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Clinopodium vulgare L. (wild basil) extract and its active constituents modulate cyclooxygenase-2 expression in neutrophils

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ABSTRACT

Clinopodium vulgare L. (wild basil) has a wide range of ethnopharmacological applications and accumulates a broad spectrum of phenolic compounds, recognized for their anti-inflammatory and anticancer properties. The triggered cyclooxygenase-2 (COX-2) expression is creating an immunosuppressive microenvironment in the inflamed tissue and considered to be the main cause of failure of even new anticancer-/immune-therapies. Nowadays, selective and novel plant-derived COX-2 inhibitors with safe profile are subject of profound research interest.

This study aimed to analyze the metabolic profile of *C. vulgare* and search for phenolic molecules with potential biological properties. By application of ^{1}H and 2D-NMR (Nuclear Magnetic Resonance) profiling, caffeic, chlorogenic acids and catechin were identified along with a bunch of primary and secondary metabolites. Further, the biological effect of *C. vulgare* extract (CVE) and its constituents on zymosan-induced COX-2 expression and apoptosis of murine neutrophils have been studied. The CVE, caffeic and chlorogenic acids inhibited zymosan-induced COX-2 expression in bone marrow neutrophils, *in vitro* and *in vivo* activated. The obtained data indicate that CVE may have a good potential to manipulate neutrophil functions, however, its action may depend on the cellular state, the inflammatory milieu and the relative content of caffeic and chlorogenic acid in the extract.

1. Introduction

Clinopodium vulgare L. (wild basil; Lamiaceae) has diverse ethnopharmacological applications and hence has been used for treatment of hemorrhagic disease, ulcer, diabetes, mastitis, prostatitis and skin inflammation (Badisa et al., 2003). Contemporary studies revealed the multiple beneficial properties of *C. vulgare* aqueous or methanolic extracts (CVEs), *i.e.* anticancer, anti-inflammatory, DNA-protective, antioxidant and antibacterial properties (Burk et al., 2009). Comprehensive investigations accentuate on the selective and tissue specific anticancer activity of the extracts against vast panel of human cancer cell lines (Badisa et al., 2003). However, to date, there is scarcity of information concerning the molecular mechanisms and targets of the anti-inflammatory and anticancer activity of CVEs referenced to specific molecule or multiple constituents that formulate the activity of the extracts.

Cyclooxygenases exist in two isoforms, and while COX-1 is constitutively expressed in the cells and has housekeeping functions, COX-2 is induced by inflammatory stimuli, which in turn accelerates the synthesis of prostaglandins, and stimulates cancer cells proliferation and their metastatic potential. Therefore, COX-2 is considered as a molecular target for the development of novel and selective (natural) anti-inflammatory drugs (Chen et al., 2019; Desai et al., 2018). Many plant-derived molecules, such as caffeic and chlorogenic acid, as well

Abbreviations: BM, Bone marrow; COX-1, Cyclooxygenase-1; COX-2, Cyclooxygenase-2; CVE, *Clinopodium vulgare* extract; HO-1, Heme oxygenase-1; iNOS, Inducible nitric oxide synthase; NF-kB, Nuclear factor-kappa B; NSAIDs, Non-steroidal anti-inflammatory drugs; Nrf2, Nuclear factor erythroid 2 p45-related factor 2; MAPK, Mitogen-activated protein kinase; MPO, Myeloperoxidase; Myd88, Myeloid differentiation primary response 88; NMR, Nuclear magnetic resonance; PGE_2, Prostaglandin E2; TLR, Toll-like receptor

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as, catechin, were found to modulate COX-2 activity and immune response *in vitro* and *in vivo* through suppression of phosphorylation of MAPKs, NF-kB p65 subunit and mRNA expression (Fechtner et al., 2017; Kulabas et al., 2018; Lee et al., 2018).

The NMR-based metabolite profiling appeared an effective and unbiased approach used to extract useful analytical data for particular molecules from the spectra of complex plant extracts (Kim et al., 2010; Wolfender et al., 2013). Applied as a holistic approach in order to distinguish the possible therapeutic agents in herbal medicine, up to date, this technique is implemented in the assessment and generation of standardized biomarkers of pharmacologically active molecules essential to ensure the quality, safety and reproducibility of the natural products (Cerulli et al., 2018; Deborde et al., 2017). In our research group, NMR-based profiling and metabolomics have been intensively applied towards identifying specific marker compounds in wide variety of medicinal plant species (Georgiev et al., 2015; Marchev et al., 2017a, 2017b; Zahmanov et al., 2015).

In this study ^{1}H and 2D-NMR profiling of *C. vulgare* has been performed. In order to reveal the anti-inflammatory potential, the effect of CVE, caffeic, chlorogenic acid and catechin on the inducible COX-2 expression in neutrophils from healthy or zymosan-injected mice has been thoroughly studied.

2. Material and methods

2.1. Plant material

The *Clinopodium vulgare* L. samples were collected in 2014 from Pirin Mountains (Bulgaria) at 1144 m a.s.l. (latitude: 41° 82′65.2´´N, longitude: 23° 37′85.1´´E). The plant species was identified by Dr. Ina Y. Aneva. The collected plants were further frozen, freeze-dried (VirTis BenchTop Pro with Omnitronics™, Genevac Ltd., UK) and stored at −20 °C prior to analyses.

2.2. Preparation of Clinopodium vulgare L. extract

Aerial parts of the plant were grounded and extracted, in triplicate, with 50% aqueous methanol (1:30 w/v), in an ultrasonic bath at 35 kHz frequency (UCI-50 Raypa® R. Espinar S.L., Barcelona, Spain) for 20 min each, at ambient temperature. The combined extracts were filtrated and evaporated till dryness under vacuum at 40 °C (IKA®-Werke GmbH & Co. KG, Germany) and further used for HPLC analysis and biological activity studies.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

The NMR analysis followed the protocol, described by Georgiev et al. (2015). The *C. vulgare* freeze-dried leaf samples (6 biological replicates, 50 mg each) were grounded and placed in Eppendorf tubes. The samples were mixed with 0.75 ml CD_3OD (99.8%) and 0.75 ml D_2O (99.9%), buffered with KH_2PO_4 (pH = 6), that contains 0.01% TSPA-d4 as an internal standard. Both deuterized solvents were purchased by Deutero GmbH (Kasbellaun, Germany). Further the samples were vortexed for 1 min at room temperature, sonicated for 20 min at 35 kHz frequency and centrifuged (12 000 rpm, at 4 °C) for 20 min more. The supernatants (0.8 ml) were transferred into 5 mm NMR tubes.

The ^{1}H NMR and 2D NMR spectra (HSQC) were recorded at 25 °C on an AVII + 600 spectrometer (Bruker, Karlsruhe, Germany) operating at frequency of 600.13 MHz with relaxation time 4.07 s and CD_3OD as an internal lock.

The resulting 1D and 2D spectra were further manually phased, baseline corrected, and referenced to the internal standard TSPA at 0.0 ppm using MestReNova software (version 12.0.1, Mestrelab Research, Santiago de Compostela, Spain). The main compounds were identified according to previously published data.

2.4. High performance liquid chromatography (HPLC)

Prior to analysis the pure compounds and the *C. vulgare* extract were dissolved in 50% aqueous methanol and filtrated through 0.45 μm syringe filters. The standard solutions were prepared at concentrations from 5 to 80 μg/ml, while the extract was 5 mg/ml. Caffeic acid (purity 99.9%) and chlorogenic acid (99.8%) were purchased from Sigma (Sigma Aldrich, St. Louis, Mo, USA), while catechin (96.0%) was supplied from Fluka (Fluka AG, Buchs, Switzerland).

The analyses were performed on HPLC system, consisting of Waters binary pump, Waters dual λ absorbance detector (Waters, Milford, MA, USA) and controlled by Breeze 3.30 software. The molecules elution was performed on a reverse-phase Kinetex® C18, 100 Å (150 × 4.6 mm, 5 μm) core-shell column (Phenomenex, Torrance, CA, USA), operating at 26 °C.

Chlorogenic and caffeic acids were determined following the procedure reported by Elmastaş et al. (2017), with some slight modifications. The mobile phase consisted of acetonitrile (phase A) and water, acidified with 0.1% formic acid (phase B) at flow rate of 1 ml/min, using the following gradient: 0–5 min, A 5/B 95; 5–20 min, A 10/B 90; 20–40 min, A 15/B 85; 40–45 min, A 5/B 95. Catechin was analyzed using isocratic mobile phase – water: methanol: phosphoric acid, at ratio 85: 15: 0.1 (v/v/v) and a flow rate of 1 ml/min (Nishitani and Sagesaka, 2004). Chlorogenic and caffeic acid were detected at λ = 360 nm, while catechin was at λ = 210 nm.

2.5. Animals

All experiments were approved by the Animal Care Committee at the Stephan Angeloff Institute of Microbiology, Sofia in accordance with the National (directive 20/01.11.2012), European rules (directive 2010/63/EU) and designed on the basis of ARRIVE guideline for animal research. ICR mice were purchased from the Slivnitza Experimental Animal Laboratory (Slivnitza, Bulgaria). Animals were housed in specific-pathogen-free (SPF) Animal Facility (license No 352/30.01.2012; registration No 11130005 issued by National Food Agency) at temperature 25 ± 2 °C, humidity 50–60%, 12 h light/dark cycle and fed with a standard chow diet and water *ad libitum*.

The ICR mice (female, 6 week-old, 25–26 g) were intraperitoneally (i.p.) injected with 1 mg/g body weight of zymosan A (ZY) from *Saccharomyces cerevisiae* (Sigma-Aldrich, Munich, Germany) or with equal amount of endotoxin-free phosphate buffer saline (PBS) in the control group. After 24 h mice were sacrificed and femur and tibia were collected as described before (Benigni et al., 2017).

2.6. Preparation of bone marrow (BM) suspension and cell purification

Bone marrow was collected by flushing of femur and tibia of mice with endotoxin-free PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Munich, Germany). Cells were centrifuged at 250 *x* g (Hettich benchtop rotor centrifuge, model Rottina 380, Tuttlingen, Germany) and washed twice with RPMI medium (Sigma-Aldrich, Munich, Germany) containing 10% fetal calf serum (FCS, Sigma-Aldrich, Munich, Germany). Neutrophils were purified by Percoll density centrifugation as previously described (Milanova et al., 2014; Swamydas et al., 2015).

2.7. Cell apoptosis

Bone marrow neutrophils from PBS-injected mice (PBS group) or ZY-injected mice (ZY group) were cultured for 18 h in the presence of increasing concentrations of CVE (10, 100, 1000 and 10 000 ng/ml). The cells were harvested, washed twice with PBS, re-suspended at concentration 1×10^5/ml in 2% FCS/PBS and subsequently incubated with antibodies against mouse Ly6G (clone 1A8; allophycocyanin (APC)-conjugated, Biolegend) and CD11b (clone M1-70; phycoerythrin

Table 1
Chemical shifts (δ) and coupling constants (J) of *C. vulgare* metabolites, identified by relevant ^{1}H and 2D-NMR spectra (Georgiev et al., 2011; Marchev et al., 2017a; Wolfender et al., 2013).

Group of compounds	Metabolite	Chemical shift (ppm)	Multiplicity/coupling constant (Hz)
Amino acids	Alanine	1.48	(d, J = 7.2)
	Glutamine	2.12/2.45	(m)/(m)
	Glutamate	2.07/2.36	(m)/(m)
	Valine	1.00/1.06	(d, J = 7.3)/(d, J = 7.0)
Sugars	α-Glucose	5.18	(d, J = 3.8)
	β-Glucose	4.58	(d, J = 7.8)
	Sucrose	5.41/4.17	(d, J = 3.8)/(d, J = 8.8)
Organic acid	Acetic acid	1.99	(s)
	Formic acid	8.45	(s)
	Fumaric acid	6.51	(s)
	Malic acid	2.81	(dd, J = 16.9; 8.2)
	Citric acid	2.74/2.56	(d, J = 16.9)/(d, J = 17.6)
Phenolic acids	Caffeic acid	7.11/6.87/7.02/7.51/6.30	(d, J = 2.1)/(d, J = 8.3)/(dd J = 8.3, 2.1)/(d, J = 16.0)/(d, J = 16.0)
	Chlorogenic acid	7.11/6.87/7.02/7.51/6.30/5.18	(d, J = 2.1)/(d, J = 8.3)/(dd, J = 8.3, 2.1)/(d, J = 16.0)/(d, J = 16.0)/(m)
	Rosmarinic acid	7.11/6.87/7.02/6.83/6.77/6.71	(d, J = 2.0)/(d, J = 8.3)/(dd, J = 8.3, 2.1)/(d, J = 2.0)/(d, J = 8.1)/(dd, J = 8.3, 2.0)
Flavonoids	Apigenin	6.68/6.33/6.54/7.89/7.02	(s)/(d, J = 3.3)/(d, J = 2.2)/(d, J = 8.9)/(d, J = 8.3)
	Kaempferol	6.33/6.54/7.98/7.02	(d, J = 3.3)/(d, J = 2.2)/(d, J = 8.9)/(d, J = 8.3)
	Catechin	4.66/4.11/2.81/2.54/5.99/6.01/ 6.91/6.87/6.71	(d, J = 7.8)/(td, J = 7.5; 5.5)/(dd, J = 16.5; 5.5)/(dd, J = 16.5; 7.5)/(d, J = 2.0)/(d, J = 2.0)/(d, J = 2.0)/(d, J = 8.3)/(dd, J = 8.1; 2.0)
Others	Choline	3.21	(s)
	Adenine	8.22	(s)
	Inositol	3.94/3.61/3.23	(m)/(m)/(m)

(PE)-labelled, Biolegend) for 15 min at 4 °C. Then the neutrophils were washed and incubated with FITC-labelled Annexin V (5 µl/sample) in binding buffer (Abcam, Cambridge, UK) for 15 min at ambient temperature. The cells were centrifuged at 200 *x* g and re-suspended in 500 µl of binding buffer. The samples were analyzed by flow cytometer (BD LSR II) using BD FCSDiva v6.1.2 Software (Becton Dickinson GmbH, San Jose, CA, USA).

2.8. Intracellular detection of COX-2

The BM cells collected from healthy or PBS-injected mice were used in the experiments for *in vitro* and *in vivo* neutrophil activation. Neutrophils purified from BM of ZY group were used in experiments for *in vivo* activation. Following purification the cells were re-suspended at concentration of 1×10^6/ml in 10% FCS/RPMI and were cultured for 4 h with the CVE or its constituents (caffeic acid, chlorogenic acid and catechin) at doses 10, 100, 1000 and 10 000 ng/ml or with the vehicle 0.03% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Munich, Germany) and where indicated were stimulated with zymosan. For optimization of the COX-2 production the purified neutrophils were re-stimulated for 2 h with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Munich, Germany) in the presence of monensim (2 µM; Sigma-Aldrich, Munich, Germany). The control group in the experiment was stimulated with LPS (100 ng/ml; Maloney et al., 1998). The cells were stained with APC-labelled antibody against Ly6G (marker for neutrophils) or isotype control and fixed with 4% paraformaldehyde (PFA, Merck KGaA, Darmstadt, Germany)/PBS. Intracellular flow cytometry was performed after permeabilization for 10 min at ambient temperature with 0.1% Triton X-100 in PBS, blocking of unspecific binding for 15 min at ambient temperature with 2% bovine serum albumin (BSA; Sigma-Aldrich, Munich, Germany) in PBS/0.5 mM EDTA, probing with polyclonal rabbit antibody against COX-2 (PA5-17614; Invitrogen, Waltham, MA USA) or control antibody at a dilution 1:80 for 1 h at 4 °C, washing with PBS, and staining with FITC-labelled anti-rabbit antibody (Biolegend, San Diego, USA) diluted 1:500 for 30 min at 4 °C. After washing with PBS, samples were analyzed by flow cytometry. COX-2 expression was presented as mean of fluorescence and was extrapolated versus background of control antibody. In some experiments COX-2 positive cells were evaluated in live mature neutrophils determined as Annexin V negative Ly6G + CD11b + upon gating (Dimitrova et al., 2012) in order to avoid the apoptotic effect of the higher dose of CVE.

2.9. Flow cytometry data analysis

After acquisition of at least 40 000 cell counts/sample and live/dead cell discrimination, data were analyzed with a BD FACSDiva v6.1.2 Software (Becton Dickinson GmbH, San Jose, CA, USA) and Flowing Software 2.2 (Cell Imaging Core, Turku Centre for Biotechnology, Turku, Finland).

2.10. Statistical analyses

Statistical analysis was accomplished by using InStat3.0 and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± standard deviation (SD). The groups variations were analyzed by one-way analysis of variance (ANOVA) test or two-tailed paired Student's *t*-test and were considered significant when $P < 0.05$.

3. Results

3.1. Metabolite profiling of C. vulgare

The phytochemical characterization of *C. vulgare* extract has been performed by ^{1}H NMR and HSQC profiling. Some distinctive for *C. vulgare* secondary metabolites, such as phenolic acids and flavonoids were detected in the CVE. According to the ^{1}H NMR spectral data the most abundant signals corresponded to caffeic, chlorogenic acid and catechin. In the aliphatic and the aromatic regions the signals of some organic acids (acetic, formic, fumaric, malic and citric acids) and amino acids (alanine, glutamine and valine) were detected, while the metabolites identified in the carbohydrate region were mainly α-, β-glucose and sucrose (Table 1).

The structures of the phenolic compounds were further confirmed by the proton-carbon single bond correlations observed in the HSQC spectra (Fig. 1) and compared with the reported data (Mohamadi et al., 2015; Wang et al., 2013; Znati et al., 2014).

Caffeic and chlorogenic acids were identified according to the three aromatic protons at δ_H 6.87–7.11 and the two trans olefinic protons at δ_H 6.30 and 7.51 (d, J = 16.0) indicating the presence of (*E*)-caffeic moiety. On the other hand the methylene protons at δ_H 5.18 (m) and the corresponding carbon resonance at δ_C 76.04 confirmed the presence of the quinic acid moiety of chlorogenic acid (Mohamadi et al., 2015).

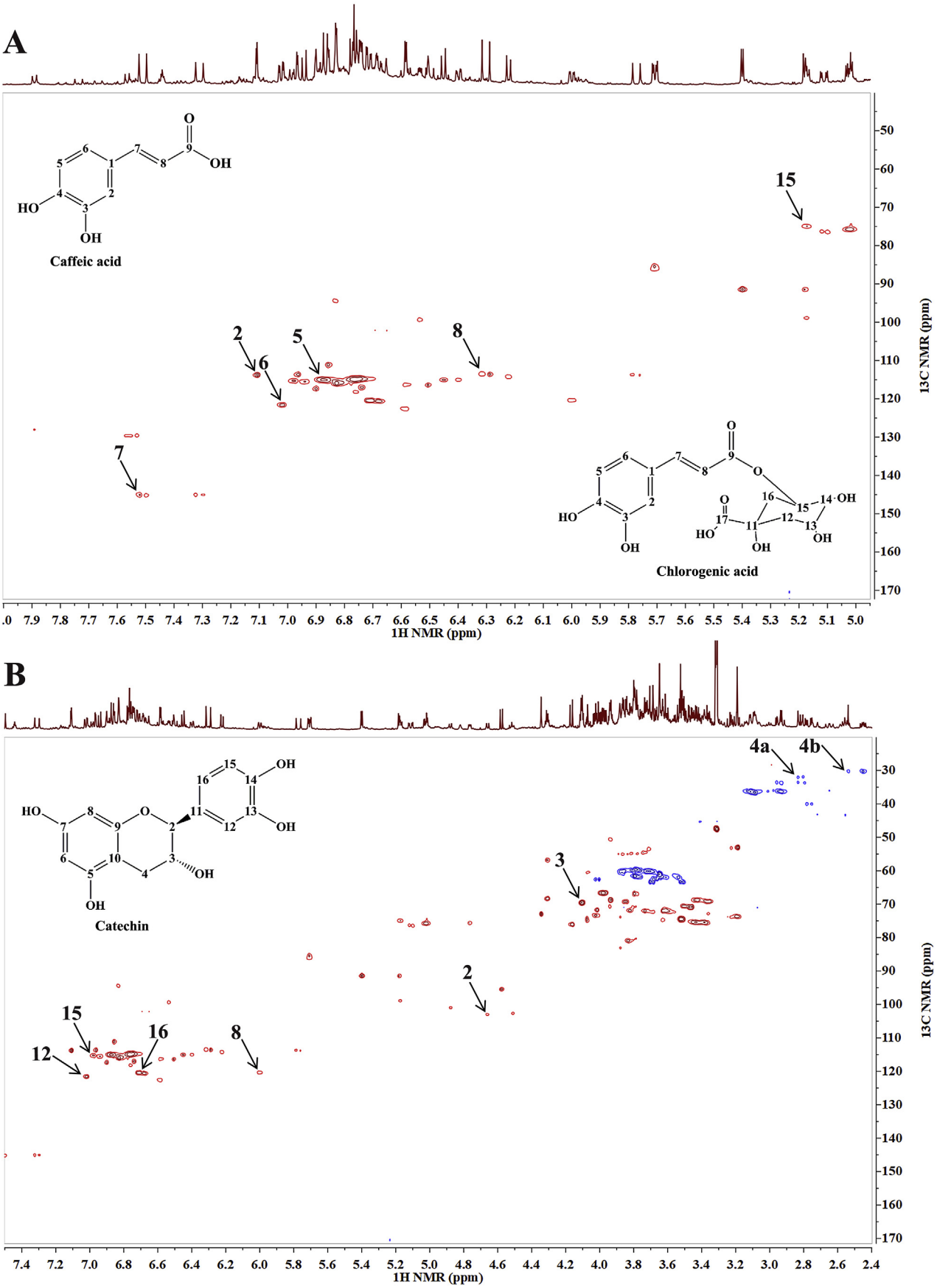

Fig. 1. Heteronuclear single quantum coherence spectroscopy (^{1}H-^{13}C HSQC) spectra of *C. vulgare* extract and the characteristic signals of A: chlorogenic and caffeic acids, and B: catechin.

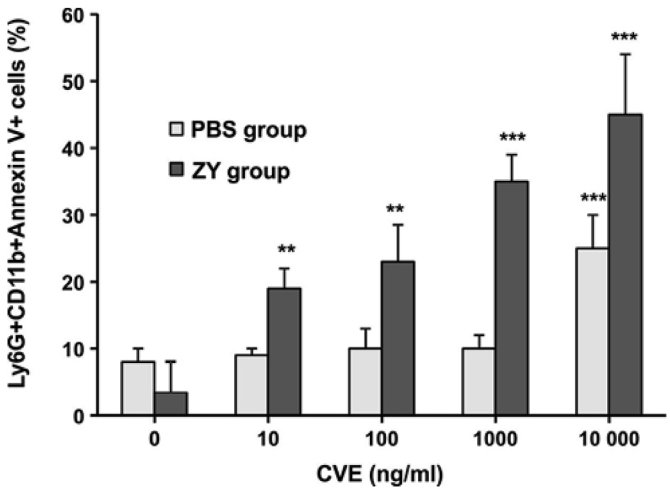

Fig. 2. Effect of CVE on apoptosis of bone marrow (BM) neutrophils from PBS-or ZY-injected mice. The data represent mean ± SD of 2 experiments involving 4 mice. ***P < 0.001, **P < 0.01 *vs* control groups incubated with the vehicle 0.3% DMSO (labelled as 0), Student t-test.

In line with previous reports, the signals of 15 carbons (seven quaternary, seven tertiary and one secondary) and nine protons were identified. Seven protons were tertiary, five of which constitute aromatic cyclic protons, *e.g.* H-6 (δ_H 5.99/δ_C 114.18), H-8 (δ_H 6.01/δ_C 121.37), H-12 (δ_H 6.91/δ_C 117.25), H-15 (δ_H 6.87/δ_C 114.83) and H-16 (δ_H 6.71/δ_C 120.36), two nonaromatic, *e.g.* H-2 (δ_H 4.66/δ_C 103.07), H-3 (δ_H 4.11/δ_C 96.77) and the other two secondary protons, *e.g.* H2-4a,b (δ_{H4a} 2.81, δ_{H4b} 2.54/δ_C 31.92) indicated the methylene group (Wang et al., 2013).

Further, the identified chlorogenic acid, caffeic acid and catechin in the CVE were quantified by HPLC and resulted to 618.90 ± 6.1, 2286.10 ± 88.7 and 34.67 ± 1.7 μg/g dry extract, respectively.

3.2. CVE increased, in a dose-dependent manner, in vitro apoptosis of bone-marrow neutrophils from zymosan-injected mice

In the first experimental settings, the effect of CVE on apoptosis of neutrophils from PBS-injected mice or mice injected with zymosan has been determined (Fig. 2). Mature BM neutrophils express highly CD11b as the integrin receptor is involved in neutrophil maturation, activation, mobilization, senescence and apoptosis (Benigni et al., 2017). Neutrophils from the ZY group were pre-activated and showed higher CD11b expression than neutrophils from the control group (mean fluorescence intensity; MFI) of 18 134 in the ZY group *vs* 9567 in the PBS group). In the population of freshly isolated cells, early apoptotic Annexin V+ neutrophils were 5.5 ± 1.5% (n = 8) in the PBS group and 5.8 ± 1.2% (n = 8) in the ZY group. After culturing for 18 h the apoptosis of Ly6G + CD11b + cells increased twice in the PBS group and 1.1 times in the ZY group suggesting that pre-activated mature neutrophils from ZY mice might be more resistant to apoptosis than neutrophils from PBS mice (Fig. 2). The CVE at concentrations of 10, 100 and 1000 ng/ml did not alter the percentage of Annexin V+ mature neutrophils from PBS mice. Neutrophils from the ZY-injected mice, however, showed significantly elevated apoptosis in the presence of CVE (Fig. 2) probably due to an interference of CVE constituents with pro- or anti-apoptotic pathways, activated in ZY neutrophils but not in PBS cells. At the highest concentration the CVE (10 000 ng/ml) accelerated significantly the apoptosis in both the control and the ZY groups.

3.3. Effect of CVE on zymosan-induced COX-2 expression in in vitro activated neutrophils

Neutrophils isolated from the PBS-injected mice were *in vitro* stimulated with increasing concentration (10, 100, 1000 and 10 000 ng/ml) of zymosan in the presence of 1000 ng/ml CVE (the apoptosis of neutrophils was 7–10%). Zymosan increased in a dose-dependent manner COX-2 expression (Fig. 3). By comparison to the vehicle, the CVE inhibited significantly the MFI for COX-2 in the ZY-stimulated group.

In the next set of experiments the purified neutrophils were stimulated with 100 ng ZY/ml in the presence of increasing concentrations of CVE (10, 100, 1000 ng/ml) and its constituents (Fig. 4). We have observed that CVE decreased in a dose-dependent manner COX-2 expression in zymosan-stimulated neutrophils. Similar effect on zymosan-induced COX-2 expression was observed by caffeic and chlorogenic acid, while catechin increased in a dose-dependent manner the intracellular COX-2 on purified neutrophils (Fig. 4).

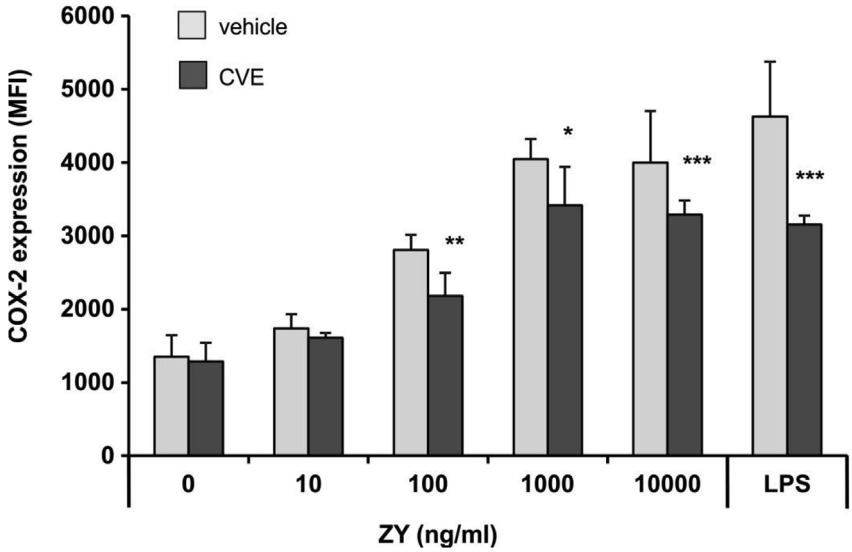

Fig. 3. Effect of CVE on COX-2 expression in neutrophils *in vitro* stimulated with zymosan. Data represent mean ± SD of MFI from 4 samples. ***P < 0.001, **P < 0.01, *P < 0.05 *vs* vehicle, ANOVA test.

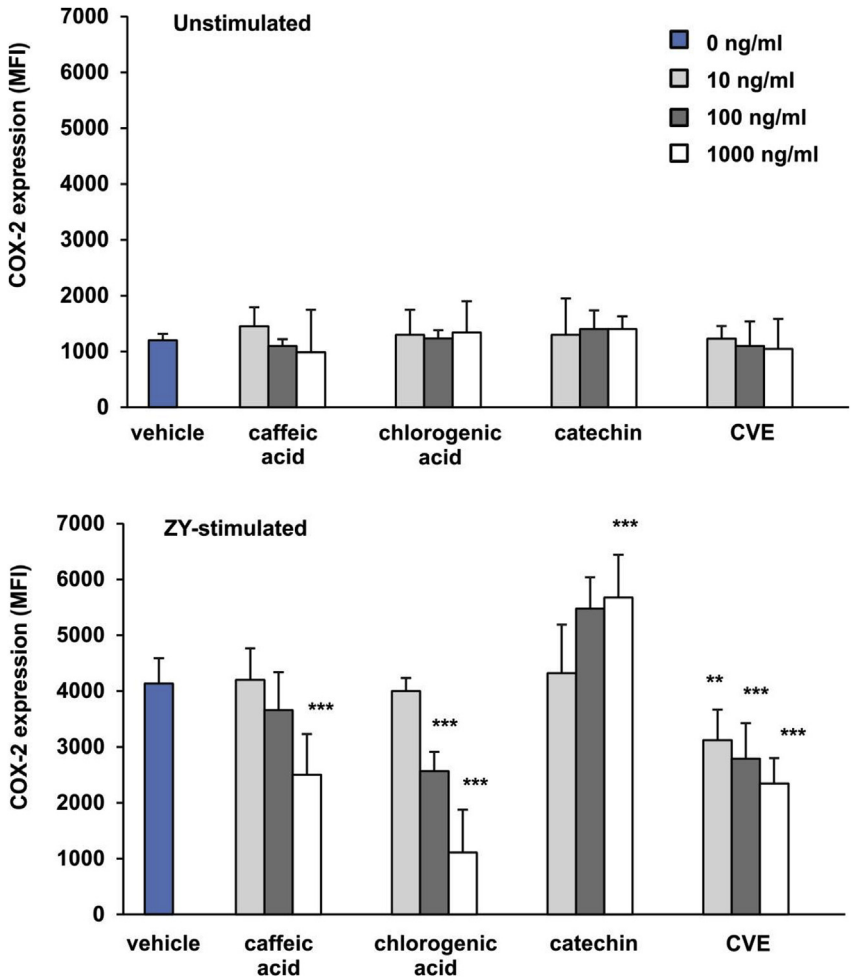

Fig. 4. Effect of the CVE and its constituents on zymosan-induced COX-2 expression in neutrophils. Data represent mean ± SD of MFI from 3 experiments. ***P < 0.001, **P < 0.01, *P < 0.05 *vs* vehicle group, ANOVA test.

3.4. Effect of CVE and its constituents on COX-2 expression in neutrophils from PBS− and ZY-injected mice

Neutrophils were purified from BM of the PBS and ZY-injected mice and were incubated with CVE, its constituents (100 ng/ml) or vehicle 0.03% DMSO for 6 h. The ZY-group showed markedly reduced frequency of Ly6G + COX-2+ cells by CVE, caffeic and chlorogenic acid and significantly increased frequency of COX-2+ neutrophils by catechin (Fig. 5).

4. Discussion

The up-regulation of COX-2 expression creates an immunosuppressive microenvironment in the cells, which in turns could reduce the effectiveness of the immunotherapies (Maturu et al., 2017). The non-steroidal anti-inflammatory drugs (NSAIDs), *i.e.* ketoprofen, naproxen sodium or ibuprofen, are among the most frequently used for suppression of PGE_2 and COX-2. In spite of the inflammation relief effect, most of the NSAIDs inhibit also COX-1 enzyme and COX-1-derived prostaglandins, causing gastrointestinal and cardiovascular complications (Thakur et al., 2018). Thus, safe and effective alternatives for treatment of inflammation processes using plant-derived molecules are being continuously sought (Koeberle and Werz, 2018).

In the current study a metabolite profiling of *C. vulgare* by 1D and 2D-NMR spectroscopy has been performed. According to the obtained data the signals of caffeic, chlorogenic acid and catechin have been found abundant; the presence of these molecules is a particular feature for *C. vulgare*. The CVE, as well as the identified phenolic molecules, have been considered as potential anticancer and anti-inflammatory agents of plant origin (Burk et al., 2009; Kulabas et al., 2018; Lee et al., 2018). Hence, further the effect of the CVE, caffeic, chlorogenic acid and catechin on inducible COX-2 expression in neutrophils from healthy or zymosan-injected mice has been studied.

The obtained results from the apoptosis study of the control group revealed that CVE extract did not induce changes in the apoptosis rates at concentrations up to 1000 ng/ml. However, in the *in vivo* pre-activated neutrophils from the ZY group the CVE dose-dependently elevated the percentage of apoptosis, probably due to an interference of CVE constituents with pro- or anti-apoptotic pathways triggered in the pre-activated ZY neutrophils. Various pathways and states of activation may determine the neutrophils' sensitivity to drug-induced apoptosis (Dimitrova et al., 2019). The pro-apoptotic mechanisms of CVE's action might be similar to those of *Clinopodium chinense* extract (Li et al., 2013; Zhu et al., 2018). It has been shown that *C. chinense* extract affected the intrinsic survival pathway, regulated by Bcl-2 (Li et al., 2013). The protein sustains neutrophil longevity and prevents constitutive apoptosis by inhibiting pro-apoptotic Bax and Bak proteins (Edwards et al., 2004). Other mechanism, described after the exposure of cells to *C. chinense* extract, was related to decreased level of p65 subunits of NF-kB that, in turn, restricted the transcription of survival proteins XIAP and A1, and hence activated caspases-3 and -9 (Fox et al., 2010; Zhu et al., 2018). The constituents of the CVE can potentiate the zymosan-induced pro-apoptotic pathways *via* interference with NF-kB signaling, mitochondrial dysfunction and caspase-3 activation (Watabe et al., 2004)

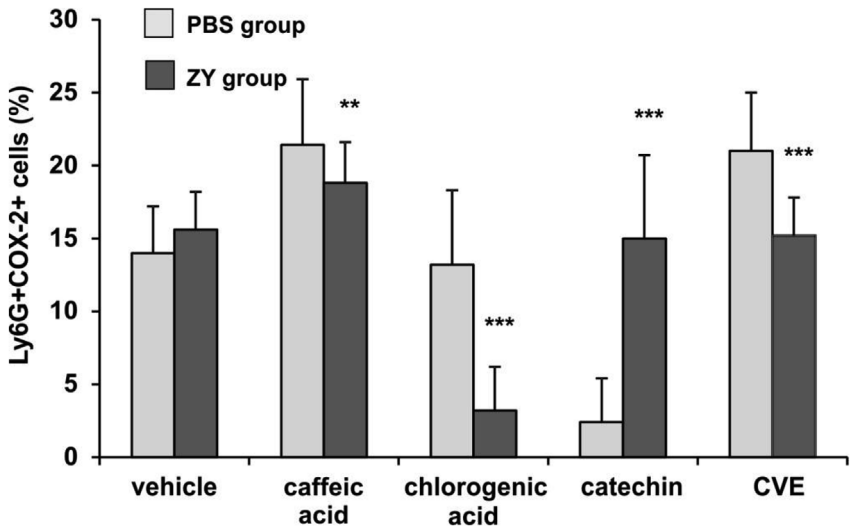

Fig. 5. Effect of CVE and pure compounds on the COX-2 production in Ly6G + neutrophils. Data represent mean ± SD of live Ly6G + COX-2+ cells from 5 mice per group. ***P < 0.001, **P < 0.01 *vs* vehicle, ANOVA test.

or *via* regulation of the expression of the death-associated protein kinase 2 (DAPK2), important for neutrophil development (Britschgi et al., 2008). However, the exact mechanisms involved in the interference of the CVE with pro-apoptotic pathways within inflammatory conditions, but not in naïve cells, need further investigation. The obtained results from the Annexin V+ assay permitted us to consider safe and proceed in the subsequent experiments with doses of the CVE and its constituents of up to 1000 ng/ml.

The CVE inhibited COX-2 expression in neutrophils induced by ZY *in vitro*. Among the studied pure compounds, caffeic and chlorogenic acids exhibited similar rates of effectiveness, thus considering them as the main contributors of the observed extract bioactivity. The levels of inhibition strongly correlated with the dose used, being the most effective at 1000 ng/ml. Caffeic acid has been considered as the active phytochemical in plant extracts, caffeic acid derivatives and was used in development of novel hybrid molecules that inhibit the expression of COX-2 (Kulabas et al., 2018). The biological effect of caffeic acid might be related to suppression of COX-2 protein at mRNA levels (Lee et al., 2018; Michaluart et al., 1999), inhibition of NF-kB pathway (Cheng et al., 2018) or activation of HO-1 pathway (Choi et al., 2018).

The pre-activated neutrophils isolated from ZY-injected mice responded to chlorogenic acid at 100 ng/ml with strongly decreased COX-2 intracellular levels and reduced number of COX-2+ cells. Similar data has been observed in LPS-activated RAW 264.7 macrophages where chlorogenic acid inhibited the mRNA expression and the *in vitro* activity of COX-2 (Guan et al., 2014). One speculative mechanism of the chlorogenic acid effects involves modulation of the myeloperoxidase-dependent lipid peroxidation (Zhang et al., 2002) and the p38 MAPK signaling pathway (Uchida, 2017). In an experimental model of osteoarthritis in rats and human chondrocytes, chlorogenic acid inhibited the COX-2/PGE_2 expression *via* the p65 NF-kB and I-kBα pathways (Lee et al., 2018; Liu et al., 2017). In neutrophils, in a similar model of inflammation as the described in this study, chlorogenic acid decreased the LPS-induced shock by interruption of MyD88-dependent early cascade triggered *via* TLRs (Park et al., 2015).

Although the CVE decreased COX-2 expression, catechin has an opposite activity on COX-2 levels and frequency of the Ly6G + COX-2+ cells. This result seems not to be surprising since pure catechin has rather demonstrated to possess COX-1 inhibitory activity and has even been combined with COX-2 inhibitors for treatment of bladder (Mohseni et al., 2004) and human breast cancer (McFadden et al., 2006). The COX-1 plays an important role in housekeeping, such as protection of gastric mucosa, regulation of gastric acid synthesis and maintenance the normal functions of the kidney by stimulating prostaglandins. Although the inhibition of the COX-1 isoform may cause gastric ulcer formation and bleeding, natural catechins are considered safe and unsubstantially affect normal tissue (Kemberling et al., 2003). In spite that some catechin-containing plant extracts had COX-1 and COX-2 inhibitory activity, the observed effect was rather due to the presence of rutin as a dominant molecule in the extracts (Gabr et al., 2018). The lack of COX-2 suppression in our study could also be explained by the molecule structure and its concentration used. For instance, the galloyl esters of the catechins had higher inhibition on COX enzymes (Seeram et al., 2003). Combination of catechin, baicalin and β-caryophyllene, at concentration of 1000 or 10 000 ng/ml for each compound, inhibited both COX isoforms (Yamaguchi and Levy, 2016).

In the current study catechin increased COX-2 expression induced by zymosan, a molecule that triggers TLR2-dependent signaling pathway in neutrophils. This finding is in contrast with other studies, where catechin was found to have inhibitory effect on neutrophil function, including cytokine production, iNOS synthesis, MPO release, IL-6 migration activity and the mRNA level of Nrf2 (Marinovic et al., 2015). The observed difference could be due to several reasons, *e.g.* Marinovic et al. (2015) performed their study in human neutrophils, which may differ in responsiveness and sensitivity from murine cells, secondly neutrophils were stimulated with LPS, which strongly triggers TLR4 and partially TLR2 signaling, and finally the applied concentrations of catechin were from 2 to 30 μM, while in the study presented here catechin was used at 100 nM. However, in the current study similar combination of the investigated molecules, as described above, or concentrations would fall into the apoptotic doses for the neutrophils. For that reason, further investigations are necessary to determine the role of catechin in modulating the neutrophils function.

5. Conclusions

The metabolite profiling of *C. vulgare* revealed the presence of some distinctive for the genus phenolic compounds, of which caffeic, chlorogenic acid and catechin were identified as the most abundant ones from the secondary metabolites pool. Their structures have been confirmed by the relevant ^{1}H- and HSQC-NMR spectra.

The CVE, caffeic and chlorogenic acid effectively inhibited zymosan-induced COX-2 expression in bone marrow neutrophils, *in vitro* and *in vivo* activated. On the other hand catechin stimulated COX-2 expression in both cases. The caffeoyl moiety in the structure of both phenolic acids eventually appeared to be essential for their anti-

inflammatory activity.

The obtained data indicated that CVE may have a good potential to manipulate neutrophil functions, however, its action may depend on the cellular state, the inflammatory milieu and the relative content of caffeic and chlorogenic acid. The anti-inflammatory activity of the CVE and its constituents indicate that they may be promising candidates in the treatment of immune diseases. The *C. vulgare* could serve then as a potential source of novel anti-inflammatory plant-derived molecules, however, further question that needs to be addressed is the mechanism of activity of chlorogenic and caffeic acid, as well as, to clarify the role of catechin in the modulation of the neutrophils function.

Conflicts of interest

The authors declare that the present work is not a subject of any potential conflict of interest.

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