

Original Research

Protective Effects of *Teucrium polium* Extract Against LPS-Induced Oxidative Stress in BV-2 Microglia: Insights from Wild-Type and *Acox1*^{-/-} Models

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Abstract

Microglial activation and the associated oxidative or inflammatory responses play a crucial role in the development of neurodegenerative diseases. In this study, we evaluated the antioxidant, cytotoxic, and anti-inflammatory properties of *Teucrium polium* (TP) ethanolic extract in murine BV-2 microglial cells, including wild-type (*Wt*) and peroxisomal β -oxidation-deficient *Acox1*^{-/-} cell lines. The TP extract exhibited high antioxidant activity as assessed via the DPPH test, with an IC₅₀ value of 73.89 μ g/mL, and contained substantial levels of total phenols (181.7 $\pm$ 2.1 mg GAE/g) and flavonoids (95.4 $\pm$ 3.1 mg QE/g). Cytotoxicity assessment revealed no detrimental effect on cell viability or proliferation at any tested concentration in either cell line. Exposure to lipopolysaccharide (LPS, 1 μ g/mL) induced significant increases in catalase activity (35% in *Wt*, 95% in *Acox1*^{-/-}) and gene expression (Cat mRNA upregulated 2-fold in *Wt* and 5-fold in *Acox1*^{-/-}), nitric oxide (NO) production, and up-regulation of inflammatory markers (*Tnf- α* , *iNos*, *Il-1 β* by ~10–15-fold) in both *Wt* and *Acox1*^{-/-} cells. Notably, co-treatment with TP extract effectively suppressed these LPS-induced changes, restoring catalase activity and expression to control levels and significantly reducing both NO (~10-fold) production and pro-inflammatory gene expression. The TP extract also modulated the



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expression of the peroxisomal transporter gene *Abcd1*, upregulating it in *Wt* cells while normalizing its expression in *Acox1*^{-/-} cells under LPS stimulation. These findings suggest that *T. polium* extract exhibits potent antioxidant and anti-inflammatory activities without cytotoxicity, and may modulate peroxisomal function and redox balance in microglial cells. The differential responses observed in *Acox1*^{-/-} cells point to the involvement of peroxisomal β -oxidation pathways in neuroinflammatory regulation. The results support the potential of *T. polium* as a neuroprotective agent in conditions associated with oxidative stress and peroxisomal dysfunction.

Keywords

Neuroglia; cell culture; anti-inflammatory; anti-oxidant; phenolics; neuroprotective effect

1. Introduction

A vast number of neurodegenerative diseases represent a growing global health burden characterized by progressive loss of neuronal structure and function. While the etiology of these disorders is multifactorial, chronic neuroinflammation has emerged as a critical contributing factor in their pathogenesis [1-3]. Microglia, the resident immune cells of the central nervous system (CNS), play a dual role in maintaining homeostasis and mediating inflammatory responses [4, 5]. Under pathological conditions, sustained microglial activation leads to the overproduction of pro-inflammatory cytokines, reactive oxygen species (ROS), and neurotoxic mediators, resulting in neuronal damage and the progression of neurodegeneration [3, 6].

The modulation of microglial activation has therefore become a promising therapeutic strategy for neurodegenerative disorders. In recent years, increasing attention has been directed toward plant-derived secondary metabolites, many of which exhibit significant anti-inflammatory, antioxidant, and neuroprotective properties [5, 7]. Phytochemicals such as flavonoids, phenolic acids, terpenoids, and alkaloids have been shown to attenuate microglial activation, inhibit inflammatory signaling pathways (e.g., NF- κ B, MAPKs), and promote neuronal survival in different *in vitro/in vivo* models [8, 9].

Among these botanicals, *Teucrium polium* L. (Lamiaceae), commonly known as felty germander, has been traditionally used in Mediterranean and Middle Eastern folk medicine for the treatment of various ailments, including inflammatory and metabolic disorders. Its extracts are rich in biologically active metabolites, such as flavonoids (apigenin, luteolin), diterpenes, and other components, which are known to exert anti-inflammatory and antioxidative effects [10, 11]. Some pharmacological studies have demonstrated that *T. polium* extracts (TP) can modulate oxidative stress and reduce systemic inflammation, suggesting a possible role in neuroprotection [12]. However, direct investigations into its effects on microglial activation and neurodegenerative pathways remain scarce.

Given the pivotal role of microglia in neurodegeneration and the pharmacological potential of *T. polium*, further research into its molecular mechanisms of action could yield valuable insights into this field. Elucidating the interaction between *T. polium*-derived compounds and

neuroinflammatory processes may contribute to the development of novel therapeutic approaches for neurodegenerative disorders.

The murine BV-2 microglial cell line is widely used as a reliable *in vitro* model to investigate microglial behavior in response to inflammatory and neurotoxic stimuli, owing to its phenotypic and functional similarities to primary microglia [13]. BV-2 wild-type (*Wt*) cells retain the capacity to produce key pro-inflammatory cytokines and reactive oxygen species upon stimulation, making them suitable for studying canonical neuroinflammatory pathways [14]. In parallel, the *Acox1*^{-/-} BV-2 line, characterized by the targeted deletion of *acyl-CoA oxidase 1*—a (ACOX) key enzyme in peroxisomal β -oxidation—offers a unique model for studying microglial dysfunction under conditions of impaired lipid metabolism and elevated oxidative stress. ACOX1 deficiency has been linked to increased peroxisomal-derived reactive oxygen species and pro-inflammatory microglial polarization, conditions that closely mimic aspects of the neurodegenerative milieu. The combined use of BV-2 *Wt* and *Acox1*^{-/-} lines enables comparative analysis of plant-derived compounds not only in standard microglial responses but also in metabolically and redox-compromised states, thereby enhancing the relevance of the findings to the contexts of neurodegeneration.

2. Materials and Methods

2.1 Plant Material

The plant material (*Teucrium polium* L.) was harvested from the Tavush region (Armenia, v. Aygedzor, 700–800 m above sea level, 40°49'32" N. L. 45°32'27" E.) during the flowering period (July 2023). The plant identification was performed in the Department of Botany and Mycology at Yerevan State University (YSU), Armenia, by Dr. Narine H. Zakaryan. The plant samples were archived at the same department's herbarium, and a voucher specimen serial number was assigned (ERCB13257).

2.2 Extract Preparation

The aerial parts of the plant were dried in the shade, then ground into a powder, and stored in a dry, dark location for further use. 1 g of powdered dried plant material was homogenized in 10 to 15 mL of 80% ethanol and left overnight at ~10°C. The extract was centrifuged for 5 min at 5000 rpm, and the supernatant was isolated. The precipitate was extracted by 4-fold, and the combined supernatant was dried by evaporation at room temperature [15]. The resulting extracts were stored at -20°C for future use.

2.3 Total Phenolic and Flavonoid Content Determination

The total phenolic content of the *T. polium* extract was determined using the Folin–Ciocalteu colorimetric assay. 500 μ L of the plant extract (1 mg/mL in dH₂O) was mixed with 100 μ L of diluted Folin–Ciocalteu reagent and incubated for 5–8 minutes. Then, 1000 μ L of 7% Na₂CO₃ solution and 900 μ L of distilled water were added to the mixture. After 2 hours of incubation at room temperature, absorbance was measured at 765 nm. Gallic acid was used to generate a calibration curve ($y = 0.0063x - 0.0718$, $R^2 > 0.99$), and the results were expressed as milligrams of gallic acid equivalent (mg GAE) per gram of dried extract [16].

The total flavonoid content of the extract was determined using the aluminum chloride (AlCl₃) colorimetric assay [13, 15]. Briefly, 500 µL of the extract solution (1 mg/mL in 80% ethanol) was mixed with 100 µL of 10% AlCl₃, 100 µL of 1 M sodium acetate (CH₃COONa), and 2800 µL of distilled water. After incubation at room temperature for 15 minutes, absorbance was measured at 415 nm using a UV–VIS spectrophotometer (GENESYS 10S, Thermo Scientific, USA). A standard calibration curve was created using quercetin (0–250 µg/mL), and the flavonoid content was expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g) [15, 17].

2.4 2,2-Diphenyl-1-Picrylhydrazyl Free Radical Scavenging Assay

The DPPH free radical scavenging assay was performed as described previously [5, 18]. For this, various concentrations of plant extracts (ranging from 31.25 to 500 µg/mL) and 0.01 mM DPPH were prepared. The absorbance of the extract and DPPH solution mixture was measured at a wavelength of 517 nm against a blank (96% ethanol) using a UV–Vis spectrophotometer (Genesys 10S, Thermo Scientific, USA) after incubation. Catechin was used as a positive control. The following formula calculated the radical scavenging activity:

$$\text{Activity (\%)} = (\text{Ab}_c - \text{Ab}_s) / \text{Ab}_c \times 100,$$

where Ab_c and Ab_s are the absorbance of the control (DPPH solution alone) and the absorbance of the sample in the presence of an extract or standard, respectively. The results were expressed as IC₅₀ values (µg/mL).

2.5 Chelating Capability of *T. polium* Extract

The metal chelating activity was evaluated based on the color change resulting from the formation of ferrozine–Fe²⁺ complexes [19]. For this, 0.4 mL of the plant extract was mixed with 1 mL of ferrous chloride solution (0.2 mM) and allowed to stand for 10 minutes. Subsequently, 0.4 mL of ferrozine solution (5 mM) was added to initiate the reaction. After a 10-minute incubation at room temperature, the absorbance was measured at 562 nm. The percentage of metal chelating activity was calculated using the following formula:

$$\text{Chelating activity (\%)} = [(A_p - A_t) / A_p] \times 100,$$

where A_p is the absorbance of the control (without extract) and A_t is the absorbance of the test sample. Ethylenediaminetetraacetic acid (EDTA) at a concentration of 22 µg/mL served as the positive control.

2.6 TBARS Assay

The determination of the antioxidant capacity of *T. polium* was carried out by studying its ability to inhibit malonaldehyde synthesis in BV-2 cells as described [19]. α-tocopherol was used as a positive standard.

2.7 Cell Cultures Used

The murine BV-2 microglial cells derived from female C57BL/6J mice (Banca-Biologica e Cell Factory, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Italy (catalog no. ATL03001)). Raas et al. [5] generated by gene editing the *Acox1*^{-/-} BV-2 cell line (PMID: 30312667). BV-2 cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (Dominique Dutscher SAS, Brumath, France). The cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. BV-2 cells are derived from C57/BL6 mice. They are immortalized by v-raf/v-myc carrying J2 retrovirus. The BV-2 microglia cell line expresses nuclear v-myc and cytoplasmic v-raf oncogene products, as well as the env gp70 antigen on its surface. It retains the morphological and functional characteristics of microglia cells. Based on the expressed characteristics of v-raf/v-myc, the metabolic and proliferation rates of BV-2 *in vitro* are significantly higher compared to other microglia [5].

BV-2 cells were seeded at a density of 2×10^4 cells per well in 96-well microplates for viability assay, and at a density of 5×10^5 cells per well in 6-well microplates - for the other applications.

Experimental groups included the following treatments: control – untreated cells, LPS only – cells treated with lipopolysaccharide (1 µg/mL), TP only – cells treated with *T. polium* extract (0.125 mg/mL), TP + LPS – cells co-treated with *T. polium* extract (0.125 mg/mL) and LPS (1 µg/mL), Positive control – depending on the assay, α-tocopherol or catechin.

2.8 Cytotoxicity Assay

T. polium extract (TPE) was tested for its cytotoxicity toward microglial cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) test. For all analyses, the extract was dissolved in 0.66% dimethyl sulfoxide (DMSO). They were treated for 24 h with various concentrations of extract (0.0625–1 mg/mL) and were incubated for 2-3 h with MTT. Then the absorbance (Abs) was measured at 570 nm. The untreated cells were considered the control.

2.9 Microglial Cell Lysate Preparation

After treating BV-2 cells with TPE, the cells were washed with Phosphate-buffered saline (PBS). They were lysed in 50 µL of RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecylsulfate, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF) and incubated at 4°C for 30 minutes. The cell lysate was centrifuged at $20,000 \times g$ for 20 min (at 4°C) [20]. The protein content was measured by BCA assay [21].

2.10 Enzymatic Activity Assay

Catalase activity was determined as described previously [20] and was expressed as % of the untreated control.

2.11 Griess Test

NO production by cells is quantified by Griess reagent (0.1% N-1-naphtylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid—Sigma-Aldrich-USA) [22]. After 24 h

incubation at 5×10^4 cells/well with different treatments (see the “Results” section), the cell activation was triggered by 1 $\mu\text{g/mL}$ LPS from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich L2880). The 50 μL of cell supernatant was placed in a 96-well plate; 50 μL of Griess reagent was added after 15 min incubation (absorbance was documented at 540 nm). For the calibration curve, dilutions of 0-50 μM NaNO_2 were used. The results were expressed as μM of released NO.

2.12 Immunoblotting

The 15 μg of protein lysate were diluted (v/v) in the loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 14% mercaptoethanol, and 0.003% Bro-mophenol blue) and heated at 100°C for 5 min, then separated on a 10% SDS-PAGE, and transferred into PVDF membrane as already described [5]. Membranes were first blocked in 5% fat-free milk in PBST (Phosphate buffer saline, 0.1% Tween 20). Then the membrane probed with the primary antibody diluted in 1% milk TBST overnight at 4°C (anti-catalase, AF3398 from R&D Systems Noyal Châtillon sur Seiche, France, dilution 1/400; anti- α -tubulin, A2228 from Sigma-Aldrich, Saint-Quentin-Fallavier, France, dilution 1/10,000). Membranes were washed in PBST and incubated with the appropriate HRP-conjugated secondary antibodies (1:5,000) in 1% fat-free milk in PBST. Membranes were washed three times in TPBS for 10 minutes, and then immunoreactivity was revealed by incubating the membranes with the HRP SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The signal was detected using the Chemidoc XRS system (Bio-Rad, Marnes-la-Coquette, France). Image processing and quantification were performed using Image Lab software (Bio-Rad, Marnes-la-Coquette, France) [5, 23].

2.13 Quantification of Gene Expression by RT-qPCR

To determine mRNA expression in the investigated cells after treatment with TPE, RT-qPCR was employed. For this cell, total RNA was isolated using the RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. The purity of nucleic acids was controlled by the ratio of absorbance at 260 nm to 280 nm, accepting a ratio between 1.8 and 2.2. Next, one μg of RNA was reverse transcribed to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) following the protocol of the manufacturer. Quantitative PCR of cDNA was performed using the FG Power SYBR Green kit (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) on an iCycler iQ Real-Time Detection System (Bio-Rad, Marnes-la-Coquette, France). Primer sequences are listed in Table 1. The thermal cycling protocol included an initial activation step at 95°C for 3 minutes, followed by 40 cycles of amplification (95°C for 10 seconds and 60°C for 1 minute). A melting curve analysis was conducted to ensure the specificity of the amplified products. Amplification efficiency for each transcript was calculated based on the slope of a standard curve derived from two-fold serial dilutions of cDNA.

Table 1 Sequences of the primers used for qPCR.

Gene Name	Accession Number	Primer Sequences
<i>Cat-F</i>	NM_009804.2	5'AGCGACCAGATGAAGCAGTG3'
<i>Cat-R</i>		5'TCCGCTCTCTGTCAAAGTGTG3'
<i>36b4-F</i>	NM_007475.5	5'CGACCTGGAAGTCCAACACTAC3'
<i>36b4-R</i>		5'ATCTGCTGCATCTGCTTG3'
<i>Tnf-α-F</i>	NM_013693.3	5'GACGTGGAAGTGGCAGAAGAG3'
<i>Tnf-α-R</i>		5'TGCCACAAGCAGGAATGAGA3'
<i>Il-1β-F</i>	NM_008361.4	5'GAGATTGAGCTGTCTGCTCA3'
<i>Il-1β-R</i>		5'AAGGAGAACCAAGCAACGAC3'
<i>iNos-F</i>	NM_010927.4	5'CCTAGTCAACTGCAAGAGAA3'
<i>iNos-R</i>		5'TTTCAGGTCACTTTGGTAGG3'
<i>Abcd1-F</i>	NM_007435.2	5'GCCAAGTTGTGGATGTGGAG3'
<i>Abcd1-R</i>		5'TTCCGCAGAGTCGGGATAGA3'

The $2^{-\Delta\Delta C_t}$ method was used to determine the relative gene expression. The results are presented as graphs of relative expression data (fold induction) [5].

2.14 Statistical Analysis

Data are expressed as the mean $\pm$ standard error or standard deviation from a minimum of three independent experiments, each conducted in triplicate. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., USA). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 The Antioxidant and Cytotoxic Properties of *T. polium* Extract

The DPPH assay demonstrated the high anti-radical activity of *T. polium* extract, with an IC_{50} value of 73.89 $\mu\text{g/mL}$ ($y = 0.1545x + 4.335$, $R^2 = 0.99$). This linear equation represents the calibration curve generated by plotting the absorbance values against different concentrations of the extract. For the positive control (catechin), this parameter value was calculated to be $13.08 \pm 0.035 \mu\text{g/mL}$ ($y = 3.4343x + 5.0693$, $R^2 = 0.99$).

The metal-chelating assay demonstrated a $21.86 \pm 2.7\%$ capacity of chelating metal ions by the TPE. For the positive control – EDTA – this capacity was evaluated to be $91.67 \pm 4.6\%$.

The reactive thiobarbituric acid (TBA) assay demonstrated that the ethanol extract of *T. polium* exhibited an inhibitory effect on malondialdehyde (MDA) synthesis, with an inhibition rate of $69.27 \pm 1.4\%$. This corresponds to a 30.73% reduction in MDA production compared to the control group (Figure 1).

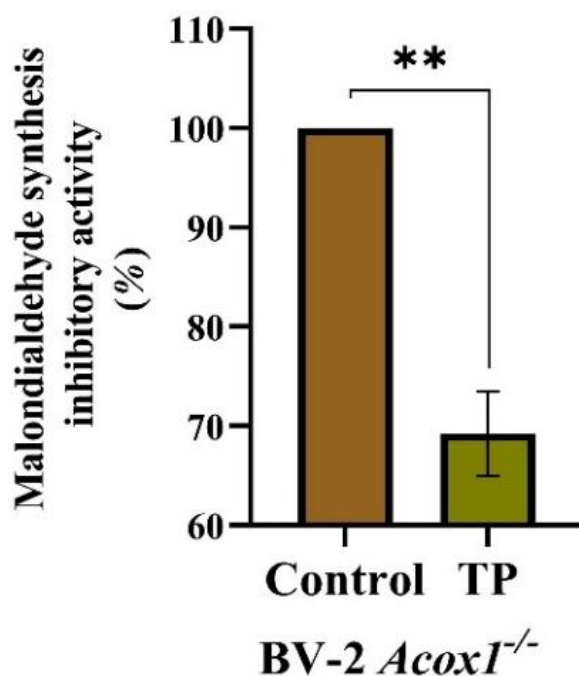


Figure 1 Representative graphs of the impact of *T. polium* to inhibit malondialdehyde synthesis in BV-2 *Acox1*^{-/-} microglial cells, Data are expressed as means $\pm$ SD from three independent experiments, each performed in triplicate (** $p < 0.001$). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test.

The quantity of total phenols was determined to be 181.7 ± 2.1 mg of GAE/g ($R^2 = 0.9905$), which corresponds to approximately 18.17% phenolic compounds in the dried extract. The total flavonoid content of the *T. polium* extract was determined to be 95.4 ± 3.1 mg of QE/g ($R^2 = 1$).

To further evaluate the biological effects of the extract, its cytotoxicity was assessed in both wild-type (*Wt*) and *Acox1*-deficient (*Acox1*^{-/-}) BV-2 cell lines. Across all tested concentrations, the extract showed no inhibitory effect on cell viability or proliferation in either cell line, with no statistically significant differences observed compared to the untreated controls (Figure 1).

These data suggest that the extract is non-cytotoxic to both wild-type and *Acox1*^{-/-} BV-2 cells, even at the highest concentrations tested. This indicates its potential safety for use in further biological or therapeutic applications.

To further evaluate the biological effects of the extract, its cytotoxicity was assessed in both wild-type (*Wt*) and *Acox1*-deficient (*Acox1*^{-/-}) BV-2 cell lines. Across all tested concentrations, the extract exhibited no inhibitory impact on cell viability or proliferation in either cell line, with no statistically significant differences observed relative to the untreated controls (Figure 2).

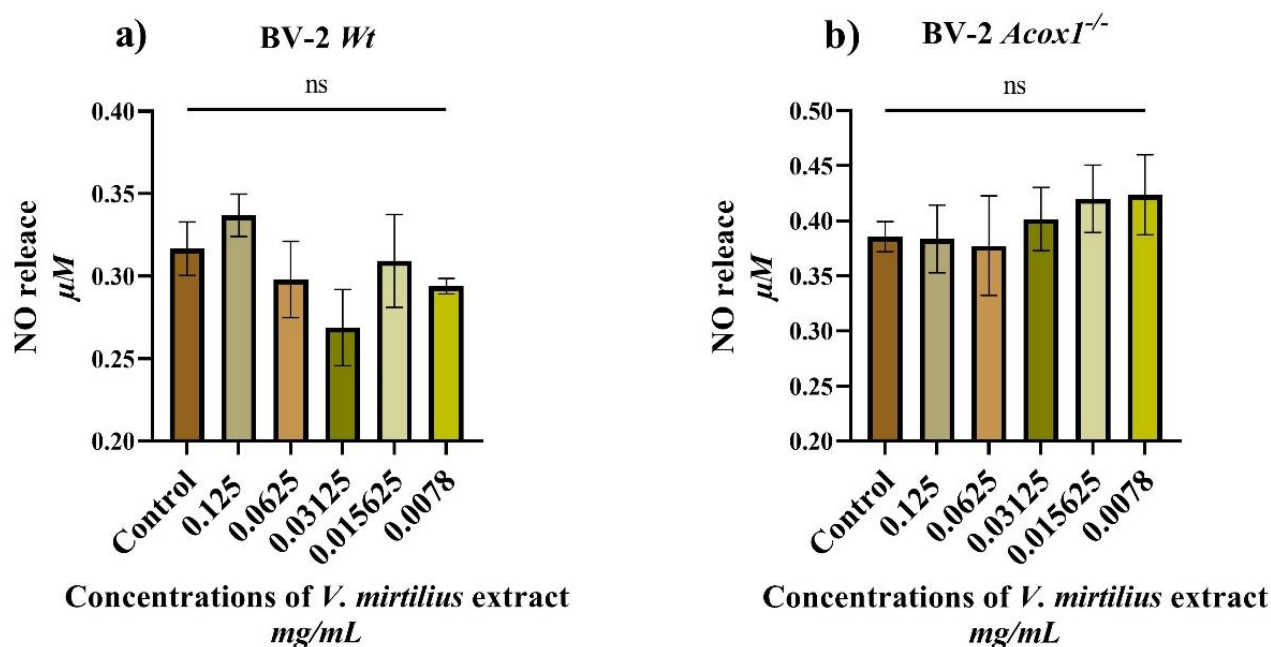


Figure 2 The impact of *T. polium* extract on the proliferation and viability of BV-2 Wt (a) and *Acox1*^{-/-} (b) microglial cells was assessed after a 24-hour treatment using the MTT assay. Untreated cells served as the control group. Data are presented as means ± SD from three independent experiments conducted in triplicate (ns, not significant). Statistical significance was evaluated using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

These data suggest that the extract is non-cytotoxic to both wild-type and *Acox1*^{-/-} BV-2 cells, even at the highest concentrations tested. This indicates its potential safety for use in further biological or therapeutic applications.

3.2 Effect of *T. polium* Extract on Peroxisomal Catalase Activity and Expression

According to our investigation, the catalase activity has not been changed under the treatment of *T. polium* extract in both cell cultures. The addition of LPS (1 μg/mL) to the cultivation medium significantly induced catalase activity in both cell lines, with increases of up to 35% in Wt cells and up to 95% in *Acox1*^{-/-} BV-2 cells compared to untreated cells (Figure 3a). The co-treatment with LPS and TP extract reduces the induced activity of catalase, bringing it to the level of the untreated control (Figure 3a).

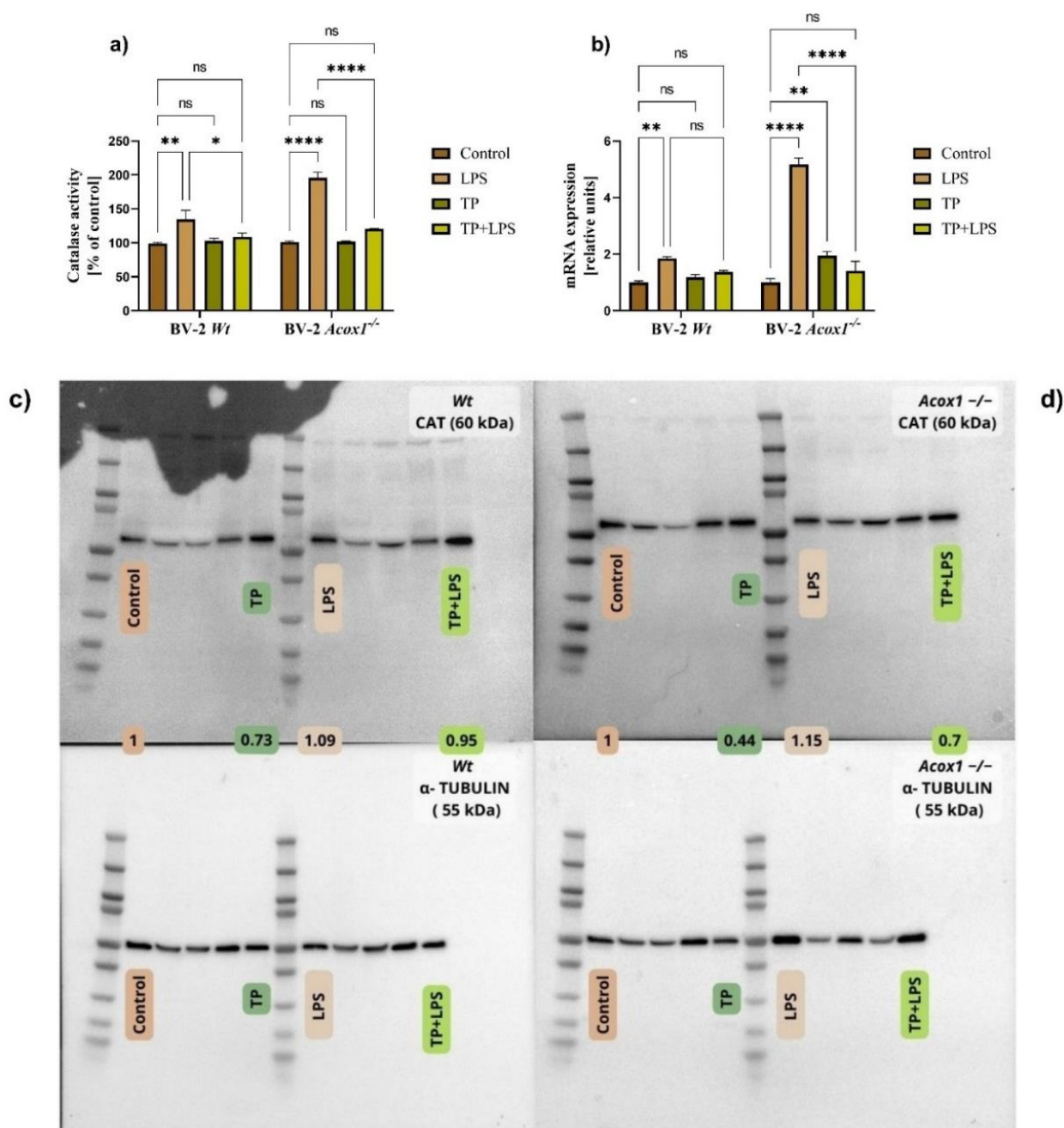


Figure 3 Representative graphs of the impact of *T. polium* extract on catalase activity (a) and Cat mRNA expression (b) were evaluated in both *Wt* and *Acox1^{-/-}* BV-2 microglial cells. Panels (c) and (d) present the immunoblotting results showing the impact of the extract on peroxisomal CAT protein expression in *Wt* (c) and *Acox1^{-/-}* (d) cells. Cells were incubated for 24 h with TP (0.125 mg/mL) in the absence or presence of LPS (1 μ g/mL). Cell lysates were analyzed by PAGE-SDS electrophoresis and subjected to immunoblotting. Band intensities were analyzed by densitometry and standardized to α -tubulin expression level. The table presents standardized densitometric values derived from the quantification of protein signal intensities (*p < 0.1; **p < 0.01; ***p < 0.001; ****p < 0.0001 and ns, not significant). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparison test.

The expression of catalase mRNA and protein levels has also been investigated, for which the 24-hour treated cells were used – both *Wt* and ACOX1 deficient (Figure 3). The LPS treatment also increased the *Cat* mRNA level in both *Wt* and *Acox1*^{-/-} cells by 2-fold and 5-fold, respectively (Figure 3b). The co-treatments of TP+LPS decreased the LPS-induced CAT gene expression, bringing it to approximately the same level as the untreated control (Figure 3b).

The immunoblotting assay reveals that the expression level of the CAT protein mirrors the changes in catalase activity observed in both cell lines (Figure 3c, d).

These findings suggest that *T. polium* extract may possess anti-inflammatory or antioxidant modulatory properties capable of counteracting LPS-induced oxidative stress responses in microglial cells. The more pronounced suppression in *Acox1*^{-/-} cells implies that peroxisomal β -oxidation might be involved in regulating catalase expression under inflammatory conditions.

3.3 Effects of *T. polium* Extract on LPS-Induced Nitric Oxide Production, the Expression of Genes Associated with Inflammatory Markers, and the Peroxisomal ABCD Transporter Protein

In order to assess the anti-inflammatory effects of TP extract, the NO production and *iNOS* gene expression levels were evaluated in the presence or the absence of LPS (Figure 4a, b).

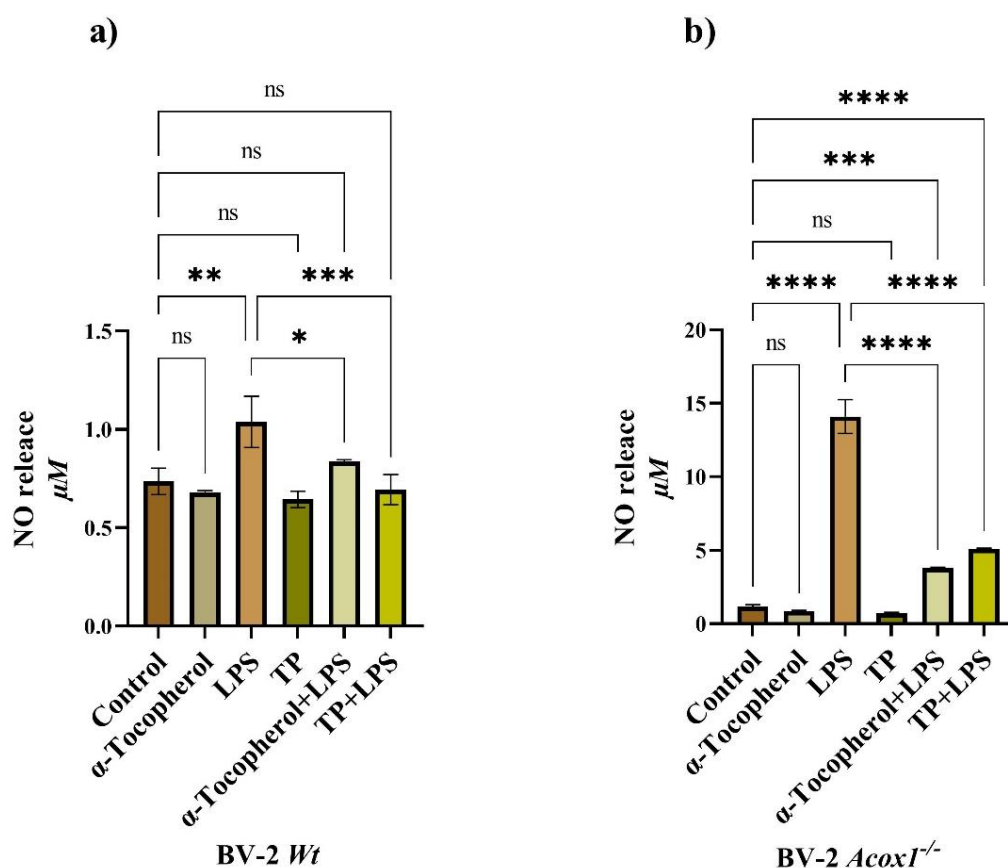


Figure 4 Representative graphs of the impact of *T. Polium* extract on the LPS-induced NO production in *Wt* (a) and *Acox1*^{-/-} (b) BV-2 microglial cells assessed by the Griess test. Cells were incubated for 24 h with TP extract (0.125 mg/mL) in the absence or the presence of 1 μg/mL LPS (*p < 0.1; **p < 0.01; ***p < 0.001; ****p < 0.0001 and ns, not significant). Statistical significance was determined using one-way ANOVA followed by Tukey's test.

Our investigation revealed that neither the positive control (α -tocopherol) nor the *T. polium* extract affected nitric oxide (NO) levels in wild-type (*Wt*) cells. In contrast, LPS stimulation resulted in approximately a 40% increase in NO production. Co-treatment with α -tocopherol and LPS normalized NO levels in *Wt* cells, maintaining them comparable to the untreated control (Figure 4a).

A similar pattern was observed in *Acox1*^{-/-} cells under treatment with α -tocopherol and *T. polium* extract alone, showing no significant impact on NO levels. However, LPS led to a dramatic 14–15-fold increase in NO production, which was partially reduced—down to approximately 10-fold—following co-treatment with α -tocopherol, though the levels did not return to baseline (Figure 4b).

Further analyses indicated that the expression levels of *iNos* and other pro-inflammatory genes, including *Il-1 β* and *Tnf- α* , responded dynamically to the pro-oxidant effects of LPS and the antioxidant activity of the TP extract (Figure 5).

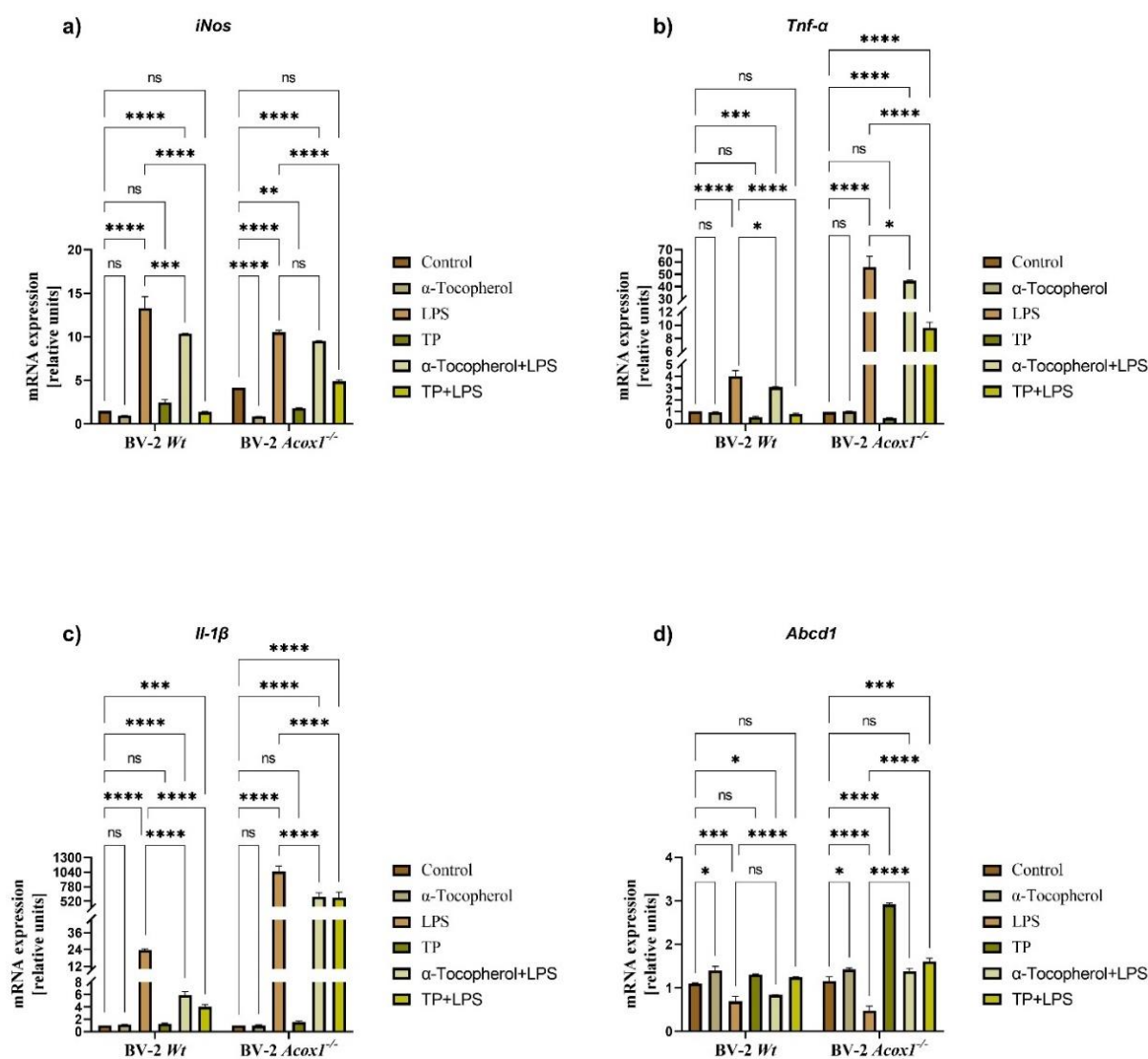


Figure 5 Representative graphs of the impact of *T. polium* extract on the gene expression of *iNos* (a), *Tnf- α* (b), *Il-1 β* (c), and *Abcd1* (d), with or without LPS stimulation, was evaluated. Data are presented as means $\pm$ SD from two independent experiments conducted in triplicate ($p < 0.1$; * $p < 0.01$; ** $p < 0.001$; **** $p < 0.0001$; ns, not significant). Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

LPS stimulation markedly elevated iNos mRNA expression in both wild-type and *Acox1*^{-/-} cells, mirroring the increase in NO production, with transcription levels rising by approximately 10- to 15-fold (Figure 5a). Co-administration of the TPE significantly suppressed this LPS-induced upregulation of iNos expression, surpassing even the positive control.

A similar trend was observed for the other pro-inflammatory markers: *Tnf-α* and *Il-1β* were strongly induced by LPS in both cell lines, whereas TP co-treatment effectively reduced their expression levels (Figure 5b, c). The extent of this reduction was quantified as follows: *Tnf-α* expression was decreased by approximately 1.3-fold in *Wt* cells and 5.9-fold in *Acox1*^{-/-} cells. *Il-1β* expression was reduced by approximately 4.2-fold in *Wt* and 1.7-fold in *Acox1*^{-/-} cells.

In contrast, the peroxisomal gene *Abcd1* displayed a distinct regulation pattern. In wild-type cells, TP treatment alone upregulated *Abcd1* mRNA, while LPS downregulated it. Notably, the TP+LPS co-treatment did not reverse the LPS-induced repression. However, in *Acox1*^{-/-} cells, TP co-treatment successfully decreased *Abcd1* expression, suggesting a protective influence of this extract under oxidative stress, as it restored *Abcd1* expression by approximately 50% compared to LPS-only treated *Acox1*^{-/-} cells (Figure 5d).

4. Discussion

In the present study, we evaluated the antioxidant, cytotoxic, and anti-inflammatory properties of *T. polium* extract using BV-2 murine microglial cell models, including both wild-type (*Wt*) and *Acox1*-deficient (*Acox1*^{-/-}) lines. The investigation focused on the anti-radical activity of the extract, its influence on cell viability, and its modulatory role in peroxisome-associated and inflammatory pathways under basal and LPS-stimulated conditions.

The DPPH assay revealed that *T. polium* extract exhibits potent antioxidant activity with an IC₅₀ value of 73.89 µg/mL, which aligns with previously reported values for plant-derived extracts rich in phenolics and flavonoids. The extract's total phenolic and flavonoid contents confirm its polyphenol-rich nature, likely contributing to its high radical-scavenging potential [24, 25]. The antioxidant profile of *T. polium* suggests sufficient efficacy to merit further investigation in oxidative stress-related models.

The cytotoxicity assessment through MTT assays in both BV-2 cell lines showed no adverse effects of the TP extract across a range of tested concentrations. The lack of cytotoxicity is a key observation, particularly for therapeutic candidates [5, 26]. The extract maintained cell viability and proliferation, thus supporting its safety profile and making it a viable candidate for neuroprotective applications.

We next explored the impact of *T. polium* on peroxisomal function, focusing on catalase (CAT), a key antioxidant enzyme predominantly located in peroxisomes [27]. Under basal conditions, TP treatment alone did not affect catalase activity or expression at the mRNA and protein levels, suggesting that the extract does not exert undue oxidative modulation in homeostatic states. However, under the LPS stimulation, which is known to induce oxidative stress and inflammatory responses in microglia [4, 28, 29], catalase activity significantly increased in both cell lines. This differential response underscores the heightened sensitivity of peroxisome-deficient cells to pro-oxidant stimuli, consistent with the known role of ACOX1 in fatty acid metabolism and redox homeostasis [30, 31].

Remarkably, co-treatment with TP extract effectively reversed LPS-induced catalase upregulation, restoring activity, transcript, and protein levels to near baseline. This regulatory effect suggests that TP may attenuate oxidative stress by indirectly modulating peroxisomal enzyme expression, rather than constitutively stimulating antioxidant defense. Given the amplified catalase response in *Acox1*^{-/-} cells, the more substantial normalizing effect observed in this line indicates that TP extract may compensate for peroxisomal dysfunction, potentially by reducing the inflammatory or oxidative burden imposed by LPS [32].

Nitric oxide is another critical marker of neuroinflammation, primarily produced by inducible nitric oxide synthase (iNOS) in activated microglia [4]. In line with this, LPS treatment induced a significant increase in NO production—approximately 40% in *Wt* cells and a striking 14–15-fold elevation in *Acox1*^{-/-} cells. These findings reflect the enhanced inflammatory response in *Acox1*-deficient microglia, likely due to impaired peroxisomal β -oxidation and disrupted redox signaling [13]. Co-treatment with α -tocopherol (positive control) or TP extract significantly mitigated LPS-induced NO production, although full normalization was only observed in *WT* cells. In *Acox1*^{-/-} cells, NO levels remained elevated, albeit reduced to about 10-fold, suggesting a partial but meaningful anti-inflammatory effect of the extract in a redox-compromised background.

This kind of suppression of NO levels corresponded closely with changes in *iNos* gene expression, which was elevated up to 15-fold upon LPS treatment and attenuated by TP co-treatment in both cell lines. Interestingly, the TP extract appeared more effective than the positive control in reducing *iNos* mRNA, hinting at the involvement of additional regulatory pathways, possibly including NF- κ B or MAPK signaling cascades [4, 33]. Similar expression patterns were observed for other canonical pro-inflammatory cytokines, *Tnf- α* and *Il-1 β* , both of which were robustly upregulated by LPS and significantly downregulated by TP in co-treatment scenarios. These findings confirm that TP extract broadly modulates inflammatory gene expression, positioning it as a potential multi-target anti-inflammatory agent.

Of particular interest is the expression pattern of the peroxisomal transporter gene *Abcd1*, which encodes a membrane protein involved in importing very-long-chain fatty acids into peroxisomes [5, 34, 35]. In *WT* cells, TP alone upregulated *Abcd1* expression, possibly as part of a mild hormetic response promoting peroxisomal function. However, LPS suppressed *Abcd1* levels, and TP was unable to rescue this repression. In contrast, *Acox1*^{-/-} cells responded differently: TP co-treatment effectively restored *Abcd1* expression to control levels following LPS exposure. This divergence suggests that TP may exert context-specific regulatory effects on peroxisomal biogenesis and function, particularly under inflammatory or genetically compromised conditions (Figure 6).

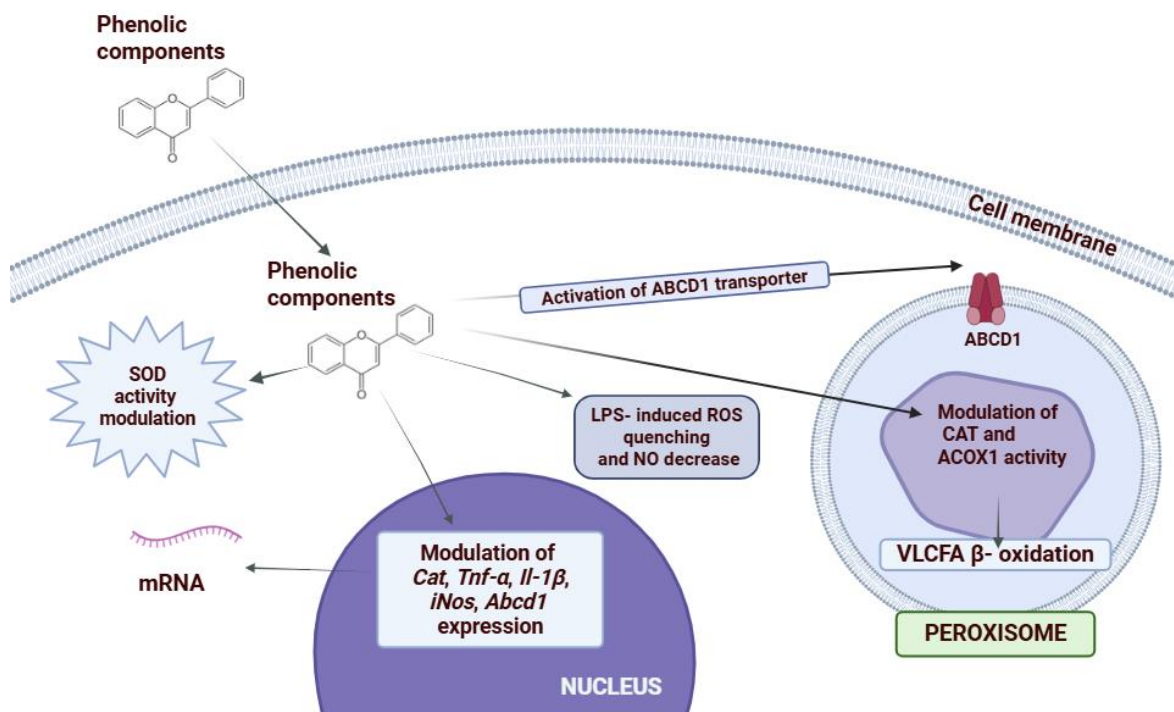


Figure 6 The schematic representation of influence of the phenolic components of TP extract on microglial BV-2 *Wt* cells.

The results underscore the dual antioxidant and anti-inflammatory potential of *T. polium* extract in microglial cells. Its non-cytotoxic nature, ability to normalize oxidative and inflammatory markers, and differential regulation of peroxisomal genes make it a promising candidate for further development in neuroinflammation and neurodegeneration models. The enhanced response in *Acox1*^{-/-} cells suggests that TP extract may be particularly beneficial in conditions involving peroxisomal dysfunction or metabolic inflammation.

5. Conclusions

The present study demonstrates that *T. polium* extract exhibits potent antioxidant capacity and exerts significant anti-inflammatory effects in BV-2 microglial cells without inducing cytotoxicity. The extract modulates LPS-induced oxidative stress by normalizing catalase activity and expression, reducing nitric oxide production, and suppressing the transcription of pro-inflammatory genes such as *iNos*, *Tnf-α*, and *Il-1β*. Additionally, its differential effect on *Abcd1* expression in wild-type cells highlights its potential role in supporting peroxisomal function under inflammatory stress. These findings suggest that *T. polium* may serve as a promising candidate for therapeutic strategies targeting neuroinflammatory and oxidative stress-related disorders, particularly in contexts of peroxisomal dysfunction.

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Author Contributions

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Competing Interests

The authors have declared that no competing interests exist.

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All data are available under reasonable request.

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The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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