

Original Research Article

Assessment Antiobesity and Anti- fatty Liver Effects of leaf Extract of *Cynara scolymus L.* in Mice under Diet-Induced Obesity

Abstract:

Objective: *Cynara scolymus L.* is an herbal medicinal product widely used in phytotherapeutics for digestive complaints, adjuvant treatment of moderate hyperlipidaemia in traditional European medicine. In this study. We conducted an animal study to investigate the effect of *Cynara* leaf extract (CLE) on visceral fat and hepatic lipid levels in mice with diet-induced obesity. In addition, we focused on gene expression profiles to investigate the anti-obesity and anti- fatty liver mechanism of CLE.

Methods: Twenty four male mice Swiss albino strain, at age of six weeks were classified into 3 groups. The first group was kept on standard normal diet and served as healthy control. Other groups received high fat diet (HFD) for 4 weeks. These animals were assigned HFD group and 5% *Cynara scolymus* leaf extract (CLE) -treated group.

Result: CLE supplementation significantly reduced body weight and white adipose tissue (WAT) weight. Moreover, in the histological analysis, CLE supplementation improved fatty liver.

Supplementation of CLE resulted in significant decrease in serum cholesterol, LDL and triacylglycerol. While serum HDL was significantly increased as compared to HFD group. Reverse transcription-polymerase chain reaction revealed that CLE supplementation up regulate acetyl co A (ACO) and hormone-sensitive lipase (HSL), in contrast, it suppressed the expression of fatty acid synthase (FAS) in the liver. For adipose tissue, the expressions of HSL in WAT was significantly upregulated while FAS was down regulate compared to HFD group.

Conclusion: These results suggest that CLE exerts anti-obesity and anti-fatty liver effects in high-fat diet-induced obese mice through suppressing lipogenesis in the liver, stimulating lipolysis in WAT.

Key wards: *Cynara scolymus*, ACO, HSL, Fatty Liver and lipid profile.

1. Introduction

Obesity, a pathological condition characterized by excessive accumulation of body lipids, is by far the most prevalent metabolic disease affecting hundreds of millions of people worldwide. The leading cause of this excessive lipid accumulation is a chronic positive energy balance combined with energy partitioning toward lipids [1].

Over the past few decades, obesity has become a global epidemic that affects diverse societies across developed and developing countries. Obesity rates correlate well with recent developments. These rapid environmental changes interact with pre-existing genetic tendencies, yet in a timescale so brief as to outstrip evolution [2].

Non-alcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver complications ranging from simple steatosis to steatohepatitis (NASH); advanced fibrosis and cirrhosis. The incidence of NAFLD is associated with obesity and has risen dramatically over the past several decades [3].

Fatty liver is a reversible condition in which triglycerides accumulate in large vacuoles in hepatocytes. Severe fatty liver is occasionally accompanied by inflammation, a situation that is referred to as steatohepatitis [4].

The potential sources of fats contributing to fatty liver include peripheral fats stored in adipose tissue that flow to the liver by way of the plasma non-esterified fatty acid (NEFA) pool (pathway 1); fatty acids newly made within the liver through de novo lipogenesis (DNL) (pathway 2); and dietary fatty acids, which can enter the liver by two means: through spillover into the plasma NEFA pool [5] (pathway 3) and through the uptake of intestinally derived chylomicron remnants (pathway 4) [6].

Abdominal adipose tissue is a complex organ and is composed of multiple distinct compartments and sub compartments, including subcutaneous fat and intra-abdominal fat. Intra-abdominal fat, which is also known as visceral adipose tissue (VAT), is considered a particularly important marker of metabolic risk [7-9].

The growing concern with obesity has led to an emphasis on the “undesirability” of white adipose tissue (WAT). Nevertheless, the tissue plays several key roles in mammalian physiology. The classical view of the function of WAT is that it provides a long-term fuel reserve which can be mobilized during food deprivation with the release of fatty acids for oxidation in other organs. Thus, the size of the adipose tissue stores increases in periods of positive energy balance and declines when energy expenditure is in excess of intake [10].

Accordingly, it is considered that both reducing WAT content and suppressing lipogenesis in the liver are very important for controlling fatty liver. Therefore, to prevent obesity, it is important to stimulate lipolysis in WAT and increase energy utilization in the liver and brown adipose tissue [11].

Artichoke (*Cynara scolymus* L.), is a plant that is widely grown in Mediterranean countries and is rich in natural antioxidants. It is not only a good food, known for its pleasant bitter taste, but also an interesting and widespread herbal drug [12]. Artichoke leaf contains up to 2% phenolic acids, mainly 3-caffeoylquinic acid (chlorogenic acid), plus 1,3-di-O-caffeoylquinic acid (cynarin), and caffeic acid; 0.4% bitter sesquiterpene lactones of which 47-83% is cynaropicrin; 0.11% flavonoids including the glycosides luteolin-7- β -rutinoside (scolymoside), luteolin-7- β -D-glucoside and luteolin-4- β -D-glucoside; phytosterols (taraxasterol); sugars; inulin; enzymes; and a volatile oil consisting mainly of the sesquiterpenes β -selinene and caryophyllene [13].

The artichoke leaf extract has been used as hepatoprotective, antimicrobial and cholesterol reducing purposes. Artichoke has been found to decrease the production of

reactive oxygen species, the oxidation of low-density lipoproteins, lipid peroxidation [14].

Thus, in the present study, we conducted an animal study to investigate the efficacy of *Cynara scolymus L.* leaf extract (CLE) on visceral fat levels and hepatic lipid accumulation in mice with high fat diet-induced obesity. In addition, we focused on the expression profiles of some genes related to beta-oxidation and lipogenesis in the liver and lipolysis in WAT to investigate the antiobesity mechanism of CLE. Histopathological investigation of liver sections was also carried out to confirm the biochemical analyses.

2. Materials and Methods

Materials

Casein was obtained from Sigma chemical company USA (Egyptian center). Starch vitamins for vitamins mixture and minerals used for salt mixture were obtained from Gomhoria chemical company Cairo, Egypt and Sigma chemical company USA (Egyptian center). *Cynara scolymus* leaf extract (CLE) was purchased from research centre of agriculture, Cairo Egypt.

The Kit used for Serum total cholesterol (TC), LDL-cholesterol (LDL-c), HDL-cholesterol (HDL-c) and triacylglycerol (TAG) determination were purchased from Diamond, Egypt.

1. Experimental Animals

Twenty four male mice Swiss albino strain, at age of six weeks weighing 16- 21g, were purchased from Helwan farm Mice were housed in care of laboratory animal house in the Faculty of Science, Ain Shames university, Cairo, Egypt. Ethics Committee at Ain Shames University, approved all the experimental procedures.

2. Test Environment

During the acclimation period, mice were housed in groups of 4 and administered normal diet). During the test period, mice were housed in individual stainless steel wire mesh cages. They were kept in a regulated environment (25 ± 1 °C, $50 \pm 2\%$ humidity), with 12 h light/dark cycles.

3. Experimental Design

Follow the acclimation for 1 week, albino mice were weighed and assigned to one of three groups so that the mean body weight of each group was uniform. The animals were divided into three groups, 10 mice each as following: Normal control: animals were fed on standard diet according to AIN-76 [15], Positive control group: animals were fed on a

high-fat diet (HFD) (table 1), and the treatment group: animals were fed on HFD containing 5% CLE (Table 1). The animals were restricted-fed and given water *ad libitum* for one month. In this study, plant extract was chosen to study its effect during 4 weeks (short term), according to the previous studies by Lion *et al.* [16].

4. Body Weight and Food Intake

During the experiment, the animals were weighed every day. Food intake was determined daily by determining the amount of feed remaining from the previous day, and the mean daily food intake for each animal was calculated.

5. Measurement of Tissue Weight

At the end of the experimental period, mice were fasten for 12 hours and they were sacrificed, blood were collected for separation of serum after clotting and centrifugation at 4000 rpm for 15 minutes. Serum samples were stored in deep freezer at -20°C in plastic vial for determination of lipid profile. Liver and interscapular brown, mesenteric, epididymal, and retroperitoneal adipose tissues were removed. All samples excluding BAT samples were weighed. Each sample was cut into small pieces, dipped in Trizol reagent (Invitrogen, Carlsbad, CA), and stored at -80°C until RNA extraction.

6. Biochemical Assays

Serum total cholesterol (TC) concentration was determined colorimetrically according to the method of Allain *et al.* [17]. Serum LDL-cholesterol (LDL) concentration was assayed colorimetrically according to the method of Assman *et al.*, [18]. Serum HDL-cholesterol (HDL) concentration was measured colorimetrically according to the method of Lopez-Virella *et al.*, [19]. Serum triacylglycerol (TAG) level was determined colorimetrically according to the method of Buccolo [20].

7. Hepatic Histological Analysis

Suitable sections of liver were fixed in 10% formalin and processed for preparation of 5 μm - thick paraffin section. These sections were sequentially stained with hematoxylin and Eosin (H & E) according to Bancroft *et al.* [21] to detect the presence of fat. This study was assessed by expert pathologists at Cairo University, Zoology Department.

8. RT-PCR

Total RNA was extracted from isolated mice organs using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from 5 μg of total RNA using a TIANscript One Step reverse transcription-PCR (RT-PCR) kit

(TIANGEN, China). The resulting cDNA was subjected to PCR reaction using EmeraldAmp® GT PCR Master Mix (Takara, Japan). Mice PCR primer sequences and conditions are demonstrated to table (2). PCR reaction were performed using denaturation temperature of 95°C for 30 seconds; annealing time of 45 seconds; and extension temperature of 72°C for 1 minute. PCR products were run on 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under U V light and photographed.

Table 1: Composition of the experimental diets.

Ingredient	HFD	HFD +CLE
Casein	20.2	20.2
Alpha-potato starch	28.2	25.2
Sucrose	13.0	13.0
Corn oil	20.0	20.0
Lard	10.0	10.0
Cellulose	4.0	4.0
Mineral mixture	3.5	3.5
Vitamin mixture	1.0	1.0
dl-Methionine	0.3	0.3
CLE	-----	5.0
Total	100.0	100.0

Mineral mixture, AIN-76; Vitamin mixture, AIN-76 (g/100 g diet).

Table (2): Mice PCR primer sequences and conditions

Gene Name	F/R	Sequence (5'-3')	Product size (bp)	Annealing temperature (°C)	Primer concentration (μ Molar)
FA S	F	CCATGGAGGAGGTGGTG ATA	380	58	0.1
	R	CGTCTCGGGATCTCTGCT AA			
β-actin	F	ACTGCCGCATCCTCTTCC TC	399	60	0.2
	R	CTCCTGCTTGCTGATCCA			

HS L	F	CATC GGAGCACTACAAACGCA AC	357	58	0.1
		TCCCGTAGGTCATAGGA			
	R	GAT GGTGGTATGGTGTCTGTA			
		CTTGA			
AC O	F	GAATCTTGGGGAGTTTAT	294	62	0.1
	R	CTGC			

9. Statistical analysis

SPSS package (version 21) was used for data analysis. All data were expressed as means standard deviation (SD) and standard error (S.E) of a minimum three or more replicates. The statistically significant differences were calculated by Student's t-test.

3. Results:

1. Body Weight and Food Intake.

Table (3) shows the body weight and amount of food intake during the experimental period. There was no significant difference between the normal control and HFD+CLE groups regarding food intake, both the final body weight and body weight gain, liver and both WAT and BAT. Body weight gain in positive control group mice fed on HFD showed a significant increase ($P<0.05$) in body weight as compared to normal control group. Group fed on HFD+CLE showed a significant decrease ($P<0.05$) in body weight as compared to positive control group. Positive control group showed a significant increase in WAT as compared to HFD+CLE group. Liver weight showed a non-significant change in all groups.

Table (3): Final body weight, weight gain, food intake, liver weight, adipose tissue weight in mice fed on standard or HFD in different studied groups.

Groups	Normal control	HFD	HFD+CLE	p value	Significance
Parameters					
Final Weight (g±SD)	20.23±0.45	26.10±0.81	21.17±0.49	0.01	**
Weight gain (g/day±SD)	2.05±.67	5.65±0.50	2.34±0.31	0.04	**

Food intake (g/day $\pm$ SD)	2.55 $\pm$ 0.43	2.48 $\pm$ 0.15	2.68 $\pm$ 0.17	0.38	NS
White adipose (g $\pm$ SD)	0.31 $\pm$ 0.1 1	0.51 $\pm$ 0.03	0.42 $\pm$ 0.02	0.02	*
Brown adipose (g $\pm$ SD)	0.16 $\pm$ 0.4 8	0.27 $\pm$ 0.06	0.26 $\pm$ 0.03	0.84	NS
Liver weight (g $\pm$ SD)	4.01 $\pm$ 0.2 1	4.87 $\pm$ 0.27	4.52 $\pm$ 0.3	0.40	NS

Table (4): Serum lipid profile in different studied groups.

Groups	TAG	TC	HDL-C	LDL-C
Parameters	(mg/dl)	(mg/dl)	(mg/dl)	(mg/dl)
<u>Control</u>	89.94- 103.31	80.64- 100.01	39.69- 48- 60	60.23- 66.10
Range	98.39 $\pm$	88.58 $\pm$	43.00 $\pm$ 1.56	63.91 $\pm$
Mean $\pm$ SE	1.92	2.30		1.4121
<u>HFD</u>	125.42- 142.55	113.19- 158.63	18.91- 21.24	88.29- 100.46
Range	138.54 $\pm$	133.67 $\pm$	18.79 $\pm$ 0.15	90.45 $\pm$ 1.34
Mean $\pm$ SE	4.21	6.1	- 56.31	41.52
% Change	40.76	50.90	0.001	0.001
P ₁ <	0.001	0.001		

<u>HFD + CLE</u>	91.71-	88.14-	40.26-	73.51-
Range	113.71	110.16	49.31	91.23
Mean± SE	97.14±	94.13±	46.33± 1.08	81.11±
% Change	2.69	1.81	7.71	0.33
P ₁ <	-1.27	6.26	N.S	21.20
P ₂ <	N.S	N.S	0.001	0.05
	0.001	0.001		0.001

P< 0.05: significant, P<0.001: highly significant.

N.S: non significant.

% change with respect to control.

P₁: in relation to control group, P₂: in relation to HFD group.

(Table 4) showed the effect of CLE on serum lipid profile in HFD and (HFD + CLE) mice in comparison with health control group. Group fed on HFD produced highly significant elevation (P<0.001) in serum triacylglycerol, total cholesterol and LDL- C levels (40.76%, 50.9% and 41.52% respectively) associated with a highly significant decline (P<0.001) in serum HDL- C level (-56.31%) in comparison with the healthy control group. On the other hand, (HFD + CLE) group showed a highly significant depletion (P<0.001) in serum TAG, total cholesterol and LDL- C levels accompanied with significant rise in serum HDL level in comparison with the HFD group.

2. Hepatic Histological Analysis

Hepatic histological analysis data after the experiment period showed that there is no specific findings were observed during the hepatohistological examination of the healthy control mice (Fig.1-a). Histopathological investigation of liver tissue slides stained with H&E in mice fed on HFD showed moderate to severe macrovascular fatty changes, which were diffusely distributed throughout the liver lobules. Parenchymal inflammation with both acute and chronic inflammatory cells accompanying focal necrosis was also observed (Fig. 1-b and 1-c). In the HFD + CLE group, the development of fatty liver was apparently suppressed (Fig.1-d).

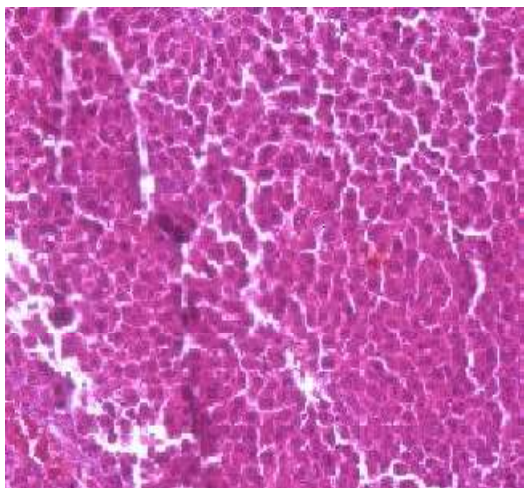


Fig 1 (a)

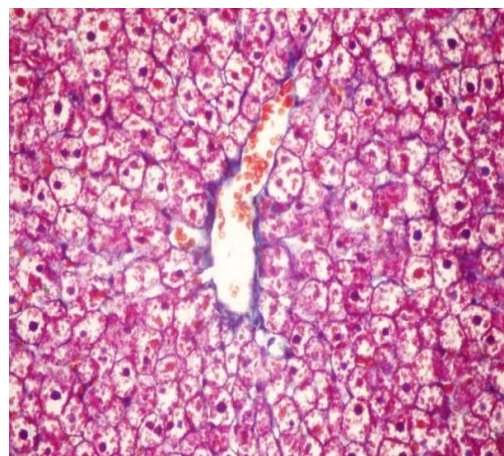


Fig 1 (b)

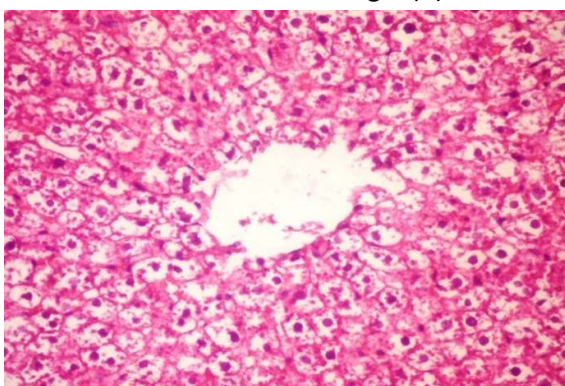


Fig. 1(c)

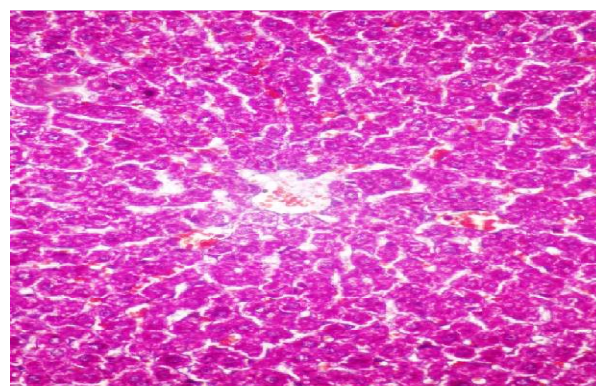


Fig. 1(d)

Fig. (1): photomicrographs of H&E stained hepatocyte sections where (a) represent a normal hepatic lobules of the healthy control mice; (b) & (c) photos represent histopathological investigation of liver tissue in mice fed on HFD showed moderate to severe macrovascular fatty changes distributed throughout the liver lobules accompanying with focal necrosis. Photo (d): represent HFD+ CLE group, showed a reduction in fat deposits in liver tissues.

3. Real-Time Quantitative RT-PCR.

Fatty acid synthase, hormone –sensitive lipase and acyl- Co A oxidase gen expression level was measured in both liver and white adipose tissue in HFD and HFD+CLE groups

using RT-PCR. Data represented in figure (2) and table (5) showed that the expression of hepatic genes involved in lipogenesis such as Fatty acid synthase (FAS) in the HFD + CLE group was significantly lower than that in the HFD group. Hormone-sensitive lipase (HSL) was significantly unregulated in liver of HFD + CLE group.

For WAT, hormone-sensitive lipase (HSL) was significantly unregulated in epididymal adipose tissue in the HFD + CLE. Expression of Fatty acid synthase (FAS) in the HFD + CLE group was significantly lower than that in the HFD group. The expression of genes related to beta-oxidation, such as acyl-CoA oxidase (ACO), was not significantly different between the 2 groups, although their expressions were higher in the HFD + CLE group. These results suggest that CLE has antiobesity effects in high-fat diet-induced obese mice through suppressing lipogenesis in the liver, stimulating lipolysis in WAT.

Table (5): Effect of CLE on mRNA levels in the liver and WAT in mice fed on high fat diet and high fat diet + CLE.

Groups			
Gens	HFD	HFD + CLE	p value
Liver			
FAS	1.00 ±0.211	0.172 ±0.032	0.018*
HSL	1.00 ±0.182	2.121 ±0.177	0.011*
ACO	1.00 ±0.172	1.370 ±0.339	0.517
white adipose tissue			
FAS	1.00 ±0.047	0.617 ±0.097	0.024*
HSL	1.00 ±0.025	1.773 ±0.229	0.028*
The data represent the mean ±S.E values (n =10). Significant different at P <0.05, P <0.01, respectively.			

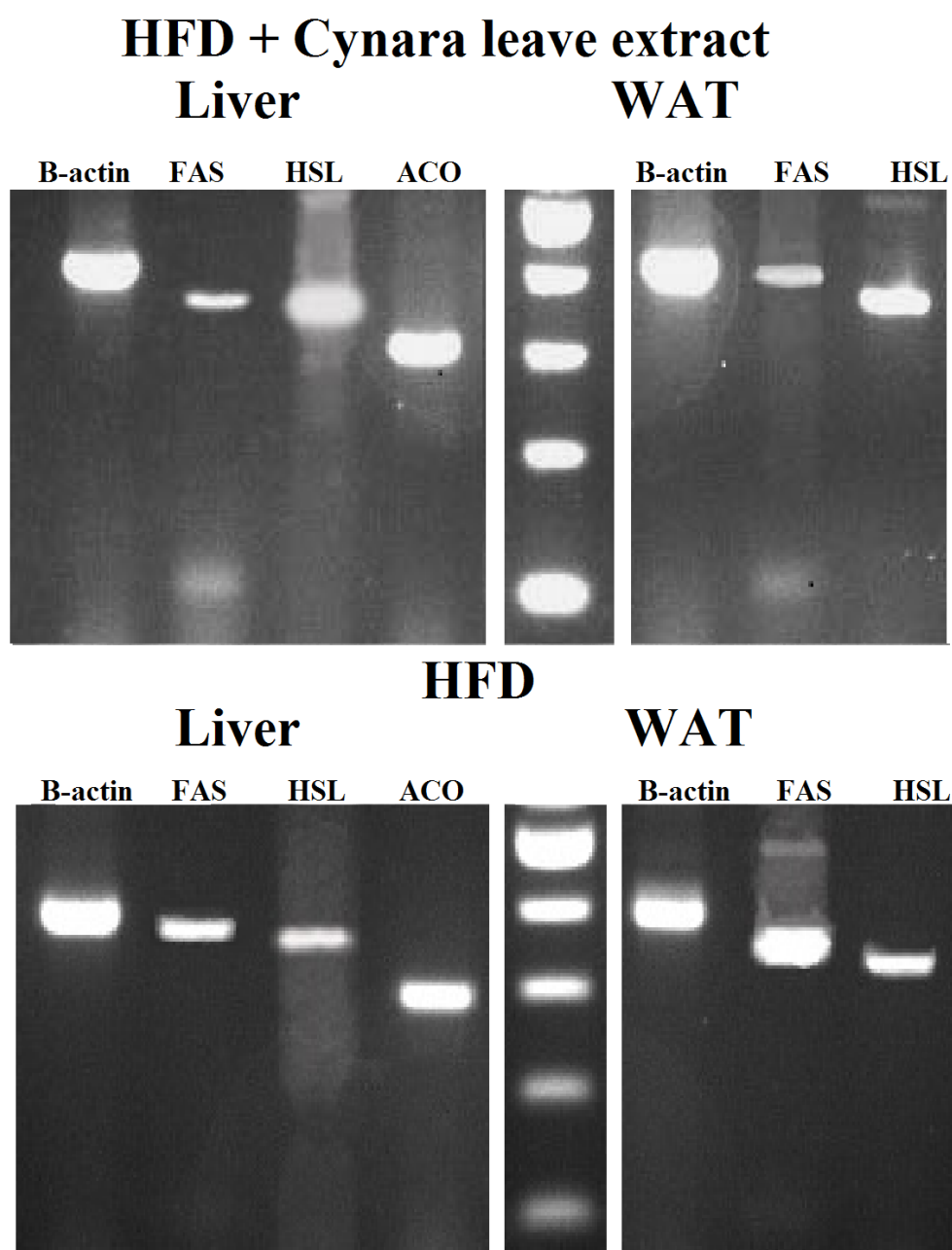


Fig. (2): Effects of (CLE) on the expressions of lipid metabolic genes in mice liver and white adipose tissue.

Discussion

Obesity is a chronic disease with serious health consequences, but weight loss is difficult to maintain through lifestyle intervention alone [22]. It is the result of a positive energy

balance, whereby energy intake exceeds expenditure, resulting in the storage of energy, primarily as lipids in white adipocytes [23].

The number of people with obesity and obesity-related diseases, such as diabetes mellitus, hypertension, coronary artery disease, fatty liver and cancers, has increased at an alarming rate all over the world [24-25]. Consequently, the idea of developing antiobesity drugs with no undesirable side effect has become a hot topic [26]. Herbal medicine has been looked at as a complementary treatment [27]. So in the present study we investigated the antiobesity effects of *Cynara scolymus* L.

In Western herbal medicine, an herb especially known for its chlorogenic acid content is artichoke leaves; the extracts are usually standardized to 15% of this compound. Cynarin is most concentrated in the leaves of artichoke [28].

Gebhardt, [29] reported that, *Cynara scolymus* L contains some constituents as Cynarin and luteolin which play a crucial role in inhibiting cholesterol and triglycerides synthesis

In this study, CLE supplementation significantly reduced body weight, body weight gain, and WAT weight without affecting energy intake (i.e., food intake). It is known that obesity develops when energy intake exceeds energy expenditure. So it's clear that the consumption of CLE for one month caused a suppressive effect on weight gain and visceral fat accumulation in mice Therefore, CLE was believed to exert antiobesity effects by increasing energy expenditure.

In the present study, feeding mice with high fat diet for (4weeks) led to increasing the serum triacylglycerol (TAG), total cholesterol (TC) and low density lipoprotein (LDL) levels were observed compared to those of the normal group (normal diet) whereas the HDL levels was decreased, with histopathological changes showed moderate to severe macrovascular fatty changes, which were diffusely distributed throughout the liver lobules. These histological assessments of hepatic injury are consistent with the elevated serum lipid concentrations.

Feeding mice on HFD with CLE improved the lipid profile by decreasing serum TAG, TC, and LDL-C levels accompanied with significant increase in serum HDL - C. Additionally, histopathological investigation of liver tissue of this group indicated a reduction in macrovesicular steatosis and microvesicular steatosis. Similar results were obtained with other previous studies [30-31]. However, highly significant decrease of serum LDL- C and an increase of HDL- C in the treated groups are agreed with Cieslik et al., [33] who reported decline tendency in total cholesterol, LDL and VLDL when diets were supplemented with *Cynara scolymus* flour.

Therefore, these extract has a strong hypo-triglyceridemic and hypo-cholesterolemic effects. This effects may be due to that *Cynara scolymus* contains some constituents as cynarin and luteolin which play a crucial role in inhibiting cholesterol and triglycerides synthesis. Luteolin by beta glucosidase in digestive tract could cause inhibition up to 60% of cholesterol synthesis [29].

These results also agree with Daniel, [33] who conclude that; this extract contains active compounds as flavonoids which have hypolipidemic effect. These compounds could not only increase the breakdown of cholesterol to bile salts and enhance their elimination through increased bile production and flow but they also inhibit the internal production of cholesterol in liver [34]. Cholesterol metabolism was associated with liver fat content independent on body weight, implying that the more fat the liver contains, the higher is cholesterol synthesis [35]. The accumulation of fat, mainly TAG, within the hepatocyte is a prerequisite for the development of NAFLD [36].

Non-alcoholic fatty liver disease (NAFLD) is one of the major causes of liver disease and is linked to the obesity and metabolic disease epidemics. NAFLD is characterized by excessive lipid accumulation in the liver accompanied by metabolic dysfunction and hepatic cell degeneration. It is considered to be the liver manifestation of insulin resistance. Hyperlipidaemia (increased blood triglycerides and/or cholesterol) is frequently associated with metabolic diseases and is also associated with fatty liver disease [37].

The current results suggest that the CLE could be helpful in decreasing the incidence of several fatty liver diseases through a reduction in TAG, TC and LDL- C and an increase in HDL level.

Moreover, we focused on gene expression profiles , Our results cleared that CLE modulates the expression of specific enzymes involved in lipogenesis and lipolysis processes in liver and WAT.

CLE leads to increase in gene expression of Acyl-CoA oxidase (ACO) (which catalyses the first and rate-determining step of the peroxisomal β -oxidation of fatty acids [38]) and Hormone-sensitive lipase (HSL) (Its the rate-limiting step in cleaving fatty acids from the triglyceride molecule [39]) and decrease the expression of fatty acid synthase (FAS) (rate-limiting enzymes in fatty acid biosynthesis, in the liver). These results revealed that Cynara leaf extract significantly up regulate the mRNA level of ACO and mRNA of HSL, in contrast, it suppressed the expression of FAS in the liver compared to HFD group

For adipose tissue, the expressions of HSL in WAT was significantly upregulated while FAS was down regulate. These results suggest that CLE exerts antiobesity and anti-fatty liver effects in high-fat diet-induced obese mice through suppressing lipogenesis in the liver, stimulating lipolysis in WAT. These results are similar to the antiobesity effects of isoflavones compounds [40-42]. Liu et al., (2011) [43] also reported the lipid-lowering potential of luteolin in palmitate-stimulated HepG2 cells. Also they record a reduction of FAS expression and an increase of CPT-1 expression, as a consequence of the luteolin-mediated AMPK activation and ROS inhibition.

Therapeutic options for NAFLD are limited to medications that reduce the risk factors. Therefore, suppressing hepatic lipid accumulation, the first step of the pathogenesis of NAFLD, appears to be very important for preventing this hepatic disorder. In this study, CLE supplementation suppressed the development of fatty liver by preventing the

accumulation of fat in hepatocytes as a results of downregulation of genes related to lipogenesis.

This result supports the possibility that CLE supplementation provides the dual effects of preventing both obesity and hepatic disorders. It is believed that isoflavones content of CLE are promising compounds for preventing obesity and fatty liver disease.

These finding seem to be in accordance with the results of Azevedo et al., (2010) [44] who has previously shown that luteolin-7-glucoside given in the diet for 1 week improved plasma lipid profile. Here we demonstrate that these effects are accompanied by an increase in liver expression of fatty acid β -oxidation related genes as well as a decrease in the rate limiting enzyme of fatty acid synthesis. These effects are consistent with the decrease in LDL and total cholesterol observed in the serum of animals fed with CLE and indicate that this extract may, in addition to improving serum lipid profile, be beneficial in the prevention of NAFLD and other metabolic diseases.

Conclusion

In the present study, the leaf extract of *Cynara scolymus* L. has been demonstrated to possess antiobesity and anti-fatty liver by in vivo assay. The current study suggest that CLE could be helpful in decreasing the incidence of several fatty liver disease through a reduction in TC, LDL and TG and an increase in HDL level. In addition, the leaf extract of *Cynara scolymus* L. appears to exert these effects through suppressing lipogenesis in the liver and promoting lipolysis in white adipose tissue.

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