Original Research Article (Experimental)

Ayurvedic anti-diabetic formulation *Lodhrasavam* inhibits alpha-amylase, alpha-glucosidase and suppresses adipogenic activity *in vitro*

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A B S T R A C T

Background: The patho-physiological cross-talk between diabetes and obesity is well established. However, the choices of drugs suitable for combined treatment of diabetes and obesity are limited. Integration of complementary and alternative medicines (CAMs), like Ayurveda, with modern medicine would be a promising strategy to fill this gap. The diagnostic principles of Ayurveda define obesity as one of the predisposing factors of *Madhumeha* (correlated as diabetes) and recommends specific formulations for managing obese-diabetes. *Lodhrasavam* is one such poly-herbal formulation prescribed for obese-diabetic patients.
Objectives: The present study is an attempt to demonstrate the possible modes of action of *Lodhrasavam*, built on the hypothesis that the formulation can exert both anti-diabetic and anti-obesity actions.
Materials and methods: *Lodhrasavam*, following simulated gastro-intestinal digestion, was monitored for inhibition of α-amylase, α-glucosidase (key digestive enzyme targets of anti-diabetic drugs) and adipogenesis using standard *in vitro* model systems.
Results: *Lodhrasavam* digest inhibited α-amylase (90%) and α-glucosidase (78%) activity as well as reduced the differentiation of 3T3-L1 fibroblasts to adipocytes. Upon fractionation, the enzyme inhibitory activity and anti-adipogenic activity of the digest were found distributed in different solvent fractions. This partly indicates a potential pharmacological networking of chemically and functionally diverse bioactive molecules in *Lodhrasavam*.
Conclusion: The study provides a possible mode of action and an experimental support for the Ayurvedic use of *Lodhrasavam* for managing obese-diabetes. Generating scientific evidences and understanding the modes of action, in contemporary scientific language, would essentially help in expanding global acceptance of potentials of CAMs in the management of life style disorders.

1. Introduction

The link between diabetes and obesity is well established in both traditional and modern systems of medicines. The World Health Organization (WHO) estimates that 44% of the diabetes cases globally are attributable to overweight and obesity [1]. Though intensive lifestyle interventions are the foundation for managing weight gain in diabetes, studies have shown that pharmacotherapies are also necessary for patients to achieve long term weight loss [2]. Despite having an evident patho-physiological cross-talk between diabetes and obesity, the choices of drugs suitable for combined treatment of diabetes and obesity are limited in modern medicine [3]. Recent examples like glucagon-like peptide-1 receptor agonists show promising results in combating the dual burden, but their long term safety is question [4,5]. Furthermore, the target based molecular drugs currently available for diabetes and obesity are associated with severe side effects such as insomnia, headache, constipation, hypoglycemia, weight gain, and renal complications [6,7]. As an alternative, integrating complementary and alternative medicines (CAMs) with modern medicine would have promising applications in managing lifestyle disorders like diabetes and obesity. However, inadequate scientific evidence plus the use of indigenous languages and epistemologies in CAMs limit their global acceptance.

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Ayurveda, an Indian traditional medicine system, prescribes several poly-herbal formulations for the treatment of diabetes and obesity. Studies have established the efficacy of some of these formulations, viz. *Chandraprabha vati*, *Arogyavardhini vati*, *Naga bhasma*, *Shilajatu*, and *Nishamalaki* using various *in vivo* models [8–13]. Though the descriptions of diabetes in Ayurveda are not in terms of modern parameters such as blood glucose, serum insulin and insulin resistance, a striking relationship between obesity (*Sthaulya*) and diabetes has been emphasized [14]. Diabetes mellitus can be correlated to *Madhumeha* (sweet urine), one of the 20 types of *Prameha* (a set of clinical disorders manifested with excess and turbid urination) described in Ayurveda [15]. *Sthaulya* (obesity), caused from unwholesome diet and sedentary lifestyle, is considered as one of the predisposing factors of *Madhumeha* as well as *Prameha*. The etiology and pathophysiology of obese-diabetes (*Sthula Prameha*) is clearly demarcated from lean diabetes (*Krusha Prameha*) [16]. As a result, Ayurvedic therapeutics have come out with specific formulations and methodologies to treat obese-diabetic patients. Generating scientific evidence and understanding their modes of action in contemporary scientific language would essentially help in expanding their global acceptance as convincing complementary strategies for managing the dual burden of obesity and diabetes.

The present study attempts to understand the possible mode of action of *Lodhrasavam*, a fermented polyherbal formulation in Ayurveda, predominantly prescribed for obese-diabetic patients [17]. *Lodhrasavam* is a complex formulation prepared from 29 plant drugs, wherein *Lodhra* (*Symplocos racemosa* Roxb.) is considered as the major ingredient (Supplementary data − 1) [18]. *Lodhra* is referred as a *medo-hara* (anti-obesity) plant and is the prime member of *Lodhradi-gana* plants, a group of plants having *medo-hara* and *kapha-hara* properties [19]. Besides the anti-diabetic and anti-obesity applications, *Lodhrasavam* is also indicated for various other diseases such as anemia, skin diseases, anorexia, hemorrhoids [18]. Owing to its broad spectrum of therapeutic benefits and the inherent properties of the herbal ingredients present, *Lodhrasavam* could be deemed to be an *Aushadha Rasayana* (a curative and wellness product) in the management of diabetes. The present study, for the first time, demonstrates the anti-diabetic and anti-obesity potentials of *Lodhrasavam* using *in vitro* experimental models.

Being an accepted oral drug for obese diabetes, the possibility of *Lodhrasavam* inhibiting the digestive enzymes viz. alpha-amylase (α-A) and alpha-glucosidase (α-G), to exert their anti-diabetic effect, is focused in this study. Digestive enzyme inhibition is considered as one of the important therapeutic strategies for controlling postprandial hyperglycemia in diabetic patients, particularly in high carbohydrate consuming populations like Indians [20,21]. Several digestive enzyme inhibitors are identified and reported from various medicinal plants [22,23]. The anti-adipogenic potential of *Lodhrasavam* has been studied using 3T3-L1 pre-adipocytes, and a well standardized cell-line model for adipogenesis and anti-obesity research *in vitro* is established [24]. The use of a static *in vitro* digestion model, to simulate the gastrointestinal digestion, adopted in the present work facilitated a realistic study of the formulation in total, than studying the individual herbal ingredients in part.

2. Materials and methods

2.1. Chemicals and reagents

Lodhrasavam, prepared in accordance with the Ayurveda text *Ashtangahrudayam* [18], was purchased from authentic commercial sources. 3T3-L1 fibroblasts were purchased from National Centre for Cell Sciences, Pune, India. All cell culture reagents used in the study were purchased from Gibco-BRL. The enzymes α-amylase (source − *Aspergillus oryzae*) and α-glucosidase (source − *Saccharomyces cerevisiae*) and other fine chemicals were purchased from Sigma−Aldrich. All other routine laboratory chemicals used in various assays were of analytical grade and purchased from SD-Fine chemicals.

2.2. Cell culture and differentiation

3T3-L1 fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in an atmosphere of 5% CO2 at 37 °C. Differentiation of 3T3-L1 fibroblasts was induced by incubating two days post-confluent (day-0) plates in differentiation induction medium containing 500 μM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 100 nM insulin (MDI). On day-3, the medium was replaced with fresh culture medium and was replaced every alternate day till the cells attained complete adipocyte morphology.

2.3. In vitro digestion of Lodhrasavam

In vitro digestion of *Lodhrasavam* was carried out following published protocols [25] with modifications to suit the samples (Fig. 1A). The electrolyte solutions of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as shown in Fig. 1B. Oral phase of the digestion was omitted in the experiment as the starting material was in liquid form. For simulated gastric digestion, 40 mL of *Lodhrasavam* mixed with 250 mL of SGF containing 2500 U/mL pepsin and 0.16 mM $CaCl_2 \cdot 2H_2O$ (pH was adjusted to 2.0 by adding 6N HCl) was incubated at 37 °C for 2 h in a shaking water bath. Following gastric digestion, the gastric chyme mixed with 250 mL SIF containing pancreatin-bile solution (final concentration of 500 μg/mL pancreatin and 3 mg/mL bile) and 0.6 mM $CaCl_2 \cdot 2H_2O$ (pH adjusted to 7.0 by adding 5N NaOH) and incubated at 37 °C in shaking water bath for another 2 h to complete the digestion. The digest of *Lodhrasavam* (hereafter referred as LD) was filtered through cotton plug and the filtrate was collected and stored at −80 °C for further use.

2.4. Bioassay guided fractionation of Lodhrasavam digest

Fractionation of LD was done as illustrated in Fig. 1A. Initially, LD was successively extracted with hexane (H), chloroform (C) and ethyl acetate (EA) and the solvent was removed using a rotary evaporator at reduced temperature and pressure. The hexane extract of LD (LDH), obtained after sequential solvent extraction, was concentrated and fractionated by column chromatography. Briefly, the chromatographic column (18 × 300 mm size) packed with 25 gm silica in hexane was inoculated with 4 mL of LDH concentrate. Elution was started with 100% hexane and continued with 10% step-gradients of chloroform, ethyl acetate, methanol and water, till 100% water. The progress of separation was monitored by thin layer chromatography (TLC) and similar fractions were pooled together. Out of the 8 fractions obtained from the column, Fr-5 showed positive results in bioassays.

LD and all its subsequent fractions were estimated for total tannins following standard Folin - Ciocalteu method [26]. Briefly, 10 μL of digest/fraction mixed with 40 μL of water, 50 μL Folin's reagent and 100 μL of 3.5% Na_2CO_3 was incubated at room temperature for 30 min. A set of gallic acid standards (50, 25, 12.5, 6.25, 3.125 μg/mL) were prepared in the same manner. The absorbance was measured at 700 nm using plate reader (xMark Microplate Spectrophotometer, BioRad, USA). The experimental concentrations of test samples were expressed as 'μg of gallic acid equivalent tannin (GAE)/mL of sample'.
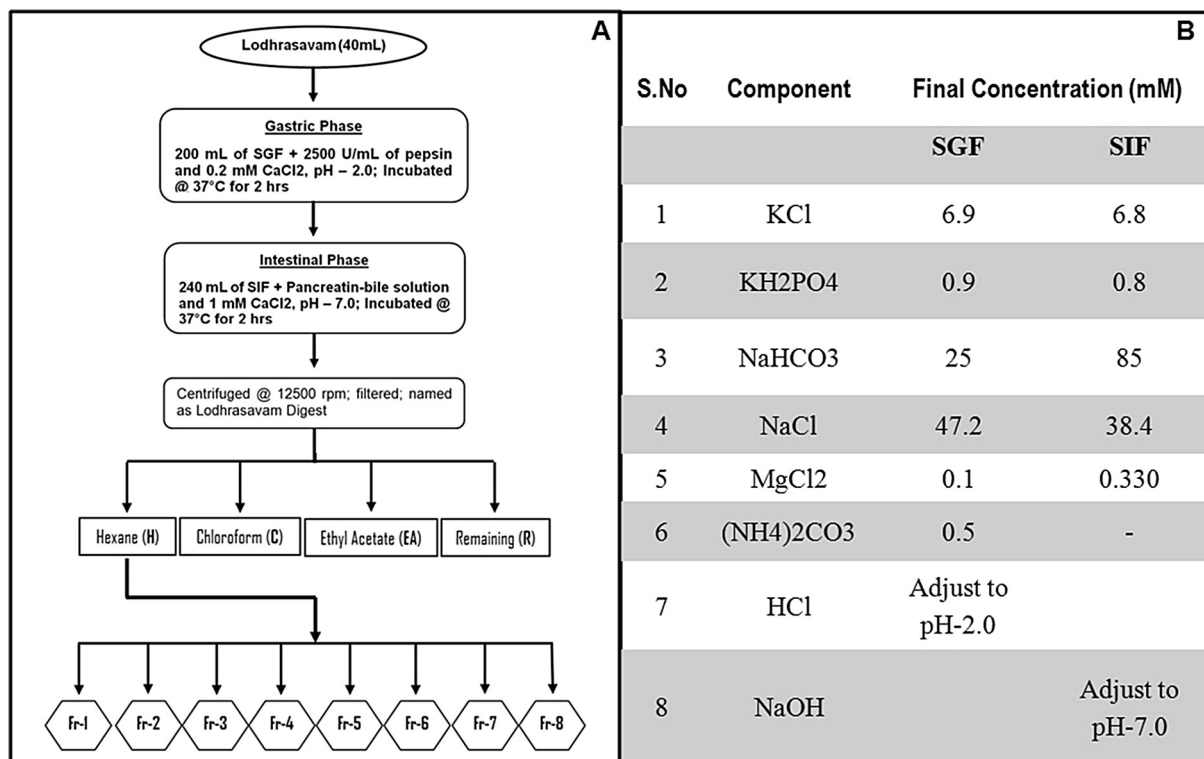
S.No	Component	Final Concentration (mM)	
		SGF	SIF
1	KCl	6.9	6.8
2	KH2PO4	0.9	0.8
3	NaHCO3	25	85
4	NaCl	47.2	38.4
5	MgCl2	0.1	0.330
6	(NH4)2CO3	0.5	-
7	HCl	Adjust to pH-2.0	
8	NaOH		Adjust to pH-7.0

Fig. 1. (A) — Diagrammatic representation of methodology adopted for fractionation of *Lodhrasavam* digest, and (B) —Table showing the composition of SGF and SIF electrolytes.

2.5. α-Amylase and α-glucosidase inhibition assay

α-A and α-G assays were carried out following standard protocol [27] with modifications to suit to 96-well plate format. For α-A, all incubations were done at room temperature. Increasing concentrations of the samples were taken and the volume was made up to 50 μL using 0.02 M sodium phosphate buffer (pH 6.9). To this, 50 μL of α-A (0.5 mg/mL) was added and incubated for 30 min. Followed by this, 50 μL of 1% starch (soluble potato starch) was added as substrate and incubated for another 10 min. The reaction was stopped by adding 50 μL of 1% dinitrosalicylic acid (DNS) and the plate was heated at boiling water bath for 5 min till the development of an orange red color.

Similarly for α-G assay, different concentrations of the samples were taken and the volume was made up to 50 μL with 0.02 M sodium phosphate buffer (pH 6.9). To this, 50 μL of α-G (0.5 U/mL) was added and incubated for 10 min at room temperature, followed by the addition of 50 μL of 3.0 mM p-nitrophenyl glucopyranoside (pNPG) as substrate and incubation for 20 min at 37 °C. The reaction was stopped by adding 50 μL of 0.1 M Na_2CO_3.

For α-A and α-G the absorbance was read at 550 and 405 nm, respectively, using plate reader. For enzyme activity, 50 μL of buffer was used as control for both α-A and α-G and a set of test samples without enzyme was used to measure the basal level of reducing sugars present in the test samples. The absorbance was subtracted from the corresponding test readings. The percentage inhibition of enzyme activity for both α-A and α-G was calculated as follows.

$$\%\text{Inhibition} = \left(\frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Test}}}{\text{Abs}_{\text{Control}}} \right) \times 100$$

2.6. Anti-adipogenesis assay and Oil-Red O staining

Anti-adipogenesis assay and Oil-Red O staining were done following published protocols with minor modifications [28]. Briefly, two days post confluent cells, grown in 48 well plates, were induced with MDI for three days in the presence of different concentrations of test samples (LD and its fractions). Following induction, the cells were replenished in normal culture medium, till the 6th day. Cells treated with MDI or vehicle alone were used as positive and negative control respectively. Oil Red O staining was done on the 6th day of differentiation. Cells were washed twice with phosphate buffer, and fixed with 4% formaldehyde for 1 h. After washing, cells were stained for 30 min in freshly diluted Oil Red O solution (0.5% Oil Red O in isopropanol diluted to 3:2 with H_2O, and filtered) at 37 °C. Images of cells stained with Oil Red were captured using phase contrast microscope (Olympus-IX-71, Olympus America Inc, USA).

2.7. Statistical analysis

A one-way ANOVA test was performed to determine the significance of test samples compared to the controls and a value of $p < 0.05$ was considered as significant.

3. Results and discussions

Ayurveda has very unique principles and procedures for designing and manufacturing therapeutic formulations [29]. Polyherbal formulations of Ayurveda are prepared through complex processes including boiling, extraction, and fermentation [14,18]. Nevertheless, they are considered as rich repository of
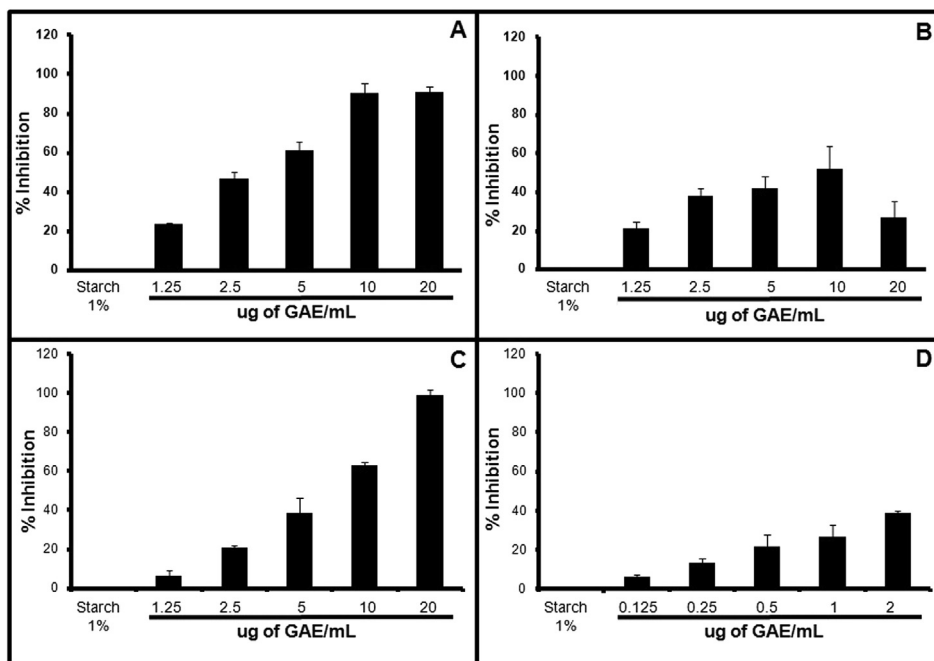
Fig. 2. Graph showing the effect of *Lodhrasavam* digest and its fractions on α-A: Percentage inhibition of α-A by (A) LD, (B) LDH, (C) LDR and (D) Fr-5. Data are mean ± SEM of three independent experiments done in duplicates and the statistical significance was calculated with controls ($p \leq 0.05$).
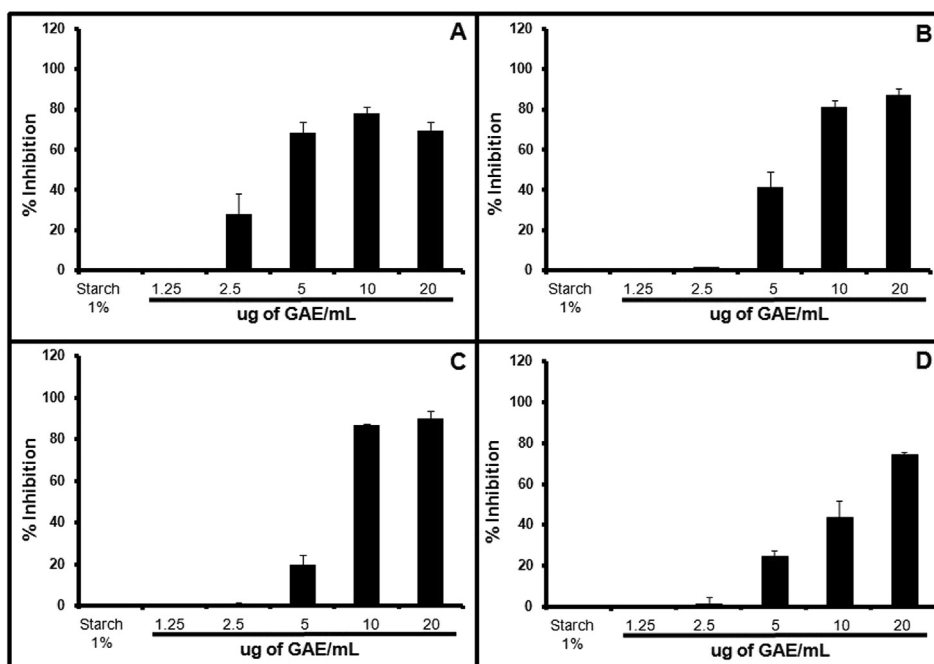

Fig. 3. Graph showing the effect of *Lodhrasavam* digest and its fractions on α-G: Percentage inhibition of α-G by (A) LD, (B) LDC, (C) LDE and (D) LDR. Data are mean ± SEM of three independent experiments done in duplicates and the statistical significance was calculated with controls ($p \leq 0.05$).

diverse bioactive molecules and are believed to exert their therapeutic effects through pharmacological-networking [30]. Studying individual component plants need not necessarily address all the possible post-digestive modifications and associated pharmacodynamic and pharmacokinetic differences that a formulation undergoes. Considering this, *Lodhrasavam* used in the present study was first subjected to a simulated *in vitro* gastro-

intestinal digestion and the digest and its fractions were used for further studies (Fig. 1).

The component plants of *Lodhrasavam* for example, *S. racemosa* Roxb., *Terminalia chebula* Retz., *Phylanthus emblica* Roxb., *Embelia ribes*, *Piper retrofractum* Vahl., have been independently studied and reported using various *in vitro* and *in vivo* models for their potential anti-diabetic and hypolipidimic effects [31—34]. But there

are no scientific studies addressing the anti-diabetic and anti-adipogenic potentials of *Lodhrasavam* as a formulation. It is hypothesized that studying the formulation in its original form is more important because the various physico-chemical processes adopted for the preparation of *Lodhrasavam* would certainly alter its phytochemistry and bioactivity compared to the component plants. However, the present study is not comparing the biological effects of *Lodhrasavam* formulation with its component plants and it is a limitation.

3.1. LD and its fractions inhibit α-A and α-G activity

Digestive enzyme inhibition has shown to have promising therapeutic effects in the management of carbohydrate uptake related disorders like diabetes and obesity. LD, obtained from simulated *in vitro* digestion, was found to inhibit α-A and α-G activity in a concentration dependent manner. LD showed a maximum of 90 ± 3% inhibition of α-A activity at the highest concentration (20 μg of GAE/mL) of the digest tested in the assay (Fig. 2A). Similarly, it showed a concentration dependent inhibition of α-G with maximum of 78 ± 3% at 10 μg of GAE/mL concentration, beyond which the inhibition reduced (Fig. 3A). The observed inhibition of α-A and α-G by LD indicates its potential to control postprandial hyperglycemia through reducing carbohydrate digestion. Besides, it also supports the reported α-G inhibitory activity of some of the component plants in the formulation [35].

3.1.1. α-A and α-G inhibition by LD fractions
Polyherbal formulations are known to exert a multi-drug, multi-target mode of action which is different from the standard lock and key mechanism of action of molecular drugs [36]. As a preliminary step to understand the nature and distribution of bioactive molecules that inhibit digestive enzymes, LD was fractionated in a bioassay guided manner, as described in materials and methods (Fig. 1A), using α-A as the primary target. The hexane (LDH) and the remaining (LDR) fractions of LD, obtained from sequential solvent extraction, were found to retain α-A inhibitory activity wherein both LDH and LDR showed a concentration dependent α-A inhibition with a maximum of 51 ± 11% (10 μg of GAE/mL) and 98 ± 3% (20 μg of GAE/mL) respectively (Fig. 2B and C). The chloroform (LDC) and ethyl acetate (LDE) fractions obtained from LD inhibited α-G activity in a concentration dependent fashion with a maximum of 87 ± 3% and 90 ± 3% inhibition for LDC and LDE, respectively, at the highest concentration of 20 μg of GAE/mL used in the assay (Fig. 3B and C).

It is interesting to note that the LDH fraction did not inhibit α-G, as well as the LDC and LDE fractions did not inhibit α-A at any of the concentrations tested in the assay (unpublished observation). However, the LDR fraction was found to inhibit α-G, in addition to α-A, in a concentration dependent manner with a maximum of 74 ± 1% at 20 μg of GAE/mL (Fig. 3D). The observed results partially support the possibility of the presence of multiple bioactive molecules, chemically and functionally distinct, exerting a network action for the gross biological effect of *Lodhrasavam* as a digestive enzyme inhibitor. Solvent extraction or liquid−liquid partitioning is the method widely used for bioassay guided fractionation of compounds from a mixture based on their polarity and solubility. In the present study use of solvents like hexane, chloroform and ethyl acetate facilitated the fractionation of bioactive compounds from LD. Considering the principles of liquid−liquid partitioning as well as the differential responses of LD fractions in α-A and α-G inhibition, it is expected that the bioactive molecules in LD would have partitioned into different solvents based on their polarity and/or solubility. However, detailed chemical analysis needs to be done to precisely understand the nature, type and structure of bioactive compounds in different fractions.

3.2. Lodhrasavam inhibits fibroblast differentiation

Being an Ayurvedic formulation of choice for treating obese-diabetes, the present study also investigated the modulatory
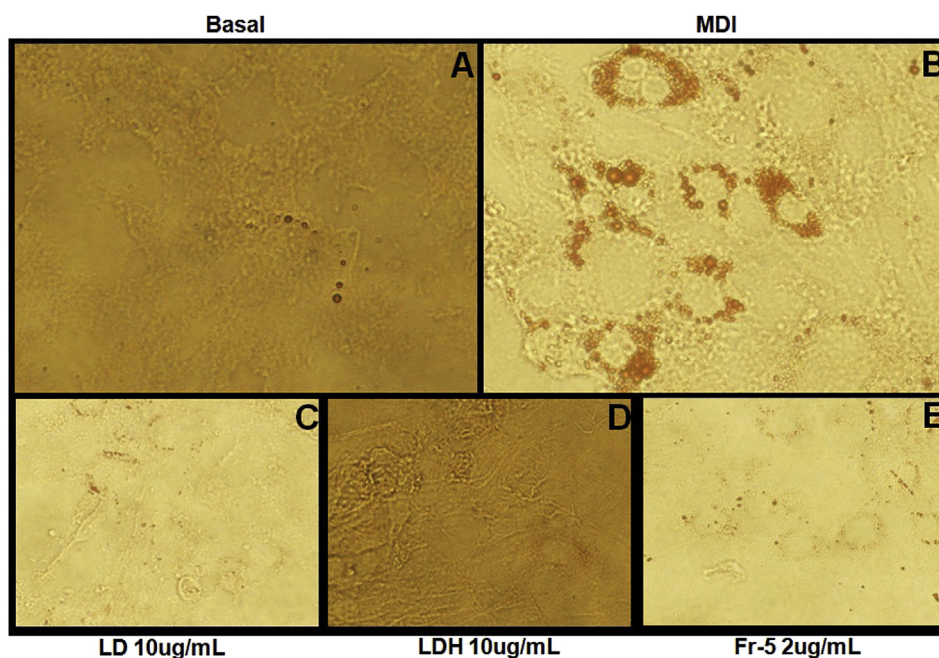

Fig. 4. Effect of Lodhrasavam digest and its fractions on adipogenesis: Oil Red O staining of 3T3-L1 fibroblasts differentiated with (A) Vehicle, (B) MDI, (C) MDI + LD, (D) MDI + LDH and (E) MDI + Fr-5 at different concentrations as indicated. Data presented are representative images of adipogenesis inhibition assay done in a concentration dependent manner. Images were captured using phase contrast microscope (Olympus-IX-71, Olympus America Inc, USA).

effect of *Lodhrasavam* on adipogenesis using 3T3-L1 fibroblast model. Inhibition of MDI induced differentiation of fibroblasts to adipocytes is considered as one of the experimental strategies for evaluating the anti-obesity action of a formulation [37]. LD, when added along with MDI, was found to inhibit adipogenesis in a concentration dependent fashion. A complete inhibition of adipogenesis qualitatively observed as reduction of oil droplet formation was obtained at a concentration of 10 µg of GAE/mL (Fig. 4C). Fig. 4 is a representative image of Oil-Red O stained adipocytes upon treatment with test samples (details are given in Supplementary Data − 2).

3.2.1. *Modulation of adipogenesis by LD fractions*

Similar to digestive enzyme inhibition the possibility of multiple bioactive compounds from *Lodhrasavam* modulating adipogenesis was taken into consideration and the fractions of LD were studied for adipogenesis inhibition. A concentration dependent inhibition of MDI mediated fibroblast differentiation was observed only with hexane (LDH) fraction (Fig. 4D). However at higher concentrations, above 10 µg of GAE/mL, all fractions including LDH were found to be cytotoxic.

The assay positive LDH fraction was further separated through column chromatography as described in materials and methods (Fig. 1A). Out of the eight fractions finally obtained, fraction 5 (Fr-5) was found to inhibit adipogenesis even at a very low concentration ranges from 0.5 to 2 µg of GAE/mL. Fr-5 showed a significant concentration dependent inhibition even at 1 µg of GAE/mL concentration (Fig. 4E). In addition, Fr-5 was also found to inhibit α-A in a concentration dependent manner. However, it showed a maximum of $38 \pm 1\%$ inhibition at the highest concentration (2 µg of GAE/mL) tested in the assay (Fig. 2D).

4. Conclusions

The study provides an experimental support for the use of *Lodhrasavam* for treating obese-diabetes. The observations from the current study indicate that *Lodhrasavam* mediated inhibition of digestive enzymes as well as adipocyte differentiation could potentially contribute to its anti-diabetic and anti-obesity properties. However, being a repository of multiple bioactive compounds, the possibility of *Lodhrasavam* modulating other anti-diabetic and anti-obesity targets cannot be ruled out. Additionally, our preliminary phytochemical studies indicate the possibility of a pharmacological networking of chemically and functionally diverse bioactive molecules contributing to the gross biological effects of *Lodhrasavam*. The study is significant in the context of diabetes and obesity as there are not many effective drugs available for managing the dual burden of obesity and diabetes. Nevertheless, the study needs to be expanded further for understanding the bioactive molecules and delineating the precise molecular mechanisms of action through which *Lodhrasavam* exerts its anti-diabetic and anti-obesity effects.

Conflict of interest

Authors declare no conflict of interest.

Sources of funding

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaim.2017.03.005.

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