0.017	-
DC	1.33±0.029^{a**}	-73
C	2.621±0.005^{b*}	97.1
E	3.12±0.017^{b*}	134.5
CE	4±0.007^{b**}	200.8
CEP	3.5±0.006^{b**}	163.2
PF	4.2±0.043^{b*}	215.7
Met	4.64±0.0008^{b**}	248.8

Values are expressed as mean ± SEM of three separate experiments

a* represents level of significance at p < 0.05 compared against NC

b* represents level of significance at p < 0.05 compared against DC

b** represents level of significance at p < 0.01 compared against DC

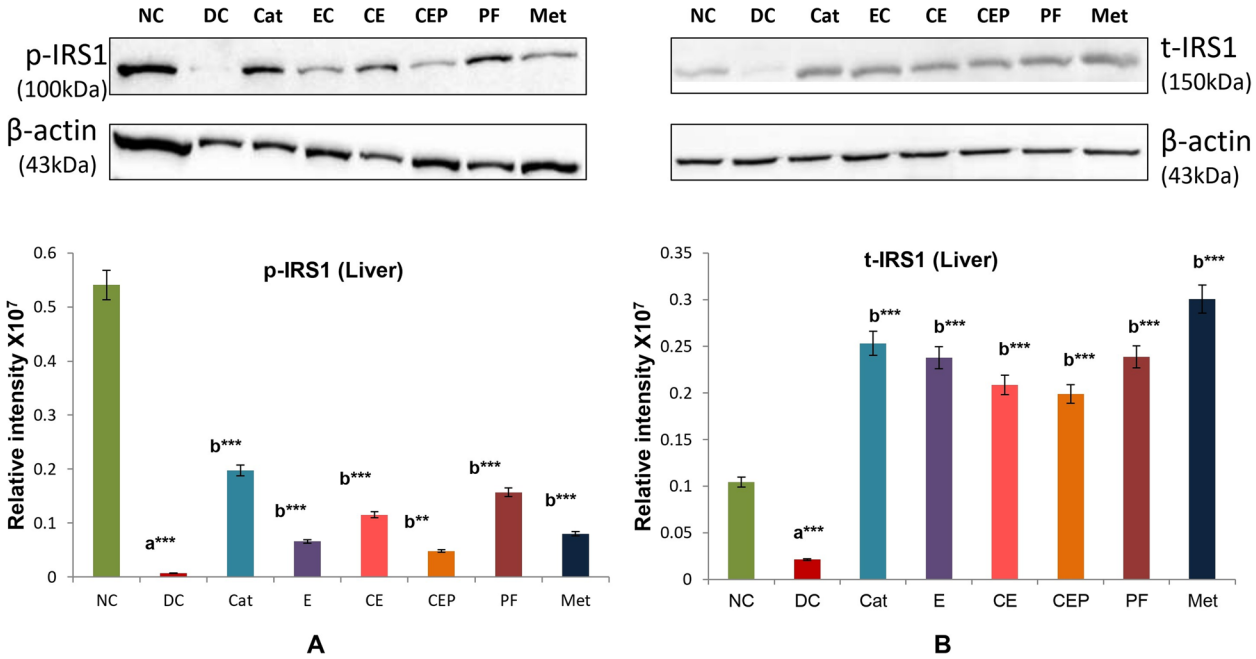

Fig. 3 Western blot analysis of the expression of (**A**). tyrosine phosphorylated IRS1 and (**B**). total IRS1 proteins in liver of NC, DC, Cat, EC, CE, CEP, PF and Met. β-actin was used as a loading control. a*** represents the level of significance at 0.001 against the normal control; b**, and b*** represents the level of significance at 0.01, and 0.001 respectively against the diabetic control. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

expression of t-Akt and p-Akt. C and E had no significant effect on t-Akt and p-Akt in the liver (Fig. 5). On the other hand, Cat and E had a very significant effect ($p < 0.0001$) on p-Akt in the skeletal muscle (Fig. 6).

Gene expression of IRS-1 and akt gene

The mRNA expression of IRS1 and Akt was similar with the protein expression. The level of t-IRS1 in liver of the DC was lowered by ~0.07fold. When compared to DC group, the gene expression was upregulated by a significant amount. The fold change in t-IRS1 expression in Cat, E, CE, CEP, PF and Met was 0.6, 0.5, 0.82, 0.7, 0.57, and 0.66 respectively (Fig. 7A). In the skeletal muscle, the fold change was 0.13, 0.24, 0.12, 0.15, and 0.12 respectively (Fig. 7B).

In Cat, E, CE, CEP, PF and Met treated groups, the relative fold change in t-Akt expression was 0.64, 0.41, 0.75, 0.58, 0.66, and 0.65 in the liver (Fig. 8A), while in the skeletal muscle the fold change was 0.04, 0.1, 0.08, 0.11, and 0.18 respectively (Fig. 8B).

Animal behavioral test: force swimming test

The Forced Swimming test showed an altered behavior in diabetic and diabetic treated mice. Diabetic mice were significantly immobile when compared to the normal control group. The altered mobility was reverted back to near normal on treatment with Cat, EC, Cat/EC, Cat/EC/P, PF and Met ($p < 0.001$). The effect of Cat, EC, and Cat/EC were similar. Also, the effect of PF and Met was comparable (Fig. 9).

Transmission electron microscopy study

TEM sections of liver in NC showed a normal nucleus with nucleolus and a distinct nuclear membrane (Fig. 10A). Significant alterations in ultrastructure were observed in the DC group. The nucleus was distorted and convoluted (Fig. 10B). Presence of tiny droplets was also seen. These aberrations were improved on treatment (Fig. 10C-H). With exception to E (Fig. 10D), the nuclear membranes of all the treated groups were intact, membrane integrity was improved, and the lipid droplets were lesser in comparison to the DC group.

Normal mice showed intact ER tubules and normal mitochondria with distinct cristaes (Fig. 10A). In contrast, diabetic mice developed a disoriented, fragmented and deteriorated ER tubules (Fig. 10B). The mitochondrial membrane was elongated and dilated. Distorted and fragmented cristaes were also seen (Fig. 10B). However, these ultrastructural changes improved considerably in the treated diabetic mice (Fig. 10C-H).

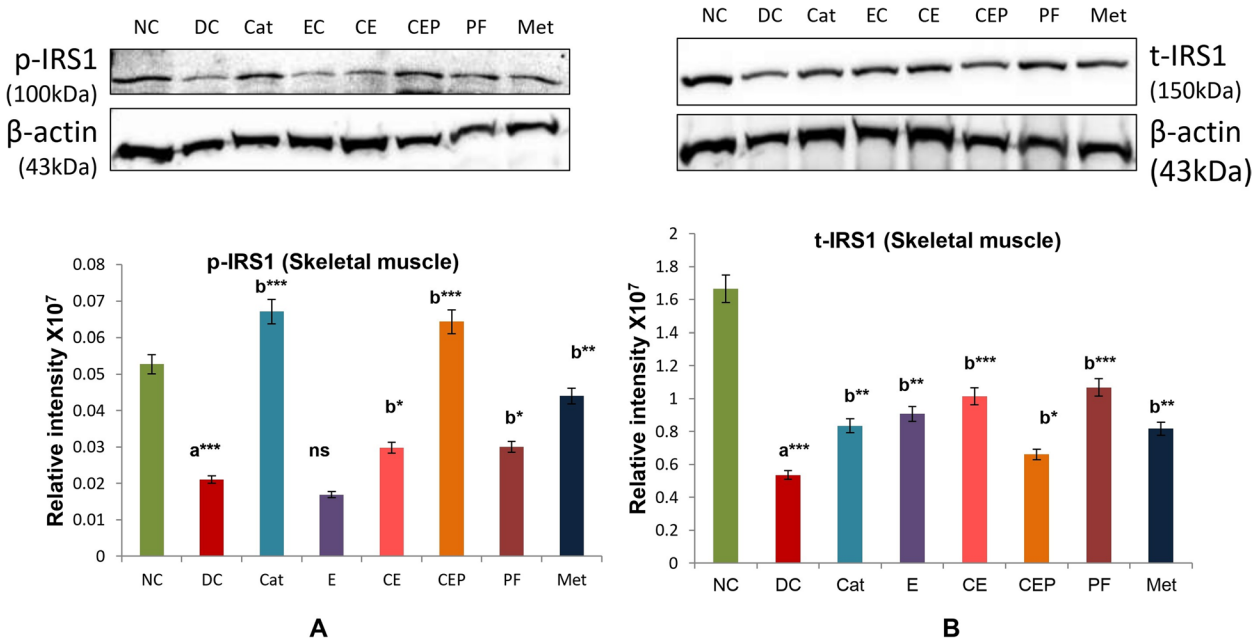

Fig. 4 Western blot analysis of the expression of (**A**). tyrosine phosphorylated IRS1 and (**B**). total IRS1 proteins in skeletal muscle of NC, DC, Cat, EC, Cat/EC, Cat/ECP, PF and Met. β-actin was used as a loading control. a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control; ns: non-significant. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

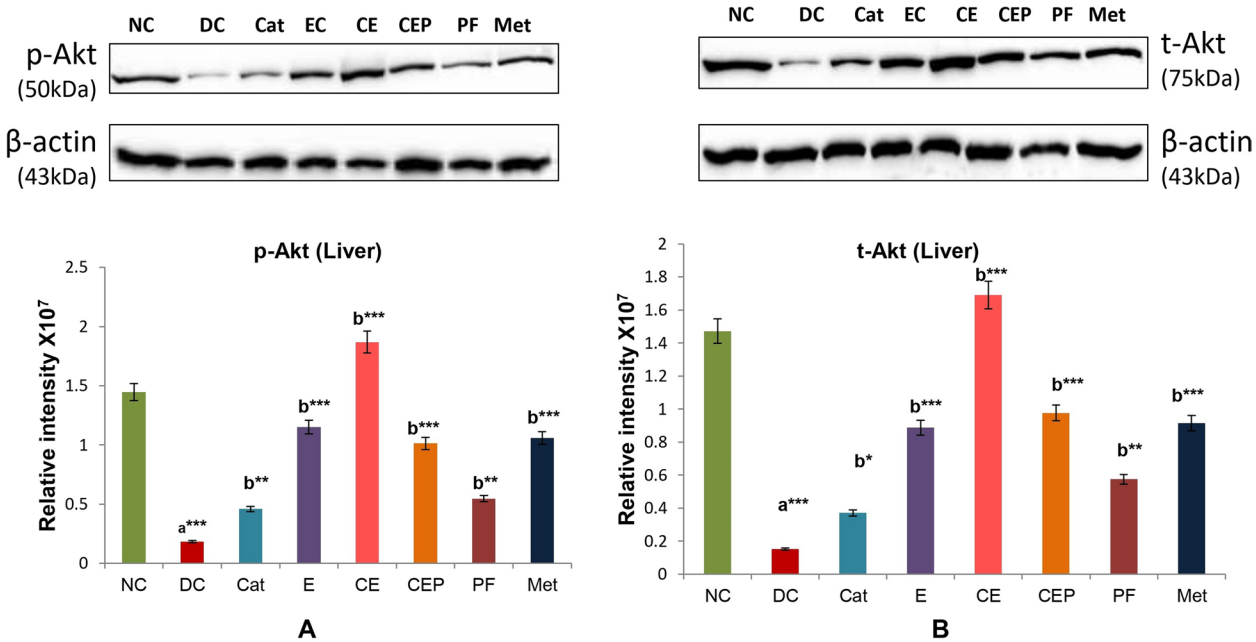

Fig. 5 Western blot analysis of the expression of (**A**). ser phosphorylated Akt and (**B**). total Akt proteins in liver of NC, DC, Cat, EC, CE, CEP, PF and Met. β-actin was used as a loading control. a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

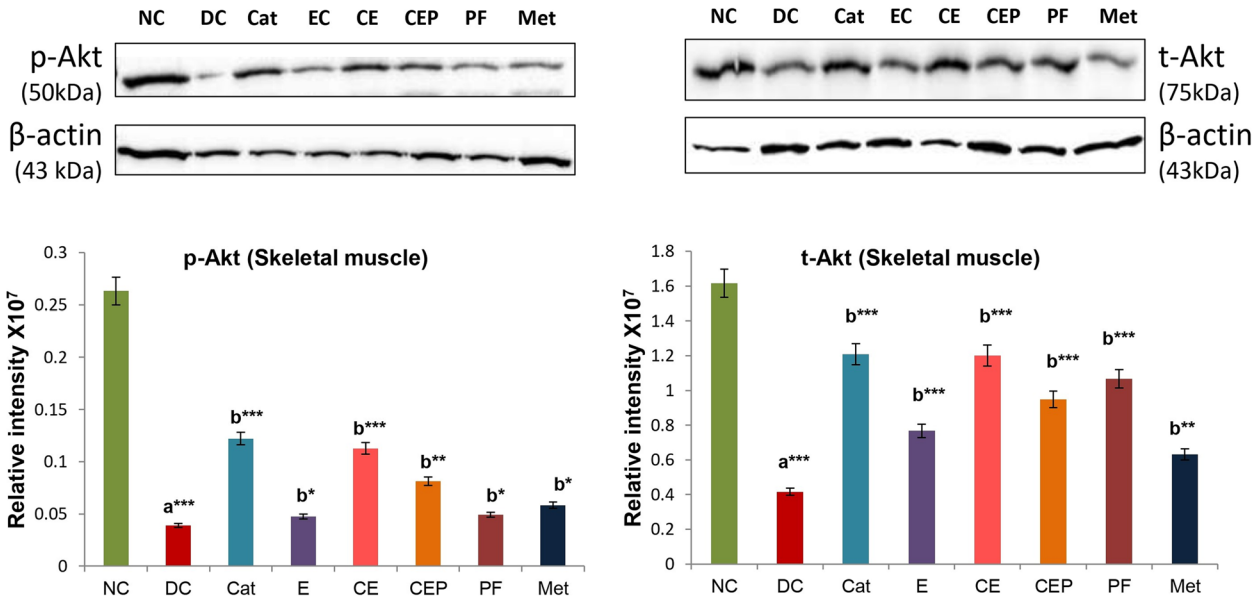

Fig. 6 Western blot analysis of the expression of (**A**). ser phosphorylated Akt and (**B**). total Akt proteins in skeletal muscle of NC, DC, Cat, EC, CE, CEP, PF and Met. β-actin was used as a loading control. a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

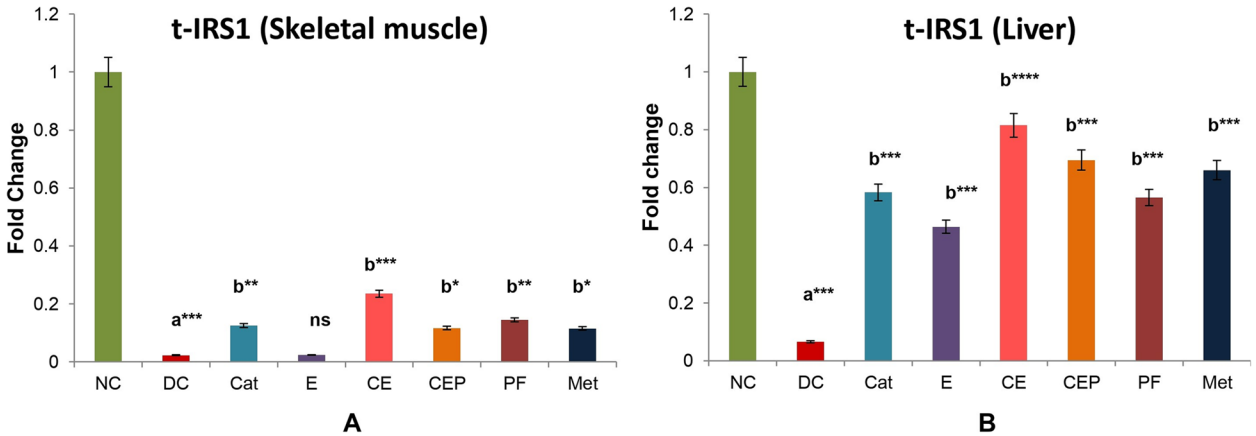

Fig. 7 Relative quantification of IRS1 gene in (**A**) liver and (**B**) skeletal muscle of NC, DC, Cat, E, CE, CEP, PF, and Met. Values are expressed as mean ± SEM. SEM: a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control; ns: non-significant. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

Docking studies

Molecular docking of the selected phytochemicals and the target ligands showed that they had effective binding energies and similar binding residues. Catechin, epicatechin, and piperine had a better binding affinity with IR tyrosine kinase than metformin (Fig. 11A; Table 4). Piperine had a betters binding affinity with the IRS1-PTB domain than metformin, catechin and epicatechin (Fig. 11B; Table 4).

Discussion

Alloxan selectively destroys pancreatic insulin secreting β-cells, resulting in a substantial increase in blood insulin level [35]. However, intravenous administration of alloxan

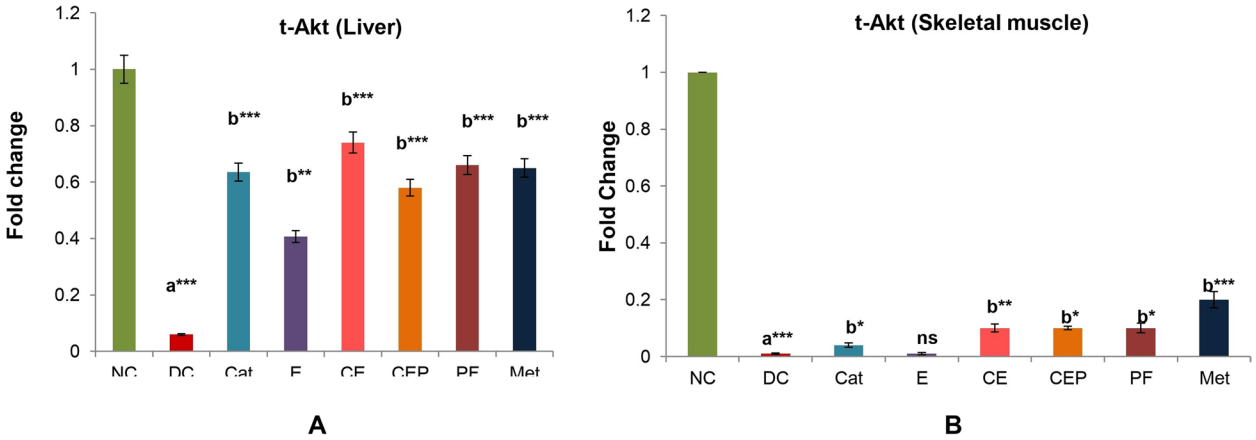

Fig. 8 Relative quantification of Akt gene in (**A**) liver and (**B**) skeletal muscle of NC, DC, Cat, E, CE, CEP, PF, and Met. Values are expressed as mean ± SEM. SEM: a*** represents the level of significance at 0.001 against the normal control; b*, b**, and b*** represents the level of significance at 0.05, 0.01, and 0.001 respectively against the diabetic control; ns: non-significant. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

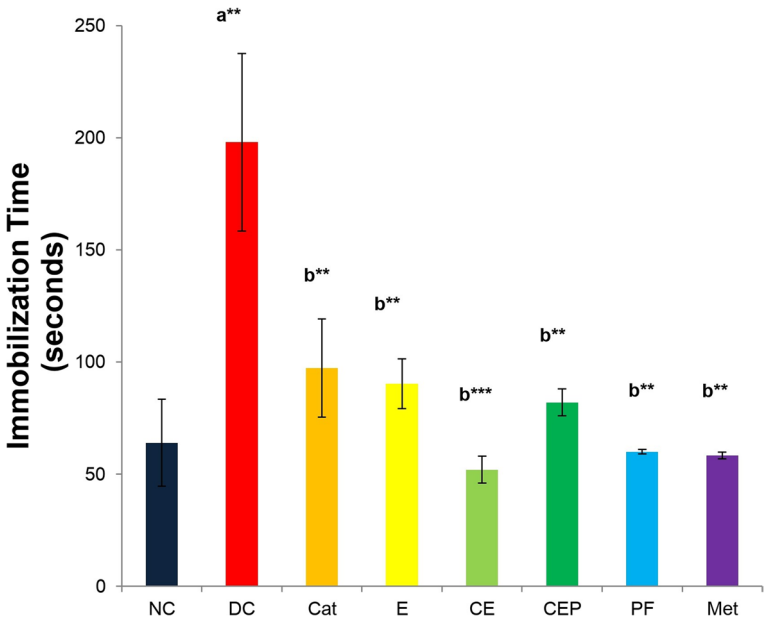

Fig. 9 Immobility time in NC, DC, Cat, EC, CE, CEP, PF and Met. Values are expressed as mean ± SEM. a** represents the level of significance at 0.01 against the normal control; b**, and b*** represents the level of significance at 0.01, and 0.001 respectively against the diabetic control. NC: normal control; DC: diabetic control; Cat: diabetic mice treated with catechin hydrate; E: diabetic mice treated with epicatechin; CE: diabetic mice treated with catechin hydrate and epicatechin; CEP: diabetic mice treated with catechin hydrate, epicatechin, and piperine; PF: diabetic mice treated with methanolic extract of *Potentilla fulgens*; Met: diabetic mice treated with metformin

at a low dose in the present study effectively induced diabetes which was confirmed by persistent elevated plasma fasting glucose for two weeks in DC mice. The results of the Antihyperglycemic study displayed the glucose tolerance effects of the plant extracts and the phytochemicals-C and E in a time- and dose-dependent manner. Maximum reduction of the glucose tolerance was at 6 h. At 10 mg/kg b.w., Cat and E lowered the blood glucose at a level similar to 20 mg/kg b.w. dose. Therefore, for further studies, 10 mg/kg b.w. of Cat and E was selected. Our results from the IPGTT also showed that the plant extracts and the phytochemicals- Cat and E suppresses

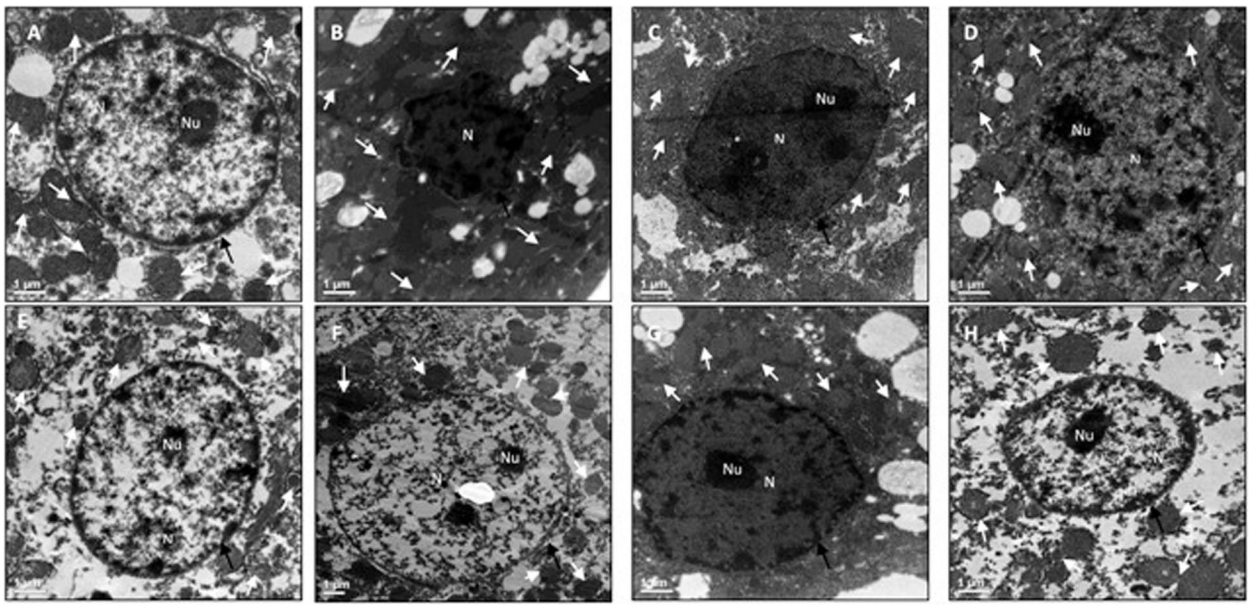

Fig. 10 TEM sections of the Liver showing nucleus (N), nuclear membrane, and mitochondria of normal, diabetic, and diabetic treated groups at 2000X magnification. **A** Normal control, **B** Diabetic control, **C** Diabetic treated with Cat (**D**) Diabetic treated with E, **E** Diabetic treated with CE, **F** Diabetic treated with CEP, **G** Diabetic treated with PF, **H** Diabetic treated with Met. Black arrows: nuclear membrane; White arrows: mitochondria

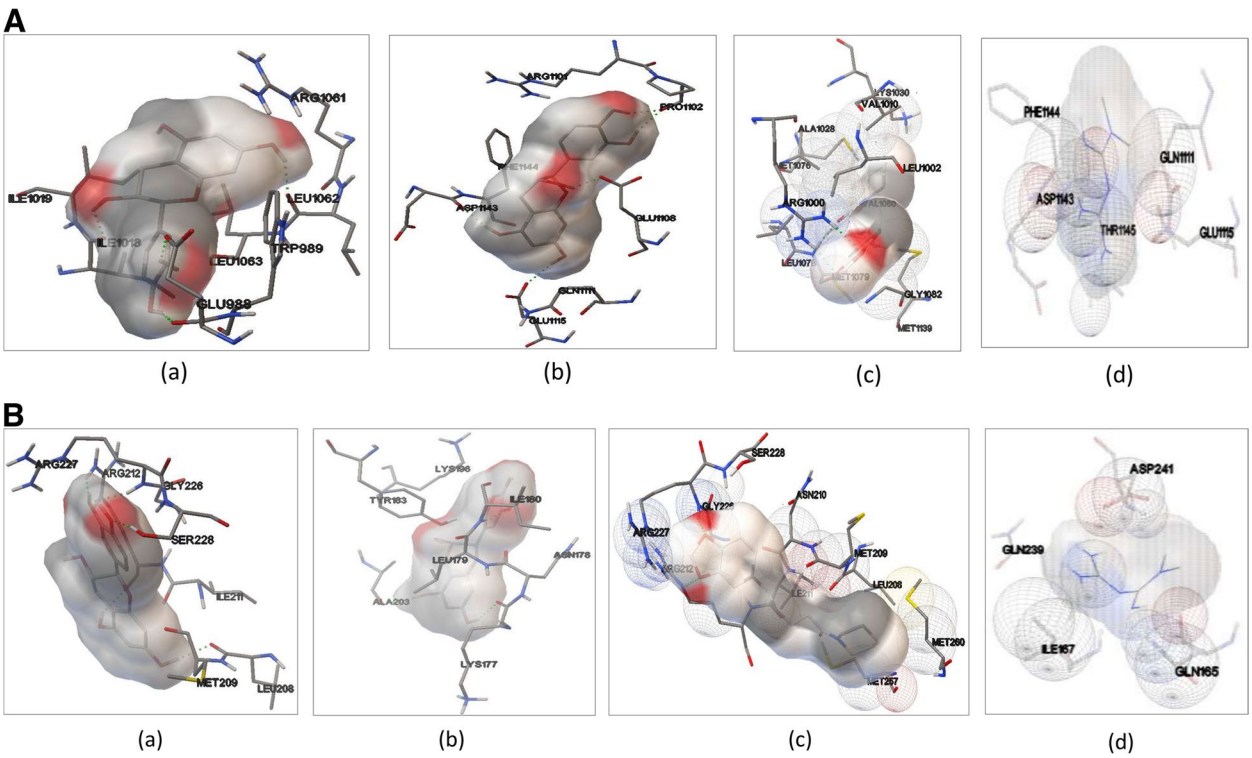

Fig. 11 Molecular docking (**A**)**.** IR tyrosine kinase and (**B**). IRS1 PTB domain with (**a**) Catechin, (**b**) Epicatechin, (**c**) Piperine, and (**d**) Metformin. Metformin was used as a positive control

Table 4 Binding energies and interactive residues in the docked complex

Compounds	Insulin receptor tyrosine kinase		IRS1 PTB domain	
	Amino acid residues	Binding energy (kCal/mol)	Amino acid residues	Binding energy (kCal/mol)
Catechin	Ile1019, Arg1061, Leu1062, Trp989, Leu1063, Glu988	-6.46	Arg1101,Pro1102,Asp1143, Phe1144, Glu1106, Gln1111, Glu1115	-5.78
Epicatechin	Arg1101, Pro1102, Asp1143, Phe1144, Glu1106, Gln1111, Glu1115	-6.93	Lys196, Tyr183,Ile180, Leu179,Asn178,Ala203, Lys17	-5.66
Piperine	Ala1028,Met1076,Arg1000,Val1010, Leu1002, Lys1030, Leu107,Met1079,Gly1082,Met1139, Val1060	-7.56	Arg227, Gly226, Ser228, Asn210, Met209,Leu206, Arg212, Met260,Met257, Ile211	-7.12
Metformin	Phe1144, Gln1111, Asp1143, Thr1145, Gln1115	-4.51	Asp241, Gln239, Ile167, Gln165	-6.26

the glucose peak. Based on the glucose area under the curve (AUG-C), all the treated groups increased the disposal of glucose significantly. The AUC-G of Cat, E and PF were comparable. The combination of CE and CEP significantly increased the glucose disposal albeit the antidiabetic drug showed a better potential at promoting glucose disposal. Nevertheless, while Met had better disposal at 500 mg/kg b.w. it is noteworthy that even at comparatively lower doses, the plant extracts and the phytochemicals could improve hyperglycemia to a large extent. The suppression of blood glucose by Cat, E, CE, and CEP was short-term, whereby we observed that after 24 h the blood glucose level increased. In case of PF, although the effect was slower in comparison to Cat, E, CE, CEP, and Met it had a long-term effect, whereby we observed that even after 24 h, the blood glucose level decreased significantly.

The present studies also showed that Cat, E, CE, CEP, Met and PF all increased the glycogen content to a significant level in the liver tissues. Insulin is the main regulator for glycogenesis in liver. The decrease of liver glycogen observed in this study may be due to low insulin action in the liver. It is well established that insulin affects gene expression through the Grb2-Sos-Ras-MAPK pathway and affects glycogen metabolism and glucose transport through the PI-3 K-PKB pathway. After 2 weeks treatment with PF, Cat, E, CE, and Cat/ECP the liver glycogen level was significantly increased. Daisy et al. [22] demonstrated that one possible way of the antidiabetogenic action of cat may be through the insulin mimetic response. Since catechin is comparatively higher in content in the plant extract, it might confer its insulin mimetic response to the plant resulting in the overall insulin sensitizing effect of the plant.

Hyperglycemia increases production of NADH which is the substrate and electron donor for mitochondrial complex I and complex I is the main site for reactive oxygen species (ROS) production [36] leading to oxidative stress (OS). As demonstrated in the results, the plant extracts and the phytochemicals reduced oxidative stress by elevating GPx and CAT activity. ROS also disrupts the intracellular signaling pathways by altering the insulin secretion and signaling [37]. Low levels of IRS1 have been observed in several insulin resistant animal models suggesting that most patients with diabetes have impaired insulin signaling including reduced tyrosine phosphorylation of IRS1 [38]. In the present study, there was a significant reduction of IRS1 and Akt at both protein and gene level indicating the presence of impaired insulin signaling in diabetic mice. Reduced tyrosine phosphorylation of IRS1 with either increased or decreased site-specific serine/threonine phosphorylation of IRS1 has been reported to be a common feature in insulin resistance and T2D [39]. The significant upregulation of IRS1 and Akt by the plant extract as well as the phytochemicals illustrates the ability of these compounds to improve insulin action and signaling. From the docking studies, we can see that these compounds bind efficiently to the IRS PTB domain, a phosphotyrosine domain that interacts directly with the juxtamembrane (JM) domain of the insulin receptors and any hindrance of these interactions by Ser/Thr phosphorylation negatively affects insulin signaling [40]. This thus supports the insulin mimetic action of these compounds in increasing the IRS1 phosphorylation and glycogen synthesis in the liver. Daisy et al. [22] illustrated the catechin mimics insulin action and since it is comparatively higher in content in PF, it might confer its insulin mimetic response to the plant resulting in overall insulin sensitizing effect of the plant.

The alterations in liver morphology can be linked to diabetic oxidative stress induced by mitochondrial damaged mediated by increase ROS production. OS which refers to an imbalance of ROS [41] was implicated by reduced GPx and CAT activity. The nucleus which influences cellular function [42] and mitochondria which modulates autophagy and apoptosis [43] are important

cell organelles. In normal mice the cells were intact and undamaged. On the otherhand, liver sections of diabetic mice microscopically exhibited nuclear envelope evagination and invagination as well as apoptotic cell nuclei. This clearly suggests that alloxan-induced diabetes causes apoptosis in liver cells. The significant effect of PF and its phytochemicals on GPx and CAT suggest the possible reason for their beneficial effect on the ultrastructural changes observed in diabetic treated mice.

Depression is one of the lifethreatening mental disorder and a major risk factor of cardiovascular and metabolic problems [44]. The FST is one of the commonly used models to study depressive-like behavior in mice [45]. The increased immobilization in DC mice could be interpreted as a sign of behavioral despair. Immobility behavior in the FST has been demonstrated to be altered in numerous animal models connected to depression pre disposition including clinical diabetes [44]. In the present study, the plant extracts as well as the phytochemicals could improve immobilizations which were otherwise elevated in diabetic mice.

Conclusions

These finding provide the biochemical basis for the antihyperglycemic activity of *P.fulgens.* The study demonstrates that *P.fulgens* and its phytochemicals-catechin and epicatechin reduce blood glucose level, possibly mediating their effects through upregulation of insulin IRS-1 and Akt.

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Authors' contributions
D.S. conceived the study; D.S. and A.B. supervised the study; S.E.S carried out the investigation and formal analysis; C.E.K. and S.B. carried out the docking studies; S.E.S., C.L.P., and D.T. analyzed the data; S.E.S., C.L.P., AND D.T. drafted the manuscript; C.L.P. and A.K.R. edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was approved by the Institutional Ethics Committee (Animal models) (IEC: Animal model/NEHU 15-12-2015) and has been performed in accordance with the ethical standards.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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