INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD)
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CYTOPROTECTIVE AND LIPID-MODULATORY EFFECTS OF CALCAREA CARBONICA 200CH ON PALMITIC ACID-INDUCED LIPOTOXICITY IN 3T3-L1 MOUSE ADIPOCYTES: AN IN VITRO STUDY

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Abstract: Palmitic acid, a saturated free fatty acid elevated in obesity and metabolic syndrome, induces lipotoxic stress in adipocytes, impairing cell viability and disrupting cholesterol/lipid handling. Calcarea carbonica is a homoeopathic drug traditionally indicated in constitutions associated with obesity and disordered fat metabolism, but its cytoprotective potential has not been characterized at the cellular level. To evaluate the cytoprotective effect and lipid transport modulation of Calcarea carbonica 200CH against palmitic acid-induced lipotoxicity in 3T3-L1 mouse adipocytes using MTT cell viability assay, phase-contrast morphological assessment, and Oil Red O staining. 3T3-L1 cells were cultured under standard conditions and exposed to palmitic acid (500 µM) to induce lipotoxic injury, followed by treatment with Calcarea carbonica 200CH at serial dilutions (1:25, 1:50, 1:100, 1:200, 1:400). Cell viability was assessed by MTT assay at 540 nm after 24 hours. Morphological changes were recorded by inverted phase-contrast microscopy. Oil Red O staining (read at 510 nm) was used to quantify percentage cholesterol transport in control, palmitic acid-only, and palmitic acid + Calcarea carbonica 200CH (1:200) groups. Data were analysed by one-way ANOVA with Dunnett's post-hoc test (mean ± SE, triplicate wells). Palmitic acid reduced cell viability to 51.19% relative to untreated control (100%; $p<0.0001$), confirming lipotoxic injury. Co-treatment with Calcarea carbonica 200CH produced a concentration-dependent protective effect, with the 1:200 dilution showing maximal protection (81.27% viability, $p<0.0001$ vs palmitic acid); the 1:100 dilution was not significantly different from palmitic acid alone (ns). Oil Red O staining showed that palmitic acid abolished cholesterol transport (0.00%) compared with control (70.48%), while co-treatment with Calcarea carbonica 200CH (1:200) partially restored transport to 54.37%. These findings suggest that *Calcarea carbonica* 200CH exerts concentration-dependent cytoprotective activity against palmitic acid-induced lipotoxicity in 3T3-L1 adipocytes and may influence lipid metabolic processes under experimental conditions. Further mechanistic studies and in vivo investigations are warranted to elucidate the underlying pathways and determine the translational relevance of these findings.

IndexTerms **- Calcarea carbonica; Cytoprotection; Homoeopathy; Palmitic acid; Lipotoxicity; 3T3-L1 adipocytes; MTT assay; Oil Red O staining.**

I.INTRODUCTION

Obesity has emerged as one of the most pressing public health challenges of the twenty-first century. Global estimates indicate that more than one billion people worldwide are now living with obesity, and the worldwide prevalence of obesity has more than doubled since 1990, with the World Health Organization formally recognizing it as a global epidemic. [1,2] This rising burden is not confined to high-income nations; low- and middle-income countries are now experiencing accelerating rates of obesity alongside persisting under-nutrition, creating a dual burden of malnutrition (3). Obesity is a major independent risk factor for cardiovascular disease, type 2 diabetes mellitus, hypertension, and several non-communicable diseases, and its downstream metabolic consequences continue to drive global mortality.[3]

At the cellular level, obesity and its complications are increasingly understood through the lens of lipotoxicity — the pathological accumulation of free fatty acids (FFAs) and lipid intermediates that exceeds the storage capacity of adipose tissue and spills over into ectopic sites, triggering oxidative stress, inflammation, and cell injury.[4] Palmitic acid, the most abundant saturated dietary fatty acid, is a well-characterized inducer of this lipotoxic state. In adipocyte models, palmitic acid has been shown to activate the local renin–angiotensin system via Toll-like receptor 4/NF-κB signalling [5], promote reactive oxygen species generation and

inflammatory changes through pathways involving membrane phospholipid remodelling [6], and induce adipocyte hypertrophy with disturbed lipid and adipokine profiles.[7] Because of this reproducible, well-defined injury profile, palmitic acid challenge is now a standard in vitro strategy for screening the cytoprotective or lipid-modulatory potential of test compounds against metabolic/lipotoxic stress.[8]

The 3T3-L1 mouse fibroblast-derived adipocyte cell line is the most extensively validated in vitro model for such studies. It reliably differentiates and accumulates intracellular lipid droplets under adipogenic stimulation, and its response to palmitic acid — reduced viability, oxidative stress, and altered lipid handling — closely mirrors adipocyte dysfunction observed in obesity.[9] Consequently, 3T3-L1 cells challenged with palmitic acid have been widely used to evaluate the anti-obesity and cytoprotective potential of natural and pharmacological agents, including plant polyphenols and phytoconstituents that attenuate palmitic acid-induced oxidative stress, inflammation, and lipid accumulation.[8]

Given the adverse effect profile and limited long-term sustainability of conventional anti-obesity pharmacotherapy, there is growing interest in complementary and alternative systems of medicine as adjunctive or safer long-term options.[8] Homoeopathy has long employed constitutional prescribing in patients with a predisposition to obesity and disordered fat metabolism, and Calcarea carbonica — prepared by trituration of the middle layer of oyster shell — is among the most frequently indicated constitutional remedies in such presentations. Classical materia medica consistently describes the Calcarea carbonica constitution as fair, flabby, chilly, and prone to obesity with sluggish digestion and impaired assimilation [10-12], and it is formally listed among remedies indicated for obesity in contemporary homoeopathic clinical guidance.[13] Despite this long clinical tradition, pre-clinical evidence characterizing the direct cellular actions of Calcarea carbonica on lipid-stressed adipocytes — as opposed to its use in whole-organism or clinical case-based reports — remains essentially absent from the literature.

This gap between longstanding clinical usage and cellular-level pharmacological evidence formed the rationale for the present study. We hypothesised that Calcarea carbonica 200CH would confer a measurable cytoprotective and lipid-modulatory effect against palmitic acid-induced lipotoxic injury in 3T3-L1 adipocytes. Accordingly, this study was designed to evaluate the effect of Calcarea carbonica 200CH, across a range of dilutions, on palmitic acid (500 µM)-induced cytotoxicity in 3T3-L1 cells using the MTT viability assay and phase-contrast morphological assessment, and to determine whether the concentration providing maximal protection also restores palmitic acid-induced disruption of intracellular cholesterol/lipid handling, assessed by Oil Red O staining

NEED OF THE STUDY.

The rapidly rising global prevalence of obesity, now affecting over one billion people worldwide, has created a serious problem of lipotoxicity-driven adipocyte dysfunction that underlies much of the metabolic disease burden associated with excess body weight. The seriousness of palmitic acid-induced lipotoxic injury has been increasingly brought to light through cellular studies demonstrating that excess saturated free fatty acids overwhelm the storage capacity of adipose tissue, spill over into ectopic sites, and trigger oxidative stress, inflammation, and cell death. Studies carried out on 3T3-L1 adipocyte models indicate that exposure to palmitic acid at concentrations in the range of 100–500 µM can reduce cell viability by approximately 40–50% and substantially disrupt normal lipid and cholesterol handling within 24 hours of exposure.

Among all pharmacological options available for managing lipid-related metabolic dysfunction, statins remain the most widely prescribed, yet a considerable proportion of patients experience statin-associated adverse effects — including muscle symptoms, new-onset diabetes, hepatic and renal injury, and cognitive effects — that limit their long-term use in a substantial subset of the population.[14] This gap has directed increasing attention toward complementary and traditional systems of medicine, including homoeopathy, in which Calcarea carbonica has long been indicated in individuals with a constitutional predisposition to obesity and disordered fat metabolism.

The knowledge of how Calcarea carbonica acts at the cellular level on lipotoxically stressed adipocytes remains inadequately established, despite its extensive traditional clinical use. Understanding this cellular action is important to improve the scientific and mechanistic basis on which such remedies are evaluated, and to determine whether their traditional indications can be corroborated through controlled experimental models. The parameters requiring focused evaluation include cell viability under lipotoxic challenge, morphological indicators of cytotoxicity, and quantitative measures of intracellular lipid/cholesterol handling — each of which was specifically assessed in the present study.

II.MATERIAL AND METHODS:

2.1 Cell line and culture conditions:

The 3T3-L1 (mouse adipocyte) cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA / Gibco-Invitrogen). Cells were cultured in 25 cm^2 tissue culture flasks in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco, USA), L-glutamine, sodium bicarbonate (Merck, Germany), and an antibiotic solution containing penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL). Cultures were maintained at 37 °C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

2.2 Test drug and induction of lipotoxicity:

Calcarea carbonica 200CH was filtered through a 0.22 µm Millipore syringe filter prior to use to ensure sterility. Palmitic acid (500 µM) was used as the lipotoxic-injury-inducing agent in all experiments.

2.3 MTT cytotoxicity/protection assay:

A two-day-old confluent monolayer of 3T3-L1 cells was trypsinised and resuspended in growth medium, and 100 µL of cell suspension (5×10^3 cells/well) was seeded into 96-well tissue culture plates and incubated at 37 °C in 5% CO_2. After attaining sufficient growth, palmitic acid (500 µM) was added to induce toxicity and incubated for one hour. Freshly prepared Calcarea

carbonica 200CH was then added at dilutions of 1:25, 1:50, 1:100, 1:200 and 1:400 (µL in DMEM), each in triplicate. Untreated control wells and palmitic acid-only wells were maintained in parallel. Plates were incubated for 24 hours at 37 °C in 5% CO_2.

2.3.1 Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed after 24 hours of treatment under an inverted phase-contrast tissue-culture microscope (Olympus CKX41 with Optika Pro5 CCD camera, 10× magnification), noting rounding, shrinkage, granulation, or vacuolization as indicators of cytotoxic change.

2.3.2 Cytotoxicity Assay by MTT Method:

Quantitative viability was determined by MTT assay. Fifteen milligrams of MTT (Sigma, M-5655) were dissolved in 3 mL PBS and filter-sterilised. After the 24-hour incubation, well contents were removed and 30 µL of MTT solution was added to each well; plates were incubated for a further 4 hours at 37 °C in 5% CO_2. The supernatant was then discarded and 100 µL of DMSO (Sigma-Aldrich, USA) was added to solubilise the formazan crystals. Absorbance was read at 540 nm on a microplate reader (Laura B. Talarico et al., 2004).[15-17]

Percentage viability was calculated as: (Mean OD of sample / Mean OD of control) × 100.

2.4 Oil Red O staining for cholesterol/lipid transport:

3T3-L1 cells were seeded at 10^5 cells/well in 24-well plates. Palmitic acid (500 µM) was added and incubated for 1 hour, followed by treatment with Calcarea carbonica 200CH at the 1:200 dilution (the concentration showing maximal protection in the MTT assay) for 24 hours at 37 °C in 5% CO_2. Untreated control and palmitic acid-only wells were maintained in parallel.

Cells were washed three times with ice-cold PBS and fixed with 10% formaldehyde (11.0 mL of 37% formaldehyde diluted with 29.0 mL PBS) for 30 minutes. After fixation, cells were washed and stained with Oil Red O working solution (0.5 g Oil Red O powder in isopropanol, stirred overnight, filtered through two-layer Whatman paper, then diluted in 60% ethanol/distilled water and re-filtered) for 15 minutes at room temperature, then washed with PBS to remove unbound stain. For quantification, DMSO was added to each well, plates were shaken for 5 minutes at room temperature, and absorbance was read at 510 nm on a spectrophotometer. Percentage cholesterol transport was calculated relative to the untreated control.

$$\text{Percentage Cholesterol Transport: } \frac{(\text{Palmitic Acid Control OD} - \text{Sample OD})}{\text{Palmitic Acid Control OD}} \text{ X } 100$$

2.5 Statistical analysis:

All experiments were performed in triplicate and results are expressed as mean ± standard error (SE). Data were analysed by one-way ANOVA followed by Dunnett's post-hoc test, comparing treatment groups against the palmitic acid-induced group. A value of $p<0.0001$ was considered statistically significant (denoted ***); ns denotes a non-significant difference.

2.6 Instruments and Reagents:

Item	Source
DMEM media	Sigma-Aldrich, USA (D5648)
Fetal bovine serum	Gibco, USA
0.25% Trypsin	Invitrogen, USA (25200-056)
Micropipettes	F1 Thermo Scientific, USA
CO2 incubator	Eppendorf, Germany
Phase-contrast microscope	Olympus, Japan, with Optika Pro5 camera
MTT reagent	Sigma-Aldrich (M5655)
Microplate / ELISA reader	ERBA, Germany
Culture plates and flasks	NUNC, Thermo Scientific, USA
Oil Red O powder	Fisher Biotech

Table 1 – Instruments and Reagents used for the study

III. RESULTS AND DISCUSSION

3.1 MTT assay: percentage viability of 3T3-L1 cells treated with palmitic acid (500 µM) with or without Calcarea carbonica 200CH:

Exposure to palmitic acid (500 µM) alone reduced 3T3-L1 cell viability to 51.19 ± 1.17% relative to the untreated control (100 ± 0%; $p<0.0001$), confirming induction of cellular stress and cytotoxicity. Co-treatment with Calcarea carbonica 200CH produced a concentration-dependent protective effect: viability was 62.94 ± 0.39% at 1:400, rising to a maximum of 81.27 ± 0.37% at 1:200 ($p<0.0001$ vs palmitic acid), before declining to 51.36 ± 0.17% at 1:100 (not significant vs palmitic acid), 36.14 ± 0.32% at 1:50,

and 17.55 ± 0.61% at 1:25 ($p<0.0001$ for both, indicating loss of protection / added toxicity at higher concentrations). The 1:200 dilution was therefore identified as the optimal cytoprotective concentration and was carried forward to the Oil Red O experiment.

Table 2. MTT assay: percentage viability of 3T3-L1 cells treated with palmitic acid (500 µM) with or without Calcarea carbonica 200CH (mean ± SE, n=3)

Group / dilution	OD I	OD II	OD III	Mean OD	% Viability (mean ± SE)
Control	0.6859	0.6715	0.6794	0.6789	100.00 ± 0.00
Palmitic acid (500 µM)	0.3415	0.3594	0.3416	0.3475	51.20 ± 1.17 ***
PA + Calcarea carb. 1:400	0.4321	0.4269	0.4228	0.4273	62.93 ± 0.39 ***
PA + Calcarea carb. 1:200	0.5550	0.5431	0.5571	0.5517	81.26 ± 0.37 ***
PA + Calcarea carb. 1:100	0.3521	0.3469	0.3471	0.3487	51.36 ± 0.17 ns
PA + Calcarea carb. 1:50	0.2516	0.2429	0.2416	0.2454	36.14 ± 0.32 ***
PA + Calcarea carb. 1:25	0.1126	0.1244	0.1203	0.1191	17.55 ± 0.61 ***

*** $p<0.0001$, ns = not significant, both vs palmitic acid-induced group (one-way ANOVA, Dunnett's post-hoc test).

Table 2 presents the MTT assay results showing the effect of *Calcarea carbonica* 200CH on palmitic acid-induced cytotoxicity in 3T3-L1 adipocytes. The control group showed **100% cell viability**, while **500 µM palmitic acid** reduced viability to **51.20%**. Among the treatment groups, the **1:200 dilution** exhibited the highest cytoprotective effect, restoring cell viability to **81.26%**, followed by the **1:400 dilution (62.93%)**. The **1:100 dilution** showed no significant effect, whereas the **1:50** and **1:25 dilutions** further reduced cell viability. These findings indicate a concentration-dependent cytoprotective effect of *Calcarea carbonica* 200CH, with the **1:200 dilution demonstrating the greatest protection** against palmitic acid-induced lipotoxicity.

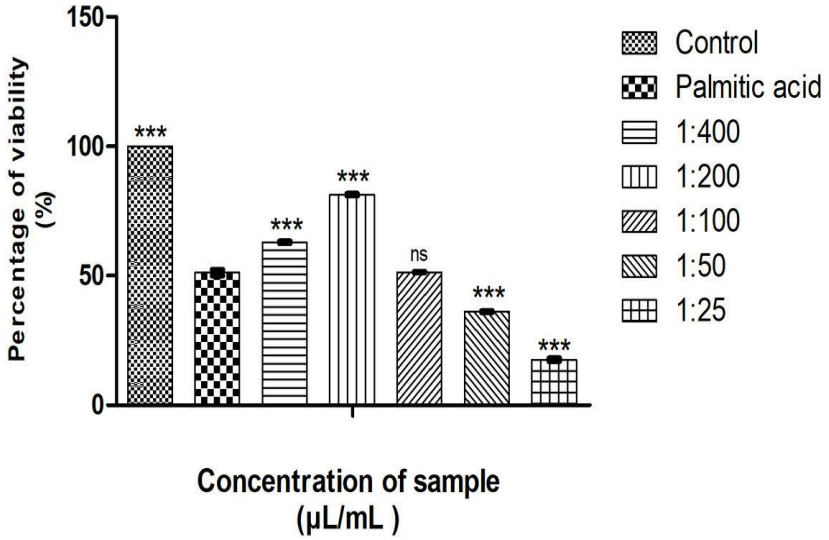

Figure 1. Cytoprotective effect of Calcarea carbonica 200CH on palmitic acid–induced cytotoxicity in 3T3-L1 cells (MTT assay). Y-axis: percentage viability; X-axis: concentration of sample. Data are mean ± SE (n=3). ***p<0.0001, ns = not significant vs palmitic acid-induced group (one-way ANOVA, Dunnett's test).

3.2 Morphological assessment by phase-contrast microscopy:

Direct microscopic observation 24 hours after treatment corroborated the MTT findings. Untreated control cells displayed a normal, healthy adherent morphology with intact confluent colonies (Fig. 2A). Palmitic acid-treated cells showed reduced cell density with scattered rounding and shrinkage consistent with lipotoxic injury (Fig. 2B). Wells treated with lower dilutions of Calcarea carbonica 200CH (1:50, 1:25) showed sparse, rounded cells with reduced adherent colony formation (Fig. 2C, 2D), in keeping with the loss of protection/added toxicity observed at these concentrations in the MTT assay.

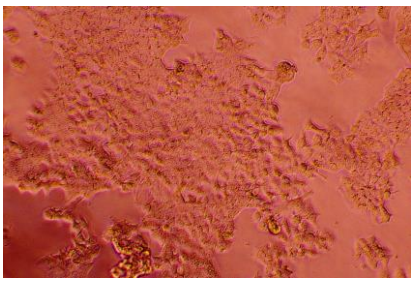

Figure 2A. Untreated control 3T3-L1 cells — healthy, confluent adherent morphology.

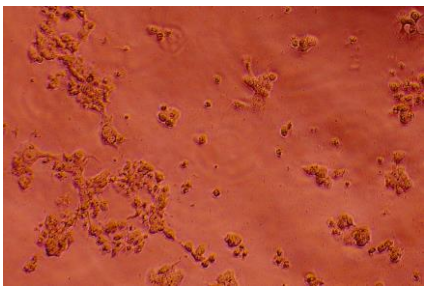

Figure 2B. Palmitic acid (500 µM)-treated cells — reduced density, rounding/shrinkage.

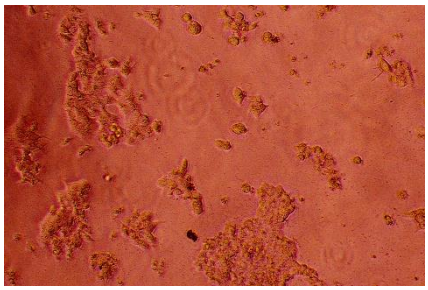

Figure 2C. Palmitic acid + Calcarea carbonica 200CH (1:400) — Moderate scattered clumps

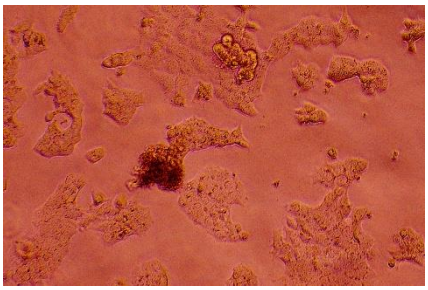

Figure 2D. Palmitic acid + Calcarea carbonica 200CH (1:200) — Scattered clumps + one dark debris spot

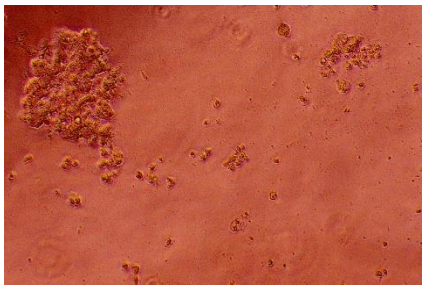

Figure 2E. Palmitic acid + Calcarea carbonica 200CH (1:100) — Denser clump upper-left + scattered debris.

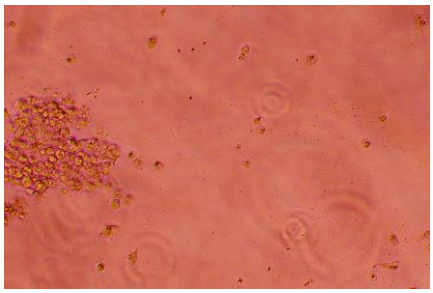

Figure 2F. Palmitic acid + Calcarea carbonica 200CH (1:50) — reduced adherent colony formation.

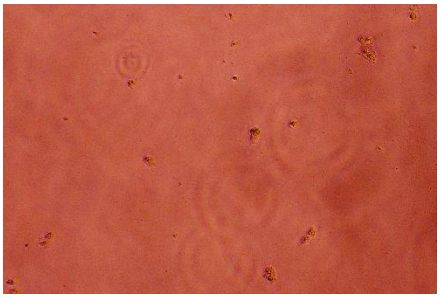

Figure 2G. Palmitic acid + Calcarea carbonica 200CH (1:25) —sparse, rounded cells (loss of protection).

Figure 2. Inverted phase-contrast micrographs of 3T3-L1 cells 24 hours after treatment (Olympus CKX41, 10× magnification).

3.3 Oil Red O staining: percentage cholesterol transport in 3T3-L1 cells:

Oil Red O staining, used as an indicator of intracellular lipid/cholesterol handling, showed that untreated control cells exhibited an optical density of 0.2549 (510 nm), corresponding to 70.48% cholesterol transport. Palmitic acid treatment abolished measurable cholesterol transport (OD 0.8636, corresponding to 0.00% transport), reflecting near-complete disruption of normal lipid handling and marked accumulation of unesterified/retained lipid. Co-treatment with Calcarea carbonica 200CH (1:200) partially restored this parameter, with an OD of 0.3941 corresponding to 54.37% cholesterol transport — approximately 77% of the control value.

Table 3. Oil Red O staining: percentage cholesterol transport in 3T3-L1 cells

Sample code	OD value (510 nm)	% Cholesterol transport
Control	0.2549	70.48
Palmitic acid (500 µM)	0.8636	0.00
Palmitic acid + Calcarea carb. 200CH (1:200)	0.3941	54.37

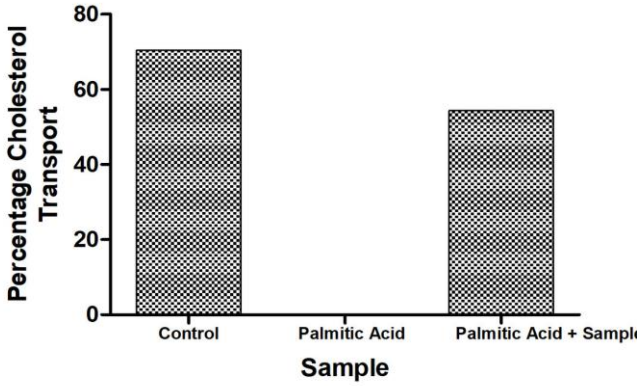

Figure 3. Percentage cholesterol transport in control, palmitic acid, and palmitic acid + Calcarea carbonica 200CH (1:200) groups (Oil Red O assay, 510 nm).

Representative Oil Red O-stained micrographs illustrated this pattern: control cells showed light, scattered lipid staining (Fig. 4A), palmitic acid-treated cells showed dense, confluent dark-red/black lipid deposits consistent with marked lipid accumulation and impaired transport (Fig. 4B), and palmitic acid + Calcarea carbonica 200CH (1:200)-treated cells showed an intermediate pattern with reduced lipid deposit density compared with palmitic acid alone (Fig. 4C, 4D).

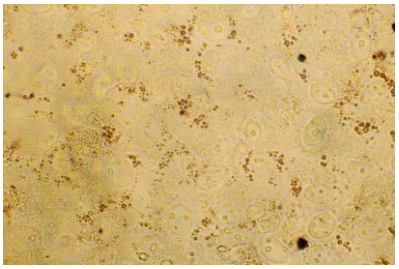

Figure 4A. Control — light, scattered Oil Red O staining.

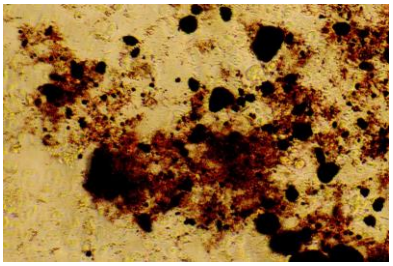

Figure 4B. Palmitic acid (500 µM) — dense, confluent lipid deposits.

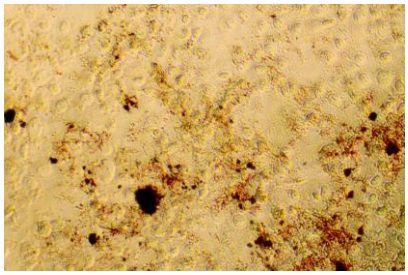

Figure 4C. Palmitic acid + Calcarea carbonica 200CH (1:200) — reduced lipid deposit density.

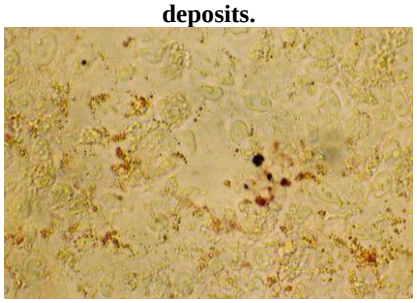

Figure 4D. Palmitic acid + Calcarea carbonica 200CH (1:200), second field.

Figure 4. Oil Red O-stained micrographs of 3T3-L1 cells across treatment groups (10× magnification).

IV. DISCUSSION

The present study was undertaken to evaluate the cytoprotective and lipid-modulatory effects of *Calcarea carbonica* 200CH against palmitic acid-induced lipotoxicity in 3T3-L1 mouse adipocytes. Lipotoxicity was induced using 500 µM palmitic acid, and the effects of different dilutions of *Calcarea carbonica* 200CH were assessed using MTT assay and Oil Red O staining. The findings demonstrated that *Calcarea carbonica* 200CH exerted a concentration-dependent cytoprotective effect, with the 1:200 dilution showing the highest restoration of cell viability and improvement in lipid transport.

In the present study, exposure to 500 µM palmitic acid significantly reduced cell viability to 51.20% and markedly impaired cholesterol transport, indicating successful induction of lipotoxicity. These findings are in agreement with Oh et al. (2023) [18], who demonstrated that palmitic acid induces adipocyte injury through lysosomal dysfunction, oxidative stress, and apoptosis. Similarly, Yudhani et al. (2023) reported that palmitic acid is widely used to establish reliable in vitro models of insulin resistance and lipotoxicity by impairing adipocyte function and cellular metabolism.[19]

Among the different dilutions tested, *Calcarea carbonica* 200CH at **1:200** exhibited the greatest cytoprotective effect, restoring cell viability to **81.26%** and improving cholesterol transport to **54.37%**. Although no previous studies have evaluated *Calcarea carbonica* 200CH in a palmitic acid-induced lipotoxicity model, the observed improvement in cell viability and lipid handling is consistent with reports that interventions reducing palmitic acid-induced oxidative stress and improving lipid metabolism enhance adipocyte survival and lipid homeostasis (Hagberg and Spalding, 2024; de Mello et al., 2025). [20]

Furthermore, the present findings are comparable with those of **Kumar et al. (2020)**, who demonstrated that *Calcarea carbonica* protected LPS-stimulated THP-1 human mononuclear cells by enhancing cell survival and suppressing inflammatory mediators, including nitric oxide (NO), TNF-α, and COX-2. Although the experimental models differ, both studies suggest that *Calcarea carbonica* possesses cytoprotective properties against cellular stress. [21] However, the precise molecular mechanisms underlying these effects remain to be elucidated through further mechanistic and in vivo studies. Further mechanistic and in vivo studies are warranted to confirm these observations and elucidate the molecular pathways involved.

Acknowledgment

I express my sincere gratitude to my mentors for their invaluable guidance, constant encouragement, constructive suggestions, and continuous support throughout the course of this research. I extend my heartfelt thanks to **Biogenix Research Center, BRMAS – Center for Molecular Biology and Applied Science, Poojappura, Thiruvananthapuram**, for providing the necessary laboratory facilities, technical support, and infrastructure to carry out this study successfully. I am deeply grateful to my parents for their unconditional love, unwavering encouragement, and sacrifices, which have been my greatest source of strength. I also sincerely thank my friends for their constant motivation, cooperation, and support during every stage of this research.

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