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Biologically valuable components, antioxidant activity and proteinase inhibition activity of leaf and callus extracts of *Salvia* sp.

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Abstract

Sage is medicinal plant, known for its antioxidant and anti-inflammatory effects. Eight extract samples were tested in this study: extract from *Salvia officinalis* L. varieties from two different geographical localities (Jaslovské Bohunice and Pobedim, Slovakia), *Salvia officinalis* L., variety "bicolor", *Salvia officinalis* L., variety "purpurescens", *Salvia apiana*, *Salvia divinorum*, and two callus cultures of *Salvia sclarea* L. and *Salvia aethiopis* L. The highest values for composite parameters were observed for extract from *Salvia apiana*. It can be concluded that prepared sage extract samples are rich on polyphenolic acids (2 950±265 $\mu g.mL^{-1}$ GAeq.) and amines (197±5.50 $\mu g.mL^{-1}$ TRPeq.). HPLC analysis confirmed the dominant content of rosmarinic acid in the extracts; the highest content was detected in the *Salvia apiana* extract (1 120±15 $\mu g.mL^{-1}$). Extract from *Salvia apiana* expressed too the highest antioxidant activity (1 710 – 4 669 $\mu g.mL^{-1}$TEAC). Similarly, the highest inhibition activity was observed for this extract on thrombin (57±3.3 %) and on other proteinases (over 80 %). Spearman correlation analysis and PCA analyses revealed a coherence between antioxidant activity of samples and their content of rosmarinic acid as well as inhibitory activity towards particular proteases, and revealed the significance of thiol based secondary metabolites. Cluster analysis demonstrates the differences of *Salvia apiana* extract from extracts of *S. officinalis* L., the group of *S. divinorum* extract and from callus cultures.

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Introduction

Sage is a common aromatic plant of the *Lamiaceae* family, widely cultivated for its medicinal and culinary purposes. Sage expresses various biological activities and medicinal properties such as antioxidant, antibacterial, antifungal, anti-inflammatory, antispasmodic, astringent and antihidrotic (Bouaziz *et al.* 2009; Khan *et al.* 2011; Hamidpour *et al.* 2014; Razavi *et al.* 2014;

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Martins *et al.* 2015). From the morphological point of view, the shrub of *Salvia apiana* (the white sage) has long, thin, velvety leaves of colour ranging from shades of grey to green. Small flowers of white sage can be purple or white with long anthers (Adams and Garcia 2005). This species can be found scattered throughout southwestern North America, with the highest abundance in Southern California (Borek *et al.* 2006). *Salvia divinorum*, commonly known as diviner's sage, is a medicinal plant native to Mexico and traditionally used by the Mazatec peoples for spiritual practices due to its hallucinogenic properties.

Salvia sclarea L. is a spicy, aromatic plant, known as anti-inflammatory, antimicrobial, antioxidant, antispasmodic, hypoglycaemic and digestive agent (Moretti *et al.* 1997; Hammer *et al.* 1999; Mantle *et al.* 2000; Miliauskas *et al.* 2004). From herbal literature, aqueous extracts of the plant were used against various digestive disorders, as well as decoction and infusion for treating polyarthritis and acute rheumatics (Peana and Moretti 2002; Rajagopal *et al.* 2013). Given the ever-increasing interest in pharmaceutically important plant-produced compounds, scientists' attention is focused on their economically viable production potential. With advances in cellular, metabolic and gene engineering, plant cells and tissue cultures are now a very promising option for producing valuable phytochemicals (Vanisree and Tsay 2004; Vanisree *et al.* 2004). In addition to the natural environment, plant material can grow under various conditions, for example be cultivated under specific conditions in closed containers as whole plants or their separate parts.

Phenolic acids and flavonoids represent the most frequent polyphenols, while soluble polyphenols can influence the positive effects of food more significantly. The polyphenolic content in sage varies with growing conditions and includes diterpenoids, flavonoids and phenolic acids and their derivatives. Commonly found phenolic acids (derivatives) in sage are carnosic acid and its derivatives, rosmarinic acid (RA), methylrosmarinate (MeR), caffeic acid (CA), salvianolic acid (SalK), syringic acid (SA), and vanillic acid (VA). It was reported, that RA derivatives from sage were more potent antioxidants in comparison to the present flavonoids, luteolin, and apigenin glycosides. Earlier studies on the antioxidant activity of sage had been limited to the involvement of diterpenoid compounds. Further, apigenin and its derivatives (e.g. apigenin 4'-methyl ether), scutellarein 6-methyl ether, isoscutellarein 8-methyl ether, luteolin and 6-OH-luteolin-6-methyl ether where published for *S. officinalis* (Veličković *et al.* 2007). The main antioxidative effect of sage, however, has been reported to relate to the presence of carnosic acid and rosmarinic acid. The level of both compounds in the dry leaves varies widely depending on genetic factors and environmental conditions. While in general the main goal of any extraction is to maximize the mass transference of targeted compound from matrix to solvent, factors that influence the extraction of polyphenols are their stability, the method of extraction, plant matrix, pH, time, temperature, type and polarity of the solvent, and particle size (Nell *et al.* 2008).

From the medicinal point of view, there are important relationships between many pathophysiological processes in the body and proteases. Trypsin (EC 3.4.21.4) acts as potential pathophysiological agent for both the acute and chronic types of pancreatitis (Whitcomb 2003). Thrombin (EC 3.4.21.5) plays a role in the coagulation disorder diseases, inflammation, and metastasis progression (Bode 2006; Scatena *et al.* 2007). Urokinase (urokinase type plasminogen activator, uPA) (EC 3.4.21.75) is an important component of the extracellular protease system specifically converting plasminogen to plasmin, participating in a number of pathophysiological processes, including Extracellular Matrix (ECM) lysis, thus tumour progression and metastasis of onco-transformed cells (Krošlák *et al.* 2016).

The main goal of this paper is to evaluate the antioxidant activity and proteinase inhibition activity in sage in relation to the content of biologically valuable component groups. Research on inhibition activities of sage extracts or components on serine proteinases are published very rarely and it is a continuing of the similar papers in this field (Fan *et al.* 2010).

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Experimental

Plant Material

The source of biological material for the experiments were leaves of *Salvia officinalis* L. from two different geographical areas – Jaslovské Bohunice and Pobedim (Slovakia), collected during year 2018. The sampled and dried sage leaves comprised the varieties "bicolor" and "purpurescens", further commercially purchased and dried leaves of *Salvia apiana* and *Salvia divinorum*.

As explantate cultures *in vitro* are known for production of secondary metabolites (either directly or more often after elicitation), callus cultures of *Salvia sclarea* L. and *Salvia aethiopis* L. were also used. Seeds harvested in Medical Plants Garden of Faculty of Pharmacy, Comenius University in Bratislava (Slovakia) were disinfected by the solution of 20 % (v/v) NaClO (Savo) for 20 min and subsequently washed three times with sterile distilled water. The seeds were placed on paper bridges in Erlenmeyer flasks containing sterile distilled water. Seeds germinated for 1 month under 16/8 h white light/dark period (22 000 lux) at 24 °C and relative humidity 70 %. Hypocotyls acquired by separation of seedlings were under aseptic conditions transferred on paper bridges in Erlenmeyer flasks with 25 mL of sterile liquid Murashige-Skoog growth medium (MS) supplemented either with kinetin (0.3 $mg.L^{-1}$) and 1-naphthyl acetic acid (NAA) (2 $mg.L^{-1}$) (*S. aethiopis* L.) or with kinetin (0.1 $mg.L^{-1}$) and 2,4-dichlorophenoxyacetic acid (1 $mg.L^{-1}$) (*S. sclarea* L.). Cultivation was performed under the conditions mentioned above. The produced calli of both *Salvia* sp. were after 1 month of sub-cultivation transferred into fresh growth media enabling the formation of sufficient amount of biomass.

Extracts from all sage samples (dried leaves and callus cultures) were prepared by crushing followed by extraction with methanol 1 : 20 (w : v). The extraction process was carried out under reflux at 70 °C for 1.5 h.

Chemicals

Common laboratory chemicals, including dimethyl sulfoxide (DMSO), methanol p.a., ethanol p.a., 2-propanol, chloroform p.a., Tween 40, *β*-carotene type IV, sodium carbonate, sodium acetate and acetic acid were supplied by Mikrochem (Slovakia). 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid (Trolox), 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB), phosphate buffer tablets, chromogenic substrate N-*α*-Cbz-L-lysine thiobenzyl ester hydrochloride (Z-L-Lys-SBzl hydrochloride), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), linoleic acid, ammonium persulfate, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and sodium dodecyl sulphate were purchased from Sigma-Aldrich (USA). All the enzymes used in the experiments were also supplied by Sigma-Aldrich. Avenanthramides A, B and C were purchased from ReseaChem (Switzerland).

HPLC analysis

HPLC separation, identification and quantification was performed using Waters HPLC system (Waters, USA) equipped with Alliance module with 2998 photodiode array (PDA) detector, Software Empower 2 and Waters Symmetry C18 column (75 × 4.6 mm i.d., 3.5 μm). A gradient elution system consisted of solvent A (0.1 % formic acid in water) and B (0.1 % formic acid in methanol). A linear gradient of 5 to 100 % solvent B over 30 min at a flow rate of 1.0 $mLmin^{-1}$ was used. The column temperature was set at 30 °C and the injection volume was 20 μL. Detection of rosmarinic acid in extract samples was performed using diode-array detector at 325 nm.

Total polyphenols content determination

Total phenolics content (TPF) in sage extracts was determined by the method described by Slinkard and Singleton (1997) modified for microplate screening system. This method based on measurement of products of the reaction of phenol hydroxyl Groups with the Folin-Ciocalteu reagent. The reaction mixture contained

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20 μL of extract/standard/ethanol (for sample/standard/blank) and 20 μL of Folin-Ciocalteu reagent. After 5 min, 200 μL of 100 $g.L^{-1}$ sodium carbonate was added. After 30 min of incubation in the dark, the optical density at 760 nm (OD_{760nm}) was measured using microplate reader OPSYS (Dynex, Chantilly, Virginia, USA). TPF was expressed as micrograms of gallic acid equivalent (GAeq.) per mL.

Total phenolic acids content determination

Total phenolic acids content (TPFA) in extracts of sage was determined by method described by Singleton *et al.* (1999), reaction with Arnov´s reagent modified for Microplate screening system. 30 μL of extract extract/standard/ethanol as blank was mixed with 150 μL of distilled water, 30 μL of 0.5 $mol.L^{-1}$ HCl, 30 μL of Arnov´s reagent (100 $g.L^{-1}$ sodium nitrite and 100 $g.L^{-1}$ sodium molybdate in distilled water), 30 μL of 1 $mol.L^{-1}$ sodium hydroxide, and 30 μL of distilled water. The optical density was measured at 490 nm (OD_{490nm}) using microplate reader OPSYS. TPFA was expressed as micrograms of caffeic acid equivalent (CAeq,) per mL.

Total flavonoid content determination

Total flavonoids content (TFL) of sage samples was determined according to Rakotoarison *et al.* (1997). Intensity of staining solution after the reaction of flavonoids with aluminum chloride is proportional to the concentration of flavonoids. The reaction mixture consisted of the extract/standard solution/ethanol as blank (100 μL) and 20 μL of 50 $g.L^{-1}$ aluminum chloride in methanol. After 30 min incubation, the optical density at 405 nm (OD_{405nm}) was measured using microplate reader OPSYS. TFL was expressed as micrograms of quercetin equivalent (Qeq.) per mL.

Total thiol content determination

For determination of total thiols content (TT) in the sample extracts, Ellman's reagent, DTNB, was used (Ellman 1959). The method is based on the reaction of sulfhydryl groups with DTNB yielding a yellow color product. Briefly, 150 μL of DTNB in 0.05 $mol.L^{-1}$ phosphate buffer, pH 7.0 was added to 20 μL of the extract/standard solution/ethanol as blank. OD_{405nm} was measured after 15 min of incubation using microplate reader OPSYS. TT was expressed as micrograms of cysteine equivalent (CYSeq,) per mL.

Total amine content determination

Total amines content (TA) was determined using reaction with ninhydrin. The reaction mixture contained 20 μL of the extract/standard/ethanol as blank and 100 μL of the ninhydrin reagent (3.5 $g.L^{-1}$ ninhydrin in 2-propanol). Optical density at 630 nm (OD_{630nm}) was measured after 45 min of incubation at 42 °C using microplate reader OPSYS. TA was expressed as micrograms of tryptophan equivalent (TRPeq.) per mL (Kaiser *et al*. 1970).

Antioxidant activity by DPPH method (DPPH)

The DPPH radical scavenging activity of sage extracts was measured by DPPH radical (•DPPH) scavenging method modified for microplate screening system. A decreased absorbance of reaction mixture indicated higher free radical scavenging potency. Aliquots of 25 μL of extract/standard/methanol for sample/standard/blank was mixed with 100 μL of DPPH solution ($3.05.10^{-4}$ $mol.L^{-1}$ in methanol). After 10 min-incubation in the dark, optical density at 540 nm (OD_{540nm}) was measured using microplate reader OPSYS. Antioxidant activity (AA_{DPPH}) was expressed as micrograms of Trolox equivalent (TROLOXeq.) per mL (Brand-Williams *et al*. 1995).

Antioxidant activity by ABTS method (ABTS)

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of sage extracts was measured by ABTS radical cation ($ABTS^{\cdot+}$) modified for microplate screening system. ABTS reagent was prepared by reaction of 7 $mmol.L^{-1}$ ABTS with 2.45 $mmol.L^{-1}$ ammonium persulfate in the volume of 10 mL, followed by incubation in the dark during 16 h,

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after that was ABTS reagent adapted on the absorbance 0.7 at 730 nm. A decreased absorbance of reaction mixture indicated higher free radical scavenging activity. 25 µL of extract/standard/ethanol (sample/standard/blank) was mixed with 100 µL of ABTS reagent. After 10 min of incubation in the dark, optical density at 630 nm (OD_{630nm}) was measured using microplate reader OPSYS. Antioxidant activity (AA_{ABTS}) was expressed as micrograms of Trolox equivalent (TROLOXeq.) per mL (Ferri *et al.* 2013).

Antioxidant activity by FRAP method (FRAP)

The ability of sage extracts to change the oxidation status of transition metals was measured by reduction of $Fe^{(III)}$-2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) complex, according to the method described by Stratil *et al.* (2007) modified for microplate screening system. $Fe^{(III)}$-TPTZ complex was prepared by mixing 10 mL TPTZ solution with 1 mL of sodium acetate buffer (pH 3.6) and 1 mL of 20 mmol.L^{-1} $FeCl_3$. An increased absorbance of reaction mixture indicated higher reducing ability. Briefly, 165 µL of $Fe^{(III)}$-TPTZ reagent was added to 35 µL of extract/standard/ethanol. After 5 min of incubation in the dark at 37 °C, optical density at 630 nm (OD_{630nm}) was measured using microplate reader OPSYS. Antioxidant activity (AA_{FRAP}) was expressed as micrograms of Trolox equivalent (TROLOXeq.) per mL.

Antioxidant activity by reducing power method (RP)

The ability to change the oxidation status of transition metals of sage extracts was measured by reduction of potassium ferricyanide, $K_3[Fe(CN)_6]$ complex, according to the method described by Jayaprakasha *et al.* (2001) modified for microplate screening system. 30 µL of extract/standard/ethanol was mixed with 100 µL distilled water, 45 µL of 1 mol.L^{-1} HCl, 45 µL of 10 g.L^{-1} $K_3[Fe(CN)_6]$ solution, 15 µL of 10 g.L^{-1} sodium dodecyl sulfate solution and 15 µL of 2 g.L^{-1} $FeCl_3$ solution. The reaction mixture was incubated for 20 min at 50 °C, followed by measuring of optical density at 630 nm (OD_{630nm}). Absorbance was measured using microplate reader OPSYS. An increased absorbance of reaction mixture indicated higher reducing ability. Antioxidant activity (AA_RP) was expressed as micrograms of Trolox equivalent (TROLOXeq.) per mL.

Proteinase inhibitory assays

For determination of proteinase inhibitory activities in the extracts, photometric methods with chromogenic substrates Z-Lys-SBzl.HCl and DTNB were used. Substrates were cleaved by trypsin (IA_TY), thrombin (IA_TR), plasmin (IA_PL) and urokinase (IA_UR) and released chromogen was detected at 405 nm. Each well contained 0.6 mmol.L^{-1} substrate, 2.817.10^{-5} mmol.L^{-1} DMSO and 20 µL of tested extract. The reaction started by the addition of the enzyme solution: 0.3 U.mg^{-1} of trypsin, 1.714 U.mg^{-1} of thrombin, 1.524 U.mg^{-1} of plasmin and 1.072 U.mg^{-1} of urokinase, each in potassium phosphate buffer (50 mmol.L^{-1}, pH 7.0). The reaction temperature was set at 37 °C. The optical density differences were measured at 405 nm using microplate reader OPSYS for each sample as (Eq. 1) (Green and Shaw 1979):

$$\delta OD = (OD_{405nm} \text{ in the 61-st min}) - (OD_{405nm} \text{ in the 1-st min}) \quad (1)$$

The inhibitory activity (IA) was calculated according to the following equation (Eq. 2):

$$IA\ [\%] = [1 - (\delta OD_{sample} - \delta OD_{blank})] / (\delta OD_{control} - \delta OD_{blank}) \times 100 \quad (2)$$

Statistical analysis

The experimental results obtained were organized into a data matrix, where the columns contained fourteen composite variables (TPC, TPAC, TFC, TTC, TAC, RA), five inhibitory (IA_TY, IA_TR, IA_UR, and IA_PL), four antioxidants (DPPH, FRAP, ABTS and RP). In the next step, statistical methods were applied in order to evaluate the relationships between the variables studied (inhibitory activities and composites) and objects (genotypes). Correlation analysis with Spearmen correlation coefficient was used to reveal statistically significant correlations ($\alpha < 0.05$; 0.01;

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0.001) between variables. The principal component analysis (PCA) and cluster analysis (CA) were chosen to evaluate relationships among all the objects (genotypes) and/or for the variables studied. Basic operations and calculations with data were performed by MS Office 2010 and the statistical approach (PCA, CA and correlation analysis) was carried out by JMP 9 software.

Results and Discussion

HPLC

Eight extract samples were analysed by HPLC on the content of selected polyphenols (rosmarinic acid, flavonoids luteolin and apigenin). Rosmarinic acid, the dominant secondary metabolite, was found in all extract samples, while detection of two flavonoids – apigenin and luteolin will be a subject of the continuing study, with regard to published data (Li *et al.* 2017; Oudjedia *et al.* 2019). The Fig. 1 represents a typical HPLC chromatogram. The content of rosmarinic acid (RA) varied from 1 120±15 μg.mL^{-1} (extract from *Salvia apiana*) to 5.02±0.04 μg.mL^{-1} (callus extract). The content of RA decreased in order *Salvia apiana* > *Salvia officinalis* L. > *Salvia divinorum* > callus culture extracts. From results, it is evident that rosmarinic acid is typical secondary metabolite produced by differenced cells, not by callus culture cells. We can observe significant differences ($p < 0.01$) between particular taxon's and varieties of sage. The particular chromatograms of eight prepared extracts are presented in Fig. 1. The overlay of spectrum of rosmarinic acid (standard) and particular peak from sample 1 (extract from *Salvia apiana*) are included.

The similar content of RA was published e.g. by Huafu *et al.* (2004), who reported that the content of rosmarinic acids in sage was 2 000 – 2 704 μg.g^{-1} in the aromatic herbs analysed. Bandoniene *et al.* (2005) described that the concentration of rosmarinic acid in *Salvia* sp. ranged from 1 000 to 930 μg.g^{-1}, which are values comparable to our results.

Habán *et al.* (2019) described that the first time of harvesting of sage with respect to RA content is more appropriate; in the second collection period (under the same soil and climatic conditions) the plants are no longer able to produce a comparable amount of rosmarinic acid. The weather conditions of the monitored varieties statistically significantly influenced the content of RA. For the industrial application and utilization of sage as a source of rosmarinic acid it should be precisely considered and argued the selection of the taxon.

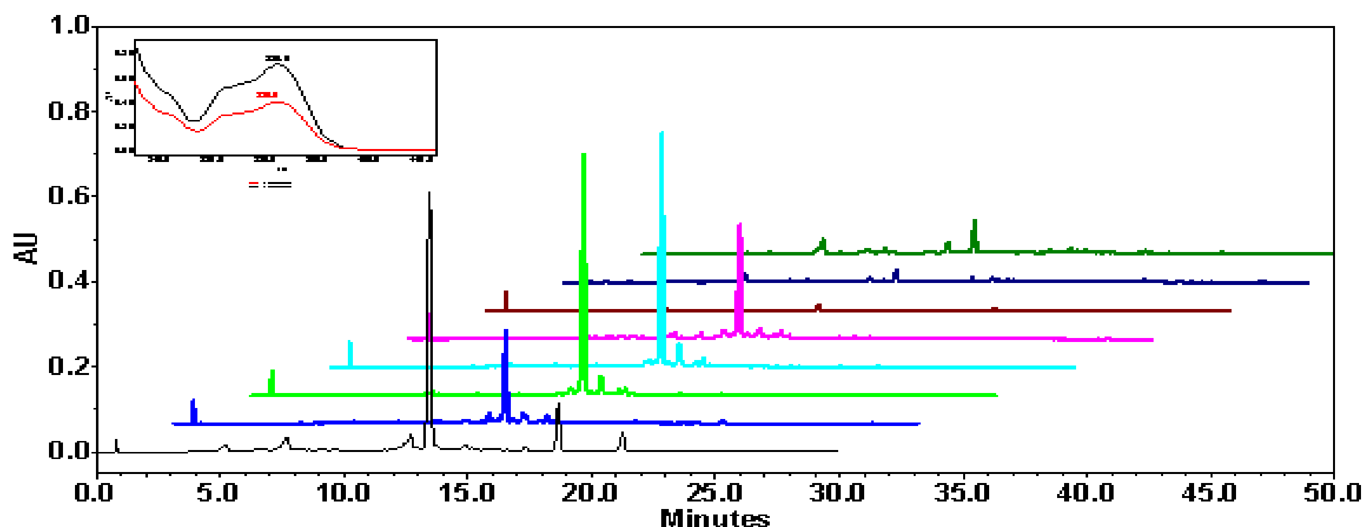

Fig. 1. Particular chromatograms of eight sage extracts. The overlay of spectrum of rosmarinic acid as standard and subjected peak from sample 1 (extract from *Salvia apiana*). The order of lines/extracts – 8 as follows: *Salvia apiana* (black line), *Salvia officinalis* L. (locality Jaslovské Bohunice, blue line), *Salvia officinalis* L., variety "bicolor"(green line), *Salvia officinalis* L., variety "purpurescens" (light blue line), *Salvia officinalis* L. (locality Pobedim, violet line), *Salvia divinorum* brown line), callus 1: *Salvia sclarea* (dark blue), callus 2*: Salvia aethiopis* (dark green).

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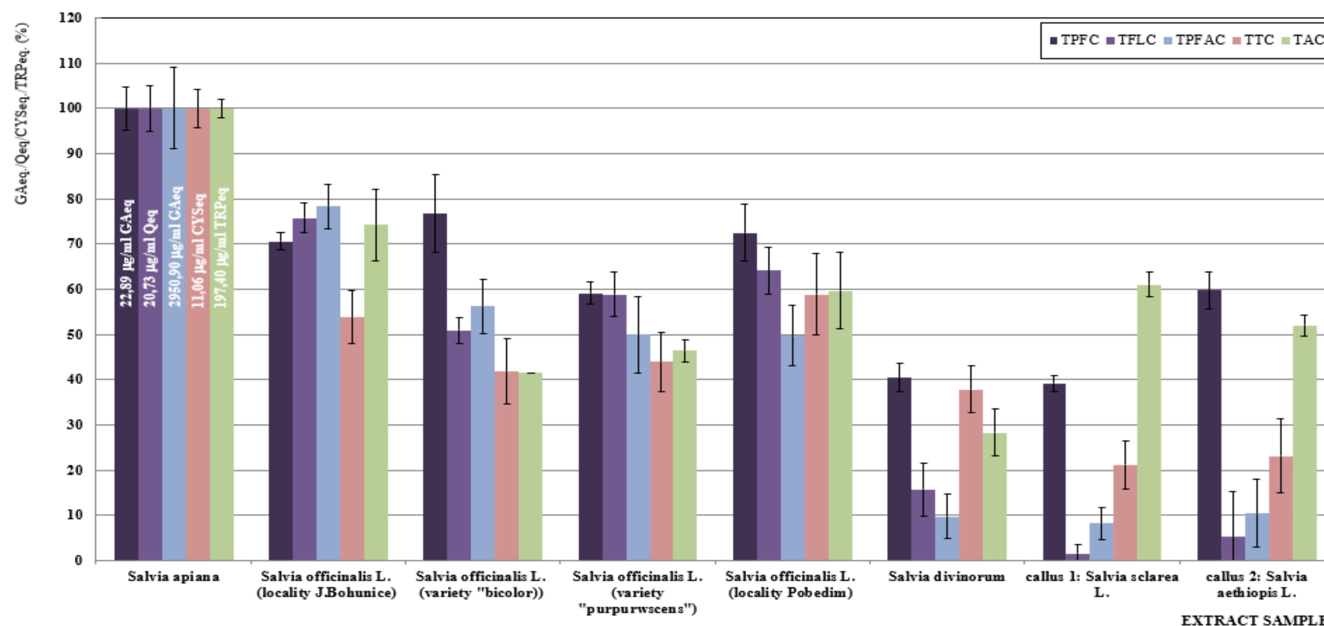

Fig. 2. The content of composite parameters in eight prepared extract samples from native matter and callus culture of *Salvia* sp. Total phenolic content (TPC) percentage expressed as GAeq., total flavonoid content (TFL) percentage expressed as Qeq., total phenolic acid content (TPCA) percentage expressed as GAeq., total thiol content (TTC) percentage expressed as CYSeq. and finally total amine content (TAC) percentage expressed as TRPeq., towards primary extract from *Salvia apiana*.

Determination of component parameters

All eight prepared extracts were tested on the content of composite parameters (see material and method section) as follows. Results are presented in Fig. 2. From results it is evident that a group of data for the measured parameters are for a particular extract sample identical and individual. The highest content of the composite parameters tested was observed for the extract from *Salvia apiana*. Relatively high content of all composite parameters was observed for four extract samples from *Salvia officinalis* L. and on the contrary, relatively low content was observed for *Salvia divinorum* and two extract samples from the both callus cultures. From general point of view, we can present that prepared sage extract samples are rich in polyphenolic acid (2 950±265 μg.mL^{-1} GAeq.) and at second on amines (197.46±5.49 μg.mL^{-1} TRPeq.). From the presented results we can conclude, that the source of biologically active compounds is the dried matter of plants, but specifically for particular taxon's. All data obtained from *Salvia officinalis* L., *Salvia divinorum* and from callus cultures are statistically significant on the level with $p < 0.05$ towards extract sample from *Salvia apiana*. The obtained data about secondary metabolites obtained during year 2018 might differ from real time values in nature as both biotic and abiotic factors affect secondary metabolites content (Murcia *et al.* 2017).

Determination of antioxidant activity parameters

The primary data matrix consists of the antioxidant activities of eight extracted samples subjected to testing by all the methods used, DPPH, ABTS, FRAP and RP. The results are shown in Fig. 3. Based on the achieved results it can be concluded that significant differences in expressed antioxidant activities among tested extract samples from native matter and callus culture of *Salvia* sp. by all applied methods have been observed. The highest antioxidant activity was recorded by RP method (4 699±18 μg.mL^{-1} TROLOXeq.) and the least antioxidant activity by the ABTS method (1 710±6 μg.mL^{-1} TROLOXeq.). All the results reached from *Salvia officinalis* L. *Salvia divinorum* extracts and from callus cultures were statistically on the level with $p < 0.05$ towards primary extract sample from *Salvia apiana*. Callus cultures and *Salvia divinorum* showed minimal antioxidant activity compared to native *Salvia apiana* and *Salvia officinalis* L. The antioxidant activity of presented extract samples correlates with content

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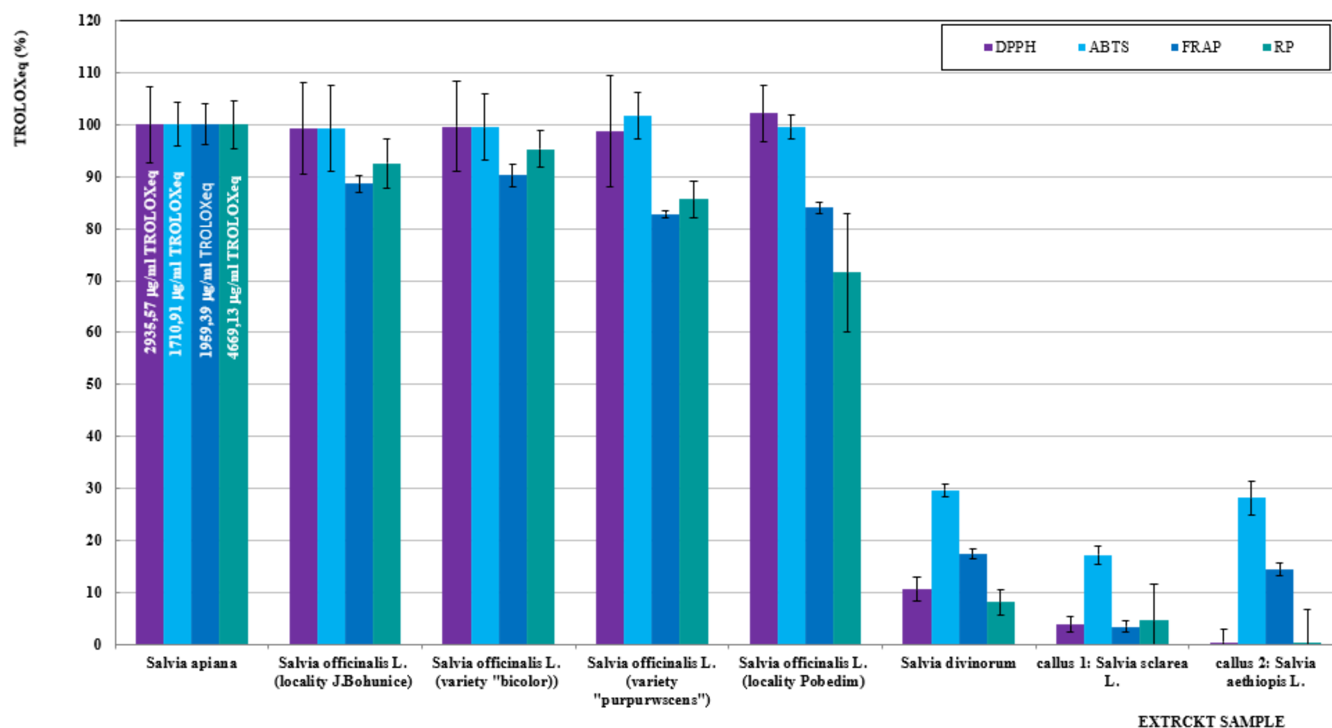

Fig. 3. The antioxidant activity of eight prepared extracts from native matter and callus culture of *Salvia* sp. Determined by DPPH, ABTS, RFAP and RP methods (respectively). Results as expressed as percentage of TROLOXeq., towards primary extract from *Salvia apiana.*

of secondary metabolites, due to fact, that secondary metabolites are major holder of antioxidant activity in plants (Lee *et al.* 2016; Pandey *et al*. 2018).

Determination of proteinase inhibition activity parameters

In our work, we focused on the inhibitory activity of selected serine proteinases against four proteinases – trypsin acting as pancreatic disorder promoter, thrombin acting as promoter of diseases associated with coagulation, urokinase acting as promoter of metastatic process of onco-transformed cells and plasmin. Plasmin-induced proteolysis had been described as a pathological mechanism for some diseases, e.g. cancer and certain viral diseases. The proteinases were selected as the third group of variables (secondary metabolites content, antioxidant activity parameters). As the result, the presented paper represents a continuity of several, yet published papers with identical methodology and alternate plant subject – barley (Maliar *et al.* 2015), oat (Krošlák *et al.* 2016) and poppy (Krošlák *et al.* 2017), enabling comparison and discussion of obtained results.

Table 1. Results of protease inhibition activity (IA) of eight extracts prepared from native matter and callus culture of *Salvia* sp. on trypsin (TY), plasmin (PL), thrombin (THR) and urokinase (UR).

Extract sample	**IA ± STD [%]**			
	IA_TY	**IA_PL**	**IA_THR**	**IA_UR**
Salvia apiana	88.67±1.01	80.16±1.57	57.33±3.30	83.82±3.52
Salvia officinalis L. (locality Jaslovské Bohunice)	55.76±3.94	80.81±1.63	59.21±4.45	64.17±4.45
Salvia officinalis L., variety "bicolor"	44.79±2.94	31.17±1.36	31.88±1.33	31.74±0.92
Salvia officinalis L., variety "purpurescens"	43.32±5.64	30.58±1.21	35.35±1.19	54.04±4.05
Salvia officinalis L. (locality Pobedim)	53.54±3.27	19.44±2.55	39.40±4.25	39.14±0.62
Salvia divinorum	50.26±2.26	0	22.63±2.05	6.12±0.25
Callus 1: *Salvia sclarea*	3.80±0.12	9.00±0.26	0.21±0.01	0
Callus 2: *Salvia aethiopis*	5.04±0.51	36.74±1.32	16.84±0.87	0.93±0.04

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Further, the selected secondary metabolites are main carriers of antioxidant activity as well as proteinase inhibition activity in the plant tissue (Meijersen *et al.* 1991; Fink *et al.* 2000; Maliar *et al.* 2015; Yu *et al.* 2018). The obtained results are presented in Table 1. The highest antioxidant activity was observed for extract from *Salvia apiana*, and IA varied from lowest on thrombin (57.33±3.30 %) up to over 80 % inhibition of other proteinases. Relatively high antioxidant activity was observed for the four extract samples from *Salvia officinalis* L., which reached values from percentile interval <20, 80>. These extracts inhibited most effectively the urokinase, however in variable manner. Opposite to this, relatively low antioxidant activity (not more than 51 %, Table 1) was observed for *Salvia divinorum* and the two extract samples from both callus cultures.

Table 2. Selected results of correlation analysis by Spearmen method between all parameters (variables).

Parameter (Variable)	IA_ TY	IA_ PL	IA_ THR	IA_ UR	AA_ DPPH	AA_ ABTS	AA_ FRAP	AA_ RP	TPF	TFL	TPFA	TT	TA
IA_THR	**0.81****	0.59	1										
IA_UR	**0.83****	0.64	**0.95****	1									
AA_DPPH	**0.76****	0.29	**0.76***	**0.71***	1								
AA_ABTS	0.48	0.38	0.64	**0.79***	0.67	1							
AA_FRAP	**0.81***	0.64	**0.81***	**0.83***	**0.83***	**0.74***	1						
AA_RP	**0.71***	0.57	0.76	**0.83***	**0.76***	**0.79***	**0.95****	1					
TPF	0.69	0.71*	0.67	0.67	**0.79***	0.60	**0.91****	**0.76***	1				
TFL	**0.91****	0.62	**0.98****	**0.98****	**0.81***	**0.71***	**0.86****	**0.81***	**0.74***	1			
TPFA	**0.74***	0.83*	**0.86****	**0.91****	0.67	**0.74***	**0.93****	**0.91****	**0.83***	**0.89****	1		
TT	**0.89****	0.52	**0.93****	**0.93****	**0.88****	**0.74***	**0.83***	**0.76***	**0,76***	**0.98****	**0.81***	1	
Contents_RA	0.62	0.50	0.67	0	**0.79***	**0.93****	**0.91****	**0.91****	**0.81***	**0.76***	**0.83***	**0.79***	1

Statistically significant values of correlation coefficients are written with bold and indicated with asterisk.

TY – trypsin; THR – thrombin; PL –plasmin; UR – urokinase; DPPH, ABTS, FRAP and RP – particular antioxidant activity methods; TPF – total polyphenol content; TFL – total flavonoid content; TPFA – total polyphenolic acid content; TT – total thiol content; TA – total amines content; RA – the content of rosmarinic acid by HPLC.

Achieved results proved significant inhibition activity of sage methanol extracts on selected proteinases. On the other hand, the measure of inhibition activity is strictly individual for particular taxon. Secondary metabolites isolated in these extracts would require further research.

Statistical processing

Statistical processing was applied on the primary data matrix. It consisted of data for eight extract samples and fourteen parameters (variables). Results are shown in Table 2. The statistically significant values of correlation coefficients are written with bold and marked with asterisk.

From the correlation analysis it is evident that several groups of parameters significantly correlate (Table 1). Except the known (published) logical correlation between proteinases (trypsin, thrombin and urokinase), antioxidant activity parameters (FRAP and RP *vs.* DPPH and ABTS) and some composite parameters (TPF, TFL, TPFA and RA), we observed interesting correlation between antioxidant activity parameters (except ABTS) and inhibition activity on trypsin and thrombin and urokinase (respectively). This fact could be explained by properties of the polyphenolic compounds in the extract samples. These are good radical scavengers and possess relatively reactive aromatic hydroxyl groups, which serve as donors and acceptors of H-bond – key feature for inhibition mechanism in catalytic cavity of proteinase (Maliar *et al.* 2004). Similarly, correlations have broadly been published between composite parameters (TPF, TFL and TPFA) in relation to antioxidant activity by all the methods used (Adomako-Bonsu *et al.* 2017). Interestingly, we found in this study a correlation between TT and inhibition activity on trypsin, thrombin and urokinase, and also an unexpected correlation between antioxidant activity (all methods) and TPF and TFL (respectively). This could indicate the presence of thio-substituted polyphenols or flavonoids in extract samples from *Salvia* sp.

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Noteworthy is also the correlation between the content of rosmarinic acid and inhibition activity on urokinase (only one from four tested proteinases), the RA with antioxidant activity (by all methods) and also the correlation to certain composite parameters (TPF, TPFA). Surprising is a correlation of RA content with TFL and TT by this statistical method.

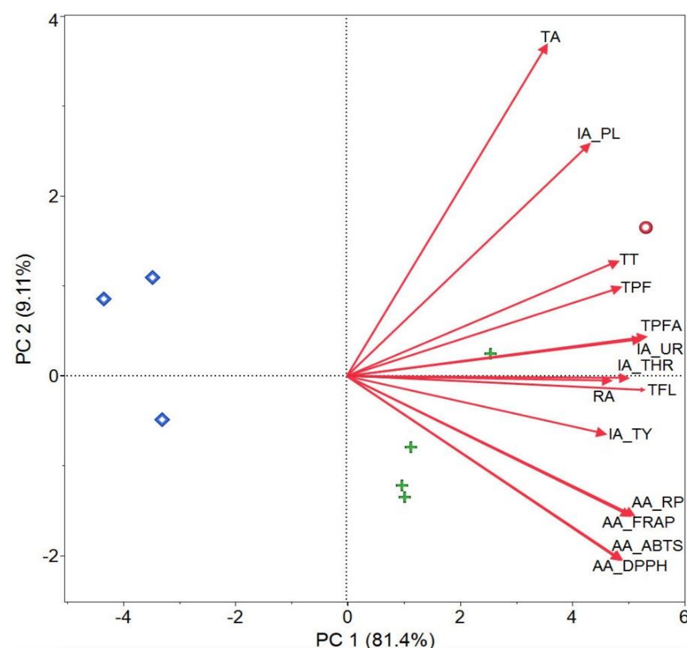

Fig. 4. Two-dimensional PCA from all primary data of eight evaluated extracts, four from *Salvia officinalis* L. of different varieties and localities (green cross), *Salvia apiana* (red circle) and extract from *Salvia divinorum* and from the both callus culture extracts (blue diamonds). Abbreviations represent metabolites or methods described in the part Experimental.

Principal Component Analysis (PCA) is a tool (method) that may provide more information about relation among parameters (variables). Two-dimensional PCA containing all primary data is given in Fig. 4. The position of each particular extract sample is represented by one point in the chart. Concerning their location it is evident that certain similarities appear between the four samples prepared from *Salvia officinalis* L. (green cross), three samples of *Salvia divinorum* and the two callus culture extracts (blue diamonds), and finally the extract sample from *Salvia apiana* presented as outlying point (red circle). From the angle of particulars vectors for each parameter it is evident the occurrence of certain relation between TPF, TPFA, IA_UR, IA_THR and RA in the first assembly and IA_TY and all four antioxidant activity parameters in the second assembly, whereas the vector for TA and IA_Pl seem to be relatively outlying. This finding differs from the conclusion of correlation analysis and suggests than rosmarinic acid possesses both proteinase inhibition activity as well as antioxidant activity. This proposal is supported by the clustering analysis (CA) of the eight evaluated extract samples and the values of 14 measured parameters (Fig. 5).

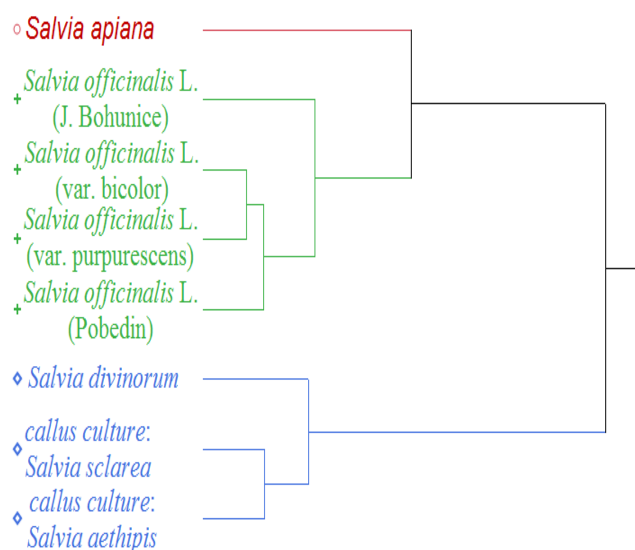

Fig. 5. Cluster analysis including all primary data of eight evaluated extracts from *Salvia officinalis* L. (green cross), *Salvia divinorum,* both callus culture extracts (blue diamonds) and extract from *Salvia apiana*. The results correspond to outputs of the PCA analysis (Fig. 4).

Cluster analysis moreover graphically presents the measure of similarity (or difference) among the extract samples from different sources. The extract from *Salvia apiana* is obviously special regarding the evaluated parameters, with best antioxidant activity, proteinase inhibition activity and content of composite parameters (Fig. 5). This extract was selected and assigned as a primary extract. Relatively homogenous is the cluster of extracts from different plants within *Salvia officinalis* L.

The extract from *Salvia divinorum* with two extracts from callus culture create an individual cluster with the lowest potent effects, though are of different nature and source.

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Conclusions

The study revealed the inhibitory activity of sage extracts from different sources on selected proteinases, which may represent a potentially pathophysiological effector in the progression of serious human diseases. Our work at first time reports that extracts of *Salvia sp*. do possess proteinase inhibition activity, in addition to being a rich source of polyphenolic acids and amines, as well as of remarkable antioxidant activity. Beside this thiols seem to be promising and active secondary metabolites in sage. Inhibition properties of RA and sage extracts on selected proteinases deserve further research.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

Adams JD, Garcia C (2005) The advantages of traditional chumash healing. Evid. Based Complement. Alternat. Med. 2: 19-23.

Adomako-Bonsu AG, Chan SL, Pratten M, Fry JR (2017). Antioxidant activity of rosmarinic acid and its principal metabolites in chemical and cellular systems: Importance of physico-chemical characteristics. Toxicol. In Vitro 40: 248-255.

Bandoniene D, Murkovic M, Petras RV (2005) Determination of rosmarinic acid in sage and borage leaves by High-Performance Liquid Chromatography with different detection methods. J. Chromatogr. Sci. 43: 372-376.

Bode W (2006) Structure and interaction modes of thrombin. Blood. Cell. Mol. Dis. 36: 120-130.

Borek TT, Hochrien JM, Irwin AN (2006) Composition of the essential oil of white sage, salvia aping. Flavour Frag. J. 21: 571-572.

Bouaziz M, Yangui T, Sayadi S, Dhouib A (2009) Disinfectant properties of essential oils from *Salvia officinalis* L. cultivated in Tunisia. Food Chem. Toxicol. 47: 2755-2760.

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of free radical method to evaluate antioxidant activity. LWT 28: 25-30.

Ellman GL (1959) Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82: 70-77.

Fan HY, Fu FH, Yang MY, Xu H, Zhang AH, Liu K (2010) Antiplatelet and antithrombotic activities of salvianolic acid A. Thromb. Res. 126:17-22.

Ferri M, Gianotti A, Tassoni A (2013) Optimisation of assay conditions for the determination of antioxidant capacity and polyphenols in cereal food components. J. Food Compos. Anal. 30: 94-101.

Fink CA, Moskal M, Firooznia F, Hoyer D, Symonsbe D, Wei D, Qia WY, Savage P, Beil M, Trapani J, Jeng AY (2000) Design and synthesis of potent thiol-based inhibitors of endothelin converting enzyme-1. Bioorg. Med. Chem. Lett. 10: 2037-2039.

Green GD, Shaw E (1979) Thiobenzyl benzyloxycarbonyl-L-lysinate, substrate for a sensitive colorimetric assay for trypsin-like enzymes. Anal. Biochem. 93: 223-226.

Habán M, Habánová M, Holovičová M, Ražná K (2019) Rosmarinic acid content in selected varieties of sage (*Salvia officinalis* L.). J. Cent. Eur. Agric. 20: 305-320.

Hamidpour M, Hamidpour R, Hamidpour S, Shahlari M (2014) Chemistry, pharmacology and medicinal property of sage (*Salvia*) to prevent and cure illnesses such as obesity, diabetes, depression, dementia, lupus, autism, heart disease and cancer. J. Tradit. Complement. Med. 4: 82-88.

Hammer KA, Carson CF, Riley TV (1999) Antimicrobial activity of essential oils and other plant extracts J. Appl. Microbiol. 86: 985-990.

Huafu W, Gordon J, Provan KH (2004) Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. Food Chem. 87: 307-311.

Jayaprakasha GK, Singh RP, Singh KK, Gáspár A, Valko M (2001) Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. Food Chem. 73: 285-290.

Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970). Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal. Biochem. 34: 595-598.

Khan, A, Najeeb-Ur-Rehman K, Alkharfy KM, Gilani AH (2011) Antidiarrheal and antispasmodic activities of *Salvia officinalis* are mediated through activation of K^+ channels. Bangladesh J. Pharmacol. 6: 111-116.

Krošlák E, Maliar T, Maliarová M, Nemeček P, Hozlár P, Ondrejovič M. Havrlentová M, Kraic J (2016) Antioxidant and protease-inhibitory potential of extracts from grains of oat. Open Chem. 14: 324334.

Krošlák E, Maliar T, Nemeček P, Viskupičová J, Maliarová M, Havrlentová M, Kraic J (2017) Antioxidant and proteinase inhibitory activities of selected poppy (*Papaver somniferum* L.) genotypes. Chem. Biodivers. 14: 176-186.

Lee SY, Abas F, Khatib A, Ismail IS, Shaari K, Zawawi N (2016) Metabolite profiling of *Neptunia oleracea* and correlation with antioxidant and α-glucosidase inhibitory activities using 1H NMR-based metabolomics. Phytochem. Lett. 16: 23-33.

Li Y, Liu H, Han Q, Kong B, Liu Q (2017) Cooperative antioxidative effects of zein hydrolysates with sage *Salvia officinalis*) extract in a liposome system. Food Chem. 222: 74-83.

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Maliar T, Jedinák A, Kadrabová J, Šturdík E (2004) Structural aspects of flavonoids as trypsin inhibitors. Eur. J. Med. Chem. 39: 241-248.

Maliar T, Slaba G, Nemeček P, Maliarová M, Benková M, Havrlentová M, Ondrejovič M, Kraic J (2015) Antioxidants, enzyme inhibitors, and biogenic compounds in grain extracts of barleys. Chem. Biodivers. 12: 1678-1695.

Mantle D, Eddeb F, Pickering AT (2000) Comparison of relative antioxidant activities of British medicinal plant species *in vitro.* J. Ethnopharmacol. 72: 47-51.

Martins N, Barros L, Santos-Buelga C, Henriques M, Silva S, Ferreira IC (2015) Evaluation of bioactive properties and phenolic compounds in different extracts prepared from *Salvia officinalis* L. Food Chem. 170: 378-385.

Meijersen M, Hoetmera AG, Lamersbl CBHW, Rovatic LC, Jansenb JBMJ, Woutersena RA (1991) Effects of the synthetic trypsin inhibitor camostate on the development of *N*-nitrosobis(2-oxopropyl)amine-induced pancreatic lesions in hamsters. Cancer Lett. 60: 205-211.

Miliauskas G, Venskutonis PR, van Beek TA (2004) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chem. 85: 231-237.

Moretti MDL, Peana AT, Satta M (1997) A study on anti-inflammatory and peripheral analgesic action of *Salvia sclarea* oil and its main components. J. Essent. Oil Res. 9: 199-204.

Murcia G, Fontana A, Pontin M, Baraldi R, Bertazza G, Piccoli N (2017) ABA and GA3 regulate the synthesis of primary and secondary metabolites related to alleviation from biotic and abiotic stresses in grapevine. Phytochemistry 135: 34-52.

Nell M, Votsch M, Vierheilig H, Steinkellner S, Zitterl-Eglseer K, Novaka ChJ (2009) Effect of phosphorus uptake on growth and secondary metabolites of garden sage (*Salvia officinalis* L.). J Sci Food Agric. 89: 1090-1096.

Oudjedia K, Manso S, Nerin C, Hassissena N, Zaidi F (2019) New active antioxidant multilayer foodpackaging films containing Algerian Sage and Bay leaves extracts and their application for oxidative stability of fried potatoes. Food Control 98: 216-226.

Pandey G, Khatoon S, Pandey MM, Ravat AKS (2018) Altitudinal variation of berberine, total phenolics and flavonoid content in *Thalictrum foliolosum* and their correlation with antimicrobial and antioxidant activities. J. Ayurveda Integr. Med. 9: 169-176.

Peana AT, Moretti MDL (2002) Pharmacological activities and applications of *Salvia sclarea* and *Salvia desoleana* essential oils. *In* Atta-ur-Rhaman (Ed.), Studies in Natural Products Chemistry, Bioactive Natural Products (Part G), Vol. 26, Elsevier Amsterdam, p. 391-423.

Rajagopal PL, Narayana Swamy VB, Kiron SS, Sreejith KR (2013) A review on anti-oxidative herbs Int. J. Pharm. Chem. Sci. 2: 1647-1650.

Rakotoarison DA, Greisser B, Trotin F, Brunet C, Dine, T, Luyckx M, Vasseur J, Cazin M, Cazin JC, Pinkas M (1997) Antioxidant activities of polyphenolic extracts from flowers, *in vitro* callus and cell suspension cultures of *Crataegus monogyna*. Pharmazie 52: 60-63.

Ravazi SMA, Cui SW, Guo Q, Ding H (2014) Some physicochemical properties of sage (*Salvia macrosiphon*) seed gum. Food Hydrocoll. 35: 453-462.

Singleton VL, Orthofer R, Lamuela-Raventós RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods Enzymol. 299: 152-178.

Scatena M, Liaw L, Giachelli CM (2007) Osteopontin: a multifunctional molecule regulating chronic inflammation and vascular disease. Arterioscler. Thromb. Vasc. Biol. 27: 2302-2309.

Slinkard K, Singleton VL (1997) Total phenol analysis: Automation and comparison with manual methods. Am. J. Enol. Viticult. 28: 49-55.

Stratil P, Klejdus B, Kubá V (2007) Determination of phenolic compounds and their antioxidant activity in fruits and cereals. Talanta 71: 1741-1751.

Vanisree M, Tsay HS (2004) Plant cell cultures-an efficient source for the production of biologically important secondary metabolites. Int. J. Appl. Sci. Eng. 1: 29-48.

Vanisree M, Lee CY, Lo SF, Nalavade SM, Lin CY, Tsay H (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue culture. Bot. Bull. Acad. Sin. 45: 1-22.

Veličković DT, Nikolova MT, Ivancheva SV, Stojanović JB, Veljković VB (2007) Extraction of flavonoids from garden (*Salvia officinalis* L.) and glutinous (*Salvia glutinosa* L.) sage by ultrasonic and classical maceration. J. Serb. Chem. Soc. 72: 73-80.

Whitcomb DC (2003) Molecular and genetic mechanisms of acute and chronic pancreatitis. Int. Congr. Ser. 1255: 49-60.

Yu ZJ, Liu S, Zhou S, Li H, Yang F, Wu L, Guo L, Li GB (2018) Virtual target screening reveals rosmarinic acid and salvianolic acid A inhibiting metallo- and serine-*β*-lactamases. Bioorg. Med. Chem. Lett. 28:1037-1042.

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