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ORIGINAL ARTICLE

Cucumis melo ssp. Agrestis var. Agrestis Ameliorates High Fat Diet Induced Dyslipidemia in Syrian Golden Hamsters and Inhibits Adipogenesis in 3T3-L1 Adipocytes

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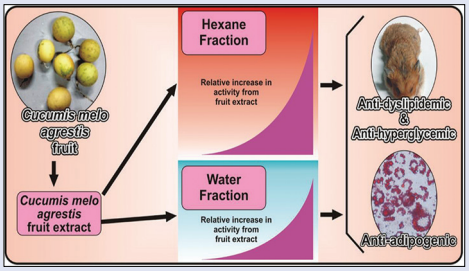
ABSTRACT

Background: *Cucumis melo ssp. agrestis var. agrestis* (CMA) is a wild variety of *C. melo*. This study aimed to explore anti-dyslipidemic and anti-adipogenic potential of CMA. **Materials and Methods:** For initial anti-dyslipidemic and antihyperglycemic potential of CMA *fruit extract* (CMFE), male Syrian golden hamsters were fed a chow or high-fat diet with or without CMFE (100 mg/kg). Further, we did fractionation of this CMFE into two fractions namely; CMA *water fraction* (CMWF) and CMA *hexane fraction* (CMHF). Phytochemical screening was done with liquid chromatography-mass spectrometry LC- (MS)/MS and direct analysis in real time-MS to detect active compounds in the fractions. Further, high-fat diet fed dyslipidemic hamsters were treated with CMWF and CMHF at 50 mg/kg for 7 days. **Results:** Oral administration of CMFE and both fractions (CMWF and CMHF) reduced the total cholesterol, triglycerides, low-density lipoprotein cholesterol, and very low-density lipoprotein-cholesterol levels in high fat diet-fed dyslipidemic hamsters. CMHF also modulated expression of genes involved in lipogenesis, lipid metabolism, and reverse cholesterol transport. Standard biochemical diagnostic tests suggested that neither of fractions causes any toxicity to hamster liver or kidneys. CMFE and CMHF also decreased oil-red-O accumulation in 3T3-L1 adipocytes. **Conclusion:** Based on these results, it is concluded that CMA possesses anti-dyslipidemic and anti-hyperglycemic activity along with the anti-adipogenic activity.

Key words: 3T3-L1 adipocytes, *Cucumis melo ssp. agrestis var. agrestis*, direct analysis in real time-mass spectrometry analysis, dyslipidemia, high-fat diet, Syrian golden hamster

SUMMARY

- The oral administration of Cucumis melo agrestis fruit extract (CMFE) and its fractions (CMWF and CMHF) improved serum lipid profile in HFD fed dyslipidemic hamsters.
- CMFE, CMWF and CMHF significantly attenuated body weight gain and eWAT hypertrophy.
- The CMHF decreased lipogenesis in both liver and adipose tissue.
- CMFE and CMHF also inhibited adipogenesis in 3T3-L1 adipocytes.



Abbreviation used: CMA: *Cucumis melo ssp. agrestis var. agrestis,* CMFE: CMA *fruit extract,* CMWF: CMA *water fraction,* CMHF: CMA *hexane fraction,* FAS: Fatty acid synthase, SREBP1c: Sterol regulatory element binding protein 1c, ACC: Acetyl CoA carboxylase, LXR α: Liver X receptor α.

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INTRODUCTION

Dyslipidemia is a lipoprotein metabolism disorder that displays increased cholesterol, triglyceride (TG) and low-density lipoprotein-cholesterol (LDL-c) along with decreased high-density lipoprotein-cholesterol (HDL-c) in blood.[1,2] Dyslipidemia is a major risk factor attributed to the development of cardiovascular diseases (CVDs). According to World Health Organization, CVDs are the number one cause of deaths worldwide. Therefore, it is a prime consideration for treatment of dyslipidemia to reduce elevated levels of lipid profile, i.e. TGs, total cholesterol (TC), and LDL-c along with an increase in HDL-c. Treatment options for these disorders include diet interventions, physical exercise, surgery, and medication. The anti-dyslipidemic drugs that are currently available in the market include statins, fibrates, niacin, ezetimibe, and bile acid binding resins.[3,4] However, a small but clinically significant population taking these medications experience adverse effects. Statins are known to increase hepatic enzymes and muscle toxicity.[5,6] Likewise, long-term therapy of fibrate in patients causes supersaturation of gallbladder bile, which increases the incidence of cholesterol gallstones.[7] Exploration of medicinal plants has always been an immense source of

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drugs and majority of the currently available drugs have been derived directly or indirectly from them. These days herbal drugs are being prescribed widely due to their effectiveness with minimum side effects and relatively low cost. The World Health Organization also considers medicinal plants as alternatives in the treatment of various diseases and the focus of research professionals for plants is increasing day by day.[8]

Cucumis melo ssp. agrestis var. agrestis (CMA) (Naudin) Pangalo var. *agrestis* Naudin, commonly known as wild melon (in English) or kachari (in Hindi) under the family cucurbitaceae.[9] CMA is a common climbing or prostrate herb, distributed almost throughout India and neighboring countries. The fruits of this plant possess the stomachic property and are also used to treat burns and abrasions. Seeds have antitussive, antioxidant, digestive, febrifuge and vermifuge properties, and seed oil extract was reported for anti-fungal activity.[10,11] Recently, we have observed that flavonoids have co-existing anti-dyslipidemic and anti-adipogenic activity, although both activities are distinct.[12] Furthermore, anti-adipogenic activity has also been reported for some of the statin classes of compound.[13,14] Syrian golden hamsters have been demonstrated as a valuable model of high fat diet (HFD) induced dyslipidemia, and it is well-suited for screening of anti-dyslipidemic agents.[12,15] In addition, hamsters also have a similarity to human plasma lipid distribution, synthesis, and excretion.[16,17] Our present study aimed to explore the anti-dyslipidemic and anti-adipogenic potential of fruit extract (excluding seed) and fractions of this plant in HFD-fed dyslipidemic hamsters. The anti-dyslipidemic activity was further assessed by gene expression and protein immunoblotting analysis in liver and adipose tissue.

MATERIALS AND METHODS

Plant materials

Ripe fruits of CMA were collected from our institute campus at Lucknow, India in July 2013. The herbarium specimen of this plant with voucher specimen number DKM24778 has been deposited in the medicinal plant herbarium of Council of Scientific and Industrial Research (CSIR)-Central Drug Research Institute (CDRI).

Chemicals

High-fat diet (Cat No. 12451) was purchased from Research Diets Inc., USA. Fenofibrate, used as positive control was purchased from Sigma-Aldrich, USA. Ethyl alcohol was procured from Merck, Germany and hexane from CDH, New Delhi, India. (4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT) and oil-red-O powder were purchased from Sigma. The kits for the assay of blood glucose, TC, TG, HDL-c, LDL-cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were purchased from Merck Specialities Pvt., Ltd., fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and ATP-citrate lyase antibodies were purchased from Cell Signaling Technology, Inc., (Beverly, MA, USA).

Extraction and fractionation

The fruits of this plant were collected, washed, and oven dried at a constant temperature of 37°C after removal of seeds. Thereafter, these were made into fine powder using a grinder and kept in an airtight amber color container protected from light. The 130 g powder was extracted in ethanol for 24 h on a mechanical shaker at room temperature. The solvent was filtered off, and the residue was macerated again with 500 ml fresh solvent consecutively 2 times for next 2 days. Finally, the residue was discarded and all the filtrates were clubbed and concentrated under reduced pressure on a rotary evaporator (BUCHI, Switzerland) at 40°C. Finally, 7 g of the crude ethanolic extract obtained and partitioning into 1:1 hexane: Water mixture. The fractions resulted in 3.29 (hexane) and 1.68 g (water) residue after solvent evaporation. Both the fractions were kept in air tight glass containers away from light and subjected to further study.

Liquid chromatography-mass kept in air tight glass containers away from light and subjected to further study LC-MS/MS analysis

The liquid chromatography-mass spectrometry (LC-MS) and LC-MS/MS analysis of fruit extract's fractions (excluding seed) of CMA was performed on waters tandem quadrupole detector triple quadrupole MS (USA). It was equipped with waters, H-Class acquity ultra performance LC (UPLC) system, and electrospray ionization (ESI) source. The column was used thermo accucore AQ C18 100 × 3 mm, 2.6 µm, and dual mode (+) LC-ESI-MS experiments were performed. 5 µl of the sample was injected through auto-sampler into UPLC flow. For the separation of individual compounds, the mobile phase used (a) methanol and acetonitrile (50:50) and (b) 5 mm ammonium acetate in 95% water. A linear gradient elution was performed at the flow rate of 0.250 ml/min as 80–40% B in 0–5 min; 40% B in 5–6 min; 20% B in 7–8 min. Nitrogen was used as the nebulizing and drying gas at flow rates of 30 and 650 L/h, respectively. The ESI source potentials were capillary voltage 3.5 kV; cone potential at 30 V for every experiment. The Source and desolvation temperature was at 120 and 350°C, respectively. The mass resolution was set approximately 1000 and the mass analyzer was scanned between 150 Th and 750 Th in 0.5 s. Online tandem mass spectra of various ions were measured by precursor ion selection in MS_1 followed by collision induced dissociation and analysis of the daughter ions by MS_2. Argon was used as collision gas and optimized at a pressure to achieve 5–10% transmission of the precursor ion. The collision energy was ramped between 10 eV and 15 eV to achieve significant fragmentation. Data acquisition and processing were carried out using MassLynx V4.1 software change note 714 software (Waters Corporation, 34 Maple Street, Milford, MA ,01757). The spectra were accumulated from the top of extracted ion chromatogram peak.

Direct analysis in real time-mass spectrometry

The direct analysis in real time (DART)-MS was recorded on a JEOL-AccuTOF JMS-T100LC MS having a DART source. The samples were subjected as such in front of DART source. Dry helium gas was used for ionization at 4 L/min flow rate and source temperature kept at 350°C. The orifice 1 was set at 28 V and spectra collected as an average of 6–8 scan.

Differentiation of 3T3-L1 adipocytes

The 3T3-L1 cell line was purchased from the American type culture collection. Cells were cultured in a humidified atmosphere at 37°C and 5% CO_2 in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum and penicillin-streptomycin antibiotics (cDMEM). For adipogenic induction, cells were seeded in 24 well-plates. Two days postconfluence, culture medium was replaced with differentiation medium [medium containing insulin 5 µg/ml, 3-isobutyl-1-methylxanthine 0.5 mM, and dexamethasone 250 nM (MDI)]. This medium was replaced after 48 h with medium containing 5 µg/ml insulin. This medium was replaced after 72 h, and cells were maintained in cDMEM. Lipid droplets started appearing from day 4, and > 90% of cells showed lipid globules 6–8 days after induction.

MTT assay

MTT assay was performed as described previously[12] and absorbance was measured at 570 nm using ELISA plate reader (BioTek, USA).

Animals and diet

Dyslipidemia was induced in 8 week old, (100–120 g body weight) Syrian golden hamsters (*Mesocricetus auratus)* by feeding a high-fat diet (Research Diets Inc., Cat. No: D12451, 45% kCal). Hamsters used in the study were taken from the colony of the Laboratory Animal

Division, CSIR-CDRI and were kept in controlled laboratory conditions of temperature (23 ± 2°C), relative humidity (50–60%), and light (300 Lx at floor level, 12/12 h light/dark cycle). All experimental procedures adopted in this study were previously approved by the Institutional Animal Ethics Committee in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals formed by the government of India. Standard pellet food and high-fat diet (Research Diets Inc., 12451), kept at 4°C was administered daily (80 g diet/cage) at a fixed time in the morning. The leftover diet of the previous day was weighed for the net food intake before being discarded. All groups were having free access to diet and water.

Experimental design for anti-dyslipidemic and anti-hyperglycemic activity of *Cucumis melo ssp. agrestis var. agrestis fruit extract*, *Cucumis melo ssp. agrestis var. agrestis water fraction* and *Cucumis melo ssp. agrestis var. agrestis hexane fraction*

The animals with identification marks were acclimatized for 7 days before the experiment. The control group of hamster was fed with normal chow diet. High-fat diet (45% kcal HFD, day 1–day 10) were given in all other groups, i.e. Vehicle, fenofibrate, CMA *fruit extract* (CMFE), CMA water fraction (CMWF), and CMA hexane fraction (CMHF). All groups were having free access to diet and water. Likewise, the body weight of the animals was recorded daily before feeding and drug administration. Fenofibrate, CMFE or CMA *water fraction* (CMWF), and CMA *hexane fraction* (CMHF) were suspended in the 0.1% gum acacia solution and gavaged orally, once daily at a fixed time for seven consecutive days (day 4–10) to treated groups and vehicle to the parallel control. The same experimental procedure was followed for assessment of anti-dyslipidemic activity of CMWF and CMHF.

Blood collection, serum analysis, and histological analysis

The blood withdrawn from the retro-orbital plexus of anesthetized animals was collected in micro-centrifuge tubes and serum was separated by centrifugation. TG, TC, HDL-c, LDL-cholesterol, glucose, ALT, AST, and creatinine were estimated using Merck selectra junior bio-analyzer (Merck Millipore). Very low-density lipoprotein-cholesterol (VLDL-c) was calculated using the formula (TG/5) of Friedewald. The sections of epididymal white adipose tissue (eWAT) and liver were fixed in 10% formalin, dehydrated, and embedded in paraffin. Further, eWAT and liver sections were stained with hematoxylin and eosin to examine the morphology.

Real time polymerase chain reaction

Total RNA was isolated from liver and eWAT using TRIZOL reagent (Invitrogen, CA, USA). First strand cDNA synthesis was performed using high-capacity cDNA reverse transcription Kit (Applied Biosystems™) and subsequently used for quantitative real time polymerase chain reaction (PCR) analysis on Light Cycler 480 (Roche Diagnostics). Statistical analysis of the quantitative real time PCR was obtained using the ($2^{-\Delta\Delta Ct}$) method, which calculates the relative changes in gene expression of the target, normalized to an endogenous reference (18S rRNA) and relative to a calibrator that serves as a control group. Gene-specific primer pairs used in these studies are listed in Table 1.

Western blotting

Liver and eWAT were freeze-thawed and triturated in liquid nitrogen and protein were isolated using mammalian cell lysis buffer supplemented with 100 mm EDTA, protease inhibitor, and phosphatase inhibitor (Sigma-Aldrich). Protein concentration was determined using the bicin-choninic acid method (Sigma) and further western blotting procedure was done as described previously.[18] To validate equal loading, actin was used as an internal loading control.

Statistical analysis

For *in vivo* studies, data were expressed as a mean + standard error of mean. Comparisons between the treatment groups and control were performed by one-way analysis of variance followed by Bonferroni's multiple comparison test. For *in vitro* studies, data were expressed as mean + standard deviation. Comparisons between the treatment groups and control were performed using Student's *t*-test. A probability value of $P < 0.05$ (*), 0.01 (**) and 0.001 (***) was used as a measure of statistical significance. Data were analyzed on Graph Pad Prism (Version 5.00, Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Cucumis melo ssp. agrestis var. agrestis fruit extract attenuates dyslipidemia and improves hyperglycemia

CMFE treatment in HFD-fed dyslipidemic hamster reduced increase in TC, LDL-c, however, we could not get any difference in TG [Figure 1a, b and d]. HFD induced hyperglycemia was also improved with treatment of CMFE [Figure 1c]. In addition, HDL-c and HDL-c to TC ratio were also improved when treated with CMFE [Figure 1e and f]. Furthermore, we examined the intracellular toxicity of CMFE in 3T3-L1 adipocytes, using the MTT assay. The result of MTT assay proves that CMA treatment is safe [Figure 1g]. Different concentrations of CMFE (25, 50, 100 µg/ml) were supplemented with MDI during differentiation. The absorbance of extracted ORO accumulated in lipid droplets shows that CMA significantly inhibits adipogenesis [Figure 1h].

Phytochemical screening

The bioactive fractions of CMA were investigated by LC-MS/MS. The previously reported data of *Cucumis species* showed the presence of carbohydrates, glycosides, phenolics, and flavonoids.[19] Similarly, the nonpolar oily fraction contains fatty acids like 16-Octadecenoic acid, methyl ester, estra-1, 3, 5 (10)-trien-17-beta-ol, hexadecanoic acid, 2-hydroxy ethyl ester, etc.[20] Our preliminary study and MS data also suggested the presence of carbohydrates, phenolics, and their glycosides. The compound eluted at 1.5 min having molecular weight 342 Da, was highly abundant in the aqueous fraction. The MS/MS spectra indicated that the molecule contains a sugar moiety (−162 Da) with the 180 Da

Table 1: Primer sequences used for real time PCR gene expression studies

Gene name	Primer pairs
LXR α	F5′ TCAGCATCTTCTCTGCAGACCGG3′
	R5′ TCATTAGCATCCGTGGGAACA3′
APOA1	F5′ ACCGTTCAGGATGAAAACTGTAG3′
	R5′ GTGACTCAGGAGTTCTGGGATAAC3′
LPL	F5′ GATTCACTTTTCTGGGACTGA3′
	R5′ GCCACTGTGCCGTACAGAGA3′
SREBP1c	F5′ GCGGACGCAGTCTGGG3′
	R5′ ATGAGCTGGAGCATGTCTTCAAA3′
LCAT	F5′ CACACAAGGCCTGTCATCCT3′
	R5′ AGCACAACCAGTTCACCACA3′

PCR: Polymerase chain reaction; LCAT: Lecithin-cholesterol acyltransferase; SREBP1c: Sterol regulatory element binding protein 1c; APOA1: Apolipoprotein A-I; LXR α: Liver X receptor α

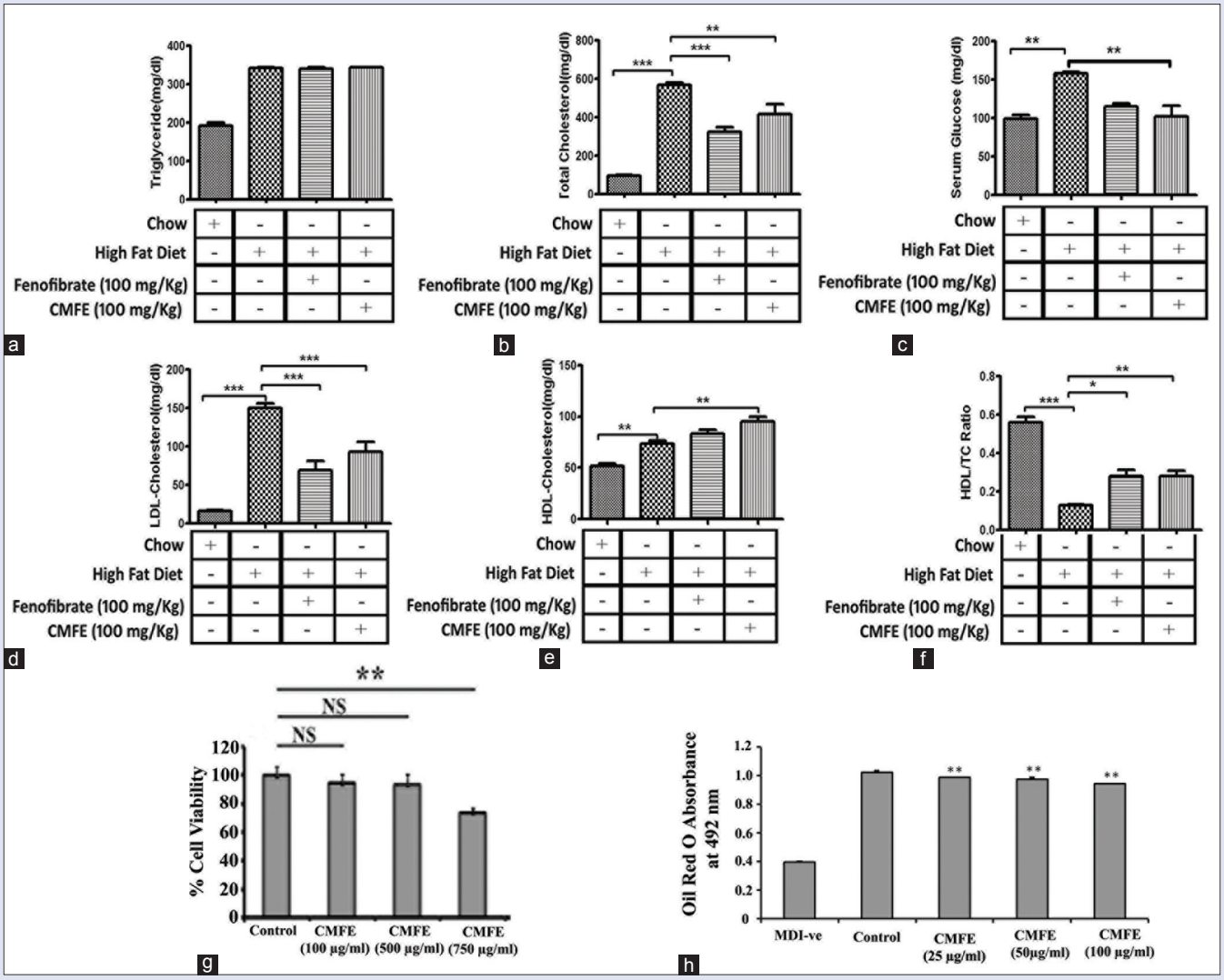

Figure 1: *Cucumis melo ssp. agrestis var. agrestis fruit extract* ameliorates dyslipidemia, improves hyperglycemia and reduces adipogenesis. Syrian golden hamster fed with chow or high fat diet, either treated orally with fenofibrate (100 mg/kg) or *Cucumis melo ssp. agrestis var. agrestis fruit extract* (100 mg/kg) for 7 days. Animals were overnight fasted, blood was isolated and further serum was separated. (a) Triglyceride (b) total cholesterol (c) serum glucose (d) low-density lipoprotein-cholesterol, (e) high-density lipoprotein-cholesterol (f) high-density lipoprotein-cholesterol/total cholesterol ratio. Mouse 3T3-L1 preadipocytes were differentiated into adipocytes with or without presence of various concentrations of *Cucumis melo ssp. agrestis var. agrestis fruit extract* (25, 50 100 µg/ml). Intracellular neutral lipids were stained with oil-red-O and absorbance was measured at 492 nm. MTT assay was performed in 3T3-L1 preadipocytes grown to confluence followed by incubation with *Cucumis melo ssp. agrestis var. agrestis* extracts (100–750 µg/ml) for 24 h. Cell viability was then determined by the MTT assay assay. (g) Cell viability using MTT assay (h) Oil-red-O staining in 3T3-L1 adipocytes. Values are means (n = 8), with their standard error of mean represented by vertical bars. Mean values were significantly different from the high fat diet diet-fed animals (one-way analysis of variance): *P < 0·05, **P < 0·001, ***P < 0.0001. *Denotes that the mean values are significantly different

unit. Thus, it could be expected as disaccharides sugar. Similarly, most of the compounds eluted subsequently shown a characteristic loss of −162 Da from parent ion that suggested the loss of a hexose unit. While the presence of indicative ion at m/z 91, 105, 107, 135 in MS/MS spectra designates the phenolic structure, and these compounds were identified as phenolic glycosides. Apart from this, compound eluted at 9.09 showed subsequent loss of the CH_2 unit with the losses of H_2O and CH_3OH. It suggested the presence of long carbon chain with the –OH and –O-CH_3 functional group. The chromatographic and MS data of detected metabolites are shown in Table 2. However, the whole metabolites detection was not possible only from ESI source. Thus, the metabolites fingerprint of both the fraction CMWF and CMHF were recorded on DART-MS and shown in [Figure 2a]. This MS can be recognized as fingerprints to discriminate the fractions metabolite and for future reference.

Cucumis melo ssp. agrestis var. agrestis fractions suppress high-fat diet-induced weight gain, improves dyslipidemia and hyperglycemia

The body weight of the HFD fed group significantly increased while CMWF and CMHF treatment significantly attenuated gain in body weight starting from 7th day to the end of the experimental period without any change in food intake throughout the experimental period [Figure 2b and c]. HFD significantly increased TC (4.1 fold), TG (2.2 fold), LDL-c (5.8 fold), VLDL-c (2.2 fold) in Syrian golden

Table 2: The chromatographic and mass spectrometric data of detected metabolites

RT	Molecular weight	Class of compounds	Precursor ion	m/z	MS/MS product ions (m/z)	Compounds detected in fraction	Glycone (neutral losses) Da
1.58	342	Disaccharide (sugar)	$[M+H]^+$	343	325 (7), 307 (9), 289 (6), 181 (12), 163 (79), 145 (100), 127 (87), 97 (28), 85 (67)	CMWF	−162
4.55	373	Phenolic glycosides	$[M+H]^+$	374	212 (35), 159 (38), 151 (59), 127 (36), 109 (67), 105 (48), 99 (30), 91 (100), 85 (69), 69 (34)	CMWF	−162
5.62	386	Phenolic glycosides	$[M+H]^+$	387	369 (0.1), 289 (0.3), 225 (18), 207 (100), 189 (9), 161 (7), 113 (4)	CMWF	−162
5.80	594	Phenolic glycosides	$[M+H]^+$	595	433 (100), 415 (50), 397 (26), 379 (14), 367 (56), 337 (49), 313 (91), 283 (34), 271 (5)	CMWF	−162
5.99	522	Phenolic glycosides	$[M+NH_4]^+$	540	378 (0.2), 344 (0.8), 325 (0.4), 237 (63), 219 (100)	CMWF	−162
6.07	530	Phenolic glycosides	$[M+NH_4]^+$	548	369 (8), 351 (100), 333 (33), 315 (9), 297 (3)	CMWF	−162
6.63	196	Phenolic derivestives	$[M+H]^+$	197	179 (100), 161 (24), 135 (65), 133 (38), 107 (73), 93 (26)	CMWF	-
6.87	288	Unidentified	$[M+H]^+$	289	253 (73), 235 (54), 216 (10), 209 (6), 173 (26), 169 (50), 161 (28), 153 (24), 135 (16), 111 (32), 97 (98), 85 (100)	CMWF	-
9.09	326	Fatty acid	$[M+H]^+$	327	309 (11), 295 (26), 277 (100), 259 (23), 185 (32), 173 (40), 163 (19), 149 (16), 135 (61), 121 (75), 119 (40), 109 (78), 107 (57), 95 (86), 93 (65)	CMHF	-

CMA: *Cucumis melo ssp. agrestis var. agrestis*; CMHF: CMA *hexane fraction*; CMWF: CMA *water fraction*; RT: Retention time

hamsters, which were decreased [TC (28.8, 41.2, and 36.9%), TG (29.3, 30.2, 38.3%), LDL-c (34.9, 31.8, 54.7%) with increased HDL-c (41.4, 30.5, 37.6%)] in serum, respectively by fenofibrate (100 mg/kg), CMWF, and CMHF (50 mg/kg) treated animals [Figure 2d-h]. Our result showed that the dyslipidemic hamsters exhibited a profound decrease in the HDL-c/TC ratio, an atherogenic marker [Figure 2i]. This atherogenicity is considered to be combined with dyslipidemia.[21] Animals fed with HFD for 11 days lead to increased serum glucose compared with chow-fed animals. CMA fractions treatment significantly suppressed the elevation of serum glucose in HFD fed hamsters [Figure 2j].

Cucumis melo ssp. agrestis var. agrestis fractions reduced adipose hypertrohy and hepatic lipid accumulation *in vivo* in Syrian golden hamsters without any toxicity

To investigate whether CMA decreases adiposity, hamsters were sacrificed and eWAT removed and weighed. The weight of eWAT was increased in the HFD-fed hamsters compared to the chow-fed hamsters, and was significantly decreased by the administration of Fenofibrate, CMWF, and CMHF [Figure 3a and b]. HFD- fed hamster showed adipocyte hypertrophy, while the adipocyte phenotype of CMA fractions (CMWF and CMHF) treated group was similar to chow diet fed animals. In addition to this, the liver histological sections of CMA fractions (CMWF and CMHF) treated animals also showed decrease hepatic lipid accumulation [Figure 3c]. The levels of AST, ALT, and creatinine that indicates liver and kidney injury, were significantly improved in CMA fractions treated animals [Figure 3d-f].In addition, the CMA treated hamsters did not induce any significant changes in the weight of liver (data not shown). Thus, the data indicate that administration of 50 mg/kg/day of CMA fractions for 7 days induced no biochemically detectable adverse toxic effects in the hamsters.

Cucumis melo ssp. agrestis var. agrestis fractions inhibited adipogenesis and lipogenesis, increased lipid metabolism and reverse cholesterol transport

Different concentrations of both CMA fractions (CMWF and CMHF at 25, 50, 100 μg/ml) were supplemented with MDI during differentiation of 3T3-l1 adipocytes. The absorbance of extracted ORO accumulated in lipid droplets shows that CMHF significantly inhibits adipogenesis [Figure 4a]. Further, FAS protein level was also reduced more prominently in CMHF treated eWAT [Figure 4b]. CMWF and CMHF treatment also decreased FAS, and ACC in the liver. Hepatic ATP citrate-lyase was reduced significantly when treated with CMHF. However, we could not get any significant change in ATP-citrate lyase protein expression when treated with CMWF [Figure 4c]. Hence, we further analyzed the mRNA expression of genes involved in lipogenesis, lipid metabolism, and reverse cholesterol transport only in CMHF treated animals. The CMHF treatment increased hepatic mRNA expression of liver X receptor α (LXR α), LPL and significantly decreased expression of sterol regulatory element binding protein 1c (SREBP1c) [Figure 4d-f]. Further, hepatic mRNA expression of lecithin-cholesterol acyltransferase (LCAT) and apolipoprotein A-I (APOA1), involved in reverse cholesterol transport were significantly enhanced by treatment of CMHF [Figure 4g and h].

DISCUSSION

Traditionally, India has very long history of using natural extracts in the form of Ayurvedic medicines. Currently, people throughout the world are recognizing the value of natural compounds.[22,23] CMA var. agrestis is a wild variety of *C. melo* and known as wild melon or musk melon. *C. melo* var. agrestis, being wild variety is economically cheaper and present in enormous quantity comparing with its original variety *C. melo*. The plants of Cucurbitaceae family had been widely used in treatments of many diseases.[24-26] The previous studies have reported the presence of alkaloids, flavonoid, terpenoids, and phenolic compounds in seed extract of *C. melo*.[11] However, to the best of our knowledge, anti-dyslipidemic activity of CMA has not been explored until date. The purpose of the present study was to assess the anti-dyslipidemic activity of CMA extract and its subsequent fractions and further evaluation of its anti-adipogenic activity. In our study, results show that CMFE attenuates dyslipidemia and improves hyperglycemia that lead us to do fractionization of this extract and further assessment of anti-dyslipidemic activity of both fractions (CMWF and CMHF). Both fractions showed significantly decreased serum lipid and glucose level in our primary assays. Many natural compounds have been previously studied in great detail for their potential to inhibit adipogenesis and/or lipid accumulation capacities such as in some noted cases of berberine, hesperidin, naringin, curcumin.[14,27-31] Our recent study also showed that the anti-adipogenic compounds also show anti-dyslipidemic activities. Hence further, we performed an anti-adipogenic activity of CMFE in 3T3-L1 adipocytes and found that CMFE decreases concentration-dependent oil-red-O accumulation, a dye used for staining of neutral TGs and lipids which indicates that CMFE causes decrease in lipid accumulation during 3T3-L1 adipogenic differentiation. Based on

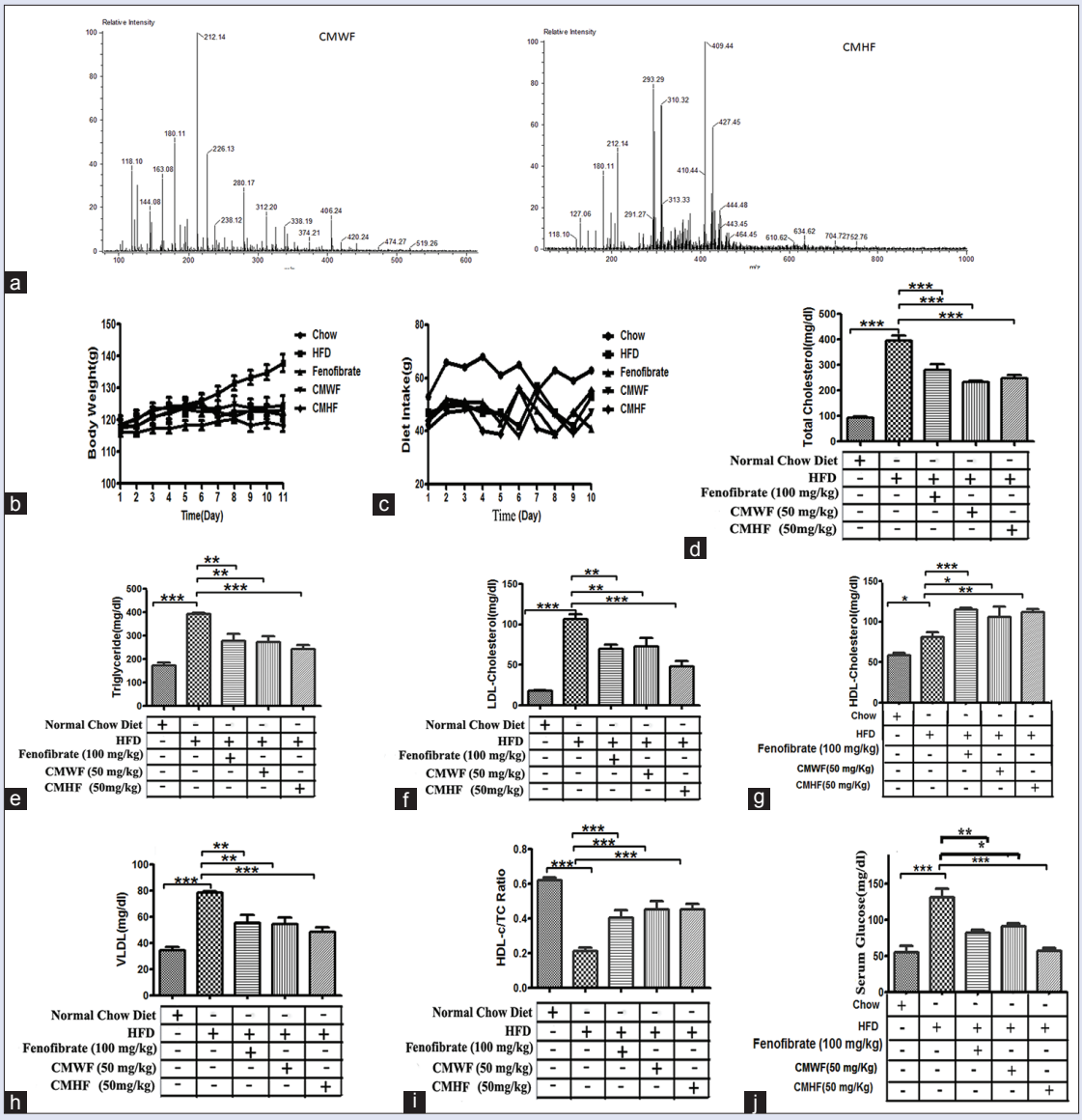

Figure 2: *Cucumis melo ssp. agrestis var. agrestis* fractions (*Cucumis melo ssp. agrestis var. agrestis water fraction* and *Cucumis melo ssp. agrestis var. agrestis hexane fraction*) attenuated body weight gain, improved dyslipidemia and hyperglycemia without altering diet intake. The direct analysis in real time-mass spectrometry was recorded on a JEOL-Accu TOF JMS-T100LC mass spectrometer having a direct analysis in real time source. The samples were subjected as such in front of direct analysis in real time source. Dry Helium gas was used for ionization at 4 L/min flow rate and source temperature kept at 350°C. The orifice 1 was set at 28 V and spectra collected as average of 6–8 scan. (a) Direct analysis in real time-mass spectrometry fingerprint of both fractions. Syrian golden hamsters ($n = 8$), fed with chow or high fat diet were kept on either fenofibrate (100 mg/kg), on *Cucumis melo ssp. agrestis var. agrestis fruit extract* (50 mg/kg) or on *Cucumis melo ssp. agrestis var. agrestis hexane fraction* (50 mg/kg) for 7 days. (b) Body weight of chow or high fat diet fed dyslipidemic hamsters treated with or without *Cucumis melo ssp. agrestis var. agrestis* fractions. Hamsters were treated with *Cucumis melo ssp. agrestis var. agrestis* fractions or fenofibrate for 7 days and body weight was measured every day in morning before providing diet. (c) Average food intake amount of chow and high fat diet diet mice in 10 days feeding. Diet intake was recorded every day. (d) Total cholesterol. (e) Triglyceride (f) low-density lipoprotein-cholesterol. (g) high-density lipoprotein-cholesterol cholesterol. (h) Very low-density lipoprotein-cholesterol. (i) High-density lipoprotein-cholesterol cholesterol/total cholesterol ratio. (j) Serum Glucose. Values are means ($n = 8$), with their standard error of mean represented by vertical bars. Mean values were significantly different from the high fat diet diet-fed animals (one-way analysis of variance): $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0.001$. *Denotes that the mean values are significantly different

the data presented in this study, CMA fractions (CMWF and CMHF) treatment significantly improved serum dyslipidemia by decreasing TG, TC, and LDL-c with increase in HDL-c, and the ratio of HDL-c to TC. Diabetic dyslipidemia is a complex cluster of abnormalities.[2] In addition to the hypolipidemic effects, CMWF and CMHF treatment also showed hypoglycemic activity in dyslipidemic hamsters. Treatment of CMA fractions also attenuated body weight and eWAT weight, a marker of obesity. Histological analysis also proved that CMA fractions decreased the hypertrophy in adipocyte, a marker of dysfunctional adipose tissue. Moreover, hepatic lipid accumulation a marker of hepatic insulin resistance is also reduced with treatment of CMWF and CMHF.[32,33] Hence taken together, CMA fractions attenuated body weight gain, ameliorated dyslipidemia, and hyperglycemia in HFD fed dyslipidemic hamsters. FAS is a very well-known lipogenic target for

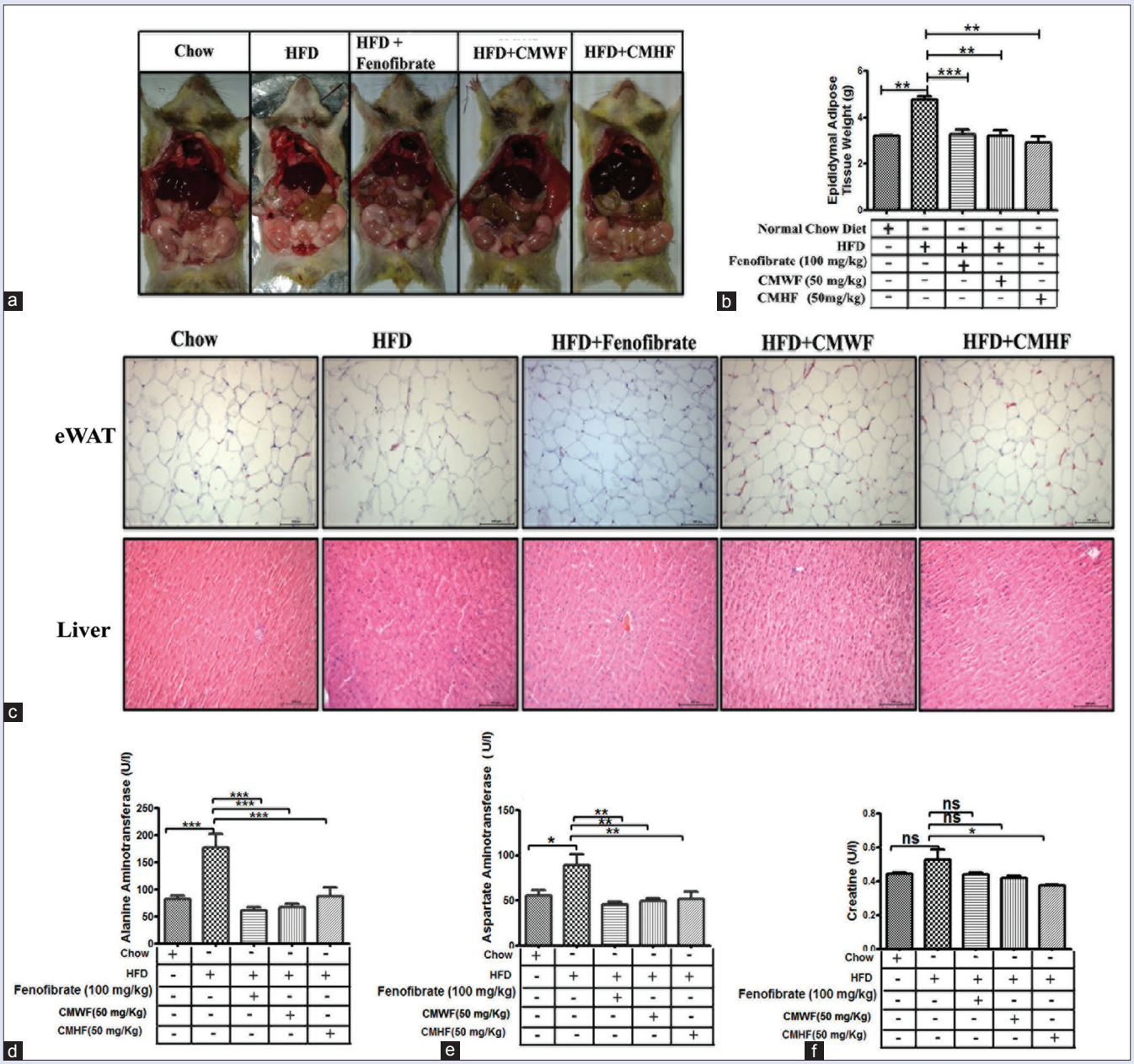

Figure 3: *Cucumis melo ssp. agrestis var. agrestis* fractions ameliorates high fat diet induced adipose tissue weight gain without any toxicity. hamsters were fed either a chow or high fat diet for 7 days in the presence of fenofibrate (100 mg/kg) or *Cucumis melo ssp. agrestis var. agrestis* fractions (50 mg/kg) ($n = 8$). (a) Images of hamster showing *Cucumis melo ssp. agrestis var. agrestis fruit extract* and *Cucumis melo ssp. agrestis var. agrestis hexane fraction* mediated decrease in epididymal white adipose tissue (b) epididymal white adipose tissue weight (c) histological analysis of the epididymal white adipose tissue and Liver after staining with hematoxylin and eosin. *Cucumis melo ssp. agrestis var. agrestis* does not cause any toxicity, *in vivo* in Syrian golden hamster (d) serum alanine aminotransferase (e) serum aspartate aminotransferase (f) serum creatinine

SREBP-1c, which was significantly decreased when treated with CMWF and CMHF in eWAT as well as in the liver, further contributing to the effect of CMA fractions on modulating circulating lipids. CMWF and CMHF treatment also decreased the protein expression of ACC. Hepatic ATP - citrate lyase was decreased when treated with CMHF, but we could not get any significant difference in ATP- citrate lyase with CMWF treatment. We could not get any significant changes in adipogenesis and other markers (e.g. ATP-citrate lyase) with CMWF. Thus, further mRNA expression analysis was performed with CMHF only. LXR α is a sensor of cholesterol excess and its activation in dyslipidemic hamsters led to an increase in reverse cholesterol transport and reduced lipid accumulation.[34] In this study, CMHF treatment in HFD-fed hamsters increased the hepatic mRNA expression of LXR α. Further, SREBP-1c is considered as a central mediator of obesity and insulin resistance and master regulator of ATP-citrate lyase, ACC, and FAS. ATP-citrate lyase is a lipogenic enzyme that converts citrate to acetyl-coenzyme A. Acetyl-coenzyme A is further converted to malonyl-CoA. Malonyl-CoA is known to play a key role for chain elongation in fatty acid biosynthesis.[35] Liver-specific ATP-citrate lyase downregulation via adenovirus-mediated RNA interference has been reported to reduce expression of peroxisome proliferator-activated receptor-gamma and the entire lipogenic program in the liver.[36] Hence, we hypothesize that

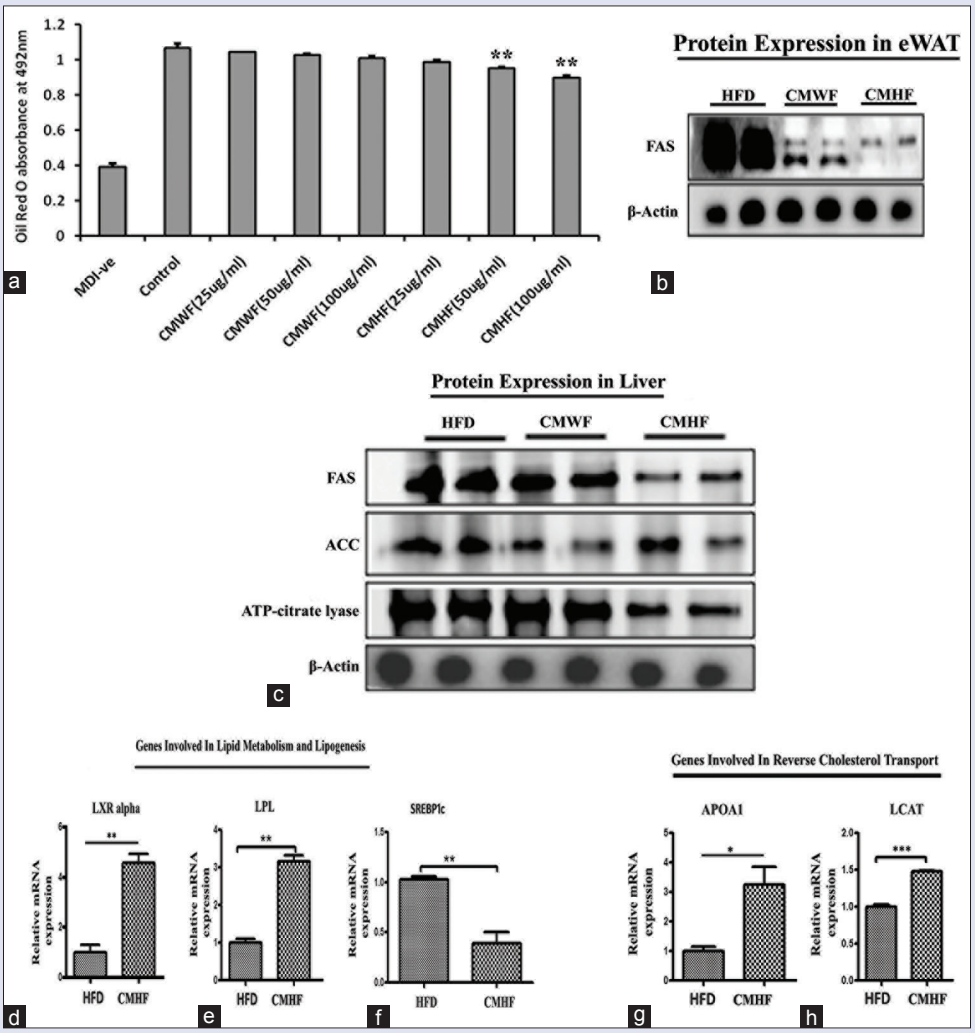

Figure 4: *Cucumis melo ssp. agrestis var. agrestis* fractions inhibited adipogenesis, lipogenesis and increased lipid metabolism and reverse cholesterol transport. Mouse 3T3-L1 preadipocytes were differentiated into adipocytes with or without presence of various concentrations of *Cucumis melo ssp. agrestis var. agrestis* fractions (*Cucumis melo ssp. agrestis var. agrestis water fraction* and *Cucumis melo ssp. agrestis var. agrestis hexane fraction* at 25, 50 100 μg/ml concentration). Intracellular neutral lipids were stained with oil-red-O and absorbance was measured at 492 nm. (a) Oil-red-O staining in 3T3-L1 adipocytes. Syrian golden hamsters kept on chow or high fat diet, were treated with fenofibrate (100 mg/kg) or *Cucumis melo ssp. agrestis var. agrestis water fraction* and *Cucumis melo ssp. agrestis var. agrestis hexane fraction* at 50 mg/kg for 7 days. At 8th day, after overnight fast, liver and epididymal white adipose tissue were collected in liquid nitrogen. (b) Protein expression of fatty acid synthase in epididymal white adipose tissue. (c) Protein expression of fatty acid synthase, ACC, ATP-citrate lyase in hepatic tissue (d) relative mRNA expression of LXR α. E. Relative mRNA of sterol regulatory element binding protein 1c. (f) Relative mRNA expression of LPL. (g) Relative mRNA expression of apolipoprotein A1. (h) Relative mRNA expression of lecithin-cholesterol acyltransferase 10. Gene expressions are relative to 18S rRNA and normalized by high fat diet-fed subgroups. Values are means (n = 8), with their standard error of the mean represented by vertical bars. Mean values were significantly different from the high-fat diets diet fed animals (one-way analysis of variance): *P < 0·05, **P < 0·01, ***P < 0·001

CMWF and CMHF increase expression of LXR α and inhibits expression of the transcription factor SREBP1c, leading to a reduced expression of its all major targets including FAS, ACC, and ATP-citrate lyase to inhibit lipogenesis in the liver as well as in adipose tissue. Overexpression of APOA-I limits progression and reduces pre-existing atherosclerosis hence, increasing the levels of APOA1 A-I has been a therapeutic target in CVDs.[37,38] CMHF treatment increased the mRNA expression of APOA1 and LCAT, the genes involved in reverse cholesterol transport, proving that CMHF not only decreases lipogenesis but also increases reverse cholesterol transport. Hence, it is concluded that apart from adipose tissue, the liver is also an important player for *in vivo* activity of CMA. Hypertriglyceridemia (>150 mg/dL) is a common lipid abnormality associated with several other metabolic disorders.[39,40] LPL plays an important role by catalyzing first reaction in TG metabolism and increase in LPL causes a reduction in hypertriglyceridemia.[41] Our results show that CMHF increased lipoprotein lipase mRNA expression in the liver that might be a reason for the decrease in serum TG level.

CONCLUSION

The administration of CMFE, CMWF, and CMHF significantly attenuated body weight gain and eWAT hypertrophy and improved serum TC, TG, and LDL-c levels in HFD-induced dyslipidemic hamsters. CMFE and CMHF inhibited adipogenesis in 3T3-L1 adipocytes. The dyslipidemic effects of CMHF altered the expression of LXR α, SREBP1c in the liver, and FAS in both liver and adipose tissue. These results suggest that CMA treatment is useful for treating metabolic diseases such as obesity and

hyperlipidemia. Our study shows the effects of CMWF and CMHF on TC, TGs, and LDL-c along with an analysis of liver and kidney adverse reactions indicates that CMA could be a cheap, efficient, and safe lipid-lowering drug in metabolic syndrome patients.

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Conflicts of interest

The authors declare no conflicts of interest.

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