INHIBITORY EFFECT OF ETHANOLIC EXTRACT OF *CITRUS PARADISI* AND NARINGIN ON LIPID ACCUMULATION IN 3T3-L1 CELL LINES

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ABSTRACT

**Background:** The aim of the study is to examine the effects of Naringin and ethanolic extract of *Citrus paradisi* on lipid accumulation in 3T3-L1 cell line. **Materials & Methods:** Various tests such as MTT assay, Lipid accumulation assay by oil red O staining, Cell proliferation assay, Measurement of free Triglycerides content, Glycerol 3 Phosphate dehydrogenase was assessed Mean standard deviation (SD). One-way analysis of variance (ANOVA) was used. The significance of differences between two groups (control and sample) was analyzed by the Student’s t-test. **Results:** Study findings show that the lipid accumulation was significantly reduced with all concentration of *Citrus paradisi* and Naringin compared with the control; *Citrus paradisi* and Naringin treatment on 3T3-L1 pre-adipocytes during proliferation decreased the cell viability in all treated groups was noted. Among the concentrations tested, 500μg/mL significantly reduced the viability of 3T3-L1 cells. There was a highly significant decrease in TG level at 250μg/ml, 500 μg/ml, and 750 μg/ml (P<0.001) dose of *Citrus paradisi* in comparison to controls. Naringin and *Citrus paradisi* inhibited GPDH activity in 3T3-L1 adipocytes in all treated groups. **Conclusion:** The lipid deposition and maturation of 3T3-L1 preadipocytes into adipocytes is prevented by treating with different doses of *Citrus paradisi* and Naringin.
KEYWORDS: Citrus paradisi, Naringin, 3T3-L1 preadipocytes, GPDH activity, MTT assay.

INTRODUCTION
Globally, the major health issues occurring among individuals is Obesity which is a disorder that is complicated with overproduction of fat in the body\cite{1}. Over the next 10 years, 20% of overall people will be affected by diabetes universally as per approximate calculation. The major threatening elements for metabolic syndrome include dyslipidemia, and atherosclerosis, diabetes that is connected with Obesity\cite{2}. The hereditary and environmental elements like nutrients that increase the number of different mature cells result in Obesity that is due to uncontrolled body fat deposits by adipocytes\cite{3-5}. The beginning and development of obesity can be avoided by clearly knowing the process by which the specific nutrient impacts the cell changes into adipocytes. The transformation of unchanged preadipocyte into distinct adipocyte is known as Adipogenesis\cite{6}. The hereditary and development elements which control the adipogenic mechanism that causes an increase in Lipid deposits\cite{7}. Adipocyte specific genes and transcription elements expression is increased during the variation of structured preadipocytes. The large spherical spindle-shaped fibroblastic cells grow into 3T3-L1 adipocytes cells which collect triglyceride droplets which stimulates marker genes of the adipocyte.

The cell line that is used generally to examine the adipocyte variation is 3T3-L1 preadipocytes\cite{8}. The transformation of preadipocytes into adipocytes can be studied by using a 3T3-L1 cell line which is the best featured and authentic prototype. The interrelation of particular genes expression and proteins are connected with adipogenesis results in the complicated mechanism of Adipocyte variation\cite{9,10}. Complete energy homeostasis of a body is made by Adipocytes which are considered to be storage of lipids passively but now it is identified as cells of dynamic sensitivity for insulin with endocrine functionalities. The major threatening elements which are responsible for the progression of metabolic syndrome and diabetes mellitus type 2 (T2DM), cardiovascular disease, and specific cancers reviewed in\cite{11,12} that cause by obesity that consists of diseased primary fats cells. Earlier Obesity is fought by controlling adipose tissue progression. Certain therapy’s that are capable of reducing or avoiding obesity is by an antiadipogenic factor that has been examined by several scholars. Adipose tissue growth can be controlled by disturbing adipocyte variation which is
the new predominant postulate that is connected to the blockage of insulin and progression of T2DM\cite{13,14}.

There is a gradual increase in epidemic obesity across the world and in the United States by the insights of financial trouble and health impacts.\cite{15} The interact of scholars and common people for vegetative and floral medicines for long-term disorders like obesity and metabolic syndrome are induced by this insight and also frustrated by traditional medicine\cite{16}. The distinct bioactive components involved in this process and its effects are not known completely.

Several mixtures like limonoids, polyphenols, and flavonoids are synthesized by \textit{Citrus paradise}\cite{17}. Cardiovascular disease can be cured by using hesperidin, naringenin, and nobiletin, naringin and hesperedin the important flavonoids which are separated from \textit{Citrus paradisi}\cite{18–20}. The fat mass in the body of human beings who are obsessed are diminished by the activity of lipolysis which are stimulated by \textit{Citrus paradisi} and its juices display activity of lipolysis in adipocytes of human beings\cite{21} as reported by more researchers\cite{22,23}. But the consequences of \textit{Citrus paradisi} on adipogenesis are not completely known.

Currently, Grapefruit and some citrus fruits that have Naringin in large quantity of aglycone are anti-hyperlipidemic\cite{24} and contains anti-hyperglycemic elements\cite{25}. It has been reported that Naringin prevented adipogenesis and likely shows impacts on adipose tissue growth and responsiveness to insulin in addition to the metabolic favoring impacts. Therefore this study evaluated the specific effects of Naringin and \textit{Citrus paradisi} on lipid accumulation through various tests.

**MATERIALS AND METHODS**

**Chemical used**

Dubelcco’s modified eagle’s medium (DMEM), 10% Bovine Calf Serum, Fetal bovine serum (FBS), Dexamethasone, Dithiothreitol (DTT), Isobutylmethyl xanthine, Insulin, were obtained from Sigma-Aldrich.

**Cell Culture**

The 3T3-L1 preadipocyte cell lines were obtained from NCCS, pune, India. The mouse embryo 3T3-L1 cells were grown to 70% confluence in Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 10% fetal bovine serum, 100 U/mL of penicillin with
streptomycin in a humidified atmosphere at 37°C with 5% CO2. Confluent dishes were trypsinized with 0.05% trypsin and the cells were resuspended in Dubelcco’s modified eagle’s medium (DMEM) media. After two day post confluent (Day 0) growth arrested 3T3-L1 preadipocytes were induced to differentiate. Cells were fed with Dubelcco’s modified eagle’s medium (DMEM) containing 10% (v/v) FBS, 1 µg/mL insulin (I), 1 µM dexamethasone (D), and 0.5 mM 3-isobutyl-1-methylxanthine (M) until day 2. The cells were fed with Dubelcco’s modified eagle’s medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1 µg/ml insulin for 2 days, after which they were fed every other day with Dubelcco’s modified eagle’s medium (DMEM) containing 10% FBS.

**Differentiation of 3T3-L1 cells to Adipocytes**

The 3T3-L1 preadipocyte were plated in 96-well plates (3x10^4 cells/well) cultured in Dubelcco’s modified eagle’s medium (DMEM) containing 25 mmol/glucose, 1 mmol/ sodium pyruvate, 4 mmole/L-glutamine, 10% heat-inactivated Fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO2. After 70% confluency, the medium was removed and fresh differentiation medium containing 0.5 mmol/lIsobutylmethyl xanthine, 2.5 µmol/l Dexamethasone (DEX), and 10µg/ml insulin was added. Test samples were added to the culture medium every 48 hours with fruit extract for 6 days and transferred to adipocyte maintaining medium (AMM). After 5 days more than 90% of the cells had differentiated into rounded cells with lipid droplet. Cells and culture medium was collected for further assay.

**Lipid accumulation assay by oil red O staining**

Oil red O staining was measured by the method of [26]. Differentiated cells was treated with extract of Citrus paradisi (Concentration 100, 250, 500, 750, 1000µg/ml) and compound Naringin (concentration 25, 50, 100, 250, 500µg/ml) and then washed gently with phosphate buffer (PBS) twice, mixed with 3.7% fresh formaldehyde (sigma) solution (60% isopropanol and 40%water). After staining of lipid droplets, the excess staining was removed and the plates were rinsed with water and dried. Cytoplasmic lipid/triglyceride droplets were visible under (40X) fluorescence microscope. To quantify the intracellular lipids the stained lipids droplets were dissolved with isopropanol (500µl/well) and the absorbance was measured at 510 nm using a micro plate reader. The optical density of fully differentiated adipocyte was taken as 100% of relative lipid content % of Oil Red O staining = (Abs Sample/Abs Control) x 100.
Cell proliferation assay
Methyl thiazoletetrazolium (MTT) assay was carried out by the method of\textsuperscript{27} done to investigate the proliferation of 3T3-L1 preadipocyte. 3T3-L1 preadipocytes were seeded at a density of $3 \times 10^6$ cells/ well into 96-well plates and maintained with Dubelcco’s modified eagle’s medium(DMEM) containing 10% bovine serum. After 24 hours of incubation, the medium was changed and the sample contains various concentrations of ethanolic extract of \textit{citrus paradisi} (concentration 100, 250, 500, 750, 1000µg/ml) and compound Naringin (concentration 25, 50, 100, 250, 500µg/ml), after 48 hours of incubation, the medium containing 10µl of MTT solution (5µg/ml) was added. The plate was wrapped with foil and incubated for 48 hours at 37°C. The supernatant was removed, and 100µl of Dimethyl sulfoxide(DMSO) was added to each well. The amount of formazan product was determined using a micro plate reader (BioRad, USA) and absorbance was read at 570nm. Experiments were done in triplicate. Unpaired t-test was used to compare the values of the \textit{Citrus Paradisi}, Naringin group, and the control group. Results were expressed as the percentage of MTT reduction.

Measurement of free Triglycerides
Cellular TG contents was measured according to\textsuperscript{26} using a commercial TG assay kit. Cells were treated with \textit{Citrus paradisi} (concentration 100, 250, 500, 750, 1000µg/ml) and compound Naringin (concentration 25, 50, 100, 250, 500µg/ml) in 6-well plates during adipocyte differentiation for 6 days. The cells were washed twice with phosphate-buffered saline (PBS), scraped in 75µl of homogenizing solution (154mM Kcl, 1mM Ethylenediaminetetra acetic acid and 50Mm Tris, pH7.4) and sonicated to homogenize the cell suspension. The residual cell lysate was centrifuged at 3000g for 5min at 25°C to remove the fat layer. The supernatants were assayed for Triglyceride and protein contents. TG was normalized to the protein concentration which was determined by the Barford assay using bovine serum albumin (BSA) as standard.

Assay of Glycerol 3 Phosphate dehydrogenase
The activity of Glycerol 3 Phosphate dehydrogenase was carried out by method of\textsuperscript{28}. \textit{Citrus paradisi} and Naringin treated with adipocyte were harvested on day 7, washed with PBS and collected using lysis buffer. 25mM Tris buffer containing 1mM Ethylenediaminetetra acetic acid (EDTA) and 1mM Dithiothreitol. (pH 7.5). The harvested cells were sonicated and then
centrifuged at 10,000 rpm for 5 min. The GPDH activity in supernatants was measured using assay.

Statistical Analysis
Data were analyzed using SPSS 21.0 software for SPSS Inc., and the values are expressed as the mean with standard deviation (SD). One-way analysis of variance (ANOVA was used). The level of statistical significance was set p<0.05. The significance of differences between two groups (control and sample) was analyzed by the Student’s t-test, with p<0.05 considered significant. Each experiment was conducted in triplicate.

RESULTS AND DISCUSSION
The cell volume of the adipocytes was mainly dependent on the triglyceride accumulation in cells. The differentiated adipocytes were stained with the Oil red O and their lipid content was determined using the Bio rad (ELISA reader). The results are shown in Plate 1.

Plate 1: Effect of Citrus paradisi on lipid accumulation in 3T3-L1 by Oil Red O Staining. Legends: Lipid content stained with Oil red O staining and magnification at 40X.

The inhibitory effect of Citrus paradisi on lipid accumulation was evaluated after the treatment with different concentrations, after adding the adipogenic hormone to induce
adipogenesis. To study adipogenesis is *Citrus paradisi* concentration (100, 250, 500, 750 and 1000µg/ml) was used. After the staining, the cells were washed and viewed under a microscope. The results showed that untreated (control) contained many droplets indicating the lipid accumulation. The treatment of adipocytes with *Citrus paradisi* inhibits the lipid accumulation in a dose dependent manner and it was observed to be highest at dose 1000µg/ml (Plate 1.5). And Naringin showed the maximum inhibition of the lipid content at the dose of 500µg (Plate 2.5).

![Plate 2: Effect of Naringin on lipid accumulation in 3T3-L1 by Oil Red O staining.](image)

Legends: Lipid content stained with Oil red O staining and magnification at 40X

All extracts were able to reduce lipogenesis in vitro. *Citrus paradisi* extract inhibited 3T3-L1 differentiation with a reduction in the number of adipocytes, confirming the effect of anti-adipocytic. In addition, the lipid accumulation was significantly reduced with all concentrations of *Citrus Paradisi* compared with the control.
Study findings show that lipid accumulation was significantly reduced with all concentration of *Citrus paradisi* compared with the control. i.e. all concentration were able to reduce lipogenesis invitro. Ethanolic extract of *Citrus paradisi* inhibited 3T3-L1 differentiation with a reduction in the number of lipid droplets, confirming this positive effect.

**Statistical Significance**

\[ p < 0.001^{***}; p < 0.01^{**}; p < 0.05^{*}, \text{NS-Non significant} \]

Figure 1a: Effect of *citrus paradisi* on lipid accumulation in 3T3-L1 by Oil Red O staining.

Values are expressed as mean±SD (n=3),

Figure 2a: Effect of Naringin on lipid accumulation in 3T3-L1 by Oil Red O staining.

Values are expressed as mean±SD (n=3),

**Statistical Significance**

\[ p < 0.001^{***}; p < 0.01^{**}; p < 0.05^{*}, \text{NS-Non significant} \]
Cell viability assay

MTT assay is a more common method used for the detection of cell viability. This method depends on the metabolic activity of the viable cells. MTT analysis was carried out to detect the effect of *Citrus paradisi* on the viability of 3T3-L1 cells during proliferation and differentiation. 3T3-L1 cells were treated with various concentrations of *Citrus paradisi* (100, 250, 500, 750 and 1000µg). *Citrus paradisi* treatment on 3T3-L1 preadipocytes during proliferation decreased the cell viability in all treated groups. The percentages of cell viability were 93%, 87%, 81.3%, 61.3% and 52 for the 100, 250, 500, 750 and 1000µg *Citrus paradisi* treatments respectively (Fig. 3). 3T3-L1 cells were treated with various concentrations of Naringin (25, 50, 100, 250 and 500µg/ml) (Fig. 4).

![Figure 3: Impact of Citrus paradisi on cell viability.](image)

Values are expressed as mean±SD (n=3),

**Statistical Significance**

p < 0.001***; p < 0.01**; p< 0.05*, NS-Non significant

The effects of *Citrus paradisi* on the viability of 3T3-L1 preadipocytes were evaluated by the MTT assay. The 3T3-L1 preadipocytes were treated with 100, 250, 500, 750 and 1000µg of extracts and control for 48 h. Exposure to the extracts had no toxic effect on the viability of 3T3-L1 cells up to a concentration of 500µg was shown in Fig 3. Among the concentrations tested, 500µg, 750µg, and 1000µg treatments significantly reduced the viability of 3T3-L1 cells against control. A study by[29] showed the effect of *Citrus aurantium* flavonoids on the proliferation of 3T3-L1 preadipocytes.
Figure 4: Impact of Naringin on cell Viability.

Values are expressed as mean±SD (n=3),

**Statistical Significance**

p < 0.001***; p < 0.01**; p < 0.05*, NS-Non significant

The effects of Naringin on the viability of 3T3-L1 preadipocytes, were evaluated by the MTT assay. The 3T3-L1 preadipocytes were treated with 25, 50, 100, 250 and 500µg of Naringin along with control at 48 h. Among the concentrations tested, 250 and 500µg significantly reduced the viability of 3T3-L1 cells.

![Figure 4: Impact of Naringin on cell Viability.](image)

Figure 5: Effect of *Citrus paradisi* on Triglyceride accumulation in 3T3-L1 cell lines

Values are expressed as mean±SD (n=3), p < 0.001***; p < 0.01**; p < 0.05*, NS-Non significant

Results show TG in *Citrus paradisi* and control. There was a significant decrease in TG level from 100µg to 1000µg (P<0.001) dose of *Citrus paradisi* in comparison to controls. However, a significant decrease in TG level was only observed at 750µg dose of *Citrus*
paradisi.\textsuperscript{[30]} Shows evidence that the effects of Citrus paradisi on cardiovascular and body weight risk factors.

![Figure 6: Effect of Naringin on Triglyceride accumulation in 3T3-L1.](image)

Values expressed as mean±SD (n=3), p < 0.001***; p < 0.01**; p< 0.05*, NS-Non significant.

Similar to Citrus paradisi, there was a highly significant decrease in TG level at 50-500µg and 500µg dose of Naringin showed a significant effect in comparison to controls. However, a significant decrease in TG level was observed from 50µg to 500µg of Naringin.

**Glycerol 3 Phosphate dehydrogenase activity**

Adipocytes treated with Citrus paradisi and Naringin were harvested on day 7, washed with PBS and collected using lysis buffer. 25mM Tris buffer containing 1mM EDTA and 1mM DTT (pH 7.5). The harvested cells were sonicated and then centrifuged at 10,000 rpm for 5 min. The GPDH activity in supernatants was measured. GPDH activity was used as a marker of differentiation in 3T3-L1 cell line since GPDH activity is expressed in differentiated mature fat cells but not in preadipocytes.

![Figure 7: Impact of citrus paradisi on Glycerol 3 Phosphate dehyogenase activity.](image)
Values represent mean ± SD. (*) p < 0.001***; p < 0.01**; p< 0.05*, NS-Non significant

Citrus paradisi increased lipolytic activity in 3T3-L1 adipocytes. The lypolytic activity of Citrus paradisi was determined by measuring glycerol levels secreted in the medium. All experiments were performed on triplicates for each treatment. Citrus paradisi inhibited GPDH activity in 3T3-L1 adipocytes in selected treated groups (p<0.05) compared with the control. This inhibition was noticed at 500µg to 1000µg of Citrus paradisi to 3T3-L1 cells resulted in a marked decrease of GPDH activity in a dose-dependent manner (Figure 7). As per the finding, it is noticed that 100µg and 250µg do not involve in inhibition process when compared to control.[31] stated the effect of expression of GPDH and shows grapefruit oil efficiently inhibits adipogenesis in cultured subcutaneous preadipocytes and adipocytes.

Figure 8: Impact of naringin on Glycerol 3 Phosphate dehydrogenase activity.

Values represent mean ± SD. (*) p< 0.001***; p < 0.01**; p< 0.05*, NS-Non significant

Naringin inhibited GPDH activity in 3T3-L1 adipocytes in treated groups (p<0.05) compared with the control. Inhibition was observed from 100 to 500µg and a marked decrease of GPDH activity in a dose-dependent manner (Figure 8).

CONCLUSION

The lipid deposition and differentiation of 3T3-L1 preadipocytes into adipocytes is prevented by treating with optimum dose of Citrus paradisi and Naringin as illustrated by our work. The study concludes that lipid accumulation was significantly reduced with all concentration of Citrus Paradisi and Naringin compared with the control. Citrus paradisi and Naringin flavonoids extract inhibited 3T3-L1 differentiation with a reduction in the number of lipid droplets, confirming this positive effect. Citrus paradisi and Naringin inhibited GPDH activity in 3T3-L1 adipocytes at concentrations. This study suggests that citrus paradisi and
naringin exert beneficial effect in *invitro* condition by prevent lipid accumulation by attenuating adipogenic differentiation.

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**REFERENCES**

9. Ntambi JM, Young-Cheul K. Adipocyte Differentiation and Gene Expression. J Nutr [Internet], Dec 1, 2000; 130(12): 3122S–3126S.


