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# An investigation on the enzyme inhibitory activities, phenolic profile and antioxidant potentials of *Salvia virgata* Jacq

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ABSTRACT

The genus *Salvia* is regarded as an important medicinal plant and also as spice worldwide. Current report might be counted as one of the first investigations to reveal the enzyme inhibitory and antiglycation activities, together with phenolic and antioxidant profiles of 80% methanolic extract prepared from the aerial parts of *S.virgata* (SVM). Furthermore, the bioavailability parameters of major phenolic compounds in SVM were evaluated. An *in vitro* human digestion simulation model composed of both gastric and intestinal phases was used for this purpose. Total phenolic, phenolic acid and flavonoid components of each of these phases were calculated in order to assess the phenolic profile of the samples. In addition, quantitative analysis of major bioactive compounds (rutin and rosmarinic acid), before and after the simulation, was practiced by employing High Performance Thin Layer Chromatography (HPTLC) system. Moreover, the potential of non-digested (ND) and bioavailable (IN) phases on the inhibition of diabetes-related enzymes ($\alpha$-amylase and $\alpha$-glucosidase) was determined. Also, the inhibitory effect of the samples on the advanced glycation end products (AGEs), formed due to hyperglycemia, was detected. In addition, cholinesterase enzyme inhibition activity of both ND and IN samples was determined. Since, the role of oxidative stress on diabetes and neurodegenerative diseases is well-known, the antioxidant potential of the samples was also estimated by different methods: DPPH and DMPD tests for free radical scavenging activity, FRAP and CUPRAC tests for metal reducing activity and total antioxidant capacity test, as well. The results showed that total phenolic contents, antioxidant potential and diabetes-related and cholinesterase enzymes inhibition of the bioavailable phase were lower than non-digested sample. Besides, *in vitro* human digestion simulation system had a declining influence on the concentrations of the major bioactive metabolites (rutin and rosmarinic acid).

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## 1. Introduction

The genus *Salvia,* commonly known as “sage”, consisting of more than 1000 species, is one of the most widely distributed members of the Lamiaceae family (Bahadori et al., 2015). Sages have been utilized from ancient times to present owing to their importance in cosmeceutical, and food industries, as well as its medicinal usage. Numerous studies revealed that *Salvia* species have antioxidant, antidiarrheal, cardio-protective, anti-inflammatory, antidiabetic, cytotoxic, antifungal and antimicrobial activity (Aghaei Jeshvaghani et al., 2015; Bahadori et al., 2017; Kalaycıoğlu et al., 2018). A wide variety of phenolic compounds such as flavonoids and phenolic acids, and also terpenoid compounds (di-and triterpenoids) have been isolated from genus *Salvia*. These various phenolic compounds found in *Salvia* species are generally considered to be responsible for the acclaimed therapeutic effects (Wang et al., 2013).

100 species of this genus grow naturally in Turkish flora, with an endemism ratio of 57%. *Salvia virgata* Jacq locally known as “fatmanaotu” or “yılancık” in Turkey, is a herbaceous and perennial plant which has approximately 30–100 cm height and an extremely branched structure (Akkol et al., 2008). *S.virgata* has been employed in folk medicine since ancient times. In Turkish folk medicine, the leaves of *S. virgata* are used in the treatment of wounds and skin diseases. Additionally, decoction obtained from aerial parts used in treatment of leukemia in Western Turkey (Coşge Şenkal et al., 2019).

As illustrated by countless studies, reactive oxygen species participate in the etiology of various chronic and metabolic disorders such as cancer, heart-related diseases, diabetes and inflammation by damaging proteins, lipids and DNA in cellular level. Accordingly, the attraction towards antioxidant molecules has a tendency to ramp up in recent years. Such compounds are acknowledged to diminish oxidation or decelerate damage to cells caused by free radicals

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(Asadi et al., 2010). There is mounting evidence documenting that extracts from *Salvia* genus show strong antioxidant activity due to the occurrence of polyphenols, (chiefly phenolic acids and flavonoids) (Farhat et al., 2013). According to the several phytochemical studies, rosmarinic acid is deemed to be the predominant phenolic compound found in the *Salvia* species (Adımcılar et al., 2019). In spite of various *in vitro* studies demonstrating the antioxidant effects of these phenolic compounds, there is few published data taking the bioavailability factor of these substances into consideration. However, activity of enzymes and pH variations along the gastrointestinal tract can affect the chemical structure and bioavailable content of phenolic compounds (Barak et al., 2019). Thus, total phenolic, flavonoid and phenolic acid contents, potential antioxidant capacity, metal reducing and radical scavenging activities of 80% methanol extract from *S.virgata* (SVM) were calculated by means of various assays before and after *in vitro* human digestion simulation method.

As stated earlier, oxidative stress might lead to increase in the prevalence of diabetes. Diabetes mellitus (DM) is a chronic, metabolic and endocrinal disorder, manifesting itself by increased blood glucose levels. The management of hyperglycemia is crucial to control DM which provokes several disorders such as retinopathy, neuropathy, nephropathy and cardiovascular diseases. DM has two main forms: Type I (insulin-dependent) and Type II (non-insulin dependent). One of the major goals of DM treatment, especially for Type 2 DM, is to decrease postprandial hyperglycemia (increased blood glucose levels after a meal (Celep et al., 2019). Postprandial hyperglycemia can be suppressed by reducing the digestion of carbohydrates, which are hydrolyzed into monosaccharides (such as fructose and glucose) by the help of digestion enzymes such as $\alpha$-amylase and $\alpha$-glucosidase. Thus, the molecules that inhibit these digestive enzymes can be beneficial to manage Type 2 DM (Nickavar and Abolhasani, 2013). Various previous studies showed that rutin and rosmarinic acid (major phenolics in this study) could be used in managing Type 2 DM due to strong digestive enzyme inhibition action (Oboh et al., 2015; Taslimi and Gulçin, 2017). Therefore, inhibition potential of SVM on $\alpha$-amylase and $\alpha$-glucosidase enzyme activity was determined. In addition, high blood glucose level is ascribed to the formation of advanced glycation end products (AGEs), irreversible heterogenic compounds formed by the non-enzymatic binding of glucose to free amino groups of an amino acid. AGEs contribute to the pathogenesis of various diabetic complications such as atherosclerosis, diabetic retinopathy, nephropathy and neuropathy, heart and kidney dysfunction, etc. (Khangholi et al., 2015). Thus, inhibition of the accumulation of AGEs is considered as one of the therapeutic approach for patients. Abbas et al. (2016) declared that various phenolic compounds such as flavonoids, phenolic acids, anthocyanins found in plants have a strong inhibition effect on the formation of AGEs. To our knowledge, although AGE inhibition studies have been conducted on some *Salvia* species (Ma et al., 2011; Safari et al., 2018), this is the first publication to declare the inhibition potential of *S.virgata* on AGEs. Besides, increased oxidative stress is also one of the most important causes of neurodegenerative diseases such as Alzheimer, Parkinson, dementia etc. (Subash et al., 2014). Acetylcholinesterase and butyrylcholinesterase are two cholinesterase enzymes that contribute to cholinergic neurotransmission by hydrolyzing acetylcholine in the central and peripheral nervous system. Therefore, cholinesterase enzyme inhibitors are broadly used in the treatment of neurodegenerative diseases (Şenol et al., 2010). Various studies showed that natural polyphenols found in the plants have an inhibitory activity of these cholinesterase enzymes (Ademosun et al., 2016). Thereby, cholinesterase inhibition activity of SVM was investigated in this study.

In this respect, the antioxidant potential, diabetes related and cholinesterase enzyme inhibition activity and inhibition potential of AGEs, together with quantification of the major phenolic compounds from the aerial parts of *S.virgata* were investigated. An *in vitro* human digestion simulation was employed in order to evaluate *in vitro* bioavailability of these phenolic compounds and its effect on the aforementioned activities. Additionally, quantitative analysis of major phenolic compounds (rutin and rosmarinic acid) were performed by HPTLC.

## 2. Material and methods

### 2.1. Chemicals

All enzymes, chemicals and references employed in the tests were afforded from Sigma Chemical Co. (St. Louis, MO, USA). The quality of all chemicals was of analytical grade.

### 2.2. Plant material

The aerial parts of *S.virgata* were collected from Kayışdağı, İstanbul, Turkey in April 2017. The botanically identification procedure of the plant samples was carried out by Prof. Dr. Erdem Yesilada. A voucher sample of the plant was kept in the Yeditepe University Faculty of Pharmacy Herbarium.

#### 2.2.1. Preparation of extract

80 g of dried and powdered aerial parts was macerated overnight with 800 mL of 80% methanol by using shaking device. After filtrating the macerates, methanol was evaporated, and the remaining residue was lyophilized (the yield was 10.98 g, 13.72%). After the lyophilization process, extract (SVM) was dissolved in water for the further tests (non- digested sample: ND).

### 2.3. In vitro human gastrointestinal digestion simulation system

*In vitro* human gastrointestinal digestion simulation system was applied as reported before (Celep et al., 2015). 2.5 mL of samples solution was mixed with certain volume of gastric environment (pH:2) which was created by pepsin enzyme and proper electrolytes. Then, peristaltic motility of the gastrointestinal system was mimicked by shaking water bath (2 h, 37, 5 °C). Afterwards, the mixture was placed into ice-bath to stop enzymatic action. 2 ml of sample was put aside as “post-gastric (PG)”. Then, the cellulosic membrane was filled with adequate amount of $NaHCO_3$ to obtain neutral environment (pH 7). Bile acids and pancreatin were introduced to the mixture and left for incubation for 2 h. After the incubation period ends, the inside content of the cellulosic tube was expressed as the “serum available” sample (IN). The collected samples were stocked at -20 °C.

### 2.4. Spectrophotometric determination of phenolic content

#### 2.4.1. Total phenolic content

The amount of total phenolic content of the samples were calculated according to Folin-Ciocalteu method which described earlier by Celep et al. (2012). Briefly, 20 $\mu$L of freshly diluted sample solution was mixed with 75 $\mu$L $Na_2CO_3$ (20%) and 100 $\mu$L of 1:10 (v/v) Folin-Ciocalteu reagent, respectively. After 30 min. incubation period at room temperature, absorbance was read spectrophotometrically at 690 nm. Gallic acid was used as the reference compound for the experiment and results were given as mg gallic acid equivalents (GAE) in 1 g dry extract.

#### 2.4.2. Total flavonoid content

Aluminium chloride method was performed for the determination of total flavonoid content of the samples described earlier by Celep et al. (2017). 150 $\mu$L 75% EtOH was added to freshly diluted sample solution. Then, 10 $\mu$L 10% $AlCl_3$ and 10 $\mu$L 1 M sodium acetate were added to the mixture. Afterwards, the mixture was incubated

for 30 min at room temperature and absorbance was measured at 415 nm. Quercetin was used as the reference compound for the experiment and results were calculated as mg quercetin equivalents (QE) in 1 g dry extract.

#### 2.4.3. Total phenolic acid content

Spectrophotometric calculation of the total phenolic acid content of the sample solutions were performed at 490 nm. A complex mixture was formed between phenolic acids and sodium nitrite – sodium molybdate mixture (Bardakci et al., 2019). Caffeic acid was employed as the reference compound for the experiment and results were estimated as mg caffeic acid equivalents (CAE) in 1 g dry extract.

### 2.5. Quantification procedure of major phenolic compounds by HPTLC

The quantitative and qualitative measurement of rutin and rosmarinic acid in three different samples (ND, PG, IN) were performed by high-performance thin layer chromatography (HPTLC) system (CAMAG, Muttenz, Switzerland) according to the validated method desrcibed earlier by Marrelli et al. (2014).

The standard solutions of rosmarinic acid and rutin (100 $\mu$g/mL) was prepared in MeOH and the concentration of sample solutions (ND, PG, IN) was arranged to 10 mg/ml. Both sample and standard solutions were filtered through a 0.45 $\mu$m syringe filter, before the application process. 1–6 $\mu$L standard solutions and 1-3 $\mu$L sample solutions were applied to normal phase glass-backed silica gel plates (20 cm x10 cm, Merck, Darmstadt, Germany) by using 100 $\mu$L syringes (Hamilton, Bonaduz, Switzerland). Linomat 5 sample applicator was used for the application procedure. The mobile phase was ethyl acetate: dichloromethane: acetic acid: formic acid: water (100: 25: 10: 10: 11; by volume) for the development process which is conducted by Automated Development Chamber (ADC 2). After development, HPTLC plates were dried using CAMAG TLC Plate Heater III at for 3 min at 100 °C. Natural Product Reagent (NPR) (1g diphenylboric acid 2-aminoethylester in 200 ml of ethyl acetate) was used for the derivatization process of the dried and heated plates by using Immersion Device. Analysis of derivatized plates were performed under UV light (366 nm) at TLC Scanner and TLC Visualizer for the quantitative and qualitative determination of major phenolics in the samples. The all HPTLC devices were connected to winCATS software. The correlation coefficients ($r^2$) were obtained above 0.99. The retention factors (Rf) and UV spectra of samples and standards were examined to prove the presence of standards in the samples. The calibration curve was created by winCATS software based on the linearity between the standard concentrations and peak areas.

### 2.6. Estimation of bioavailability index of major phenolic compounds

The bioavailability index (BAvI) of rutin and rosmarinic acid were calculated employing the theoretical equation reported by Barak et al. (2019). According to this equation:

$$BAvI = C_{IN}/C_{ND}$$

Mathematical ratio of amount of phenolics in the absorbed phase (IN) to the phenolic content in non-digested phase (ND) was defined as the “bioavailability index (BAvI)”.

### 2.7. Determination of free radical-scavenging activity

#### 2.7.1. DPPH radical-scavenging activity test

Freshly diluted sample solutions were combined with methanolic DPPH solution (100 $\mu$M) in order to determine DPPH (2.2-diphenyl-1-picrylhydrazyl) radical-scavenging activity. After the incubation interval at room temperature for 45 min, the absorbance was calculated at 517 nm. Calibration curve was obtained by using butylated hydroxyl toluene (BHT) as the reference compound. $EC_{50}$ values of results were stated as mg/mL (Celep et al., 2013).

#### 2.7.2. DMPD radical-scavenging activity test

DMPD+ (N,N-dimethyl-p-phenylendiamine) radical-scavenging activity test was determined according to method reported earlier by Kurt-Celep et al. (2020). 100 mM acetate buffer (pH: 5.25) and 50 mM ferric chloride were combined with 100 mM DMPD solution to obtain DMPD+ reagent. The absorbance of DMPD solution at 505 nm must be 0.900 ± 0.100 in order to show uninhibited signal. Freshly prepared DMPD solution was combined with sample solutions. After the 10 min incubation period, absorbance was measured at 505 nm. Trolox was used to obtain a standard curve and the results were expressed as mg trolox equivalents in 1 g dry extract.

### 2.8. Determination of metal-reducing activity

#### 2.8.1. Ferric reducing antioxidant power (FRAP) test

25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), and 2.5 mL of $FeCl_3.6H_20$ (20 mM) were mixed to obtain FRAP reagent. Afterwards, 10 $\mu$L of samples were added to 260 $\mu$L of FRAP reagent, and diluted to 300 $\mu$L with distilled water in a 96 well-plate. After 30 min incubation period at 37°C, the measurement of absorbance was performed at 593 nm. BHT was employed as a reference compound. Ferrous chloride solution (0.25–2 mM) was used to develop a standard curve and the results were given as mM FeSO4 in 1 g dry extract (Alizadeh, 2013) .

#### 2.8.2. Cupric reducing antioxidant capacity (CUPRAC) test

CUPRAC activity test was estimated according to the method described before by Apak et al.,(2004). Equal volumes of 10 mM $CuSO_4$, neocupraine and ammonium acetate buffer (85 $\mu$L) were mixed in a 96-well plate. After that, 51 $\mu$L distilled water and 43 $\mu$L of sample solutions were added to the mixture respectively. After the incubation period of 20 min, the absorbance was calculated at 450 nm. The results were stated as mg ascorbic acid equivalent in 1 g dry extract.

### 2.9. Determination of total antioxidant capacity test

The total antioxidant capacity test was calculated according to the phosphomolybdenum method explained earlier by Barak et al. (2020). Firstly, 28 mM sodium phosphate monobasic, 4 mM ammonium molybdate and 600 mM $H_2SO_4$ were mixed in order to obtain TOAC solution. Then, 300 $\mu$L of TOAC solution were mixed with 30 $\mu$L of sample solutions in a 96-well plate. After the incubation period at 95 °C for 90 min, the absorbance was read at 695 nm. Ascorbic acid was used to obtain a standard curve and the results were calculated as mg Trolox equivalents in 1 g dry extract.

### 2.10. Inhibitory activity on diabetes-related enzymes

#### 2.10.1. $\alpha$-amylase inhibition activity

$\alpha$- amylase inhibition potential was determined colorimetrically by employing 3,5-dinitrosalicylic acid (DNS) reagent. According to the method, starch is converted into maltose by $\alpha$ –amylase. Maltose released from starch is measured by the reduction of 3,5-dinitrosalicylic acid. Yellow color of alkaline 3, 5-dinitro salicylic acid (DNS) is reduced to the orange-red color by formed maltose from starch. The intensity of the color is proportional to the concentration of maltose present in the sample (Balan et al., 2017). Results were calculated as percentage of inhibitory activity of ND and IN samples in 1000 $\mu$g/mL and 500 $\mu$g/mL concentration. 200 $\mu$g/mL, 500 $\mu$g/mL, 250 $\mu$g/mL, 125 $\mu$g/mL, 62.5 $\mu$g/mL concentrations of acarbose was used as positive control to obtain a standard curve.

*2.10.2. α-glucosidase inhibition activity*

α-glucosidase inhibition activity was calculated by method described earlier by Celep et al. (2019). 100 mM phosphate buffer was prepared by mixing monosodium phosphate and disodium phosphate. 20 μL of samples was diluted with 170 μL of 100 mM potassium phosphate buffer (pH: 7.0) containing α-glucosidase solution (0.2 U/ml) was incubated in 96-well plates at 37 °C for 15 min. After pre incubation, 20 μL of 2.5 mM p-nitrophenyl-α-D-glucopyranoside solution in 100 mM potassium phosphate buffer (pH 7.0) and 80 μL 0.2 M sodium carbonate was added to mixture. After incubation period for 15 min at 37 °C for, the absorbance was determined at 405 nm. Results were calculated as percentage of inhibitory activity in 1000 μg/mL and 500 μg/mL concentrations of ND and IN samples. 200 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL concentrations of quercetin was used as positive control to obtain a standard curve.

*2.10.3. Inhibition activity of advanced glycation end products (AGEs) with a BSA-glucose method*

AGEs inhibition activity of ND and IN samples at different concentrations was measured according to the method described earlier by Starowicz and Zieliński (2019). In the AGEs procedure, freshly prepared 0.5 mg/mL, and 1 mg/mL concentrations of ND and IN samples were used. 1 mL of 10 mg/ mL concentration of bovine serum albumin (BSA) (prepared with phosphate buffer (0.1 M, pH 7.4)) and 1 mL of 0.5 M glucose (in 0.1 M pH 7.4 phosphate buffer) were added to 1 mL of ND and IN sample solutions of different concentrations. Blank samples were prepared without adding 0.5 M glucose solution and the control samples were prepared without adding ND and IN samples. Different concentrations (1000–62.5 μg/mL) of quercetin were used as reference material. After the experimental groups were prepared, they were incubated for 40 h by shaking at 100 rpm in a 55 °C-degree water bath. Following the end of the incubation period, fluorescence intensity was measured in the Thermo Scientific™ Varioskan™ LUX multimode microplate reader in the 370 nm excitation / 440 nm emission range.

*2.11. Inhibitory activity on acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) enzymes)*

AChE and BChE inhibitory activities of the sample solutions were studied by scarcely modified Ellman's spectrophotometry based method (Ellman et al., 1961). AChE (Type-VI-S; EC 3.1.1.7, Sigma, St. Louis, MO, USA) and Horse serum BChE (EC 3.1.1.8, Sigma, St. Louis, MO, USA) were used as enzymes. Acetylthiocholine iodide (AChI) and Butyrylthiocholine chloride (BChC) were used as the substrate of enzymes. Adequate amounts of phosphate buffer, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) solution, enzyme and test substance were mixed in 96 well-plate and kept in 25 °C for 15 minutes for incubation period. Then, substrates were added to mixture and results were immediately measured at 412 nm via well-plate reader. Results were calculated as percentage of inhibitory activity of ND and IN samples in 1000 μg/mL and 500 μg/mL concentration. 1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL concentrations of galanthamine was used as positive control to obtain a standard curve for both assays.

*2.12. Statistics*

All experiments and tests were performed in triplicate. The 6.1 version of the GraphPad Prism software program was used for all data, and after determining the parametric or non-parametric distribution of the data, the data showing the parametric distribution were compared with each other. 1 way ANOVA Tukey's Multiple comparisons analysis section was used for the results of TPC, TFC, Total Phenolic Acid, FRAP, CUPRAC, TOAC, DPPH, DMPD experiments. 2 way ANOVA Sidak's Multiple comparisons analysis section was used for the results of α-amylase, α-glucosidase, and inhibition activity of AGE. $p < 0.05$ was found as statistically significant difference for all tests

## 3. Results

*3.1. Determination of the phenolic profile of the samples*

As shown in Table 1, total phenolic, phenolic acid and flavonoid contents of the non-digested, post-gastric and serum-available samples were calculated. The results indicated that all of total amount of phenolic compounds were decreased after human digestion simulation system. Most dramatic decrease was seen in total phenolic content from ND sample (125.11 ± 5.44 mg/g GAE) to IN sample (68.71 ± 6.43 mg/g GAE).

The HPTLC plate and chromatograms were presented to exhibit the presence of major phenolic compounds (rutin and rosmarinic acid) in Figs. 1 and 2, respectively. Quantitative analysis of rutin and rosmarinic acid was shown in Table 2.

*3.2. Determination of antioxidant potential of the samples*

Mechanistically different assays were implemented in order to estimate the antioxidant capacities of the samples. DPPH and DMPD assays were performed in order to evaluate free radical scavenging activity. As showed in Table 3, no meaningful differences were seen between ND and PG samples in terms of both DPPH and DMPD experiments. However, significant decline was detected in IN fraction for free radical scavenging activity. Same descending trend in IN fraction was also observed in metal reducing capability assays (CUPRAC and FRAP) and total antioxidant capacity test.

*3.3. Diabetes-related enzyme inhibition activity*

α-amylase and α-glucosidase inhibition activity of ND and IN samples of SVM were showed in Table 4. Results showed that both ND and IN samples had a strong α-amylase and α-glucosidase inhibition activity. ND sample had a better activity than IN sample in terms of percentage inhibition of α-amylase and α-glucosidase in both 1 mg/mL and 0,5 mg/mL concentrations. 1 mg/mL concentration of ND sample (81.2%) showed higher α-amylase inhibition effect than 1 mg/mL acarbose (77.7%). In addition, 1 mg/mL ND sample (70.2%) had higher α-glucosidase inhibition activity than 1 mg/mL quercetin (63.7%). Moreover, the α-amylase and α-glucosidase percentage

**Table 1**
Spectrophotometric determination of phenolic profile of SVM* samples and their bioavailability indices.

| *Samples* | *ND*[A] | *PG* | *IN* | *BAvI (%)* |
|---|---|---|---|---|
| Total phenolic content[B] | $125.11^{a} \pm 5.44$ | $94.02^{b} \pm 2.25$ | $68.71^{d} \pm 6.43$ | 54.91% |
| Total phenolic acid content[C] | $273.7^{a} \pm 0.36$ | $245,6^{b} \pm 1.28$ | $186,11^{b} \pm 0.08$ | 67.99% |
| Total flavonoid content[D] | $28.03^{a} \pm 0.44$ | $26.29^{b} \pm 1.39$ | $20.62^{bc} \pm 1.03$ | 73.56% |

[A] The abbreviations for samples are *ND: non-digested, PG: postgastric, IN: bioavailable, BAvI: Bioavailability index*
[B] Results were stated as the mean of triplicates ± standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.
[C] Results were stated as the mean of triplicates ± standard deviation (S.D.) and as mg caffeic acid equivalents (CAE) in 1 g sample.
[D] Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as mg quercetin equivalents (QE) in 1 g sample.
* SVM: 80 % methanolic extract of *Salvia virgata*.
**Different letters in the same row indicate significance ($p < 0.05$).

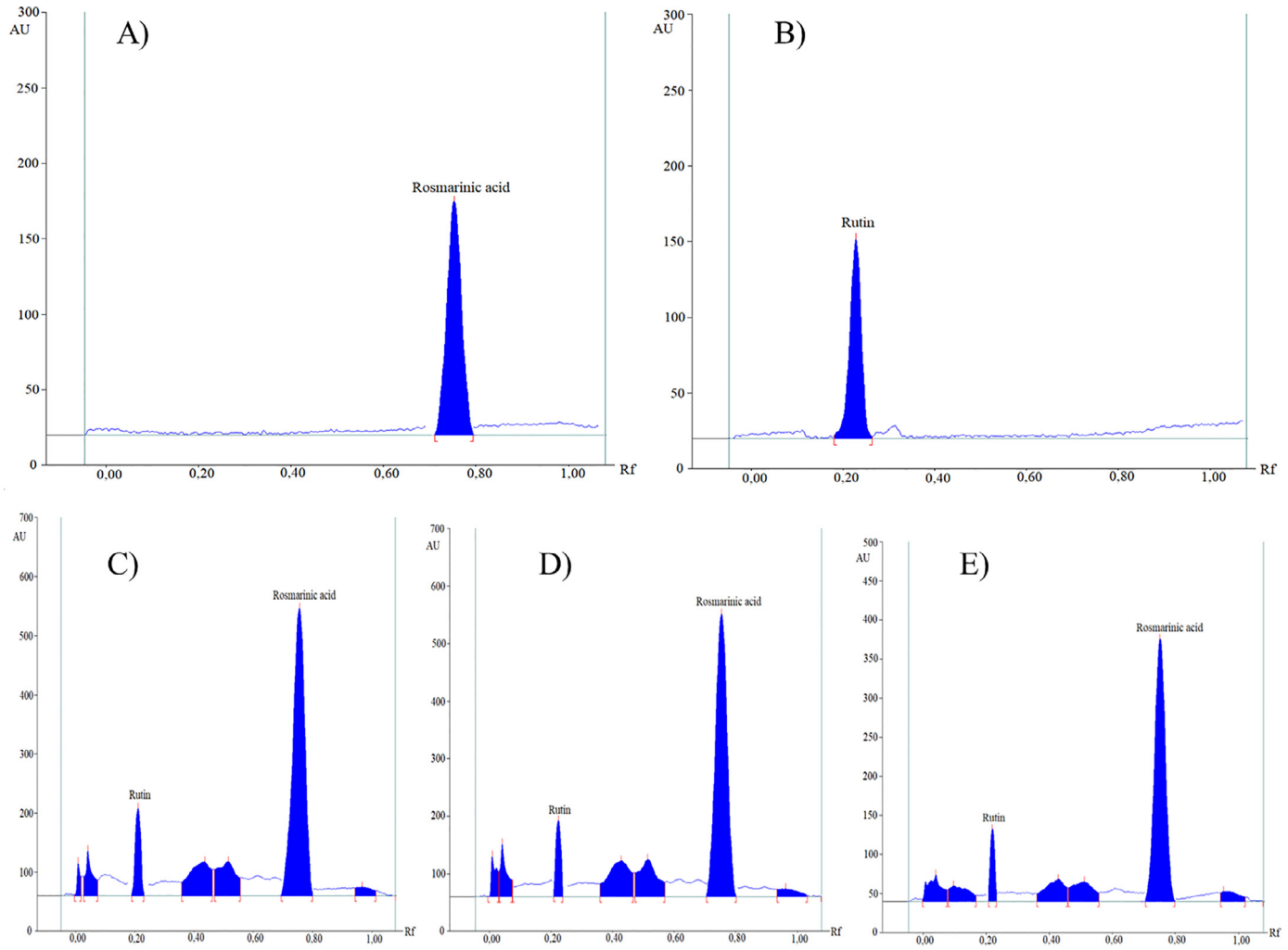


**Fig. 1.** Spectra of A) Rosmarinic acid, B) Rutin, C) SVM ND, D) SVM PG, E) SVM IN at 366 nm. Mobile phase: EtOAc/ $CHCl_2$/ $CH_3COOH$/ HCOOOH/ $H_2O$ (100:25:10:10:11); Derivatization: NPR reagent.

inhibition activity of ND and IN samples appeared to increase from 0.5 mg/mL to 1 mg/mL concentration.

### 3.4. Inhibition potential of AGE

AGE inhibition activity of ND and IN samples of SVM was demonstrated in Table 5. Results showed that both ND and IN samples had a strong inhibition activity of AGE. ND sample had a better activity than IN sample in terms of percentage inhibition of AGE in both 1 mg/mL and 0.5 mg/mL concentrations. For both concentrations, inhibition potentials of AGE were decreased significantly after digestion (for 1 mg/mL: 86.2 ± 5.85 to 48.26 ± 2.38, for 0.5 mg/mL: 55.5 ± 2.22 to 39.46 ± 3.92). Reference substance quercetin showed potent inhibition activity of both 1 mg/mL and 0.5 mg/mL concentrations when compared to samples of SVM.

### 3.5. Cholinesterase enzymes inhibition activity

AChE and BChE inhibition activity of ND and IN samples of SVM were demonstrated in Table 4. Results showed that both ND and IN samples had a moderate to low cholinesterase inhibition activity. ND sample had a better activity than IN sample in terms of percentage inhibition of AChE and BChE in both 1 mg/mL and 0.5 mg/mL concentrations. For both concentrations, both AChE and BChE inhibition potentials were dropped significantly after digestion (For 1 mg/ml: 16.45 ± 0.93 to 12.29 ± 0.48, 25.98 ± 1.02 to 15.26 ± 0.49, respectively). Reference substance galanthamine showed potent inhibition activity when compared to fractions of SVM.

## 4. Discussion

A growing body of evidence reveals that oxidative stress prompts the formation of a set of severe chronic disorders, including cancer, Alzheimer's, diabetes etc. Despite the presence of both enzymatic and non-enzymatic protective systems in our body, this antioxidant defense system may display weakness due to factors such as smoking, alcohol usage, regular drug usage, stress, etc. Thus, the importance of secondary plant metabolites in today's scientific approach has a tendency to rise. Various studies demonstrated that phenolic substances including flavonoids, phenolic acids, anthocyanidins, etc. are widely distributed secondary metabolites in plants and beneficial constituents to prevent oxidative stress and its harmful consequences (Ting et al., 2015). Yet, an overwhelming majority of these studies neglected the bioavailability of such compounds. Bioactive metabolites in plants are likely to go through significant structural changes in gastrointestinal system resulting from various factors such as enzyme activity, pH value, body temperature, etc. It is a fact that metabolites should be absorbable into blood circulation in order to display pharmacological effect (Barak et al., 2019). Thus, *in vitro* human digestion simulation systems are broadly used in order to assess the bioavailability concept. Obviously, clinical and animal trials should be practiced to acquire more precise results. Nevertheless,

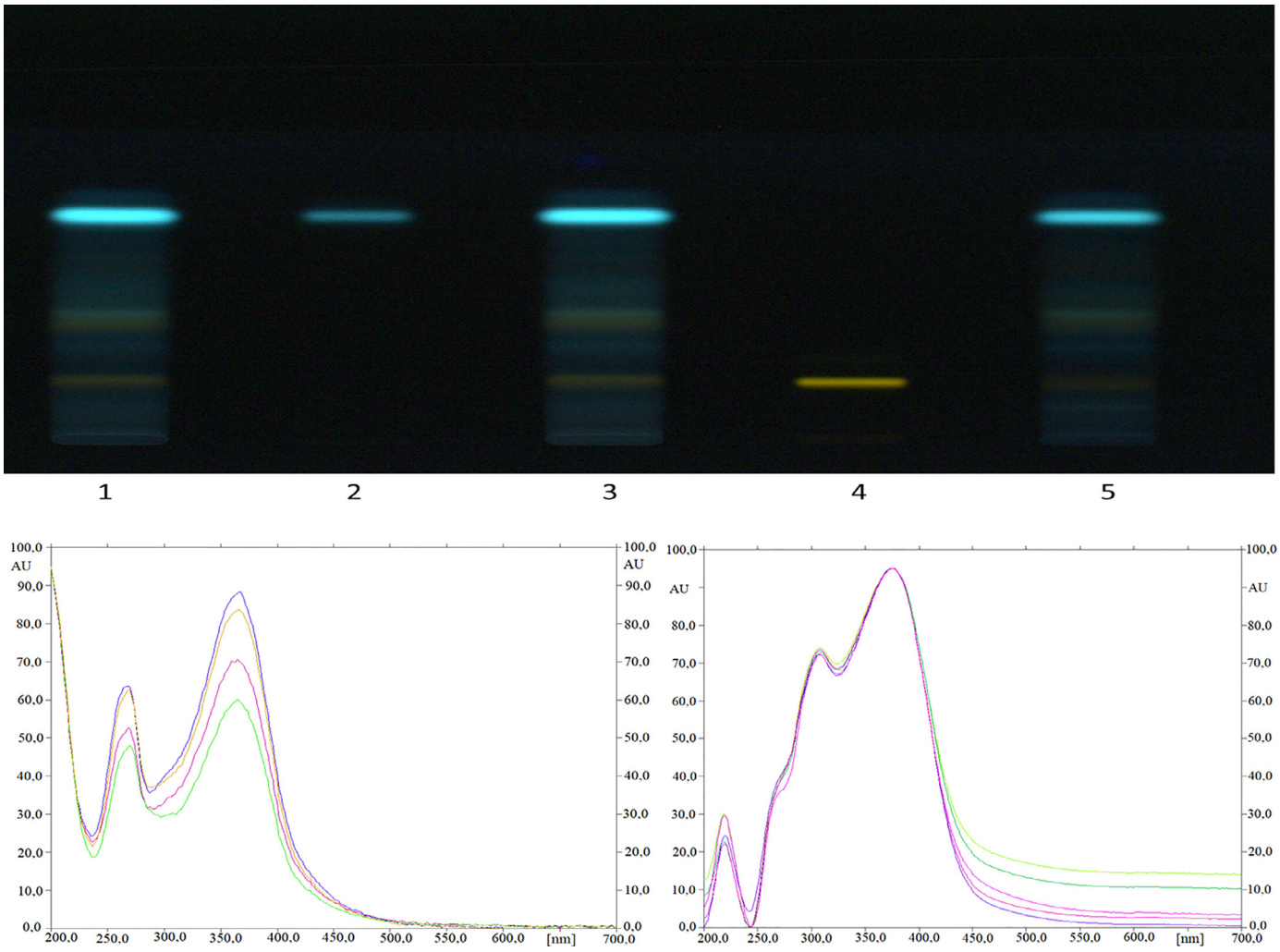


**Fig. 2.** A) HPTLC chromatograms of: 1.SVM ND, 2.Rosmarinic acid 3.SVM PG, 4.Rutin 5. SVM IN Mobile phase: EtOAc/ $CHCl_2$/ $CH_3COOH$/ HCOOOH/ $H_2O$ (100:25:10:10:11); Derivatization: NPR reagent. Visualization: 366 nm. B) UV spectra of rutin and same Rf value of tracks at 366 nm. C) UV spectra of chlorogenic acid and same Rf value of tracks at 366 nm.

such studies may have some restrictions such as ethical concerns, expensiveness, excessive amount of time required. Since *in vitro* studies provide reproducible results without raising much of the mentioned concerns, they are widely touted as a substitute to *in vivo* models (Alminger et al., 2014).

Several research groups have delved into the phenolic content, *in vitro* antioxidant potential and anti-diabetic activity of various extracts prepared from aerial parts of *S.virgata* (Akkol et al., 2008; Kalaycıoğlu et al., 2018; Koşar et al., 2011; Tepe et al., 2007). To best our knowledge, this paper is the first study concentrating on the bioavailability of major phenolic substances applying an *in vitro* digestion simulation model together with its influence on the antioxidant potential. The amount of total phenolic, flavonoid and phenolic acid contents of 80% methanolic extract of *S.virgata* aerial parts were demonstrated in Table 1. However, total phenolic acid content of *S.virgata* has been detected for the first time according to our knowledge. Table 1 presented that serum-available (IN) fraction has the lowest total content in the all assays. As shown in Table 2, the decrease in the amount of rutin and rosmarinic acid in the IN fraction may have caused a decrease in total content measurements. In addition, the decline in the concentrations of phenolic compounds may be ascribed to the basic medium in the intestinal phase. Also, the descending trend in total content assays can be reasoned by pH alterations and the considerable enzyme activity leading to the biotransformation of the phenolics. Besides, in a previous study of ours, opposite results to the present study were obtained, revealing that the results of total content assays are unique to the investigated sample since the plant matrix may vary from between species (Celep et al., 2017).

**Table 2**
Quantification of the major bioactive compounds by HPTLC and their bioavailability indices.[B]

| *References* | *ND*[A] | *PG* | *IN* | *BAvI (%)* |
|---|---|---|---|---|
| Rutin | $9.58^{b} \pm 0.74$ | $9{,}01^{b} \pm 0.62$ | $2.51 \pm 0.43$ | 26.2% |
| Rosmarinic acid | $37.93^{b} \pm 1{,}42$ | $35.26^{b} \pm 1{,}71$ | $18.05 \pm 0.83$ | 47.58% |

[A] The abbreviations for samples are *ND: non-digested, PG: postgastric, IN: bioavailable, BAvI: Bioavailability index*

[B] Results are expressed as mg/g dry extract with standard deviations.

*Different letters in the same row indicate significance ($p < 0.05$).

As mentioned earlier, in accordance with the previous reports, rosmarinic acid and rutin were detected as the major metabolites found in the current study. Potential oral bioavailability of these phenolic metabolites was measured individually. Rosmarinic acid (an ester of 3,4-dihydroxyphenyllactic acid and caffeic acid) is one of the most abundant phenolic compounds present in the Lamiaceae family plants. According to the reports of Koşar et al. (2011) and Akkol et al. (2008), rosmarinic acid was determined as the major metabolite in the aerial parts of *S.virgata* collected from Turkish flora. As seen in Table 2, the concentration of rosmarinic acid diminished slightly in

**Table 3**
*In vitro* antioxidant activity of SVM* samples before and after simulated human digestion.

| *Name of the analysis* | *ND*[A] | *PG* | *IN* |
|---|---|---|---|
| DPPH[B,#] | $0.57^{a} \pm 0.02$ | $0.58^{b} \pm 0.05$ | $0.79^{a} \pm 0.1$ |
| DMPD[C,§] | $178.15^{a} \pm 1.11$ | $174.4^{ab} \pm 4.17$ | $135.78^{c} \pm 2.88$ |
| FRAP activity[D,##] | $1.43^{a} \pm 0.18$ | $1.32^{b} \pm 0.09$ | $1.08^{c} \pm 0.12$ |
| CUPRAC activity[E,§§] | $416.89^{a} \pm 6.99$ | $356.73^{b} \pm 6.82$ | $256.79^{d} \pm 2.51$ |
| Total antioxidant capacity[E] | $51.13^{a} \pm 7.37$ | $32.77^{b} \pm 2.44$ | $25.18^{b} \pm 0.72$ |

P.S. 1) EC50 value of the reference compound "butylated hydroxytoluene (BHT)" in DPPH scavenging activity is found to be 0.67 ± 0.04.
2) FRAP activity of the reference compound "butylated hydroxytoluene (BHT)" is found to be 4.24 ± 0.48mM FeSO4 eq.in 1 g sample.
[A] The abbreviations for samples are *ND: non-digested, PG: post-gastric, IN: bioavailable*
[B] Results were stated as the mean of triplicates ± standard deviation (S.D.) and DPPH activity was expressed as EC50 in $\mu$g/mL equivalents.
[C] Results were stated as the mean of triplicates ± standard deviation (S.D.) and as mg Trolox equivalents (TE) in 1 g sample.
[D] Results were stated as the mean of triplicates ± standard deviation (S.D.) and as Mm $FeSO_4$ equivalents in 1 g sample.
[E] Results were stated as the mean of triplicates ± standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.
* 80% methanolic extract of *Salvia virgata*.
**Different letters in the same row indicate significance ($p < 0.05$).
[#] 2,2-diphenyl-1-picrylhydrazyl.
[§] N,N-dimethyl-p-phenylendiamine.
[##] Ferric reducing antioxidant power.
[§§] Cupric reducing antioxidant capacity.
P.S FRAP activity of the reference compound "butylated hydroxytoluene (BHT)" is found to be 4.18 ± 0.26 mM $FeSO_4$ eq. in 1 g sample.

gastric conditions. Also, the same trend was observed in the studies of Celep et al. (2018) and Costa et al. (2014). Although, some researchers detected significant declines in rosmarinic acid contents under gastric conditions (Giao et al., 2012; Zorić et al., 2016), it was observed to be quite stable in the present study. Besides, a dramatic decrease of rosmarinic acid content was seen in serum available (IN) fraction, parallel to many previous studies (Celep et al., 2018; Gayoso et al., 2016; Gonçalves et al., 2019; Pellegrini et al., 2018). This phenomenon might arise due to various conditions. While, Gayoso et al. (2016) claimed that phenolic compounds undergo degradation or transformation into other compounds because of the alkaline conditions in the large intestine, Celep et al. (2018) emphasized the possible interactions among the intercellular substances. Descending level of rosmarinic acid may be linked to many factors arisen during the simulative digestion method including plant matrix, enzyme variation, pH differences, osmotic distribution of the dialysis membrane etc. Besides, bioavailability index of rosmarinic acid was calculated as 47.58% in the current study in spite of the negative influence of simulative digestion, which was greater than pure rosmarinic acid (46%), described by Gayoso et al. (2016).

**Table 4**
Enzyme inhibitory potential of ND and IN samples of SVM*.

| *Name of the sample* | $\alpha$-amylase[B] | $\alpha$-glucosidase[C] | AChE**,[D] | BChE***,[D] |
|---|---|---|---|---|
| ND 1 mg/mL[A] | 81.2 ± 1.6 [§] | $70.2^{d} \pm 2.51$ | 16.45 ± 0.93 | 25.98 ± 1.02 |
| ND 0.5 mg/mL | $64.4^{a} \pm 1.53$ | 55.7 ± 1.2 | 11.87 ± 0.62 | 17.85 ± 0.78 |
| IN 1 mg/mL | 66.4 ± 0.52 | 60.39 ± 0.72 | 12.29 ± 0.48 | 15.26 ± 0.49 |
| IN 0,5 mg/mL | 60.75 ± 0.1 | 41.4 ± 1.2 | 8.42 ± 0.51 | 11.02 ± 0.35 |

* 80% methanolic extract of *Salvia virgata*.
** Acetylcholinesterase
*** Butyrylcholinesterase
[A] The abbreviations for samples are *ND: non-digested, IN: bioavailable*
[B] Acarbose was used as control group with 77.7 ± 0.4% inhibition at 1 mg/mL, 73.4 ± 0.5% inhibition at 0.5 mg/mL.
[C] Quercetin was used as control group with 63.7 ± 0.9% inhibition at 1 mg/mL, 60.5 ± 1.1% inhibition at 0.5 mg/mL.
[D] Galantamine was used as control group with 89.58 ± 2.36% inhibition at 1 mg/mL, 74.37 ± 1.68% inhibition at 0.5 mg/mL for AChE and 81.27 ± 2.03% inhibition at 1 mg/mL, 69.55 ± 2.46% inhibition at 0.5 mg/mL for BChE.
[§] Different letters in the same row indicate significance ($p < 0.05$).

**Table 5**
AGEs inhibitory potential of ND and IN samples of SVM*

| *Name of the sample* | AGEs[B] |
|---|---|
| ND 1 mg/mL[A] | 86.2 ± 5.85 |
| ND 0.5 mg/mL | 55.5 ± 2.22 |
| IN 1 mg/mL | 48.26 ± 2.38 |
| IN 0.5 mg/mL | 39.46 ± 2.92 |

* 80% methanolic extract of *Salvia virgata*.
[A] The abbreviations for samples are *ND: non-digested, IN: bioavailable*
[B] Quercetin was used as control group with 93.36 ± 3.32% inhibition at 1 mg/mL, 89.88 ± 2.57% inhibition at 0.5 mg/mL.

Rutin, also known as quercetin 3-O-rutinoside, was found as the second major metabolite in the present study. Even though, rutin was detected in various alcoholic extracts of numerous *Salvia* species before, to our knowledge this is the first study detected rutin in the SVM. Similar to many studies, the concentration of rutin remained significantly unchanged after gastric conditions (PG) in contrast to serum available fraction (IN) in which significant decline was observed (Tenore et al., 2013). It might be claimed that flavonoid glycosides have resistance to acid hydrolysis in the stomach and moving the colon without forming any chemical changes. As mentioned earlier, rutin amount was decreased considerably after the intestinal phase. Dey (2019) reported that C-ring fussion occured in flavonoid glycosides lead to hydrolysis of A and B rings, then biotransformation by dihydroxylation results in low bioavailiability index. Moreover, Lucas-Gonzalez et al. (2016) stated that alkaline conditions provoke the transformation, oxidation and polymerization on the structure of the flavonoid glycosides which render them unstable and less bioavailable. In addition, Saura-Calixto et al. (2007) also agreed with the low bioavailability of rutin in the serum-available phase since it forms complexes with proteins, fibers and metal ions in the plant matrix. Furthermore, rutin displayed lower bioavailability then rosmarinic acid in the present study. The claim that flavonoid glycosides have a lower bioavailability compared to phenolic acids seems to be consistent with our previous study (Celep et al., 2018).

Antioxidant potential of SVM was determined by employing mechanistically different assays to obtain more precise results. DPPH and DMPD tests were conducted to detect free radical scavenging activity. Moreover, metal reducing capacity was determined by both CUPRAC and FRAP tests. In addition, total antioxidant capacity was calculated in the current study. According to results presented in Table 3, ND and PG samples showed analogous antioxidant activity by means of all assays. However, significant decrease was seen in IN fraction in terms of antioxidant activity. Thus, it can be argued that this descending trend in the antioxidant activity may be related to total content assays in which similar decrease was also detected during the course of digestion (Table 2). As mentioned earlier, the amounts of major metabolites were also dminished dramatically in serum-available fraction. It is a well-established phenomenon, proved by various studies, that antioxidant activity was directly affected by phenolic contents. The results of Gayoso et al. (2016) also showed that intestinal fraction of both rutin and rosmarinic acid have the lower antioxidant activity than non-digested fraction.

As previously described, postprandial hyperglycemia might be decreased by the inhibition of digestive enzymes such as $\alpha$-amylase and $\alpha$-glucosidase. In the present study, $\alpha$-amylase and $\alpha$-glucosidase inhibition activity of ND and IN samples of SVM were examined. According to the results in Table 4, it was shown that both ND and IN samples possess a strong $\alpha$-amylase and $\alpha$-glucosidase inhibition

activity. Nickavar and Abolhasani (2013) also demonstrated high inhibition activity of *S.virgata* ethanolic extract on $\alpha$-amylase enzyme. This activity was attributed to a flavone compound (chrysoeriol) isolated from ethyl acetate fraction. In addition, a correlation between inhibition of $\alpha$-amylase and $\alpha$-glucosidase activities and total phenolic contents was reported, suggesting that phenolic compounds present in the extracts were responsible for the inhibition of carbohydrate–digestive enzymes (Sayah et al., 2017). Thus, the decrease in enzyme-inhibition activity of IN fraction might be considered on account of having lower total phenolic content and concentrations of rutin and rosmarinic acid than ND fraction. Oboh et al. (2015) hypothesized that hydrogen bonds are formed by interaction between hydroxyl groups of rutin and the polar groups in the allosteric site of enzymes. As a result, molecular configuration and hydrophilic and hydrophobic characteristics of enzyme would be changed by this interaction, led to a decline in enzyme activity. McCue and Shetty (2004) also stated that an increase in the $\alpha$-amylase inhibition was observed depending on the concentration of rosmarinic acid.

AGEs are the consequences of the chronic DM that arise from the interaction of excess blood glucose and free amino acids of proteins. Consistently formed AGEs may induce the alterations of the structures and functions of the proteins. Therefore, inhibition of the glycation pathways might have a positive effect on the pathogenesis of DM and its complications (Starowicz and Zieliński, 2019). Meanwhile, oxidative stress causes the augmentation of diabetes-related complications and formation of AGEs by activating protein kinase C and transcription factors. Investigations of new potent AGE inhibitors have been conducted by various researchers in recent years. Attraction of medicinal plants on anti-glycation studies are growing owing to their low side effects and potentials (Safari et al., 2018). Therefore, inhibition activity of AGEs of SVM was investigated in this study. To our knowledge, although some *Salvia* species have been studied on the inhibition potential of AGEs, this is the first investigation conducted on *S.virgata* (Asadi et al., 2010; Ma et al., 2011). According to the results presented in Table 5, both ND and IN samples of SVM displayed a strong inhibition activity of AGEs. Inhibition percentage of 1 mg/mL concentration ND sample (86,17%) was adjacent to the inhibition percentage of 1 mg/mL of the reference compound quercetin (93,36%). In addition, inhibition activity decreased after human digestion simulation such as 1 mg/mL IN sample had a 48,26% inhibition percentage. As mentioned earlier, total phenolic, phenolic acid and flavonoid contents had a descending trend from ND to IN sample. Various researchers agree that phenolic compounds supress AGE formation and have strong anti-glycation properties. Safari et al. (2018) suggest that neighboring –OH groups found in the phenolics are responsible for the anti-glycation activity. Yeh et al. (2017) also declare that three or more –OH groups in the phenolic acids have better anti-glycation activity and carboxylic group placement may develeop different consequences. Furthermore, flavonoids such as quercetin, rutin and luteolin have a significant anti-glycation effect particularly on the early phase of glycation process. According to the study of Wu and Yen (2005), these three flavonoids demonstrated better anti-glycation activity than the AGEs inhibitor aminoguanidine. As a result, the decline in the anti-glycation activity after human digestion simulation method may be attributed to the decreased levels of total phenolic,phenolic acid and flavonoid concentrations. Moreover, rutin and rosmarinic acid were declared as the powerful glycation inhibitors by the various research groups as well (Ma et al., 2011; Ou et al., 2017; Wu and Yen, 2005). As demonstrated in Table 2, concentration of rutin and rosmarinic acid concentrations had a tendency to decrease after human digestion model. Thus, this circumstance may be another reason for the diminish anti-glycation activity of the bioavailable phase.

Cholinesterase inhibitors were used in the treatment of neurodegenerative diseases for a long period of time (Li et al., 2018). In addition, it is a well-known fact that antioxidant activity may be beneficial against neurodegenerative disorders (Subash et al., 2014). For these reasons, AChE and BChE inhibition activity of SVM and its alteration after simulated digestion method were investigated in this study. In a previous study, dichloromethane and ethylaceate extracts of *S. virgata* were investigated for their AChE and BChE inhibition activity. Results demonstrated no activity up to 100 $\mu$g/ml concentration for the studied extracts (Şenol et al., 2010). However, in this study, methanolic extract of *S. virgata* was investigated in meaningfully higher doses and results showed a moderate inhibition activity in non-digested phase (Table 4). Various extracts of *Salvia* species from Turkey have shown *in vitro* AChE and BChE inhibition activity in previous studies (Orhan et al., 2007). Nonetheless, prior *in vitro* studies were neglecting the effects of human digestion on the bioactivity. In our study, results revealed that there was a significant reduction in AChE and BChE inhibition activity after simulated digestion. Scientific literature also demonstrates that rutin and rosmarinic acid have potent cholinesterase inhibition activity (Ademosun et al., 2016; Gülçin et al., 2016). It can be hypothesized that decline of rutin and rosmarinic acid concentration in IN phase might be triggered the downgrade for inhibition bioactivity.

To sum up, influence of *in vitro* human digestion on the bioavailability of phenolic profile, antioxidant potential, cholinesterase enzymes inhibition activity and anti-diabetic properties of *Salvia virgata* were investigated. The bioavailability index of rutin and rosmarinic acid contents was calculated. Results have demonstrated that phenolic contents, antioxidant potential, diabetes-related and cholinesterase enzyme inhibition activities and contents of major phenolic compounds were significantly influenced by *in vitro* digestion method. Although bioavailability of major phenolic compounds showed descending trend after human digestion, SVM would be regarded as potent antioxidant and anti-diabetic extract in terms of practiced experiments in the present study.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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