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Effect of *Ichnocarpus frutescens* (L.) R.Br. hexane extract on preadipocytes viability and lipid accumulation in 3T3-L1 cells

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ABSTRACT

Objective: To investigate the crude extracts of *Ichnocarpus frutescens* (*I. frutescens*) for antiobesity effect. **Methods:** Leaves of *I. frutescens* were sequentially extracted with hexane, ethyl acetate, and methanol and their effect on viability of 3T3-L1 preadipocytes were evaluated. Based on this the apoptosis on preadipocytes was confirmed by DNA fragmentation and LDH (Lactate dehydrogenase) leakage assays. Anti-adipogenesis was performed by oil red O (ORO) staining and free glycerol release in the medium of differentiated adipocytes. **Results:** The hexane extract of *I. frutescens* (IFHE) inhibited cell viability in a time- and dose-related manner. An increased release of LDH, as a marker of membrane integrity, was observed at a dose of 200 μg/mL. The discontinuous DNA fragments on agarose gel electrophoresis showed the apoptotic effect of the IFHE. Morphological observations of cells stained with ORO showed a decrease in cellular lipid content at the concentrations tested compared to the induced control cells. In the experiment of lipolytic activity, treatment with IFHE enhanced glycerol secretion with the rates of approximately 28%, 55%, and 46% at the concentrations of 100, 200 and 300 μg/mL, respectively. **Conclusions:** The observed properties clearly revealed the medicinal property of *I. frutescens* in the treatment of obesity.

1. Introduction

Obesity remains a major global public health issue because of its increasing prevalence, cutting across all sex, age-groups, ethnicity or race. Obesity, characterized by excess accumulation of adipose tissue, is detrimental to many systems and is involved in the pathogenesis of multiple human diseases[1,2]. Obesity and dyslipidemia are disorders of metabolic syndrome[3]. This chronic metabolic disorder that results from the imbalance between energy intake and energy expenditure is characterized by enlarged fat mass and elevated lipid concentration in blood[4]. The amount of fat mass is increased when the number and/or size of adipocytes are multiplied by proliferation and differentiation. Adipocytes are active sites of lipid metabolism involving storage, mobilization, and transport of lipids as well as utilization of lipid-mediated signaling pathways. Recently, evidence from several *in vivo* and in vitro studies has indicated that apoptosis is a significant factor in adipocyte depletion during weight reduction[5]. Induction of death of adipocytes through apoptosis may emerge as a promising strategy for prevention and treatment of obesity because removal of adipocytes via this mechanism will result in reducing body fat.

Natural compounds can be screened for their ability to induce adipocyte apoptosis, allowing the identification of potential obesity drugs, which provides a potential target for treatment of obesity. Plants and plant based medications have been man's prime therapeutic tools since the dawn of civilization and are still among the frontline treatments for a variety of ailments[6]. Traditional medicine is a comprehensive term used to refer both to codified systems of medicine such as Ayurveda and also to various forms of indigenous medicine (empirical knowledge). Worldwide more than 35 000 plants are used for medicinal purposes. India and China are the herbal giants of the world by holding more than 40% of the medicinal species in the world herbal market[7].

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I. frutescens is a profusely branched straggling shrub distributed commonly in scrub jungles of South-East Asia up to 1100 MSL[8]. The vernacular names of this plant are 'Udarkodi' in Tamil and "Krsnasariba" in Sanskrit[9]. The leaves of this plant contain apigenin, luteolin, vitexin, isovitexin, vanilic, syringic, sinapic acids[10], triterpene, and glycosides[11]. The stem contains friedelin, friedelinol, lupeol acetate, and oleonolic acid[12]. The roots are used as a diuretic and diaphoretic[13]. The whole plant is used to treat burning sensation, fever, nephrolithiasis, leprosy, and general weakness[14].

The Siddis of Uttara Kannada district of Karnataka, India[15] and the tribals of Madhya Pradesh, India[16] use the flowers and roots to treat diabetes. The aqueous extract of the root[17] and methanol extract of the leaves[18] showed antidiabetic effect. Polyphenol rich fraction of *I. frutescens* prevented the progression of diabetic renal failure[19] and showed antitumor properties[20]. The apoptotic and antiadipogenic activities of *I. frutescens* were evaluated in this study.

2. Materials and methods

2.1. Chemical reagents

MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], oil red O (ORO), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), antibiotic mixture (penicillin-streptomycin), dexamethasone (DEX) and insulin (INS), were purchased from Himedia Chemicals (Chennai, India). Free glycerol reagent, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Aldrich (Bangalore, India). All other chemicals were of reagent grade.

2.2. Plant material and preparation of crude extracts

Leaves of *I. frutescens* were collected in Chennai, Tamil Nadu. The plant material was authenticated by the taxonomist at the Department of Botany, Loyola College, Chennai, Tamil Nadu, India. A voucher specimen (ERIS-2) was deposited in the herbarium of the Entomology Research Institute.

Powdered leaf material (3 kg) was soaked sequentially in hexane (IFHE), ethyl acetate (IFEAE), and (IFME) methanol for 72 h, respectively with intermittent shaking. After 72 h the solution was filtered and the filtrate was concentrated under reduced pressure using rotary evaporator. The extracts were stored in airtight containers at -4 ℃ and used. Preliminary phytochemical analysis was performed qualitatively using hexane extract.

2.3. Cell culture

3T3-L1 preadipocyte purchased from National Centre for Cell Sciences (Pune, India) were cultured in 24 well plate at a density of 5×10^4 cells/well at 37 ℃ in a humidified atmosphere of 5% CO_2 in DMEM containing 10% FBS, 2 mM glutamine, 20 mM Hepes, 50 U/mL penicillin, and 50 mg/mL streptomycin sulfate. After 80% confluency, cells were cultured with differentiation medium (DMEM with 10% FBS, 0.5 mM IBMX, 2 mM DEX and 1.7 mM INS). After 48 h of stimulation, cells were cultured in DMEM supplemented with 10% FBS with/without IFHE and changed every two days up to 8 d.

2.4. MTT assay

Cell viability was assessed by the MTT assay[21]. 3T3-L1 preadipocytes were plated into 96-well microtiter plate at a density of 1×10^4 cells/well and allowed to adhere overnight. After 24 h, the culture medium was replaced by 200 μL serial dilutions (0-500 μg) of IFHE, IFEAE, and IFME. The cells were incubated for 24, 48, and 72 h; untreated cells acted as control. The final concentration of the solvent was less than 0.1% in the cell culture medium. Culture solutions were then removed and replaced by 90 μL of culture medium. Ten microliters of a sterile, filtered MTT solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) was added to each well to reach a final concentration of 0.5 mg MTT/mL. After 5 h, the unreacted dye was removed, and then the insoluble formazan crystals were dissolved in DMSO (200 μL/well) and measured spectrophotometrically at 570 nm. The cell population growth percentage (%) was expressed as the percentage of cell growth compared to the control, and it was calculated by A_{570} nm [AM]/A_{570} nm [control] × 100.

2.5. Detection of DNA fragmentation by gel electrophoresis

Preadipocytes were cultured in 6 well plates at a density of 3×10^6 cells/well. After reaching confluency, cells were treated with IFHE (0-300 μg) for 48 h. Cells were extracted with 500 μL of lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl, pH 8.0) at room temperature for 15 min and centrifuged at 16 000 *g* for 10 min. DNA was extracted twice with phenol:chloroform (1:1), precipitated with ethanol, and resuspended in Tris/EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA was analyzed after separation by gel electrophoresis (2% Agarose)[22].

2.6. Oil red O staining

3T3-L1 adipocytes were cultured as described previously. Differentiated adipocytes were incubated with 0-300 μg IFHE for 48 h up to 6 d and maintained at 37 ℃ in a humidified 5% CO_2 incubator. Cells were washed twice with PBS and then fixed with 10% neutral formalin for at least 20 min at room temperature. After the 10% neutral formalin was removed, wells were washed with 60% isopropyl alcohol and then washed exhaustively with water. Cells were stained with oil red O working solution for 20 min and the staining dye of cells was extracted with 100% isopropyl alcohol and measured spectrophotometrically at 510 nm[23]. The oil red O-stained material (OROSM, %) was compared to control wells containing cell culture medium without IFHE was calculated by A_{510} nm [AM]/A_{510} nm [control] × 100.

2.7. Measurement of glycerol release

The glycerol level secreted by adipocytes was determined using the enzymatic reagent, free glycerol reagent (Sigma, India), directed by the protocol of GPO-TRINDER (Sigma, India).

2.8. Lactate dehydrogenase activity (LDH)

3T3-L1 cells were seeded in 24 well plate at a density of 5×10^4 cells/well and allowed to adhere overnight. IFHE in culture media was added at the 72 h and untreated cells acted as control. Cell-free supernatant was obtained by centrifugation at 400 *g* for 10 min. Two millilitres of Tris-EDTA-NADH buffer (pH 7.4, 50 mmol/L Tris buffer, 5 mmol/L EDTA, 150 μmol/L NADH) and 50 μL cell-free supernatant were mixed in a 24 well plate and incubated at 37 ℃ for 10 min. Pre-warmed (37 ℃) pyruvate solution (200 μL) was added to each well and the reaction velocity was measured in the first 3 min by a spectrophotometric micro-plate reader at 340 nm at 37 ℃[24].

2.9. Statistical analysis

Statistical evaluation of the data was done by one-way analysis of variance followed by the Student's *t*-test. The results were expressed as mean ± SEM using Graph Pad Prism (version 5.0), and *P* values ≤ 0.05 were considered significant.

3. Results

3.1. Preliminary phytochemical analysis

The preliminary phytochemical analysis of the hexane extract of *I. frutescens* showed the presence of phytoconstituents, such as steroids, terpenoids, alkaloids and wax.

3.2. Cell viability and LDH release

To assess the effect on proliferation as well as apoptosis over time, fibroblasts were cultured and treated with different concentrations (0-500 μg) of IFHE (Figure 1A), IFEAE (Figure 2), and IFME (Figure 3) for 24, 48, and 72 h. After treatment, the number of live cells was determined by MTS assay. IFHE reduced viability of preadipocytes time dependently between 100 and 300 μg doses compared with the vehicle. These concentrations of IFHE were selected for subsequent assays. IFEAE and IFME extracts did not exhibit significant reduction in cell numbers. IFHE treatment at 200 μg concentration significantly increased the release of lactate dehydrogenase (LDH), a marker of membrane integrity, after 48 and 72 h compared to 24 h (Figure 4).

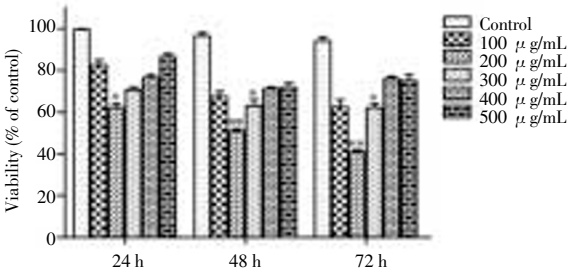

Figure 1. Effect of *I. frutescens* hexane extract on the viability of 3T3-L1 preadipocytes.
3T3-L1 preadipocytes were treated with 0-500 μg IFHE for 24, 48, and 72 h. Values are mean ± SEM (n = 5); *$P<0.05$; **$P<0.005$ compared with the control.

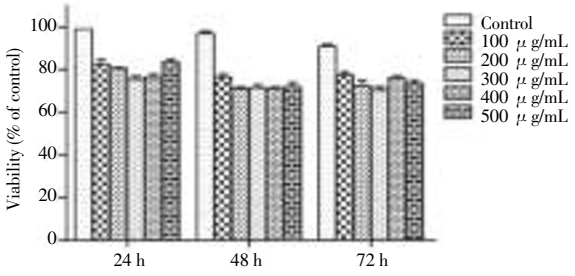

Figure 2. Effect of *I. frutescens* ethyl acetate extract on the viability of 3T3-L1 preadipocytes.
3T3-L1 preadipocytes were treated with 0-500 μg IFEAE for 24, 48, and 72 h. Values are mean ± SEM (n = 5); compared with the control.

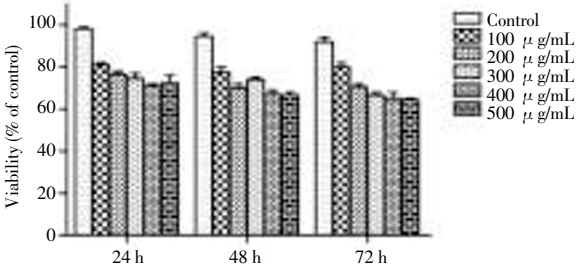

Figure 3. Effect of *I. frutescens* methanol extract on the viability of 3T3-L1 preadipocytes.
3T3-L1 preadipocytes were treated with 0-500 μg IFME for 24, 48, and 72 h. Values are mean ± SEM (n = 5); compared with the control.

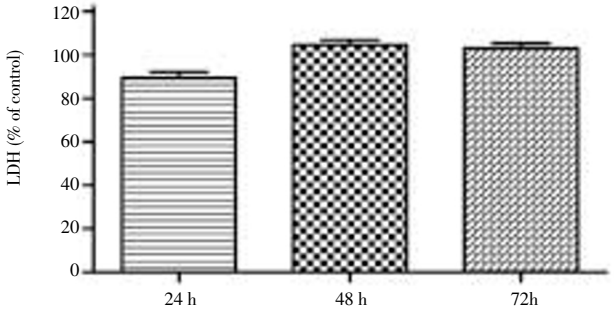

Figure 4. Effect of IFHE on LDH release from 3T3-L1 adipocytes.
LDH release from 3T3-L1 cells treated with IFHE at a concentration of 200 μg/mL for 24, 48, and 72 h. Values are expressed as mean ± SEM (n=5) and as a percentage of untreated control cells.

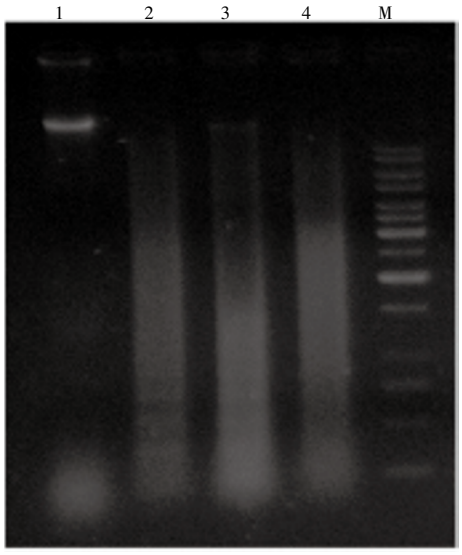

Figure 5. Agarose gel electrophoresis of DNA fragmentation in IFHE treated preadipocytes.
Demonstration of preadipocytes apoptosis by gel electrophoresis. 3T3-L1 preadipocytes were incubated with IFHE at various concentrations for 48 h. Total cellular DNA was extracted and analyzed by gel electrophoresis as described in Materials and methods. Lane 1: control; lane 2: 100 μg IFHE; lane 3: 200 μg IFHE; lane 4: 300 μg IFHE; lane M-DNA marker.

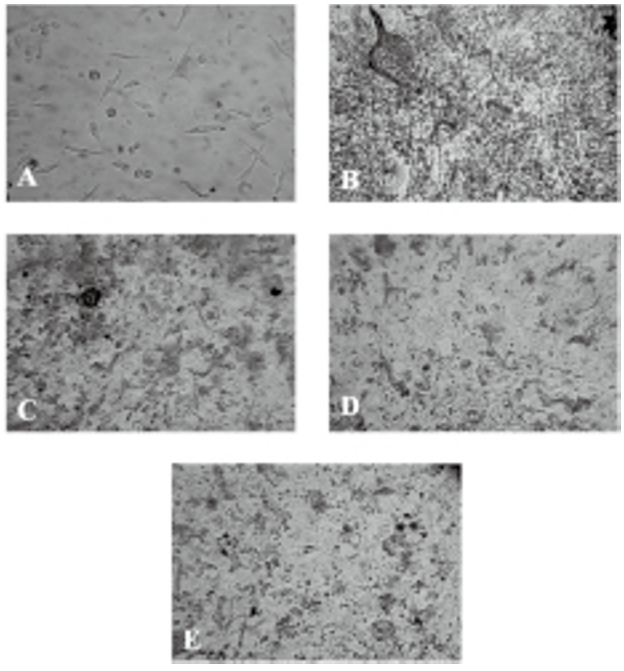

Figure 6. Effect of IFHE on ORO staining of 3T3-L1 adipocytes.
Photomicrograph of ORO-stained adipocytes (10 × magnification). IFHE dissolved in DMSO was added with standard adipogenic medium and treated at the concentrations 0-300 μg/mL during days 0 to 2, 2 to 4, and 4 to 6. On day 6, cells were stained with ORO. The experiments were performed in triplicate and repeated at least twice. (A) Normal control cells (DMSO as vehicle); (B) Induced control cells (adipogenic medium); (C) IFHE 100 μg + adipogenic medium treated cells; (D) IFHE 200 μg + adipogenic medium treated cells; (E) IFHE 300 μg + adipogenic medium treated cells.

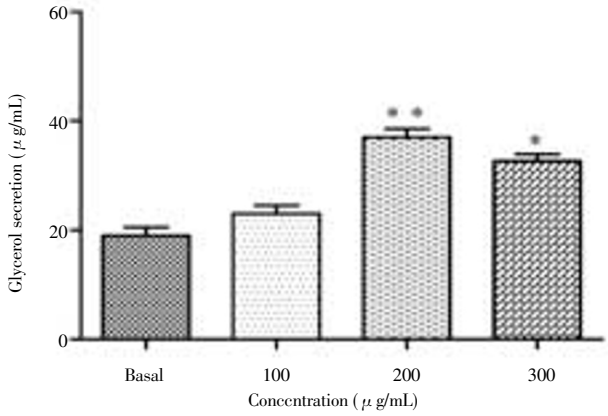

Figure 7. Effect of IFHE on glycerol secretion in 3T3-L1 adipocytes.
3T3-L1 adipocytes were differentiated for 6 d and treated with IFHE (100-300 μg/mL) for 24 h. Media were collected for glycerol measurement. Values are mean ± SEM (n = 3); *P<0.05; **P<0.005 compared with the fully differentiated control adipocytes (Basal).

3.3. IFHE induced apoptosis

To assess whether the reduction in cell number was caused by apoptosis, we studied the exposure of adipocytes to IFHE for 48 h, which resulted in the induction of apoptosis significantly between 100 and 300 μg doses. DNA extracts from adipocytes treated with IFHE displayed a characteristic apoptotic ladder pattern of discontinuous DNA fragments on agarose gel electrophoresis (Figure 5).

3.4. Effect of IFHE on the inhibition of adipocyte differentiation

During the differentiation of 3T3-L1 preadipocytes to adipocytes, the cells were treated with IFHE during day 0-6. The degree of adipocyte differentiation was assessed by morphological changes based on ORO staining of cellular triglyceride contents. Mature adipocytes were determined by the presence of cells in round nature and increased cytoplasmic lipid droplets. The differences in lipid accumulation in treated adipocytes were photomicrographed at 10× magnification (Figure 6). The cell size and intracellular fat drops appeared to be plenty in induced control group, and comparatively adipogenesis and cell viability were decreased between 100 and 300 μg treated groups; the inhibitory effect of 200 μg was more potent. These results indicated that IFHE, at these doses, decreased adipogenesis and reduced cell number.

Reduction in amount of lipid accumulation represented the decrease in triglycerides, which was due to lipolytic activity of IFHE. In order to examine whether the reduction in triglyceride content was associated with lipolysis, the

lipolytic effect of IFHE in 3T3-L1 adipocytes was determined by measuring the glycerol level secreted in the medium. Glycerol secretion increased with the rates of approximately 28%, 55%, and 46% at the concentrations of 100, 200 and 300 μg/mL, respectively (Figure 7).

4. Discussion

Adipocytes are the primary site of energy storage and accumulate triacylglycerol during nutritional excess. It has been reported that adipocyte dysfunction plays an important role in the development of obesity and insulin resistance. Decrease in adipose tissue mass involve the loss of lipids through lipolysis and may also involve the loss of adipocytes via apoptosis, a highly regulated form of cell death[25]. Apoptosis of adipocytes may emerge as a promising strategy for prevention and treatment of obesity because removal of adipocytes via this mechanism will result in reducing body fat and can result in long-lasting maintenance of weight loss[26].

The results of the MTT assay in the present study clearly indicated that treatment of preadipocytes with IFHE during the cell proliferative stage inhibited cell viability in a time- and dose-related manner. IFHE significantly reduced cell number and showed apoptosis at 200 μg at maximum between 48 and 72 h after the culture initiation. The studies on the effect of capsaicin[27], epigallocatechin-gallate[28], and esculetin[29] on 3T3-L1 apdipocytes apoptosis were reported. A quantitative analysis of LDH activity was used to determine the percentage of dead cells. An increased release of LDH, a marker of cellular membrane integrity, was observed from treated cells. Similar observations were seen in the effect of triterpene saponins derived from gingseng in cancer cell models[30] and the effect of triterpenoid extract of bitter melon in 3T3-L1 preadipocytes[31-32].

DNA fragmentation is one of the confirmations of apoptosis, where nuclear condensation and chromatin fragmentation ensues cell death. In this study, a characteristic apoptotic ladder pattern of discontinuous DNA fragments was seen on agarose gel electrophoresis of IFHE treated preadipocytes. Adipocytes play an important role in lipid homeostasis and energy balance by relating to triglyceride storage and release of free fatty acids. Adipocyte differentiation and the amount of fat accumulation are associated with the occurrence and development of obesity[33]. However, recent reports indicate that one of the critical events for the accumulation of body fat is the differentiation of preadipocytes into adipocyte in the adipose tissue[34].

Adipogenesis, the development of mature fat cells from preadipocytes, is an intensely studied model of cellular differentiation. In the present study, we observed the impedance of adipocytes differentiation by IFHE at the later stage of adipogenesis (Days 4 to 6). The cells have gained the characteristics of mature adipocytes by days 4 to 6, leading to their increased susceptibility to apoptosis. Morphological observations of cells stained with ORO, a lipid stain, showed a decrease in cellular lipid content at the concentrations tested when compared to untreated control cells. Therefore, one can postulate that inhibition of adipogenesis and viability with IFHE during the late period is due to apoptosis. Xanthohumol and its isomer isoxanthohumol of *Humulus lupulus* were reported to induce apoptosis and inhibit adipogenesis in mature 3T3-L1 adipocytes[35]. In a study, genistein showed antiadipogenic effect and strongly promoted lipolysis but naringenin promoted epinephrine-induced lipolysis to a greater extent[36]. We have shown that IFHE enhanced secretion of glycerol in 3T3-L1 adipocytes in a dose-dependent manner as caused by lipolysis. This indicated that IFHE inhibited adipogenesis of adipocytes undergoing differentiation through reduction in lipid accumulation.

In conclusion, the results of this study clearly showed that IFHE inhibited the population growth and induced apoptosis of 3T3-L1 preadipocytes. These results demonstrated that IFHE efficiently suppresses adipogenesis in 3T3-L1 adipocytes. The effects on adipocyte viability, apoptosis, and adipogenesis provide a basis for proposing that IFHE may have applications in the treatment of obesity.

Conflict of interest statement

The authors declare no conflict of interest.

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