BULGARIAN ACADEMY OF SCIENCES

INSTITUTE OF MICROBIOLOGY

"STEPHAN ANGELOFF"

MPharm Martina Stoyanova Savova

MECHANISM OF MODULATION OF OBESITY IN *IN VITRO* MODEL OF HUMAN ADIPOCYTES THROUGH APPLICATION OF BIOACTIVE COMPOUNDS

SUMMARY

On a dissertation for the award of educational and scientific degree

"Doctor" in the field of 5.11. Biotechnology

(Technology of biologically active molecules)

Scientific supervisor: Prof. Dr. Milen I. Georgiev

Members of the scientific jury: Prof. Dr. Lyudmila Kabaivanova
Assoc. Prof. Dr. Petya Dimitrova
Prof. Dr. Albert Krastanov, DSc
Prof. Dr. Tsvetalina Tankova, MD, DSc
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Plovdiv, 2023

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The dissertation is consisted of 131 pages, 38 figures and 9 tables. The literature sources cited are 218.

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The PhD student was authorised with the right to defend the doctoral thesis by order № I-136/27.09.22 of the Director of IMicB – BAS, Prof. Dr. Penka Petrova.

The dissertation defense will take place at ..... from ..... hours in the seminar hall of the Institute of Microbiology "Stephan Angeloff" – BAS (IMicB), Sofia, Bulgaria. Sofia.

The defense materials are available at IMicB – BAS and are published on the institutional website.

Content:

Abbreviations and symbols

$_{\Delta}$G - free energy of binding
Ki - affinity constant
aak-2 - orthologue of human AMPK
ACC - acetyl-CoA carboxylase
acs-2 - acyl-CoA synthetase
ADIPOQ - adiponectin
AKT - protein kinase B
AMPK - adenosine monophosphate - activated protein kinase
atgl-1 - fasting-responsive adipose triglyceride lipase
C/EBP - CCAAT/enhancer-binding protein
cDNA - complementary DNA
FASN - fatty acid synthase
fat - fatty acid desaturases
GLUT4 - glucose transporter type 4
hlh-11 - helix loop helix-11
HO-1 - heme oxygenase-1
HPLC - high performance liquid chromatography
IL - interleukin
lipl-3 - liposomal lipase-3
LOD - limit of detection
LOQ - limit of quantification
mRNA - informational RNA
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NGM - nematode growth media
NMR - nuclear magnetic resonance
NR - Nile red
nhr-49 - nuclear hormone receptor 49
ORO - oil red O
PGC1A - peroxisome proliferator-activated receptor gamma co-activator 1 alpha
PI3K - phosphoinositide 3-kinase
pod-2 - polarity and osmotic sensitivity defect
PPAR - peroxisome proliferator-activated receptor
RPL13A - ribosomal protein L13a
RT-qPCR - real-time quantitative reverse transcription polymerase chain reaction
sbp-1 - sterol regulatory element binding protein-1
SEM - standard error of the mean
SGBS - Simpson-Golabi-Behmel syndrome
SREBP - sterol regulatory element-binding protein
TGF - transforming growth factor
TUBB - tubulin
UCP1 - uncoupling protein 1

I. INTRODUCTION

The obesity prevalence has reached epidemic proportions in the recent decades (Okunogbe et al., 2022). The annual death rate associated with overweight and obesity exceeds 2.8 million people and accounts for a significant proportion of total mortality from socially significant lifestyle-related diseases (Kumanyika et al., 2020). Therefore, immediate measures are needed to prevent and address this growing global health problem.

Plants are sources of structurally diverse bioactive molecules whose potential for obesity control has not been studied in a sufficient depth. The multi-component chemical composition of plant extracts, comprising a rich array of natural molecules, confers a notable advantage over synthetic drugs in the modulation of multiple molecular mechanisms (Martel et al., 2017).

The complex pathophysiological mechanisms involved in the development of obesity require prevention and therapy approach that target more than one molecular mechanism. Therefore, modulation of signaling pathways related to adipogenesis, energy metabolism, intercellular and intracellular communication may provide a novel approach for the prevention of overweight and obesity. Potential molecular targets for obesity management include the major adipogenic transcription factors CCAAT/enhancer-binding protein alpha (C/EBPα) and peroxisome proliferator-activated receptor gamma (PPARγ), as well as their downstream regulatory factors that stimulate lipogenesis – sterol regulatory binding protein 1 (SREBP1), fatty acid synthase (FASN) and acetyl-CoA carboxilase (ACC; Ramirez et al, 2020). Stimulation of lipolysis, is an alternative approach for obesity prevention (Arner et al., 2019). Another key signaling pathway for the energy metabolism is the phosphoinositide 3 kinase (PI3K)/protein kinase B (AKT) cascade, involved in multiple cellular functions, including differentiation and insulin-stimulated glucose uptake in adipocytes (Foukas et al., 2013; Jung et al., 2020; Mandl et al., 2019; Song et al., 2015). Furthermore, obesity is also associated with systemic low-grade inflammation, which alleviation could be an approach to favor metabolic homeostasis during obesity.

The research strategy in this doctoral thesis includes the use of an *in vitro* platform to evaluate the effect of plant extracts and their metabolites on the fat cells physiology and modulation of the inflammatory response. Combination between molecular pharmacology, ethnopharmacology and phytochemical analysis provides detailed elucidation of the therapeutic potential of medicinal plant extracts and identification of phytochemicals with anti-obesity activity. The established cell-based model system for assessment of anti-adipogenic activity also provides insights into specific molecular mechanisms of action. The

potential anti-obesogenic effect of the most prominent secondary metabolite have been studied *in vitro* in human cell line and further validated *in vivo* in the model organism *Caenorhabditis elegans* as a suitable model system for evaluation of the effects of bioactive substances on lipid metabolism (Li et al., 2020a; Yue et al., 2021a).

II. AIM AND OBJECTIVES

1. Aim

The aim of this dissertation is to investigate the effect of extracts from *Z. jujuba* (jujube), *P. aviculare* (common knotgrass), *P. hydropiper* (water pepper) and their secondary metabolites on the processes of adipogenesis and lipid accumulation *in vitro* in in human adipocytes. Additionally, it aims to validate the effect of the most promising treatment in an *in vivo* model of obesity using nematodes – *C. elegans*. The experimental strategy used is summarized in Figure 1.

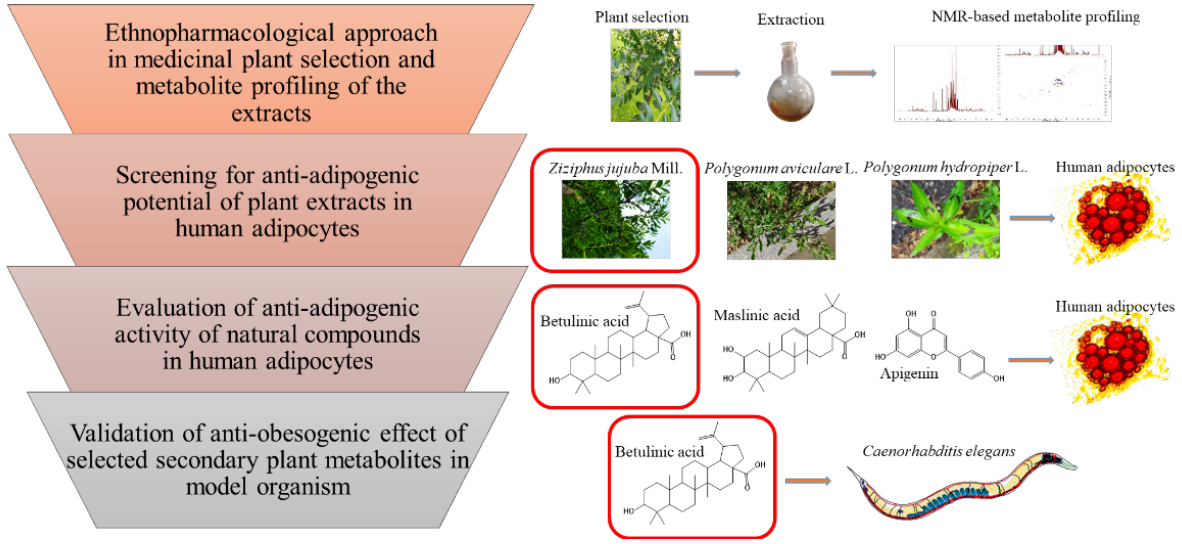

Figure 1. Experimental strategy of the dissertation.

2. Objectives

2.1. Selection of plants species according to available ethnopharmacological data for their application in obesity management.

2.2. Determination of the phytochemical composition of selected plant extracts by metabolic profiling using nuclear magnetic resonance (NMR).

2.3. Assessing the influence of extracts of *Z. jujuba*, *P. aviculare* and *P. hydropiper* on the processes of adipogenesis and lipolysis in an *in vitro* obesity model.

2.4. Evaluation of the mechanism of anti-adipogenic action of *Z. jujuba*, *P. aviculare* and *P. hydropiper* extracts in human adipocytes.

2.5. *In silico* docking simulation utilizing natural molecules selected in accordance with the phytochemical composition data obtained from the studied extracts.

2.6. Establishing the mechanism of action of selected secondary metabolites - apigenin, betulinic and maslinic acid in an *in vitro* model of human adipocytes.

2.7. Validating the anti-obesogenic effect of betulinic acid and elucidating its molecular mechanisms in *C. elegans* as a model organism for obesity.

III. MATERIALS AND METHODS

1. Collection of plant material and extraction

The leaf mass of *Z. jujuba* and the aerial part of *P. hydropiper* were collected from the area of the village of Zvanichevo, and the aerial parts of *P. aviculare* – from the town of Sandanski. The collected plant material was identified and deposited in the botanical collection of BAS. The collected plant material was further frozen, freeze-dried and grounded. 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. Nuclear magnetic resonance spectroscopy

Spectral data from ^{1}H NMR and 2D NMR were recorded at 25 °C on a Bruker AVII+600 spectrometer (Karlsruhe, Germany), with an operating frequency of 600.13 MHz and a relaxation time of 4.07 s according to the protocol detailed by Georgiev et al. (2015).

3. High performance liquid chromatography (HPLC)

The content of apigenin in the *Z. jujuba* extract was determined by HPLC as described by Bardakci et al. (2019). The method for detection and quantification of apigenin was validated for linearity and sensitivity by determining the limit of detection (LOD) and limit of quantification (LOQ).

4. *In silico* docking analysis

Docking calculations were performed using Autodock Vina software on PyRx 0.8. The crystal structures of the target proteins were extracted from the Protein Data Base (PDB, www.wwpdb.org) with PDB IDs as follows: 1NWQ for C/EBPα; 2P4Y for PPARγ, 1O6L for AKT, 5ITD for PI3K.

5. *In vitro* evaluation of anti-adipogenic potential

5.1. Cell cultivation and treatment

Human preadipocytes isolated from a patient with Simpson-Golabi-Bemel syndrome (SGBS) were cultured and differentiated according to optimal conditions (Wabitsch et al., 2001). Along with the induction of adipogenic differentiation (day 0), cells were treated within each media renewal on days 4th and 8th, respectively (Fig. 2). All concentrations used were selected after

assessing cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The extracts of *Z. jujuba* (jujube), *P. aviculare* (common knotgrass) and *P. hydropiper* (water pepper) were added to the culture medium at final concentrations of 5, 10 and 25 μg/mL, while the pure substances – apigenin, betulinic and maslinic acid, were added at concentrations of 0.25, 0.5 and 1 μM, rosmarinic acid – 1, 5 and 25 μM and orlistat (used as a positive control) at a concentration of 5 μM. A group treated with 0.02% DMSO was included as a control to exclude the influence of vehicle (Vehicle). All analyses were performed 24 h after the last treatment – on day 9th of differentiation, respectively (Fig. 2).

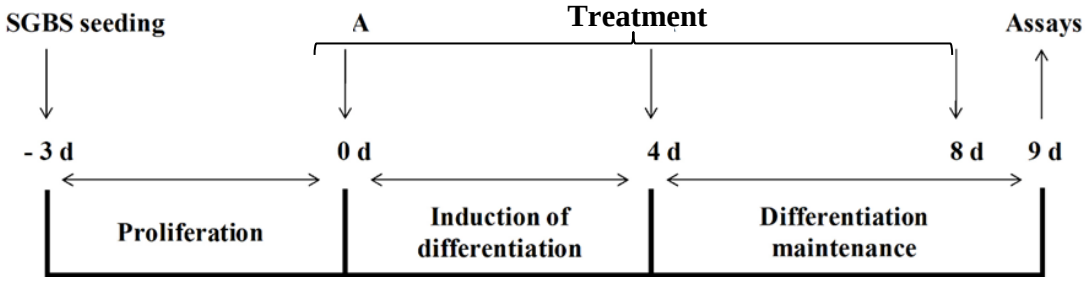

Figure 2. Schematic respesentation of the adipogenic differentiation process of Simpson-Golabi-Behmel syndrome (SGBS) human adipocytes, indicating the treatment intervals in days (d).

5.2. Assay for intracellular lipid accumulation (adipogenesis) and glycerol release (adipolysis)
Oil red O (ORO) lipid staining is used to quantify accumulated lipids. The effect on basal lipolysis was assessed by determining the concentration of glycerol released into the culture medium.

5.3. Real-time polymerase chain reaction (RT-qPCR) of mRNA
Total RNA from mature adipocytes was isolated with a Quick-RNA Miniprep kit from Zymo Research (#R1055, Ivrine, CA, USA). Reverse transcription was performed using the cDNA Canvax FirstStrand kit (#PR008, Cordoba, Spain). Expression of genes essential for the adipocyte differentiation was examined by RT-qPCR using Sso EvaGreen SuperMix (#1725204) on a CFX96 system (Bio-Rad).

5.4. Immunoblot
The protein extraction was performed using RIPA lysis buffer. Equivalent amounts of total protein lysate were separated by vertical electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with the respective primary

antibody for one day. To visualize primary antibodies against the target proteins, incubation with species-specific secondary antibody was performed followed by fluorescence intensity measurement on a ChemiDoc MP imaging system (Bio-Rad). The data of the selected protein levels were normalized to a reference protein using Image Lab 6.0.1 software (Bio-Rad).

6. *In vivo* model of glucose-induced obesity in *C. elegans*

6.1. Induction of lipid accumulation and treatment in *C. elegans*

The wild type N2 Bristol *C. elegans* and *Escherichia coli* OP50 were purcheased by the Caenorhabditis Genetic Centre (CGC, University of Minnesota, MN, USA). The nematodes were cultured at 20 °C according to standard procedures on petri dishes with solid nematode growth medium (NGM) with or without 2% glucose and seeded with *E. coli* OP50 as a food source. For the experiments, an age-synchronized larval population was obtained. During the first two larval stages, the food source was *E. coli* OP50, after which the nematodes were transferred onto new NGM medium petri dishes, similarly, with or without glucose but pretreated with betulinic acid (10, 25 and 50 μM) or vehicle. Orlistat at a concentration of 12 μM was used as a lipid-lowering control.

As a nutrient source, heat-inactivated *E. coli* was used during treatments to decrease the influence of bacterial metabolism.

After reaching the fourth larval stage (L4), nematodes were collected with M9 buffer and analyses, including phenotypic assays, viability assessment, lipid staining, confocal microscopy and RNA isolation were performed.

6.2. Viability assessment and phenotypic assays

The performed phenotypic analyses confirmed no significant effect on vitality, reproductive capacity, locomotor activity and lifespan in the model organism *C. elegans*. Thus, indicating that the treatment concentrations used are non-toxic and safe for the nematodes.

6.3. Lipid staining

Staining of accumulated lipids enables visualization and quantification of lipid depots. Two lipid dyes were employed. For the ORO staining, the amount of accumulated lipids was represented as normalized pixel intensity relative to a control group of nematodes grown on 2% glucose medium. For lipid staining with Nile red (NR), microscopic preparations were made and imaged using a confocal microscope.

6.4. Confocal microscopy

Confocal fluorescence images of nematodes from each group were captured on the Stellaris 5 confocal system equipped with a Dmi8 inverted microscope from Leica (Wetzlar, Germany). The images were analyzed using ImageJ software by converting the fluorescencet signal to corrected total cell fluorescence (CTCF) and the resulting values were normalized to the control group of nematodes grown on 2% glucose medium (Stuhr et al., 2022).

6.5. Real-time polymerase chain reaction of mRNAs and microRNAs

Total RNA isolation was performed with the phenolic reagent PureZol (#7326809, Bio-Rad). Reverse transcription was performed with a complementary DNA (cDNA) synthesis kit - First strand cDNA synthesis kit (Canvax, Cordoba, Spain). Revert Aid H Minus First Strand cDNA synthesis kit (#K1632; Thermo Fisher Scientific, Waltham, MA, USA) and stem-loop primers (Chen et al., 2005) were used for reverse transcription of the selected microRNAs.

7. Statistical analysis

Statistical data processing was performed in SigmaPlot v11.0 from Systat Software GmbH (Erkrath, Germany). Data are presented as mean ± standard error of the mean (SEM). Differences between experimental groups were calculated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistically significant differences between groups were considered at p values < 0.05. Survival curves of different groups were compared by log-rank test to detect statistical significance between them. All data presented were obtained from at least three independent experiments.

IV. RESULTS

1. Phytochemical characterization of experimental extracts of *Z. jujuba*, *P. hydropiper* and *P. aviculare*

Metabolic profiling of plant extracts is crucial for identifying natural compounds responsible for the observed biological activity. In the present work, leaf extracts of *Z. jujuba* (ZJL), aerial parts of *P. hydropiper* (PHA) and *P. aviculare* (PAA) were subjected to phytochemical characterization.

1.1. Metabolic profiling by NMR

The extracts from the species of the genus *Polygonum* expectedly showed similar phytochemical profile in NMR-based metabolic profiling. Spectral data from the proton NMR spectra (Fig. 3) were compared with those from the literature (Georgiev et al., 2011; Georgiev et al., 2015; Gowda et al., 2015; Wolfender et al., 2013; Yang et al., 2014). The primary and secondary metabolites identified in the extracts of *P. aviculare* and *P. hydropiper* are summarized in Table 1. Based on NMR metabolic profiling of *Z. jujuba* extract, by comparing the obtained spectra with data from the literature (Georgiev et al., 2011; Georgiev et al., 2015; Gowda et al., 2014; Wolfender et al, 2013), characteristic signals were identified for some primary and secondary metabolites, summarized in Table 2. The presence of apigenin (Moharram et al., 2021; Wolfender et al., 2013) in *Z. jujuba* extract was confirmed by analysis of the obtained proton (^{1}H) and two-dimensional heteronuclear correlation (H^{1}-C^{13} HSQC) NMR spectra (Fig. 4).

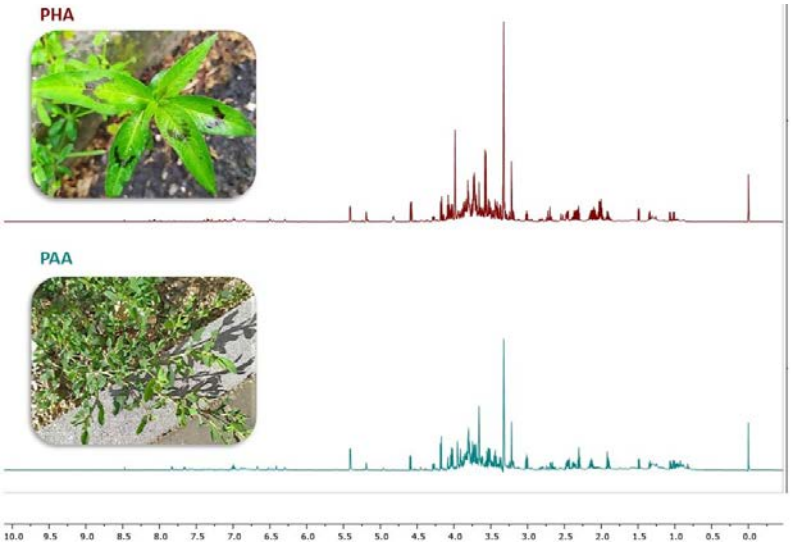

Figure 3. Proton NMR spectra of extracts of aerial parts of *P. aviculare* (PAA) and *P. hydropiper* (PHA).

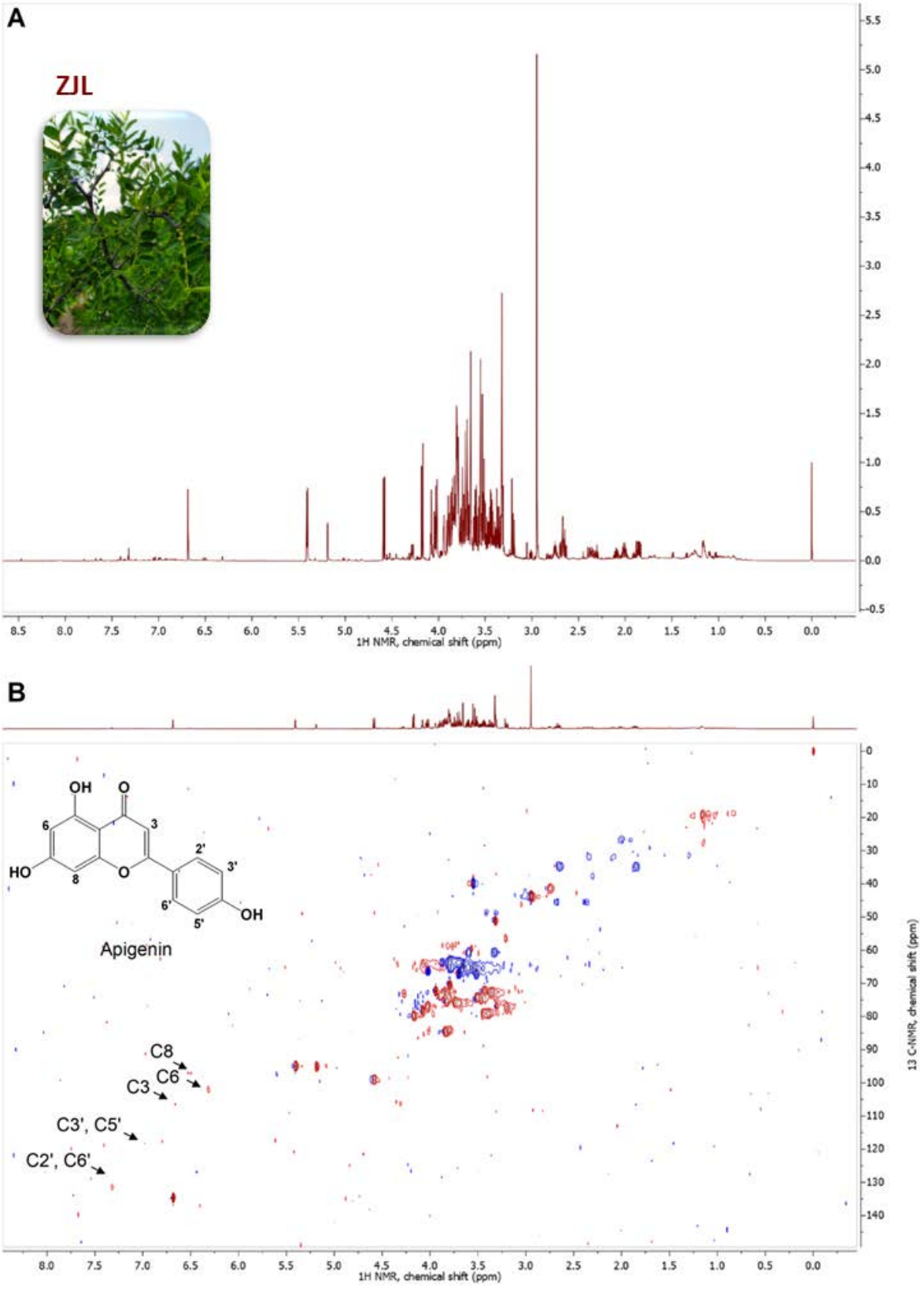

Figure 4. Metabolic profiling of extract from *Z. jujuba* leaves (ZJL). Proton NMR spectrum of *Z. jujuba* extract (**A**). Two-dimensional heteronuclear correlation (H^1-C^{13} HSQC) spectrum of *Z. jujuba* extract and characteristic apigenin signals (**B**).

Table 1. Chemical shift (δ) and coupling constant (*J*) of metabolites identified by analysis of proton NMR spectra of extracts of *P. aviculare* and *P. hydropiper*.

Metabolites	***P. aviculare*, (δ, ppm; *J*, Hz)**	***P. hydropiper* (δ, ppm; *J*, Hz)**
Amino acids		
Alanine	1.49 (d, *J* = 7.2)	1.49 (d, *J* = 7.27)
Aspragic acid	2.68 (dd, *J* = 15.3, 3), 2.81 (dd, *J* = 17.3, 3.6), 3.88 (dd, *J* = 12.2, 2.81)	-
Valin	1.01 (d, *J* = 7.1), 1.07 (d, *J* = 7.1)	1.01 (d, *J* = 7.0), 1.06 (d, *J* = 7.0)
Glutamine	-	2.13 (m), 2.46 (m)
Glutamic acid	2.11 (m), 2.46 (m), 3.75 (dd, *J* = 10.5, 8.8)	-
Levtsin	0.97 (d, *J* = 6.4), d 0.99 (d, *J* = 6.5)	-
Treonin	1.34 (d, J = 6.5)	-
Carbohydrates		
α-Glucose	5.19 (d, *J* = 3.7)	5.19 (d, *J* = 3.73)
β-Glucose	4.58 (d, *J* = 7.9)	4.59 (d, *J* = 7.9)
Sucrose	5.41 (d, *J* = 3.8)	5.41 (d, *J* = 3.83)
Fructose	4.17 (d, *J* = 8.7)	4.18 (d, *J* = 8.67)
Organic acids		
γ-Amino butyrate	1.9 (m), 2.31 (t, *J* = 7.2), 3.01 (t, *J* = 7.3)	1.9 (m), 2.31 (t, *J* = 7.35), 3.01 (t, *J* = 7.4)
Gallic acid	7.03 (s)	-
Formic acid	8.48 (s)	-
Fumaric acid	6.54 (s)	6.53 (s)
Flavonoids		
Kaempferol	6.28 (d, *J* = 2.0), 6.49 (d, *J* = 2.0), 8.07 (d, *J* = 8.9), 7.00 (d, *J* = 8.5)	6.28 (d, *J* = 2.0), 6.49 (d, *J* = 2.0), 7.00 (d, *J* = 8.5)
Quercetin	6.3 (d, *J* = 1.7), 6.49 (d, *J* = 2.0), 7.75 (d, *J* = 2.3), 6.99 (d, *J* =	-

	8.9), 7.7 (dd, *J* = 8.6, 2.2)	
Myricetin	6.3 (d, *J* = 2.0) 6.49 (d, *J* = 2.0), 7.34 (s)	-
Myricitrin	0.98 (d, *J* = 6.1), 3.35 - 3.93 (m), 5.35 (d, *J* = 1.9), 6.3 (d, *J* = 2.0), 6.97(s)	-
Rutin	6.29 (d, *J* = 2), 6.49 (d, *J* = 2.4), 7.68 (d, *J* = 2.1), 6.98 (d, *J* = 8.5), 7.57 (dd, *J* = 8.5, 2.1), 5.02 (d, *J* = 7.5), 4.53 (d, *J* = 1.7)	6.3 (d, *J* = 2), 6.49 (d, *J* = 2.3), 6.98 (d, *J* = 8.5) 7.62 (dd, *J* = 8.5, 2.1)
Hyperoside	7.87 (d, J=1.2), 6.85 (d, *J=8*.6), 6.43 (s), 6.30 (d, J=2.5), 5.17 (d, J=7.9)	-

Table 2. Chemical shift (δ) and coupling constant (*J*) of metabolites identified by proton NMR spectrum analysis of *Z. jujuba* leaf extract.

Metabolites	**Chemical shift (δ, ppm)**	**Coupling constant (*J*, Hz)**
Amino acids		
Alanine	1.49	(d, *J* = 7.2)
Valin	1.01/1.07	(d, *J* = 7.3)/(d, *J* = 7.1)
Glutamine	2.14/2.39	(m)/(m)
Treonin	1.33	(d, *J* = 6.9)
Carbohydrates		
α-glucose	5.19	(d, *J* = 3.8)
β-glucose	4.59	(d, *J* = 7.9)
Sucrose	5.41	(d, *J* = 3.9)
Fructose	4.18	(d, *J* = 8.6)
Organic acids		
γ-amino butyrate	1.90/2.30/3.01	(m)/(t, *J* = 7.5)/(t, *J* = 7.3)
Formic acid	8.47	(s)
Acetic acid	1.90	(s)
Succinic acid	2.40	(s)
Fumaric acid	6.48	(s)

Flavonoids		
Apigenin	6.66/6.31/6.52/8.08/6.98	(s)/(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)

1.2. Quantification of apigenin in *Z. jujuba* leaf extract

The content of apigenin was determined by HPLC and corresponds to 0.17 ± 0.02 mg/g dry extract. The sensitivity of the method was calculated as LOD = 3.3σ/S and LOQ = 10σ/S. The calibration curve ($y = 8.89 \times 10^4 x + 4.28 \times 10^4$) showed a good linear relationship in the region between 5 and 100 μg/mL, with $R^2 = 0.9952$. The values obtained for LOD and LOQ are 8.02 and 24.30 μg/mL, respectively.

2. *In vitro* screening for assessment of the anti-adipogenic potential of *Z. jujuba*, *P. aviculare* and *P. hydropiper* extracts

The selected plants (*Z. jujuba*, *P. aviculare* and *P. hydropiper*) are used in Bulgarian traditional medicine for treatment of obesity and metabolic disorders. The obtained extracts were subjected to evaluation of their anti-adipogenic potential in *in vitro* obesity model in human adipocytes.

2.1. Cell viability assessment

The results of the cell viability assay demonstrated that within the concentration range of 1-100 μg/mL, these extracts did not exibit a significant decrease in the viability of preadipocytes after 24 hours treatment. These findings allowed subsequent experiments to be performed using this model system.

2.2. Analysis of intracellular lipid accumulation and effect on adipolysis

Representative microscopic images of stained adipocytes (Fig. 5B) and the quantification of lipid depots (Fig. 5A) revealed a significant and concentration-dependent reduction in lipid

accumulation in the presence of the studied extracts.

The obtained results demonstrate a moderate decrease in glycerol concentration when treated with the *Z. jujuba* extract. In contrast, extracts from both plants of the genus *Polygonum* exibit a more substantial reduction in secreted glycerol, indicating an inhibition of basal lipolysis (Fig. 6).

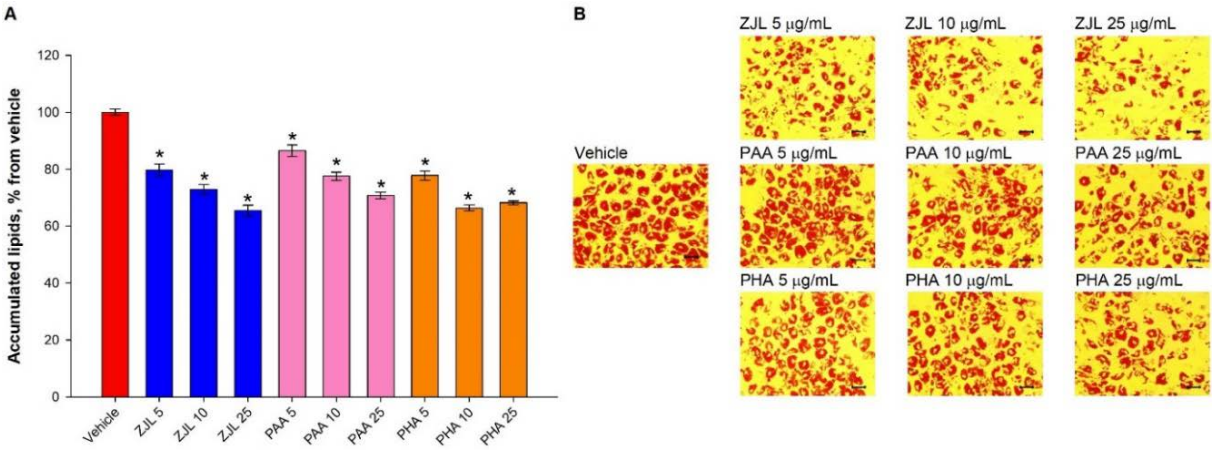

Figure 5. *Z. jujuba* leaf extract (ZJL), *P. aviculare* (PAA) and *P. hydropiper* (PHA) suppressed adipogenesis and lipid accumulation in human adipocytes. Spectrophotometric determination of accumulated lipids presented as percentage of the differentiated control group (**A**). Representative microscopic images at 20× magnification (50 μm scale bar) from ORO staining on day 9 of adipocyte differentiation (**B**). Data are presented as mean ± SEM. *p<0.05 compared with control group (Vehicle).

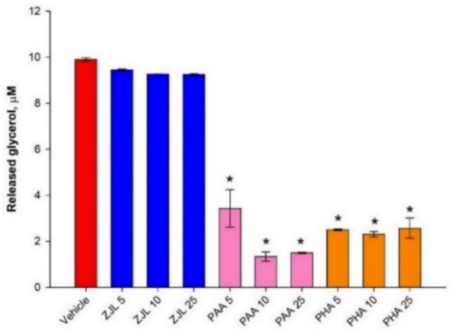

Figure 6. Significant inhibition of adipolysis was observed upon *P. aviculare* (PAA) and *P. hydropiper* (PHA) extracts treatments, compared to *Z. jujuba* (ZJL). Graphical representation of the concentration of glycerol released into the culture medium (μM) on day 9 of adipocyte differentiation. Data are presented as mean ± SEM, each experimental group consisted of at least six parallel samples in three independent experiments. *p<0.05 compared with the differentiated control group (Vehicle).

2.3. Real-time polymerase chain reaction of mRNA

A decrease in *PPARG* mRNA levels (Fig. 7F) was reported as a result of the treatment with the *Z. jujuba* extract, whereas *CEBPA* expression (Fig. 7D) was affected by all three tested extracts.

Similarly, *Z. jujuba* extract dose-dependantly decreased the expression of adiponectin-encoding gene (*ADIPOQ*, Fig. 7A) and *ACC* (Fig. 7C). Gene expression of *AKT* (Fig. 7B) and *PI3KCA* (Fig. 7E) was significantly decreased only upon *Z. jujuba* extract treatment.

In summary, these data indicate that *Z. jujuba* extract affects the expression of key genes involved in adipo- and lipogenesis.

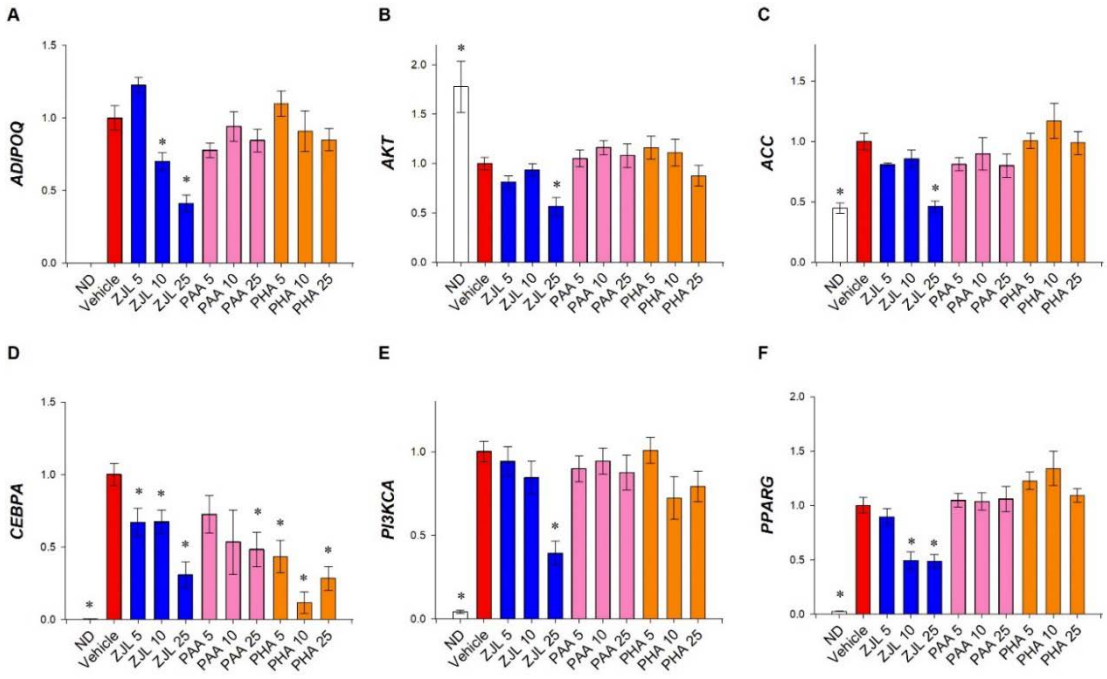

Figure 7. Expression of genes essential for adipogenic differentiation is repressed by *Z. jujuba* (ZJL) extract treatment, compared to application of *P. aviculare* (PAA) and *P. hydropiper* (PHA). Relative mRNA expression normalized to the differentiated control group (Vehicle) for the following genes *ADIPOQ* (**A**), *AKT* (**B**), *ACC* (**C**), *CEBPA* (**D**), *PI3KCA* (**E**), *PPARG* (**F**), obtained by RT-qPCR analysis. *RPL13A* and *TUBB* were used as reference genes. Data from three independent experiments are presented as mean ± SEM. *p<0.05 compared to control group.

2.4. Immunoblot for determination of protein expression

The results of the immunoblot analysis revealed that the *Z. jujuba* extract treatment, decreased the PPARγ protein abundance (Fig. 8A), whereas the extracts of *P. aviculare* and *P. hydropiper* only affected its expression when treated with 25 μg/mL. Suppression of C/EBPα (Fig. 8 D) as

well as adiponectin (Fig. 8 C) expression was observed only upon *Z. jujuba* extract. A decrease in PI3K protein expression (Fig. 8E) was observed after application of each of the extracts examined. Regarding to the treatment with *Z. jujuba* extract, only 25 μg/mL significantly decreased AKT levels (Fig. 8 B).

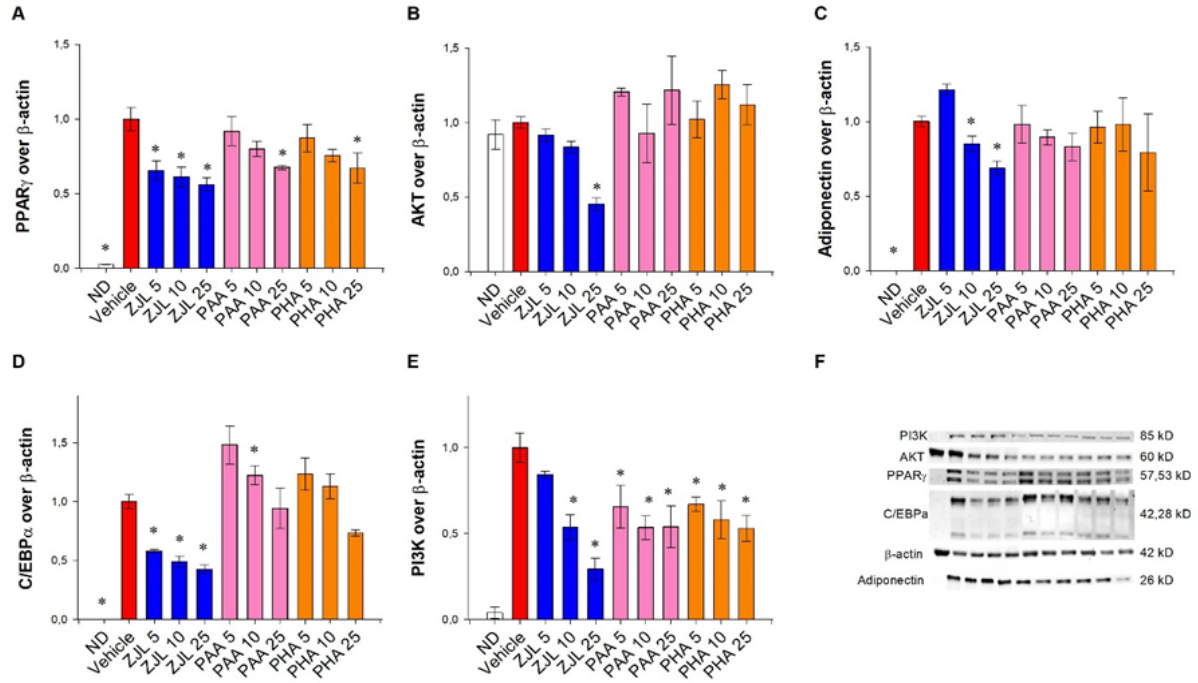

Figure 8. Protein expression of the studied adipogenic factors was suppressed to the greatest extent when *Z. jujuba* (ZJL) extract was applied, compared to the treatment with *P. aviculare* (PAA) and *P. hydropiper* (PHA) extracts. Immunoblot analysis was performed to assess the effect on PPARγ (**A**), AKT (**B**), adiponectin (**C**), C/EBPα (**D**) and PI3K (**E**) levels. Representative images of the blots used for detection of the respective proteins (**F**). β-actin was used in normalization as a reference protein. Data are presented as mean ± SEM of three independent experiments. *p<0.05 compared with the differentiated control group (Vehicle).

Based on the results, it can be summarized that the anti-adipogenic effect of *Z. jujuba* extract was attributed to the inhibition of PPARγ, C/EBPα, adiponectin and PI3K/AKT signaling pathway. On the other hand, the extracts of *P. aviculare* and *P. hydropiper* only affected PI3K and PPARγ. Since the *Z. jujuba* extract demonstrated an impact on the expression of key adipogenic factors at both transcriptional and translational levels, this extract was selected for subsequent experiments in the obesity cell model employed in the present work.

3. *In vitro* determination of the anti-adipogenic mechanism of selected secondary metabolites from *Z. jujuba* extract, namely apigenin, betulinic and maslinic acid

Three secondary metabolites (apigenin, betulinic and maslinic acid) were selected among the natural compounds present in the *Z. jujuba* leaf extract. They were further subjected to assessment of their anti-adipogenic potential and investigation of their molecular mechanism of action.. The application of these selected secondary metabolites was found not to statistically affect preadipocyte viability in the concentration range chosen for treatment (0.25-1 μM).

3.1. *In silico* docking analysis

The affinity of selected natural molecules for target proteins involved in adipogenesis and insulin signaling is expressed by free binding energy ($_{\Delta}$ G) and affinity constant (Ki ; Table 3). Possible interaction with PI3K and PPARγ in the treatment concentration range (0.25-1 μM) is presented in Figure 9. Overall, the *in silico* simulation highlights PI3K and PPARγ as potential target protein structures for the effects of anpgenin, betulinic and maslinic acid.

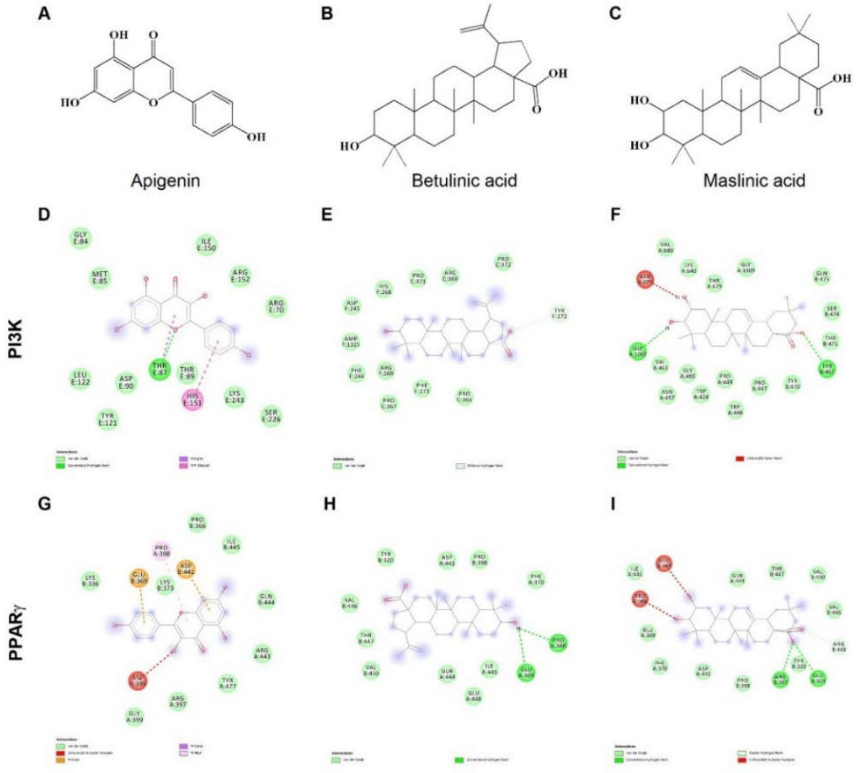

Figure 9. Computer docking simulation of the spatial orientation of apigenin, betulinic and maslinic acid and their binding sites to PI3K and PPARγ. Structures of apigenin (APG, **A**), betulinic (BA, **B**) and maslinic acid (MA, **C**). The binding sites between the amino acid residues of the corresponding protein structure (PI3K or PPARγ) and the functional groups of

the selected natural molecules (APG, BA, and MA) are represented as follows: APG-PI3K (**D**), BA-PI3K (**E**), MA-PI3K (**F**)**,** APG-PPARγ (**G**), BA-PPARγ (**H**), and MA-PPARγ (**I**).

With the sole exception of apigenin binding to C/EBPα, all compounds showed high affinities in the low micromolar range (0.02-17.4 μM) relative to all target proteins tested.

Table 3. Theoretically calculated free energy (Δ G, kcal/M) and affinity constant (K_i , μM) for the interaction between apigenin, betulinic and maslinic acid and selected target protein structures.

Target protein	Apigenin	Betulinic acid	Maslinic acid
ACT	-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
PI3K	-8.1 kcal/M; 1.2 μM	-8.8 kcal/M; 0.4 μM*	-10.4 kcal/M; 0.02 μM*
PPARγ	-7.2 kcal/M; 5.4 μM	-8.2 kcal/M; 1.0 μM*	-8.8 kcal/M; 0.4 μM*

*K_i values in the range of treatment concentrations (0.25-1 μM).

3.2. Analysis of intracellular lipid accumulation and effect on adipolysis

The anti-adipogenic potential of the secondary metabolites from the extract, that showed the greatest potential, was assessed by lipid staining (Fig. 10). Representative microscopic images of stained adipocytes (Fig. 10B) and quantification of accumulated lipids (Fig. 10A) demonstrated that betulinic acid posses the highest anti-adipogenic effect among the tested compounds.

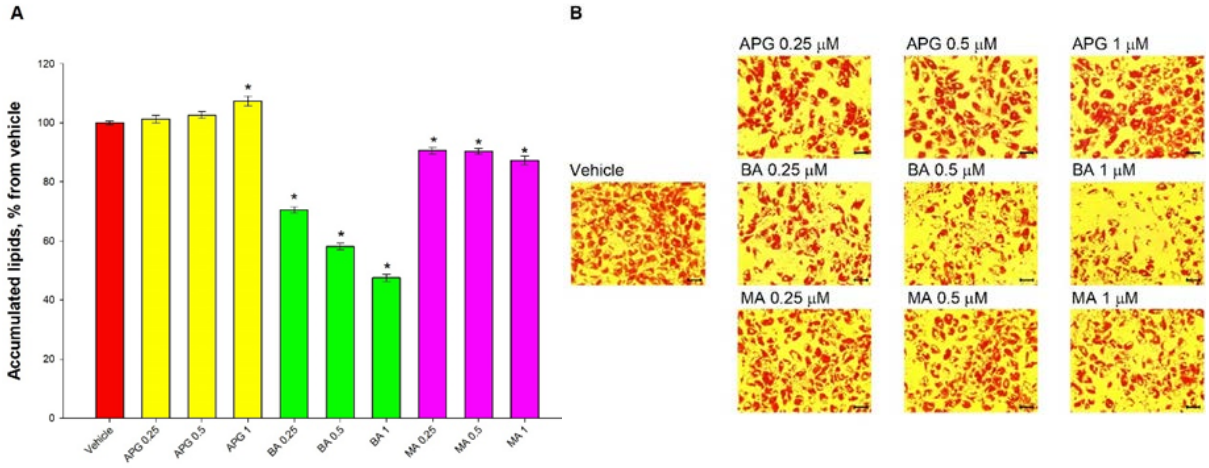

Figure 10. Betulinic (BA) and maslinic (MA) acid significantly inhibit fat cell

differentiation and lipid accumulation, compared with apigenin (APG). Spectrophotometric determination of accumulated lipids presented as percentage of differentiated control group (**A**). Representative microscopic images at 20× magnification (50 μm scale) from ORO staining on day 9 of adipocyte differentiation (**B**). Data are presented as mean ± SEM, each experimental group consisted of at least six parallel samples in three independent experiments. *p<0.05 compared with control group (Vehicle).

The results obtained indicate increase in the basal lipolysis only in the apigenin and maslinic acid treatments (Fig. 11).

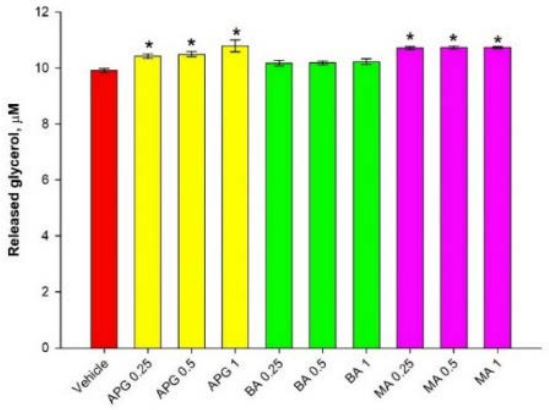

Figure 11. Triglyceride hydrolysis in human adipocytes is increased by treatment with apigenin (APG) and maslinic acid (MA). Concentration of released glycerol (μM) in culture medium on day 9 of adipocyte differentiation. Data are presented as mean ± SEM, each experimental group consisted of at least six parallel samples in three independent experiments. *p<0.05 compared with the differentiated control group (Vehicle).

3.3. Real-time polymerase chain reaction of mRNA

Analysis of the gene expression was performed to elucidate the molecular mechanism of action of the secondary metabolites selected from the composition of the *Z. jujuba* extract. The gene expression of crutial for the fat cell differentiation regulatory factors involved was determined in human adipocytes treated with apigenin, betulinic and maslinic acid (Fig. 12). Decreased expression of *PPARG* (Fig. 12A) was observed in betulinic and maslinic acid treatments. Suppression at the mRNA level of *CEBPA* (Fig. 12D) was only observed at the highest concentrations of apigenin and maslinic acid. Expression of *ADIPOQ* (Fig. 12C) was suppressed only at 1 μM apigenin and 0.25 μM betulinic acid. Furthermore, a decrease in *ACC* gene expression was observed for all the substances used (Fig. 12F).

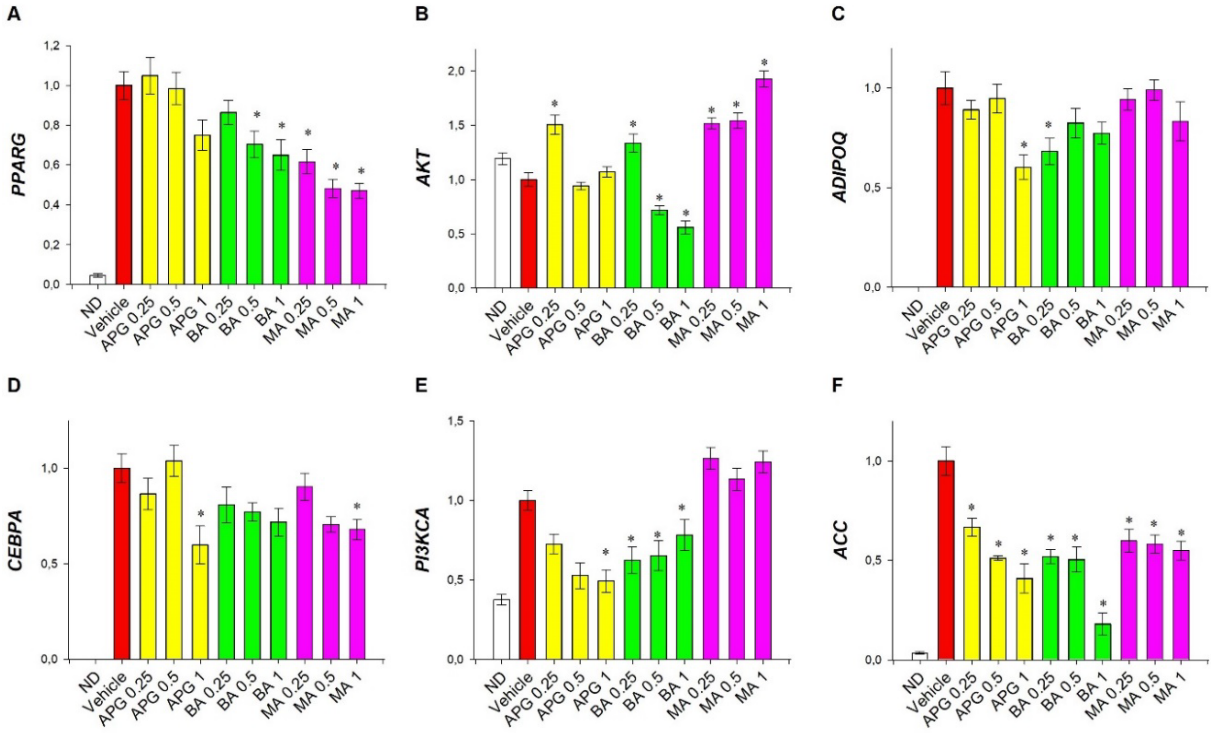

Figure 12. Expression of genes key for adipogenic differentiation is repressed to the greatest extent upon betulinic acid (BA), compared with apigenin (APG) and maslinic acid (MA) treatment. Relative mRNA expression normalized to the differentiated control group (Vehicle) for the following genes: *PPARG* (**A**), *AKT* (**B**), *ADIPOQ* (**C**), *CEBPA* (**D**), *PI3KCA* (**E**), *ACC* (**F**) obtained by RT-qPCR analysis. *RPL13A* and *TUBB* were used as reference genes. Data from three independent experiments are presented as mean ± SEM. *p<0.05 compared to the differentiated control group.

Subsequent gene expression analysis of *PI3KCA* and *AKT* was performed to clarify whether this molecular pathway is involved in the anti-adipogenic effect of the selected secondary metabolites. The observed changes in *AKT* gene expression (Fig. 12B) upon administration of betulinic acid exhibited a bidirectional pattern. Specifically, an increase in *AKT* gene expression was observed at 0.25 μM, whereas asuppression at 0.5 and 1 μM. Further, the expression of *AKT* (Fig. 12B) was increased in maslinic acid and apigenin treatments at 0.25 μM. During adipogenic differentiation *PI3KCA* expression is upregulated (Ortega-Molina et al., 2015). Supplementation of apigenin and betulinic acid to the culture medium during adipogenesis, downregulates *PI3KCA* expression (Fig. 12E).

Despite the different directional patterns observed, it is noteworthy that all treatments modulated PI3K/AKT signaling pathway during adipocyte differentiation.

3.4. Immunoblot for determination of protein expression

To complement the data obtained from RT-qPCR analysis, protein expression of selected regulatory factors for adipocyte differentiation was evaluated by immunoblotting (Fig. 13).

Protein levels of PPARγ (Fig. 13A) were decreased as a result of betulinic and maslinic acid treatments. With respect to the inhibitory effect on PPARγ expression induced by these natural compounds, the present results are in agreement with the literature data (Ormazabal et al., 2018; Kim et al., 2019) as well as docking assay data showing the affinity of betulinic and maslinic acid for the PPARγ structure. Apigenin treatment affected the level of PPARγ (Fig. 13A) only at the 1 μM concentration.

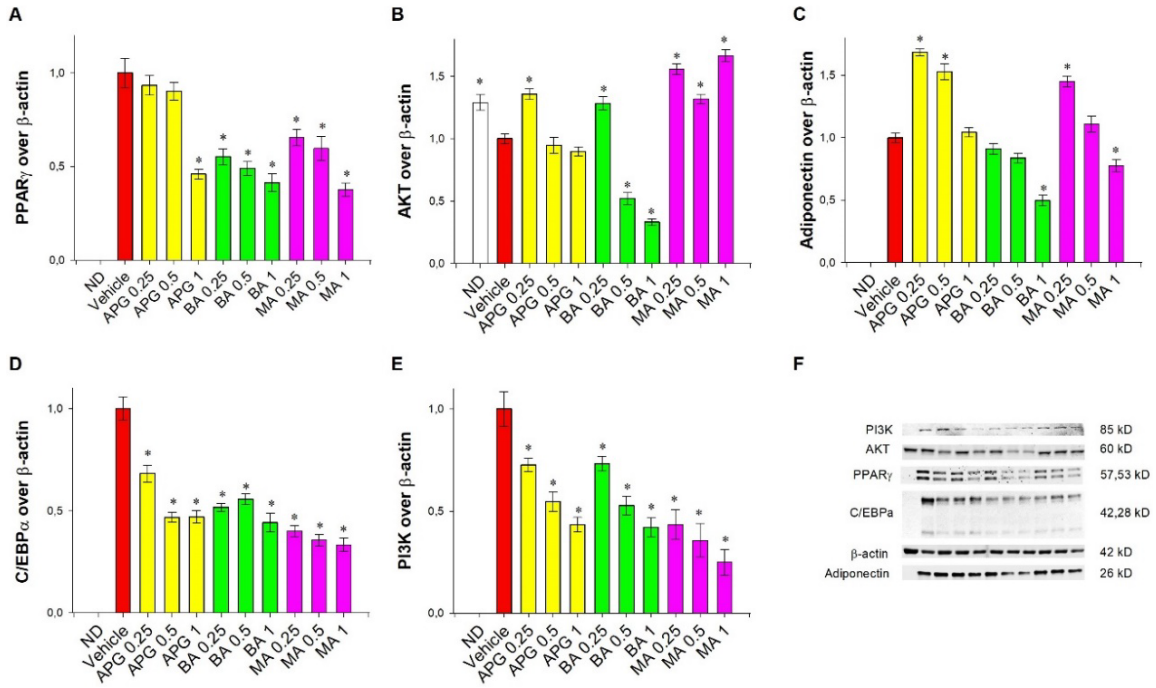

Figure 13. Protein expression of the studied regulators of fat cell differentiation, was considerably suppressed by application of betulinic acid (BA), in contrast wth apigenin (APG), and maslinic acid (MA). Immunoblot analysis was performed to assess the effect on PPARγ (**A**), AKT (**B**), adiponectin (**C**), C/EBPα (**D**) PI3K (**E**) levels. Representative images from the blots (**D**). Data are presented as mean ± SEM from three independent experiments. *p<0.05 compared with control group (Vehicle).

All selected compounds demonstrated a decrease in C/EBPα protein levels (Fig. 13D). This pronounced effect on C/EBPα expression can be attributed to the negative regulation of PPARγ (Fig. 13A) upon apigenin, betulinic and maslinic acid treatment. Data from the immunoblot analysis for adiponectin (Fig. 13C) revealed that its expression was upregulated

in apigenin 0.25 and 0.5 μM treatments. Application of betulinic acid resulted in a decrease in adiponectin protein levels (Fig. 13C), whereas maslinic acid showed a bidirectional effect (increase with 0.25 μM and suppression at 0.5 μM; Fig. 13C). The promising results exhibited by betulinic and maslinic acid in modulating all the studied proteins provide strong support for the hypothesis of their significant potential for application in obesity management (Kim et al., 2019; Liu et al., 2019; Torre et al., 2020). For all natural molecules studied, inhibition of PI3K expression was observed (Fig. 13E). Regarding immunoblot analysis, apigenin and betulinic acid at concentrations of 0.5 and 1 μM downregulated AKT expression (Fig. 13B).

Among the secondary metabolites studied in the *Z. jujuba* extract, betulinic acid demonstrated the most prominent anti-adipogenic effect by modulation of the molecular pathways responsible for adipocyte differentiation. Based on these findings, betulinic acid was selected for *in vivo* validation of its biological activity in a model of obesity using *C. elegans.*

4. Effect of rosmarinic acid on adipogenesis and concomitant inflammation in an *in vitro* model of obesity

Obesity is a complex disease in characterized by disruption in the physiology of adipose tissue, metabolic homeostasis of the entire organism, as well as inflammatory and immune response. Chronic low-grade inflammation associated with obesity is the major contributor to complications and multimorbidity (Boutens et al., 2018; Lin et al., 2020). In this regard, targeting more than one molecular mechanism is more rational than affecting adipogenesis or lipolysis alone. To address the complex nature of obesity and its associated inflammatory response, rosmarinic acid, a natural molecule known for its anti-inflammatory effects, has been chosen to investigate its impact on human adipocyte differentiation.

4.1. Determination of cell viability

The results of the MTT assay showed that at concentrations up to 100 μM rosmarinic acid did not affect the cell viability in human preadipocytes, ensuring the absence of toxicity.

4.2. Analysis of intracellular lipid accumulation and effect on adipolysis

The ORO lipid staining was employed to evaluate the effect of rosmarinic acid on adipogenesis, while the mesuarement of the glycerol released in the culture medium served as an indicator of lipid hydrolysis. Concentration-dependent suppression of fat cell differentiation was observed in respose to rosmarinic acid treatment, compared to the control group (Fig. 14). Basal lipolysis was increased upon rosmarinic acid treatment, with a difference of more than 30% with increasing concentration compared to the differentiated control group (Fig. 15).

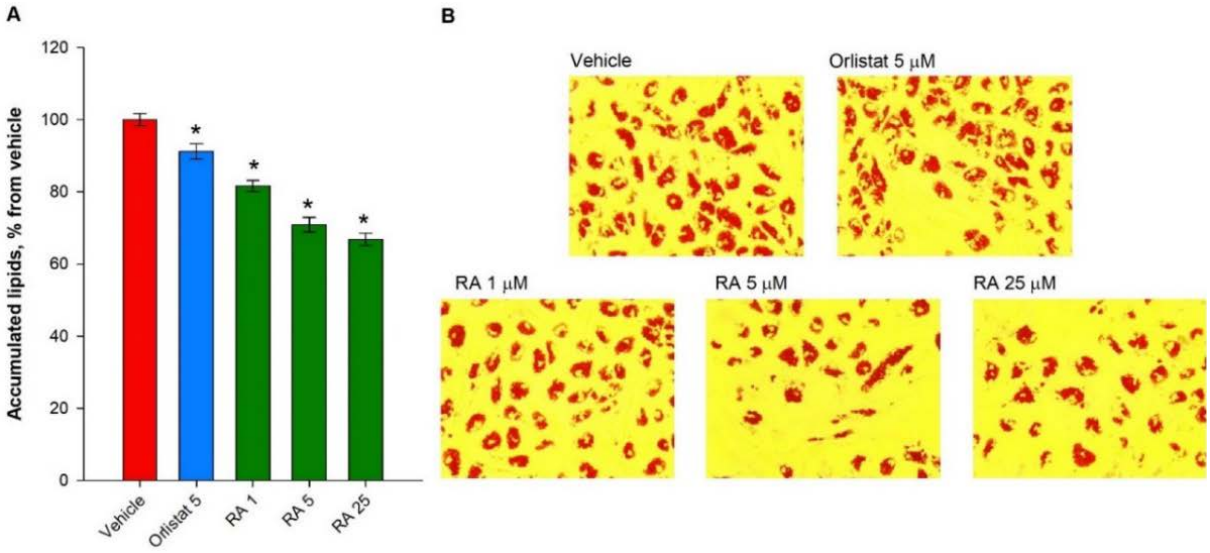

Figure 14. Rosmarinic acid (RA) inhibits lipid accumulation in human adipocytes. Spectrophotometric determination of accumulated lipids presented as percentage of the differentiated control group (Vehicle, **A**). Representative microscopic images at 20× magnification (50 μm scale bar) from oil red O staining at day 9 of adipocyte differentiation (**B**). Data are presented as mean ± SEM. *p<0.05 compared with the control group.

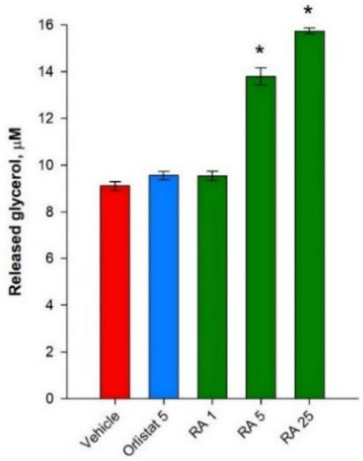

Figure 15. Rosmarinic acid (RA) stimulate basal lipolysis in human adipocytes. Concentration of secreted glycerol (μM) at day 9 of adipocyte differentiation. Data are presented as mean ± SEM, each experimental group consisted of at least six parallel samples in three independent experiments. *p<0.05 compared with the differentiated control group (Vehicle).

4.3. Real-time polymerase chain reaction of mRNA

The expression of genes related to the adipogenesis was examined by RT-qPCR. Treatment with rosmarinic acid resulted in a significant reduction in the expression of *ACC* (Fig. 16A)

and *FASN* (Fig. 16D) as well as *FABP4* (Fig. 16E) compared to the differentiated control and orlistat.

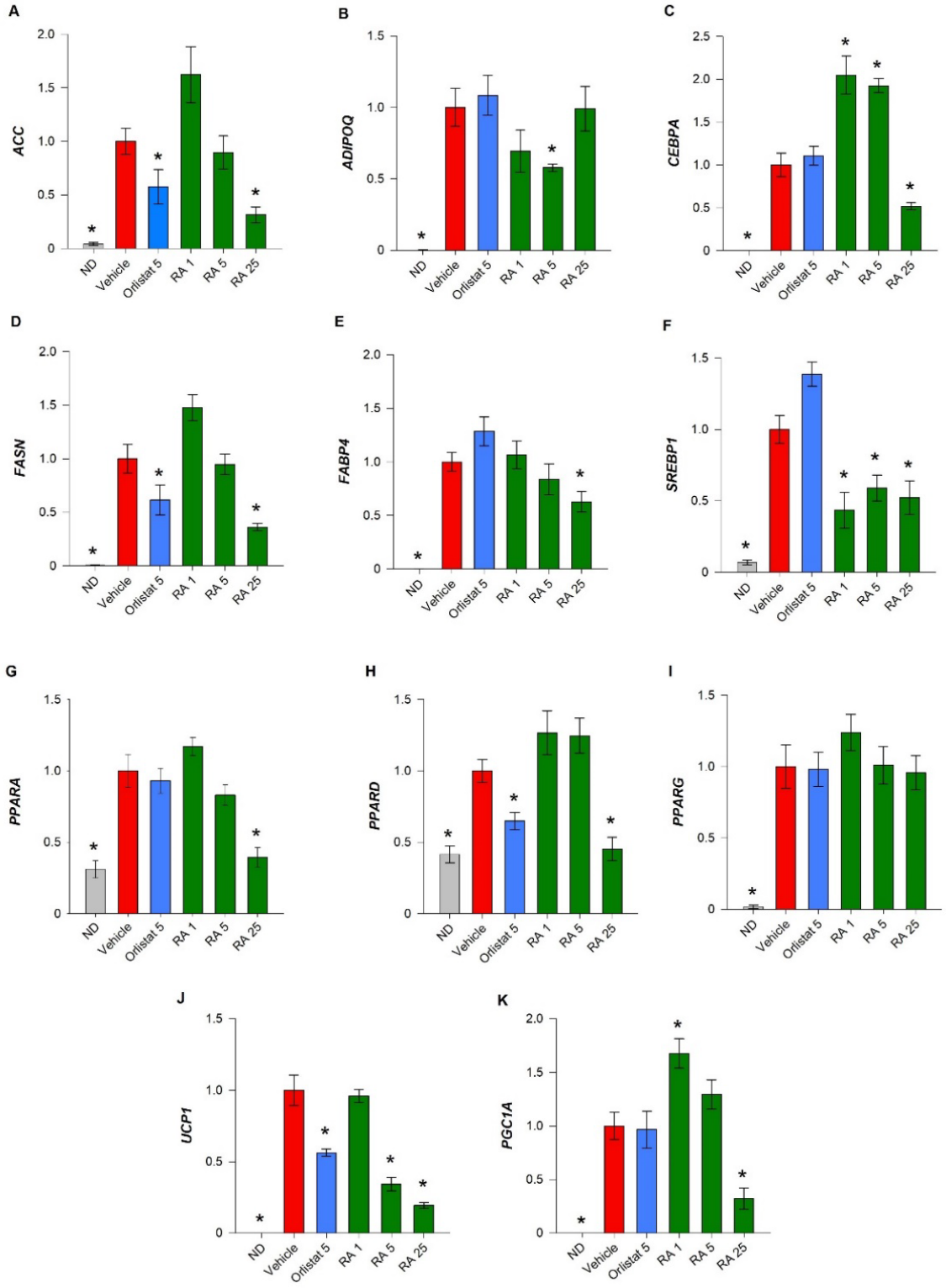

Figure 16. Rosmarinic acid (RA) downregulates the expression of genes related to adipogenic differentiation. Relative expression of mRNA normalized to the differentiated control (Vehicle) group for the following genes: *ACC* (**A**), *ADIPOQ* (**B**), *CEBPA* (**C**), *FASN*

(**D**), *FABP4* (**E**), *SREBP1* (**F**), *PPARA* (**G**), *PPARD* (**H**), *PPARG* (**I**), *UCP1* (**J**) and *PGC1A* (**K**) obtained by RT-qPCR analysis. *RPL13A* and *TUBB* were used as reference genes. Data from three independent experiments are presented as mean ± SEM. *p<0.05 compared to control group.

The effect of rosmarinic acid on adiponectin (Fig. 16B) and *CEBPA* (Fig. 16C) gene expression did not follow a concentration dependence. *ADIPOQ* expression was significantly suppressed in the group treated with 5 μM rosmarinic acid and less affected at the other concentrations used. In contrast, a suppression in *CEBPA* level was reported at the highest concentration applied (25 μM), while 1 and 5 μM led to the opposite effect. The *SREBP1* (Fig. 16F), which is a key for *de novo* lipogenesis transcription factor was upregulated in orlistat-treated adipocytes, whereas rosmarinic acid significantly decreased its expression.

Additionally, the expression of two major "browning" genes, uncoupling protein 1 (*UCP1*) and peroxisome proliferator-activated receptor gamma co-activator 1 alpha (*PGC1A*) was investigated. The activation of *UCP1* and *PGC1A* is a marker of the transdifferentiation of white fat cells into brown fat cells, a process known as "browning".A significant decrease in response to rosmarinic acid treatment was observed for both *UCP1* (Fig. 16I) and *PGC1A* (Fig. 16K), therefore, excluding the hypothesis for induction of "browning" in adipocytes.

Regarding anti-inflammatory mediators, increased gene expression of *interleukin* (*IL*) *17A* (Fig. 17C) and *IL10* (Fig. 17D) was observed in the groups differentiated in the presence of rosmarinic acid. Meanwhile, the levels of genes encoding proinflammatory *IL1B* (Fig. 17A) and transforming growth factor beta (*TGF*) *1B* (Fig. 17B) were significantly downregulated by rosmarinic acid treatment.

According to the RT-qPCR analysis, rosmarinic acid inhibited adipogenesis by repressing the expression of key adipogenic and lipogenic genes. In addition, the results provide evidence that rosmarinic acid alleviates obesity-associated inflammation by affecting the autocrine inflammatory response in adipocytes.

4.4. Immunoblot for determination of protein expression

Changes in protein levels of C/EBPα, PPARγ and adiponectin following rosmarinic acid treatment were detected by immunoblot (Fig. 18). The obtained results demonstrated a significant decrease in C/EBPα, PPARγ (Fig. 18A,B) and adiponectin (Fig. 18C) protein abundance in the rosmarinic acid-treated groups in contrast with orlistat and the control group.

Therefore, these regulators, essential for adipocyte differentiation, serve as target protein structures for the action of rosmarinic acid in the fat cells.

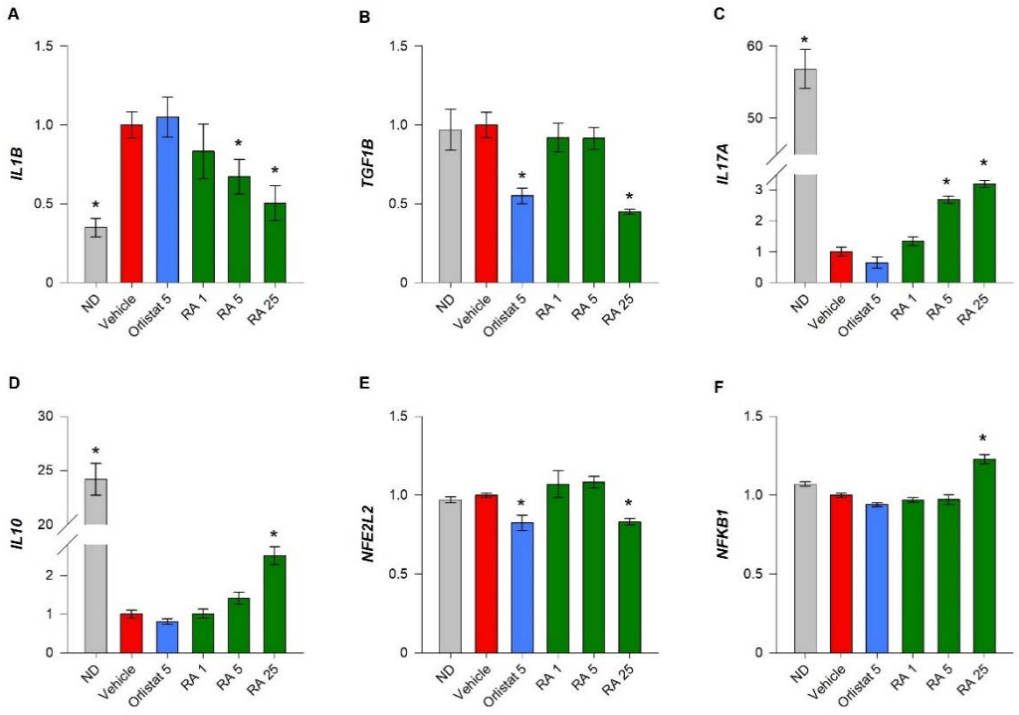

Figure 17. Rosmarinic acid (RA) benefits the inflammatory response in human adipocytes. Relative gene expression of selected cytokines: *IL1B* (**A**), *TGF1B* (**B**), *IL17A* (**C**), *IL10* (**D**), *NFE2L2* (**E**), *NFKB1* (**F**). Data from RT-qPCR analysis were normalized to the control (Vehicle) using *RPL13A* and *TUBB* as reference genes. Results for each group are presented as mean ± SEM of three independent experiments with three technical replicates. *p<0.05 compared with the control group.

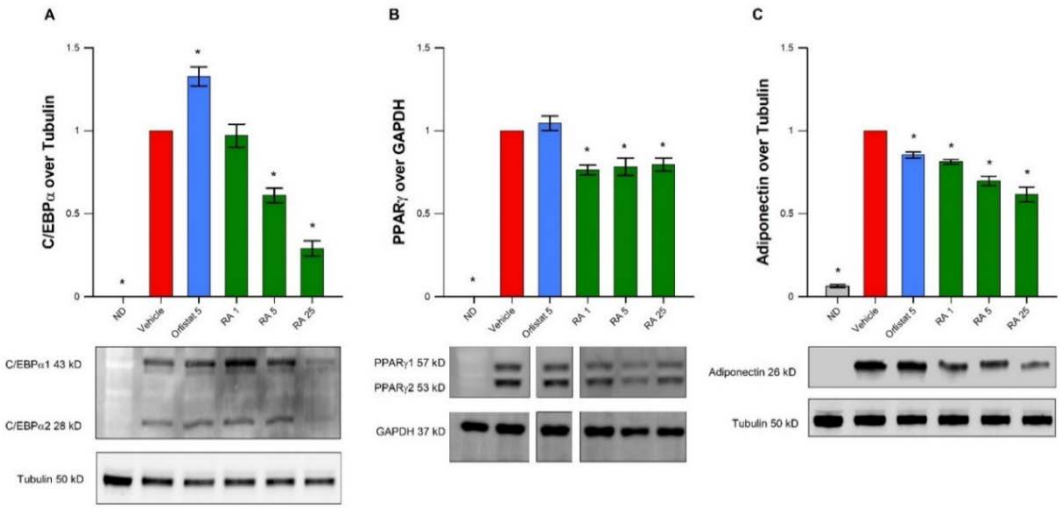

Figure 18. Rosmarinic acid (RA) suppresses protein expression of C/EBPα, PPARγ and

adiponectin in human adipocytes. Immunoblot analysis was performed to assess the effect on C/EBPα (**A**), PPARγ (**B**) and adiponectin (**C**) levels. Tubulin or GAPDH were used in normalization as reference proteins. Data are presented as mean ± SEM from three independent experiments. *p<0.05 compared with differentiated control group (Vehicle).

In summary, the data obtained reveal the anti-adipogenic potential of rosmarinic acid. The pronounced effect of this natural molecule on the processes of adipogenesis and adipolysis is complemented by the beneficial influence on the inflammatory profile of human adipocytes.

5. *In vivo* validation of the lipid-lowering effect of betulinic acid and determination of the molecular mechanism of action in *C. elegans*

The promising biological activity exhibited by betulinic acid in *in vitro* studies on human adipocytes, along with the identification of key molecular mechanisms associated with obesity involving PPARγ and PI3K/AKT signaling, necessitates further validation trough *in vivo* experiments.

5.1. Vitality assessment of nematodes and phenotypic analyses

The results of the experiments carried out showed that the concentrations of betulinic acid (10, 25 and 50 μM) and orlistat (12 μM) used did not affect the vitality and the phenotypic parameters studied - locomotion activity, reproductive capacity and lifespan.

5.2.Staining of accumulated lipids

Compared to humans, *C. elegans* does not posses dedicated fat cells. Instead, lipid depots are primarily found in the gut and hypodermis of the nematode in the form of lipid droplets (Yue et al., 2021a).

Lipid staining results of nematodes treated with 10, 25 and 50 μM betulinic acid showed a statistically significant decrease in ORO (Fig. 19C) and NR (Fig. 19B), which did not exceed the effect obtained from the control-orlistat used, (Fig. 19B,C).

Lipid staining results confirmed the potential of betulinic acid as a natural compound affecting glucose-induced lipid accumulation in *C. elegans*. Subsequent analysis of the expression of genes key to lipid metabolism was performed to determine the molecular mechanism of action of betulinic acid in the *in vivo* model of obesity.

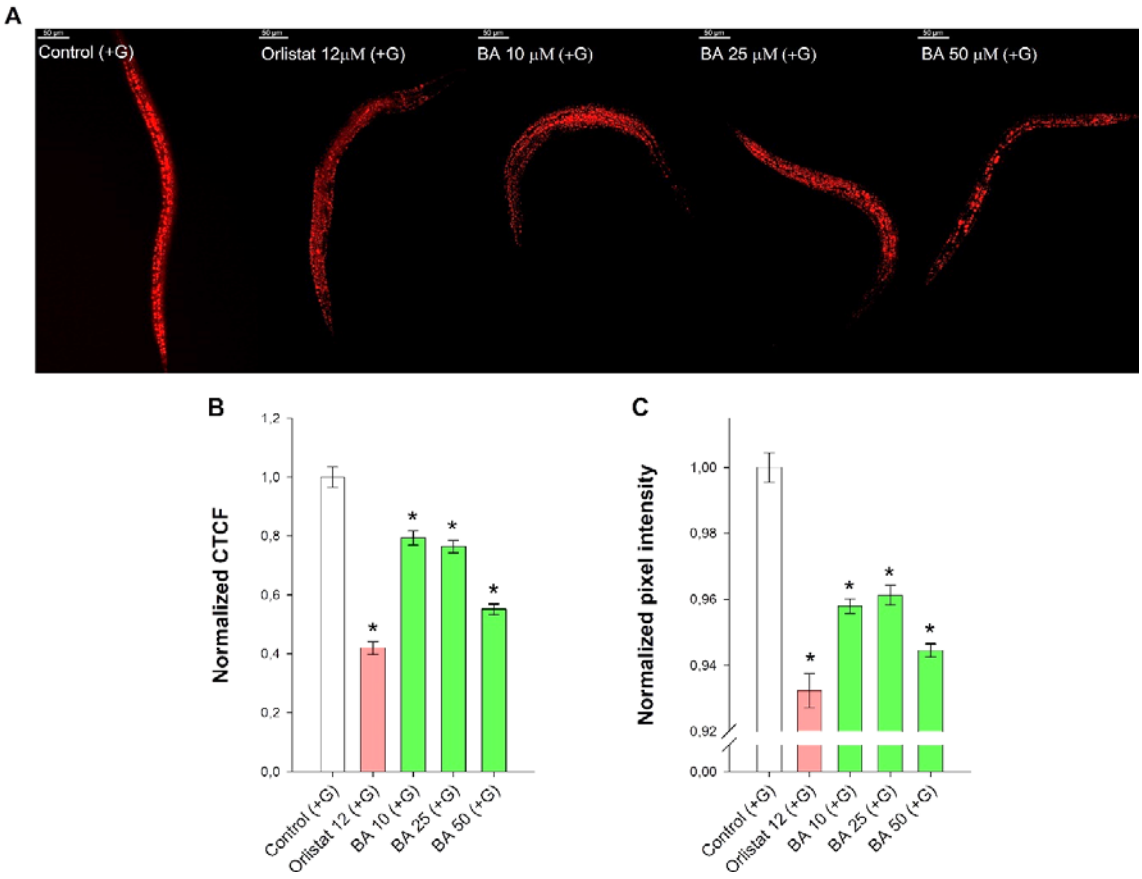

Figure 19. Betulinic acid (BA) inhibits lipid accumulation in *C. elegans* induced by the supplementation with 2% glucose (+G). Representative confocal microscopy images at 20x magnification (50 μm scale bar) from Nile red staining (**A**). Quantification of fluorescence intensity reported from lipid staining with Nile red as a normalized value for corrected total cell fluorescence (CTCF), number of nematodes n = 70-80 per group (**B**). Quantification of intensity reported upon lipid staining with Oil red O as normalized value for pixel intensity, number of nematodes n = 30-40 per group (**C**). Data are presented as mean ± SEM of three independent experiments. *p<0.05 compared with control group (Control+G).

5.3. Real-time polymerase chain reaction of mRNAs and microRNAs

The lipid metabolism of *C. elegans* involves diverse and tightly regulated complex processes. In the present study, the expression of key transcription factors for lipid homeostasis such as *sbp-1* and the human C/EBPs homologue, *cebp-2* was investigated. In addition, the functional homologue of human PPARs nuclear hormone receptor 49 (*nhr-49*) has been investigated. Interestingly, this transcription factor stimulates two different aspects of fat metabolism in the nematode, lipogenesis and β-oxidation (Wan et al., 2022). Moreover, the involvement of *nhr-49* in lipogenesis is mediated by upregulating the expression of some of the fatty acid

desaturases (*fat-2, -5, -6* and *-7*), which are nematode-specific enzymes involved in *de novo* lipogenesis (Ow et al., 2021). As an ortholog of human C/EBPs in *C. elegans*, *cebp-2* (Xu et al., 2015) also activates enzymes involved in fatty acid desaturation. Another key transcription factor for lipid biogenesis is the ortholog of the human SREBP , *sbp-1*, which regulates fatty acid synthesis *via* the aforementioned desaturases. In addition, *sbp-1* directly activates the enzymes involved in *de novo* lipogenesis, *pod-2* (homolog of ACC) and *fasn-1* (Yue et al., 2021a). The expression of the helix loop helix-11 (*hlh-11*) essential for lipid catabolism was examined. This transcription factor inhibits lipid catabolism in the presence of sufficient nutrient resources by affecting adipose triglyceride lipase (*atgl-1*), lysosomal lipase (*lipl-3*), hydrolyzing lipid droplets to fatty acids, and acyl-CoA synthetase (*acs-2*), involved in β-oxidation. The relative expression of *aak-2* as an ortholog of AMPK was also investigated to determine whether it is involved in the mechanism of action of betulinic acid (Li et al., 2020a).

Betulinic acid significantly reduces glucose-stimulated lipid accumulation in nematodes (Fig. 20). At the lowest concentration applied, 10 μM, the expression of *aak-2* (Fig. 20G) and *acs-2* (Fig. 20 H) was upregulated. The effect on *nhr-49* revelaed biphasic transcriptional response (Fig. 20 F), as betulinic acid (10 μM) significantly increased its expression, whereas 50 μM significantly suppressed it. A similar biphasic response at the transcriptional level, for the highest and lowest concentrations of betulinic acid applied, was observed for *atgl-1* (Fig. 20 C). Treatments with 25 and 50 μM significantly decreased the expression of enzymes involved in lipogenesis (*fat-5*, *fat-6* and *fat-7*, Fig. 20 I-K) and *pod-2* (Fig. 20 A), while the expression of *cebp-2* (Fig. 20 E) was upregulated.

On the other hand, an increase in *hlh-11* expression (Fig. 20 D) was recorded at all applied concentrations of betulinic acid. No statistically significant effect on transcriptional level was reported for the following genes *sbp-1*, *fat-2* and *fasn-1* (Fig. 20 L-N).

Orlistat treatment significantly suppressed gene expression of *pod-2*, *atgl-1*, *hlh-11*, *nhr-49*, and *acs-2* (Fig. 20 *)*, whereas *lipl-3*, *cebp-2*, *aak-2*, *fat-5*, *fat-6*, *fat-7*, *fat-2*, *fasn-1*, and *sbp-1* were not affected (Fig. 20).

Significant transcriptional change in some of the selected genes indicates that both betulinic acid and orlistat affect lipid metabolism in *C. elegans*. Subsequent RT-qPCR analysis of selected microRNAs was performed to reveal the complex posttranscriptional control of betulinic acid and orlistat treatment in glucose-supplemented nematodes.

Treatment with 10, 25 and 50 μM betulinic acid induced a dose-dependent decrease in *miR-60* expression (Fig. 21A). In the present study, *lin-4* expression was also downregulated

(Fig. 21 B). The expression of *miR-786* (Fig. 21 D) was only suppressed in the 10 μM treatment, whereas the expression of *let-7* (Fig. 21 C) was only downregulated in the 50 μM treatment. Among the microRNAs examined, *miR-34* and *miR-80* were not affected by betulinic acid treatment (Fig. 21 E,F). Orlistat (12 μM) significantly increased the expression of *miR-34* only (Fig. 21 E), whereas no significant effect was observed for *miR-60*, *lin-4*, *let-7*, *miR-786*, and *miR-80* (Fig. 21).

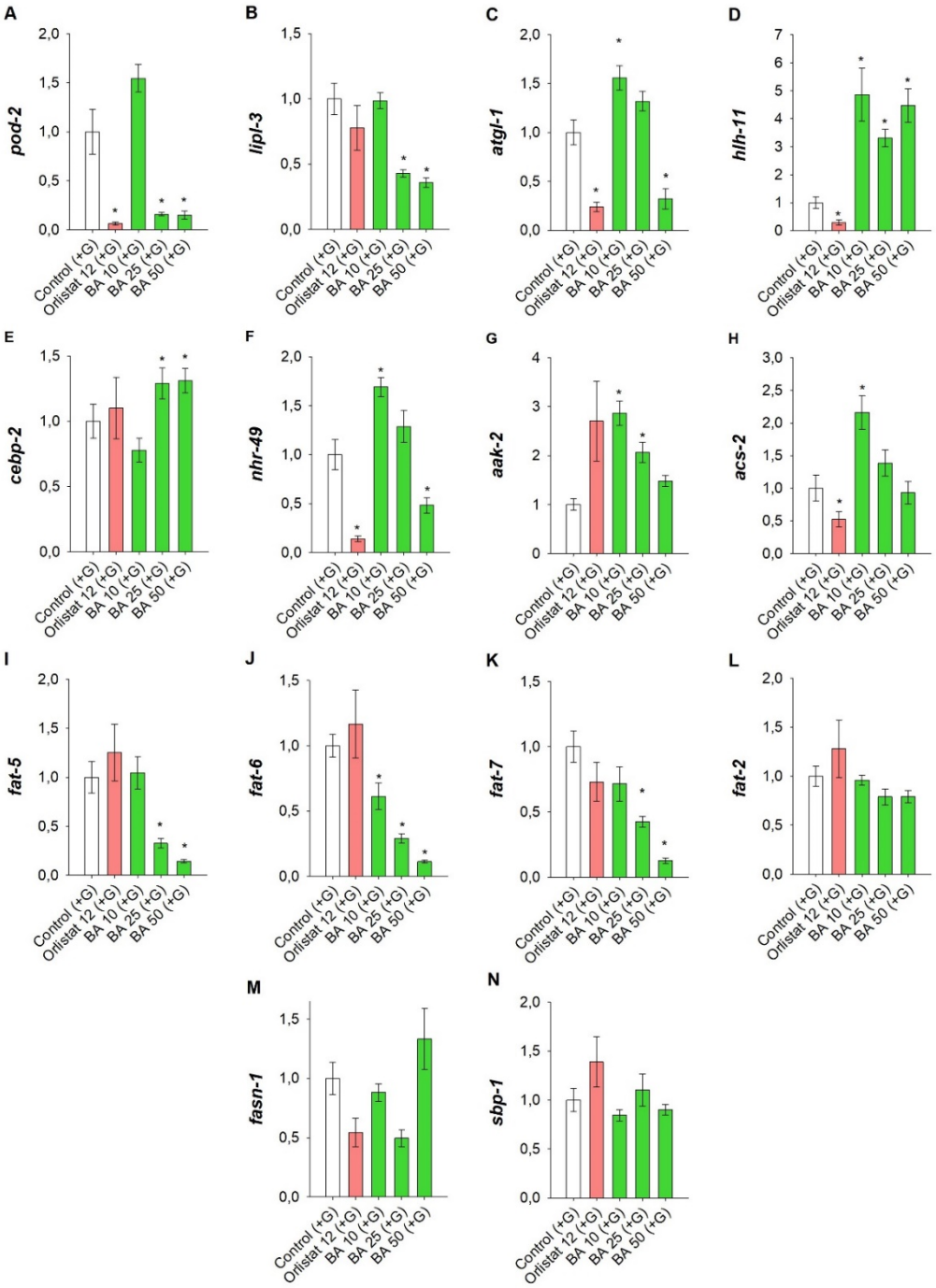

Figure 20**. Betulinic acid (BA) affects the expression of genes key to lipid metabolism in**

***C. elegans*.** Relative expression of mRNAs normalized to control group for the following genes: *pod-2* (**A**), *lipl-3* (**B**), *atgl-1* (**C**), *hlh-11* (**D**), *cebp-2* (**E**), *nhr-49* (**F**), *aak-2* (**G**), *acs-2* (**H**), *fat-5* (**I**), *fat-6* (**J**), *fat-7* (**K**), *fat-2* (**L**) *fasn-1* (**M**) and *sbp-1* (**N**) obtained by RT-qPCR analysis. Data from three independent experiments are presented as mean ± SEM. *p<0.05 compared with the control group.

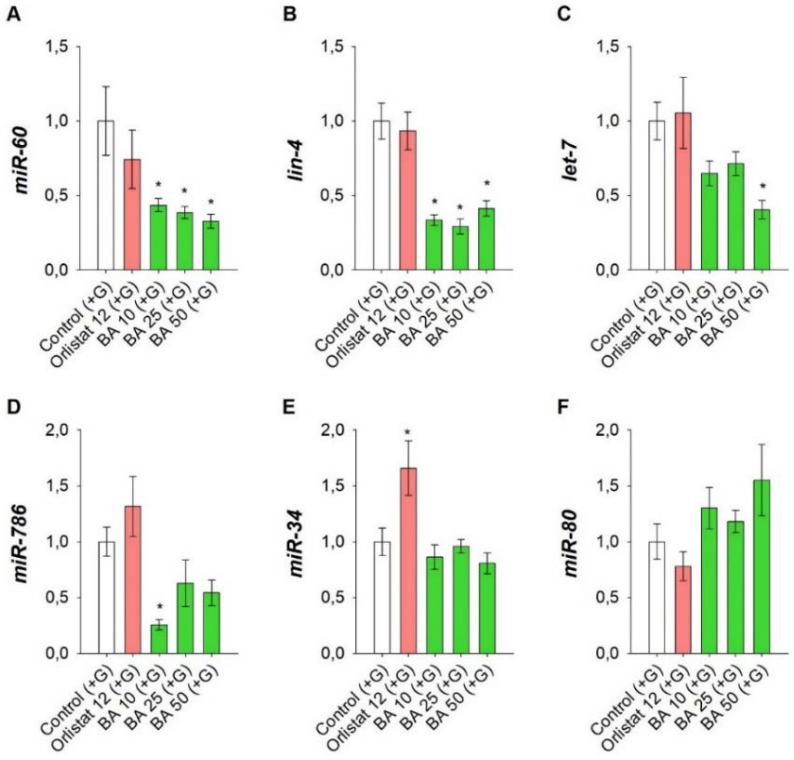

Figure 21. Betulinic acid (BA) represses *miR-60* and *lin-4* expression in *C. elegans*. Relative expression of microRNAs normalized to control group for: *mir-60* (**A**), *lin-4* (**B**), *let-7* (**C**), *miR-786* (**D**), *miR-34* (**E**) and *miR-80* (**F**) obtained by RT-qPCR analysis. Data from three independent experiments are presented as mean ± SEM. *p<0.05 compared to control group.

Computer-assisted target identification for microRNAs and mRNAs is essential to elucidate their biological role. On the basis of the observed significant alteration of the expression profiles of *miR-60* and *lin-4* following treatment with betulinic acid, their target mRNAs were inferred by bioinformatics analysis using the TargetScanWorm database (Jan et al., 2011). Among the proposed target mRNAs (8mer+1) for *miR-60* - aquaporin-7 (*aqp-7*) , *nhr-34* and *acs-4* are directly related to lipid metabolism and possibly involved in the mechanism of action of betulinic acid. Regarding *lin-4*, two targed mRNAs, *hlh-30* and

mediator-15, (mdt- *15*), were identified, both of which are involved in lipid metabolism (O'Rourke et al., 2013). The identified targets of these affected microRNAs are potential players in the molecular mechanism of action of betulinic acid.

In conclusion, among the plant extracts and natural molecules studied, the most pronounced anti-adipogenic effect was shown by *Z. jujuba* extract and betulinic acid, which at the cell lineage stage suppressed the expression of key factors in fat cell differentiation. Subsequent experiment at the organismal level confirmed that betulinic acid affects various aspects of lipid metabolism in the glucose-predisposed model of obesity in *C. elegans*. The results of gene expression analysis indicate that the transcription factor *nhr-49* plays an essential role in the mechanism of action of betulinic acid. Treatment at a concentration of 10 μM increased *nhr-49* expression, which correlated with the reported expression of enzymes associated with lipid hydrolysis (*atgl-1*) and subsequent lipid oxidation (*acs-2* and *aak-2*), whereas the use of 50 μM betulinic acid resulted in a decrease in *nhr-49* expression and in the expression of lipogenic genes (*pod-2, fat-5, -6*, and -7) that are regulated by *nhr-49*.

V. DISCUSSION

During the experimental work for this dissertation, a combinatorial approach between molecular pharmacology and ethnopharmacology has been applied to identify plant secondary metabolites with the potential to beneficially affect obesity and determine their molecular mechanism of action.

In the following, the results of the present work are commented, divided into several sections according to the logical and chronological sequence of their execution.

1. *Z. jujuba* extract exhibits the most pronounced anti-adipogenic activity in an *in vitro* model of obesity

Based on the approach taken in the present work to target the underlying pathophysiological mechanisms leading to obesity, by suppressing adipogenesis and stimulating adipolysis, the extract of *Z. jujuba* leaves was selected for subsequent work analyses. The extract demonstrated a relatively weaker suppression of adipolysis but affected the expression of all adipogenesis players examined, the PI3K/AKT signaling pathway and following inhibition of PPARγ and C/EBPα. On the basis of the metabolic profiling of this extract and a thorough literature review of its chemical composition, apigenin, betulinic and maslinic acid were selected and their anti-adipogenic activity and molecular mechanism was investigated *in vitro* obesity model system.

2. Betulinic acid exerts the most promising inhibition of lipid accumulation at the cellular level

Plant secondary metabolites are known to have multiple beneficial effects on health status (Atanasov et al., 2021), including influencing obesity and its comorbidities (Bhardwaj et al., 2021; Li et al., 2022; Martel et al., 2017).

Based on the correlation between the activated PI3K/AKT signaling pathway and the increased expression of PPARγ and C/EBPα during adipogenesis (Lopez-Guadamillas et al., 2016; Min et al., 2013; Nagai et al., 2018; Ortega-Molina et al., 2015), the results suggest the involvement of these mediators in the mechanism of action of apigenin, betulinic and maslinic acid. Further studies are needed to determine whether the selected compounds directly interact with PPARγ or C/EBPα. Regarding the role of PI3K/AKT in insulin signaling (Foukas et al., 2013; Mandl et al., 2020; Ortega-Molina et al., 2015), the decrease in PI3K protein levels in all experimental treatments modulated this signaling pathway in all treatments. Despite the essential role of insulin signaling in maintaining insulin sensitivity, inhibition of PI3K has been reported to have beneficial metabolic effects during adipogenic differentiation (Foukas et al.,

2013; Ortega-Molina et al., 2015). Summarizing the results of RT-qPCR and immunoblot analyses, the inhibition of PI3K/AKT signaling pathway and the subsequent downregulation of PPARγ and C/EBPα is the main mechanism involved in the anti-adipogenic effect of *Z. jujuba* extract and apigenin, betulinic and maslinic acid (Fig. 22).

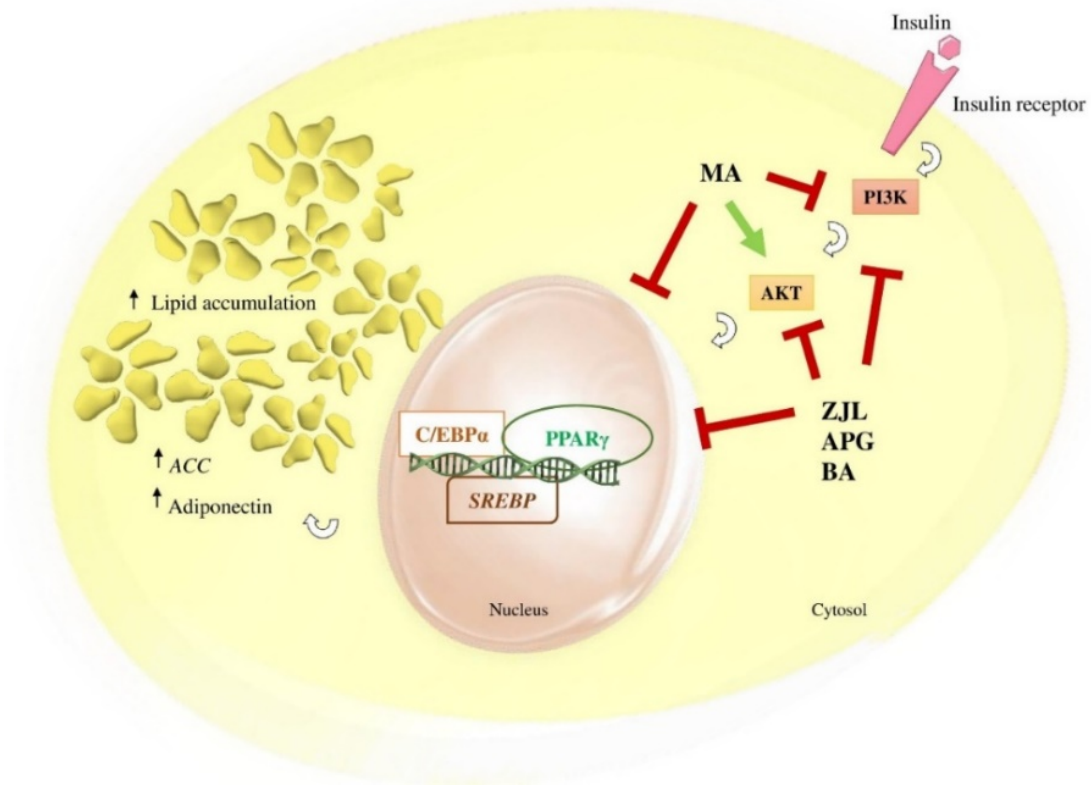

Figure 22. *Z. jujuba* extract (ZJL), apigenin (APG), betulinic (BA), and maslinic (MA) acids modulate the PI3K/AKT signaling pathway and solicit key transcription factors for adipocyte differentiation. Apigenin and betulinic acid showed an effect on gene expression of the signaling pathways studied similar to that observed with the application of the *Z. jujuba* extract. Interestingly, maslinic acid inhibited PI3K but upregulated AKT, indicating that maslinic acid modulates PI3K/AKT signaling by a mechanism distinct from that of betulinic acid, apigenin, and *Z. jujuba* extract.

The results of the performed analyses provide evidence for the anti-adipogenic potential and molecular mechanism of action of the *Z. jujuba* extract and the natural molecules studied - apigenin, betulinic and maslinic acid in an *in vitro* model of human adipocytes. Among the selected secondary metabolites of *Z. jujuba* extract, betulinic acid was reported to have the highest potential to influence the process of adipogenic differentiation *in vitro*, warranting its subsequent study in an *in vivo* model of obesity.

3. Rosmarinic acid discloses an anti-adipogenic potential and ammeliorates the inflammatory response in human adipocytes

Treatment of human adipocytes with rosmarinic acid inhibits lipid accumulation, increases lipolysis, and suppresses the expression of genes key to fat cell differentiation.

Regarding the adipogenic transcription factors addressed in this work, the results of the immunoblot analysis indicate that rosmarinic acid suppresses the protein expression of C/EBPα, indicating that this transcription factor is responsible for the biological response reported as a result of the treatment. The significant downregulation of *SREBP1* expression is consistent with the previous statement, as this factor is directly associated with *CEPBA* during adipocyte differentiation. In addition, inhibition of SREBP1-regulated signaling pathways in adipocytes is associated with reduced production of tumor necrosis factor-α, IL-6, adiponectin, and resistin (Okuno et al., 2018). Drawing a parallel with the correlations reported in the literature, the observed downregulation of *CEBPA* and *SREBP1* expression likely results in the suppression of adiponectin and *FABP4* expression. Consistent with the suppressed adipogenesis, a decrease in the expression of *ACC* and *FASN*, which are essential enzymes for *de novo* lipogenesis under direct control by *SREBP1,* has also been reported with rosmarinic acid treatment (Boutens et al., 2018; Lin et al., 2020; Okuno et al., 2018).

Previous studies on the effects of rosmarinic acid suggest that modulating signaling pathways related to inflammatory response and oxidative stress are among the molecular targets of this natural molecule. Such signaling pathways include NFκB/MAPK (Joardar et al., 2019), AMPK/TGF-β (Yang et al., 2012; Zhang et al., 2018b), and NRF2/ heme oxygenase 1 (HO-1; Gerogianni et al., 2018; Joardar et al., 2019). The anti-inflammatory activity of rosmarinic acid, through influencing NRF2 and NFκB, has been demonstrated in cell types from various tissues including neurons (Gerogianni et al., 2018; Rong et al., 2018), nephrons (Joardar et al., 2019) and keratinocytes (Georgiev et al., 2012).

In summary, the results show that rosmarinic acid suppresses gene expression of key adipogenic factors as well as downstream protein synthesis at C/EBPα, PPARγ and adiponectin (Fig. 23), leading to the observed anti-adipogenic effect. Based on the unaffected expression of *UCP1* and *PGC1A* in the present study, it cannot be proposed that the observed decrease in lipid accumulation is due to "browning" of adipose tissue. The beneficial effect on cytokines and key transcription factors of inflammatory pathways is one of the mechanisms by which rosmarinic acid exerts its anti-adipogenic effect. The results obtained support that the observed decrease in lipid accumulation with rosmarinic acid treatment is due to both the suppressed

differentiation of fat cells and the anti-inflammatory action of the natural molecule studied. Among the regulatory factors investigated in the present work, the most significant effect was found for C/EBPα, on the basis of which it can be proposed that affecting this transcription factor is a key mechanism in the anti-adipogenic effect of rosmarinic acid. In addition, an anti-inflammatory effect of rosmarinic acid was observed in the treatments by modulating the TGF-β/IL-17A signaling pathway during adipogenic differentiation.

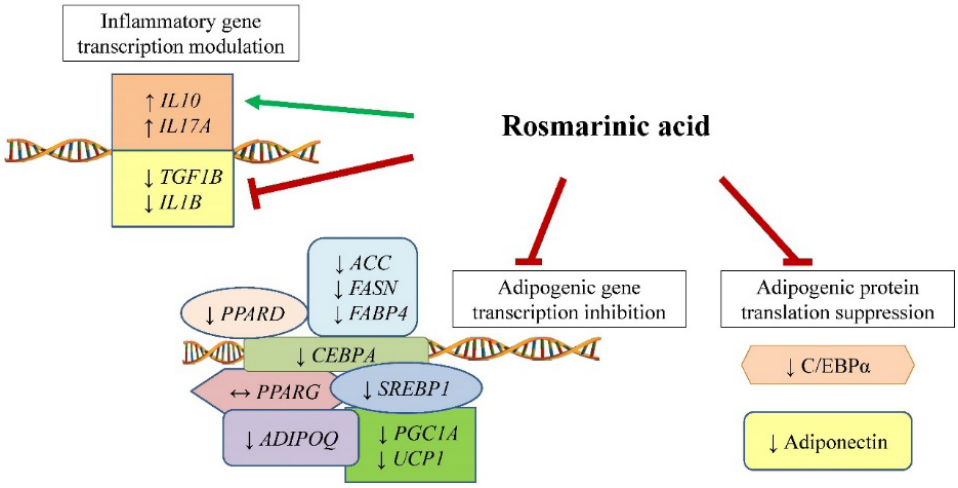

Figure 23. Rosmarinic acid exerts its anti-adipogenic effect by suppressing adipogenesis and lipogenesis, in parallel with reducing the inflammatory response during human adipocyte differentiation.

4. Betulinic acid restricts lipid synthesis and stimulates energy expenditure in an *in vivo* model of obesity in *C. elegans*

The last experiment included in this work aims to confirm the results of a natural molecule selected on the basis of *in vitro* experiments in an *in vivo* model of obesity in *C. elegans*. It is important to note that, to date, the potential of betulinic acid to influence lipid metabolism has not been investigated in *C. elegans* as a model system of obesity. In the present experiment, orlistat, an approved drug for the treatment of obesity, was used as a control substance to compare its effects on lipid accumulation. The observed decrease in lipid accumulation with orlistat treatment is consistent with data from the literature (Aranaz et al., 2020; Navarro-Herrera et al., 2018; Reboredo et al., 2021). A similar trend was confirmed after its administration in mouse models subjected to a high-fat diet, along with improved serum lipid values (Reboredo et al., 2021; Yi et al., 2021; Yue et al., 2021b; Zakaria et al., 2021).

Betulinic acid is a plant secondary metabolite possessing valuable immunoregulatory, anti-inflammatory (Lin et al., 2022) and hepatoprotective (Gu et al., 2019; Zhao et al., 2022)

properties. Phenotypic analyses have shown that betulinic acid does not affect lifespan, locomotion rates, and reproductive capacity of *C. elegans*. As betulinic acid concentration increased, a statistically significant suppression of lipid accumulation was observed in nematodes. The data confirm the reported potential of betulinic acid to favorably affect obesity in *in vitro* (Brusotti et al., 2017; Mohsen et al., 2019) and *in vivo* (Kim et al., 2019; Melo et al., 2009) models.

The results of lipid accumulation quantification indicated that, at all concentrations applied, betulinic acid inhibited glucose-induced lipid accumulation in nematodes. Despite the observed trend of decrease in accumulated lipids, analysis of *nhr-49* and *atgl-1* gene expression suggested the involvement of different mechanisms mediating the observed 10 and 50 μM effect. Treatment with betulinic acid at 10 μM, increased *nhr-49*, *atgl-1*, *acs-2*, and *aak-2* expression, suggesting that fat hydrolysis and β-oxidation were increased. On the other hand, the highest concentration of betulinic acid, 50 μM, markedly inhibited both lipid synthesis and lipolysis, proposed by the decrease in the expression of *pod-2*, *nhr-49*, *fat-5*, *-6* and *-7*, as well as the lipases *lipl-3* and *atgl-1*.

Betulinic acid treatment affects various aspects of lipid metabolism in *C. elegans*, depending on the concentration administered. The transcription factor *nhr-49* is a functional homologue of a family of nuclear receptors, PPARs, whose isoform, PPARγ, is affected in the cell model system. In summary, a molecular mechanism by which betulinic acid reduces lipid accumulation in *C. elegans* (Fig. 24). Treatment with 10 μM stimulated lipid hydrolysis and subsequent lipid oxidation, whereas the inclusion of 50 μM markedly inhibited lipid synthesis.

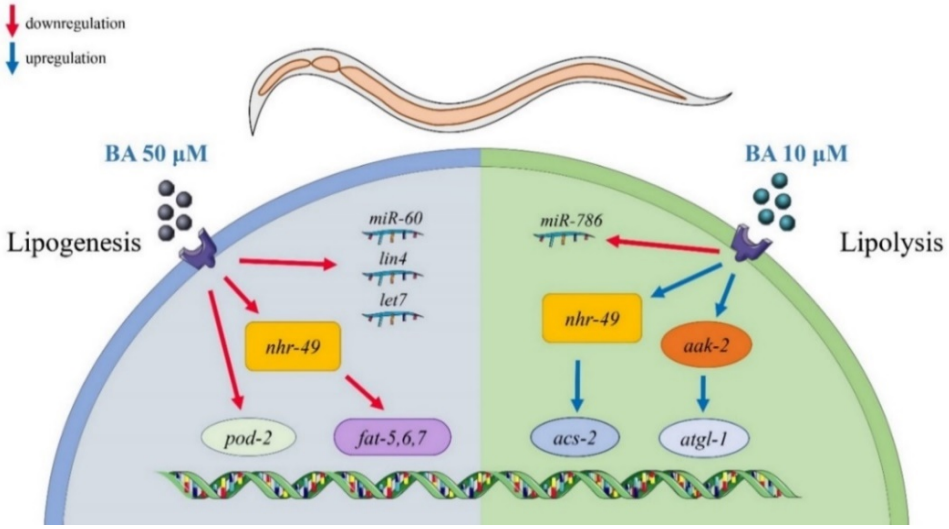

Figure 24. Anti-obesogenic mechanism of action of betulinic acid (BA) model of obesity in *C. elegans* due to biphasic interference with *nhr-49* expression. Betulinic acid treatment

under conditions of elevated glucose in the culture medium prevented the accumulation of triglycerides in the lipid depots of nematodes. Judging by the increased expression of *nhr-49*, *atgl-1*, *acs-2* and *aak-2* upon treatment with 10 μM, it is proposed that lipolysis is stimulated at this concentration. In contrast, the mechanism affected by the 50 μM treatment was inhibition of lipid synthesis by suppressing the expression of *pod-2*, *nhr-49* and its associated desaturases, *fat-5*, *-6* and *-7*.

5. Conclusion and future perspectives

In this dissertation a model of obesity in human adipocytes is adapted for the first time in Bulgaria. Screening for the effects on adipogenesis and lipolysis of extracts of medicinal plants used in herbal combinations for the treatment of metabolic disorders as well as selected natural compounds was performed. The experimental platform structured in this way allows testing for anti-adipogenic activity and establishing the molecular mechanism of action of natural molecules. Among the natural molecules investigated, the potential to influence selected molecular signaling pathways of the most promising has been validated at the organismal level in a glucose-induced obesity model in *C. elegans.* This *in vivo* model system has also been introduced for the first time in the context of investigating the complex network of molecular mechanisms underlying obesity. The results of the experiments conducted in this work provide, for the first time, data on the mechanism of action of *Z. jujuba* leaf extract in a model of obesity in human adipocytes. Inhibition of the PI3K/AKT signaling pathway was identified as the major molecular mechanism of the effect of *Z. jujuba* extract, apigenin and betulinic acid on adipogenesis. Furthermore, the observed influence of PPARγ and C/EBPα expression upon treatment with *Z. jujuba* extract and selected molecules indicates the involvement of these transcription factors in the mechanism of action of the established anti-adipogenic effect. In conclusion, it is possible to argue that all the plant secondary metabolites studied (apigenin, betulinic and maslinic acid) are involved in the anti-adipogenic effect shown by the application of the extract through PI3K/AKT signaling, which appears to be their common molecular mechanism. However, the *Z. jujuba* extract exerted the most prominent effect on the reduction of accumulated lipids in the cell model. This justifies the persistence of the extract as a source of valuable bioactive substances with the potential to influence lipid metabolism.

Among the natural molecules selected from the composition of the *Z. jujuba* extract, betulinic acid showed the most notable anti-adipogenic potential, in the low concentration range of treatment used. The results indicate that betulinic acid interacts with the PI3K/AKT

signaling cascade and modulates PPARγ in a model of obesity in human adipocytes. Based on the cell model data, we can summarize that betulinic acid is the most promising, among the natural molecules studied in this dissertation, whose effect has been validated at the organosome level.

Additionally included in the study, rosmarinic acid exhibited its anti-adipogenic effect through inhibition of key adipogenesis factors such as C/EBPα, PPARγ and adiponectin The anti-inflammatory effect was found, by influencing cytokines and key transcription factors of inflammatory pathways, is an outstanding advantage of rosmarinic acid, as it affects, besides adipogenesis, inflammation as a complication accompanying obesity. These results indicate the potency of rosmarinic acid as a component of future combinations studied in the established *in vivo* model system.

The final experiment validates the potency of betulinic acid to affect pathological lipid accumulation in glucose-induced lipid accumulation in *C. elegans*. Despite differences in concentration response at the gene expression level, the ultimate effect of betulinic acid treatment was a marked reduction in lipid accumulation. Furthermore, at the highest concentration used, the suppression of fat accumulation approximated the effect induced by orlistat, which is indicative of the potential of the studied terpenoid for application in obesity therapy. The results obtained from the *in vivo* experiment confirm the hypothesis that betulinic acid is a natural compound with potential for application in the pharmacotherapeutic approach for the treatment and prevention of obesity.

The results of the present work provide detailed mechanistic information on the molecular pathways affected upon treatment with selected plant extracts and pure natural molecules. After a detailed analysis of the obtained data, future directions for further work in the scientific field of molecular pharmacology of obesity are formed. The *Z. jujuba* extract, which showed the most pronounced activity in the cellular model system of obesity, deserves further development in order to identify other potential bioactive secondary metabolites from its composition. Among the pure molecules from the *Z. jujuba* extract investigated, betulinic acid demonstrated an outstanding potential for lowering the lipid accumulation, both in the cell model and at the organismal level used. At the highest concentration tested, betulinic acid inhibited lipid accumulation to a degree comparable to the results achieved with the reference drug orlistat, which is commonly used for the treatment of obesity. Although the potential synergism of the hybrid combination between orlistat and betulinic acid has not been confirmed in terms of reduction of lipid accumulation, it is reasonable to plan different hybrid

combinations between betulinic acid and other synthetic substances in order to enhance the observed effect and develop innovative products for obesity control. The concept of multitarget therapy is centered on the objective of simultaneously targeting multiple effector structures to achieve an optimal therapeutic effect. The data obtained from the investigation of the mechanism of action of the individual pure substances in the present work provide a scientific basis for considering a future combination therapy involving betulinic and rosmarinic acid. The rationale behind this potential combination is to encompass a wider range of mechanisms that can be targeted to effectively address obesity, including adipogenesis, adipolysis, and low-grade inflammation. By combining these two molecules, it is hypothesized that a synergistic or additive effect can be achieved, leading to a more comprehensive and potentially more effective therapeutic approach. Such a combination therapy holds promise for targeting multiple aspects of obesity pathology and may offer greater therapeutic benefits compared to individual molecule-based treatments. However, further research and experimental validation are needed to confirm the potential synergistic effects and optimal dosing regimens of betulinic acid and rosmarinic acid in combination therapy for obesity management.

VI. CONCLUSIONS

1. Phytochemical profile data have been enriched of leaf extracts from *Z. jujuba*, aerial parts of *P. aviculare* and *P. hydropiper* by NMR-based metabolic profiling.
2. A platform for *in vitro* screening of anti-adipogenic potential of plant extracts and natural molecules in human SGBS adipocytes has been successfully structured.
3. The three studied extracts revealed inhibitory effects on the processes of adipogenesis and lipolysis, with the most prominent anti-adipogenic activity detected for the extract of *Z. jujuba*.
4. Betulinic acid, a characteristic compound found in *Z. jujuba* extract, demonstrates the most notable anti-adipogenic action and influence key signaling pathways such as PI3K/AKT in an *in vitro* model of human adipocyte obesity.
5. Betulinic acid restrains lipid metabolism *in vivo* in a model organism *C. elegans* by modulating the transcription factor *nhr-49*, which regulates the processes of lipid synthesis and hydrolysis.
6. Betulinic acid regulates insulin-mediated adipocyte differentiation *via* the PI3K/AKT signaling pathway, expression of the transcription factor nhr-49/PPARs and microRNAs associated with lipid metabolism.

VII. CONTRIBUTIONS

With scientific and fundamental character:

1. The anti-adipogenic activity of leaf extracts of *Z. jujuba* and aerial parts of *P. aviculare* and *P. hydropiper* in an *in vitro* model of obesity in human adipocytes.
2. The molecular pathways involved in the mechanism of anti-adipogenic action of apigenin, betulinic and maslinic acid in adipocytes have been determined.
3. The effect of rosmarinic acid in SGBS adipocytes on adipogenesis and lipolysis as well as on the expression of inflammatory factors during adipocyte differentiation was characterized.
4. The inhibitory effect on lipid depots by administration of betulinic acid (10 μM) was shown to be associated with stimulation of *nhr-49* and *acs-2* expression, whereas at a concentration of 50 μM betulinic acid affected genes related to lipid hydrolysis and the action of desaturases in *C. elegans*.
5. The effect of betulinic acid on the expression of microRNAs whose target genes are involved in lipolysis and lipogenesis in *C. elegans*.

Scientific and applied:

1. *An in vitro* model of adipocyte differentiation was introduced and optimized as a screening platform for the anti-adipogenic potential of plant extracts and natural molecules.
2. An adapted model for studying the phenotype and lipid accumulation of obesity in nematodes of *C. elegans*, which provides an *in vivo* platform to assess the anti-obesogenic potential of molecules of different origins.
3. The data obtained on the molecular mechanism of action of betulinic acid may serve as a basis for the development of product(s) for weight control and obesity prevention.

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Published materials on the dissertation

Scientific publications

1. **Savova M.S.**, Todorova M.N., Apostolov A.G., Yahubyan G.T., Georgiev M.I., Betulinic acid counteracts the lipid accumulation in *Caenorhabditis elegans* by modulation of *nhr-49* expression, *Biomed. Pharmacother.* 156 (2022) 113862 (**IF$_{2021}$ 7.419; Q1**).
2. **Savova M.S.**, Apostolov A.G., Mihaylova L.V., Georgiev M.I., Modulation of adipogenesis by *Polygonum hydropiper* L. and *P. aviculare* L. extracts, *Macedonian Pharm. Bull.* 68 (2022) 155-156.
3. Georgiev M.I., Vasileva L.V., **Savova M.S.**, Antiobesity molecules of natural origin, *Food Front.* 2 (2022) 23-24.
4. **Savova M.S.**, Vasileva L.V., Mladenova S.G., Amirova K.A., Ferrante C., Orlando G., Wabitsch M., Georgiev M.I., *Ziziphus jujuba* Mill. leaf extract restrains adipogenesis by targeting PI3K/AKT signaling pathway, *Biomed. Pharmacother.* 141 (2021) 111934 (**IF$_{2021}$ 7.419; Q1**).
5. Vasileva L.V., **Savova M.S.**, Tews D., Wabitsch M., Georgiev M.I., Rosmarinic acid attenuates obesity and obesity-related inflammation in human adipocytes, *Food Chem. Toxicol.* 149 (2021) 112002 (**IF$_{2021}$ 5.572; Q1**).
6. Vasileva L.V., **Savova M.S.**, Amirova K.M., Dinkova-Kostova A.T., Georgiev M.I., Obesity and NRF2-mediated cytoprotection: Where is the missing link? *Pharmacol. Res.* 156 (2020) 104760 (**IF$_{2020}$ 7.658; Q1**).

Total impact factor - 28

Participation in scientific conferences

Oral reports

1. **Savova M.S.**, Mihaylova L.V., Mladenova S.G., Georgiev M.I., Betulinic acid affects lipid accumulation during differentiation of human adipocytes. *International Scientific Conference on Plant Biodiversity and Sustainability*, 13-14 October 2022, Online conference.
2. Georgiev M.I., Vasileva L.V., **Savova M.S.,** Anti-obesity molecules of natural origin. *4th International Symposium on Phytochemicals in Medicine and Food*, 30 November - 04 December 2020, Xi`an, China.

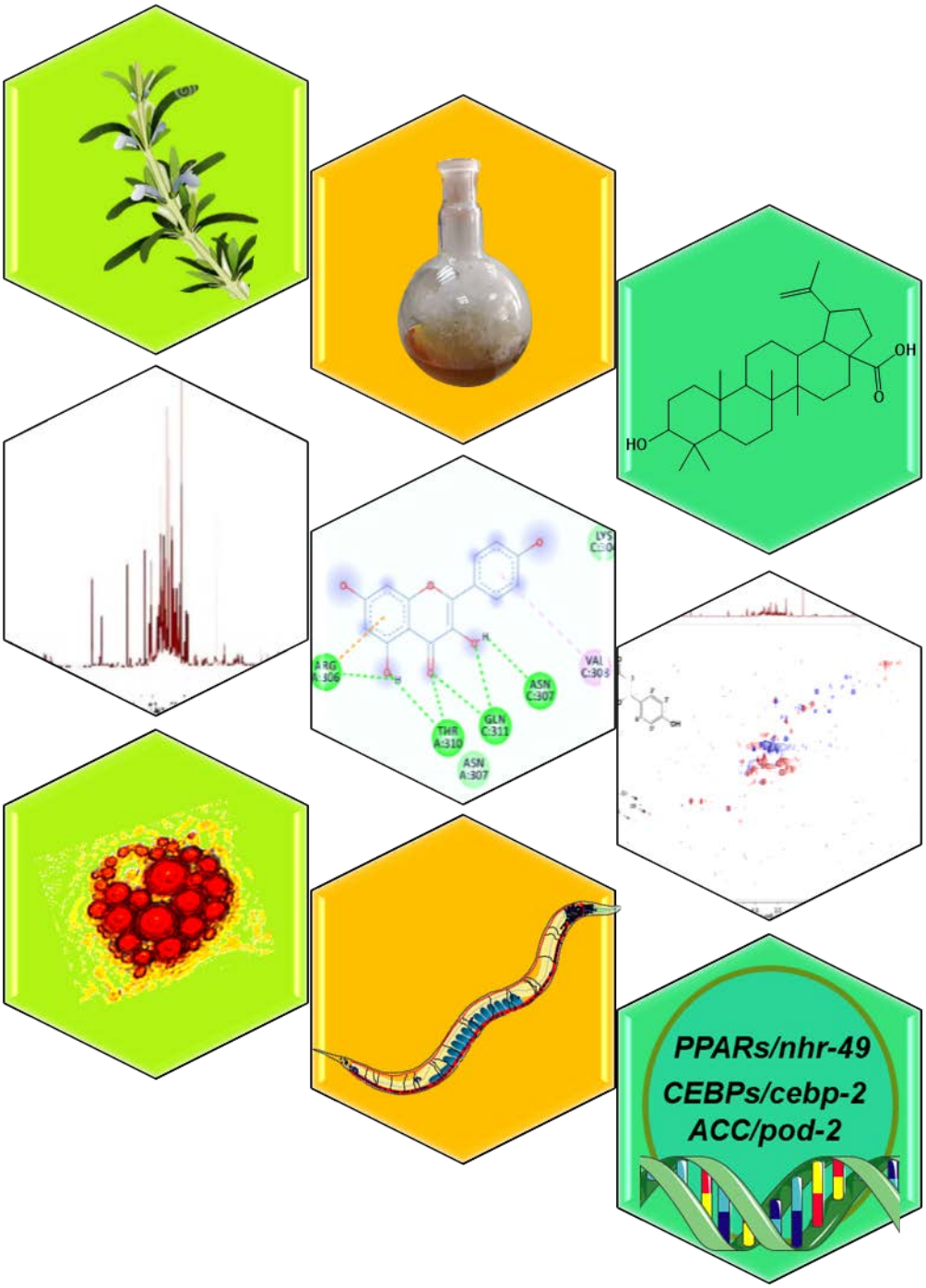
Poster presentations

3. **Savova M.S.**, Apostolov A.G., Mihaylova L.V., Georgiev M.I., Modulation of adipogenesis by *Polygonum hydropiper* L. and *P. aviculare* extracts. *11th Conference on Medicinal and Aromatic Plants of Southeast European Countries*, 6-10 October 2022, Ohrid, Republic of North Macedonia.
4. **Savova M.S.**, Todorova M.N., Apostolov A.G., Yahubyan G.T., Georgiev M.I., Betulinic acid decreased glucose-induced lipid accumulation in *Caenorhabditis elegans via* modulation of *nhr-49* expression. Natural Products in Drug Discovery and Development - Advances and Perspectives, 19-22 September 2022, Iasi, Romania.
5. **Savova M.S.**, Vasileva L.V., Mladenova S.G., Amirova K.M., Blacheva-Sivenova Z.P., Georgiev M.I., Betulinic acid affects lipid accumulation during differentiation of human SGBS adipocytes. *1st International Conference on Plant Systems Biology and Biotechnology*, 14-17 June 2021, Golden Sands Black Sea resort, Bulgaria.
6. **Savova M.S.**, Balcheva-Sivenova Z.P., Mladenova S.G., Amirova K.M., Vasileva L.V., Georgiev M.I., Extract of *Ziziphus jujube* Mill. leaves reduces lipid accumulation in adipocytes. *1st International Conference on Plant Systems Biology and Biotechnology*, 14-17 June 2021, Golden Sands Black Sea resort, Bulgaria.

Received awards

“Ivan Evstratiev Geshov” award for youngest scientists for achievements in scientific division "Biomedicine and Quality of Life", competition 2021.

Citations of publications related to the dissertation work excluding self-citations – 79 (Scopus, accessed on 06.06.2023); Index of Harish – 7.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O
HO
ASN C:307
GLN C:311
THR A:310
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PPARs/nhr-49
CEBPs/cebp-2
ACC/pod-2