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Antiobesity and lipid lowering effects of *Glycyrrhiza* chalcones: Experimental and computational studies

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ABSTRACT

Twelve flavonoids (**1–12**), isolated from *Glycyrrhiza glabra* roots were evaluated for their pancreatic lipase (PL) inhibitory activity *in vitro*. The structures of the isolated compounds were elucidated by spectroscopic methods. Amongst all the compounds **7**, **8**, **10** and **11** showed strong inhibition against PL with IC_{50} values of 7.3 μM, 35.5 μM, 14.9 μM and 37.6 μM, respectively. Molecular docking studies on the most active compound **7** revealed that it binds with the key amino acid residues of the PL active site. *In silico* absorption, distribution, metabolism and excretion (ADME) parameters were also computed on the active compounds to determine their preliminary pharmacokinetic properties. Further, investigations were carried out to determine the antiobesity and lipid lowering effects of **7** and **10** in high fat diet (HFD) fed male SD rats. In the rats supplemented with compound **7** the body weight increase was only 23.2 ± 3.6 g as compared to 64.2 ± 0.5 g in the HFD control group while in the rats treated with compound **10** showed 23.2 ± 3.6 g weight gain only. Compound **7** decreased the levels of plasma total cholesterol (TC) to 84.6 ± 1.4 mg/dl and plasma total triglycerides (TG) to 128.8 ± 6.0 mg/dl. Compound **10** also lowered the plasma TC and TG levels considerably. The results indicate the potential of the chalcone scaffold as a source of PL inhibitors for preventing obesity.

Introduction

Physiologically, obesity is a disarray of energy balance and primarily considered as a disorder of lipid metabolism (Strader et al., 1998). A growing number of enzymes involved in lipid metabolic pathways are being identified and characterized. They represent a rich pool of potential therapeutic targets for obesity (Shi and Burn, 2004; Melnikova and Wages, 2006). Inhibition of PL (triacylgycerol acyl hydrolase), the principal lipolytic enzyme, synthesized and secreted by pancreas (Mukherjee, 2003) is one of the approaches for the development of newer antiobesity drugs. Tetrahydrolipstatin (Orlistat), a commercial anti-obesity drug, is a known pancreatic lipase inhibitor (Borgstrom, 1988; Hadvary et al., 1991). Previously, we have given an account of the reported plants with antiobesity properties (Birari and Bhutani, 2007) and the various PL inhibitors reported from these natural sources (Bhutani et al., 2007). Recently, much interest has been shifted on plant flavonoids that might be beneficial in reducing the risk of obesity (Peluso, 2006). Dietary catechins and anthocyanins significantly decrease the weight of abdominal adipose tissues (Murase et al., 2002; Tsuda, 2008). Accordingly, investigation on the metabolic effects of plant flavonoids might lead to more effective strategies for the treatment of obesity. The health hazards like diabetes, obesity and metabolic related disorders are related to the dietary habits and most of the nutraceutical on the market focuses on these areas. Anthocyanin-rich berries or derived extracts, procyanidins rich grape seed, bilberry and cranberry extract are well known for their antioxidant and lipid lowering ability (Espin et al., 2007).

Roots of *Glycyrrhiza glabra* (Fabaceae/Papilionaceae), also known as licorice and sweet root has been used medicinally for the past 4000 years (Duval et al., 2007; Iritani, 1992). Historically, the dried rhizome and root of this plant were employed medicinally by the Egyptian, Chinese, Greek, Indian, and Roman civilizations as an expectorant and carminative. Several pharmacological activities, such as antiulcer, antiinflammatory, antidiuretic, antiepileptic, antiviral, antiallergic and antioxidant properties have been attributed to the licorice compound glycyrrhizin and glycyrrhizic acid (Visavadiya et al., 2009; Nassiri Asl and Hosseinzadeh, 2008). Licorice flavonoid oil (LFO) suppresses abdominal fat accumulation by regulation of rate-limiting enzyme activities related to fatty acid synthesis and oxidation in the liver (Nakagawa et al., 2004).

Licorice extract and its primary constituent glycyrrhizin are extensively used amongst US population and are considered as Generally Recognized as Safe (GRAS) for use in foods by the U.S. FDA (Isbrucker and Burdock, 2006; Nakagawa et al., 2008a,b).

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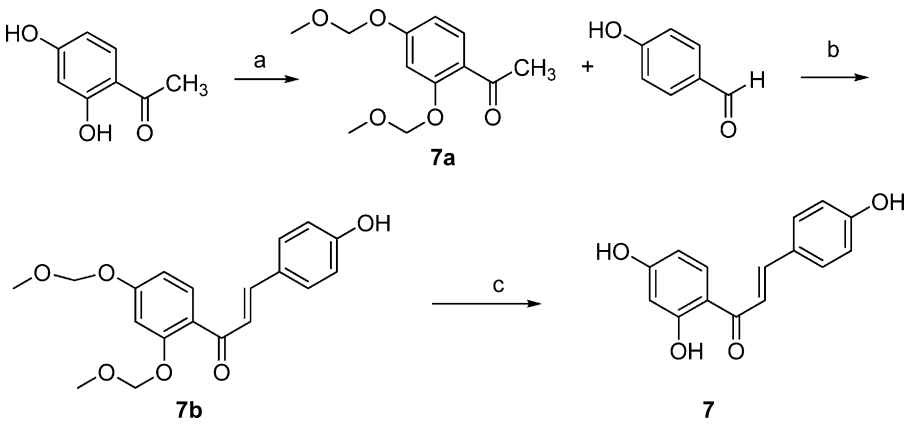

Scheme 1. Key steps in synthesis of LiOH·H_2O. Reaction conditions: (a) dry acetone, K_2CO_3, MOMCl, reflux 7 h; (b) MeOH, LiOH·H_2O, reflux 4 h and (c) 3NHCl, reflux, 30 min.

Various genotoxic studies have indicated that licorice extracts and glycyrrhizin is neither teratogenic nor mutagenic, and may even possess anti-genotoxic properties under certain conditions (Nakagawa et al., 2008a,b; Kaur et al., 2009). Clinical studies have shown that LFO is safe because no clinically significant adverse events occurred when it was given daily to healthy or overweight subjects for up to 12 weeks. These reports suggest the relative safety of licorice extracts and the chalcone when administered orally in animals as well as humans (Aoki et al., 2007). Isoliquiritigenin the major chalcone from *G. glabra* also has several pharmacological effects such as antioxidant, anti-inflammatory, anti-tumor, anti-platelet, and anti-peptic ulcer actions (Haraguchi et al., 1998). Recently it has shown to have protective effects on mitochondrial cells against arachidonic acid and iron induced oxidative stress (Choi et al., 2010). Isoliquiritigenin was reported as quite safe and well tolerated when given orally (Lee et al., 2008).

In our continuing research on identification of newer antiobesity leads from natural sources, many plant extracts have been screened for their PL inhibition (Birari et al., 2009). Recently our group has published the antiobesity and lipid lowering effects of *Murraya koenigii* (L.). Spreng leaves extracts and mahanimbine on high fat diet induced obese rats (Birari et al., 2010). In the present work, an integrative *in vivo-in vitro-in silico* approach was followed to study the antiobesity properties of *Glycyrrhiza* flavonoids. Twelve flavonoid aglycones and glycosides isolated from *Glycyrrhiza* roots were tested for their PL inhibitory action *in vitro*. In order to understand the mode of binding of these compounds with the active site of the PL enzyme, molecular docking approach was employed. *In silico* ADME parameters were also computed on the active compounds to determine their preliminary pharmacokinetic properties. Animal studies on most active compounds **7** and **10** demonstrated the potential of these types of compounds in the development of antiobesity therapeutics.

Materials and methods

Plant material

G. glabra (root) was procured from local market of Chandigarh, India and identified by qualified botanist and voucher specimen has been preserved in the herbarium of Department of Natural Products, NIPER, SAS Nagar, India.

Animals and grouping

Forty-eight male SD rats of weight 175 ± 15 g were procured from Central Animal Facility, NIPER. The animals were housed under standard environmental conditions (temperature 22 ± 2 °C; humidity 55 ± 5%) with a 12 h light/dark cycle at the animal house. All the animals were housed in polypropylene cages in groups of 3 per cage and had free access to water *ad libitum*. The protocol of this experiment was approved by the Institutional Animal Ethics Committee (IAEC) and the experiments were carried out in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA, India) given on animal experimentation.

Extraction and isolation

Dried and powdered roots of *G. glabra* (1 kg) were subjected to sequential extraction with hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) to prepare the respective extracts. The DCM extract of *G. glabra* is expected to contain the prenylated flavonoids and hence, a reported method was followed (Vaya et al., 1997) to give compound **1**–**4**.

EtOAc extract (GGE; 10 g) was purified on silica gel (#60-120). Elution was carried out using hexane, $CHCl_3$ and MeOH in increasing order of polarity. Fractions were pooled according to TLC pattern to give 5 fractions. Non-polar fractions (GGE-1 to GGE-3) were further purified on silica gel (#60-120) to give aglycones **5–9**. The polar fractions (GGE-4 and GGE-5) were purified on Sephadex LH-20 to give the glycosides **10**–**12**.

MeOH extract (45 g) was dissolved in water and partitioned with butanol (BuOH). BuOH fraction (27 g) was taken for purification. It was fractionated by vacuum liquid chromatography on silica gel G (#200-400), using $CHCl_3$/MeOH gradient to yield 5 major fractions. Purification of Fraction 4 (FrM4) yielded compound **10**.

For *in vivo* studies large quantity of compound **7** was required hence its synthesis was carried out. A mixture of 2,4-dihydroxyacetophenone (0.76 g, 5 mmol), anhydrous K_2CO_3 (2.070 g, 15 mmol), and MOM chloride (0.48 g, 12 mmol) was refluxed in dry acetone (25 ml) for 7 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated and the residue subjected to column chromatoghraphy with pet ether/EtOAc 1:1 as an eluent to yield **7a** (68%). Base catalyzed Claisen condensation of **7a** with 4-hydroxy benzaldehyde in presence of LiOH·H_2O (Bhagat et al., 2006) gave compound **7b** which upon deprotection (Vogel et al., 2008) yielded compound **7** (Scheme 1).

Measurement of PL inhibitory activity

PL inhibitory activity was measured using p-nitro phenol palmitate (PNPP) as a substrate. The samples were screened against the PL using standard protocol previously published from our labora-

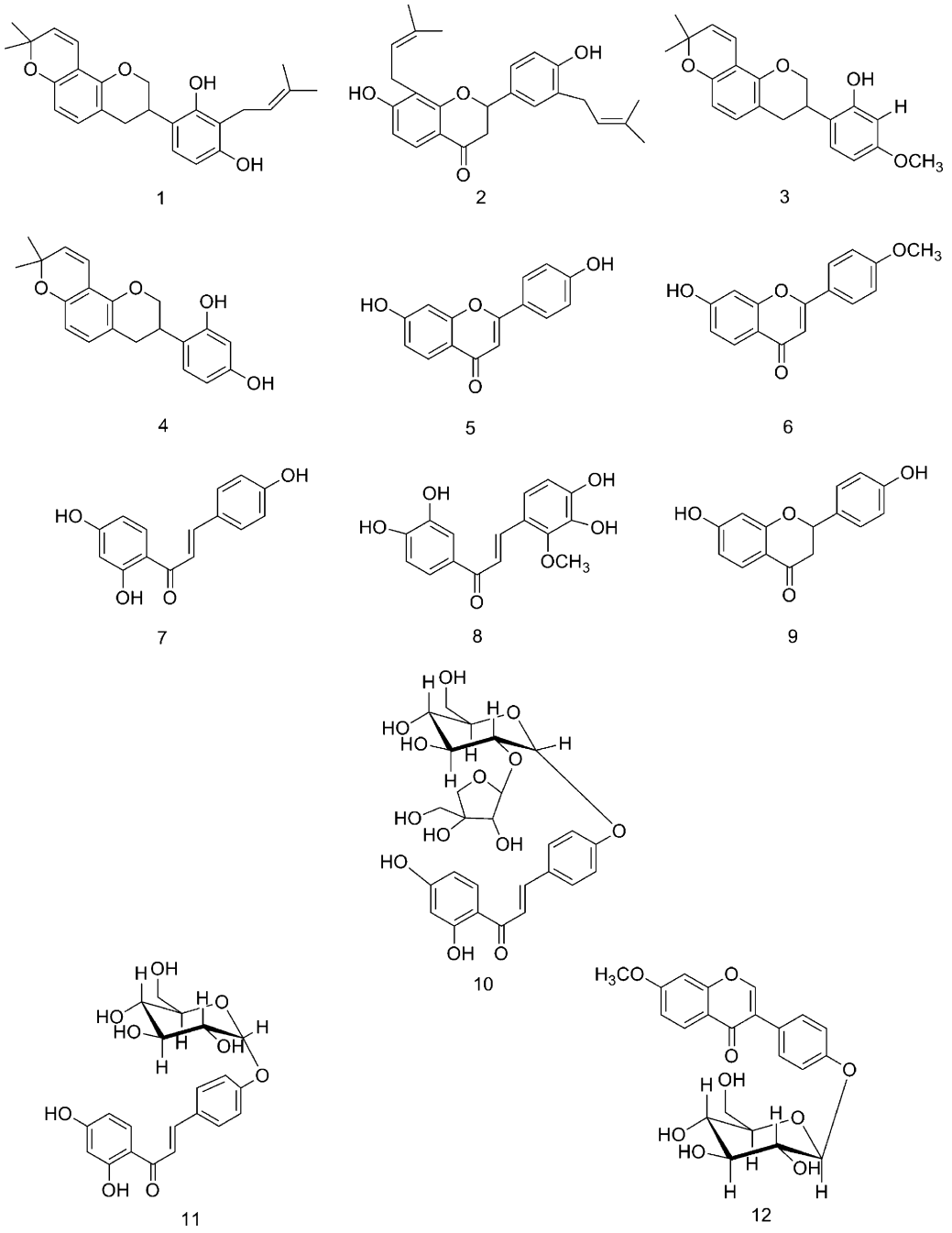

Fig. 1. Structures of compounds isolated from *G. glabra* roots.

tory (Birari et al., 2009). Orlistat was used as a standard in the assay. For bioactivity assay, all studies were done in triplicate, and mean values and standard error of mean were calculated.

Computational study

In order to understand the binding affinity of the compound with the PL enzyme, compounds **3**, **4**, **7**, **8**, **10** and **11** were subjected for docking analysis using FlexX program. These compounds were initially built on SYBYL7.1 and energy minimized by Powell's method using Tripos force field with 0.05 kcal/mol energy gradient convergence criterion. FlexX is a fast, flexible docking method that uses an incremental construction algorithm to place ligands into the active site of the protein. The crystal structure of PL-Colipase complex taken from protein databank (PDB code: 1LPB) was used for docking study, and the active site for docking was defined with the amino acids Ser152, Asp176 and His263 falling within 9.5 Å radius of the co-crystallized ligand methoxyundecylphosphinic acid (Egloff et al., 1995). ADME analysis of all the active compounds was performed by the Discovery Studio 2.5 (Accelrys) software and QikProp module of Schrödinger software.

Induction of obesity and hyperlipidemia

After 7 days of acclimation, animals were randomly divided into 8 groups ($n = 6$): one normal control group, one HFD control and remaining 3–8 as treatment groups. Animals in normal control group were fed with normal pellet diet (NPD) while the other groups were fed with HFD (Srinivasan et al., 2005; Gaikwad et al., 2007) *ad libitum*, respectively, throughout the experiment.

Table 1
PL inhibitory activity and docking analysis of the isolated compounds of *G. glabra* roots.

Compounds	Percent inhibition at 250 μg/ml	IC_{50} (μM)	Docking score (kcal/mol)	Residues making hydrogen bonding
1	43.8 ± 2.4	ND	ND	ND
2	59.3 ± 1.9	ND	ND	ND
3	61.4 ± 1.8	404.9	−10.3	Arg256, Trp114
4	41.3 ± 2.9	485.6	−14.7	Arg256, Ser152, His263
5	3.3 ± 1.6	ND	ND	ND
6	2.7 ± 1.1	ND	ND	ND
7	99.9 ± 1.9	7.3	−23.7	His263, Phe215, Asp79, Arg256
8	98.8 ± 2.4	35.5	−17.1	Asp79, Arg256, Asp257, Phe215
9	2.0 ± 3.1	ND	ND	ND
10	93.3 ± 2.8	14.9	−21.9	Gly76, Asp79, His263, His151, Ser152, Cys181, Thr115
11	98.2 ± 2.1	37.6	−17.4	Asp79, Ser152, His263, His75, Cys181
12	6.9 ± 3.0	ND	ND	ND

ND, not determined.

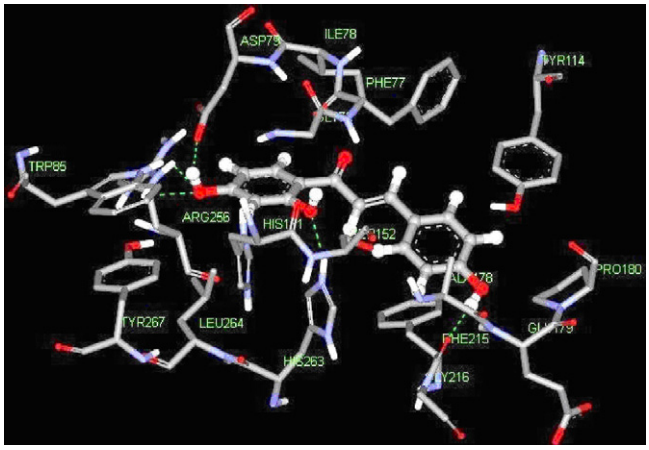

Fig. 2. Molecular docking derived binding pose of the most potent compound **7** in PL enzyme. The inhibitor is shown as ball and stick model and FlexX software was used to derive the binding mode. Compound **7** forms one strong hydrogen bond with the hydrogen atom of the imidazole ring of His263, two strong hydrogen bonds with the guanidine hydrogen atoms of Arg256, one hydrogen bond with the delta oxygen atom (O_δ) of Asp79 and one hydrogen bond with amide bond carbonyl oxygen (C=O) atom of Phe215.

Treatment

Treatments were started from 29th day and continued for two weeks. The treatment groups 3–5 were given the DCM, EtOAc and MeOH extracts of *G. glabra* respectively at 300 mg/kg/day of body weight by oral root. Treatment groups 6–8 were given compounds **7**, **10** and standard drug orlistat respectively at 30 mg/kg/day of body weight by oral root. During the course of treatment the treatment groups were continued to feed with HFD.

Determination of body weight and food intake

The body weight of each rat was measured once each week and the total amount of food consumed was recorded 3 times per week.

Determination of lipid profile

Blood samples were collected from tail vein of the rats into micro centrifuge tubes containing heparin (10 μl, 1000 IU ml^{-1}). The plasma was separated by centrifugation (10 min, 10,000 rpm) and was analyzed for plasma glucose, TG and TC using commercially available colorimetric diagnostic kits.

Statistical analysis

Statistical analysis was carried out by using commercially available software sigmastat 3.5. Values are expressed as mean ± SEM. For multiple comparisons, one way ANOVA was used followed by Tuckey and/Dunnet's test. p value < 0.05 was considered to be significant.

Results and discussion

G. glabra is a rich source of phenolic class of compounds. Four flavan types of constituents (**1–4**) were isolated from DCM fraction of acetone extract. Bioactivity-guided fractionation of the EtOAc extract of *G. glabra*, led to the isolation of eight known secondary metabolites (**5–12**). The compounds (Fig. 1) were identified by comparison of their physicochemical and spectroscopic data (^1H, ^{13}C NMR, 2D NMR and Mass Spectrometry) with those of literature data as, Hispaglabridin A (**1**), Glabrol (**2**), 4′-O-methoxy glabridin (**3**), Glabridin (**4**) (Vaya et al., 1997), 4′,7-dihydroxy flavone (**5**), 7-hydroxy-4′-methoxy flavone (**6**) (Yoo et al., 2004), Isoliquiritigenin (**7**) (Ma et al., 2005), 3,3′,4,4′-tetrahydroxy-2-methoxychalcone (**8**) (Nowakowska, 2007), Liquiritigenin (**9**), Licuroside (**10**), Isoliquiritoside (**11**) (Hatano et al., 1998) and Isoononin (**12**) (Jayaprakasam et al., 2009) respectively.

The PL inhibitory activities of the all the compounds were calculated as percent inhibition at 250 μg/ml (Table 1). Six compounds inhibited PL activity in a dose-dependent manner. Amongst, the compounds tested, **7**, **8**, **10** and **11** exhibited the strong inhibitory activities, with IC_{50} values of 7.3 ± 0.7 μM, 35.5 ± 0.5 μM, 14.9 ± 0.8 μM and 37.6 ± 0.7 μM, respectively. Interestingly, all

Table 2
ADME properties of active compounds showing PL inhibition.

Compound no.	ASL	A log P	PSA	log S	%H_O_Abs	log D	MWt	HBA	HBD	Rot_Bond
3	2	4.5	41.2	−5.3	100	4.2	338.4	3	1	2
4	2	3.7	55.6	−4.8	100	3.7	324.4	3	2	2
7	3	1.9	90.4	−3.0	77	1.9	256.2	3	2	7
8	3	1.0	116.4	−2.7	66	1.9	302.3	6	4	9
10	3	−1.4	236.7	−3.6	0	−0.4	550.5	17	7	16
11	3	−0.1	68.9	−2.7	33	0.6	418.4	12	5	13

ASL, ADMET_solubility_level; PSA, polar surface area; %H_O_Abs, percent human oral absorption; MWt, molecular weight; HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; Rot_Bond, number of rotatable bonds.

Table 3
Effects of HFD on body weight.

Treatment groups[a]	Weights (g)			
	Initial weight	28th Day	43rd Day	Weight gain during treatment
Normal control	176.0 ± 4.9	271.2 ± 2.5	307.4 ± 2.2	36.2 ± 0.8[b]
HFD control	163.8 ± 2.6	323.4 ± 6.3	387.6 ± 6.1	64.2 ± 0.5[c]
GG-DCM	164.6 ± 2.1	306.6 ± 6.3	323.8 ± 8.4	17.2 ± 2.4[c]
GG-EtOAc	168.2 ± 5.4	329.8 ± 10.5	343.4 ± 12.8	13.6 ± 3.5[c]
GG-MeOH	172.4 ± 3.0	322.6 ± 10.3	345.2 ± 11.3	22.6 ± 4.8[c]
10	174.2 ± 9.1	322.0 ± 7.0	350.2 ± 6.2	28.2 ± 1.6[c]
7	167.8 ± 7.8	308.8 ± 2.3	332.0 ± 4.7	23.2 ± 3.6[c]
Orlistat	168.4 ± 7.4	321.4 ± 5.8	295.4 ± 11.8	−26.0 ± 10.5[c]

One way ANOVA followed by Tuckey's multiple comparison test.
[a] $n = 6$ per group; values are expressed in mean ± SEM.
[b] $p < 0.05$.
[c] $p < 0.001$.

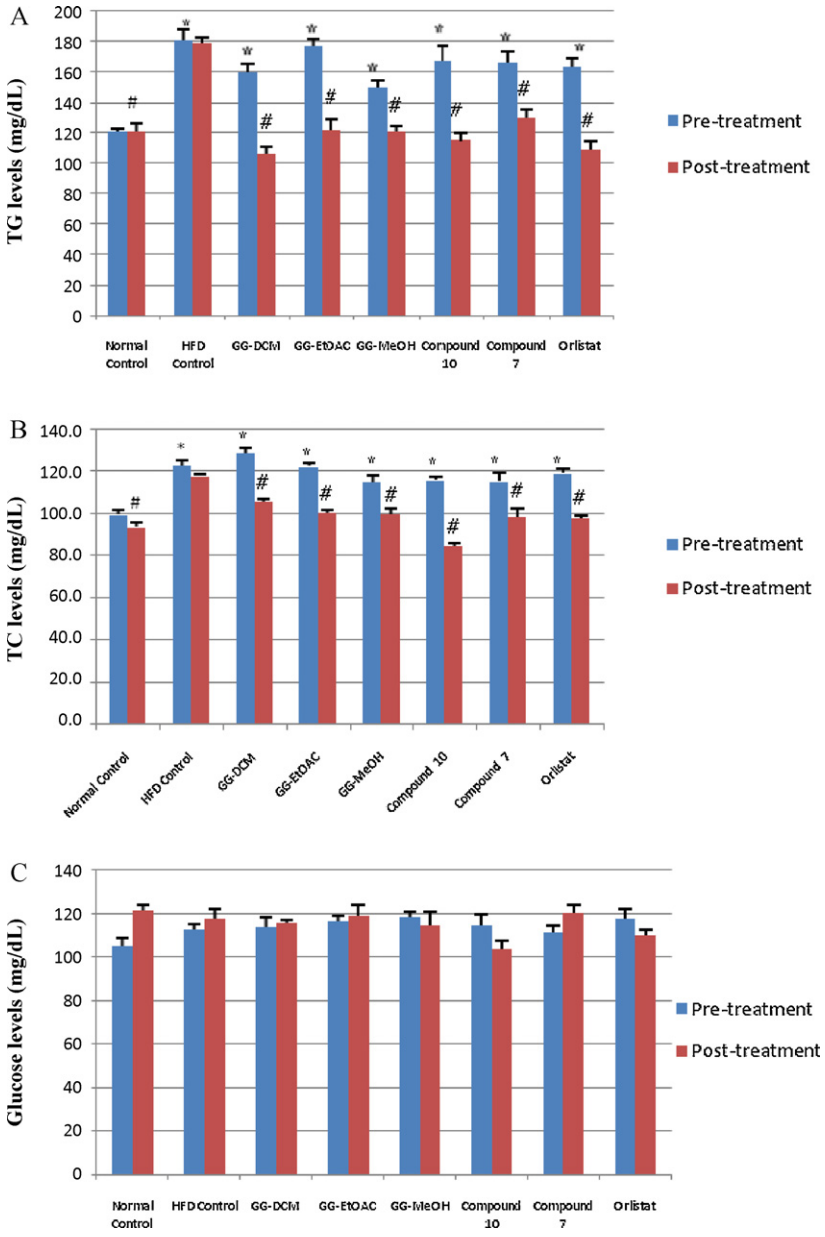

Fig. 3. Effects of treatment groups on the lipid profile of the HFD fed male SD rats. (A) Significant decrease in levels of total TG was observed in post-treatment groups as compared to normal and HFD control. (B) Significant decrease in levels of TC was observed in post-treatment groups as compared to normal and HFD control. (C) No significant effects were observed in blood glucose levels of pretreatment and post-treatment groups. $n = 6$ per group, values are expressed in mean ± SEM. $^{*}p < 0.05$ when compared with the normal control in pretreatment groups, $^{\#}p < 0.05$ when compared with the HFD control in post treatment groups.

these compounds are chalcone class of compounds, which shows the potential of this type of naturally occurring scaffold for the PL inhibition. Orlistat was found to be a potent inhibitor of PL with calculated IC_{50} value of 0.015 ± 0.1 μM. The flavan class of compounds (**1–4**) moderately inhibited the PL, while the flavone and dihydroflavone aglycones (**5**, **6** and **9**) and flavone glycoside (**12**) showed a weak PL inhibition.

Molecular docking was performed on compounds **3**, **4**, **7**, **8**, **10** and **11** to understand the affinity and mode of binding with the PL enzyme active site residues. Docking analysis showed a high binding potential of compound **7** towards the active site of PL with total docking score of −23.7 kcal/mol. The docking score of other compounds **3**, **4**, **8**, **10** and **11** along with the hydrogen bonding interaction residues are illustrated in Table 1. In human PL, N-terminal domain residues Ser152, Asp176, and His263 form the catalytic triad while C-terminal domain binds to co-lipase, the cofactor required for the activity (Luthi-Peng et al., 1992). To achieve an active conformation, the open lid structure of the PL requires the interactions of Arg257 (Arg256 in PDB: 1LPB) and Asp258 (Asp257 in PDB: 1LPB) with the core residues (Lowe, 2002). Any disruption of the interactions with these residues prevents the lid from attaining an optimal conformation. From the docking pose of ligand as shown in Fig. 2 within the PL enzyme active site, it is clearly observed that compound **7** formed strong hydrogen bonding interactions with the active site amino acid residues of the enzyme (Table 1). The compounds binding to these catalytic and other nearby residues are expected to play an important role in PL inhibition (Luthi-Peng et al., 1992; Lowe, 2002), supporting the stronger PL inhibitory activity of the compound **7** in our *in vitro* assay (Table 1). Interestingly, the trend of variation of the PL inhibitory activity (IC_{50}) and docking score of these compounds was excellent, and highly significant correlation coefficient of 0.85 was obtained.

It is well known that the poor pharmacokinetic properties are one of the main reasons for terminating the development of drug candidates in advance stages. Hence, all the active compounds were subjected for *in silico* ADME evaluation. Specifically, some key parameters along with the Lipinski's rule of five (Lipinski et al., 2001) that are examined as part of multiple property optimizations are molecular weight (MW), octanol/water partition coefficient ($\log p$), computed aqueous solubility ($\log S$) and polar surface area (PSA). All the active compounds (except compounds **10** and **11**, due to sugar moieties) followed the entire Lipinski parameters (Table 2). In the particular case of the PL inhibitors like Orlistat, the site of action is in the lumen and thus systemic absorption is not needed for activity. Orlistat has minimal systemic absorption (Zhi et al., 1996). Interestingly, *in silico* analysis of the most active compound **7** showed good oral bioavailability. Though there is no advantage of good oral bioavailability of compound **7** inhibiting the PL, it may result in lesser activity *in vivo* as it will get absorbed in the body and will not be fully available for the action on PL. Its diglycoside, licuroside (compound **10**), which also strongly inhibited PL *in vitro*, showed minimal or no absorption in the *in silico* analysis (Table 2). This indicates that chemical modifications of compound **7** may provide stronger PL inhibitor with necessary pharmacokinetic properties.

On the basis of results obtained from *in vitro* PL inhibitory assay as well as the predictions from *in silico* analysis, we performed animal studies to further prove the antiobesity effects of compound **7** and **10**. Table 3 shows a significant increase in the body weight in the HFD fed groups as compared to the normal group receiving only NPD before treatment (first 28 days). Interestingly, continuous supplementation of the DCM, EtOAc, MeOH extracts and the compounds (**7** and **10**), for two weeks, considerably decreased the weight gain as compared to the HFD control group. In the rats supplemented with compound **7** the body weight increase was only 23.2 ± 3.6 g as compared to 64.2 ± 0.5 g in the HFD control group while in the rats treated with compound **10** showed 23.2 ± 3.6 g weight gain only. Fig. 3 shows the plasma TG, TC and glucose levels of the experimental animals. Significant increase in the levels of plasma TG and TC levels were observed in the HFD fed groups for the initial 28 days while no significant effects on plasma glucose levels were observed. After the treatment, however, the plasma TG and TC levels were considerably decreased in the treatment groups while glucose levels did not changed significantly. Compound **7** decreased the levels of plasma TC to 84.6 ± 1.4 mg/dl and plasma TG to 128.8 ± 6.0 mg/dl. Compound **10** also lowered the plasma TC and TG levels considerably. Neither of these compounds altered the plasma glucose levels significantly suggesting that they may not have any effect on glucose metabolism. This indicates that the extracts as well as the compounds do not have hypoglycemic or hyperglycemic effects in the treated animals. These results clearly suggest that these extracts/compounds have definite effect on lipid metabolism and their effects may be due to inhibition of PL reducing the intestinal absorption of dietary fats.

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