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Original Research

Possible Preventive Effect of *Ziziphora clinopodioides* Lam. Essential Oil on Some Neurodegenerative Disorders

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Abstract

The present article describes some characteristics of the effect of essential oil (EO) extracted from *Ziziphora clinopodioides* harvested from Armenian highlands on microglial cell lines (BV-2 wild-type (WT) and acyl-CoA oxidase1 (ACOX1)-deficient (*Acox1*$^{-/-}$) cells). The mutant cell line was used as a model to investigate cellular oxidative damage following EO treatment. The main components of the tested EO were pulegone, isomenthone, 1,8-cineole, piperitone, and neomenthole, with concentrations of 42.1%, 9.7%, 8.22%, 7.35%, and 5.9%, respectively, in plants harvested from the high-altitude Armenian landscape. The IC_{50} value of the EO in the DPPH assay was 7.025 µL/mL. The sub-cytotoxic concentrations (based on the MTT assay) for both cell lines were 5×10^{-1} µL/mL. The catalase activity of the WT cells was decreased following 24-h treatment with the EO, but that of *Acox1*$^{-/-}$ BV-2 cellswas increased. ACOX1 activity was decreased (up to 49%) at 72hof treatment. These results show the protective effect of the tested EO on Acox1$^{-/-}$ mutantcells.

Keywords

Ziziphora; pulegone; microglia; catalase; acetyl-CoA oxidase 1; cytotoxicity

1. Introduction

Organic substances produced by plants are structurally and functionally diverse metabolites that are not only important for the adaptation and survival of plants but are also beneficial to humans in several aspects. These primary and secondary metabolites play many different roles in plant growth and development and in response to external factors [1-3]. Plants of the Lamiaceae family are the most diverse and widespread in terms of ethnomedicine and are among the most thoroughly studied ones. These plants possess a broad spectrum of biological activity, including antibacterial and antioxidant activities [4-7]. *Ziziphora clinopodioides* Lam. is a widely distributed perennial aromatic plant belonging to this family. It has been used in folk medicine since ancient times [8]. The medicinal value of this plant is mainly based on the yield of essential oil (EO) and its chemical composition [8, 9]. The main components of this plant EO are thymol, pulegone, menthone, isomenthone, 1,8-cineole, and piperitone. According to the literature, all these metabolites possess antibacterial and antioxidant activities [4, 9, 10]. Benabdallah et al. (2018) showed the acetylcholinesterase inhibitory activity of pilegone, one of the main compounds of the investigated EO [11]. Sedighi et al. (2019) reported the neuroprotective effect of *Z. clinopodioides* hydroalcoholic extracts on a rat model of Alzheimer's disease [12]. Other studies have also provided evidence of the neuroprotective effect of different extracts of this plant and their individual components based on their antioxidant activity [13, 14].

On the basis of the abovementioned findings, we aimed to investigate the effect of the *Z. clinopodioides* EO components on the activity of antioxidant enzymes of the specialized central nervous system macrophages, namely microglial cells, which play a role in brain development regulation. For this purpose, the following two neuronal microglial cell lines were used: BV-2 wild-type (WT) cells and acyl-CoA oxidase 1 (ACOX1)-deficient (*Acox1*$^{-/-}$) cells [15, 16]. The mutant cells show accumulation of very long-chain fatty acids (VLCFAs) and generation of proinflammatory cytokines, revealing their promising application as a model to investigate changes in peroxisomal β-oxidation on oxidative stress, inflammation, and cellular oxidative damage [17, 18]. The selected cell models meet three essential criteria for their application: they are well studied, are easy to cultivate, and play a central role in the development of oxidative stress-induced changes.

2. Materials and Methods

2.1 Plant Material

Z. clinopodioides plants were collected during the flowering season (July 2017–2019, Kotayk region, 1500–1600 m a.s.l.). The plants were identified at the Institute of Botany, National Academy of Sciences of Armenia, Yerevan, Armenia, and a voucher specimen number was provided. Plant samples are included in the herbarium of the same department and are available upon request.

2.2 Extraction of EO

EOs were extracted from air-dried plant material (aerial parts alone) by hydro distillation using a Clevenger-type apparatus as described previously [19].

2.3 Determination of Radical Scavenging Activity

Free radical scavenging activity of the tested EO was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [20]. Catechin was used as a positive reference. The sample solution contained 125 µL (1 mM) DPPH, 375 µL ethanol, and 500 µL of test solution (EOs or catechin at different concentrations). In the control solution, the test solution was replaced with ethanol. The absorbance was measured at 514 nm [21].

2.4 Determination of EO Chemical Composition

Gas chromatography (GC)-mass spectrometry (MS) analysis of the EOs was performed using a Hewlett-Packard 5890 Series II gas chromatograph as described previously [21, 22].

2.5 Cell Cultures

The test cell lines (BV-2, *Acox1*$^{-/-}$mutants and WT cells) were provided by the Laboratory BioPeroxIL: Laboratoire de Biochimie du Peroxysome, Inflammation et Métabolisme Lipidique, Université de Bourgogne, Dijon, France.

2.6 BV-2 Microglia Cell Culture

Murine microglial BV-2 cell lines (BV-2, *Acox1*$^{-/-}$mutants and WT cells) were cultivated as described previously [23].

2.7 MTTAssay

Cell proliferation (mitochondrial activity) was measured by 3-(4,5-dimethyltrazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded onto 96-well plates and then treated with various concentrations of *Z. clinopodioides* EO (50 to 5×10^{-4} µL/mL) for 24–72 h [23]. This test was performed to clarify the effect of the EOnon microglial cell viability.

2.8 BV-2 Cell Lysate Preparation

Following the treatment of BV-2 microglial cells with the EO, the cells were washed with phosphate-buffered saline (PBS) and lysed by the addition of 50 µL of radioimmunoprecipitation (RIPA) buffer as described previously [23]. The protein content was measured according to the method of Smith et al.(1985) [24].

2.9 Enzymatic Activity Measurement

Catalase and ACOX1 activities in fresh cell lysate were measured according to the method of Cherkaoui-Malki et al. [25] and Oaxaca-Castillo D. et al. (2007) [26]. Data are expressed as units/mg of protein.

2.10 Data Processing

Statistical analysis was conducted using Student's t-test (Excel software), and the differences were considered statistically significant at $p< 0.05$.

3. Results

3.1 Chemical Composition and Antioxidant Activity of Z. clinopodioides EO

The average yield of the EO was approximately1%. The EO of *Z. clinopodioides* harvested from the high-altitude Armenian landscape was rich in pulegone (42.1%), isomenthone (9.7%), 1,8-cineole (8.22%), piperitone (7.35%), and neomenthole (5.9%) (Figure 1).

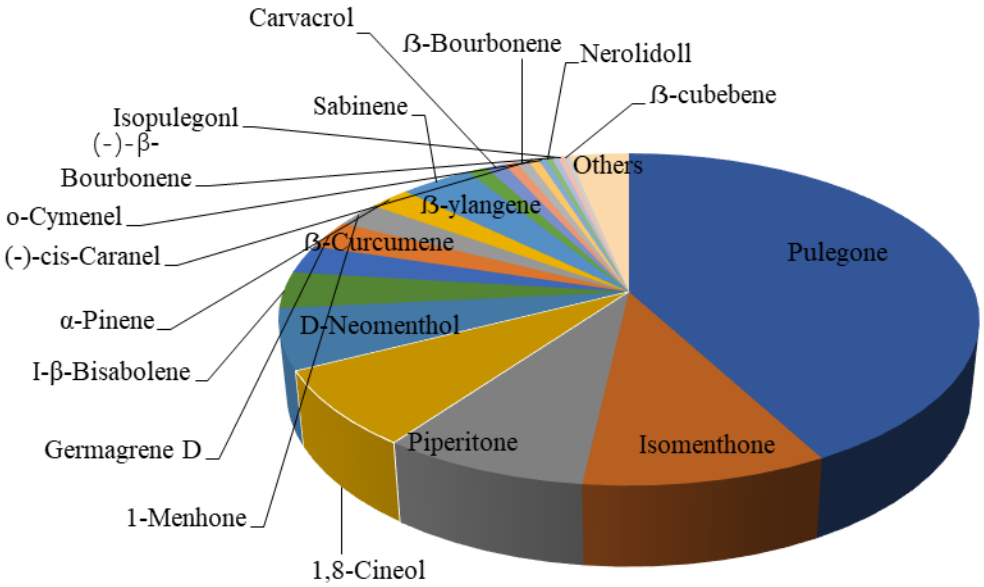

Figure 1 Chemical composition of *Z. clinopodioides* essential oil.

Our experiments confirmed the scientific literature data that the EO of *Z. clinopodioides* shows high antioxidant activity in chemical-based tests; it showed low IC_{50} value (7.025 ±0.9 µL/mL) ((R^2 = 0.851) (data not shown)) as expected (IC_{50} value of the positive control catechin was 12.62 ±0.8 µg/mL) ((R^2 = 0.899) (data not shown)), which indicates its high potential in scavenging free radicals [19, 27].

3.2 Effect of Z. clinopodioides EO on BV2 Cell Viability (MTT Assay)

The sub-cytotoxic concentration of EO for both cell lines was determined as 5×10^{-1} µL/mL (Figures 2A, 2B) ($p< 0.05$). This concentration was used in further analyses. This concentration also allowed to avoid the toxic effect of the EO and to determine only its activity on antioxidant enzymes.

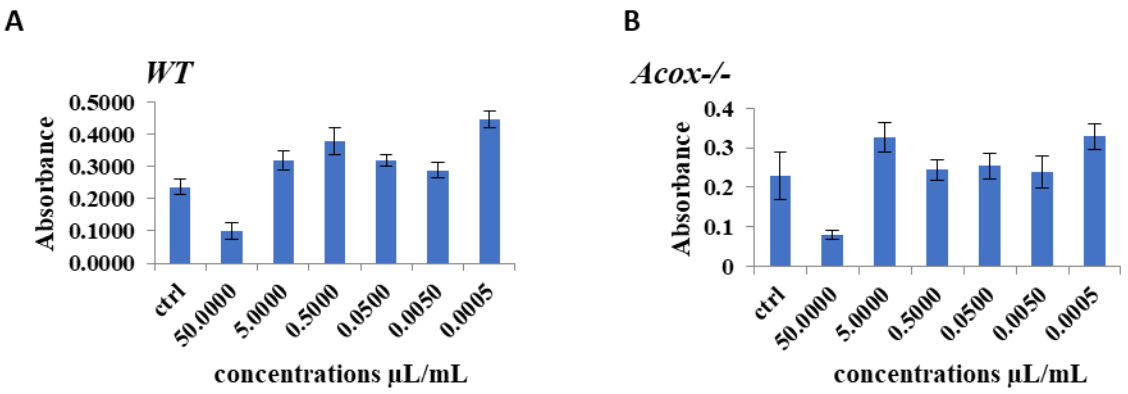

Figure 2 Effects of *Z. clinopodioides* EO on the viability of BV-2 WT and *Acox1*$^{-/-}$ microglial cells (MTT assay; A and B, respectively). Cells were treated for 24 h with the EO at various concentrations (50 to 5×10^{-4} µL/mL). Results are expressed as mean ±SD of three repetitions ($p < 0.05$ for both BV-2 cell lines).

Cell viability of WT cells increased following treatment with the EO for 24 and 48h, while the cell viability of the mutant cells increased only after 24hof treatment (Figure 3). No statistically significant changes were observed for other cases ($p > 0.05$).

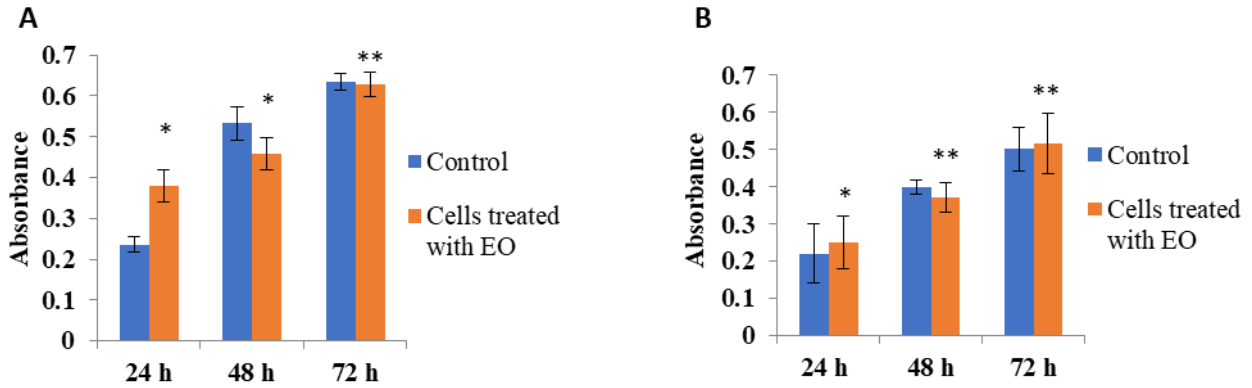

Figure 3 Influence of the *Z. clinopodioides* EO on the cell viability of BV-2 WT and *Acox1*$^{-/-}$microglial cells (MTT assay; A and B, respectively). Cells were treated with the EO at 5×10^{-1} µL/mL concentration. Results are expressed as mean ±SD of three repetitions (* indicates $p < 0.05$, ** indicates $p > 0.05$, for both cell lines).

3.3 Effect of Z. clinopodioides EO on Peroxisomal Functions in BV-2 Cells

BV-2 WT cells showed a decrease in catalase activity at 24 h of treatment with EO, while *Acox1*$^{-/-}$ BV-2 cells showed an increase in catalase activity after treatment. Although the activity of this enzyme was increased in both cell lines at 72hof treatment with the EO, the difference was not statistically significant ($p > 0.05$) (Figure 4).

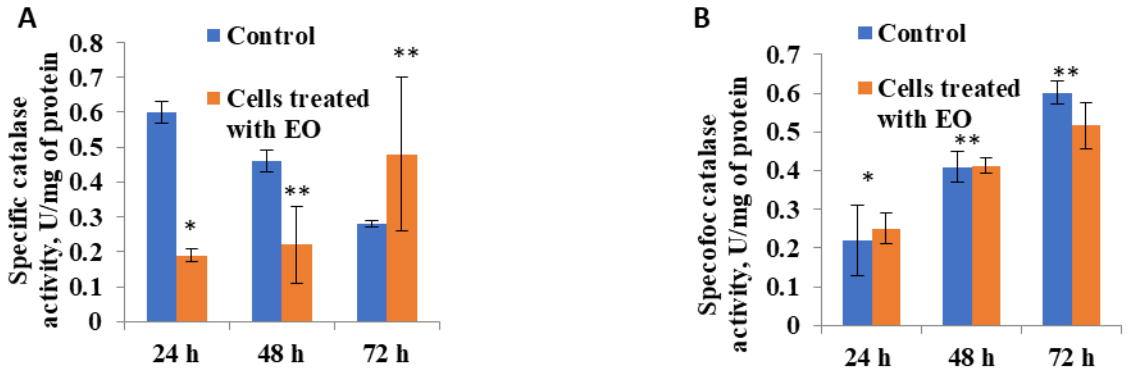

Figure 4 Effect of the EO on the catalase activity of BV-2 WT and mutant microglial cells (A and B, respectively). *$p < 0.05$; **$p > 0.05$. Cells were treated with the EO at 5×10^{-1} µL/mL concentration.

The *Z. clinopodioides* EO caused a considerable decrease in ACOX1 activity (up to 40%) at 72hof treatment (Figure 5) ($p< 0.05$). We also observed an increase in the catalase activity of BV-2 WT cells (up to 20%) only after 48h of treatment ($p > 0.05$).

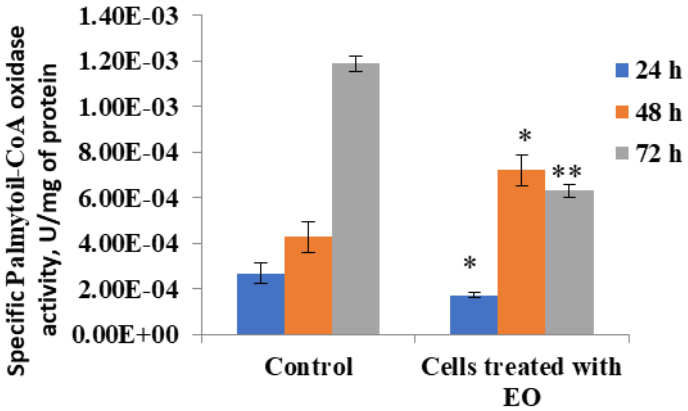

Figure 5 Effect of *Z. clinopodioides* EO on palmitoyl-CoA oxidase type 1 activity of BV-2 WT cells. Cells were treated with the EO at 5×10^{-1} µL/mL concentration. Results are expressed as mean ±SD of 3 repetitions (*$p < 0.05$; **$p > 0.05$).

4. Discussion

Z. clinopodioides EO is rich in pulegone, isomenthone, piperitone, and 1,8-cineole [8]. As reported previously [5], our present study results confirmed these findings data and revealed that the chemical composition of the EO of *Z. clinopodioides* from high-altitude Armenian landscape was very similar to those described in literature. This implies that despite the differences between the elevation of the growing area of *Z. clinopodioides* and climatic conditions, this plant produces approximately the same components of EO [5, 8, 19]. In contrast to the literature data [9, 10], wherein thymol was reported to be one of the main components in the EO extracted from *Z. clinopodioides* of Armenian flora, the concentration of this component in the present study did not exceed 0.35%. All these components show reducing capability [28], which implies that they can alter the activity of different enzymes [29].

Ahmadi et al. (2021) [8] reported that the *Z. clinopodioides* EO shows a high cytotoxic effect on human lymphocytes at 1–10 µL/mL concentration. As mentioned earlier, we used a lower concentration of the EO in our experiments, which did not have a significant effect on the viability of the tested cell lines but could exert antioxidant activity in chemical-based assays.

The evaluation of the activity of ACOX1 – the rate-limiting enzyme involved in peroxisomal β-oxidation– and of the activity of catalase – the main peroxisomal antioxidant enzyme in the tested microglial cells– after treatment with *Z. clinopodioides* EO is of interest as these enzymes play a crucial role in maintaining redox balance of cells [30]. Peroxisomes also contain enzymes that catalyze the production of hydrogen peroxide (H_2O_2), superoxide (O_2^-), or nitric oxide (NO) as part of their normal catalytic cycle. These molecules react with other molecules to form other reactive oxygen species and reactive nitrogen species [23]. Some of the main antioxidant enzymes, such as catalase and superoxide dismutase, play critical and sometimes even a central role in the cellular antioxidant defense process by catalyzing the dismutation and subsequent quenching of H_2O_2, including those, which form from the enzymatic oxidation by the action of ACOX1 [15].

Our present study results showed that the tested concentration of *Z. clinopodioides* EO influenced the activity of the main cellular antioxidant enzymes catalase and ACOX1. Catalase activity was decreased in WT cells, which indicated that the treatment did not induce stress in these cells. Catalase activity was increased in ACOX1-deficient cells at 24hof treatment. This tendency was, however, not retained with the increase in EO treatment duration. This phenomenon can serve as an indicator of the protective effect of the *Z. clinopodioides* EO components on mutant cells [27, 29].

5. Conclusion

In summary, *Z. clinopodioides* EO shows remarkable antioxidant properties in chemical-based assays, and it can also alter the activity of cellular antioxidant enzymes such as catalase and ACOX1. The findings of the present study confirm that the EO extracted from *Z. clinopodioides* harvested from the high-altitude Armenian landscape affects the cellular antioxidant activity of microglial cells and can prevent neurodegenerative changes such as the accumulation of VLCFA in cells. Because of this favorable property, the tested *Z. clinopodioides* EO can be used in preventive medicine.

List of Abbreviations

ACOX1	acyl-CoA oxidase type 1
DMEM	Dulbecco's modified Eagle medium
DPPH	1,1-diphenyl-2-picrylhydrazyl
EDTA	ethylenediamine tetraacetic acid
EO	essential oil
FBS	fetal bovine serum
GC MS	chromatography mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIST	National Institute of Standards and Technology
PBS	*Phosphate-buffered* saline
RIPA	radioimmunoprecipitation

SOD	Superoxide dismutase
VLCFA	very-long-chain fatty acids
WT	wild type

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Author Contributions

Conceptualization, N.S. and M.P.; methodology, N.S.; investigation, N.S.; data curation, N.S., N.S..; writing—review and editing, N.S and M.P; supervision, N.S. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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