

Effects of *Zygodphyllum gaetulum* Aqueous Extract on Erythrocytes Lipid Peroxidation and Paraoxonase 1 Activity in Hypercholesterolemic Rats

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ABSTRACT

Currently, herbal medicines are experiencing a resurgence of interest and their popularity is increasing in developing and developed countries due to their natural origin and minor side effects. This study aimed to investigate the effects of a lyophilized aqueous extract of *Zygodphyllum gaetulum* (Zg) on erythrocytes lipid peroxidation and paraoxonase 1 (PON 1) activity in rats fed a high-cholesterol diet. Twelve hypercholesterolemic male Wistar rats (cholesterolemia value > 3mmol/L) weighing 176±5g were divided into two groups fed a high cholesterol diet (1% cholesterol+0.5% cholic acid) supplemented (HC-Zg) or not (HC) with Zg aqueous extract (1%), for 4 weeks. In the HC-Zg group compared with the HC group, plasma total cholesterol (TC) and non-HDL cholesterol levels were respectively 1.5- and 2.2- fold lower, whereas HDL-cholesterol was 2-fold higher. Atherogenic indexes, TC/HDL-C and TC-HDL-C/HDL-C were respectively decreased by 71% and 66%. In erythrocytes, thiobarbituric acid reactive substances (TBARS) concentrations tended to decrease but not significantly. The activities of superoxide dismutase (SOD) and catalase (CAT) were increased by 42% and 38%, respectively, while those of glutathione peroxidase (GSH-Px) and glutathione reductase (GSSH-Red) showed no significant difference. Moreover, reduced glutathione content (GSH) was similar in both groups. Plasma PON 1 activity was 1.5-fold higher in the hypercholesterolemic group treated with Zg compared with the untreated group. In conclusion, in the hypercholesterolemic rat, the treatment with *Z. gaetulum* extract induced a cholesterol-lowering effect but didn't improve erythrocytes lipid peroxidation despite stimulation of antioxidant enzymes activities. Moreover, Zg extract increased plasma PON 1 activity suggesting a possible lipoprotein protection from oxidation.

Keywords: Antioxidant enzymes; Erythrocytes; Hypercholesterolemia; Lipid peroxidation; paraoxonase 1; Rat; *Zygodphyllum gaetulum*.

INTRODUCTION

Cardiovascular diseases (CVDs) remain the biggest cause of deaths worldwide¹. Several factors such as high caloric diet, age, lack of exercise, smoking, alcohol consumption and genetic predisposition have been linked with CVD^{2,3}. Dyslipidemia is one of the important causes of cardiovascular disease related mortality and morbidity. Recently, it has become a significant problem in public health of developing countries^{4,5}. Hypercholesterolemia, a condition characterized by very high levels of cholesterol in blood⁶, is implicated in atherosclerosis and other cardiovascular diseases^{6,7}. The modification of lipid concentration has been found to be a useful approach to decrease cardiovascular mortality through prevention of atherosclerotic diseases development^{8,9}. Hypercholesterolemia is also associated with enhanced oxidative stress related to increased lipid peroxidation¹⁰. Overproduction of oxidized LDL is a major factor in the vascular damage associated with high cholesterol levels¹¹. Hence, the inhibition of oxidative stress under hypercholesterolemic conditions is considered to be an important therapeutic approach and efforts have been

made to identify the antioxidative functions of various medicinal plants¹⁰. Paraoxonase 1 (PON1) is a hydrolase enzyme closely associated with HDL in plasma. It has been postulated to play a protective effect on low density lipoprotein oxidation¹². Moreover, this enzyme has been reported to be an important contributor to the antioxidant and anti-inflammatory activities of HDL. PON1 impedes oxidative modification of LDL. Serum PON1 activity is related to systemic lipid peroxidation stress and prospective cardiovascular risk¹³. The erythrocyte is considered a prime target for free radical attack due to the presence of high contents of polyunsaturated fatty acid in their membrane and the oxygen transport, which are potent promoters of reactive oxygen species (ROS)¹⁴. Erythrocytes have been used as a model to investigate oxidative damage due to their high concentration of O₂ and their known sensitivity to endogenous ROS, especially to peroxy radicals (ROO·) that may attack membrane components, inducing changes in membrane rheology, conformation of membrane proteins, cellular morphology, protein cross-linking, and hemolysis^{15,16}. Numerous varieties of plants have been used traditionally in the

treatment of hypercholesterolemia and cardiovascular diseases. Indeed, plants are rich in bioactive compounds that have therapeutic and prophylactic effects against many diseases, including CVD¹⁷. *Zygophyllum gaetulum* (Zygophyllaceae) locally known as "Aggaya" or "El Barraya" in Algeria and Morocco is widely used in traditional medicine for its many virtues. It essentially acts as anti-diabetic¹⁸, anti-inflammatory¹⁹ and anti-diarrheal²⁰. Also, in our previous study, it was found that *Z. gaetulum* aqueous extract improved hypercholesterolemia and attenuated oxidative stress in plasma and tissues of rats fed a high cholesterol diet²¹. Thus, in the present study, we investigated the effect of an aqueous extract of *Z. gaetulum* on erythrocytes lipid peroxidation and plasma paraoxonase 1 activity in hypercholesterolemic rats.

MATERIALS AND METHODS

Plant Material and Preparation of Zygophyllum gaetulum Aqueous Extract

Zygophyllum gaetulum (Zygophyllaceae) plant was collected in Adrar (south Algeria) in May 2015. The plant was identified taxonomically and authenticated by the Botanical Research Institute of Oran University. The preparation of *Zygophyllum gaetulum* aqueous extract was realized as previously described²¹. Briefly, 50 g of *Z. gaetulum* powder were suspended in 500 mL of distilled water, heated under reflux for 30 min and filtered. The filtrate was frozen at -70°C and lyophilized. The crude yield of the lyophilized *Z. gaetulum* extract was approximately 30% (wt/wt). It was stored at ambient temperature until further use.

Animals and Dietary Treatment

Male Wistar rats (Pasteur Institute, Algiers, Algeria) weighing 176 ± 5 g were housed in stainless steel cages in a room with controlled lighting (12 hour light/dark cycle), constant temperature (24°C) and relative humidity (60%). We followed the general guidelines on the use of living animals in scientific investigations²², and the protocol and use of rats were approved by our institutional committee on animal care and use. Experimental hypercholesterolemia was induced by feeding a high-cholesterol diet (HCD, 1% cholesterol + 0.5% cholic acid) to rats. Hypercholesterolemic rats ($n=12$, cholesterolemia value= 5.71 ± 0.62 mmol/L) were then divided into two groups: The hypercholesterolemic untreated group (HC) was fed the HCD and the hypercholesterolemic treated group was fed the same diet supplemented with *Zygophyllum gaetulum* lyophilized aqueous extract (1g/100g of the diet) (HC-Zg), for 4 weeks. The 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; cholesterol, 10; cholic acid 5.

Blood and Erythrocytes Samples

After 4 weeks of experiment and an overnight fasting, six rats from each group were anesthetized with sodium pentobarbital solution (60 mg/kg body weight) and euthanized with an overdose. Blood was collected from the abdominal aorta into tubes containing EDTA and plasma was obtained by low-speed centrifugation (1,000g for 20

min, 4°C). The erythrocyte sediment was washed twice with ice-cold distilled water (1/4, v/v) and centrifuged (1000g for 10 min, 4°C). Plasma and erythrocytes samples were stored at -70°C until analysis.

Determination of Plasma and Lipoprotein Cholesterol Levels

Plasma total cholesterol (TC) concentrations were assayed by standard enzymatic methods (kit Biocon, Germany). Plasma HDL cholesterol (HDL-C) was determined after precipitation of apolipoprotein B-containing lipoproteins (kit Spinreact, Spain). Plasma non-HDL-C was obtained by calculating the difference between TC and HDL-C values.

Determination of Hemoglobin, Plasma Albumin and Uric Acid

Erythrocyte hemoglobin was estimated by the cyanmethemoglobin method (Kit Chronolab, Spain). Plasma albumin and uric acid were measured by enzymatic methods (Kit Biolabo, France and kit Spinreact, Spain, respectively).

Assay for Lipid Peroxidation in Erythrocytes

Erythrocyte samples (100 μL) were diluted with 900 μL of PBS buffer and incubated for 60 min at 37°C in the presence of 100 μL of hydrogen peroxide (H_2O_2) (1.15%)²³. The reaction was inhibited with 1 mL of trichloroacetic acid (20%). After centrifugation at 1,000g for 10 min, 100 μL of butylated hydroxytoluene (2%) was mixed with supernatant sample. Erythrocyte lipid peroxidation was then estimated to the method of Quantanilha et al.²⁴.

Erythrocytes Antioxidant Enzymes Activities Determination

Superoxide dismutase (SOD, EC. 1.15.1.1) was assayed using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (kit Cayman, USA). Glutathione peroxidase assay measures GSH-Px (EC. 1.11.1.9) activity indirectly by a coupled reaction with glutathione reductase (kit Cayman, USA). Glutathione reductase (GSSH-Red, EC. 1.6.4.2) activity was estimated by measuring the rate of NADPH oxidation (kit Cayman, USA). Catalase (CAT, EC. 1.11.1.6) method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 (kit Cayman, USA).

Erythrocytes Reduced Glutathione Analysis

Reduced glutathione (GSH) concentration was measured according to the method of Sedlak and Lindsay²⁵. GSH reacts with DTNB [5,5'-dithio-bis (2-nitrobenzoic acid)] to form TNB (5-thio-2-nitrobenzoic acid) which can be quantified at 412 nm. 1mL of erythrocytes was mixed with 800 μL of ice-cold distilled water and 200 μL of 50% trichloroacetic acid (TCA) and incubated for 15 min. After centrifugation at 1,200g for 15 min, 400 μL of the supernatant were mixed with 800 μL of Tris buffer (0.4 mol/L, pH=8.9) and 20 μL of DTNB reagent (0.01mol/L). After 5 min of incubation, the absorbance of the reaction mixture was measured at 412 nm and reduced glutathione was used as a standard.

Determination of Plasma Paraoxonase 1 (PON 1) Activity

Paraoxonase activity measurement was performed in the absence (basal activity) and presence of NaCl (salt-stimulated activity). The rate of paraoxon hydrolysis (diethyl-p-nitrophenylphosphate) was measured by monitoring the increase of absorbance at 270 nm and 25°C. For this assay, 10 µL of plasma was added to 1 mL of a solution containing 10 mM of phenyl acetate in 20 mM Tris/HCl pH 8.0 and 1 mM CaCl₂. The activity was calculated relative to the molecular extinction coefficient of the phenyl (1310 M⁻¹.cm⁻¹)²⁶.

Statistical Analysis

Data are expressed as means ± SEM for six rats per group. Statistical analysis was carried out by Student's t-test. The calculations were performed using STATISTICA (Version 6.1, Statsoft, USA). A difference of p<0.05 was considered significant between the both hypercholesterolemic groups treated (HC-Zg) or not (HC) with *Z. gaetulum* aqueous extract.

RESULTS

Plasma and Lipoprotein Cholesterol Levels, Atherogenic Indexes and Plasma Paraoxonase1 (PON 1) activity

Plasma total cholesterol (TC) and non-HDL cholesterol levels were respectively 1.5 - and 2.2- fold lower in Zg treated than in untreated hypercholesterolemic rats (Table 1). However, HDL cholesterol (HDL-C) concentrations were 2-fold higher in the HC-Zg group compared with the HC. TC/HDL-C and TC-HDL-C/HDL-C ratios were respectively decreased by 71% and 66% in HC-Zg vs HC. Plasma PON1 activity was 1.5-fold higher in the hypercholesterolemic group treated with Zg extract compared with the untreated group.

Erythrocytes Hemoglobin and Plasma Albumin and Uric Acid Concentrations

There was no significant difference in erythrocytes hemoglobin and plasma albumin levels in the HC-Zg group compared with the HC group (Table 2). However, plasma uric acid concentration was decreased by 20% in Zg treated than untreated hypercholesterolemic rats.

TBARS Concentrations, Antioxidant Enzymes Activities and Reduced Glutathione Content in Erythrocytes

Z. gaetulum aqueous extract treatment had a tendency to reduce erythrocytes TBARS concentrations but not significantly (Table 3). The activities of superoxide dismutase (SOD) and catalase (CAT) were respectively increased by 42% and 38%, while those of glutathione peroxidase (GSH-Px) and glutathione reductase (GSSH-Red) showed no significant difference. In addition, the content of reduced glutathione (GSH) was similar in the both groups.

DISCUSSION

The aim of the present study was to investigate the effect of *Zygophyllum gaetulum* (Zygophyllaceae) lyophilized aqueous extract on erythrocytes lipid peroxidation and antioxidant status as well as on paraoxonase 1 (PON 1) activity in rats fed a high-cholesterol diet. High cholesterol diet is regarded as a crucial factor in the development of hypercholesterolemia, atherosclerosis and ischemic heart disease^{27,28}. After 4-week of treatment with *Zygophyllum*

gaetulum extract, plasma total cholesterol and non-HDL cholesterol concentrations decreased significantly. Several lines of evidence showed that plants with phenolic compounds had anti-lipidemic activities and contribute to prevent the development of atherosclerosis^{29,30}. Indeed, saponins are reported to precipitate cholesterol from micelles and interfere with enterohepatic circulation of bile acids making it unavailable for intestinal absorption, this forces liver to produce more bile from plasma cholesterol and hence the reduction in plasma cholesterol level³¹. Furthermore, tannins are known to lower plasma total cholesterol concentrations³². HDL carries cholesterol and cholesterol esters from the peripheral tissues and cells to the liver, where cholesterol is metabolized into bile acids. This pathway plays a very important role in reducing cholesterol levels in the blood and peripheral tissues, and in inhibiting atherosclerotic plaque formation in the aorta³³. The results our study show significant increases of HDL-cholesterol levels in hypercholesterolemic rats treated with Zg aqueous extract compared with untreated. Qualitative analysis of *Zygophyllum gaetulum* plant had shown the presence of sterols, flavonoids, saponins and tannins³⁴. The association of serum TC and low-density lipoprotein cholesterol (LDL-C) with developing coronary heart disease (CHD) has been well established, and low serum high-density lipoprotein cholesterol (HDL-C) is considered a major risk factor for CHD^{35,36}, both responsible for increased risk of cardiovascular disease³⁷. The atherogenic indexes, a coronary risk marker³⁸ indicate the presence of foam cells, lipids or a plaque in heart, coronary artery, aorta, kidney and liver. Moreover, these indices are more high risk of oxidative alterations in these organs is important³⁹. In the hypercholesterolemic rats treated with Zg aqueous extract compared to the untreated controls, the levels of LDL-HDL₁ were reduced. Moreover, the atherogenic ratios, LDL-HDL₁-C/HDL-C, TC/HDL-C and TC-HDL/HDL-C were lowered. Taken together, these results are in favor of prevention against the risk occurrence of CVD. These findings are in accordance to those of Ikewuchi *et al.*⁴⁰ who showed that treatment with an extract of *Chromolaena odorata* (L.) decreased total cholesterol, LDL-C and atherogenic index, and increased HDL-C in rats consuming a diet supplemented with 1% cholesterol. Similarly, a decrease in LDL-C and an increase in HDL-C, due to the decrease of apo B/apoA ratio were reported by Asgary *et al.*⁴¹ in hypercholesterolemic rabbits treated with alcoholic extract of *Hypericum perforatum*.

In our experiment, plasma uric acid levels were significantly diminished in hypercholesterolemic group treated Zg extract compared with untreated group. There is some evidence that uric acid is one of the most important antioxidants that can eliminate up to 60% of free radicals⁴². However, its excess in plasma is often associated with CVD and is considered pro-oxidant⁴³. In Zg treated rats, the decline of plasma TBARS levels suggesting that *Zygophyllum gaetulum* decreases oxidative stress in HC rats. These finding are in agreement with those obtained in hypercholesterolemic rats treated with garlic extract⁴⁴. Several recent studies indicate that some medical herbs

Table 1: Plasma and lipoprotein cholesterol levels (mmol/L), atherogenic indexes and plasma paraoxonase 1 (PON 1) activity in hypercholesterolemic rats treated or not with *Zg* aqueous extract.

	HC	HC-Zg
TC	3.89±1.13	2.65±0.59*
HDL-C	0.59±0.01	1.19±0.34**
Non-HDL-C	3.25±0.02	1.45±0.01*
TC/HDL-C	5.22±0.32	1.52±0.17***
TC-HDL-C/HDL-C	2.10±0.22	0.71±0.43*
PON 1 (U/mL)	3.78±0.39	5.78±1.17*

Values are expressed as means±SD for six rats per group. HC: Untreated hypercholesterolemic rats. HC-Zg: *Zygodphyllum gaetulum* treated hypercholesterolemic rats. TC: total cholesterol, HDL-C: HDL cholesterol. *P<0.05, **P<0.01 and ***P<0.001, HC-Zg vs HC group.

Table 2: Erythrocytes hemoglobin content and plasma albumin and uric acid concentrations in hypercholesterolemic rats treated or not with *Zg* aqueous extract.

	HC	HC-Zg
Hemoglobin (mg/dL)	10.33±0.90	10.24±1.64
Albumin (g/L)	27.44±1.05	26.36±0.90
Uric acid (µmol/L)	91.18±10.71	72.64±14.16*

Values are expressed as means±SD for six rats per group. HC: Untreated hypercholesterolemic rats. HC-Zg: *Zygodphyllum gaetulum* treated hypercholesterolemic rats. *P<0.05, HC-Zg vs HC group.

Table 3: Thiobarbituric acid reactive substances (TBARS) concentrations, antioxidant enzymes activities and reduced glutathione (GSH) content in erythrocytes of hypercholesterolemic rats treated or not with *Zg* aqueous extract.

	HC	HC-Zg
TBARS (µmol/mL)	9.57 ± 5.06	8.88±2.35
SOD (U/mL)	120.57±6.88	209.40±2.21*
CAT (pmol/min/mL)	1.17±0.24	1.88±1.36*
GSH-Px (nmol/min/mL)	12.25±2.06	11.89±1.03
GSSH-Red (nmol/min/mL)	48.35±1.88	48.35±7.02
GSH (µmol/mL)	34.38±16.67	34.46±1.48

Values are expressed as means±SD for six rats per group. HC: Untreated hypercholesterolemic rats. HC-Zg: *Zygodphyllum gaetulum* treated hypercholesterolemic rats. SOD: Superoxide dismutase, CAT: catalase, GSH-Px: glutathione peroxidase, GSSH-Red: glutathione reductase. *P<0.05, HC-Zg vs HC group.

have both a lipid-lowering ability and antioxidative activities to suppress lipid peroxides production and then

eventually may contribute to their effectiveness in preventing hypercholesterolemia^{45,46}. Furthermore, our results indicated that TBARS of red blood cells were not sensitive to *Zygodphyllum gaetulum* treatment. Erythrocytes antioxidants such as GSH, SOD, CAT and GSH-Px are normally the first line of defense against (ROS). Glutathione (GSH) is regarded as the non-protein molecule having a thiol group the most abundant in mammalian cells. GSH plays an important role in many cellular processes such as cell differentiation, proliferation and apoptosis and is known for its powerful antioxidant properties. During oxidative stress, GSH levels were decreased in cells⁴⁷. In erythrocytes glutathione plays a central role in co-ordinating the antioxidant defense processes.

In our study revealed that in hypercholesterolemic rats, *Zygodphyllum gaetulum* aqueous extract raises erythrocytes SOD activity. Our data suggest that significant SOD activity is not enough in itself to protect red blood cells from the deleterious effects of reactive oxygen species. However, GSH level was similar in the both groups. These observations are in agreement with those of Bouderbala *et al.*⁴⁸ which showed an increase the SOD activity with no sensitive GSH content in erythrocytes of rats hypercholesterolemic treated with an aqueous extract of *Ajuga iva*. Paraoxonase-1 (PON1), an HDL-associated enzyme has been shown to possess antioxidant/anti-inflammatory properties to attenuation and protect against of atherogenic low density lipoprotein (LDL) oxidation^{49,50}. Our results demonstrate that in hypercholesterolemic rats, *Zygodphyllum gaetulum* elevate plasma PON1 activity. As PON1 is known to be tightly bound with HDL-C, consequently, these data suggest that the significant increase in PON1 activity could be a consequence of an enhanced synthesis and/or secretion of HDL-C concentration. Pezeshkian *et al.*⁵¹ showed that cholesterol-rich diet (2%) in rabbit decreased serum level of PON1 and the enzyme inhibitor helps accelerating the development of atheroma. In conclusion, our results shows that aqueous extract of *Zygodphyllum gaetulum* induces a cholesterol-lowering effect but does not improve erythrocyte lipid peroxidation, despite stimulation of SOD and CAT activities. Moreover, *Zg* extract increased plasma PON1 activity suggesting lipoprotein protection from oxidation.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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