

ACHILLEA SANTOLINA REDUCED OXIDATIVE STRESS IN THE LIVER OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

Amin Ardestani, Raziieh Yazdanparast *

*Institute of Biochemistry and Biophysics, P. O. Box 13145-1384,
University of Tehran, Tehran, Iran*

Summary

The aim of this study was to evaluate the effect of *Achillea santolina* extracts on lipid peroxidation, protein oxidation and antioxidant defense system such as superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) in the liver of streptozotocin (STZ)-induced diabetic rats. The extract (100 mg/kg of body weight/day) was given orally for 30 consecutive days to the STZ-treated rats. Under diabetic condition, malondialdehyde (MDA) and protein carbonyl (PCO) indices of lipid and protein oxidation, respectively, were both increased. Moreover, our data indicated liver GSH depletion along with significant reduction in the activities of the antioxidant enzymes such as CAT and SOD in the same group of rats. The oral administration of extracts to the diabetic rats significantly alleviated various oxidative stress parameters in the liver of diabetic rats. The elevated levels of liver MDA and PCO were significantly reduced in diabetic rats fed the extract. In addition, the decreased levels of antioxidant enzyme (SOD and CAT) and glutathione were significantly improved with the extract. Furthermore, a protection against STZ induced liver injury was mediated through inhibition in the modulation of liver markers such as, alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) in serum. Based on these observations, it can be concluded that *Achillea santolina* has protective effect on liver tissue in STZ-induced oxidative stress probably due to its high antioxidative potential.

Key words: *Achillea santolina*, oxidative stress, diabetes, streptozotocin, liver

*Corresponding author:
Raziieh Yazdanparast
Inst. Biochem. Biophys
The University of Tehran
P.O.Box 13145-1384
Tehran, Iran
Tel: +98 21 66956976
Fax: +98 21 66404680
E-mail address: yazdan@ibb.ut.ac.ir

Introduction

Oxidative stress has been defined as an imbalance between the elevated level of reactive oxygen species (ROS) and/or impaired function of antioxidant defense system. ROS usually include superoxide anion, hydroxyl radical and hydrogen peroxide that are capable of damaging various molecular targets including DNA, proteins and lipids (1,2). There are convincing experimental and clinical evidences that the generation of reactive oxygen species (ROS) is increased in both type of diabetes and that the onset of diabetes is closely associated with oxidative stress (3,4). Some of the factors involve in oxidative stress in diabetes include the increased generation of ROS by glycation and lipid peroxidation, shifts in redox balance resulting from altered carbohydrate and lipid metabolism, and impaired antioxidant defense systems (5). Streptozotocin (STZ), an antibiotic isolated from *Streptomyces achromogenes* is a well-known genotoxic agent and one of the most commonly used substances to induce diabetes in experimental animals (6). On the other hand, STZ has drawn attention as a potential source of oxidative stress. STZ cause ROS-dependent cellular injuries including membrane lipid peroxidation, protein oxidation and DNA damage in many tissues such as liver (7,8). Intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes which degrade superoxide and hydroperoxides. Such antioxidant systems prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures (9).

Renewed attention in recent decades to alternative medicines and natural therapies has stimulated a new wave of research interest in traditional practices. The plant kingdom has become a target for the search of new drugs and biological, active "lead" compounds (10). Protective effects of exogenously administered antioxidants have been extensively studied in diabetic animal models in recent years, thus providing beneficial effects of antioxidants mainly from plant sources (11). It has been documented that antioxidants and free radical scavengers such as catalase (12), melatonin (13), vitamin C (8) and polyphenols (14) protect rat tissues against cytotoxic effects of STZ. Accordingly, there has been increasing interest regarding the role and use of natural antioxidants as means of preventing damages in diabetes due to high oxidative stress.

Achillea santolina L. (compositae) is a traditional plant used as an herbal remedy for anti-diabetic and anti-inflammatory purposes (15) in many parts of Iraq. Recently, we found that oral administration of *Achillea santolina* extract for four weeks to STZ-induced diabetic rats significantly reduced their serum glucose levels (unpublished data). However, the effect of the extract on oxidative stress during diabetes is not known. In this study, the efficacy of *Achillea santolina* extract in relieving the oxidative stress associated with diabetes was examined in liver of STZ-induced diabetic rat model.

Materials and methods

Plant material:

Aerial parts of the plant were collected from Tikrit in May, 2005. The plant was characterized as: *Achillea santolina* L. by Dr.khalil I. Al-Shemmary (Biology Dep., Faculty of Sciences, Tikrit University, Iraq) and a voucher specimen (No. 5625) was deposited at the herbarium of the Faculty of sciences, Tikrit University.

Extraction:

The powdered plant material (50 g) was extracted three times with ethanol-water (7:3, v/v), at room temperature. The combined extracts were concentrated under reduced pressure and the volume was adjusted to 500 ml (equivalent to 100 mg plant powder per ml). The concentrated extract was divided into 25 ml aliquots and kept at -20 °C for further investigation.

Antioxidant capacity by the phosphomolybdenum method:

The total antioxidant capacity of *Achillea santolina* extract was evaluated by the method of Prieto et al (16). An aliquot of 0.1 ml of sample solution (equivalent to 100 µg) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). In the case of the blank, 0.1 ml of methanol was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm. The antioxidant capacity was expressed as equivalent of ascorbic acid (mg ascorbic acid / g dried extract).

Preparation of model diabetic rats:

Male wistar albino rats (n=30), 5-7 months old with a weight of 200-250 g (purchased from Pasteur Institute, Tehran, Iran) were housed under conventional conditions and were allowed free access to food and water ad libitum. Streptozotocin (STZ; Sigma, USA; 40 mg/kg body weight) was dissolved in 0.1 M sodium citrate buffer at pH 4.5 just before use and injected intraperitoneally (i.p) to 22 rats. Control rats (group1, n=8) received with the same route of administration an equivalent volume of citrate buffer. Three days after STZ administration, the diabetic rats with blood glucose levels higher than 280 mg/dl were selected and distributed in two groups (2 and 3). The plant extract was administered by gavages (i.g.) to 7 rats of group 3 in a dose of 1 ml/rat (equivalent to 100 mg plant powder / kg body weight) for 30 consecutive days. The control healthy rats (group 1, n=8) and the control diabetic rats (group 2, n=7) received the same volume of distilled water (i.g). The blood glucose levels of rats in each group were determined every ten days, using glucose oxidase kit according to manufacture's instructions (Pars azmoon, Tehran, Iran). In the final day, the animals were killed by cervical dislocation followed by decapitation. The whole liver was quickly dissected off and put in ice-cold saline. The homogenate, using 0.1 M phosphate buffer (pH 7.4), was then centrifuged at 5000 g for 30 minutes to remove debris. The supernatant was used for all the assays. The protein

concentration was determined by the method of Lowry et al (17) using bovine serum albumin as the standard.

Lipid peroxidation assay:

Malondialdehyde (MDA) levels, an index of lipid peroxidation, were measured by the double heating method (18). The method is based on spectrophotometric measurement of the purple color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose, 2.5 ml of trichloroacetic acid solution (10%, w/v) was added to 0.5 ml supernatant of the tissue preparation in each centrifuge tube and tubes were placed in a boiling water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at 1000 g for 10 min and 2 ml of each sample supernatant was transferred to a test tube containing 1 ml of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm. The concentration of MDA was calculated based on the absorbance coefficient of the TBA-MDA complex ($\epsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and it was expressed as nmol/mg protein.

Determination of protein carbonyl content:

Protein carbonyls (PCO) were measured by using the method of Reznick and Packer (19). Briefly, 1 ml of supernatant was placed in each of the two glass tubes. Then 2 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl was added to one of the tubes, while 2 ml HCl (2.5 mM) was added to the second tube. Tubes were incubated for 1h at room temperature. Samples were vortexed every 15 min. Then 2.5 ml TCA (20%, w/v) was added and the tubes were left on ice for 5 min followed by centrifugation for 5 min to collect the protein precipitates. The pellet was then washed three times with 2 ml ethanol-ethyl acetate (1:1, v/v). The final precipitate was dissolved in 1 ml of guanidine hydrochloride solution (6 M) and it was incubated for 10 min at 37 °C while mixing. The absorbance of the sample was measured at 370 nm. The carbonyl content was calculated based on the molar extinction coefficient of DNPH ($\epsilon = 2.2 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$) and expressed as nmol/mg protein.

Determination of catalase activity:

Catalase (CAT) activity was measured according to the method of Aebi (20) by following the decrease in absorbance of H₂O₂ at 240 nm for 1 min. The enzyme activity (U) was expressed as $\times 10^{-3}$ k/sec/mg protein, where k is the rate constant of the first order reaction of CAT.

Determination of superoxide dismutase activity:

Superoxide dismutase (SOD) activity was measured based on inhibition of the formation of amino blue tetrazolium formazan in nicotineamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium (NADH-PMS-NBT) system, according to method of Kakkar et al (21). A one unit of enzyme activity was expressed as 50% inhibition of NBT reduction/min/mg protein.

Determination of reduced glutathione:

Reduced glutathione (GSH) was determined by the method of Ellman (22). The supernatant (0.5 ml) was treated with 0.5 ml Ellman's reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid, DTNB, in 100 ml of 0.1% sodium nitrate) and 3 ml of phosphate buffer (0.2 M, pH 8). The absorbance was read at 412 nm. GSH was expressed as mg/100 g tissue.

Liver function tests:

ALP, ALT, and AST were determined by standard automated technique using Autoanalyser (Carry 100 Bio, Australia).

Statistical analysis

Statistical analyses were performed using student's t-test. All data are presented as means \pm S.D., and statistical significance was achieved when $P < 0.05$.

Results

A significant increase in the level of blood glucose and a decrease in body weight were observed in diabetic rats when compared to control rats. Administration of ASE to diabetic rats significantly decreased the level of blood glucose and increased body weight gain to near control level (Table 1). Total antioxidant activity was measured by the phosphomolybdenum method. Phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds and the formation of green Mo(V) complex with a maximal absorption at 695 nm (16). The antioxidant capacity of ASE was estimated to be equivalent to 150.3 mg ascorbic acid per g of the dried extract.

Table 1. Serum glucose levels and body weight of control, diabetic and ASE-treated rats. Each measurement has been done at least in triplicate and values are the means \pm S.D. for six rats in each group.

Parameters	Control	Diabetic	ASE-treated
Initial body weight (g)	240 \pm 11	210 \pm 6	215 \pm 7
Final body weight (g)	305 \pm 24	154 \pm 7	252 \pm 7
Initial serum glucose (mg/dl)	95 \pm 19	284 \pm 9*	305 \pm 21
Final serum glucose (mg/dl)	88 \pm 14	294 \pm 10*	121 \pm 8**

* significantly different from normal rats ($P < 0.05$).

** significantly different from STZ-treated rats ($P < 0.05$).

In order to explore the effect of *Achillea santolina* extract on the liver function of the diabetic rats, lipid peroxidation, protein oxidation and antioxidant defense system capabilities were evaluated according to appropriate methods reported in methods section.

Figure 1 show that MDA level, the end product of lipid peroxidation and a marker of free radical generation, in the liver was significantly decreased (by 82%) upon treatment with the extract, whereas diabetic rats showed enhanced levels of lipid peroxidation. Moreover, Figure 1 also indicates that the liver protein carbonyl (PCO) levels are enormously higher in diabetic rats compared to the control subjects. Treatment with the plant extract resulted in reduction of PCO compared to the untreated diabetic rats (by almost 47%). This clearly indicated that ASE, by decreasing oxidative stress, may be effective in preventing oxidative protein damages which are thought to be involved in liver damages under the diabetic condition (23).

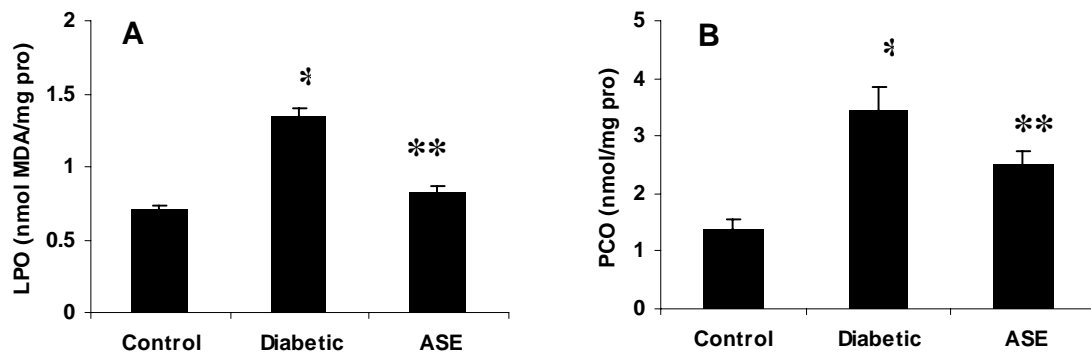


Fig 1. Liver malondialdehyde (MDA) (A) and protein carbonyl (PCO) (B) in control, diabetic and ASE-treated diabetic group. For experimental details see materials and methods section. Each value represents the mean \pm S.D. (n=6). * significantly different from normal rats ($P < 0.05$). ** significantly different from STZ-treated rats ($P < 0.05$).

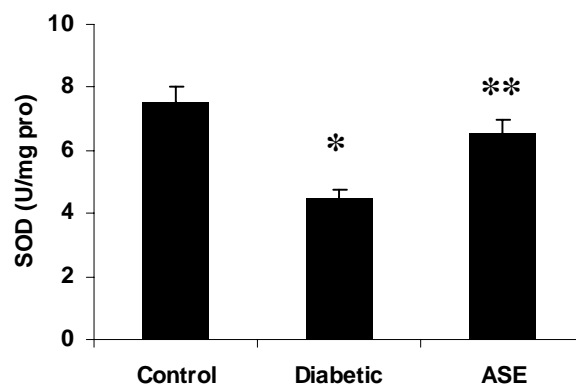


Fig 2. Liver superoxide dismutase (SOD) in control, diabetic and ASE-treated diabetic group. One unit (U) of enzyme activity was expressed as 50% inhibition of NBT reduction/min/mg protein. For experimental details see materials and methods section. Each value represents the mean \pm S.D. (n=6). * significantly different from normal rats ($P < 0.05$). ** significantly different from STZ-treated rats ($P < 0.05$).

For further evaluation of the oxidative status of the liver, the activities of some of the antioxidative enzymes (e.g., SOD and CAT) and also the GSH level were measured. As shown in Figures 2-4, the activity of SOD and CAT and also the pool size of GSH were all lower in the diabetic liver. However, treatment of the diabetic rats with the crude extract significantly increased the CAT and SOD activities and enhanced the GSH content by 38, 46, and 86%, respectively relative to the control diabetic rats.

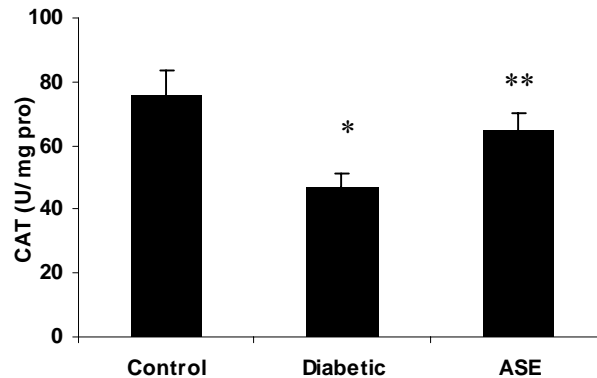


Fig 3. Liver catalase (CAT) in control, diabetic and ASE-treated diabetic group. The enzyme activity (U) was expressed as $\times 10^{-3}$ k/sec/mg protein, where k is the rate constant of the first order reaction of CAT. For experimental details see materials and methods section. Each value represents the mean \pm S.D. (n=6). * significantly different from normal rats ($P < 0.05$). ** significantly different from STZ-treated rats ($P < 0.05$).

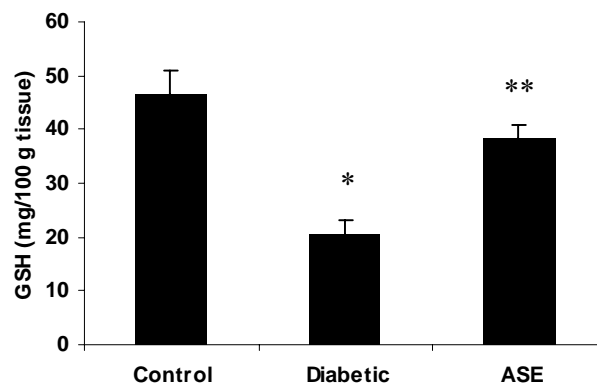


Fig 4. Reduced glutathione (GSH) levels in control, diabetic and ASE-treated diabetic rats. For experimental details see materials and methods section. Each value represents the mean \pm S.D. (n=6). * significantly different from normal rats ($P < 0.05$). ** significantly different from STZ-treated rats ($P < 0.05$).

Liver function tests were done to assess STZ-induced hepatic injury. STZ modulated most of the investigated liver function parameters (Table 2). STZ elevated plasma levels of ALP, ALT and AST. Treatment with the plant extract significantly reduced these enzymes after 4 weeks of treatment implying that the plant has executed a protective effect against STZ-induced liver damage.

Table 2. Effect of *Achillea santolina* extract on the STZ-induced modulation in the levels of serum ALP (alkaline phosphatase), ALT (alanine transaminase), and AST (aspartate transaminase).

Parameters	Control	Diabetic	ASE
ALP	100 ± 7	278 ± 20*	147 ± 8**
ALT	100 ± 13	255 ± 19*	155 ± 17**
AST	100 ± 19	217 ± 21*	146 ± 10**

Activities of ALP, ALT, and AST are expressed as % of control. Each value represents the mean ± S.D. (n=6).

*significantly different from normal (P< 0.05)

**significantly different from STZ-treated group (P< 0.05)

Discussion

Increased oxidative stress is believed to be involved in the development and progression of diabetes and its complication. Diabetes is usually accompanied by increased production of free radicals or impaired antioxidant defenses (24). STZ, a genotoxic agent, is a well-known ROS generating system. Therefore, various antioxidants capable of scavenging ROS have been tested on STZ-induced diabetic rats to examine their effects on glucose metabolism and on oxidative stress during diabetes (25).

Biomarkers of ROS reactions have the potential not only to determine the extent of oxidative injury, but also to predict the potential efficiency of therapeutic strategies aimed at reducing the oxidative stress. Lipid peroxidation is an important biological consequence of oxidative cellular damage which increases drastically in diabetes. The polyunsaturated fatty acyl side chains, because of their susceptibility to oxidative damage in membrane phospholipids, pose a constant threat to cellular integrity and function (26). Oxidative stress associated with diabetes elevates the peroxidation of cellular lipids, which in turn are reflected by liver MDA levels. The oral administration of the extract significantly decreased the MDA levels, suggesting a reduction in the oxidative stress. The protein oxidation provoked by reactive free radicals has been demonstrated to play a significant role in diabetes and its complications (27,28). Accumulation of modified proteins disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways (29). Major molecular mechanisms leading to structural changes in proteins are free radical-mediated protein oxidation characterized by protein carbonyl formation (19). The elevated levels of the liver PCO in diabetic rats were quenched by the plant extract.

The decrease in antioxidant enzymes such as SOD, CAT and glutathione peroxidase appears to be responsible for the oxidative stress in some diseases such as diabetes (3,30). To maintain the balance between oxidative and antioxidative process and to avert oxidative stress, an external source of the antioxidant protection system is consequently crucial (25,30). The activities of liver SOD and CAT were both lower in the diabetic situation. A decreased in the activities of these enzymes can lead to an excess availability of superoxide and hydrogen peroxide in the biological systems, which in turn generate hydroxyl radicals involved in initiation and propagation of lipid peroxidation (31). However, treatment of the diabetic rats with the crude extract significantly increased the CAT and SOD activities. GSH is a major intracellular redox buffer that may approach concentrations up to 10 mM. GSH functions as a direct free radical scavenger and as cosubstrate for glutathione peroxidase activity (32). The decrease in GSH level in liver during diabetes is probably due to its increased utilization by the hepatic cell. This may be due to the attempt by the hepatocytes to counteract the increased formation of lipid peroxides (33). In our study, a significant rise in the liver GSH level was observed in the plant-treated diabetic rats.

Mechanisms that contribute to the oxidative stress in diabetes include lipid peroxidation, stimulation of protein glycation, polyol pathway, and inactivation of antioxidant enzymes (3-5). Antioxidants such as vitamin C and E, melatonin and flavonoids have been shown to reduce the oxidative stress in experimental diabetes (34,35). Therefore, the antioxidant activity of *Achillea santolina* might be due to similar antioxidative agents in this plant. Previous studies in our lab using STZ-induced diabetic rats showed that the hydroalcoholic extracts of *Achillea santolina* reduced the blood glucose levels. The results of this investigation might set up a correlation between the hypoglycemic and antioxidant activity of the plant. This conclusion however, does not exclude the possibilities of other mechanisms by which the plant exerts its effects such as improving insulin sensitivity. Further work is required to disclose this point. To come up with such a correlation, we need to have access to the purified agents of the plant responsive for its antidiabetic and antioxidative properties. These types of activities are under our present investigation.

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