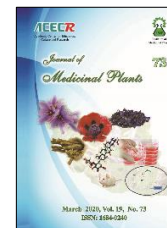




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Research Article

Effective fraction of *Teucrium polium* suppressed polyol pathway through inhibiting the aldose reductase enzyme: strategy to reduce retinopathy

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ABSTRACT

Background: Several metabolic pathways are involved in the complications of diabetes like polyol pathway. Aldose reductase (AR) is a key enzyme in the polyol pathway, which catalyzes the conversion of glucose to sorbitol. AR inhibitors are appropriate to prevent and treat the diabetes complications. **Objective:** This study was designed to investigate the effect of different fractions of *Teucrium polium* on the AR activity. **Methods:** Fifty cow's eye lenses were prepared and AR enzyme was purified according to the Hyman-Kinoshita method. The enzyme activity in the presence of the crude extract and different fractions of *Teucrium polium* (1, 5, 10, 20 and 100 µg/ml) was measured. In addition, IC₅₀ content of fractions was also measured for the neutralization of DPPH free radical. Since some AR inhibitors are phenolic compounds, the phenolic and flavonoid contents have been investigated. **Results:** Results showed that the highest phenol and flavonoid content and the lowest IC₅₀ value (3.67 µg/ml) for AR inhibition were related to the ethyl acetate fraction. Line weaver-Burk plot showed that ethyl acetate fraction acts as a non-competitive enzyme inhibition. **Conclusion:** Thus, *T. polium* can be proposed as a therapy to prevent or treat chronic complications of diabetes in the future.

1. Introduction

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic disorders with long-term complications such as retinopathy, neuropathy, and nephropathy [1].

Diabetic retinopathy is one of the earliest critical secondary complications of diabetes that

affect eyes. It is characterized by cloudiness or opacification of the lens that is responsible for focusing light and producing clear and sharp images [2]. Several metabolic pathways are involved in retinopathy, like activation of polyol pathway, non-enzymatic glycation of eye lens proteins and oxidative stress [3]. Upon cell

Abbreviations: AR, Aldose reductase; CAE, Catechin equivalents; DM, Diabetes mellitus; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FCR, Folin-Ciocalteu reagent; GAE, Gallic acid equivalents; G6P, glucose-6-phosphate; NADPH, Nicotinamide adenine dinucleotide phosphate; ROS, Reactive oxygen species; *T. polium*, *Teucrium polium*

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entry, glucose is converted to glucose-6-phosphate (G6P) by hexokinase to enter the glycolysis pathway, and only a small amount of non-phosphorylated glucose enters the polyol pathway; however, under hyperglycemia conditions where hexokinase is saturated with glucose, about one-third of intracellular glucose enters the polyol pathway, which is converted to sorbitol and fructose by the AR enzyme (EC 1.1.1.21) and sorbitol dehydrogenase, respectively [4, 5]. The subsequent intracellular accumulation of sorbitol leads to the hyperosmotic effect, which in turn can alter the membrane permeability and cell damage [6, 7]. AR is a key enzyme in polyol pathway, catalyzes the rate limiting step of the polyol pathway, and nicotinamide adenosine dinucleotide phosphate-dependent enzyme [4, 8]. On the other hand, increased hyperglycemia and insulin resistance increase free radical production and oxidative stress in diabetes. Hyperglycemia may increase ROS production by activating the polyol pathway and increasing glucose auto-oxidation mechanisms. Therefore, hyperglycemia-induced oxidative stress increases risk of diabetes-related complications. So, inhibiting AR enzyme is a major strategy in the prevention or treatment of chronic complications of diabetes [9, 10]. AR inhibitors are classified into various groups, including carboxylic acid derivatives (e.g., zopalrestat, tolrestat, ponalrestat), phenol derivatives, and spiro hydantoin, and related cyclic amines (e.g., sorbinil and fidarestat). Some of these inhibitors have entered advanced clinical trials [11]. Plant-based AR inhibitors are important considering their easy access, low cost, and fewer side effects. Flavonoids are a large group of plant compounds that are found in almost all fruits and vegetables and medicinal plants [12]. These antioxidants compounds have an effect on

oxidative stress in type 1 and type 2, diabetes, and have been considered as protective agents [13, 14]. Several studies reported that flavonoids have an inhibitory effect on the activity of the aldose reductase purified from rat lens [13-19]. *Teucrium polium* (*T. polium*) is a perennial shrub, 20-50 cm high, distributed widely in the dry and stony places of the hills and deserts of almost all Mediterranean countries, South Western Asia, Europe and North Africa. It also has high anti-fever, antibacterial, anti-inflammatory, and antioxidant effects. *T. polium* tea is used for the treatment of abdominal pain, indigestion, colds, type 2 diabetes and genitourinary diseases in Iranian traditional medicine. In addition, *T. polium* is used to treat digestive diseases, inflammation, diabetes, and rheumatism in the Mediterranean countries [13, 14]. It is also used as an agent with anti-scar, and blood pressure lowering, antispastic, anorexia, and antifever properties. Up to now, more than 134 active substances with wide structural and chemical diversities have been isolated and characterized from the aerial parts, roots and seeds of *T. polium*. In that line, components of the volatile oil including diterpenoids, flavonoids, steroidal compounds, caffeic acid and its derivatives have been identified [14].

Previous studies have shown that the aqueous extract of the dried aerial parts of *T. polium* is used by many type 2 diabetic patients particularly in the Southern Iran as an antidiabetic drug [20, 21]. Ardestani and Yazdanparast (2007) evaluated the antioxidant and antiglycation activities of several organic fractions of the *T. polium* extract [22]. So far, there is no study on effect of this herb on AR activity. This study was done to investigate the effect of crude extract and different fractions of the plant on the activity of bovine lens AR enzyme.

2. Materials and Methods

2.1. 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 form 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.

2.2. Plant material

The flowering branches of *T. polium* 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.

2.3. Preparation of crude extract and organic fractions of *Teucrium polium*

The crude extract and organic fractions of *T. polium* were prepared based on Ardestani and Yazdanparast method [22]. One hundred grams of the powder was extracted four times (overnight) with 1 L of mixture of ethanol: water (7:3 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 were 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.

2.4. Determination of the phenolic and flavonoid contents

The phenolic content of the crude extract and different fractions of the *T. polium* 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 [23]. The flavonoid content of the crude extract and different fractions of the *T. polium* was measured using a calorimetric method described in scientific papers with some changes. The results were expressed as mg cathicine equivalents (GAE) per gram of the dried extract or fraction [24].

2.5. Antioxidant activity using DPPH radical scavenging

The DPPH radical scavenging activity of crude extract and different fractions was measured by using the method of Bahramikia and Yazdanparast [25]. 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, IC₅₀ value of crude extract and different fractions was

calculated and compared with IC₅₀ of vitamin C, which is an indicator of antioxidant activity measurement.

2.6. Enzyme preparation from lens homogenates

Fifty cow's eyes were prepared from the nearest slaughterhouse, and AR was purified according to the Hyman-Kinoshita method with slight modifications and stored in the freezer (-70 °C) for further use [26, 27]. A lique of the purified enzyme was used to determine the enzyme activity.

The enzyme activity in the presence of the crude extract and different fractions (1, 5, 10, 20 and 100 µg/ml) and DL-glyceraldehyde as substrate and NADPH as cofactor was measured and compared the control without plant.

2.7. 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 (1, 5, 10, 20 and 100

µg/ml). The concentration of crude extract and different fractions, which inhibits 50% of the enzyme activity (IC₅₀), was determined by the regression line curve showing concentration versus activity [28, 29].

2.8. Determining the type of AR inhibition

To determine the AR inhibiting activity, 0.3 ml of ethyl acetate fraction (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), AR enzyme and different concentrations of glyceraldehyde (0.05-0.3 µM) as substrate. 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).

2.9. 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.

Table 1. The phenolic and flavonoid contents in crude extract and different fractions of *T. polium*

Sample	¹ Total Phenolic Content	² Total flavonoid Content
Crude extract	101.36 ± 0.83	61.34 ± 2.48
Diethyl ether fraction	62.02 ± 1.76	24.21 ± 0.79
Ethyl acetate Fraction	104.18 ± 2.56	82.55 ± 0.73
water fraction	84.1 ± 1.31	73.17 ± 3.52

¹ The total amount of phenolic compounds expressed as milligrams of gallic acid equivalents per gram of dry weight.

² The total amount of flavonoid compounds was expressed as milligrams f catechin equivalents per gram of dry weight. The results of the three independent tests were expressed as mean ± SD.

3. Results

3.1. 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 104.18 mg gallic acid equivalents/g dried fraction/extract and 82.55 mg catechin equivalents/g dried fraction/extract, respectively.

3.2. DPPH radical scavenging activity

The DPPH test is widely used to evaluate the free-radical scavenging capacity of antioxidants. The effect of 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 μ g/ml.

Table 2. DPPH radical scavenging activity (IC_{50}) of the crude extract and different fractions of *Teucrium polium*

Samples	IC_{50} (μ g/ml)
Crude extract	24.33 ± 2.1
Diethyl ether fraction	144.0 ± 6.1
Ethyl acetate fraction	115.0 ± 4.7
Water fraction	52.3 ± 1.9
Ascorbic acid	9.56 ± 0.9

The results of the three independent tests were expressed as mean $\pm$ SD.

3.3. Determining IC_{50} of crude extract and different fractions of *Teucrium polium* for the inhibition of AR activity

The effects of the crude extract and different fractions for the inhibition of AR activity were measured, using D-glyceraldehyde as a substrate. Their inhibitory activates and IC_{50}

values on the AR enzyme 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 ($IC_{50} = 3.67$).

Table 3. The IC_{50} content of total extract and fractions of *Teucrium polium* for inhibition of AR Enzyme

Samples	IC_{50} (μ g/ml)
Crude extract	6.97 ± 1.5
Diethyl ether fraction	3.67 ± 1.1
Ethyl acetate fraction	6.19 ± 1.7
Water fraction	285.3 ± 25.1
Ascorbic acid	1.40 ± 0.2

3.4. Determining of AR inhibition type

Kinetic analysis of AR inhibition was performed by ethyl acetate fraction using the lineweaver-burk plot showing $1/V$ vs. $1/S$ (Fig. 1). The results showed noncompetitive inhibition of AR by the ethyl acetate fraction of the *T. polium*. The present study investigated the inhibitory effect of total extract and fractions of the *T. polium* on the AR activity.

4. Discussion

Present study was done to find a major strategy to inhibit AR to treat diabetes complications. As above mentioned, AR is a key enzyme in the polyol pathway, catalyzes the rate limiting process, and any elevated activity causes diabetes complications in various tissues [2, 3]. AR inhibitors include a variety of different structural compounds such as plant extracts and specific small molecules [30]. Increased blood glucose increases centralized glucose autooxidation, which in turn produces free radicals.

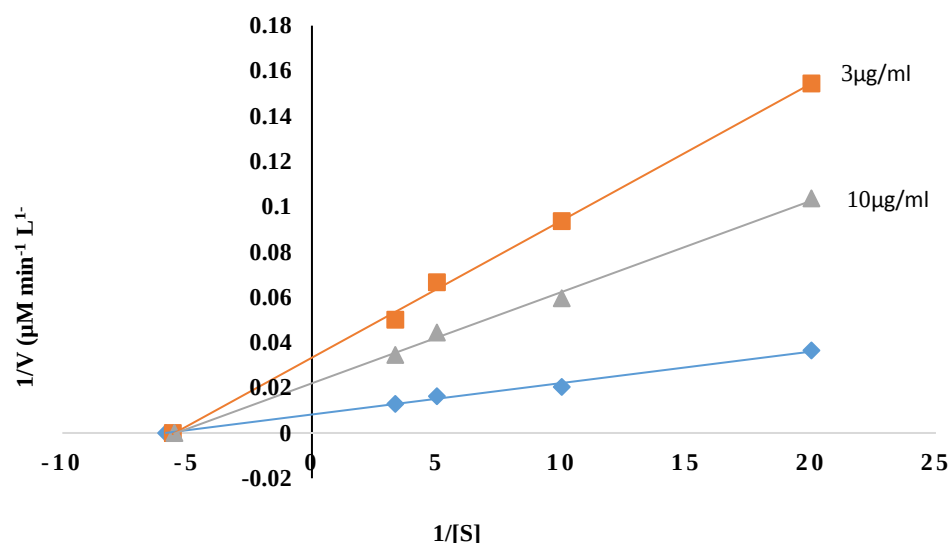


Fig. 1. Noncompetitive inhibition of aldose reductase (AR) by ethyl acetate fraction of *Teucrium polium* (concentrations of 3 and 10 $\mu\text{g/ml}$)

In this case, many free radicals are produced, which cannot be eliminated by the antioxidants defense systems of the body, which in turn causes complications of diabetes. As well as, antioxidant-based treatment, like food antioxidants such as vitamin E and lipoic acid, is effective to reduce oxidative stress. Studies have shown that antioxidant-enriched food supplements are capable to prevent and treat complications and treat those occurred [31]. Polyphenols and flavonoids in plant extracts, as strong and natural antioxidants, are the main factors that limit the adverse effects of oxidative stress in the body. They also are important to prevent and treat many common inflammatory diseases by inhibiting free radicals, such as diabetes, and reducing their associated complications [31]. So, research studies have documented the ethnopharmacological, phytochemical, and antioxidant researches on the anti-diabetic properties of native vegetables and herbs. In 1975, it was reported that flavonoids have an inhibitory effect on the

activity of the aldose reductase purified from rat lens [15].

Quercetin is main flavonoids in the studies, which reduces the sorbitol accumulation in the lens and thus prevents cataract formation [16-19]. AR enzyme also causes oxidative stress combined with chronic diabetes complications; therefore, a good AR inhibitor must have both an inhibitory effect on enzyme activity and antioxidant activity. Regarding the antioxidant and inhibitory properties of phenol and flavonoid compounds extracted from different plants, the amount of these compounds was first measured in total extract and different fractions of *T. polium* [18]. The results showed that the phenol and flavonoid content of the plant is acceptable. The highest phenol and flavonoid content was obtained from ethyl acetate fraction (phenol content = 104.18 ± 2.56 VS. flavonoid content = 82.55 ± 0.73). Since flavonoid compounds have also antioxidant effects, the different fractions were successful in DPPH radical scavenging and inhibited it to a large

extent. Due to high phenol and flavonoid content and the high antioxidant activity of the plant, attempts were made to investigate the effect of each of the fractions on inhibition of AR activity. Results showed that Ethyl acetate fraction of *T. polium* ($IC_{50} = 3.67 \mu g / ml$) had the highest inhibitory activity, which was superior to quercetin as a positive control ($IC_{50} = 1.65 \mu g / ml$).

Since this fraction has the highest phenol and flavonoid content, and due to studies on common feature of a good inhibitor which is having a hydrophobic region to bind the acidic group of enzymes, as well as, capability of flavonoids to meet such need, so, it can be concluded that the ethyl acetate fraction can be used as a good AR inhibitor [15-19]. Our results were in agreement with a previous study where indicated *Ginkgo biloba* extract, quercetin and its derivative rutin reduced the activity if this rate limiting enzyme, while quercetin proved itself to be the most potent flavonoid by having the most significant inhibitory effect [19]. As a result, ethyl acetate fraction of *T. polium* demonstrated a non-competitive inhibition. These inhibitors can bind the enzyme and enzyme-substrate complex, which reduces V_m , while K_m remains constant. In these inhibitions, there is no competition between the inhibitor and substrate to bind the enzyme, and the high substrate concentration cannot overcome inhibition; thus the binding of the inhibitor to

the enzyme-substrate complex leads to its deactivation.

5. Conclusion

Results of this study indicated that ethyl acetate fraction of *T. polium* acts as a non-competitive enzyme inhibition. This type of inhibition is an advantage, since this inhibitor can be successful even in high blood glucose levels. Thus, *T. polium* can be proposed as a therapy to prevent or treat chronic complications of diabetes in the future.

Author contributions

Somayeh Amraee carried out the experiment and contributed to the data collection. Seifollah Bahramikia contributed to the study design, preparation, revision, manuscript writing and the approval of the final version of manuscript to be published. Abdelnasser Mohammadi contributed to data analysis and interpretation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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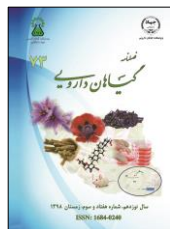
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فراکسیون مؤثر گیاه مریم‌نخودی مسیر پلی ال را از طریق مهار آنزیم آلدوز ردوکتاز کاهش می‌دهد: استراتژی برای کاهش رتینوپاتی

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چکیده	اطلاعات مقاله
مقدمه: چندین مسیر متابولیکی در ایجاد عوارض دیابت نقش دارند که یکی از آنها مسیر پلی ال است. آنزیم کلیدی این مسیر آلدوز ردوکتاز می‌باشد که تبدیل گلوکز به سوربیتول را کاتالیز می‌کند. از جمله مواردی که می‌تواند در پیشگیری و درمان عوارض دیابت مؤثر باشد، مهارکننده‌های آلدوز ردوکتاز می‌باشد. هدف: در این مطالعه اثر فراکسیون‌های مختلف گیاه مریم‌نخودی بر فعالیت آنزیم آلدوز ردوکتاز بررسی شد. روش بررسی: ۵۰ عدد لنز چشم گاو از کشتارگاه تهیه و آنزیم آلدوز ردوکتاز با استفاده از روش Hyman-Kinoshita method تخلیص شد. فعالیت آنزیم در حضور غلظت‌های مختلف (۱، ۵، ۱۰، ۲۰ و ۱۰۰ میکروگرم بر میلی‌لیتر) عصاره و فراکسیون‌های آلی گیاه مریم‌نخودی بررسی شد. میزان IC_{50} فراکسیون‌های مختلف برای خنثی سازی رادیکال آزاد DPPH نیز اندازه‌گیری شد. علاوه بر آن چون گروهی از مهارکننده‌های این آنزیم ترکیبات فنولی هستند میزان ترکیبات فنولی و فلاونوئیدی این گیاه مورد بررسی قرار گرفت. نتایج: طبق نتایج به دست آمده بیشترین مقدار فنول و فلاونوئید و پایین‌ترین میزان IC_{50} برای مهار آنزیم آلدوز ردوکتاز با مقدار ۳/۶۷ میکروگرم بر میلی لیتر مربوط به فراکسیون اتیل استاتی با بود مطالعات کیتیکی نشان داد که نوع مهار فراکسیون‌ها از نوع غیررقابتی بود. نتیجه‌گیری: با توجه به این یافته‌ها با جداسازی ترکیبات مؤثر گیاه و تأثیر آنها بر فعالیت آنزیم آلدوزردوکتاز می‌توان در آینده از این گیاه برای پیشگیری و یا درمان عوارض مزمن دیابت استفاده کرد.	کلواژگان: مریم‌نخودی آلدوز ردوکتاز فراکسیون اتیل استاتی مسیر پلی ال

مخفف‌ها: (AR) Aldose reductase; (CAE) Catechin equivalents; (DM) Diabetes mellitus; (DPPH) 2,2-diphenyl-1-picrylhydrazyl; (FCR) Folin-Ciocalteu reagent; (GAE) Gallic acid equivalents; (G6P) glucose-6-phosphate; (NADPH) Nicotinamide adenine dinucleotide phosphate; (ROS) Reactive oxygen species; (*T. polium*) *Teucrium polium*.
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