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ORIGINAL ARTICLE

Plant Extract-Derived Betulinic Acid Chalcone Inhibits the Development of Type 2 Diabetes Mellitus Via Targeting Peroxisome Proliferator-Activated Receptor-γ-Regulated Gene Expression

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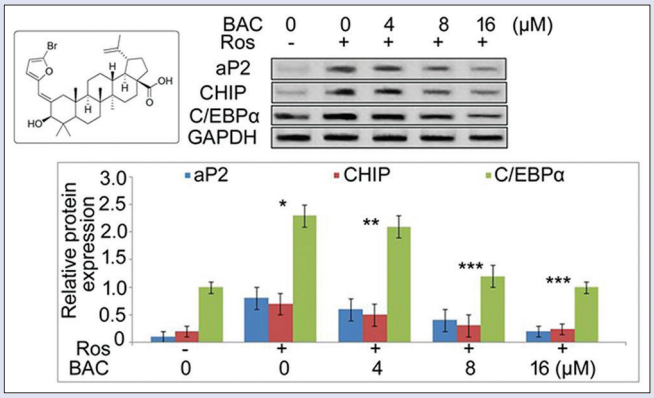
ABSTRACT

Aim: Diabetes mellitus is a syndrome related to metabolism having complicated pathogenesis and its morbidity rate is quickly increasing every year. In the present study, the antagonistic effect of betulinic acid chalcone for peroxisome proliferator-activated receptor-γ (PPARγ) was explored with possible implications in diabetic treatment. **Materials and Methods:** The adipocyte differentiation following betulinic acid chalcone treatment was evaluated using Oil Red O staining. Reverse transcription–polymerase chain reaction was employed for gene expression and Western blot for analysis of differentiation linked protein expression. **Results:** Betulinic acid chalcone repressed PPARγ-ligand-binding domain level and transcriptional property of retinoid X receptor α-PPARγ in 293T cells. The rosiglitazone suggestively ($P < 0.01$) augmented grease droplet accumulation in adipocytes in comparison to control adipocytes. The improved grease droplet accumulation by rosiglitazone in adipocytes was suppressed on treatment with betulinic acid chalcone. The surge in grease droplet accumulation by rosiglitazone was lessened completely on treatment with 16-μM betulinic acid chalcone. Treatment of adipocytes with betulinic acid chalcone suppressed rosiglitazone-induced expression of fatty acid synthase (FAS), CCAAT/enhancer-binding protein-α (C/EBPα), adipocyte fatty acid-binding protein 2 (aP2), and HMG-CoA genes pointedly. Treatment of adipocytes with betulinic acid chalcone instigated a marked decrease in rosiglitazone-induced expression of aP2, carboxyl terminus of the Hsc70-interacting protein (CHIP), and C/EBPα. The suppressive effect of rosiglitazone on expression of p-Akt/t-Akt, PPARa, p-FoxO1/t-FoxO1, and p-AMP protein kinase (AMPK)/t-AMPK was expressively ($P < 0.01$) assuaged in the adipocytes by betulinic acid chalcone. **Conclusion:** The present study established that betulinic acid chalcone suppressed PPARγ activity and adipocyte differentiation. Moreover, the activation of FoxO1/Akt/AMPK was upregulated and FAS/EBPα/aP2/HMG-CoA expression was subdued by betulinic acid chalcone in the adipocytes. Therefore, betulinic acid chalcone may be appraised further for a possible role in the treatment of diabetes.

Key words: Adipocytes, antidiabetic, differentiation, glucolipids, peroxisome proliferator

SUMMARY

- The study proves that betulinic acid chalcone blocked PPARγ activity and adipocyte differentiation. The activation of FoxO1/Akt/AMP protein kinase was upregulated and fatty acid synthase/enhancer-binding protein-α/adipocyte fatty acid-binding protein 2/HMG-CoA expression was inhibited by betulinic acid chalcone in the adipocytes. Therefore, betulinic acid chalcone may be appraised further for potential role in the diabetes treatment.



Abbreviations used: DM: Diabetes mellitus; PPARγ: Peroxisome proliferator-activated receptor-γ; DMEM: Dulbecco's modified Eagle's medium; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction.

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INTRODUCTION

Diabetes mellitus is a syndrome connected with metabolism having complicated pathogenesis and its morbidity rate is quickly increasing every year. The distinguishing feature of diabetes mellitus is chronic hyperglycemia and loss of beta-cells in pancreas ensuing in deficiency of insulin.[1,2] Till date, effective treatment free from side effects has not been advanced for diabetes mellitus.[3] The abnormally raised sugar level and metabolism of lipids caused by the deficiency in insulin secretion or high glucagon level are the protuberant factors tangled in diabetes mellitus type 2 development.[4] There is hyperglycemia as well as hyperlipidemia in the diabetes mellitus patients clinically.[4] Diabetes

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mellitus is measured to be a developing health problem globally and a load on the societies because it mostly affects the life standard of the people. It has been suggested by the American and European Diabetes Association that the level of glycosylated hemoglobin in 7% of the patients with diabetes should be studied to classify undesired glucose in blood.[5] The oral antidiabetic drugs are associated with several harmful effects and the insulin administration triggers increase in body weight and hypoglycemia. This highlights the need to develop efficient and innovative treatment tactic for diabetes mellitus type 2 urgently.[6]

The metabolism of glucolipids in body is controlled by peroxisome proliferator-activated receptor-γ (PPARγ).[7] The PPARγ belongs to the family of nuclear receptors and is dependent on ligands for its activity.[8] The receptor on ligation followed by activation combines with retinoid X receptor-α (RXRα) to afford heterodimer.[9] The effect of the expression of various genes on PPAR response element is measured by the the formation of heterodimer.[9] The thiazolidinedione class of chemotherapeutics like rosiglitazone was employed clinically for diabetes mellitus type 2 treatment reduction hypoglycemia by acting as agonists for PPARγ.[10] The clinical use of these chemotherapeutics has led to cardiovascular disorders and augmented body weight.[10] Recently, antidiabetic studies have engrossed on knocking out of PPARγ gene or targeting its expression at an intermediate stage to eradicate resistance of insulin to fatty foods for the development of effective strategy.[11,12] The use of PPARγ antagonists for diabetes mellitus has no side effects and is therefore favored over earlier treatments.[13-15] Betulinic acid, a triterpenoid compound, has structural geographies appropriate for diversity of chemical modifications.[16,17] The transformation of this molecule has been the source of molecules keeping various activities.[16,17] Chalcones are the precursors in biosynthesis of flavonoids and have been established to hold a wide range of biological activities comprising the antidiabetic property.[18] Several therapeutic targets through which chalcones apply their effect have been identified which comprise PPARγ, dipeptidyl peptidase-4, α-amylase, α-glucosidase, aldose reductase, and protein tyrosine phosphatase 1B.[18] Therefore, chalcones unquestionably act as a promising antidiabetic agent. In the present study, the antagonistic effect of betulinic acid chalcone [Figure 1] for PPARγ was examined with possible implications in antidiabetic treatment.

MATERIALS AND METHODS

Cell lines and culture

The preadipocyte, 3T3-L1, and 293T cells were attained from the American Type Culture Collection, Manassas, VA, USA. Maintenance of the cells was made in Dulbecco's modified Eagle's medium (DMEM) which controlled fetal bovine serum (FBS) (10%) as well as antibiotics (penicillin-streptomycin). The cell lines were cultured for 24 h under an atmosphere of 5% CO_2 at 37°C.

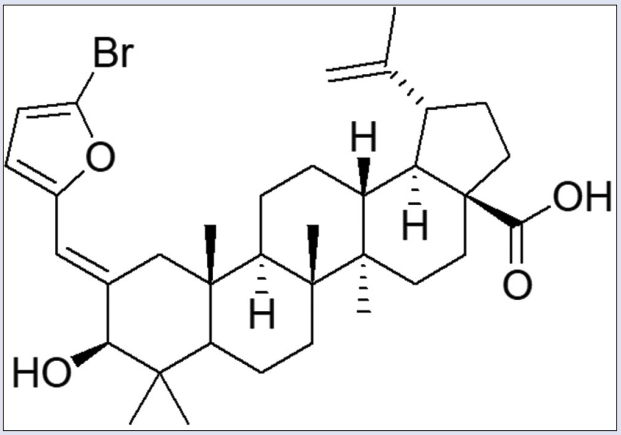

Figure 1: Chemical structure of betulinic acid chalcone

Synthesis of betulinic acid chalcone

Betulinic acid chalcone was arranged by refluxing the betulinic acid (1 mmol) with benzaldehyde (1 mmol) at 100°C. The reaction process was monitored by thin-layer chromatography in 3:1 petroleum ether and ethyl acetate. After 12 h, the reactants got entirely disbursed and the product formed was purified by column chromatography.

Determination of luciferase activity

The effect of betulinic acid chalcone on rosiglitazone-mediated variations in RXRα-PPARγ transcription and expression of PPARγ was analyzed by luciferase activity assay.[2] The 293T cells (2×10^5) were detached in 24-well plates in DMEM till reaching 70%–80% confluence. The plasmids for PPARγ-ligand-binding domain (LBD) – forward 5'-GTATTA GGATCC GAGTCCGCTGACCTCCG-3' and reverse 5'-GATACA CTCGAG CTAGTACAAGTCCTTGTAGATC-3' (Promega Corporation, Madison, WI, USA) – were transfected in the cells by employing Ca/PO_4 transfection kit (Sigma-Aldrich). The cells following 8 h of plasmid transfection were transferred in medium mixed with 2-, 4-, 8-, and 16-μM betulinic acid chalcone. Following 18 h, the medium was poured and PBS was employed for plate washing twice. The lysis of cells was carried out on adding lysis buffer 130 μL followed by 25-min incubation. The luciferase activity was measured using luciferase kits available commercially (Dual-Luciferase®, Promega Corporation). The fluorescein was employed as control.

Analysis of differentiation in 3T3-L1 preadipocyte

The effect of betulinic acid chalcone and rosiglitazone on preadipocyte differentiation was scrutinized by known methodology.[19] The 3T3-L1 cells were put in 6-well plates at 2×10^5 cells per well concentration and preserved till reaching 100% confluence. The cells were transferred in medium which contained a mixture of dexamethasone (0.4 mg/l), and insulin (0.9) mg/l on the 4th day. Following 3-day culture, medium was swapped by new medium encompassing insulin (0.9 mg/l) and incubation for 3 more days was sustained. The adipocytes after differentiation were cultured in DMEM plus 10% FBS for 48 h. The adipocytes were exposed to 2-, 4-, 8-, and 16-μM betulinic acid chalcone, rosiglitazone, or only dimethyl sulfoxide (DMSO) (control) for 6 days. The PBS washing of cells was completed following the removal of differentiation medium. The accumulation of fats in adipocytes was evaluated on staining with Oil Red O using commercial kits (Nanjing Jiancheng Bioengineering Institute). The BX50 microscope (Olympus Corporation, Tokyo, Japan) was employed to observe the cell for differentiation.

Quantitative polymerase chain reaction

The 3T3-L1 adipocytes at 2×10^5 cells per well concentration were deferred in 6-well plates after differentiation. The adipocytes were exposed to 2-, 4-, 8-, and 16-μM betulinic acid chalcone, rosiglitazone, or only DMSO (control) for 24 h. The total RNA extraction from adipocytes was carried out on treatment with TRIzol reagent (Invitrogen). The 20-μg samples were exposed to cDNA synthesis using PrimeScript™ RT kit (Takara Biotechnology, Co., Ltd., Dalian, China) by reverse transcription (RT). The Applied Biosystems 7300 instrument was employed for amplification of the cDNA solution consisting of total RNA (100 ng) according to manual directions. The reaction mixture used contained forward and backward primers

(200 nM), targeted cDNA-specific probes (100 nM), and Universal Master Mix (TaqMan). ABI Prism 7700 Sequence Detector was employed for conducting polymerase chain reaction (PCR) reaction using the conditions: 52°C for 1 min, 93°C for 4 min, then 42 cycles of 93°C for 10 s, and 58°C for 1 min. The Detection software (PE Biosystems, Japan) was used for inspection of the data. The primers used were as follows: GAPDH forward, 5'-GTATGACTCCACTCACGGCAAA-3' and reverse, 5'-GGTCTCGCTCCTGGAAGATG-3'; fatty acid synthase (FAS) forward, 5'-CTGAGATCCCAGCACTTCTTGA-3' and reverse, 5'-GCCTCCGAAGCCAAATGAG-3'; CCAAT/enhancer-binding protein-α (C/EBPα) forward, 5'-CTGAGAT CCCAGCACTTCTTGA-3' and reverse, 5'-CACGGCTCA GCTGTTCCA-3'; adipocyte fatty acid-binding protein 2 (aP2) forward, 5'-CATGGCCAAGCC CAACAT-3' and reverse, 5'-CGCCCAGTTTGAAGG AAATC-3'; and HMG-CoA forward, 5'-CATGCAGATTCT GGCAGTCAGT-3' and reverse, 5'-CGG CTTCACAAACCA CAGTCT-3'.

Western blot analysis

The adipocytes following differentiation were cleaned in ice-cold PBS and consequently lysed in TNN buffer (containing of Triton X-100 [1.2%], Nonidet P-40 [1.2%], along with inhibitors for phosphatases and proteases, $PhCH_2SO_2F$, sodium fluoride [110 mM], sodium vanadate [450 mM], and sodium phosphate [450 mg/mL]). The cells were transferred to Eppendorf tubes, centrifuged at 4°C for 15 min at 12,000 ×g. The BCA kit was used for analysis of protein concentration. The samples of 10 μg were put into wells which contained 8%–10% sodium dodecyl sulfate-polyacrylamide gel and resolved by electrophoresis. Transfer of proteins to polyvinylidene difluoride membrane was followed by blocking at room temperature with PBS encompassing Tween-20 (0.05%) and dry milk (5%) for 1.2 h. Incubation was carried out overnight at 4°C with aP2, CHIP, p-Akt, t-Akt, PPARa, p-FoxO1, t-FoxO1, p-AMP protein kinase (AMPK), t-AMPK, and C/EBPα primary antibodies. Following membrane washing, incubation at room temperature was done with HRP-conjugated goat anti-rabbit. Visualization and quantification were made using improved chemiluminescence reagent on Super RX-N film and ImageJ software, respectively.

Statistical analysis

The data articulated are mean ± standard deviation. The Student's *t*-test and ANOVA with Tukey's *post hoc* multiple comparison test were used for comparison of data attained. The data were evaluated using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The values were taken significantly at $P < 0.05$.

RESULTS

Peroxisome proliferator-activated receptor-γ antagonistic property of betulinic acid chalcone

Betulinic acid chalcone blocked PPARγ-LBD level in 293T cells in a dose-based manner [Figure 2a]. Although rosiglitazone suggestively ($P < 0.01$) promoted PPARγ-LBD in 293T cells, the surge was reversed by betulinic acid chalcone. The transcriptional property of RXRα-PPARγ was augmented noticeably on administration of rosiglitazone in 293T cells [Figure 2b]. However, betulinic acid chalcone lightened the rosiglitazone-induced transcriptional potential of RXRα-PPARγ in a concentration-based manner. These data recommended the suppression of RXRα-PPARγ transcriptional potential by betulinic acid chalcone, thereby illuminating its antagonistic property for PPARγ transcription.

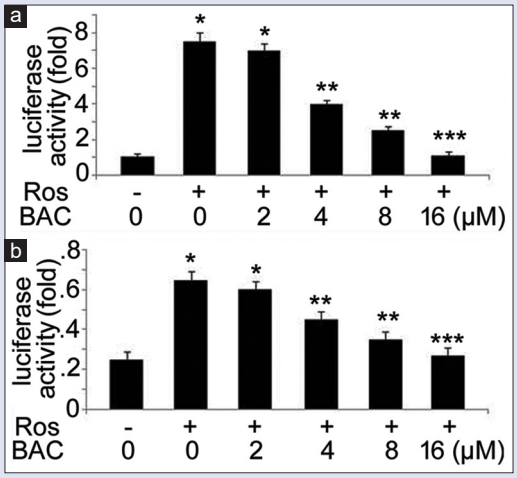

Figure 2: Betulinic acid chalcone suppressed rosiglitazone persuaded (a) peroxisome proliferator-activated receptor-γ-ligand-binding domain activation and (b) PPRE-Luc transcription in 293T cells. The cell line 293T UAS-TK-Luc comprising an integrated reporter in which the luciferase gene is under the control of Gal4-binding site (UAS) upstream of the thymidine kinase promoter was employed. The cells were transfected with plasmids for peroxisome proliferator-activated receptor-γ-ligand-binding domain and pRL-SV40 (reference) followed by incubation with betulinic acid chalcone at 6 h of transfection. The luciferase activity was investigated at 24 h of betulinic acid chalcone treatment using the kit luciferase. Data are accessible as the means ± standard deviation ($n = 6$) (*$P < 0.01$ and **$P < 0.001$ vs. control group)

Differentiation of 3T3-L1 preadipocytes is suppressed by betulinic acid chalcone

The effect of rosiglitazone alone and in combination with betulinic acid chalcone on differentiation of 3T3-L1 preadipocytes was evaluated by Oil Red O staining assay [Figure 3]. The rosiglitazone significantly ($P < 0.01$) augmented grease droplet accumulation in adipocytes in comparison to control adipocytes. The boosted grease droplet accumulation by rosiglitazone in adipocytes was suppressed on treatment with betulinic acid chalcone. The suppression of rosiglitazone-induced grease droplet accretion in adipocytes by betulinic acid chalcone was dependent on concentration. The increase in grease droplet accumulation by rosiglitazone was lessened completely on treatment with 16-μM betulinic acid chalcone.

Betulinic acid chalcone targets lipid metabolism-associated genes

The lipid metabolism connected with gene expression was evaluated in adipocytes following treatment with betulinic acid chalcone by RT-PCR [Figure 4]. The expression of genes such as FAS and HMG-CoA was knowingly ($P < 0.01$) promoted in adipocytes by rosiglitazone. Moreover, rosiglitazone also improved C/EBPα and aP2 expression in adipocytes. Treatment of adipocytes with betulinic acid chalcone blocked rosiglitazone-induced expression of FAS, C/EBPα, aP2, and HMG-CoA genes. The suppression of genes by betulinic acid chalcone was noteworthy ($P < 0.01$) at 2 μM and maximum at 16 μM.

Betulinic acid chalcone suppresses adipocyte fatty acid-binding protein 2, CHIP, and CCAAT/enhancer-binding protein-α expression

The expression of aP2, CHIP, and C/EBPα was evaluated in adipocytes following treatment with betulinic acid chalcone by Western

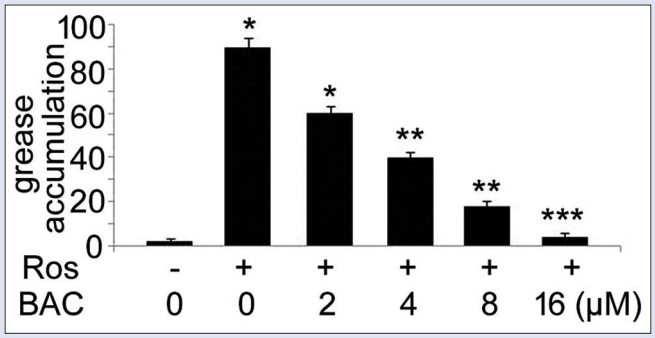

Figure 3: Suppression of adipocyte differentiation by betulinic acid chalcone. The adipocytes were treated with dimethyl sulfoxide (control), rosiglitazone, and 2-, 4-, 8-, and 16-μM betulinic acid chalcone. The adipocyte differentiation was examined at 24 h of betulinic acid chalcone treatment using the Oil Red O staining assay by BX50 microscope (Olympus Corporation, Tokyo, Japan). Data are obtainable as the means ± standard deviation (n = 6) (*P < 0.05, **P < 0.01, and ***P < 0.001 vs. control group)

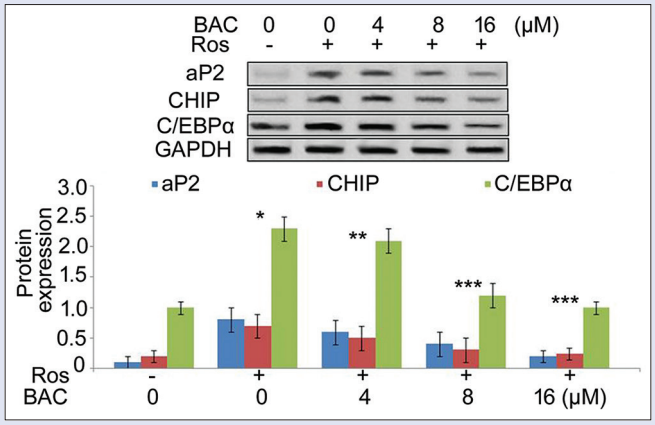

Figure 5: Effect of betulinic acid chalcone on aP2, CHIP, and CCAAT/enhancer-binding protein-α. The adipocytes were treated with dimethyl sulfoxide (control), rosiglitazone, and 2-, 4-, 8-, and 16-μM betulinic acid chalcone. Western blotting was employed for valuation of aP2, CHIP, and dimethyl sulfoxide protein expression in adipocytes. Data are accessible as the means ± standard deviation (n = 6) (*P < 0.05, **P < 0.01, and ***P < 0.001 vs. control group)

blotting [Figure 5]. The protein expression conforming to aP2, CHIP, and C/EBPα was decidedly boosted by rosiglitazone in adipocytes. However, treatment of adipocytes with betulinic acid chalcone caused a noticeable reduction in rosiglitazone-induced expression of aP2, CHIP, and C/EBPα. The suppression of aP2, CHIP, and C/EBPα by betulinic acid chalcone was noteworthy from 2-μM concentration.

Expressions of p-Akt, p-forkhead transcription factor Foxo1 (phospho-FoxO1), p-AMP protein kinase, and peroxisome proliferator-activated receptor-α

The expression of p-Akt/t-Akt was expressively (P < 0.01) lowered by rosiglitazone in the adipocytes [Figure 6]. After treatment of adipocytes with betulinic acid chalcone, the rosiglitazone-mediated reduction of p-Akt/t-Akt was suggestively (P < 0.001) lessened. Betulinic acid chalcone reversed rosiglitazone-mediated reduction of p-Akt/t-Akt at

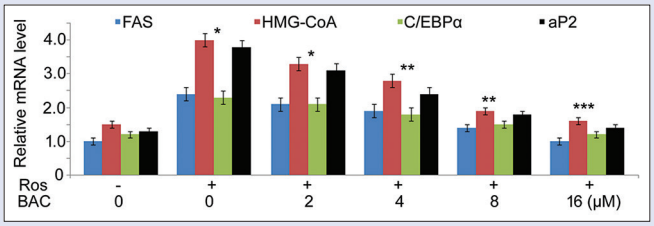

Figure 4: Effect of betulinic acid chalcone on lipid metabolism-associated genes. The adipocytes were treated with dimethyl sulfoxide (control), rosiglitazone, and 2-, 4-, 8-, and 16-μM betulinic acid chalcone. Reverse transcription–polymerase chain reaction assay was employed for analysis of fatty acid synthase, CCAAT/enhancer-binding protein-α, adipocyte fatty acid-binding protein 2, and HMG-CoA mRNA expression in adipocytes. Data are accessible as the means ± standard deviation (n = 6) (*P < 0.05, **P < 0.01, and ***P < 0.001 vs. control group)

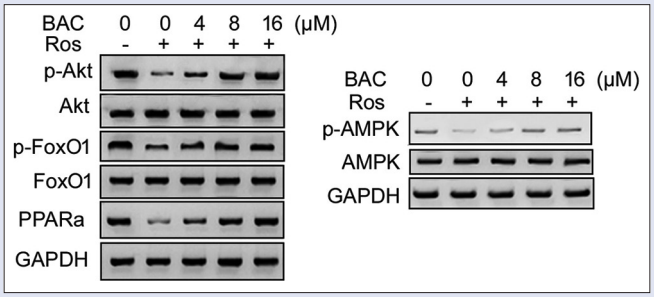

Figure 6: Effect of betulinic acid chalcone on p-Akt/t-Akt and peroxisome proliferator-activated receptor-α, p-FoxO1/t-FoxO1, and p-AMP protein kinase/t-AMP protein kinase in adipocytes. The adipocytes were treated with dimethyl sulfoxide (control), rosiglitazone, and 2-, 4-, 8-, and 16-μM betulinic acid chalcone. Western blotting was employed for valuation of protein expression in adipocytes (p-Akt [Ser473], p-FoxO1 [Ser256], and AMP protein kinase [Thr172])

2, 4, 8, and 16 μM. The rosiglitazone-induced lessening of PPARa was also relieved in adipocytes on treatment with betulinic acid chalcone. The level of p-FoxO1/t-FoxO1 in the adipocytes was also lowered expressively (P < 0.01) by rosiglitazone. Treatment of the adipocytes with betulinic acid chalcone weakened rosiglitazone-induced reduction on p-FoxO1/t-FoxO1. The level of p-AMPK/t-AMPK was evidently suppressed in adipocytes by rosiglitazone. Again, betulinic acid chalcone treatment pointedly reversed the rosiglitazone-mediated decrease of p-FoxO1/t-FoxO1 in adipocytes.

DISCUSSION

The current investigated whether betulinic acid chalcone, a benzylidene derivative of betulinic acid, could regulate som e of the hyperlipidemic parameters. The possible mechanism of betulinic acid chalcone for suppression of lipid metabolism-related genes was also discovered. In the modern societies, the occurrence of diabetes mellitus type 2 has exposed an exponential augmentation and its morbidity rate has also radically augmented.[1,2] The hyperglycemic patients progress multiple complications like diabetic nephropathy followed by diabetic foot at the progressive stage of diabetes mellitus which deteriorates their life standard.[20] The clinicians are making incessant efforts to comprehend the molecular mechanism of diabetes mellitus.[21-23] The reduction in glucose absorption in body by inadequate insulin secretion leads to improved lipolysis and finally hyperglycemia.[24] Another reason for

hyperglycemia includes the production of glucagon unduly by the α-cells in pancreas leading to boosted gluconeogenesis and abnormality in glucolipid metabolism.[25] The antidiabetic drugs recommended currently have shown many side effects clinically, and administration of insulin leads to hypoglycemia along with weight gain risk.[26,27] Mechanistically, oral antidiabetics fitting to the thiazolidinedione family of compounds improve insulin sensitivity through PPARγ, a nuclear receptor activation.[28] These drug molecules exhibit injurious effects containing cardiovascular disorders and increase body weight.[29] Studies have shown that downregulation of PPARγ activity through mutation or genetic knock down of PPARγ triggers the reduction of high fat-induced insulin resistance.[30] The present study found that betulinic acid chalcone suppressed PPARγ level and downregulated RXRα-PPARγ transcription in rosiglitazone-administered 293T cells. This initial observation designated antagonist potential of betulinic acid chalcone for PPARγ. Further, studies using Oil Red O staining assay on 3T3-L1 preadipocytes exposed that betulinic acid chalcone inhibited the accumulation of grease droplets. Therefore, betulinic acid chalcone treatment led to a clear reduction of adipocyte differentiation potential. Studies have shown that PPARγ plays the main role in the process of adipogenesis as well as lipogenesis.[31] The present study displayed that rosiglitazone administration augmented PPARγ activity in 293T cells decidedly. However, rosiglitazone-mediated enlarged PPARγ activity was blocked in 293T cells on treatment with betulinic acid chalcone. The improved GLUT4 translocation to the cell membrane is related to the activation of AMPK pathway.[32] The GLUT4 is convoluted in the uptake of glucose and thereby plays an important role in antidiabetic therapies.[32] The study has exposed that membrane GLUT4 level is abridged in the animal model of diabetes.[33] The data of the present study disclosed that betulinic acid chalcone boosted p-AMPK/t-AMPK level in adipocytes administered with rosiglitazone. The Akt phosphorylation was blocked in adipocytes markedly by rosiglitazone administration. The level of GLUT4 was also endorsed in the rosiglitazone-administered adipocytes on treatment with betulinic acid chalcone. It is known that high blood glucose catalyzes insulin release which then activates PI3K followed by Akt and FoxO1 phosphorylation.[34] The irreversibly phosphorylated FoxO1 helps the inhibition of glucose metabolism by insulin and production of glucose in liver.[35] Thus, gluconeogenesis is repressed by insulin through Akt-mediated FoxO1 phosphorylation.[36] In the present study, p-Akt/t-Akt level was evidently enhanced by betulinic acid chalcone in the rosiglitazone-administered adipocytes. The p-FoxO1/t-FoxO1 level in adipocytes was suppressed on administration of rosiglitazone, but the effect was lessened by betulinic acid chalcone. It is apparent from the existing study that betulinic acid chalcone displays an antidiabetic effect in adipocytes by activation of Akt/AMPK pathways.

CONCLUSION

The present study established that betulinic acid chalcone inhibited PPARγ activity and adipocyte differentiation. Moreover, the activation of FoxO1/Akt/AMPK was upregulated and FAS/EBPα/aP2/HMG-CoA expression was subdued by betulinic acid chalcone in the adipocytes. Therefore, betulinic acid chalcone may be assessed further for potential role in the treatment of diabetes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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