Clinical Phytoscience

ORIGINAL CONTRIBUTION

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An investigationof the inhibitory effects of dichloromethane and methanol extracts of *Salvia macilenta*, *Salvia officinalis*, *Salvia santolinifola* and *Salvia mirzayanii* on diabetes marker enzymes, an approach for the treatment diabetes

Hasan Javid[1,2], Soheila Moein[1,2*] and Mahmoodreza Moein[3,4]

Abstract

Background: Diabetes mellitus is a type of metabolic disease characterized by elevated blood sugar. The main strategy for its treatment is to inhibit carbohydrate-hydrolyzing enzymes, including α-amylase and α-glucosidase. The aim of the present study was to evaluate the efficacy of *Salvia* extracts in inhibiting diabetes marker enzymes.

Materials and methods: This experimental study was performed in vitro. The studied plants included *Salvia mirzayanii*, *Salvia macilenta*, *Salvia officinalis* and *Salvia santolinifola* and inhibitory effects of their methanolic and dichloromethane extracts were investigated. After calculating the percentage of α-amylase inhibition and IC_{50} of the extracts, Km and Vmax were also determined using prism7.Statistical analysis was performed employing with Graph Pad instat3 software.

Results: The results here in revealed that methanol extracts of *Salvia santolinifola* (with $IC_{50} = 54.72 \pm 9.6$ µg / ml) and *Salvia officinalis* with ($IC_{50} = 54.87 \pm 5.7$ µg / ml) and dichloromethane extract of *Salvia officinalis* with ($IC_{50} = 71.20 \pm 14.3$ µg / ml) had the greatest inhibitory effect on α-amylase comparing to acarbose with ($IC_{50} = 42.94 \pm 3.8$ µg / ml) as a standard. Tukey test results showed that there is a significant difference between IC_{50} of acarbose comparing to methanol extract of *Salvia mirzayanii* and dichloromethane extracts of *Salvia mirzayanii* and *Salvia santolinifola* with *P* value $^{<}$0.001 in α-amylase inhibition.

Conclusion: The extracts had significant inhibitory effects on α-amylase inhibition. Among the extracts of the studied species, methanol extract of *Salvia santolinifola* demonstrated the greatest inhibitory effect on α-amylase.

Keywords: Diabetes, α-Amylase, *Salvia* extracts, Inhibition percentage, Kineticof inhibition

Background

Diabetes mellitus is one of the most important public health problems in the world. According to the International Diabetes Federation reports, there are 415 million adults with diabetes, which is likely to reach 612 million by 2040 [1]. Unless action is taken, it is predicted that by 2030 at least 350 million people will have type 2 diabetes.

*Correspondence: Soheila_9@yahoo.com
[2] Department of Biochemistry, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
Full list of author information is available at the end of the article

Some *Lamiacea*,such as *Salvia mirzayanii*, are used to treat diabetes [2]. Diabetes mellitus is an endocrine and metabolic disorder characterized by chronic hyperglycemia caused by adefect in insulin secretion or its function. Diabetic patients in many countries around the world suffer from postprandial hyperglycemia (PPHG) due to changes in their diet and lifestyle and high carbohydrate intake. This can progress to type 2 diabetes. Current strategies for the treatment and control of postprandial hyperglycemia included inhibition of α-glucosidase and α-amylase resulting in delayed digestion and absorption of carbohydrates. α-amylase and α-glucosidase inhibitors such as acarbose and voglibosecan potentially reduce the progression of the diabetes and prevented from the neuropathy, nephropathy, and retinopathy. However, these drugs have side effects such as diarrhea, abdominal pain, and gastrointestinal complications; therefore, many efforts have been made to replace gastrointestinal inhibitors [3].

Glucosidases are a group of carbohydrate digestive enzymes that break down carbohydrates into monosaccharides. A number of traditional Iranian medicinal plants, especially *Salvia mirzayanii(S.mirzayanii*), act as α-amylase inhibitor in the prevention and management of diabetes [4]. Inhibition of α-amylase and α-glucosidase specifically reduced postprandial hyperglycemia and thus prevented the progression of diabetes [5]. Vegetables and plants have been consumed by humanas food and for the treatment of degenerative diseases. Scientific evidence proves that many of these herbs and herbal materials have medical properties that reduce symptoms of the diseases and prevent from these diseases. Research on medicinal plants for diabetes management in recent years was the interest of specialists and it has been found that some of them have anti-hyperglycemic properties, acting by the inhibition of carbohydrate hydrolyzing enzymes. This property is due to the presence of biological active compounds such as phenols and flavonoids. Phenols also have antioxidant properties due to hydroxyl groups [6].

Salvia is the largest genus of *Lamiacea* with more than 900 species, spreading throughout the world, especially in the temperate and warm areas. In Iran, *Salvia* contains 58 species, 17 of which are endemic. Extracts of *Salvia* species have various biological activities including antioxidant, antibacterial, anti-tumor, anti-inflammatory and anti-diabetic. In traditional Iranian medicine, some species of *Salvia* have antidiabetic effects [7]. Medicinal plants have always been an important source of natural compounds with high therapeutic potential [8]. The first goal of diabetic patients treatment is to control hyperglycemia [9]. In this study, we investigated α-amylase inhibition bydifferent species of *Salvia*,as an approach for the treatment of diabetes.

Materials and methods

3, 5 dinitro salicylic acid (DNSA) and α-amylase enzyme from porcine pancreas and acarbose were purchased from Sigma Aldrich USA and other chemicals were obtained from Merck Company in Germany.

Specimen collection and preparation

Salvia mirzayanii (*S.mirzayanii*), *Salvia officinalis (S.officinalis*), *Salvia macilenta (S.macilenta*) and *Salvia santolinifola (S.santolinifola*) plants were collected by Mr. Asadipourin March 2018, from around Bandar Abbas. The voucher numbers were deposited in Medicinal Plants Processing Research Center, School of Pharmacy, Shiraz University of Medical Sciences. The voucher numbers of *S.mirzayanii*, *S.santolinifola*, *S.officinalis* and *S.macilenta* are MPPRC-93-6, MPPRC-97-2,MPPRC-90-18 and MPPRC-93-1, respectively. The leaves of all plants were dried, chopped, and extracted with dichloromethane and methanol using a rotary evaporator, respectively. After that, *Salvia* extracts were prepared and stored at 4 °C.

The inhibition of α-amylase (EC:3.2.1.1) was evaluated with minor modifications by concentrationsof 0.0025, 0.1,0.2 and 0.5 mg / ml dichloromethane and methanol extracts of *S.mirzayanii*, *S.macilenta*, *S. officinalis* and *S. santolinifola* according to the method reported by Maccu and Shetty, [10]. In this method, the amount of inhibition is determined using starch (45 λ,1%) as a substrate and 3,5 dinitro salicylic acid as a reagent. First, *Salvia* extracts (45 λ) are mixed with distilled water and starch. The reaction begins with the addition of α-amylase (10 λ) and the same amount of sodium phosphate buffer (PH = 6.9) was added. The mixture was incubated at 37 °C for 15 min and 100 λ of dinitro salicylic acid and sodium potassium tartrate (as stop solution) was added.

After 15 min incubation at 85 °C, the activity of α-amylase was measured by measurement of absorbance at 540 nm (Biotek Corporation's synergy HTX multi-mode reader). To measure enzyme inhibition by the control, the above mentioned procedure was performed, only DMSO (*Dimethyl sulfoxide)* is used instead of the *Salvia* extract. In this study, acarbose is employed as a standard.

The percentage of enzyme inhibition by the samples and acarbose was determined by the following formula.

$\%I = 100\text{-}((OD\ control\text{-}OD\ sample)\ /OD\ control) \times 100.$

Each experiment was repeated 3 times .

Blank contained sodium phosphate buffer instead of the enzyme. We calculated the IC_{50} of α-amylase inhibition by *Salvia* extracts. Then Km and Vmax of the enzyme were calculated.

Kinetics of α-amylase enzyme

To determine the type of inhibition and to calculate Km and Vmax, different concentrations of substrate (starch) (0.25%, 0.5%, 1%, 2%, 4% and 6%) were prepared. The extract concentration, which caused 50% inhibition, was used. The experiment was started with a low concentration of substrate, (starch). The experiment was started (according to the mentioned procedure described previously) with low substrate concentration until Michaelis Menten curve reaches saturation or plateau (this plateau showed maximum velocity). Then, Km and Vmax were calculated using Graph pad prism software. Comparison of Km and Vmax in the absence of inhibitor and in the presence of inhibitor indicates the type of inhibition.

Statistical analysis

After calculating IC_{50} by Excel 2019 software, Graph Pad prism 7 and Graph Pad instat 3 softwares were used for statistical analysis. Mean ± SD values of the obtained data were calculated using Graph Pad instat3 and One-way ANOVA with Tukey posttest. If P value was $^{<}$ 0.05 then the difference is considered as significant. Graph Pad prism6 software was also used to calculate Km and Vmax values.

Results

The average percentageof α-amylase inhibition by *Salvia* species and acarbose was determined. Figure 1 shows a comparison of the mean percentage of α-amylase inhibition. As shown, acarbose had the highest inhibition rate (94%) as a standard at 0.1 mg / ml. In addition, at the concentration of 0.2 mg / ml, methanol extract of *S.santolinifolia* with 91% and methanol extract of *S.officinalis* with 87.1% showed the highest inhibition percentage. At 0.5 mg / ml concentration, the inhibitory effect of methanol extract of *S.macilenta* (93.69%) was higher than other species.

The results of the calculated IC_{50} (the concentration of *Salvia* extract which inhibits α-amylase 50%) ± SD by GraphPad instat3 software are presented in Table 1 with respect to repeated experiments.

Concentration and activity values were then entered in Graphpad prism software and Km and Vmax values were calculated. The results of Km ± SD and Vmax ± SD are presented in Table 2.

Comparison of Km and Vmax with and without inhibitor determines the type of inhibition. The inhibition of the enzyme by acarbose, is competitive (Vmax is constant and Km increased). The inhibition of the enzyme by *Salvia* extracts is mixed, (Vmax decreased and Km increased).

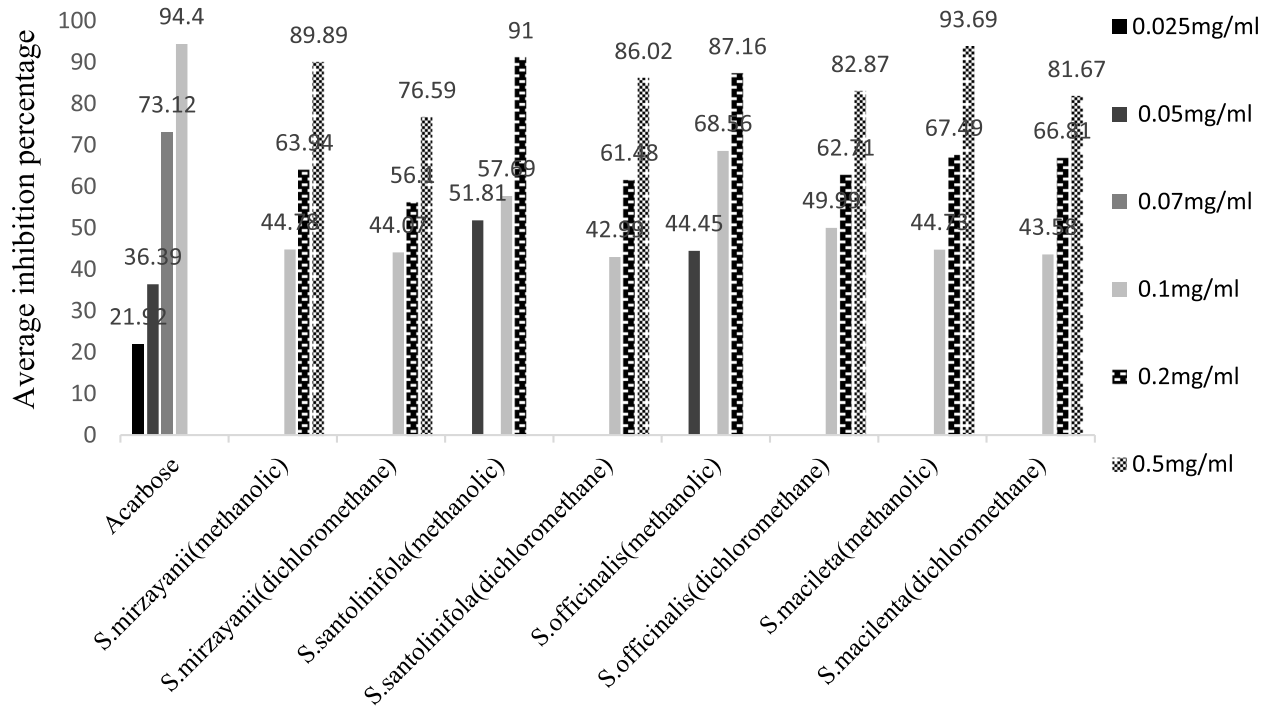

Fig. 1 Comparison of mean percentage of α-amylase inhibition by different concentrations of the extracts and acarbose

The saturation diagram (Michaelis Menten) of the α-amylase for the one-time trial drawn by Excel 2019 software and was shown in Figs. 2 and 3.

As is known, the activity without inhibitor was higher than that of the studied *Salvia* species. To convert absorbance to the activity, maltose standard diagram was drawn. Then, we calculate the amount of activity using the line equation of maltose standard diagram. (The standard maltose curve with four points for converting OD to activity is shown in Fig. 4). Then, concentration and activity values were entered in Graphpad prism software and values of Km and Vmax were calculated.

Table 1 $IC_{50} \pm SD$ of the extracts and acarbose (μg/ml)

Samples	α-amylase inhibition (IC_{50}, μg/ml)	Samples	α-amylase inhibition (IC_{50}, μg/ml)
S. mirzayanii (methanolic)	114.8 ± 11.1	*S. officinalis* (methanolic)	54.9 ± 5.7
S. mirzayanii (dichloromethane)	150.9 ± 6.7	*S. officinalis* (dichloromethane)	71.2 ± 14.4
S. santolinifola (methanolic)	54.7 ± 9.6	*S. macilenta* (methanolic)	103.7 ± 1.1
S. santolinifola (dichloromethane)	132.9 ± 15.8	*S. macilenta* (dichloromethane)	100.9 ± 10.8
Acarbose	42.9 ± 3.9		

Table 2 Km ± SD and Vmax ± SD and the mode inhibition of the studied samples and acarbose

Extract	Km ± SD)mmol(	Vmax±SD)μmol/min(	Kind of inhibition
Acarbose (standard)	1.264 ± 0.301↑	0.0076 ± 0.0007(constant)	Competitive
Salvia mirzayanii (methanolic)	1.969 ± 0.142↑	0.0075 ± 0.0002↓	Mixed
Salvia mirzayanii (dichloromethane)	2.398 ± 0.736↑	0.0074 ± 0.0009↓	Mixed
Salvia santolinifola (methanolic)	1.351 ± 0.208↑	0.0075 ± 0.0003↓	Mixed
Salvia santolinifola (dichloromethane)	1.082 ± 0.242↑	0.0073 ± 0.0004↓	Mixed
Salvia officinalis (methanolic)	1.665 ± 0.219↑	0.0075 ± 0.0003↓	Mixed
Salvia officinalis (dichloromethane)	1.343 ± 0.245↑	0.0075 ± 0.0004↓	Mixed
Salvia macilenta (methanolic)	2.339 ± 0.315↑	0.0074 ± 0.0004↓	Mixed
Salvia macilenta (dichloromethane)	2.456 ± 0.541↑	0.0073 ± 0.0006↓	Mixed
Without inhibition	1.01 ± 0.213	0.0076 ± 0.0006	–

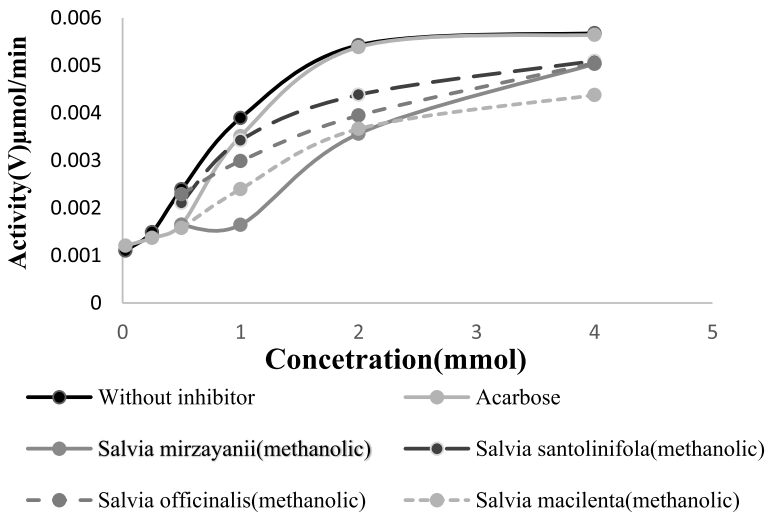

Fig. 2 Michaelis Menten curves of methanolic extracts of *Salvia* species in comparison to reaction without inhibitor and acarbose as a standard

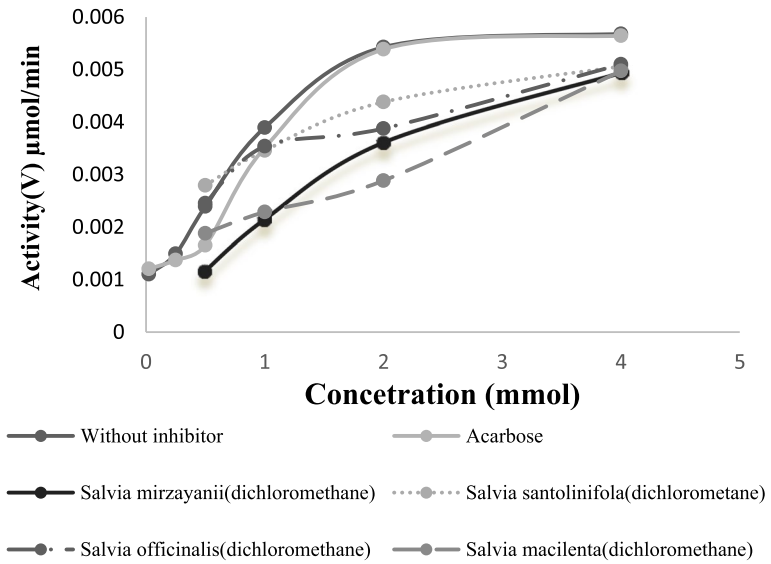

Fig. 3 Michaelis Menten curves of dichloromethane extracts of *Salvia* species in comparison to reaction without inhibitor and acarbose as a standard

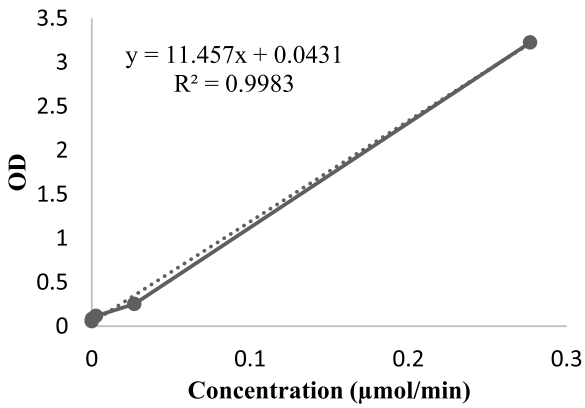

Fig. 4 Standard curve of maltose

Tukey post hoc test, compares the IC_{50} of the studied species and acarbose. Based on these results, there are significant differences between the IC_{50} of acarbose and methanol, and dichloromethane extracts of *S.mirzayanii* and dichloromethane extract of *S.santolinifola* (*P* value < 0.001) in α-amylase inhibition. Also, there was not a significant difference between methanol, dichloromethane extracts of *S.officinalis* and acarbose (P value> 0.05). Also, there is a significant difference between the of IC_{50} of acarbose, methanol and dichloromethane extracts of *S.macilenta* (P value < 0.01). Between different samples, methanolic extract of *S.officinalis* ($IC_{50} = 54.9 \pm 5.7$ µg/ml) and methanolic extract of *S.santolinifola* ($IC_{50} = 54.7 \pm 9.6$ µg/ml) possessed the highest α-amylase inhibition, respectively.

Discussion

Type 1 diabetes is insulin dependent and type 2 diabetes is non-insulin dependent. About 90% of people suffer from type 2 diabetes. Although, with insulin injection, type 1 diabetes is treatable, finding drugs to treat and manage type 2 diabetes is an important area of research. Pancreatic α-amylase and intestinal α-glucosidase are key enzymes that convert carbohydrates to monosaccharides. Therefore, they are responsible for elevation blood sugar; and inhibiting these enzymes is an appropriate way to treat diabetes [11]. The α-amylase enzyme has three domains (A, B, C) and has 496 amino acids. The catalytic site of α-amylase (PA) contains Asp197-Glu223-Asp300 amino acids [12]. *Salvia* has been so popular that it has been the subject of numerous chemical studies. These plants are rich in polyphenols and contain more than 160 polyphenols, some of which are unique to the genus *Salvia*. Several phenolic acetophenone glycosides have only been identified in *S.officinalis* [13]. There is a strong correlation between plant phenol content and inhibitory activities of α-amylase and α-glucosidase. Polyphenols are considered as potent antioxidants due to their redox properties in their hydroxyl groups [14]. One of the important functions of polyphenols is to inhibit digestive enzymes, especially carbohydrate hydrolyzing enzymes such as α-amylase and α-glucosidase. Inhibitors of enzymes capable of digestion carbohydrates decrease glucose absorption. Polyphenolic α-amylase and

α-glucosidase inhibitors derived from natural sources have been reported to be beneficial in reducing postprandial hyperglycemia [15].

The results of this study showed that dichloromethane and methanol extracts of *S.mirzayanii* inhibit α-amylase. In addition, according to Table 1, their mean IC_{50} (IC_{50} = 150.9 ± 6.7 µg / ml, IC_{50} = 114.8 ± 11.1 µg / ml) respectively,is higher than the standard IC_{50} acarbose (IC_{50} = 42.9 ± 3.9 µg / ml). In other words, their levels of α-amylase inhibition were lower than acarbose. These results are consistent with the findings of a study by Dr. Moein et al. (2012) which showed that phenolic compounds in *S.mirzayanii* may be responsible for free radical scavengingand the prevention of disease such as cancer and cardiovascular disease, diabetes and aging [16]. It is also in line with Dr. Moein's 2018 study, which showed that aqueous-methanol fractions of *S.mirzayanii* inhibited the α-amylase, but further studies were needed to determine the type of inhibition [17]. Study by Mohammad Reza Zarshenas in 2014 is consistent with the above mentioned results on diabetic model of rats, which showed that injection alcoholic extractof *S.mirzayanii* decrease blood glucose levels [18]. The results of this study also showed that dichloromethane (IC_{50} = 71.2 ± 14.4 µg/ml, Table 1) and methanol (IC_{50} = 54.9 ± 5.7 µg/ml, Table 1) extracts of *S.officinalis* lower than acarbose (IC_{50} = 42.9 ± 5.7 µg/ml). These results are consistent with the findings of Ahmad Ghorbani et al. (2016), who identified the phytochemical compounds of *S.officinalis* flowers, leaves and stems. These compounds included alkaloids, carbohydrates, fatty acids, glycoside derivatives such as flavonoid glycosides, phenolic compounds and terpenoids that had anti-mutagenic, anti-inflammatory effects and anti-hypoglycemic activity and increased memory [19]. More than 120 compounds have been characterized in the essential oil of aerial parts of *S. officinalis*. The major components of this oil include borneol, camphor, caryophyllene, cineole, elemene, humulene, ledene, pinene, and thujone. Oxidative stress plays a significant role in the initiation and progression of several diseases, such as diabetes. The most effective antioxidant components of *S. officinalis* are carnosol, rosmarinic acid, carnosic acid, caffeic acid, rosmanol, rosmadial, genkwanin, and cirsimaritin [19]. It is also in line with studies by Rahman Mahdizadeh et al. (2018) who stated *S.officinalis* reduces blood sugar levels in mice and inhibits gluconeogenesis and glycogenolysis in the liver [20]. *S. officinalis* decreases blood glucose like acarbose, and also inhibits the activity of the intestinal maltase and sucrase and therefore possesses antidiabetic activity [20]. These results are in line with the results of Eidey et al. (2009) who reported that methanolic extract of *S.officinalis* significantly reduced blood glucose in hyperglycemic rats. In other study, ethanol extract of *S.officinalis* leaves decreased blood glucose in rat diabetic model [21].

The results of this study showed that dichloromethane (IC_{50} = 100.9 ± 10.8 µg / ml, Table 1) and methanol (IC_{50} = 103.7 ± 1.1 µg / ml, Table 1) extracts of *S.macilenta* inhibited α-amylase, lower than acarbose (IC_{50} = 42.9 ± 3.9 µg / ml, Table 1). Vasil Georgiev et al. (2017) reported that *S.macilenta* is rich in phenolic acid and polyphenols, flavonoids and terpenoids. These compounds have hypoglycemic properties [22]. It is also Mujtaba Asadullah's reported that (2017) extracts of *S.macilenta* and *S.officinalis* are used in traditional Iranian medicine for treating many disorders such as diabetes [23]. The results of this study showed that dichloromethane (IC_{50} = 132.9 ± 15.88 µg / ml, Table 1) and methanol (IC_{50} = 54.7 ± 9.6 µg / ml, Table 1) extracts of *S.santolinifola* inhibited α-amylase, lower than acarbose (IC_{50} = 42.9 ± 3.9 µg / ml, Table 1).

Also, S.Asadietal. (2011) reported that DNA damage by free radicals is inhibited by *Salvia* species [24]. Dichloromethane solvent extracts diterpenes, and methanol solvent extracts polyphenols. Polyphenols are generally presented in plants, and these polyphenolic compounds have been reported to be α-amylase inhibitors [4].

Based on Table 2, acarbose showed competitive inhibition with (Km = 1.264 ± 0.301 and Vmax = 0.0076 ± 0) compared with no inhibitors with (Km = 1.01 ± 0.213 and Vmax = 0.0076 ± 0.0006).

All the studied extracts showed mixed inhibition and Km and Vmax of these samples are shown in Table 2.

Uncompetitive and non-competitive inhibitors have the ability to inhibit the enzyme more than acarbose as a competitive inhibitor and, unlike acarbose, their inhibitory potency is not affected by substrate concentration [25, 26]. In 2017, Dr. Homaei and Soodeh Bahramian used the Michaelis Menten equation to determine Km, Vmax of plant extractsand type of α-amylase inhibition [27].

Conclusion

The extracts had significant inhibitory effects on α-amylase. Among the extracts of the studied species, methanolic extracts of *S.santolinifola* and *S.officinalis* had moreinhibitory effects on α-amylase. The type of inhibition of *Salvia* species, is mixed with decreasing Vmax and increasing Km, and the studied samples could inhibit α-amylase independent of the substrate concentration. However, the type of acarbose inhibition is competitive, which is affected by the substrate concentration.

Abbreviations
IC_{50}: the concentration of the extract inhibits enzyme 50%; kg: kilogram; ml: millimeter; ANOVA: Analysis of Variance; C: Centigrade degree; g: gram; Km: Michaelis Menten constant; Vmax: MaximumVelocity; S: Salvia.

Acknowledgements
The authors thank Hormozgan University of Medical Sciences (Grant NO.165504) and Shiraz University of Medical Sciences, (Grant NO.20578).

Authors' contributions
SM and MM designed the study. SM helped in writing research paper and revised the final manuscript. SM statistically analyzed the obtained data. HJ carried out all parts of the experiments and writing the research paper. MM was involved in selection of plants and their extraction. This research was supervised by SM. The authors read and approved the final manuscript.

Funding
This study was funded by HormozganUniversity of Medical Sciences (Grant NO 970305) and Shiraz University of Medical Sciences (Grant NO 17727).

Availability of data and materials
All the data is contained in the manuscript.

Declarations

Ethics approval and consent to participate
The study protocol was approved by institutional ethical committee (No:IR.HUMS.REC.2018.270).

Consent for publication
Not applicable.

Competing interests
The authors declare that there are no conflicts of interest for publication this manuscript.

Author details
[1]Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran. [2]Department of Biochemistry, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran. [3]Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. [4]Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Received: 28 April 2020 Accepted: 16 January 2022
Published online: 28 January 2022

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