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Antioxidant capacity, cytotoxicity and post-treatment effect of *Myrtus communis* L. leaf extract in a 6-hydroxydopamine-induced SH-SY5Y cell model

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Abstract

Purpose: Oxidative stress drives the loss of dopaminergic neurons in Parkinson's disease, which is usually diagnosed after many of these neurons are lost. Agents acting after injury are therefore of interest. This study measured the phenolic content and antioxidant capacity of an ethanolic *Myrtus communis* L. leaf extract, defined its non-cytotoxic concentration range, and tested whether the extract, applied after 6-hydroxydopamine (6-OHDA) injury, improves the viability of human SH-SY5Y neuroblastoma cells.

Method: Leaves of *M. communis* were dried, powdered, and macerated in ethanol for 3 days. Total phenolic (Folin–Ciocalteu) and total flavonoid contents, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, and ferric reducing antioxidant power (FRAP) were measured. Cytotoxicity was assessed in L929 fibroblasts and SH-SY5Y cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 and 48 h. SH-SY5Y cells were exposed to 200 µM 6-OHDA for 24 h and then treated with the extract (10–1000 µg/mL) for 24 h. Groups were compared by one-way analysis of variance (ANOVA) and Dunnett's test.

Findings: The extract (yield 16.87%) contained 256.00 mg gallic acid equivalents (GAE)/g total phenolics and 48.00 mg quercetin equivalents (QE)/g total flavonoids, and its FRAP value was 285.66 mg GAE/g extract. The half-maximal inhibitory concentrations (IC_{50}) for DPPH and ABTS were 75.97 and 106.7 µg/mL (gallic acid: 45.25 and 64.41 µg/mL). By the ISO 10993-5 criterion (≥70% viability), L929 cells tolerated up to 31.25 µg/mL for 48 h. SH-SY5Y cells were about five times more sensitive than L929 cells (48-h IC_{50} 31.16 vs 158.4 µg/mL). 6-OHDA reduced SH-SY5Y viability slightly at 24 h (87.5% at 200 µM) and by about half at 48 h (IC_{50} 227.7 µM). Post-treatment increased the MTT signal only at 75 µg/mL (116.0% of 6-OHDA-only cells, $p = 0.016$), whereas concentrations ≥200 µg/mL lowered it to 54.0–73.1%.

Conclusion: The extract showed moderate radical-scavenging activity and was tolerated within a relatively narrow concentration range, and SH-SY5Y cells were more sensitive to it than fibroblasts. Post-treatment increased the MTT signal at a single concentration. This preliminary finding should be supported by independent viability assays and mechanistic studies.

Keywords: Myrtle, Phenolic compounds, Neuroprotection, Parkinson's disease, MTT assay

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Myrtus communis L. yaprak ekstresinin antioksidan kapasitesi, sitotoksisitesi ve 6-hidroksidopamin ile indüklenen SH-SY5Y hücre modelinde hasar sonrası uygulama etkisi

Özet

Amaç: Oksidatif stres, Parkinson hastalığında dopaminerjik nöron kaybının temel nedenlerinden biridir ve hastalık genellikle bu nöronların önemli bir kısmı kaybedildikten sonra teşhis edilir. Bu nedenle hasar sonrasında etki gösterebilen ajanlar özellikle önemlidir. Bu çalışmada *Myrtus communis* L. yapraklarından hazırlanan etanollü ekstrenin fenolik içeriğinin ve antioksidan kapasitesinin ölçülmesi, sitotoksik olmayan konsantrasyon aralığının belirlenmesi ve 6-hidroksidopamin (6-OHDA) hasarından sonra uygulanan ekstrenin insan SH-SY5Y nöroblastoma hücrelerinin canlılığını artırıp artırmadığının test edilmesi amaçlanmıştır.

Metod: *M. communis* yaprakları kurutulup toz haline getirilmiş ve 3 gün etanolde maserasyona bırakılmıştır. Toplam fenolik (Folin–Ciocalteu) ve toplam flavonoit içerikleri, 2,2-difenil-1-pikrilhidrazil (DPPH) ve 2,2′-azino-bis(3-etilbenzotiyazolin-6-sülfonik asit) (ABTS) radikal süpürücü aktivite ile demir indirgeyici antioksidan güç (FRAP) ölçülmüştür. Sitotoksisite, L929 fibroblast ve SH-SY5Y hücrelerinde 24 ve 48 saat sonunda 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) testiyle değerlendirilmiştir. SH-SY5Y hücreleri 24 saat boyunca 200 µM 6-OHDA'ya maruz bırakıldıktan sonra, 24 saat boyunca ekstre (10–1000 µg/mL) ile muamele edilmiştir. Gruplar tek yönlü varyans analizi (ANOVA) ve Dunnett testiyle karşılaştırılmıştır.

Bulgular: Ekstre (verim %16,87) 256,00 mg gallik asit eşdeğeri (GAE)/g toplam fenolik ve 48,00 mg kuersetin eşdeğeri (QE)/g toplam flavonoit içermiş, FRAP değeri 285,66 mg GAE/g ekstre bulunmuştur. DPPH ve ABTS için yarı maksimal inhibisyon konsantrasyonu (IC_{50}) değerleri 75,97 ve 106,7 µg/mL'dir (gallik asit: 45,25 ve 64,41 µg/mL). ISO 10993-5 ölçütüne (≥%70 canlılık) göre L929 hücreleri 48 saatte 31,25 µg/mL'ye kadar ekstreyi tolere etmiştir. SH-SY5Y hücreleri L929 hücrelerinden yaklaşık beş kat daha duyarlı bulunmuştur (48 saat IC_{50} 31,16 ve 158,4 µg/mL). 6-OHDA, SH-SY5Y canlılığını 24 saatte az (200 µM'de %87,5), 48 saatte ise yaklaşık yarı yarıya azaltmıştır (IC_{50} 227,7 µM). Hasar sonrası uygulama MTT sinyalini yalnızca 75 µg/mL'de artırmış (yalnız 6-OHDA grubuna göre %116,0; $p = 0,016$), ≥200 µg/mL ise %54,0–73,1'e düşürmüştür.

Sonuç: Ekstre orta düzeyde radikal süpürücü aktivite göstermiş ve görece dar bir konsantrasyon aralığında tolere edilmiştir; SH-SY5Y hücrelerinin ekstreye fibroblastlara göre daha duyarlı olduğu görülmüştür. Hasar sonrası uygulama, tek bir konsantrasyonda MTT sinyalinde artış sağlamıştır. Bu ön bulgunun bağımsız canlılık testleri ve mekanistik çalışmalarla desteklenmesi önerilmektedir.

Anahtar kelimeler: mersin, fenolik bileşikler, nöroproteksiyon, Parkinson hastalığı, MTT testi

1. Introduction

Oxidative stress is a central mechanism in many toxic and degenerative processes, and it plays a key role in Parkinson's disease (PD). PD is one of the most common neurodegenerative disorders and one of the fastest-growing neurological conditions worldwide [1]. In PD, dopaminergic neurons of the substantia nigra degenerate progressively. This loss is closely linked to mitochondrial dysfunction, weakened antioxidant defenses and oxidative damage to lipids, proteins and DNA [2]. Because PD is usually diagnosed only after many of these neurons have already been lost [3], an agent that only prevents oxidative injury would come too late for most patients. It is therefore worth asking whether plant antioxidants can also help cells survive after the damage has occurred.

Phenolics and flavonoids are among the plant compounds studied for this purpose, and *Myrtus communis* L. (Myrtaceae) is a rich source of both. This evergreen Mediterranean shrub, known in Türkiye as "Mersin", "Murt" or "Hambeles", is used in Anatolian folk medicine for gastrointestinal and respiratory complaints, hypertension and skin disorders, among others [4–6]. Its leaves contain phenolics, flavonoids and tannins, and its essential oil is rich in monoterpenes such as α-pinene, 1,8-cineole and linalool [6]. Myrtle phenolics scavenge reactive oxygen species (ROS) and chelate pro-oxidant metal ions, which is thought to underlie the antioxidant, anti-inflammatory and cytoprotective effects reported for its extracts [7–9]. Recent phytochemical and *in vitro* studies on *M. communis* have further characterized its phenolic profile and reported antioxidant, enzyme inhibition and other biological activities [10, 11]. While the antioxidant capacity of myrtle leaves is well documented [7–9, 12], some myrtle preparations are also strongly cytotoxic [12]. Any protective use therefore first requires a defined non-cytotoxic concentration range.

Testing such a use also requires an oxidative-injury model, and 6-hydroxydopamine (6-OHDA) is the most widely used one. This hydroxylated dopamine analog is taken up selectively by catecholaminergic neurons through the dopamine and noradrenaline transporters. Inside the cell, it auto-oxidizes to hydrogen peroxide, superoxide and reactive quinones and impairs mitochondrial respiration, reproducing the oxidative injury seen in PD [13]. Because its toxicity is driven mainly by oxidative stress, it is well suited to testing whether an antioxidant-rich extract can counter this type of injury. It is used both in vivo and in vitro, including in the human SH-SY5Y neuroblastoma line, which expresses tyrosine hydroxylase and other catecholaminergic markers and is one of the most common cell models in PD research [13, 14]. Many plant extracts have shown neuroprotective effects in such *in vitro* models, although their translation to clinical use remains limited [15]. Evidence for a neuroprotective effect of *M. communis* in this model comes from a single study, in

which a myrtle berry by-product extract was given to rat PC12 cells 12 h before 6-OHDA exposure [16]. Whether the leaf extract can act after the injury, in human neuron-like cells, has not been tested.

The present study addresses this gap. It differs from earlier work in three ways: it uses the leaf rather than the fruit and human SH-SY5Y cells rather than rat PC12 cells, and it applies the extract only after 6-OHDA injury, testing rescue rather than prevention. The hypothesis was that a phenolic-rich ethanolic leaf extract would improve the viability of 6-OHDA-injured SH-SY5Y cells within its non-cytotoxic range. To test this, (i) the phenolic and flavonoid contents and the antioxidant capacity of the extract (2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [ABTS] radical scavenging and ferric reducing antioxidant power [FRAP]) were measured, (ii) its non-cytotoxic concentration range was determined in L929 and SH-SY5Y cells, and (iii) its effect on SH-SY5Y viability was tested when it was applied after 6-OHDA injury.

2. Materials and methods

2.1. Chemicals and reagents

DPPH was purchased from TCI Chemicals (Tokyo, Japan); ABTS and gallic acid from Sigma-Aldrich (St. Louis, MO, USA); and Folin–Ciocalteu reagent, quercetin, aluminum chloride, potassium acetate and ethanol from Merck (Darmstadt, Germany). 6-OHDA (CAS 28094-15-7), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and fetal bovine serum (FBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM/F12 medium and penicillin–streptomycin were from VivaCell Biosciences (Denzlingen, Germany), trypsin from Capricorn Scientific (Ebsdorfergrund, Germany) and phosphate-buffered saline (PBS) from Serana (Pessin, Germany). Ascorbic acid, potassium persulfate, sodium carbonate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), iron(III) chloride hexahydrate, dimethyl sulfoxide (DMSO) and all other chemicals were of analytical grade. Absorbance in the microplate assays was read on a microplate reader (EPOCH, BioTek Instruments, Winooski, VT, USA).

2.2. Plant material

Leaves of *M. communis* were collected from Delibekirli village, Kırıkhan, Hatay, Türkiye (525 m a.s.l.) on 16 November 2023. A voucher specimen was deposited in the Herbarium of the Ankara University Faculty of Pharmacy (AEF 31026). Additionally, the plant's leaves have been dried for the purpose of preparing an extract.

2.3. Preparation of the extract

Dried, crushed leaves (100 g) were macerated in 96% ethanol (1000 mL) at room temperature for 3 days. The extract was filtered daily, and the plant material was re-extracted with ethanol. The combined filtrates were evaporated to dryness under reduced pressure at 40 °C in a rotary evaporator (R-215, Büchi, Flawil, Switzerland). The extraction yield was 16.87% (w/w, dry leaf basis). For the chemical assays, the extract was dissolved at 1 mg/mL in the appropriate solvent. For the cell assays, a stock solution was prepared in serum- and glutamine-free DMEM/F12 medium.

2.4. Total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method [17]. Briefly, 100 µL of the extract solution (1 mg/mL) was mixed with 3.9 mL of distilled water, 250 µL of Folin–Ciocalteu reagent and 750 µL of 20% Na_2CO_3. The mixture was incubated at 40 °C for 30 min, and absorbance was measured at 760 nm with the microplate reader. TPC was calculated from a gallic acid calibration curve (15.625–500 µg/mL) and expressed as mg gallic acid equivalents per g of extract (mg GAE/g extract).

2.5. Total flavonoid content

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method [18]. Briefly, 0.5 mL of the extract solution (1 mg/mL) was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After 30 min at room temperature, absorbance was measured at 415 nm with the microplate reader. TFC was calculated from a quercetin calibration curve (15.625–500 µg/mL) and expressed as mg quercetin equivalents per g of extract (mg QE/g extract).

2.6. Antioxidant activity

All three antioxidant assays were run in 96-well plates. **DPPH radical scavenging.** DPPH radical-scavenging activity was determined by the method of Brand-Williams et al. [19] with minor changes, as described previously [20]. Briefly, 20 µL of each sample was mixed with 180 µL of freshly prepared 0.1 mM DPPH solution and kept in the dark at room temperature for 2 h, and absorbance was read at 517 nm. The extract was tested at 31.25, 62.5, 125, 250 and 500 µg/mL, with gallic acid (20–300 µg/mL) as the positive control. Scavenging activity was calculated as:

$$Scavenging\ activity\ (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control and A_1 the absorbance of the sample. IC_{50} values, the concentrations giving 50% scavenging, were calculated from the concentration–response curves; lower IC_{50} values indicate stronger activity.

ABTS radical scavenging. ABTS radical cation scavenging was measured by the method of Re et al. [21]. The radical cation was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate and leaving the mixture in the dark for 12–16 h. The solution was then diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample (20 µL) was mixed with 180 µL of this working solution, and absorbance was read at 734 nm after 6 min at room temperature. Extract and gallic acid concentrations, scavenging activity and IC_{50} values were as described for the DPPH assay.

FRAP assay. FRAP was measured by the method of Benzie and Strain [22] with minor modifications. The FRAP reagent was prepared fresh by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ (10:1:1, v/v/v). Each sample (20 µL) was mixed with 180 µL of the reagent and incubated at room temperature for 4 min, and absorbance was read at 593 nm. Results were expressed as mg GAE/g extract using a gallic acid calibration curve (20–300 µg/mL).

2.7. Cell culture

L929 mouse fibroblast cells (ATCC® CCL-1™) and SH-SY5Y human neuroblastoma cells (ATCC® CRL-2266™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used at passages 6–7. Cells were grown in DMEM/F12 medium with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 (CO_2 incubator, Sanyo, Japan). For all cell assays, cells were seeded in 96-well plates at 1×10^4 cells/well and left to attach for 24 h before treatment.

2.8. Cytotoxicity (MTT assay)

Cell viability was measured by the MTT assay [23]. L929 cells were treated with the extract at 1.95–1000 µg/mL for 24 and 48 h. SH-SY5Y cells were treated at 3.91–2000 µg/mL for 24 h and at 1.95–1000 µg/mL for 48 h. At the end of treatment, MTT was added at a final concentration of 0.5 mg/mL for 4 h. The formazan crystals were dissolved in DMSO, and absorbance was read at 570 nm. After subtraction of the blank absorbance, viability was expressed as a percentage of the untreated control. Following ISO 10993-5, viability below 70% of the untreated control was considered cytotoxic [24]. This standard was developed for medical devices but is widely used as a conventional threshold for plant extracts.

2.9. 6-OHDA-induced injury and post-treatment

To choose the injury concentration, SH-SY5Y cells were exposed to 6-OHDA (10–400 µM) for 24 and 48 h. 6-OHDA was dissolved immediately before use in sterile distilled water containing 0.2% (w/v) ascorbic acid. In the post-treatment (rescue) experiment, cells were first exposed to 200 µM 6-OHDA for 24 h. The medium was then replaced with fresh medium containing the extract (10–1000 µg/mL), and the cells were incubated for another 24 h. Control cells were exposed to 6-OHDA in the same way and then kept for 24 h in extract-free medium. Viability was measured by the MTT assay in both experiments. In the rescue experiment, it was expressed relative to cells treated with 6-OHDA alone (100%).

2.10. Statistical analysis

Results of the chemical assays are given as mean ± standard deviation (SD) of three independent measurements. Cell viability results are given as mean ± standard error of the mean (SEM) of replicate wells (n = 2–6 per concentration) from three independent experiments. In each experiment, every concentration was compared with the corresponding control by one-way analysis of variance (ANOVA) followed by two-sided Dunnett's test. Before ANOVA, normality of the residuals was checked with the Shapiro–Wilk test and homogeneity of variances with the Brown–Forsythe test. Both assumptions were met in all data sets, except for unequal variances in L929 cells at 48 h. For this data set, Welch's

ANOVA with Bonferroni-corrected Welch's *t*-tests was used as a check and gave the same results. These tests were run in Python 3 (SciPy 1.18). IC_{50} values and their 95% confidence intervals (CIs) were calculated in GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA) by nonlinear regression of the normalized data against log-transformed concentrations (log[inhibitor] vs. normalized response, variable slope). If 50% viability was not reached within the tested range, the IC_{50} is reported as greater than the highest concentration tested. To compare the two cell lines, a sensitivity ratio (SR) was calculated as IC_{50}(L929)/IC_{50}(SH-SY5Y); an SR above 1 means that SH-SY5Y cells are more sensitive to the extract. Differences were considered significant at $p < 0.05$ (two-sided).

3. Results

3.1. Phenolic and flavonoid contents and antioxidant activity

Ethanolic extraction gave a yield of 16.87%. The extract contained 256.00 ± 6.16 mg GAE/g total phenolics and 48.00 ± 11.41 mg QE/g total flavonoids, and its FRAP value was 285.66 ± 21.92 mg GAE/g extract (Table 1). It scavenged DPPH and ABTS radicals in a concentration-dependent manner, with almost complete scavenging at 250–500 µg/mL (Figure 1A). The IC_{50} values were 75.97 µg/mL (95% CI 65.12–119.5) for DPPH and 106.7 µg/mL (95% CI 95.9–128.5) for ABTS, about 1.7 times those of gallic acid (45.25 and 64.41 µg/mL; Figure 1B; Table 1).

Table 1. Extraction yield, total phenolic and flavonoid contents, ferric reducing antioxidant power, and radical-scavenging IC_{50} values of *M. communis* leaf extract and gallic acid

Parameter	Extract	Gallic acid
Extraction yield (%, w/w)	16.87	N/A
TPC (mg GAE/g extract)	256.00 ± 6.16	N/A
TFC (mg QE/g extract)	48.00 ± 11.41	N/A
FRAP (mg GAE/g extract)	285.66 ± 21.92	N/A
DPPH IC_{50} (µg/mL)	75.97 (65.12–119.5)	45.25 (42.47–47.95)
ABTS IC_{50} (µg/mL)	106.7 (95.9–128.5)	64.41 (60.54–68.49)

Values are mean ± SD (n = 3); IC_{50} values are given with 95% confidence intervals. GAE, gallic acid equivalents; QE, quercetin equivalents; TPC, total phenolic content; TFC, total flavonoid content; FRAP, ferric reducing antioxidant power; N/A, not applicable.

3.2. Cytotoxicity in L929 and SH-SY5Y cells

The extract reduced cell viability in a concentration- and time-dependent manner in both cell lines (Figure 2; Table 2). In L929 cells at 24 h, viability fell to 78.7% at 3.91 µg/mL, stayed close to the ISO 10993-5 threshold (67.0–71.7%) from 7.81 to 250 µg/mL, and dropped to 58.8% and 31.2% at 500 and 1000 µg/mL (Figure 2A). At 48 h, L929 viability was unchanged up to 7.81 µg/mL (97.5–103.6%), stayed ≥70% up to 31.25 µg/mL (77.0% at 15.63 and 70.2% at 31.25 µg/mL), and declined steadily from 62.8% at 62.5 µg/mL to 19.0% at 1000 µg/mL (Figure 2B).

In SH-SY5Y cells, the MTT signal rose slightly but significantly at 7.81 µg/mL at both time points (117.6% at 24 h and 116.9% at 48 h). At 24 h, viability stayed ≥70% up to 62.5 µg/mL (71.5%) and fell to 67.8% at 125 µg/mL and 27.6% at 2000 µg/mL (Figure 2C). At 48 h, viability was maintained up to 15.63 µg/mL (104.3%) but dropped sharply to 50.2% at 31.25 µg/mL and to 16.8% at 1000 µg/mL (Figure 2D).

The 48-h IC_{50} values were 158.4 µg/mL (95% CI 123.9–204.0) for L929 and 31.16 µg/mL (95% CI 29.76–46.56) for SH-SY5Y cells (SR ≈ 5.1), so SH-SY5Y cells were clearly the more sensitive line (Table 2). At 24 h, the IC_{50} for SH-SY5Y cells was 502.2 µg/mL (95% CI 348.1–724.3), whereas the L929 value lay above the highest tested concentration (>1000 µg/mL), because viability plateaued across the middle concentrations.

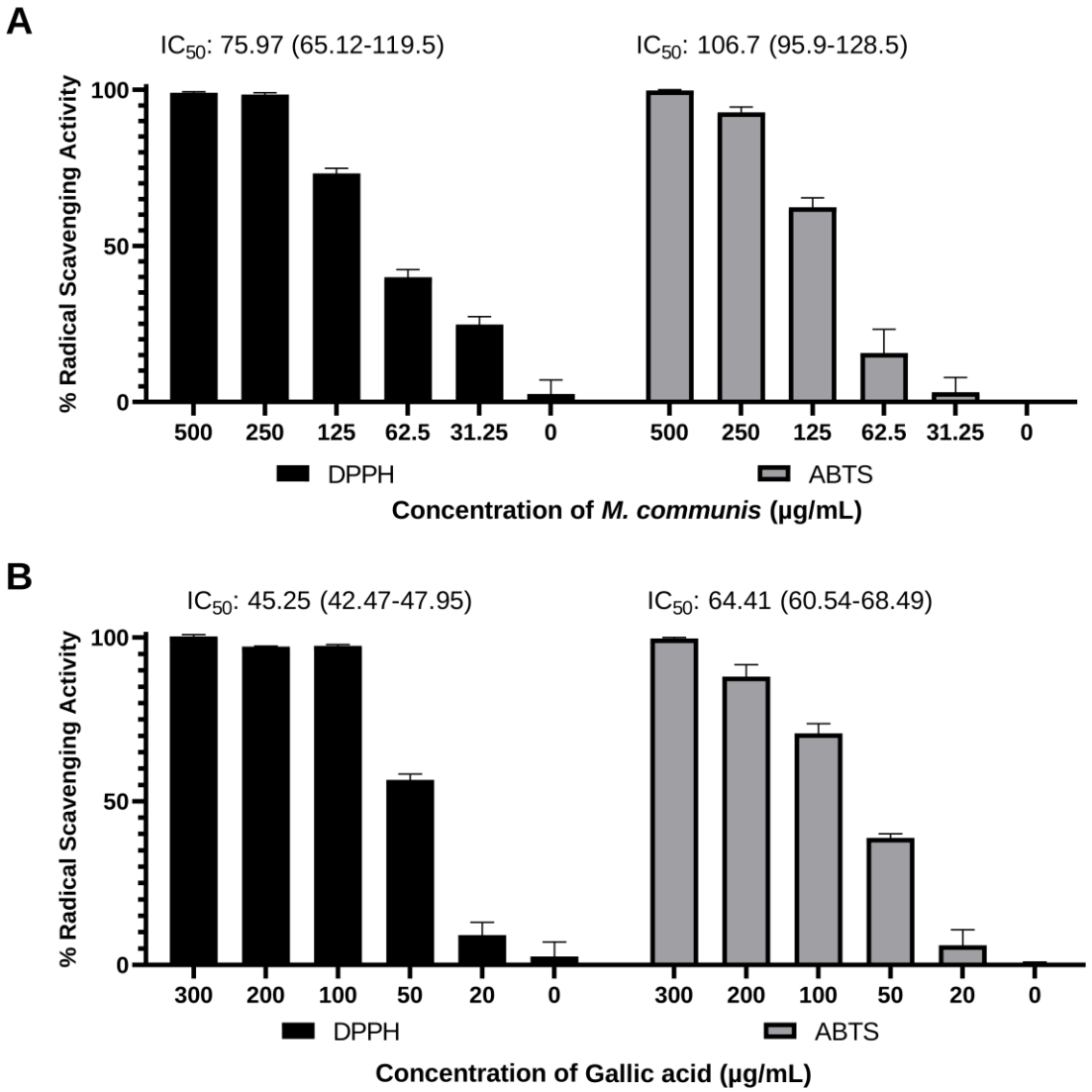

Figure 1. DPPH and ABTS radical-scavenging activity of (A) *M. communis* leaf extract and (B) the positive control gallic acid. Bars show mean ± SD; IC_{50} values are given in µg/mL with 95% confidence intervals

3.3. 6-OHDA-induced injury and post-treatment with the extract

6-OHDA reduced SH-SY5Y viability in a time-dependent manner (Figure 3A, B; Table 2). The effect at 24 h was small: viability was significantly lower than control only at 20, 200 and 400 µM (90.0%, 87.5% and 85.1%; p = 0.021, 0.002 and <0.001) and ranged from 92.1% to 100.3% at the other concentrations, so no IC_{50} could be estimated. At 48 h, all concentrations reduced viability significantly ($p < 0.001$), from 79.0% at 10 µM to 46.7–47.6% at 200–400 µM, with an IC_{50} of 227.7 µM (95% CI 183.5–296.4). Based on these results, 200 µM, which lies within the 95% CI of the 48-h IC_{50}, was chosen for the rescue experiment.

When the extract was applied for 24 h after a 24-h exposure to 200 µM 6-OHDA, its effect did not follow a simple concentration–response pattern (Figure 3C). Viability at 10–150 µg/mL (92.3–106.9% of the 6-OHDA-only level) did not differ from cells treated with 6-OHDA alone, except for a significant increase in MTT signal to 116.0% at 75 µg/mL (p = 0.016). At ≥200 µg/mL, viability fell significantly below the 6-OHDA-only level (73.1% to 54.0%; $p <$ 0.001), in line with the extract's own cytotoxicity, which may have been enhanced by the preceding injury (see Section 4).

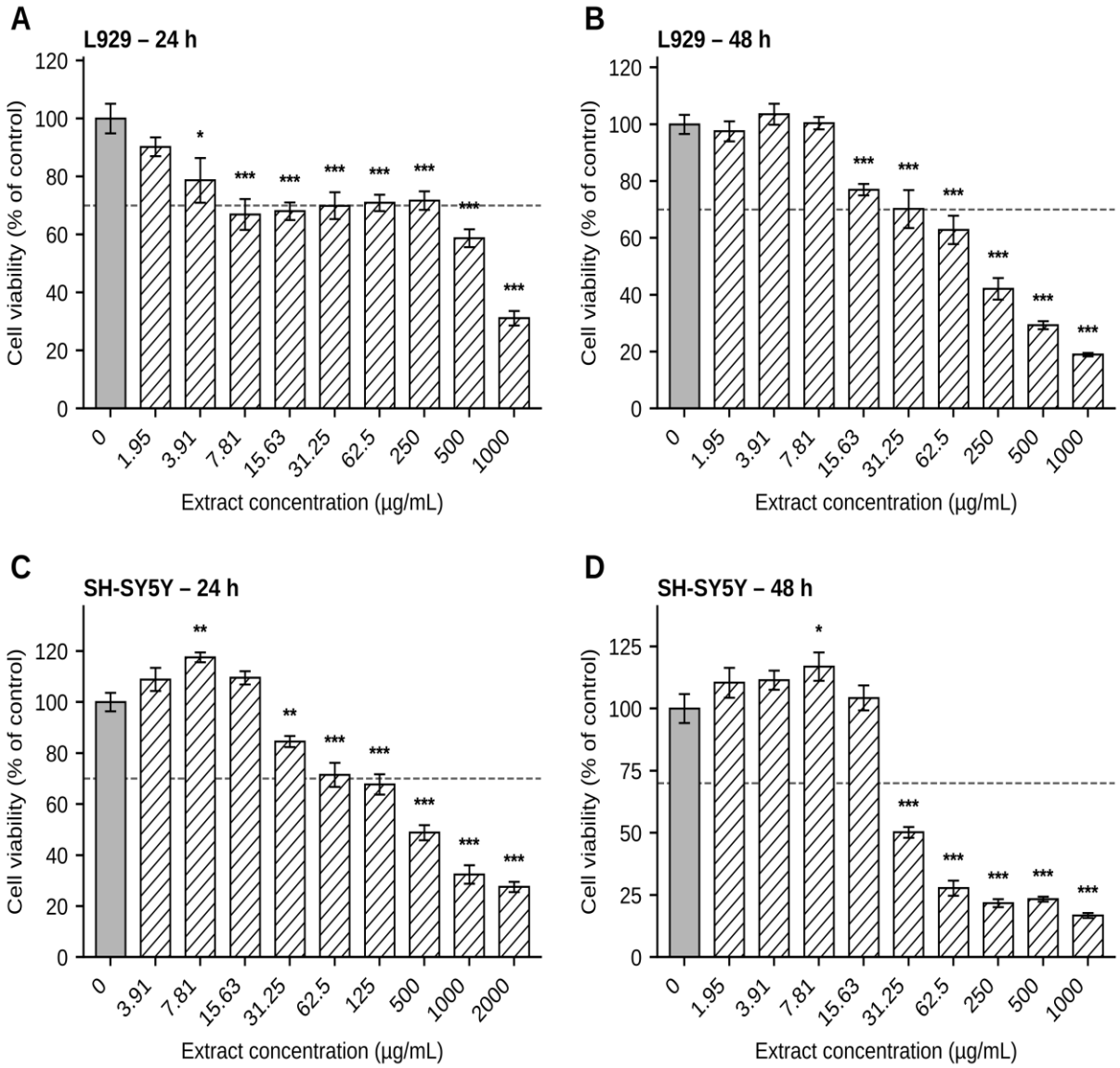

Figure 2. Effect of *M. communis* leaf extract on the viability of L929 cells after (A) 24 h and (B) 48 h and of SH-SY5Y cells after (C) 24 h and (D) 48 h (MTT assay). Bars show mean ± SEM; the dashed line marks the ISO 10993-5 cytotoxicity threshold (70% viability). *p < 0.05, **p < 0.01, ***p < 0.001 vs control (Dunnett's test)

Table 2. One-way ANOVA results and IC_{50} values for the cell-viability (MTT) experiments

Experiment	F (df_1, df_2)	p (ANOVA)	IC_{50} (95% CI)
L929, extract, 24 h	17.37 (9, 44)	<0.0001	>1000 µg/mL
L929, extract, 48 h	68.64 (9, 46)	<0.0001	158.4 (123.9–204.0) µg/mL
SH-SY5Y, extract, 24 h	112.17 (9, 34)	<0.0001	502.2 (348.1–724.3) µg/mL
SH-SY5Y, extract, 48 h	116.94 (9, 40)	<0.0001	31.16 (29.76–46.56) µg/mL
SH-SY5Y, 6-OHDA, 24 h	5.72 (9, 43)	<0.0001	not reached
SH-SY5Y, 6-OHDA, 48 h	48.57 (9, 50)	<0.0001	227.7 (183.5–296.4) µM
SH-SY5Y, 200 µM 6-OHDA + extract post-treatment, 24 h	35.93 (11, 48)	<0.0001	–

df, degrees of freedom; CI, confidence interval. Each concentration was compared with its control by two-sided Dunnett's test; the results are shown in Figures 2 and 3

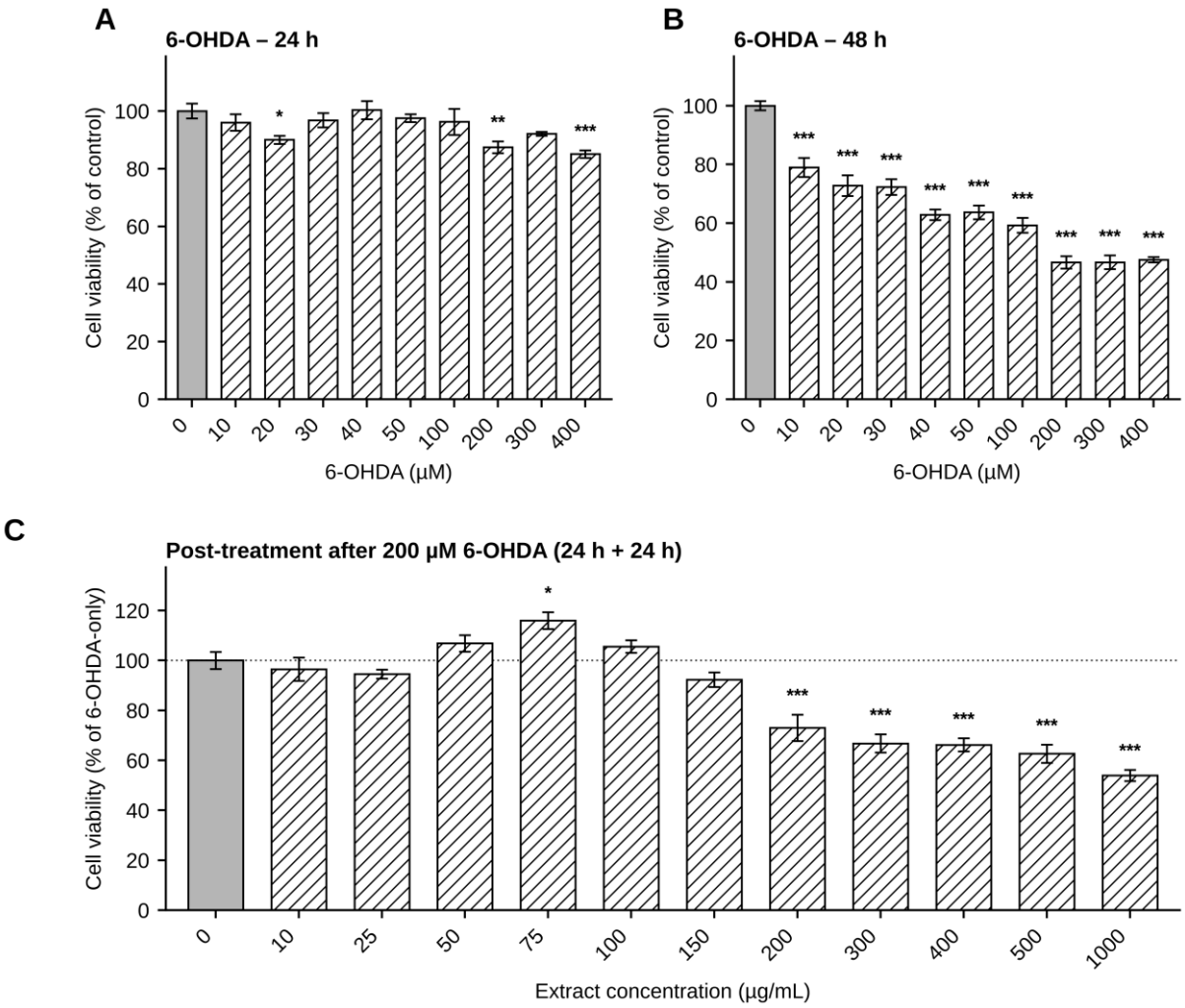

Figure 3. Effect of 6-OHDA on SH-SY5Y cell viability after (A) 24 h and (B) 48 h, and (C) effect of 24-h post-treatment with *M. communis* leaf extract on SH-SY5Y cells previously exposed to 200 µM 6-OHDA for 24 h; in (C) viability is expressed relative to cells exposed to 6-OHDA alone (0 µg/mL, dotted line). Bars show mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 vs the respective control (Dunnett's test)

4. Discussion

This study asked whether an ethanolic *M. communis* leaf extract, applied after 6-OHDA injury, could increase the viability of human SH-SY5Y cells. The data gave only limited support for this hypothesis. The extract was rich in phenolics and showed concentration-dependent radical-scavenging and reducing activity, but it was also cytotoxic within a fairly narrow concentration range, especially to SH-SY5Y cells. Given after 6-OHDA injury, it increased the MTT signal at only one concentration (75 µg/mL; 116.0% of the 6-OHDA-only level), while higher concentrations reduced viability, in line with its own cytotoxicity.

The extract scavenged DPPH and ABTS radicals and reduced Fe^{3+} in a concentration-dependent manner, though less strongly than gallic acid. The phenolic content and antioxidant activity of myrtle leaves vary with plant part, origin, extraction method and solvent [7–9]. Reported leaf TPC values range from about 29–36 mg GAE/g for Tunisian and Moroccan extracts [7, 8] to 415.85 mg GAE/g for a methanolic extract from Algeria [9]. Our TPC (256.00 mg GAE/g extract) lies within this range, well above the value for a Moroccan ethanolic leaf extract (29.0 mg GAE/g) [8] but below the Algerian value. Our TFC (48.00 mg QE/g extract) was also much lower than the 285.80 mg QE/g reported by Belahcene et al. [9]. In line with this, our DPPH and ABTS IC_{50} values (75.97 and 106.7 µg/mL) were two to five times higher than those of the Algerian extract (33.08 and 22.83 µg/mL) [9], indicating weaker scavenging. These comparisons are only approximate, since extraction conditions, solvent, plant origin and the units used to express results differ among studies. Still, total phenolic content correlates strongly with antioxidant activity in myrtle extracts [8], which suggests that phenolics account for much of the activity seen here.

The extract was tolerated only within a limited concentration range that depended on cell type and exposure time. L929 fibroblasts tolerated up to 31.25 µg/mL for 48 h, whereas SH-SY5Y viability fell below 70% at ≥31.25 µg/mL after 48 h and at ≥125 µg/mL after 24 h. At 48 h, SH-SY5Y cells, the intended target, were about five times more sensitive

than L929 fibroblasts, leaving a small margin between potentially protective and cytotoxic concentrations. L929 fibroblasts are one of the reference cell lines recommended in ISO 10993-5 for in vitro cytotoxicity testing [24]. Strong cytotoxicity has also been reported for other *M. communis* preparations, for example for Moroccan myrtle essential oils in MCF-7 breast cancer cells (IC_{50} 4–6.25 µg/mL) [12], although essential oils and polar extracts differ greatly in composition. The small rise in MTT signal at 7.81 µg/mL in SH-SY5Y cells resembles a low-dose stimulatory (hormetic) response. It may, however, simply reflect higher reductase activity rather than more cells, and it was not examined further.

Because the rescue experiment used a 24-h post-treatment, the relevant tolerance range is that of the 24-h exposure, in which SH-SY5Y viability stayed ≥70% up to about 62.5 µg/mL. The only concentration with an effect (75 µg/mL) lies just above the highest concentration that was clearly tolerated at 24 h (62.5 µg/mL) and was not itself tested in uninjured cells, and the losses at ≥200 µg/mL are in the range of the extract's own cytotoxicity. Concentrations of 100–150 µg/mL did not lower viability below the 6-OHDA-only level, although similar concentrations reduced the viability of uninjured cells. The two experiments differ in their reference group (6-OHDA-only vs untreated cells) and in the cells' exposure history, which may explain this difference. The lower viability at ≥200 µg/mL may, however, not reflect the extract's own cytotoxicity alone. Cells injured by 6-OHDA carry an oxidative burden and weakened antioxidant defenses [2], and at high concentrations polyphenols can act as pro-oxidants rather than antioxidants. Injured cells may therefore have been more vulnerable to the extract, so that its toxicity added to, or interacted synergistically with, the residual 6-OHDA injury. Because no extract-only group was included in the same experiment, additive and synergistic effects cannot be distinguished in the present data. Pre-treatment with a myrtle berry by-product extract (10–100 µg/mL, 12 h before 6-OHDA) protected PC12 cells against 6-OHDA and lowered ROS levels [16]. In that study, 6-OHDA was used at its IC_{50} and killed about half of the cells, whereas in our dose-finding experiment a 24-h exposure to 200 µM reduced viability by only about 12%. Our data extend that observation to the leaf, to human neuroblastoma cells and to treatment after injury, although the effect was limited to one concentration.

The increase in MTT signal after post-treatment may be linked to the antioxidant constituents of the extract. Myrtle leaf extracts contain phenolic acids, flavonoids and tannins, which contribute to their radical-scavenging and reducing activity [6, 10]. These compounds may have reduced the oxidative stress that persists after 6-OHDA exposure or helped maintain the metabolic activity of the cells [16]. Neither possibility was measured here. Because the extract was given after the injury, the rise in MTT signal should be read as a higher signal relative to injured cells, not as evidence of cell protection or neuronal repair. Many plant extracts that protect neuronal cells in vitro have not yet shown a clinical benefit, and in vitro findings of this kind need careful interpretation [15]. Measurements of oxidative stress and cell death will be needed to clarify the mechanism behind the observed effect.

This study has several limitations. The 6-OHDA concentration was based on the 48-h IC_{50}, but the rescue design used a 24-h exposure, which reduced viability by only about 12% in the dose-finding experiment and left little room to detect recovery. The rescue experiment was also normalized to the 6-OHDA-only group, so recovery toward the viability of uninjured cells could not be quantified. Viability was measured only by the MTT assay, which reflects mitochondrial reductase activity. Extracts rich in redox-active phenolics can reduce MTT directly and overestimate viability [25], so an independent viability or cell-death assay and cell-free controls are needed to confirm the finding. In addition, 75 µg/mL was not tested in uninjured cells, so its effect on its own is unknown. Undifferentiated SH-SY5Y cells were used, although differentiated cells resemble mature dopaminergic neurons more closely [14], and the extract was not chemically profiled. Future work should measure ROS, apoptotic signaling, mitochondrial function and dopaminergic markers, compare pre- and post-treatment designs, and optimize the dose.

5. Conclusions

The leaf extract of *M. communis* contained phenolics and flavonoids and showed moderate, concentration-dependent antioxidant activity. It was non-cytotoxic only within a limited concentration range, and at 48 h SH-SY5Y cells were about five times more sensitive to it than L929 fibroblasts, leaving a small margin between potentially protective and cytotoxic concentrations. Applied after 6-OHDA injury, the extract increased the MTT signal relative to injured cells at a single concentration (75 µg/mL), whereas higher concentrations were cytotoxic. This exploratory finding provides only limited support for the hypothesis of post-injury cytoprotection and requires confirmation with independent viability assays, appropriate controls, and stronger injury and mechanistic endpoints before any neuroprotective claim can be made.

Artificial Intelligence Statement

( ) This article did not use artificial intelligence.

(X) This article does use artificial intelligence. During the preparation of this manuscript, the authors used Claude (Anthropic) to improve English phrasing and grammar. The authors reviewed and edited the content as needed and take full responsibility for the framework and validity of the final paper.

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Ethical statement: This study used commercially obtained cell lines and did not involve human participants or experimental animals; therefore, it does not require ethical approval.

Data Availability Statement
( ) Option A (Public Repository): "The research data supporting this study are publicly available in [Name of Repository: e.g., Zenodo, Figshare, Dryad] with the permanent DOI link [URL/DOI]."
(X) Option B (Upon Request): "The raw data supporting the findings of this study are available from the corresponding author (S.Ç.) upon reasonable request and within ethical boundaries."
( ) Option C (Third-Party Data): "The data used in this study were obtained from [Name of Institution/Source] and are subject to third-party restrictions regarding redistribution."
( ) Option D (No Data): "No new data were created or analyzed in this study. Data sharing is not applicable to this article."

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