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Research Article

Inhibitory Effect of *Heracleum persicum* and *Ziziphus jujuba* on Activity of Alpha-Amylase

Reza Afrisham,[1] Mohammad Aberomand,[2] Mohammad Ali Ghaffari,[2] Amir Siahpoosh,[3] and Mostafa Jamalan[2]

[1]*Department of Clinical Biochemistry, Faculty of Medicine, Student Research Committee, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 15794-61357, Iran*
[2]*Department of Clinical Biochemistry, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 15794-61357, Iran*
[3]*Department of Pharmacognosy, Faculty of Pharmacy, Herbal Medicine & Natural Product Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz 15794-61357, Iran*

Correspondence should be addressed to Mohammad Aberomand; aberumand@yahoo.com

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Postprandial hyperglycemia plays an important role in the development of type 2 diabetes. Inhibition of alpha-amylase was led to a delay in breaks down of starch and glycogen and prevented a rapid rise in blood sugar. Alpha-amylase was isolated by gel filtration chromatography Sephadex G-75 from bovine pancreas. Then, total methanolic extracts of plants were prepared and IC_{50} values of extracts on alpha-amylase were obtained and compared with acarbose IC_{50}. The polyphenolic content of extracts and antioxidant capacity were determined by Folin-Ciocalteu test and DPPH test, respectively. The specific activity of alpha-amylase was 48.2 U/mg. For inhibition of alpha-amylase, IC_{50} values of *H. persicum*, *Z. jujuba*, and acarbose were 307, 827, and 113 μg/ml, respectively. For inhibition of DPPH radical, IC_{50} values of extracts were 235 and 701 μg/ml. Total phenolic contents of methanol extracts were 73.8 ± 3.2 and 44.2 ± 1.8 μg tannic acid equivalent/mg extract. Acarbose causes gastrointestinal symptoms and liver toxicity, but *H. persicum* and *Z. jujuba* decrease these side effects and prevent gastrointestinal disorders. Due to the high polyphenolic content and antioxidant capacity of these plants and significant inhibitory effect of the plants on alpha-amylase, these plants can be proposed for treatment of diabetic patients.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia, resulting from insufficient or inefficient insulin secretion, with alterations in carbohydrate, lipid, and protein metabolism [1]. Diabetes mellitus is cause of morbidity and mortality worldwide, with an estimation of 382 million adult patients and 5.1 million deaths in the year of 2013 [2]. In diabetes mellitus, postprandial phase is characterized by a rapid increase in blood glucose and its high levels. These postprandial "hyperglycemic spikes" may be relevant to the pathophysiology of late diabetic complications that is recently receiving much attention [3]. Type 2 diabetes receives more attention than type 1 diabetes because it is considered to be a preventable disease. Type 2 diabetes is caused by an imbalance between blood sugar absorption and insulin secretion. Postprandial hyperglycemia plays an important role in the development of type 2 diabetes [4].

However, oxidative stress is caused by reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) that play an important role in the pathogenesis of various degenerative diseases such as diabetes, chronic liver disease, aging, and obesity [5]. Postprandial oxidative stress is associated with a higher risk for diabetes, obesity, high blood pressure, and atherosclerosis. Therefore, the use of agents that reduce postprandial hyperglycemia and oxidative stress may be a treatment for diabetic patients [6].

Human alpha-amylase is secreted by the salivary gland and the pancreas that plays an important role in the digestion

of starch and glycogen [7]. Alpha-amylase is a calcium metalloenzyme that catalyzes the hydrolysis of glycosidic α-D-(1,4)-glycosidic bonds in starch, amylose, amylopectin, glycogen, and different maltodextrins and is converted to shorter oligosaccharides. Other enzymes are also involved in process of starch breakdown, but alpha-amylase is necessary to start the process of hydrolysis [8]. Reduction in the rate of starch digestion can delay the absorption rate of glucose. Inhibition of mammalian alpha-amylase in the intestine led to a delay in breakdowns of starch and oligosaccharides to monosaccharides before they can be absorbed. This process decreases the absorption of glucose and consequently reduces blood sugar levels after postprandial phase [9].

A group of the enzyme inhibiting drugs is considered in the management of type 2 diabetes. In the past two decades, these drugs are considered as potent therapeutic agents for the treatment of type 2 diabetes. Examples of these types of drug include acarbose, miglitol, and voglibose [10, 11]. However, some side effects of these enzyme inhibitors, including liver disorders, abdominal pain, kidney tumors, flatulence, liver damage, acute hepatitis, diarrhea, and abdominal fullness, have been reported [1]. Despite development in production of synthetic drugs for treatment of diabetes, there is an interest in the use of herbal treatments due to their high efficacy, limiting side effects, easy access, and reasonable price. Today, more than 1,200 plant species are used for the treatment of diabetes; half of these plants are used traditionally [12].

Golpar (*Heracleum persicum*) is a herbaceous perennial plant of the family Umbelliferae, with aromatic members and divided and serrated leaves. It is traditionally used as an antiseptic, carminative, digestive, and food aromatic [13]. This plant reduces LDL cholesterol [14] and has strong anti-inflammatory, analgesic, anticonvulsant, antibacterial, cytotoxic, antifungal, and immunomodulatory effects [15]. Study showed that different parts of *H. persicum* contain terpenoids, terry terpenes, furanocoumarins and flavonoids, alkaloids, and volatile metabolites [16].

Another plant, *Ziziphus jujuba*, belongs to the family of Rhamnaceae [17]. *Ziziphus* species are used commonly in traditional medicine to treat various diseases such as gastrointestinal disorders, fatigue, obesity, liver pain, urinary tract problem, skin infections, diabetes, loss of appetite, fever, bronchitis, pharyngitis, anemia, diarrhea, and insomnia [18]. In particular, *Z. jujuba*'s fruits are rich in sugar, vitamin C, bioflavonoids, fibers, and minerals. Pharmaceutical ingredients in *Z. jujuba* fruits have been reported such as sterols, alkaloids, serotonin, saponins, flavonoids, polyphenols, terry terpenes, and c-GMP. The methanol extract of *Z. jujuba* fruits has pharmacological antioxidant effects. In addition, it can suppress cancer cell proliferation and protect the liver [19]. Flavonoids are plant secondary metabolites that show a large group of polyphenolic compounds. These materials have a wide range of biological activities such as anti-inflammatory, antiulcer, antitumor, antibacterial, and antithrombotic effect [20]. Flavonoids cannot be made by humans; as a result, they must come from the daily diet. Evidence suggests that flavonoids play an important biological role in eliminating reactive oxygen species [21]. Therefore, they can protect the body from free radicals and prevent the progression of some chronic diseases. Today, the antioxidant activity of flavonoids is considered for potential therapeutic applications [22]. Flavonoids and polyphenol contents of *H. persicum* and *Z. jujuba* can be useful in reducing oxidative stress and alpha-amylase inhibition. To date, no report has yet been published on the *H. persicum* and *Z. jujuba* effects on inhibition of the alpha-amylase enzyme. So, the purposes of this study were to investigate the inhibitory effects of *H. persicum* and *Z. jujuba* on the activity of alpha-amylase enzyme and also determine the polyphenolic content and antioxidant capacity of these plants.

2. Materials and Methods

2.1. Chemicals and Reagents. Acarbose was purchased from Fluka (Germany). Folin-Ciocalteu reaction, 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), Sephadex G-75, phenyl methyl-sulfonyl fluoride (PMSF), starch, and sucrose were acquired from Sigma (USA). Other materials, such as tannic acid, methanol, sodium carbonate, potassium phosphate, ammonium sulfate, sodium chloride, and calcium chloride, were obtained from Merck (Germany).

2.2. Extraction of Plants. The *H. persicum* and *Z. jujuba* fruits were purchased from one of the reputable shops in Ahvaz. The fruits were confirmed by a registered pharmacologist as *Heracleum persicum* (with the code of A140100301FP) and *Ziziphus jujuba* (with the code of A142430301FP) species, respectively. After drying, *Z. jujuba* cores were removed and fruits were converted by the electric mill and kept in the refrigerator until extracting. For the preparation of methanolic extracts, 100 g of edible powder of fruits was solved in 1000 mL of total methanol; then, the desired solution was mixed for three days at the temperature of 20–25°C using an electrical sieve shaker. After 48 hours, the solution was passed through Whatman's filter paper. Filtered solution was put up in the Ben Murray to evaporate the solvent. The extracts obtained were kept at a temperature of −20°C until analysis [23].

2.3. DPPH Radical Scavenging Assay of Extracts. The DPPH quenching ability of methanol extracts was measured according to Brand-Williams et al. [24]. Subsequently, 3.9 mL of DPPH Stoke was poured into Covet and absorption was read at 515 NM by Shimadzu's UV-Vis spectrophotometer. Then, the methanol DPPH solution was mixed with 100 μL serial dilutions (156–1250 μg/mL) of the extracts and after 30 min, the absorbance was read at 515 NM. The antiradical activity was expressed as IC_{50} (the minimum dose necessary to create a 50% inhibition) and the ability to scavenge the DPPH radical was calculated using the following:

$$\text{DPPH scavenging effect} = 100 \times \frac{(A_0 - A_s)}{A_0}. \quad (1)$$

That A_0 is the absorbance of the control at 30 min and A_s is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

2.4. Measurement of Total Phenolic Content. For measurement of total phenolic content, 0.5 mL of the extracts (concentration of 1 mg/mL) was added to 2.5 mL of Folin-Ciocalteu reagent (diluted with water at a ratio of 1 to 10); after five minutes, 2 mL Na_2CO_3 7.5 (%w/v) was added. The resulting solutions were kept in the dark at room temperature for two hours and then the absorbance was read at 765 NM. Tannic acid was used as the standard [25]. All samples were analyzed in triplicate.

2.5. Partial Purification of Alpha-Amylase from Bovine Pancreas

Step 1. 65 g of the bovine pancreas was weighed and, after washing with physiologic serum, was held in −70°C until the time of use.

Step 2. The pancreatic tissue (65 g) was homogenized in 650 mL buffer A (200 mM phosphate, pH = 7, $CaCl_2$ 3 mM, and NaCl 10 mM) and PMSF 0.3 mM, by homogenizer device, for 5 minutes at 4°C.

Step 3. The homogenate was centrifuged in 10000 g for 20 minutes at 4°C (Sigma centrifuge, model 3-30KS, Germany), the pellet was discarded, and the supernatant was separated.

Step 4. The supernatant was subjected to fractionation of ammonium sulfate precipitation. The alpha-amylase precipitated in a saturation range of 50–80% was centrifuged at 10,000 ×g for 20 min. Ammonium sulfate was added under stirring conditions in 4°C.

Step 5. After centrifugation, the precipitate was redissolved in 30 mL of buffer A and then it was dialyzed overnight against the same buffer for 48 hours and the buffer was replaced every 12 hours [26, 27].

2.6. Gel Filtration Chromatography on Sephadex G-75

Step 1. The dialyzed ammonium sulfate fraction was applied to the column (2.5x approximately 50 cm) of gel filtration Sephadex G-75, which had been equilibrated with buffer A. Flow rate of buffer from the column was adjusted to 1 mL/min.

Step 2. Collected fractions (3 mL per fraction) absorbance was read at 280 NM. Finally, homogeneity of alpha-amylase was extracted from bovine pancreas, and the fractions containing enzyme activity were pooled.

Step 3. The enzyme activity prepared from Step 2 was concentrated to a volume of 5–7 mL by sucrose.

Step 4. The concentration of the enzyme was indicated by using PAGE electrophoresis and staining with Coomassie Brilliant Blue [28].

2.7. Concentration Measurement of Soluble Protein. At each stage of extraction, concentration measurement of soluble protein was determined by Bradford method, and bovine serum albumin was used as standard [29].

2.8. Measurement of Alpha-Amylase Activity. At each stage of extraction, amylase activity was measured with the dinitrosalicylic acid method using soluble starch as substrate. The reaction mixture contained 200 μL of diluted enzyme and 200 μL of buffer A containing 1% (w/v) of soluble starch. The reaction was stopped by 400 μL of dinitrosalicylic acid color reagent and tubes were incubated at 100°C for 5 min. Then, the reaction was diluted with 1600 μL distilled water, and the absorbance was read at 540 NM. The amylase activity was calculated using maltose solutions as reference. One amylase unit (U) is defined as the amount of amylase releasing 1 μmol reducing sugar as maltose per minute [26].

2.9. Measurement of Alpha-Amylase Activity Inhibition. Different concentrations (25–3200 μg/mL) of plant extracts were prepared in DMSO. Equal volumes (100 μL) of alpha-amylase and extracts were incubated in Microtubes for 10 min at 37°C. Then, 100 μL of 1% soluble starch dissolved in buffer A was added to each of Microtubes and the samples were incubated for 30 min at 37°C. The reaction was stopped by 200 μL of dinitrosalicylic acid color reagent and Microtubes were incubated at 100°C for 5 min. Once samples had cooled in room temperature, 50 μL was removed from each Microtube and transferred to the wells of 96-well microplate. The reaction mixture was diluted by adding 200 μL of water to each well and the absorbance was measured by microplate reader (Hiperion, model MPR4$^+$, Germany) at 540 NM. Blank readings (no enzyme) were subtracted from each well and the results were compared to the positive control (acarbose) [30]:

$$\%\ \text{inhibition} = 100 \times \frac{(A_0 - A_s)}{A_0}. \quad (2)$$

That A_0 is the absorbance of control, and A_s is the absorbance of plant extracts. All samples were analyzed in triplicate.

2.10. Data Analysis. The results of three replicates were pooled and expressed as mean ± standard deviation (SD). IC_{50} values were calculated using linear diagram (by regression coefficient above 0.9) of % inhibition against logarithm of different concentrations of extracts.

3. Results

Measuring of alpha-amylase activity and protein in different stages of purification is shown in Table 1. According to the results in the last stage of purification, after gel filtration chromatography, the purification factor reached 45 with a recovery yield of 1.28% of the amylase activity. The total activity of alpha-amylase, total protein, and specific activity of the enzyme were obtained: 149.6 units, 3.1 mg, and 48.2 mg/unit, respectively. For valuation of alpha-amylase purification, PAGE electrophoresis was performed. Figure 1 indicates that the sample before chromatography (dialysis) showed several

Table 1: Measuring of alpha-amylase activity and protein at different stages of purification.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Crude extract	10820	11600	1.07	100	1
$(NH_4)_2SO_4$ precipitation (50–80%)	4130	9810	2.37	84.5	2.21
Sephadex G-75 chromatography	3.1	149.6	48.2	1.28	45

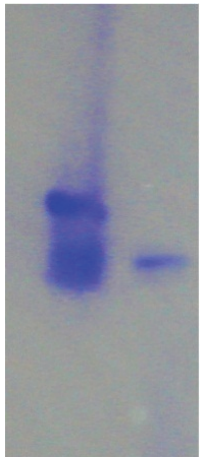

Figure 1: PAGE electrophoresis in order to compare the samples before and after chromatography; the sample before chromatography (dialysis) showed several bands; however, a distinct band was observed after chromatography.

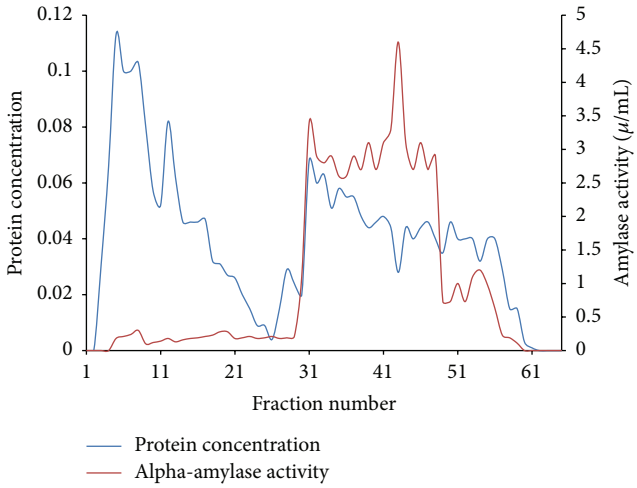

Figure 2: Evaluation of protein concentration and alpha-amylase activity of gel filtration chromatography.

bands, but distinct band was observed after chromatography. Figure 2 shows evaluation of protein concentration and alpha-amylase activity of gel filtration chromatography.

Figures 3 and 4 indicate a linear diagram of alpha-amylase % inhibition that is drawn against logarithm of different concentrations of *H. persicum* and *Z. jujuba*, respectively. For

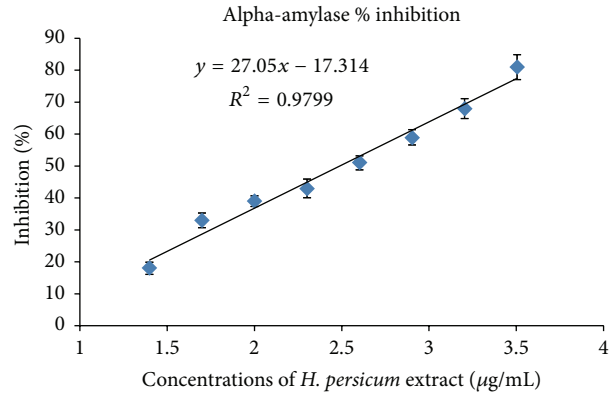

Figure 3: A linear diagram of inhibition percentage of alpha-amylase against logarithm of different concentrations of *H. persicum*; the IC_{50} value of the extract was 307 μg/mL. Values are presented as mean ± SD.

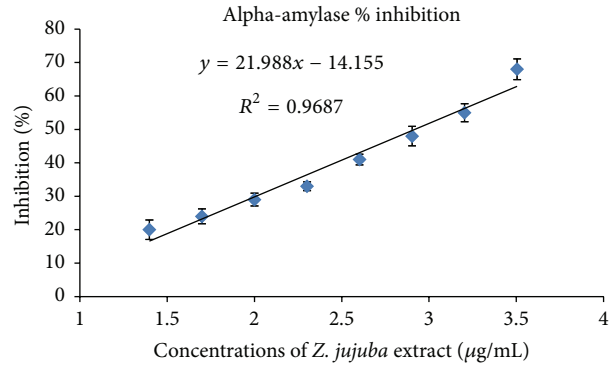

Figure 4: A linear diagram of the inhibition percentage of alpha-amylase against logarithm of different concentrations of *Z. jujuba*; the IC_{50} value of the extract was 867 μg/mL. Values are presented as mean ± SD.

alpha-amylase enzyme inhibition, the calculated IC_{50} values of extracts were 307 and 867 μg/mL, respectively.

The results for alpha-amylase inhibition assay of *H. persicum*, *Z. jujuba*, and acarbose are shown in Figure 5. The concentration of 50% inhibition of acarbose was 113 μg/mL.

Figures 6 and 7 indicate linear diagram of DPPH radical % inhibition that is drawn against logarithm of different concentrations of *H. persicum* and *Z. jujuba*, respectively. For inhibition of DPPH radical, IC_{50} values of extracts calculated were 235 and 701 μg/mL, respectively. Table 2 indicates that the total phenolic contents of methanol extracts

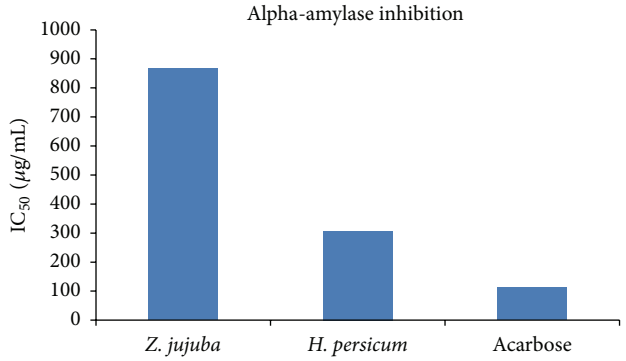

Figure 5: Comparison of alpha-amylase inhibition of *H. persicum*, *Z. jujuba*, and acarbose; the IC_{50} values of extracts were 307, 867, and 113 μg/mL, respectively.

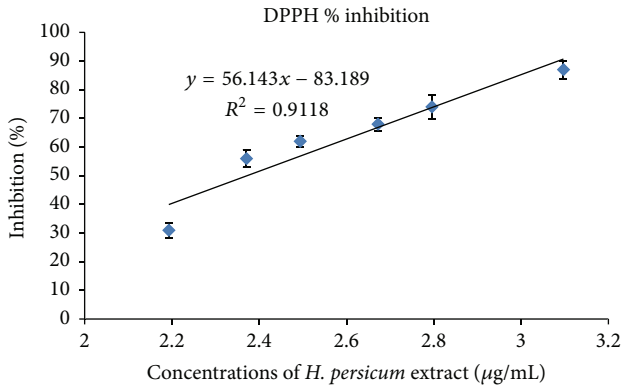

Figure 6: A linear diagram of the inhibition percentage of DPPH radical against logarithm of different concentrations of *H. persicum*; the IC_{50} value of the extract was 235 μg/mL. Values are presented as mean ± SD.

of *H. persicum* and *Z. jujuba* were 73.8 ± 3.2 and 44.2 ± 1.8 μg tannic acid equivalent/mg extract, respectively.

4. Discussion

The purpose of this study was to investigate the inhibitory effect of *Heracleum persicum* and *Ziziphus jujuba* on the activity of alpha-amylase. The results of this study showed that methanolic extracts of *H. persicum* and *Z. jujuba* have a high inhibitory effect on activity of alpha-amylase enzyme with IC_{50} 307 and 867 μg/mL, respectively. This inhibitory effect is comparable with acarbose that is lowering drug of blood glucose in diabetic patients. The results of this study are consistent with previous studies.

Studies indicate that compounds in plants can have an inhibitory role; for example, Luteolin in strawberry and polyphenols in green tea inhibit the enzyme α-amylase [31]. In 2014, Oboh et al. studied the inhibitory effect of polyphenol-rich aqueous extract of *Theobroma cacao* bean on alpha-amylase and alpha-glucosidase. The results revealed that the EC_{50} values of the extract were 1.81 ± 0.22 mg/mL for alpha-amylase and 1.84 ± 0.17 mg/mL for alpha-glucosidase

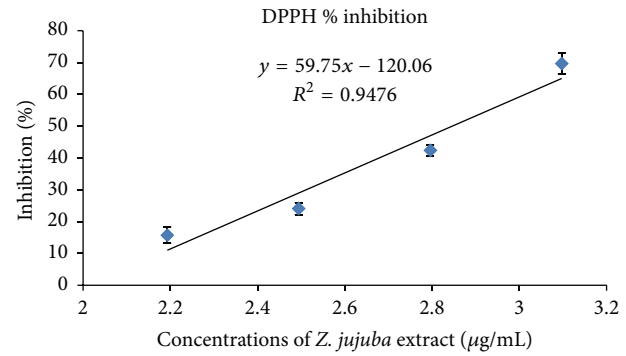

Figure 7: A linear diagram of the inhibition percentage of DPPH radical against logarithm of different concentrations of *Z. jujuba*; the IC_{50} value of the extract was 701 μg/mL. Values are presented as mean ± SD.

Table 2: Total phenolic contents of methanolic extracts.

Methanolic extracts	Total phenolic content
Heracleum persicum	73.8 ± 3.2
Ziziphus jujuba	44.2 ± 1.8

[32]. In 2004, Abu Soud et al. studied the inhibitory effect of 13 plants on alpha-amylase activity. For example, *Aloe vera* and *Paronychia argentea* plants had IC_{50} values of 0.08 and 0.2 mg/mL, respectively. *Aloe vera* was more effective because of *cinnamic acid* derivatives, and *Paronychia argentea* due to its flavonoids [33].

In another study (2000), Kim et al. studied the inhibitory effect of flavonoids, such as Luteolin, Amentoflavone, and Luteolin-7-o-glucoside, on alpha-amylase and alpha-glucosidase. The results showed that 0.5 mg/mL Luteolin inhibited 36% activity of alpha-glucosidase enzyme that this effect was stronger than acarbose. However, this flavonoid inhibited alpha-amylase enzyme with high power but lower than acarbose [34]. In 2009, Shobana et al. examined the inhibitory effect of phenolic compounds in the methanolic extract of *Eleusine coracana* L. seed peel on alpha-glucosidase and alpha-amylase enzymes. These phenols included phenolic acids and polyphenols that showed a potent inhibitory effect on both enzymes and IC_{50} values were 16.9 and 23.5 μg/mL, respectively. This study indicated the therapeutic potential of plants phenolic content in the treatment of postprandial hyperglycemia [1]. In another study, evaluation of the antioxidant activity of aqueous and methanol extracts prepared from the bark of stems, leaves, and roots of dry zone mahogany indicated that an extract of the root has antioxidant activity. The effect of this extract on the enzyme kinetics of alpha-glucosidase and alpha-amylase indicated that the extract acts as a competitive inhibitor of alpha-glucosidase and noncompetitive inhibitor of alpha-amylase [35].

Therefore, in the study, the antioxidant capacity and polyphenolic content of extracts of *H. persicum* and *Z. jujuba* were determined such that IC_{50} values of extracts were 235 and 701 μg/mL for antioxidant capacity and total phenolic content of methanol extracts was 73.8 ± 3.2 and 44.2 ± 1.8 μg

tannic acid equivalent/mg extract, respectively. Similarly, in 2007, Çoruh et al. determined the antioxidant capacity of *H. persicum* methanolic extract by DPPH test such that IC_{50} value was 438 μg/mL and polyphenolic content of its methanolic extract was 59.6 μg gallic acid equivalent/mg extract [36]. In addition, in 2013, Kim et al. measured the antioxidant capacity of ethanol extract of black *Z. jujuba* (aged for 72 hours) by DPPH test and IC_{50} value was 540 μg/mL, and its polyphenolic content was 13.79 mg/g. Furthermore, inhibitory effect of black *Z. jujuba* (aged for 72 hours) at the concentration of 3.33 mg/mL (ethanol extract) on alpha-glucosidase was 80% [19].

These results show the high polyphenolic content of *H. persicum* and *Z. jujuba* that implies the potent capacity of the plants in the clearing of oxidants.

As we know, the oxidative stress is caused by reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl radical (OH); moreover, ROS plays an important role in the pathogenesis of various degenerative diseases such as diabetes, obesity, chronic liver disease, and aging [5]; similarly, postprandial oxidative stress is associated with a higher risk for diabetes, obesity, high blood pressure, and atherosclerosis [6]; in addition, Shobana et al. (2009) indicated the inhibitory effect of phenolic compounds on alpha-glucosidase and alpha-amylase enzymes [1]; therefore, *H. persicum* and *Z. jujuba* can be used as potent antioxidants and as a strong inhibitor of alpha-amylase in the improvement of type 2 diabetes patients.

Acarbose causes gastrointestinal symptoms and liver toxicity [1] but *Ziziphus* species are used commonly in traditional medicine to treat various diseases such as gastrointestinal disorders, liver pain, obesity, urinary, diabetes, and diarrhea [18]. In addition, in 2010, Sharififar et al. showed that administration of *H. persicum* had no toxic effect on the liver, because the normal levels of serum ALT and AST [37] and *H. persicum* are an anti-bloating that are used in traditional medicine for treatment of digestive disorders [13]. Thus, *H. persicum* and *Z. jujuba* can be proposed as a replacement for acarbose drug in order to inhibit the alpha-amylase in type 2 diabetes. Studies on some alkaloids of *H. persicum* have noted their anticonvulsant and cytotoxic effects [15]. In addition, essential oil of *H. persicum* in comparison with potassium dichromate (positive control) exhibited the most cytotoxicity. The essential oil of *H. persicum* analyzed by gas chromatography- mass spectrometry (GC-MS) showed that the major constituents were hexyl butyrate and octyl acetate [38]. Also, the in vitro cytotoxicities of the triterpenic acids extracted from *Z. jujuba* were tested against tumor cell lines. The lupane-type triterpenes and 3-O-p-coumaroyl alphitolic acids showed high cytotoxic activities. These results suggest that the *H. persicum* and *Z. jujuba* may play an important role in the cytotoxicity [39]. Therefore, for elimination of side effects, further research should be performed on the components isolated from *H. persicum* and *Z. jujuba* in order to inhibit alpha-amylase and antioxidant activity; the components isolated from *H. persicum* and *Z. jujuba* may lead to chemical entities for clinical application.

5. Conclusions

The results showed that acarbose causes gastrointestinal symptoms and liver toxicity, but *H. persicum* and *Z. jujuba* decrease these side effects and prevent the gastrointestinal disorders. Due to the high polyphenolic content and antioxidant capacity of these plants and significant inhibitory effect of the plants on alpha-amylase enzyme, *H. persicum* and *Z. jujuba* can be proposed for treatment of diabetic patients.

Conflict of Interests

The authors have no conflict of interests.

Authors' Contribution

Study concept, design, and critical revision of the paper for important intellectual content were done by Mohammad Aberomand and Mohammad Ali Ghaffari. Amir Siahpoosh was pharmacognosy adviser. Mostafa Jamalan was biochemistry adviser. Drafting of the paper and conducting the experiments were done by Reza Afrisham.

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