Multicomponent Ayurveda formulation Lodhrasavam ameliorates steatosis and lipotoxicity in HepG2 cell model of NAFLD

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Abstract:

Background: Non-alcoholic fatty liver disease (NAFLD) is a complex, multifactorial and multi-system disorder. It is one of the major contributors of liver disease worldwide. Among the many factors involved in the pathogenesis of NAFLD, free fatty acids (FFAs) such as palmitic acid induced lipotoxicity promotes steatosis, oxidative stress and insulin resistance that activate apoptotic cascades leading to tissue damage and inflammation. Since NAFLD is a multifactorial metabolic disorder, conventional target-based drug therapies have limited success. Therefore, the use of multicomponent ayurveda herbal formulations could be a promising alternative due to their multitargeted mechanisms of action. The present study investigates the effects and underlying mechanism of actions of an ayurveda formulation, Lodhrasavam (TDU-LS-1), in the in-vitro model of NAFLD.

Methods: Lipotoxicity was induced in HepG2 cells by treating the cells with 1mM palmitic acid for 24 hrs followed by drug (TDU-LS-1) treatment for another 24 hrs. The effect of TDU-LS-1 on lipotoxicity was evaluated by MTT assay. The effect of TDU-LS-1 on steatosis was studied by estimating intracellular triglycerides, lipid droplets formation and expression of genes involved in lipid metabolism. Further, to examine the antioxidant activity, DPPH scavenging assay was performed.

Results/discussion: TDU-LS-1 was found to increase the antioxidant activity in a concentration dependent manner with an IC50 of 16.45 μg GAE/ml. Palmitic acid induced lipotoxicity in HepG2 cells was reduced by lower concentrations of TDU-LS-1. Also, the results from triglyceride (TAG) assay, Oil-Red-O staining and BODIPY 493/503 confocal imaging suggest that TDU-LS-1 reduces the palmitate induced triglyceride deposition and lipid droplet
accumulation in HepG2 cells. Further, the qRT-PCR analysis of TDU-LS-1 at a concentration of 32 μg/GAE revealed that it modulates the expression of SREBP, FASN, SCD1, ACOX, and PPARγ that are relevant in hepatic lipid metabolism. Our results suggest that TDU-LS-1 can reduce de novo lipogenesis, peroxisomal lipid peroxidation as well as lipotoxicity in the in vitro palmitate-induced NAFLD model in HepG2 cells.

**Keywords:** NAFLD, Lodhrasavam, lipotoxicity, HepG2, steatosis

**Introduction:**

Non-alcoholic fatty liver disease (NAFLD) is a complex, multisystem disease, extending its effect on extrahepatic organs and regulatory pathways (Byrne and Targher, 2015). High-calorie diet and sedentary lifestyle are typical risk factors for NAFLD. However, many other factors including, but not limited to, type 2 diabetes, insulin resistance, obesity, hypertension, metabolic syndrome, genetic variations, alterations in gut permeability and microbiome are also playing a crucial role in its development and progression (Carr et al., 2016; Friedman et al., 2018; Younossi et al., 2018). It is one of the major contributors of liver disease worldwide with an estimated prevalence of 25% in the adult population (Younossi et al., 2019). In India its prevalence varies between 9% in rural and to 35% in urban populations (Duseja, 2010). When left untreated, NAFLD leads to deleterious liver physiology like steatohepatitis, fibrosis and cirrhosis that can further increase the risk of liver cancer and end-stage liver disease (Haas et al., 2016; Michelotti et al., 2013). Also, the renaming of NAFLD as Metabolic-Associated Fatty Liver Disease (MAFLD) by a consensus arrived by a panel of experts is the reflection of its complex etiopathology and patient heterogeneity as well as the multidirectional relationship with other metabolic disorders (Eslam et al., 2020).

Despite its high clinical and socioeconomic burden there are no approved therapeutics for NAFLD and the current clinical management strategies are largely centered around controlling the predisposing factors like diabetes and obesity (Mundi et al., 2020). One of the reasons for this is the inadequacy of conventional single target-based drugs to address the complex etiopathology of NAFLD as well as the limited knowledge we have on the systemic biology of this disease. Therefore, frontiers of biology started looking at innovative systemic strategies for addressing the multifactorial pathophysiology of NAFLD. This is where a transdisciplinary approach of integrating biomedicine’s molecular perspective of NAFLD with its systemic
counterpart from traditional medicines like Ayurveda become relevant and can probably open up new opportunities for better holistic strategies for the prevention and management of NAFLD. One of the characteristics of NAFLD is the accumulation of lipid droplets (LDs) in the hepatocytes leading to a condition referred to as hepatic steatosis, and it happens via flux of free fatty acids (FFAs) from adipose tissue due to increased lipolysis, de novo lipogenesis (DNL), excess dietary fat intake, decreased export of triglycerides and reduced beta-oxidation (Alkhouri et al., 2009; Schulze and McNiven, 2019). Although Ayurveda has an epistemologically and ontologically different understanding of NAFLD from biomedicine, both schools emphasize the involvement of metabolic imbalances and the role of diabetes (Prameha) and obesity (Sthoulya) as predisposing factors in NAFLD. Previous studies from our laboratory by Butala et al., 2017 reported the anti-diabetic and anti-obesity effects of Lodhrasavam, a polyherbal formulation in Ayurveda prescribed for obese-diabetic patients. According to Ayurvedic pharmacology principles, the key herbal ingredients of this formulation belong to the Lodhradi-gana group of plants, a group having medo-hara (anti-obesity) and kapha-hara properties and both are essential for the management of glucose and lipid homeostasis in the body. Considering the profound ant-obesity and anti-lipidemic properties of Lodhrasavam as well as its rich herbal-ingredient diversity the present study aimed at evaluating its potential effect on reducing lipotoxicity and steatosis in free fatty acid (FFAs) induced liver model.

Materials and methods

Chemicals and Reagents
HepG2 human hepatoma cell line (NCCS, Pune, India); Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, New York); fetal bovine serum (FBS) (GIBCO, Grand Island, New York); penicillin/streptomycin (GIBCO, Grand Island, New York); fatty acid free bovine serum albumin (BSA) (Genei, Bangalore, India); sodium palmitate (PA) (Sigma-Aldrich, St. Louis, MO, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (SRL, India); triglyceride kit (BeneSphere, Avantor, USA); 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA); Ascorbic Acid (Sigma-Aldrich, St. Louis, MO, USA); Folin – Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA); gallic acid (Sigma-Aldrich, St. Louis, MO, USA); BODIPY 493/503 (Cayman chemical 25892); Hoechst 33342 (Cayman chemical 15547); Oil-Red-O stain (Sigma-Aldrich, St. Louis, MO, USA); trizol reagent (Takara...
Bio, Shiga, Japan); PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan); PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan)

**Determination of total tannin content**

TDU-LS-1 was estimated for total tannins following standard Folin - Ciocalteu method (Ainsworth and Gillespie, 2007). Briefly, 10 μL of TDU-LS-1 with 40 μL of water, 50 μL FC reagent (previously diluted with water 1:10 v/v) and 100 μL of 3.5% Na₂CO₃ 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 760 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.

**Determination of DPPH radical scavenging activity**

Free radical scavenging ability of different concentrations of TDU-LS-1 was tested by DPPH radical scavenging assay (Rahman et al., 2015). DPPH produces violet/purple color in ethanol solution and fades to shades of yellow color in the presence of antioxidants. A solution of 0.1 mM DPPH in ethanol was prepared, and 2.4 mL of this solution was mixed with 1.6 mL of TDU-LS-1 in ethanol to get a concentration gradient (128, 64, 32, 16, 8, 4, 2 μg GAE/ml). The reaction mixture was vortexed thoroughly and left in the dark at room temperature (RT) for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference. Percentage DPPH radical scavenging activity was calculated by the following equation: % DPPH radical scavenging activity= \{(A₀− A₁)/A₀\} ×100; where A₀ is the absorbance of the control, and A₁ is the absorbance of TDU-LS-1 or the standard. The % of inhibition was plotted against concentration, and from the graph IC₅₀ was calculated. The experiment was performed in triplicates and repeated three times. Ascorbic acid Equivalent Antioxidant Capacity (AEAC) was calculated using the formula: IC₅₀ (AA) /IC₅₀ (sample). Where, IC₅₀(AA) is IC₅₀ of standard (i.e., Ascorbic acid) and IC₅₀(sample) is IC₅₀ of sample.

**Cell Culture and establishment of palmitic acid induced NAFLD model**

HepG2 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were seeded in multi-well plates and grown for 48 hrs. The medium was changed to DMEM (serum free) containing 5% fatty-
acid-free bovine serum albumin (BSA) with or without 1mM palmitic acid (PA) and grown for 24 h. Further, the cells were treated with various concentrations of TDU-LS-1 in the same BSA-medium and incubated for 24 h. The changes in lipid droplet formation were observed and quantified using various methods as described in the below sections.

**Cytotoxicity assay**

The cytotoxic effects of various concentrations of TDU-LS-1 in HepG2 cells were studied using MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. HepG2 cells were seeded in 96-well plates at the density of $1.5 \times 10^4$ cells/well and cultured in 100μL of DMEM containing 10% FBS overnight. The medium was then discarded and was replaced with 128, 64, 32, 16, 8, 4, 2, 0 μg GAE/ml TDU-LS-1 diluted in the culture medium, followed by incubation for a further 24 h at 37°C. 500 μg/ml of MTT working solution were then added to each well followed by incubation for 4 h at 37°C. The violet formazan crystal in each well was dissolved in DMSO and the absorbance of each well was measured at 570 nm using a microplate reader (xMark Microplate Spectrophotometer, BioRad, USA). HepG2 cells were treated with 1mM PA in 5% BSA media for 24 h at 37°C, followed by treatment with 128, 64, 32, 16, 8, 4, 0 μg GAE/ml TDU-LS-1 for 24 h at 37°C. Further, to assess they cytotoxicity effect MTT assay was performed as described.

**BODIPY 493/503 staining of lipid droplets**

HepG2 cells were seeded in 35mm confocal dishes, treated with 1 mM PA for 24 h, and then cultured with 32 μg GAE/ml TDU-LS-1 for 24 h at 37°C. Cells were stained with 10μM BODIPY 493/503 dye and 10μg/ml Hoechst dye and incubated for 30 mins at 37°C (Qiu and Simon, 2016). Cells were washed with PBS, visualized under the LSM880 confocal live cell imaging system, (Carls Ziess, Germany) and images were taken at 40x magnification.

**Oil Red O (ORO) staining of intracellular lipid accumulation**

HepG2 cells were seeded in six-well plates (1.2×10^6 cells/well), treated with 1 mM PA for 24 h, and then cultured with 32 μg GAE/ml TDU-LS-1 for 24 h at 37°C. Cells were washed twice with PBS, and fixed with 4% formaldehyde for 30 mins. After washing, cells were stained for 20 min in freshly diluted ORO solution (0.5% ORO in isopropanol diluted to 3:2 with H_2O, and filtered) at 37 °C. After staining, the cells were thoroughly washed with double distilled water to remove
the unbound staining solution and images of cells stained with Oil Red were captured using phase contrast microscope (Olympus-IX- Olympus America Inc, USA). To quantify the ORO contents, isopropanol was added to each well and the optical density of the samples at a wavelength of 450 nm was measured using a microplate reader (xMark Microplate Spectrophotometer, BioRad, USA) (Mehlem et al., 2013).

**Triglyceride (TG) estimation**
To analyze the content of cellular triglycerides, the cells were washed with PBS, scraped into 250μl 0.1% PBST and pulse sonicated for 45 % amplitude for 45 seconds. The lysates were assayed for their total triglyceride content using assay kit and cellular protein was estimated using the Bradford method. The results were expressed as μg of triglyceride per mg of cellular protein.

**Quantification real-time PCR**
Total RNA was isolated from treated HepG2 cells using trizol reagent according to the manufacturer’s instructions, and the absorbances of the extracted RNAs at 260 nm and 280 nm were determined spectrophotometrically (Nanodrop, Thermofisher Scientific). RNA samples with purity ratios (A260/A280) between 1.8 and 2.0 were used for synthesizing single-strand cDNA by means of PrimeScript 1st strand cDNA Synthesis Kit for quantitative real-time polymerase chain reaction (qPCR). Gene-specific primers were designed (Table 2) (Bioserve, Hyderabad, India) and qPCR was performed to detect the relative mRNA expression levels using PrimeScript RT-PCR Kit as described in the protocol. To determine the specificity of the amplification, melting curve analysis was performed for all final PCR products. Relative changes in gene expression were calculated and expressed as fold changes using the relative quantification (ΔΔCt) method. The relative abundance of each transcript was normalized to that of GAPDH. Each sample was run in triplicate.

**Statistical analysis**
All results are expressed as the means ± standard deviations (SDs). 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.

**Results**
TDU-LS-1 increases DPPH radical scavenging activity in a concentration dependent manner

The antioxidant effect of TDU-LS-1 was studied using DPPH assay and the results showed a concentration dependent increase in radical scavenging activity by the formulation. The highest concentration of 128 \( \mu \text{g GAE/ml} \) tested in the assay showed 90.40\% \pm 1.39\% inhibition of DPPH assay whereas 15.33\% \pm 5.38\% inhibition was observed at the lowest concentration of 2 \( \mu \text{g GAE/ml} \) (Fig-1). IC\textsubscript{50} of TDU-LS-1 is 16.45 \( \mu \text{g GAE/ml} \) and IC\textsubscript{50} of ascorbic acid is 24.51 \( \mu \text{g/ml} \). AEAC (Ascorbic acid equivalent antioxidant capacity) was found to be 1.49 (Fig 1).

TDU-LS-1 reduces cytotoxicity of HepG2 cells in palmitic acid induced NAFLD model

Different concentrations of TDU-LS-1 were tested on HepG2 cells for assessing the cytotoxicity of the formulation. Results of 24 hr treatment showed that only the highest concentration (128 \( \mu \text{g GAE/ml} \) used in the experiment caused any significant cytotoxicity in HepG2 cells. It showed 25.67\% \pm 10.48\% viability whereas the lowest concentration tested (2 \( \mu \text{g GAE/ml} \) showed 92.66\% \pm 9.43\% viability after 24 h treatment (Fig-2A). An in vitro model of NAFLD was established in HepG2 cells by inducing the cells with palmitic acid for 24 hrs. HepG2 cells were treated with various concentrations of PA and observed for cytotoxicity effects. Cells treated with 1mM PA reduced the viability of cells to 37.64\% \pm 5.71\% along with increasing lipid and triglyceride accumulation. In order to study the effect of TDU-LS-1 on protecting the PA-induced cytotoxicity of HepG2 cells. On the other hand, TDU-LS-1 treatment post 1mM palmitic acid induction was found to reverse the palmitic acid-induced cytotoxicity, especially at lower concentrations of the formulation like 4, 8, 16 and 32 \( \mu \text{g GAE/ml} \). These concentrations of TDU-LS-1 increased the viability of PA treated cells to 67.46\% \pm 4.9\%, 64.81\% \pm 5.06\%, 60.69\% \pm 3.44\% and 41.64\% \pm 3.70\% respectively. Although 64 \( \mu \text{g GAE/ml} \) TDU-LS-1 was non-toxic in the MTT assay, this concentration was found to be ineffective in the palmitic acid treatment (Fig 2 A-B).

TDU-LS-1 reduces intracellular lipid droplet and triglyceride

The in vitro model of PA induced hepatic steatosis was used to investigate the efficacy of TDU-LS-1 against hepatic steatosis. BODIPY 493/503 staining showed a 1.72-fold increase in mean fluorescence intensity in 1mM PA treated cells when compared to untreated cells (Fig 3 E-F). Further, it was found that 32\( \mu \text{g GAE/ml} \) TDU-LS-1 significantly reduced the mean fluorescence
intensity by 0.72-fold. Similar to BODIPY staining, cells stained with ORO also showed that PA treatment for 24 h significantly induced significant intracellular lipid accumulation in HepG2 cells. The absorbance of the stained cells in the PA-treated model group was 1.2 times that of the cells in the control group. However, the intracellular lipid accumulation in 32μg GAE/ml TDU-LS-1 treated cells significantly reduced the lipid droplet accumulation by 1.14-fold in comparison to PA induced cells (Fig 3 A-D). Further, in the PA treated cells the TG levels increased 1.5-fold when compared to untreated cells. However, 32 μg GAE/ml TDU-LS-1 treated cells significantly reduced the TG levels by 1.10-fold in comparison to PA induced cells (Fig 3 G).

**TDU-LS-1 attenuates lipogenic mRNA expressions induced by PA**

To investigate how TDU-LS-1 ameliorated fat deposition in the liver, the expressions of genes associated with lipid metabolism in HepG2 cells were examined using qRT-PCR. The mRNA expression levels of *PPARγ*, *SREBP-1C*, *FASN*, *SCD1* and *ACOX1* significantly increased in PA treated cells by 6.2-fold, 3.2-fold, 2.0-fold, 1.9-fold and 2.36-fold respectively in comparison to untreated cells. 32 μg GAE/ml TDU-LS-1 significantly reduced the expression of *PPARγ* (2.62-fold), *SREBP-1C* (2.21-fold), *FASN* (1.39-fold), *SCD1* (1.54-fold) and *ACOX1* (1.75) when compared with PA treated cells (Fig 4A-C).

**Discussion**

In previous studies conducted in our lab, *Lodhrasavam* has shown potential anti-diabetic and anti-adipogenic effects. It inhibited α-amylase and α-glucosidase activity by 90% and 78% respectively and reduced adipocyte differentiation significantly (Butala et al., 2017). It has not been explored for its anti-steatosis and antioxidant activities. In the present study we aimed at evaluating the effect of TDU-LS-1 on saturated fatty acid (palmitate) induced hepatic steatosis and lipotoxicity.

In a case study conducted by Sahu et al., 2022, it was found that *Ayurveda* formulations (*Avipattikara Churna, Punarnava Mandura, Sankha Bhasma, Tarunikusumakara Churna, Triphala Churna and Kutaki Churna*) have immense potential in treating grade 2 fatty liver disease. A similar effect was seen in a randomized placebo-controlled clinical trial of a multiherbal formulation, *Sharapunkhadi* powder on subjects with grade 1-3 fatty liver disease (Remya et al., 2020). Livogrit, a tri-herbal *Ayurvedic* medicine, was studied for its
hepatoprotective effect on methionine and cysteine deficient NASH models in both HepG2 spheroids and rat primary hepatocytes. It reduced fat accumulation and ROS production and increased lipolysis hence attenuating NASH (Balkrishna et al., 2022).

NAFLD is characterized by the accumulation of lipid droplets (LDs) in the hepatocytes, referred to as hepatic steatosis. It is considered as the “first-hit” in the development of NAFLD. LDs can accumulate in the liver via three mechanisms: a) flux of free fatty acids (FFAs) from adipose tissue due to increased lipolysis, increased de novo lipogenesis (DNL), and dietary fat intake; b) decreased export of triglycerides and c) reduced beta-oxidation. Elevated plasma free fatty acid (FFA) levels play an etiological role in the pathogenesis of NAFLD. In particular, the saturated fatty acid palmitate, which makes up 30%-40% of high plasma FFA concentration, is a major contributor to an increase in intrahepatic triglyceride (Cacicedo et al., 2005; Schroeder-Gloeckler et al., 2007). In the present study, 1 mM PA was used to induce cellular steatosis (Gómez-Lechón et al., 2007). We found that the accumulation of lipid droplets and the intracellular TG content significantly increased in FFA-treated HepG2 cells. TDU-LS-1 treatment of HepG2 cells exhibited an inhibitory effect on FFA-induced hepatic steatosis.

To investigate the mechanism of TDU-LS-1 inhibition of hepatic steatosis, we evaluated the expression of key genes involved in lipogenesis, including SREBP-1c, FASN and SCD-1 (Parlati et al., 2021). SREBP-1c is a master regulator of lipogenesis, known to transcriptionally regulate downstream genes involved in de novo lipogenesis, including those encoding ACC and FAS (Osborne, 2000). In a study by Motoyuki et al., 2007, it showed an increase in expression of SREBP-1c, ACC and FAS in NAFLD patients. It has been reported that SREBP-1c activation is associated with increased lipogenesis in NAFLD (Yan et al., 2014). In mice, HFD caused fat accumulation and induced SREBP-1c mRNA expression which further contributes to the progression of hepatic steatosis (Kim et al., 2010; Zhu et al., 2019). Li et al., 2011 showed that inhibiting SREBP-1c activity can exert an anti-hepatic steatosis effect in diet-induced obese mice. SCD-1 is an endoplasmic reticulum bound rate limiting enzyme that catalyzes the formation of mono-unsaturated fatty acids from saturated fatty acids (Paton and Ntambi, 2009). Liver specific knock-out of SCDI in mice fed with high-carbohydrate diet showed reduction in hepatic steatosis (Miyazaki et al., 2007) (Jiang et al., 2005) in their experiments using antisense oligonucleotide inhibitor of SCD1 showed inhibition of lipogenesis and promoted lipid oxidation in both primary mouse hepatocytes and HFD fed mice. In the current study, 24hr incubation with
1mM PA increased the expression of these genes. Following which TDU-LS-1 significantly downregulated the expression of SREBP-1c, FAS and SCD-1.

The hepatic PPARγ plays a putative role in the progression of fatty liver disease in the NAFLD patients. PPAR-γ is up-regulated in the liver of obese patients with NAFLD, and recently, the expression of PPAR-γ is considered as an additional reinforcing lipogenic signal, assisting SREBP-1c to trigger the development of hepatic steatosis (Pettinelli and Videla, 2011). We showed that PA increased PPAR-γ mRNA expression in HepG2 cells, which was significantly reduced by TDU-LS-1.

When there is an excess of fatty acids in hepatocytes, alternative pathways of fatty acid oxidation are activated, such as β-oxidation in peroxisomes. The peroxisomal acylCoA oxidases ACOX is one of the first and rate-limiting enzymes of β-oxidation pathways in peroxisomes (Reddy and Hashimoto, 2001). Expression of ACOX increase in NAFLD patients indicating that peroxisomal β-oxidation is compensatively enhanced in NAFLD (Motoyuki et al., 2007). In our study we observed an increase in ACOX gene expression in PA treated cells, whereas in TDU-LS-1 treated cells a significant decrease in its expression was shown. TDU-LS-1 reduced the β-oxidation in peroxisomes indicative of its role in lowering the excessive ROS production which occurs as a result of excessive oxidation of fatty acids. Further studies need to be performed to understand the effect of TDU-LS-1 in lowering oxidative stress induced by palmitic acid.

**Conclusion**

Excessive accumulation of lipid droplets in the hepatocytes is the primary hallmark of NAFLD. Therefore, reducing the amount of lipid droplets and inhibiting de novo lipogenesis (DNL) are considered as important treatment strategies for NAFLD. Our study provides experimental evidence for the anti-steatosis effect of TDU-LS-1. It exerts its effects through reducing the lipid droplet formation in hepatocytes and inhibiting key genes involved in DNL. In addition, radical scavenging activities of TDU-LS-1 makes it more beneficial in addressing the complexities of NAFLD pathophysiology. Further studies need to be conducted to understand the holistic mechanism of action of this complex multicomponent Ayurveda formulation for managing NAFLD.

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**Conflict of interest**

Authors declare no conflict of interest.

**Figure legends**

**Figure 1:** TDU-LS-1 increases DPPH radical scavenging activity in a concentration dependent manner. IC50 of TDU-LS-1 is 16.45 μg GAE/ml.

**Figure 2:** TDU-LS-1 reduces cytotoxicity of HepG2 cells in palmitic acid induced NAFLD model. A) In the 24 hr treatment of HepG2 cells the highest concentration of 128 μg GAE/ml showed 25.67% ± 10.48% viability while the lower concentrations did not significantly affect the viability of the cells. B) 1mM PA reduced the viability of cells to 37.64% ± 5.71% and treatment with various concentrations of TDU-LS-1 gradually increased the viability as the concentration decreased.

**Figure 3:** TDU-LS-1 inhibits intracellular lipid droplet and triglyceride accumulation. A-C) After ORO staining it was observed that 32 μg GAE/ml of TDU-LS-1 reduced the lipid droplets induced by 1mM PA; D) the intracellular lipid accumulation in 32 μg GAE/ml TDU-LS-1 treated cells significantly reduced by 1.14-fold in comparison to PA induced cells; E) Untreated cells (E1-E3); 1mM PA treated cells (E4-E6); 32 μg GAE/ml TDU-LS-1 treated cells (E7-E9). Live cells stained with BODIPY 493/503 (Green) and Hoechst dyes (Blue); F) 1.72-fold increase in mean fluorescence intensity in 1mM PA treated cells when compared to untreated cells and 32 μg GAE/ml TDU-LS-1 significantly reduced the mean fluorescence intensity by 0.72-fold was observed.

**Figure 4:** Treatment with 1mM PA increased the expression of the lipogeneic genes while 32 μg GAE/ml TDU-LS-1 significantly reduced their expression (A-B). 32 μg GAE/ml TDU-LS-1
treated cells significantly decreased the expression of ACOX gene while it increased the expression of CPT1A (C)

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The diagrams illustrate the relative mRNA expression levels of various genes under different conditions:

**A**
- **PPARG**
- **SREBP**

**B**
- **FASN**
- **SCD1**

**C**
- **ACOX**
- **CPTIA**

The conditions compared are:
- Untreated
- 1mM PA
- 1mM PA+32 µg GAE/ml TDU-LS-1

Significance levels indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.
Table 1. List of plant ingredients present in *Lodhrasavam*

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<td>Cinnamomum tamala</td>
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<td>Neopicrorhiza scrophulariiflora</td>
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<td>Inula racemosa C.B.Clarke</td>
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<td>Cyclea peltata</td>
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<td>Piper mullesua</td>
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<td>28</td>
<td>Terminalia bellerica Gaertn.</td>
<td>Bibhitaka</td>
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<td>29</td>
<td>Phyllanthus Emblica Roxb.</td>
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Table 2: List of primers

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