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Original Article

Cichorioside a biocoumarin modulates lipid and glucose storage on 3T3-L1 cell lines: *In vitro* and *in silico* approach

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ABSTRACT

Natural coumarins are reputed to demonstrate several biological effects to improve adverse health conditions including; obesity. This study was conducted to explore the potential *in vitro* lipid and glucose modulatory activity of cichorioside on 3T3-L1 cell lines. **Methods:** The 3T3-L1 cell lines were cultured and cell viability was assessed. Glucose content in medium of cultured cells was measured. Differentiation of 3T3-L1 cells from pre-adipocytes to adipocytes was evaluated upon addition of cichorioside. Expression of mRNA of the peroxisome proliferator-activated receptor gamma (PPARγ) was estimated. **Results:** Survival of around 90% of cultured cells was observed at 10μM cichorioside. Cichorioside inhibited glucose uptake from the medium by the 3T3-L1 cell lines. Cichorioside considerably inhibited pre-adipocyte differentiation and the lipid content in intercellular storage. Cichorioside demonstrated an upregulation of the mRNA expression of PPARγ. Moreover, the docking studies supported the results *via* the deep interaction of cichorioside with amino acids residue of PPAR-γ. Taken together, these findings are the first report on *in vitro* evaluation of cichorioside to modulate the lipid storage and glucose uptake of cultured 3T3-L1 cell lines.

Key words: Cichorioside, 3T3-L1cell lines, Oil red O staining, PPARγ.

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INTRODUCTION

Based on the reports of the World Health Organization, approximately from each three adults one person is overweight and with a ratio of 1:10 of adults are suffering from severe obesity.[1] The incidence of obesity increased and is becoming a worldwide epidemic condition. Patients suffering from obesity are liable to serious health issues specially, metabolic issues including dyslipidemia, diabetes, fatty liver, renal and cardiovascular dysfunctions. Consequently, it represents enormous socio-economic and negative impacts on patient's social and psychological attitude.[2-6] Currently, several therapeutic plans are implemented for the treatment and/or prevention of obesity including lifestyle, nutrition education, and anti-obesity medications.[7,8] However, it is reported that weight management through physical exercises are not sufficient to produce marked treatment.[9] Moreover, the limited use of approved synthetic anti-obesity drugs due to its harmful side effects.[10,11] On the other hand, the surgery is not recommended for management of obesity due to the hazards of complications.[12] Accordingly, the discovery of alternative medications of safe, and effective properties is necessary.[13,14] Numerous plant based drugs revealed noticeable anti-obesity properties.[1,2,15] Coumarins are phenolic compounds that are found naturally plants, bacteria, and fungi, and their synthetic analogs can be readily synthetized.[16,17] In addition, coumarins showed broad pharmacological activities.[18,19] Coumarins and their derivatives demonstrated various therapeutic properties including antioxidant,[20] anti-inflammatory,[21] anticancer,[22] anti-diabetic[23] and anti-obesity.[24] These talented findings provoked us to evaluate the anti-obesity activity of the coumarin glucoside, namely Cichorioside (cichoriin). Based on our previous report of *in vivo* anti-obesity and antidiabetic activities of cichoriin,[25,26] this study aimed to assess the *in vitro* modulatory activity of cichorioside on lipid and glucose storage in 3T3-L1 cell lines as a complementary investigation. In addition, assessment of the molecular mechanism that governs the activity was assessed and confirmed *via* computational studies.

MATERIALS AND METHODS

Cell culture

The 3T3-L1 cell line was obtained from College of Science, King Faisal University, Saudi Arabia. To maintain the viability of cells, they were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 25 mM HEPES buffer at 37 °C with 5% CO_2. Mixture of 10 μg/mL insulin, 1 μM dexamethasone and 10 μM rosiglitazone differentiation medium (IM), was used as inducer for the differentiation of adipocytes. Cichorioside was added for 7 days. During culture process, the DM was replaced every 36 hours.[27]

Measurement of glucose content in medium

The 3T3-L1 cells were cultured in 96-well plates up to differentiation. The cell-free supernatants were collected, and total glucose level was measured using (Cayman chemicals) in accordance to the manufacturer's procedures. The results were expressed in mg/dL.

Oil Red O staining

After seventh day of differentiation of 3T3-L1 cell, cells treated or not treated with cichorioside

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were washed with phosphate buffered saline and fixed with 4% paraformaldehyde for 30 minutes, before staining with Oil Red O at 4 °C for one hour.[28] After the staining process, the cells were aspirated twice with phosphate buffered saline and then fixed using 8% methanol. The lipid content in the cells was captured using an XLcore life technologies microscope at magnification power of 200× with EVOS XL core imaging. In summary, cell supernatants were removed, and the lipid-loaded Oil Red O stain was dissolved using isopropanol. Then, 200 µL aliquots from each well were transferred to a new 96-well plate; and the OD value was measured at 490 nm.

RT-qPCR for quantification of mRNA

The cichorioside treated cells were cultured for 8 days in a CO_2 incubator. The treated 3T3-L1 cells were washed with ice-cold phosphate buffered saline, and the cell-free supernatant was removed. Total mRNA was extracted using the modified trizol method. The extracted RNA was quantified using Nano-Drop, and 300 ng of mRNA was used for cDNA preparation using the MQ basic cDNA synthesis kit.[29] The primers sequence for the peroxisome proliferator-activated receptor gamma (PPARγ) gene and the glyceraldehyde3-phosphate dehydrogenase (GAPDH) housekeeping gene is summarized in table 1. mRNA expression was quantified using ΔΔCt values.

Computational studies

The computational study was performed to explicate the molecular interactions of cichorioside (CID-442101) and PPARγ (PDB ID: 5u5l). The docking analysis was carried out using Auto dock tools (ADT) v1.5.4 and Autodock v4.2 programs. Chemical structure of ligands was downloaded from Pubchem database (http://www.pdb.org).[30] Ligand was docked to target protein complexes with the molecule considered as a rigid body and the ligand being flexible. The search was extended over the whole receptor protein used as blind docking. The search was carried out with the Lamarckian Genetic Algorithm; populations of 150 individuals with a mutation rate of 0.02 were evolved for 5 generations. The results were evaluated by sorting the different complexes with respect to the predicted binding energy. A cluster analysis based on root mean square deviation values, with reference to the starting geometry, was subsequently performed and the lowest energy conformation of the more populated cluster was considered as the most trustable solution. Docked ligand-receptor interactions were visualized and analyzed using Discovery Studio 2021 Client trial version.

RESULTS AND DISCUSSIONS

Effect of cichorioside on cell viability of 3T3-L1 cell lines

The percentage cell viability of 3T3-L1 cells following the administration of cichorioside was determined, with a 90% of survival was observed at 10 µM cichorioside (Figure 1). The 3T3-L1 cell line was screened *in vitro* for adipocyte differentiation. IM containing rosiglitazone is widely applied to induce differentiation in the 3T3-L1 cell line. In the current study, media containing rosiglitazone, insulin, and dexamethasone was applied to prompt lipogenesis in the 3T3-L1 cells. The effect of cichorioside on glucose uptake, intracellular lipid content, and activation of the transcriptional cascade was assessed. cichorioside affected the differentiation of 3T3-L1 cells from pre-adipocytes to adipocytes, with an IC_{20} of 10 µM. The dose concentration was determined, and the functional concentration was followed for the glucose update assay.

Effect of cichorioside on glucose uptake of 3T3-L1 cell lines

The level of glucose in the culture medium was decreased following rosiglitazone-induced adipocyte differentiation. Furthermore, the glucose concentration (media) in the cichorioside treatment was increased compared to the induction medium group (Figure 2). Specifically, it was observed that glucose level in the medium was two folds increased in response to cichorioside treatment compared to the IM group. These findings indicate that cichorioside inhibited glucose uptake from the medium by the 3T3-L1 cell line. Taken together, cichorioside demonstrated a good reduction in glucose level compared to the control group, suggesting antidiabetic activity, as well as a lower uptake of glucose compared to the IM group, demonstrating a potentially lower lipogenic effect on 3T3-L1 cells.

Effect of cichorioside on 3T3-L1 pre-adipocyte differentiation to adipocytes

Cichorioside significantly affected the differentiation of 3T3-L1 cells from pre-adipocytes to adipocytes upon being added to

Table 1: Real-time PCR primer details.

Primer name	Forward	Reverse
PPARγ	GAAAGACAACGGACAAATCACC	GGGGGTGATATGTTTGAACTTG
GAPDH	CGTCCCGTAGACAAAATGGT	TTGATGGCAACAATCTCCAC

(PPARγ) peroxisome proliferator-activated receptor gamma; (GAPDH) glyceraldehyde 3-phosphate dehydrogenase

Table 2: Interactions of cichorioside and amino acid residues of PPARγ.

Binding energy	Ligand efficiency	Intermole energy	Ligand atoms	Docked amino acid residue (bond length)
			- Conventional hydrogen bond: 'Sugar moiety' C4-O	
			C3´-H	CYS`285'(2.79 Å)
			C3´-H	MET`364'(2.77 Å)
			C4´-H	TYR`327'(2.17 Å)
			C6´-H	HIS`323'(2.77 Å)
-6.58	-0.27	-6.31	- Pi-Cation:	
			'Ring A`-O	ARG`288'(3.53 Å)
			- Pi-alkyl hydrophobic interaction: O	
			Ring A, B	ARG`288'(3.53 Å), (3.76 Å)
			Ring A, B	ALA`292'(4.23 Å), (5.21 Å)
			Ring A, B	ILE`326'(4.82 Å), (5.19 Å)
			Ring A, B	LEU`330'(5.17 Å), (4.65 Å)

CYS; Cysteine, MET; Methionine, TYR; Tyrosine, HIS; Histidine, ARG; Arginine, ALA; Alanine, ILE; Isoleucine, LEU; Leucine

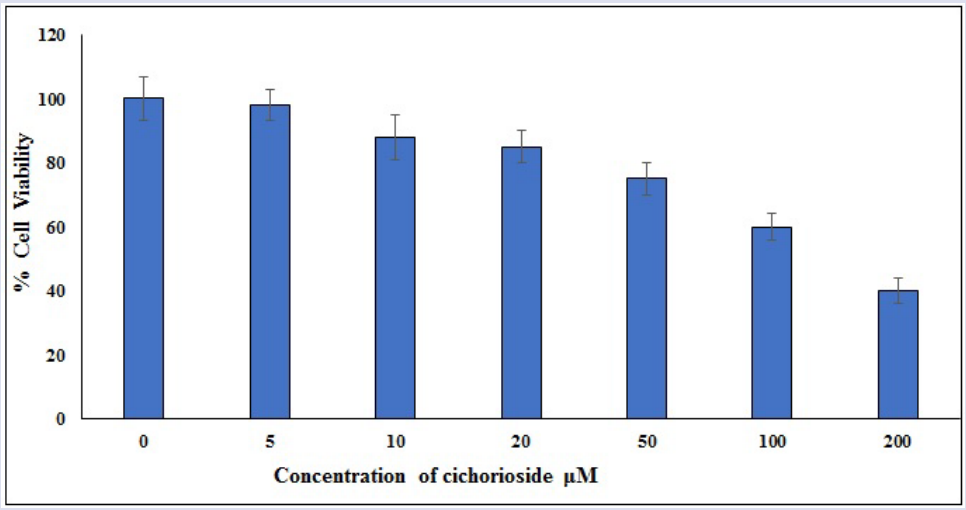

Figure 1: Effect of cichorioside on cell viability of 3T3-L1 cell line.

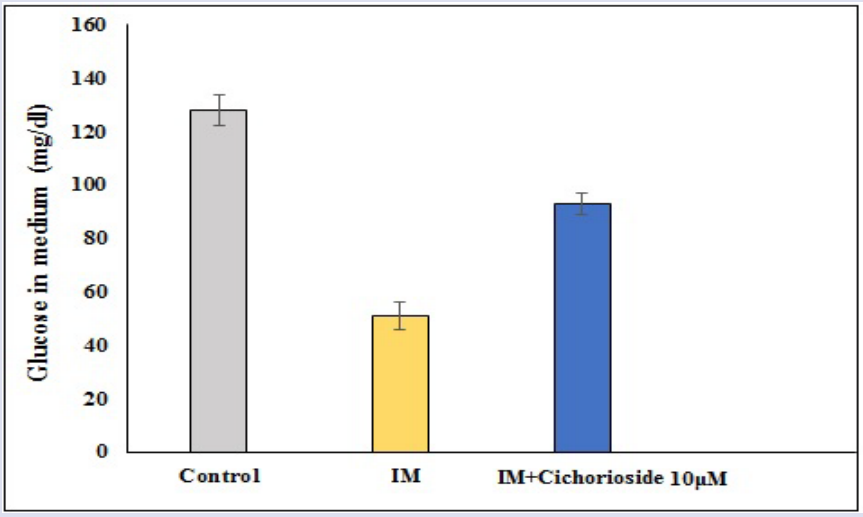

Figure 2: Effect of cichorioside on glucose uptake of 3T3-L1 cell line .

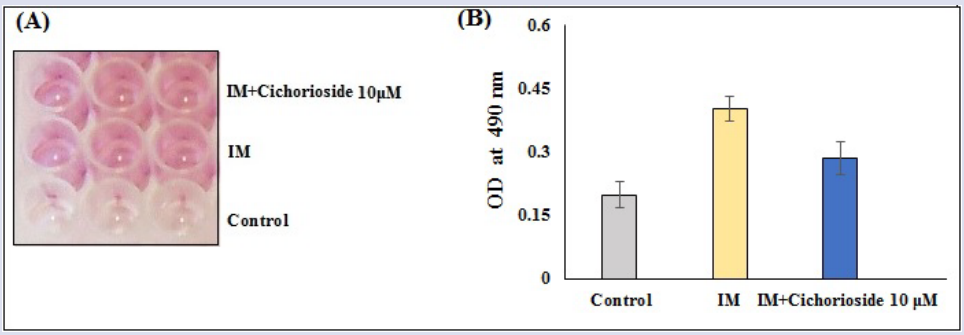

Figure 3: On 3T3-L1 pre-adipocyte differentiation to adipocytes. (A) Oil Red O staining on day 8 of culture; (B) the OD values were measured at 490 nm.

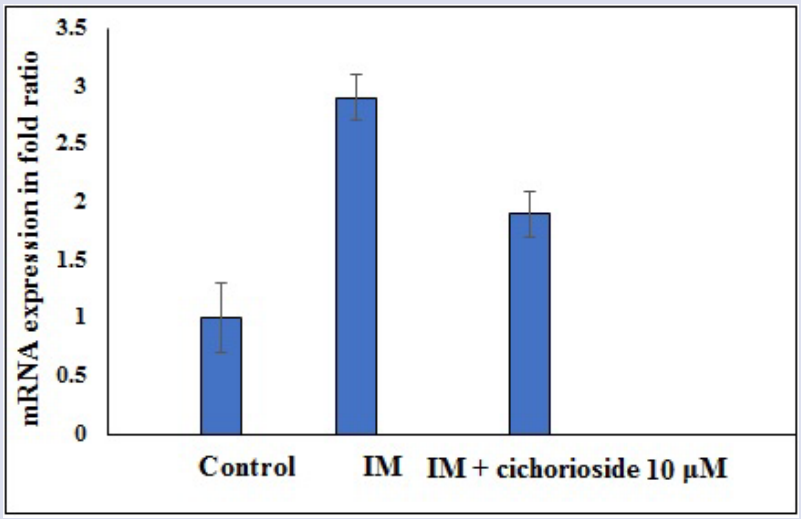

Figure 4: Effects of cichorioside on the mRNA expression of PPARγ marker in 3T3-L1 cells evaluated by real-time PCR.

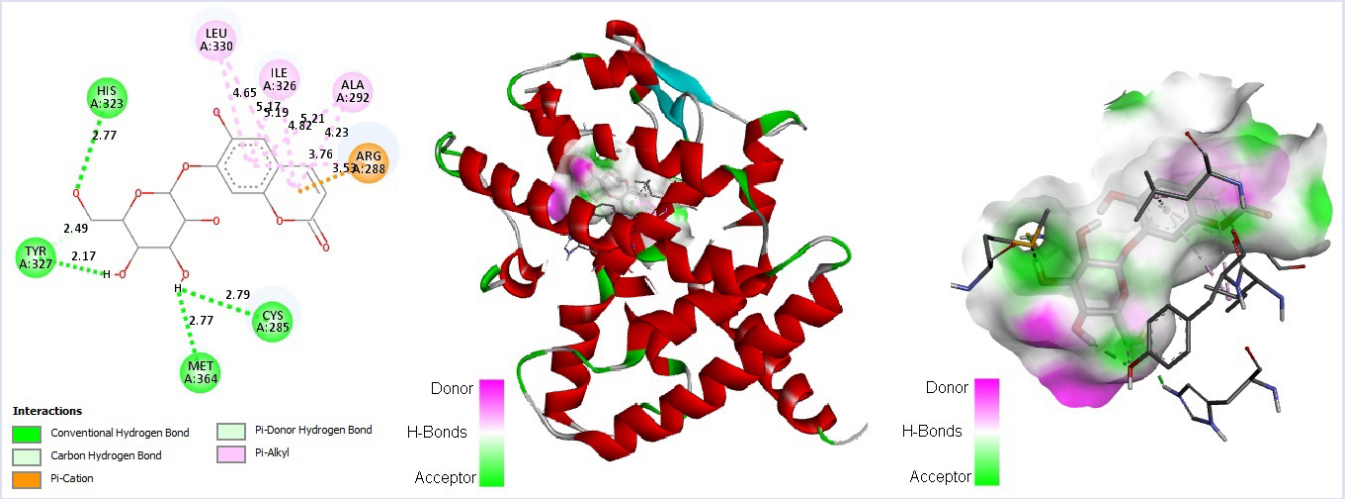

Figure 5: *In silico* docking and binding interactions of cichorioside with PPARγ receptor, showing the analysis of amino-acid interactions and their length, together with the binding pocket of ligand–receptor interactions.

the differentiation medium. On day 8 of culture, as shown by Oil Red O staining, cichorioside significantly inhibited pre-adipocyte differentiation and the lipid content in intercellular storage. Lipid content was quantified by adding isopropanol to each well to dissolve the Oil Red O, followed by measuring the OD at 490 nm. The results revealed that cichorioside led to a remarkable decrease in the OD as the concentration increased. Specifically, treatment led to significant decreases in lipid storage content when compared to the IM group (Figure 3).

Effect of cichorioside on expression of PPARγ in 3T3-L1 cells

Transcriptional markers play a major role in differentiation and functional adipogenesis including peroxisome proliferator-activated receptor gamma (PPARγ). Hence, PPARγ was quantified as anti-adipogenesis targets. The results demonstrated that cichorioside significantly downregulated adipogenesis in the 3T3-L1 cell line (Figure 4). The results showed that the mRNA expression of PPARγ was upregulated in the IM group, while cichorioside treatment downregulated its expression. Cichorioside was highly potent in controlling the expression of adipogenic marker, in addition to inhibiting lipid storage and glucose uptake in the 3T3-L1 cell line.

In silico binding of cichorioside to PPARγ adipogenic marker in 3T3-L1 Cells

The *in silico* binding of cichorioside to PPARγ receptor was evaluated to determine its effect on the regulatory amino acids. The interaction of cichorioside demonstrated a binding energy of −6.58 kcal/mol and intermolecular energy of −6.31 kcal/mol toward PPARγ through interactions with eight different amino acids namely; cysteine, methionine, tyrosine, histidine, arginine, alanine, isoleucine and leucine between residues 285 and 364, including conventional hydrogen , Pi-cation and pi–alkyl hydrophobic interactions (Figure 5 and table 2).

CONCLUSION

The current study demonstrated that cichorioside treatment suppressed lipids and glucose accumulation in 3T3-L1 cell lines. In addition, cichorioside showed upregulated expression of mRNAof PPARγ. The results displayed that cichorioside is an interesting candidate for the management of lipid and glucose storage in metabolic related

dysfunction. The study recommends further clinical studies for more exploration of cichorioside's possible importance as a strategy for management of lipid and glucose storage.

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