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RESEARCH ARTICLE

Inhibitory effect of effective fraction of *Salvia officinalis* on aldose reductase activity: strategy to reduce complications of type 2 diabetes

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Abstract
Aldose reductase (AR) is the key enzyme of the polyol pathway, which plays an important role in the pathogenesis of diabetic complications. AR inhibitors can be used as an important strategy in the treatment of diabetic complications. The aim of the present study was to investigate effect of different fractions of *Salvia officinalis* on the bovine lens aldose reductase activity. For this purpose, the phenolic and flavonoid contents, IC_{50} values of different fractions of the *S. officinalis* to neutralize the DPPH free radicals were first measured. Then, attempts were made to investigate the effect of these fractions on the AR enzyme activity. Results indicated that ethyl acetate fraction had the highest of phenolic and flavonoid contents by 412.6 ± 1.55 and 372.5 ± 6.47 mg/ml, respectively. Also, the ethyl acetate fraction showed the lowest IC_{50} content of 1.18 µg/ml for scavenging of the free radicals and 9.25 µg/ml for the inhibition of AR activity. According to the Lineweaver–Burk plot, the ethyl acetate fraction acts as an uncompetitive enzyme inhibitor. These findings revealed that all fractions showed inhibitory effect on AR activity, where in ethyl acetate fraction it was found to be maximum which may be due to its high phenolic and flavonoid content.

Keywords Aldose reductase · Diabetic complications · *Salvia officinalis* · Ethyl acetate fraction

Introduction

Diabetes mellitus (DM) is a complex metabolic disorder caused by the lack of insulin production or insulin resistance (IR) (Tang et al. 2012). Increasing blood sugar levels in diabetics leads to long-term complications of diabetes. Although the development of diabetic complications can be prevented by controlling the blood sugar levels, it is difficult to maintain normal blood sugar during the life of a person with diabetes. Cataract is one of the hyperglycemia complications, which occurs due to sorbitol accumulation. When the level of blood sugar is high, excess sugar enters the polyol pathway, which are them converted to sorbitol by the AR enzyme and sorbitol to fructose by sorbitol dehydrogenase. Since sorbitol cannot cross the membrane, it accumulates inside the cell and results in hyperosmotic effect, which causes cell damage and changes in permeability of the membrane (Lee et al. 2010). The AR is the key enzyme of the polyol pathway with EC 1.1.1.21, the cofactor of which is NADPH (Yoon et al. 2013). In addition to its important role in the development of cataract, the role of AR has been proved in the pathogenesis of diabetic complications such as neuropathy, nephropathy and retinopathy (Moon et al. 2006). Thus, AR inhibition can help prevent complications of diabetes. The identification of various chemical and plant compounds have been started to inhibit the AR enzyme since several decades ago. Although pharmaceutical companies are seeking to produce AR inhibitors, the primary inhibitors were produced about one quarter of a century ago. Primary inhibitors showed little effect or high toxicity. So far, hundreds of inhibitor molecules have been produced, some of them were associated with problems in the clinical field, including sorbinil and epalrestat, which either had little or no useful effect. On the other hand, tolrestat was an effective inhibitor that was introduced to the market, which was later abandoned. The only inhibitor used today is epalrestat. However, an AR inhibitor should have beneficial antioxidant effects and inhibitory property.

Salvia officinalis is the most valuable type of the mint family (Lamiaceae) and researches showed that it contains bitter substances, tannins, volatile essential oils (cineole,

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camphor, alpha and beta-thujone), flavonoids (apigenin and luteolin), glycosylated substances, tocopherols, rosmarinic acid and ascorbic acid. In the past, this herb was used as a diuretic agent, coagulation factor, and an antiperspirant (Arabi et al. 2014; Glisic et al. 2010). The *S. officinalis* extract has an anti-diabetic and lipid-lowering effect (Bower et al. 2016). *Salvia officinalis* is effective in improving memory and has been used to treat Alzheimer's in traditional Chinese medicine (Perry et al. 1998). Several separate studies, one of which was conducted in Canada, investigated the effect of the *S. officinalis* extract on a number of people with dementia and results showed that this herb has an inhibitory effect on cholinesterase, anti-Alzheimer's effect and improves the nervous system in the target population (Howes and Perry 2011; Imanshahidi and Hosseinzadeh 2006). The results of another study in Israel showed that *S. officinalis* has anti-inflammatory, anti-bacterial and antiviral effects, as well as direct activity in the respiratory tract, cough reflux, and nasal airflow (Rakover et al. 2008). The results of a study in Brazil proved that *S. officinalis* hydroalcoholic extract has beneficial effects in reducing damages caused by oxidative stress such as neurological disorders, inflammation and cancer (Garcia et al. 2016). So far, there has been no study on effect of this herb on AR activity. In this study, the effect of crude extract and different fractions of *S. officinalis* on the activity of bovine lens AR were investigated.

Materials and methods

Chemical materials

DPPH and Folin–Ciocalteu reagent (FCR) were obtained from Sigma-Aldrich Chemical Co. Ltd. (England). Gallic acid, ascorbic acid, quercetin, catechin, and sulfate ammonium were obtained from Sigma (St. Louis, MO, USA). DL-glyceraldehyde, the reduced from of nicotinamide adenine dinucleotide phosphate (NADPH), diethyl ether, ethyl acetate and ethanol were obtained from Merck Co. All other chemicals used were analytical grade. Glass double distilled water was used in all experiments.

Plant material

The flowering branches of *S. officinalis* were collected from the lands in Kuhdasht city of Lorestan province between June and August of 2016. The collected material were separated and dried in the shade away from sunlight. After being dried, the materials were separately milled and stored in the refrigerator until use.

Preparation of crude extract and organic fractions of *Salvia officinalis*

One hundred grams of the powder was extracted four times (overnight) with 1 L of mixture of ethanol: water (8:3 ratios) at 60 °C. The extracts were filtrated, concentrated using a rotary evaporator and then dried to a residue by lyophilization. The average yield of the extracts was 22%. The residue re-dissolved in water and divided into two aliquots. One aliquot was kept at − 20 °C and the other aliquot was subjected to fractionation processes. The aliquot was extracted first with diethyl ether for four times at room temperature. The extracted liquid phase was then re-extracted with ethyl acetate for four times. The resulting three fractions (diethyl ether, ethyl acetate and water) were evaporated under vacuum to dryness to give the diethyl ether, ethyl acetate, and water fractions, respectively. They were quantitatively re-dissolved in ethanol to a 10 mg/ml concentration. The stock solutions were kept at − 20 °C in the dark for future analyses.

Determination of the phenolic and flavonoid contents

The phenolic content of the crude extract and different fractions of the *S. officinalis* was determined using Folin–Ciocalteu reagent (FCR) according to published methods with some modifications. The results were expressed as mg gallic acid equivalents (GAE) per gram of the dried extract or fraction (Slinkard and Singleton 1977). The flavonoid content of the crude extract and different fractions of the *S. officinalis* was measured using a calorimetric method described in scientific articles with some changes. The results were expressed as mg cathicine equivalents (GAE) per gram of the dried extract or fraction (Zhishen et al. 1999).

Antioxidant activity using DPPH radical scavenging

The DPPH radical scavenging activity of crude extract and different fractions was measured by using the method of Blois (1958). Briefly, 0.2 mM solution of DPPH in ethanol was prepared and 1 ml of this solution was added to 1 ml of different concentrations (25–400 µg/ml) of the extract and different fractions. The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Vitamin C was used as the positive control. In this study, IC_{50} value

of crude extract and different fractions was calculated and compared with IC_{50} of vitamin C, which is an indicator of antioxidant activity measurement.

Enzyme preparation from lens homogenates

Fifty cow's eyes were prepared from the nearest slaughterhouse, and according to the Hyman–Kinoshita method with slight modifications, its AR was purified and stored in the freezer (−70 °C) for further use (Hayman and Kinoshita 1965; Lee et al. 2008a, b). A liquate of the purified enzyme was used to determine the enzyme activity.

Measurement of AR activity

The enzyme activity was determined by measuring the reduction of the NADPH absorption in 340 nm, at every 30 s intervals for 5 min. Each ml of cuvette contains 100 µl enzyme, 0.5 mM phosphate buffer (500 µl) (pH = 6.2), 200 µl DL-glyceraldehyde as substrate at 0.05–0.3 mM concentrations and NADPH 0.6 mM with or without crude extract and different fractions. The concentration of crude extract and different fractions, which inhibits 50% of the enzyme activity (IC_{50}), was determined by the regression line curve showing concentration versus activity (Lee et al. 2008a, b).

Determining the type of AR inhibition

To determine the AR inhibiting activity, 0.3 ml of crude extract and different fractions (5 and 10 µg/ml) from various stock solutions was added to the reaction mixture consisted of 0.5 ml phosphate buffer with pH = 6.2, NADPH (0.6 mM), enzyme and different concentrations of glyceraldehyde (0.05–0.3 µM) as substrate (Kim et al. 2013). The AR activity was measured based on the decrease in NADPH absorption at 340 nm after adding the substrate based on BioTek power wave XS spectrophotometer (BioTek Instruments, VT, USA).

Statistical analysis

All values are expressed as mean ± S.D. The significance of differences between the means of the treated and untreated groups have been calculated by unpaired Student's *t* test and *p* values less than 0.05 were considered significant.

Results

The phenolic and flavonoid contents

The content of phenolic and flavonoid compounds in the crude extract and different fractions were determined and expressed in terms of gallic acid and catechin equivalents (Table 1). Among these fractions ethyl acetate fraction showed the highest phenolic and flavonoid contents by 412.60 mg gallic acid equivalents/g dried fraction/extract and 372.49 mg catechin equivalents/g dried fraction/extract, respectively.

Table 1 The phenol and flavonoid contents of the crude extract and different fractions of *Salvia officinalis*

Sample	Crude phenolic content[a]	Crude flavonoid content[b]
Crude extract	165.2 ± .41	1.89 ± 160.07
Diethyl ether fraction	1.12 ± 141.08	89.38 ± 1.39
Ethyl acetate fraction	412.60 ± 1.55	372.49 ± 6.47
Water fraction	184.76 ± .94	174.79 ± 5.08

The results of the three independent tests were expressed as mean ± SD

[a]The crude amount of phenolic compounds expressed as milligrams of gallic acid equivalents per gram of dry weight

[b]The crude amount of flavonoid compounds was expressed as milligrams of catechin equivalents per gram of dry weight

Table 2 DPPH radical scavenging activity of the crude extract and fractions of *Salvia officinalis*

Sample	IC_{50} (µg/ml)
Crude extract	20.16 ± 1.2
Diethyl ether fraction	20.41 ± 1.3
Ethyl acetate fraction	1.18 ± 0.2
Water fraction	15.72 ± 1.1
Ascorbic acid	9.56 ± 0.4

The results of the three independent tests were expressed as mean ± SD

DPPH radical scavenging activity

The DPPH test is widely used to evaluate the free-radical scavenging capacity of antioxidants. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. The effective concentrations of crude extract, different fractions and vitamin C required to scavenge 50% of the DPPH radicals, the IC_{50} values; are presented in Table 2. As shown in this Table, the ethyl acetate fraction has the highest activity and has a much better performance than vitamin C with IC_{50} of 9.56 µg/ml.

Determining IC_{50} of crude extract and different fractions of *Salvia officinalis* for the inhibition of AR activity

The effects of the crude extract and different fractions for the inhibition of AR activity were estimated, using

D-glyceraldehide as a substrate. Their inhibitory potencies and IC_{50} values on the AR enzyme were estimated and were showed in Table 3. Results indicated that all fractions were found to inhibit lens AR activity. Among the different fractions and crude extract, the ethyl acetate fraction had the highest AR inhibitory activity ($IC_{50} = 9.25$).

Determining of AR inhibition type

Kinetic analysis of AR inhibition was performed by ethyl acetate fraction using the lineweaver–burk plot showing 1/V versus 1/S (Fig. 1). The results showed uncompetitive inhibition of AR by the ethyl acetate fraction of the *S. officinalis*.

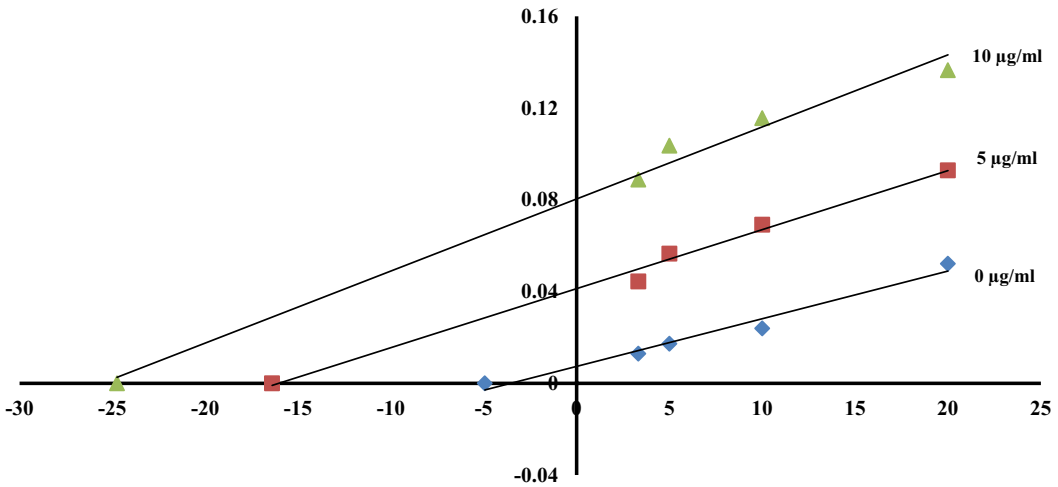
Table 3 The IC_{50} value of crude extract and different fractions of *Salvia officinalis* for the inhibition of AR enzyme

Sample	IC_{50} (µg/ml)
Crude extract	23.08 ± 2.5
Diethyl ether fraction	9.25 ± 1.0
Ethyl acetate fraction	14.13 ± 2.2
Water fraction	228.75 ± 15.1
Quercetin	1.65 ± 0.2

The results of the three independent tests were expressed as mean ± SD

Discussion

One of the mechanisms that is involved in the development of chronic diabetic complications is the polyol pathway, the key enzyme of which is aldose reductase, which catalyzes the rate limiting step. On the other hand, increased AR activity causes oxidative stress associated with diabetes in the lens, peripheral nerve and kidney cortex through the elimination of major non-enzymatic antioxidants, ascorbate, taurine and changes in redox glutathione levels (Obrosova et al. 2010; Suzen and Buyukbingol 2003). Various researches have shown that the elimination of antioxidants is prevented or reversed by AR inhibition or any defect in its gene (Obrosova 2002, 2005; Valko et al. 2007). In one study, sorbinil-based AR inhibition resulted in the expression normalization of 71% of oxidative stress genes in retinal vessels of diabetic rats (Livingstone et al. 1995). Therefore, AR inhibition is a suitable solution to reduce or prevent the chronic complications of diabetes. One of the groups that are used as an AR inhibitor is phenolic compounds, including flavonoids. Flavonoids have strong antioxidant effects and the scavenging of hydroxyl radicals, superoxide anions and proxy lipids radicals (Bors et al. 1997; Lean et al. 1999; Varma et al. 1975). Flavonoids and other antioxidants have the potential to prevent and treat many diseases. It was reported in 1975 that flavonoids have inhibitory activity against the purified rat lens AR enzyme (Varma et al. 1975). One the example of



	I=0	I=10 µg/ml
1/vm	0.0073	0.0819
1/km	3.47	24.81
Vm	136.98	12.21
Km	0.288	0.04

Fig. 1 Uncompetitive inhibition of AR by ethyl acetate fraction of *Salvia officinalis*

the flavonoid group that has been investigated frequently is quercetin, which reduces the sorbitol accumulation rate and causes delay in the onset of cataract formation (Obrosova and Fathallah 2000; Obrosova 2002; Varma et al. 1975). According to the foregoing, AR enzyme causes oxidative stress in addition to the chronic complications of diabetes; therefore, a good AR inhibitor must have an inhibitory effect on the activity of the enzyme as well as an antioxidant activity. Considering the antioxidant and anti-inhibitory properties of phenolic and flavonoid compounds extracted from different plants, the amount of these compounds in crude extract and different fractions of the *S. officinalis* was first measured. The results showed that the phenolic and flavonoid content of the plant is at the acceptable level. Among crude extract and different fractions, the highest phenolic and flavonoid content was obtained from ethyl acetate fraction with phenolic and flavonoid contents of 412.60 ± 1.55 and 372.49 ± 6.47, respectively. Also, the most antioxidant activity was related to ethyl acetate fraction with $IC_{50} = 1.18$. Considering the high phenolic and flavonoid contents of the ethyl acetate fraction of the *S. officinalis* in this study, and the low IC_{50} value of this fraction in the naturalization of DPPH free radicals and the confirmation of the antioxidant property of the plant, the effect of each of them on the inhibition of the AR activity was separately investigated.

According to the tests performed, the ethyl acetate fraction of the *S. officinalis* with IC_{50} of 9.25 µg/ml had the highest inhibitory activity, which had excellent activity as compared with the quercetin as a positive control with $IC_{50} = 1.65$. Since this fraction has the highest phenolic and flavonoid content on the one hand, and pilot studies, which referred to having a hydrophobic region for binding to the acidic group of enzymes as a common feature of a good inhibitor, and flavonoids can meet this need on the other, it was concluded that the ethyl acetate fraction could be used as a good AR inhibitor. The results also showed uncompetitive inhibition of AR by the ethyl acetate fraction of the *S. officinalis*. In this type of inhibition, the inhibitor compound only binds to the enzyme–substrate complex and both of Vm and Km are reduced accordingly. In this type of inhibition, increased substrate concentration cannot overcome the inhibition, since as the substrate concentration increase; better conditions in which enzyme inhibitor binds to the complex formed between the enzyme and the substrate. Considering the foregoing, the presence of uncompetitive inhibition between the ethyl acetate fraction of the *S. officinalis* with aldose reductase enzyme is considered as an advantage, because this inhibitor can be successful even in high blood sugar levels.

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Compliance with ethical standards

Ethical statement N/A.

Conflict of interest This manuscript described has not been published before; not under consideration for publication anywhere else; and has been approved by all co-authors.

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