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Ziziphus jujuba Mill. leaf extract restrains adipogenesis by targeting PI3K/AKT signaling pathway

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ABSTRACT

The escalation in the global prevalence of obesity has focused attention on finding novel approaches for its management. *Ziziphus jujuba* Mill. (ZJL) leaf extract is reported as a traditional remedy for diverse pathological conditions, including obesity. The present study investigated whether ZJL affects adipogenic differentiation in human adipocytes. Additionally, following metabolite profiling of the extract, apigenin (APG), betulinic acid (BA) and maslinic acid (MA) were selected for biological activity evaluation. The possible interactions between APG, BA, MA and target proteins with a central role in adipogenesis were assessed through molecular docking. The potential mechanisms of ZJL, APG, BA and MA were identified using transcriptional analysis through real-time quantitative PCR and protein abundance evaluation by Western blotting. The obtained results revealed a concentration-dependent reduction of accumulated lipids after ZJL, BA and MA application. The key adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα) were strongly decreased at a protein level by all treatments. Moreover, the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway was found to be involved in the anti-adipogenic effect of ZJL, APG and BA. Collectively, our findings indicate that ZJL and its pure compounds hampered adipocyte differentiation through PI3K/AKT inhibition. Among the selected compounds, BA exhibits the most promising anti-adipogenic activity. Furthermore, being a complex mixture of phytochemicals, the ZJL extract could be utilized as source of yet unknown bioactive leads with potential implementation in obesity management.

1. Introduction

The prevalence of obesity has reached epidemic proportions, which has led to a higher incidence of its comorbidities, such as type 2 diabetes, atherosclerosis, hypertension, dyslipidaemia, and subsequent increase in the healthcare costs [1–3]. Moreover, persistent low-grade chronic inflammation, typical for obesity, contributes to the exacerbation of existing complications [4–6]. Mortality associated with overweight and obesity exceeds 2.8 million people every year and makes up a substantial portion of the overall mortality from lifestyle-related diseases [7]. Therefore, immediate measures are required in prevention and management of this expanding global problem.

Discovery of a safe and an effective anti-obesity therapy is an urgent necessity, considering the serious adverse side effects accompanying the approved anti-obesity drugs [3]. Plants are natural sources of various bioactive compounds with yet insufficiently explored potential in obesity management. The complex chemical composition of the plant extracts could provide potent compounds that exhibit biological effect *via* more than one mechanism, which is an important advantage over synthetic medicines [8,9].

Modulation of signaling pathways related to adipogenesis, energy metabolism, inter- and intracellular communication could provide a

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novel approach for the prevention of overweight and obesity. The major adipogenic transcription factors, peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα), as well as their downstream regulatory enzymes involved in lipid synthesis are among the possible targets for obesity therapy [10,11]. Furthermore, modulation of lipolysis rate coupled with elevated capacity of energy expenditure is an additional mechanism favouring the control of overweight and obesity [1,12]. Another key metabolic pathway is the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling which is involved in numerous cellular functions, including differentiation and insulin-stimulated glucose uptake in adipocytes [13–16]. Previous studies suggested that inhibition of the PI3K/AKT signaling pathway leads to decreased adiposity and improved metabolic health [16–21].

Ziziphus jujuba Mill. (Rhamnaceae) is a deciduous tree of common occurrence, cultivated as a source of nutritious fruits [22–24] which are reported to affect the lipid metabolism *in vitro* in murine adipocytes [25. Besides the fruits, other parts, *i.e.* roots, stem bark, seeds and leaves are employed in traditional medicine [26–28]. Ethnopharmacologically the leaves of *Z. jujuba* are described as a remedy for typhoid fever, furuncle, ulcerative skin infections [26,29], anxiety [27] and obesity [30]. Additionally, several contemporary studies have evaluated the hepatoprotective [26], anti-inflammatory and antibiofilm [27] properties of *Z. jujuba* leaf (ZJL) extract. However, the potential effect of ZJL extract on human adipocyte function in the context of obesity has not been adequately addressed.

The present study hypothesized the anti-adipogenic potential of ZJL in an *in vitro* obesity model. Metabolite profiling was performed *via* nuclear magnetic resonance (NMR) based approach to explore the phytochemical content of the ZJL extract and to select pure compounds for further biological evaluation. Subsequently, an *in silico* molecular docking analysis of the selected secondary metabolites, namely, apigenin (APG), betulinic acid (BA), maslinic acid (MA) against adipogenesis-related proteins was executed. Further, the modulatory potential of ZJL, pure APG, BA and MA on adipogenic differentiation was assessed in human adipocytes and real-time quantitative PCR (RT-qPCR) and Western blotting techniques were used to identify key regulatory genes and pathways altered by ZJL and its compounds. Integration of the obtained data resulted in a proposed mechanism of action of ZJL, APG, BA and MA in human adipocytes.

2. Materials and methods

2.1. Materials

Apigenin (molecular weight 270.24 g/M; purity ≥ 95%; #89159) was obtained from Phytolab (Vestenbergsgreuth, Germany), betulinic acid (molecular weight 456.71 g/M; purity ≥ 98%; #0031 S) and maslinic acid (molecular weight 472.71 g/M; purity ≥ 98%; 2320 S) were purchased from Extrasynthese (Genay, France). Deuterated methanol and water were purchased by Deutero GmbH (Kasbellaun, Germany). Standard chemicals and cell culture media were obtained from Merck KGaA (Darmstadt, Germany). All chemicals and reagents used to perform electrophoresis, immunoblotting and RT-qPCR were acquired from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Antibodies for Western blotting were purchased as follows: rabbit anti-C/EBPα (#2295 S), anti-PPARγ (#2443 S); anti-AKT (#9272) and anti-PI3K (#4257) from Cell Signaling Technology (Leiden, The Netherlands); anti-adiponectin (#GTX112777) from GeneTex Inc. (Ivrine, CA, USA); hFAB Rhodamine anti-actin beta antibody (#AHP2417) and secondary StarBright Blue 700-conjugated goat anti-rabbit IgG (#12004162) from Bio-Rad.

2.2. Plant material and extraction

Leaves from *Z. jujuba* were collected in 2019 from Zvanichevo, Bulgaria (latitude: 42°18`75``N; longitude: 24°25`09``E). Following botanical identification, a voucher specimen (№ SOM1396) was transferred to the botanical collection of the Bulgarian Academy of Sciences. The collected plant material was further frozen, freeze-dried and ground. Single ultrasound-assisted extraction was performed with 50% aqueous methanol (1:30 w/v) for 20 min at 20 °C. The obtained extract was concentrated *via* rotary vacuum evaporator at 40 °C, further freeze-dried and stored at −20 °C prior to biological assays.

2.3. NMR-based metabolomics and chromatographic analysis

The NMR-based analysis was performed as described previously [31,32]. Recording of ^{1}H NMR and ^{2}D NMR spectra (heteronuclear single quantum coherence spectroscopy, HSQC) was carried out on an AVII+ 600 spectrometer from Bruker (Karlsruhe, Germany). The received spectral data were subjected to manual phase and baseline correction, and further referenced to 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as an internal standard at 0.0 ppm and normalized to its peak, scaled to 1.0, using MestReNova software version 12.0.0 from Mestrelab Research (Santiago de Compostela, Spain). The main compounds were identified according to previously published data. Selected secondary metabolites were quantified through high-performance liquid chromatography (HPLC) analysis presented as mg/g dry extract (Supplementary material).

2.4. Cell culture and treatment

Human Simpson-Golabi-Behmel syndrome (SGBS) preadipocyte cell strain was cultured according to previously described conditions [12,33]. Briefly, near-confluent preadipocytes were subjected to adipogenic differentiation in the presence of ZJL (5, 10 and 25 μg/mL), pure APG (0.25, 0.5 and 1 μM), BA (0.25, 0.5 and 1 μM), MA (0.25, 0.5 and 1 μM) or 0.02% DMSO as vehicle. Treatments were applied with every media replacement on day 0, 4 and 8 of differentiation, respectively. The experimental concentrations were chosen upon cell viability evaluation (Supplementary Fig. S1). Finally, 24 h after the last treatment the cells were subjected to sample collection and subsequent analyses. Each assay was performed in three technical replicates from three independent experiments.

2.5. In silico molecular docking

Docking calculations were conducted through the Autodock Vina of PyRx 0.8 software according to previously described conditions and parameters [12]. The crystal structures of the target proteins were derived from the protein data bank (PDB, www.wwpdb.org) with PDB IDs as follows: 1O6L for AKT, 1NWQ for C/EBPα, 3PCU for glucose transporter type 4 (GLUT4), 3BU6 for insulin receptor (ISR), 5ITD for PI3K, 2P4Y for PPARγ, 1OEM for protein tyrosine phosphatase 1B (PTP1B). Autodock4 and Lamarckian genetic algorithms were used to dock 250 conformations for each test compound (Molinspiration Database, www.molinspiration.com). Discovery studio 2020 Visualizer was employed to investigate the protein to ligand non-bonding interactions.

2.6. Quantification of intracellular lipids and basal lipolysis

Accumulated lipids in differentiated adipocytes were stained with an Oil red O (ORO) solution as described previously [11]. Representative photographs were taken using an Oxion Inverso OX.2053-PLPH inverted microscope equipped with DC.10000-Pro CMEX camera from Euromex (Arnhem, The Netherlands). Lipid staining was extracted from adipocytes and absorbance at 495 nm was measured on Biochrome Anthos Zenyth 340 multiplate reader (Cambridge, UK). The content of the accumulated lipids was calculated through the absorbance of the extracted ORO dye and results were presented as percentage accumulated lipids, compared to the vehicle control group.

Influence on basal lipolysis by experimental treatments was quantified as concentration of released glycerol in the culture media through an Adipolysis assay kit (#MAK313) from Merck KGaA according to the instructions provided by the manufacturer. Glycerol concentration (μg/mL) was calculated according to the obtained standard curve.

2.7. RT-qPCR analysis

Total RNA samples were obtained as previously described [11] with Quick-RNA miniprep kit (#R1055) from Zymo Research (Irvine, CA, USA). Reverse transcription to copy DNA was performed with First strand cDNA synthesis kit (PR008-XL) from Canvax (Córdoba, Spain). Reaction mix using SsoFast EvaGreen Supermix (#1725204, Bio-Rad) and gene-specific primers (Supplementary Table S1) was prepared and analyzed on C1000 Touch thermal cycler with CFX96 detection system (Bio-Rad). Gene expression was measured by comparative threshold cycle ($_{\Delta\Delta}$Ct) method. Ribosomal protein L13a (*RPL13A*), hypoxanthine guanine phosphoribosyl transferase (*HPRT*) and β-tubulin (*TUBB*) were used as reference genes. Gene expression data analysis was performed with CFX Maestro software (Bio-Rad).

2.8. Western blotting

Cell lysates were extracted in an ice-cold RIPA lysis buffer supplemented with 1% protease and phosphatase inhibitors prior to the procedure, and the total protein concentration was determined using Bradford reagent. Equivalent amount of protein samples (30 μg per lane) was resolved on a sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes [12]. Membrane blocking for an hour with 5% (w/v) skim milk in Tris buffered saline at room temperature was followed by overnight incubation with primary antibodies against PPARγ, C/EBPα, adiponectin, AKT or PI3K. Chem-iDoc MP Imaging System (Bio-Rad) was used for multiplex fluorescent detection. Whole uncropped images of the original Western blots are provided in Supplementary Fig. S3. Normalization was done over β-actin as a housekeeping protein on Image Lab 6.0.1 software (Bio-Rad).

2.9. Statistical analysis

The obtained data were statistically processed with SigmaPlot v11.0 from Systat Software GmbH (Erkrath, Germany) software and presented as mean ± SEM. Differences between groups were determined by one-way analysis of variance (ANOVA), followed by a Bonferroni's *post hoc* test. A value of $^{*}p < 0.05$ was considered significant.

3. Results

3.1. NMR-based metabolite profiling

The characterization of the metabolite profile of ZJL has been recently subjected to elevated interest [24,25,29,34], which has expanded the existing knowledge about its chemical content. Major classes of compounds detected in the plant extract are flavonoids, pentacyclic triterpenes, saponins and cyclopeptide alkaloids [23,25,29]. For instance, the application of *Z. jujuba* fruit extract fraction, rich in terpenoids, on murine pre-adipocytes, resulted in inhibited adipogenic differentiation [25]. The authors have hence proposed that the inhibition of PPARγ and C/EBPα is related to the abundance of triterpenoic acids such as betulinic acid in the extract [25]. Several studies report that terpenoic acids are found in the *Z. jujuba* leaf extract as well [23,24,29,30]. Therefore, we have targeted two terpenoid acids, BA and MA, to evaluate their contribution to the biological activity of the ZJL extract in human adipocytes.

Further, the most abundant flavonoids reported in *Z. jujuba* extracts are apigenin, quercetin and kaempferol derivatives [23,24,26]. We have employed an NMR-based approach for the identification of common primary metabolites and several secondary metabolites in ZJL (Table 1) and the spectral data were further compared to the literature [31,32,35].

The presence of apigenin in the ZJL extract was confirmed by analysis of the obtained ^{1}H NMR and HSQC spectra (Supplementary Fig. S2) through comparison with previously reported data [35,36]. According to the HPLC quantification, the content of APG resulted to 0.17 ± 0.02 mg/g dry weight extract. In addition to the two terpenoids BA and MA, we have selected the flavonoid compound apigenin to examine its potential to modulate the adipogenic differentiation in our *in vitro* model system.

3.2. In silico molecular docking

The protein affinity of small molecules can be predicted, with favourable accuracy, through docking calculations which facilitate the clarification of the underlying molecular mechanisms affected by a given compound [12,37]. Therefore, in the present study, docking runs were performed to determine the putative affinities of APG, BA and MA towards selected protein targets, involved in adipogenesis and insulin signaling.

Adipogenic differentiation is regulated by a complex network of external and internal factors. Signaling molecules such as insulin, growth factors, corticosteroids and retinoids induce white adipogenic program in the precursor fat cells [2,5]. Upon insulin activation the ISR transmits the signal through PI3K/AKT pathway which in turn elevates the C/EBPα and PPARγ levels [16,17,20]. Subsequently, during the process of maturation of the adipocyte the expression of GLUT4 is increased [14,16,38]. Protein phosphatases, such as PTP1B, are essential regulators of insulin signaling and energy metabolism in adipocytes [39]. The reported improved insulin sensitivity resulted from inhibited PTP1B activity shed light on possibility of pharmacological inhibition of PTP1B aiming improved insulin signaling [40].

The affinity of the selected phenolic compounds towards the aforementioned target proteins was expressed in terms of binding free energy ($_{\Delta}$G) and affinity constant (Ki; Table 2). With the sole exception of the

Table 1
Chemical shifts (δ) and coupling constants (J) of the metabolites identified by their relevant ^{1}H NMR spectra [31,32,35,36].

Metabolite	Chemical shift (δ, ppm)	Coupling constant (*J*, Hz)
Amino acids		
Alanine	1.49	(d, $J = 7.2$)
Glutamine	2.14/2.39	(m)/(m)
Threonine	1.33	(d, $J = 6.9$)
Valine	1.01/1.07	(d, $J = 7.3$)/(d, $J = 7.1$)
Carbohydrates		
α-Glucose	5.19	(d, $J = 3.8$)
β-Glucose	4.59	(d, $J = 7.9$)
Fructose	4.18	(d, $J = 8.6$)
Sucrose	5.41	(d, $J = 3.9$)
Organic acids		
Acetic acid	1.90	(s)
Formic acid	8.47	(s)
Fumaric acid	6.48	(s)
Succinic acid	2.40	(s)
Flavonoids		
Apigenin	6.66/6.31/6.52/8.08/6.98	(s)/(d, $J = 2.0$)/(d, $J = 2.0$)/(d, $J = 9.0$)/(d, $J = 8.5$)
Kaempferol	6.32/6.50/8.08/7.00	(d, $J = 2.0$)/(d, $J = 2.1$)/(d, $J = 9.0$)/(d, $J = 8.5$)
Quercetin	6.32/6.50/7.72/7.00/7.62	(d, $J = 2.0$)/(d, $J = 2.1$)/(d, $J = 2.0$)/(d, $J = 8.5$)/(dd, $J = 8.5;2.1$)
Rutin	6.32/6.50/7.67/6.82/7.62/5.02/4.54	(d, $J = 2.0$)/(d, $J = 2.1$)/(d, $J = 2.2$)/(d, $J = 9.3$)/(dd, $J = 8.5;2.1$)/(d, $J = 7.5$)/(d, $J = 1.3$)
Others		
Choline	3.22	(s)
γ-Amino-butyrate	1.90/2.30/3.01	(m)/(t, $J = 7.5$)/(t, $J = 7.3$)

Table 2
Theoretically calculated free energy of binding ($_{\Delta}$G, kcal/M) and affinity constant (Ki, μM) for apigenin, betulinic and maslinic acids to the selected protein structures for AKT, C/EBPα, GLUT4, ISR, PI3K, PPARγ and PTP1B.

Target protein	Apigenin	Betulinic acid	Maslinic acid
AKT	-6.5 kcal/M; 17.4 μM	-7.1 kcal/M; 6.3 μM	-7.6 kcal/M; 2.7 μM
C/EBPα	-5.6 kcal/M; 79.5 μM	-6.5 kcal/M; 17.4 μM	-6.9 kcal/M; 8.9 μM
GLUT4	-7.8 kcal/M; 1.9 μM	-7.0 kcal/M; 7.5 μM	-7.6 kcal/M; 2.7 μM
ISR	-7.5 kcal/M; 3.2 μM	-7.9 kcal/M; 1.6 μM	-7.8 kcal/M; 1.9 μM
PI3K	-8.1 kcal/M; 1.2 μM	-8.8 kcal/M; 0.4 μM[a]	-10.4 kcal/M; 0.02 μM[a]
PPARγ	-7.2 kcal/M; 5.4 μM	-8.2 kcal/M; 1.0 μM[a]	-8.8 kcal/M; 0.4 μM[a]
PTP1B	-6.5 kcal/M; 17.4 μM	-6.9 kcal/M; 8.9 μM	-7.3 kcal/M; 4.5 μM

[a] Ki within the treatment range (0.25–1 μM).

APG binding to C/EBPα, all compounds showed putative affinities in the low micromolar range (0.02–17.4 μM) against all tested proteins. According to calculated Ki both terpenic acids are going to interact with PI3K and PPARγ within the treatment concentration range (0.25–1 μM).

The orientation of APG, MA and BA at the protein binding sites of the PI3K and PPARγ proteins is illustrated in Fig. 1. Further, the binding affinity of APG towards PI3K (Fig. 1D) was found to be very close to the treatment concentration range, providing further rationale for its evaluation at a molecular level. Inhibition of PTP1B [39] and PPARγ [38] has previously been reported for BA, similar to the suggested interactions by our docking especially for PPARγ (Table 2; Fig. 1I). Regarding MA, the higher affinity displayed towards PI3K (Fig. 1J), could be a result of multiple binding interactions, namely hydrogen bonds, Van der Waals and pi-interactions.

Collectively, the *in silico* molecular docking simulation imposed PI3K and PPARγ as potential protein targets for the molecular action of APG, BA and MA.

3.3. Z. jujuba leaf extract and betulinic acid mitigated adipogenic lipid accumulation

Adipose tissue expansion is characterized by formation of lipid droplet-enriched white adipocytes and/or overgrowth of already differentiated cells [2,10,11]. The anti-adipogenic potential of ZJL and its selected pure compounds was assessed through ORO lipid staining. Representative microphotographs of the stained adipocytes (Fig. 1A) and quantification of the total lipids (Fig. 1B) revealed significant and concentration-dependent reduction of lipid accumulation upon ZJL (79.7%, 72.9% and 65.4%, for 5, 10 and 25 μg/mL), BA (70.5%, 58.1% and 47.5%, for 0.25, 0.5 and 1 μM) and MA treatment (90.5%, 90.3% and 87.2% for 5, 10 and 25 μM), respectively. Interestingly, even though APG has been reported to affect adipogenesis in other *in vitro* obesity models [41–43], within the present investigation we did not observe changes in lipid accumulation upon its application.

Assuming that the modulation of lipolysis is a promising approach for obesity management and decrease in adipogenesis could correlate to changes in basal lipolysis rates [1,5], the concentration of free glycerol released in the culture media was used to quantify the effect on basal unstimulated lipolysis. The obtained results suggested moderate effect on glycerol concentration, such as elevation by APG and MA and reduction by ZJL (Fig. 2C).

Lipid accumulation in SGBS were significantly and dose-dependently decreased upon ZJL treatment. A similar pattern of inhibition of lipid accumulation was observed for BA in human adipocytes and to a lesser extent for MA. We then focused on the role of adipogenic factors that could be potentially mediating the observed reduction in adipogenesis.

3.4. Z. jujuba leaf extract and its constituents modulated key adipogenic transcription factors

The complex regulatory network of adipocyte differentiation provides opportunities for targeted modulation of adipogenesis. Thus, determination of the relative mRNA expression of the main adipogenic transcription factors PPARγ and C/EBPα, as well as, of adiponectin (an adipokine related to the differentiation of white adipocytes) and one of the crucial enzymes of fatty acid biogenesis - acetyl-CoA carboxylase (ACC), were evaluated upon treatment with ZJL, APG, BA or MA during adipogenic differentiation. In addition, to further clarify the molecular mechanism, the protein abundance of PPARγ, C/EBPα and adiponectin was detected.

Downregulation in the mRNA levels of *PPARG* (Fig. 3A) was observed upon ZJL, BA and MA treatment. Similarly, the results of the Western blot analysis revealed that ZJL and both terpenic acids decreased the protein levels of PPARγ in a concentration-dependent manner (Fig. 3E). Regarding the inhibitory effects induced by these natural compounds on PPARγ, the present findings are consistent with the literature data [38,44] as well as the data from the molecular docking predictions (Fig. 1H; 1I). The APG treatment affected the protein abundance of PPARγ only at its highest concentration used, in contrast to previously published data by Li et al. [41]. These discrepancies could be a result from either the different treatment concentration range or the employed cell model.

Suppression on *CEBPA* mRNA level (Fig. 3B) and respective protein abundance (Fig. 3F) following ZJL treatment was observed. Intriguingly, all tested pure compounds diminished the C/EBPα protein abundance (Fig. 3F) without significant silencing at a transcriptional level (Fig. 3B). This strong effect on C/EBPα protein could be explained as a consequence of the negative regulation on PPARγ by APG, BA and MA or other overarching inhibitory signals.

Similarly, the ZJL extract concentration-dependently diminished *ADIPOQ* gene (Fig. 3C) and protein expression (Fig. 3G) while upon treatment with the pure compounds, alterations were detected only at a protein level. The data from the Western blot analysis for adiponectin revealed that its protein expression was increased by APG 0.25 and 0.5 μM (Fig. 3G). Treatment with BA resulted in concentration-dependent reduction in adiponectin (Fig. 3G) while MA produced bidirectional effect (elevation upon treatment with MA 0.25 μM and suppression above 0.5 μM; Fig. 3G).

In the presence of all experimental treatments, significant and concentration-dependent reduction in gene expression of *ACC* (Fig. 3D) was revealed by the RT-qPCR analysis. The observed suppression of this marker of *de novo* lipogenesis is in positive correlation with the decrease in PPARγ (Fig. 3E) and C/EBPα (Fig. 3F) proteins.

Taken together, these data demonstrate that ZJL and its selected pure compounds concentration-dependently reduce adipogenic proteins with emphasis on the modulation of PPARγ as a master regulator of adipogenesis. Of noteworthy interest is the higher efficacy displayed by BA and MA in modulating all tested proteins, which support their potential implementation in obesity management as pure compounds [45–47]. Furthermore, our findings provide evidence that all tested compounds take part in the anti-adipogenic action of ZJL extract.

3.5. PI3K/AKT signaling is involved in the anti-adipogenic effect of Z. jujuba leaf extract and its pure active compounds

Activated PI3K/AKT signaling pathway is engaged in the insulin-mediated induction of adipogenesis [15,48], hence, its inhibition might hamper the adipocyte differentiation and prevent obesity exacerbation [16,17,19,21]. Therefore, we next examined the PI3K and AKT relative mRNA levels and protein abundance (Fig. 4) in order to clarify whether this molecular pathway is involved in the anti-adipogenic effect

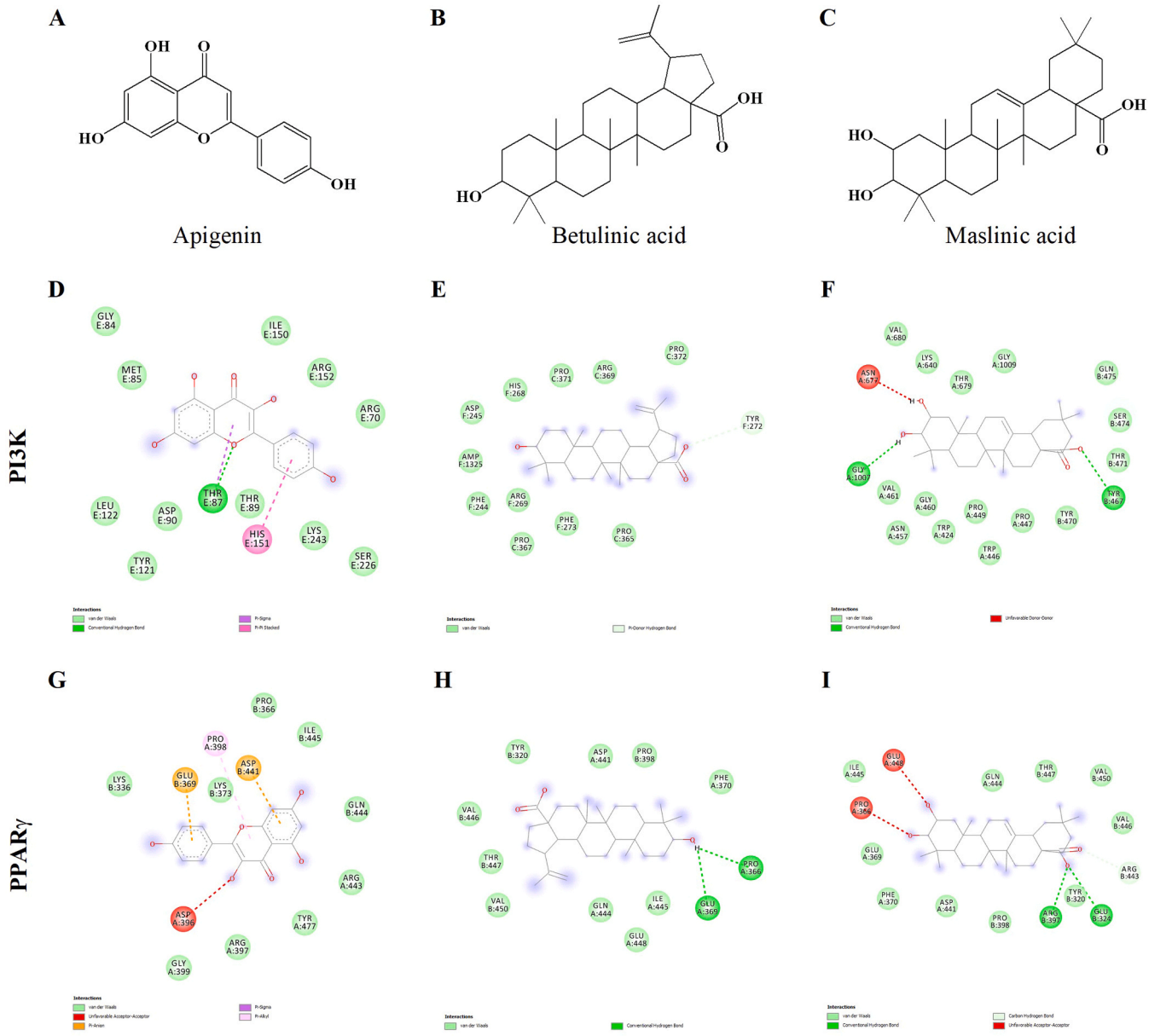

Fig. 1. Chemical structures of apigenin (A), betulinic acid (B) and maslinic acid (C). Docking patterns of PI3K and PPARγ with APG, BA and MA are visualized as follows: APG-PI3K (D), BA-PI3K (E), MA-PI3K (F), APG-PPARγ (G), BA-PPARγ (H), and MA-PPARγ (I).

of ZJL and its selected secondary metabolites.

The detected changes upon ZJL and BA treatments in the gene expression of *AKT* (Fig. 4A) are consistent with the lowered protein abundance (Fig. 4D). In contrast, the other terpenic acid, MA elevated AKT (Fig. 4A; 4D) at both transcriptional and protein levels. Regarding the insignificant interaction between APG and AKT the data from the RT-qPCR (Fig. 4A) and the Western blot (Fig. 4C) analyses are in line with the predicted low affinity from the molecular docking calculations (Table 2).

Adipogenic differentiation is characterized by overexpression of PI3K [21]. Downregulation of the *PI3KCA* mRNA level (Fig. 3B) and its corresponding protein abundance (Fig. 4D) was detected upon ZJL treatment in human adipocytes. Additionally, modulatory effects were induced by BA and MA on the changes in protein levels of PI3K (Fig. 4D) in correlation with the predicted interactions from the docking analysis (Fig. 1E; 1 F). However, the pattern of gene expression, following BA and MA treatment, does not parallel to the variations of the protein steady state level, as shown by the Western blot analysis, thus indicating direct interactions of MA and BA with the active sites of the protein. Further, our results indicate possible interaction between APG and PI3K, based on the lowest calculated value for Ki = 1.2 μM and $_{\Delta}G$ = -8.1 kcal/M between tested proteins (Table 2), confirmed in reduction of PI3K gene expression (Fig. 4B) and protein abundance (Fig. 4D).

Despite the varying degrees in their modulatory effect, all studied treatments influenced the PI3K/AKT signaling pathway in mature adipocytes. Therefore, we could speculate that ZJL extract exerts its anti-adipogenic effect through PI3K/AKT and its selected compounds contribute to this mechanism of action, with BA being the most potent one.

3.6. *Proposed mechanism of action of Z. jujuba leaf extract and its bioactive pure compounds in human adipocytes*

The precise balance between lipid deposition and lipid elimination, termed as lipid turnover, is one of the key processes in the regulation of weight maintenance [1,2]. It is reported that lipid elimination rate

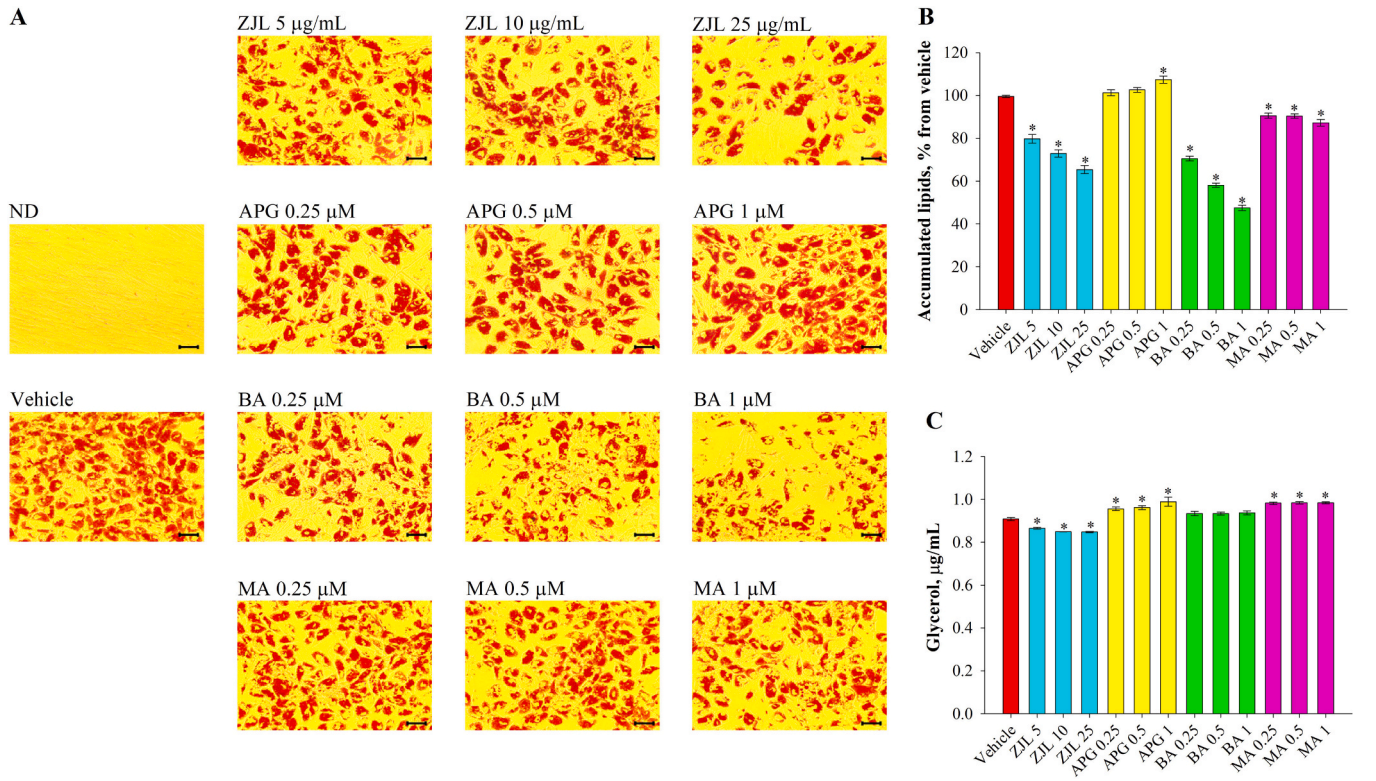

Fig. 2. *Z. jujuba* leaf (ZJL) extract, betulinic acid (BA) and maslinic acid (MA) but not apigenin (APG) decreased lipid accumulation in human adipocytes. Representative microscopic photographs (20 × magnification, scale bar 50 µm) of non-differentiated preadipocytes (ND), vehicle treated adipocytes and the experimental groups captured on day 9 of differentiation after ORO lipid staining (A). Quantitative assessment of the accumulated lipids, expressed as percentage of the vehicle treated group (B). Concentration of glycerol (µg/mL), released in the culture media (C). Values are shown as mean ± SEM, each experimental group included six technical replicates, performed in three independent experiments. *p < 0.05 compared to vehicle control group.

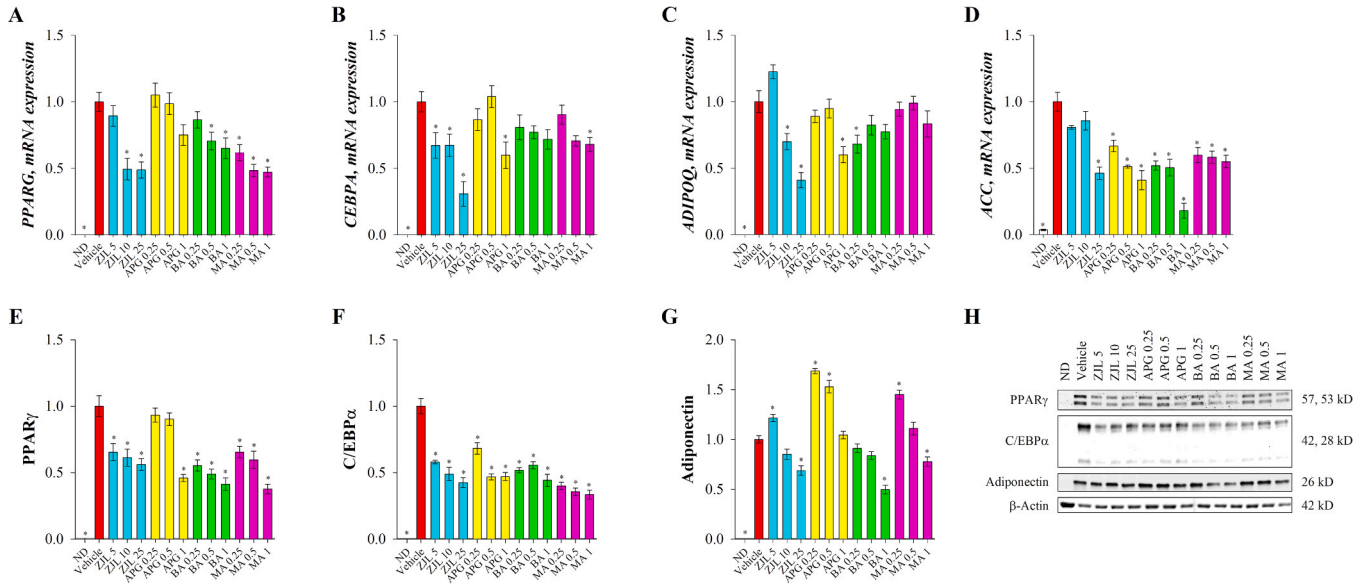

Fig. 3. *Z. jujuba* leaf extract (ZJL), apigenin (APG), betulinic acid (BA) and maslinic acid (MA) inhibited adipogenesis through a modulation of major adipogenic regulators. Relative mRNA expression for the following adipogenic genes: *PPARG* (A), *CEBPA* (B), *ADIPOQ* (C) and *ACC* (D) normalized to *RPL13A*, *HPRT* and *TUBB* as reference genes. Protein expression of PPARγ (E), C/EBPα (F), Adiponectin (G) normalized to β-actin. Representative images of the Western blots (H). Data are representative for from three independent experiments (mean ± SEM). *p < 0.05 compared to vehicle group.

declines with age, facilitating weight gain and development of overweight and obesity [1,3]. Considering obesity is a growing health threat, the influence of lipid turnover through modulation of adipogenic differentiation is a reasonable approach for obesity management [3,5,10].

Our findings shed light on the molecular mechanism of action of ZJL in human adipocytes. Considering the correlation between activated PI3K/AKT signaling pathway and PPARγ, and C/EBPα transcription factors during adipogenesis [16–21], our results suggest implication of

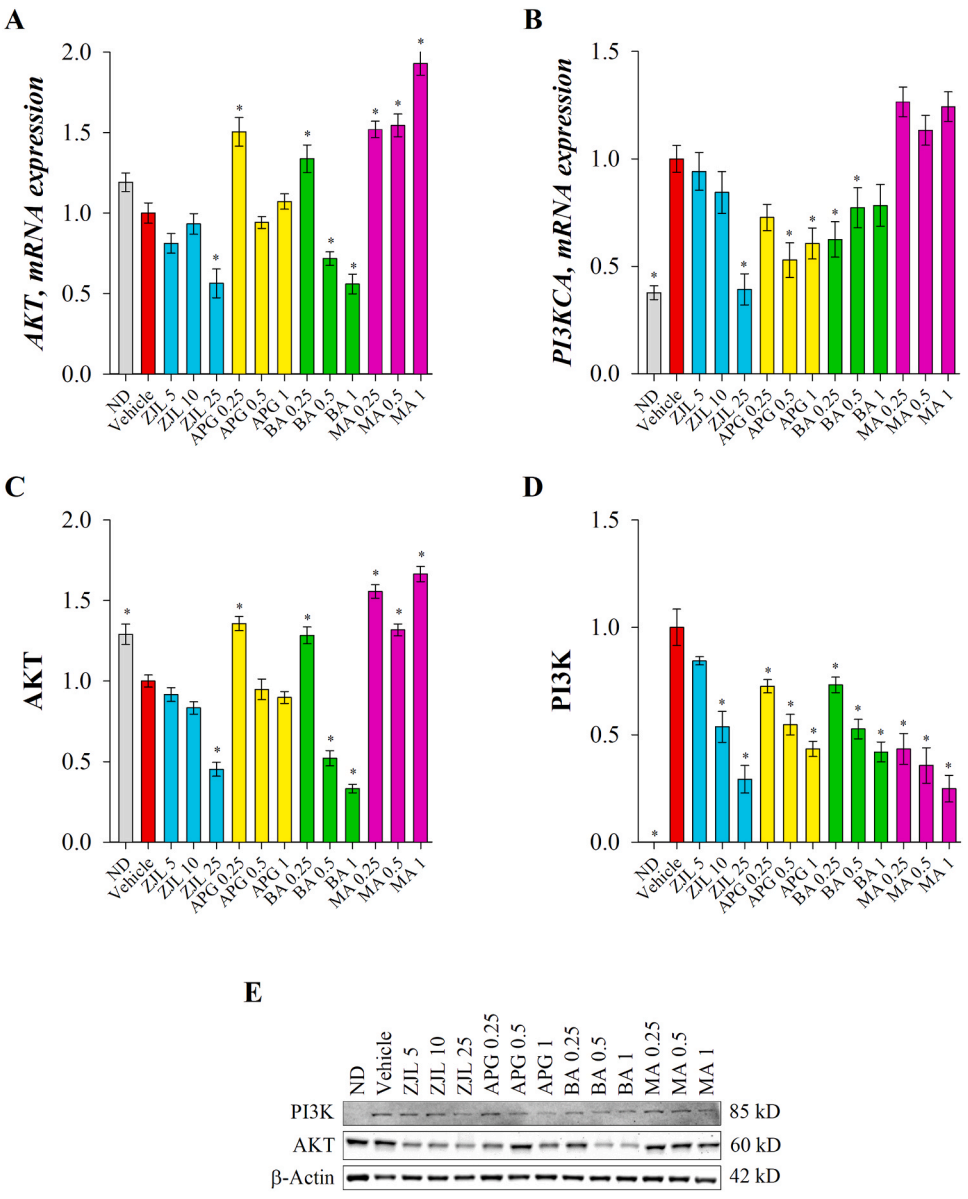

Fig. 4. The anti-adipogenic effect of *Z. jujuba* leaf extract (ZJL), apigenin (APG), betulinic acid (BA) and maslinic acid (MA) is mediated *via* PI3K/AKT signaling. Relative mRNA expression of *PI3KCA* (A) and *AKT* (B) normalized to *RPL13A*, *HPRT* and *TUBB* as reference genes. Protein abundance of PI3K (C) and AKT (D) normalized over β-actin. Representative images from the Western blotting analysis (E). Data are presented as mean ± SEM from three independent experiments. *p < 0.05 compared to vehicle group.

these mediators in the mechanism of ZJL, APG, BA and MA. Further investigations are required in order to determine whether selected compounds directly interact with PPARγ or C/EBPα. Regarding the role of PI3K/AKT in insulin signaling [13,14,18–21], decreased protein abundance of PI3K by all treatments suggests eventually a modulation of insulin signaling. Despite the importance of insulin signaling in preserving insulin sensitivity, inhibition of PI3K is reported with beneficial metabolic effect during adipogenic differentiation [13,16]. Moreover, calculated low values of Ki between APG, BA or MA and ISR refer to a possible interaction with ISR as an upstream regulator of PI3K/AKT signaling pathway. Obtained docking results for BA against PTP1B are consistent with reported enzyme inhibition [39] and suggest improved insulin sensitivity. Integration of the results from RT-qPCR and Western blotting analyses suggests that inhibition of the PI3K/AKT-mediated trigger of adipogenesis - and subsequent downregulation of PPARγ and C/EBPα - is the main mechanism involved in the effect of ZJL extract on adipocyte function (Fig. 5). Further, among the selected pure compounds APG and BA exhibited similar effect on a translational level with the latter inducing inhibition in adipogenesis in a manner close to that observed upon the extract application. Interestingly, the other terpenoid, MA, inhibited PI3K yet elevated AKT, which indicates that it modulates the PI3K/AKT signaling *via* different mechanism than BA, APG and the ZJL extract.

Taken together, we could consider that all studied pure compounds are involved in the ZJL extract anti-adipogenic effect and modulation through PI3K/AKT signaling is their common underlying molecular pathway. However, the ZJL extract itself exerts the most potent lipid reducing activity in human adipocytes reflected by the adipogenesis assay. This suggests that it could be exploited as a source of other potent bioactive leads.

4. Discussion

Severity of obesity correlates to the volume and the distribution of white adipose tissue, as the main lipid-storage organ, which results from either adipose tissue expansion (adipocyte hyperplasia) or abnormal hypertrophy [3,5,10]. Under specific stimuli, such as insulin, glucocorticoids, cAMP inducers, *etc.* [14] preadipocytes undergo differentiation into mature adipocytes. For instance, insulin triggers adipogenic differentiation through ISR that leads to activation of PI3K/AKT and subsequent upregulation in specific adipogenic transcription factors [18,21,48]. The early phase is governed by C/EBPβ and the later by PPARγ,

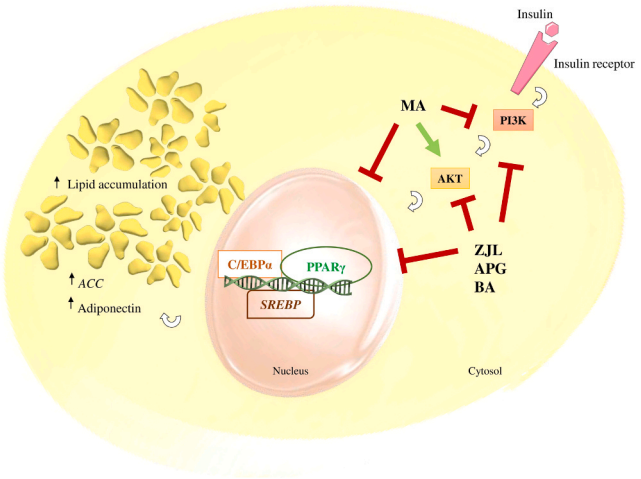

Fig. 5. Prospective mechanism of action of the *Z. jujuba* leaf extract (ZJL), apigenin (APG), betulinic acid (BA) and maslinic acid (MA) in human adipocytes. The ZJL, pure APG and pure BA extract hampers adipogenic lipid accumulation through inhibition in the PI3K/AKT signaling, decrease in the C/EBPα, PPARγ and subsequent downregulation of adiponectin and ACC. Treatment with MA produces similar changes at a molecular level, except for a significant AKT activation instead of its downregulation.

C/EBPα and sterol regulatory element-binding protein 1 (SREBP1) which further activate their downstream lipogenic enzymes such as ACC and fatty acid synthase [11,17,20].

Several studies suggest that ZJL extract possess hepatoprotective and anti-inflammatory potential [26,27], however, investigation of its effect on adipocyte function has not been evaluated to date. In order to clarify the mechanism of action of ZJL in human adipocytes, we have examined the effect on lipid accumulation, basal unstimulated lipolysis, as well as, the relative mRNA and protein expression of PI3K and AKT and the adipogenic master regulators - PPARγ and C/EBPα. Mechanistically, the observed anti-adipogenic potential was mediated through a concentration-dependant inhibition of PI3K/AKT signaling and subsequent downregulation of PPARγ and C/EBPα. The pure natural compounds APG, BA and MA were found to contribute to the decreased rate of differentiation and lipid accumulation induced by the ZJL extract.

Plant secondary metabolites are recognized to have countless beneficial health effects [8], including modulation of adipogenesis [3,9,12]. Plant extracts are characterized with chemical complexity and their biological activity is commonly attributed to a synergistic combination of more than one active compound [3,8,9,18]. However, based on the metabolite profiling of ZJL extract and the previously reported biological activity of APG, BA and MA with regards to obesity [41,42,44,46] we have selected these compounds to be examined in our investigation for their anti-adipogenic potential. Several reports provide evidence that APG could modulate adipogenic differentiation through PPARγ modulation [41,42]. Additionally, among the terpenoids we have selected BA and MA as they are components of the ZJL fruit, leaf and root extract and have been investigated for their activity in experimental obesity models [23–25,29,30,34]. We have employed a molecular docking approach to screen for a potential interaction between adipogenic regulatory proteins and APG, BA and MA. The *in silico* simulations predicted high affinity in the low micromolar range against PI3K and PPARγ, which directed our attention towards the evaluation of these target proteins at a molecular level in the *in vitro* obesity model.

Hepatoprotective [49,50], as well as, anti-inflammatory, anti--allergic and skin-protective effects [51] are reported for APG application in various *in vivo* and *in vitro* model systems. In addition, a number of studies have reported its anti-adipogenic effect in murine adipocytes and in animal models of obesity [41,42,52–54]. Several reports suggest that APG promotes browning of white fat cells [4,42,55] which supports its modulatory activity on adipocyte differentiation. Moreover, clinical investigations have determined the positive outcome of APG consumption in the daily diet to diminish the risk of coronary heart disease development in adults [56,57]. In the present study, we have demonstrated unaffected lipid accumulation and activation of lipolysis as reported by Gómez-Zorita et al. [58]. Administration of APG alleviated diet-induced obesity and insulin resistance *via* enhanced lipolysis and improved fatty acid oxidation mediated by adenosine monophosphate-activated protein kinase (AMPK)-dependent mechanism [55]. Our findings revealed strong reduction in PPARγ protein level as a result of the treatment with 1 μM APG, which correlates to modulatory activity reported by Feng et al. [4]. Additionally, in the context of the inflammatory environment in obesity, APG is identified as PPARγ modulator that resolves the inflammation in adipose tissue and liver in HFD and *ob/ob* mice [4]. The anti-adipogenic effect of APG 20 μM in bone marrow-derived mesenchymal stem cells involves inhibition of the early adipogenic transcription factor C/EBPβ [43], while concentrations of 1–25 μM did not exert such effects in a similar model [58]. However, at a higher concentration APG (40 μM) effectively decreased lipid accumulation and downregulated PPARγ and C/EBPα in 3T3-L1 murine adipocytes [41]. Our results partially correlate with the literature data, which might be a consequence of differences in the model system sensitivity towards the analyzed substances and the significantly lower concentration range (below 1 μM). Interestingly, significant inhibition was observed for C/EBPα in our model system as a result of APG application that is inconsistent with the weak binding affinity (Ki = 79.5 μM) calculated for APG. Additionally, previous data reported the null effect induced by this compound on C/EBPα gene expression [58] that overall scales back the importance of direct interaction with this protein in mediating the pharmacological effects induced by APG. Our findings suggest PI3K as the most probable direct target for APG-induced modulation in adipocytes function. The concentration-dependent reduction in the PI3K protein levels upon APG treatment correlates with the low value of the calculated affinity constant (Ki = 1.2 μM), hence, downregulation in the other adipogenic factors could be subsequent to a direct PI3K inhibition.

Betulinic acid is a pentacyclic lupane-type triterpene that exists widely in food and medicinal herbs including ZJL [23,24]. The biological activity of BA has been well described for its hepatoprotective [59], anti-neoplastic and proapoptotic potential [60]. Several investigations suggest an anti-adipogenic effect upon BA treatment in both *in vitro* and *in vivo* obesity models and report decreased size of fat depots and adipocyte lipid droplets and improved serum parameters [38,39,46,61]. Administration of BA in a HFD-murine model resulted in reduction of weight gain, amount of abdominal fat, normalized serum glucose, triglycerides and total cholesterol and restored impaired insulin, leptin and ghrelin levels [61]. Our findings regarding adipogenesis revealed inhibition of intracellular lipid accumulation. Consistent with the previously published data [38,46], an inhibition of PPARγ, C/EBPα and their downstream targets *ACC* and adiponectin was demonstrated by the RT-qPCR and the Western blotting analyses as a result of BA treatment. In addition, the PI3K/AKT signaling pathway was suppressed at both transcriptional and protein levels by pure BA to a degree similar to the effect of the ZJL extract. Collectively, our data support the notion that BA could produce significant anti-adipogenic activity as a pure compound even in the low micromolar range of application. Mechanistically, we could speculate that BA interacts with the PI3K/AKT signaling and potentially modulates PPARγ in human adipocytes.

Maslinic acid is a pentacyclic triterpenoid distributed mainly in the olive peel [49] that is also identified in the ZJL extract [23]. Previous investigations found that MA exhibits anti-inflammatory [41,62], cardioprotective [45], anti-arthritic [63], anti-adipogenic [44] and hepatoprotective [47] activities. The anti-adipogenic effect of MA reported in 3T3-L1 adipocytes included enhanced glucose uptake, decreased lipid content in accordance with decreased transcription of PPARγ and fatty acid-binding protein 4 [44]. Similar results were further discussed in an

in vivo model of non-alcoholic fatty liver disease in mice, indicating decrease in weight gain, alleviated hepatic lipid accumulation, increased adiponectin plasma levels and identifying AMPK activation as the anti-steatogenic mechanism of MA [47]. In the present investigation, inhibition of adipogenesis upon MA treatment in human adipocytes was revealed that could be attributed to a downregulation in PPARγ and PI3K as suspected direct targets which was further supported by the docking predictions. The observed decrease in C/EBPα protein levels and the bidirectional effect on adiponectin level observed in MA treated adipocytes are most probably subsequent to the PPARγ modulation.

Taken together, our findings provide evidence for the anti-adipogenic potential of ZJL extract in human adipocytes. The modulation in adipocyte differentiation and involvement in the ZJL biological activity of APG, BA and MA, as pure compounds, were confirmed by the molecular docking, gene and protein expression analyses. The inhibition of the PI3K/AKT signaling pathway was identified as the main molecular mechanism of the ZJL, APG and BA effect on adipogenesis. Further, modulation of PPARγ and subsequently C/EBPα was found to be involved within the suppression of adipocyte maturation by the extract and the selected pure compounds.

5. Conclusions

Collectively, the present study revealed that ZJL extract supresses adipogenesis in human adipocytes. Following NMR-based phytochemical analysis of ZJL extract, APG, BA and MA were selected as potential secondary metabolites contributing to the observed anti-adipogenic effect of the extract. The docking simulation revealed that the selected pure compounds would interact with the highest affinity towards PI3K and PPARγ. The mechanistic investigation exposed the PI3K/AKT signaling pathway as the central component in the anti-adipogenic effect of ZJL extract and its active compounds. Simultaneously, reduction in PPARγ and C/EBPα was detected suggesting that both proteins are implicated in the ZJL, APG, BA and MA action in human adipocytes. Among the studied pure compounds, BA exerts the most potent anti-adipogenic activity. However, the ZJL extract, itself, induces superior inhibition of adipocyte differentiation and lipid accumulation than its secondary metabolites. Ultimately, the ZJL extract with its complex phytochemical profile and promising biological activity could be further exploited as a source of novel natural compounds with application in obesity management. Identifying plant extracts and pure natural phytochemicals with potent anti-adipogenic action, such as that of ZJL and BA, can open up novel ways of targeting adipocyte function and preventing obesity.

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CRediT authorship contribution statement

Martina S. Savova: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization, Investigation. **Liliya V. Vasileva**: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Visualization. **Saveta G. Mladenova:** Writing – original draft, Investigation. **Kristiana M. Amirova:** Formal analysis, Data curation, Writing – original draft, Visualization. **Claudio Ferrante**: Software, Data Curation, Writing – original draft, Visualization. **Giustino Orlando**: Software, Writing – review & editing. **Martin Wabitsch**: Methodology, Writing – review & editing. **Milen I. Georgiev**: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – review & editing.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111934.

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