Fifty Percent Ethanolic Extract of *Momordica charantia* Inhibits Adipogenesis and Promotes Adipolysis in 3T3-L1 Pre-Adipocyte Cells

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Abstract

Background: Natural products have gained importance recently for the treatment of obesity and its complications, partly because of the side effects of modern drugs.Hence, we aimed to study and compare the effect of varying concentrations of *Momordicacharantia*on adipogenesis and adipolysis using 3T3-L1 pre-adipocyte cell lines.

Methods: 3T3-L1 pre-adipocytes were procured from the National Center for Cell Sciences, Pune, and cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1 mM L-glutamine. An ethanolic extract of *M. charantia* (EEMC)was prepared by the graded ethanol fractionation method and the total phenol content (TPC) determined using the Folin-Ciocalteau (F-C) assay. The cytotoxic dose was determined by the sulforhodamine-B (SRB) assay. The adipogenesis and adipolysis assays (Cayman chemicals, Ann Arbor, USA) were performed according to the manufacturer's protocols.

Results: The 3T3-L1 pre-adipocytes treated with increasing concentrations of EEMC during (*p= 0.012*) and after (*p= 0.026*) differentiation demonstrated significant reduction in lipid droplet accumulation. There was a significant decrease in glycerol release during differentiation (*p= 0.018*) and a significant increase in glycerol release after differentiation (*p= 0.0007*) with increasing concentrations of EEMC. However, the effect of EEMC on adipogenesis and adipolysis was greater during 3T3-L1 pre-adipocyte differentiation than after.

Conclusions: The data showed that the 50% EEMC is potent inhibitor of lipogenesis and stimulator of lipolysis in 3T3-L1 pre-adipocytes. Further analyses will be performed to determine the key antioxidant compound(s) in the extract by phenolic acid profiling using high performance liquid chromatography (HPLC). Also, the mechanism of action of EEMC on adipogenesis and adipolysis will be elucidated.

Keywords: Adipogenesis, Adipolysis, *Momordicacharantia*, Glycerol release, 3T3-L1 pre-adipocytes, Oil red staining

Introduction

Obesity is a fast-spreading epidemic of the 21st century, and a major cause of many metabolic disorders. Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired (1). Rapid urbanization, modernization, and adoption of lifestyles with reduced physical

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activity and increased caloric intake have resulted in rising obesity rates in several developing countries, including India. Obese individuals are prone to type-2 diabetes, cardiovascular disease, hypertension, and cancer. These four diseases account for 60% of deaths worldwide (2). The global prevalence of over weightness and obesity increased from 857 million in 1980 to 2.1 billion in 2013, which is one-third of the world's population (3). In humans, induction of obesity is either 'genetic' or 'lifestyle' related and the latter is a complex intermix of sedentary lifestyles and high-calorie diets amounting to nutritional overload.

Obesity is the result of increased adipogenesis and down regulation of lipolysis. Adipogenesis is the development of pre-adipocytes to mature adipocytes with the accumulation of lipid droplets (4). The differentiation of pre-adipocytes into mature insulin-sensitive adipocytes, which produce adiponectin, is regulated by two families of transcription factors: peroxisome proliferator-activated receptors (PPARs) and CCAAT/enhancer binding proteins (C/EBPs) (5). Other positive regulators include sterol-regulatory element-binding protein (SREBP), Krox20, signal transducer and activator of transcription 5 (STAT5), and members of the Klf family. The combined actions of these transcription factors result in a complex transcriptional cascade that causes adipocyte differentiation and tissue formation (6).

Lipolysis, or mobilization, in adipocytes results from the breakdown of adipose triacylglycerols (TAG) into nonesterified fatty acids (NEAF). Hormones that induce lipolysis are epinephrine, norepinephrine, ghrelin, growth hormone, testosterone, and cortisol. Dave *et al.* evaluated the lipolytic response of stem bromelain (SBM) on 3T3L1 cells by measuring the expression levels of perilipin, hormone sensitive lipase (HSL), and tumor necrosis factor-alpha (TNF-α) during adipocyte differentiation. Stem bromelain downregulated perilipin while upregulating TNF-α with no appreciable HSL expression changes (7). The induction of lipolysis and inhibition of TG synthesis in white adipocytes have been considered therapy targets for the prevention and treatment of obesity and its related disorders (8).

Momordicacharantia (MC), known as bitter melon or bitter gourd, a popular plant used by many diabetics, is widely grown in tropical areas. The active components of MC include charantin (a steroid glycoside), vicine, polypeptide-p or plant insulin, mormordin, vitamin C, carotenoids, flavonoids, polyphenols, quercetin, and gallic acid, among others. *M. charantia* effects include increased insulin-like effects, stimulation of pancreatic secretion leading to decreased hepatic gluconeogenesis, increased hepatic glycogen synthesis, increased peripheral glucose oxidation, increased glucose transporter type 4 (GLUT4) protein content in muscle plasma membrane, and decreased glucose absorption, (9). *M. charantia*-induced decrease in blood glucose appears to be a recognized effect, and no serious adverse effects on humans have been reported. Animal studies indicate that bromelain juice (BMJ) was also effective in reducing weight gain, possibly due to reduced adipose hypertrophy, inhibition of lipogenic genes, and increased plasma catecholamines (10, 11). With the focus on its hypoglycemic action, the hypolipidemic effects of MC have been overlooked until recently. With the emerging evidence that MC affects glucose and lipid metabolism, MC or its extract(s) has the potential of being a dietary adjunct for weight reduction and glycemic control.

Few studies have addressed the effect of MC extract on lipid metabolism in obesity; therefore, we analyzed the anti-adipogenic effect of MC extract on pre-adipocytes.

Materials and Methods
Preparation of ethanolic extract of Momordicacharantia (EEMC)
The ethanolic extract of MC was prepared by a graded ethanol fractionation method as described earlier (12). In brief, MC fruit was made into a paste using a regular household mixer with no additional water. The paste was lyophilized on a freeze dryer (Alpha 2-4 LD Plus from Christ, GmbH) and 50% EEMC was produced using 50 gm of this lyophilized powder. The solution was concentrated using a Rotovapor R-215 (Buchi, Switzerland) and then filtered using Whatmann's filter paper number 1. The concentrated extracts

were lyophilized on a freeze dryer (Alpha 2-4 LD Plus from Christ, GmbH) and the lyophilized powder was stored at -80ºC. For further analysis an EEMC stock was prepared by dissolving the lyophilized powder in phosphate-buffered saline (PBS).

Based on the total phenol content of 50% lyophilized powder reported by us in previous studies, the working stock standard was prepared by serially diluting the stock to a concentration range of 0.195-100µg/ml (2X) (13).

3T3-L1 pre-adipocyte cells were procured from NCCS Pune, India and maintained in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin-streptomycin. in adherent and treated tissue culture flasks and allowed to grow to 80% confluency.

Cytotoxicity assay

The sulforhodamine-B (SRB) assay was performed to evaluate the cytotoxic effect of 50% EEMC on the pre-adipocyte cells. The cells were grown according to the manufacturer's instructions using an Adipogenesis Assay Kit, (item no. 10006908, Cayman Chemical). Ten thousand cells were seeded in wells of 96-well plates and maintained in 100 µL of DMEM supplemented with 20% FBS, 1% glutamine, and 1% penicillin-streptomycin and incubated at 37°C with 5% CO_2 until confluent. The wells were divided into five categories consisting of media blank (without cells), non-differentiated (no treatment), control (treated with induction and insulin media, available in the kit), "during" differentiation (treated with induction and insulin media and EEMC until day 7), and "after" differentiation (treated with induction and insulin media until day 7 and then treated with EEMC on day 7).

Two days post-confluence (day 0), the growth medium was replaced with 100 µl of induction medium in the control and "during" and "after" differentiation groups and 100 µL of 50% EEMC at concentrations ranging from 0.09 to 50 µg/ml (1X) was added to the "during" differentiation group and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2. On day 3, the induction medium was replaced with insulin

medium and 100 µL of 50% EEMC at concentrations ranging from 0.09 to 50µg/ml (1X) was added again to the "during" differentiation group. The insulin medium and EEMC were replaced every two days until day 7. On day 7 the media was replaced with fresh insulin medium and 100 µL of EEMC at the above concentrations (1X) in both the "during" and "after" differentiation groups and the cells were incubated for another 48 hours. On day 9, the reaction was stopped and cytotoxicity was evaluated by the SRB assay.

Cultures were fixed with trichloroacetic-acid (TCA) and stained with 0.4% SRB dissolved in 1% acetic acid. Sulforhodamine-B dyes bind with membrane proteins of live cells. Unbound dye was removed by washing with 1% acetic acid. Finally, membrane protein-bound dye was extracted with 10 mM unbuffered Tris base [tris (hydroxymethyl) aminomethane] and the optical density was measured at 564 nm using a multimode reader (Enspire from PerkinElmer). Because the color intensity of SRB dye-treated wells is directly related to the number of viable cells, the cytotoxic effect of the compound can be evaluated; a decrease in absorbance signifies a decrease in the number of viable cells and an increase in cytotoxicity.

From the SRB assay, it was found that concentrations of EECM ranging from 0.09 to 3.725µg/ml had no cytotoxic effect on 3T3-L1 pre-adipocytes. Based on this result, concentrations g from 0.25 to 4 µg/ml (2X) of 50% EEMC were used for both the adipogenesis and adipolysis assays.

Adipogenesis assay

Cells were grown as described above for the cytotoxicity assay from days 0 to 9. On day 9, adipogenesis was evaluated by Oil Red O Staining according to the manufacturer's protocol (Cayman chemical, Adipogenesis Assay kit, no. 10006908). Cells were stained at room temperature. Most of the medium was removed from the wells. To each well 75 µl of diluted Lipid Droplets Assay Fixative was added and the cells were incubated for 15 minutes. Then the wells were washed with 100 µl of wash solution two times for five minutes each. After the wells were dry, 75 µl of Oil Red O working solution was added to all the wells, including the background wells containing no cells, and incubated for 20

minutes. After the incubation period, the Oil Red O Solution was removed and cells were washed with distilled water several times until the water contained no visible pink color. Next the wells were washed with 100 µl of wash solution two times for five minutes each and allowed to dry. After the wells were dry, 100 µl of dye extraction solution was added to each well and the plates were incubated for 30 min at room temperature. The absorbance was then read at 520 nm with a multimode reader (Enspire from PerkinElmer).

Adipolysis assay
Cells were grown as described above for the cytotoxicity assay from days 0 to 9. On day 9, adipolysis was evaluated according to the manufacturer's protocol (Cayman chemical, Adipolysis Assay kit no. 10009381) using a glycerol standard. Twenty-five µl of glycerol standards at concentrations from 7.8 to 125µg/ml were added to a new 96-well plate. Twenty-five µl of the cell culture supernatants were collected and added to the corresponding wells of the new 96-well plate. To this, 100 µl of diluted Free Glycerol Assay Reagent was added per well, incubated for 15 minutes at room temperature, and absorbance was read at 540 nm.

The standard curve was plotted as a function of glycerol concentration and to determine the equation of the line. The glycerol concentration was determined using the formula: Glycerol concentration (µg/ml) = **[**A540 - (y-intercept)**]** **/** slope **x** sample dilution.

Statistical analysis:
All statistics were analyzed using GraphPad Prism, Prism 5 for Windows, version 5.01. Data were expressed as mean values ± SEMs. A one-way ANOVA model was used to compare means between the groups. Each sample was analyzed in triplicate. *Post hoc* pairwise multiple comparisons were evaluated using the Bonferroni post-test, after ANOVA. Results were considered significant at *p < 0.05.*

Results
The SRB assay showed similar patterns of cell death in both the "during" and "after" differentiation groups. The data obtained during differentiation showed that a dose of around 1.560µg/ml is effective for the treatment of 3T3-L1 cell lines with minimal cell death. No inhibition was seen at the four lowest concentrations, and inhibition was near maximal at 6.250 µg/ml of EEMC (Fig.1).
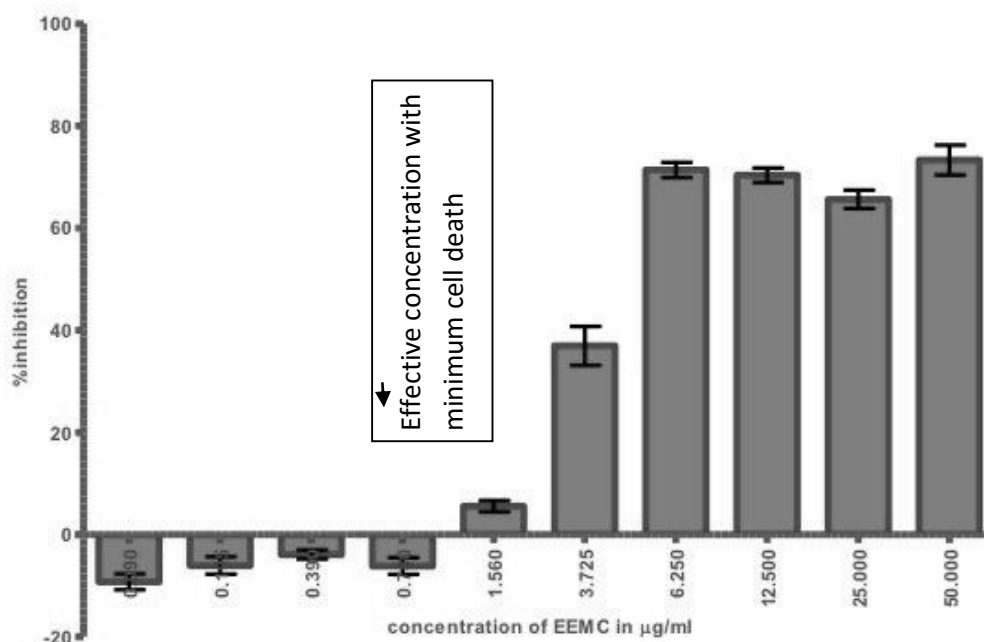

Fig. 1. Cytotoxic effect of increasing concentrations of 50% EEMC during differentiation on 3T3-L1 pre-adipocyte cells.

In The adipogenesis assay a dose-dependent response was seen on treatment of 3T3-L1 cells with increasing concentrations of 50% EEMC, with lower concentrations having more lipid accumulation than higher concentrations (Fig. 2).
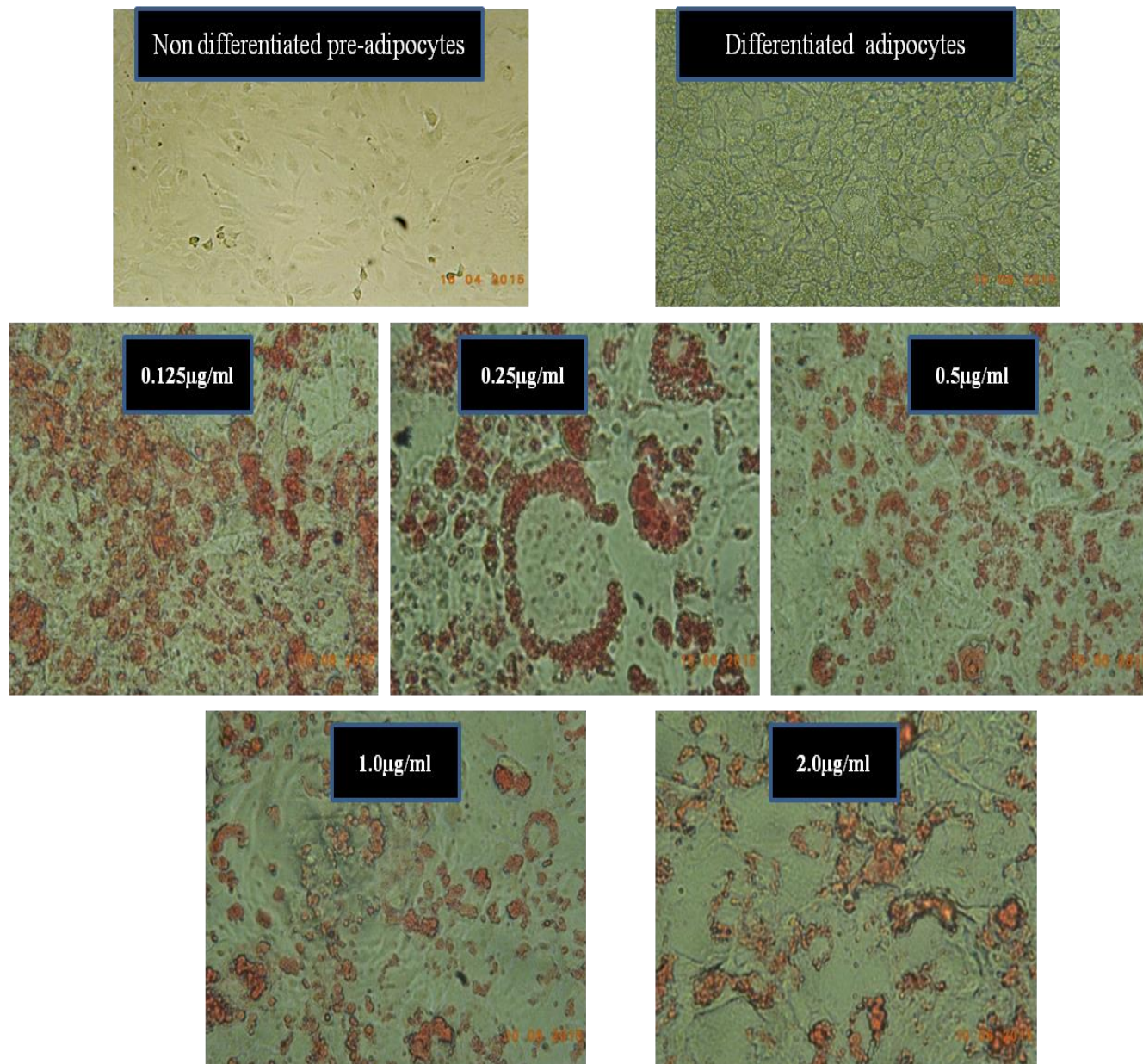

Fig. 2. Effect of increasing concentrations of 50% EEMC on adipogenesis in 3T3-L1 pre-adipocytes assessed by oil red staining.

The adipogenesis assay showed that increasing the EEMC concentration led to a decrease in lipid accumulation in adipocytes both during (Fig. 3a) ($p < 0.012$ - control versus cells treated with 2 µg/ml of EEMC) and after (Fig. 3b) ($p < 0.026$ -control versus cells treated with 2 µg/ml of EEMC) differentiation, with a near 50% reduction at 2.0 µg/ml of EECM in both cases. However, the decrease in the percentage of adipogenesis was greater during differentiation than after.
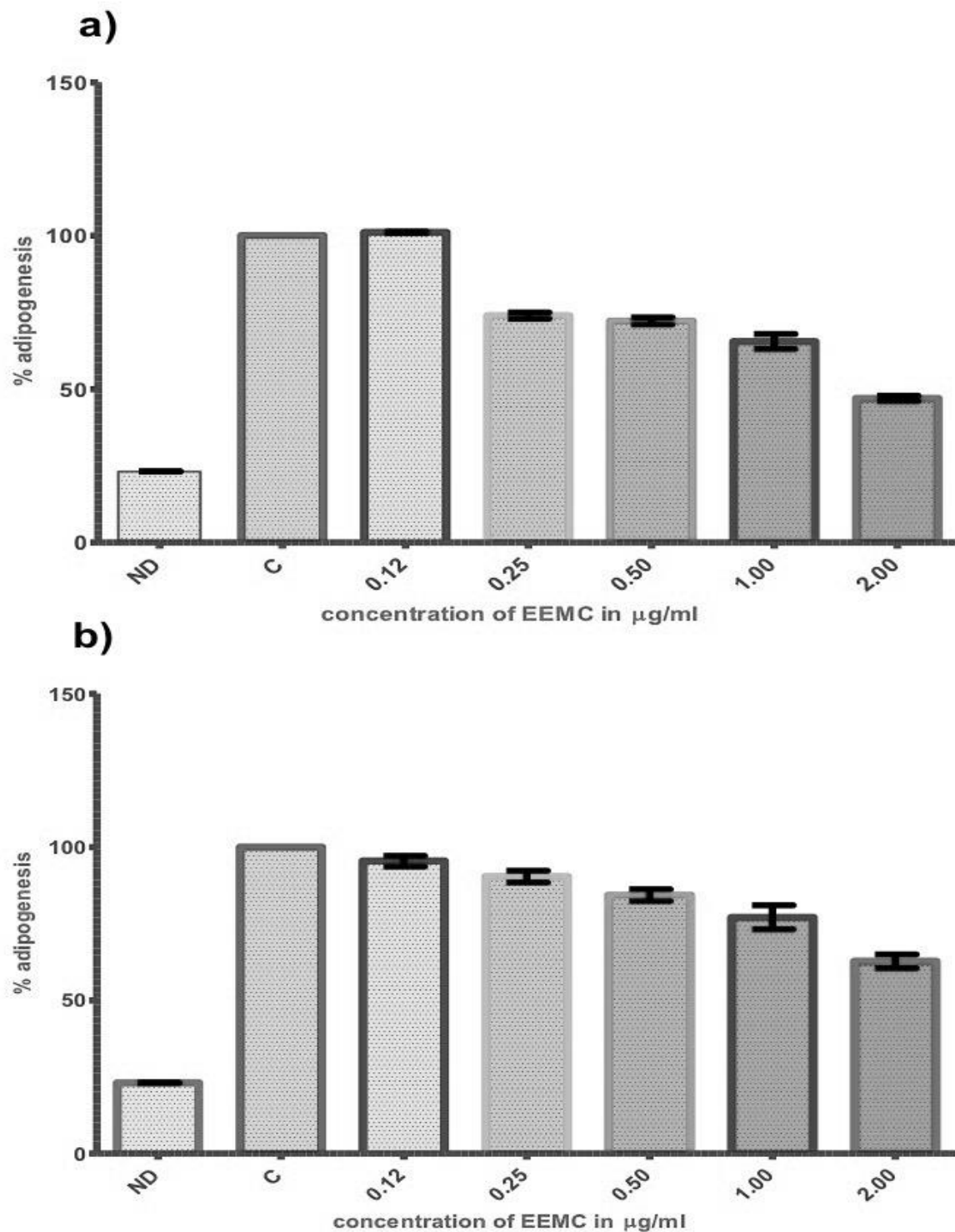
Fig. 3. Effect of increasing concentrations of 50% EEMC on adipogenesis during (a) and after (b) differentiation of 3T3-L1 pre-adipocytes (*P* value of control versus cells treated with 2µg/ml of EEMC).

The glycerol release was determined in the conditioned media after treating the cells with increasing concentrations of 50% EEMC during differentiation from days 0 to 7. As the EEMC concentration increased, the glycerol release decreased. The decreased glycerol release may be due to decreased lipid accumulation at higher EEMC concentrations (Fig. 4a) However, when compared to the control there was increased release of glycerol even from cells treated with 2 µg/ml of EEMC ($p < 0.018$ - control versus cells treated with 2 µg/ml of EEMC).

However, after differentiation, glycerol release increased at higher EEMC concentrations. The amount of lipid accumulated on day 9 was similar in all the cells and the greatest glycerol release was seen in the cells that received the highest concentration of EEMC (Fig. 4b) ($p < 0.0007$ - control versus cells treated with 2 µg/ml of EEMC).
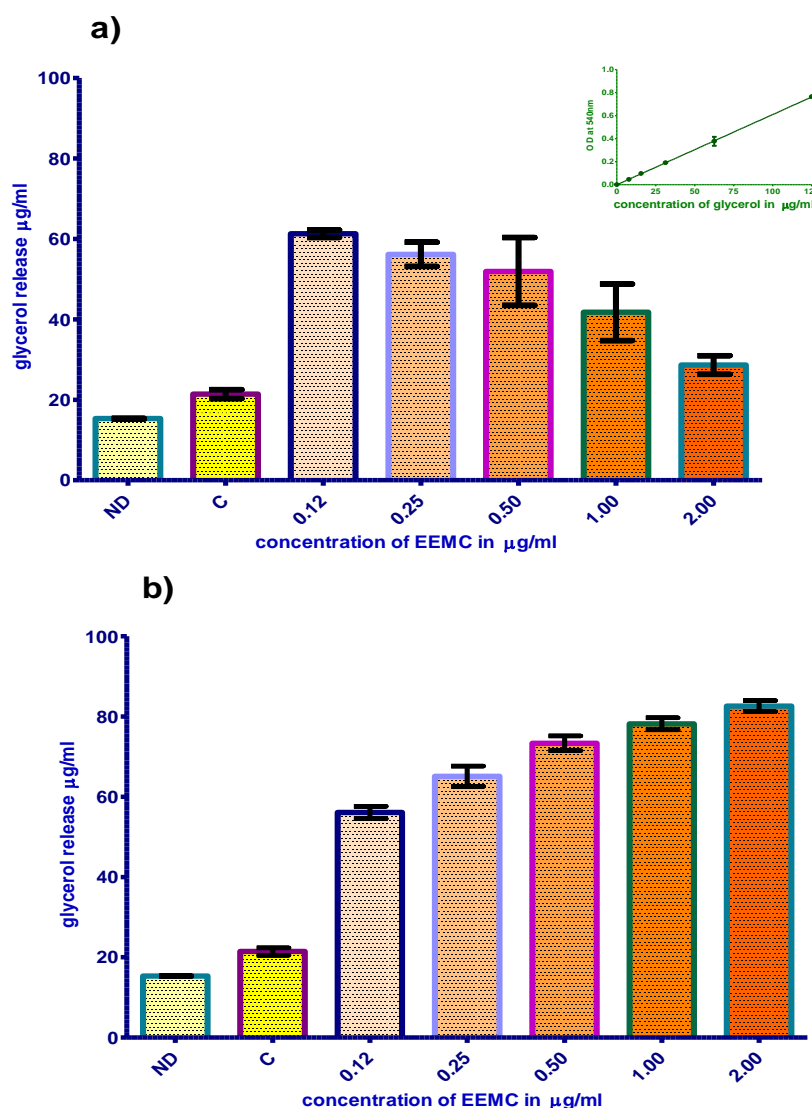

Fig. 4. Effect of increasing concentrations of 50% EEMC on adipolysis during (a) and after (b) differentiation of 3T3-L1 pre-adipocytes. (P value of control versus cells treated with 2µg/ml of EEMC)

Discussion

Obesity has affected various aspects of human life and society and has been evident in the human record for over 20,000 years (14). The treatment of obesity is complex and needs a multifaceted approach that includes exercise programs, diet, and behavioral modifications, with or without pharmacotherapy. Increasing obesity rates have resulted in a renewed interest in the use of herbs

and alternative medicines (15). Natural products have gained importance for the treatment of obesity and its complications recently because of their greater efficacy and lesser side effects than modern drugs. *M. chirantia* is extensively used in folk medicines as a remedy for diabetes and its complications, especially in India, Southeast Asia, Africa, and South America. Animal studies have shown that the effect of MC juice in reducing weight gain may be due to reduced adipose hypertrophy, inhibition of lipogenic genes, and increased plasma catecholamines (16-19). The medicinal value of MC has been attributed to its high antioxidant properties, which are related to phenols, flavonoids, isoflavones, terpenes, anthroquinones, and glucosinolates, which also confer the bitter taste (20).

Phenolics, or phenols, are major secondary metabolites present in both edible and non-edible plants, and have been reported to have multiple biological effects, including antioxidant activity. Antioxidants are essential for plant growth and reproduction, and are produced as a defense response against plant injury and pathogens (21).Phenols are antioxidants due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (22).*M. charantia* is a good source of phenols (23).Semiz A *et al.* showed that *in vivo* treatment of rats with MC fruit extract enhanced both antioxidant enzyme (AOE) and glutathione S-transferase (GST) activities (24). Hamissou M *et al.* showed that MC was 82.05% as effective as ascorbic acid in inhibiting the free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and has higher total phenolic compounds than zucchini, indicating that MC is a high antioxidant-containing vegetable (25).

Based on our previous study, which showed that 50% EEMC had the highest total phenol content, we used 50% EEMC for further analyses.

Adipose tissue represents a dynamic endocrine organ that not only maintains energy balance but also controls lipid homeostasis by two processes: lipogenesis and lipolysis (26). The cytotoxicity study on 3T3L-1 pre-adipocytes was performed by SRB assay using 50% EEMC, and the percentage inhibition was calculated to determine the highest concentration of EEMC in which cells were viable. The result obtained from the study showed that a

dose of around 1.560 µg/ml is effective for the treatment of 3T3L-1 cell lines causing minimum cell death (Fig 1). Based on this dose adipogenesis and adipolysis were analyzed. The concentrations of 50% EEMC were fixed at, 0.125, 0.25, 0.5, 1.0, and, 2.0 µg/ml.

Adipogenesis is a complex process in which adipocyte growth and differentiation contribute to overall adipose mass (26). The experimentally-induced adipocyte differentiation was characterized by plumping of adipocytes due to accumulation of lipid droplets and higher percentage of adipocyte differentiation.

The differentiation of pre-adipocytes into adipocytes is regulated by a complex network of transcription factors including PPARγ, and C/EBPα and β. PPARγ and C/EBPα activate the expression of genes involved in adipogenesis to trigger fatty acid and triglyceride syntheses via activation of fatty acid synthase (FAS), lipoprotein lipase (LPL), adipocyte differentiation-related protein (ADRP), adipocyte-specific binding protein 2 (aP2), CD36, perilipin, and others during the terminal stage of pre-adipocyte differentiation (27-29),whereas C/EBPβ activates genes involved in the initial stage of differentiation.

LI85008F, a novel herbal formulation, has been shown to antagonize PPARγ through Ser112 phosphorylation via mitogen activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) activation. It also downregulates the expression of PPARγ-responsive gene products such as adipocyte differentiation related protein (ADRP), CD36, adipocyte-specific-binding protein (aP2), and perilipin. These observations also showed that LI85008F treatment resulted in decreases in fat storage and availability of the fatty acid transport system, and increased susceptibility to hormone-sensitive lipase (HSL) for lipolytic breakdown of stored fat (30).Nerurkar PV *et al.,* showed that pre-adipocytes treated with varying concentrations of MC during differentiation demonstrated a significant reduction in lipid content and mRNA expression of adipocyte transcription factors including PPARγ, SREBP-1c, adipocytokine, and resistin (15). Also, adipocytes treated with MC for 48 h showed reduced lipid content, perilipin mRNA expression, and increased lipolysis (31).

In our study, the qualitative analysis by Oil Red O staining of adipogenesis both during and after differentiation showed a decrease in accumulation of lipid droplets with increased EEMC concentrations (Fig. 2).

The result of the quantitative analyses of both "during" ($p = 0.012$) and "after" ($p = 0.026$) adipocyte differentiation also showed that as EEMC concentration increased, adipogenesis decreased (Figs. 3a and 3b). The percentage of adipogenesis was less during adipocyte differentiation than after. The results of both the quantitative and qualitative analyses may be due to downregulation of the transcription factors C/EBP and PPAR family members, which slow the de novo synthesis of fatty acids and triglycerides and inhibit the early differentiation of pre-adipocytes and adipogenesis in mature adipocytes.

Adipolysis is the breakdown of lipids, which involves hydrolysis of triglycerides into glycerol and free fatty acids (FFAs). Hormone-sensitive lipase, a rate-limiting enzyme in adipose tissue lipolysis, hydrolyzes the stored triglycerides into glycerol and FFAs (32). Catecholamines stimulate adipocyte lipolysis by increasing intracellular cAMP, an important second messenger in the signaling pathways that mobilize fat stores and activate cAMP-dependent protein kinase A (PKA). Protein kinase A then phosphorylates both perilipins and HSL, which is associated with an increase in hydrolytic activity of the enzyme and the translocation of HSL from the cytosol to the lipid droplet. It has been shown that berberine, a hypoglycemic agent, increases phosphodiesterase (PDE) 3B and PDE4 activity by reducing the inhibition of PDE, leading to a decrease in cAMP and HSL phosphorylation, which is independent of the adenosine monophosphate-activated protein kinase (AMPK) pathway (33). The cytokine TNF-α, an important mediator of lipid metabolism, downregulates the anti-lipolytic genes PDE3B and $G_i\alpha_1$ (7). This regulation is influenced by the glucose concentration to which the cells are exposed and one or more glycolytic intermediates (34).

The adipolysis was analyzed using glycerol as the standard ($R^2 = 0.9963$). Treating the cells during differentiation showed that as the EEMC concentration increased, glycerol release decreased. This may be due to decreased lipid accumulation in the cells which were under the influence of EEMC during differentiation. However, treating the cells with increasing concentrations of EEMC after differentiation increased the glycerol release, since there was equal lipid accumulation in all the cells before they were exposed to EEMC. This result obtained may be attributed to a decrease in HSL phosphorylation or TNF-α productions. These findings provide evidence for the potential of EEMC in preventing adipocyte differentiation and lipid accumulation, and promoting lipolysis.

Hence we conclude that 50% EEMC reduced lipid droplet accumulation and increased glycerol release on treating 3T3-L1 pre-adipocyte cells both during and after differentiation. However, the effect of EEMC on adipogenesis was greater in during differentiation than after.

We plan to identify the bioactive compound(s) present in the 50% EEMC using high performance liquid chromatography and mass spectrophotometry. The active compound, once isolated, will be re-tested on the both adipogenesis and adipolysis. The mechanism of action of EEMC on adipogenesis and adipolysis will be studied by Western blot analysis and RT-PCR assay. Further experiments will use primary human pre-adipocytes and animals to establish the therapeutic index of the purified active compound.

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