

Effect of *Lamium Album* on Mitochondrial Oxidative Stress in Diabetic Rats

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Abstract

Background: Diabetes mellitus (DM) is characterized by the presence of hyperglycemia. It has been documented that oxidative stress and reactive oxygen species (ROS) production have a key role in the pathogenesis of diabetes and its complications. Neutrophils as a part of immune system produce ROS, neutrophils function might be altered in diabetes. *Lamium album* is known to have antioxidant, and free radical scavenging actions. The aim of the present study was to evaluate the potential effect of *L. album* on mitochondrial ROS production from circulating neutrophils in diabetic rats.

Materials and Methods: Twenty-one male Wistar rats were randomly divided into three groups: normal control rats receiving daily saline; diabetic control rats receiving daily saline; and diabetic rats treated daily with hydroalcoholic extract of *L. album* (100 mg/kg) for 28 days. On the 28th day of treatment, whole blood samples were obtained and mitochondrial ROS of neutrophils were measured by dihydrorhodamine (DHR) flow cytometric method. Also, fasting blood sugar (FBS) was measured.

Results: Mitochondrial ROS didn't show any significant differences among diabetic rats treated with *L. album* extract, diabetic control rats, and normal control rats (P=0.8). Serum glucose in diabetic control was significantly higher than normal control rats (P=0.0001). However, *L. album* caused a remarkable decrease in serum glucose of diabetic rats (P=0.03).

Conclusion: According to the present findings, it seems that *L. album* at a dose of 100 mg/kg could not decrease mitochondrial ROS production from neutrophils in diabetic rats. Further studies considering higher concentrations of *L. album* are appreciated to evaluate its impact on the production of mitochondrial ROS along with extracellular ROS in diabetes condition.

Keywords: *Diabetes mellitus* (DM), *Lamium album*, *Reactive oxygen species* (ROS), *Dihydrorhodamine* (DHR) test

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Introduction

Diabetes mellitus (DM) is the most common endocrine disorder characterized by the presence of hyperglycemia. DM is the result of insulin deficiency, insulin resistance, or both and leads to major problems such as abnormalities in carbohydrate, lipid and protein metabolism. These abnormalities could

damage to the main organs including liver, kidney and pancreas (1, 2).

The number of patients with diabetes is expected to increase to 366 million by the year 2030 (3). Since the incidence rate of this complex disease worldwide is rising considerably (4), there is still an urgent need

for effective therapeutic interventions with low side effects and low associated costs.

DM is a multifactorial disorder (5); it has been suggested that increased oxidative stress plays an important role in the pathogenesis of diabetes (6) as shown in both experimental animals and subjects with diabetes (7, 8). It is well documented that oxidative stress is the result of an imbalance between free radical production and antioxidant defense system. Hyperglycemia results in enhanced formation of free radicals such as reactive oxygen species (ROS), and many studies have suggested that diabetes complications might be the result of increased formation of free radicals and ROS production (9-13). It is well documented that the main source of ROS in most cells are mitochondria (14). Neutrophils as a part of immune system produce considerably more ROS than other cells, neutrophils function might be altered in diabetes (15, 16).

Since some studies have shown the beneficial antioxidant activity of some plant extracts like Curcumin (17) and Genistein (18) in diabetes, finding other plant extracts with more powerful activity is appreciated.

Lamium album or non-stinging nettle, is known to present several beneficial properties containing antiproliferative, anti-inflammatory, antioxidant, and free radical scavenging actions (19-22).

Information about antioxidant efficiency of *L. album* on mitochondrial ROS production is missing, so the present study was conducted to evaluate the potential effect of *L. album* on mitochondrial ROS production from circulating neutrophils in diabetic rats.

Materials and methods

Animals

During this experimental study, adult male Wistar rats (250-300g) were used. The selected animals were housed under normal laboratory conditions (12 h light: 12 h dark photoperiod; at 22-26 °C) with free access to their appropriate diet and water. All procedures were performed in accordance with the internationally accepted principles for laboratory animal care and use as found in the US guidelines (NIH publication #85-23, revised in 1985). This study was approved by the research committee at Guilan University of Medical Sciences (Rasht, Iran) (No 93112004)

Diabetes induction

The rats were fasted overnight and treated with a single intraperitoneal (IP) injection of STZ (50 mg/kg body weight freshly prepared in sodium citrate buffer) (23) (Sigma- Aldrich Diagnostic Ltd). Normal rats received the same volume of sodium citrate buffer. Diabetes was confirmed by measuring blood

glucose by glucometer (Accu chek, Roche, Germany) three days after injection. Rats with fasting blood glucose more than 300 mg/dl were accepted as diabetic rats (23).

Plant material and extraction

Plant material

Aerial part of *L. album* was collected from suburbs of Rasht city (Guilan province) in spring 2016 and the species were authenticated at the herbarium unit of Pharmacognosy Department, Pharmacy Faculty (Guilan University of Medical Sciences ; Herbarium number: 202HGUM).

Plant extraction and chemical assessment

The aerial part of *L. album* was dried in the shade. Preparation of the extract was done as previously described (24). The total phenolic content of the nettle extract was determined using the Folin-Ciocalteu reagent (25). Gallic acid was used as standard and the result was expressed as mg gallic acid (GAL)/gr plant extract; total phenolic content of *L. album* was 0.61(mg GAL/gr).

Total flavonoid content was determined using aluminium chloride (AlCl₃) (26). Quercetin was used as a standard and the result was expressed as mg quercetin (QE)/gr plant extract; Total flavonoid content of *L. album* extract was 2.10 (mgQE/gr)

Study Design

All rats were randomly divided into three groups, each group containing seven rats (27, 28) as follows: Group I: normal control rats receiving daily saline; Group 2: diabetic control rats receiving daily saline; Group 3: Diabetic rats treated daily with 100 mg/kg of hydroalcoholic extract of *L. album*.

Treatment began three days after diabetes induction by IP injection and all the rats were maintained for 28 days on their respective treatments (24).

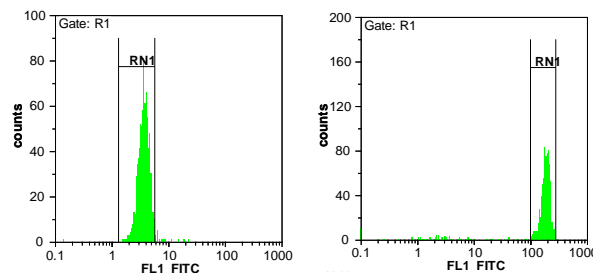


Figure 1. Unstimulated (left panel) and PMA-stimulated sample (right panel).

On the 28th day of treatment, whole blood samples were obtained and used for dihydrorhodamine (DHR) flow cytometric test by Partec flow cytometry as

previously described (29). Briefly, DHR (a non-fluorescent compound) enters the cells and is changed to rhodamine (highly fluorescent) as a result of the action of mitochondrial ROS. We performed the test in whole blood and measured the test in neutrophils by flow cytometry gating. Stimulation index was calculated by division of mean channel fluorescence between phorbol myristate acetate (PMA)-stimulated to unstimulated samples (Figure 1). Also, fasting blood sugar (FBS) was measured. DHR, catalase and PMA were purchased from Sigma-Aldrich.

Statistical Analysis

Data are presented as mean \pm SD or SEM as appropriate. Inter-group comparisons were performed using the one-way analysis of variance (ANOVA). For all tests, $P < 0.05$ was considered statistically

significant. Data were analyzed using SPSS software version 16.

Ethics Statements

All authors hereby declare that all experiments have been performed and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Results

Effect of L. album on mitochondrial ROS

Mitochondrial ROS didn't show any significant differences among diabetic rats treated with *L. album* extract, diabetic control rats, and normal control rats ($P=0.8$) (Table 1).

Table 1. Effect of *Lamium album* on mitochondrial oxidative stress and serum glucose in streptozotocin-induced diabetic rats on the 28th day of treatment.

Groups	Stimulation Index ^a	Serum glucose(mg/dl) ^b	Weight (g) ^a
Normal control	48.57 \pm 9.34	107.71 \pm 7.80	275.14 \pm 24.81
Diabetic control	48.04 \pm 9.87	466.50 \pm 41.14 ^c	212.29 \pm 37.05 ^c
Diabetic+ <i>Lamium</i>	41.69 \pm 10.34	333.43 \pm 67.08 ^{c,d}	204.86 \pm 26.79 ^c

^aValues are given mean \pm SD. ^bValues are given mean \pm SEM.

^c $P < 0.01$ by comparison with normal control rats.

^d $P < 0.05$ by comparison with diabetic rats.

Effect of L. album on serum glucose and weight

On day 28, serum glucose of diabetic control rats was significantly higher than that of normal control rats ($P=0.0001$). However, *L. album* caused a remarkable decrease in serum level of glucose in diabetic rats ($P=0.03$) (Table 1). Also, weight decreased significantly in the diabetic control rats ($P=0.003$) and the diabetic rats treated with *L. album* ($P=0.001$) (Table 1).

Discussion

The main finding of the present study was that mitochondrial ROS from neutrophils were not statistically significant among diabetic rats exposed to *L. album* (at dose of 100 mg/kg), diabetic control and normal control rats.

Our finding was partially in agreement with that of the study conducted by Marin et al. (15) in which Astaxanthin (ASTA) at one dose was not effective in decreasing intracellular ROS production from neutrophils in alloxan-induced diabetic rats. It should be noted that in our study only one dose of *L. album* extract was used; it is likely that using higher concentrations of the extract could be more useful than the present therapeutic procedure.

In contrast to the present study, Rossoni-Júnior et al. (16) showed that ROS production in peripheral neutrophil was significantly lower in diabetic rats treated with Annatto extract compared with non-diabetic and diabetic rats. It was concluded that Annatto extract might be beneficial at controlling ROS production possibly by modulation of NADPH oxidase activity or gene expression. Guerra et al. (30) found that diabetic rats receiving 2% acai (as diet supplementation) had significantly lower production of ROS in neutrophils. They concluded that the reducing effect of acai on the production of ROS might be commonly due to its polyphenols. It has been demonstrated that polyphenols are able to directly deactivate pre-oxidant reactive species and indirectly activate the gene transcription of antioxidant enzymes (31). In Rossoni-Júnior et al. (16) and Guerra et al. (30) studies, ROS production was quantified by chemiluminescence method; it is demonstrated that this assay mainly determines $O_2\bullet^-$ (32). In the present study, the intervention of *L. album* in diabetic rats was assessed and it didn't cause decrease in ROS compared with diabetic control rats. Mitochondrial ROS production was determined using DHR flow cytometric test. Through

flow cytometric technique, prominently intracellular H₂O₂ production was assessed (33, 34). Although, blood samples were not subjected to isolation of polymorphonuclear leukocytes (PMNs) but the test was measured in PMNs by flow cytometry gating.

In the present study, mitochondrial ROS production from neutrophils had no significant difference in diabetic rats as compared to normal control rats. Our finding was partly in line with that of Nakanishi et al. study (34) in which baseline level of mitochondrial ROS in diabetic patients was not significantly different from healthy subjects. It has been suggested that hyperglycemia condition could induce mitochondrial ROS production by activation of protein kinase C (PKC) (35, 36), however, PKC might not always be activated in diabetic patients (34).

In the present study, phytochemical analysis of this plant extract has determined polyphenols and flavonoids (20). The hypoglycemic effect of *L. album* could be in part attributed to its polyphenol compounds (37).

Our study had some limitations: 1) we didn't measure plasma catecholamines and cortisol, since these hormones could affect neutrophil distribution (38, 39). 2) Using DHR dye, the degree of mitochondrial ROS might be affected by the unsolicited alterations in the mitochondrial membrane potential (34); therefore, an additional probe is needed to confirm the results (33).

Overall, according to our findings, it seems that *L. album* at a dose of 100 mg/kg could not decrease mitochondrial ROS production from neutrophils in diabetic rats. Clearly, in view of the limits of our study, the interpretation of *L. album* influence appears to be problematic. Therefore, more studies using higher concentrations of *L. album* are appreciated to evaluate its impact on the production of mitochondrial ROS along with extracellular ROS in diabetes condition.

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Authors' Contributions

K.K and RJS designed the study, wrote the protocol, performed the interpretation of data and wrote the first draft of the manuscript. MA, RG, and MN managed the acquisition of data. K.K and RJS performed critical revision of the manuscript and managed the literature searches. MA, RJS and RG performed analysis and administrative, technical and

material support. All authors read and approved the final manuscript.

Conflicts of Interest

All authors state that there is no conflict of interest in the present study.

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