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Liv.52 up-regulates cellular antioxidants and increase glucose uptake to circumvent oleic acid induced hepatic steatosis in HepG2 cells

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ABSTRACT

HepG2 cells were rendered steatotic by supplementing 2.0 mM oleic acid (OA) in the culture media for 24 h. OA induced hepatic steatosis in HepG2 cells was marked by significant accumulation of lipid droplets as determined by Oil-Red-O (ORO) based colorimetric assay, increased triacylglycerol (TAG) and increased lipid peroxidation. It was also marked by increased inflammatory cytokines TNF-α and IL-8 with decreased enzymic and non-enzymic antioxidant molecules and decreased cell proliferation associated with insulin resistance and DNA fragmentation. Addition of Liv.52 hydro-alcoholic extract (LHAE) 50 μg/mL to the steatotic cells was effective in increasing the insulin mediated glucose uptake by 3.13 folds and increased cell proliferation by 3.81 folds with decreased TAG content (55%) and cytokines. The intracellular glutathione content was increased by 8.9 folds without substantial increase in GSSG content. LHAE decreased TNF-α and IL-8 by 51% and 6.5% folds respectively, lipid peroxidation by 65% and inhibited DNA fragmentation by 69%. The superoxide dismutase, catalase and glutathione peroxidase activities were increased by 88%, 128% and 64% respectively. Albumin and urea content was increased while the alanine aminotransferase (ALAT) activity was significantly decreased by LHAE. Hence, LHAE effectively attenuate molecular perturbations associated with non-alcoholic fatty liver disease (NAFLD) indications in HepG2 cells.

Introduction

Non-alcoholic steatohepatitis (NASH), the inflammatory form of non-alcoholic fatty liver disease (NAFLD) is a chronic disease that occurs in individuals without significant alcohol consumption (Ludwig et al. 1980). Liver biopsy remains the cornerstone for the diagnosis of NASH, with macrovesicular fat infiltration and lobular inflammation being characteristic (Luyckx et al. 2000). Patients with primary NASH typically have the insulin resistance syndrome (Knobler et al. 1999). Initially, the cause of NASH was unknown and there was no defined therapy. More than 2 decades later, this clinical syndrome is better understood, but still there is no Food and Drug Administration – approved therapy (Falck-Yerrer et al. 2001). NASH is increasingly recognized as a major cause of cryptogenic cirrhosis and an indication for liver transplantation. The pathogenesis of NASH is complicated, and the prevailing theory is the "two hits" hypothesis proposed by Day and James (1998). The "first hit" is the deposition of liver free fatty acid and triglyceride in hepatocytes (steatosis). The second "hit", steatosis progresses to NASH and this progress is associated with factors such as oxidative stress, mitochondrial dysfunction, and cytokines capable of inducing inflammation, fibrosis, or necrosis (Day and James 1998). The elevated cytokine interactions with oxidative stress mediators and lipid peroxides have been postulated to play a role in induction of steatohepatitis in both alcoholic and non-alcoholic origin. TNF-α is an important cytokine in the development of many forms of liver injury (Day and James 1998; Valenti et al. 2002; Wigg et al. 2001).

Oxidative stress has been recognized to be mainly involved in the etiology of liver diseases such as hepatocellular carcinoma, viral and alcoholic hepatitis, NASH, alcoholic steatohepatitis *etc.* It is known that reactive oxygen species [ROS] and reactive nitrogen species play a crucial role in disease induction and progression (Adachi and Ishii 2002). Oxidative stress results from an imbalance between pro-oxidant and antioxidant chemical species that leads to oxidative damage of cellular macromolecules (Browning and Horton 2004). It may be secondary to the release of inflammatory mediators which are the prime mediator of cell injury. ROS including oxygen ions, free radicals and peroxides are the main pro-oxidants in the body. The ROS are generated physiologically during oxidative phosphorylation (Robertson et al. 2001).

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Antioxidants from herbal and dietary origin have been well documented to have therapeutic effect to counteract liver damage (Scalbert et al. 2005; Park et al. 2011; Cai et al. 2011). Lipotoxicity has been implicated mainly in the pathogenesis of NAFLD, and free fatty acids appear to be important contributors of lipotoxicity (Feldstein et al. 2004a,b). The agents with the ability to prevent or attenuate free fatty acids induced hepatoxicity and oxidative stress induced damage represent a promising therapeutic choice for NAFLD.

In this context, Liv.52 a novel herbal formulation is known to play a pivotal role in combating liver disorder due to various infections and damage due to biological and chemical toxicants (Husseini et al. 2005; Mitra et al. 2008; Vidyashankar et al. 2010; Vidyashankar and Patki 2010) in humans, animals as well *in vitro* models. Liv.52 is rich in phenolic compounds and in particular polyphenols are believed to be at least in part, responsible for such effects (Vidyashankar et al. 2010). The HepG2 cells retain and mimic many of the specialized functions, which characterize normal human hepatocytes and used extensively to study the phase I, phase II and antioxidant enzymes ensuring that they constitute a good model to study cytoprotective, genotoxic and antigenotoxic effects of compounds *in vitro* (Mersch-Sundermannn et al. 2004). Hence, HepG2 cells were rendered steatotic with oleic acid and Liv.52 hydro-alcohol extract (LHAE) was tested for its possible beneficial effect.

Materials and methods

Chemicals

Ammonium acetate, Bradford reagent, cytochalasin-B, cytochrome-C, DPPH, Dulbecco's Minimum Essential Medium Eagle (DMEM), fetal bovine serum (FBS), trypsin, EDTA, glutathione, hydrogen peroxide, insulin, MTT, NADPH, oleic acid, thiobarbituric acid, xanthine and xanthine oxidase were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Deoxy-[^{3}H]D-glucose was purchased from the Department of Atomic Energy, Mumbai, India. Methanol, acetonitrile and water were procured from Merck (LC–MS grade). All other solvents reagents used were of analytical grade.

Composition of Liv.52

The Liv.52 is the approved proprietary medicine by drug regulatory authority Department of AYUSH, Ministry of Health and Family Welfare, Government of India. The Liv.52 formulation contains extracts of the following medicinal plants in definite proportions – Himsra (*Capparis spinosa*) 65 mg, Kasani (*Cichorium intybus*) 65 mg, Kakamachi (*Solanum nigrum*) 32 mg, Arjuna (*Terminalia arjuna*) 32 mg, Kasamarda (*Cassia occidentalis*) 16 mg, Biranjasipha (*Achillea millefolium*) 16 mg, Jhavuka (*Tamarix gallica*) 16 mg. The good agricultural and collection practices (GACP) were employed for plants used in the formulation. Plants were identified and certified by Botanist and a voucher specimen of each constituent plant has been archived in the herbarium of R&D, The Himalaya Drug Company, Bangalore, India.

Liv.52 hydroalcoholic extract (LHAE)

Liv.52 granules (100 g) was ground into powder under liquid nitrogen with a mortar and pestle, and extracted with 1000 mL of ethanol–water (70:30 v/v) at RT for 30 min. The mixture was then extracted in a shaking water bath (300 rpm) at 37 °C for 24 h. After cooling to room temperature, the slurries were centrifuged at 1000 rpm for 15 min, and the supernatant was collected. The solution was then evaporated under reduced pressure to obtain the ethanol extract, with the yield being 2.3% of the dry weight of Liv.52. Thus obtained Liv.52 hydroalcoholic extract was used to evaluate the inhibitory effect on OA-induced fatty liver model *in vitro*.

Liquid chromatography–mass spectrometer analysis

The LC–MS/MS instrument consisted of an HPLC (Shimadzu LC-20AD) coupled with API-2000 mass spectrometer-MS/MS [Applied Biosystem/MDS SCIEX, Canada]. The 20 μL LHAE at the concentration of 2 mg/mL in methanol was injected through SIL-HTC Shimadzu auto sampler for phytochemical screening. The Luna RP-C_{18} (5 μm, 250 mm × 4.6 mm) (Phenomenex Torrance, CA, USA) column was used for separation and analysis of LHAE. The column oven temperature was maintained at 40 °C throughout the analysis by CTO-10ASVP column oven. The binary mobile phase is the combination of A (10 mM ammonium acetate and 0.1% formic acid in water) and B (acetonitrile). The gradient was varied linearly from 3% to 9% (B) in 0–5 min, 9% to 16% (B) in 5–15 min, 16% to 50% (B) in 15–45 min, 50% to 90% (B) in 45–48 min, and held with 90% (B) at 48–51 min followed by 90% to 3% (B) in 51–60 min delivered at a flow rate of 600 μL/min with split out 200 μL/min to mass spectrometer. An API-2000 mass spectrometer coupled with electron spray ionization (ESI) interface was used to obtain the MS/MS data using Analyst 1.5 version software. The ionization conditions were optimized and the following conditions were adopted – ionization voltage was −4500 V; curtain gas (CUR) 25 psi; focussing potential (FP) −300 V; entrance potential (EP) −2 V; declustering potential (DP) −20 V; ionization source temperature 420 °C; ion source gas 1 (GS1) 55 psi and ion source gas 2 (GS2) 65 psi. Collision energy (CE) for MRM of precursor to product ion was optimized by multiple run through LC until most intense precursor to product ion transition state is obtained. The data was recorded in negative multiple reaction monitoring (MRM) mode. Compounds identified by LC–MS/MS (Fig. 1) were characterized according to Rabaneda et al. (2003) and given in Table 1.

Cell culture

HepG2 cells (hepatocellular carcinoma cell line), obtained from the National Center for Cell Science (NCCS) Pune, India, were maintained in culture in 25 cm^2 polystyrene flasks (Tarsons) with DMEM containing 10% FBS, 1% antibiotic–antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO_2 at 37 °C with 95% humidity.

LHAE cytotoxicity

HepG2 cells were plated in 96-multiwell culture plates at 1×10^5 cells per well. To study *LHAE* cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing *LHAE* at various concentrations was added. At different time points, cellular viability was determined by the MTT assay (Mosmann 1983).

Oleic acid induced hepatic steatosis and its inhibition by LHAE

The confluent HepG2 in 96 well culture plate were washed in PBS and added with medium containing 0–2.0 mM oleic acid–bovine serum albumin (OA–BSA) complex (molar ratio 4:1). Then the cells were further incubated for 24 h. The medium with only BSA was selected as the control. The extent of steatosis was quantified by oil-red-O (ORO) based colorimetric assay (Cui et al. 2010) and measuring triacylglycerol content at various time intervals using triglyceride estimation kit (Pointe Scientific, Mumbai, India). To study the hepatic steatosis inhibitory effect of LHAE, the confluent HepG2 cells were added 2.0 mM (OA–BSA) complex