Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism.

A THESIS SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE PARTIAL FULFILMENT OF THE AWARD OF THE DEGREE OF
M.Sc. LIFE SCIENCES (AYURVEDA BIOLOGY)

BY

DR SWATHI R HEGDE

UNDER THE GUIDANCE OF
DR C.N. VISHNUPRASAD

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, BENGALURU

JULY 2024

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
Private University Established in Karnataka by ACT 35 of 2013
BENGALURU - 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis "**Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism**" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr C. N. Vishnuprasad. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.

Place: Bengaluru

Signature of the Candidate

Date: 19-06-24

Name of candidate: DR SWATHI R HEGDE

Reg. No.: 2022MScAB04

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013
BENGALURU - 560064

CERTIFICATE FROM THESIS SUPERVISOR

This is to certify that the work incorporated in this thesis **"Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism"** submitted by Dr. Swathi R Hegde was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

Dr. C. N. Vishnuprasad 19.06.2024
Associate Professor,
TDU Bangalore.

ACKNOWLEDGEMENT

Firstly, I would like to thank The University of Trans-disciplinary Health Sciences and Technology for providing the facilities and academic environment. I would like to express my sincere gratitude to my coordinators Dr C. N. Vishnuprasad and Dr Megha for their support and guidance. I extend my gratitude to Dr. Subramanya Kumar for his guidance. A special heartfelt thanks to Ms. Sania Kouser for supporting me constantly throughout the project. I sincerely thank Mr. Abhi. V. Badiger, Ms. Priyanka Gladys Pinto, Dr Sanket Sharma and Dr Arun Bhanu for their support.

SUMMARY

The Ayurvedic formulation Lodhrasavam, known to be helpful in the management of Sthoulya was tested for its effect on 3T3L1 cell model. Experiments were done to see the inhibitory action of Lodhrasavam on adipogenesis in 3T3L1 fibroblasts (i.e.,3T3L1 differentiation) and to see the lipolytic action of Lodhrasavam in mature 3T3L1 adipocytes. TAG assay, Oil O Red staining, and RT-PCR were done to estimate the effect. The results show that Lodhrasavam can significantly inhibit adipogenesis in 3T3L1 fibroblasts by inhibiting the expression levels of adipogenic genes like PPAR-ɣ, SREBP, FABP4 and CEBPA and can accelerate lipolysis in mature 3T3L1 adipocytes.

Lodhrasavam inhibits adipogenesis and accelerates lipolysis in the adipocytes via *Agni* modulation i.e., the integrated effect of the individual ingredients of Lodhrasavam results in the modulation of both poshana and pachana functions of medo-dhatwagni which is directly involved in the manifestation of Sthoulya.

Further in the study, the HepG2 cells were treated with the conditioned media from Lodhrasavam treated 3T3L1 cells and TAG assay was performed with the cell lysate. This study was done on the basis of the previous study done in our lab showing the protective effect of Lodhrasavam against palmitate toxicity in HepG2 cell model. However, being a preliminary study, the results did not satisfy the hypothesis of the current study in HepG2 cell model. Further studies need to be done for a better understanding on these results.

PERSONAL REFLECTION

It was a great experience working in the lab doing cell biology, bio-assays and molecular work being a person from an Ayurveda background. I could learn a part of how contemporary bio-medical drug research is done. Some of the technical skills I could learn as a part of my project are:

- Cell culture work - 3T3L1 fibroblasts and HepG2 cells in particular.
- Induction of adipogenesis – 3T3L1 cell differentiation and Drug treatment.
- Performing TAG assay, Protein estimation assay and Gallic acid estimation assay.
- Performing gene expression studies by running RT-PCR.
- Oil-O-Red cell staining
- Understanding the concepts of Ayurveda through the lens of contemporary biology.

Other than the technical skills, time management, patience and planning are the major learnings from this project.

Apart from the skills learnt from the project, I got an opportunity to learn and be a part of the animal studies in which the lab was working on.

I could also observe and learn the other experiments being done in the lab like Western blotting.

Since my friends were working in other labs, I could hear and learn interesting things about drosophila and C. elegans too.

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1. INTRODUCTION

In Ayurveda, *Agni* is a crucial element that governs digestion and metabolism within the body. It transforms food into energy that is essential for all vital bodily functions. *Agni* is therefore regarded as the source of life, influencing attributes such as complexion, strength, health, nourishment, radiance, vitality, energy, and life force. Table 1. shows the various types of *Agni* described in the three classical texts of Ayurveda.

Acharya Charaka noted that the cessation of *Agni*'s function leads to death. When *Agni* operates in a balanced state, it promotes optimal health, enabling an individual to enjoy a long, fulfilling, and healthy life. Conversely, if *Agni* becomes imbalanced or disturbed, it disrupts the body's metabolic processes, leading to various health issues and diseases. Thus, *Agni* is considered the fundamental basis of life (Cha. Chi. 15/3-4) (P. V. Sharma, 2001). One such disease caused by the malfunctioning of *Agni* is Sthoulya.

Charaka Samhita	**Sushrutha Samhita**	**Ashtanga Hridaya**
Jatharagni	*Pachakagni*	*Bhutagnis-5*
Bhutagni-5	*Ranjakagni*	*Dhatvagnis-7*
Dhatvagni-7	*Alochakagni*	*Dhoshagnis-3*
	Sadhakagni	*Malagnis-3*
	Bhrajakagni	
	Bhutagnis-5	

Table 1. Classification of *Agni* described in *brihatrayees* (P. V. Sharma, 2001; Srikantha Murthy, 1995), (P. V. Sharma, 2001).

1.1. Sthoulya

In Ayurveda, *Sthoulya* is seen as an imbalance in the body's metabolism, often linked to lifestyle factors and genetic predispositions. The primary manifestations of obesity in Ayurveda are described through specific signs like *Medomamsa Ativruddhi* (Excessive accumulation of fatty tissue), *Chala Sphik* (Flabby buttocks), *Chala Udara* (Flabby abdomen), *Chala Stana* (Flabby breasts), *Ayathaopachaya* (Disproportionate body growth) *Anutsaha* (General lack of enthusiasm and energy). *Sthoulya* is associated with several significant health complications, often described as the eight major defects. They are

Ayushohrasa (Reduced lifespan), *Javoparodha* (Decreased physical agility and interest in activities), *Kricchra Vyavaya* (Difficulty in engaging in sexual activity), *Dourbalya* (General debility or weakness), *Dourgandhya* (Unpleasant body odor), *Swedaabadha* (Excessive sweating), *Kshudhatimatra* (Excessive and persistent hunger) and *Pipasatiyoga* (Excessive thirst) (P. V. Sharma, 2001).

1.1.1. Importance of *Agni* in *Sthoulya*

It is said that all diseases are manifested as a result of *Agnimandya* (deranged *Agni* function) (Srikantha Murthy, 1995). Table 2. shows the various types of *Agnimandya* described in Ayurveda texts. In the context of *Sthoulya*, the *Agnimandya* refers to increased *Jatharagni* leading to excessive hunger and overeating, and a malfunctioned *Medo-dhatwagni* (the *Agni* present in the *Medo-dhatu* and responsible for *poshana* (nourishment/anabolism) and *pachana* (digestion/catabolism) of *Medo-Dhatu*) leading to excessive *poshana* of *Medo-dhatu* (fatty tissue) and reduced *poshana* of the *uttarottara dhatus* like *asthi*, *majja*, and *shukra dhatus*.

Type	Dosha predominance	Description
Mandagni	*Kapha*	Refers to a slow and weak digestive fire. Individuals with *Mandagni* have a sluggish digestion process, even with small amounts of food, and this condition is typically related to the *Kapha Dosha*.
Teekshnagni	*Pitta*	This denotes an excessively strong digestive fire, leading to rapid digestion of food, regardless of its nature. This is usually associated with *Pitta Dosha*.
Vishamagni	*Vata*	Characterized by irregular digestion, sometimes fast and sometimes slow, usually influenced by the *Vata Dosha*. This can lead to several digestive disorders.
Samagni	*Samadosha*	This balanced state of *Agni* digests and assimilates food efficiently and at the right time. Individuals with *Samagni* enjoy good health and robust tissue quality.

Table 2. Description of the types of *Agnimandya* (deranged *Agni* function)

1.1.2. Sthoulya Chikitsa

The approach includes dietary regulations, detoxification procedures, specific medications, and lifestyle modifications to restore balance and reduce excess body fat.

Guru Ahara (Consuming heavy foods) and *Apatarpana Chikitsa* (Adopting a treatment, diet, and lifestyle that do not overly nourish or provide excess calories) are generally adopted measures to treat *Sthoulya*. (P. V. Sharma, 2001) Table 3. shows selected dietary practices for the purpose. A list of medicines used for *Sthoulya* are given in Table 4. *Lodhrasavam* is one such formulation that is used to treat *Sthoulya*.

Pathya	***Apathya***
Old rice Green gram Kodo millet Barnyard millet Barley Green gram Pointed gourd Leafy vegetables Bitter-tasting vegetables Honey Buttermilk Warm water etc	New rice Black gram Starchy vegetables Sweet-tasting vegetables Sweet fruits Milk and dairy products Sugarcane products etc

Table 3. *Pathya-Apathya Ahaara* for *Sthoulya*.

Eka Moolika Prayoga	*Guduchi Bhadramusta Haritaki Vibhitaki Amalaki Agnimantha Guggulu etc*	***Rasa Yogas***	*Trayushanadi Loha Rasabhasma Yoga Vadavagni Rasa Trimurti Rasa Mahalakshmi Vilasa Rasa Vidangadi Loha etc*
Churna Yogas	*Trikatu Churna Vidangadi Churna Nishamalaki churna etc*	***Kashaya Yogas***	*Aragvadadi Kashaya Varunadi Kashaya Triphaladhya Kashaya Brihatmanjistadi Kashaya etc*
Vati Yogas	*Navaka Guggulu Amritadya Guggulu Taramandoora Guda etc*	***Asava-Arista***	*Lodhrasava Takrarishta Loharista etc*

Table 4. Internal medicines used for *Sthoulya*.

1.2. Lodhrasavam

रोध्रमूर्वाशठीवेल्लभार्गीनतनख़प्लवान्।
कलिङ्गकुष्ठक्रमुकप्रियङ्ग्वतिविषाग्निकान्॥२५॥
द्वे विशाले चतुर्जातं भूनिम्बं कटुरोहिणीम्।
यवानीं पौष्करं पाठां ग्रन्थिं चव्यं फलत्रयम्॥२६॥
कर्षांशमम्बुकलशे पादशेषे स्रुते हिमे।
द्वौ प्रस्थौ माक्षिकात्क्षिप्त्वा रक्षेत्पक्षमुपेक्षया॥२७॥
रोध्रासवोऽयं मेहर्शःश्वित्रकुष्ठारुचिकृमीन्।
पाण्डुत्वं ग्रहणीदोषं स्थूलतां च नियच्छति॥२८॥

In the context of *Prameha Chikitsa*, Acharya Vagbhata has described *Lodhrasavam* for the treatment of *Prameha, Arsha, Shwitra, Kushtha, Aruchi, Krimi, Pandu, Grahani* and *Sthoulya*. Most of the ingredients of *Lodhrasavam* are considered to be *kaphahara* and *medohara* in the classics (Table 5, 6.1 and 6.2).

Table.5. A compilation of the properties of each of the ingredients of *Lodhrasavam* (LS) as mentioned in the classical texts of Ayurveda.

Ingredients of LS	Properties of each ingredient described in the classical texts of Ayurveda		
	Dhanwantari Nighantu	**Bhava Prakasha**	**Others**
Lodhra	sheeta, kashaya, trishna-arochaka-vatakaphasrajit, chakshyshya, vishahara.	grahi, laghu, sheeta, chakshushya, kaphapittahara, raktapittasruk-jwara-atisara-shothahara-tilvakahara	
Murva	swadurasa, ushna, hridroga-kapha-vatajit, kushtha-kandu-vami-moha-vishamajwara nashini.	sara, guru, swadu, tikta, pittasra-mehanut, tridosha-trishna-hridroga-kandu-kushtha-jwarapaha.	
Shati	tikta, tikshna, ushna, sannipatajwarapha, kapha-ugravrana-kasaghni, vaktrashuddhikara.	kashaya, grahi, laghu, tikta, tikshna, katuka, ushna, aasyamalanashini, shotha-kasa-vrana-shwasa-shoola-sidhma-grahapaha.	
Vella		katu, teekshna, ushna, rooksha, vahnikara, laghu, shoola, adhmana-udara- shleshmakrimi- vata-vibandhanut.	
Bharangi	tikta, ushna, shwasa-kaphapaha, gulma-jwara-asrukvataghni, yashmapeenasahara.	rooksha, katu, tikta, ruchya, ushna, pachani, laghu, deepani, raktagulmahara, shotha-kasa-kaphashwasa-peenasa-jwaravatahara.	
Nata	kashaya, ushna, snigdha, tridoshahara, drik-sheersha-vishadoshaghnam, bhoota-apasmaranashanam.	kushthahara, ushna, swadu, snigdha, laghu,visha-apasmara-shoola-akshiroga-tridoshahara.	
Nakha			katu, ushna, shwasa-kasa-tridoshahara. (kaiyadeva nighantu)
Plava	tikta, kashaya, agnikara, shishira, shleshmaraktajit, pitta-jwara-atisaraghni, trishna-krimivinashini.	katu, hima, grahi, tikta, deepana-pachana, kashaya, kaphapittasruk-jwara-aruchi-jantuhara.	
Kalinga		tridoshaghna, sangrahi, katu, sheetala, jwara-atisara-arsha-vami-visarpa-kushthanut, deepana, gudakeelasra-vatasra-shleshmashoolajit, rooksha, hima.	

Ingredients of LS	Properties of each ingredient described in the classical texts of Ayurveda		
	Dhanwantari Nighantu	**Bhava Prakasha**	**Others**
Kushtha		ushna, katu, swadu, shukrala, tikta, laghu, vatasra-visarpa-kasa-kushtha-maruta-kaphahara.	
Kramuka		guru, hima, rooksha, kashaya, kaphapittajit, mohanam, deepanam, ruchyam, aasyavairasyanashanam, tridoshachedi.	
Priyangu	sheetala, tikta, moha-dahavinashini, jwara-vantihara, raktaprasadana	vatapittahara, raktatisara-dourgandhya-sweda-daha-jwarapaha, gulma-trishna-visha-mohaghni, vibandha-adhmana-balakrut, sangrahi, kaphapittajit.	
Ativisha	katu, ushna, tikta, kaphapittajwarapaha, amatisara-kasaghni, visha-chardi vinashini.	ushna, katu, tikta, pachana-deepana, kaphapittaatisara-amavisha-kasa-vami-krimihara.	
Agni	ushna, vatahara, panduhara, shopha-shleshma-agnimandya-aama-vibandha nashana	shwayathu-pandu-kaphavatahara, deepana.	
Vishala	tridoshahara, hridya, deepana, sara, shoola-gulma-krimi-kushthahara	vrana-shleshma-vata-kushtha-asra-jantujit, deepana, shwasa-gulmaghna, snigdha-ushna,sara.	
Ela		sookshma, kapha-shwasa-kasa-arsha-mootrakrichra-vatahara, sheeta, laghu.	
Patra	kapha-vata-arsha-hrillasa-arochakapaha.	madhura, kinchit teekshna, ushna, pichchila, laghu, kapha-vata-arsha-hrillasa-aruchi-peenasahara.	
Nagakeshara	alposhna, laghu, tikta, kaphapaha, bastiruk-visha-vatasra-kandughnam, shophanashanam.	kashaya, ushna, ruksha, laghu, amapachana, jwara-kandu-trishna-sweda-chardi-hrillasanashanam, dourgandhya-kushtha-visarpa-kapha-pitta-vishapaham.	

Table.5. A compilation of the properties of each of the ingredients of LS as mentioned in the classical texts of Ayurveda.

Continued...

Ingredients of LS	Properties of each ingredient described in the classical texts of Ayurveda		
	Dhanwantari Nighantu	**Bhava Prakasha**	**Others**
Bhunimba			tiktarasa, laghu, rooksha, katu vipaka, ushna veerya, kaphapittashamaka.
Katuki	pittajit, tikta, katu, sheetarsadahajit, balasa-arochaka-vishamajwaranashini.	katupaka, tikta, rooksha, hima, laghu, bhedani, deepani, hridya, kaphapittajwarapaha, prameha-shwasa-kasa-asra-daha-kushtha-krimihara.	
Yavani	katu, tikta, ushna, vata-shleshma-vishamaya-gulma-udara-shoolahara, aashudeepana.	pachana, ruchya, teekshna, ushna, katu, laghu, deepana, tikta, pittala, shukrashoolahara, vatashleshma-udara-aanaha-gulma-pleeha-krimihara.	
Pushkara	tikta, katu, ushna, kapha-vata-jwarapaha, shwasa-hikka-arochana-kasa-shopha-pandu-adhmananashana.	katu, tikta, ushna, vata-kapha-jwara-kasa-aruchi-shwasa-parshwashoolanut.	
Patha	tikta, vrishya, vishaghna, kushtha-kandu-chardi-hridroga-jwara-tridoshashamani, atisara-shoola-kaphapittajwarapaha.	ushna, katu, teekshna, vatashleshmahara, laghu, shoola-jwara-chardi-kushtha-atisara-hrudruja-daha-kandu-visha-shwasa-krimi-gulma-gara-vranan hanti.	
Granthi	katu, swadupaka, hima, snigdha, tridoshahara, trishna-jwara-udara-jantu-ama nashini, rasayani.	deepani, vrishya, swadupaka, rasayani, anushna, katu, snigdha, vatashleshmahara, laghu, rechani, shleshma-kasa-udara-jwara-kushtha-prameha-gulma-arsha-pleeha-shoola-ama-marutan hanti, pippali madhusamyutaa- medakaphavinashini, shwasa-kasa-jwarahara, medhya, agnivardhana.	

Continued...

Ingredients of LS	Properties of each ingredient described in the classical texts of Ayurveda		
	Dhanwantari Nighantu	**Bhava Prakasha**	**Others**
Chavya	katu, ushna, jantuhara, deepana, kaphodrekahara, vataprakopashamana.	kanamoolagunam visheshat gudajapaham.	
Haritaki		rooksha-ushna, deepana, medhya, rasayana, chakshushya, laghu, ayushya, brumhana, anulomana, shwasa-kasa-prameha-arsha-kushtha-shotha-udara-krimihara	
vibhitaki	katupaka, laghu, vaiswaryajit, sara, kasa-akshiroha-mukharoga-krimihara, keshavridhdhikara, rooksha, kaphasrajit.	ushnaveerya, kaphapittanut, bhedhana, kasanashana, rooksha, netrahita, keshya, krimi-vaiswaryanashanam.	
Amalaki	vayastha, vrushya, hima, sara, tridoshahara, jwaraghna, rasayana.	hareetakisamam, raktapitta-pramehaghnam, vrushyam, rasayanam, tridoshaghna.	
Madhu			madhura-kashaya rasa, laghu-vishada-rooksha, madhurapaka, ushnaveerya, kaphahara, medohara.

Table.6.1. Summarizing the *Gana* categorization of Lodhrasavam (LS) ingredient plants for their Kaphahara property (Srikantha Murthy, 1995)

INGREDIENTS	Kaphahara gana (A.H.Su.15)						
	Aragwadhadi gana	Arkadi gana	Mushkakadi gana	Asanadi gana	Surasadi gana	Mustadi gana	Vatsakadi gana
Lodhra							
Murva							+
Shati							
Vella					+		+
Bharangi		+			+		+
Nata					+		
Nakha							
Plava						+	
Kalinga	+			+			+
Kushtha						+	
Kramuka				+			
Priyangu							
Ativisha						+	+
Agni	+		+			+	+
Vishala							
Ela						+	+
Lavanga							
Patra							
Nagakeshara							
Bhunimba	+						
Katuki						+	+
Yavani							+
Pushkara							
Patha	+					+	+
Granthi							+
Chavya							+
Haritaki			+			+	
Vibhitaki			+			+	
Amalaki			+			+	
Madhu							

Table.6.2. Summarizing the *Gana* categorization of Lodhrasavam (LS) ingredient plants for their Kaphamedohara property (Srikantha Murthy, 1995)

INGREDIENTS	Kaphamedohara-A.H.Su.15							
	Patoladi gana	Varunadi gana	Rodhradi gana	Vacha-haridradi gana	Priyangu-ambashthadi gana	Nyagrodhadi gana	Eladi gana	Shyamadi gana
Lodhra			+		+	+		
Murva	+							
Shati								
Vella								
Bharangi					+			
Nata							+	
Nakha							+	
Plava			+	+				
Kalinga				+				
Kushtha							+	
Kramuka								+
Priyangu								
Ativisha				+				
Agni		+						
Vishala								
Ela							+	
Lavanga								
Patra							+	
Nagakeshara							+	
Bhunimba								
Katuki	+							
Yavani								
Pushkara								
Patha	+							
Granthi								
Chavya								
Haritaki		+		+				
Vibhitaki								
Amalaki								
Madhu								

Table.7. A compilation of the ingredient plants of Lodhrasavam (LS) for their individual effect on lipid metabolism.

Ingredient plants of LS	References for their effect on lipid metabolism
Symplocos cochinchinensis	The hexane extract of *Symplocos cochinchinensis* notably decreased total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), while significantly elevating high-density lipoprotein cholesterol (HDL-C) levels, compared to the hypolipidemic control group. (Sunil et al., 2012)
Kaempferia galanga	Kaempferol was observed to inhibit lipid accumulation in both adipocytes and zebrafish. Using Oil Red O and Nile Red staining techniques, a reduction in the number of intracellular lipid droplets was evident in adipocytes and zebrafish treated with kaempferol (Cao et al., 2023; Lee et al., 2023).
Embelia ribes	Embelin, derived from *Embelia ribes*, demonstrated preventive effects on lipid metabolism and oxidative stress in rats with high-fat diet-induced obesity. Additionally, the extract exhibited significant lipid-lowering activity in these animals. (Bhandari et al., 2002).
Valeriana jatamansi	In studies, triglyceride (TG) serum levels significantly dropped in groups receiving low, medium, and high doses of IRFV (Isolated Root Fraction of Valeriana) and the simvastatin group, compared to the model group. Similarly, the serum levels of high-density lipoprotein cholesterol (HDL-C) markedly increased across all dosages of IRFV and in the simvastatin-treated group. (Deng et al., 2022; Liu et al., 2022).
Saussurea costus	Rats fed a high-fat diet for 12 weeks exhibited markedly increased serum levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C), alongside reduced levels of high-density lipoprotein cholesterol (HDL-C). However, treatment with hispidulin at doses of 60 or 100 mg/kg/day for 12 weeks significantly lowered serum TC, TG, and LDL-C levels and raised HDL-C levels compared to the model group. (Wu & Xu, 2016)
Areca catechu	Supplementation with Areca extract notably reduced triglyceride absorption and plasma lipid levels. After administering an oral dose of [9,10(n)-(3)H] triglyceride, the amount of triglyceride absorbed and detected in the bloodstream was significantly lower in the Areca nut extract-supplemented rats compared to the control group. (Byun et al., 2001; Qu et al., 2021)
Aconitum heterophyllum	The methanol fraction of *Aconitum heterophyllum* Wall ex Royle demonstrated a significant hypolipidemic effect in rats with diet-induced obesity. (Subash & Augustine, 2012)
Plumbago zeylanica	Plumbagin combats obesity by inhibiting pancreatic lipase activity and preventing the differentiation of adipocytes. (Pai et al., 2018).
Citrullus colocynthis	*Citrullus colocynthis* demonstrates antiplatelet and profibrinolytic activities in both normal and high-fat diet-induced obese rats (Alhawiti, 2018).

Elettaria cardamomum	Consumption of cardamom (*Elettaria cardamomum* (L.) Maton) seeds enhances energy expenditure and decreases fat mass in mice by influencing neural circuits that control lipolysis in adipose tissue and promote mitochondrial oxidative metabolism in the liver and skeletal muscles. (Delgadillo-Puga et al., 2023)
Cinnamomum verum	Gold nanoparticles functionalized with bioactive compounds from *Cinnamomum verum* prevent obesity by reshaping the gut microbiota. (V. K. Sharma et al., 2022)
Cinnamomum tamala	The ethanolic extracts of *Cinnamomum tamala* and *Aloe vera* significantly reduced lipid levels and increased HDL-c concentrations. (Singh et al., 2015)
Mesua ferrea	Cobra's saffron significantly inhibited lipid peroxidation (LPO) (Yadav & Bhatnagar, 2010)
Swertia chirayita	Swertiamarin significantly reduces the accumulation of lipids, indicating its potential to prevent fat buildup. (Wang & He, 2019)
Cuminum cyminum	Arq Zeera and its primary components exhibit strong anti-obesity effects in high-fat diet-induced obese rats, achieved through actions including hypolipidemic, hypoglycemic, hypoinsulinemic, hypoleptinemic effects, and inhibition of pancreatic lipase. (Haque & Ansari, 2018)
Inula racemosa	IrA reduced total cholesterol, triglycerides, low-density lipoprotein cholesterol, and the atherogenic index, while increasing high-density lipoprotein cholesterol compared to the positive control. (Mangathayaru et al., 2009)
Cyclea peltata	The aqueous extract primarily lowers glucose and lipid levels by enhancing insulin levels. (Bose & Sarma, 1975)
Piper longum	Piperine exhibits numerous pharmacological effects, including antiproliferative, antitumor, antiangiogenic, antioxidant, antidiabetic, anti-obesity, cardioprotective, antimicrobial, antiaging, and immunomodulatory effects in various in vitro and in vivo experimental trials. (Haq et al., 2021)
Terminalia chebula	Anti-Obesity Effects of Terminalia chebula Fruit Extract on High-Fat Diet-Induced Obese Mice (Subramanian et al., 2021)
Terminalia bellerica	Effectiveness of Triphala Extracts on Alterations in the Fecal Microbiome and Metabolome of an Obese Human Gut Model (Kwandee et al., 2023)
Phyllanthus emblica	Effectiveness of Triphala Extracts on Alterations in the Fecal Microbiome and Metabolome of an Obese Human Gut Model (Kwandee et al., 2023)
Honey	Honey and its components lower blood sugar levels, enhance insulin sensitivity, and improve lipid metabolism by decreasing triglycerides, total cholesterol, and LDL levels, while increasing HDL levels. These effects help prevent excessive weight gain and reduce the risk of obesity and its associated complications. (Zulkifli et al., 2022).

1.3. Correlating *Sthoulya* to Obesity

Overweight and obesity are abnormal or excessive fat accumulation that presents a health risk. A body mass index (BMI) over 25 is considered overweight, and over 30 is obese. (WHO) *Sthoulya* is the increase in body mass due to the accumulation of fatty tissue and has similar symptoms and manifestations as that of obesity.(Verma et al., 2019) The accumulation of excessive body fat induces various changes in mechanical functions, the activity of the sympathetic nervous system, cellular dynamics, and metabolic processes. These changes play a significant role in the development of chronic diseases, heightening the risk of conditions such as cancer, insulin resistance, and cardiovascular disorders. Several common obesity-related conditions include degenerative joint disease (DJD), increased levels of free fatty acids (FFA), gastroesophageal reflux disease (GERD), hypertension (HTN), obesity hypoventilation syndrome (OHS), obstructive sleep apnea (OSA), and disturbances in the renin-angiotensin-aldosterone system (RAAS).(Busebee et al., 2023) Classification of obesity is given in Table 6. Commonly adapted interventions to overcome obesity are given in Table 7.(Aaseth et al., 2021) The long-term usage of anti-obesity drugs poses significant challenges due to potentially life-threatening side effects. Several promising weight-loss medications have been withdrawn from the market because of these adverse effects. Some examples include aminorex, linked to pulmonary hypertension; fenfluramine and dexfenfluramine, associated with cardiac valvulopathy; phenylpropanolamine, implicated in stroke; rimonabant, which has been linked to suicidal ideation and behavior; sibutramine, associated with myocardial infarction and stroke; and more recently, lorcaserin, which has raised concerns regarding cancer risk. (Tak & Lee, 2021) Complications of bariatric surgery: Tachycardia, surgical complications like fistula, bleeding, herniation, gastric erosion, bowel obstructions, etc., deep vein thrombosis and pulmonary embolism, post-operative pneumonia, nutritional complications like anemia, vitamin D & b12 deficiency, etc., hepato-biliary complications like gall stones, etc., gastric ulcers, dumping syndrome, food blockade by a ring, mesenteric vein or portal system thrombosis, neuropathy, myopathy and encephalopathy, & infertility(Kassir et al., 2016)

The issue of obesity has grown to epidemic proportions, with over 4 million people dying each year as a result of being overweight or obese in 2017 according to the global burden of disease. The incidence of overweight and obesity is rising steadily among both adults and children. Globally, the proportion of children and adolescents aged 5 to 19 who are classified as overweight or obese surged from 4% in 1975 to 18% in 2016. In India, the number of adults aged 20 to 69 classified as overweight is expected to more

than double from 2010 to 2040, while the rate of obesity is predicted to triple. By 2040, the prevalence of overweight and obesity among Indian men is projected to reach 30.5% (ranging from 27.4% to 34.4%) and 9.5% (ranging from 5.4% to 13.3%), respectively. Among women, these rates are anticipated to hit 27.4% (ranging from 24.5% to 30.6%) for overweight and 13.9% (ranging from 10.1% to 16.9%) for obesity.(Luhar et al., 2020)

Classification of Obesity based on BMI	
Type	**description**
Class I	BMI - 30 to less than 35
Class II	BMI - 35 to less than 40
Class III	BMI - 40 and higher
Classification of Obesity based on Metabolic status	
Metabolically Unhealthy	Individuals with obesity and metabolic dysfunction, based on criteria such as dyslipidaemia, hypertension, insulin resistance, and elevated inflammatory markers, are considered metabolically unhealthy
Metabolically Healthy	Individuals with obesity without metabolic dysfunction
Other classifications	
Central Adiposity	fat accumulation in the abdominal region
Sarcopenic Obesity	concurrent obesity and decreased muscle mass or function

Table 8. Classification of Obesity

Dietary interventions	Drug interventions	Surgical interventions
Low-fat diet (LFD) Low carbohydrate-high fat diet (LFDHF) Low carbohydrate-high protein (LFDHP) The Paleo diet (high in protein) The 5:2 intermittent fasting diet Etc.	Liraglutide Semaglutide Orlistat Bupropion Bupropion-naltrexone Phentermine/ topiramate Etc.	Bariatric surgery Etc.

Table 9. Interventions in Obesity

1.4. Impaired lipid Metabolism in Obesity

Lipid metabolism is the process of synthesis, break down, and storage of fat in cells for energy. Key mechanisms involved in lipid metabolism include lipolysis (Stored triglycerides in fat cells are broken down into glycerol and free fatty acids by enzymes called lipases; and hormones like adrenaline and glucagon activate lipase enzymes, particularly hormone-sensitive lipase (HSL), initiating lipolysis during energy demand), beta-oxidation, ketogenesis, de novo lipogenesis and its storage as triglycerides, cholesterol synthesis (key enzymes include HMG-CoA reductase, which is a major control point and target for cholesterol-lowering drugs (statins)), and lipoprotein metabolism (lipoproteins like chylomicrons and VLDL are formed to transport triglycerides and cholesterol in the blood; LDL delivers cholesterol to cells via receptor-mediated endocytosis, while HDL helps remove excess cholesterol from tissues and returns it to the liver for excretion or recycling.). Insulin promotes fat storage by enhancing lipogenesis and inhibiting lipolysis. In contrast, glucagon and adrenaline stimulate lipolysis and fatty acid oxidation. Molecular pathways such as acetyl-CoA carboxylase, PPAR-ɣ, FASN, FABP4, CEBP-α, leptin, adiponectin, DPP4, etc., (for formation) and hormone-sensitive lipase (for breakdown) are tightly regulated to maintain metabolic balance. These pathways are fundamental for maintaining energy homeostasis and supporting various cellular functions. Disruptions can lead to metabolic diseases like obesity, diabetes, and cardiovascular disorders. Understanding these processes is critical for developing therapeutic strategies against such conditions. Additionally, the free fatty acids released into the blood as a result of lipolysis happening in the adipose tissue are taken up by the liver for various downstream

processes and storage purposes. When this fat storage in the liver increases, it gradually leads to fatty liver-like conditions. (Godoy-Matos et al., 2020)

1.5. *Lodhrasavam* in Obesity and lipid metabolism

With an increase in the complications of obesity management in medical practice, there is a need for innovative strategies for obesity management. Approaches exploring the benefits of traditional systems of medicine for the treatment of obesity is promising in this context. Ayurveda is one such system of holistic medicines that focuses on preserving health and treating diseases. As mentioned, *Agni,* a key physiological phenomenon in Ayurveda, plays a crucial role in the manifestation as well as the management of Obesity *(Sthoulya)*. In the Ayurvedic classics, *Lodhrasavam* is said to have the potential to manage obesity via *Agni* modulation. It is also considered an *Oushadha Rasayana*. Previous studies from our lab has shown the anti-diabetic and anti-adipogenic effect of *Lodhrasavam* using in vitro model systems (Butala et al., 2017). Yet, the probable molecular mechanism of action of this polyherbal formulation has not been studied and it is imperative for a better understanding of its biology and its applications in the management of obesity. Previous studies in our lab had also shown that Lodhrasavam alleviates steatosis and lipotoxicity in a HepG2 cell model of non-alcoholic fatty liver disease (NAFLD) (Kouser et al., 2023)

1.6. In vitro models for studying lipid metabolism

Adipose tissue is composed of a variety of cell types, including endothelial cells, blood cells, fibroblasts, preadipocytes, macrophages, and other immune cells, alongside mature adipocytes. When the population of adipocytes grows significantly, they dominate the cellular composition, leading to the formation of adipose tissue. There are two types of body fat: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT primarily serves as an energy reservoir; it stores surplus energy in the form of triglycerides (TG) through a process called lipogenesis and mobilizes these reserves during periods of fasting through lipolysis. An overabundance of energy intake results in an increase in both the number and size of adipocytes, leading to the expansion of WAT, which can contribute to the development of obesity.(Tung et al., 2017)

To investigate the molecular mechanisms behind adipocyte differentiation, researchers employ various cell culture models. These models include preadipocyte cell lines and primary cultures derived from stromal vascular precursor cells of adipose tissue. Studying preadipocyte differentiation within living

organisms presents significant challenges because human and animal fat tissues are complex mixtures of small blood vessels, nerve fibers, fibroblasts, and preadipocytes at different developmental stages. This complexity, coupled with issues like contamination, limited lifespan, and the low proportion of preadipocytes in total adipose tissue, complicates the use of primary preadipocyte cultures. As a result, the molecular mechanisms of adipogenesis are predominantly explored using in vitro methods, particularly through clonal preadipocyte cell lines from mice or rats. Among these, the 3T3-L1 cell line, developed by Green and Kehinde, is a well-established and robust model. Derived from 17- to 19-day-old Swiss 3T3 mouse embryos, the 3T3-L1 line closely mimics the differentiation of preadipocytes into adipocytes as observed in animal tissues. Upon reaching confluence, some 3T3-L1 cells can spontaneously differentiate into rounded cells containing large triglyceride droplets. These cells exhibit significant increases in de novo lipogenesis and heightened activities of enzymes such as ATP-citrate lyase, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), glycerophosphate acyltransferase, and malic enzyme. This spontaneous adipocyte conversion typically occurs 2 to 4 weeks post-confluence. However, the differentiation process can be expedited by treating the cells with an adipogenic cocktail comprising insulin, dexamethasone, and 3-isobutyl-1-methylxanthine. This treatment prompts the expression of key transcription factors within one hour, leading to full differentiation within 10 to 12 days. (Tung et al., 2017)

HepG2 cells were among the earliest hepatic cell lines to demonstrate the essential traits of hepatocytes. Initially identified and characterized as hepatocellular carcinoma (HCC) in 1975, these cells have since been a pivotal model for liver cancer research. (Arzumanian et al., 2021) The hepG2 cell model is also used to study lipid metabolism (uptake, storage and toxicity) in liver.(Sandoval et al., 2023)

1.7. Hypothesis of the current study

The hypothesis is that *Lodhrasavam* being indicated for *Sthoulya*, inhibits adipogenesis in 3T3L1 fibroblasts and induces lipolysis in mature 3T3L1 adipocytes via *Agni* modulation, and also affects the lipid uptake by liver cells (HepG2 cell model) and has a hepatoprotective activity.

2. MATERIALS AND METHODS

Lodhrasavam (Kottakkal Arya Vaidya Sala) with Batch No. 524174 was procured from the market and was filtered using a 0.2µm filter before being used for the experiments.

2.1. Literature review

A PUBMED search was done to find the effect of each of the ingredients of LS in obesity and lipid metabolism using their botanical names and also to find the previous studies done on LS in obesity. Ayurvedic classical texts were referred to find the pharmacological actions of each ingredient separately, as well as to study the description of *Sthoulya* and *Agni* in Ayurveda.

2.2. 3T3L1 cell culture

3T3-L1 fibroblasts were purchased from National Centre for Cell Sciences, Pune, India. 3T3-L1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% penicillin and 1% streptomycin supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in a 5% CO_2 humidified atmosphere.

Materials required: DMEM, TE, PBS, culture plate, 15ml tube, laminar air flow, aspirator, pipette, tips, incubator, centrifuge, Neubauer chamber.

Method: Take out the culture plate from the incubator; observe under the microscope for confluency and contamination (if any). Take the culture plate in the laminar airflow. Keep the culture plate in the appropriate position and remove the medium using an aspirator. Wash with PBS, add 1 ml of TE, and close the lid; keep it in the incubator at 37 degrees Celsius for 2 minutes. After 3 mins of incubation, take the plate into the laminar airflow; Add 3 ml of fresh DMEM to it using a pipette; mix it slowly to get all the cells suspended in the medium; transfer it to a 15 ml tube using a pipette. Centrifuge the tube with cells at 2,700 rpm for 2 minutes. Meanwhile, take a new plate and add 8 ml of fresh DMEM to it. After centrifugation, a pellet of cells is formed. Take the tube inside the laminar airflow and aspirate the liquid portion of the tube; add 1 ml of fresh DMEM to the tube and mix it slowly to get a homogeneous suspension of the cells. Take the suspension in the Neubauer chamber, count the number of cells, and seed the required number of cells in each well of the experiment plate and maintenance plate. Observe the cells under the microscope and keep them in the incubator at 37 degrees Celsius for growth.(Butala et al., 2017)

2.3. Gallic Acid Estimation Assay

Materials required: gallic acid standard solution, folin-ciocalteu reagent 3.5% Sodium carbonate solution, 1M Sodium-potassium buffer solution, 96-well plate, Distilled water, Test tubes, Spectrophotometer, Pipettes Vortex mixer or shaker, Lodhrasavam.

Reagents preparation:

Gallic acid standard solution: 1 mg/ml concentration. (Weigh 1 mg of gallic acid salt & vortex in 1 ml 0.02M sodium potassium buffer). For 250 µgm/ml concentration aliquote 250µl from the above solution & make up to 1ml.Folin-Ciocalteu reagent: Prepare a 1:1 ratio of Fc reagent with water according to the number of wells and volume required. 3.5% Sodium carbonate solution: add 3.5 gm of Sodium carbonate in 100 ml of nice water. For 25 ml of sodium carbonate: add 0.875 gm in 20 ml nice water. Prepare 1M Sodium-potassium buffer.

Procedure:

Add 40 µL of 0.02M NAP buffer to the wells in triplicate. Add 10 µl of Lodhrasavam to the wells in triplicate. Add 50µl of Fc reagent to the sample and mix it well except the control group, while adding fc reagent lights should be turned off. Add 50 µL of 0.02 M of NAP buffer to the control. Incubate the plate for 30 minutes in a dark chamber. Add 3.5 % of 100 microliters of Sodium carbonate to all the wells and mix it well. Read the plate under a microplate reader against 700 nm wavelength (200 path length).

2.4. MTT cell viability assay

Principle: A rapid colorimetric assay based on the cleavage of the tetrazolium ring of MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in active mitochondria of living cells as an estimate of viable cell number.

Method: 3T3L1 cells were seeded in a 48-well plate with 20,000 cells in each well. (half of the plate was seeded on Day 0(to be treated with LS after 72hrs of seeding) and the other half on Day 2 (to be treated with LS after 24hrs of seeding)) On Day 3, the cells in all the wells were treated separately with 48LS, 40LS, 32LS, 28LS, 24LS, and 16LS. On Day 4 (i.e., after 24hrs of treatment with LS) MTT was added to all the wells. [Weigh 7.5mg of MTT and dissolve it in 1ml of 1X PBS. Once it dissolves completely, mix it with 14ml of DMEM to get a working concentration of 0.5mg/ml. Add 300 µL of MTT working solution to each well in the plate and incubate at 37 degrees Celsius in dark for 2 hours. After 2 hrs, aspirate out

the MTT working solution; add 300 μL of DMSO and mix well. Read 570nm wavelength using a spectrophotometer. Calculate the cell viability using the obtained OD values.]

2.5. Induction of Adipogenesis (3T3L1 cell differentiation)

The 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS, and 1% PS at 37 °C in a humidified 5% CO_2 atmosphere. Cells were maintained in the same medium until growth arrest. After 48 hours of confluency (Day 5), differentiation was induced in a differentiation initiation medium comprising DMEM containing 10% FBS, 1%PS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 50nM insulin, and 250 μM dexamethasone. The differentiation medium was withdrawn on Day 8 and switched to differentiation progression medium (DPM) comprising DMEM supplemented with 10% FBS, 1%PS, and 50nM insulin. On Day 10, cells were switched to a post-differentiation medium (PDM) containing 10% FBS and 1% PS which was kept until the experiment day (TAG, Oil O Red, RNA isolation). (Butala et al., 2017)

For studying the effect of Lodhrasavam on mature adipocytes, the same induction procedure was followed but without treatment with Lodhrasavam till maturity. On Day 12, the cells were treated with various concentrations of Lodhrasavam and the TAG levels were measured after 48 hours and 72 hours for lipolysis (TAG levels in the culture media).

2.6. TAG assay

Principle: The triglyceride assay detects triglyceride levels by measuring glycerol that is released from an enzymatic reaction with a lipase: one mole of glycerol per mole of triglyceride. Glycerol is measured in a coupled reaction scheme that links the production of NADH to the activation of a pro luciferin that produces light with luciferase.

The amount of triglyceride is determined from the difference of glycerol measured in the absence (free glycerol) and presence (total glycerol) of lipase. Lipase converts triglyceride (TAG) to glycerol. Glycerol kinase and glycerol-3-phosphate dehydrogenase are used to generate NADH. In the presence of NADH, Reductase enzymatically reduces a pro-luciferin Reductase Substrate to luciferin. The amount of light produced is proportional to the amount of glycerol in the sample. The triglycerides kit from Benesphera was used for the assay.

Preparation of reagents: 1% PBST: 100ul of Tween-20 in 10ml of 1X PBS.

Preparation of glycerol standard: Glycerol: PBST = 1: 7 (50ul glycerol in 350µL PBST)

Sample preparation: Remove the media and wash with a PBS buffer (500 ul/ml of PBS). Remove the PBS buffer & add 250µL of PBST. Scrape the cells and aliquot them in an Eppendorf tube. Sonicate until it appears homogeneous. Vortex and spin at 4000 rpm for 1 min. 200µL of supernatant is added to another tube for TAG assay; the remaining 50ul is kept for Bradford's assay.

Method: TAG assay samples are kept in a hot water bath at 80 degrees Celsius for 10 mins. Take glycerol standard and PBST & serially dilute (50µL in each well). Add samples in different wells as well. Then add 100µL of TAG reagent in each well. Incubate at 37 degrees Celsius for 30 mins. Read at 546 nm wavelength.

2.7. BCA (Bicinchoninic Acid) protein estimation assay

Principle: The BCA assay relies on the reduction of Cu^{2+} ions to Cu^{1+} ions by protein in an alkaline medium. The Cu^{1+} ions then react with the bicinchoninic acid (BCA) and form a complex that absorbs light at 562 nm, which can be measured using a spectrophotometer. The QPRO-BCA kit standard from Cynagen was used for the assay.

Materials: BCA working reagent (see recipe below), BSA standard, Protein sample, 96-well microplate & Microplate reader

BCA Working Reagent Recipe:

BCA reagent A: 50 mg/mL of bicinchoninic acid in 2% sodium carbonate solution

BCA reagent B: 4% cupric sulfate solution in 0.4 N sodium hydroxide

Mix 50 parts of BCA reagent A with 1 part of BCA reagent B. Mix well and incubate at room temperature for 30 minutes before use.

BSA (2mg/ml)- weigh 2 mg of BSA and add 1 ml of extraction buffer (PBST) to it and vortex.

Method: Prepare the BCA working reagent by mixing 50 parts of BCA reagent A and 1 part of BCA reagent B. Prepare a series of BSA standard solutions with known concentrations ranging from 0 to 2 mg/mL. Use distilled water to prepare the dilutions. Pipette 10 µL of each BSA standard solution and protein sample into separate wells of a 96-well microplate. Add 80 µL of the BCA working reagent to each well. Incubate the plate at 37°C for 30 minutes. Measure the absorbance at 562 nm using a microplate reader. Plot a standard curve using the absorbance values of the BSA standards. Calculate the protein concentration of the samples using the standard curve.

2.8. Oil-O-Red staining

The principle is that the solubility of ORO in neutral lipids is higher than that in the dye solution. During staining, ORO is transferred from the dye solution to fat, so that the lipid droplets can be stained red.
Preparation of reagents: 0.5% stock solution- dissolve 0.5gram of ORO in 100ml of isopropanol. Keep in water bath until the dye dissolve or keep on magnetic stirrer as an alternative. Working solution- 30ml of ORO stock diluted with 20ml of distilled water (keep on magnetic stirrer until homogeneous).
Cell Staining: 1. Fixation – Remove the medium and gently wash the cells twice with PBS. Add Formalin (10%) to the cells and incubate for 30 minutes to 1 hour. Note: Do not add formalin directly onto the cells. Pipette onto the wall and mix by gently rotating. 2. Discard the formalin and wash the cells twice using water. Add 60% isopropanol to the cells and incubate for 5 minutes. 3. Discard 60% isopropanol and cover the cells evenly with Oil Red O Working Solution. Rotate the plate or dish, and incubate for 10–20 minutes. 4. Discard the Oil Red O solution and wash the cells 2–5 times with 1X PBS until no excess stain is seen. Cover the cells with 1X PBS and view them under the microscope. Lipid droplets appear red and nuclei appear blue.
Quantification of Oil Red O staining:
The stain was extracted in 250 µl isopropanol and 200 µl was used to measure Oil Red O stain in a 96-well plate reader at 500 nm.

2.9. RNA isolation

Cell lysis: Set the centrifuge for fast cooling (4°C) 10 mins before scraping of cells. Remove media from wells and wash cells once with 1X PBS. Add 500µl of 1X PBS to the washed cells and scrape them. Transfer the scraped cells into labelled 1.5ml Eppendorf tubes. Centrifuge the tubes at 4000 rpm for 10 mins. Carefully remove the supernatant without disturbing the pellet. Add TRIzol reagent (Total RNA isolation reagent) according to the pellet amount (200-1000µl). (TRIzol maintains RNA integrity while disrupting cells and dissolving cell components) The RNAiso Plus from TaKaRa was used for lysis purpose.

Isolation: Gently lyse the cells with a pipette tip and vortex until clear visibility. Add chloroform in a 1:5 ratio with TRIzol (Ex-300:60) and vortex for 30 secs (Use DEPC treated tips and Eppendorf tubes after this step). Allow to rest at room temperature for 3 mins, then centrifuge the tubes at 14000 rpm for 15

mins. Carefully transfer the upper transparent phase to new labelled DEPC treated Eppendorf tubes. Add double the ice-cold 100% isopropanol volume to the transferred phase. Mix well. Centrifuge the tubes at 14,000 rpm for 40 mins, discard the supernatant and retain the pellet. Add 500µl of 70% DEPC Ethanol to the pellet (Prepare 70% with DEPC treated water). Free the pellet attached to the tube by gentle agitation. Centrifuge the tubes at 12,000 rpm for 10 mins. Carefully remove the supernatant without disturbing the pellet. Repeat this step once again. Allow the tubes to air dry to remove ethanol. Add 10-15µl of DEPC-treated water to dissolve the RNA pellet. Quantify the RNA using a Nanodrop.

2.10. cDNA synthesis

First, quantify the RNA to see the purity. Once quantified, calculate the volume of RNA as required 500 ng of cDNA (divide the concentration of RNA by 500). Calculate the volume of autoclaved distilled water by subtracting the volume of RNA with 6.5ul Master Mix has to be prepared in a 1.5ml tube & add 3.5µL of Master Mix to each sample. Master Mix composition for one sample is Buffer – 2ul, Enzyme-0.5ul, Primer-0.5ul, & Hexamer-0.5ul (Total-3.5ul). Vortex the Master Mix; Take the PCR tubes 1st add water then add RNA & then add 3.5ul Master Mix to each tube. Then vortex it, keep it for synthesis in PCR and label the PCR tubes & store it at -20 degrees.

2.11. Primer designing

Visit the NCBI Gene website; enter the gene symbol as per the gene of interest; Select the organism's name and select the gene. On the next page scroll down until the Transcript table & click on NMID. Click on send to & we will have options in that options select format FASTA. In the same tab click on FASTA. Once we select format FASTA the file will download & open the file with Notepad. Now open NCBI Primer blast & choose the option primer for the target on one template. Now paste the copied sequence. Keep the product size minimum:100 & maximum: 250; Melting temperature: Minimum: 57 & Optimum: 60; Annealing Temperature (Tm): Maximum: 63 & Minimum: 3; Primer return: 10. Select the primers with the specificity of binding. Copy the following information in the EXCEL: Species name, Gene name, Gene symbol, Gene ID, NMID, Forward primer (Temperature & sequence), Reverse primer (Temperature & sequence), Product length.

1	PPAR γ	Forward Primer	TTTTCAAGGGTGCCAGTTTC
		Reverse Primer	AATCCTTGGCCCTCTGAGAT
2	SREBP-1c	Forward Primer	GTGAGCCTGACAAGCAATCA
		Reverse Primer	GGTGCCTACAGAGCAAGAGG
3	FASN	Forward Primer	TGGGTTCTAGCCAGCAGAGT
		Reverse Primer	ACCACCAGAGACCGTTATGC
4	Leptin	Forward Primer	GCTCATGTCCCTGTGGTTAG
		Reverse Primer	GCCCTGAAATGCGGTATGTA
5	FABP4	Forward Primer	AAGGTGAAGAGCATCATAACCCT
		Reverse Primer	TCACGCCTTTCATAACACATTCC
		Reverse Primer	CGCCCTTAGTTGGTCAGAAG
6	GAPDH	Forward Primer	ACCCAGAAGACTGTGGATGG
		Reverse Primer	CACATTGGGGGTAGGAACAC

2.12. RT-PCR

Take out the Primers & Samples from -20 degrees, thaw them, and keep them on ice. For each primer prepare Master Mix. [Water- 2.2 µL, Forward Primer – 0.4 µL, Reverse Primer- 0.4ul, SYBR Green- 5ul]. Then make a PCR plate plan. Then add 2ul of cDNA sample to the wells. Now add the SYBR green to each primer labelled Master Mix tube and mix well; then add 8ul of Master Mix to each well. Check the plate for air bubbles. Then cover the plate, seal it & run the sample in the qRT-PCR machine. Machine - Set the samples and targets; Then set the methodology & temperature: 50 degrees – 2 minutes, 95 degrees- 3 minutes, 95 degrees- 10 seconds, 60 degrees- 30 seconds, 95 degrees -15 seconds, 60 degrees- 1 minute, 95 degree- 15 seconds, then place the plate inside qRT-PCR. Then run the protocol for the given time.

2.13. Lipase inhibition assay

Materials required: 96-well plate, Distilled water, Test tubes, Spectrophotometer, Pipettes Vortex mixer, or shaker.

Reagents required: 1M sodium Phosphate buffer (1M stock), 50 Mm sodium phosphate buffer (50 Mm working solution), 10mg/ml lipase, substrate PNPB.

Reagents preparation: Prepare 50 mM of sodium phosphate buffer of pH 8.0. Weigh 200 mg of lipase aliquot, and 10 ml of 50 mM NaP buffer in two different tubes add 100 mg of weighed lipase into each tube and vortex it for 4 minutes. Then centrifuge it at 4000rpm for 10 minutes. Then collect the supernatant in a separate vial. Then prepare lipase buffer mix by adding 1.5 ml of prepared lipase to 3.5 ml of 50 mM NaP buffer.

PNPB substrate preparation: This should be prepared freshly before the addition. Add 3µl of PNPB in 15ml of 50mM NaP buffer.

Procedure:

Serially dilute the Lodhrasavam with 128µg/GAE as the highest concentration. Then add 150µl of lipase buffer mix to one set of duplicates and for the other set add 150µl of 50mMNap buffer (color control). Then keep the plate for incubation for 30 minutes at 37°c. Then add 90µl of freshly prepared subsatrate to all the wells. Then read the plates under a spectrophotometer against 405nm for 45 minutes.

2.14. HepG2 cell culture and treatment

It is done by the same procedure as that of 3T3L1 cell culture as explained except for the trypsinization step where 3 ml of TE has to be added unlike for 3T3L1 cells. The cells were seeded in 12 well plates with 80,000 cells in each well. After reaching confluency these cells were treated with the conditioned media from 3T3L1 adipocytes which were previously treated with LS for the inhibition of adipogenesis in pre-adipocytes and increasing lipolysis in mature adipocytes. This was done to know the indirect effect of treating adipocytes with LS on hepatocytes. TAG levels in HepG2 cells were measured after 24 hours and 48 hours of treatment with conditioned media from the Lodhrasavam-treated (for inhibition of adipogenesis) wells. TAG levels in HepG2 cells were measured after 48 hours and 72 hours of treatment with conditioned media from Lodhrasavam-treated (for increasing lipolysis) wells.

2.15. Statistical analysis

Mean and standard deviation; single factor ANOVA.

3. RESULTS

3.1. Cell viability assay

When treated after 24 hours of seeding, cells show 36.3%, 74.4%, 88.1%, 89.9% & 91.5% viability to 48LS, 40LS, 32LS, 24LS & 16LS respectively with control having 100% viability; whereas, those which were treated after 72 hours of seeding, show 61%, 76.4%, 88.9%, 92.5% & 90.5% viability to 48LS, 40LS, 32LS, 24LS & 16LS respectively with control having 100% viability. (Fig.1)

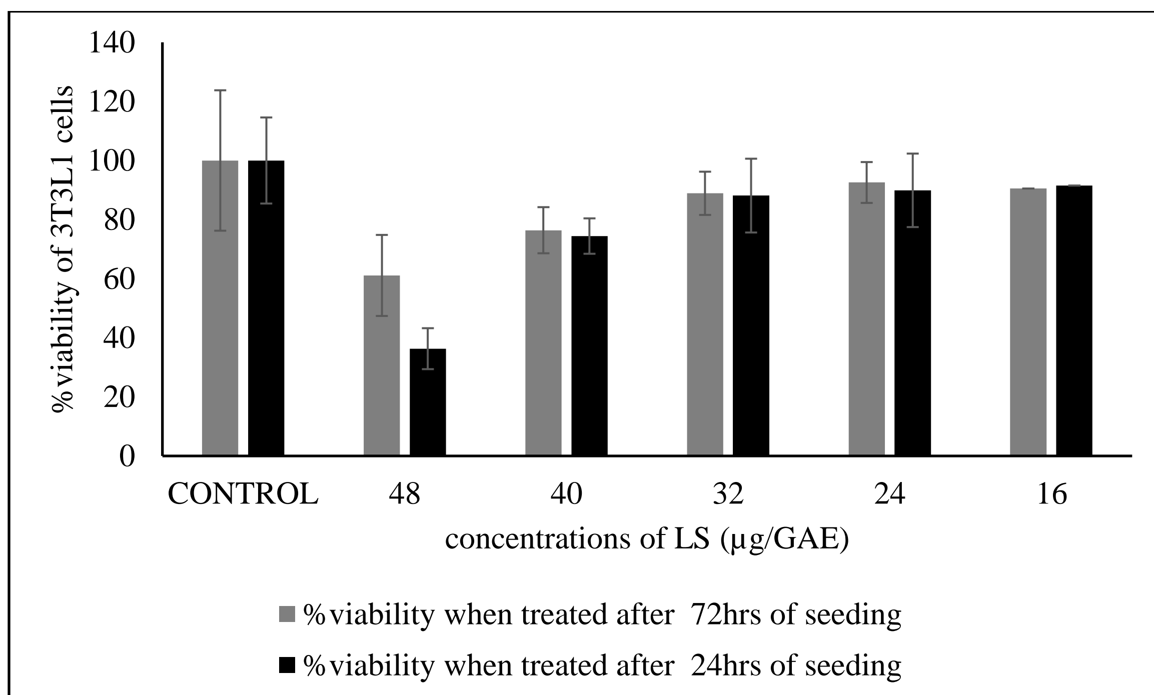

Fig.1. All tested concentrations of LS showed >76% cell viability when treated after 72 hours of seeding; while treatment after 24 hours of seeding showed >74% cell viability with all the concentrations except for 48ug/GAE concentration. (which showed 36.3% viability)

3.2. LS inhibits adipogenesis by affecting the TAG levels in the 3T3L1 cell model

When treated with 32LS, 28LS, 24LS, and 16LS the cells showed the TAG levels of 28, 36, 55, and 72 µg/mg protein respectively with MDI-treated cells and control showing 92 and 16µg/mg protein respectively. 32LS showed a significant inhibition of adipogenesis. (Fig.2)

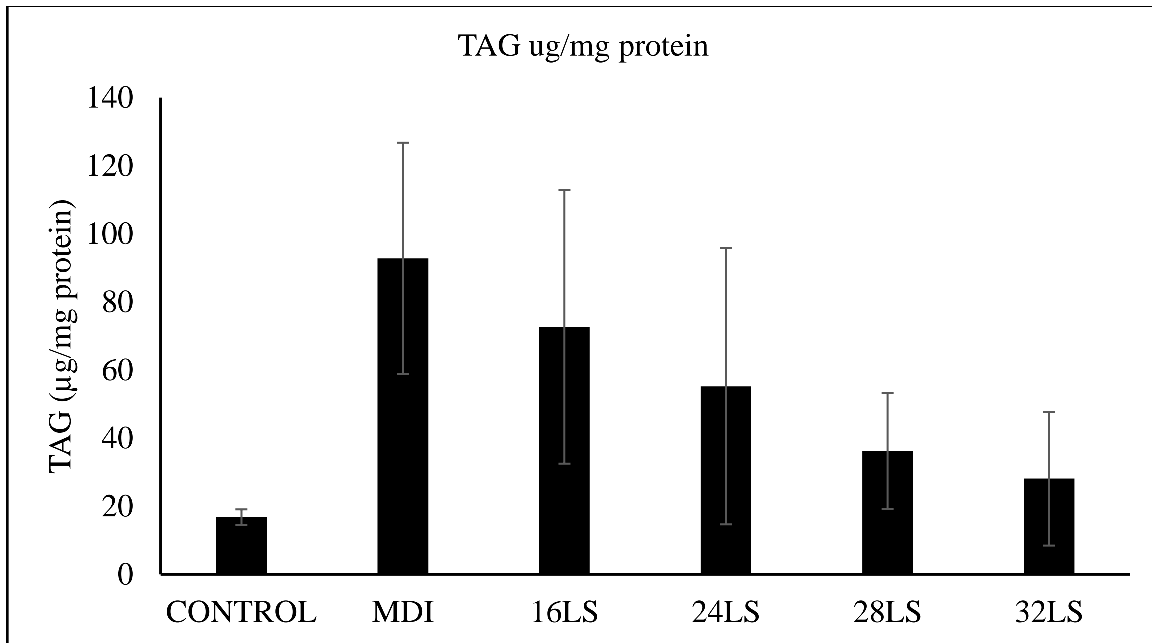

Fig.2(a). MDI shows a 5.5-fold increase in Triglyceride (TAG) accumulation compared to control. 32LS shows a 3.3-fold, 28LS a 2.6-fold, 24LS a 1.7-fold, and 16LS a 1.3-fold decrease in Triglyceride (TAG) accumulation compared to MDI.

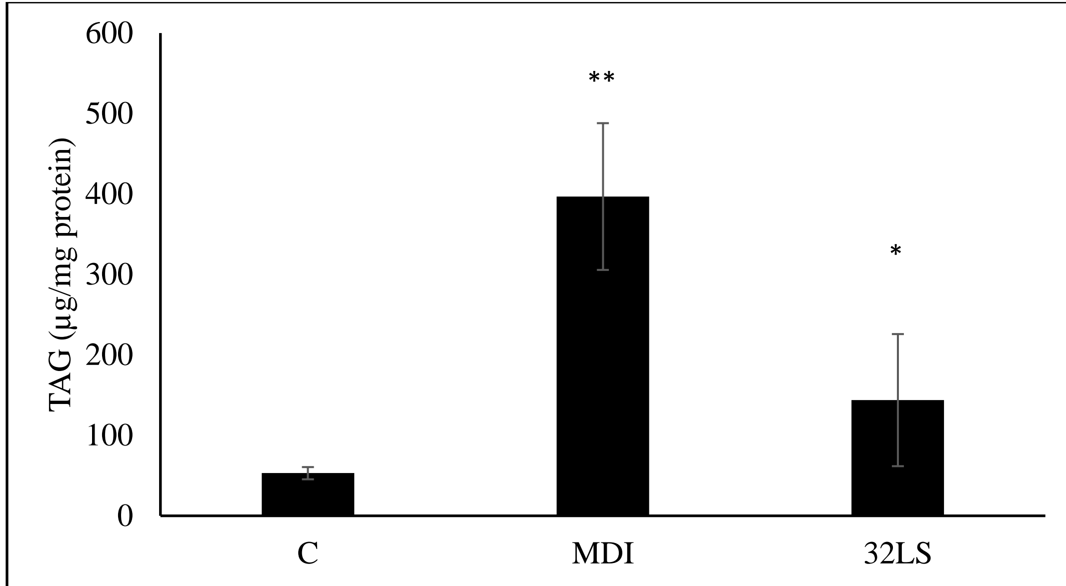

Fig.2(b). MDI shows a 7.5-fold increase in Triglyceride (TAG) accumulation compared to control. 32LS shows a 2.8-fold decrease in Triglyceride (TAG) accumulation compared to MDI.

3.3. LS inhibits adipogenesis by affecting the lipid droplet accumulation in the 3T3L1 cell model:

When treated with 32LS, 28LS, 24LS, and 16LS the cells showed lipid droplet accumulation levels of 83.9±10.6, 94.4±6, 105.2±.8 and 107.7±29.8 respectively with MDI-treated cells and control showing 125.4±15.4 and 100±0 respectively. 32LS showed a significant inhibition of adipogenesis. (Fig.3)

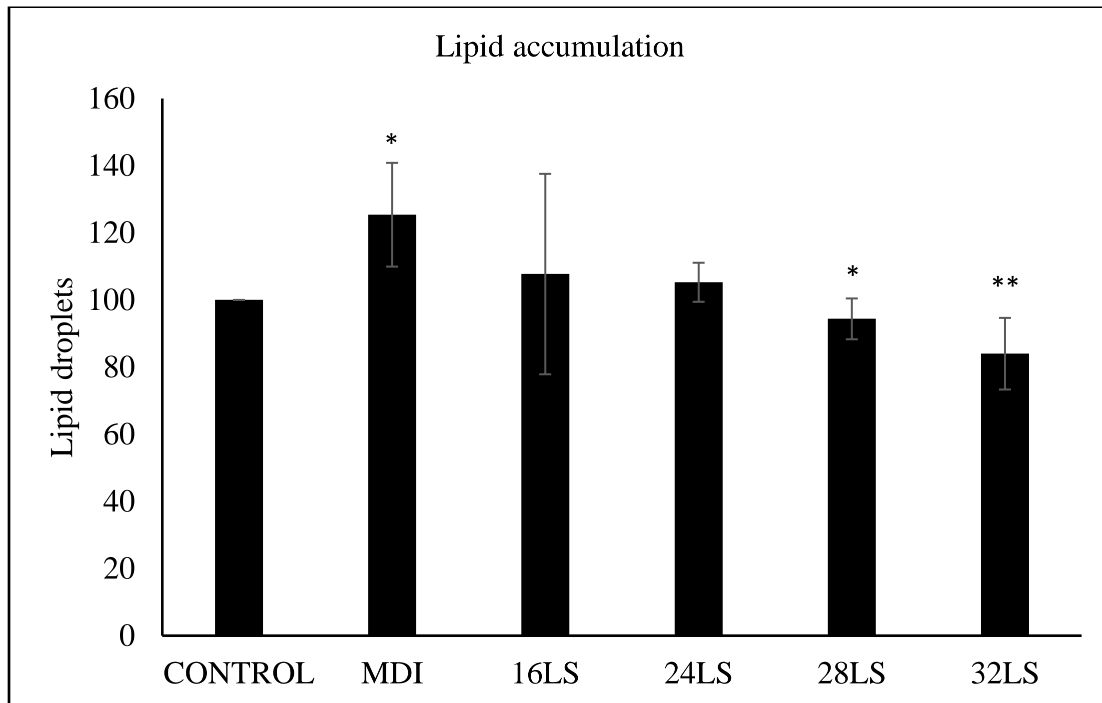

Fig.3(a). MDI shows a 1.25-fold increase in lipid droplet accumulation compared to control. 32LS shows a 1.5-fold, 28LS a 1.3-fold, 24LS a 1.19-fold, and 16LS a 1.16-fold decrease in lipid droplet accumulation compared to MDI.

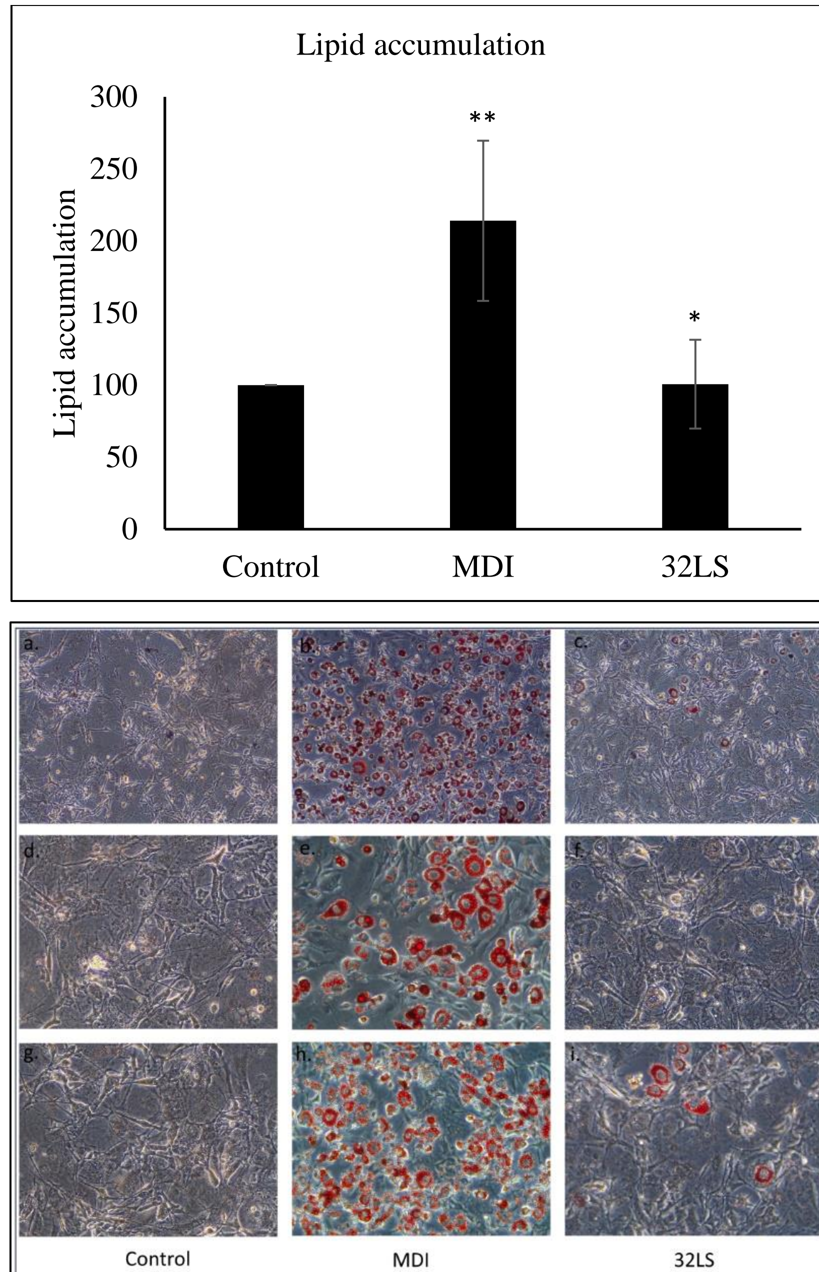

Fig.3(b&c). MDI shows a 2.13-fold increase in lipid droplet accumulation compared to control. (p-value 0.006.) 32LS shows a 2.12-fold decrease in lipid droplet accumulation compared to MDI. (p-value 0.01)

3.4. LS inhibits the levels of free fatty acids being released by 3T3L1 adipocytes

The TAG levels in the media of cells treated with 32LS, MDI, and control were 54±6.8, 153±34.5, and 44±4.9 respectively. (Fig.4)

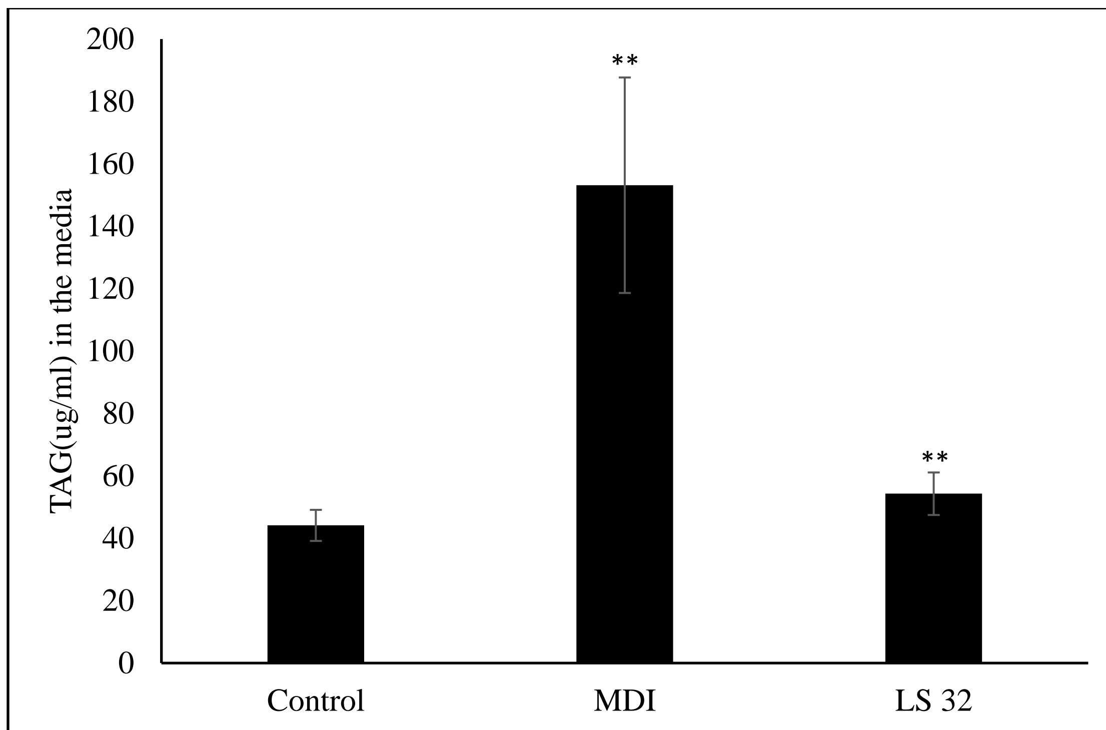

Fig.4. MDI shows a 3.47-fold increase in FFA release into the media compared to the control. (p-value 0.005.) 32LS shows a 2.8-fold decrease in FFA release into the media compared to MDI. (p-value 0.008)

3.5. LS inhibits the expression of the genes involved in adipogenesis in the 3T3L1 cell model

PPAR-ɣ, SREBP, FABP4, CEBPA, FASN, and DPP4 show 18.9-fold, 4.25-fold, 6.7-fold, 6.5-fold, 6.9-fold, and 2-fold lesser expression levels in the cells treated with 32LS when compared to those treated with MDI alone. (Fig.5)

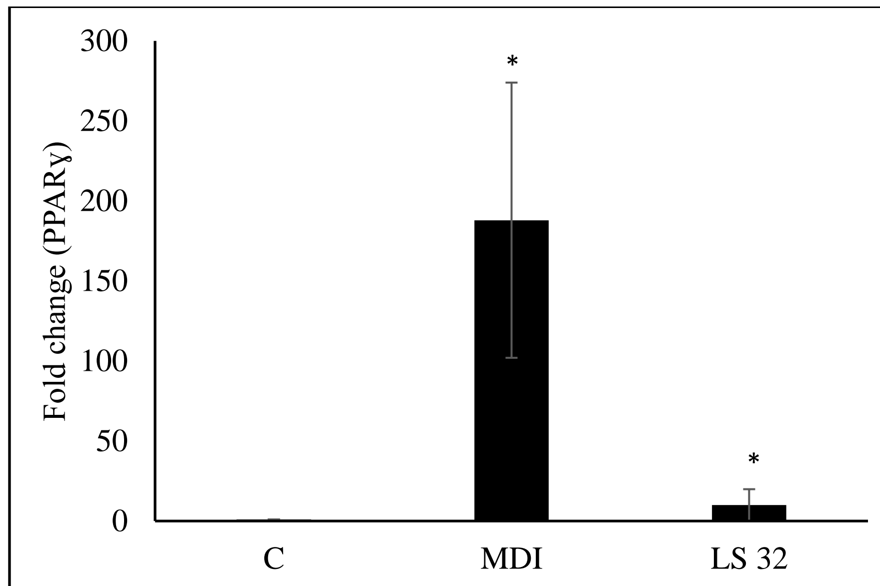

Fig.5(a). MDI shows a 187.9-fold increase in the expression levels of PPAR-ɣ compared to control. 32LS shows an 18.9-fold decrease in the expression levels of PPAR-ɣ compared to MDI.

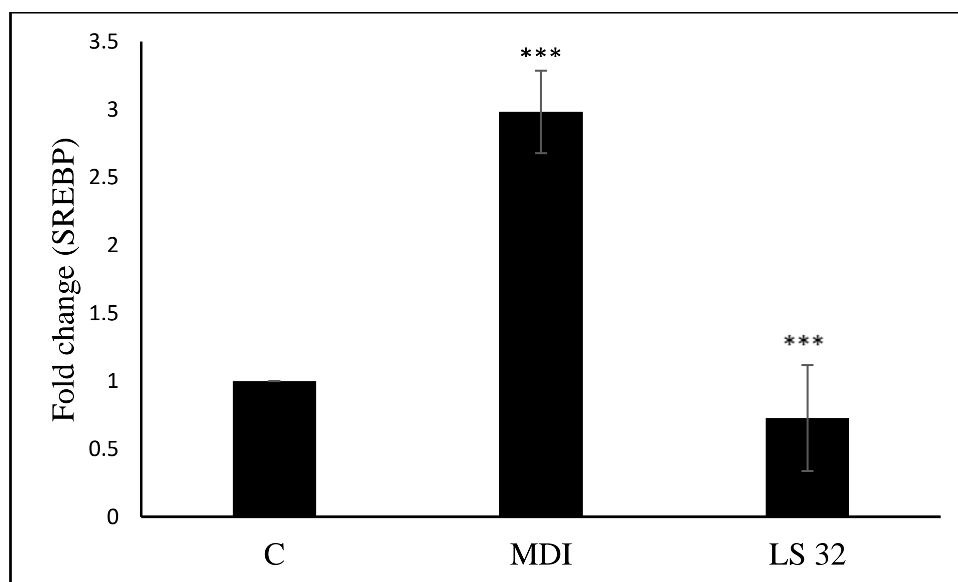

Fig.5(b). MDI shows a 3-fold increase in the expression levels of SREBP compared to control.

32LS shows a 4.25-fold decrease in the expression levels of SREBP compared to MDI.

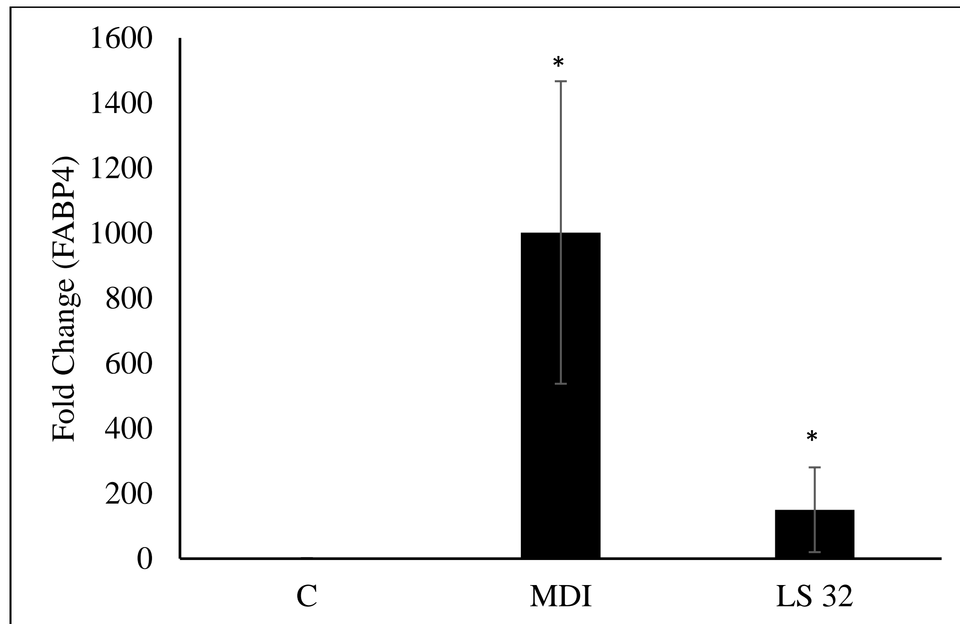

Fig.5(c). MDI shows a 1002-fold increase in the expression levels of FABP4 compared to the control. 32LS shows a 6.7-fold decrease in the expression levels of FABP4 compared to MDI.

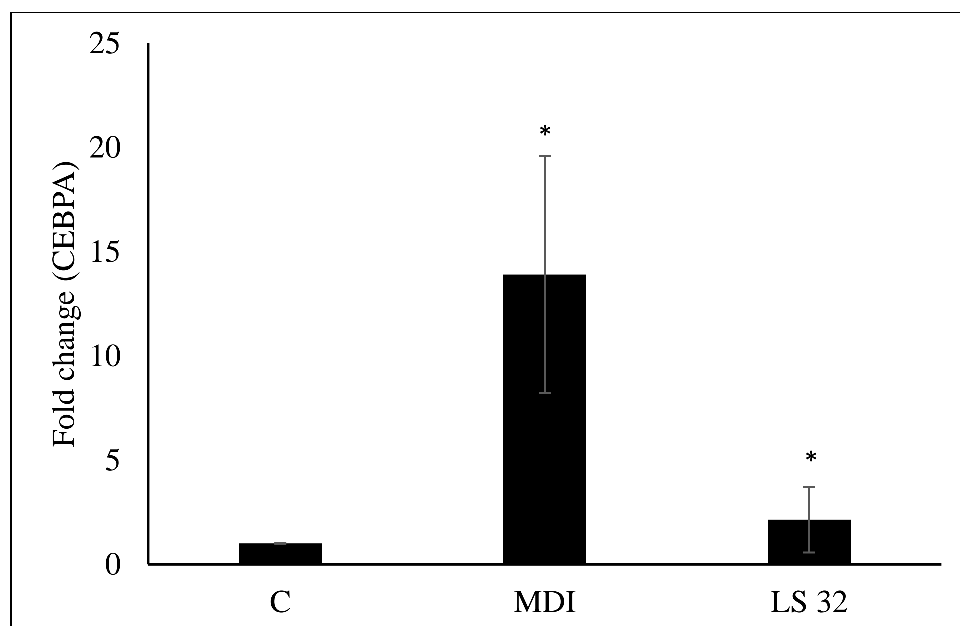

Fig.5(d). MDI shows a 13.9-fold increase in the expression levels of CEBPA compared to the control. 32LS shows a 6.5-fold decrease in the expression levels of CEBPA compared to MDI.

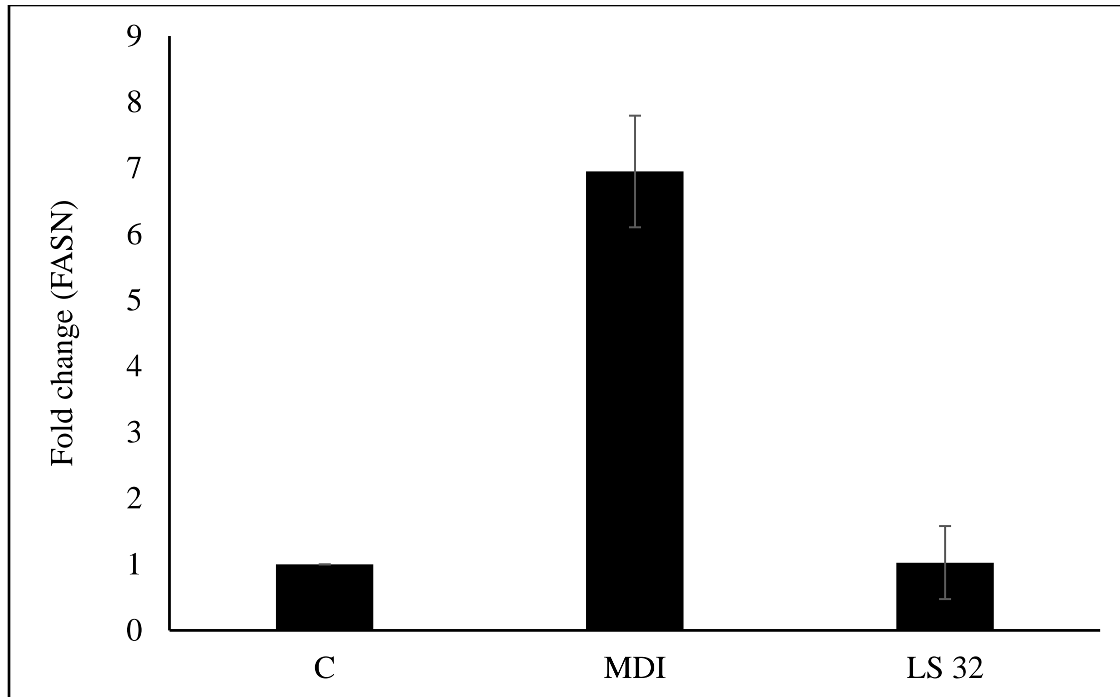

Fig.(e). MDI shows a 6.9-fold increase in the expression levels of FASN compared to the control. 32LS shows a 6.9-fold decrease in the expression levels of FASN compared to MDI.

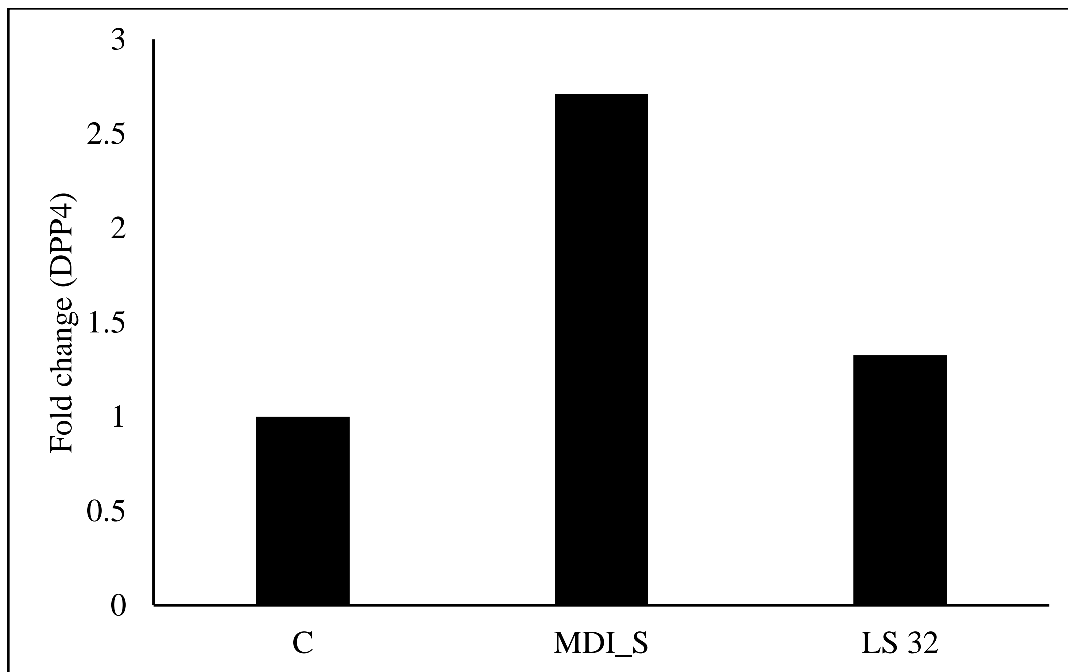

Fig.5(f). MDI shows a 2.7-fold increase in the expression levels of DPP4 compared to the control. 32LS shows a 2-fold decrease in the expression levels of DPP4 compared to MDI.

3.6. Effect on liver cells

HepG2 cells, when treated with the conditioned media from the Lodhrasavam-treated 3T3L1 cells (for its inhibitory action on adipogenesis) for 24 hours-32LS, 28LS, 24LS, and 16LS showed the accumulation of 155, 157, 112, and 135 µg/mg protein TAG respectively with control and MDI showing 124 and 96 µg/mg protein TAG respectively whereas, when treated for 48 hours- 32LS, 28LS, 24LS, and 16LS showed the accumulation of 215, 200, 260 and 191 µg/mg protein TAG respectively with control and MDI showing 219 and 228 µg/mg protein TAG respectively. (Fig.6)

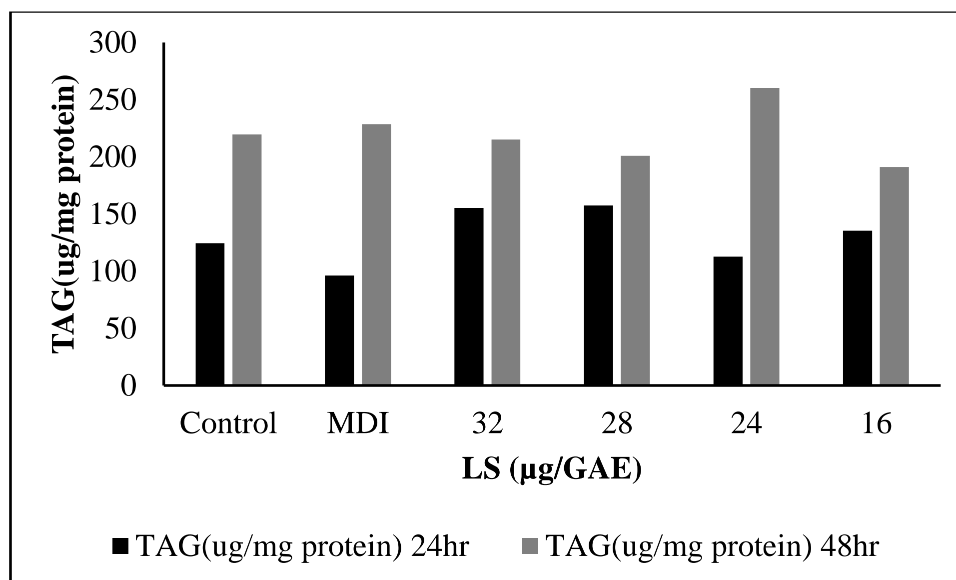

Fig.6. TAG levels in the HepG2 cells after 24 and 48 hours of treatment with the conditioned media taken from LS-treated 3T3L1 cells.

3.7. Lodhrasavam shows dose-dependent inhibition of lipase activity

128LS, 64LS, 32LS, 16LS, and 8LS show 43±5, 22±2.5, 13±3.6, 6±0.7, and 2.59±1.9 percentage inhibition of lipase activity. (Fig.7)

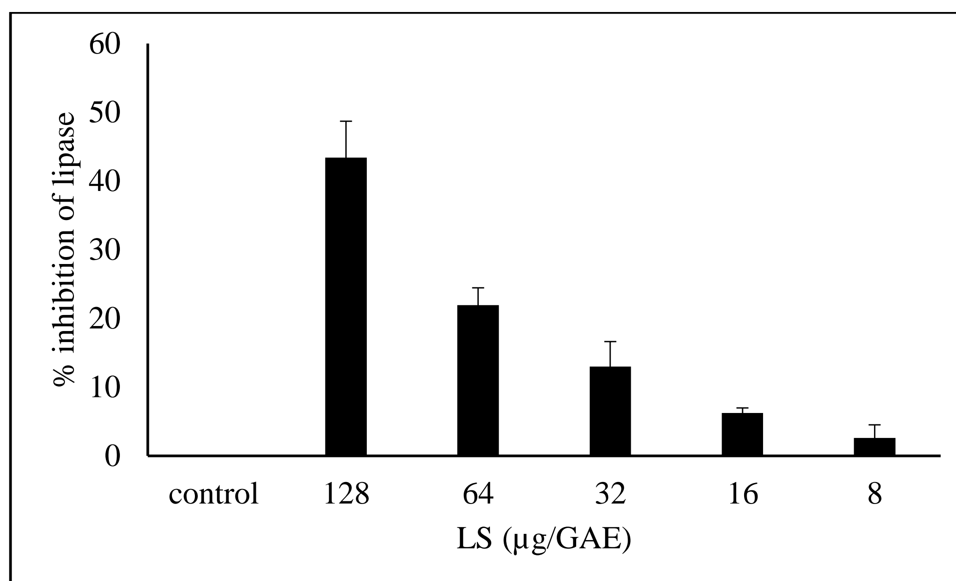

Fig.7. Lodhrasavam shows a dose-dependent inhibition of lipase activity.

3.8. Lodhrasavam showed an increase in lipolysis in mature adipocytes

3T3L1 mature adipocytes treated with 32LS, 28LS, 24LS, and 16LS for 48hours show lipolysis levels of 134.6±19.5, 134.9±18, 132.4±12.6 and 131±4.5 µg/ml respectively with control and MDI showing 66.6±26.7 and 161.9±58.7 µg/ml respectively and those treated for 72hours show 261.8±3, 265.8±8, 266.2±4 and 271.6±4.5 µg/ml respectively with control and MDI showing 122±11.6 and 197.3±46 µg/ml respectively. (Fig.8)

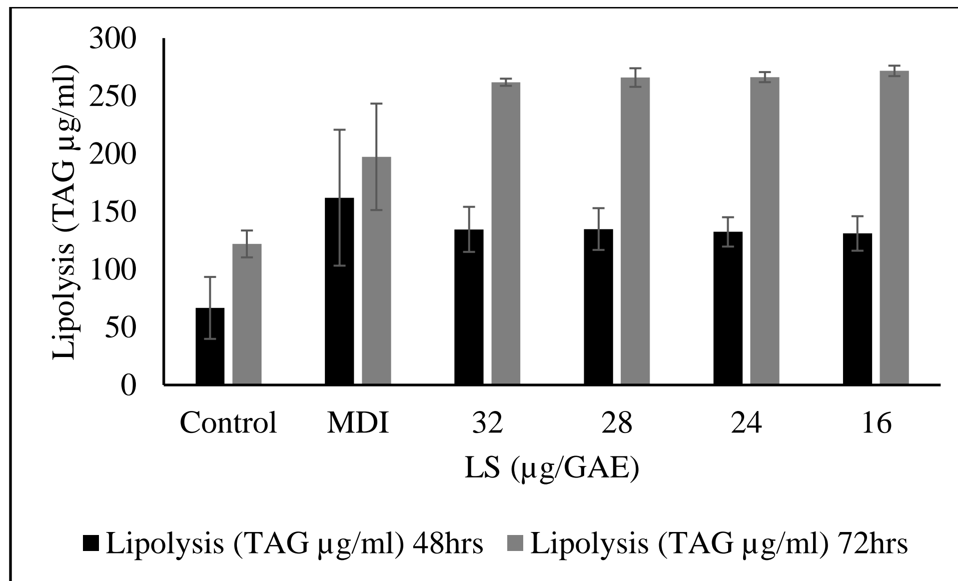

Fig.8. Lodhrasavam increases lipolysis after 72 hours of treatment in mature adipocytes.

3.9. Effect on liver cells

HepG2 cells, when treated with the conditioned media from the Lodhrasavam-treated mature 3T3L1 cells for 24 hours-32LS, 28LS, 24LS, and 16LS showed the accumulation of 219, 205, 167, and 167 µg/mg protein TAG respectively with control and MDI showing 115 and 127 µg/mg protein TAG respectively whereas, when treated for 48 hours- 32LS, 28LS, 24LS, and 16LS showed the accumulation of 135, 137, 107 and 106 µg/mg protein TAG respectively with control and MDI showing 115 and 137 µg/mg protein TAG respectively while when treated for 72 hours- 32LS, 28LS, 24LS, and 16LS showed the accumulation of 115, 120, 105 and 92 µg/mg protein TAG respectively with control and MDI showing 49 and 48 µg/mg protein TAG respectively. (Fig.9)

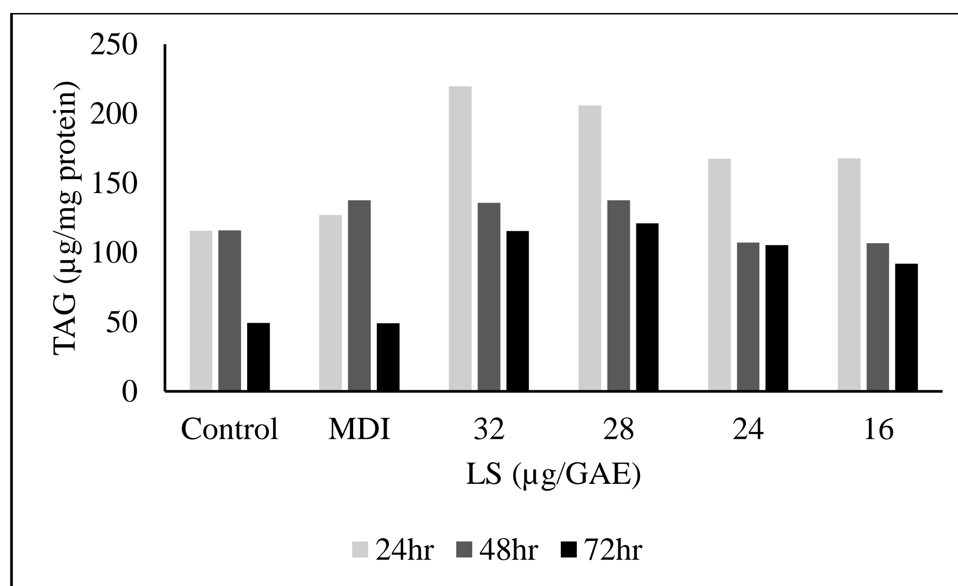

Fig.9(a). TAG levels in the HepG2 cells after 24 hours, 48 hours, and 72 hours of treatment with the conditioned media taken from LS-treated mature 3T3L1 adipocytes.

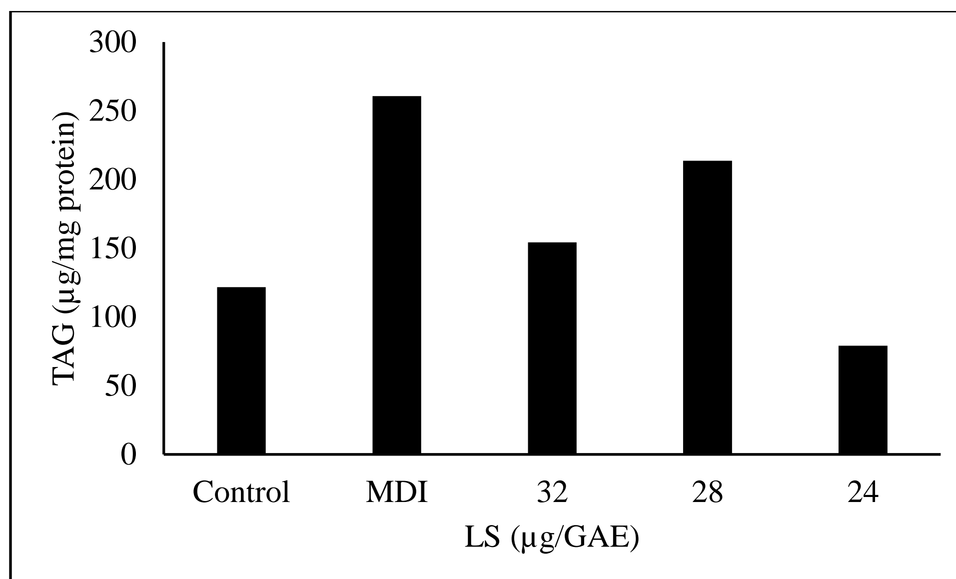

Fig. 9(b). 32LS, 28LS & 24LS showed a decrease in TAG levels in 3T3L1 mature adipocytes (cell lysate) after 72 hours of treatment

4. DISCUSSION

When treated after 24 hours of seeding, cells show 36.3%, 74.4%, 88.1%, 89.9% & 91.5% viability to 48LS, 40LS, 32LS, 24LS & 16LS respectively with control having 100% viability; whereas, those which were treated after 72 hours of seeding, show 61%, 76.4%, 88.9%, 92.5% & 90.5% viability to 48LS, 40LS, 32LS, 24LS & 16LS respectively with control having 100% viability. Only 48LS showed less than 50% viability when treated after 24 hours of seeding whereas all the tested concentrations showed no toxicity in 3T3L1 adipocytes.

When treated with 32LS, 28LS, 24LS, and 16LS the cells showed the TAG levels of 28, 36, 55, and 72 µg/mg protein respectively with MDI-treated cells and control showing 92 and 16µg/mg protein respectively. 32LS showed a significant inhibition in the accumulation of TAG in 3T3L1 cells. When treated with 32LS, 28LS, 24LS, and 16LS the cells showed lipid droplet accumulation levels of 83.9±10.6,

94.4±6, 105.2±.8 and 107.7±29.8 respectively with MDI-treated cells and control showing 125.4±15.4 and 100±0 respectively. 32LS showed a significant inhibition of adipogenesis. LS reduces the expression of the genes involved in adipogenesis in the 3T3L1 cell model. PPAR-ɣ, SREBP, FABP4, and CEBPA show 18.9-fold, 4.25-fold, 6.7-fold, and 6.5-fold lesser expression levels in the cells treated with 32LS when compared to those treated with MDI alone. This potential of *Lodhrasavam* can be utilized in the preventive aspects of obesity and *Sthoulya* during its poorvaroopa stage as well for its *medohara*, *pachana*, and *agnideepana* properties (Table 5, 6.1 & 6.2). The TAG levels in the media of cells treated with 32LS, MDI, and control were 54±6.8, 153±34.5, and 44±4.9 respectively by reducing the levels of free fatty acids being released by 3T3L1 adipocytes. This reduction in the FFA release from the adipocytes could be because of the inhibition of adipogenesis. It is believed that wherever there is adipogenesis, lipolysis will also happen simultaneously to maintain homeostasis as a part of metabolism. But when the mature 3T3L1 adipocytes were treated with the same concentrations of *Lodhrasavam*, there was an increase in the levels of TAG in the culture media denoting an increase in lipolysis in the treated cells. This indicates that *Lodhrasavam* increases the breakdown of lipids in the in vitro disease model of obesity showing its potential role in the management of obesity. However further trials need to be done for deeper insights. *Medo dhatwagni*- the *Agni* which is present in the *medo dhatu* (which can be correlated to adipose tissue) is responsible for both *poshana* (nourishment) and *pachana* (digestion/conversion) in *medo dhatu* and is said to be responsible for lipid metabolism in adipose tissue. In adipogenesis- the genes responsible for adipogenesis like PPARG, FABP4, CEBPA, SREBP, DPP4, FASN, etc., can be correlated to a part of the function of *Agni* which is responsible for the *poshana* (nourishment) of *medo dhatu*. It can also be said as *brumhana* (nourishment) of *medo dhatu*. *Ati-brumhana* (over-nourishment) of *medo dhatu* will lead to *medo dhatu vriddhi* and *Sthoulya* (Obesity). The inhibition in the expression of these adipogenic genes can be related to the *Agni* modulation to avoid *ati-brumhana* of *medo dhatu* leading to *Sthoulya* or Obesity. Since most of the ingredients of *Lodhrasavam* are of *tikta-kashaya rasa* and are of *ushna veerya* (hot potency) which are responsible to enhance *Agni*, it can be said that *Lodhrasavam* affects lipid metabolism via *Agni* modulation. *Lodhrasavam* also shows a dose-dependent inhibition of lipase enzyme activity. Lipases are necessary for the breakdown of fat to be taken up by the cells. When these lipases are inhibited, the amount of fat being taken up by the cells will be less hence preventing adiposity. Most of the ingredients of *Lodhrasavam* along with *Madhu* (honey) are considered to be *kaphahara* and *medohara* in the classics (Table 6.1 &6.2). *Lodhrasavam* has ingredients that are mostly ushna *veerya*, having *tikta-katu-kashaya rasa*, and has the property of *Agni deepana* and *ama pachana* (Table 5). Some of the ingredients also have targeted action on *Medo-dhatu* as per the classical categorization (i.e., *Medohara*

property) (Table 6.1 & 6.2). So, it can be inferred that *Lodhrasavam* inhibits adipogenesis (*Medohara* action) by enhancing the *medo-dhatwagni*.

When the HepG2 cells were treated with the conditioned media from *Lodhrasavam*-treated 3T3L1 cells, as a preliminary study to know the indirect effect of LS treatment on liver cells, there was an increase in the lipid uptake by HepG2 cells compared to the untreated ones. This could be because of an increased levels of lipids in the LS treated conditioned media compared to the untreated ones. However, the lipid levels have decreased over time i.e., after 48 hours and 72 hours in all the concentrations including the untreated ones. Although this is not satisfying the hypothesis of the current study, further studies need to be done for a better understanding of this result.

5. CONCLUSION

- *Lodhrasavam* significantly inhibits adipogenesis in 3T3L1 cell model indicating its potential as a preventive medicine for obesity. This inhibition takes place via its effect on the nourishing (*poshana*) function of *medo-dhatwagni*. It can be used in conditions of impaired lipid metabolism prior to the phenotypic manifestation of obesity.
- *Lodhrasavam* increases the breakdown of triacyl glycerides accumulated in the mature adipocytes showing its potential anti-obesity action. This catabolic action is because of its effect on the *pachana* effect of *medo-dhatwagni*.
- Considering the results showing the inhibitory effect of *Lodhrasavam* on the genes involved in adipogenesis, the changes in the expression levels of these genes can be said to be because of the *Agni* modulation by *Lodhrasavam*.

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APPENDIX

PROGRESS REPORT- 1

Title: Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism.

Objectives:

- Defining the role of *Agni* in modulating lipid metabolism in liver and adipose tissue.
- Identification and mapping of pathways that can be correlated to *Agni* modulation.
- Delineating the modulatory effect of *Lodhrasavam* on *Agni* in lipid metabolism using HepG2 and 3T3 cell models.

Progress made in the objectives:

- Cell culture- Splitting and seeding of HepG2 and 3T3 cell models.
- Performing cell viability assay (MTT).
- Performing TAG assay and Bradford's protein estimation assay on HepG2 cells treated with Lodhrasavam.

Skills learned:

- Handling cells independently - splitting and seeding of cells
- MTT assay
- TAG assay
- Bradford's protein estimation
- RNA isolation
- Using Nanodrop spectrophotometer

Challenges faced:

- Giving the title for the project was bit difficult.
- Writing precise objectives for the study took a lot of thinking.
- Handling cells for the first time was difficult.
- Could get the R^2 value as 0.999 in TAG assay only after few trials.

PROGRESS REPORT- 2

Title: Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism.

Objectives:

- Defining the role of *Agni* in modulating lipid metabolism in liver and adipose tissue.
- Identification and mapping of pathways that can be correlated to *Agni* modulation.
- Delineating the modulatory effect of *Lodhrasavam* on *Agni* in lipid metabolism using HepG2 and 3T3 cell models.

Progress made in the objectives:

- Experiments show the inhibitory effect of *Lodhrasavam* on adipogenesis in 3T3L1 cell model (TAG assay, BCA, Oil O Red staining and q-RTPCR techniques were used); however, these experiments have to be repeated. Adipogenesis was induced using MDI (IBMX, Insulin & Dexamethasone). Treatment with various concentrations of Lodhrasavam was done along with the MDI media after 5 days of seeding. Cells treated with MDI alone was kept as a positive control for adipogenesis. Insulin media change was done on 3rd day after induction with MDI, followed by normal media change on 5th day after induction. TAG assay, Oil O Red staining and RNA isolation was done on 7th day after induction i.e., 13th day after seeding.
- Literature work on *Lodhrasavam* and its ingredients for its modulatory effect on *Agni* and *medas*.
- MTT viability assay to know the toxicity of *Lodhrasavam* on 3T3L1 cell model is ongoing.

Skills learned:

- Induction of adipogenesis using MDI
- cDNA conversion
- q-RTPCR

Challenges faced:

- Difficulty in induction of adipogenesis
- Pipetting errors in q-RTPCR
- Interpreting the results in terms of Ayurveda

PROGRESS REPORT – 3

Title: Exploring the molecular correlates of *Agni* modulation by *Lodhrasavam* in adipose and liver tissue lipid metabolism.

Objectives:

- Defining the role of *Agni* in modulating lipid metabolism in liver and adipose tissue.
- Identification and mapping of pathways that can be correlated to *Agni* modulation.
- Delineating the modulatory effect of *Lodhrasavam* on *Agni* in lipid metabolism using HepG2 and 3T3 cell models.

Progress made in the objectives:

- Defining the role of Agni in modulating lipid metabolism in and adipose tissue and liver.
- Identification of genes involved in lipid metabolism that can be correlated to Agni modulation.
- Lodhrasavam significantly inhibits the induction of adipogenesis in 3T3L1 cell model. This shows the obesity preventing potential of Lodhrasavam.
- Defining the inhibitory effect of Lodhrasavam on adipogenesis through Agni modulation on the basis of classical literature.
- Experiments to be done to see the effect of Lodhrasavam on mature adipocytes to see its therapeutic effect in obesity.

Challenges faced:

- Contamination issues during cell culture
- Bringing in the concepts of ayurveda with lipid metabolism and building an explanation for the effect of Lodhrasavam on adipocytes.