Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 15 (2), 2024; 399-403

Anti- adipogenic actions of *Cyperus rotundus* L. in 3T3L-1 Cells

Research Article

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Abstract

Obesity can be described as the "New World Syndrome". It is a multi-factorial disorder, which is often associated with many other significant diseases such as diabetes, hypertension and other cardiovascular diseases, osteoarthritis and certain cancers. The management of obesity will therefore require a comprehensive range of strategies focussing on existing weight problems and obesity complications. Herbal drugs are an integral part of the traditional medicines due to presence of wide range of nature made bioactive compounds. *Musta* (*Cyperus rotundus* L.) is one such drug practiced for management of obesity in Ayurveda and other traditional system of medicines. The present study reports the cytotoxicity and anti-adipogenic effect of aqueous and ethanolic extracts of *C. rotundus* in 3T3L1 cell line at concentration 1, 10 and 100 µg /ml. MTT (3-(4,5-dimetrhylthiazole-2-yl)-2,5-diphenyl tetrazolium) assay revealed non-toxic nature of both extracts. Inhibition of lipid accumulation in cells was observed significant in ethanolic extract. Significant reduction in lipid accumulation at 100 µg /ml concentration was observed in ethanol extract sample. The anti-adipogenic effect of the *Musta* is attributed to the reduced expression of PPARg (Peroxisome proliferator-activated receptor gamma), and increased expression of GLUT4 (Glucose transporter protein type-4) in 3T3 L1 cells as assessed by the RT-PCR. The *Cyperus rotundus* L. in ethanolic extract proves a potential herb for the management of obesity and related non-communicable diseases.

Keywords: Adipogenesis*, Cyperus rotundus, Musta,* Obesity, 3T3-L1, SDG 3.4.

Introduction

The prevalence rate of obesity is rising day by day and is expected to reach 167 million individuals worldwide by the year 2025 which requires new add on therapeutic safe intervention. (1) Obesity is one of the leading causes of mortality. Obesity and overweight are defined as inappropriate or excessive deposition of fats in the body. (2) Rapid urbanisation, sedentary lifestyles, poor dietary habits, restricted physical activities, etc. contribute to obesity, which is commonly seen habits nowadays. Obesity is a common risk factor for type 2 diabetes, cardiovascular diseases, hyperlipidaemia, certain types of cancer, etc. (3) For the management of obesity and associated risks, one should follow healthy diet, adequate exercise, medications, surgery and appropriate counselling.

Obesity treatment is a long-term protocol. To take synthetic medicines for long term is risk bearing deal. Long term administration of anti-obesity medication, are reported for side effects like insomnia, tremor, hypertension, tachycardia, headache, palpitation, constipation, abdominal pain, bloating, flatulence, diarrhea, oily stools etc.

Herbal medicines are most commonly used complementary and alternative medicines therapies for weight loss worldwide. (4,5) It has been reported that, herbal medicines as a treatment option is preferred by people due to reasons such as dissatisfaction with conventional treatment, past positive experiences, and family traditions. (6)

Ayurveda*;* the life science have laid down principles of disease management. *Acharya Charaka* described *Lekhaniya Mahakashya*, a group of ten medications that affect obesity in various ways. These herbal drugs are *Musta* (*Cyperus rotundus* L.), *Kustha* (*Saussarea lappa* C.B.Clarke), *Haridra* (*Curcuma longa* L.), *Daruharidra* (*Berberis aristata* DC.), *Vacha* (*Acorus calamus* Linn.), *Ativisha* (*Aconitum heterophyllum* Wall.), *Katurohini* (*Picrorhiza kurroa* Royle. Ex. Benth.), *Chitraka* (*Plumbago zeylanica* Linn.), *Chirbilv*a (*Holoptelea integrifolia* Planch.), *Hemvati* (*Iris germenica* L.) (7) Among these drugs, some drugs are used individually or in combination with other herbal drugs.

Several studies have been reported on the drugs having anti-obesity potential such as the combination of *Musta* (*Cyperus rotundus* L.), *Haimavati* (*Iris versicolor* L.) and *Chirbilva* (*Holoptelea integrifolia* Planch.) (8), *Haridra* (*Curcuma longa* L.) (9), *Triphala* (*Terminalia chebula* L., *Terminalia bellerica* L., and *Phyllanthus emblica* L.) , etc.

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Musta (*Cyperus rotundus* Linn.) is one of the best medicines used for management of obesity. (10) The present study investigated the potential of *Musta* as an anti-obesity therapeutic candidate by using 3T3-L1 cells. (11)

Materials and method

Collection of test drug

Cyperus rotundus Linn. (Cyperaceae) rhizomes were collected from Pune, India. The sample was authenticated from pharmacognosy expert. The dried rhizome powder (50 g) was extracted in 100% ethyl alcohol (400 mL) and distilled water (400 mL) by Soxhlet extraction method. The extracts were dried by using water bath technique. The 10 mg of dried forms of extract were dissolved in 1 ml of distilled water and 1 ml of ethanol.

Procurement of cell lines

3T3- L1 cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India.

Procurement of chemicals

All chemicals used namely Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, Fetal bovine serum (FBS), Dexamethasone (DEX), 3-iso butyl-1-methyl xanthine (IBMX), Indomethacin (IT), Insulin, Antibiotic-antimycotic solution, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Phosphate buffer solution (PBS), Oil red O stain, etc, were purchased from Sigma Chemicals Co., St. Louis, MO. USA were of laboratory grade.

Cell culture

P-13 (passage 13) of 3T3- L1 was used for the culture. The cells were trypsinized in a T-25 flask containing 0.25% trypsin before being seeded at a density of 1×10^3 cells per well in 96-well plates. The remaining cell suspension was then moved to a T-75 flask for the rest of the experiment. The cells were then kept at 37^{0}C and 5% CO_2 for another 24 hours.

Cytotoxicity assay

Cell viability was anlysed using MTT assay. 3T3-L1 cells were transferred into 96-well plates and allowed to grow until 80% confluency. When cells reached the required confluency, they were treated for 24 hours with different concentrations (1, 10, 20, 50 and 100 µg/ml) of *Musta* (*Cyperus rotundus* Linn.) extract in aqueous and ethanol. The culture media was taken out after 24 hours, and 50 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide MTT solution was added to the well plates. The plates were then incubated at 37^{0}C with 5% CO_2 for 4 hours. In order to dissolve the purple formazan crystals, 100 µl of DMSO (Dimethyl sulfoxide) was added to each well after the MTT solution had been incubated. The enzyme-linked immunosorbent assay (ELISA) reader was used to detect the absorbance at the optimal density of 570 nm.

3T3-L1 cell culture and differentiation

Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% antimycotic-antibiotic solution and incubated at 37^{0}C temperature, 5% CO_2. After cells were attended 80-90% confluency, they were treated with adipogenic differentiation cocktail i.e., 1.0 µM dexamethasone (DEX), 0.5 mM isobutyl methyl xanthine (IBMX) and 200 µM indomethacin (IT) with ethanol extract of *Cyperus rotundus* L. (Day 0). The cells were allowed to incubate for 48 hours at 37^{0}C with 5% CO_2. On day 3, the previously inserted media was replaced with adipogenesis progression media (DMEM with 10 µl/ml Insulin). Cells were treated with a concentration of 1,10 and 100 µg ethanol extract of *C. rotundus* L. and incubated for 48 hours. Cells were examined under microscope. On the 5th and 7th days, the freshly prepared media was added by removing the previous media. Then further incubation was done at 37^{0}C with 5% CO_2. On 8th day, cells were examined under microscope to determine whether they were differentiating into adipocytes.

Oil red O staining

The differentiated cells were subjected to staining procedure. The cells were rinsed with PBS, and 4% formaldehyde was added, followed by 15 minutes of incubation. After further washing with PBS, the oil-red O stain dissolved in isopropanol (200 µl) was inserted in the well plate, followed by incubation for 15 minutes. After that, PBS was used twice more to rinse the cells, and the plate were observed under a microscope for red staining, which indicates lipid accumulation. Quantitative estimation of the lipid-stained area was performed by using Image J analysis software.

Quantitative real-time polymerase chain reaction (RT-PCR)

The differentiated 3T3-L1 cells were collected and lysed with 300 µl Trizol. The collected cell lysate was mixed with chloroform and centrifuged to produce the RNA fraction. The tube containing cells was centrifuged at 12000 rpm for 15 minutes at 4^0 C. Then, the supernatant layer was transferred to another tube, and in that same tube, an equal amount of Isopropanol was added, mixed gently, and centrifuged as previously mentioned. In that precipitated RNA, DEPC (Diethyl Pyrocarbonate) water was added and incubated in water bath at 55^{0}C for 1 hour. For DNA synthesis, master mix solution was prepared as mentioned in Table 1. Each tube was filled with 13.2 µl of RNA sample and 6.8 µl of master mix solution. Quantstudio 5 and a high-capacity cDNA synthesis kit from Applied Biosciences were also used. The gene expression levels were evaluated by quantitative real time-PCR (Applied Biosystem) which was carried out as per the standard protocol mentioned by manufacturer (ABI, USA). The primers used for gene expression analysis of PPARg were Forward primer (FP) {5'agaccactcgcattcctttg3'}, Reverse primer (RP) {5'atcgcactttggtattcttgg3'}; for GLUT4 FP (GTAACTTCATTGTCGGCATGG), RP

Published online in http://ijam.co.in ISSN No: 0976-5921

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(AGCTGAGATCTGGTCAAACG) and β-actin RP (5'ttgcgttacacccttttcttg3'), RP (5'ttgcgttacacccttttcttg3'), β-actin was used as normal control. The relative gene expression was calculated by using ΔΔCT technique.

Results

Effect of *C. rotundus* L. extract on 3T3-L1 cell viability

After the treatment of both extracts of *C. rotundus* at a concentration of 1, 10, 20, 50 and 100 µg/ml, we found that there was no toxicity in cells, hence were chosen for further adipogenesis evaluation. (Figure 1 & 2)

Figure 1: Graph of Cell viability of 3T3 cell line with concentration of aqueous extract *C. rotundus* L.

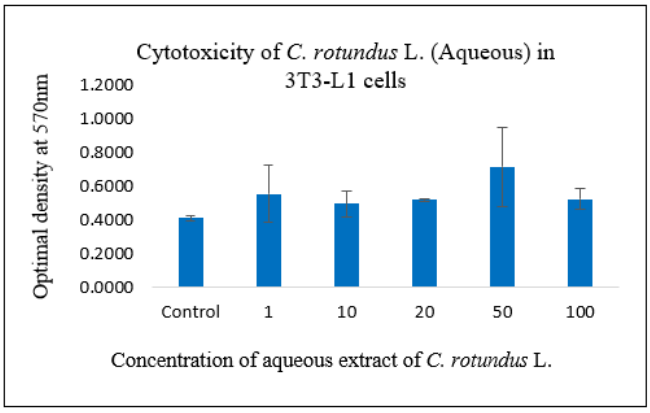
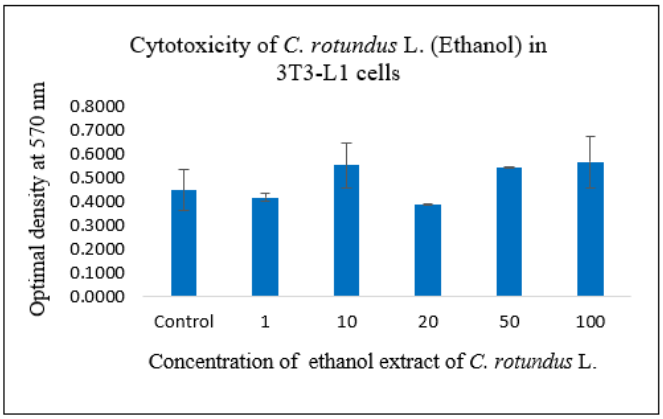


Figure 2: Graph of Cell viability of 3T3 cell line with concentration of ethanol extract of *C. rotundus* L.



Effect of *C. rotundus* L. extract on 3T3-L1 cell differentiation

3T3-L1 cells were differentiated into adipocytes along with 1, 10, and 100 µg/ml of *C. rotundus* extract for 8 days. The result observed after staining with oil red O stain showed inhibition of lipids accumulation *C. rotundus* treated cells at abovesaid concentrations. (Figure 3) Treatment with ethanol extract *C. rotundus* L. showed an 85.88 %, 90.33 %, and 90.59 % decrease in the lipid content at concentrations of 1, 10, and 100 µg/ml, respectively, which was statistically significant (P< 0.001) from the standard control i.e., adipogenic cocktail. (Figure 4) At 100 ug/ml concentration, relatively higher inhibition to lipid accumulation was observed as compared to the other concentrations through staining studies, hence the study on 100 µg/ml for evaluated for adipogenic genes expression.

Effect of *C. rotundus* L. on gene expression

The mechanism of ethanol extract of *C. rotundus* L. was investigated using the relative mRNA expression of genes involved in the adipogenic process. We studied mRNA expression of adipogenic genes such as PPARg and GLUT4 to evaluate the decrease in the lipid accumulation. The expression of PPARg was decreased by 5 folds at a concentration of 100 µg/ml as compared to standard control. (Figure 5) The GLUT4 expression showed 14.3-fold increase at a concentration of 100 µg/ml when compared to standard control. (Figure 6)

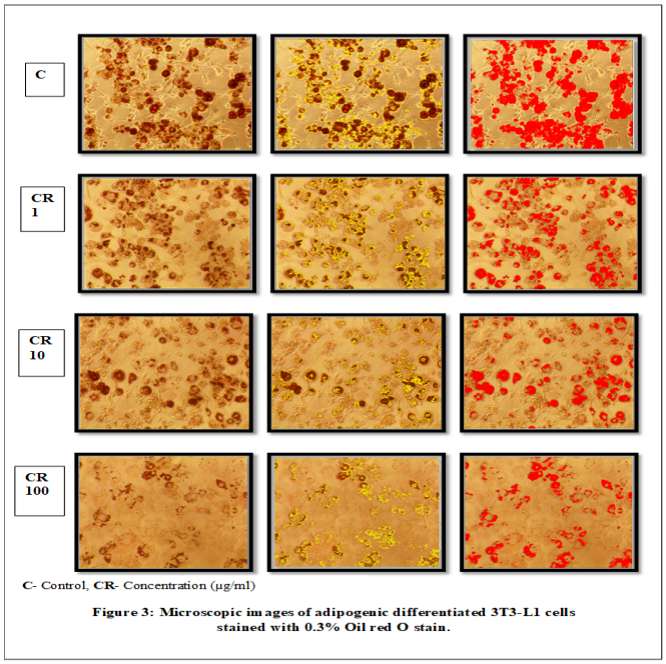
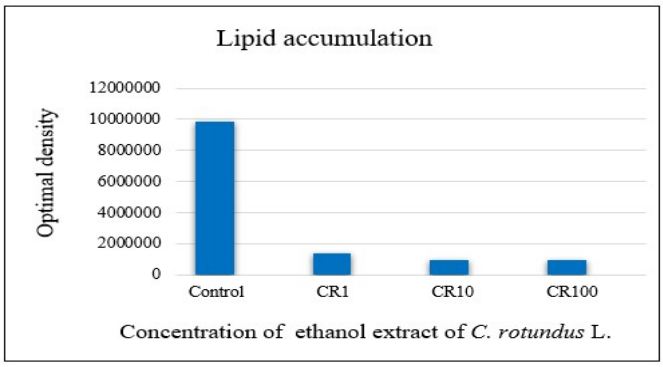


Figure 3: Microscopic images of adipogenic differentiated 3T3-L1 cells stained with 0.3% Oil red O stain.

Figure 4: Graph representing the lipid accumulation at various concentration of *C. rotundus* L. in 3T3-L1 adipocytes



[p value≤0.05, **p-value≤0.01 by one-way Anova.]

Figure 5: Mean fold expression of PPARg at concentration of 100µg/ml of *C. rotundus* L. in 3T3-L1 adipocytes

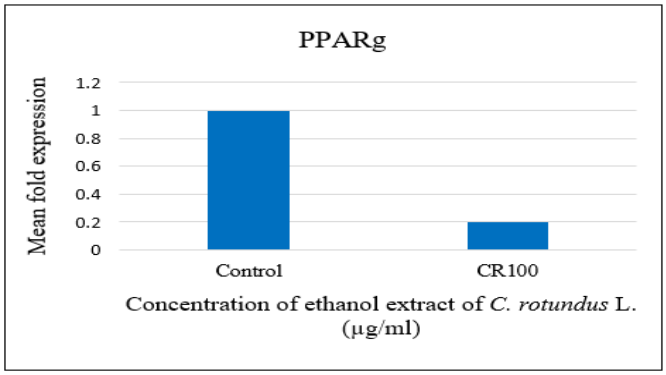
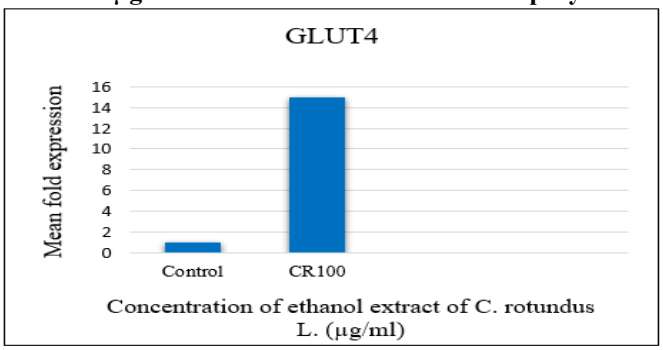


Figure 6: Mean fold expression of GLUT4 at concentration of 100µg/ml of *C. rotundus* L. in 3T3-L1 adipocytes



Discussion

The current study reports the antiadipogenic potential of *C. rotundus* L. in 3T3-L1 cell line model. Adipose tissue has a distinct function in the human body and has two main types. White adipose tissue (WAT) stores surplus energy in the form of triglycerides, whereas brown adipose tissue (BAT) utilises energy through thermogenesis. By promoting BAT development and suppressing WAT development, obesity may be managed. (12) According to many research studies, one of the negative effects of excessive adipogenesis is obesity, which can be avoided by preventing the differentiation of 3T3-L1 cells. (13) The inhibition of adipogenesis may be helpful to prevent obesity, diabetes, atherosclerosis and other related health issues. 3T3-L1cells have ability to produce homogenous population in situ and are similar in morphology and biochemically. (14)

The differentiation of preadipocytes to fully differentiated adipocytes with increasing lipid accumulation during the process is followed by an increase in the expression of several transcription factors and adipocyte-specific genes. (15) Several transcription factors such as PPARg, *fatty acid synthase (FAS), CCA AT/enhancer-binding protein (C/EBP), etc. plays important role in adipogenesis. (16) C. rotundus downregulates the expression of PPARg which is* initially regulated by C/EBP. Adipogenesis is suppressed due to blockade of C/EBP. (16) Thus *C. rotundus* further stop expression of genes involved in the intake and storage of fat is increased in adipose tissue by PPARg activation. (17) Thus, it further assists GLUT4 in the cell's signalling and functioning. (18) The GLUT4 glucose transporter is an essential regulator of the body's overall glucose homeostasis and plays a vital role in mediating the removal of glucose from the circulation. (19) GLUT4 dysfunction leads to inflammation, insulin resistance, metabolic reprogramming, cancer, and many chronic illnesses. (20) *C. rotundus* at 100 µg/ml increases the expression of GLUT4 as well.

The results of the MTT experiment demonstrate that both *C. rotundus* aqueous and ethanolic extracts were less cytotoxic. However, the amount of lipid accumulation in cells were observed less in the ethanolic extract. When cells treated with various concentrations of the ethanolic extract of *Musta* (*C. rotundus* L.) were compared to controls, it was found that the concentrations of 1 µg /ml and 10 µg /ml appeared to have comparable lower levels of lipid accumulation than controls, while the concentration of 100 µg /ml showed a reduction of lipid accumulation. Oil red O staining was slightly greater at concentrations of 1 µg /ml and 10 µg /ml of *Musta* (*C. rotundus* L.) extract compared to the control group at 100 µg /ml.

Conclusion

C. rotundus L. acts as anti-adipogenic by reducing expression of PPARg, increasing GLUT4 in 3T3 L1 cells and lowering lipid accumulation in adipocytes thus providing a lead for management of obesity and other metabolic illnesses.

Acknowledgment

Authors acknowledges to management Dr D Y Patil College of Ayurved & Research Centre and Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College and hospital, D.Y. Patil Vidyapeeth (Deemed to be University), Pimpri, Pune - 411018 for constant support and encouragement for conducting research work.

Financial support and sponsorship: NA

List of Abbreviations

Abbreviation	Elaboration
C. rotundus	*Cyperus rotundus* Linn
DEPC	Diethyl Pyrocarbonate
DEX	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FP	Forward primer
GLUT4	Glucose transporter protein type-4
IBMX	3-isobutyl-1-methyl xanthine
ITS	Indomethacin
MTT	3-(4,5-dimetrhylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffer saline
PPARg	Peroxisome proliferator-activated receptor gamma
Rf value	Retardation factor value
RP	Reverse primer
rpm	Revolution per minute
SDG	Sustainable Development Goal
ΔΔCT	Delta delta cycle threshold

Reference

1. https://www.who.int/. Last accessed on 05/11/2023.
2. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight. Last accessed on 10/11/2023.
3. https://www.fic.nih.gov/. Last accessed on 12/11/2023.
4. Barnes PM, Bloom B, Nahin RL. Complementary and alternative medicine use among adults and

children: United States. *Natl Health Stat Report*. 2008;1–23.
5. Eldalo AS, Alotaibi MN, et al. Use of herbal medicines in the treatment of obesity in taif, Saudi Arabia. *Saudi J Med Med Sci*. 2017;5(2):149–54.
6. Welz AN, Emberger-Klein A, Menrad K. Why people use herbal medicine: insights from a focus-group study in Germany. BMC Complement Altern Med. 2018; 18(1):92.
7. Shastri Kashinath, Shastri Gorakhanatha, Charaka Samhita, Chaukhambha Bharati Academy, Varanasi, Reprint:2009, Chapter 4, Pg-72.
8. Bambhole V.D. Effect of some medicinal plant preparations of adipose tissue metabolism. *Anc Sci Life*. 1988;8(2):117-124.
9. Hwang, Jung Seok & LEE, et al. Anti-adipogenic effect of a turmeric extract-loaded nanoemulsion in 3T3-L1 preadipocytes and high fat diet-fed mice. Food Science and Technology. 2019; 39.
10. Pirzada AM, Ali HH, et al. Cyperus rotundus L.: Traditional uses, phytochemistry, and pharmacological activities. J Ethnopharmacol. 2015; 174:540-560.
11. Tung Yen-Chen, Hsieh Pei-Hsuan, et al. Cellular models for the evaluation of the antiobesity effect of selected phytochemicals from food and herbs. Journal of Food and Drug Analysis. 2017;25(1): 100-110.
12. Lee, J. E., Schmidt, H., Lai, B., & Ge, K. Transcriptional and Epigenomic Regulation of Adipogenesis. Molecular and cellular biology. 2019; 39(11), e00601-18.
13. Ayyappan Prathapan, Krishna Mahesh, et al. A. Modulation of adipogenesis and glucose uptake by Curcuma longa extract in 3T3L1 and L6 cell lines -An in vitro study. Asian Pacific Journal of Tropical Disease. 2012.
14. Sarjeant Kelesha, Stephens Jacqueline M. "Adipogenesis." Cold Spring Harbor perspectives in biology. 20121 Sep;4(9).
15. Tang Qi Qun, Lane M Daniel. Adipogenesis: from stem cell to adipocyte. Annu Rev Biochem. 2012; 81:715-736.
16. Rosen, E. D., Hsu, C. H., et al. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes & development. 2002;16(1), 22–26.
17. Liao, W., Nguyen M. T., et al. Suppression of PPAR-gamma attenuates insulin-stimulated glucose uptake by affecting both GLUT1 and GLUT4 in 3T3-L1 adipocytes. American journal of physiology. Endocrinology and metabolism. 2007;293(1), E219–E227.
18. Naowaboot J., Chung C. H., et al. Mulberry leaf extract increases adiponectin in murine 3T3-L1 adipocytes. Nutrition research (New York, N.Y.). 2012;32(1), 39–44.
19. Huang S, and Czech Michael P. "The GLUT4 glucose transporter." Cell metabolism. 2007;5(4): 237-52.
20. Chang, Y. C., Chan, M. H., Yang, Y. F., Li, C. H., & Hsiao, M. Glucose transporter 4: Insulin response mastermind, glycolysis catalyst and treatment direction for cancer progression. Cancer letters. 2023: 563.

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