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Research Article

Favorable Effects of *Globularia alypum* L. Lyophilized Methanolic Extract on the Reverse Cholesterol Transport and Lipoprotein Peroxidation in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

The beneficial health effects of plant polyphenols were frequently attributed to their powerful hypolipemiant and antioxidant effects. We hypothesized that administration of *Globularia alypum* (*Ga*) lyophilized methanolic extract would ameliorate glycemia, lipid parameters, reverse cholesterol transport, as well as lipoproteins peroxidation, in diabetic rats. Diabetes was induced in male Wistar rats, weighing 250±10 g by a single intraperitoneal injection of streptozotocin (STZ) (55 mg/kg body weight). Diabetic rats (n=20) were divided into two groups, and fed during 4 weeks diets containing 20% casein (D) or a casein diet supplemented with *a Ga extract* (1 g/kg BW) (D*Ga*). At d28, in D*Ga vs* D group, glycemia was lowered by 81%, while insulinemia was markedly increased by 72%. Liver and serum lipid values were significantly decreased ($P< 0.05$). Lecithin:cholesterol acyltransferase (LCAT) activity was improved (+48%). Indeed, HDL_3-PL (enzyme substrate) and HDL_3-UC (acyl group acceptor) concentrations were respectively reduced by 50 and 52%, whereas HDL_2-CE values (product of LCAT reaction) were increased (+35%). Atherogenicity ratios VLDL-LDL-C/HDL-C, TC/HDL-C and apoB/apoA were lowered respectively by 50%, 25% and 71%. Moreover, VLDL-LDL and HDL_3 peroxidation was decreased by 47 and 75%, respectively.

In STZ-induced diabetic rat, *Globularia alypum* extract reduces glycemia, liver and serum lipids values, VLDL-LDL and HDL_3 lipid peroxidation and ameliorates the reverse cholesterol transport. Therefore, *Ga* extract may be useful for preventing diabetes and lipid disorders.

Keywords: *Globularia alypum;* Rat; Diabetes; Streptozotocin; Lipids; LCAT; HDL_2; HDL_3; Lipid peroxidation

INTRODUCTION

The prevalence of diabetes is increasing worldwide. The World Health Organization estimates that more than 180 million people worldwide have diabetes and this number is likely to more than double by 2030[1]. Dyslipidemia is more frequent in diabetics than age- and sex-matched non-diabetic individuals and can match any of the lipid profiles seen in the general population. However, diabetic dyslipidemia is mainly attributable to increased total cholesterol and triglyceride levels and impaired lipoprotein profile[2]. Furthermore, emerging evidence suggests that the functional properties of HDL-C are not fully assessed by its serum concentration level alone. HDL is not a single entity, but rather a complex composition of lipoprotein particles with a variety of functions[3]. The different HDL sub-fractions have different biological functions and play different roles in the efficiency of reverse cholesterol transport (RCT) and cellular cholesterol efflux. The modification of any enzyme, receptor or HDL subfraction can affect the ability of HDL to promote RCT[4]. The major cardiovascular protective effects of HDL are attributed to their role in RCT and cholesterol efflux. Structural modification and compositional alteration of HDL particles as a result of chronic inflammation and acute phase responses may adversely affect or reverse their normal biological function[5].

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43)

Table 1: Phytochemical components screening of *G. alypum* methanolic extract by thin-layer chromatography (TLC)

Compounds	Presence
Terpenoids	++
Phenolic acids	++
Flavonoids	+++
Anthracene and derivatives	-
Free quinones	-
Alkaloids	-

All tests were performed six times.
+++: Very high amount; ++: High amount; +: Low amount; -: Not detected

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Table 2: Body weight (BW), food intake, glycemia and insulinemia in diabetic rats treated or not with *Ga* extract

	D	D*Ga*
BW (g)	200.90 ± 9.77	195.10 ± 8.62
Food intake (g/d per rat)	34.75 ± 1.62	19.30 ± 1.09*
Glycemia (mmol/l)	27.41 ± 0.21	5.21 ± 0.83*
Insulinemia (µUI/ml)	5.20 ± 0.42	18.32 ± 2.29*

Values are mean ± SEM of 10 rats per group. Statistical analysis was performed using the Student t-test.
**P<0.05, diabetic rats treated with the Ga extract (DGa) vs. Untreated diabetic rats (D)*

Table 3: Liver and serum lipid concentrations in diabetic rats treated or not with *Ga* extract

	D	D*Ga*
Liver (mmol/g)		
TC	1.56 ± 0.12	1.00 ± 0.08*
TG	2.10 ± 0.18	1.28 ± 0.25*
PL	3.96 ± 0.34	2.86 ± 0.20*
Serum (mmol/L)		
TC	2.69 ± 0.18	2.10 ± 0.13*
VLDL-LDL-C	0.35 ± 0.04	0.20 ± 0.03*
HDL-C	1.18 ± 0.05	1.47 ± 0.03*
TG	0.84 ± 0.14	0.45 ± 0.08*
PL	1.01 ± 0.12	1.04 ± 0.13

Values are mean ± SEM of 10 rats per group. Statistical analysis was performed using the Student t-test.
**P<0.05, diabetic rats treated with the Ga extract (DGa) vs. Untreated diabetic rats (D)*

Table 4: Lecithin: cholesterol acyltransferase activity, apolipoprotein A and B and atherogenicity ratios

	D	D*Ga*
LCAT activity (nmol/mL/h)	10.02 ± 1.93	19.36 ± 3.38*
apo A (g/L)	1.35 ± 0.22	2.51 ± 0.33*
apo B (g/L)	2.14 ± 0.36	1.15 ± 0.29*
Atherogenecity ratios		
VLDL-LDL-C/HDL-C	1.02 ± 0.10	0.51 ± 0.08*
TC/HDL-C	2.02 ± 0.14	1.51 ± 0.12*
apoB/apoA	1.64 ± 0.46	0.47 ± 0.14*

Data are shown as mean ± SEM for 10 rats per group. Statistical analysis was performed using the Student t-test. The values were considered to be different at P< 0.05.
**P<0.05, diabetic rats treated with the Ga extract (DGa) vs untreated diabetic rats (D)*

is synthesized exclusively in the liver. This serum enzyme circulates in association with HDL particles, particularly HDL_3, and is activated by apolipoprotein A-1 (apo A-1)[6]. LCAT enlarges the HDL size by removing 2-acyl group from lecithin or phosphatidyl ethanolamine to free cholesterol. LCAT activity, therefore results in the generation of cholesteryl esters which migrate to the inner core of HDL particles[7]. Therefore, cholesterol esterification contributes to larger HDL_2 formed from small HDL_3 particles. Recent investigations have demonstrated that the HDL-associated enzyme LCAT may play a significant role in lipoprotein modification and hence atherogenesis and normalization of both the quantity and quality of HDL particles[8], and especially of the function of small, dense HDL thus constitutes a novel approach to attenuating atherosclerosis in dyslipidemic individuals with metabolic disease.

In vivo oxidative stress, inflammatory load as well as circulating levels of oxidized low density lipoprotein (ox-LDL) are higher in patients with diabetes mellitus compared with controls[9]. Whereas, the precise mechanism of atherogenesis still needs further investigations, oxidative modification of LDL is considered to be an essential process in the activation of the inflammatory pathway[10].

Renewed attention to alternative medicine, more than 400 different plants and plant extracts have been described in the diabetic patient. Many medicinal plants contain large amounts of antioxidants such as polyphenols and flavonoids, which can play an important role in adsorbing and neutralizing free radicals. Furthermore, these components are known to improve lipid parameters, inhibit lipid peroxidation and to scavenge free radicals[11].

Globularia alypum L. (*Globulariaceae*, now *Plantaginaceae*), locally named "Tasselgha" is a medicinal plant commonly used in Algerian traditional medicine, shows some benefits in the treatment of diabetes, renal, cardiovascular diseases, and various cancerous lesions of the stomach, colon, rectum, liver, and esophagus[12]. Furthermore, phytochemical analysis of the hydromethanolic from the aerial part of *Ga* extract revealed the presence of high amounts of phenolic compounds, which are known to possess strong hypoglycemic and antioxidative activities[13]. Recently, in rats fed a high-fructose diet, the *Globularia alypum* aqueous extract supplementation showed a beneficial effect on plasma triglycerides and lipid peroxidation[14].

However, no detailed information regarding *Globularia alypum* effect on LCAT activity and its action on HDL_3-HDL_2 conversion have been undertaken. Therefore, in the present study the effects of *Globularia alypum* L.

Table 5: HDL_2 amounts and composition in diabetic rats treated or not with *Ga* extract

	D	D*Ga*
Amount (g/L)	4.35 ± 0.35	6.25 ± 0.44*
Apolipoproteins (g/L)	1.83 ± 0.31	2.82 ± 0.51*
Triacylglycerols (mmol/L)	0.09 ± 0.01	0.04 ± 0.01*
Phospholipids (mmol/L)	0.16 ± 0.02	0.32 ± 0.05*
Unesterified cholesterol (mmol/L)	0.28 ± 0.04	0.43 ± 0.02*
Cholesteryl esters (mmol/L)	0.46 ± 0.07	0.71 ± 0.04*

Values are means ± S.E.M of 10 rats per group. Statistical analysis was performed using the Student t-test. The values were considered to be different at P< 0.05.
**P<0.05, diabetic rats treated with the Ga extract (DGa) vs.Untreated diabetic rats (D)*

lyophilised methanolic extract was investigated on glycemia, lipid contents and reverse cholesterol transport as well as on the lipoprotein peroxidation, in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Plant material: *Globularia alypum* was collected from farmland in north eastern Algeria (Douar Bouni, Béjaïa), between February and March 2012, identified taxonomically and authenticated by the Botanical Research Laboratory of Oran University (voucher specimen number KR1965). The plant material was stored at room temperature in a dry place before use. Fresh aerial parts (leaves) of the *Ga* plant were dried at ambient temperature (24°C) for 7 days and ground to a powder. The *Ga* extract was prepared as follows: 50 g of the powdered aerial parts were refluxed at 60-70°C in 500 ml methanol for 30 minutes and the decoction was filtered with cotton wool. The filtrate was concentrated at 65°C by a rotavapor (Buchi Labortechnink AG, Postfach, Switzerland) under a reduced pressure and frozen at -70°C before lyophilization (Christ, alpha 1-2 LD). The crude yield of the lyophilized extract was approximately 30% (wt/wt). It was stored at ambient temperature until further use.

Phytochemical analysis by thin-layer chromatography (TLC): To identify the main chemical groups, the extract was resuspended in MeOH and separated by the silica gel G_{60} F_{254} (0.25 mm thickness) on an aluminum support (Merck). The following solvent systems are used: AcOEt-HCOOH-AcOH-H_2O (100:11:11:26) (flavonoids, phenolic acids), AcOEt-MeOH-H_2O (100:13.5:10) (anthracene derivatives), AcOEt-MeOH-H_2O (100:17:13) (free quinons), CH_2Cl_2-MeOH-NH_4OH (90:10:1) (alkaloids) and $CHCl_3$-MeOH-H_2O (64:40:8); (65:35:10 lower phase) (terpenoids). For detection of the components, the following spray reagents were utilized: Neu (flavonoids), Folin-Ciocalteu (phenolic acids), sulfuric anisaldehyde (terpenoids), KOH (anthracene derivatives, free quinons), Dragendorff's reagent (alkaloids), Komarowsky (saponins)[15,16] Before and after revelation, chromatograms were viewed under a UV lamp at 254 and 365 nm.

Animals and treatment: Male Wistar rats (Iffa Credo, l'Arbresle, Lyon, France), weighing 240-260 g were housed in stainless steel cages at temperature of 23±1°C with a 12-hours light/dark cycle and relative humidity of 60%. The ingredient composition of the diet (expressed in g/kg) was: casein, 200 (95% purity, Prolabo, Paris, France), sunflower oil, 50; sucrose, 40; cellulose, 50; cornstarch, 590; minerals 40; vitamins, 20. Food and tap water were provided *ad libitum*. The General Guidelines on the Use of Living Animals in Scientific Investigations Council of European Communities[17] were followed, and the protocol and use of rats were approved by our institutional committee on animal care and use. All efforts were made to minimize animal suffering and the number of animals used.

Induction of diabetes in rats: After one week of acclimatization, the rats were subjected to a 12 hour fast. Diabetes was induced by a single intraperitoneal injection of freshly prepared STZ (Sigma, St. Louis, MO, USA) at a dose of 55 mg/kg body weight (BW). The STZ was freshly dissolved in citrate buffer (0.01M, pH 4.5) and the injection volume was prepared to contain 1.0 ml/kg. Forty-eight hours after streptozotocin administration, the diabetic state was assessed by measuring glycemia using a glucometer (using a one-touch glucometer Accu-Chek Active, Germany). Only rats in which fasting blood glucose levels greater than 16 mmol/L within 3 days after STZ injection were considered diabetic.

STZ-induced diabetic rats (n=20) were randomly divided into two groups. The untreated diabetic group (D) received a diet containing 20% casein and the treated group (D*Ga*) received the same diet supplemented with the *Ga* extract (1 g/kg BW), for 28 days.

Blood and liver samples: After 4 weeks of the experiment and overnight fasting, 10 rats from each group were anesthetized with sodium pentobarbital (60 mg/kg BW) and euthanized with an overdose. Blood was obtained from the abdominal aorta of rats and collected into tubes containing ethylenediaminetetraacetic acid-Na_2 (Sigma, St Louis, Mo). Serum was prepared by low speed centrifugation at 1000g for 20 min at 4°C. The liver was removed, rinsed with cold saline, and weighed. Aliquots of serum and 50 to 100 mg of liver was stored at -70°C until analyzed.

Glycemia and insulinemia: Fasting glycemia was determined at days 0, 7, 14, 21 and 28 as described above. Insulin was measured at d28 using an enzyme immunoassay (EIA) based on the competition between unlabelled rat insulin and acethylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites (EIA kit, SpiBio, Montigny-le-Bretonneux, France). The coloration intensity was determined spectrophotometrically at 405nm.

Table 6: HDL_3 amount and compositions in diabetic rats treated or not with *Ga* extract

	D	D*Ga*
Amount (g/L)	6.46 ± 0.36	5.05 ± 0.51*
Apolipoproteins (g/L)	4.82 ± 0.34	4.23 ± 0.51
Triacylglycerols (mmol/L)	0.04 ± 0.01	0.06 ± 0.01
Phospholipids (mmol/L)	0.22 ± 0.03	0.11 ± 0.02*
Unesterified cholesterol (mmol/L)	0.25 ± 0.02	0.12 ± 0.01*
Cholesteryl esters (mmol/L)	0.41 ± 0.04	0.20 ± 0.01*

**P<0.05, diabetic rats treated with the Ga extract (DGa) vs. Untreated diabetic rats (D)*

Table 7: Lipoprotein peroxidation in diabetic rats treated or not with *Ga* extract

	D	D*Ga*
Lipoprotein peroxidation (nmol TBARS/24 h.mg protein)		
VLDL-LDL	5.62 ± 0.62	2.96 ± 0.42*
HDL_2	0.34 ± 0.06	0.29 ± 0.05
HDL_3	1.01 ± 0.08	1.25 ± 0.03*

*Data are shown as mean ± SEM for 10 rats per group. Statistical analysis was performed using the Student t-test. The values were considered to be different at P< 0.05. *P<0.05, diabetic rats treated with the Ga extract (DGa) vs. Untreated diabetic rats (D)*

Serum and liver lipids: Total cholesterol (TC) and triacylglycerols (TG) were determined by enzymatic method (kits Biocon, Germany) and phospholipids (PL) by kit Biomérieux, France).

Determination of apolipoproteins A-1 (apo A-1) and B (apo B): Serum apo A-1 and apo B concentrations were determined by immunoturbidimetry (kit Orion Diagnostica, Espoo, Finland).

Lecithin: cholesterol acyltransferase (LCAT) activity assay: Serum LCAT activity was assayed by the conversion of unesterified [^{3}H] cholesterol to esterified [^{3}H] cholesterol, according to the method of Glomset and Wright[18] modified by Knipping[19].

Cholesterol and egg phosphatidylcholines (PC) were used for the preparation of liposomes. Specifically, 2 mg cholesterol and 16 mg egg PC in chloroform-methanol (2:1, v/v) were evaporated to dryness under nitrogen stream. After adding 1.0 ml of (10 mM Tris-HCl, 150 mMNaCl and 1.0 mM EDTA, pH 7.4), the solution was sonicated for 30 minutes at 100 W and 25°C. LCAT activity was determined using liposomes as substrate. The cholesterol esterifying activity was expressed as nanomoles of esterified cholesterol/h/ml of serum.

Isolation and characterization of serum lipoprotein fractions: At d28, serum lipoproteins (VLDL-LDL and HDL) were isolated by precipitation with dextran sulfate and phosphotungstate (Burnstein[20]). HDL_2 and HDL_3 were isolated by differential dextran sulphate magnesium chloride precipitation according to the method of Burstein et al.[21]. To estimate the validity of this method, ultracentrifugation was performed according to Havel et al.[22]. In each lipoprotein fraction, CT, TG and PL were assayed by methods as previously described. Unesterified cholesterol (UC) contents were determined using enzymatic method (kit Biocon, Germany). Esterified cholesterol (EC) concentrations were obtained by calculating the difference between TC and UC values. Cholesteryl esters (CE) levels were estimated as 1.67 times the esterified cholesterol content. Total apolipoprotein concentrations were measured using bovine serum albumin as a standard (Sigma Chemical Company, St Louis, MO, USA), according to the method of Lowry et al.[23].

Lipoprotein peroxidation assay*:* As described by Frémont et al.[24], 100 µL (250 µg protein) of each lipoprotein fraction (VLDL-LDL, HDL_2 and HDL_3) was added to 900 µL phosphate buffered saline (PBS) supplemented with 2.5mM EDTA to prevent oxidation, and incubated for 24 h at 37°C with 20 µL $CuSO_4$ (0.25 mmol/L). The production of thiobarbituric acid reactive substances (TBARS) was determined by the spectrophotometric method. Malondialdehyde, prepared by tetraethoxypropane hydrolysis, was used to establish a standard curve. Results were expressed as nmol TBARS produced/mg of protein in 24 h.

STATISTICAL ANALYSIS OF DATA

All data are presented as means ± SEM of 10 rats per group. The calculations were performed using STATISTICA 6.1 (for Windows, StatSoft Inc. software, Tulsa, OK, USA). Statistical analysis was carried out by Student *t*-test. A difference of $P<0.05$ was considered significant between the both groups treated and untreated with *Globularia alypum* methanolic extract. Linear regression analysis was used to determine correlation coefficients between LCAT activity and HDL_2, HDL_3 amounts and composition

RESULTS

Phytochemical screening: The phytochemical analysis of the methanolic extract of *Globularia alypum* revealed the presence of terpenoids, phenolic acids, flavonoids, and the absence of anthracene derivatives, free quinones and alkaloids (Table 1). The positive tests were more intense for flavonoids than for the other compounds.

Body weight, food intake, glycemia and serum insulin concentrations: At d28, the body weight was unchanged between the both diabetic groups, in spite of a decreased food intake (-44%) by diabetic group treated with *Ga*, compared to the untreated group. Moreover, *Ga* treatment lowered markedly glycemia by 81%, whereas fasting insulinemia was improved (+72%) (Table 2).

Lipid concentrations in liver, serum and lipoproteins: In *Ga* treated compared with untreated diabetic rats, liver total cholesterol, triacylglycerols and phospholipids contents were respectively decreased by 36, 39 and 28% (Table 3).

Serum total cholesterol and VLDL-LDL-C values were respectively 1.3- and 1.7-fold lower, whereas HDL-C concentrations were 1.2-fold higher in Ga treated than untreated diabetic rats. Moreover, serum triacylglycerols values were decreased by 46%, whereas phospholipids levels remained unchanged between the both groups.

LCAT activity, apolipoprotein concentrations and atherogenicity ratios: *Globularia alypum* treatment increased significantly LCAT activity by +48% (Table 4). In *Ga* treated than untreated diabetic rats, apo A-1 values were 1.8-fold higher, while those of apo B were 1.8-fold lower. VLDL-LDL-C/HDL-C, TC/HDL-C and apoB/apoA ratios were respectively reduced by 50, 25 and 71%, in D*Ga* compared to D rats (Table 4).

Serum HDL_2 and HDL_3 amounts and composition: In *Ga* treated compared with untreated diabetic rats, HDL_2 amount, which was the sum of apolipoproteins (apos), triacylglycerols (TG), phospholipids (PL), unesterified cholesterol (UC) and cholesteryl esters contents, were significantly increased (+30%) (Table 5). Indeed, total apolipoproteins, PL and UC contents were 1.5-fold higher and CE values were 2-fold higher. However, HDL_2-TG concentrations were decreased by 56%.

HDL_3 amount was significantly lower (-22%) in *Ga* treated than untreated diabetic rats (Table 6). This decrease was concomitant with a low PL (-50%), UC (-52%) and CE (-51%) concentrations. However, no significant difference was observed in total apos and TG values in both *Ga* treated and untreated diabetic rats.

Correlation analysis: In diabetic rats treated with *Ga* extract a positive correlation was noted between LCAT activity and HDL_3-PL ($r=0.67$, $P<0.05$), LCAT activity and HDL_2-CE ($r=0.81$, $P<0.05$), LCAT activity and apo A-1 ($r=0.71$, $P<0.05$). Also, an inverse relationship was noted between LCAT activity and hypertriglyceridemia ($r=-0.95$, $P<0.05$).

Lipoproteins peroxidation: *Globularia alypum* treatment decreased significantly lipid peroxidation in VLDL-LDL (-47%) and HDL_3 (-75%) fractions; whereas no significant difference was observed in HDL_2 (Table 7).

DISCUSSION

The present study evaluated the effects of *Globularia alypum* (*Ga*) methanolic extract on glycaemia, reverse cholesterol transport as well as lipoprotein peroxidation in STZ-induced diabetic rats.

STZ-induced diabetes is characterized by apoptosis of pancreatic β-cells and by attenuation of insulin gene expression and reduction of insulin synthesis, which is induced by oxyradicals. Pancreatic β-cells normally maintain glycemia concentrations within a narrow range by modulating their insulin secretion rate in response to the glucose concentration in blood[25,26]. In our experiment, *Globularia alypum* exhibited a remarkable hypoglycemic action at d28; blood glucose level reached a mean value of 5.32±0.15 mmol/L, compared to 27.83±0.41 mmol/L obtained in the diabetic group. Moreover, the variation in insulinemia levels showed an opposite trend to that of glucose. The fundamental mechanism underlying hyperglycemia in diabetes mellitus involves the over-production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilization of glucose by the tissues. Skim et al.[27] indicated that this plant might act by increasing the peripheral metabolism of glucose and insulin secretion. In addition, reduced glucose absorption from the gastrointestinal tract[28], blocking renal tubular reabsorption of glucose[29], or inhibition of endogenous glucose production by the liver[30] are possible mechanisms for the hypoglycemic action of the *Ga* extract.

In our study, increased serum insulin levels in *Ga*-treated diabetic rats may possibly due to the insulinotropic substances present in *Ga* extract that induce the intact functional beta cells of the Langerhans islet to produce insulin, or the protection of the functional beta cells from further deterioration so that they remain active and produce insulin. In the literature was reported a bio-guided fractionation of the aerial parts of *Ga* phytochemicals which led to the isolation of flavonoids (6-hydroxyluteolin 7-O-laminaribioside, eriodictyol 7-O-sophoroside and 6'-O-coumaroyl-1'-O-[2-(4-hydroxyphenyl)ethyl]β-D-glucopyranoside), flavonoid glycosides (luteolin 7-O-sophoroside, 6-hydroxyluteolin 7-O-sophoroside, 6-hydroxyteolin 7-O-beta-D-glucopyranoside), phenylethyl glycosides (2-(3,4-dihydroxyphenyl) ethyl O-α-rhamnopyranosyl-(1→3)-4-O-[(E)-caffeoyl]-6-O-[(E)-p-coumaroyl]-β-glucopyranoside, 2-(3,4-dihydroxyphenyl) ethyl O-α-rhamnopyranosyl-(1→3)-4-O-[(E)-caffeoyl]-6-O-[(E)-feruloyl]-β-glucopyranoside and 2-(3,4-dihydroxyphenyl) ethyl O-α-rhamnopyranosyl-(1→3)-4-O-[(E)-caffeoyl]-6-O-menthiafoloyl-β-glucopyranoside), phenylethanoids glycoside (acteoside, isoacteoside and forsythiaside), lignan glycoside (syringaresinol 4'-O-□-glucopyranoside), phenylpropanoid glycoside (syringing) and iridoid glycosides (catalpol, globularioside, globularin, globularicisin, globularidin, globularinin, globularimin, lytanthosalin, alpinoside)[13,31].

Lipids play an important role in the pathogenesis of diabetes mellitus. Usually, high concentration of blood lipids and lipoproteins is characteristic of uncontrolled diabetes[32]. Moreover, it is known that the rise in blood sugar is accompanied by a marked increase in total cholesterol (TC), LDL-C, TG and a reduction of HDL-C in diabetic animals. In our study, the marked rise in the serum and liver lipid concentrations were found in STZ induced diabetic rats and are mainly due to increased mobilization of free fatty acids (FFAs) from peripheral depots[33]. Triglyceride-rich lipoproteins (TRLs) are increased, leading to hypertriglyceridemia, this effect is mainly due to decreased lipoprotein lipase activity (LPL)[34].

In our experiment, *Globularia alypum* methanolic extract treatment prevented against hypertriglyceridemia by reducing TG in liver and serum. These effects might be

explained by the increased of serum LPL activity, an enzyme involved in the hydrolysis of serum TRL triacylglycerols and/or the reduction of hepatic VLDL synthesis. Also, the lipid lowering activities of the *Ga* extract could be due to its antioxidant compounds.

The degree of hypercholesterolemia is directly proportional to the diabetes severity[35]. In *Ga* treated rats, the decline in serum cholesterol levels could be due to the presence of flavonoids in this extract, since these compounds have been reported to have hypocholesterolemic effects. Indeed, many herbs and plant products have been shown to possess, in addition to their hypoglycemic effects, hypocholesterolemic activities, in the STZ-induced diabetic rat[36]. Recently, Merghache et al.[37] have shown that *Globularin*, an iridoid glucoside of *Ga*, reduced serum total cholesterol and triglyceride concentrations in diabetic rat. The possible mechanism by which the *Ga* extract could exert its cholesterol lowering activities was not elucidated. At this stage of the study, several fundamental mechanisms can be proposed to explain our results. *Globularia alypum* may act by decreasing the cholesterol biosynthesis especially by decreasing the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase activity and/or by reducing the NADPH required for fatty acids and cholesterol synthesis. On the other hand, it can be suggested that *Ga* extract led to a decrease in cholesterol absorption from the intestine and may have the ability to inhibit the intestinal absorption of bile acids and neutral steroids and to enhance hepatic cholesterol 7α-hydroxylase activity.

Plasma HDL particles are, however, highly heterogeneous in structure, composition and biological function. In our study, decreased HDL_3-UC as well as increased HDL_2-CE was observed and promoted reverse cholesterol transport. In addition, the fact that apo A-I concentrations were higher in rats treated with *Ga* extract confirmed that increased LCAT activity was due to the high value of its essential activating cofactor. Moreover, there was a positive correlation between LCAT activity and apo A-1 amounts (r=0.71, $P<0.05$). LCAT uses cholesterol and HDL_3-PL as substrate to form cholesteryl esters (CE), producing HDL_2[38]. Cholesteryl esters are then transferred from HDL_2 to triglyceride rich-lipoproteins, such as VLDL and HDL_2. The structural modification and compositional alteration of HDL particles as a result of chronic inflammation and acute phase responses may adversely affect or reverse their normal biological function (Ragbir & Farmer, 2010). In addition, our findings showed that *Ga* extract led to improved LCAT activity, ensuring HDL_3-HDL_2 conversion and the subsequent hepatic uptake. Insulin acts also on HDL metabolism by activating LCAT and hepatic lipase activities[39].

Other important applications of plasma lipid markers, that are relevant to CVD risk, are the atherogenicity ratios VLDL-LDL-C/HDL-C TC/HDL-C and apoB/apoA. In our experiment, these ratios were significantly diminished in diabetic rats treated with *Ga* and this effect could be attributed to the antioxidant property of this plant. Atherogenecity ratios indicate the deposition of foam cells or plaque or fatty infiltration or lipids in heart, coronaries, aorta, liver and kidney. Inversely, higher values of these ratios obtained in diabetic rats indicate an elevated atherosclerotic and oxidative damage of these organs.

A growing body of evidence suggests that HDL exerts part of its antiatherogenic effect by counteracting LDL oxidation. When inflammation is localized without a generalized systemic inflammatory response, HDL is protective against atherosclerosis by enhancing RCT, inhibiting plaque formation, reducing LDL oxidation and generation of inflammation factors (*i.e.* C reactive protein, IL-6) and macrophage adherence factors (*i.e.* VCAM-1, ICAM-1)[3]. In STZ-diabetic rats, our investigation indicates the reduction of VLDL-LDL lipoprotein susceptibility to oxidation with the *Ga* extract. These data suggested that the phytoconstituents of this plant appeared to may have the ability to protect VLDL-LDL against oxidation. Moreover, atheroprotective properties of HDL_3 particles are consistent with results of animal studies[40]; however, its precise molecular features remain indeterminate. HDL_3 display potent capacity to protect LDL from free radical-induced oxidative damage[41] and to inhibit oxLDL-induced apoptosis of endothelial cells[42].

To conclude, *Globularia alypum* lyophilised methanolic extract appears to contribute to alleviating the adverse effect of diabetes mellitus by enhancing lipid metabolism as well as the reverse cholesterol transport and lipoprotein peroxidation. Also, *Ga* treatment may be beneficial for correcting hyperglycemia and preventing diabetic complications. This experiment also suggests the possible mechanism of the hypoglycemic action of *Ga* extract which is from potentiating the insulinemia effect or increasing either the insulin pancreatic secretion from the existing beta cells or its release from the bound form. Since terpenoids, flavonoids and phenolic acids were detected as the main phytochemical compounds, they might be partly responsible for the activity of the MeOH extract. Therefore, other studies will be needed in the future in order to determine which one (or more) of its active constituents has the main antidiabetic, hypolipidemic and antioxidant effects, the mechanism of action and to assess the safety of this plant.

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COMPETING INTERESTS

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