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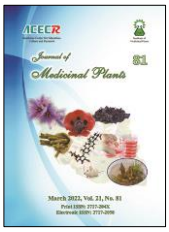

Research Article

The α-amylase and α-glucosidase inhibitory effects of some traditional antidiabetic prescriptions based on bioautography using LC-ESI/MSMS

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ABSTRACT

Background: Diabetes, one of the most common metabolic diseases in many societies, has influenced the quality of human life for many years. Traditional, alternative, and complementary medicine use individual or mixed plant prescriptions to reduce adverse consequences of diabetes. This is based on the human experience of treating and managing disease complications in different geographic areas, over a hundred years. **Objective**: This research tries to find and recommend the most influential traditional medicine prescription for the inhibition of key enzymes associated with diabetes. **Methods:** Considering the full and widespread use of the medicinal plants, in this research, 15 most reliable Iranian and Iraqi herbal prescriptions in controlling diabetes were studied for the inhibition of α-amylase and α-glucosidase enzymes. Chemicals of the most effective prescription for the inhibition of these enzymes were separated by using the HPTLC method. For chromatogram development, a mobile phase consisting of ethyl acetate: toluene: methanol: formic acid was used. A direct enzyme inhibitory assay on the HPTLC plate was used to investigate the most effective molecules. Thereafter, the influential molecules were identified by using LC-ESI/MSMS. **Results:** Based on the results, prescription No. 3, (from the Iraqi herbal prescription) containing the extract of *Prunus mahaleb* L. and *Prunus dulcis* (Mill.) D.A.Webb was identified as the best α-amylase and α-glucosidase inhibitor. **Conclusion:** The chemical and molecular analysis of this extract which was performed by the HPTLC method and further by LC-ESI/MSMS, indicates two compounds of catechin and epicatechin.

1. Introduction

Diabetes is an insulin-dependent metabolic disease in which the body is unable to properly use blood glucose due to insufficient function or secretion of insulin [1]. The main factor in keeping blood glucose levels is the insulin

Abbreviations: HPTLC, High Performance Thin Layer Chromatography; LC-ESI/MSMS, Liquid Chromatography-Electrospary Ionization/Tandem Mass Spectrometry; DMSO, Dimethyl Sulfoxide; CID, Collision Induced Dissociation; ECD, Electron Capture Dissociation; IT, Ion Trap

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hormone. If there is any abnormality in the function or level of this hormone, glucose absorption will be impaired and blood glucose levels will rise, which can lead to diabetes [2]. According to the International Atlas of Diabetes (IDF), the number of diabetes in the Middle East is ~73 million in 2021, which is projected to increase by 87 % to ~136 million in 2045 [3].

Patients with insulin problems and diabetes, in addition to the problems of this enzyme deficiency, suffer from complications and problems caused by fluctuating blood sugar, which greatly affects the quality and longevity of daily life [4]. In addition to using medications and treatments, these patients ought to control their lifestyle in terms of activity and nutrition [5]. Recent research has shown that the best and fastest way to control and treat this disease is to inhibit the basic enzymes like α-amylase and α-glucosidase in the metabolism of sugars in the body. [6, 7]. The activity of digestive enzymes, especially α-amylase and α-glucosidase, from the moment of onset, is up to 2 hours after eating. During this period, glucose enters the circulatory system and thus blood glucose levels rise [8]. A diabetic person's body is unable to control blood glucose level fluctuation. Therefore, patients with diabetes must prevent a sudden rise in their blood sugar concentration. One of these ways is to use drugs such as Acarbose, Miglitol, and Voglibose that act as inhibitors of α-amylase and α-glucosidase enzymes. These drugs have side effects such as bloating and gastrointestinal, decreased group B vitamins, liver enzyme dysfunction, diarrhea, abdominal pain, and rash [9, 10].

Many diabetics are interested in controlling the negative effects of blood sugar fluctuations and complications of diabetes with traditional medicine and herbs instead or in addition to using chemical drugs. Besides chemical methods, they can use complementary medicine methods to improve and control living conditions and blood sugar levels [11]. There is a wide range of plants in traditional medicine of different ethnic groups and countries whose extracts have the property of inhibiting enzymes of glucose metabolism [8]. For example, plants called *Allium sativum*, *Artemisia pallens*, *Areca catechu*, *Biophytum sensitivum*, *Brassica juncea*, *Eugenia uniflora*, *Eugenia jambolana*, and *Capparis decidua* have been identified to show inhibitory effects on diabetic mice [12, 13]. The natural active ingredients in these plants are often the main building blocks of current diabetes medications [14].

Different geographical areas with ancient civilizations have disease control methods with medicinal plants that have been used for many years. Islamic traditional medicine (related to the geographical region of Iran and Iraq) with the support of scientists such as Avicenna and Ismail Gorgani have many interesting prescriptions to control blood sugar levels and complications in diabetic patients [15, 16].

In this study, inhibition of the isolated compounds from Traditional prescriptions on diabetes-related enzymes (α-amylase and α-glucosidase) was performed by using the HPTLC plate based on bioautography. This method is used to evaluate the biological/chemical activity of isolated constituents of natural creatures based on the selected special technique. This technique has a simple operation with low cost and high levels of sensitivity and specificity, which combines TLC separation with the biological/chemical activity on-plate determination [17].

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This study tries to answer that, which of the prescriptions and recommendations of traditional medicine has the greatest impact on the inhibition of enzymes associated with diabetes. Moreover, which of the isolated compounds from these prescriptions has the greatest effect on enzyme inhibition. As a consequence, the enzymatic inhibition of the most important Iraqi herbal medicinal prescriptions for controlling blood sugar and diabetes complications has been investigated by using the bioautographic method with HPTLC. Thereafter, active compounds of the most effective prescription for the inhibition of α-amylase and α-glucosidase enzymes have been identified by using mass spectrometry.

2. Materials and Methods

Fig. 1 shows the entire route of this study schematically. After extraction of each prescription, the effect of enzymatic inhibition of the extract and optimizing the separation of the extract compounds were performed simultaneously by the HPTLC method. Then, the inhibition percentage of the separated compounds isolated on the plate was investigated and enzyme inhibitory compounds were identified. Enzyme inhibitory compounds were isolated from the HPTLC plate and identified by using mass spectrometry.

2.1. Plant materials and sample preparations

Prescriptions were selected based on the two main sources of Iranian and Iraqi traditional medicine, namely *The Canon of Medicine* (by Avicenna) and *Zakhireye Khwarazmshahi* (by Ismail Gorgani). Dried plants of 15 common and consumption types of diabetes control prescriptions were collected from the different areas of Iran and Iraq (Table 1).

They were identified and approved in the HAB herbarium of Iraq natural history museum, Iraq. To prepare the extract of each version, according to the recommended method, aqueous extract (at a temperature of 25 °C, for 30 min) and methanol extract (which is a modern scientific basis for extraction) were performed. In addition, for the prescriptions that used boiling water, extraction was performed with hot water (30 min, temperature 97 ± 0.5 °C) and the percentage of dry extract efficiency was obtained. To obtain the percentage of dry extract efficiency, the ratio of dry extract (g) to the initial powder was calculated and expressed as a percentage. Then, certain amounts of each extract were prepared in DMSO (Sigma-Aldrich Co., Germany) solvent up to a concentration of 1 mg/L and used for enzymatic testing steps. After powdering and mixing all samples, 20 g of each sample was suspended in 70 ml of water, hot water (97 ± 0.5 °C), and methanol, respectively. They were placed in an ultrasonic bath (Takta Co., Iran) for 15 min for extraction. The extracts were filtered (0.22 µm) and dried by using a freeze-dryer (Pishtaz Co., Iran). All extractions were performed once without replication.

2.2. Chemicals

Dimethyl sulfoxide was obtained from Carlo Erba Co., (France). Methanol, formic acid, ethyl acetate, dichloromethane, acetic acid, and HPTLC plates were purchased from Merck Co. (Germany). The α-amylase (Accession: P04746) and α-glucosidase (Accession: P10253) enzymes, Na_2HPO_4, NaH_2PO_4, toluene, and acetone were purchased from Sigma Aldrich Co. (Germany).

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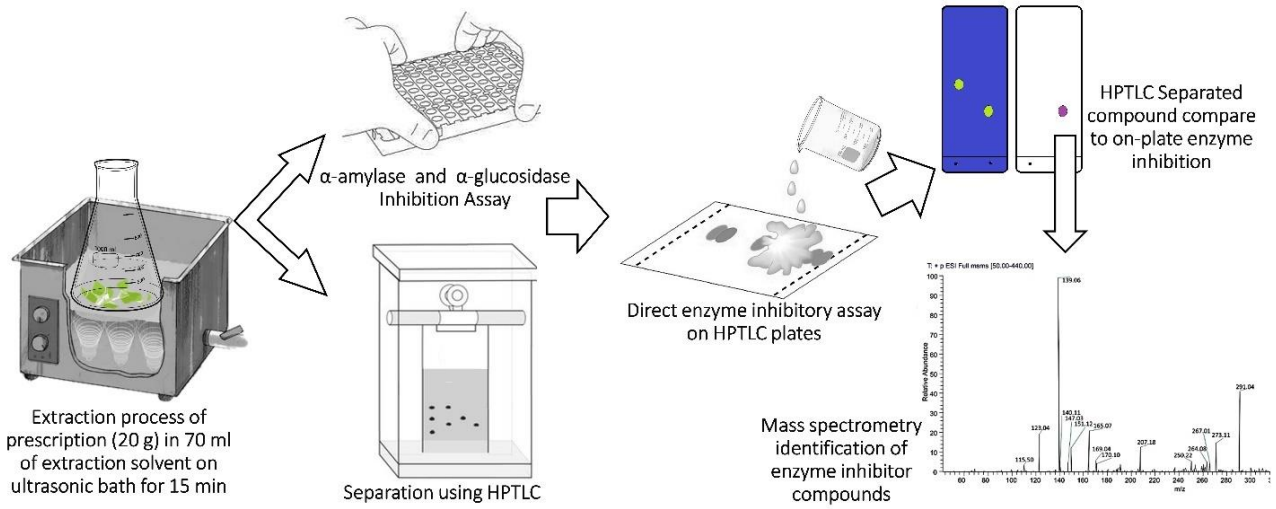

Fig 1. Schematic sketch of the study steps

Table 1. Plants and their weight ratio in the selected prescription

Sample No.	Prescription (the type and amounts of prescribed plants)
1	*Nigella sativa* L. -HAB:90223*, *Hordeum vulgare* L. -HAB:00984, *Triticum aestivum* L. -HAB:03341 (5 g of each)
2	*Triticum aestivum* L. -HAB:67112, *Nigella sativa* L. - HAB:90223 (4.2 g of each), 2.1 g *Trigonella foenum-graecum* L. -HAB:22390 and 1.05 g of *Laurus nobilis* L. -HAB:45450
3	18.2 g of *Prunus mahaleb* L. -HAB:23116 and 1.8 g of *Prunus dulcis* (Mill.) D.A. Webb-HAB:23412 powder
4	13.3 g of *Salvia officinalis* L. -HAB:46112 and 6.7 g of *Urtica dioica* L. -HAB:87001
5	*Artemisia absinthium* L. -HAB:40103 and *Artemisia annua* L. -HAB:40232 (4.5 g of each), 2.27 g of *Teucrium fruticans* L. -HAB:60231 s, 2.7 g of *Cyperus rotundus* L. -HAB:80120, 3.6 g of *Petroselinum crispum* (Mill.) Fuss -HAB:70023 and 2.27 g of *Piper nigrum* L. -HAB:49231
6	*Nigella sativa* L. - HAB:90223, *Trigonella foenum-graecum* L. -HAB:22390 and *Lepidium sativum* L. -HAB:57691 (4.6 g of each), 3.8 g of *Pimpinella anisum* L. -HAB:23801
7	*Tribulus terrestris* L. -HAB:11238, *Berberis vulgaris* L. -HAB:46012, *Citrus aurantiifolia* (Christm.) Swingle -HAB:78012, *Echium amoenum* Fisch. & C.A.Mey. -HAB:55281 and *Portulaca oleracea* L. -HAB:56001 seed (4 g of each)
8	*Saccharum ravennae* (L.) L. -HAB:60602 and *Coriandrum sativum* L. -HAB:21348 (10 g of each)
9	*Anethum graveolens* L. -HAB:01003 seed, *Artemisia absinthium* L. -HAB:40103, *Tribulus terrestris* L. -HAB:11238, *Echium amoenum* Fisch. & C.A.Mey. -HAB:55281, *Cichorium intybus* L. -HAB:40982, *Achillea millefolium* L. -HAB:60921 and *Urtica dioica* L. -HAB:87001 (2.9 g of each)
10	*Lens culinaris* subsp. *microsperma* (Baumg.) N.F.Mattos -HAB:32400 and *Trigonella foenum-graecum* L. -HAB:22390 (6.6 g of each)
11	20 g of *Citrullus colocynthis* (L.) Schrad.s-HAB:90331
12	*Saccharum ravennae* (L.) L. -HAB:60602 and *Glycyrrhiza glabra*-HAB:00032 (1.4 g of each) *Portulaca oleracea* L. -HAB:56001 seed, *Lactuca sativa* L. -HAB:70841 seed and *Coriandrum sativum* L. -HAB:21348 (4.3 g of each), *Citrus* × *aurantium* L. -HAB:50327 and *Tulipa suaveolens Roth* -HAB:99201 (0.9 g of each), *Santalum album* L. -HAB:00921, *Punica granatum* L. -HAB:80023, *Rhus coriaria* L. -HAB:70345 and *Gomma arabica* (0.6 g of each) and 0.1 g *Cinnamomum camphora* (L.) J. Presl -HAB:40972

Table 1. Plants and their weight ratio in the selected prescription (Continued)

Sample No.	Prescription (the type and amounts of prescribed plants)
13	*Saccharum ravennae* (L.) L. -HAB:60602, *Portulaca oleracea* L. -HAB:56001 seed, *Lactuca sativa* L. -HAB:70841 seed, *Rosa* × *damascena* Herrm. -HAB:00378, *Tulipa suaveolens Roth* -HAB:99201 and *Punica granatum* L. -HAB:80023 (4 g of each)
14	*Plantago major* L seed
15	5 g of *Tulipa suaveolens Roth* -HAB:99201, *Punica granatum* L. -HAB:80023, *Peganum harmala* L. -HAB:09205 seed, *Phyllanthus emblica* L. -HAB:03002 and *Coriandrum sativum* L. -HAB:21348 (4 g of each)

*All codes are HAB herbarium of Iraq natural history museum.

2.3. Separation Instrumentations

Sample solutions for HPTLC analyses were spotted (20 µl) in the form of bands of width 8 mm with a Camag 100 ml syringe on precoated silica gel aluminum plate 60F254 (2 × 10 cm) with 200 mm thickness from Merck Co. (Germany) and a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 200 nl/s was employed that the space between the two bands was 1 cm. The spots of the desired plant extracts were measured using different solvent systems. The best solvent system is ethyl acetate: formic acid: methanol: toluene in the ratio of 3: 3: 0.2: 0.8.

2.4. Enzyme Assay Inhibition

For α-amylase inhibition assay, 100 µl of each sample was mixed with 200 µl of the starch substrate solution (10 % w/v starch and 17 mmol/L NaCl in phosphate buffer 20 mmol/L; Sigma-Aldrich Co., Germany) at 20 °C and pH 6.9. Then, 200 µl of α-amylase solution (2 mg/ml in phosphate buffer 20 mmol/L) was added. The mixture was incubated for 3 min at 20 °C. After adding 200 µl of DNS solution (3,5-Dinitrosalicylic acid; Sigma-Aldrich Co., Germany), the vials were incubated at 100 °C for 15 min, and finally, 1100 µl of distilled water was added to all vials. The absorbance of 250 µl of each sample (in 3 repetitions for each sample) was studied at 540 nm. The DMSO (Sigma-Aldrich Co., Germany) was used as a negative control. The amount of α-amylase inhibition was measured by Eq. 1 where Abln and Asmp represented the absorption of negative control and sample average, respectively [18-20].

$$\alpha - \text{amylase inhibition } \% = \left(\frac{A_{bln} - A_{smp}}{A_{bln}}\right) \times 100 \quad \text{(Eq. 1)}$$

For α-glucosidase inhibition assay, 40 µl of each sample (in DMSO at the concentration of 1mg/L) was mixed with 80 µl of α-glucosidase solution (0.5 Unit/ml in phosphate buffer 20 mmol/L; Sigma-Aldrich Co., Germany) and 480 µl phosphate buffer (100 mmol/L) which were incubated for 15 min at 37 °C. Then, 80 µl of PNGP (p-nitrophenyl from *p*-nitrophenyl-b-D-glucopyranoside, 5 mmol/L; Sigma-Aldrich Co., Germany) substrate solution was added and incubated for 15 min. The reaction with 320 µl sodium carbonate (0.2 mmol/L) was finalized. The absorbance of 250 µl of each sample (in 3 repetitions for each sample) was studied at 405 nm. The DMSO was used as a negative control. The amount of α-glucosidase inhibition was measured by Eq. 2 where Abln and Asmp represented the absorption of negative control and sample average, respectively [18-20].

$$\alpha - \text{glucosidase inhibition } \% = \left(\frac{A_{bln} - A_{smp}}{A_{bln}}\right) \times 100 \quad \text{(Eq. 2)}$$

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2.5. Direct enzyme inhibitory assay on HPTLC plates

For the determination of α-amylase inhibitory activity, the iodine test was performed. The HPTLC separation plates were dried and placed in an α-amylase solution (0.2 % w/v in phosphate buffer 20 mmol/L) for 5 sec. After drying, the plates were placed in the starch solution (1% w/v) as an enzyme-substrate for 10 sec and dried (at room temperature). Finally, the plates were placed in a vertical chamber and iodine pieces were placed in the chamber. Over the iodine sublimation, appeared spots on the plate having α-amylase inhibitory activity, become violet and visualized.

2.6. Liquid Chromatography-Mass Spectrometry Identification

For the mass spectrometry, a much larger amount of the extract with the best separation and the highest enzyme inhibition on the plate had to be prepared. Therefore, a preparative plate with a band of aqueous extract of sample No. 3 (with a volume of 300 µl) was placed on a 10 × 20 cm plate to obtain a sufficient amount of material for LC/MS analysis. After separation, the appropriate position was shaved from the plate and dissolved in methanol: water in the ratio of 30:70, and completely vortexed for 1 minute. It was then centrifuged (10 min at 10,000 rpm) and the supernatant was used for injection into the LC/MS.

HPLC system model LCPacking Dionex, with UltiMate binary pump, Prefused C18 Dionex PreMap precondition column (id. 300 µm × 5 mm), autosampler at 35 °C with 5 µl loop and 1/2 injection volume and Fused silica C18 Capillary PerpMap100 column (3 µm, 100 Aº, id. 75 µm × 150 mm) made by Dionex Co., (California, USA) were used for chromatographic analysis. Mobile phase including eluent A: a mixture of water with 2 % acetonitrile and 0.1 % trifluoroacetic acid, and eluent B: a mixture of acetonitrile with 20 % water and 0.08 % trifluoroacetic acid were used.

Washing began with eluent A and within 5 min the percentage of eluent B increased to 25 %. Then, during 5 min, the percentage of solvent B increased to 100 % and washing was continued for 10 min. finally, the percentage of solvent A was increased to 100 % within 5 min, and washing was performed with this eluent for 10 min. The flow rate was 200 nl/min and the total separation time was 30 min using a UV detection at 370 nm. The LC-ESI-MS system which was used in the present study and included PicoTip®emitter made by New Objective (Woburn, MA, USA) and used as a spray nozzle. Mass spectrometry apparatus model Finigan LTQFT Ultra which was made (Thermo Fisher Scientific., Germany) equipped with (NESI) ion generator, Nano Electro Spray Ionization, the ability to break ions by two methods (CID) Collision Induced Dissociation and (ECD) Electron Capture Dissociation and Ion Trap (IT) mass detector with ability separation of FWHM 100 Da and accuracy higher than 0.5 ppm were connected to HPLC and used to identify metabolites. Instrument control, data acquisition, and processing were conducted by the Xcalibur software. Typical negative ESI-MS conditions were: capillary voltage 2.0 kV and skimmer cone voltage 20 V.

2.7. Molecular Docking Studies

The protein structure of human pancreatic alpha-amylase (1BSI) and human lysosomal alpha-glucosidase (5NN8) have been retrieved from Protein Data Bank. SwissDock program was used to simulate the ligand-enzyme interaction [21]. The best ligand-enzyme structure was chosen based on the full fitness factor [22]. The structure which had the highest

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full fitness was selected as the best-simulated model. The acquired structures were further processed with UCSF Chimera software [23].

3. Results

3.1. The extraction efficiency of prescriptions

As mentioned in the previous section, the selection of prescriptions for controlling the condition of diabetic patients and reducing their blood sugar was done from the sources of Iranian and Iraqi traditional medicine. Based on the documents, aqueous extracts (at 25 and 97 °C) and methanol extracts were prepared, their solvents were dried and the percentage of extraction efficiency was obtained (Table 2).

According to Table 2, the aqueous extracts of No. 2, 3, 8, and 13, which had the maximum inhibition of both enzymes, were chosen to study their enzyme inhibitory compounds. For this purpose, extracts were washed on HPTLC plates by using different washing systems to reach the best separation solvent system. Fig. 2 shows the separation condition, the results of each extract, and the optimal separation mobile phase systems.

Table 2. The extraction efficiency of each sample of medical prescriptions and their inhibition degree of α-amylase and α-glucosidase

Sample	Extraction solvent (ml)			Enzyme Inhibition % (□ α-Amylase ■ α-Glucosidase)
	Water	Hot water	Methanol	0 10 20 30 40 50 60 70 80 90
No.1	15.40 ± 0.02	16.60 ± 0.03	7.80 ± 0.01	W H M
No. 2	21.60 ± 0.04	27.40 ± 0.01	21.40 ± 0.02	W H M
***No. 3**	10.01 ± 0.11	-**	***69.60 ± 0.06***	W H
No. 4	16.00 ± 0.03	-	12.00 ± 0.04	W M
No. 5	12.80 ± 0.01	-	5.20 ± 0.01	W M
No. 6	17.20 ± 0.02	-	10.00 ± 0.02	W M
No. 7	24.05 ± 0.12	2.00 ± 0.01	8.00 ± 0.01	W H M

* An extract that showed a maximum inhibition percentage of both enzymes
** Extraction with hot water, only for the samples that used hot water in the traditional medicine prescription to prepare medicine
W Water extract enzyme inhibition %
H Hot water extract enzyme inhibition %
M Methanol extract enzyme inhibition %

Table 2. The extraction efficiency of each sample of medical prescriptions and their inhibition degree of α-amylase and α-glucosidase (Continued)

Sample	Extraction solvent (ml)			Enzyme Inhibition % (□ α-Amylase ■ α-Glucosidase)
	Water	Hot water	Methanol	0 10 20 30 40 50 60 70 80 90
No. 8	32.80 ± 0.13	-	15.00 ± 0.03	W M
No. 9	***68.11 ± 0.20***	***40.60 ± 0.08***	38.20 ± 0.07	W H M
No. 10	41.00 ± 0.17	-	***70.00 ± 0.05***	W M
No. 11	16.00 ± 0.02	-	48.11 ± 0.11	W M
No. 12	16.23 ± 0.03	-	10.00 ± 0.01	W M
No. 13	10.00 ± 0.07	-	12.02 ± 0.12	W M
No. 14	4.02 ± 0.01	-	5.00 ± 0.00	W M
No. 15	16.00 ± 0.01	-	10.03 ± 0.02	W M

* An extract that showed a maximum inhibition percentage of both enzymes
** Extraction with hot water, only for the samples that used hot water in the traditional medicine prescription to prepare medicine
W Water extract enzyme inhibition %
H Hot water extract enzyme inhibition %
M Methanol extract enzyme inhibition %

Fig. 2 shows the separation of extract compounds which are having the highest enzyme inhibition on the HPTLC plate. In this figure, on each plate, four different extracts that had the highest inhibition of both enzymes were isolated for further studies. The studied extracts are water extract of samples No. 3 (a), 13 (b), 2 (c), and methanol extract of sample No. 8 (d). As can be seen in Fig. 2, the mobile phases A and B are not able to move materials and separate them on the stationary phase. Increasing the hydrophilicity of mobile phase by increasing water (Fig. 2C), methanol (Fig. 2D), or formic acid (Fig. 2E) has been able to increase the mobility of materials on the stationary phase.

But the separation capability is still very weak. In Fig. 2F, Fig. 2G, and Fig. 2E, the mobile phase ratio was optimized. Finally, the mobile phase consisting of toluene: ethyl acetate: formic acid: methanol in the ratio percentage of 45: 45: 2: 8 provided the best separation of compounds (Fig. 2E). The direct on plate enzyme inhibition was performed on this mobile phase (Fig. 2I).

In the mass spectrometry study, the extracted HPTLC band was injected into a mass spectrometry liquid chromatography apparatus. Then, the amount of separation, as well as the type of separated materials were examined. Fig. 3 shows the HPLC (Fig. 3A) and mass spectrometry (Fig. 3B and Fig. 3C) analyses of the two peaks which were observed in chromatogram 3a. As can be seen, the compound on the HPTLC plate with the enzyme inhibition effect contains two compounds with very close retention times. This can also be seen with a little attention in the semi-preparative HPTLC plate. These two compounds (with HPLC retention time of: 9.09 and 10.51 min) were separated by liquid chromatography and their mass spectrometric spectra were obtained. The MS spectrum of both compounds has a very similar pattern, but the frequency of detected peaks is different. The most important identified adduct ion peaks are $[M+Na]^{+\bullet}$, $[M+K]^{+\bullet}$ $[M+H]^{+\bullet}$, $[M+H-H_2O]^{+\bullet}$, $[M+H-3H_2O]^{+\bullet}$.

These ions and fractures are suggested based on a study by the Metlin Online Library. According to the mass spectrometry publication and based on software suggestions, the extracts of *P. mahaleb* and *P. dulcis*, these two compounds are flavonoid structures of catechin and epicatechin. These two structures are so similar with only some spatial structure differences. For better identification of CID, MS was performed and the results were confirmed both suggested structures (Fig. 3D and Fig. 3E). Based on the comparison of publications and the interpretation of two spectra in Fig. 3D and Fig. 3E, it is indicated that the spectral pattern includes fractions of 179.02, 205.08, 245.19, and 289.08 m/z related to catechin composition. Also, the spectral pattern includes fractures of 124.04, 139.06, 151.12, 165.07, 207.18, and 291.04 m/z related to epicatechin composition. Catechin and epicatechin are phenolic compounds that have both antioxidant and enzymatic inhibitory effects.

Our results showed that epicatechin is bound to human pancreatic alpha-amylase by attaching to aspartic acid (D300) (Table 3). Interestingly, it works as an essential amino acid in this enzyme [24]. It usually acts as a transition state stabilizer [20], making hydrogen bonds with its substrate. Therefore, it will help the substrate bind to the enzyme and increase enzyme efficiency (Fig. 4 and Fig. 5). Consequently, attaching epicatechin to this amino acid prevents any substrate from binding to the enzyme properly and inhibits enzyme activity. Furthermore, some hydrophobic amino acids around epicatechin (Y52, F55, W58, W59, Y62, and Y151) will help it to be stabilized [19]. Therefore, the hydrophobic interaction between epicatechin and surrounding amino acids can stabilize the attachment and potentiate enzyme activity inhibition.

Besides, our results illustrated that catechin is directly bound to aspartic acid (D356) and asparagine (N350) in human pancreatic alpha-amylase. Although catechin did not bind directly to the active site or enzyme's binding site, it seems it affects one of the binding site amino acids (N298), which is near to the binding site of catechin and enzyme. It can change the protein's conformation and configuration, which leads to prevention or decreasing substrate-binding efficiency.

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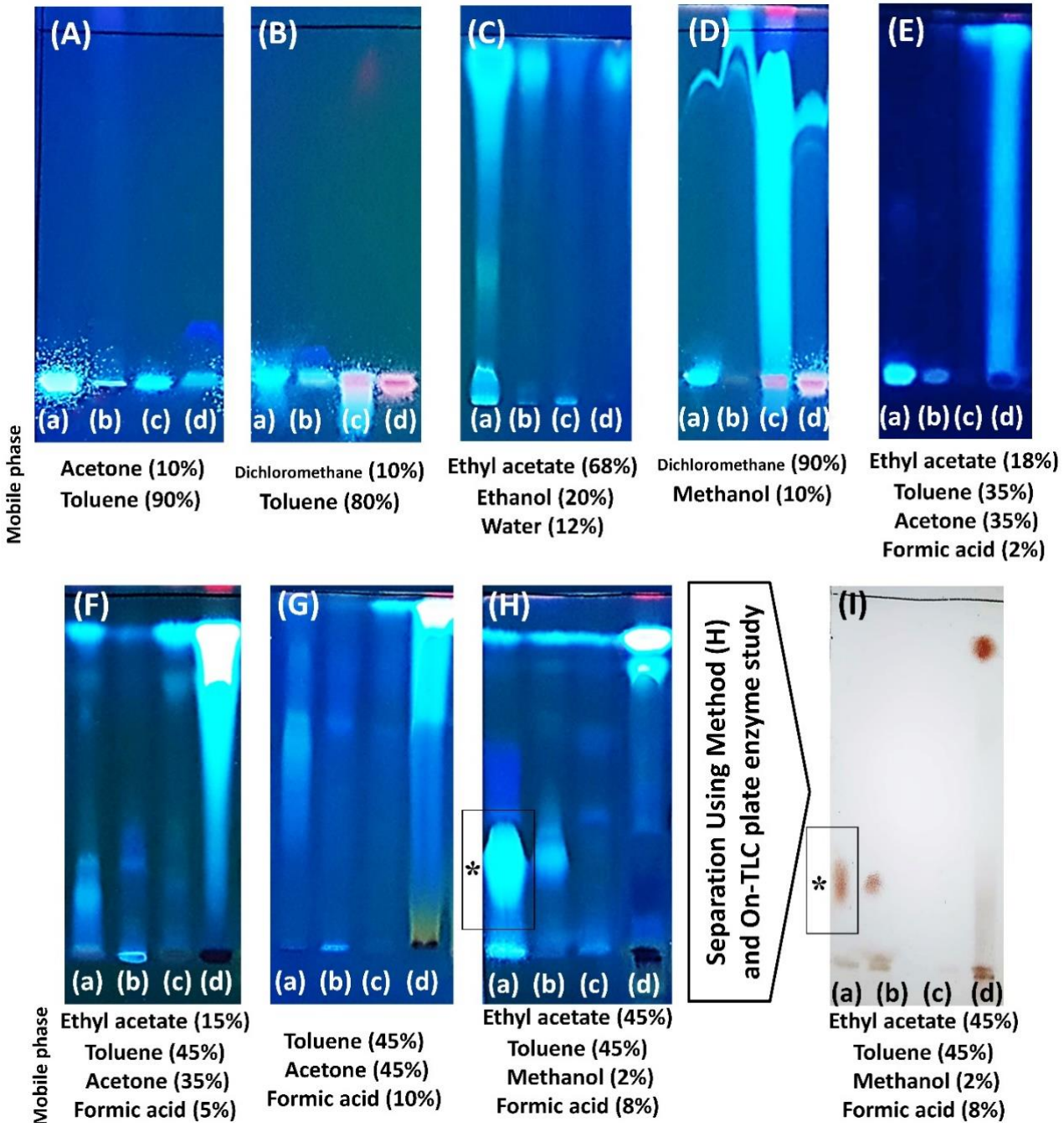

Fig. 2. Image of HPTLC separation (at 366 nm) optimization of the aqueous extract of samples No. 3 (a), 13 (b), 2 (c), and methanol extract of sample No. 8 (d) using a variety of mobile phases (A-H) and On-plate enzyme inhibition of the aqueous extract based on method H separation condition. The symbol (*) demonstrated the extracted band that was a subject of LC-MS analysis.

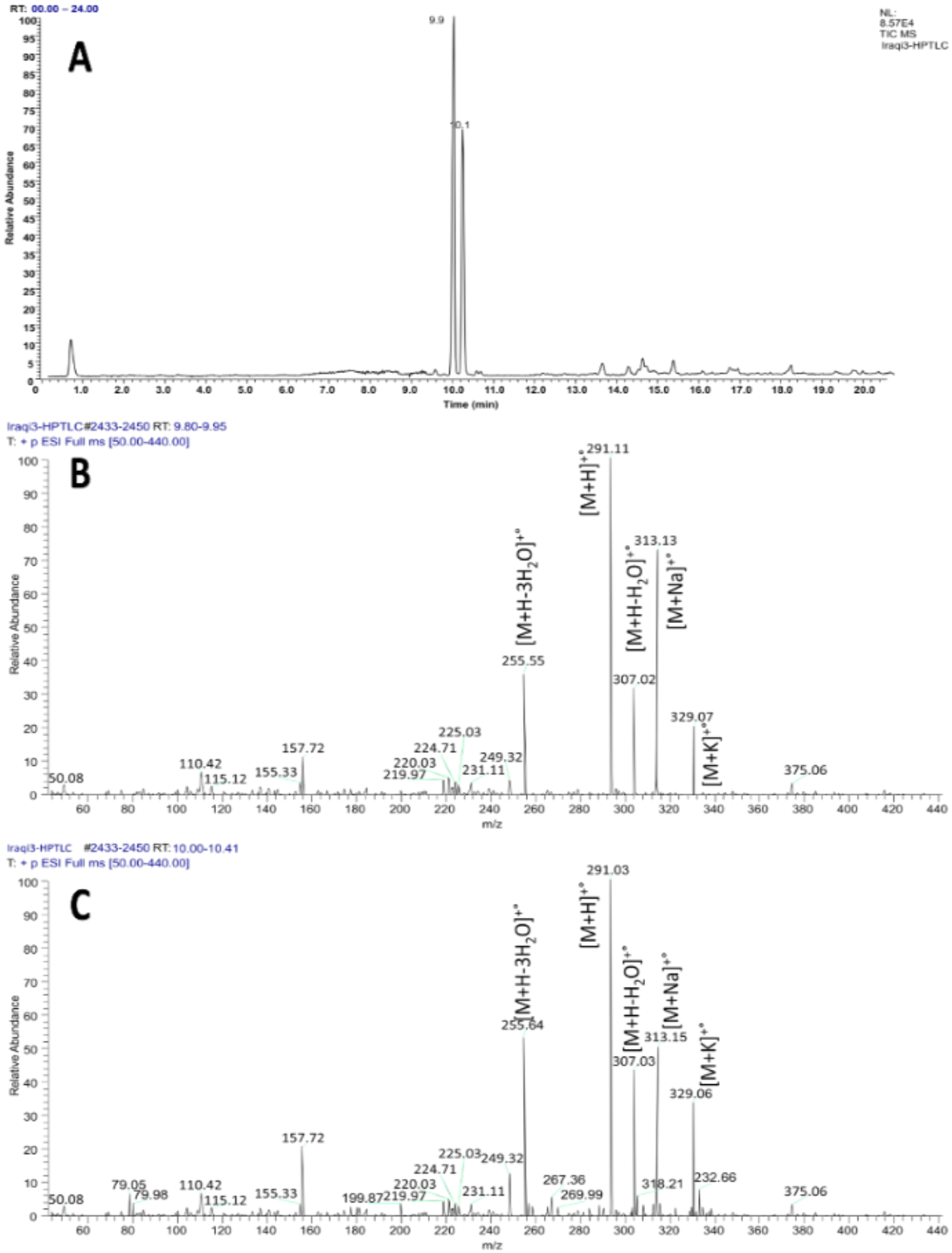

Fig. 3. HPLC separation of HPTLC separation (spot * on Fig. 2H): (A), Adduct mass spectrum pattern of the peak with a retention time of 9.09: (B), and the peak with a retention time of 10.51: (C), The pattern of CID mass spectrum of the peak with a retention time of 9.09: (D), and the peak with a retention time of 10.51: (E).

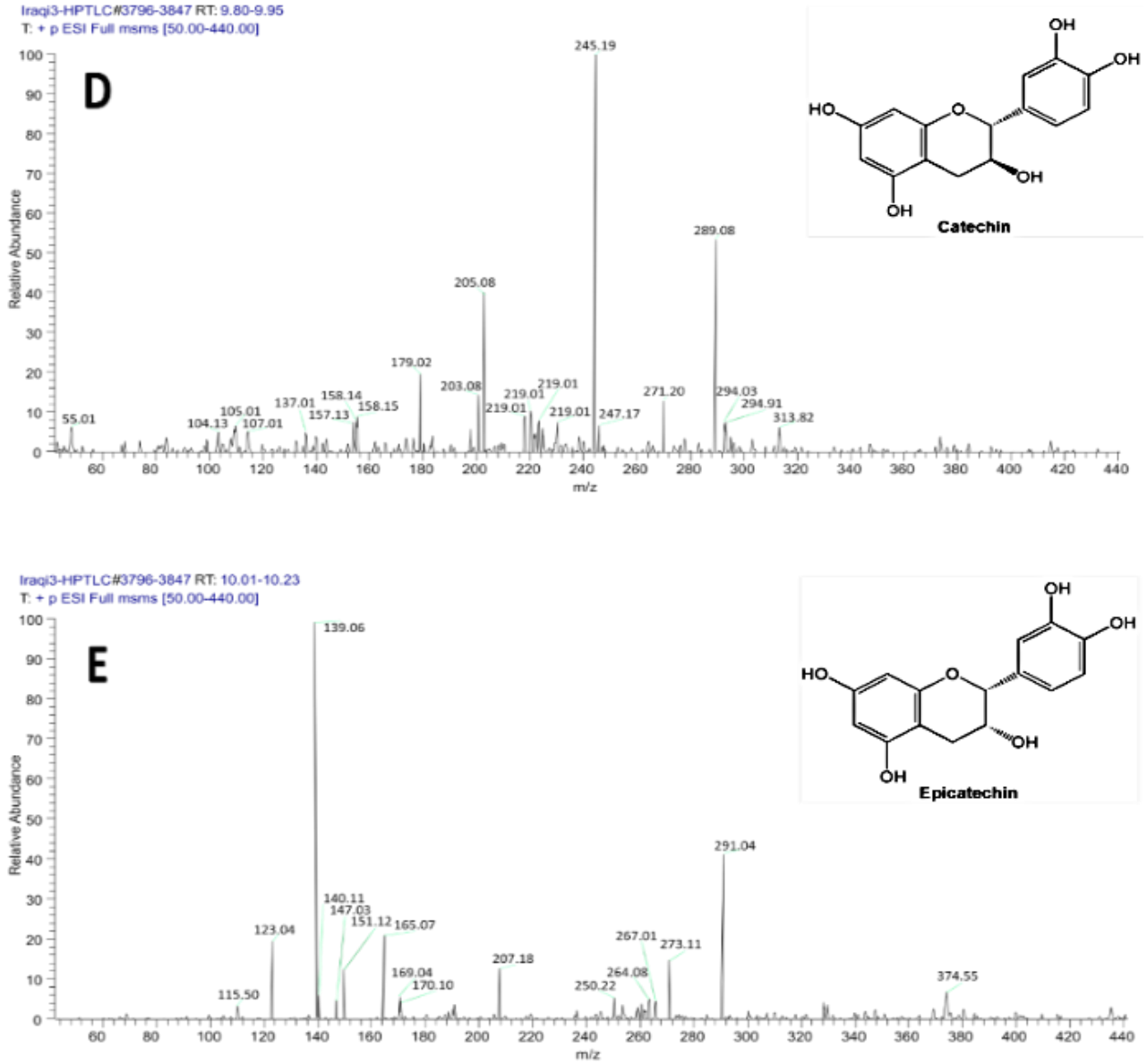

Fig. 3. HPLC separation of HPTLC separation (spot * on Fig. 2H): (A), Adduct mass spectrum pattern of the peak with a retention time of 9.09: (B), and the peak with a retention time of 10.51: (C), The pattern of CID mass spectrum of the peak with a retention time of 9.09: (D), and the peak with a retention time of 10.51: (E).

Table 3. Molecular docking results from human pancreatic alpha-amylase and human lysosomal alpha-glucosidase with catechin and epicatechin

Enzyme	Ligand	Number of hydrogen bonds	Interacting residue of the enzyme	Hydrogen bond distance(Å)
α-Amylase	Epicatechin	1	D300	2.13
	Catechin	2	N350, D356	1.985, 1.847
α-Glucosidase	Epicatechin	1	D513	1.809
	Catechin	1	E192	1.911

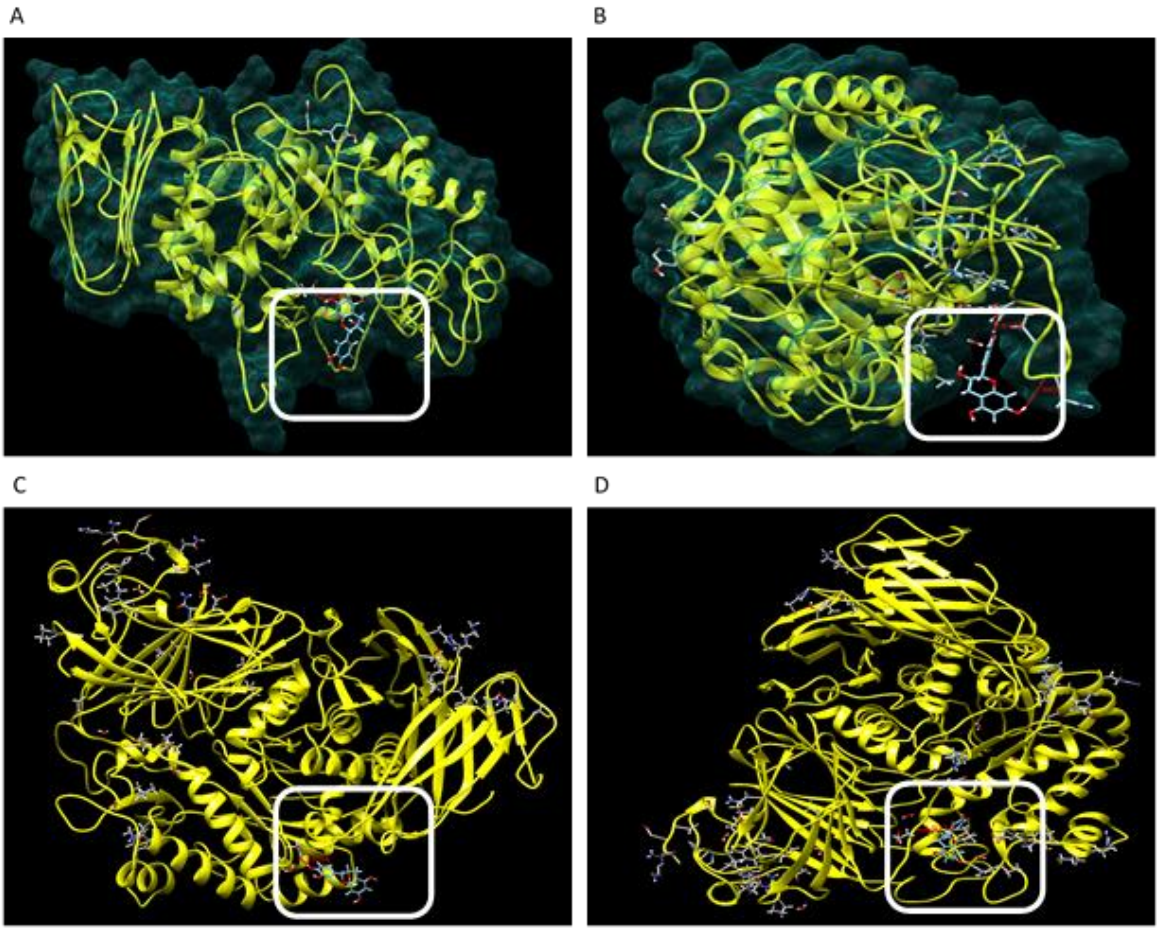

Fig. 4. The interaction of human pancreatic alpha-amylase with A) epicatechin and B) catechin was modeled by the SwissDock program. The interaction of human lysosomal alpha-glucosidase with C) epicatechin and D) catechin was modeled by the same program.

4. Discussion

As can be seen, the highest extraction efficiency percentage is related to samples No. 3 (69.6 %) and 10 (70.0 %). The highest extraction efficiency percentage of aqueous extract (at 25 and 97 °C) is related to sample No. 9 at 25 °C (68.11 %) and 97 °C (40.6 %). α-amylase and α-glucosidase inhibitory activity of each extract were investigated. As can be seen in Table 2, the enzyme inhibition percentage of α-amylase and α-glucosidase are shown by white and black bars. In this table, letters W and H mean aqueous extracts which were prepared at 25 and 97 °C, respectively, and the letter M refers to methanol extract. As can be seen, the maximum inhibition of α-amylase was performed by aqueous extract of sample No. 3 (40.2 %), followed by aqueous extract of sample No. 13 (35.2 %), hot water extract of sample No. 7 (32.6 %), and methanol extract of sample No. 8 (31.1 %). The maximum inhibition of α-glucosidase was performed by aqueous extract of samples No. 3 (83.3 %), 15 (88.4 %), and 13 (77.3 %), as well as methanol extract of samples No. 10 (76.2 %), 13 (75.1 %), and 15 (63.3 %). Extracts of samples No. 6, 9, and 11 inhibited only α-amylase, and extracts of samples No.12 and 15 inhibited only α-glucosidase. In addition, the aqueous extract of sample No. 3 showed the highest activity inhibition of both enzymes.

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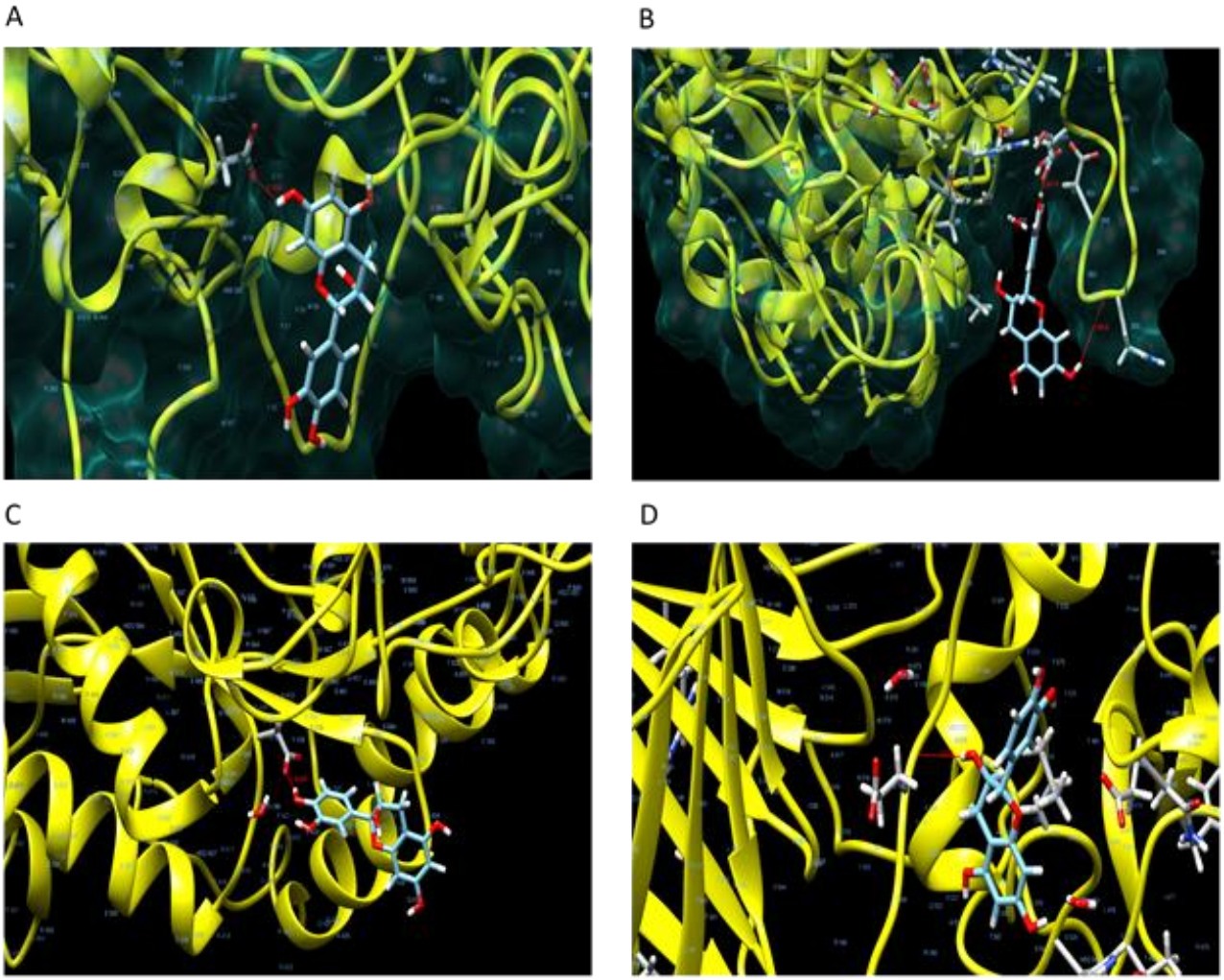

Fig. 5. The magnified images of the interaction of human pancreatic alpha-amylase with A) epicatechin and B) catechin which were modeled by the Swiss Dock program. The magnified images of the interaction of human lysosomal alpha-glucosidase with C) epicatechin and D) catechin which were modeled by the same program.

As can be seen in Fig. 2I, extracts (a) and (d) have enzymatic inhibition. In addition, as methanol extract No. 8 (d) has much more contaminations and less enzymatic inhibition than aqueous extract of sample No.3 (a), further study to identify inhibitory compounds was performed on aqueous extract of sample No.3 (a) (*). Thus, as mentioned in section 2.7, an appropriate amount of substance * in aqueous sample No. 3 was prepared using semi-prepared HPTLC separation. Ultimately, the compound with an enzyme inhibitory effect was removed for mass spectrometry analysis (Fig. 6.).

The interactions of the identified enzymes and compounds were modeled and investigated. Our results demonstrated that epicatechin is bound to aspartic acid (D513) in human lysosomal alpha-glucosidase. It is worth mentioning that the binding site of epicatechin to the enzyme is close to the enzyme's active site (D518 and E521). The attachment of epicatechin to the enzyme can change the conformation and configuration of the active site and hinder the substrate-binding efficiency.

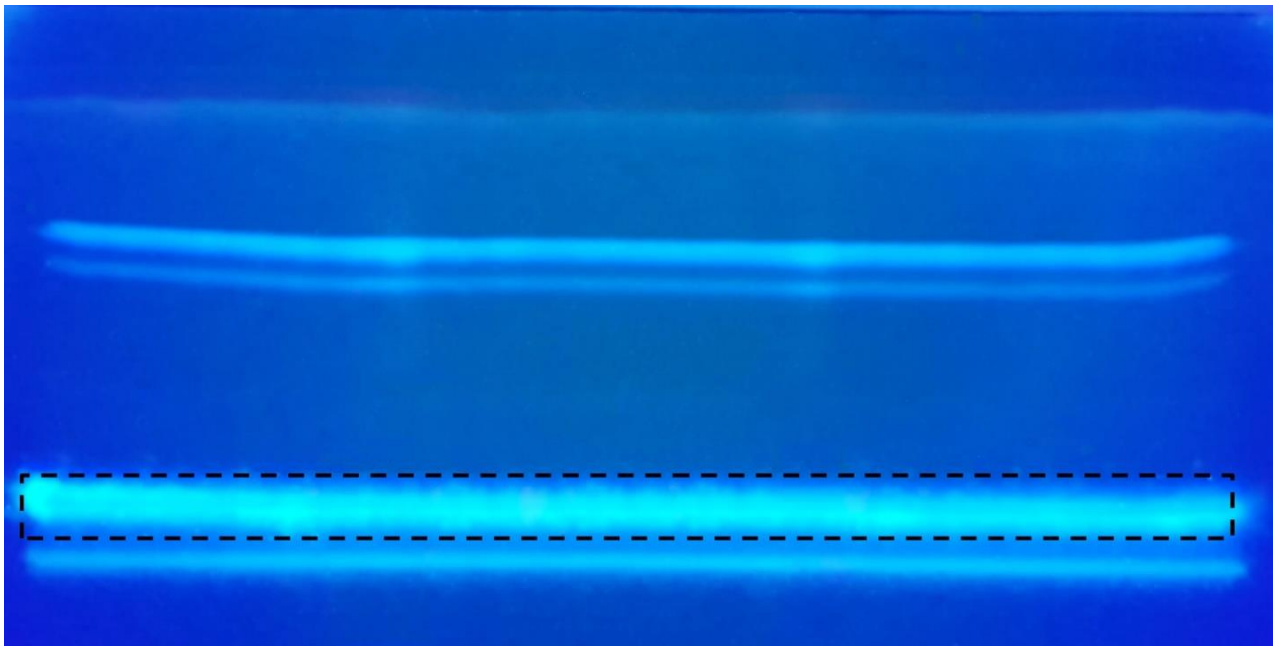

Fig. 6. The compound with an enzyme inhibitory effect

Moreover, our results demonstrated that catechin is bound to glutamic acid (E192) in human lysosomal alpha-glucosidase. In this case, similar to catechin's attachment to human pancreatic alpha-amylase, catechin is not bound to any active site or binding site amino acids of the enzyme. However, it seems that it is close enough to some binding site amino acids of the enzyme that can perturb their activity and decrease the enzyme's substrate-binding efficiency.

To sum up, our results are consistent with previous publications [25, 26] that catechin and epicatechin can efficiently decrease the activity of human pancreatic alpha-amylase and human lysosomal alpha-glucosidase. However, more investigations are needed to confirm these assumptions.

5. Conclusion

The results of different medicinal plant prescriptions showed that some of them have the ability to inhibit α-amylase and α-glucosidase enzymes. These results presented that the extract, which is containing mahaleb and sweet almond and belonging to the same family, contains the chemicals catechin and epicatechin. These substances are able to inhibit metabolic enzymes due to their chemical structures. The results of this study can be summarized as:

1- The aqueous extracts are the best α-amylase and α-glucosidase inhibitors.

2- The best enzyme inhibitory extract contains *P. mahaleb* and *P. dulcis*.

3- In this paper, a suitable method for HPTLC On-Plate Enzyme Assay was introduced.

4- Two compounds of catechin and epicatechin have been separated from the aqueous extract of sample No. 3. These two compounds are enzymatic inhibitors, according to the articles published by other researchers.

Author contributions

RAB: Experimentation; HR: HPTLC experimental analysis and; AH: Enzyme assay; SMM: Molecular modeling and bioinformatics

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study; FM: Original idea presentation, study design, study supervision, data interpretation, and revision of the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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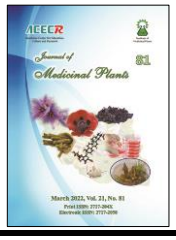

مقاله تحقیقاتی

بررسی فعالیت بازدارندگی آلفا-آمیلاز و آلفا-گلوکوزیداز برخی از نسخه‌های سنتی گیاهی با استفاده از روش بیواتوگرافی/ طیف‌سنجی جرمی

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اطلاعات مقاله

گل‌واژگان:
دیابت
آلفا-آمیلاز
آلفا-گلوکوزیداز
طب سنتی عراقی
محلب
Prunus dulcis
HPTLC

چکیده

مقدمه: دیابت، یکی از شایع‌ترین بیماری‌های متابولیک در بسیاری از جوامع بوده که بر زندگی انسان و کیفیت آن تأثیر داشته است. طب سنتی جایگزین و مکمل، از نسخه‌های گیاهی به صورت مفرده یا مرکبه برای کاهش عوارض دیابت استفاده می‌کند. این روش‌های قدیمی با بیش از صد سال قدمت، مبتنی بر تجربه انسانی در درمان و مدیریت عوارض بیماری در مناطق جغرافیایی مختلف می‌باشند. **هدف:** این پژوهش سعی دارد مؤثرترین نسخه طب سنتی را در مهار آنزیم‌های مرتبط با دیابت پیدا و توصیه کند. **روش بررسی:** با توجه به استفاده کامل و گسترده از گیاهان دارویی، در این تحقیق، ۱۵ نسخه گیاهی ایرانی و عراقی معتبر در کنترل عوارض دیابت برای مهار آنزیم‌های آلفا آمیلاز و آلفا گلوکوزیداز مورد بررسی قرار گرفتند. ترکیبات شیمیایی موثرترین نسخه در مهار این آنزیم‌ها با استفاده از روش HPTLC جدا شدند. برای توسعه کروماتوگرام از فاز متحرک حاوی اتیل استات: تولوئن: متانول: اسید فرمیک استفاده شد. برای بررسی موثرترین مولکول‌ها از روش مهار آنزیمی روی صفحه HPTLC استفاده شد. پس از آن، مولکول‌های تاثیرگذار با استفاده از روش LC-ESI/MSMS شناسایی شدند. **نتایج:** بر اساس نتایج، نسخه شماره ۳ (از نسخه گیاهی عراقی) حاوی عصاره دو گیاه *Prunus mahaleb* L. (محلب) و *Prunus dulcis* (Mill.) D.A.Webb به عنوان بهترین مهارکننده‌های آنزیم‌های آلفا آمیلاز و آلفا گلوکوزیداز شناخته شد. **نتیجه‌گیری:** آنالیز شیمیایی و مولکولی این نسخه به روش HPTLC و سپس توسط LC-ESI/MSMS انجام شد که نشان‌دهنده دو ترکیب کاتچین و اپی‌کاتچین می‌باشد.

مخفف‌ها: HPTLC، کروماتوگرافی لایه نازک با کارایی بالا؛ LC-ESI/MSMS، کروماتوگرافی مایع- یونیزاسیون الکترواسپری متصل به طیف‌سنج جرمی؛ DMSO، دی متیل سولفوکسید؛ CID، تفکیک ناشی از برخورد؛ ECD، تفکیک گرفتن الکترون؛ IT، تله یون

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