IN VITRO EVALUATION OF THE ANTI-OBESITY POTENTIAL OF CRASSULA OVATA STEM EXTRACT IN 3T3-L1 ADIPOCYTES

[1]Sundhararajan R, [2]Sanjukta Sainath Singh, [3]Ahmed Haji V M*

[1]Principal, Department of Pharmaceutical Chemistry, Mohamed Sathak A.J. College of Pharmacy

[2]Associate Professor, Department of Pharmacology, Mohamed Sathak A.J. College of Pharmacy

[3]Department of Pharmacology, Mohamed Sathak A.J. College of Pharmacy

The Tamilnadu DR.M.G.R Medical University, India

Corresponding Author: Ahmed Haji V M

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Abstract

Obesity is a major metabolic disorder associated with excessive lipid accumulation and adipokine dysregulation. The present study investigated the in vitro anti-obesity potential of *Crassula ovata* stem extract using 3T3-L1 preadipocyte and adipocyte models, focusing on cytotoxicity, lipid accumulation, and adiponectin secretion. The stems were extracted using ethanol by Soxhlet extraction, yielding 18.34% w/w. Cytotoxicity was evaluated using the MTT assay, which demonstrated high cell viability at 250, 500, and 1000 µg/mL, with values of 93.13%, 90.97%, and 88.91%, respectively, confirming the safety of the extract for further studies. Successful differentiation of 3T3-L1 preadipocytes into mature adipocytes was achieved by day 8 using IBMX, dexamethasone, and insulin. Oil Red O staining demonstrated a concentration-dependent reduction in intracellular lipid accumulation following treatment with *C. ovata* stem extract. Quantitative analysis showed lipid accumulation values of 86.07 ± 3.01, 70.94 ± 2.31, and 57.64 ± 1.33 at 250, 500, and 1000 µg/mL, respectively, compared with 99.9 ± 0.15 in the control group ($p < 0.0001$). Furthermore, adiponectin secretion increased dose-dependently from 0.3241 ± 0.0040 to 0.5764 ± 0.0040 ng/mL, with the highest concentration exceeding the effect of berberine (0.4975 ± 0.0020 ng/mL). The combined inhibition of lipid accumulation and enhancement of adiponectin secretion indicates a dual anti-obesity effect. These findings suggest that *C. ovata* stem extract may modulate adipocyte lipid metabolism and endocrine function, supporting its potential as a natural therapeutic candidate for obesity and associated metabolic disorders.

Key Words: Obesity, MTT assay, Soxhlet extraction, metabolic disorders

INTRODUCTION

Obesity is a chronic, multifactorial metabolic disorder characterized by an abnormal or excessive accumulation of body fat that poses a significant risk to health [1]. It is most commonly assessed using the Body Mass Index (BMI), where a BMI ≥30 kg/m² is classified as obesity, while BMI between 25–29.9 kg/m² is considered overweight. Although BMI is widely used, it does not fully reflect body fat distribution, and central (visceral) obesity, measured using waist circumference or waist-to-hip ratio, is more strongly associated with metabolic complications.

Obesity has reached pandemic proportions, affecting both developed and developing countries, including India, due to rapid urbanization, sedentary lifestyles, unhealthy dietary patterns, and genetic predisposition. The condition is no longer limited to adults; childhood and adolescent obesity have emerged as major public health concerns, increasing the risk of early-onset metabolic diseases. Obesity is a major risk factor for a wide range of non-communicable diseases, including type 2 diabetes mellitus, cardiovascular diseases, hypertension, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), polycystic ovarian syndrome (PCOS), certain cancers, and musculoskeletal disorders. Beyond physical health, obesity is associated with psychological issues such as depression, low self-esteem, and reduced quality of life.

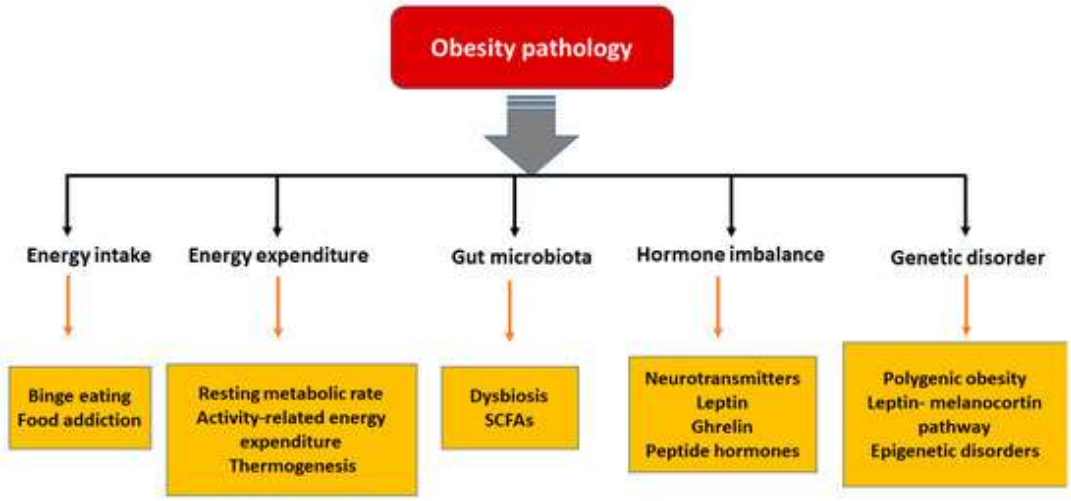


Figure 1: Pathophysiology of Obesity

PLANT PROFILE

1.1. Botanical Classification

- **Kingdom:** Plantae
- **Clade:** Tracheophytes
- **Clade:** Angiosperms
- **Clade:** Eudicots
- **Order:** Saxifragales
- **Family:** Crassulaceae
- **Genus:** *Crassula*
- **Species:** *Crassula ovata* (Miller) Druce
- *Crassula ovata* belongs to the family **Crassulaceae**, which is well known for succulent plants adapted to arid and semi-arid environments, often exhibiting Crassulacean Acid Metabolism (CAM).

Figure 2: Stems of *Crassula ovata*

Aim

The present study aimed to evaluate the anti-obesity potential of *Crassula ovata* stem extract using in vitro assays, by investigating its effects on adipocyte differentiation, lipid accumulation, and adiponectin secretion in 3T3-L1 preadipocyte cells.

Objectives

- To prepare the ethanolic extract of *Crassula ovata* stem using the Soxhlet extraction method.
- To evaluate the cytotoxicity of the stem extract and determine a safe and effective concentration using the MTT assay on 3T3-L1 preadipocytes.
- To induce the differentiation of 3T3-L1 preadipocytes into mature adipocytes and assess the effect of the stem extract on adipogenesis.
- To assess intracellular lipid accumulation in differentiated and treated adipocytes using Oil Red O staining and quantitative analysis.
- To determine the effect of the stem extract on adiponectin secretion in treated adipocytes using ELISA, thereby elucidating its role in metabolic regulation and lipid homeostasis.
- To perform appropriate statistical analyses to validate and interpret the experimental findings.

MATERIALS AND METHODS

Collection, authentication, and preparation of *Crassula ovata* extract

Fresh stems of *Crassula ovata* were collected and carefully rinsed with distilled water to eliminate surface contaminants. The cleaned stems were shade-dried at ambient temperature until complete moisture removal was achieved. The dried stems were then pulverized using a mechanical grinder and passed through a sieve to obtain a uniformly coarse powder. About 100 g of the powdered stem material was loaded into a Soxhlet apparatus and extracted with 95% ethanol (250 mL) as the extraction solvent. The extraction process was continued for approximately 8 hours until the siphoning solvent appeared clear, indicating maximum extraction of soluble phytochemicals. The resulting ethanolic extract was concentrated by solvent removal under reduced pressure using a rotary evaporator maintained at 40–45 °C. The concentrated extract was further dried in a desiccator to eliminate residual moisture, weighed to determine the extraction yield, and stored in airtight containers at 4 °C for subsequent experimental analysis.

MTT Cytotoxicity Assay

The cytotoxic effect of the *Crassula ovata* stem extract on 3T3-L1 preadipocytes was assessed using the MTT colorimetric assay. Cells were plated in 96-well culture plates at a density of 1×10^4 cells per well and incubated overnight to allow proper attachment. Subsequently, the cells were exposed to varying concentrations of the stem extract (250, 500, and 1000 µg/mL) and maintained for 24 h under controlled conditions (37 °C in a humidified atmosphere containing 5% CO_2). Following treatment, 20 µL of MTT solution (5 mg/mL prepared in PBS) was added to each well and the plates were incubated for an additional 4 h to allow the formation of formazan crystals by viable cells. The resulting crystals were solubilized using 150 µL of dimethyl sulfoxide (DMSO), and the absorbance was recorded at 570 nm using a microplate reader. Cell viability was expressed as a percentage in comparison with untreated control cells, and concentrations showing minimal cytotoxicity were selected for further experimental studies.

Cell Culture and Differentiation

3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution, and maintained at 37 °C in a humidified incubator with 5% CO_2. Cells were allowed to proliferate until complete confluence and then maintained for an additional two days to attain growth arrest, which was designated as day 0. Adipogenic differentiation was initiated by replacing the culture medium with an induction medium comprising 0.5 mM isobutylmethylxanthine (IBMX), 1 µM dexamethasone, and 10 µg/mL insulin, and cells were incubated for two days (day 2). Following induction, the medium was substituted with a differentiation maintenance medium containing 10 µg/mL insulin and maintained for another two days (day 4). Thereafter, the cells were cultured in post-differentiation medium and continued up to day 8 to ensure full maturation into adipocytes. Throughout the differentiation process, cells were treated with *Crassula ovata* stem extract at concentrations of 250, 500, and 1000 µg/mL. The extract solution (250 µL) was added to each well containing 1.25 mL of culture medium to achieve a final volume of 1.5 mL. A standard reference drug was included under identical experimental conditions for comparative evaluation

Oil Red O Staining of Cells

Intracellular lipid deposition in differentiated and extract-treated adipocytes was evaluated using Oil Red O staining. On the eighth day of differentiation, the cells were gently washed twice with phosphate-buffered saline (PBS) and subsequently fixed with 10% formalin for 30 minutes at room temperature. Following fixation, the cells were thoroughly rinsed with distilled water to remove residual fixative and then incubated with freshly prepared 60% Oil Red O staining solution for 30 minutes. Excess stain was removed by washing the cells repeatedly with distilled water. Stained lipid droplets within the cells were observed and documented using a light microscope. For quantitative analysis, the bound dye was solubilized by adding isopropanol to each well, and after an incubation period of 30 minutes, the extracted dye solution was collected. The absorbance was measured at 490 nm using a microplate reader to determine the relative lipid content.

Statistical Analysis

All experimental results were expressed as the mean ± standard error of the mean (SEM) of triplicates. Statistical analysis was performed using GraphPad Prism software version 8.0. Differences between groups were analyzed, and values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

1.1.Extraction of *Crassula ovata* flowers

The ethanolic extraction of *Crassula ovata* stem using the Soxhlet apparatus yielded a dark greenish-brown semisolid extract. From 100 g of dried and powdered stem material, the percentage yield of the ethanolic extract was calculated to be 18.34% (w/w). The relatively high yield indicates efficient extraction of ethanol-soluble phytoconstituents present in the stem of *C. ovata*.

The use of 95% ethanol as the extraction solvent played a crucial role in achieving this yield, as ethanol is known for its ability to dissolve a broad spectrum of phytochemicals, including phenolics, flavonoids, tannins, glycosides, and certain terpenoids. Soxhlet extraction further ensured continuous contact between the solvent and plant material, facilitating exhaustive extraction of bioactive compounds.

1.1.1. Significance of Extraction Yield

The percentage yield obtained in the present study (18.34%) is considered pharmacognostically significant, particularly for a succulent plant such as *Crassula ovata*. Succulent stems are generally rich in water-retaining tissues; however, the observed yield suggests the presence of substantial quantities of ethanol-extractable secondary metabolites beyond structural polysaccharides.

A higher extraction yield is often correlated with:

- Increased availability of phytochemicals for biological screening
- Improved reproducibility in in vitro assays
- Enhanced feasibility for scale-up and formulation development

In phytopharmacological studies, yield serves as a preliminary indicator of the plant's chemical richness and extraction efficiency. The yield obtained in this study supports the selection of *C. ovata* stem as a suitable plant part for further biological evaluation.

1.1.2. Influence of Extraction Method

The Soxhlet extraction technique used in the present investigation offers several advantages, including continuous solvent recycling, uniform heat distribution, and effective penetration of solvent into plant matrices. These factors collectively contribute to improved extraction efficiency compared to cold maceration or simple percolation techniques.

The extraction duration of approximately 8 hours was sufficient to exhaustively extract the phytoconstituents, as evidenced by the colorless appearance of the siphoning solvent. This observation confirms that prolonged extraction beyond this duration may not significantly enhance yield and could potentially lead to thermal degradation of heat-sensitive compounds.

Additionally, concentration of the extract under reduced pressure at 40–45 °C minimized the loss of volatile compounds and prevented thermal decomposition, ensuring preservation of the chemical integrity of the extract.

Although limited scientific literature is available specifically on the extraction yield of *Crassula ovata* stem, studies on other members of the Crassulaceae family have reported

moderate to high yields using polar solvents such as ethanol and methanol. Plants belonging to this family are known to contain:

- Polyphenols
- Organic acids
- Flavonoids
- Triterpenes
- Polysaccharides

The yield obtained in the present study is comparable with ethanolic extracts of other medicinal succulents, indicating that *C. ovata* stem may serve as a promising source of bioactive constituents.

Post-extraction drying in a desiccator and storage at 4 °C in airtight containers were employed to preserve the stability of the extract. These conditions are essential to prevent oxidative degradation, microbial growth, and moisture absorption, which could otherwise compromise the quality and biological activity of the extract.

The semisolid nature and uniform consistency of the extract further indicate proper solvent removal and suitability for dissolution in appropriate vehicles for in vitro testing.

1.2.MTT assay

1.2.1. Effect of *Crassula ovata* Stem Extract on Cell Viability

The cytotoxic potential of the ethanolic stem extract of *Crassula ovata* was evaluated in 3T3-L1 preadipocytes using the MTT assay, a widely accepted method for assessing mitochondrial metabolic activity and cellular viability. The results demonstrated a concentration-dependent reduction in cell viability following 24 h exposure to the extract; however, the reduction remained within acceptable limits, indicating minimal cytotoxicity.

Table 2: Effect of *Crassula ovata* Stem Extract on Cell Viability (%)

Concentration (µg/mL)	Berberine (% Cell Viability, Mean ± SD)	*Crassula ovata* (% Cell Viability, Mean ± SD)
250	97.64 ± 0.19	93.13 ± 0.29
500	95.25 ± 0.20	90.97 ± 0.32
1000	91.32 ± 0.71	88.91 ± 0.45

At a concentration of 250 µg/mL, the *C. ovata* stem extract exhibited 93.13 ± 0.29% cell viability, suggesting that the majority of cells retained normal metabolic activity. When the concentration was increased to 500 µg/mL, cell viability decreased slightly to 90.97 ± 0.32%, while at 1000 µg/mL, viability was recorded as 88.91 ± 0.45%. These findings indicate that even at the highest tested concentration, the extract did not induce severe cytotoxic effects on 3T3-L1 preadipocytes.

Berberine, used as the standard reference compound, showed comparatively higher cell viability across all concentrations, with values of 97.64 ± 0.19%, 95.25 ± 0.20%, and 91.32 ± 0.71% at 250, 500, and 1000 µg/mL, respectively. Although berberine demonstrated slightly superior cytocompatibility, the difference between berberine and *C. ovata* extract was not substantial, highlighting the relative safety of the plant extract.

1.2.2. Selection of Non-Cytotoxic Concentrations

In cytotoxicity screening for adipogenesis studies, concentrations maintaining ≥85–90% cell viability are generally considered safe for further functional assays. In the present study, all tested concentrations of *C. ovata* stem extract maintained viability above 88%, confirming their suitability for subsequent experiments involving adipocyte differentiation, lipid accumulation, and adipokine secretion.

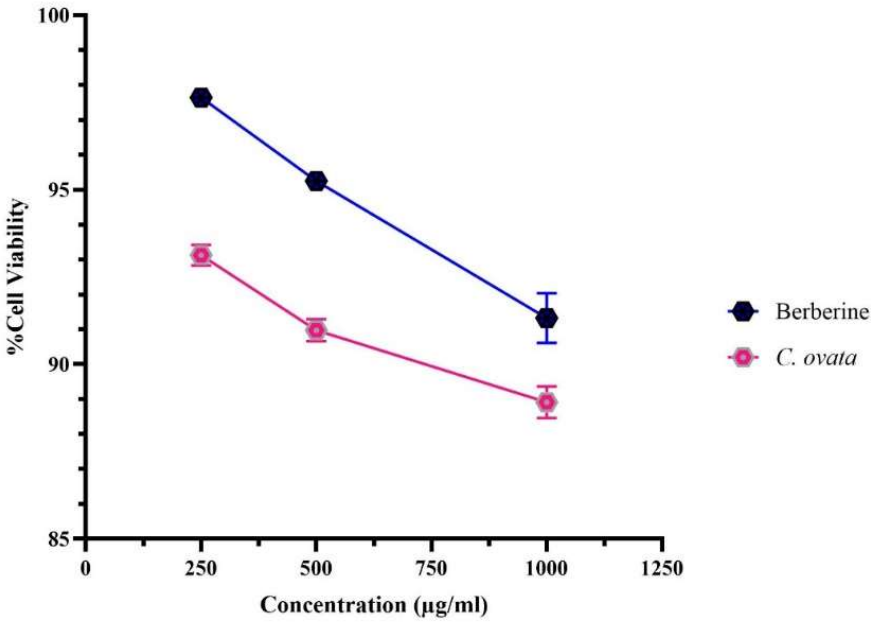


Figure 3: Effect of *Crassula ovata* Stem Extract on Cell Viability (%)

Based on these observations, 250, 500, and 1000 µg/mL were selected as non-toxic working concentrations for downstream anti-obesity evaluations. The results confirm that any observed anti-adipogenic or lipid-modulating effects in later experiments are unlikely to be attributed to nonspecific cytotoxicity.

1.1.1. Mechanistic Basis of the MTT Assay

The MTT assay is based on the ability of mitochondrial succinate dehydrogenase enzymes in viable cells to reduce the yellow tetrazolium salt (MTT) into insoluble purple formazan crystals. The quantity of formazan formed is directly proportional to the number of metabolically active cells.

The preservation of high absorbance values at 570 nm in extract-treated cells indicates that the *C. ovata* stem extract does not significantly impair mitochondrial enzymatic activity at the tested concentrations. This suggests that the extract does not induce mitochondrial dysfunction, oxidative stress-mediated cell death, or membrane destabilization under the experimental conditions.

1.1.2. Possible Cellular Mechanisms Underlying Low Cytotoxicity

The minimal cytotoxicity observed with *C. ovata* stem extract may be attributed to the presence of bioactive phytochemicals with cytoprotective properties, such as:

- Phenolic compounds, known to scavenge reactive oxygen species (ROS)
- Flavonoids, which stabilize mitochondrial membranes and modulate cellular redox balance
- Terpenoids and organic acids, which may regulate intracellular signaling without inducing apoptosis

These compounds may collectively contribute to maintaining cellular integrity and metabolic function, thereby preventing cytotoxic damage in preadipocytes.

Furthermore, plants belonging to the Crassulaceae family are known for their antioxidant potential, which may counteract oxidative stress and support cell survival. This protective effect is particularly important in adipocyte studies, as oxidative stress plays a critical role in adipogenesis and metabolic dysregulation.

1.2. Effect of *Crassula ovata* Stem Extract on Adipocyte Differentiation

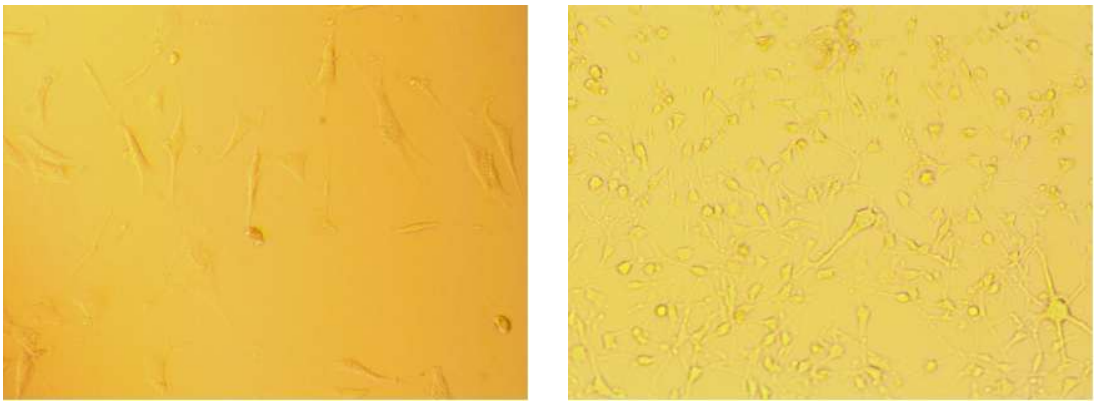
The effect of the ethanolic stem extract of *Crassula ovata* on adipogenic differentiation was evaluated using the well-established 3T3-L1 preadipocyte model. Under control conditions, untreated 3T3-L1 cells underwent normal differentiation following hormonal induction, exhibiting typical morphological changes such as increased cell size and intracellular lipid droplet formation by day 8, confirming successful adipocyte maturation.

Treatment with *C. ovata* stem extract during the differentiation period resulted in a concentration-dependent inhibition of adipocyte differentiation. Cells exposed to the extract at 250 µg/mL showed a noticeable reduction in adipocyte maturation compared to untreated differentiated controls. This effect became more pronounced at 500 µg/mL, where fewer cells exhibited adipocyte-like morphology, and lipid droplet formation appeared markedly reduced. At the highest concentration (1000 µg/mL), differentiation was substantially suppressed, with cells retaining a fibroblast-like appearance and showing minimal morphological features characteristic of mature adipocytes.

These observations indicate that *C. ovata* stem extract effectively interferes with the adipogenic program in 3T3-L1 cells without inducing cytotoxicity, as confirmed by prior MTT assay results.

1.2.1. Role of the Extract During Different Stages of Differentiation

Adipocyte differentiation is a tightly regulated, multistep process involving growth arrest, early transcriptional activation, and terminal lipid accumulation. The continuous presence of *C. ovata* stem extract throughout the differentiation period suggests that the extract may act on both early and late stages of adipogenesis.



Preadipocyte Adipocytes

Figure 4: Differentiation of preadipocytes to adipocytes

During the early induction phase (day 0–2), the hormonal cocktail (IBMX, dexamethasone, and insulin) normally activates key adipogenic transcription factors. The observed reduction in differentiation upon extract treatment suggests possible inhibition of early signaling pathways responsible for initiating adipogenesis. Additionally, the persistence of reduced adipocyte maturation during later stages (day 4–8) indicates that the extract may also interfere with lipid biosynthesis and storage mechanisms essential for terminal differentiation.

1.2.2. Proposed Mechanism of Action

The anti-adipogenic effect of *C. ovata* stem extract may be attributed to multiple complementary mechanisms:

a) Modulation of Adipogenic Transcription Factors

Adipocyte differentiation is primarily regulated by transcription factors such as ***peroxisome proliferator-activated receptor gamma (PPARγ)*** and ***CCAAT/enhancer-binding protein alpha (C/EBPα)***. These factors drive the expression of adipocyte-specific genes involved in lipid uptake and storage.

Bioactive phytochemicals present in **C. ovata** stem extract—particularly flavonoids and phenolic compounds—may suppress the activation or expression of these transcription factors, thereby inhibiting the adipogenic cascade. Downregulation of PPARγ activity is known to result in reduced adipocyte formation and lipid accumulation.

a) Interference with Insulin Signaling

Insulin plays a crucial role in adipogenesis by promoting glucose uptake and lipogenesis. The extract may attenuate insulin-mediated signaling pathways, leading to reduced activation of downstream molecules such as AKT and SREBP-1c, which are essential for adipocyte maturation and lipid synthesis.

b) Activation of Energy-Regulating Pathways

Plant-derived polyphenols have been reported to activate ***AMP-activated protein kinase (AMPK)***, a key regulator of cellular energy homeostasis. Activation of AMPK suppresses adipogenesis by inhibiting lipid synthesis and promoting fatty acid oxidation. The observed anti-adipogenic effect of **C. ovata** stem extract may therefore be mediated, at least in part, through AMPK activation.

c) Antioxidant-Mediated Regulation

Oxidative stress has been implicated in adipocyte differentiation and metabolic dysfunction. The antioxidant constituents of **C. ovata** stem extract may reduce intracellular oxidative stress, thereby modulating redox-sensitive signaling pathways involved in adipogenesis.

The inhibition of adipocyte differentiation is a key therapeutic target in obesity management, as excessive formation of adipocytes contributes to increased fat mass and metabolic

imbalance. The ability of **C. ovata** stem extract to suppress adipogenesis in 3T3-L1 cells suggests its potential role in preventing adipose tissue expansion.

By targeting early transcriptional events and metabolic pathways involved in adipocyte maturation, the extract may help regulate lipid storage and improve metabolic homeostasis. These findings support the traditional and emerging interest in plant-based interventions for obesity and metabolic disorders.

1.1. Lipid accumulation

The accumulation of intracellular lipids is a defining characteristic of adipocyte maturation and a critical indicator of adipogenesis. In the present study, lipid deposition in differentiated 3T3-L1 adipocytes was assessed using Oil Red O staining following treatment with ethanolic stem extract of *Crassula ovata*. Qualitative microscopic observation and quantitative spectrophotometric analysis collectively demonstrated a marked, concentration-dependent reduction in lipid accumulation in extract-treated cells.

Untreated differentiated control cells exhibited intense Oil Red O staining, characterized by abundant and densely packed red lipid droplets occupying a significant portion of the cytoplasmic area. This confirms successful adipogenic differentiation under standard hormonal induction conditions. In contrast, cells treated with *C. ovata* stem extract showed visibly reduced staining intensity, indicating decreased intracellular lipid storage.

1.1.1. Quantitative Analysis of Lipid Content

Quantitative measurement of Oil Red O dye eluted from the cells revealed a statistically significant reduction in lipid accumulation in all treatment groups when compared with the control. The control group exhibited a lipid content of 99.90 ± 0.15%, representing maximal lipid accumulation. Treatment with the standard drug berberine resulted in a substantial reduction in lipid content to 69.41 ± 2.14%, validating the sensitivity and reliability of the experimental model.

The *C. ovata* stem extract demonstrated a dose-dependent inhibitory effect on lipid accumulation. At 250 µg/mL, lipid content was reduced to 86.07 ± 3.01%, indicating moderate inhibition of adipocyte lipid storage. Increasing the concentration to 500 µg/mL led to a pronounced reduction to 70.94 ± 2.31%, closely comparable to the effect observed with

berberine. At the highest concentration tested (1000 µg/mL), lipid accumulation was significantly suppressed to 57.64 ± 1.33%, representing the strongest inhibitory effect among the extract-treated groups.

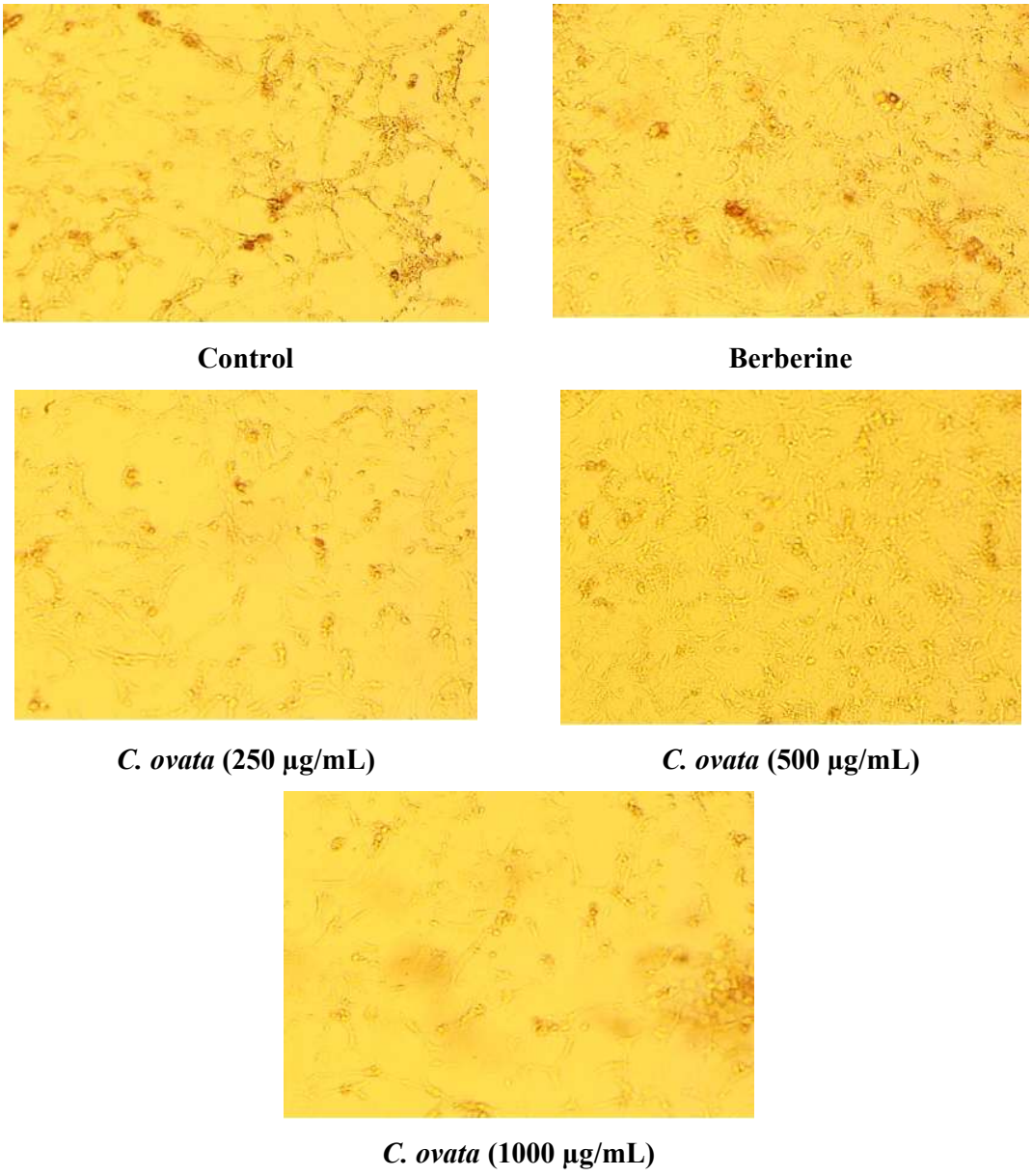


Figure 5: Oil red O staining of adipocytes with *C. ovata* treatment

Statistical analysis revealed that all treatment groups showed **highly significant differences (**$p < 0.0001$) when compared with the control group, confirming the robustness and reproducibility of the observed effects.

1.1.2. Morphological Correlation with Quantitative Findings

The quantitative results were strongly supported by microscopic observations. Control adipocytes displayed large, coalesced lipid droplets typical of mature adipocytes. In contrast, extract-treated cells exhibited fewer and smaller lipid droplets, with a noticeable reduction in droplet density as extract concentration increased. At 1000 µg/mL, lipid droplets were sparse and poorly developed, and cells retained a morphology closer to undifferentiated fibroblast-like cells.

This morphological evidence confirms that the reduction in lipid accumulation was not an artifact of dye elution or measurement but reflected genuine suppression of intracellular lipid synthesis and storage.

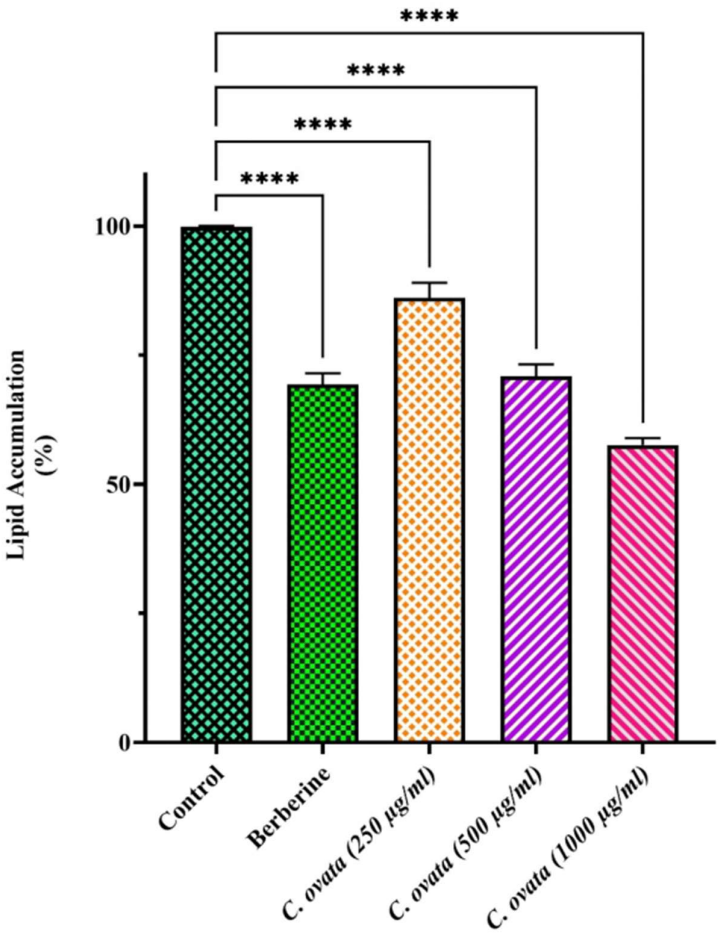


Figure 6: Effect of *Crassula ovata* Stem Extract on lipid accumulation (%)

1.1.3. Mechanistic Basis for the Reduction of Intracellular Lipid Accumulation by *Crassula ovata* Stem Extract

a) Modulation of Adipogenic Transcription Factors (PPARγ and C/EBPα)

Adipocyte differentiation is a highly regulated biological process governed primarily by the coordinated activation of adipogenic transcription factors, particularly peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα). These transcription factors act as master regulators of adipogenesis by initiating and sustaining the expression of genes involved in lipid uptake, triglyceride synthesis, and adipocyte maturation. In differentiated 3T3-L1 adipocytes, PPARγ and C/EBPα synergistically promote the transcription of adipocyte-specific markers such as adipocyte fatty acid–binding protein (aP2), lipoprotein lipase (LPL), and fatty acid synthase (FASN), ultimately leading to extensive intracellular lipid accumulation.

Table 3: Effect of *Crassula ovata* Stem Extract on lipid accumulation (%)

Treatment / Concentration (µg/mL)	Mean (%)	SD
Control	99.90	0.1482
Berberine	69.41	2.1422
C. ovata (250 µg/mL)	86.07	3.01
C. ovata (500 µg/mL)	70.94	2.31
C. ovata (1000 µg/mL)	57.64	1.3275

The significant, dose-dependent reduction in Oil Red O–stained lipid droplets observed following treatment with *Crassula ovata* stem extract strongly suggests an inhibitory effect on the activation or expression of these transcription factors. Phytochemicals such as flavonoids, phenolic acids, and terpenoids—commonly reported in succulent plants—are known to interfere with transcriptional regulation by modulating nuclear receptor activity. By suppressing PPARγ and C/EBPα signaling, the extract may disrupt the adipogenic cascade at an early stage, preventing the commitment of preadipocytes to the adipocyte lineage and reducing the expression of genes essential for lipid storage. This transcriptional suppression ultimately manifests as decreased triglyceride accumulation, as evidenced by the reduced Oil Red O staining intensity.

Importantly, inhibition of adipogenic transcription factors does not require cytotoxicity, and the maintained cell viability observed in the MTT assay supports the hypothesis that *C. ovata* stem extract exerts a targeted regulatory effect rather than inducing cell death. This mechanism is particularly relevant in anti-obesity research, as selective inhibition of adipogenesis is considered a safer and more sustainable therapeutic strategy compared to cytotoxic approaches.

b) Inhibition of De Novo Lipogenesis and Triglyceride Synthesis

De novo lipogenesis is a critical metabolic pathway in adipocytes, responsible for converting excess glucose and other substrates into fatty acids, which are subsequently esterified into triglycerides and stored within lipid droplets. Key enzymes involved in this process include acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), both of which are transcriptionally regulated by adipogenic signaling pathways. Enhanced activity of these enzymes leads to increased lipid droplet formation and adipocyte hypertrophy, a hallmark of obesity.

The pronounced reduction in intracellular lipid content following *C. ovata* stem extract treatment suggests a strong inhibitory effect on lipogenic pathways. Phytochemicals such as polyphenols and flavonoids are well documented to suppress lipogenic enzyme activity either directly, by inhibiting enzyme function, or indirectly, by downregulating gene expression. By limiting the synthesis of fatty acids and triglycerides, the extract effectively reduces lipid droplet expansion within adipocytes.

Moreover, reduced lipid accumulation at higher extract concentrations indicates that the extract may influence both early lipid synthesis and later stages of triglyceride storage. This dual action is particularly advantageous, as it prevents not only the formation of new lipids but also the excessive accumulation of existing lipid reserves. The close similarity between the lipid-reducing effect of *C. ovata* stem extract and the standard drug berberine further supports the likelihood of shared lipogenesis-inhibitory mechanisms.

c) Activation of AMPK and Regulation of Energy Homeostasis

AMP-activated protein kinase (AMPK) functions as a central metabolic sensor that maintains cellular energy balance. When activated, AMPK suppresses anabolic processes such as lipid and cholesterol synthesis while promoting catabolic pathways including fatty acid oxidation.

In adipocytes, AMPK activation leads to inhibition of ACC and downregulation of lipogenic gene expression, thereby reducing triglyceride synthesis and lipid storage.

The substantial suppression of lipid accumulation observed with *C. ovata* stem extract, particularly at higher concentrations, strongly indicates potential activation of AMPK signaling pathways. Many plant-derived bioactive compounds, including flavonoids and phenolics, are known AMPK activators and exert anti-obesity effects through this mechanism. By activating AMPK, the extract may shift cellular metabolism away from lipid storage toward energy utilization, thereby limiting adipocyte hypertrophy.

Activation of AMPK also antagonizes PPARγ-mediated adipogenesis, further reinforcing the extract's ability to suppress adipocyte differentiation and lipid accumulation. This integrated metabolic regulation is particularly significant, as AMPK activation not only reduces fat accumulation but also improves insulin sensitivity and glucose homeostasis, both of which are commonly impaired in obesity.

d) Modulation of Insulin Signaling and Glucose Utilization

Insulin plays a pivotal role in adipogenesis by stimulating glucose uptake and promoting lipogenesis within adipocytes. Enhanced insulin signaling increases intracellular glucose availability, which serves as a substrate for fatty acid and triglyceride synthesis. Dysregulated insulin signaling is therefore closely associated with excessive lipid accumulation and adipocyte enlargement.

The reduced lipid deposition observed following *C. ovata* stem extract treatment suggests possible modulation of insulin-mediated pathways. By attenuating insulin signaling or reducing glucose flux into adipocytes, the extract may limit substrate availability for lipogenesis. This mechanism aligns with previous reports of plant-derived compounds that reduce adipocyte lipid accumulation by improving insulin sensitivity while simultaneously suppressing lipogenic pathways.

Such modulation of insulin signaling is particularly relevant for metabolic health, as it may prevent insulin resistance—a key pathological feature of obesity and type 2 diabetes.

e) Antioxidant-Mediated Regulation of Adipogenesis

Oxidative stress has emerged as an important contributor to adipocyte differentiation and lipid accumulation. Elevated reactive oxygen species (ROS) levels promote adipogenic signaling pathways and enhance lipid synthesis. Antioxidant compounds counteract these effects by reducing oxidative stress and suppressing redox-sensitive transcription factors involved in adipogenesis.

Crassula ovata stem extract is likely to contain antioxidant phytochemicals that mitigate oxidative stress within adipocytes. By lowering ROS levels, the extract may indirectly inhibit adipogenic differentiation and lipid accumulation. This antioxidant-mediated mechanism complements the direct inhibition of transcriptional and metabolic pathways, resulting in a comprehensive anti-adipogenic effect.

1.1.4. Relevance of Lipid Accumulation Inhibition to Obesity Management

Obesity is characterized by excessive expansion of adipose tissue resulting from both increased adipocyte number (hyperplasia) and enlarged adipocyte size (hypertrophy). Intracellular lipid accumulation is central to both processes, as it reflects enhanced adipogenesis and lipid storage capacity. Persistent lipid overload in adipocytes leads to adipose tissue dysfunction, chronic low-grade inflammation, insulin resistance, and metabolic syndrome.

The ability of *Crassula ovata* stem extract to significantly reduce intracellular lipid accumulation directly addresses a fundamental pathological feature of obesity. By suppressing lipid deposition within adipocytes, the extract may limit adipose tissue expansion and prevent the progression of obesity-related metabolic complications. Unlike cytotoxic agents that reduce adipocyte number through cell death, the extract appears to act by modulating metabolic and transcriptional pathways, offering a safer and more physiologically relevant approach.

Furthermore, reduced lipid accumulation in adipocytes is closely associated with improved adipokine secretion profiles, including enhanced adiponectin levels, which play a protective role in metabolic regulation. By limiting adipocyte hypertrophy, the extract may help maintain adipose tissue functionality and systemic metabolic balance.

The dose-dependent and highly significant reduction in lipid accumulation (****$p < 0.0001$) observed in this study highlights the strong therapeutic potential of *C. ovata* stem extract as a

natural anti-obesity agent. Its efficacy comparable to, or exceeding, that of berberine at higher concentrations further underscores its relevance for plant-based obesity management strategies.

1.2. *Role of Adiponectin*

Adiponectin secretion by differentiated 3T3-L1 adipocytes was significantly modulated following treatment with *Crassula ovata* stem extract, as quantified by ELISA. Untreated control cells exhibited a basal adiponectin level of 0.2974 ± 0.0034 ng/mL, reflecting normal adipokine secretion under standard differentiation conditions. Treatment with the reference compound berberine resulted in a marked elevation of adiponectin levels (0.4975 ± 0.0020 ng/mL), confirming its established adiponectin-enhancing and insulin-sensitizing activity.

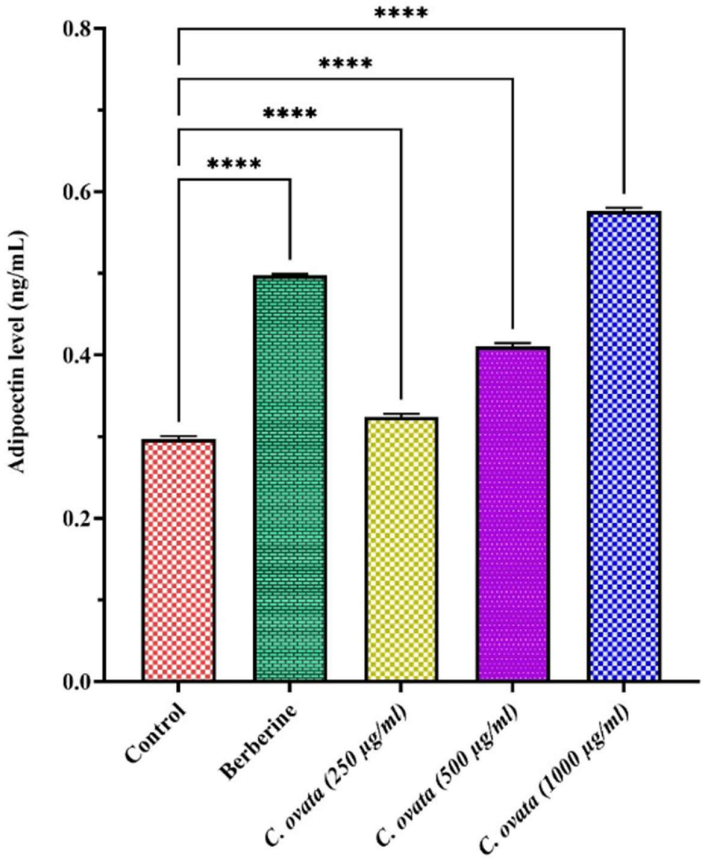


Figure 7: Effect of *Crassula ovata* Stem Extract on adiponectin

Cells treated with *C. ovata* stem extract demonstrated a dose-dependent increase in adiponectin secretion. At 250 µg/mL, adiponectin levels rose moderately to 0.3241 ± 0.0040 ng/mL, showing a statistically significant increase compared with the control group (****$p < 0.0001$).

Further elevation was observed at 500 µg/mL, with adiponectin levels reaching 0.4107 ± 0.0040 ng/mL, indicating enhanced adipocyte functionality and improved adipokine secretion. The highest concentration tested (1000 µg/mL) resulted in the greatest increase in adiponectin levels (0.5764 ± 0.0040 ng/mL), surpassing even the standard drug treatment.

Table 4: Effect of *Crassula ovata* Stem Extract on adiponectin

Treatment / Concentration (µg/mL)	Mean (ng/mL)	SD
Control	0.2974	0.0034
Berberine	0.4975	0.0020
C. ovata (250 µg/mL)	0.3241	0.0040
C. ovata (500 µg/mL)	0.4107	0.0040
C. ovata (1000 µg/mL)	0.5764	0.0040

All treatment groups exhibited **highly significant differences (**$p < 0.0001$) when compared to the untreated control, demonstrating that *C. ovata* stem extract effectively stimulates adiponectin production in mature adipocytes.

1.2.1. Effect of Crassula ovata *on Adiponectin Secretion*

Adiponectin is a crucial adipocyte-derived hormone that plays a central role in regulating glucose metabolism, lipid homeostasis, and insulin sensitivity. Reduced adiponectin levels are strongly associated with obesity, insulin resistance, metabolic syndrome, and type 2 diabetes. Therefore, therapeutic strategies aimed at restoring or enhancing adiponectin secretion are considered highly valuable in obesity management.

In the present study, treatment with *Crassula ovata* stem extract significantly enhanced adiponectin secretion in differentiated 3T3-L1 adipocytes in a concentration-dependent manner. The progressive increase in adiponectin levels observed with increasing extract concentrations suggests that bioactive constituents present in the stem exert a stimulatory effect on adipocyte endocrine function rather than causing cytotoxic stress. This finding aligns with earlier MTT assay results, which confirmed the non-toxic nature of the extract at the tested concentrations.

The increase in adiponectin levels at 250 µg/mL indicates early modulation of adipocyte signaling pathways, while the pronounced elevation at 500 and 1000 µg/mL reflects a strong enhancement of adipocyte metabolic health. Notably, the adiponectin levels observed at the highest concentration exceeded those produced by berberine, suggesting that *C. ovata* stem extract may possess potent adiponectin-regulatory activity.

1.2.2. Mechanisms Involved in Obesity Regulation through Adiponectin

a) Adiponectin–AMPK Axis in Obesity

One of the most critical mechanisms linking adiponectin to obesity regulation is its ability to activate the ***AMP-activated protein kinase (AMPK) pathway***, a master regulator of cellular energy homeostasis. AMPK functions as an intracellular energy sensor that responds to changes in the AMP/ATP ratio, switching cellular metabolism from anabolic to catabolic processes during energy deficit. Adiponectin binding to its receptors, AdipoR1 and AdipoR2, leads to phosphorylation and activation of AMPK, particularly in skeletal muscle and liver tissues.

In lean individuals, adequate adiponectin levels maintain continuous AMPK activation, promoting fatty acid oxidation, enhancing glucose uptake, and inhibiting lipid synthesis. This metabolic balance prevents excessive lipid storage and ensures efficient utilization of energy substrates. However, in obesity, circulating adiponectin levels are significantly reduced, leading to impaired AMPK signaling. As a result, fatty acid oxidation is suppressed, while lipogenesis is enhanced through increased activity of enzymes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). This metabolic shift contributes directly to adipocyte hypertrophy and ectopic lipid accumulation.

Furthermore, reduced AMPK activity in obesity diminishes GLUT4 translocation to the plasma membrane in skeletal muscle, impairing glucose uptake and exacerbating hyperglycemia and insulin resistance. The decline in AMPK signaling also disrupts mitochondrial biogenesis and function, leading to reduced energy expenditure and increased oxidative stress. Thus, adiponectin deficiency establishes a metabolic environment favorable to fat accumulation, impaired glucose homeostasis, and progression of obesity-related complications.

Restoration of adiponectin levels has been shown to reactivate AMPK signaling, reverse lipid accumulation, and improve insulin sensitivity. Therefore, modulation of the adiponectin–AMPK axis represents a pivotal therapeutic target in obesity management.

b) Role of Adiponectin in Regulating PPARα and Fatty Acid Oxidation

Another crucial mechanism through which adiponectin exerts anti-obesity effects is the regulation of ***peroxisome proliferator-activated receptor alpha (PPARα)***, a nuclear receptor involved in lipid metabolism. PPARα controls the transcription of genes responsible for mitochondrial and peroxisomal β-oxidation of fatty acids, thereby promoting lipid catabolism and preventing lipid accumulation.

Adiponectin enhances PPARα expression and activity, particularly in hepatic tissue, leading to increased fatty acid oxidation and reduced triglyceride storage. In obesity, reduced adiponectin levels result in diminished PPARα activation, causing a decline in fatty acid oxidation capacity. This promotes hepatic steatosis and contributes to the development of non-alcoholic fatty liver disease (NAFLD), a common obesity-associated disorder.

The impairment of PPARα signaling also leads to reduced mitochondrial efficiency and decreased energy expenditure. Consequently, excess fatty acids are redirected toward adipose tissue storage, exacerbating adipocyte enlargement and metabolic stress. The inability to efficiently oxidize fatty acids further contributes to lipotoxicity in insulin-sensitive tissues, aggravating insulin resistance.

By restoring adiponectin-mediated activation of PPARα, fatty acid oxidation can be enhanced, lipid burden reduced, and metabolic flexibility improved. This mechanism highlights adiponectin's role as a metabolic switch that determines whether fatty acids are stored or utilized for energy, making it central to obesity regulation.

c) Anti-Inflammatory Mechanism of Adiponectin in Obesity

Chronic low-grade inflammation is a hallmark of obesity and plays a decisive role in the development of insulin resistance and metabolic dysfunction. Adiponectin possesses strong ***anti-inflammatory properties***, distinguishing it from other adipokines such as leptin and resistin, which promote inflammation. In healthy adipose tissue, adiponectin suppresses inflammatory signaling pathways, thereby maintaining metabolic homeostasis.

Mechanistically, adiponectin inhibits nuclear factor-kappa B (NF-κB) signaling, reducing the expression of pro-inflammatory cytokines including TNF-α, IL-6, and MCP-1. It also promotes the polarization of macrophages toward the anti-inflammatory M2 phenotype, thereby limiting immune cell infiltration into adipose tissue. In obesity, reduced adiponectin levels remove this protective anti-inflammatory control, resulting in sustained activation of inflammatory cascades.

Inflammation directly suppresses adiponectin gene expression, creating a vicious cycle wherein decreased adiponectin amplifies inflammation, which in turn further reduces adiponectin production. This inflammatory milieu disrupts insulin signaling by inducing serine phosphorylation of insulin receptor substrates, thereby impairing glucose uptake and promoting insulin resistance.

By restoring adiponectin levels, inflammatory stress within adipose tissue can be attenuated, improving insulin responsiveness and metabolic function. This mechanism underscores the importance of adiponectin as a molecular bridge linking obesity, inflammation, and metabolic disease.

1.2.3. Adiponectin and Insulin Sensitivity in Obesity

Adiponectin plays a direct and critical role in enhancing ***insulin sensitivity***, making it a central regulator in obesity-associated insulin resistance. Adiponectin improves insulin signaling by activating the IRS-1/PI3K/Akt pathway, which enhances glucose uptake and suppresses hepatic glucose production.

In obesity, hypoadiponectinemia impairs insulin receptor signaling, leading to decreased glucose uptake in muscle and adipose tissue and increased gluconeogenesis in the liver. This contributes to hyperglycemia, compensatory hyperinsulinemia, and eventual β-cell dysfunction. The reduction in adiponectin further aggravates insulin resistance by increasing lipid accumulation and inflammation, both of which interfere with insulin signaling.

Restoration of adiponectin improves insulin responsiveness across multiple tissues, highlighting its therapeutic relevance in obesity-associated diabetes. This mechanism demonstrates that adiponectin deficiency is not merely a marker of obesity but a causal factor in metabolic dysregulation.

1.2.4. Adiponectin in Adipocyte Differentiation and Endocrine Function

Adiponectin is essential for maintaining healthy adipocyte differentiation and endocrine function. It promotes the formation of smaller, insulin-sensitive adipocytes rather than hypertrophic, dysfunctional fat cells. In obesity, reduced adiponectin disrupts adipocyte maturation, leading to enlarged adipocytes with impaired metabolic activity.

Dysfunctional adipocytes exhibit reduced adiponectin secretion and increased release of pro-inflammatory cytokines and free fatty acids, further worsening metabolic imbalance. This altered adipocyte phenotype contributes to systemic insulin resistance and lipid dysregulation.

By supporting proper adipocyte differentiation and endocrine balance, adiponectin helps maintain adipose tissue health and prevents obesity-associated complications.

SUMMARY AND CONCLUSION

- The study evaluated the anti-obesity potential of *Crassula ovata* stem extract using 3T3-L1 preadipocyte and adipocyte models to assess cytotoxicity, lipid accumulation, and adiponectin secretion.
- Ethanolic Soxhlet extraction of *C. ovata* stems yielded 18.34% w/w, indicating efficient extraction of ethanol-soluble bioactive constituents.
- The MTT assay demonstrated high cell viability in 3T3-L1 preadipocytes following extract treatment, confirming the non-toxic nature of the extract.
- At concentrations of 250, 500, and 1000 µg/mL, cell viability remained above 88%, with values of 93.13%, 90.97%, and 88.91%, respectively.
- The standard drug berberine showed slightly higher viability (97.64–91.32%), validating the assay and serving as a positive control.
- Based on cytotoxicity results, all tested concentrations of *C. ovata* extract were considered safe for adipocyte differentiation studies.
- Differentiation of 3T3-L1 preadipocytes into mature adipocytes was successfully achieved by day 8 using IBMX, dexamethasone, and insulin induction.
- Treatment with *C. ovata* stem extract during differentiation modulated adipocyte lipid metabolism without impairing cell morphology or viability.
- Oil Red O staining qualitatively showed a visible reduction in intracellular lipid droplets in extract-treated adipocytes compared to control cells.

- Quantitative Oil Red O analysis revealed reduced lipid accumulation at 250 µg/mL (86.07 ± 3.01) and 500 µg/mL (70.94 ± 2.31) relative to control (99.9 ± 0.15).
- At 1000 µg/mL, lipid content was further decreased (57.64 ± 1.33), indicating strong suppression of intracellular triglyceride deposition.
- All extract-treated groups showed **high statistical significance (**$p < 0.0001$) when compared with the control group.
- Adiponectin secretion in untreated adipocytes was 0.2974 ± 0.0034 ng/mL, representing basal adipokine levels.
- Berberine treatment significantly increased adiponectin levels to 0.4975 ± 0.0020 ng/mL, confirming its adiponectin-enhancing effect.
- *C. ovata* stem extract increased adiponectin secretion in a dose-dependent manner to 0.3241 ± 0.0040, 0.4107 ± 0.0040, and 0.5764 ± 0.0040 ng/mL at 250, 500, and 1000 µg/mL, respectively.
- The adiponectin levels at 1000 µg/mL exceeded those of the standard drug, indicating potent adipocyte endocrine modulation.
- The increase in adiponectin suggests improved insulin sensitivity, enhanced fatty acid oxidation, and restoration of adipocyte metabolic function.
- Mechanistically, the observed effects may involve activation of AMPK signaling, regulation of PPARα-mediated lipid metabolism, and suppression of lipogenic pathways.
- The combined reduction in lipid accumulation and elevation of adiponectin highlights a dual anti-obesity mechanism targeting both adipogenesis and adipokine dysregulation.
- Overall, *Crassula ovata* stem extract demonstrated significant anti-obesity potential in vitro, supporting its future development as a natural therapeutic agent for obesity and metabolic disorders.

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