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ORIGINAL ARTICLE

Transcriptional analyses of the effects of *Catharanthus roseus* L. medicinal plant extracts on some markers related to obesity and inflammation in 3T3-L1 mouse cell lines

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Abstract

Catharanthus roseus L. (*C. roseus*) is one of the medicinal plants used to treat diabetes. In this study, 3T3-L1 preadipocyte cell lines which are fully differentiated into adipocytes were utilized and these were treated with extracts derived from above ground part of *C. roseus.* The effect of these extracts on obesity and inflammation markers in cells was examined at the transcriptional level. Adipocyte lipid contents were measured by Oil Red O staining. Analyses, including changes related to adiposity and inflammation, were evaluated by measuring the relative mRNA expression levels of the genes of interest by the Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method. The appropriate dose and durations for *C. roseus* were determined to be 12.5 μg/mL and 24- and 48 h respectively. The expression of the inflammation marker Interleukin-6 (Il-6) was decreased when *C. roseus* extract was administered to fully differentiated 3T3-L1 cells according to the determined dose and durations. Lipoprotein lipase (Lpl) and Fatty acid synthase (Fasn) gene expressions in fully differentiated cells decreased compared to the control after 24 h however, this effect was not observed after 48 h. Collagen V, has also been shown to be affected by treatment of fully differentiated 3T3-L1 cells with plant extracts in both 24- and 48-h periods.

Keywords *Catharanthus roseus*-diabetes-inflammation-medicinal plants-obesity

Abbreviations

C.roseus	*Catharanthus roseus* L
MCP	Monocyte chemoattractant protein
CLS	Crown like structures
IL-1β	Interleukin-1β
Il-6/IL-6	Interleukin-6
Tnf-α/TNF-α	Tumor necrosis factor α
Lpl	Lipoprotein lipase
LPS	Lipopolysaccharide
Fasn	Fatty acid synthase
TIAs	Terpenoid indole alkaloids
WHO	World Health Organization
ATCC	American type culture collection
IBMX	3-isobutyl-1-methylxanthine
DMEM	Dulbecco's modified eagle medium
SGBS	Simpson Golabi Behmel Syndrome
ColV	Collagen V
18S	18S ribosomal RNA
Pln1	Perilipin
NCBI	National Center for Biotechnology Information
Fabp 4	Fatty acid-binding protein 4
SD	Standard deviation
Atgl	Adipose triglyceride lipase
ns	Not significant
Glut 4	Glucose transporter type 4
ECM	Extracellular matrix
VLDL	Very low-density lipoprotein
RT-PCR	Reverse transcription-polymerase chain reaction

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Introduction

Obesity is an overexpansion of adipose tissue stores resulting from chronic calorie consumption that exceeds an individual's energy needs (Hardy et al. 2012). Obesity has reached epidemic proportions all over the world today because of its

association with many diseases (Goossens et al. 2011). In 2014, more than 600 million adults worldwide (13% of the total adult population) were classified as obese and it has been reported that the global obesity prevalence increased by about 2% every 10 years (Abarca-Gómez et al. 2017; Pineda et al. 2018). According to World Health Organization (WHO), contrary to what is known, obesity is not only limited to industrialized societies, over 115 million people in developing countries are estimated to suffer from obesity related problems. The prevalence of obesity has increased threefold in the last two decades in Europe, and the obesity prevalence is estimated to be 23% (Uerlich et al. 2016; Pineda et al. 2018). In the United States, 68.5% of the adult population has overweight or obesity problems (Bray et al. 2018). Although there are not any publications about the distribution of obesity mediated inflammation worldwide to our knowledge, it is well established that there is a tight relation between obesity and inflammation. There are reports showing that obesity is associated with chronic systemic inflammation and that there are components of signaling pathways involved in inflammatory responses which is conditioned by the innate immune system activation in adipose tissue and promotes an increase in the production and release of pro-inflammatory cytokines in response that contribute to the triggering of the systemic acute-phase response which is characterized by elevation of acute-phase protein levels (Olefsky 2009; Rodríguez-Hernández et al. 2013). So, it therefore is probable that distribution of obesity mediated inflammation correlates positively with that of obesity.

Increased obesity rates have emerged as a major health problem and cause economic burden in all countries (Engin 2017). Although there are behavioral approaches and pharmacological treatments for obesity, it is not possible to say that they are very effective in healing. Although it is effective in reducing body weight, associated comorbidities and improving quality of life, bariatric surgery is associated with many complications (Qasim et al. 2018). It has been reported by previous studies that obesity is characterized as a state of systemic chronic low inflammation (Park et al. 2005; Goossens et al. 2011; Liu et al. 2016). In response to excessive intake of nutrients and energy, metabolic signals and inflammatory responses damaging homeostasis are initiated by the cells (Gregor and Hotamisligil 2011). The secretion of pro-inflammatory cytokines by both adipocytes and adipose tissue macrophages contributes to the development of insulin resistance and atherosclerosis (Zieger et al. 2018). 3T3-L1 preadipocyte cell line that is derived from mouse cells and has the capability to differentiate into an adipocyte-like phenotype under appropriate conditions is originally of fibroblast cell type and has therefore a fibroblast-like morphology (Green and Kehinde 1975). The number of articles published on adipogenesis and the biochemistry of adipocytes where these cell lines were utilized reached a significant value. Although 3T3-L1 cells have the potential to convert from fibroblastic phenotype to adipocytes, this alteration does not occur under normal circumstances, if necessary stimulating agents are required. Only in the presence of the agents insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX) at certain concentrations, fibroblast-like morphology having 3T3-L1 preadipocyte cells convert to fully differentiated adipocyte cells. There are reports about the protocols of how to effectively differentiate 3T3-L1 cells to adipocytes which indicates that the differentiantion of 3T3-L1 cells to adipocytes requires an optimal conversion condition (Zebisch et al. 2012). Once 3T3-L1 preadipocyte cells are stimulated to differentiate into adipocytes, adipogenesis is triggered and this in turn leads to the secretion of adipocytokines. Adipocytokine secretion plays a crucial role in the generation of an inflammatory microenvironment. In a study where human preadipocyte Simpson Golabi Behmel Syndrome (SGBS) cells were converted to differentiated SGBS cells, it was shown that the increase in adipogenesis correlated positively with an accumulation of inflammatory markers such as Monocyte chemoattractant protein (MCP), (IL-1β) and IL-6 in the supernatant of differentiated SGBS cells. There are also a significant number of reports showing that adipocytes are secreting pro-inflammatory components such as IL-6 and TNF-α (Smitka and Marešová 2015; Nickel et al. 2018; Fuggetta et al. 2019). Based on these information we hypothesised that the creation of an inflammatory microenvironment without making use of additional treatments with agents such as (LPS) and TNF-α by solely due to the conversion of preadipocyte 3T3-L1 cells to adipocytes would be sufficient to result in an enrichment of pro-inflammatory markers and this would mimic an in vitro chronic inflammation state to a mild chronic inflammation of an adipose tissue in vivo.

C. roseus is a perennial plant belonging to the family of Apocynaceae (Espejel-Nava et al. 2018). It is widely distributed throughout the world due to its high ability to survive in various habitats and its use as ornamental plant (Al-Shaqha et al. 2015). It produces more than 130 different terpenoid indole alkaloids (TIAs), some of which exhibit strong and important pharmacological activities (Almagro et al. 2015). We in one of our previous studies have investigated the alkaloids of this plant in callus and multiple shoots obtained from *C. roseus* (Altan and Duru 2017). It is used in traditional practice for the treatment of diabetes in many countries such as Malaysia, India, China, South Africa and Mexico. Studies to investigate oral hypoglycemic effects have led to the discovery of the first clinically used natural anticancer agents vinblastine and vincristine (Tiong et al. 2015). Extract of *C. roseus* flower dissolved in ethanol has been reported to have wound healing activity (Nayak and Pereira 2006). There are also studies showing the hypolipidemic and anti-inflammatory activity of *C. roseus* (Patel et al. 2011; Rasineni et al. 2013; Wahjuni et al. 2015; Rajabi et al. 2018). Based on these observations, the ability of *C. roseus* to inhibit

inflammation and adipogenesis in 3T3-L1 cells was investigated. The results support the view that the plant may be a potential alternative treatment for obesity and associated inflammation.

Materials and methods

Plant material

The *C. roseus* plant to be used for the study was obtained commercially from a wholesaler in Köyceğiz/Muğla. The plants were dried for 5 weeks in a cool and moisture-free environment. The above-ground parts of the dried plants were ground to a powder. The plant powder was incubated in absolute ethanol for 48 h at room temperature (Shanmugaraju and Bhakyaraj 2016). After the extract was filtered, the solvent was removed by evaporation. The stock solutions were dissolved in ethanol to a final concentration of 20 mg/mL and stored at −20 °C until use.

Cell culture and differentiation

3T3-L1 mouse cell lines were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified eagle medium (DMEM) (Capricorn, Cat. no. DMEM-HA) supplemented to 10% with newborn calf serum (Capricorn, Cat. no. DMEM-HA), 1% penicillin/streptomycin (Multicell, Cat. no. 450–201-EL). For experiments the cells were grown to a confluence of 70% and stimulated to differentiate in predifferentiation medium containing 1 μM dexamethasone (Sigma, BCBV5460), 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma,STBF6061V), 1.0 μg/mL insulin (Sigma, SLBV1793) (Zebisch et al. 2012). The predifferentiation medium then was replaced after 48 h with differentiation medium in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin, 1.0 μg/mL insulin (Sigma, SLBV1793) for 7–10 days.

Evaluation of changes in morphology and viability of cells

The cells were placed onto petri dishes and incubated within a media containing 12.5 μg/mL, 25 μg/mL and 50 μg/mL *C. roseus* extracts for 24-, 48- and 72 h (van de Venter et al. 2008). The adherence and morphology of the cells on petri dishes were evaluated using light microscopy.

Staining of the 3T3-L1 cells with oil red O dye

The differentiation of 3T3-L1 adipocytes was done as described previously. Oil Red O staining was done according to manufacturer's instructions using the Biovision Oil Red O Staining kit. The procedures that adipocytes went through can be summarized so that they were initially fixed at room temperature for 20 min in 3.7% formaldehyde followed by rinsing them with distilled water and staining them for 2 h at room temperature. The dye was extracted for 30 min in 0.5 mL of dye extraction solution. The procedure ended by the aspiration of the dye and rinsing of the plates that after with distilled water. Removal of the dye was performed using 0.5 mL of dye extraction buffer for 30 min.

Total RNA isolation and real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from adipocyte cells was extracted using a RiboEx™ (Cat. No.301–001) total RNA isolation solution from GeneAll (Cat. No.301–902). Ultraviolet light spectrophotometry followed by formaldehyde- agarose gel electrophoresis was used to determine the quality and quantity of the isolated RNA. Five hundred nanograms of total RNA was reverse transcribed using oligo-dT primers with EasyScript Plus cDNA synthesis kit from ABM Alfagen (Cat. No. G236). The amplification of reverse transcribed RNA was achieved with the use of Ampliqon RealQ Plus 2 × Master Mix Green in the presence of 0.3 mM gene-specific forward and reverse primers by a thermocycling on a Roche Light Cycler 96 Real Time PCR machine for 45 cycles. The temperatures and durations for denaturation, annealing and extension were 95 °C for 30 s; 55–58 °C for 30 s and 72 °C for 30 s, respectively. The normalization of the differences in individual samples was done by the use of amplified 18S expression as a standard control. The description of the mouse Lipoprotein lipase (Lpl), Fatty acid synthase (Fasn), Collagen V (ColV), Interleukin-6 (Il-6) and 18S ribosomal RNA (18S) primer sequences are given below in Table 1. All expression data for mouse Lpl, Fasn, ColV and Il-6 were interpreted by relating them to 18S RNA. Pooled cDNA of the samples being assayed were used to generate standard curves. This enables the accurate comparison of samples within each assay but do not necessarily enable accurate comparison of samples between different assays represented as arbitrary units. The analyses of all samples were done in duplicates; DNA contamination was eliminated by designing primers spanning an intron. The primers were designed based on the information of the sequences of the genes of interest using The National Center for Biotechnology Information (NCBI 2018) as a source. The information about the primer sequences used for Real Time PCR and the accession numbers of the genes analyzed are shown in Table 1.

Statistical analysis

The comparison of groups with respect to continuous variables and comparison of baseline and posttreatment

Table 1 Sequences, lengths, melting temperatures and NCBI accession numbers for primers designed for real time PCR reactions

Gene	Primer sequence (5′ 3′)	Primer length (bases)	Tm (°C)	GenBank accession number
18S	Forward: TTCGAACGTCTGCCCTATCAA	21	63	NR_003278.3
	Reverse: ATGGTAGGCACGGCGACTA	19	63	
Lpl	Forward: ACTCGCTCTCAGATGCCCTA	20	65	NM_008509.2
	Reverse: TTGTGTTGCTTGCCATTCTC	20	60	
Fasn	Forward: CTGAGATCCCAGCACTTCTTGA	22	64	NM_007988.3
	Reverse: GCCTCCGAAGCCAAATGAG	19	63	
Il-6	Forward: ACCAAACTGGATATAATCAGGAAA	24	56	NM_031168.2
	Reverse: GAGAGCATTGGAAATTGGGG	20	57	
ColV	Forward:CTCAGGGGTAACGAAAACCA	20	60	NM_015734.2
	Reverse:GGAGAAGTCCTCGGGAAAAC	20	60	

measurements were done by using Student's two-sample t-test and a paired t-test respectively. Differentially expressed genes with significance were identified using a P value <0.03.

Results

The effect of *Catharanthus roseus* extract on cell viability

To observe the effect of *C. roseus* extracts on undifferentiated 3T3-L1 cells, cell morphology and cell survival circumstances were examined. 3T3-L1 cells are adherent cells and they need to be attached to the surface to maintain their viability and the tendency of them to detach will be indicative of a deteriorated cell morphology. Therefore, it was investigated whether the doses of 12.5 μg/mL, 25 μg/mL and 50 μg/mL of ethanol-dissolved extracts obtained from the above-ground parts of the plant along with the solvent itself alone had unfavorable effects on the cells. Cells were exposed to these determined doses for 24-, 48- and 72 h. At the end of the treatment of cells with the solvent itself alone in all time durations, it was observed that the solvent used had no toxic effect on the cells and was suitable for the experiment since there was neither a detachment of the cells from surface nor a change in cell morphology (Fig. 1a, b, c). The treatment of the cells with the extract for 72 h, regardless of concentration and time, both affected the cells' adherence to the surface to which they were attached and caused deterioration in their morphology. Based on this, it was concluded that the application of extracts for 72 h had a negative effect on cell viability (Fig. 1f, i, l). It was also demonstrated in this study that the treatment of the cells with extracts at doses of 25 μg/mL and 50 μg/mL for 48 h, resulted in cell detachment and morphology deterioration. Thus, it was concluded that the treatment of cells with an extract dose of 25 μg/mL and above for 48 h and more had no favorable effect on cell viability (Fig. 1g–l). It was observed that treatment of the cells with 12.5 μg/mL extract for 24 h and 48 h did not cause a significant decrease in cell attachment to the surface and did not result in a negative effect on cell morphology. Based on all these evaluations, it was concluded that the best condition for the cells to be treated with *C. roseus* extract is 12.5 μg/mL dose application for 24- and 48 h (Fig. 1d, e).

The information about the size of the cells both in Fig. 1 and Fig. 2 were estimated using the bar graphs shown in each figure introduced by using the ImageJ from http://rsb.info.nih.gov/ij/download.html (National Institutes of Health, (NIH) 2020). It is well established that obesity is a low grade chronic inflammation, and obese individuals undergo an increase in lipid accumulation in their adipocytes which in turn leads to the enlargement of their adipose tissue. Extraordinary lipid accumulation of adipocytes causes the transformation of monocytes in heterogeneous adipose tissue to macrophages and these macrophages infiltrate adipocytes and form crown like structures (CLS) in which the case obesity has now become an inflammatory syndrome. In this study, cells were used as an in vitro model and preadipocyte 3T3-L1 cells were first transformed into fully differentiated adipocytes to mimic the fat accumulation state of adipocytes in obese individuals and thus to evaluate the markers of inflammation. The results show that the differentiation conditions used to treat cells with extract were appropriate and that undifferentiated preadipocyte cells were converted to fully differentiated adipocyte cells as expected (Fig. 2).

Alterations of lipogenic Lpl and Fasn enzyme expressions due to the treatment with *Catharanthus roseus* extract

Gene expression measurements of lipogenic Lp1 and Fasn genes were performed to understand the molecular mechanism underlying the hypolipidemic effect of *C. roseus* extract. Treatment of fully differentiated 3T3-L1 adipocyte cells with the extract at a dose of 12.5 μg/mL for 24 h resulted in a significant decrease in Fasn mRNA level. Exposure of adipocyte cells to extracts at a dose of 12.5 μg/mL for 24 h resulted in a reduction of Fasn gene expression levels in these cells by

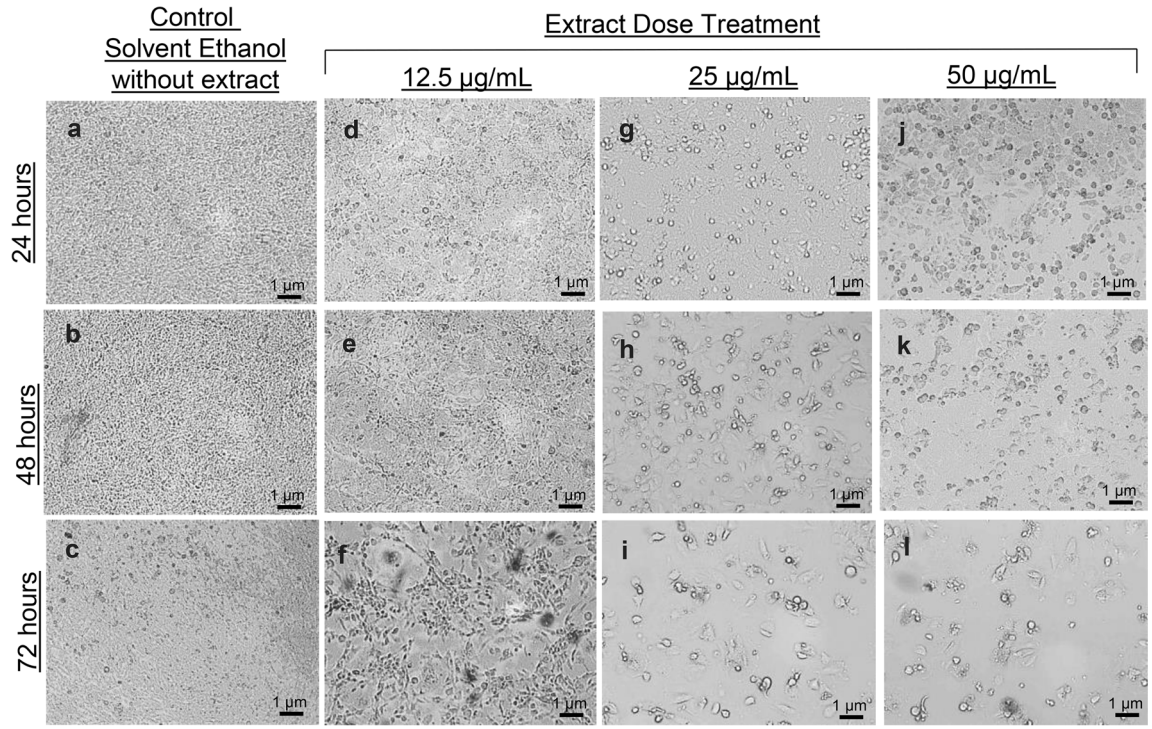

Fig. 1 Microscopic visualization and evaluation of changes in morphology and viability of 3T3-L1 cells treated with plain solvent or *C. roseus* extracts with a dose of 12.5 µg/mL, 25 µg/mL and 50 µg/mL for 24 h (**a**, **d**, **g** and **j**), 48 h (**b**, **e**, **h** and **k**) and 72 h (**c**, **f**, **i** and **l**) by light microscopy. (Scale bars in each micrograph represent a length of 1 µm)

53% compared to their control counterparts. Although the administration of the extract at this dose for 48 h appears to show an increase in the Fasn mRNA level of the cells, this increase is statistically not significant. Thus, it was concluded that treatment of adipocyte cells with extract at a dose of 12.5 µg/mL had an effect on Fasn mRNA expression in the first 24 h, which reduced gene expression, and then this effect disappeared (Fig. 3a).

Following the same experimental setup, the gene expression pattern obtained by looking at the mRNA levels of another lipogenic enzyme Lpl revealed a great parallelism with the expression pattern of the Fasn gene. In this case, treatment of fully differentiated 3T3-L1 cells with extract at a dose of 12.5 µg/mL for 24 h significantly reduced the Lpl mRNA level too. After 24 h, Lpl gene expression decreased by 61%. Although the treatment of cells with extract for 48 h seemed to reduce Lpl gene expression, this decrease was not significant when statistical analyzes were considered. These results concluded a significant reduction in the lipogenic enzyme Lpl gene expression when the extract used was administered to fully differentiated adipocytes at a dose of 12.5 µg/mL for 24 h, but this reduction effect disappeared after 48 h at the same dose (Fig. 3b).

The data obtained regarding the alterations of 3T3-L1 Fasn and Lpl expression profiles in response to 12.5 µg/mL *C. roseus* extract treatments for 24 h and 48 h in arbitrary units is summarized in Table 2. Values shown in bold indicate that the percent changes analyzed are of statistical significance. The values having the descriptions "(ns)" indicate that these values are statistically not significant.

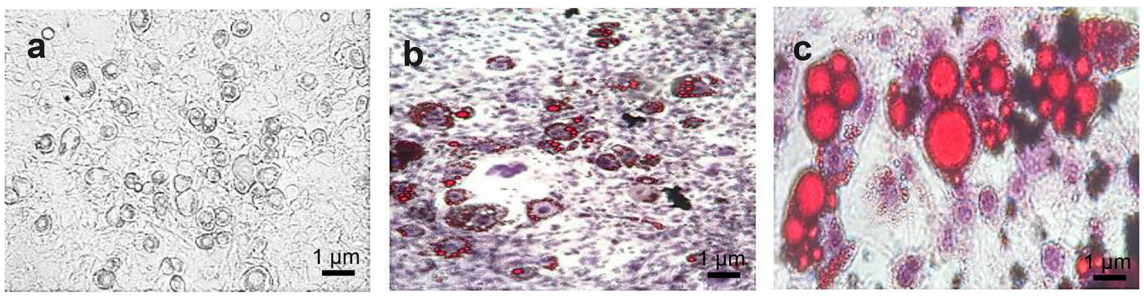

Fig. 2 Oil Red O Staining to determine the lipid accumulation in fully differentiated 3T3-L1 cells. Cells were visualized before staining and after staining in a 10 × magnitude (**a**, **b**) respectively and after staining at a 40 × magnitude (**c**) by light microscopy. (Scale bars in each micrograph represent a length of 1 µm)

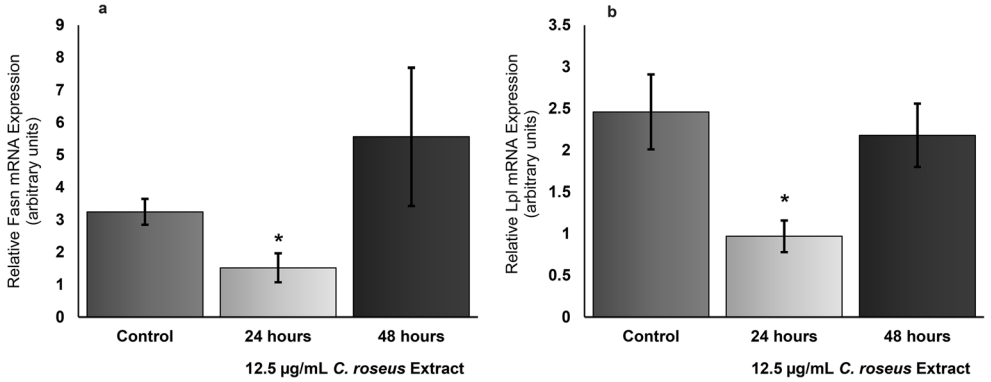

Fig. 3 Quantitative measurement of Fasn and Lpl mRNA in 3T3-L1 cells. Fasn and Lpl quantitation of mRNA in 3T3- L1 adipocytes differentiated with IBMX, Dexamethasone and insulin were done by Real Time RT-PCR using duplicate measurements in three separate experiments. The changes in Fasn mRNA and Lpl mRNA expression in fully differentiated adipocytes indicated with asterisks were significant ($P < 0.03$). (Error bars show standard deviation (SD))

Effects on expression of extracellular matrix component collagen V and pro-inflammatory marker Il-6 after treatment with *Catharanthus roseus* extract

In addition to the metabolic complications, obesity appears as a condition that manifests itself with some changes in the remodeling of the extracellular matrix (ECM) components in adipose tissue. The extracellular matrix components of adipose tissue undergo a constant remodeling so that the shapes and functions of adipocytes and their precursor cells respond to nutritional cues. It is well established in the literature that obesity causes accumulation of ECM components and their modifiers in adipose tissue (Lin et al. 2016). Collagens are the most abundant scaffolding in ECM. Collagen V has been chosen to investigate the potential changes in ECM remodeling as a consequence of an increase in adiposity since it is essential for fibrillation of types I and III collagen, and consequently for optimal fibrillary formation and tissue quality. Changes in Collagen V mRNA levels were evaluated to get an opinion about the role of *C. roseus* extract on extracellular matrix remodeling. Exposing fully differentiated 3T3-L1 adipocyte cells to the extract at a dose of 12.5 µg/mL for both 24 h and 48 h resulted in a significant decrease in Collagen V mRNA expression by 97% and 91% respectively (Fig. 4a).

The molecular mechanisms underlying the relationship between obesity and inflammation has been uncovered to a significant extent. The change in the interaction of adipocytes and monocytes of heterogeneous adipose tissue due to obesity, causes the secretion of some inflammatory cytokines which in turn results in the conversion of native monocytes to pro-inflammatory macrophages infiltrating adipocytes and consequently form "crown like structures" (CLS). An adipose tissue under such stress results in a state of mild chronic inflammation. When this is the case, knowing what effect *C. roseus* has in terms of reducing the inflammatory condition that will arise due to the increase of adiposity has been one of the questions sought for this study. For this purpose, *C. roseus* extracts were applied to fully differentiated 3T3-L1 cells at a dose of 12.5 µg/mL for 24 h and 48 h periods. At this dose, pro-inflammatory Il-6 mRNA level used as a marker in both time periods decreased by approximately 40% (Fig. 4b).

The data obtained regarding the alterations of 3T3-L1 ColV and Il-6 expression profiles in response to 12.5 µg/mL *C. roseus* extract treatments for 24 h and 48 h in arbitrary units is summarized in Table 3. Values shown in bold indicate that the percent changes analyzed are statistically significant.

Table 2 Fully differentiated 3T3-L1 gene expressions and percent changes of Fasn and Lpl in response to 12.5 µg/mL *C. roseus* treatment for 24- and 48 h in comparison to control. Plus (+) indicates an increase and minus (−) a decrease in gene expression. Bold labeling mean that the alterations are statistically significant whereas "(ns)" indicates that the change is not of significance

Samples genes of interest	Relative mRNA expression (arbitrary units)				
	Control	12.5 µg/ml *C. roseus* extract			
		24 h	% change to control	48 h	% change to control
Fasn	3.25 ± 0.40	1.52 ± 0.45	**−53%**	5.56 ± 2.13	+71% (ns)
Lpl	2.46 ± 0.45	0.97 ± 0.19	**−61%**	2.18 ± 0.38	−11% (ns)

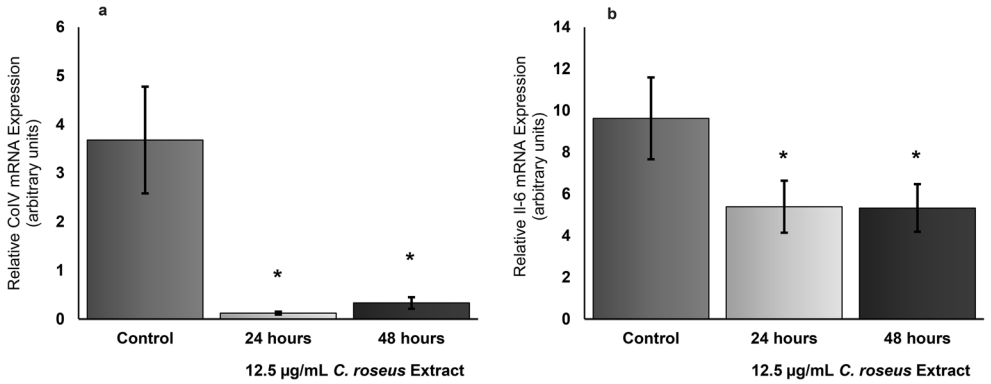

Fig. 4 Quantitative measurement of ColV and Il-6 mRNA in 3T3-L1 cells. ColV and Il-6 quantitation of mRNA in 3T3- L1 adipocytes differentiated with IBMX, Dexamethasone and insulin were done by Real Time RT-PCR using duplicate measurements in three separate experiments. The changes in ColV mRNA and Il-6 mRNA expression in fully differentiated adipocytes indicated with asterisks were significant ($P < 0.03$). (Error bars show standard deviation (SD))

Discussion

Today, obesity is a global disease that affects individuals from each age group in developing and developed countries (Ofei 2005). Studies have been able to relate obesity to many health problems such as insulin resistance, cancer, heart and respiratory diseases and hypertension. The risk of morbidity and premature death is directly related to excessive accumulation and distribution of fat in the body (Greco et al. 2002). When health expenditures are taken into consideration, it has been demonstrated that expenditures for obese individuals are 1.5–1.8 times higher than for non-obese individuals (Cox et al. 2015). Many pharmacological drugs used to combat obesity have also been reported to have significant side effects (Kang and Park 2012; Huang et al. 2019). Considering all these, medicinal plants stand out as potential useful alternatives to pharmacological drugs due to their cheapness, reliability and low side effects. Preadipocyte 3T3-L1 cells acquire adipocyte morphology after differentiation induction, accumulate large amounts of triacylglycerol and become sensitive to lipolytic and lipogenic hormones, thereby providing an excellent model system to study the biochemical and molecular mechanisms involved in adipose tissue development and lipid metabolism (Moustaid and Sul 1991). In adipogenesis, the interaction of proteins such as fatty acid-binding protein with lipid metabolizing enzymes such as lipoprotein lipase and fatty acid synthase induces various transcription factors to mediate adipocyte differentiation (Kim and Spiegelman 1996; Tsai et al. 2017). Therefore, as an important approach for the prevention and/or treatment of obesity, it is important to suppress both adipocyte cell count (hyperplasia) and cell size (hypertrophy) induced adipose tissue expansion. *C. roseus* is an important plant in this sense because there are previous studies showing the effect of it decreasing lipid parameters. Rasineni et al. showed in their study where they used a high-fat diet fed mice model that *C. roseus* leaf extract solution given to the mice at a certain dose on a daily basis (100 mg/kg body weight/day) reduced the amount of free fatty acid in the tissue by providing a decrease in the activity of Fasn (Rasineni et al. 2013). In our study, we observe a positive correlation between this decrease in Fasn activity and our finding of the decrease of mRNA expression obtained by applying *C. roseus* plant extracts to adipocytes.

It is well established that adipogenesis plays a prominent role in the expansion of a healthy adipose tissue which in turn leads to obesity (Kershaw and Flier 2004; Vishvanath and Gupta 2019). It is also very well known that diabetes and obesity are two diseases that are significantly interdependent (Golay and Ybarra 2005). *C. roseus* is a medicinal plant that is commonly used to treat diseases in a wide spectrum including

Table 3 Fully differentiated 3T3-L1 gene expressions and percent changes of ColV and Il-6 in response to 12.5 µg/mL *C. roseus* treatment for 24- and 48 h in comparison to control. Plus (+) indicates an increase and minus (−) a decrease in gene expression. Bold labeling mean that the alterations are statistically significant

Samples genes of interest	Relative mRNA expression (arbitrary units)				
	Control	12.5 µg/ml *C. roseus* extract			
		24 h	% change to control	48 h	% change to control
ColV	3.68 ± 1.10	0.12 ± 0.03	**−97%**	0.33 ± 0.12	−91%
Il-6	9.63 ± 1.97	5.39 ± 1.24	**−40%**	5.33 ± 1.14	−40%

diabetes all over the world. Although there are quite a number of reports regarding antihyperglycemic effects of *C. roseus*, the influence of this plant on adipogenesis and obesity has not been studied extensively. It is important to know whether metabolic improvements mediated through *C. roseus* might be linked to its effects on adipogenesis. In a recent study it has been reported that 1α, 25-dihydroxy Vitamin D3 containing fractions of *C. roseus* leaf aqueous extract inhibited preadipocyte differentiation and induced lipolysis in 3T3-L1 cells. The authors showed in their study in detail that the differentiation of 3T3-L1 preadipocytes was inhibited and the lipid accumulation in differentiating 3T3-L1 cells was reduced upon *C. roseus* extract administration (Borah et al. 2019). This is the first report to our knowledge that provides a direct answer to the question whether *C. roseus* extracts have an effect on adipogenesis. They also showed in their study using RT-PCR analysis that the genes such as Perilipin 1 (Pln1), Fatty acid-binding protein 4 (Fabp 4), Adipose triglyceride lipase (Atgl), Lipoprotein lipase (Lpl), Glucose transporter type 4 (Glut4), and Adiponectin which they called "adipocyte factors" were downregulated upon *C. roseus* extract treatment. The downregulation of Lpl after *C. roseus* treatment that we obtained in our current study is consistent with this report.

Adipocytes have the property of storing fatty acids that are generated during the process starting with the metabolism of circulating exogenous fat as triglycerides and also fatty acids as the result of endogenous fatty acid biosynthesis. Lipoprotein lipase hydrolyzes triacylglycerol-rich portions of both very low-density lipoprotein (VLDL) and of chylomicrons, resulting in fatty acid release. These nonesterified fatty acids are then imported by fat cells and mainly serve as a precursor for fat synthesis (Kim and Spiegelman 1996; Mead et al. 2002). Studies in diabetic mice and pigs fed on a high-fat diet treated with *C. roseus* leafs dissolved in ethanol have been reported to reduce the levels of triglyceride and cholesterol levels in blood (Ara et al. 2009; Patel et al. 2011; Muralidharan 2014). This decrease in lipid levels may be associated with the plant down-regulating Lpl mRNA expression, which is increased in adipose tissue.

120 alkaloids were shown to be produced in *C. roseus* of which 70 were pharmacologically active (Barrales-Cureño et al. 2019). Pharmacokinetics of alkaloids are changing from one to another, which in turn may influence their effect on the biological question of interest. Aconitum is one of the plants, which is shown to contain alkaloids and in a study it was reported that the concentration of all of the six analyzed Aconitum alkaloids were decreased within a time frame of 24 h in rat plasma (Zhang et al. 2019). There are also similar studies that characterized the pharmacokinetics of *C. roseus* alkaloids such as vinblastine (van Tellingen et al. 1993), vindoline and catharanthine (Lin et al. 2015). In all these reports it was consistently shown that the plasma levels of all of the alkaloids decreased within 24 h. For some alkaloids present in *C. roseus* this effect was even observed between 8- and 24 h, We, in this study, observed that the downregulatory effect of *C. roseus* extract on 3T3-L1 cells after 24 h was no longer the case after 48 h of administration. In line with previous reports, we believe that the reason of the attenuation of the inhibitory effects of *C. roseus* on the expression of Lpl and Fasn is likely to be the consequence of the much reduced amounts of *C. roseus* taken into cells between 24- and 48 h, hence decreasing its effect further. In addition, as a result of the increase in the incubation period due to the mixed multiple componential nature of plant extracts, the structures of some substances in the extract may deteriorate or change and lose their effectiveness, so the expected effect may not be seen. Apart from this, our plant extract may also have blocked gene expression via a cytoplasmic signaling pathway.

Adipose tissue also secrets cytokines that can affect energy metabolism. Tumor necrosis factor (Tnf-α) and interleukin-6 (Il-6) are prominent pro-inflammatory cytokines (Zieger et al. 2018). Dysregulated and continuous synthesis of Il-6 is associated with chronic inflammation and autoimmune disorders (Tanaka et al. 2014). Increased cytokine levels such as of Tnf-α and Il-6 were observed in the blood of mice fed with a high-fat diet for 6 weeks. *C. roseus* leaf extract led to a decrease in the levels of these cytokines. In our study, plant extract provided a decrease in the mRNA levels of the inflammatory agent Il-6, consistent with the observed drop in blood serum levels mentioned in the study above.

An increase in inflammatory macrophages, fibrosis, many ECM components including Collagen VI and thrombospondin has been reported to be the characteristics of adipose tissues obtained from obese, insulin resistant rodents and humans (Spencer et al. 2011). In an experiment conducted by Spencer et al. (2011), it was found that there was a significant increase in both mRNA and protein levels of Collagen V in fat tissue of obese individuals compared to lean individuals. In our study, *C. roseus* plant rearranged ECM components and reduced Collagen V mRNA expression in 3T3-L1 cells that have been dysregulated due to obesity.

Conclusion

This study focused on molecular changes in gene regulation at the transcriptional level in order to reveal the underlying mechanisms of the results obtained in studies in the literature, which were rather in vivo studies in which the effects of *C. roseus* plant extracts were studied. Although we observed that the results obtained by in vivo studies are mostly in positive correlation with the results we received at the transcriptional level, there is a need for more detailed studies to understand the molecular mechanisms underlying these changes. In this sense, we believe that our study is important at the

molecular level and also constitutes a basis for conducting more detailed studies.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interest.

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