ORIGINAL ARTICLE

α-Glucosidase Inhibitors from *Marrubium astracanicum*: Isolation and Molecular Docking

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Abstract

Marrubium astracanicum Jacq., Lamiaceae, is an herbaceous plant distributed in Iran, Turkey, and southern Caucasia. In the present study, α-glucosidase inhibitory principles of *M. astracanicum* aerial parts were investigated by a bioassay-guided approach. The extraction procedure from the plant aerial parts was carried out successively using petroleum ether, ethyl acetate, and methanol. The ethyl acetate extract, with the highest *in vitro* α-glucosidase inhibitory activity, was subjected to further chemical investigation. The structures of the isolated compounds were elucidated by ^{1}H-NMR and ^{13}C-NMR spectral analyses as apigenin-7-*O*-(3″-(*E*)-*p*-coumaroyl)-β-D-glucoside, apigenin-7-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside, martynoside, apigenin-7-*O*-β-D-glucoside, and verbascoside. In addition, the antidiabetic potential of the isolated compounds was evaluated in an *in vitro* α-glucosidase inhibitory assay, and kinetic and molecular docking studies were done for the most active compound. Apigenin-7-*O*-(3″-(*E*)-*p*-coumaroyl)-β-D-glucoside and apigenin-7-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside showed the most potent *in vitro* α-glucosidase inhibitory activity with IC$_{50}$ values of 57.4 ± 1.2 and 186.4 ± 2.5 μM, respectively, which was about 4 to 13 times lower than the IC$_{50}$ value of the reference compound acarbose (751.2 ± 0.4 μM).

Keywords Flavonoid · Phenylethanoid · Phenolic glycoside · Diabetes · Competitive inhibitor · *In silico* study

Introduction

Diabetes mellitus type II which is also called adult type or non-insulin-dependent diabetes mellitus is a metabolic disease characterized by chronic hyperglycemia resulting from reduced response of the organs to insulin. It has been predicted that the number of patients with diabetes increases from 415 million in 2015 to approximately 642 million by 2040 (Carracher et al. 2018). This global growth in prevalence of diabetes mellitus type II has made it as one of the world's greatest health problems (Khan et al. 2020). α-Glucosidase inhibitors such as acarbose, voglibose, and miglitol are one of the drug classes used in the management of diabetes mellitus type II (Derosa and Maffioli 2012). The enzyme α-glycosidase is located in the brush border of the small intestine mucosa that acts to hydrolyze carbohydrates containing α-(1→4) glucose linkage (Hossain et al. 2020). α-Glucosidase inhibitor drugs such as acarbose are efficient in suppressing postprandial blood glucose levels by the inhibition of the carbohydrate degradation to free absorbable glucose and help to prevent oxidative stress–induced damages caused by increase in blood glucose levels (Wright Jr et al. 2006). Different classes of natural products especially phenolics have been reported to possess α-glucosidase inhibitory effects (Kumar et al. 2011; Proença et al. 2020). Therefore, plants are considered as a rich source of biologically natural product for development of new α-glucosidase inhibitor drugs with diminished side effects.

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The genus *Marrubium* L. from the family Lamiaceae consists of 49 species mainly distributed in Mediterranean and Irano-Turanian phytogeographic regions (Talebi et al. 2019). Among the *Marrubium* taxa, *M. vulgare* L., *M. cuneatum* Banks & Sol., *Ballota deserti* (Noë) Jury, Rejdali & A.J.K.Griffiths, syn. *M. deserti* (Noë) Coss., and *M. globosum* ssp. *libanoticum* (Boiss.) P.H.Davis have been mentioned for their medicinal use as antidiabetic agents in folk medicine of some countries (Rigano et al. 2007; Loizzo et al. 2008; Laouer et al. 2009; Hamza et al. 2019). Previous studies have demonstrated the *in vitro* α-glucosidase inhibitory activity of the methanolic extract of *M. vulgare* aerial parts (IC$_{50}$ 12.66 μg/ml), as well as significant dose-dependent blood glucose–lowering effects of its aqueous extract in alloxan-induced diabetic rats (Boudjelal et al. 2012). In 2008, Loizzo et al. reported the methanolic extract of *M. radiatum* aerial parts as one of the most potent extracts with α-glucosidase and α-amylase inhibitory activities (IC$_{50}$ 68.8 and 61.1 μg/ml, respectively), between different extracts obtained from some plants used in Lebanon traditional medicine against diabetes. In another study on *M. alysson* L., a diterpenoid lactone, called marrubiin, was isolated from its ethyl acetate extract with a strong inhibitory effect on α-glucosidase enzyme with IC$_{50}$ value of 16.62 μg/ml compared with acarbose as positive control with IC$_{50}$ value of 64.14 μg/ml (El-Mohsen et al. 2014).

Marrubium astracanicum Jacq. is one of eleven *Marrubium* species represented in the flora of Iran (Jamzad, 2012). The infusion of the aerial parts is used by indigenous people in Iran and Turkey for alleviation of stomachache and joint pains, as antipyretic and for the treatment of colds (Mosaddegh et al. 2012; Altundag and Ozturk 2011). Sesquiterpenes such as β-caryophyllene, germacrene D, and bicyclogermacrene have also been identified as main compounds of essential oil extracted from this plant aerial part (Javidnia et al. 2007; Teimori et al. 2008). Regarding traditional uses and α-glucosidase inhibitory potentials of *Marrubium* species, the present study was designed to isolate the α-glucosidase inhibitory principles of *M. astracanicum* in a bioassay-guided isolation approach.

Materials and Methods

Plant Material

The flowering aerial parts of *Marrubium astracanicum* Jacq., Lamiaceae, were collected from the Pol-e Zanguleh region (Chalus County, Mazandaran Province, Iran) in June 2018. The plant was authenticated by botanist Dr. Yousef Ajani (Research Institute of Forests and Rangelands, Karaj, Iran) and a voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran (7085-TEH).

Extraction and Isolation

The air dried and grinded plant aerial parts (0.6 kg) were macerated, successively, with petroleum ether, ethyl acetate, and methanol (3× 4 l each) at room temperature. The extracts were concentrated using a rotary evaporator at 40 °C and dried in a vacuum oven (40 °C). The ethyl acetate extract (15 g) was subjected to CC on a silica gel (650 g, 230–400 mesh, Merck, Germany) and eluted with CH$_2$Cl$_2$-EtOAc (100:0 to 0:100%), and then EtOAc-MeOH (80:20) to get twenty fractions (E1-20). Compounds **1** (23 mg) and **2** (18 mg) were obtained as yellow precipitates from fractions E2 and E5, respectively. Fraction E10 (200 mg) was chromatographed on a Sephadex LH-20 column (20 g, Fluka, Switzerland) with MeOH to get compounds **3** (22 mg) and **4** (18 mg). Compound **5** (9 mg) was purified from fraction E20 *via* Sephadex LH20 column chromatography (10 g) using MeOH as the eluent. In all steps, column chromatography was monitored by TLC under UV at 254 and 366 nm, following ammonia vapor exposure. The structures of the isolated compounds were elucidated using ^{1}H-NMR and ^{13}C-NMR spectral analyses (Varian- INOVA, 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR), as well as comparing with published data (see Supporting Information).

α-Glucosidase Inhibition Assay

α-Glucosidase inhibitory activity of the extracts and isolated compounds were evaluated in an *in vitro* colorimetric assay (Asgari et al. 2019). The α-glucosidase enzyme (EC3.2.1.20, *Saccharomyces cerevisiae*, 20 U/mg) solution (1 U/ml, 20 μl), potassium phosphate buffer (50 mM, pH 6.8, 135 μl), and sample solution (500 μg/ml for the extracts and 750 μM for the pure compounds, 20 μl) were added to a 96-well plate and incubated at 37 °C for 10 min. After that, *p*-nitrophenyl-α-D-glucoside (PNPG) solution (4 mM, 25 μl) was added to the mixture and the plate was incubated for 20 min at 37 °C. Finally, the absorbances were recorded by a spectrophotometer (Gen5, PowerWave XS2, BioTek) at 405 nm and the percentage of enzyme inhibition was determined in comparison with the negative control (10% dimethyl sulfoxide). Acarbose is the antidiabetic drug used as positive control. The IC$_{50}$ value was determined for the tested compound with inhibition percentage higher than 50% in preliminary assay. Each sample was tested in triplicate and results were analyzed using Sigma Plot 11.0 software and expressed as mean ± SD.

Enzyme Kinetic Study

The inhibition mode of the two most potent compounds against α-glucosidase enzyme activity was investigated (Asgari et al. 2019). For this purpose, α-glucosidase inhibitory activity was measured in the absence and presence of

different concentrations of the tested compounds in combination with different concentrations of PNPG (1–10 mM) as the substrate. A Lineweaver-Burk plot was generated to identify the type of inhibition and the Michaelis-Menten constant (K_m) value was determined from plot between reciprocal of the substrate concentration (1/[S]) and reciprocal of enzyme rate (1/V) over various inhibitor concentrations. Experimental inhibitor constant (K_i) value was constructed by secondary plots of the inhibitor concentrations [I] versus K_m.

Molecular Docking Study

The interaction of α-glucosidase protein and compound **2** was simulated by Auto Dock 4 software and Auto Dock Tools (ADT 1.5.6) using the hybrid Lamarckian genetic algorithm (LGA). In human's intestine, maltase-glucoamylase (MGAM) catalyzes the hydrolysis of disaccharides. Therefore, the *C*-terminal subunit of maltase-glucoamylase enzyme (GH31) was used for docking to better understand the isolated compounds-enzyme interactions. The three-dimensional (3D) crystal structure of human intestinal α-glucosidase (PDB code 3TOP) with a resolution of 2.88 °A was taken from the RCSB Protein Data Bank (Sakayanathan et al. 2018). The Hyperchem.8.0.10 software was used to develop and optimize 3D structures of selected compounds for molecular docking. The cubic grid box was built by 50 °A size (x, y, z) in the center with coordination of $x = -29.9365$, $y =$

34.8669, $z = 25.8692$ which was determined according to the position of the co-crystalized ligand (acarbose). All other parameters were left to default settings of Auto Dock 4. For validation of results, root mean-square deviation (RMSD) less than 2.0 °A was used as threshold. The RMSD was calculated in comparison to re-docking of original inhibitor, acarbose, that co-crystallized with 3TOP enzyme. The most favorable conformation by lowest free energy of binding was chosen and their target residues were determined.

Results and Discussion

Isolation and Structural Elucidation

Chemical investigation of ethyl acetate extract of *M. astracanicum* aerial parts resulted in the isolation of three flavone glycosides, apigenin-7-*O*-(3″-(*E*)-*p*-coumaroyl)-β-D-glucoside (**1**), apigenin-7-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside (**2**), and apigenin-7-*O*-β-D-glucoside (**4**), together with two phenylethanoid glycosides, martynoside (**3**) and verbascoside (**5**). Molecular structures of the isolated compounds were elucidated through ^{1}H-NMR and ^{13}C-NMR spectral analyses. This is the first report on isolation of these phenolic derivatives from the aerial parts of *M. astracanicum*.

1

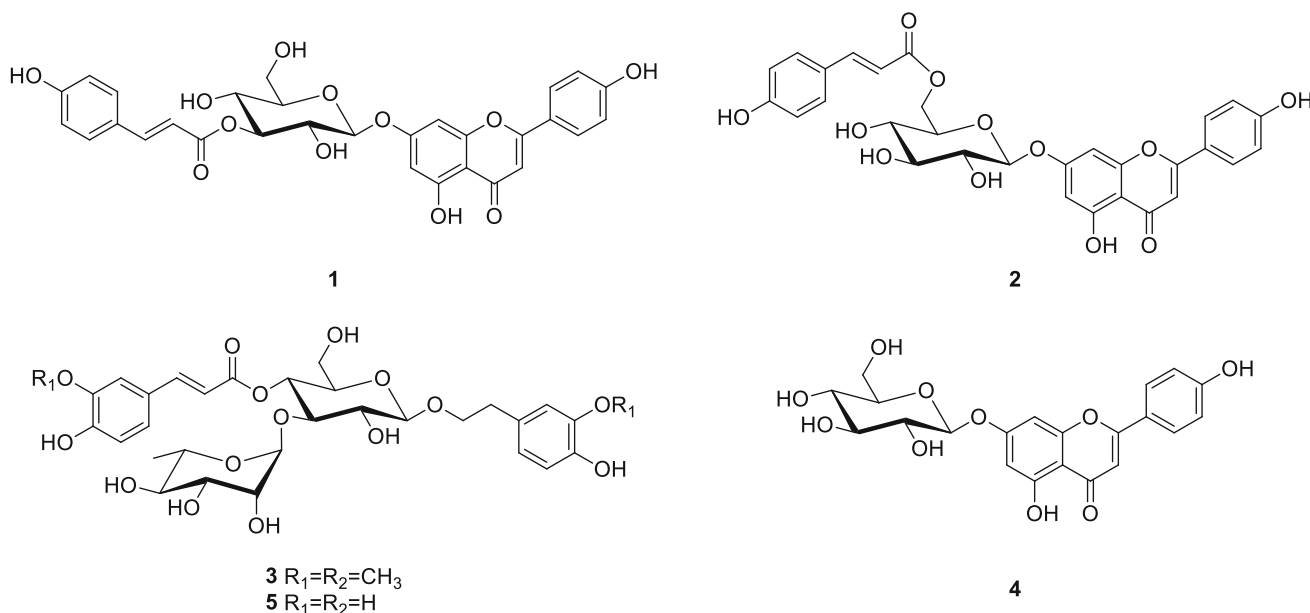**2**

3 R₁=R₂=CH₃
5 R₁=R₂=H

4

Table 1 α-Glucosidase inhibitory effects of the extracts and isolated compounds from the aerial parts of *Marrubium astracanicum* (mean $\pm$ SD)

Samples	Inhibition [a] (%)	IC$_{50}$ value (μM)
Petroleum ether extract	*Trace*	-
Ethyl acetate extract	43.5 $\pm$ 0.7	-
Methanol extract	12.1 $\pm$ 0.2	-
Apigenin-7-O-(3″-(E)-p-coumaroyl)-β-D-glucoside (**1**)	-	57.4 $\pm$ 1.2
Apigenin-7-O-(6″-(E)-p-coumaroyl)-β-D-glucoside (**2**)	-	186.4 $\pm$ 2.5
Martynoside (**3**)	-	366.1 $\pm$ 0.3
Apigenin-7-O-β-D-glucoside (**4**)	-	926.1 $\pm$ 1.0
Verbascoside (**5**)	-	286.3 $\pm$ 0.6
Acarbose (positive control)	-	751.2 $\pm$ 0.4

[a] Extracts were tested in concentration of 500 μg/ml. A dash (-) indicates not determined

α-Glucosidase Inhibition Assay

The inhibitory potentials of the extracts and isolated compounds **1**–**5** were evaluated *in vitro* against the α-glucosidase enzyme in comparison with acarbose, as a standard drug. As shown in Table 1, among the tested extracts, ethyl acetate extract demonstrated the most α-glucosidase inhibitory activity with the 43.5 $\pm$ 0.7% inhibition in concentration of 500 μg/ml. The α-glucosidase inhibition assay of the compounds **1**–**5** isolated from ethyl acetate extract revealed that except compound **4**, all the compounds were more potent than the standard drug against α-glucosidase enzyme (Table 1). Compounds **1** and **2** with IC$_{50}$ values of 57.4 $\pm$ 1.2 and 186.4 $\pm$ 2.5 μM, respectively, were the most potent α-glucosidase inhibitors in comparison with acarbose (IC$_{50}$ 751.2 $\pm$ 0.4 μM). Compound **1** showed a potent *in vitro* α-glucosidase inhibitory effect which was about thirteen times stronger the reference compound acarbose.

Enzyme Kinetic Study

The most potent compounds, apigenin-7-*O*-(3″-(*E*)-*p*-coumaroyl)-β-D-glucoside (**1**) and apigenin-7-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside (**2**), were subjected to a kinetic study to identify their mechanism of actions. As can be seen in Fig. 1, the K_m and V_{max} values increased by increasing the

Fig. 1 The inhibition kinetic of α-glucosidase enzyme by **1**. The Lineweaver-Burk plot in the absence and presence of different concentrations of **1** (μM)
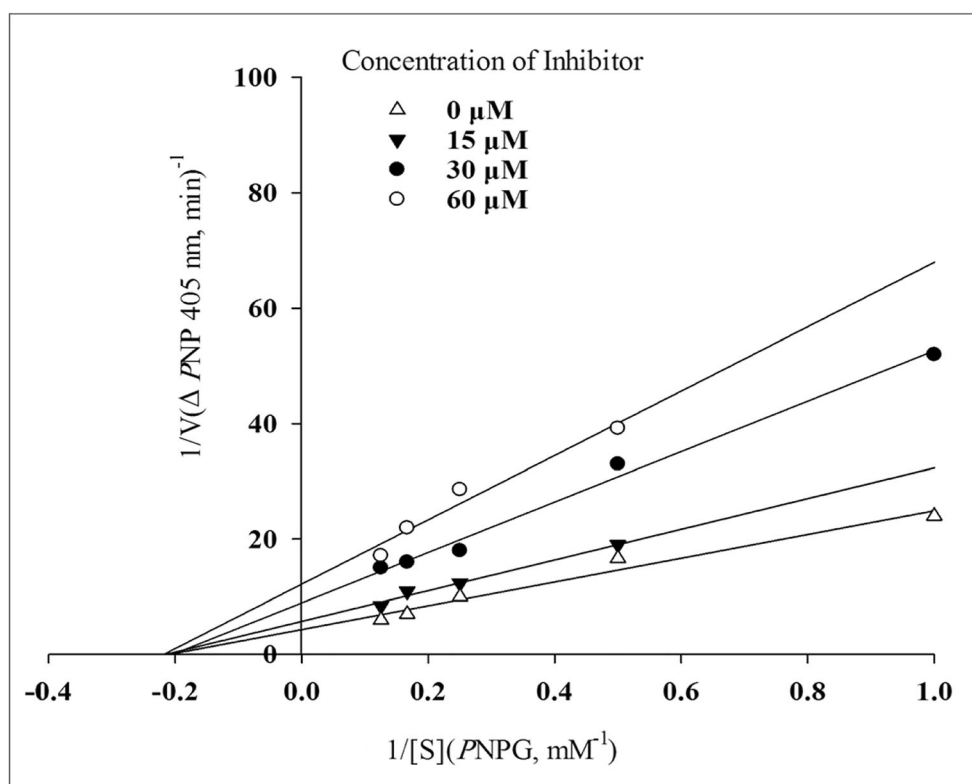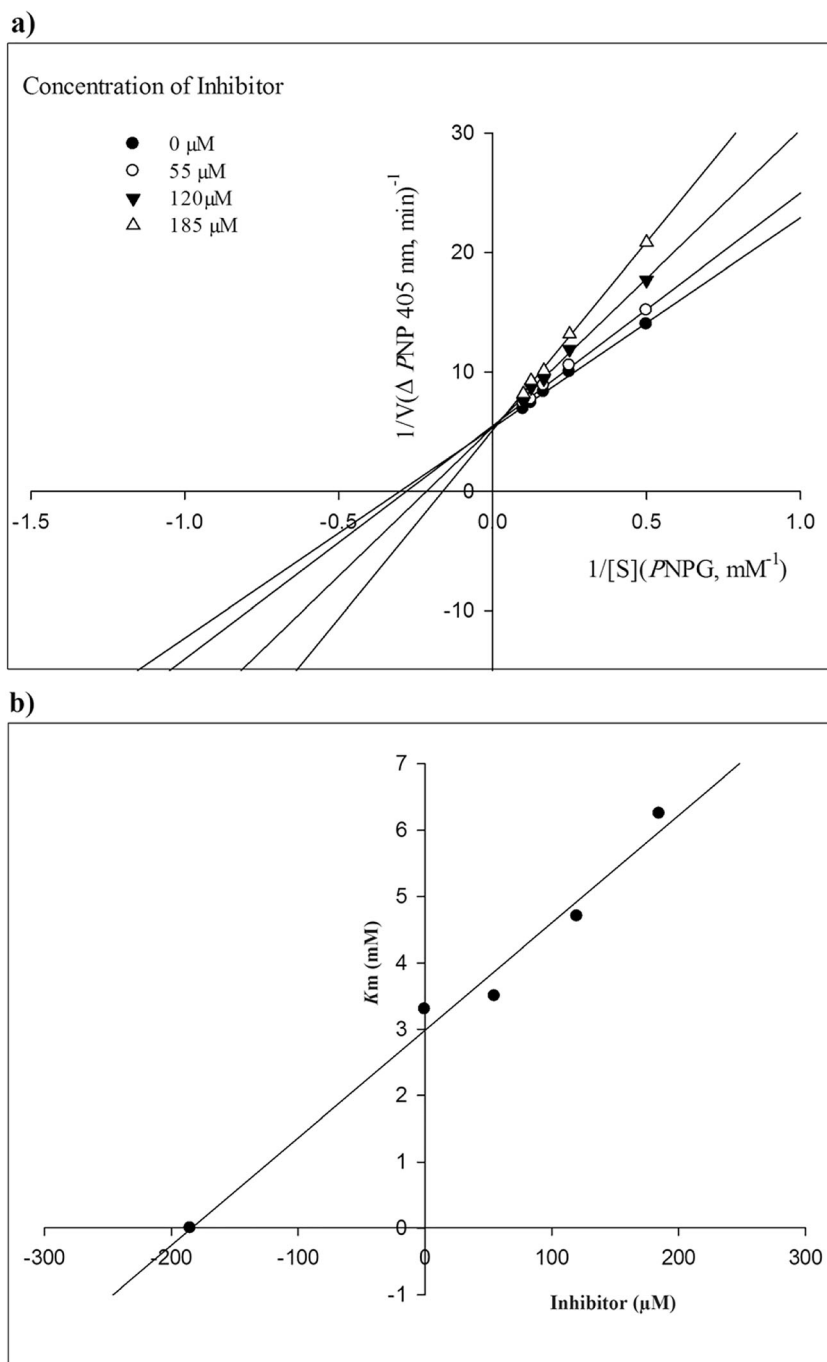
Fig. 2 The inhibition kinetic of α-glucosidase enzyme by compound **2**. (**a**) The Lineweaver-Burk plot in the absence and presence of different concentrations of **2** (μM). (**b**) The secondary plot between K_m and various concentrations of **2**


concentrations of compound **1** (0-60 μM), which indicated a non-competitive mode of inhibition. The enzyme kinetic study of apigenin-7-O-(6″-(E)-p-coumaroyl)-β-D-glucoside (**2**), however, showed that the K_m value increased, and V_{max} was not affected by increasing the inhibitor's concentrations (0–185 μM) (Fig. 2a). This result showed that compound **2** binds to the enzyme active site in a competitive mode of inhibition. Furthermore, the K_i value of compound **2** was determined as 185 μM (Fig. 2b).

Molecular Docking Study

For analyzing the interactions between the isolated compounds and α-glucosidase enzyme, molecular docking study was performed by Auto Dock Tools (version 1.5.6). Previous studies demonstrated a significant homology between *S. cerevisiae* α-glucosidase (G13) and human maltase-glucoamylase enzyme (GH31). They are similar in the catalytic domain (Rigden 2002). Therefore, the crystal structure of

Fig. 3 Acarbose (cyan) and the most potent compound **2** (purple) superimposed in the active site pocket
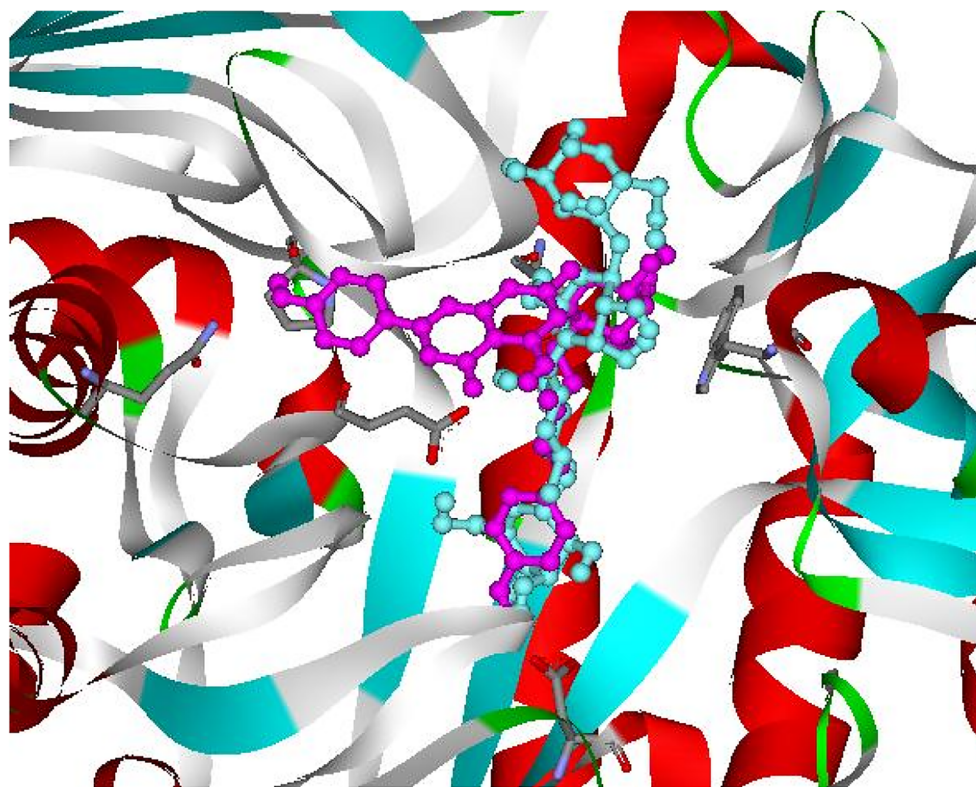
C-terminal domain of human intestinal α-glucosidase (PDB code: 3TOP) was used for docking simulations (Liu et al. 2019). In Fig. 3, the structures of acarbose and compound **2** (apigenin-7-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside), which was one of the most potent inhibitors among the isolated compounds with competitive mode of action, were superimposed in the active site of enzyme. The docking poses of the acarbose and the isolated compounds in the active site of enzyme are shown in Fig. 4. Results for docking studies of the isolated compounds, as well as acarbose in the studied protein, including free binding energy (FBE), inhibition constant (K_i), and interacted residues of active site are also summarized in Table 2 and according to the results, the lowest binding energy for compound **2** was calculated as −7.61 kcal/mol.

As shown in Figs. 3 and 4, compound **2** fitted to the active site pocket of enzyme in a slightly different manner in comparison to acarbose. The OH in 4′ position of the B ring in compound **2** interacts with GLN1533 (2.77 Å) and PRO1160 (1.76 Å) residues of the active site. As previous reports claimed, the hydroxyl groups in 3′ and 4′ positions (B ring) of flavonoids can bond to the active site residues of α-glucosidase. In addition, the type of flavonoid structure (aromatic rings and specific distributing of electron clouds) facilitates the proton donation of the C-3′ or C-4′ OH groups. Also, the present results showed that the hydrogen of the C-4′ OH group in compound **2** donated the proton to form a hydrogen bond with PRO1160 and the O of the same position made additional

hydrogen bond with GLN1533 residue of the α-glucosidase enzyme. On the other hand, compound **2** interacts with ASP1157 and ASP 1279 *via* C-4″ OH (in the sugar moiety) and 4‴ OH (hydroxyl of *p*-coumaroyl), respectively. These interactions and amino acid residues are in agreement with previous reports (Liu et al. 2019; Singh et al. 2018). Finally, a review on IC$_{50}$ values of isolated apigenin derivatives **1**, **2**, and **4** (Table 1) shows that a significant increase in α-glucosidase inhibitory activity is observed by additon of a *p*-coumaroyl group on the glucosyl moiety of apigenin-7-O-β-D-glucoside, among which *p*-coumaroylation of the OH at C-3″ position was found to be more effective to enhance enzyme inhibition.

A literature review revealed that there are limited reports on antidiabetic potentials of coumaroylated flavonoid glycosides (Ma et al. 2015; Ikechukwu and Ifeanyi 2016; Li et al. 2019). An investigation on red onion bulb (*Allium cepa* var. *cepa* L., Alliaceae) resulted in isolation of kaempferol-3-*O*-(6″-(*E*)-*p*-coumaroyl)-β-D-glucoside as the bioactive compound of the aqueous extract with considerable hypoglycemic activity at the dose of 25 mg/kg in alloxan-induced diabetic rats (Ikechukwu and Ifeanyi 2016). Furthermoe, Ma et al. (2015) reported the α-glucosidase inhibitory activity of some phenolics including seven flavonoid derivatives from the flowers of *Edgeworthia gardneri* (Wall.) Meisn., Thymelaeaceae, which is used by indigenus people in Tibet as herbal tea for diabetes and hyperlipidemia (Ma et al. 2015). They showed that oral

Fig. 4 Docking poses of the acarbose and the isolated compounds **1**–**5** in the active site of the enzyme
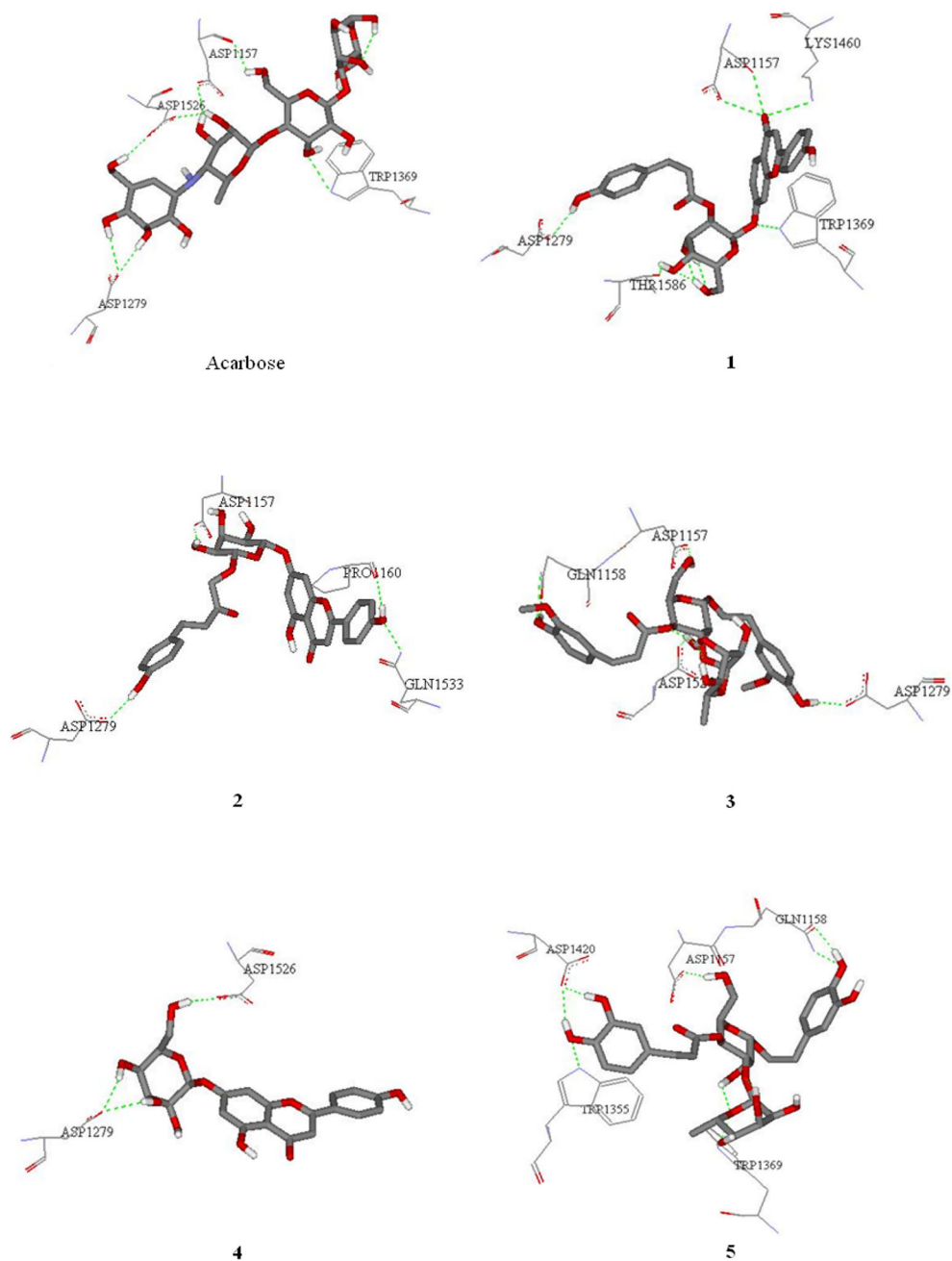

Table 2 Results for docking study of the isolated compounds in human intestinal α-glucosidase (PDB code: 3TOP)

Compounds	- Log FBE (kcal/mol)	K_i (μM)	Residues with polar interaction
Apigenin-7-O-(3″-(E)-p-coumaroyl)-β-D-glucoside (**1**)	− 7.56	2.88	ASP1157 – ASP1279 – TRP1369 – LYS1460 – THR1586
Apigenin-7-O-(6″-(E)-p-coumaroyl)-β-D-glucoside (**2**)	− 7.61	2.66	ASP1157 – PRO1160 – ASP1279 – GLN1533
Martynoside (**3**)	− 6.17	29.87	ASP1157 – GLN1158 – ASP1279 – ASP1526
Apigenin-7-O-β-D-glucoside (**4**)	− 7.99	1.39	ASP1279 – ASP1526
Verbascoside (**5**)	− 5.89	48.43	ASP1157 – GLN1158 – TRP1355 – TRP1369 – ASP1420
Acarbose (positive control)	− 7.92	1.55	ASP1157 – ASP1279 – TRP1369 – ASP1526

administration of kaempferol-3-O-(6″-(E)-p-coumaroyl)-β-D-glucoside (tiliroside), the main compound isolated from *E. gardneri* flowers with IC_{50} value of 202 ± 12 μg/ml in α-glucosidase inhibitory assay, significantly decreased the postprandial blood glucose levels of normal and streptozotocin-induced diabetic mice at the dose of 300 mg/kg. In the mentioned study, quercetin (3,3′,4′5,7-pentahydroxyflavone) was identified as the most potent α-glucosidase inhibitor principle of *E. gardneri* flowers with IC_{50} value of 5.1 ± 0.3 μg/ml (IC_{50} value of acarbose 465 ± 37 μg/ml). The addition of second coumaroyl moiety can enhance the affinity of flavonoid glycoside toward α-glucosidase enzyme. In 2019, some dicoumaroylated flavonol rhamnosides were reported as α-glucosidase inhibitor principles of the ethyl acetate extract of *Machilus litseifolia* S.K. Lee., Lauraceae, of which 4′-O-methyl-2″,4″-di-(Z)-p-coumaroyl afzelin with IC_{50} value of 5.9 μM was found 91 times more potent than standard drug acarbos (IC_{50} 266 μM) (Li et al. 2019).

Beside the mechanisms including regeneration of pancreatic B cells, regulation of insulin secretion, and delaying glucose absorption, the antioxidant activity of plant extracts and natural products is also contributed to antidiabetic effect by alleviating the oxidative stress damages induced by diabetes (Kang et al. 2020). In a study on the antioxidant activity of 24 Lamiaceae species growing in Iran, the methanol extract of *M. astracanicum* was found to have considerable antioxidant activity in DPPH free radical scavenging (IC_{50} 618.3 ± 54.7 μg/ml) and ferric-reducing antioxidant power (FRAP value 16.4 ± 1.5 quercetin equivalent/g extract) assays (Firuzi et al. 2010). Moreover, apigenin-7-O-(6′-(E)-p-coumaroyl)-β-D-glucoside (**1**), the most potent α-glucosidase inhibitor isolated from *M. astracanicum* in the present study, has also been reported to possess a potent antioxidant activity with IC_{50} value of 7.69×10^{-4} mg/ml in DPPH free radical scavenging assay (Delazar et al. 2005).

Conclusions

The bioassay-guided isolation of the constituents of *M. astracanicum* aerial parts resulted in isolation of five phenolic glycosides (**1**–**5**) from its ethyl acetate extract, among which apigenin-7-O-(3″-(E)-p-coumaroyl)-β-D-glucoside (**1**) and apigenin-7-O-(6″-(E)-p-coumaroyl)-β-D-glucoside (**2**) demonstrated potent inhibition toward α-glucosidase. These coumaroyl flavone glycosides could be considered appropriate natural candidates in antidiabetic drug development research due to their potential as α-glucosidase inhibitors in controlling postprandial blood glucose levels in diabetic patients. The present study also highlights the beneficial potentials of *Marrubium astracanicum* as a helpful herbal supplement for diabetic patients. However, further pharmacological and toxicological studies are needed to establish its efficacy and safety in management of diabetes mellitus type II.

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