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ORIGINAL ARTICLE

SUNCA Standardized Extract from *Helianthus annus* L Exerts Enhanced Digestive Enzyme Activity and Subsides Obesity through Inhibition of C/EBP-α and PPAR-γ Expression Both in *in vitro* and *in vivo* Model

K. Gouthamchandra[1], Sudeep HV[1], Amritharaj[1], Lingaraju H B[2], Shyam Prasad K[1,2]

Department of [1]Biomedicinal Research and [2]Phytochemistry, R&D Centre for Excellence, Vidya Herbs Pvt. Ltd, Bangalore, Karnataka, India

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ABSTRACT

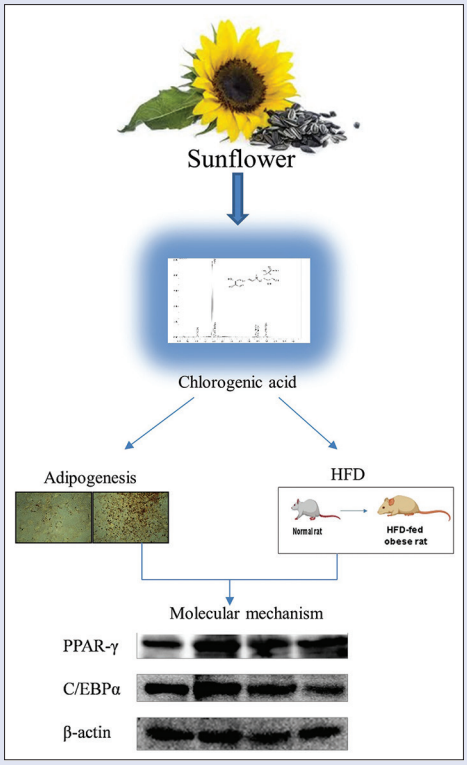
Background: *Helianthus annuus* L. (sunflower) is well known for its edible oil, sunflower seed has been used as folk remedy for several diseases including cancer. **Materials and Methods:** In this study, SUNCA, a standardized extract enriched with chlorogenic acid (CGA) from sunflower seed was investigated for *in vitro* antidiabetic activity using α-amylase and α-glucosidase enzyme inhibition assay. Further, for preventing obesity, we demonstrated anti-adipogenesis in 3T3-L1 cell culture system and on body weight gain and adiposity in rat fed high-fat diet (HFD). **Results:** SUNCA showed significant inhibitory activity toward digestive enzymes such as α-amylase and α-glucosidase. On the other hand, SUNCA significantly inhibits lipid accumulation during adipogenesis in a dose-dependent manner. The treatment with 100 μg/kg b.w of SUNCA apparently prevented the body weight gain and the increase of triglyceride and total cholesterol level in rat fed an HFD for 6 weeks. Further immunoblotting studies reveal that SUNCA significantly down-regulates the expression of transcription factors peroxisome proliferator-activated receptor gamma and CCAT/enhancer-binding protein alpha, both in adipocytes and adipose tissues. **Conclusion:** The findings reported here provide the therapeutic potential of SUNCA, a standardized extract from *Helanthus annus* L.

Key words: 3T3-L cells, adipogenesis, chlorogenic acid, high-fat diet, sunflower

SUMMARY

- SUNCA strongly inhibited both α-amylase and α-glucosidase activity
- SUNCA efficiently subsided adipocyte differentiation by suppressing the expression of adipogenic transcription factor peroxisome proliferator-activated receptor gamma and CCAT/enhancer-binding protein alpha
- SUNCA controls weight gain and improved serum lipid profile, with reduction in serum triglycerides, total cholesterol, low-density lipoprotein, and elevation in high-density lipoprotein when compared to the high-fat diet control group.

Abbreviations used: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PPAR-γ: Peroxisome proliferator-activated receptor gamma; CEBP-α: CCAT/enhancer-binding protein alpha; HFD: High-fat diet; TC: Triglycerides; LDL-C: Low-density lipoprotein-cholesterol; VLDL-C: Very low-density lipoprotein cholesterol.



Correspondence:

Dr. K. Gouthamchandra,
Department of Biomedicinal Research,
R&D Centre for Excellence, Vidya Herbs Pvt. Ltd, #14A, Jigani I Phase, Bengaluru - 560 105, Karnataka, India. E-mail: goutham06@gmail.com

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INTRODUCTION

Obesity is abnormal depositions of fat in the adipose tissue due to unhealthy food habits and reduced physical activity. Obesity has become an epidemic and associated with many other health problems, such as cardiovascular disease, hypertension, and type II diabetes. The occurrence of obesity is rising radically among all ages with the changes in lifestyles dietary fat intake. The recommended pharmaceuticals include orlistat, sibutramine, fluoxetine, and topiramate.[1-4] However,

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these medications should be used with caution, especially in patients with cardiovascular disorders, because it may cause adverse side effects.[5,6] Currently, obesity remedies based on dietary supplements are popular, suggesting that phytotherapy could modulate the expression of proteins involved in adipocyte differentiation have attracted much attention and serve as strategies in obesity treatment and prevention.[7,8] There are several studies, for example, polyphenol-rich extract obtained from bilberry *Vaccinium myrtillus* L. cranberry Vaccinium macrocarpon Aiton fruits, raspberry *Rubus idaeus* L. and chokeberry *Aronia melanocarpa* (Michx.) Elliot, its underlying molecular mechanisms were investigated in differentiated 3T3-L1 adipose cells, and results revealed that the analyzed extract may be a promising source of bioactive compounds. Quercetin and rutin ameliorate lipid accumulation in the fructose-fed rats.[9,10] *Helianthus annuus* L. is grown as a crop primarily for its seeds, which is one of the rich sources of edible oil in the world and it has been conventionally used for several diseases such as cough, diarrhea, fever, flu, dysentery, inflammations, and bronchitis, laryngitis, malaria, rheumatism, scorpion stings, snakebite and wounds.[11-13] It has been reported that sunflower seed contains massive number of nutritious components, including protein, unsaturated fats, selenium, copper, zinc, folate, iron, fiber, and vitamins (especially E). The sunflower seed harvested for oil production, ranking in the 4th position at world. The residues resulting from oil extraction are valuable and nutritious by-product with high proteins. Besides the high-protein contents, the residues originating from oil extraction are rich in phenolic compounds which account for 1%–4% of the total mass with chlorogenic acid (CGA) being the predominant component.[14,15] CGAs represent a large family of phenolic compounds present in plants. CGA is majorly found in green coffee beans and has several benefits, including obesity, type 2 diabetes, and anticancer activities.[16-18] In the present study, SUNCA standardized extract, enriched with CGA prepared from *H. annuus* L seeds were investigated for anti-diabetic and anti-obesity activity using different experimental models.

MATERIALS AND METHODS

Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, oil red O stain, isopropanol, and formalin were purchased from Sigma Aldrich.

Sample preparation

SUNCA standardized extract enriched with CGA [Supplementary Figure 1] prepared from *Helianthus annus* L. seed was procured from the Department of Phytochemistry, Vidya Herbs Pvt. Ltd., Bengaluru. Subsequently, it was dissolved in distilled water for appropriate concentration.

In vitro α-glucosidase activity inhibition assay

The inhibition of α-glucosidase activity of SUNCA was determined by adopting the standard method.[19]

In vitro α-amylase activity inhibition assay

The assay was carried out by adopting the standard protocol described.[20]

Cell culture

3T3-L1 preadipocyte cells were obtained from the National Centre for Cell Sciences (NCCS, Pune, India) and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin (100 µg/mL at 37°C in 5% CO_2 environment according to the standard recommended protocols.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

MTT assay was carried out by adopting the standard protocol. Briefly, the cells (5×10^3 per 96 well) were incubated at 37°C in 5% CO_2 and 95% air with different concentration of SUNCA. After 48 h, a 20 µL aliquot of MTT (a yellow tetrazole; 5 mg/mL in phosphate-buffered saline) was added to the wells and incubated further for 4 h at 37°C. The supernatant was removed carefully, 100 µl of dimethyl sulfoxide was added and mixed and the absorbance was read at 563 nm to determine the formazan concentration which is proportional to the number of live cells.

Adipocyte differentiation and lipid accumulation

3T3-L1 mouse adipocytes were cultured in DMEM-containing 10% FBS until confluent and were then maintained in the same medium for an additional 2 days. Differentiation was induced 2 days postconfluence (day 0 of differentiation) by adding 0.5 mM IBMX, 1 mM dexamethasone and 5 mg/mL insulin in DMEM with 10% FBS (MDI). After 2 days of incubation, the culture medium was changed to fresh DMEM-containing 10% FBS and 5 mg/mL insulin. Two days later, the medium was replaced with DMEM supplemented with 10% FBS and incubated for another 2 days. SUNCA was added 2 days after confluence (day 4) and maintained another 2 days (6 days) at which time >90% of the cells were mature adipocytes with accumulated fat droplets.

Oil red O staining

Intracellular lipid accumulation was measured using oil red O. The oil red O working solution was prepared as described by Ramírez-Zacarías *et al*.[21] The 3T3-L1 cells were fixed with 10% formalin and then stained for 1 h with a filtered solution of 60% oil red O in 100% aqueous 2-isopropanol. To quantify the intracellular lipids, the stained lipid droplets were dissolved in isopropanol (3 mL per well). The extracted dye was transferred into a 96-well plate, and the absorbance was read with a Multiscan Ex microplate reader (Thermofischer) at 500 nm.

Protective effect of SUNCA on high-fat-induced obesity

Animals and diets

Thirty-two male 4-week-old Sprague–Dawley rats were purchased from Biogen (Bengaluru, India). Following a 1-week acclimatization period, the animals were randomly divided into four groups $n = 6$ each, standard chow diet (normal control), high-fat diet (HFD) alone (HFD-control), HFD plus SUNCA 50 and HFD plus SUNCA 100. The animals except the control group were given in-house prepared HFD with or without SUNCA (50 and 100 mg/kg b.w). The diets were given in the form of pellets. SUNCA was administered orally each day for 6 weeks. The animals were housed in a room with controlled atmosphere (temperature, 22°C ± 2°C, humidity, 55% ± 5%; 12 h light/dark cycle) throughout the experiment. The animal experiments were carried out with due clearance from Committee for the purpose of control and supervision of experiments on animals (a statutory committee established under the Prevention of Cruelty to Animals Act, 1960 in India) (Approval No. VHPL/PCL/IAEC/05/13).

Determination of body weight and food intake

Throughout the experimental period, the weight gain of rats was monitored weekly and the food intake was monitored every 2nd day. The average weight of the animals in a group is represented in the

results. Initial weight means the weight of the animals at the end of 6th week and final weight means, the weight of the animals at the end of 15 weeks. The total amount of food consumed by a rat was measured as:

Consumption of feed = Total quantity of feed given to rat – leftover feed.

Biochemical analysis

At the end of the treatment period, the animals were sacrificed and blood collected by cardiac puncture. The blood samples were allowed to coagulate at room temperature for 15 min. Serum was collected by centrifuging at 3000 rpm for 10 min at 4°C. The serum lipid levels such as total cholesterol (TC), triglycerides (TGs), high-density lipoprotein-cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-C) were estimated using an Automatic Chemistry Analyzer.

Histological analysis of epididymal adipose tissue

Adipose tissue samples were immediately retrieved and washed in saline, fixed in 10% formalin and embedded in paraffin. Tissue sections were cut at a thickness of 5 mm and stained with hematoxylin and eosin and imaged with light microscopy.

Western blotting

After 8-day differentiation in the presence of SUNCA, 3T3-L1 adipocytes were collected and lysed in ice-cold RIPA lysis buffer for 30 min. Similarly, on the other hand, adipose tissue collected from HFD fed rats was prepared. Protein concentrations were determined using a Bradford reagent. An equal amount of protein for each sample was loaded and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred to a nitrocellulose membrane using a semi-dry transfer and blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. The nitrocellulose membranes were then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for h. Immunoreactive proteins were detected using the chemiluminescent enhanced chemiluminescence assay, and images were captured using Image Quant, GE health care.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance ANOVA followed by Dunnett's multiple comparisons to analyze the significance of differences between control and treated groups. All statistical tests were performed at the $P < 0.05$ level of statistical significance. All the measurements were made in triplicate; data were reported as a mean ± standard error.

RESULTS

In vitro anti-diabetic enzyme inhibition assays

To determine the antidiabetic potential of SUNCA, α-glucosidase and α-amylase enzyme activity inhibition assay were performed. It was noticed that SUNCA demonstrated dose-dependent α-glucosidase inhibition activity, interestingly, SUNCA at the concentration of 0.1 mg/mL showed 89.10% α-glucosidase activity inhibition [Figure 1a]. Similarly, the α-amylase inhibitory activity was also assessed [Figure 1b]. The inhibition of amylase by SUNCA (0.1–1 mg/mL) showed concentration-dependent α-amylase inhibition activity. At the concentration of 1 mg/mL, SUNCA showed 55.14% of α-amylase inhibition.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

Effect of SUNCA on 3T3L adipocyte cell viability was measured using MTT reduction method and the result is indicated in Figure 2a. Results revealed that SUNCA at concentrations of 0.25–0.5 mg/mL did not affect cell viability, whereas, at higher concentration, 2 mg/mL showed significantly decreased cell viability compared to the control group. Therefore, the concentration range of 0.25 and 0.5 mg/mL was chosen for further experiments.

Effects of SUNCA on lipid accumulation

To investigate the effects of SUNCA on preadipocyte differentiation, the lipid accumulation was measured by an oil red O staining assay. The 3T3-L1 cells were treated with 250 and 500 μg/mL of SUNCA during differentiation. As shown in Figure 2b and c, interestingly, SUNCA suppressed lipid accumulation in 3T3-L1 adipocytes at levels that were statistically significant ($P < 0.05$), suggesting that SUNCA inhibits adipogenesis in 3T3-L1 cells.

Effect of SUNCA on the expression of peroxisome proliferator-activated receptor-gamma and CCAT/enhancer-binding protein alpha in 3T3-L1 adipocytes

To investigate whether SUNCA suppresses adipogenesis through a peroxisome proliferator-activated receptor gamma (PPAR-γ) pathway, we have carried out western blot experiments; it is very clear that protein expressions of PPAR-γ and CCAT/enhancer-binding protein alpha (CEBP-α) significantly up-regulated in differentiated 3T3-L1 cells. After the treatment of fully differentiated cells with 250 and 500 μg/mLof SUNCA. We observed that the expressions of PPAR-γ and CEBP-α were dose-dependently inhibited by SUNCA. PPAR-γ and CEBP-α protein levels were significantly reduced by treatment with 500 μg/mLof SUNCA [Figure 3a].

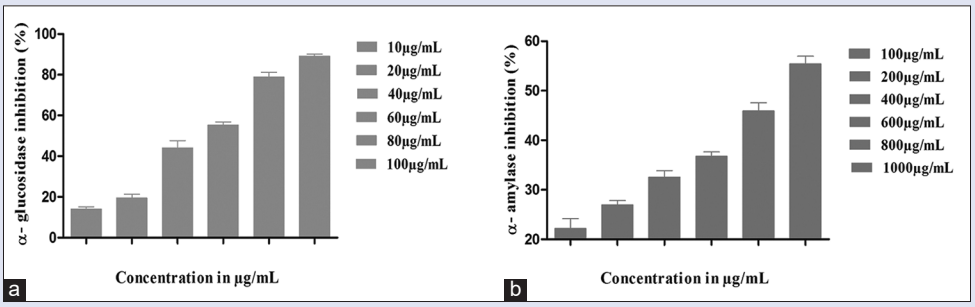

Figure 1: Effect of SUNCA on antidiabetic enzymes. (a) % inhibition of α- Glucosidase enzyme by SUNCA. (b) % inhibition of α- Amylase enzyme activity by SUNCA. Data represents mean ± SEM, $n = 3$

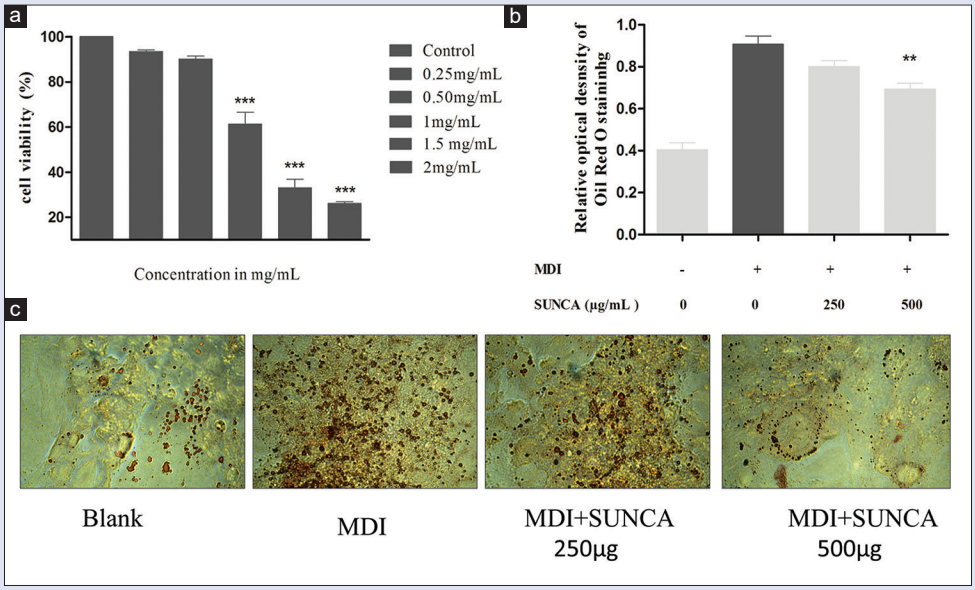

Figure 2: (a) Effect of SUNCA on cell viability in 3T3-L1 adipocytes (b) Effect of SUNCA on lipid accumulation in 3T3-L1 cells, the lipid content was quantified by measuring the absorbance. (c) 3T3-L1 cells were differentiated in the absence or in the presence of SUNCA (250 and 500µg/mL) for 8 days. Lipid droplets were measured by Oil Red O staining. All values are the means ± SEM of three independent experiments. $^{\#}P < 0.05$, vs. undifferentiated control cells; $^{*}P < 0.05$, vs. differentiated control cells

Effect of SUNCA administration on increase in body weight gain and food intake of high-fat diet rats

There was a significant increase in the mean body weight after a 6-week consumption of HFD compared to control ($P < 0.001$). Oral administration of SUNCA significantly reduced the mean body weight gain of HFD rats in a dose-dependent manner (50 mg/kg, $P < 0.01$ and 100 mg/kg, $P < 0.001$) compared to HFD group. Further, it was observed that there was a significant increase in the mean feed intake among the HFD rats. SUNCA treated group showed a significant reduction in feed consumption as compared to HFD rats ($P < 0.01$) at 100 mg/kg dose [Figure 4a and b].

Effect of SUNCA on blood lipid profile in high-fat diet rats

The serum analysis showed a significant alteration in the lipid profile of HFD rats compared to control animals ($P < 0.001$) [Figure 5]. SUNCA administration alleviated the blood lipid profile in a dose-dependent manner. SUNCA effectively reduced the TC at 50 ($P < 0.01$) and 100 mg/kg ($P < 0.001$) doses as compared to HFD rats. The TG level was restored significantly in 100 mg/kg SUNCA treated rats ($P < 0.05$). Further, there was a considerable decrease in the LDL-C level among the rats treated with 50 and 100 mg/kg of SUNCA ($P < 0.05$ and $P < 0.001$, respectively). SUNCA treatment at 100 mg/kg increased the serum HDL-cholesterol significantly as compared to HFD group ($P < 0.001$).

Inhibitory effect of SUNCA on peroxisome proliferator-activated receptor γ and a CCAT/enhancer-binding protein alpha expression in adipose tissue

As shown in Figure 4b, supplementation with different concentration of SUNCA significantly reduced the expression of adipogenic

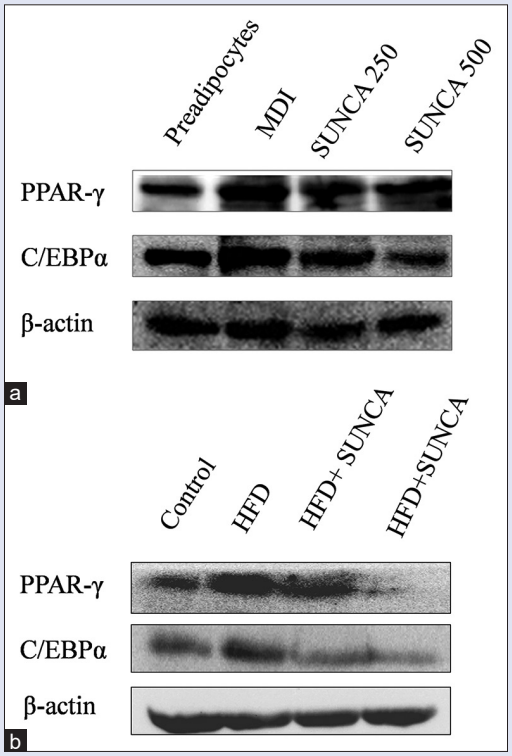

Figure 3: Effect of SUNCA on the expression of adipogenesis-related factors in differentiated 3T3L cells and adipose tissue of HFD induced rats (A) Post-confluent 3T3-L1 cells were differentiated in the absence or presence of SUNCA (250 and 500µg/mL) for 6 days and protein expression of PPARγ and C/EBPα protein expressions were analyzed by western blot. (B) Relative changes protein expression PPARγ and C/EBPα in the adipose tissue of HFD induced rats were measured by western blot

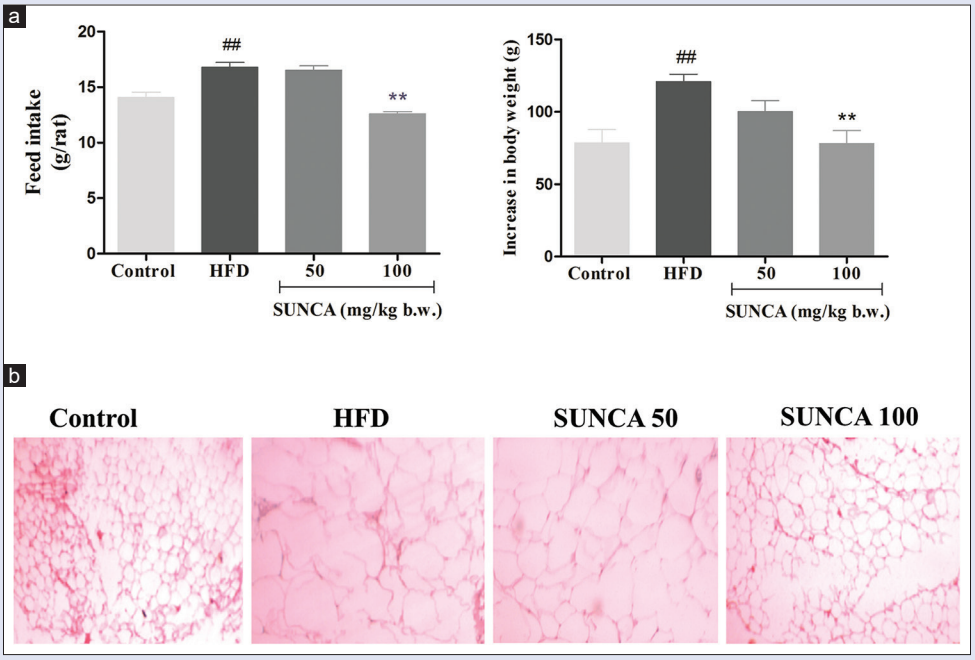

Figure 4: Effect of SUNCA on (a) feed intake (b) body weight and (c) histology of adipose tissues in high-fat diet-induced obese rats

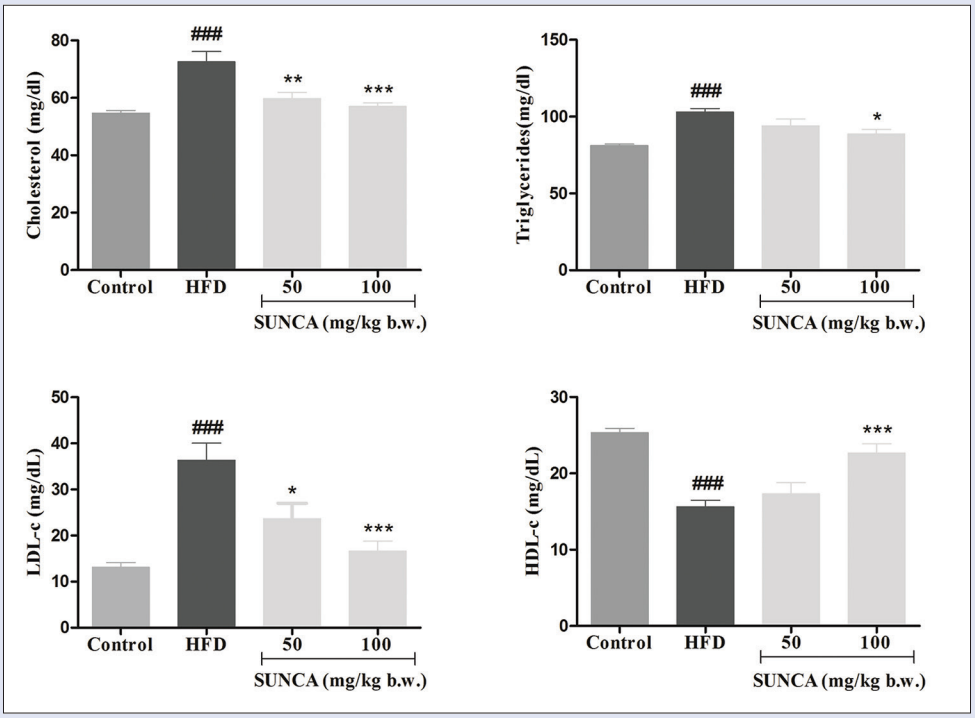

Figure 5: Effects of SUNCA on biochemical parameters in the serum of high-fat diet-induced obese rats

proteins. PPAR-γ is highly expressed in adipose tissue and is a key regulator of adipocyte differentiation and a potent modulator of whole-body lipid metabolism. SUNCA significantly suppressed the HFD induced expression of PPAR-γ in epididymal adipose tissue of HFD plus SUNCA-fed Rats. The expression of PPAR-γ in epididymal adipose tissue of HFD-fed Rats was highly elevated compared with control rats. SUNCA treatment clearly showed decreased PPAR-γ and CEBP-α expression.

Histopathology

Histopathological examination of epididymal adipose tissue sections showed significantly increased adipocyte size in the HFD-control rats

compared with normal controls [Figure 4b]. The increase in adipocyte area was attenuated by SUNCA.

DISCUSSION

The sunflower seed is a remarkable source of nutrients, minerals, antioxidants, and vitamins possessing antioxidant, antimicrobial and antidiabetic, antihypertensive, anti-inflammatory, and wound-healing activity.[13] In this study, we proposed for the first time the SUNCA standardized extract of *H. annus* L. is able to inhibit diabetic enzymes and adipogenesis both *in vitro* and *in vivo*. The treatment goal of diabetic patients is to maintain near-normal levels of glycemic control, in both pre and postprandial conditions. α-amylase and β-glucosidase enzyme activities in the body are responsible for postprandial hyperglycemia. Alpha-amylase catalyzes the hydrolysis of alpha-1,4-glycosidic linkages of starch, glycogen and various oligosaccharides. Alpha-glucosidase further breaks down the disaccharides to simple sugars, readily available for intestinal absorption. The inhibition of α-amylase and β-glucosidase enzyme which are particularly useful in postprandial glycemic control, are good candidates for the prevention of diabetic complications.[22-24] In the present study, we have observed that SUNCA exhibited potent alpha-amylase and alpha-glucosidase inhibitory activity which might be due to the presence of CGA and other polyphenols present in the standardized extract. Inhibition of digestive enzymes activity by polyphenols is a reasonable approach to control hyperglycemia and calorie intake. Currently, several studies have reported that rutin, quercetin, and catechin are a group of flavonoids showed blood glucose lowering properties in animal model and being potent compounds in the inhibition of alpha-amylase.[25]

It is well known that the 3T3-L1 cell line has been recognized as an ideal cell line to study adipogenesis. Several studies have confirmed that adipocyte differentiation and the amount of fat accumulation are associated with the occurrence and development of obesity. Therefore, the inhibition of adipocyte differentiation is one of the strategies for the treatment of obesity. In our study, lipid accumulation in 3T3-L1 preadipocytes, differentiated in the presence of insulin, dexamethasone, and IBMX. Oil red O staining and lipid measurement results showed that SUNCA treatment inhibited lipid droplet accumulation in a dose-dependent manner. Further to demonstrate anti-adipogenic mechanism we have carried out Western blot experiments to check the expression level of master regulators of adipogenesis PPAR-γ and CEBP-α both in *in vitro* and *in vivo*.[26] PPAR-γ and CEBP-α were upregulated in fully differentiated 3T3-L1 cells and in adipose tissue in the HFD-fed obese rats. Our result clearly indicates that treatment with SUNCA decreased the expression of PPAR-γ and CEBP-α protein levels both in adipocytes and adipose tissue, this clearly indicates that SUNCA inhibits early adipogenic processes and lipid accumulation by downregulating the expression of CEBPs and PPAR-γ. Several studies on the inhibitory activity of bioactive compounds isolated from plants on adipocyte differentiation have also revealed that the expression of both CEBP-β and PPAR-γ were significantly inhibited leading to a decrease in the lipid content of adipocytes.[27,28] Our results are consistent with previous reports.

Further, high fat-induced rat model of obesity was used to investigate the antiobesity potential of SUNCA. Increase in body weight is considered as an index of obesity. The body weight of all groups was increased during the study period, especially higher in case of HFD fed groups compared to that of the normal control group. The administration of SUNCA for 6 weeks showed a significant decrease in mean body weight gain compared to HFD rats. The evident justification for the decrease in body weight is due to significant reduction in the feed consumption among treated with 100 mg/kg of SUNCA. Loss of appetite is correlated with reduction in body weight due to alteration in metabolism and delaying absorption of nutrients during digestion, which have been reported in the literature.[29] It has been reported that higher levels of TC and TG found in HFD-induced obesity model. During HFD, intake conversion of glucose into fatty acids takes place from which TGs are formed and transported to bloodstreams very LDL-C (VLDL-C) and stored as fat in adipose tissue. In the HFD rat model of obesity, high levels of serum TC and TG were reported. During HFD intake, liver can convert glucose into fatty acids, from which TGs are formed, which gets transported to the bloodstream as VLDL-C and stored as fat in the adipose tissue.[30] Treatment with SUNCA caused significant decrease in TC, TG, and LDL-C levels. This could be due to the presence of CGA and other phytosterols in the standardized extract, as they possess more affinity for micelles than cholesterol and reduce incorporation of cholesterol in micelles in the intestine.[31] From the present findings, it was observed that SUNCA regulated the blood lipid profile ameliorating the hyperlipidemia caused by HFD. Histological analysis revealed a greater number of hypertrophied cells in the adipose tissue of the HFD group, whereas the SUNCA treatment suppressed adipocyte size in HFD-induced adipose tissue. Thus, the results of the present study depict that the SUNCA standardized extract enriched with CGA have a promising role in therapy of diabetes and high-fat-induced obesity. Further, the findings of the present study provide scientific evidence for the use of *H. annuus* L in traditional medicine for treating obesity.

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Conflicts of interest

There are no conflicts of interest.

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