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GLOBULARIA ALYPUM AQUEOUS EXTRACT PREVENTS LIPOPROTEINS LIPID PEROXIDATION AND ENHANCES CATALASE ACTIVITY IN RED BLOOD CELLS OF RATS FED A HIGH-FRUCTOSE DIET

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Abstract

Aim

Globulariaalypum aqueous extract (*Ga*) (0.5%) improves antioxidant status of some tissues in rats fed high-fructose diet. The present study was designed to explore the effect of *Ga*on lipid peroxidation of serum, lipoproteins and red blood cells (RBC), as well as antioxidant enzymes activities of RBC in rats fed a high-fructose diet (61%).

Materials and methods

Male Wistar rats (n=24) weighing 241±13 g were divided into 4 groups and fed, during 14 weeks, diets containing 20% casein and 61% cornstarch (control diet, C) or fructose (F), and *Ga* supplementation (0.5%; C*Ga* and F*Ga*).

Results

Compared to control group, thiobarbituric acid reactive substances (TBARS) contents were increased in RBC (+60%), VLDL (+87%), LDL-HDL_1 (+30%), and inversely, were lowered in HDL_2 (-39%) in rats fed high-fructose. Moreover, RBC catalase activity was reduced by 83%. *Ga* aqueous extract supplementation in rats fed high-fructose diet induced low values of TBARS in RBC (-59%), VLDL (-60%) and LDL-HDL_1 (-44%). Furthermore, compared with C group, *Ga* supplementation to high-fructose diet or control diets enhanced RBC catalase activities.

In conclusion, *Globulariaalypum* aqueous extract is more effective to prevent lipoproteins lipid peroxidation and to improve RBC antioxidant capacity.

Keywords: Rat, High-fructose diet, *Globulariaalypum*, Lipoproteins,Red blood cells, Antioxidant status.

1. Introduction

Fructose rich diet (FRD) can induce metabolic alterations, the most prominent being disturbance of plasma lipid profile, hepatic *de novo*lipogenesis and lipotoxicity, oxidative stress and hyperuricemia[1].During oxidative stress and

in many pathological events, enhanced levels of thiobarbituric acid reactive substances (TBARS), which are useful parameter for the assessment of the lipid peroxidation extent, have been reported[2].Studies have shown that high-fructose diet results in insulin resistance (IR) and excessive generation of reactive oxygen species (ROS). Normally, erythrocytes are well equipped to degrade ROS *via* the actions of natural antioxidant enzymes superoxide dismutase (SOD). SOD (EC. 1. 15.1.1) is a metalloenzyme that catalyzes dismutation of superoxide anion to molecular oxygen and hydrogen peroxide (H_2O_2). Catalase (CAT) (EC. 1.11.1.6) and glutathione peroxidase (GPx) (EC. 1.11.1.9) catalyze the reduction of hydroperoxides, including hydrogen peroxide, and function to protect the cell from oxidative damage. In red blood cells, CAT and GPx jointly protect hemoglobin from oxidative damage[3].

Traditionally, phytotherapy seems to be the best possible alternate medicine for treating many metabolic and vascular disorders as natural products from herbs or medicinal plants. The usefulness of natural antioxidants from plant materials have been evaluated for therapeutic efficiency against diseases related to oxidative stress. *Globulariaalypum*(*Ga*), locally named "Tasselgha", is a medicinal plant commonly used in Algerian popular medicine, shows some benefits in the treatment of several diseases[4].Furthermore, it has been shown in our previous study, that *Ga*aqueous extract supplementation in rats fed a high-fructose diet exerted a beneficial action against oxidative stress in kidney and muscle, by lowering lipid peroxidation and improving SOD activity in muscle and GPx in kidney. Moreover, *Ga* could prevent oxidative stress in heart by increasing SOD and GPx activities[5].

However, no detailed reports regarding the effect of *Ga*aqueous extract on serum, lipoproteins and red blood cells (RBC) lipid peroxidation, as well as antioxidant enzymes activities of RBC, in rats fed a high-fructose diet, have been undertaken. The first objective was to study the consequences of high-fructose feeding compared with control diet on serum, lipoproteins and RBC lipid peroxidation and antioxidant enzymes of RBC. The second objective was to assess the influence of a concomitant *Ga* aqueous extract supplementation, known to have antioxidant properties.

2. Materials and Methods

2.1 Animals and Dietary Treatments

Twenty-four male Wistar rats (Iffa Credo, l'Arbresle, Lyon, France) weighing 242±13 g were housed in stainless cages at temperature of 24°C with a 12-hour light/dark cycle and relative humidity of 60%. The animals were randomly divided into 4 groups of six rats each and submitted for 14 weeks to diets containing 20% casein and 61% cornstarch: control diet (C), or fructose (F) and supplemented with *Ga* aqueous extract (0.5%; C*Ga*, F*Ga*). Preparation of *Ga* lyophilized aqueous extract was realized from plant material, as previously described[5]. Food and

tap water were freely available. Body weight and glycemia (using a one touch glucometer Accu-Chek® Active, Germany) were recorded weekly. The diet composition (expressed in g/100 g) was casein, 20 (95% purity; Prolabo, Paris, France); 61, (fructose or cornstarch), sunflower oil, 5; sucrose, 4.5; cellulose, 5; minerals, 3.5; vitamins, 1 as previously described[5]. The general guidelines for the care and use of laboratory animals recommended by the Council of European Communities[6] were followed, and the protocol and use of rats were approved by our Institutional Committee on Animal Care and Use(Regn.No 314/*Ga*/11/CPCSEA).

2.2 Blood Samples and Isolation of Red Blood Cells

After 14 weeks of the experiment, rats were fasted overnight and anesthetized with chloral hydrate 10% (3mL/kg BW) and euthanized with an overdose. Blood was obtained from the abdominal aorta of rats and collected into tubes containing ethylene-diamine-tetra-acetic acid-Na_2 (EDTA) (Sigma, St Louis, Mo). Blood was centrifuged at 1000 xg for 10 min at 4°C. To obtain the red blood cells, the top serum was separated and the layer (leukocytes) was removed. Separated RBC was then washed 3 times by resuspending in 0.9% NaCl solution and repeating the centrifugation. The washed cells were lysed in four times its volume of ice-cold HPLC-grade water. The mixture was centrifuged at 10,000 x g for 15 min; the supernatant was removed to measure lipid peroxidation and antioxidant enzymes activities. Serum and RBC were stored at -70°C until use.

2.3 Serum Lipids Measurements

Enzymatic methods were used for determination of total cholesterol (TC), triacylglycerols (TG) (kits BioconDiagnostik, Vöhl/Marienhagen, Germany) and phospholipids (PL) (kit Biomérieux Marcy-l'Etoile, France).

2.4 Serum Lipoproteins Isolation and Characterization

Serum very low-density lipoproteins (VLDL) and low density lipoprotein-high-density lipoprotein$_1$ (LDL-HDL_1) were isolated by precipitation using $MgCl_2$and phosphotungstate (Sigma Chemical Company, France) according to the method of Burstein *et al*.,[7]. HDL_2 and HDL_3were isolated by differential dextran sulfate magnesium chloride precipitation according to the method of Burstein *et al*.,[8].To estimate the validity of this method, ultracentrifugation was performed according to Havel *et al*.,[9]. In each lipoprotein fraction, CT, TG and PL were assayed by kits methods. Unesterified cholesterol (UC) contents were determined using enzymatic method (kit Wako, 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.*,[10].

2.5 Lipid Peroxidation

As a marker of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) of serum, lipoproteins and RBC were measured according to the method of Quintanilha*et al.*[11],using malondialdehyde (Sigma–Aldrich, l'Isled'Abeau, France) as a standard. One milliliter of diluted sample (protein concentration about 2mg/mL) was added to 2 mL of thiobarbituric acid (final concentration, 0.017 mmol/L), plus butylatedhydroxytoluene (concentration 3.36 µmol/L) (Sigma-Aldrich Chemie, Germany). The mixture was incubated for 15min at 100°C. After cooling and centrifugation, the supernatant absorbance was measured at 535 nm. Data were expressed as µmoLof TBARS produced/mL of serum.

2.6 Antioxidant Enzymes Activities Measurements

Superoxide dismutase assay uses a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (kit Cayman, Ann Arbor USA). One unit of SOD was defined as the amount of enzyme needed to exhibit 50% of superoxide radicaldismutation. SOD activity in RBC was measured at 440 to 460 nm using plates readers and was expressed as U/mL.

Glutathione peroxidase assay (kit Cayman, Ann Arbor USA) measures GPx activity indirectly by a coupled reaction with glutathionereductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, was recycled to its reduced state by glutathione reductase and NADPH (nicotinamide adenine dinucleotide phosphate, reduced form). The oxidation of NADPH to $NADP^+$ was accompanied by a decrease in absorbance at 340 nm. One unit of GPx was defined as the amount of enzyme that catalyzed the oxidation of 1nmoLof NADPH per min at 25°C.GPx activity in RBC was measured at 340 nm using plates readers and was expressed as nmol/min/mL.

Catalase (EC. 1.11.1.6) activity was determined by the method of Aebi[12]by measuring the rate of decomposition of H_2O_2 at 240 nm. In RBC, CAT activity was assayed in 250 µL of supernatant and was measured according to the method as previously described[5].

2.7 Data and Statistical Analysis

All data are presented as means ± SEM of 6 rats per group. Statistical analysis was carried out by STATISTICA (version 4.1; Statsoft, Tulska, Okla). The significance of differences in response to fructose and *Ga* supplementation

was analyzed by 2-way analysis of variance followed by Tukey honestly significant difference test. Statistical significance was set at $p < 0.05$:Fructose effect: [a]F*vs* C; [d]F*Gavs*C*Ga*and*Ga* effect: [b]F*Gavs*F; [c]F*Gavs* C; [e]*CGavs* C.

3. Results

3.1 Effect of *Ga* extract on serum lipids and lipoproteins contents

After 14 weeks, serum total cholesterol concentration was not sensitive to the consumption of a high-fructose diet. In contrast, when compared with the control diet, high-fructose-fed animals exhibited an increase in serum triacyglycerols value (Table 1). This hypertriglyceridemia was concomitant with high TG values of VLDL, LDL-$HDL_1$and HDL_2. Moreover, TG/HDL-C ratio was 2.4-fold higher in F group compared with C group. In rats fed a high-fructose diet, the amounts of lipoproteins, which represent the sum of apolipoproteins (apos), unesterified cholesterol (UC), cholesteryl esters (CEs), triacylglycerols (TGs) and phospholipids (PL) contents, showed no significant difference in HDL_2 and HDL_3, whereas, VLDL and LDL-HDL_1 amounts were increased respectively by 23% and 21%. Indeed, high values of apo (+33%), UC (+50%) and TG (+45%) in VLDL, and only apo (+44%) and TG (+66%) Concentrations were increased in LDL-HDL_1(figure 1).

Table-1: Serum lipids concentrations (mmol/L) and atherogenicity ratios.

	C	**C*Ga***	**F**	**F*Ga***
Triacyglycerols	0.40 ± 0.12	0.44 ± 0.14	0.77 ± 0.24 [a]	0.43 ± 0.18 [b]
Total cholesterol	2.03 ± 0.51	1.62 ± 0.36	1.74 ± 0.33	1.94 ± 0.32
HDL-C	1.38 ± 0.21	1.14 ± 0.11	1.07 ± 0.09	1.13 ± 0.24
Atherogenicity ratios				
TG/HDL-C	0.30 ± 0.12	0.39 ± 0.12	0.72 ± 0.22 [a]	0.38 ± 0.11 [b]
CT/ HDL-C	1.48 ± 0.37	1.42 ± 0.24	1.63 ± 0.27	1.83 ± 0.70

Values are means ± SEM of 6 rats per group. Rats were fed diets containing 20% casein and 61% cornstarch (control diet, C), or 61% fructose (F), and *Ga* aqueous extract supplementation (0.5%) (C*Ga*, F*Ga*) for 14 weeks The significance of differences in response to the fructose and *Ga* supplementation was analyzed by 2-way analysis of variance followed by Tukey honestly significant difference test. Statistical significance was set at *p<0.05*: [a] Fructose effect: F *vs* C; [b]*Ga* effect: F*Gavs* F.

*G alypum*aqueous extract supplementation to rats fed a high-fructose diet inducedreduction in TG/HDL-C ratio (-47%) (Table 1) with a significant decrease in triglycerides contents of serum (-44%) and VLDL (-68%), whereas,

there was no significant changes in LDL-HDL_1-TG. Moreover, F*Ga* group showed a significant decrease in VLDL amounts (-24%), whereas that of LDL-HDL_1 tended to be lower but not significantly, compared with F group.

3.2 Effect of *Ga* extract on lipid peroxidation in serum, lipoproteins and RBC

As shown in Table 2, in F group compared with C group, TBARS levels were increased in RBC (+60%), whereas, there was no significant difference in serum values. *Globulariaalypum*aqueous extract supplementation to rats fed a high-fructose diet showed a significant decrease of TBARS in RBC (-59%).

Compared with control group, lipid peroxidation was significantly increased in VLDL by 87% and LDL-HDL_1 by 30%, and inversely, it was lowered in HDL_2 by 39%, in rats fed a high-fructose diet (Table 2). *Ga*aqueous extract supplementation to rats fed high-fructose diet, showed that TBARS values were decreased by 60% in VLDL and 44% in LDL-HDL_1.

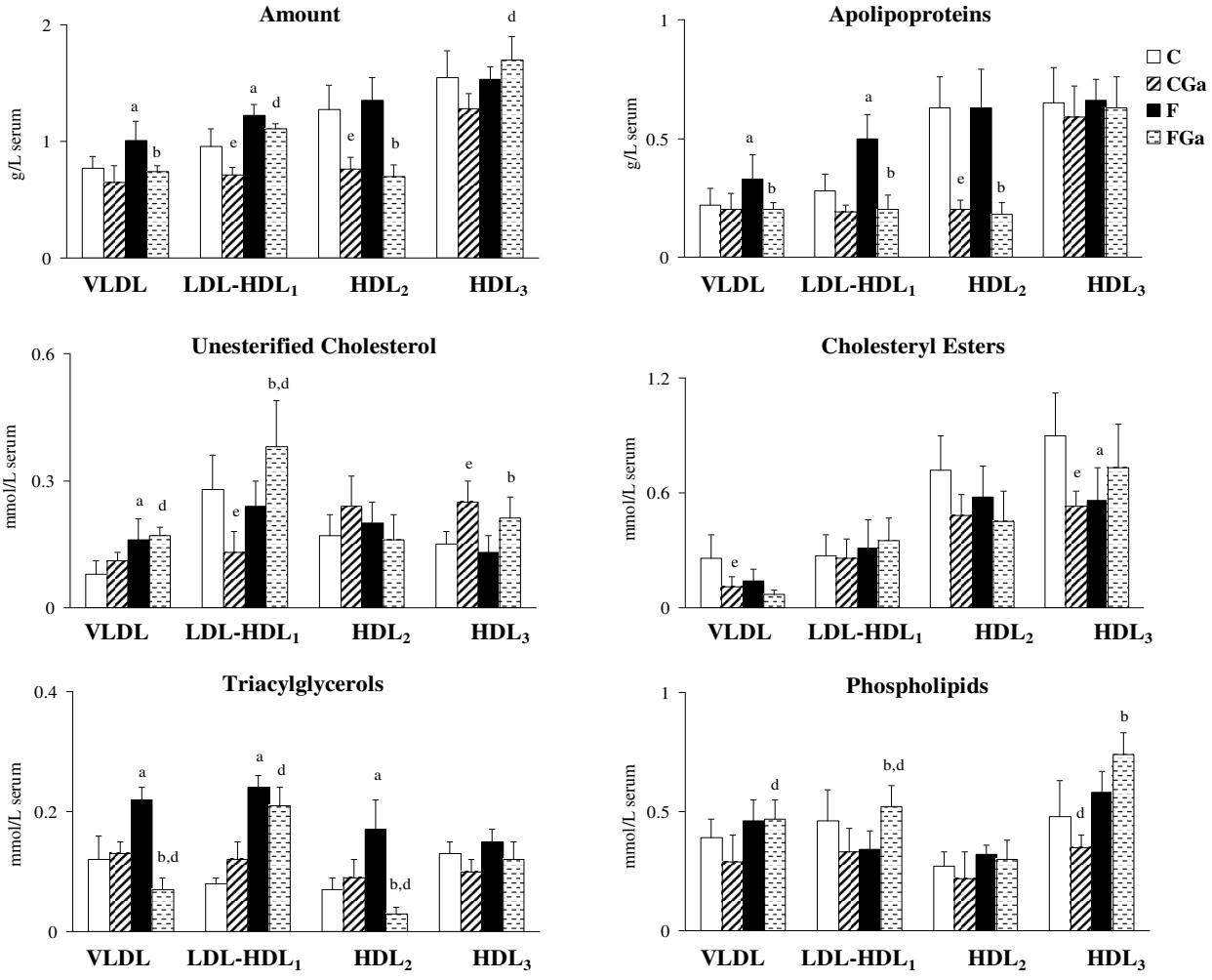

Figure-1: Lipoproteins amounts and composition.

Values are means ± SEM of 6 rats per group. The significance of differences in response to the fructose and *Ga*supplementation was analyzed by 2-way analysis of variance followed by Tukey honestly significant difference test. Statistical significance was set at $p < 0.05$:

Fructose effect: [a]F *vs*C and [d] F*Gavs*C*Ga*

*Ga*effect:[b] F*Gavs*F and [e] C*Ga*vs C

Moreover, in C group, *Ga* supplementation to control diet (C*Ga*) induced significant decrease in TBARS values of LDL-HDL_1 by 75% and 70%, compared with C and F*Ga* groups, respectively. There was no significant changes in HDL_3TBARS by fructose diet, whereas *Ga* aqueous extract supplementation in this diet showed a significant decrease of TBARS concentrations (-51%) compared to F group (Table 2).

Table-2: Thiobarbituric acid reactive substances (TBARS) levels in RBC, serum and lipoproteins (µmol/L).

	C	C*Ga*	F	F*Ga*
RBC	34.87±14.85	31.23±12.08	87.79±15.79 [a]	35.22±11.86[b]
Serum	40.43±9.15	33.35±8.92	37.89±13.67	33.62±10.71
Lipoproteins				
VLDL	1.01±0.37	0.69±0.17[d]	7.61±2.26[a]	3.07±1.19[b]
LDL-HDL_1	7.63±1.485.88	1.88±0.63[d,e]	10.86±2.27 [a]	6.12±1.76 [b]
HDL_2	±1.23	5.18±1.54	3.56±1.54 [a]	5.00±1.26
HDL_3	8.00 ±1.58	7.01±1.68	7.97±1.27	3.86±0.95[b,d]

The significance ofdifferences in response to the fructose and *Ga* supplementationwas analyzed by 2-way analysis of variance followedby Tukey honestly significant difference test. Statisticalsignificance was set at $p < 0.05$:

[a] Fructose effect: F *vs* C; [b]*Ga* effect: F*Gavs* F.

[d] Fructose effect:F*Gavs*C*Ga*; [e]*Ga* effect: C*Ga*vs C

3.3 Effect of *Ga* extract on antioxidant enzymes activity in RBC

In F group compared with C group, RBC catalase (CAT) activity was 6-fold lower, whereas there was no significant difference in superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities (Table 3). However, compared with F group, *Ga* supplementation to fructose diet induced a significant increase of CAT in RBC by + 84%. Moreover, the *Ga*aqueous extract enhanced CAT activity by + 35% in control diet (C*Ga*) compared with C and F*Ga* groups.

Table-3: Antioxidant enzymes activities in RBC.

	C	C*Ga*	F	F*Ga*
SOD (U/mL)	196.04±30.28	139.70±29.40	188.00±32.50	174.04±19.16
GPx (nmol/min/mL)	03.55±2.85	379.32±12.85	385.40±12.80	418.69±17.13
CAT (U/mL)	1.10±0.21	1.69±0.47[e,d]	0.18±0.04 [a]	1.13±0.15 [b]

Values are means ± SEM of 6 rats per group. The significance ofdifferences in response to the fructose and *Ga* supplementationwas analyzed by 2-way analysis of variance followedby Tukey honestly significant difference test. Statisticalsignificance was set at $p < 0.05$.

[a] Fructose effect: F *vs* C; [b]*Ga* effect: F*Gavs* F.

[d] Fructose effect:F*Gavs*C*Ga*; [e]*Ga* effect: C*Ga*vs C

4. Discussion

Until now most of the studies on diets enriched in fructose have been focusing on effects induced by very high content of dietary fructose. In animal studies, fructose was a source of 45–66% of the energetic value of fodder and in human studies the value even rose to 90%[13]. In the present study, after 14 weeks, we examined whether the *G alypum* aqueous extract supplementation might improve the lipid profile and oxidative damage resulting from a high fructose diet (60%) in rats.

High fructose feeding stimulates hepatic triglycerides production, by promoting reesterification of circulating nonesterified fatty acids and by stimulating *de novo* fatty acid synthesis[14].The low serum TG observed with *Ga* supplementation in fructose diet resulted from reduced synthesis of liver triacylglycerols and their transport *via* VLDL. Indeed, decreased amounts of VLDL were reflected by their low TG concentrations, suggesting a smaller number of VLDL particles. Jarouliya*et al*.,[15]reported that administration of Spirulina maxima was effective in normalizing the triglyceride levels in Wistar rats fed a fructose diet. This could be due to decreased VLDL triglyceride production or increased VLDL clearance in the peripheral tissues. The reduced TG levels could be due to their hydrolysis by lipoprotein lipase activity, a key enzyme in triglyceride rich lipoproteins metabolism.Recently, Merghache*et al.,*[16]have shown that *Globularin*, an iridoidglucoside of *Ga*, reduced serum total cholesterol and triglycerides concentrations in diabetic rat.Our results showed that neither HDL_2 (figure 3) nor HDL_3 (figure 4)amounts were sensitive to fructose diet.However, *G alypum* aqueous extract supplementation induced a significant reduction in HDL_2 (-39%), probably, by an increase in hepatic triglyceride lipase (HTGL), since TG-HDL_2 values were significantly reduced. Another remarkable effect of the*Ga* supplementation in the fructose diet was a distinct reduction in TG/HDL-C ratio. The ratio TG/HDL-C, initially proposed by Gazano*et al.,*[17]is an atherogenic index which was an indicator of development of coronary heart disease development in humans[18].

Lipid peroxidation of cell membranes and serum lipoproteins represents a primary event in the oxidative stress establishment[19]. Moreover, susceptibility of LDL to oxidative modifications depends on its fatty acid composition and cellular and extracellular antioxidants, which serve to trap reactive oxygen species and to inhibit the chain reaction of free radicals [20]. In our present study, *Ga* supplementation prevented high-fructose induced hypertriglyceridemia by reducing TG concentrations in serum and VLDL fraction. The antioxidant potential of

*Ga*aqueous extract was effective to reduce lipid peroxidation in VLDL and LDL-HDL_1, and was able to prevent peroxidation in HDL_3subfractions. These decreases, observed with *Globulariaalypum*, could be considered as good indicators of lowered lipid peroxidation. Studies have demonstrated that triglyceride-rich lipoproteins, *e.g*. VLDL are more susceptible to oxidation than HDL[21], suggesting a direct oxidation of lipoproteins by ROS. This oxidation would take place in tissues, rather than in serum, where high antioxidants might prevent it. Lipid peroxidation products, such as MDA, could also be directly provided by tissues. They would be subsequently released into the blood circulation and distributed into serum components, especially the LDL/VLDL fraction [22].Several other studies have shown that sucrose or fructose produce an increase in VLDL triacylglycerols associated with a decrease in serum vitamin E and increased lipid peroxidation [23]. Vitamin E depletion in sucrose- or fructose-fed rats predispose VLDL and LDL enriched with triacylglycerols to subsequent oxidative stress [20], which is one of the critical mechanisms involved in the progression of atherosclerosis. Dornas*et al*.,[24]showed in rats models that increased ROS production and/or impaired antioxidant defense may in turn lead to excessive peroxidation of polyunsaturated fatty acids contained in LDL particles.

Phytochemicals studies on aerial parts of *Globulariaalypum*(*Ga*) have revealed the presence of several phenylethanoids flavonoids, and iridoids, as the major constituents of the extract, and their antioxidant activities have been determined[25]. The results of Chograni*et al*.,[26]showed that tunisian*Globulariaalypum*leaves exhibited the highest phenol and flavonoid contents, and displayed the highest antioxidant activity, based on radical-scavenging activitiy. Our previous data have shown that in rats fed high-fructose diet for 14 weeks, prooxidant effects were observed in some selected tissues, and *Ga*supplementation was effective in improving antioxidant status by reducing lipid peroxidation and enhancing antioxidant enzymes in these tissues[5].Oxidative stress, defined as an overproduction of free radicals, or a decrease of antioxidant defense mechanisms, determines cellular damage with functional alterations of the involved tissue. Free radicals can attack any biochemical cell component, but lipids are a major target. Compared to controls, serum TBARS concentrations, which were a marker of lipid peroxidation, remained unchanged in rats after 14 weeks of high-fructose feeding. However, oxidative stress was observed in RBC with a significant increase in TBARS levels and a low catalase (CAT) activity, whereas, there was no significant difference in SOD and GPx activities. These results suggested that serum had a stronger capacity than RBC to reduce lipid peroxidation induced by high-fructose diet. Therefore, there are many reports in the literature describing an increase in plasma lipid peroxides resulted from a decline in cellular, non enzymatic and enzymatic

antioxidant potential in fructose-fed rats. Indeed, Bhagya*et al*.,[27]showed that free fatty acids (FFA) concentrations were elevated in plasma which are the substrates for lipid peroxidation in the plasma of fructose-induced hypertension rats. Moreover, Girard *et al*.,[28]reported that hyperglycemia in spontaneously hypertensive rats (SHR) that were fed fructose diet, was associated with a decrease in total antioxidant capacity of whole blood which could result from both associated factors: increased plasma and VLDL-LDL TBARS concentrations and decreased SOD and GPx activities of RBC.

Our data showed an antioxidant potential of *Ga* aqueous extract which was effective against RBC oxidative stress induced by fructose overfeeding, leading decreased lipid peroxidation and increased CAT activity. This indicated that *Ga*administration involved lower hydrogen superoxide concentrations and its decomposition, and subsequently reduced oxidative stress induced by high-fructose diet. It could be suggested that during oxidative stress, polyphenols could form stable complexes with catalase-rich blood cells and also acted synergistically with plasma albumin to decompose ROS[29, 30]. Therefore, even if only micromolar amounts of polyphenols reached plasma, these might still be effective as radical scavengers[30].

5. Conclusion

These findings indicate that feeding a high-fructose diet for 14 weeks leads to dyslipidemia and decreased antioxidant defense system, especially in RBC catalase activity, without affecting lipid susceptibility to peroxidation in serum. *Globulariaalypum*aqueous extract supplementation in rats fed a high-fructose diet is able to correct lipid profile and to prevent lipid peroxidation in VLDL, LDL-HDL_1 and HDL_3. In addition, it also improves antioxidant status in RBC by lowering lipid peroxidation and enhancing antioxidant enzymes. However, further investigations to fully identify the biologically active ingredients and to define the precise molecular mechanism (s) of these effects are required.

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Conflict of Interest

The authors declare that there are no financial/commercial conflicts of interest.

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