Journal of Infection and Public Health 12 (2019) 726–732

Contents lists available at ScienceDirect

Journal of Infection and Public Health

journal homepage: http://www.elsevier.com/locate/jiph

Betulinic acid lowers lipid accumulation in adipocytes through enhanced NCoA1–PPARγ interaction

Al-Mutary Mohsen G[a], Gasem Mohammad Abu-Taweel[a], Rajakrishnan Rajagopal[b], Kim Sun-Ju[c], Hak-Jae Kim[d,*], Young Ock Kim[c], Ramzi A. Mothana[e], Shine Kadaikunnan[b], Jamal M. Khaled[b], Nasir A. Siddiqui[e], Adnan J. Al-Rehaily[e]

[a] Department of Basic Sciences, College of Education, Imam Abdulrahman Bin Faisal University, P.O. Box 2375, Dammam, 31451, Saudi Arabia
[b] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia
[c] Department of Bio-Environmental Chemistry, College of Agriculture and Life Sciences, Chungnam National University, 99 Daehak-Ro,Yuseung-Gu, Daejeon 34134, Republic of Korea
[d] Department of Clinical Pharmacology, College of Medicine, Soonchunhyang University, Cheonan, Republic of Korea
[e] Department of Pharmacognosy, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia

ARTICLE INFO

Article history:
Received 26 March 2019
Received in revised form 4 May 2019
Accepted 12 May 2019

Keywords:
Adipogenesis
Betulinic acid
NMR
PPAR gamma
Tectona grandis

ABSTRACT

Background: Investigation for a naturally occurring anti-obesity drug has become the need of society all over the world. Betulinic acid (BA) is a lupane-type pentacyclic triterpene and is sourced from various organisms. This high potential biologically active molecule is reported to have anti-obesity effect. In this study, we report the molecular mechanism of action of BA that underlies anti-obesity activity and also an improved method of its isolation common teak tree.
Methods: Mouse pre-adipocyte cells were used to develop hyperlipidemic conditions in vitro. Change in expression of genes associated to adipogenesis was checked using quantitative real-time PCR (qPCR). Co-factor specificity of PPAR gamma was analyzed through immune precipitation and immunoblot.
Results: Betulinic acid was found to be effective in reducing the lipid content in 3T3L1 cells. Level of PPAR gamma and LXR alpha was reduced in connection to reduced adipogenesis. Change in steroid responsive co-activators (SRCs) during BA treatment proved that the compound can impart profound change in co-factor selectivity, which is crucial in determining the activity profile of PPAR gamma. BA treatment enhanced the SRC-1 interaction with PPAR gamma while reducing the levels of SRC-3.
Conclusion: Present study has proved that betulinic acid, a promising candidate in anti-obesity drug development, has potential in regulating the activity of PPAR gamma through co-factor modulation.

Introduction

Obesity has become a major health problem in worldwide in 21st century. According to the World Health Organization (WHO), 1.2 billion people worldwide are officially classified as overweight. Obesity is a major health threat in both low- and high income countries. Kingdom of Saudi Arabia is showing a high prevalence to obesity and overweight over the past few decades due to many factors such as changed life style induced by westernization, less physical activity, increase in stress and also of high socio-economic status. There are studies published recently pointing out the increase in obesity associated diseases such as diabetes, CVD and cancer among Saudi population [1]. An immediate action in many aspects is required to address the issue from academicians, public health departments and social workers. The primary level treatment strategies for obesity mainly rely on diet control, exercise and public awareness. Even though these are low cost and minimal risk options, they do not generally end up in marked or sustainable weight loss. Effective psychological therapies, like cognitive behavioral therapy, which requires a dedicated lengthy in-person counseling and planned steps cannot be easily implicated on a mass scale [2]. The search for why Asian-Indian communities are more susceptible to the condition still continues. The concept of 'thrifty genotypes', epigenetic factors that influence health during *in-utero* environment, socio-economic factors, maternal nutrition etc. are suspected to drive this metabolic evil [3,4].

* Corresponding author.
E-mail address: Hak3962@sch.ac.kr (H.-J. Kim).

https://doi.org/10.1016/j.jiph.2019.05.011

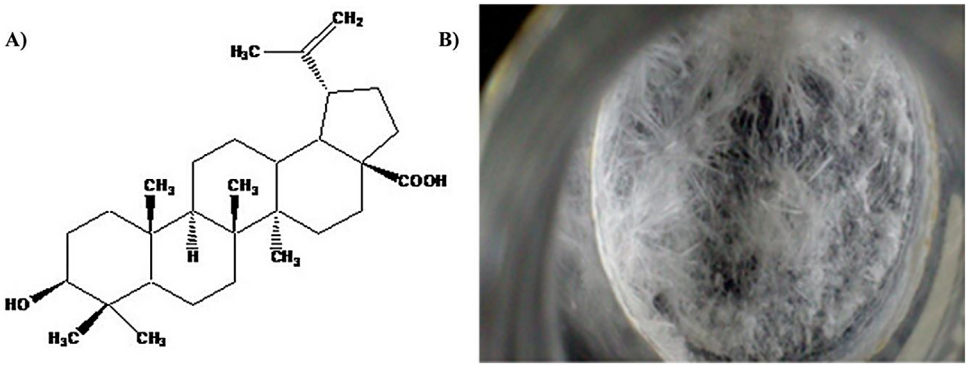

Fig. 1. Chemical structure of betulinic acid.

Adipose tissue comprised of adipocytes, is a loose connective tissue found in stereotypical depots throughout the body. Adipose tissue is a complex and heterogenic tissue exhibiting high variability and appears to have multiple functions, especially in metabolic regulation. Most of our understanding on molecular basis of adipocyte differentiation and adipogenesis comes from in vitro studies of fibroblasts and pre-adipocytes [5]. Mature white adipocytes comprise of large lipid droplets which cover majority of cell volume, leaving cytoplasm and nucleus to periphery. Increased energy expenditure and fasting leads to hydrolysis of fatty acids [6].

Natural ingredients and medicinal plant preparations may enhance satiety, boost metabolism, and speed up weight loss [7,8]. Betulinic acid (BA) is a naturally occurring pentacyclictriterpene. In this study, we isolated betulinic acid from *Tectona grandis*. The purity and structure was confirmed by spectroscopic methods. BA is reported to have anti-obesity effect and can act as a PPAR gamma antagonist [9]. Being a versatile nuclear factor, it is well documented that the activity spectrum of PPAR gamma is regulated by timely interaction of co-activators and co-repressors [10]. To prove the effect of betulinic acid in reducing adipogenesis through PPAR gamma modulation, we checked the change in co-factor specificity effect due to BA. Physical interaction of co-factors with PPAR gamma and the changes correlated to BA activity were analysed. BA treatment enhanced the SRC-1 interaction with PPAR gamma while reducing the levels of SRC-3.

Materials and methods

Extraction, purification and structural elucidation of betulinic acid

The plant material after collection was washed, shade dried, powdered and stored in moisture free condition for further processing. 200 g of finely powdered bark was used for extraction process using soxhlet apparatus. Extracts taken with hexane (0.49%, w/w), chloroform (1.2%, w/w), ethyl acetate (0.95%, w/w) and methanol (1.66%, w/w) were concentrated by lyophilization and stored at −200°C. Chloroform extract of bark was selected for isolation of toxic compound based on preliminary studies. After de-fatting with hexane, the extract was dissolved in chloroform and methanol (1:1). By centrifugation at 8000 rpm for 30 min, insoluble particles were removed from the extract. Supernatant collected and dried in vacuo. It was then re-dissolved in CHCl3–MeOH (2:1). On drying at RT, crystalline structures developed were removed by filtration with ordinary filter paper. The process was repeated three times for effective crystallization and purification of compound (Fig. 1B). NMR spectra including 1H, 13C and DEPT experiments were recorded on 500 MHz spectrometer (AvanceII 500, Bruker) with TMS as internal standard. Fab-Mass spectrum was recorded with JMS 600H (JOEL, Japan). IR spectrum of compound was taken in Spectrum 100 (Perkin Elmer, Beaconsfield, UK).

Animal cell culture

Cell lines selected for the study include Chick Embryo Fibroblast (CEF), Mouse fibroblast (L929), Mouse pre-adipocyte (3T3L1), Mouse myoblast (C2C12) and four cancerous cell lines– Human colon cancer (HCT 119), Naso-pharyngeal (KB), Liver carcinoma (HepG2), MCF and HEK293. CEF cells were dissected out from 8 to 12 days old chick embryo under aseptic conditions. Cells were cultured in MEM with 1% antibiotic-antimycotic mix and 10% fetal calf serum. HEK 293 and KB cells were grown in DMEM supplemented with 10% serum and 1% antibiotic-antimycotic mix. All cultures were maintained at 37 °C in a humidified atmosphere in a 5% CO_2 incubator.

MTT assay

As part of preliminary analysis, cytotoxic effect of crude extracts from bark was checked against CEF and HEK 293 cells. MTT assay was carried out as previously described protocol [11]. Briefly, 5×10^4/ml cells were seeded at log phase in 24 well plates and incubated overnight. Growth media was removed and fresh media added with extracts at different concentration (0.5, 5, 12.5, 25 and 50 μg/ml) or DMSO as control and incubated. Isolated pure compound was checked for activity within 1–200 μM concentrations. Volume of DMSO with sample was restricted to 0.1% to avoid solvent-induced cytotoxicity. Sample containing media was removed and cells incubated with MTT for 3–4 h. Crystals developed were solubilized by shaking in DMSO. Absorbance measured at 570 nm using UV–vis. spectrophotometer (UV-1700, Shimadzu, Japan). Percentage of cell viability was calculated and plotted against concentration. BA was checked for cytotoxicity against CEF, HEK 293, KB and HCT 119 cell lines by MTT assay.

Adipocyte differentiation and experimental set up

3T3-L1 cells were cultured in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/mL of streptomycin and 0.25 μg/ml of amphotericin B, denoted as Media M1, in 5% CO_2 incubator at 37 °C in a humidified chamber. 3T3L1 cells were subjected to differentiation as per the protocol described elsewhere [12]. Briefly, cells were differentiated in media designated M2 (M1+ insulin (1.7 μM), dexamethasone (10 μM) and IBMX (500 μM) and was changed to media M3 (M1+ insulin (1.7 μM) only) after every 48 h. Effect of betulinic acid was checked at 1, 5 and 10 μM concentration at D0 and D10 of differentiation.

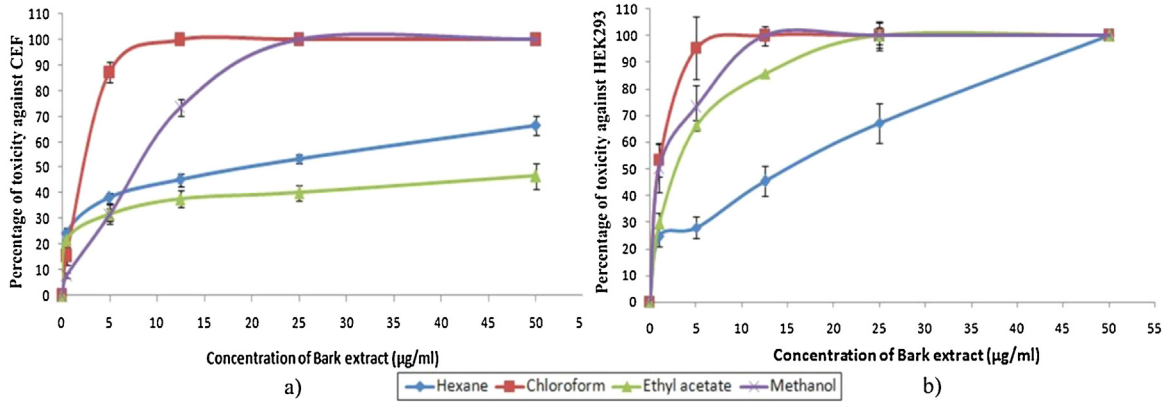

Fig. 2. (a) Cytotoxicity of different extracts of teak bark against chick embryo fibroblast cells and (b) HEK293. Experiments were performed in triplicate and values expressed as mean ± SD.

Oil red O staining

To check the lipid droplet formation, 3T3L1 cells were differentiated for 8 to 10 days in media M3 under different experiment conditions. With slight modifications from previously described protocol, lipid droplets were stained with oil red O [12,13]. Briefly, stock solution was prepared by dissolving 0.5 g of dye in 100 % isopropanol. After dissolving, 60 volume of stock was mixed with 40 volume of water to prepare the working solution. The solution was mixed well and filtered with Whatman No.1 filter paper after one hour incubation at room temperature. Cells were washed twice with PBS and fixed with paraformaldehyde. Following 15 min incubation, cells were washed with PBS and 60% isopropanol in water. Plates were allowed to dry in airflow for a few minutes. Working concentration of oil red O stain was added and incubated for one hour at 37 °C. After incubation, the remaining solution was pipette off and cells washed with PBS. Images were taken with camera attached microscope (Eclipse *Ti*, Nikon, Japan) and the lipid bound stain was dissolved in 100% isopropanol with shaking for one hour. Absorbance was read at 560 nm using microplate reader (Infinite 100, Tecan, USA). All measurements were done in triplicate and values plotted as average with SD.

RNA isolation and RT-PCR

For RNA isolation, cells were isolated for 10 cm petri dishes. RNA was isolated using Trizol reagent (Ambion, Carlsbad, USA) as per the manufacturer's instruction.

Immunoblot analysis

Protein levels of nuclear factors like PPARγ2 and LXRα and nuclear co-factors such as NCoA-1 and 3 were assessed after separation of total cell lysate by SDS-PAGE. After separation, proteins were transferred to immobilion-P-PVDF membranes. Concentration of antibodies and procedure was done as per the protocol described previously [12].

Immuno-cytochemical studies using confocal microscopy

Cells were seeded (2×10^4/well) in 96 well black walled bioimaging plate (BD Bioscience, USA) and incubated to confluence. Differentiated cells with and without BA treatment (10, 50 μM) were stained with PPARγ2 antibody. As a positive control, cells differentiated in presence of rosiglitazone were also kept. Undifferentiated cells were grown in Dulbecco's Modified Eagle's Media (DMEM) devoid of differentiation mix. Immunocytochemical staining was performed as per standard protocol [14] (Maldie, 2008). Briefly, cells were washed twice in Kreb's Ringer Buffer (KRB, pH 7.2). After fixing in 4% formaldehyde, cells were washed in glycine containing buffer. For permeabilization and blocking, cells were incubated in KRB with 3% goat serum and 0.1% saponin for 45 min. Cells were washed thrice and incubated for 60 min in presence of mouse raised anti PPAR-γ antibody at 1:300 dilution. After washing thrice in KRB pH-7.2, cells were incubated for 60 min in goat raised anti-mouse IgG-FITC conjugate. Cells were washed thrice in KRB and kept in HEPES containing buffer for spinning disc confocal imaging (Pathway 855, BD Bioscience, CA, USA). Images were analysed with Image Data Explorer (BD-IDE, BD Bioscience, USA).

Statistical analysis

The means and standard errors were calculated from three independent experiments. The statistical significance was determined by ANOVA (student's t-test). A difference in P-value of <0.05 is considered as significant.

Results and discussion

Structural characterization of betulinic acid

Results from published studies indicate that chloroform extract of bark was highly toxic among hexane, chloroform, ethyl acetate and methanol extract of leaf, bark and wood of teak [15]. Based on that, chloroform extract was selected for activity guided fractionation and led to the isolation of a pure compound with potent cytotoxicity. Comparing UV spectra, NMR, FAB-MS and IR data with reference led us to conclude that the compound is a pentacyclic lupane type triterpene, 3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid) (Fig.1).

Betulinic acid (BA): λ_{max}: 240 (0.05), ν_{max}: 3456 (—OH), 2936, 1684 (C = 0), 1449, 1235, 1031 (—OH), 882 cm^{-1} (Fig. 3.10); Fab+-MS m/z (rel. int.): 456 [M+] (10), 438 [M+-H2O] (5), 440 [438 + 2H+] (5), 410 [438-C = O] (4), 394 [438-CO-CH3] (5), 189 [M+ —C14H5O] (35), 173,145,135, 119, 105 (Supplementary Fig. S1). Proton NMR (500 MHz, CDCl3) gave peaks at δH: 0.75, 0.81, 0.93, 0.95 and 0.97 corresponding to vinyl methyl, δ1.68 for exo-methylene at C-30, δ1.2-2.2 (m, other aliphatic ring protons), δ3.19 (dd, 1H), δ4.726 and 4.729 (br s, 1H each, on methylene group at C29). 2.25, 3.0, 3.19 (dt, —H on C13, C19 and C-3 respectively) (Supplementary Fig. S2). 13C NMR (δ, ppm): 38.69 (C-1); 27.13 (C-2); 78.92 (C-3); 38.78 (C-4); 55.32 (C-5); 18.25 (C-6); 34.29 (C-7); 40.65 (C-8); 50.51 (C-9); 37.09 (C-10); 21.85 (C-11); 25.5 (C-12); 38.27 (C-13); 42.41 (C-14); 30.56 (C-15); 32.22 (C-16); 56.19 (C-17); 46.93 (C-

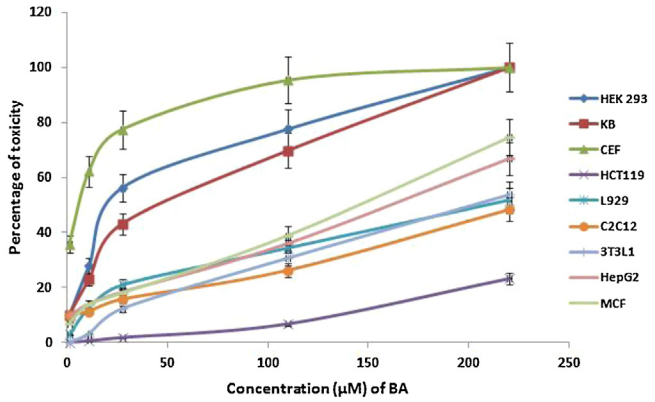

Fig. 3. Cytotoxicity of BA against a panel of normal and cancerous cell lines. Different concentrations of BA showing toxicity against cell lines are expressed in percentage of toxicity. Values expressed as mean ± SD.

18); 49.19 (C-19); 150.66 (C-20); 29.64 (C-21); 37.14 (C-22); 27.88 (C-23); 15.3 (C-24); 15.8 (C-25); 16.05 (C-26); 14.62 (C-27); 179.24 (C-28); 109.46 (C-29); 19.27 (C-30). Counts of —CH3 (6), —CH2 (11) and —CH (6) (Supplementary Fig. S3) were also compared based on DEPT results (Supplementary Fig. S4).

Cytotoxicity of bark extracts and BA

Toxicity of extracts from bark of teak was tested against Chick Embryo Fibroblast (CEF) and Human Embryonic Kidney (HEK 293) cells as a preliminary analysis. Chloroform extract was found to be more active against both cells with more than 85% inhibition against CEF and more than 95% inhibition against HEK 293 at minimum concentration compared to other extracts (Fig. 2a & b)

Based on activity guided fractionation of bark extract, BA was isolated and its antiproliferative effect was checked within 1–200 μM concentrations. The cell lines selected for the study include four normal cell lines viz., CEF, L929, C2C12, 3T3L1; four cancerous cell lines namely KB, HCT119, HepG2, MCF and one immortalized normal cell line, HEK293. IC_{50} value of the compound was determined by plotting percentage of toxicity against concentration of compound tested (Fig. 2). Percentage of cell viability was calculated by MTT assay. The compound was able to reduce the viability of CEF (IC_{50} – 5 μM), HEK293 (IC_{50} – 49 μM) and KB (IC_{50} – 130 μM) cells very effectively. It was able to show toxicity against most of the cell lines treated and is given in decreasing order of toxicity – MCF 7 (IC_{50} – 142.5 μM)>HepG2 (IC_{50} – 160 μM)>3T3L1 (IC_{50} – 192 μM) > L929 (IC_{50} – 197 μM) > C2C12 (IC_{50} > 200 μM) > HCT119 (IC_{50} > 200 μM).

A study on cytotoxicity of bark extracts of the *T. grandis* has been reported by few other groups [15–17]. Detailed work on the phytochemical constituent leading to cytotoxicity has not yet been reported from this plant. A study conducted by Neamatallah et al. [18] on bark dust of teak revealed the presence of juglone with antimicrobial activity. Yield of this compound from bark of *T. grandis* has been calculated as 1.3 g Kg^{-1} dried powder. Apart from the solvent extraction, partition and chromatographic separation method which has been reported in earlier studies [18–20], this paper describes an improved method of isolating BA in pure form by solvent wash of extract and crystallizing.

Dose dependent inhibitory effect of BA on adipogenesis

To evaluate the anti-obesity effect of BA, concentration below 10 μM (1, 2, 5, 10 μM) was taken and administered along with differentiation mix as mentioned in materials and methods. Results showed that BA was able to inhibit lipid accumulation in a dose-dependent pattern compared to differentiated control and rosiglitazone treatment (Fig. 4). Lipid droplets and cellular hypertrophy was reduced up on increase in concentration of BA. Representative images and those stained with oil red O stain are shown in Fig. 4A (i–v) and 4C (I & II). Earlier, Kim et al. [21] has reported the pancreatic lipase inhibitory effect and adipocyte lipolysis potential of betulinic acid after oral administration in mice models. BA is shown to have PPAR gamma antagonistic effect resulting in reduced adipogenesis and promoting osteogenesis [9]. Binding potential of betulinic acid and two structurally modified forms towards PPARγ has showed that the modified forms of BA was able to activate PPARγ more effective than the parent molecule in pancreatic cancer cells [22].

Betulinic acid restored pre-adipocyte morphology of 3T3L1 after MDI induced adipogenesis

We investigated the ability of BA to reverse MDI induced morphological changes in 3T3L1 preadipocyte. Cells were grown as mentioned in the previous section. Growth was continued for 8 days. Lipid droplet accumulation was clearly visible on microscopic analysis. From the 9th day onwards, post differentiation media was continued with 10 μM BA and continued till 14th day. Results showed a very significant reduction in lipid droplet content compared to MDI treated control cells (Fig. 4D).

The results proved the potential of BA as a potent anti-obesity drug as it was able to reverse the lipid content in adipocyte. Both the experiments have shown that BA can inhibit adipogenesis and also can reverse the phenotype within short period of time. Thiazolidinediones (TZD) are currently used antidiabetic drugs that increase insulin sensitivity in peripheral organs [23]. Various studies on nonhypoglycemic effect of TZDs revealed its side effect on increasing LDL, cholesterol and thereby obesity [24]. The effect of BA on reducing the lipid content in MDI treated cells resembles its antiobesity potential in vivo.

Betulinic acid alters nuclear factor and co-factor gene expression leading to reduced adipogenesis

Being evident with the results, we proceeded to elucidate the gene level mechanism of action of BA during adipogenesis.Expression of PPAR gamma and LXR alpha was enhanced in all experiment conditions. Compared to MDI and rosiglitazone assisted differentiation, cells grown in presence of BA showed reduction in expression of nuclear factors. PPAR alpha and delta did not showed statistically significant change in activity compared to MDI treated groups eventhough the (Fig. 5A). Nuclear co-factors that are critical in modulating the activity of PPARg come under p160 co-activators. Nuclear co-activator/steroid responsive co-activators – 1, 2 and 3 are such major types of co-regulators. Expression analysis of nuclear **co-**activators (NCoA) showed a dose dependent variation in expression with respect to betulinic acid (Fig. 5B). NCoA 1 and 3 recorded an increase in expression at day 10 and is higher than MDI and rosiglitazone groups. NCoA 1 and 3 are nuclear co-factors known to reduce adipogenesis. Studies done by Picard et al. [25] have showed that the energy balance between white and brown fat are end results of balance between NCoA 1 and 2. On molecular level, it can be said that the absence of NCoA2 can reduce obesity and absence of NCoA1 can induce obesity.

Confocal staining and western blots proved the cellular reduction of PPARγ2 protein in presence of BA

Adipocyte differentiation is a well orchestrated cellular mechanism involving various nuclear factors and co-factors. As the effect of betulinic acid in reducing the differentiation potential and gene

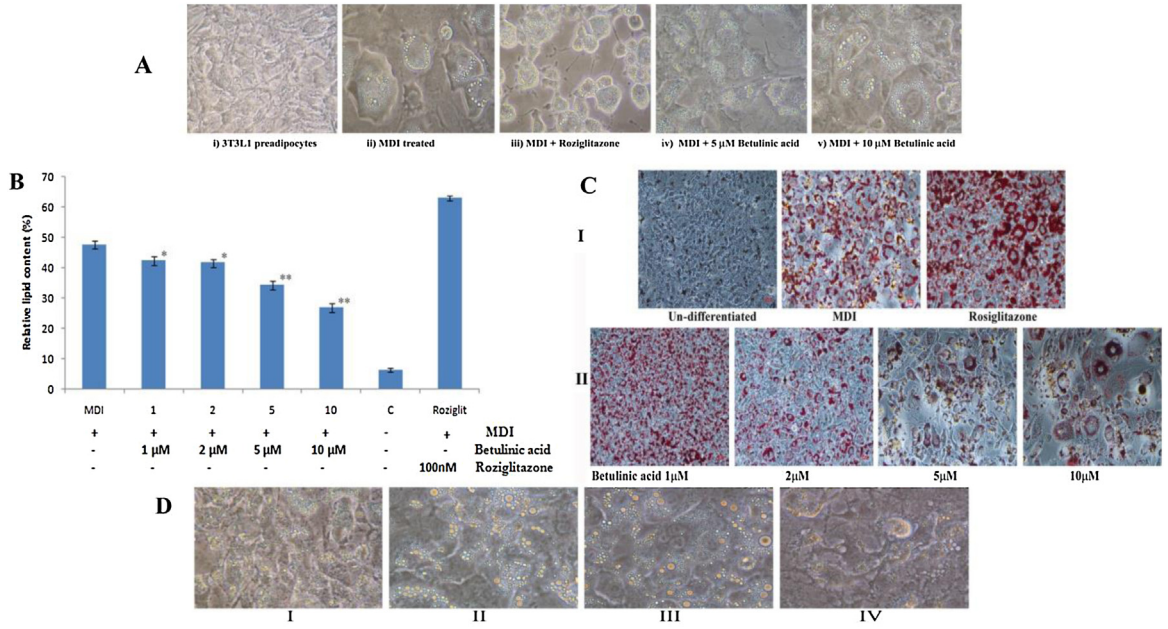

Fig. 4. BA inhibited lipid droplet accumulation in differentiated adipocytes. Compound was treated at 1–10 μM concentration. (A) Picture showing reduction in lipid droplet formation in concentration dependent manner. Difference in cell size and lipid droplets in (i) undifferentiated (ii) differentiated (iii) differentiated in presence of roziglitazone (iv and v) and differentiated in presence of 5 and 10 μM betulinic acid is clearly visible. (B) Graphshowing the change in lipid droplet content as recorded by oil red O staining (C) Differentiated 3T3L1 cells in presence of rosiglitazone and varying concentration of BA are stained with oil red O. (D) Reversal of lipid droplet content after administration of BA from 9th day of maturation till day 14. The change in lipid droplets are seen in image D-III and D-IV where 5 and 10 μM BA was given respectively. (For interpretation of the references to colour in the figure legend and text, the reader is referred to the web version of this article.)

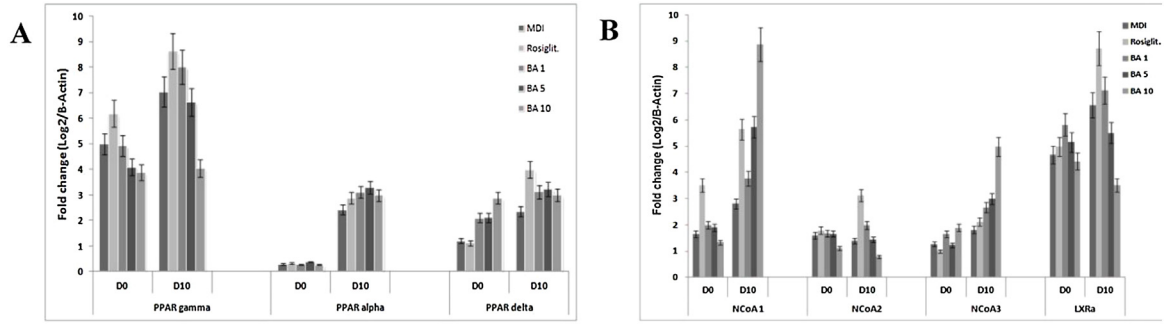

Fig. 5. Gene expression analysis of differentiated cells, differentiated in presence of rosiglitazone and betulinic acid (1, 5 and 10 μM) are shown. 5A represents fold change in nuclear factors under PPAR and 5B represents fold change in nuclear co-factors and LXR alpha.

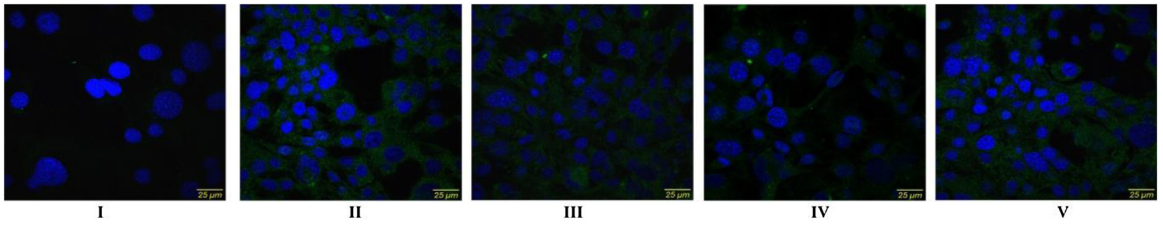

Fig. 6. Confocal images of PPARγ2 stained cells differentiated in presence of BA. Undifferentiated cells (5-I) were compared with cells differentiated without (5-II) or with BA at 5 (5-III) and 10μM concentration (5-IV). Rosiglitazone treated cells are imaged and shown in 5-V. Blue — nucleus; green – PPAR γ2. (For interpretation of the references to colour in the figure legend, the reader is referred to the web version of this article.)

level changes in adipocytes were evident from previous experiments, we checked the cellular content of PPAR gamma respective to 5 and 10 μM concentrations of BA.

Fig. 6 shows the PPARγ2 content was reduced in presence of betulinic acid and was concentration dependent. Rosiglitazone enhanced the fluorescence in cells compared to MDI treated alone group. Previous studies have reported the poor binding potential of betulinic acid on PPAR gamma compared to its structural variants [26] and also on PPAR gamma antagonistic activity [22]. Luciferase based transactivation assay done previously has proved that BA, even though showed affinity towards PPAR gamma, but was not able to activate it [9].

Level of nuclear factors were checked with culture lysate from differentiated cells and those in presence of rosiglitazone and 5 and 10 μM BA compared to undifferentiated control (Fig. 7). Level of PPAR gamma and LXR alpha was reduced in proportional to BA

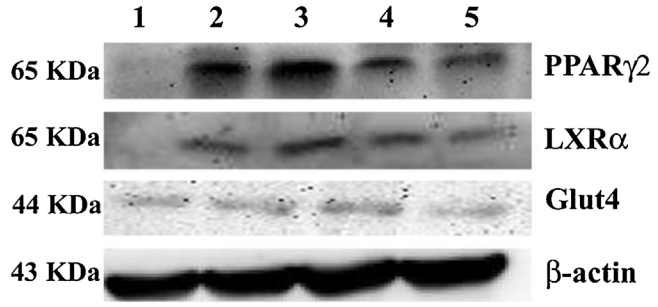

Fig. 7. Western blot image showing the change in protein levels with respect to BA treatment. Reduction in PPAR gamma and LXR alpha due to BA treatments are clearly evident in lanes 4 and 5. Lane 1 = undifferentiated control, 2 = MDI, 3 = MDI + rosiglitazone, 4 = MDI + 5 μM BA, 5 = MDI + 10 μM BA.

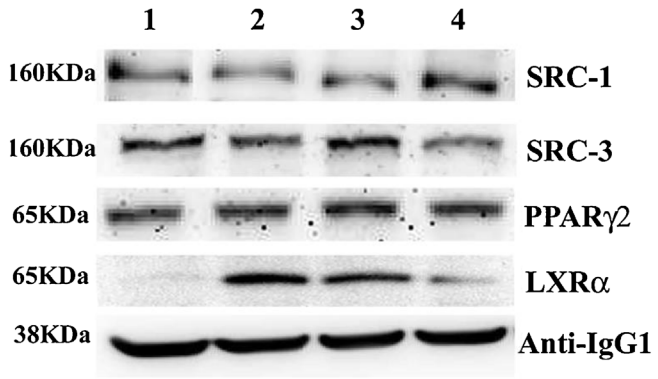

Fig. 8. Western blot showing images of co-factor associated to PPAR gamma captured after immuno-precipitation in presence and absence of BA. Lane 1 contain proteins from un-diffferentiated well, Lane 2 with rosiglitazone treated, Lanes 3 and 4 are with proteins from 1 and 10 μM BA treated cells respectively.

which shows that the reduction in adipogenesis is associated to nuclear factor concentration.

Considering the present results, it can assumed that the reduction in PPAR gamma and further on differentiation process may not be due to direct binding of BA on PPAR gamma, but through gene level alterations.

Betulinic acid can alter co-factor specificity of PPAR gamma resulting in reduced adipogenesis

Nuclear co-factors and chromatin modulating protein play major role in designing the activity spectrum of most of nuclear factors. For a highly versatile protein like PPAR gamma, the co-factor interactions and timely activation are very crucial [27]. Steroid receptor co-activators/nuclear co-activators are among the first discovered co-activator proteins that can interact with nuclear factors and enhance activity. These are major cofactors in regulating metabolism and associated physiological processes [10]. Results from the experiments so far proved the potential of BA in reducing adipogenesis and is affected through reducing the gene and protein levels of PPAR gamma. Now we planned to analyse the change in interaction of co-factors with PPAR gamma with respect to betulinic acid.

PPAR gamma was immuno-precipitated at different experiment conditions — undifferentiated, differentiated with rosiglitazone/10 μM betulinic acid. Results showed that the interaction of SRC-1 was enhanced in presence of betulinic acid (Fig. 8). Similar trend was shown by LXRα also. This protein is a major nuclear factor playing crucial role in lipid metabolism. It is still controversial about the trans-activation of PPARg and LXRa in promoting lipid synthesis and transport. From the results presented here, it can be concluded that the binding of BA on PPARg may inhibit the interaction with LXRa leading to reduced lipid content. Similarly, studies on adipocyte differentiation has shown that SRC1 as an antiobesity co-factor while SRC3 null mice showed considerable reduction in WAT volume [28]. Interaction of SRC-1 with PPARg was also reduced in presence of BA and shows a concentration dependent change in intensity. SRC3 showed a reduction in intensity compared to rosiglitazone and MDI wells. Previous reports states that the reduction in SRC-3 led to reduced adipocity and subsequently led to undetectable levels of PPARγ2 in epididymal fat pads [29]

Conclusion

Betulinic acid, a pentacyclic lupane type triterpene, is a high potent phytochemical with various reported biological activity. Due to cytotoxicity and reduced solubility in aqueous media, the compound has not been taken up for clinical trials. Here in this paper, we have reported a simple and modified method of isolation from *T. grandis*, commonly known as teak. The anti-obesity effect of betulinic acid is studied in detail in this paper with precise molecular mechanism of action elucidated. Betulinic acid was able to reduce the gene and protein expression of PPAR gamma and also in regulating co-factor specificity leading to alterations in adipogenesis. It has also proved that the compound was able to induce the activity within biologically safe concentration, which enhance the valuation of this molecule to enter next phase of drug research.

Funding

Deanship of Scientific Research at King Saud University.

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgement

The authors extend their sincere appreciations to the Deanship of Scientific Research at King Saud University for funding the work through the research group project no. (RGP-073).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jiph.2019.05.011.

References

[1] De Nicola E, Aburizaiza OS, Siddique A, Khwaja H, Carpenter DO. Obesity and public health in the Kingdom of Saudi Arabia. Rev Environ Health 2015;30(3):191–205.
[2] Balachandran C, Duraipandiyan V, Emi N, Ignacimuthu S. Antimicrobial and cytotoxic properties of *Streptomyces* sp. (ERINLG-51) isolated from Southern Western Ghats. South Indian J Biol Sci 2015;1:7–14.
[3] Kinra S, Rameshwar SKV, Ghafoorunissa Mendu VV, Ravikumar R, Mohan V, Wilkinson IB, et al. Effect of integration of supplemental nutrition with public health programmes in pregnancy and early childhood on cardiovascular risk in rural Indian adolescents: long term follow-up of Hyderabad nutrition trial. BMJ 2008;337:a605, http://dx.doi.org/10.1136/bmj.a605.
[4] Arokiyaraj S, Saravanan M, Badathala V. Green synthesis of Silver nanoparticles using aqueous extract of Taraxacum officinale and its antimicrobial activity. South Indian Journal Of Bioogical Sciences 2015;2:115–8.

[5] Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 2006;7(12):885–96.
[6] Lefterova MI, Lazar MA. New developments in adipogenesis. Trends Endocrinol Metab 2009;20(3):107–14.
[7] Larson NI, Story MT, Nelson MC. Neighborhood environments: disparities in access to healthy foods in the US. Am J Prev Med 2009;36(1):74–81.e10.
[8] McCrory MA, Hamaker BR, Lovejoy MC. Pulse consumption, satiety, and weight management. Adv Nutr 2010;1(1):17–30.
[9] Brusotti G, Montanari R, Capelli D, Cattaneo G, Laghezza A, Tortorella P, et al. Betulinic acid is a PPARγ antagonist that improves glucose uptake, promotes osteogenesis and inhibits adipogenesis. Sci Rep 2017;7(1):5777, http://dx.doi.org/10.1038/s41598-017-05666-6.
[10] Stashi E, York B, O'Malley BW. Steroid receptor coactivators: servants and masters for control of systems metabolism. Trends Endocrinol Metab 2014;25(7):337–47.
[11] Wilson AP. Cytotoxicity and viability assays. In: Masters JRW, editor. Animal cell culture: a practical approach. Oxford Univ Press; 2000. p. 175–219.
[12] Krishna MS, Kumar AA, Jaleel KAA. Time-dependent alterations in mRNA, protein and microRNA during in vitro adipogenesis. Mol Cell Biochem 2018;448(1–2):1–8, http://dx.doi.org/10.1007/s11010-018-3307-y. Epub 1 February 2018.
[13] Kraus NA, Ehebauer F, Zapp B, Rudolphi B, Kraus BJ, Kraus D. Quantitative assessment of adipocyte differentiation in cell culture. Adipocyte 2016;5(4):351–8.
[14] Maldie D. Methods in molecular biology. In: Ailhaud G, editor. Adipose tissue protocols. Totowa, NJ: Humana Press Inc; 2008. p. 54–6.
[15] Krishna MS, Nair AJ. Antibacterial, cytotoxic and antioxidant potential of different extracts from leaf, bark and wood of *Tectona grandis*. Int J Pharm Drug Res 2010;2(2):155–8.
[16] Khan RM, Mlungwanab SM. 5-Hydroxylapachol: a cytotoxic agent from *Tectona grandis*. Phytochem 1999;50(3):439–42.
[17] Mukherjee D, Kumar NS, Khatua T, Mukherjee PK. Rapid validated HPTLC method for estimation of betulinic acid in *Nelumbo nucifera* (Nymphaeaceae) rhizome extract. Phytochem Anal 2010;21(6):556–60.
[18] Neamatallah A, Yan L, Dewar SJ, Austin B. An extract from Teak (*Tectona grandis*) bark inhibited Listeria monocytogenes and methicillin resistant *Staphylococcus aureus*. Lett Appl Microbiol 2005;41(1):94–6.
[19] Galgon T, Hoke D, Drager B. Identification and quantification of betulinic acid. Phytochem Anal 1999;10:187–90.
[20] Haque ME, Shekhar HU, Mohamad AU, Rahman H, Islam AMI, Hossain MS. Triterpenoids from the stem bark of *Avicennia officinalis*. Dhaka Univ J Pharm Sci 2006;5(1):53–7.
[21] Kim J, Lee YS, Kim CS, Kim JS. Betulinic acid has an inhibitory effect on pancreatic lipase and induces adipocyte lipolysis. Phytother Res 2012;26(7):1103–6.
[22] Chintharlapalli S, Sabitha P, Shengxi L, Indira J, Gayathri C, et al. 2-cyano-lup-1-en-3-oxo-20-oic acid, a cyano derivative of betulinic acid, activates peroxisome proliferator-activated receptor g in colon and pancreatic cancer cells. Carcinogenesis 2007;28(11):2337–46.
[23] Kahn CR, Chen L, Cohen SE. Unraveling the mechanism of action of thiazolidinediones. J Clin Invest 2000;106(11):1305–7.
[24] Hussein H, Wentworth JM, Nankervis AJ, Proietto J, Colman PG. Effectiveness and side effects of thiazolidinediones for type 2 diabetes: real-life experience from a tertiary hospital. Med J Aust 2004;181(10):536–9.
[25] Picard F, Gehin M, Annicotte J, Rocchi S, Champy MF, O'Malley BW, et al. SRC1 and TIF2 control energy balance between white and brown adipose tissues. Cell 2002;111(7):931–41.
[26] Genet C, Strehle A, Schmidt C, Boudjelal G, Lobstein A, Schoonjans K, et al. Structure-activity relationship study of betulinic acid, a novel and selective tgr5 agonist, and its synthetic derivatives: potential impact in diabetes. J Med Chem 2010;53(1):178–90.
[27] Rollins RA, Coppo M, Rogatsky I. Minireview: nuclear receptor coregulators of the p160 family: insights into inflammation and metabolism. Mol Endocrinol 2015;29(4):502–17.
[28] Louet JF, O'Malley BW. Coregulators in adipogenesis: what could we learn from the SRC (p160) coactivator family? Cell Cycle 2007;6(20):2448–52.
[29] Louet JF, Coste A, Amazit L, Tannour-Louet M, Wu RC, Tsai SY, et al. Oncogenic steroid receptor coactivator-3 is a key regulator of the white adipogenic program. Proc Natl Acad Sci U S A 2006;103(47):17868–73.