VOLUME 11 ISSUE 2 2025

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 11, No. 2, 693-702 (2025)

International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Evaluation of the Anti-obesity Effect by Inhibiting Pancreatic Lipase Activity and Assessing the Anti-Adipogenic Impact on 3T3-L1 Preadipocytes, of a Polyherbal Formulation Sukkumalli Kashayam

Priya J.*, Safreen K. and Monika K.

Department of Biochemistry, Ethiraj College for women (Autonomous) (Affiliated to University of Madras), Chennai 600 008, Tamil Nadu, India

**Corresponding Author*

Received: 2nd January, 2025; **Accepted:** 15th April 2025; **Published online:** 19th October, 2025

https://doi.org/10.33745/ijzi.2025.v11i02.066

Abstract: Pancreatic lipase is one of the key lipolytic enzymes in the gut that actively promotes the breakdown and absorption of dietary triglycerides and cholesteryl esters. Inhibiting this enzyme has been the subject of extensive research to determine the potential ability of natural products to prevent the absorption of dietary fat. Despite being regarded as one of the most dependable targets for the treatment of obesity and dyslipidemia, to date, orlistat is the only known FDA-approved, effective, oral pancreatic lipase inhibitor available for clinical use apart from the centrally acting anti-obesity agents. However, it is known to be associated with adverse gastrointestinal and renal complications. The purpose of this investigation was to evaluate the inhibitory potentials of porcine pancreatic lipase of polyherbal formulation Sukkumalli kashayam (SMK) by *in vitro* means. The research was extended further to explore and evaluate the inhibition of lipid accumulation in 3T3-L1 preadipocyte .The findings revealed that SMK inhibited pancreatic lipase with IC_{50} value of 0.84±0.0016 mg/ml compared to selected standard orlistat with IC_{50} value of 0.25±0.0016 mg/ml and inhibited the activity of porcine pancreatic lipase in a dose dependent manner. It also significantly reduced lipid accumulation by 67.86 % at 100 µg/ml in 3T3-L1 adipocytes, suggesting the anti-obesity activity. The study result implies that SMK could be used as therapeutic agent and functional food with respect to treatment and prevention of obesity.

Keywords: Sukkumalli kashayam, Porcine Pancreatic lipase, 3T3-L1 adipocytes, Oil red O staining, Anti-obesity

Citation: Priya J., Safreen K. and Monika K.: Evaluation of the anti-obesity effect by inhibiting pancreatic lipase activity and assessing the anti-adipogenic impact on 3T3-L1 preadipocytes, of a polyherbal formulation Sukkumalli kashayam. Intern. J. Zool. Invest. 11(2): 693-702, 2025.

https://doi.org/10.33745/ijzi.2025.v11i02.066

Introduction

Obesity, characterized by an excessive accumulation of adipose tissue, has emerged as a global health crisis of unprecedented proportions. Its prevalence has reached epidemic levels, affecting people of all ages, genders, and socioeconomic backgrounds. According to the

World Health Organization (WHO), more than 650 million adults were obese in 2016, and the numbers continue to rise unabated. This escalating trend is deeply concerning, since obesity is linked to numerous harmful health consequences, including cardiovascular diseases, type 2 diabetes, hypertension, sleep apnea, respiratory disorders, certain cancers, and a reduced quality of life (Fruh, 2017). The multifaceted nature of obesity presents a formidable challenge in its management. It is not merely a matter of excess body weight but involves intricate interactions between genetics, metabolism, lifestyle, and environmental factors. Numerous scientific fields have shown a growing interest in the molecular mechanisms that govern triglyceride production and in medical strategies aimed at decreasing fat absorption and accumulation through phytochemicals, which offers a promising chance for the identification of novel anti-obesity medications. One common strategy used to reduce excessive energy intake is to inhibit the absorption of dietary fats.

Pancreatic lipase enzyme (triacylglycerol acylhydrolase) secreted from pancreas is a key enzyme related to the dietary triglycerides absorption and catalyzes the digestion of dietary triglycerides (Veeramachaneni *et al*., 2015). Hydrolysis of 50-70% dietary fats is done by pancreatic lipase (Birari and Bhutani, 2007) and reduction of fat absorption via pancreatic lipase inhibition is recognized to help in obesity management (Ahn *et al*., 2012). The FDA has authorized orlistat (Xenical), a relatively successful drug for the long- term management of obesity and it works by inhibiting the pancreatic lipase enzyme, which stops around 30% of dietary fat from being absorbed (Seyedan *et al*., 2015). However, its usage is restricted due to major gastrointestinal side effects (De La Garza *et al*., 2011).

Adipose tissues are classified as either brown (BAT) or white (WAT) and extra energy is stored in adipocyte lipid droplets as neutral lipids. The aberrant growth of WAT associated with obesity consists of an increase in the size of adipocytes or an increase in the number of adipocytes through adipocyte differentiation (Haczeyni *et al*., 2018). The process of precursor cells of adipocytes proliferating and differentiating into mature adipocytes is called adipogenesis (Haider and Larose, 2019). Adipogenesis is strongly controlled by the transcriptional signalling and cascade pathways that involves transcription factors such as C/EBPα, C/EBPβ, C/EBPδ, PPARγ, fatty acid synthase (FAS) and sterol regulatory element binding protein (SREBP1). Reducing adipogenic transcription factors could be an effective technique for preventing adipose tissue development, as obesity progresses (Jakab *et al*., 2021).Among the most widely utilized *in vitro* models for studying the biology of adipose tissue is the differentiation of the 3T3-L1 preadipocyte fibroblast cell line isolated from the embryo of a mouse. The preadipocyte develops into mature adipocyte and on differentiation, the cell line displays the white adipose tissue's shape and gene expression (Morrison and McGee, 2015). It is a common tool used for analysis of subcellular pathway involved in preadipocytic cell differentiation (Cave and Crowther, 2019).

In light of the negative side effects of synthetic medications, herbal remedies are now preferred owing to their efficacy in controlling obesity and a variety of other chronic conditions. The need for alternative and complementary strategies has led to an increased interest in traditional herbal remedies, which have been used for centuries in various cultures to treat a wide range of ailments, including obesity (Hasani-Ranjbar *et al*., 2009).The World Health Organization (WHO, 2002) defines "traditional medicine" as "health practices, approaches, knowledge, and beliefs that incorporate medicines based on plants, animals, minerals, spiritual therapies, and manual techniques used either alone or in combination to treat, diagnose, and prevent illnesses or maintain wellbeing.

Many of these medicinal practices and formulations were passed down orally to our

ancestors, and many Indian families still use specific herbal formulations as home remedies to cure common ailments like colds, pains, and coughs. One such polyherbal formulation is Sukkumalli kashayam (SMK) which is consumed as tea or coffee, with or without milk, to treat cold, coughs, and to strengthen immunity. Sukku, which means dry ginger and Malli, which means coriander seeds, are the main constituents of kashayam, followed by clove, cumin seeds, and black pepper. Although there is no documented proof of the effectiveness of medication, kashayam has countless health benefits in managing sickness and strengthening the immune system. This study aimed to investigate the *in vitro* inhibitory potentials of porcine pancreatic lipase of polyherbal formulation Sukkumalli kashayam (SMK) by *in vitro* means and was extended further to explore and evaluate the inhibition of adipogenesis in 3T3-L1 preadipocytes.

Materials and Methods

Collection of Samples

Ginger, Coriander seeds, Cumin, Black Pepper, and Clove were purchased from an organic store at Pudupet, Chennai, Tamil Nadu, India. Dry samples of Ginger, Coriander seeds, Cumin, Black Pepper, and Clove in ratio of 10:10:5:5:1 were grinded, and the coarse powder obtained was stored in a sterile airtight container for further study.

Preparation of Extract

The Sukkumalli kashayam extract (SMK) was prepared by decoction process by dissolving 25 g of powder in 400 ml of distilled water and heated at 50-60 °C till the mixture gets reduced to half of the volume (200 ml). After cooling the mixture, it was filtered using muslin cloth and the filtrate was concentrated in an incubator at 45 °C and then the extract was stored in a sterile container at 2 to 5 °C until the completion of the study. The yield of the extract was calculated as follows:

$$\% \text{ yield} = [\text{Weight of the extract} / \text{Weight of the sample}] \times 100$$

In-Vitro Anti-Obesity Activity

Pancreatic Lipase Inhibitory Activity

Lipase inhibitory activity of SMK was determined using a modified assay method of Etoundi *et al*. (2010). Briefly, a suspension containing 1% (v/v) triolein and 1% (v/v) tween 40 in 0.1 M phosphate buffer (pH 8) was prepared and emulsified. Assay was then initiated by adding 1600 µl of the triolein emulsion to 200 µl of porcine pancreatic lipase (0.5 g pancreatin in 15 ml of 0.1 M phosphate buffer at pH 8.0) and 200 µl of sample at different concentrations (200-1000 µg). The test tubes containing reaction mixture were incubated at 37°C for 30 min and then the absorbance was recorded at 450 nm. Orlistat is used as positive control and the % inhibition of pancreatic lipase was calculated using the following formula:

$$\% \text{ inhibition of Pancreatic lipase} = (Abs_{Control} - Abs_{Extract}) / Abs_{control} \text{ X100}$$

In-Vitro Anti-Adipogenic Activity

Preparation of 3t3-L1 Preadipocytes Cell Suspension

A subculture of 3T3-L1 preadipocytes in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the disaggregated cells in the flask 25 ml of DMEM with 10% FCS was added. The cells were suspended in the medium by gentle passage with the pipette and the cells homogenized.

Seeding of Cells

1 ml of the homogenized cell suspension was added to each well of a 96 well culture plate along with the different concentrations of samples of SMK and Orlistat (0 to 200 µg/ml) and incubated at 37°C in a humidified CO_2 incubator with 5% CO_2. After 48 h of incubation, the cells were observed under an inverted tissue culture microscope. With an 80% confluence of cells cytotoxic assay was carried out.

Cell Vaibility Assay

The cell viability assay was carried out using (3-(4, 5-dimethyl thiazol-2yl) -2, 5- diphenyltetrazolium bromide (MTT). MTT is cleaved by mitochondrial succinate dehydrogenase and reductase of

Table 1: Porcine Pancreatic Lipase Inhibitory Activity of SMK and Orlistat

	CONCENTRATION (µg/ml)					
	200	**400**	**600**	**800**	**1000**	**IC_{50}**
Orlistat	47.70±0.66	58.45±0.42	63.44±0.20	74.12±0.41	82.20±0.31	0.25±0.0016
SMK	23.40±0.54	27.92±0.21	37.92±0.23	48.93±0.31	57.90±0.74	0.84±0.0016

n=3

viable cells, yielding a measurable purple product formazan. This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. After 48 h incubation, the wells were added with MTT and left for 3 h at room temperature and the content of the wells were removed using the pipette and 100 µl SDS in DMSO was added to dissolve the formazan crystals. The absorbance was then read in Lark LIPR-9608 microplate reader at 540 nm (Mosman *et al*., 1983).

Seeding of Cells and Differentiation

3T3-L1 preadipocytes were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS at 37°C in an atmosphere containing 5% CO_2. To induce adipocyte differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h (day 2) with an inducer (10 µg/ml insulin, 2.5 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine) including samples SMK and Orlistat at different concentrations (i.e 0, 25, 50 and 100 µg/ml).On day 2 (Day 2) medium replaced with DMEM supplemented with 10% FBS and 10 µg/ml insulin. After 2 days (Day 4) the cells were re-fed with fresh DMEM with10% FBS. On day 6 (Day 6) the fully differentiated 3T3-L1 cells were examined for each experiment.

Oil Red O Staining of Intracellular Triglycerides

On day 6, fully differentiated 3T3-L1 adipocytes were washed with PBS and fixed with 10% formalin for 60 min. After three washes with distilled water, the cells were stained for at least 1 h at room temperature in a freshly filtered Oil Red O solution (0.5% Oil Red O in 60% isopropanol). After the cells were washed thrice with distilled water, the stained cells were photographed under a microscope. Finally, the dye retained in the 3T3-L1 cells was eluted with isopropanol and quantified by measuring the absorbance at 510 nm.

Results

Pancreatic Lipase Inhibitory Activity

The result of pancreatic lipase inhibitory activity of SMK and positive control orlistat tested at various concentrations (200 µg/ml,400 µg/ ml,600 µg/ ml,800 µg/ ml,1000 µg/ ml) demonstrated an increase in inhibitory activity of pancreatic lipase with an increase in the concentration of test extract (Table 1). In the analysis of IC_{50}, SMK showed a reported value of 0.84±0.0016 µg/ml, while orlistat had a value of 0.25±0.0016 µg/ml. A lower IC_{50} value indicates a greater effectiveness in inhibiting pancreatic lipase. The findings of this study demonstrate a notable anti-obesity effect of SMK.

Cell Viability Assay in 3t3-L1 Cells

The results of the cell viability assay in 3T3-L1 cell lines of SMK was determined by MTT assay using orlistat as positive control at various concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml). The findings highlighted that SMK and orlistat showed decrease in cell viability with increase in concentration of extract (Fig. 1). However, the experimental study highlighted that SMK and the positive control orlistat exhibited 80% of cell viability (Fig. 1) which determines its low cytotoxic effect on 3T3-L1 preadipocytes (Figs. 2, 3).

Lipid Inhibition and Triglyceride Extraction in 3t3-L1 Cell Lines

The inhibitory effect of SMK on triglyceride accumulation in 3T3-L1 cells was determined by Oil Red O Staining at various concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml). The study result

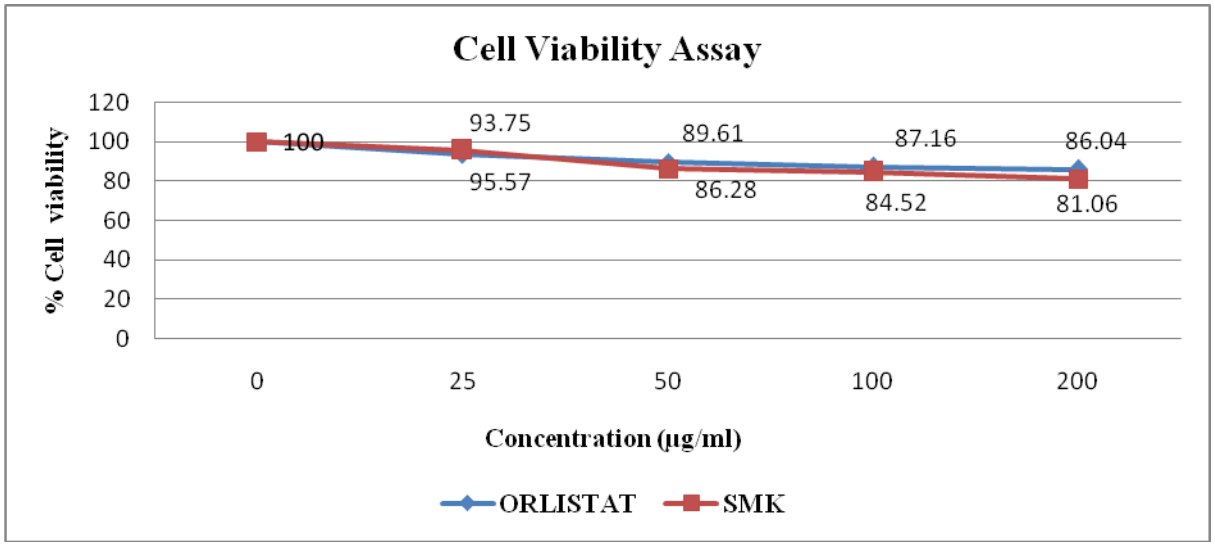

Fig. 1: Cell viability assay of SMK and Orlistat in 3T3-L1 adipocytes cells.

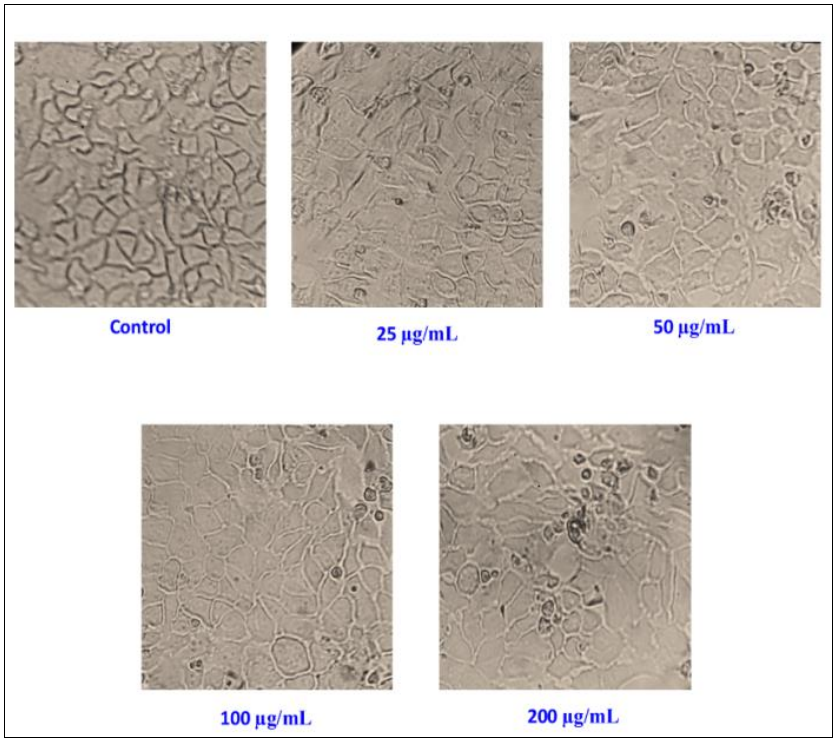

Fig. 2*:* Cytotoxicity of Orlistat on the 3T3-L1 pre-adipocyte cell line.

reported that SMK inhibited triglyceride accumulation in 3T3-L1 cells in a dose dependent manner (Fig. 4) which showcases the anti-adipogenic activity of the test extract (Figs. 5, 6).

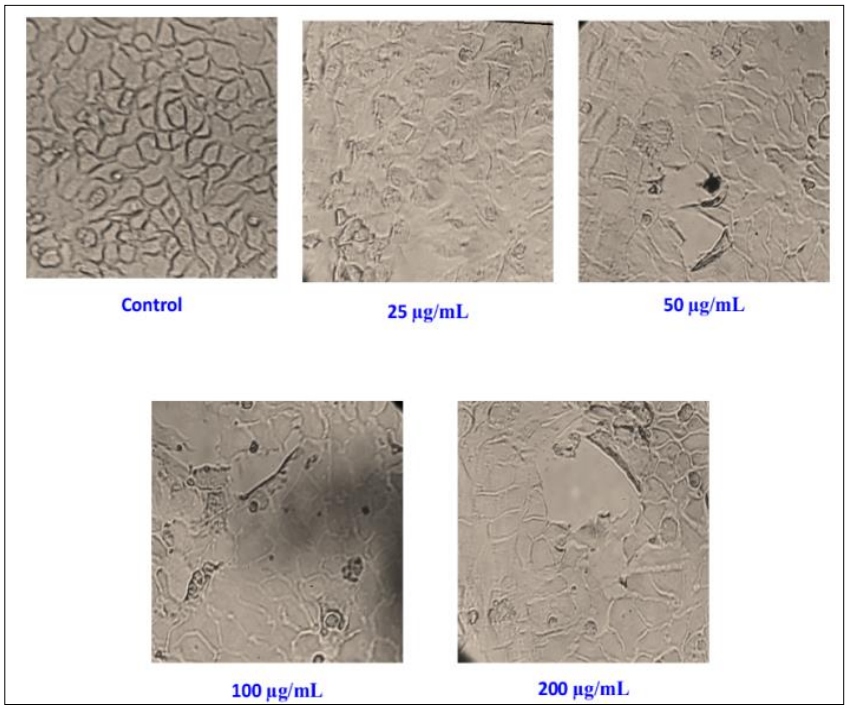

Fig. 3: Cytotoxicity of SMK on the 3T3-L1 pre-adipocyte cell line.

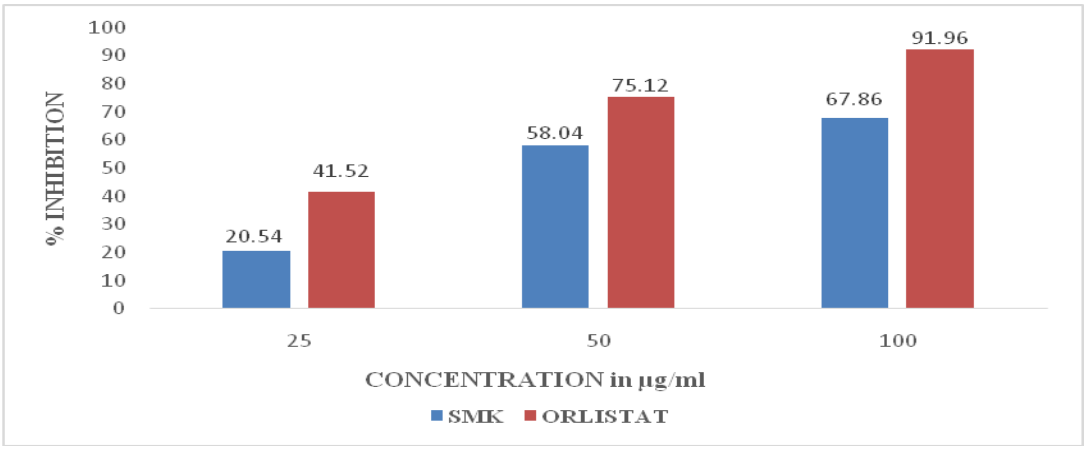

Fig. 4: Inhibitory effect of SMK and Orlistat on lipid accumulation in 3T3-L1 adipocytes cells.

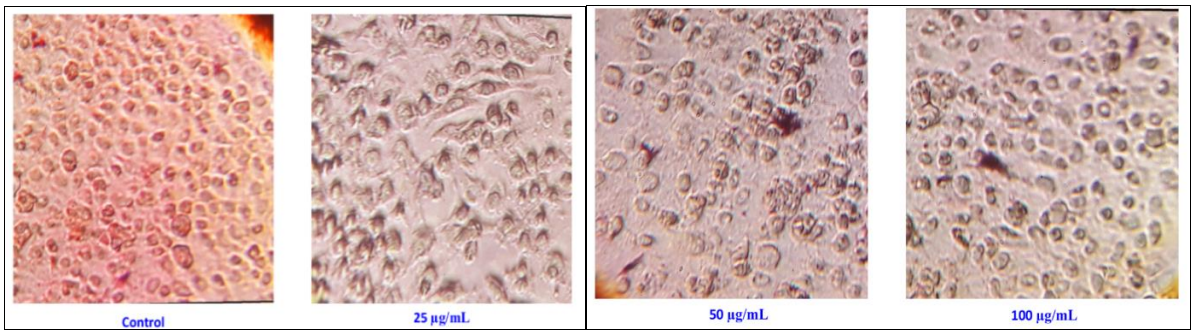

Fig. 5: Effect of Orlistat on lipid accumulation in differentiated 3T3-L1 cells as measured with Oil Red O staining.

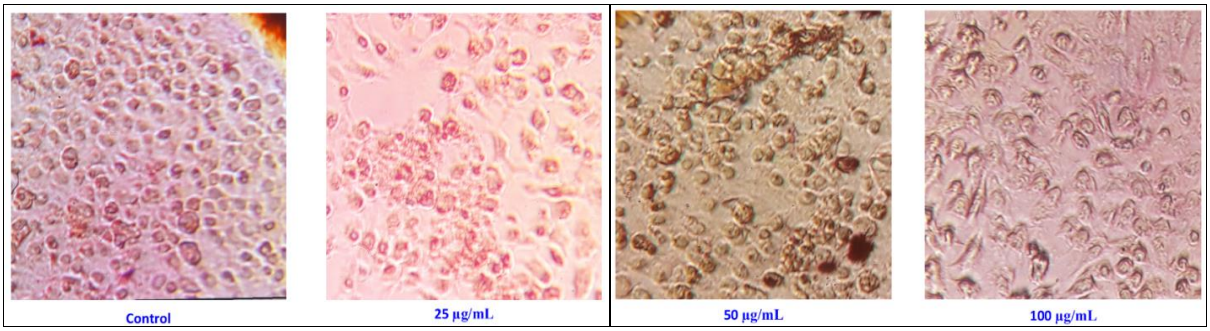

Fig. 6: Effect of SMK on lipid accumulation in differentiated 3T3-L1 cells as measured with Oil Red O staining.

Discussion

Obesity results from the formation of fat cells and the buildup of fats within those cells; therefore, reducing the amount of fat stored in adipocytes is essential for both preventing and addressing obesity. Various natural plant extracts and their active ingredients have been demonstrated to be effective in reducing the formation of fat cells (Youn *et al*., 2017).

Porcine Pancreatic Lipase Inhibitory Assay

Pancreatic lipase is an essential enzyme that plays a vital role in lipid breakdown, being produced and secreted by the pancreas. This enzyme is necessary for the efficient absorption of dietary triacylglycerols, as it transforms them into monoacylglycerols and fatty acids. These products are formed through lipid hydrolysis and subsequently create mixed micelles with bile salts, cholesterol, and lysophosphatidic acid. The resulting micelles are absorbed by enterocytes, where they contribute to the synthesis of triglycerides (TG). Ultimately, adipocytes store triglycerides as their main energy reserve (Lim *et al*., 2022). Recently, innovative strategies for treating obesity have sought to lower energy consumption through gastrointestinal means while maintaining essential bodily functions. One method involves inhibiting pancreatic lipase, leading numerous researchers to investigate the possible effectiveness of natural substances as anti-obesity medications (Yun, 2010). There was a strong relationship between lipases and plasma TG levels, with triacylglycerol absorption efficiency being one of the major factors contributing to plasma TG levels; however, dietary TG is not as well absorbed until it is hydrolyzed to fatty acids by triacylglycerol lipases. Hyper-lipidemia can be avoided by preventing TG absorption from the small intestine to enterocytes. Thus, an inhibitor of digestive lipase that limits intestinal fat absorption could be used to treat hyperlipidemia and has tremendous potential as an anti-obesity treatment (Mahmoud and Elnour, 2011). Inhibition of digestion and absorption of dietary lipids, through an inhibitory action on pancreatic lipase, can be targeted for development of anti-obesity agents. The present study reported that SMK significantly inhibited pancreatic lipase in a dose dependent manner of up to 57.90% (1000 µg),while positive control orlistat reported 82.20% (1000 µg). Literature review has revealed anti-obesity activity in ginger (Mahmoud and. Elnour, 2013; Danış *et al*., 2015; Yoshioka *et al*., 2019; Rahayu *et al*., 2019; Wafa, 2019;) coriander seeds (Ado *et al*., 2013; Fernando *et al*., 2018; Hameed *et al*., 2020; Eid *et al*., 2022; Maser *et al*., 2023;) black pepper (McCrea *et al*., 2015), cumin seeds (Ado *et al*., 2013; Fernando *et al*., 2018) and cloves (McCrea *et al*., 2015; Fernando *et al*., 2018; Chimbetete *et al*., 2019; Kamandloo *et al*., 2023), which supports the present study. The inhibitory action of SMK on pancreatic lipase may be linked to the secondary metabolites and the combined effect of the components found in the polyherbal formulation.

Anti-Adipogenic Activity

Cell Viability Assay and Lipid Inhibition in 3T3-L1 Cells

To eliminate the possibility that the extract impacts cell viability, a toxicity assessment was essential to determine the concentration at which the extract becomes harmful before testing the extracts/compounds with the 3T3-L1 cell line (Castillo *et al*., 2019). In this investigation, the cytotoxicity of SMK was evaluated using the MTT assay on 3T3-L1 preadipocytes, which measures cell viability through the activity of the mitochondrial enzyme succinate dehydrogenase. The screened SMK exhibited relatively low cytotoxicity toward 3T3-L1 preadipocyte cells, and cell viability was above 80% at a concentration of 200 µg/ml.

To evaluate the impact of SMK extracts on the adipogenesis of 3T3-L1 cells, the differentiation of 3T3-L1 pre-adipocytes into mature adipocytes was stimulated with various concentrations (25 µg/ml, 50 µg/ml, and 100 µg/ml) of the SMK extract, along with standard orlistat (for 0–2 days), and this treatment continued for a total of 6 days. The fat accumulation was assessed in fully differentiated adipocytes by Oil Red O staining. This stain is an optimized process commonly used for intracellular lipid staining and tissue staining that stains neutral fats, mainly triglycerides with an orange-red tint. Cells were initially examined and photographed under a microscope, and the optical density values were determined to measure intracellular triglyceride and computed as a percentage of the control (%). The result of the study showed the reduced TG accumulation of SMK reported 67.86% inhibition at 100 µg/ml and positive control, orlistat reported 91.96% inhibition at 100 µg/ml against 3T3-L1 cells. The microscopic images of cells stained with Oil Red O dye showed that size of lipid droplets was smaller in SMK at 100 µg/ml against 3T3-L1 cell lines as compared to that of standard and also inhibitory activity was observed to be dose dependent. Therefore, the present study reported reduced lipid accumulation in differentiated adipocytes as evidenced by Oil Red O staining, suggesting anti-obesity activity. A review of the literature has identified anti-adipogenic properties in ginger (Suk *et al*., 2015; Suk *et al*., 2017; Wei *et al*., 2017; Seo *et al*., 2021; Li *et al*., 2023; Gembe-Olivarez *et al*., 2023), coriander seeds (Ngamdokmai *et al*., 2022), pepper (Lahrita *et al*., 2015), and cloves (Jung *et al*., 2012), which aligns with our current research findings and suggests a potential synergistic effect among the components of SMK. This synergistic interaction could lead to improved therapeutic outcomes or benefits compared to using each individual plant alone.

Conclusion

In conclusion, the research on the anti-obesity and anti-adipogenic activity of the polyherbal mixture SMK presents a promising avenue for addressing the global epidemic of obesity and the associated health risks. Furthermore the study suggests that this formulation does not affect cell viability, making them potentially suitable for long-term use as anti-obesity agents. To our knowledge, this is the first study exploring the anti-obesity and anti-adipogenic properties of the polyherbal formulation; however, further investigation is necessary before endorsing it for therapeutic use.

Ethical Statement

No animal or microbes have been used or sacrificed for this study, hence Ethical Approval not required.

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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