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Research Article

Antioxidant and enzyme-inhibiting activity of lyophilized extract from *Clinopodium vulgare* L. (Lamiaceae)

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Abstract

Clinopodium vulgare L. (Lamiaceae) was used in the traditional Bulgarian medicine for treatment of wounds, diabetes and gastric ulcers. The aim of the present study was to evaluate the antioxidant capacity of the extract (CVE) and fractions from *C. vulgare* (CV) using DPPH, ABTS and FRAP methods. Enzyme inhibitory activity against acetylcholinesterase, α-glucosidase and α-amylase was also investigated. Rosmarinic acid was used as a positive control. The fraction CV3 demonstrated the highest radical scavenging activity with IC_{50} values of 0.02 mg/ml (DPPH) and 0.0002 mg/ml (ABTS), as well as the strongest ferric reducing potential (FRAP) of 0.89 mM TE/mg dw. The crude aqueous-methanol extract of *C. vulgare* also showed high activity with IC_{50} values of 0.05 mg/ml (DPPH), 0.04 mg/ml (ABTS) and 0.89 mM TE/mg dw (FRAP). Moreover, CV3 demonstrated moderate α-glucosidase and α-amylase inhibitory potential.

Keywords

Introduction

Clinopodium vulgare L. (Lamiaceae) is a perennial herbaceous plant widespread in Bulgaria. Aerial parts are used in the Bulgarian folk medicine for treatment of diabetes, gastric ulcers and cancer. The genus *Clinopodium* L. consist of flowering plants, widely distributed in southern and southeastern Europe, North America, Latin America and Asia (Saltos et al. 2014). The herbal drug alleviates symptoms associated with mastitis, prostatitis, skin irritation and swelling. Previous investigations revealed a variety of beneficial effects of *C. vulgare* extracts (CVE) i.e. anti-bacterial, anti-inflammatory, antioxidant and anticancer activities (Tepe et al. 2007; Burk et al. 2009; Stefanovic et al. 2011; Batsalova et al. 2017). Recently it was established the acute and subacute toxicity of *C. vulgare* lyophilized water extract (Zheleva-Dimitrova et al. 2019)

Plants have been used for many years in the traditional medicine to treat various diseases and conditions. Over the last decades it was established that the oxidative stress is involved in the initial development of many diseases including Alzheimer's disease, (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) (Smith et al. 2007; Niedzielska et al. 2015). Reactive oxygen species

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(ROS), naturally formed during normal metabolism, can damage biological structures such as proteins, lipids or DNA. Polyphenolic compounds, naturally present in vegetal sources, can contribute to decrease the pathological influence of oxidative stress.

Based on accurate masses, MS/MS and comparison with standards, a variety of flavonoids, caffeic acid oligomers and saponins were tentatively elucidated in CVE. Rosmarinic acid (RA) was the major compound. Prevoius UHPLC-HRMS analysis revealed CVE as a new rich source of water soluble caffeic acid oligomers (Zheleva-Dimitrova et al. 2019).

In the present study we provided evidence for the antioxidant and enzyme-inhibiting effects of the investigated lyophilized extract (CVE) and fractions from *C. vulgare* (CV).

Materials and methods

Plant material

C. vulgare aerial parts were collected in July 2017 from region of German village near Sofia, Bulgaria (voucher specimen SO 107606). Air-dried powdered aerial parts (50 g) were triplicate extracted with water (500 ml) by ultrasound assisted extraction (15 min each time). A lyophilized *C. vulgare* extract (CVE) (5 g) was used for further phytochemical and pharmacological assays.

CVE fractionation and identification

1 g of the obtained crude CVE was dissolved in 5 ml of distilled water and applied to a low-bar liquid chromatographic system (Lobar, RP18, Merck). Elution was performed with 100 ml of distilled water solution and increasing concentrations of methanol (0 → 70%). Fractions of 30 ml were collected. The composition of the fractions was monitored by HPLC-UV, on an RP 18 reversed phase column and water-methanol gradient elution. Fractions of similar composition were combined. The procedure was repeated three times. 3 combinations with similar composition were selected for the present work – CV1, CV2, CV3, as well as the raw CVE. Rosmarinic acid was found to be the main compound in CVE (Zheleva-Dimitova et al. 2019). CVE, CV fractions, and the positive control RA were used for further experiments at concentrations in the range 1 μg – 1.0 mg/ ml. Main compounds in the most active fraction were identified by ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS). The method was previously described in (Zheleva-Dimitova et al. 2019) with some modifications (Gevrenova et al. 2020).

Chemicals and reagents

2,2'-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3ethylbenzothiazine-6-sulfonic acid) (ABTS), 6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), $FeCl_3 \cdot 6H_2O$, sodium acetate, potassium persulphate, acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, acetylthiocholine iodide (AChI) were purchased from Sigma-Aldrich. All the others chemicals including the solvents were of analytical grade.

Methods

Antioxidant activity measurements

DPPH method for determination of radical scavenging activity

DPPH assay was done according to the method of (Zengin et al. 2014) with some modifications. 150 μl of methanolic DPPH solution (0.005 mg/ mL) was added to 200 μl of the CVE, or the fraction of CV in various concentrations. For the control, 200 μl of pure methanol was added to 150 μl of DPPH. The mixture was shaken and left in the dark for 15 minutes. The absorbance of the resulting solutions was measured at 517 nm against MeOH. Decreased absorption indicates higher DPPH radical scavenging activity. At pronounced radical scavenging activity, the IC_{50} concentration at which the % DPPH radical scavenging activity decreased by 50% was also calculated.

ABTS radical scavenging activity

One mL of a 7 mM solution of 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was mixed with 1 ml of a 2.4 mM $K_2S_2O_8$ solution. This mixture was left in the dark for approximately 12 hours, after which 60 ml of MeOH were added to a few ml of it to obtain an absorption of about 0.7 at 734 nm. Then, 0.3 ml of this solution was added to 0.3 ml of the solution of CVE or CV fraction. The absorbance of the mixture was measured after 7 minutes at 734 nm. Mixture of ABTS and methanol served as a control. Decreased absorption due to the discoloration of the ABTS solution indicates higher radical scavenging activity. At pronounced radical scavenging activity, the IC_{50} concentration at which the % ABTS radical scavenging activity decreased by 50% was also calculated (Zengin et al. 2014).

Ferric reducing/antioxidant power (FRAP method)

Total antioxidant activity – FRAP, was conducted by the method of (Zheleva-Dimitrova 2013) with minor modifications. 25 ml of acetate buffer (pH 3.6) was mixed with 2.5 ml of 10 mM solution of 2,4,6-tripyridyl-S-triazine (TPTZ in 40 mM HCl) and 2.5 ml of 20 mM solution of $FeCl_3 \times 6H_2O$, and was heated to 37 °C. 15 μl from different concentrations of the selected fractions of *C. vulgare* and 15 μl of different concentrations of RA were placed in 96-well plates. 285 μl of FRAP solution were then added. The mixture was left in the dark and after 30 minutes the absorbance at 593 nm was measured. The results of this experiment were expressed as μM Trolox equivalent (TE)/ g (μM TE/ g).

Determination of the antioxidant activity in a linoleic acid system

Inhibition of lipid peroxidation of the CVE and CV fractions was performed by so-called ammonium-thiocyanate method (Zheleva-Dimitrova 2013), including ammonium thiocyanate, iron dichloride and linoleic acid. During lipid peroxidation, linoleic acid peroxides oxidize Fe^{2+} to Fe^{3+}. Fe^{3+} then forms with SCN^- a colored, red complex that has an absorption maximum at 500 nm. Thus, the intensity of the staining, indicated a significant process of lipid peroxidation. The decrease in absorption indicates antioxidant activity.

Methods for determining enzyme inhibitory activity

AChE inhibition assay

The acetylcholinesterase inhibitory activity of the CVE or of selected fractions of *Clinopodium vulgare* and rosemary RA acid was determined by the method of (Ellman 1961). Briefly in 96-well plate, 10 µl solution of the CVE or the fractions, 15 µl solution of AchE in phosphate buffer (0.03 U/ml) and 200 µl buffer (pH 8) were mixed. After 30 minutes incubation 15 µl solution of DTNB (0.3 mM) and 15 µl solution of acetylthiocholine iodide (1.8 mM) were aded. The mixture was incubated for another 15 minutes at room temperature and the absorbance at 403 nm was measured. Absorption was recorded on a Microplate Reader Biochrom EZ 800. Galantamine was used as a positive control.

Determination of α-glucosidase inhibitory activity

Alfa-glucosidase inhibitory activity was determined by the method described by (Zengin et al. 2014). To 50 µl of different concentrations of CVE and CV fractions 50 µl of α-glucosidase in phosphate buffer (pH 6.8) and PNPG (4-nitrophenyl-α-D-glucopyranoside) were added. The solutions were incubated for 15 minutes at 37 °C. The reaction was stopped by the addition of sodium carbonate – 50 µl, 0.2M. The absorbance of the sample and the blank were measured at 400 nm. The alfa-glucosidase inhibitory activity was expressed as millimoles equivalent of acarbose (mM ACE/ g extract).

Determination of α-amylase inhibitory activity

Alfa-amylase inhibitory activity was determined by the Caraway-Somogyi method with minor modifications (Zengin et al. 2014). 20 µl of the methanolic solution of CVE and fractions of CV – were mixed with 50 µl solution of the enzyme α-amylase in phosphate buffer (pH 6.9) and incubated for 10 minutes at 37 °C. The reaction was initialized by the addition of starch (0.05%). The control was prepared in an analogous manner by adding all reaction agents without the enzyme α-amylase. The reaction mixture was re-incubated for 10 minutes at 37 °C, then stopped by the addition of HCl (20 µl, 1 M). 100 µl solution of iodine in potassium iodide (Lugol's solution) was added. Sample and control absorption were measured spectrophotometrically at a wavelength of 630 nm. The absorbance of the blank is subtracted from that of the sample and the amylase inhibitory activity was expressed as millimoles equivalent of acarbose (mM ACE/ g extract).

The reference standard rosemary acid (RA) and butyl-hydroxytoluene (BHT) were used as positive controls.

Statistical analysis

For all the experiments all the assays were carried out in triplicate. Results were expressed as a mean ± SD (n = 3). Values of $p < 0.05$ were considered statistically significant. The differences between the groups were analyzed using one-way analysis of variance (ANOVA).

Results and discussion

Determination of antioxidant activity

Various antioxidant activity tests were performed to determine the antioxidant profile of the crude plant extract CVE and 3 different fractions. Tests based on different mechanisms were used in the research. The results for DPPH, ABTS and FRAP activity are presented in Table 1. The combined fraction CV3 showed the highest radical scavenging activity with corresponding IC_{50} values of 0.02 mg/ml and 0.002 mg/ml for DPPH and ABTS test respectively, as well as the strongest iron reducing potential FRAP 0.89 mM TE/mg dw. The crude CVE also showed high activity with IC_{50} of 0.05 mg/ ml, 0.04 mg/ml and 0.89 mM TE/mg dw for DPPH, ABTS, and FRAP respectively. However, they do not exceed the activity of the positive control – RA, which has been reported in the literature to have an effective DPPH radical scavenging activity (SC_{50} of 5.5 ± 0.2 µg/ mL) (Zhu et al. 2014). Sarikurkcu et al. (2015) studied various extracts of *C. vulgare* – aqueous, methanolic and acetone and found very good antioxidant properties for this plant species. They have shown that the aqueous extract exhibited the highest radical scavenging activity with 81.72 mg TE/g extract for DPPH test. The ABTS cation capture assay showed superiority of the methanol extract (51.45 mg TE/ g extract), which also possessed the strongest reducing activity in FRAP assay.

Inhibition of lipid peroxidation of the combined fraction CV3 and the crude CVE are presented in Figure 1. The experiment was performed within five days and the figure shows that the fraction, extract and pure rosemary acid weakly inhibit lipid peroxidation, compared to the control, containing a pure methanol. In comparison, the other positive control of BHT significantly slowed down

Table 1. DPPH, ABTS и FRAP activity of the fractions and raw extract of *Clinopodium vulgare*.

Sample	DPPH	ABTS	FRAP
	IC_{50} mg/ml	IC_{50} mg/ml	mM TE/mg dw
CV1	0.19	0.03	0.69
CV2	0.1	0.05	0.44
CV3	0.02	0.002	0.89
CVE	0.05	0.04	0.85
RA	0.0004	0.007	1.9

the formation of linoleic acid peroxides. The low activity of the extract, fraction and RA was probably due to their low lipid solubility. According to Son and Lewis (2002), the antioxidant activity of phenolic compounds depends not only on the hydroxyl groups or catechol rings, but also on the partition coefficient (log P) or hydrophobicity of the compounds. Hydrophobic antioxidants tend to show better activity. Polar fractions are more active free radical scavengers, while non-polar fractions are more effective in protecting linoleic acid peroxidation (Koşar et al, 2011). However, studies obtained by Popov et al. (2013) show that RA is significantly superior to trolox, ascorbic acid and dihydroquercetin in tests to inhibit linoleic acid peroxidation. According to the Popov study, test substances can be ranked according to their activity, in the following order: rosmarinic acid > dihydroquercetin trolox > ascorbic acid. The contradiction with our study probably could be explained with the different concentrations used in their experiments.

Evaluation of acetylcholinesterase, α-glucosidase and α-amylase inhibitory activity

In *in vitro* assays for acetylcholinesterase (AchE) inhibitory activity, the extract, fractions and pure RA did not show
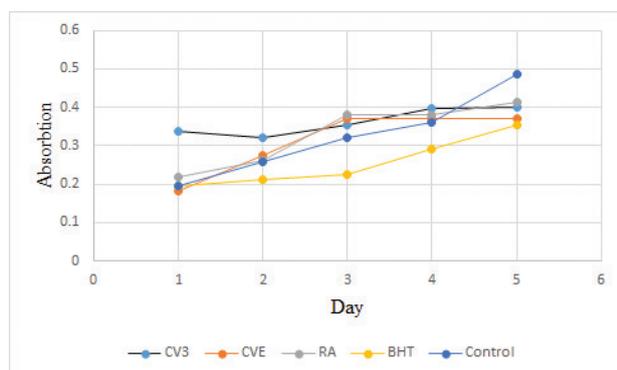
Figure 1. Inhibition of lipid peroxidation by CVE, CV3 fraction and rosmarinic acid.

inhibitory activity at the concentrations tested. Therefore, the test was performed with higher concentration (10 mg/ml) for CV3, CVE and rosemary acid, but the results also showed a lack of acetylcholinesterase inhibitory activity.

In our previous *in vivo* experiments (Zheleva-Dimitrova et al. 2019), the application of lyophilized extract of *C. vulgare*, in doses in the range of 400–2000 mg/ kg caused respiratory distress and ataxia in experimental animals, mice and rats. It is well known that selective inhibition of AChE can cause accumulation of ACh in the synaptic cleft, leading to overstimulation and disruption of nerve impulses and ultimately causing symptoms such as ataxia, central respiratory paralysis, seizures, coma and death. Therefore, we hypothesized that the aqueous metanol extract of *C. vulgare* and CV3 may inhibit acetylcholinesterase, which was not established in the present *in vitro* study. This discrepancy could be due to the different

ezyme AchE type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, used in the present *in vitro* study. In the study, performed by Sarikurkcu et al. (2015), the acetone extract of *C. vulgare* showed remarkable inhibitory activity on acetylcholinesterase and butyrylcholinesterase. There is much evidence in the literature for the inhibitory effect of rosmarinic acid on these enzymes. Gülçin et al. (2016) found that the AChE inhibitory effect was mainly related to aromatic compounds and to a lesser extent to aliphatic compounds. In their study, AChE was very effectively inhibited by rosemary acid with a Ki value of 42.52 pM. On the other hand, rosemary acid inhibits BChE with a Ki value of 121.60 pM. The lack of such an inhibitory potential of RA in our study is probably related to the concentration used or to the reduced AChE activity, used in our experiments.

On the other hand, all samples tested showed significant α-glucosidase inhibitory activity. Fraction CV3 again showed the highest activity, higher than rosmarinic acid and comparable to that of acarbose. This is also established after the calculations of the IC_{50} of the studied fractions. CV3 and CVE exhibited the most pronounced statistically significant inhibitory activity (Table 2). For rosmarinic acid, as a major component in the crude extract and one of the major in CV3, α-glucosidase inhibitory activity has been demonstrated. Zhu et al. (2014) found a significant inhibitory effect of rosemary acid extract with IC_{50} 0.23 ± 0.01 mg/ mL.

Table 2. Alfa-glucosidase inhibitory activity (IC_{50}) of CVE, CV3 and RA.

Sample	IC_{50} mg/ml
CV3	0.03
CVE	0.05
RA	0.13
Acarbose	0.027

In addition, it is likely that the other components in the composition of the crude extract and CV3 have such activity. Zeng et al. (2016) in their study on various *C. chinense* compounds demonstrated that apigenin, luteolin, ethyl rosmarinate and clinopodium acid B, isolated from this Chinese species have a potent α-glucosidase inhibitory effect with IC_{50} values ranging from 0.6 up to 2.0 μM. In our study, these compounds were also identified (Zheleva-Dimitrova et al. 2019). In the study of Sarikurkcu et al. (2015), the highest inhibitory activities for alpha amylase and alpha glucosidase were found in methanolic and aqueous extracts of *C. vulgare*.

Data on α-amylase inhibitory activity are presented in Table 3. Again, the CV3 fraction showed the highest activity, comparable to that of RA, but significantly lower than the positive control acarbose, which is comonly used drug in the treatment of diabetes type II. Our results show that CV3 and the RA reacted with porcine pancreatic amylase, inhibiting its enzymatic activity against starch as a substrate *in vitro*. A study conducted by McCue and Kalidas (2004) illustrated that α-amylase activity had decreased depending on the RA content of the extract. Ninety-seven
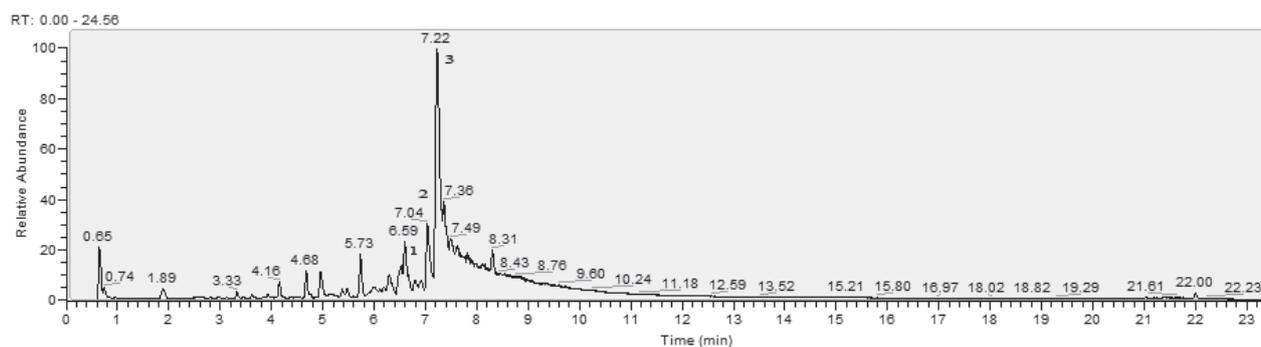
Figure 2. Total ion chromatogram of CV3 in negative ion mode; **1**- salvianolic acid A; **2**- salvianolic acid L; **3**- clinopodic acid K.

Table 3. Alfa-amylase inhibitory activity (%) of CVE, CV, and RA.

Sample	Concentration (mg/ml)	Inhibitory activity (%)
CV1	1.88	12.52
CV3	1.56	18.56
CVE	1.12	16.61
RA	1.10	17.32
Acarbose	1.29	84.78

percent (97%) RA showed the strongest reactivity, followed by lemon balm-based extract with 50% RA, and then by oregano-based extract with 7% RA. Surprisingly, the oregano-based extract with 7% RA showed amylase inhibiting activity that was only slightly less than the extract with 50% RA, suggesting that other phenolic components of the oregano extract may support the anti-amylase activity, perhaps through synergistic mechanisms. From the UHPLC-HRMS analysis of the crude extract and the most active fractions, in our study, we found a wide variety of such phenolic compounds (Zheleva-Dimitrova et al. 2019).

Yi and Lee (2018) investigated RA-rich fractions of *Orthosiphon stamineus* on α-glucosidase and α-amylase inhibitory activity. They found that the crude extract and its fractions rich in rosemary acid showed dose-dependent inhibition of both enzymes. Fractions containing higher amounts (above 50% RA) have comparable inhibitory acti-

vity to standard rosmarinic acid as an inhibitor of both enzymes. Approximately 62.50 mg/ mL and 5 mg/ mL of the richest RA fraction are sufficient to achieve almost 100% inhibition of α-amylase and α-glucosidase, respectively.

The most active fraction CV3 was analyzed by UHPLC-HRMS. Based on the retention times, MS and MS/MS accurate masses, fragmentation patterns and comparison with reference standards, the main compounds in CV3 were identified as clinopodic acid K, followed by salvianolic acid A and salvianolic acid L (Fig. 2) (Zheleva-Dimitrova 2019).

Conclusions

In conclusion, the studied extract and fractions from *C. vulgare* containing a variety of phenylpropanoids possess a moderate antioxidant and enzyme inhibitory potential that could be explored in further *in vivo* experiments

Acknowledgement

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