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Anti-obesity effects of *Garcinia indica* high pressure ethanolic extract *in vitro*

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Abstract
Obesity is one of the most common metabolic diseases that often lead to cardiovascular diseases, type II diabetes, cancer, and osteoarthritis. Inhibition of adipogenesis is a therapeutic target for reducing obesity and obesity-related disorders. We have investigated the anti-adipogenic effect of a supercritical ethanolic extract of dried fruits of *Garcinia indica in-vitro*. Chemical analysis of the high pressure ethanolic (HPE) extract showed that it has 36.7% hydroxy citric acid (HCA), 2.5% garcinol, 1.5% xanthochymol, 0.38% isogarcinol, and trace amounts of α-mangostin and cyaanidin-3-0-glucoside. *G. indica* HPE extract has an IC_{50} values of 112 ug/ml in 3T3-L1 pre-adipocytes and 140 ug/ml in differentiated adipocytes in the cytotoxicity assays. The HPE extract inhibited the adipocyte differentiation significantly in a dose-dependent manner with increasing concentrations of extract in 3T3-L1 preadipocytes. Treatment of HepG2 liver cells with *G. indica* HPE extract down regulated mRNA expression of mTOR, GLUT3 and HIF-1α genes and up regulated AMPKα genes very significantly. Similarly, down regulation of mTOR, HIF-1α and AKT1 mRNA expression was noticed in extract-treated 3T3-L1 pre-adipocytes. On the other hand, SIRT1 and AMPKα mRNA mRNA expression were up regulated in 3T3-L1 pre-adipocytes. *G. indica* HPE extract also down regulated genes such as FXR and SHP, and up regulated PPAR-γ gene that are associated with fat metabolism in HepG2 cells. These results show that the anti-obesity effect of *G. indica* HPE extract is mainly through signaling pathways affecting the expression of genes associated with weight control and fat metabolism.

Keywords: *Garcinia indica*, obesity, adipocytes, cytotoxicity, differentiation, gene expression

1. Introduction
Obesity, one of the most common metabolic diseases in humans has a complex etiology; however, a basic underlying feature involves excess energy intake relative to energy expenditure, leading to excessive fat accumulation [1]. Excessive accumulation of body lipid may have adverse effects on overall health leading to diseases including hypertension, type II diabetes, cardiovascular disease, cancer, and osteoarthritis [2]. Consequently, the modulation of adipogenesis is now recognized as adesirable target for ameliorating obesity [3]. Adipogenesis is the process of differentiation of preadipocytes into lipid accumulating mature adipocytes [4]. For decades, studies have been conducted on many natural products to regulate the process of adipogenesis [3]. Among these are *Garcinia* extracts which have demonstrated promising anti-adipogenic properties. *Garcinia cambogia* is considered as one of the most popular supplements for inhibiting adipogenesis [5]. The hydroxy citric acid (HCA) in *G. cambogia* inhibits adenosine triphosphate citrate lyase which is required for fatty acid and cholesterol biosynthesis [6]. However, concerns have been raisedon the prolonged use of *G. cambogia* extract (GE) due to a possible hepatotoxicity [7]. Like *G. cambogia*, other species of *Garcinia* such as *G. indica* also contain significant HCA content in the fruits and are sometimes used interchangeably with *G. cambogia*. The genus *Garcinia* (Clusiaceae family) includes about 200 species throughout the world. Of these, 36 species have been reported from India, and among these *G. indica* and *G. cambogia* species are endemic to Western Ghats in South India [8-9]. *G. indica*, named Kokum in Hindi and referred to as Vrikshamala in Ayurveda, is a rich antioxidant with high HCA content in the fruits. *G. indica* fruits have been used for centuries in Asian countries for culinary purposes as a condiment and flavoring agent in place of tamarind or lemon and to make meals sourer and filling [10-12]. Besides its use as a culinary agent, the dried rind of *G. indica* combined with salt and other organic acids can help to lower the pH and thus provides a bacteriostatic effect in curing fish. A myriad of health effects has been attributed to *Garcinia* (including *G. cambogia*, *G. atroviridis*, and *G. indica*) includinganti-obesity [12-13], antiulcerogenic [14-15], antioxidative [16-17], antihyperglycemic [18], antimicrobial [19-20], anti-inflammatory [21-22] and anticancer effects [23-24]. In particular, the anti-obesity effects of *Garcinia* or more specifically of its HCA content have been elucidated with unprecedented clarity over the last few decades.

Besides its efficacy in the reduction of body weight and food intake, Garcinia/HCA has been proven to be beneficial in ameliorating obesity-related complications such as inflammation, oxidative stress, and insulin resistance [16]. However, the efficacy of Garcinia/HCA remains a subject of debate. Despite the evidence about HCA's efficacy in promoting weight loss and suppressing food intake, the marketing of a plethora of over-the-counter slimming aids containing HCA has taken place. In the present study we investigated the anti-obesity effects of a high pressure ethanolic extract of dried fruits (rind) of *G. indicain vitro*.

2. Materials and Methods

2.1. Cells and culture: Human liver cell line (HepG2) and mouse pre-adipocyte cell line (3T3-L1) were purchased from American Type Culture Collection, Rockville, MD) and grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 µg/ml of penicillin in a 5% CO_2 humidified incubator maintained at 37 °C.

2.2. Preparation of *G. indica* high pressure ethanol (HPE) extract: The dried fruit pericarp of *Garcinia indica* Choisy (Kokum) was purchased from authorized retail distributor (Double Horse, Kerala, India). In addition to botanical confirmation, species identity was also confirmed by extract composition. The material was dried for 24 h at 40°C, resulting in 7.4% loss of weight. Afterwards, it was passed through a cutting mill with 6 mm sieve and mixed in a ratio of 1 + 1 with kieselguhr. The powder was filled in a pressure vessel and percolated with 10 kg of ethanol (95%)/kg at 110 bar and 70 °C. The HPE extract solution was evaporated to dryness by a vacuum rotary evaporator to leave a dark viscous extract representing 53% of the starting material.

2.3. Extract analysis: HPE extract was analyzed by Reversed Phase (RP) HPLC. Reference substances such as α-mangostin, cyanidin-3-O-glucoside (syn, chrysanthemin) and (-)-hydroxy citric acid (HCA) (syn. +/-garcinia acid) were purchased from Sigma-Aldrich, and garcinol (camboginol) was purchased from Cayman Chemical. HPLC grade solvents were purchased from Th. Geyer GmbH & Co. KG (Rehlingen, Germany). The HPLC instrument was a VWR Hitachi Elite LaChrom system consisting of autosampler L-2200, pump L-2130, PDA detector L-2455 and column oven L-2350; data acquisition software EZChrom Elite, version 3.3.2 SP2, build 3.3.2.1037. Separation of all analytes was achieved on a RP-18 HPLC column, Lichrospher 100 RP-18e, 250-4 mm with pre-column 4-4 mm supplied by Merck KG aA (Darmstadt, Germany). For α-mangostin, Eluent A was deionized wateracidified with 1% acetic acid and eluent B was acetonitrile;eluent composition: 0-3 min 25% A + 75% B, 3-25 min gradient to 100% B;detection-245 nm; column oven40°C; injection volume10 µl. For cyanidin-3-O-glucoside eluents were same as for α-mangostin;eluent composition: 0-20 min gradient from 95% A + 5% B to 80% A + 20% B detection: 520 nm, column oven 40°C, injection volume 30 µl. For (-) hydroxy citric acid eluent A was ionized water acidified with 0.1 % acetic acid, eluent B was acetonitrile; eluent composition: 0-20 min 95% A + 5% B (isocratic) detection 210 nm, column oven40°C, injection volume 10 µl.For garcinol, xanthochymol, isogarcinol eluent A wasdeionizedwater acidified with 0.1 % orthophosphoric acid, eluent B was acetonitrile; eluent composition: 0-20 min gradient from 15 % A + 85 % B to 100 % B;detection: 215 nm, column oven 40°C; injection volume 10 µl.

2.4. Cytotoxicity: Both HepG2 and 3T3-L1 cells were treated with increasing concentrations of *G. indica* high pressure ethanolic extract for 72 h and cytotoxicity analyzed using cell proliferation kit I (Roche Biochemicals, IN) [25,26].

2.5. Analysis of adipolysis in 3T3-L1 cells: 3T3-L1 pre-adipocytes were plated in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml penicillin and100 units/ml streptomycin in 24 well BIOCOAT plates (BD Biosciences, San Jose, CA) at a cell density of 10^5 cells/well in 2 ml of medium. On the next day, when cells were attached well, medium was replaced with differentiation medium [26] and treated with increasing concentrations of *G. indica* extract for 72 h in a CO_2 incubator maintained at 37°C. Subsequently, the differentiation medium was replaced with DMEM containing 1 µM insulin (insulin medium) with increasing concentrations of *G. indica* extract for 48 h and on the fifth day, insulin medium was aspirated and replaced with fresh insulin medium [27]. On the seventh day, medium was removed from the plate and 500 ul of 10% formalin was added into each well. The plate was incubated for 5 min and later replaced with 10% fresh formalin solution (500µl) for 1 h. The wells were washed with 500 ul 60% isopropanol and the wells allowed to dry completely. The cells were stained with 200 µl of 0.6% Oil Red O solution for 10 min and washed four times with 2 ml of water each time. The plates were allowed to dry completely, and Oil Red O was eluted from each well in 750 µl 100% isopropanol for 10 min. The eluted solution was transferred to 1.5 ml spectrophotometric cuvettes and the absorbance was measured at 500 nm wavelength in a Beckman spectrophotometer. The decrease in absorbance compared to untreated sample indicates the inhibition of adipocyte differentiation.

2.6. Gene expression studies: mRNA expression of genes associated with obesity (AMPKα, mTOR, SIRT1, AKT1, HIF-1α, GLUT1and GLUT3) and fat metabolism such as liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator-activated receptor-gamma (PPAR-γ), small heterodimer partner (SHP), and bile acid binding protein (BABP) were analyzed by reverse transcriptase-polymerase chain reaction assay [28]. The relative expression of each gene was quantified from the gel pictures using the UN-SCAN-IT gel software (Silk Scientific Corporation, Orem, UT). The house keeping gene (β-actin) was included as control for gene expression investigations.

2.7. Statistical analysis: Mean and standard deviation values were calculated, and graphs were prepared by Microsoft Excel. Gene expression data was analyzed statistically by Students *t*-test against expression of house-keeping gene, significance level was determined and incorporated into the graphs.

3. Results

3.1. Chemical analysis of G. indica HPE extract: The HPE extract used for efficacy testing contained 36.7% of HCA,2.5 % of garcinol, 1.5 % of xanthochymol and 0.38 % of isogarcinol as well as trace components of 36 ppm α-mangostin and 3 ppm of cyanidin-3-O-glucoside. Even if this relatively unstable constituent is quite low, its presence is used to differentiate between *G. indica* and *G. cambogia* (syn *G. hanbury*) [29]. Both contain high amount of HCA and garcinol in the fruit rind but only *G. indica* contains red anthocyanin pigments, cyanidin-3-O-glucoside and cyanidin-3-O-sambubioside in a ratio of 4:1. The total content of

anthocyanin pigments by spectroscopy at 535 nm wavelength was 0.15%.

3.2. Cytotoxicity of *G. indica* HPE extract on 3T3-L1 pre-adipocytes and adipocytes

G. indica HPE extract has a IC_{50} value of 112 µg/ml forpre-adipocytes is whereas that for adipocytes was 141µg/ml (Fig. 1&2). This indicates that the pre-adipocytes are more sensitive to HPE extract than adipocytes. The highest concentration of *G.indica* extract (200 µg/ml) induced death in 75% of pre-adipocytes whereas only 60% adipocytes were killed by 200 µg/ml *G.indica* extract.

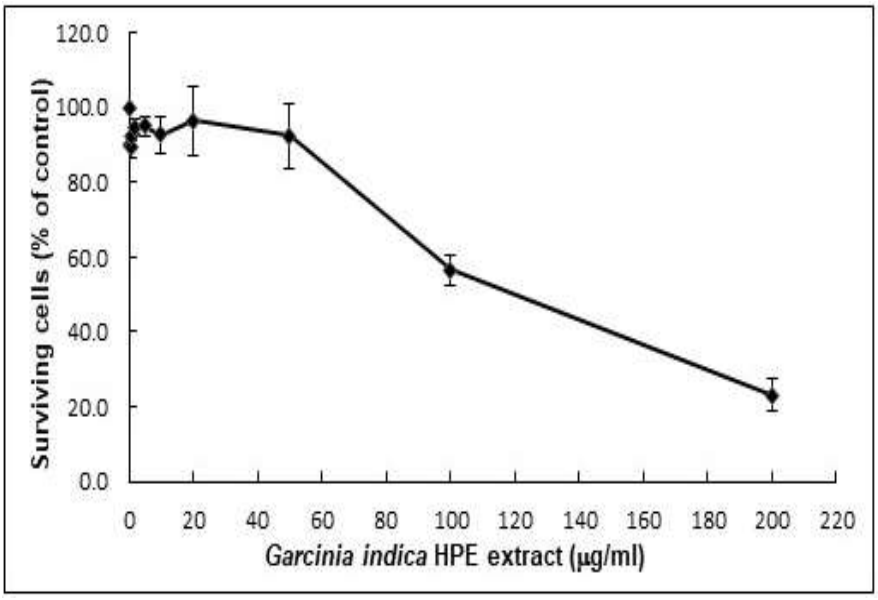

Fig 1: Cytotoxicity of *Garcinia indica* high pressure ethanolic (HPE) extract in 3T3-L1 mouse pre-adipocytes

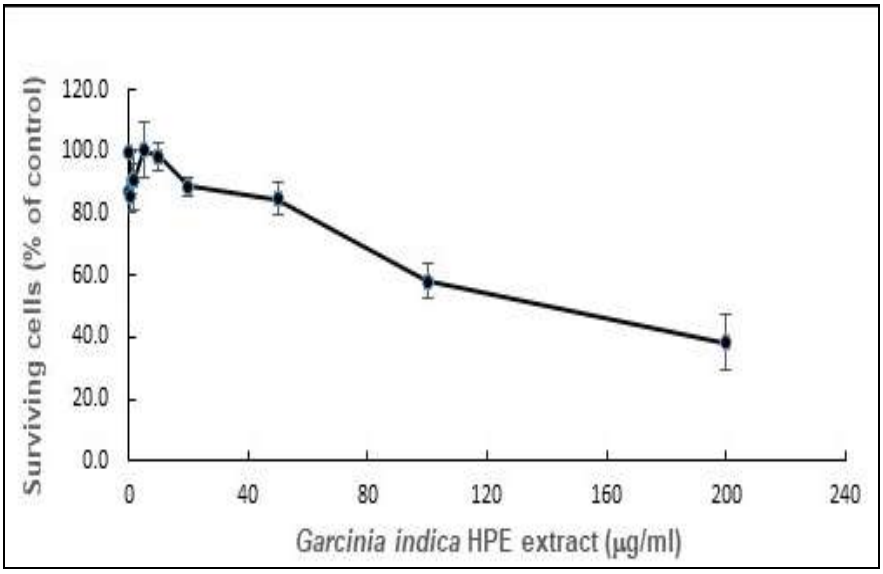

Fig 2: Cytotoxicity of *Garcinia indica* high pressure ethanolic (HPE) extract in 3T3-L1 differentiated mouse adipocytes

3.3. Effect of *G. indica* HPE extract on adipocyte differentiation

HPE extract can inhibit the adipocyte differentiation significantly and a dose-dependent inhibition of adipocyte differentiation was quite visible with the extract. Differentiation was inhibited to an extent of 20-25% in 50-100 µg extract treated cells compared to untreated control cells (Fig. 3 and 4).

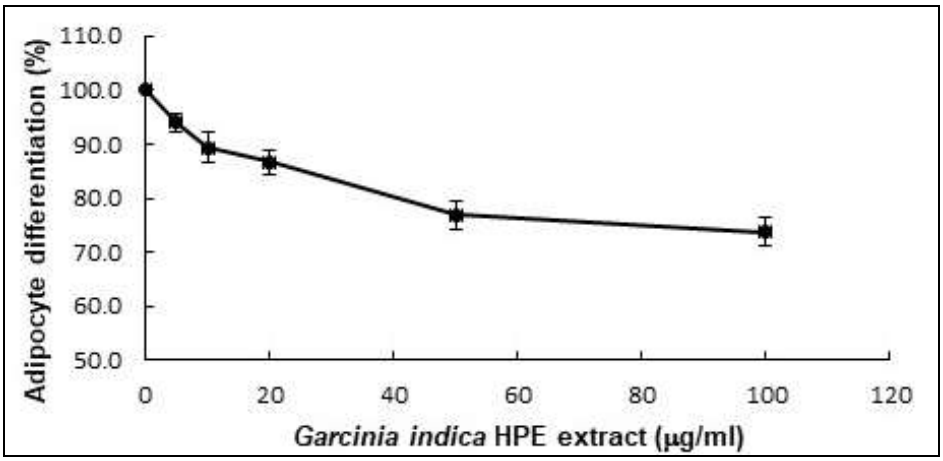

Fig 3: Inhibition of adipocyte differentiation by *Garcinia indica* high pressure ethanolic (HPE) extract in 3T3-L1 pre-adipocytes

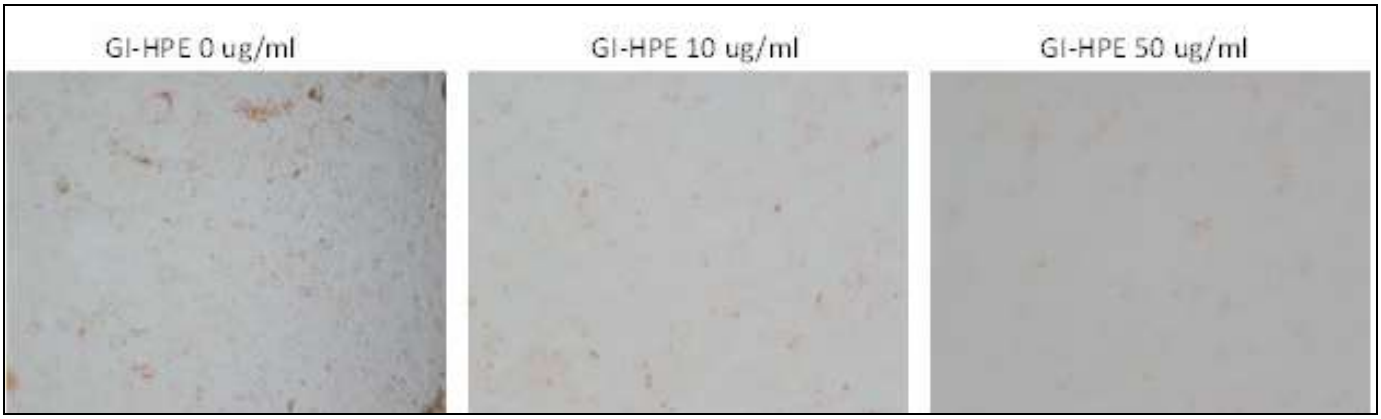

Fig 4: Inhibition of oil-o-red staining in 3T3-L1 pre-adipocytes indicating the inhibition of adipocyte differentiation by *Garcinia indica* (GI) H PE extract.

3.4. Effect of *G.indica* HPE extracton mRNA expression of genes associated with weight control

Treatment of HepG2 liver cells with *G. indica* extract down regulated mRNA expression of HIF-1α, mTOR, and GLUT3 significantly and up regulated AMPKα mRNA expression very significantly in HepG2 human liver cells (Fig. 5a &b). Similarly, in 3T3-L1 pre-adipocytes *G. indica* extract down regulated mTOR, HIF-1αand AKT1mRNA expression. On the other hand, it up regulated SIRT1 and AMPKαmRNA expression very significantly (Fig. 6a&b).

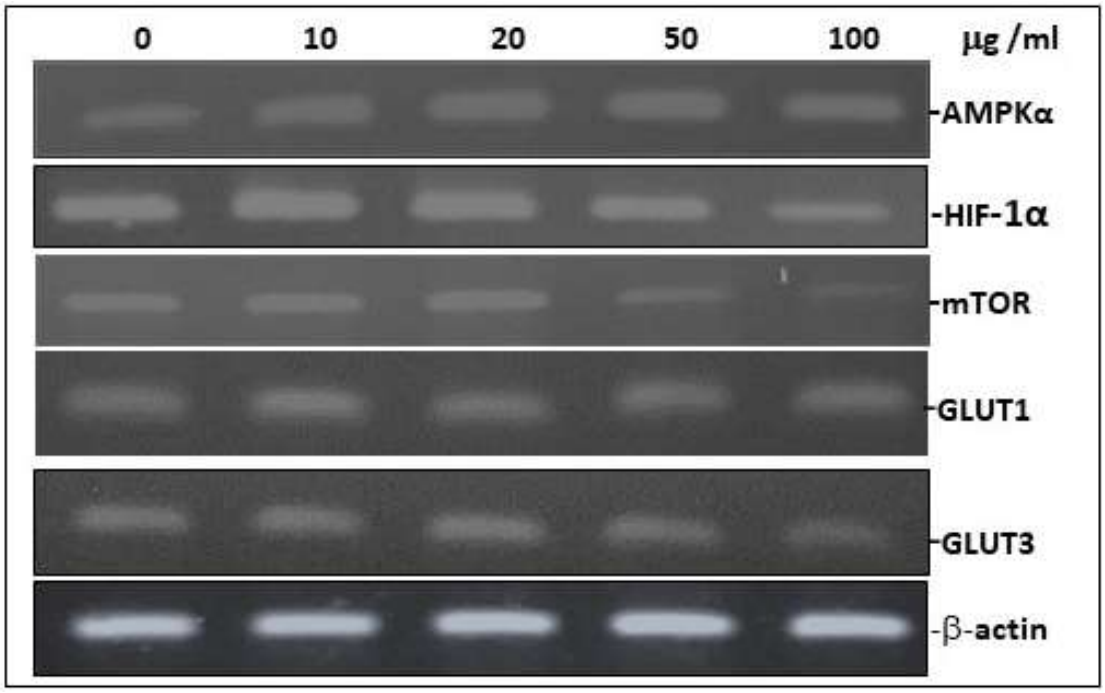

Fig 5a: Effect of with *Garcinia indica* HPE extract on the mRNA expression of genes associated with obesity in HepG2 human liver cells

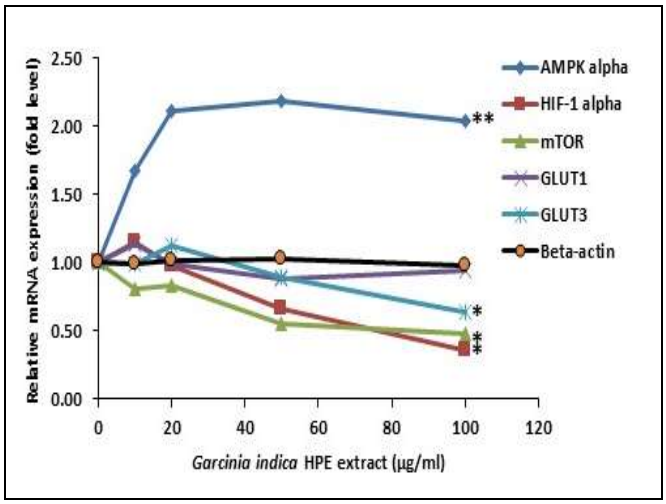

Fig 5b: Quantification of mRNA expression of genes associated with obesity in HepG2 cells treated with *Garcinia indica* HPE extract. The relative expression (fold level) was calculated based on the untreated control sample (*p <0.05, **p <0.01).

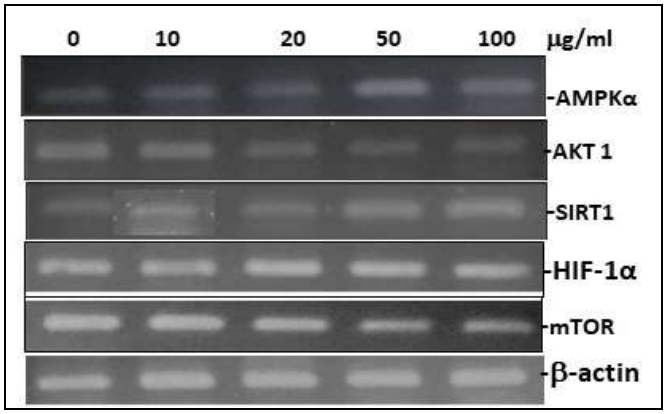

Fig 6a. Effect of *Garcinia indica* HPE extract on mRNA expression of genes associated with obesity in 3T3-L1 mouse pre-adipocytes

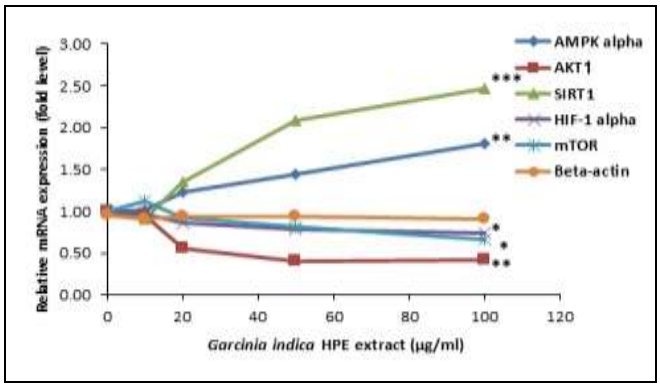

Fig 6b: Quantification of mRNA expression of genes associated with obesity in 3T3-L1 mouse pre-adipocytes treated with *Garcinia indica* HPE extract. The relative expression (fold level) was calculated based on the untreated control sample ("p<0.05, "pc0.01, mp<0.001).

3.5. Effect of *G. indica*-HPE extract on mRNA expression of genes associated with fat metabolism

In HepG2 liver cells, *G. indica* HPE extract down regulated genes associated with fat metabolism such as FXR, and SHP and it up regulated very significantly PPAR-γmRNA expression(Fig. 7a&b).

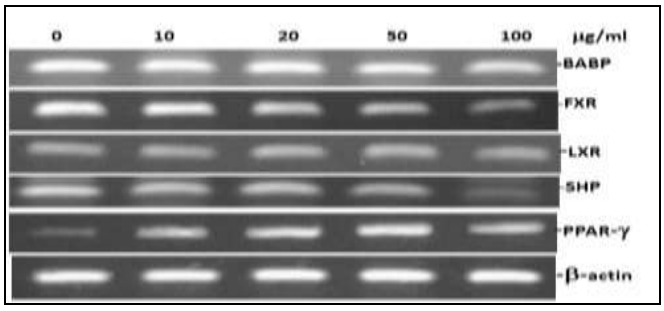

Fig 7a: Effect of *Garcinia indica* HPE extract on mRNA expression of genes associated with fat metabolism in HePG2 human liver cells

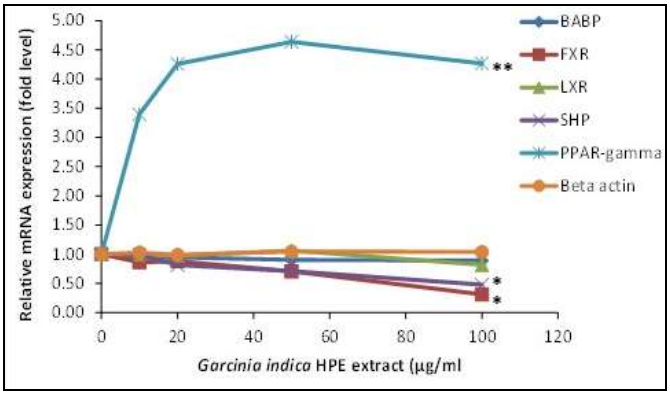

Fig 7b: Quantification of mRNA expression of genes associated with fat metabolism in HepG2 human liver cells treated with Garcinia indica HPE extract. The relative expression (fold level) was calculated based on the untreated control sample (*p<0.05, "p<0.01).

4. Discussion

Over the past few decades, an increasing trend of obesity as well as obesity-related comorbidity and mortality are observed throughout the world [30-31]. In the United States, the International Association of the Study of Obesity (IASO) reported that 35.5% of men and 35.8% women are obese (Body Mass Index -BMI ≥ 30)[30,32]. According to the World Health Organization (WHO) standard, overweight subjects are diagnosed with BMI values in the range of 25–29.99. Obesity itself, defined as BMI ≥ 30, is generally associated with several chronic and debilitating health problems including hyperlipidemia, hypertension, coronary heart disease, diabetes, cancer, disease of the gall bladder, osteoarthritis, shortage of breath, abnormal dilation of the veins, backache, and even psychological problems [31-33]. Even though some drugs are available in the market to ameliorate or prevent obesity, there are concerns related to costs, efficacy, and side effects associated with these drugs [34-36]. For example, the currently available pharmacological agents, Sibutramine, Rimonabant, Orlistat, and Phentermine which are licensed for weight reduction therapy, appear to possess some adverse effects [34-35]. Phentermine, for instance, has been reported to cause dry mouth, insomnia, headache, dizziness, fatigue, and palpitation [35]. In the year 2010, FDA had announced the market withdrawal of Meridia (Sibutramine) due to its risk of serious cardiovascular events [35-36]. Natural products and plant-based dietary supplements have been used by people for centuries and evidence is beginning to emerge supporting the consumption of these herbs as an effective strategy for disease treatment and health maintenance. Several ethnobotanical studies have reported on

the positive application of these herbs in the treatments for obesity also [37] and specifically the anti-obesity effects of *Garcinia* have been well described [12]. General extracts and specific compounds isolated from *G. indica* have been studied extensively for various bioactivities like antioxidant, antibacterial, antifungal, anti-obesity, antidiabetic, gastroprotective and anticancer activities [12]. Pharmacological studies of *G. indica* have shown that benzophenones, anthocyanins, and organic acids are the major bioactive constituents reported in *G. indica.* Among the different bioactivities reported, antioxidant properties are perhaps the most important activity for *G. indica* [38-39]. Chloroform extracts of *G. indica* fruit rinds exhibited excellent antioxidant activities in β-carotene-linoleate and DPPH assays [40-41]. Organic acids like citric acid and malic acid from *G. indica* also act as good antioxidants [41].

The chemical analysis of *G. indica* HPE extract contains 36.7% HCA in it in addition to small amounts of garcinol, isogarcinol, xanthochymol, anthocyanin and minute quantities of alpha-mangostin and cyanidin-3-O-glucoside in it. (-)-HCA has inhibitory effect on ATP-citrate lyase (ATP citrate oxaloacetatelyase, EC 4.1.3.8). This enzyme plays influential role in fatty acid synthesis from carbohydrates. It catalyzes cleavage of citrate to acetyl-CoA and oxaloacetate. The ultimate source of carbon for fatty acids is acetyl-CoA which is an important molecule in formation of fats from carbohydrates. By limiting the availability of acetyl-CoA, (-)-HCA plays important role in regulating fatty acid synthesis [41-42].

The fruit juice of *G. indica* is very acidic with a pH 1.5 to 2.0 and contains high concentration of acids. Major portion of organic acids in *Garcinia indica* is hydroxy citric acid (HCA) (1, 2 dihydroxypropane-1, 2, 3-tricarboxylic acid). Rinds contain about 20-30% of (-)-HCA on dry basis [41]. HCA is an anti-obesity agent, attributed with reduced food intake, increased energy expenditure, suppression of fatty acid synthesis and an enhancement of glycogen synthesis in liver [42-43]. Cytotoxicity data with *G. indica* HPE extract on 3T3-L1 pre-adipocytes and differentiated adipocytes indicated that the extract is more cytotoxic in pre-adipocytes than adipocytes. However, both pre-adipocytes and adipocytes were killed and removed by the extract. These results indicate that fat cells may be removed from the body with *G. indica* HPE extract use. Several studies conducted by Sullivan and colleagues had confirmed the inhibition of *in vivo* and *in vitro* rates of lipogenesis in several tissues involved in the conversion of carbohydrate into fatty acids (such as liver, adipose tissue, and small intestine), when HCA was predominantly given to rodent models [44-45]. Lowenstein [46] demonstrated that HCA greatly inhibited *in vivo* fatty acid synthesis in rat liver. In the present investigation we have seen that in addition to cytotoxic effect, *G. indica* HPE extract also significantly inhibited the differentiation of preadipocytes into adipocytes. Inhibition of lipogenesis by HCA and *G. indica* have also been reported earlier by other investigators [41-43]. In the present investigation, treatment of HepG2 liver cells with *G. Indica* HPE extract induced upregulation of AMPKα mRNA expression and down regulation of HIF-1α, mTOR and GLUT3 mRNAs. Similarly, treatment of 3T3-L1 cells with the extract showed an upregulation of AMPKα and SIRT1 mRNAs and down regulation of AKT1, HIF-1α and mTOR mRNAs. It has been shown that activation of AMPKα occurs during adipolysis and therapeutic AMPKα activation protects the cells against obesityand related metabolic dysfunction [47-48]. Also, Picard et al.[49] showed that in differentiated fat cells, upregulation of SIRT1 triggers lipolysis and loss of fat. Transcriptional down regulation genes such as AKT1, HIF-1α, mTOR and GLUT3 during adipolysis induced by various agents have been described in the literature [50-54]. Among the five genes that are directly connected with fat metabolism, PPAR-γtranscription was upregulated where as FXR and SHP was down regulated significantly. These genes are expected to be regulated in this manner based on the results reported by other investigators [55-57]. These results show that *G. indica* HPE extract can induce adipolysis and facilitate their removal through its pronounced effect on different signaling pathways involving AKT1, AMPKα, mTOR, SIRT1 and PPAR-γ. Further*in vivo* research on the *G. indica* HPE extract would be worthwhile for its development as an anti-obesity nutraceutical.

Conflict of interest: The authors declare the following conflict of interest with respect to the research, authorship, and/or publication of this article. Dr. Steven J. Melnick is the founder of Dharma Biomedical LLC, which is an evidence-based ethnobotanical and evochemical drug discovery and nutraceutical company operating on a for-profit basis. Dr. Karl-Werner Quirin is the Chief Executive Officer of Flavex Naturextrakte GmbH, Rehlingen, Germany, a company producing specialty botanical extracts for cosmetics and food supplements based on supercritical CO_2 extraction. Dr. Cheppail Ramachandran is an employee of Dharma Biomedical LLC.

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