# Ursolic acid and luteolin-7-glucoside improves rat plasma lipid profile and increases liver glycogen content through glycogen synthase kinase-3


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Original paper

**Title:** Ursolic acid and luteolin-7-glucoside improves rat plasma lipid profile and increases liver glycogen content through glycogen synthase kinase-3

**Short title:** UA and L7G improves lipid profile and increases liver glycogen

**Authors:** Marisa F. Azevedo[1], Çagri Camsari[1,a], Carla M. Sá[1], Cristovao F. Lima[2], Manuel Fernandes-Ferreira[2], Cristina Pereira-Wilson[1,*]

**Affiliations:**

[1] CBMA– Centre of Molecular and Environmental Biology/Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

[2] CITAB – Centre for the Research and Technology of Agro-Environmental and Biological Sciences/Department of Biology, University of Minho, 4710-057 Braga, Portugal.

* Author to whom correspondence should be addressed [Phone: +351 253604318; Fax +351 253678980; e-mail: cpereira@bio.uminho.pt].

[a] Present address: Department of Physiology, Faculty of Veterinary Medicine, University of Ankara, 06110 Ankara, Turkey.

## Abstract

In the present study, two phytochemicals - ursolic acid (UA) and luteolin-7-glucoside (L7G) - were assessed *in vivo* in healthy rats regarding effects on plasma glucose and lipid profile (total cholesterol, HDL and LDL), as well as liver glycogen content, in view of their importance in the aetiology of diabetes and associated complications. Both UA and L7G significantly decreased plasma glucose concentration. UA also significantly increased liver glycogen levels accompanied by phosphorylation of glycogen synthase kinase-3 (GSK3). The increase in glycogen deposition induced by UA (mediated by GSK3) could have contributed for the lower plasma glucose levels observed. Both compounds significantly lowered total plasma cholesterol and low-density lipoprotein levels, and in addition UA increased plasma high-density lipoprotein levels. Our results show that particularly UA may be useful in preventable strategies for people at risk of developing diabetes and associated cardiovascular complications by improving plasma glucose levels and lipid profile, as well as by promoting liver glycogen deposition.


**Keywords:** Lamiaceae species; ursolic acid; luteolin-7-glucoside; lipid profile; liver glycogen; functional foods.

## INTRODUCTION

Glucose is a major source of energy for humans and its concentration in the blood is tightly regulated by the pancreatic hormone insulin. Diabetes mellitus is a metabolic disorder characterised by chronically elevated blood glucose associated with the impairment of insulin secretion and/or a deficient action on peripheral tissues (Klover

and Mooney, 2004). Once absorbed after a meal, rising plasma glucose stimulates insulin release and, in tissues such as liver and skeletal muscle, glucose uptake and glycogen synthesis is promoted, which constitutes an important mechanism of glycaemic control. In diabetic patients this mechanism of plasma glucose clearance is less efficient, originating hyperglycaemia (Postic *et al.*, 2004).

In addition to hyperglycaemia, diabetic patients often show an abnormal plasma lipid profile with high levels of low-density lipoprotein (LDL) cholesterol and lower than desirable levels of high-density lipoprotein (HDL) cholesterol, a combination implicated in the development of cardiovascular complications (Kastelein, 2005; Mooradian, 2009). Although not totally elucidated, insulin resistance have been implicated in the pathogenesis of diabetic dyslipidemia (Mooradian, 2009). By reducing insulin resistance as well as by lowering plasma glucose and LDL cholesterol on the one hand, and increasing HDL cholesterol levels on the other, an improvement of the diabetic health status may be achieved that would also help prevent cardiovascular complications. The fact that the number of cases of diabetes is increasing worldwide and the realisation that the therapeutic drugs currently in use are not a hundred percent efficient motivates the search for new active principles among natural compounds from medicinal plants. In addition, type 2 diabetes mellitus (T2DM) has been considered a preventable disease through lifestyle changes that includes diet interventions (Costacou and Mayer-Davis, 2003). Natural compounds may be well accepted and considered for the production of enriched added value functional foods and included in dietary strategies for prevention of T2DM and/or health improvement of diabetic patients.

Based on ethnopharmacological data and pharmacological studies, the genus *Salvia* (Lamiaceae family) has been suggested to possess antidiabetic properties

(Baricevic and Bartol, 2000). Previous studies have demonstrated *Salvia officinalis* tea to decrease fasting blood glucose levels in healthy animals (Lima *et al.*, 2006a). Moreover, methanolic and ethanolic extracts of *S. officinalis* as well as an infusion of *S. fruticosa* showed to have hypoglycaemic effects in diabetic experimental animals (Perfumi *et al.*, 1991; Alarcon-Aguilar *et al.*, 2002; Eidi *et al.*, 2005). Luteolin-7-glucoside (L7G) and ursolic acid (UA) (Fig.1) are a flavonoid and a triterpenoid, respectively, present in many plants and particularly abundant in *Salvia* species (Lima *et al.*, 2005; Janicsak *et al.*, 2006), and may contribute to these plants' biological effects (Baricevic and Bartol, 2000).

Many effects attributed to natural compounds, in particular to flavonoids, result from *in vitro* studies, which may not be observed *in vivo* due to biotransformation reactions and low bioavailability. Therefore, it is of great interest to assess possible beneficial effects of isolated natural compounds in *in vivo* studies.

In the present study, the effects of L7G and UA were monitored *in vivo* in healthy rats regarding plasma glucose, plasma lipid profile (total cholesterol, HDL and LDL) and liver glycogen content in view of their importance in the aetiology of diabetes and associated complications. The expression of glycogen synthase kinase-3 (GSK3) in the liver was also measured since it is an important enzyme involved in the insulin sensitive glycogen synthesis pathway (Lee and Kim, 2007).

## MATERIAL AND METHODS

**Chemicals.** Bradford Reagent and ursolic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin-7-*O*-glucoside was purchased from Extrasynthese (Genay, France). Glucofix was purchased from A. Menarini Diagnostics (Firenze, Italy).

Commercial kits to measure total cholesterol, LDL cholesterol and HDL cholesterol were acquired from Spinreact (Girona, Spain). All others reagents were of analytical grade.

**Animals.** Male Wistar rats (6 weeks old) were purchased from Charles River Laboratories (Barcelona, Spain) and acclimated to our laboratory animal facilities for at least one week before the start of the experiment. During the experimental period, animals were maintained on a natural light/dark cycle at 20 ± 2ºC and given food and tap water *ad libitum*. Animals were kept and handled in accordance with our university regulations that follow NIH guidelines (NIH Publication No. 80-23; revised 1978) for the experimental use and care of laboratory animals.

**Experimental design.** Fifteen male Wistar rats were divided into three groups: control; UA-supplemented diet and L7G-supplemented diet. Test compounds were administered orally, once a day, for 7 consecutive days, well mixed in a small piece of food (control group received vehicle only) in a daily dose of 2 mg of compound per kg of animal body weight. The dose of the compound administered was based on estimations of physiological concentrations by Hertog *et al.* (1993). During the experiment water was given *ad libitum* to the animals. The administration of each compound did not change food and beverage consumption, as well as animal body weights when compared to the control. At the end of the treatment, animals were sacrificed by decapitation. Blood samples were collected and plasma kept for the measurement of glucose and lipid parameters (total cholesterol, LDL cholesterol, HDL cholesterol). Liver samples were also collected, frozen in liquid nitrogen and kept at -80ºC for further analyses.

**Quantification of plasma glucose, total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol levels.** The amount of glucose in rat plasma was measured using a colorimetric enzymatic method - Glucofix - following manufacturer's specifications.

The plasma total cholesterol, LDL cholesterol and HDL cholesterol levels were measured in rat plasma using spectrophotometric commercial kits from Spinreact following manufacturer's specifications.

**Liver glycogen quantification.** The liver glycogen content was quantified by the amyloglucosidase method as described by Keppler and Decker (1974). Dilutions of the liver homogenate were used to ensure that the determination was done within the linear phase. Liver glycogen content is expressed in µmol glucose per g of liver.

**Liver homogenates and western blot analysis.** A piece of liver was homogenized in cold lysis buffer (0.5% NP-40 in 50 mM $Na_2HPO_4$, pH 7.4, 150 mM $NaCl_2$, 2 mM EDTA,) containing protease (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin) and phosphatase (20 mM NaF, 20 mM orthovanadate) inhibitors added just before use. The homogenate was then centrifuged at 10,000 ×*g* at 4ºC for 10 min and the supernatant collected. The amount of protein was measured using Bradford Reagent following manufacturer instructions, using BSA as a standard.

For western blot, 25 µg of protein of each sample were separated by SDS-PAGE, transferred onto Hybond-P polyvinylidene difluoride membranes (GE Healthcare, UK) and then blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in

PBS). The membranes were then probed using a rabbit polyclonal antibody to rat phospho-GSK3α/β (Ser21/9) (Cell Signalling Technology, Inc., Danvers, MA, USA), mouse monoclonal antibody to rat total GSK3α/β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse monoclonal anti-β-actin (Sigma-Aldrich, Inc). After incubation with the secondary antibody, immunoreactive bands were developed using a chemiluminescence detection system, the Chemi Doc XRS (BioRad Laboratories, Inc.). Band area intensity was quantified using the Quantity One software from Bio-Rad.

**Statistical Analysis.** Results are expressed as the mean ± standard error of the mean (SEM) for the number of animals in the group. Statistical significances between groups were determined using the one-way ANOVA followed by the Newman-Keuls multiple comparison test. $P$ value < 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

In the present study, we aimed to identify active principles among plants' constituents to be used in dietary approaches that offer low-cost alternatives in interventions to prevent diabetes or limit its progression. For that, two individual constituents (UA and L7G) present in *Salvia* species, plants to which antidiabetic properties are attributed, were used at relatively low concentrations (to mimic the normal intake in the human diet) in order to evaluate *in vivo* effects on plasma glucose, lipid profile and liver glycogen in healthy rats.

After 7 days treatment (with 2 $mg.kg^{-1}.day^{-1}$ of UA or L7G), plasma glucose levels were significantly decreased from 176.1 mg/dl (9.78 mM) in control group to 158.1 mg/dl (8.78 mM) in UA and 157.6 mg/dl (8.76 mM) in L7G (Fig. 2A). A

previous study reported that UA prevents glucose intolerance in high-fat-fed mice (Jayaprakasam *et al.*, 2006), corroborating our data that this triterpenoid may interfere with glucose clearance mechanisms. UA and L7G also increased the glycogen content in liver, which was significant for UA (Fig. 2B). Therefore, the increase in glycogen deposition induced mainly by UA could have contributed for the lower plasma glucose levels observed. Recently, *in vitro* studies performed by Jung *et al.* (2007) in cultured adipocytes and CHO/IR cells showed that UA is an insulin sensitizer, leading to an increase in insulin receptor (IR)*β* auto-phosphorylation and a subsequent activation of downstream phosphatidylinositol 3-kinase (PI3K) signalling pathway activation. The activation of this pathway would result in phosphorylation and inactivation of GSK3, leading to an increase glycogen synthase activation and glycogen synthesis (Lee and Kim, 2007). In agreement with this, the increase of liver glycogen induced by UA observed in the present study was accompanied by an increase of liver phospho-GSK3 expression (Fig. 3), indicating that *in vivo* this compound may also be activating IR and the PI3K signalling. It seems, therefore, that UA may be useful in the prevention of T2DM, and probably in its treatment where patients show a decrease in insulin response due to lower IR and insulin receptor substrate-1-phosphorylation with decreased PI3K activity (Goodyear *et al.*, 1995; Pratipanawatr *et al.*, 2001).

Although to a smaller extent, L7G also induced the phosphorylation of GSK3 ($P = 0.06$, Fig. 3). Flavonoids have shown different effects with respect to the regulation of insulin signalling pathway, which suggest the effects may depend on compounds' concentration and be cell type and context specific. For example, certain flavonoids such as luteolin (the aglycone of L7G), quercetin, apigenin and kaempferol showed inhibitory effects on insulin signalling pathway in mouse adipose cells by suppressing

insulin receptor phosphorylation and subsequent inhibition of PI3K and Akt activation (Nomura *et al.*, 2008). Moreover, in colon cancer cells the flavonoids luteolin and quercetin, as well as the triterpenoid UA, inhibited the PI3K/Akt pathway (Xavier *et al.*, 2009). On the other hand, kaempferol-3-neohesperidoside stimulates glycogen synthesis in rat soleus muscle, possibly through PI3K/GSK3 pathway (Cazarolli *et al.*, 2009). As well, epigallocatechin-gallate showed stimulatory effects on insulin pathway inhibiting gluconeogenesis and GSK3 activity in hepatocytes through the activation of PI3K/Akt-dependent pathway (Waltner-Law *et al.*, 2002; Lin and Lin, 2008).

In addition to plasma glucose, plasma lipids are important predictors of T2DM progression and development of cardiovascular complications. In order to assess possible effects of the UA and L7G on lipid profile, levels of plasma lipids (total cholesterol, LDL and HDL) were measured. As shown in Fig. 4A, total plasma cholesterol levels were significantly reduced by both treatments (by 28.5% with UA and by 29.2% with L7G), which was accompanied by a consistent reduction of plasma LDL levels (by 36.2% and 39.5%, respectively, Fig. 4B). In addition, a 51.1% increase of HDL plasma levels was induced by UA treatment while L7G was not active on this parameter (Fig. 4C). Both UA and L7G increased significantly the HDL/total cholesterol ratio (HTR in %), although effects were more pronounced for UA (Fig. 4D). Thus, both compounds showed relevant effects on the control of plasma lipids, by diminishing total cholesterol and LDL with UA additionally increasing HDL levels. Interestingly, in a pilot study where human volunteers at risk to develop T2DM were treated with *S. officinalis* tea (L7G being the main flavonoid present in the extract) an improvement of lipid profile was also observed by decreasing plasma total cholesterol and LDL while increasing HDL levels (Sá *et al.*, 2009). Statins and fibrates are

pharmaceutical drugs currently used in the treatment of diabetic dyslipidaemia (Kastelein, 2005). Since statins act mainly by reducing LDL cholesterol levels whereas fibrates increase HDL cholesterol levels, combination therapies have been used to control dyslipidaemia and to diminish the risk of cardiovascular diseases and associated morbidity and mortality (Kastelein, 2005). UA administered alone produced this double effect in rats, which warrants future studies in diabetic/obese animals and humans.

Thiazolidinediones (TZDs), also used in the treatment of T2DM patients, act through peroxisome proliferator-activated receptor-γ improving insulin sensitivity and lipid profile (Levetan, 2007; Kersten, 2008). In addition, recently Ciaraldi *et al.* (2006) showed that the TZD troglitazone also inhibited GSK3 in skeletal muscle of obese T2DM patients. In the present study, although probably through different mechanisms, UA was also effective on both the inhibition of GSK3 activity (with a concomitant rise in liver glycogen synthesis) and on the improvement of lipid profile. Since insulin resistance may have a central role in the development of diabetic dyslipidemia (Mooradian, 2009), the possible induction of insulin sensitivity by UA may be also indirectly involved in the improvement of lipid profile.

The effects of UA and L7G, in particular those of UA, suggest, therefore, that these compounds could contribute positively to prevention and also the control of dyslipidaemia and hyperglycaemia observed in T2DM. These beneficial effects of UA and L7G may be assisted by their known effects on the enhancement of cellular antioxidant defences (Lima *et al.*, 2006b; Yin and Chan, 2007; Ramos *et al.*, 2008) known to be overwhelmed by the inherent excess of reactive oxygen species production observed in diabetes.

In conclusion, we observed that L7G showed effects *in vivo* on plasma glucose and lipid profile, whereas UA in addition to these effects also increased liver glycogen deposition and plasma HDL levels. Considering that T2DM and cardiovascular diseases can be prevented through lifestyle changes including dietary strategies, these two phytochemicals may be good candidates for the production of functional foods and food supplements due to their beneficial properties on relevant parameters for the prevention and control of these disorders. In addition, since these compounds are present in many *Salvia* species, they may account for the health benefits attributed to this genus, in particular for its antidiabetic reputation.

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**Abbreviations used**

GSK3, glycogen synthase kinase-3, HDL, high-density lipoprotein; IR, insulin receptor; L7G, luteolin-7-*O*-glucoside; LDL, low-density lipoprotein; PI3K, phosphatidylinositol 3-kinase; T2DM, type 2 diabetes mellitus; TZD, thiazolidinedione, UA, ursolic acid.

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## FIGURE CAPTIONS

**Figure 1.** Chemical structures of the compounds used in this study: the flavonoid luteolin-7-glucoside and the pentacyclic triterpenoid ursolic acid.

**Figure 2.** Effect of 7 days diet supplied with the test compound (UA or L7G) on rat plasma glucose concentration (**A**), and liver glycogen content (**B**). Values are expressed as mean ± SEM ($n$ = 5). $^{**}$, $P < 0.01$ when compared with the control group.

**Figure 3.** Western blot analysis of phospho-GSK3-β protein in liver homogenates of rats (A), with representative immunoblots and corresponding loading control ($\beta$-actin) from a pair of animals from each treatment (B). Values are expressed as mean ± SEM (n = 5). $^{*}$ $P < 0.05$ when compared with the control group. $^{a}$ $P = 0.06$ when compared with the control group (in this case analysed by the Student t-test).

**Figure 4.** Effects of 7 days diet supplied with the test compound (UA or L7G) on rat plasma total cholesterol (**A**), LDL cholesterol (**B**) and HDL cholesterol (**C**). HTR (%) = (HDL/Total cholesterol) × 100. Values are expressed as mean ± SEM ($n$ = 5). $^{***}$, $P < 0.001$; $^{**}$, $P < 0.01$ and $^{*}$, $P < 0.05$ when compared with the control group.

## Conflicts of Interest

The authors have no conflicts of interest.

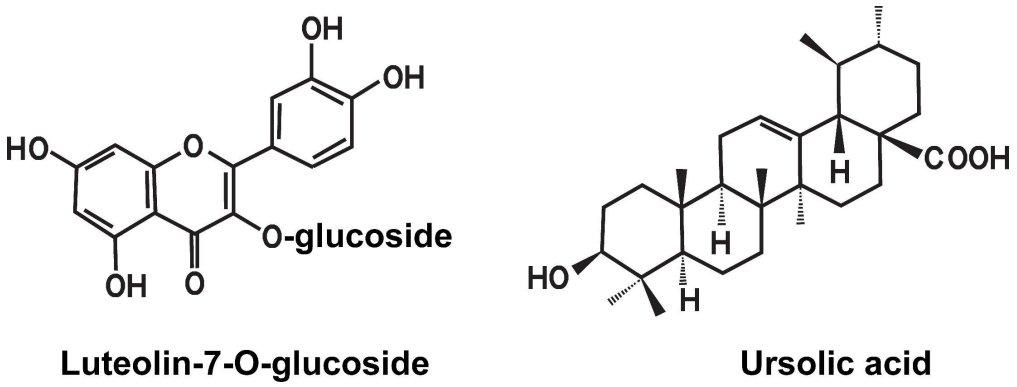


Figure 1. Chemical structures of the compounds used in this study: the flavonoid luteolin-7-glucoside and the pentacyclic triterpenoid ursolic acid.
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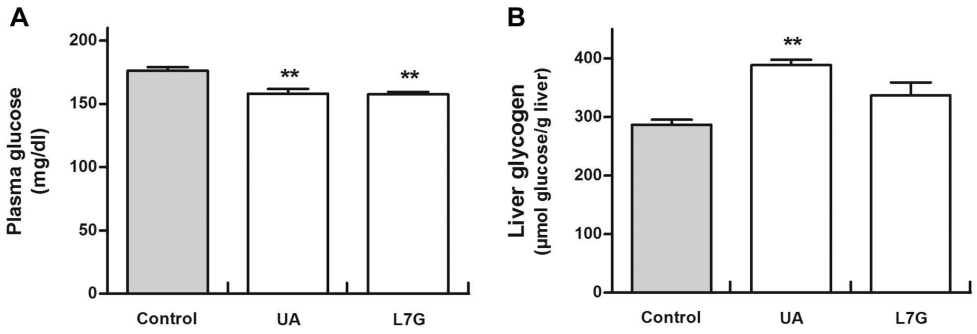


Figure 2. Effect of 7 days diet supplied with the test compound (UA or L7G) on rat plasma glucose concentration (A), and liver glycogen content (B). Values are expressed as mean ± SEM (n = 5). **, P < 0.01 when compared with the control group.
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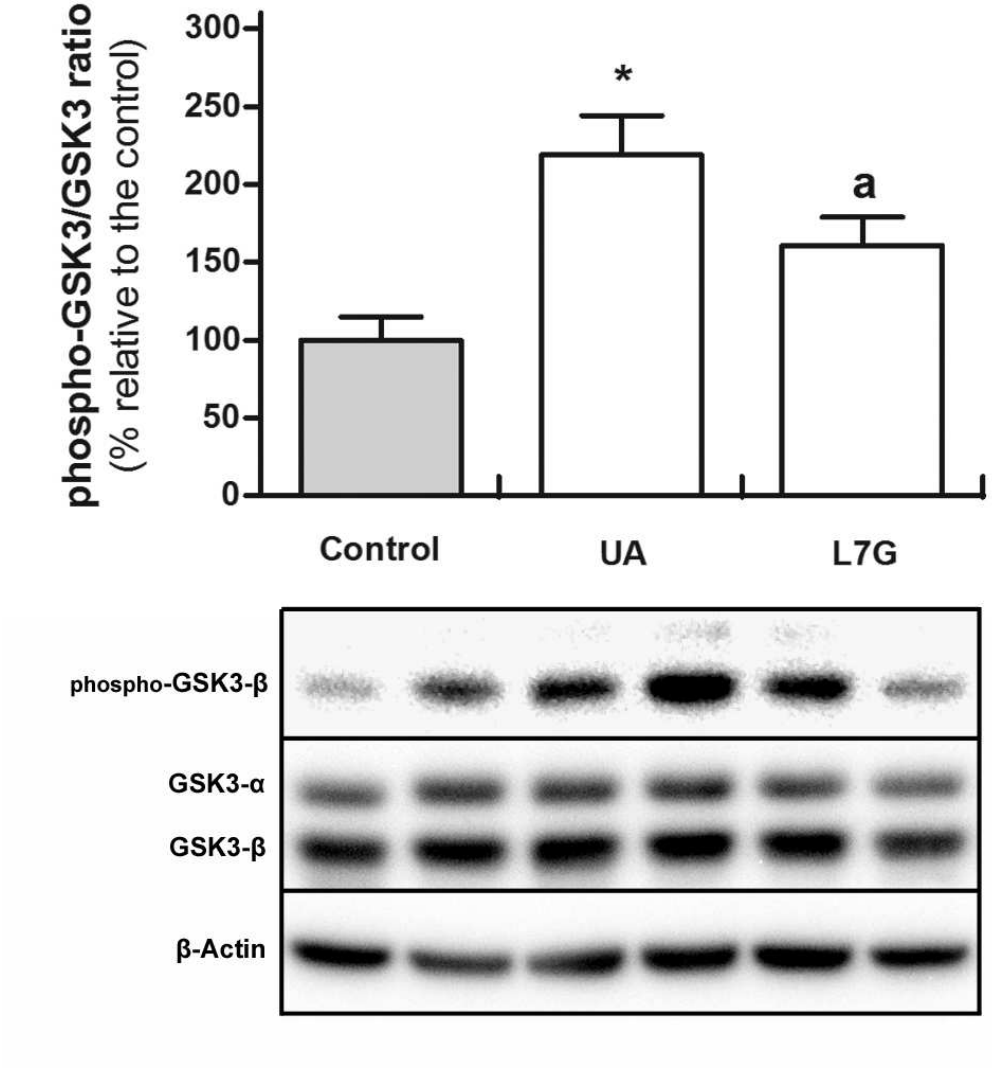


Figure 3. Western blot analysis of phospho-GSK3-β protein in liver homogenates of rats (A), with representative immunoblots and corresponding loading control (β actin) from a pair of animals from each treatment (B). Values are expressed as mean ± SEM (n = 5). * $P < 0.05$ when compared with the control group. [a] $P = 0.06$ when compared with the control group (in this case analysed by the Student t-test).
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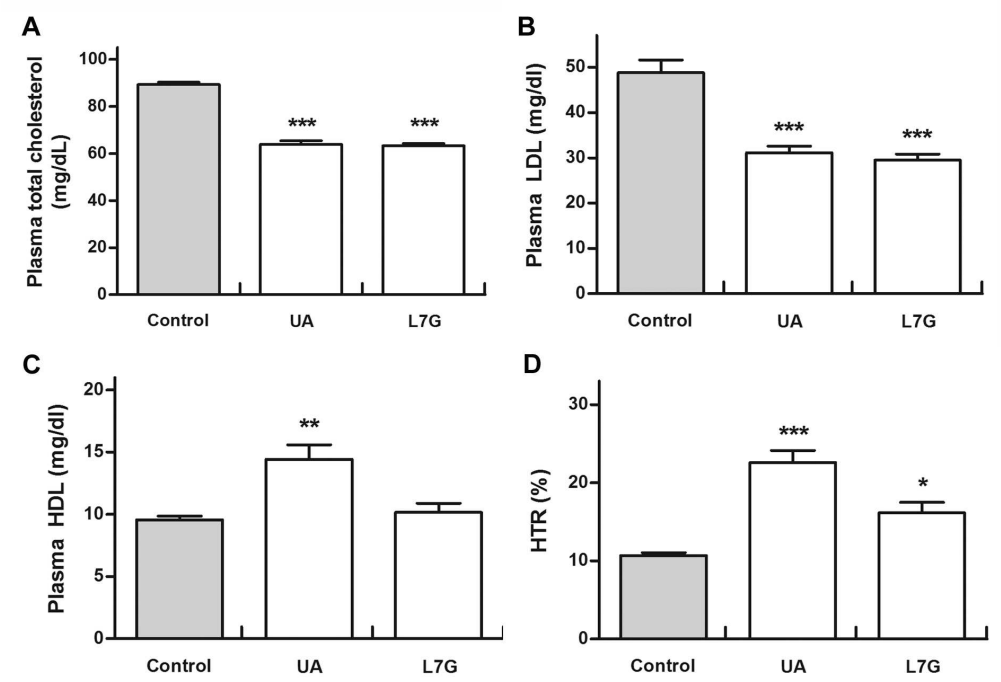


Figure 4. Effects of 7 days diet supplied with the test compound (UA or L7G) on rat plasma total cholesterol (A), LDL cholesterol (B) and HDL cholesterol (C). HTR (%) = (HDL/Total cholesterol) × 100. Values are expressed as mean ± SEM (n = 5). ***, $P < 0.001$; **, $P < 0.01$ and *, $P < 0.05$ when compared with the control group.
160x112mm (300 x 300 DPI)