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Original Article

Pharmacology Section

Anti-hypertriglyceridemic Activity of *Cornus Mas* in Diabetic Rats

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ABSTRACT

Introduction: Hypertriglyceridemia is among the multiple metabolic derangements seen in diabetes mellitus. Cornelian cherry *(Cornus mas* L.), belongs to the family Cornacea and has been shown to be helpful in treating hyperlipidemia.

Aim: The study investigates mechanisms of action of dietary Cornelian Cherry fruit Dried Powder (CCDP) for ameliorating hypertriglyceridemia in diabetic rats.

Materials and Methods: An experimental study with parallel controls was performed. Fifty six male adult rats were randomly assigned into 8 equal groups and treated as follows for 4 weeks. Negative control: Normal rats, basic diet; positive control: streptozotocin-induced diabetic rats, basic diet; T1 to T4 groups: diabetic rats fed with diets containing 0.25, 0.5, 1 and 2 g/ 100 g $_{BW}$ CCDP, respectively; T5: diabetic rats fed with basic diet plus 100 mg/kg $_{BW}$ of fenofibrate in drinking water and T6: normal rats fed with basic diet containing 1 g/100 g $_{BW}$ CCDP. Selected serum biochemical parameters, Lipoprotein Lipase (LPL) level as well as Adipose Tissue Hormone Sensitive Lipase (HSL) and Hepatic Peroxisome Proliferator-Activated Receptor Alpha (PPARα) levels were assayed. Analysis of data was performed by one-way ANOVA followed by Tukey's test with $p<0.05$ as the significant level.

Results: Treatment with CCDP at all dosages as well as fenofibrate decreased serum triglycerides and VLDL levels as compared to positive control. Serum AST significantly decreased in T1, T3 and T4 groups as compared to positive control rats. Serum LPL levels in the diabetic positive control group decreased significantly as compared to negative control. Rats in T1, T2, T4 and T5 groups showed increased serum LPL levels as compared to positive control. No significant difference was observed in hepatic PPARα levels among CCDP-treated and negative or positive controls. Positive control rats showed a significant decrease in adipose tissue HSL levels. Administration of CCDP in T3 group significantly increased HSL values as compared to positive control. CCDP in healthy rats did not change any of these parameters.

Conclusion: Findings of the present study confirms positive effects of *C. mas* fruit on some liver function enzymes and hypertriglyceridemia and clarifies that increased serum LPL levels is at least partly responsible for hypotriglyceridemic effect of the fruit in rats with STZ-induced diabetes mellitus.

Keywords: Cornelian cherry, Diabetes mellitus, Triglycerides

INTRODUCTION

Diabetes mellitus is one of the most commonly encountered metabolic diseases in many parts of the world and is considered as an important health issue due to its relatively high prevalence and severe complications [1]. Hypertriglyceridemia is among the common metabolic derangements seen in people with type 2 diabetes [2] and also may be a complication of uncontrolled type 1 diabetes [3]. Hypertriglyceridemia causes atherosclerosis when Triglyceride (TG) levels are markedly increased [2]. Changes in the activity of enzymes involved in the maintenance of TGs homeostasis has a major role in this regard; for example increased lipolysis by HSL in the adipocytes due to lack of insulin synthesis or response in diabetes leads to increased release of fatty acids from adipocytes which increases VLDL synthesis in liver and consequently its higher concentration in bloodstream. Moreover, reduced scavenging of VLDL in type 1 diabetes occurs due to LPL dependency on insulin [4]. Peroxisome PPARα that regulates transcription of genes involved in lipid metabolism is also very important. PPARα activation leads to an increase in production of LPL associated with capillary endothelial cells in adipose tissue and consequently higher scavenging of VLDL. On the other hand, activation of PPARα in the liver stimulates oxidation of fatty acids, which reduces TG reserves and VLDL synthesis [5].

Although different classes of medications including Fibrates, Niacin, Statins *etc*., are currently used in the management of major TG elevations, the outcome is still far from perfect and sometimes the side effects are considerable. For instance, Fibrates as agonists of PPARα, decrease TG levels, however clinical trial results have been unconvincing with regards to their benefit [6] and consumption of Niacin has been associated with increased insulin resistance [2].

Therefore, finding safe and multifaceted agents with the potential to be used at least as adjunct therapy or included in the diet is persuaded. Cornelian cherry, botanically named *Cornus mas* L., belongs to the family Cornacea and the order Cornales. This plant grows in the Northern hemisphere, East Asia, East and North West America, East Africa, and Europe as well as Turkey, Iran, and Azerbaijan [7].

Among different positive health effects, this fruit has been shown to be helpful in treating hyperlipidemia [8]. In a small clinical trial by Soltani et al., daily consumption of the fruit extract reduced serum TG level in type 2 diabetic adult patients [9]. This motivated us to investigate the action mechanism of *C. mas* in ameliorating hypertriglyceridemia in diabetes with regard to serum LPL, adipose tissue HSL and hepatic PPARα levels in diabetic rats.

MATERIALS AND METHODS

An experimental study with parallel controls was accomplished at Shahrekord University of Medical Sciences (Shahrekord, Iran) and School of Veterinary Medicine, Shiraz University (Shiraz, Iran) from August 2015 to December 2016.

Preparation of Cornelian Cherry Dried Powder (CCDP)

Cornelian cherry fruits were bought from the market in August-September, 2015. The quality, genus and species were confirmed by a botanist and a sample is available in the Medicinal Plants Research Herbarium of the Shahrekord University of Medical Sciences (herbarium code: 201). Fruits were washed and dried by a freeze dryer system. The fruit powder was then mixed with ground standard feed of different groups.

Phytochemical evaluation: Folin-Ciocalceu reagent and colorimetric method were used to measure the amounts of phenolic compounds and flavonoids (in terms of rutin) in *C. mas* extract [10,11].

Total anthocyanin amount in *C. mas* was measured using a spectrophotometric differential pH method and total anthocyanin content was expressed in mg cyanidin 3-glucoside/mL [12].

The 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) and Reducing Power Assay (RPA) were used for measurement of antioxidant activity of this fruit [13] which was expressed as IC_{50} that indicates the concentration of compound that causes 50% inhibition in oxidant capacity.

Animals and study design: Fifty six male Wistar rats weighing 230±30 g were housed under standard light and temperature conditions with free access to commercial feed and tap water for two weeks in order to adapt. They were then randomly assigned into 8 equal groups and fed with the following dietary regimens for 4 weeks.

Negative control (normal rats, corn-soy based diet as basic diet); positive control (diabetic rats, basic diet), T1 to T4 groups: diabetic rats that were fed with basic diet containing 0.25, 0.5, 1 and 2 g/100 g_{BW} CCDP, respectively; T5 (comparative control): diabetic rats that were fed with basic diet plus 100 mg/kg_{BW} of fenofibrate (Sigma-Aldrich, St. Louis, USA) in drinking water [14] and T6: normal rats that were fed with basic diet containing 1 g/100 g_{BW} CCDP. The analysis of basic diet is presented in [Table/Fig-1].

Humidity (%)	9.9
Dry matter (%)	90.1
Crude fat (%)	3.4
Crude protein (%)	21.5
Crude fiber (%)	3.4
Ash (%)	5.35
NaCl (%)	0.37
Calcium (%)	0.93
Phosphorus (%)	0.73
Nitrogen-free extract (%)	56.4
Total digestible nutrients (%)	75.5
Digestible energy (Kcal/Kg)	3330
Metabolisable energy (Kcal/Kg)	2910

[Table/Fig-1]: The analysis of basic diet.

Diabetes was induced by intraperitoneal (i.p.) Injection of Streptozotocin (STZ) (Sigma Aldrich Co., St Louis, USA) dissolved in 0.1 M cold sodium citrate buffer, pH 4.5, (60 mg/kg body weight) as previously described [15]. Blood sampling by tail clip was performed on day 7 after STZ administration and Fasting Blood Sugar (FBS) level was determined by a glucometer (Biotech Co., Ltd., Hsinchu, Taiwan). Rats with blood glucose level >300 mg/dL were considered diabetic and were included in the study. This procedure was also repeated at the end of the experiment (28 days later).

After 4 weeks, the animals were anesthetized and then euthanized with 10% chloral hydrate (Sigma Aldrich Co., St Louis, USA) (0.3 mL/100 g_{BW}, i.p.) after an overnight fasting. Blood samples were collected from the heart during anesthesia and liver and visceral fat samples were immediately removed and stored at -70°C until analysis.

All procedures used in the present study were in accordance with institutional ethical guidelines for care and use of laboratory animals in experiments which are compatible with European convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Determination of Biochemical Parameters

Sera were harvested by centrifugation of blood samples at 3000 rpm for 10 min. serum concentrations of TG, VLDL, Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), were measured by commercially available kits (ParsAzmoon Co., Iran) in an auto analyser (Hitachi 902, Japan).

Measuring Serum LPL Level

Rat LPL ELISA kit (Zellbio, Germany) was used for measuring LPL. This assay was based on biotin double antibody sandwich technology using anti-rat LPL monoclonal antibody. Absorbance values were read at 450 nm and concentrations were calculated accordingly. The assay range of the kit was 0.1-40 U/L. Intra-assay and inter-assay precision of the kit (CV %) were <10% and <12%, respectively.

Measuring Hepatic PPARα and Adipose Tissue HSL Levels

Cellular membrane disintegration of adipose and hepatic tissue samples was accomplished by liquid nitrogen freeze-thawing procedure. One hundred mg of the tissue was homogenized in 1 mL of PBS (pH=7.4, 100 Mm). The prepared suspension was centrifuged at 4000-6000 rpm for 10 minutes and supernatant was carefully removed. The amounts of PPARα in the liver and adipose tissue HSL were measured by rat sandwich ELISA kits (Zellbio, Germany) at 450 nm. The assay ranges of the kits were 0.5-40 ng/mL (PPARα) and 0.3-90 ng/mL (HSL). Intra-assay and inter-assay precision (CV %) for both kits were <10% and <12%, respectively.

STATISTICAL ANALYSIS

Analysis of data were performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test in SPSS software and the significant level for differences was considered to be $p<0.05$.

RESULTS

Phytochemical Parameters

The assayed amounts were 13.6 mg/g for phenolic compounds, 4.82 mg/g for flavonoids and 15 mg/mL for anthocyanins with IC50 of 1.89 µg/mL.

Biochemical Parameters

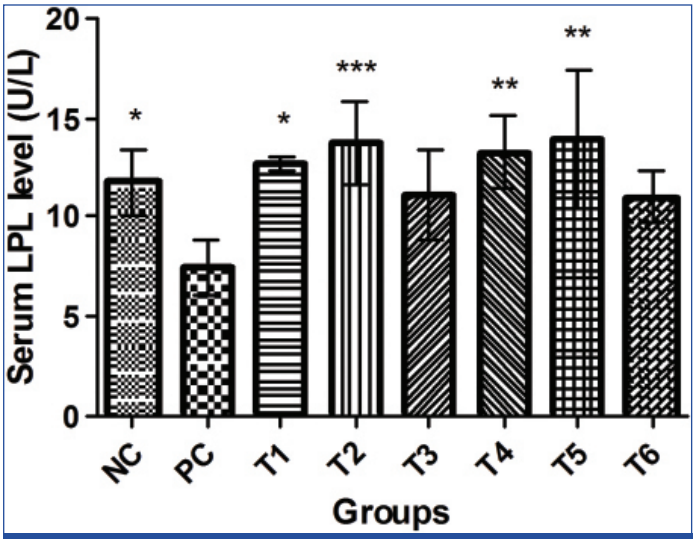
As shown in [Table/Fig-2], serum levels of FBS, TGs, VLDL, ALT, and AST in the diabetic control group increased significantly as compared to negative control rats ($p<0.001$ for all comparisons). Treatment with 0.25, 0.5, 1, and 2 g/100 g BW of CCDP as well as fenofibrate caused a significant decrease in serum TG and VLDL levels in the diabetic rats as compared to positive control animals ($p<0.001$). According to the findings, different dosages of CCDP and fenofibrate caused statistically similar decrease in TG and VLDL levels, but serum TG and VLDL levels in *C. mas*-treated healthy rats did not change as compared to the healthy control rats. CCDP did not significantly change blood glucose level in diabetic or healthy rats ($p>0.05$). CCDP at 0.25, 1, and 2 g/100 g BW as well as fenofibrate caused a significant decrease in AST in the diabetic rats as compared to positive control group ($p<0.001$). CCDP had no effect on AST levels in the healthy rats. The results showed statistically the same effect of fenofibrate and 0.25, 1, and 2 g/100 g BW of CCDP on serum ALT and AST levels in the diabetic rats. CCDP had no significant effect on serum levels of ALT in diabetic rats as compared to positive control as well as normal rats as compared to negative control animals ($p>0.05$).

	NC	PC	T1	T2	T3	T4	T5	T6
FBS (mg/dL)	159±19.0*	506±115	494±45.7	460±100	508±43.2	448±102	501±74.9	147±32.5*
TG (mg/dL)	60.1±13.7*	694±242	123±42.9*	47±10.8*	57.2±18.2*	43.1±8.29*	54.7±9.63*	55.4±12.5*
VLDL (mg/dL)	12.02±2.75*	138±48.5	24.6±8.58*	9.40±2.16*	11.4±3.65*	8.62±1.65*	14.8±7.67*	11.1±2.50*
ALT (U/L)	202±98.2*	627±324	546±59.3	523±312	348±86.3	388±149	134±83.5*	187±51.3*

[Table/Fig-2]: Serum biochemical parameters (mean±SD) of different groups. NC: Negative control (normal rats, basic diet); PC: positive control (diabetic rats, basic diet), T1 to T4 groups: diabetic rats that were fed with basic diet containing 0.25, 0.5, 1 and 2 g/100 g $_{BW}$ CCDP, respectively; T5 (comparative control): diabetic rats that were fed basic diet plus 100 mg/kg $_{BW}$ of fenofibrate and T6: normal rats that were fed with basic diet containing 1 g/100 g $_{BW}$ CCDP.
Asterisk sign is used to demonstrate significant difference with PC group (p<0.05).

Serum LPL Levels

As illustrated in [Table/Fig-3], LPL levels in the diabetic positive control group decreased significantly as compared to negative control group (p=0.021). Treatment with 0.25, 0.5, and 2 g/100 g BW of CCDP and 100 mg/kg of fenofibrate in diabetic rats caused a significant increase in serum LPL as compared to positive control group (p=0.014, p<0.001, p=0.001 and p=0.003, respectively) while CCDP alone had no appreciable effect in healthy rats as compared to negative control animals (p>0.05). No significant difference in LPL level was observed among fenofibrate-treated and CCDP-treated diabetic groups (p>0.05).

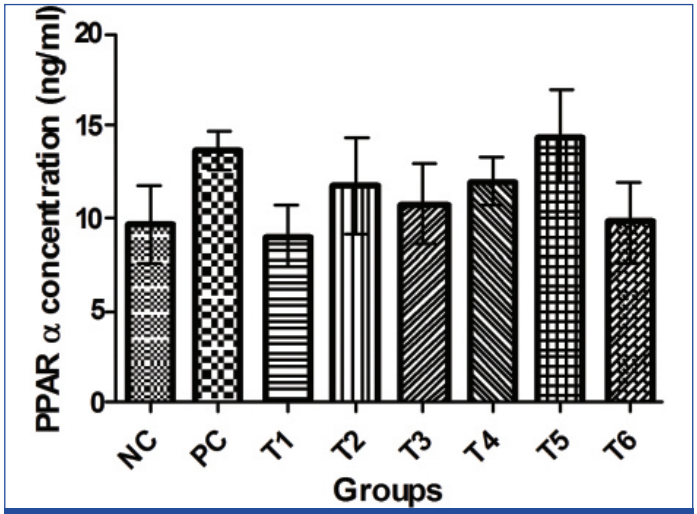


[Table/Fig-3]: Serum lipoprotein lipase (LPL) levels (mean±SD) in different groups. NC: Negative control (normal rats, basic diet); PC: positive control (diabetic rats, basic diet), T1 to T4 groups: diabetic rats that were fed with basic diet containing 0.25, 0.5, 1 and 2 g/100 g $_{BW}$ CCDP, respectively; T5 (comparative control): diabetic rats that were fed basic diet plus 100 mg/kg $_{BW}$ of fenofibrate and T6: normal rats that were fed with basic diet containing 1 g/ 100 g $_{BW}$ CCDP.
Asterisk sign is used to demonstrate significant difference with PC group (*p<0.05, **p<0.01, ***p<0.001).

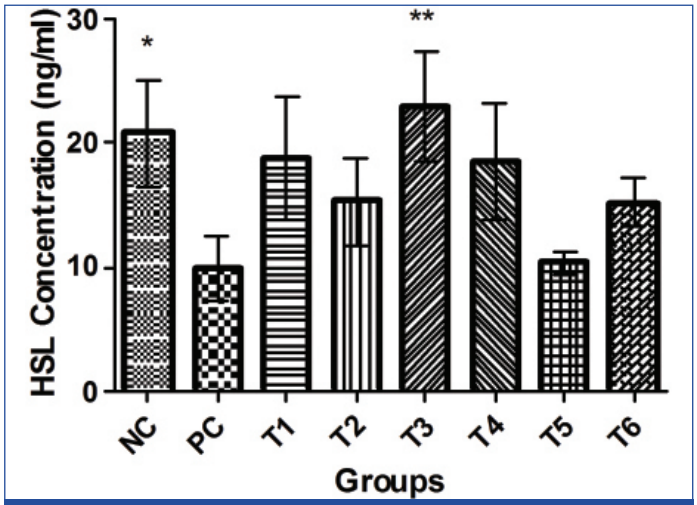
Liver PPARα and Adipose Tissue HSL Levels

[Table/Fig-4] shows liver concentration of PPARα in different groups. Induction of diabetes resulted only in a trivial increase in PPARα level in liver of rats in positive control group as compared to negative control rats (p>0.05). No significant difference was observed among CCDP-treated and negative or positive control rats (p>0.05). Interestingly, fenofibrate administration was associated with an appreciable increase in PPARα concentration as compared to negative control, T1 and T6 animals (p=0.011, p=0.027 and p=0.018, respectively).

As demonstrated in [Table/Fig-5], diabetes was associated with a significant decrease in adipose tissue HSL levels as compared to negative control group (p=0.021). Although administration of CCDP at all four dosages increased HSL value as compared to positive control group, the increase was only significant in 1 mg/100 g BW (T3) group (p=0.007) and other CCDP-treated groups had statistically the same values of HSL as compared to both negative and positive control groups (p>0.05). Fenofibrate administration had no appreciable effect on HSL values as compared to positive control rats (p>0.05) and rats in this group showed significant difference with negative control group (p=0.003). CCDP alone did not significantly change HSL level as compared to negative control group (p>0.05).



[Table/Fig-4]: Concentrations of peroxisome proliferator-activated receptor α (PPARα) (mean±SD) in liver of different groups. NC: Negative control (normal rats, basic diet); PC: Positive Control (diabetic rats, basic diet), T1 to T4 groups: diabetic rats that were fed with basic diet containing 0.25, 0.5, 1 and 2 g/100 g $_{BW}$ CCDP, respectively; T5 (comparative control): diabetic rats that were fed basic diet plus 100 mg/kg $_{BW}$ of fenofibrate and T6: normal rats that were fed with basic diet containing 1 g/100 g BW CCDP.
No significant difference was observed between PC rats and other groups (p>0.05).



[Table/Fig-5]: Concentrations of Hormone-Sensitive Lipase (HSL) (mean±SD) in adipose tissue of different groups. NC: Negative control (normal rats, basic diet); PC: positive control (diabetic rats, basic diet), T1 to T4 groups: diabetic rats that were fed with basic diet containing 0.25, 0.5, 1 and 2 g/100 g $_{BW}$ CCDP, respectively; T5 (comparative control): diabetic rats that were fed basic diet plus 100 mg/kg $_{BW}$ of fenofibrate and T6: normal rats that were fed with basic diet containing 1 g/100 g $_{BW}$ CCDP.
Asterisk sign is used to demonstrate significant difference with PC group (*p<0.05, **p<0.01).

DISCUSSION

Hypertriglyceridemia is a metabolic derangement in type 2 diabetes [2] and also may be a complication of uncontrolled type 1 diabetes [3]. Unfortunately, currently available hypolipidemic drugs are associated with relatively serious side effects and do not consistently keep the normolipidemic states nor completely prevent late complications of

diabetic hyperlipidemia [16]. With alarmingly increased prevalence of diabetes and associated costs, interest in alternative therapies including medicinal plants has grown extensively [17]. Chemical compounds isolated from medicinal plants provide opportunities to further develop therapeutic strategies. Cornelian cherry fruits have various nutritional and medicinal benefits [18]. The fruit is rich in anthocyanins, phenolic and flavonoid compounds, the amounts of which depend on plant genotype, weather and geographical conditions as well as fruit ripeness [19]. As previously stated in the current study, the amounts of these main chemical compounds in our samples were relatively high with 13.6 mg/g (phenolic compounds), 4.82 mg/g (flavonoids) and 15 mg/mL (anthocyanins) with antioxidant capacity (IC50) of 1.89 µg/mL. Other researchers have reported different values; for example Pawlowska AM et al., reported that the amount of flavonoids present in *C. mas* gathered in Italy in September was 22.1 mg/g [20]. A study by Tural S et al., demonstrated that the amounts of phenolic compounds and anthocyanins, as well as antioxidant capacity of *C. mas* gathered in Turkey in September were 4.37 mg/g, 1.97 mg/g, and 0.5 mg/ml, respectively [21]. The relative inconsistency in these findings is probably due to the effect of the region and time of gathering the samples.

It has been demonstrated that in STZ-induced diabetic rats triglyceride levels of liver and kidney shows a significant reduction which may be due to an increased mobilisation of lipids from these tissues or a decrease in fatty acid uptake and storage capacity that increase serum TGs [22]. This can describe changes in serum TGs and VLDL levels in diabetic rats as observed in the current study.

In this study, administration of 0.25, 0.5, 1, and 2 g/100 g BW of CCDP caused significant decrease in serum VLDL and TGs levels as compared to positive control rats. This is consistent with the study performed by Rafieian-Kopaei M et al., where administration of cornelian cherry in diet resulted in a reduction of TGs levels of atherosclerotic rabbits [23]. Unfortunately the mechanism of this effect was not investigated by these authors.

Lipoprotein lipase has a pivotal role in lipid metabolism. It hydrolyzes TGs in lipoprotein particles and provides fatty acids for peripheral tissues. Serum level of preheparin LPL reflects LPL production mainly in adipocytes [24] and correlates negatively with serum TG and positively with High-Density Lipoprotein-Cholesterol (HDL-C) [25]. Decreased activity of LPL is important in diabetic dyslipidemia [26]. In the present study, STZ-induced diabetes resulted in a significant decrease in serum LPL level which was reversed by fenofibrate administration. It is well established that PPARα and PPARγ agonists are transcriptional regulators of LPL expression [27] which describes the effect of fenofibrate (a PPARα agonist) on LPL level in our study. Interestingly, treatment of diabetic rats with CCDP at all four dosages also reversed this change. Therefore, increased LPL level may be considered as a mechanism for hypotriglyceridemic activity of cornelian cherry. Increased expression of LPL has also been previously described as a hypolipidemic activity of herbs; for instance Chao CY et al., showed that wild bitter gourd extract up-regulates mRNA expression of LPL in the epididymal adipose tissue of mice [28].

In vitro investigations have indicated that flavonoids increase expression of LPL in adipose tissue and muscle cells [29]. Besides, high doses of phenolic compounds have been demonstrated to prevent hyperlipidemia in rats through significant increase in LPL activity [30]. Therefore, effect of CCDP on LPL can be explained by the presence of flavonoids and poly phenolic compounds in this fruit.

PPARα is highly expressed in liver and its activation is associated with increased hepatic lipid uptake and oxidation [31]. As previously stated in our study hepatic PPARα level only showed a tendency to increase in diabetic rats as compared to negative control animals (p=0.092) and neither CCDP nor fenofibrate appreciably affected this parameter as compared to positive control rats, although hepatic PPARα levels were slightly lower in CCDP groups. Studies that have investigated hepatic PPARα levels in diabetic models are scarce. In a very recent study, Tong J et al., reported that treating HepG2 cells with glucose increases PPARα mRNA levels which is down regulated by a fraction of *Cichorium glandulosum* seed extract [32]. This report may be at least partially consistent with our results although different methods and study conditions may be important considerations.

Hormone sensitive lipase is an intracellular lipase enzyme which is highly expressed in adipose tissue and is capable of hydrolyzing triacylglycerols, diacylglycerols, monoacylglycerols, and cholesteryl esters, as well as other lipid and water soluble substrates [33]. We did not find a study that had evaluated the HSL protein concentration in insulin deficient models; however previous studies show that HSL protein and mRNA expression is decreased in adipose tissues of humans with insulin resistant states [34]. HSL activity is acutely controlled by reversible phosphorylation which is antagonized by insulin [35]. Therefore, HSL activity increases in insulin deficiency which at least hypothetically can lead to reduced HSL mRNA and protein expression. This can describe the reduction which was observed in adipose tissue HSL protein level of diabetic rats of our study, although it remains to be confirmed in future studies. Administration of CCDP increased HSL level especially in rats treated with 1 g/100 g BW (T3). This may be related to a change in insulin level which unfortunately was not assayed in our study.

Toxic and destructive effects of STZ have not only been detected in pancreatic islet beta cells but may also involve other organs such as the liver [36]. Liver is one of the main targets of insulin action and plays an important role in maintaining and stabilizing blood glucose levels [37]. Changes observed in the liver are not exclusively due to toxic effects of STZ; rather, they are induced mainly by complications due to diabetes. STZ causes hepatotoxicity via producing free radicals and subsequently lipids peroxidation in hepatocytes membrane [38].

Increased activity of plasma ALT and AST is most probably due to liver dysfunction [39] and leakage of these enzymes into the bloodstream during diabetes [40]. ALT and AST are good markers to measure the rate of hepatocytes damage [41]. In the early steps of liver destruction, hepatocytes cytoplasmic enzymes probably leak from the cells into the bloodstream and membrane permeability increases [42]. Moreover, increased catabolism of proteins alongside gluconeogenesis and urea production that occur in diabetes is probably responsible for increase in these transaminases in the blood. In addition, because insulin suppresses gluconeogenetic enzyme-producing genes and ALT is a gluconeogenic enzyme, the production of this enzyme increases during diabetes when insulin signaling becomes defective. This condition can be even unrelated to liver injury [43].

In the present study, two liver enzymes, i.e., ALT and AST, increased significantly in the diabetic rats as compared to the negative control group. Treatment with CCDP improved serum levels of AST. Consistently, it has been previously shown that administration of hydro alcoholic extract of cornelian cherry fruit decreases serum ALT and AST levels in diabetic rats [44].

LIMITATION

Possible effects on the function of the enzymes which are involved in metabolism of TGs, apolipoprotein synthesis and metabolism, TG absorption from gastrointestinal tract etc., are main determinants that could be considered as potential mechanisms of the hypotriglyceridemic activity of *C. mas* which are not assayed in the current study and remain to be evaluated in future.

CONCLUSION

In conclusion, findings of the present study confirms positive effects of *C. mas* fruit on some liver function enzymes and hypertriglyceridemia and clarifies that increased serum LPL levels is at least partly responsible for hypotriglyceridemic effect of the fruit in rats with STZ-induced diabetes mellitus.

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