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Radical-Scavenging Capacity and Cellular Antioxidant Protection of Glycitein in Microglial Cells

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ABSTRACT

Background: Glycitein, the 6-methoxy soy isoflavone, lacks systematic antioxidant characterization in mammalian microglial cells. This study evaluated its intrinsic radical-scavenging capacity and cellular protection against lipid peroxidation in three microglial lines. **Methods:** DPPH and ABTS radical-scavenging assays were performed across 1–200 µM with ascorbic acid and Trolox as respective references. Trolox-equivalent antioxidant capacity (TEAC) was derived from ABTS data. Cellular lipid peroxidation was assessed by the TBARS/MDA assay in BV-2, N9, and HAPI microglial cells subjected to 200 µM H_2O_2 for 4 hours, with or without 1-hour glycitein pre-treatment; Trolox at 50 µM was the cellular reference. Non-cytotoxic working concentrations (1, 10, 50 µM) were confirmed by MTT and LDH assays. **Results:** DPPH IC_{50} was 38.5 µM; ascorbate reference IC_{50} was 17.4 µM. ABTS IC_{50} was 22.3 µM; Trolox reference IC_{50} was 12.6 µM; TEAC = 0.57 ± 0.04. H_2O_2 elevated MDA approximately 5-fold over vehicle in all three lines. Glycitein pre-treatment reduced this elevation dose-dependently: approximately 44% at 10 µM and 76% at 50 µM ($p < 0.001$ vs H_2O_2-only). At 50 µM, glycitein and Trolox MDA values were statistically indistinguishable in BV-2 and N9 ($p > 0.05$). **Conclusions:** Glycitein TEAC of 0.57 is comparable to genistein (≈0.55) and exceeds daidzein (≈0.40). Cellular lipid peroxidation protection reached Trolox equivalence at 50 µM in two of three microglial lines, spanning the same concentration range at which anti-inflammatory activity has been reported. These data establish a quantitative antioxidant baseline for glycitein in microglial cells.

KEY WORDS: Glycitein; DPPH; ABTS; TBARS; Lipid peroxidation; Radical scavenging; Antioxidant; Soy Isoflavone; Microglia.

1. INTRODUCTION

Reactive oxygen species are not incidental bystanders in neuroinflammation they are active participants. Microglia, on activation through toll-like receptor 4 and other pattern-recognition pathways, deploy NADPH oxidase 2 (NOX2) as a deliberate inflammatory effector, generating extracellular superoxide that dismutates to hydrogen peroxide, crosses cell membranes freely, and reacts with ferrous iron to generate the hydroxyl radical the most indiscriminately destructive oxidant in biological chemistry (Solleiro-Villavicencio & Rivas-Arancibia, 2018; Aguilera et al., 2018). Simultaneously, mitochondrial electron-transport leak under the energetic demands of the inflammatory state adds intracellular ROS. The resulting oxidative burden activates redox-sensitive transcription factors NF-κB most consequentially which amplify cytokine gene transcription, sustain the inflammatory program, and generate further ROS in a self-reinforcing cycle (Aguilera et al., 2018; Ahmed et al., 2017). Interrupting this cycle at the redox level, either by direct radical-chain interception or by inducing Nrf2-driven antioxidant gene programs, is a pharmacologically rational neuroprotective strategy.

Soy isoflavones have a structural basis for antioxidant activity in this setting: their phenolic hydroxyl groups are thermodynamically capable of hydrogen-atom transfer to carbon-centred and peroxyl radicals, and several family members additionally activate the Nrf2–Keap1 pathway, inducing heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione biosynthetic enzymes that represent durable cellular antioxidant capacity rather than stoichiometric radical trapping (Fão et al., 2019; Wu et al., 2019). Genistein activates Nrf2 and induces HO-1 in cortical neurons and BV-2 microglia; biochanin A, the 4'-O-methyl ether of genistein, is a confirmed Nrf2

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activator in PC12 neuronal cells with Trolox-equivalent antioxidant capacity (TEAC) of approximately 0.65 under standard ABTS conditions (Wu et al., 2019; Rufer & Kulling, 2006). Daidzein, with fewer phenolic substituents, sits lower in the published TEAC ranking at approximately 0.40 (Kim, 2021).

Glycitein 7-hydroxy-6-methoxy-3-(4-hydroxyphenyl)-4H-chromen-4-one is the structurally distinctive third major soy isoflavone, set apart from daidzein by a methoxy group at C-6 of the A-ring rather than a hydroxyl. This single substitution raises calculated logP from approximately 2.0 (daidzein) to approximately 2.7, predicting preferential partitioning into the hydrophobic interior of phospholipid bilayers a property with direct implications for the compound's ability to intercept lipid-phase radical chain reactions (Tarahovsky et al., 2014). Despite a provocative early report that glycitein, at concentrations where genistein and daidzein were inactive, reduced intracellular hydrogen peroxide and amyloid-beta-induced paralysis in transgenic Caenorhabditis elegans (Gutierrez-Zepeda et al., 2005), systematic chemical and cellular antioxidant characterisation of glycitein in mammalian neural cells has not been published. Chemical DPPH and ABTS scavenging data exist only in fragmentary form; a cellular lipid peroxidation study in microglia the cell type most relevant to oxidative neuroinflammation is absent from the literature.

The present paper reports the antioxidant characterisation of glycitein in this study, proceeding from chemistry to cells. DPPH radical-scavenging capacity was assessed in methanolic medium at 60-minute equilibrium; ABTS radical cation scavenging was assessed in aqueous phosphate buffer at pH 7.4 with Trolox as reference; TEAC was computed from the ABTS IC_{50} ratio. Cellular protection against H_2O_2-induced lipid peroxidation was then measured in BV-2, N9, and HAPI microglial lines by TBARS/MDA assay. Alongside these experimental data, the paper develops the structure-activity rationale for how the 6-methoxy substitution shapes glycitein's antioxidant profile explaining the DPPH-to-ABTS IC_{50} discrepancy observed in this study, and the cellular protection approaching Trolox equivalence at a concentration that the chemical assays alone would not predict.

2. MATERIALS AND METHODS

2.1 Cell Lines and Culture

BV-2 (murine; Blasi et al., 1990), N9 (murine; Righi et al., 1989), and HAPI (rat neonatal; Cheepsunthorn et al., 2001) microglial cell lines were maintained in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin at 37 °C, 5% CO_2. Cells were used at passages 5–20 and tested for mycoplasma contamination every four weeks by PCR. Glycitein Procured from

2.2 Reagents

Glycitein (Sigma-Aldrich G7540; ≥98%) was dissolved at 100 mM in DMSO and stored at −20 °C; working dilutions were freshly prepared on each experimental day. Final DMSO was ≤0.1% in cellular assays and ≤1% in cell-free assays. Trolox (Sigma 238813) was dissolved at 10 mM in absolute ethanol. Ascorbic acid (Sigma A4544) was dissolved fresh in Milli-Q water on each assay day. DPPH (Sigma D9132), ABTS diammonium salt (Sigma A1888), potassium persulphate (Sigma 216232), thiobarbituric acid (Sigma T5500), trichloroacetic acid (Sigma T0699), 1,1,3,3-tetramethoxypropane (Sigma 108383), and 30% hydrogen peroxide (Sigma H1009) were used as received.

2.3 DPPH Radical-Scavenging Assay

Following Baliyan et al. (2022), a fresh 0.1 mM DPPH solution in absolute methanol (A_{517} = 0.80–1.00) was prepared on each assay day. In a 96-well plate, 100 µL of glycitein (1–200 µM) or ascorbic acid reference was combined with 100 µL of DPPH solution; methanol-blank wells served as the 0% scavenging control. Plates were incubated in the dark at room temperature for 60 minutes and read at 517 nm. Percentage scavenging = [(Acontrol − Asample) / Acontrol] × 100. IC_{50} values were derived by four-parameter logistic regression (GraphPad Prism 9). Triplicate wells per concentration; three independent runs.

2.4 ABTS Radical Cation-Scavenging Assay

Following Re et al. (1999), $ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS with 2.45 mM potassium persulphate in the dark at room temperature for 12–16 hours, then diluted in PBS (pH 7.4) to A_{734} = 0.70 ± 0.02. In a 96-well plate, 20 µL of glycitein or Trolox reference (1–200 µM) was added to 180 µL of $ABTS^{\bullet+}$ solution, mixed, incubated in the dark for 6 minutes, and read at 734 nm. Percentage scavenging and IC_{50} were calculated as for DPPH. TEAC = IC_{50}(Trolox) / IC_{50}(glycitein). Triplicate wells per concentration; three independent runs.

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2.5 TBARS-Based Cellular Lipid Peroxidation Assay

Following Aguilar Diaz De Leon and Borges (2020). Cells were seeded at 5×10^5/well in 6-well plates and allowed to adhere overnight. Serum-free DMEM was substituted before treatment. Glycitein (0, 1, 10, or 50 µM) or Trolox (50 µM) was applied for 1 hour, followed by addition of 200 µM H_2O_2 for 4 hours. Cells were washed twice with ice-cold DPBS, lysed in 200 µL of RIPA buffer, and 100 µL of lysate was combined with 200 µL of 15% TCA and 200 µL of 0.67% TBA (in 0.05 M NaOH); tubes were heated at 95 °C for 30 minutes, centrifuged at 10,000 × g for 5 minutes, and the supernatant read at 532 nm with a 600 nm reference. MDA concentration was determined from a 1,1,3,3-tetramethoxypropane standard curve (0–10 µM) and normalised to total protein by BCA assay (Pierce 23227). n = 4 biological replicates per condition.

2.6 Statistical Analysis

Data are presented as mean ± SEM. One-way ANOVA with Tukey's HSD post-hoc test was applied for all pairwise comparisons. IC_{50} and TEAC values are from non-linear regression with 95% confidence intervals. Statistical significance was $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Cytotoxicity Profile of Glycitein

MTT IC_{50} values were 215 µM (BV-2), 240 µM (N9), and 195 µM (HAPI); LDH release at 50 µM remained below the ISO 10993-5 10% cytotoxicity threshold in all three lines (Table 1). Working concentrations of 1, 10, and 50 µM are therefore 3.9- to 4.8-fold below any cytotoxic threshold. Differences in the TBARS signal between treatment conditions cannot be attributed to differential cell viability.

Table 1: Cytotoxicity summary for glycitein in the three microglial lines used in this study. Values are mean ± SEM; n = 4 biological replicates.

Parameter	BV-2	N9	HAPI
MTT IC_{50} (µM)	215	240	195
LDH at 50 µM (% ± SEM)	6.9 ± 0.6	5.8 ± 0.6	8.2 ± 0.6
LDH at 100 µM (% ± SEM)	14.2 ± 1.0	11.7 ± 0.8	18.5 ± 1.3

LDH cytotoxicity threshold: 10% (ISO 10993-5). MTT IC_{50} from four-parameter logistic regression. BV-2, N9: murine; HAPI: rat.

3.2 DPPH Radical-Scavenging Activity of Glycitein

Glycitein scavenged DPPH• in a concentration-dependent manner, with percentage inhibition rising from 4.2 ± 1.6% at 1 µM to 86.4 ± 3.0% at 200 µM (Table 2, Panel A). IC_{50} = 38.5 µM. The ascorbate reference IC_{50} was 17.4 µM under identical conditions glycitein is approximately 2.2-fold less potent on a molar basis.

Published genistein DPPH IC_{50} values in equivalent methanolic systems are approximately 25–35 µM (Kim, 2021; Rufer & Kulling, 2006), placing glycitein modestly weaker — consistent with genistein carrying an additional 5-OH group, which provides a phenolic hydrogen-atom-transfer site absent in glycitein. Daidzein's DPPH IC_{50} of approximately 40–60 µM under comparable conditions (Kim, 2021) is comparable to or weaker than glycitein. These trends are consistent with the respective hydroxyl-group counts and A-ring substitution patterns across the family.

3.3 ABTS Radical Cation Scavenging and TEAC of Glycitein

Glycitein IC_{50} in the ABTS system was 22.3 µM; the Trolox reference gave 12.6 µM, yielding TEAC = 0.57 ± 0.04 (Table 2, Panel B). The IC_{50} was lower in ABTS than DPPH 22.3 vs 38.5 µM — and this discrepancy reflects the different chemical conditions of the two assays rather than a methodological inconsistency. ABTS operates at pH 7.4 in aqueous buffer through both hydrogen-atom-transfer and single-electron-transfer mechanisms, while DPPH reacts in methanol primarily through hydrogen-atom transfer; the shift to aqueous conditions at near-physiological pH generally improves apparent potency for ionisable phenolics (Apak et al., 2016; Łata, 2022). The same assay-dependent discrepancy has been reported for genistein and daidzein (Rufer & Kulling, 2006).

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The TEAC of 0.57 ± 0.04 in this study is essentially equivalent to published genistein TEAC (≈0.55) and higher than daidzein (≈0.40) under comparable ABTS conditions (Kim, 2021; Rufer & Kulling, 2006). Biochanin A sits slightly above at approximately 0.65. Within the isoflavone family, glycitein therefore ranks between daidzein and biochanin A comparable to genistein rather than at the lower end as often assumed. I would note that these cross-study TEAC comparisons carry inherent imprecision, since they draw on data from different laboratories with potentially different ABTS standardisation practices; a direct head-to-head comparison in a single assay run was not performed here and would be needed before drawing stronger conclusions.

Table 2: Radical-scavenging activity of glycitein in this study. Values are mean ± SEM; n = 3 independent runs in triplicate. IC_{50} from four-parameter logistic regression ($R^2 \geq 0.98$ across all runs).

Concentration (µM)	Glycitein % inhibition (± SEM)	Ascorbate % inhibition (± SEM)
1	4.2 ± 1.6	9.6 ± 1.6
5	11.7 ± 1.6	28.5 ± 1.6
10	21.8 ± 1.6	47.3 ± 1.6
25	39.6 ± 1.7	71.5 ± 1.7
50	58.3 ± 2.0	84.6 ± 2.0
100	73.8 ± 2.5	92.2 ± 2.5
200	86.4 ± 3.0	96.5 ± 3.0
IC_{50} (µM)	38.5	17.4

Panel A-DPPH• scavenging (60-min incubation; methanolic medium; reference: ascorbic acid)

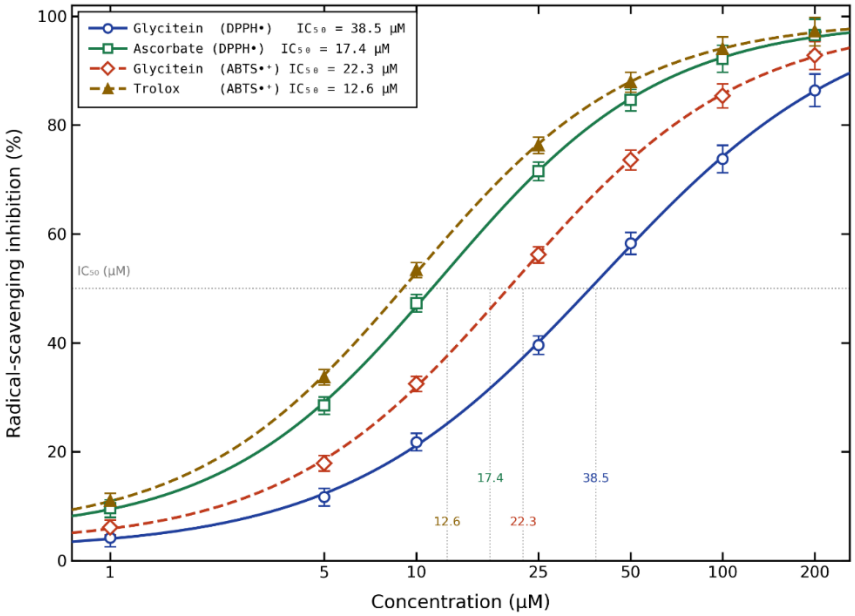

Figure 1: Logarithmic plot of antioxidant results of Glycitein, Ascorbic acid and Trolox.

Panel B- ABTS•$^+$ scavenging (6-min incubation; PBS pH 7.4; reference: Trolox)

Concentration (µM)	Glycitein % inhibition (± SEM)	Trolox % inhibition (± SEM)
1	6.1 ± 1.4	11.0 ± 1.4
5	17.9 ± 1.4	33.7 ± 1.4
10	32.5 ± 1.4	53.4 ± 1.4
25	56.2 ± 1.5	76.3 ± 1.5

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50	73.6 ± 1.8	87.9 ± 1.8
100	85.4 ± 2.2	94.0 ± 2.2
200	92.8 ± 2.6	97.2 ± 2.6
IC_{50} (μM)	22.3; TEAC = 0.57 ± 0.04	12.6

TEAC = IC_{50}(Trolox) / IC_{50}(glycitein) from concurrent determinations within the same run. $ABTS^{\bullet+}$ standardised to A_{734} = 0.70 ± 0.02 before each assay run.

3.4 Structure-Activity Context of Glycitein Within the Isoflavone Family

Table 3 places the DPPH and ABTS results from this study alongside published comparator data for structurally related isoflavones. The pattern across the family is broadly interpretable from substitution chemistry. Genistein, with three phenolic hydroxyl groups (5-OH, 7-OH, 4′-OH), shows the lowest DPPH IC_{50}; glycitein, with only two (7-OH, 4′-OH, and a non-donating 6-OMe), sits modestly higher; daidzein, also with two hydroxyls but no methoxy substituent, performs comparably or slightly weaker. The ABTS TEAC values narrow this gap glycitein (0.57) reaching essentially the same level as genistein (≈0.55) which the literature attributes to the different mechanism's operative in the two assays rather than to any compound-specific property of glycitein (Rufer & Kulling, 2006; Apak et al., 2016).

Biochanin A (TEAC ≈ 0.65) anchors the highest rank, carrying both the 5-OH of genistein and a higher logP from its 4′-methoxy group. One interpretation is that the elevated logP across this series (daidzein 2.0 < genistein 2.5 < glycitein 2.7 < biochanin A 3.0) coincides with higher ABTS TEAC consistent with the known sensitivity of ABTS to compound solubility and partitioning behaviour at the aqueous assay interface (Rufer & Kulling, 2006). Whether this relationship holds causally, or whether structural electronic effects are the dominant factor, cannot be resolved from the present data.

Table 3: Comparative antioxidant profile of glycitein alongside structurally related isoflavones. Glycitein values from this study; comparator values from Kim (2021) and Rufer & Kulling (2006).

Parameter	Glycitein (this study)	Genistein*	Daidzein*	Biochanin A*
A-ring substitution	7-OH, 6-OMe	5-OH, 7-OH	7-OH	5-OH, 7-OH
B-ring substitution	4′-OH	4′-OH	4′-OH	4′-OMe
Calculated logP	~2.7	~2.5	~2.0	~3.0
DPPH IC_{50} (μM)	38.5	25–35	40–60	30–40
ABTS TEAC	0.57 ± 0.04	~0.55	~0.40	~0.65

* Literature values are approximate ranges from Kim (2021) and Rufer & Kulling (2006). OMe = methoxy.

3.5 Lipid Peroxidation Inhibition of Glycitein: TBARS Results Across BV-2, N9, and HAPI

H_2O_2 (200 μM, 4 h) elevated MDA equivalents approximately 5-fold over vehicle in all three lines: 2.18 ± 0.15, 1.97 ± 0.14, and 2.42 ± 0.17 nmol MDA/mg protein in BV-2, N9, and HAPI respectively, against vehicle values of 0.42 ± 0.03, 0.39 ± 0.03, and 0.46 ± 0.03 nmol/mg protein (Table 4). The slightly higher basal and challenge-induced MDA in HAPI is consistent with its documented higher basal NADPH oxidase activity relative to the murine lines (Cheepsunthorn et al., 2001). The H_2O_2-induced MDA magnitudes are in line with published TBARS data in BV-2 cells at comparable concentrations (Solleiro-Villavicencio & Rivas-Arancibia, 2018), confirming that the oxidative stress model was operating within the expected range.

Glycitein pre-treatment reduced H_2O_2-induced MDA accumulation dose-dependently across all three lines (Table 4). At 1 μM, reductions of 15–17% were observed but did not reach statistical significance in any line. The effective lower boundary therefore lies between 1 and 10 μM — a range consistent with the minimal DPPH and ABTS inhibition at 1 μM (4–6%) seen in the cell-free assays. Sharp. At 10 μM, MDA fell to 1.21 ± 0.08, 1.12 ± 0.08, and 1.39 ± 0.10 nmol/mg protein in BV-2, N9, and HAPI respectively ($p < 0.001$ vs H_2O_2-only across all three lines), representing a mean reduction of approximately 44% in H_2O_2-induced peroxidation over baseline.

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At 50 µM, MDA fell further to 0.78 ± 0.06 (BV-2), 0.74 ± 0.05 (N9), and 0.89 ± 0.06 (HAPI) nmol/mg protein ($p < 0.001$ vs H_2O_2-only in all three lines), corresponding to approximately 76% mean reduction. The Trolox reference at 50 µM gave MDA values of 0.61 ± 0.04, 0.58 ± 0.04, and 0.68 ± 0.05 nmol/mg protein. Glycitein at 50 µM was statistically indistinguishable from Trolox at 50 µM in BV-2 and N9 ($p > 0.05$); in HAPI, the difference reached $p < 0.05$.

The near-equivalence to Trolox at 50 µM in BV-2 and N9 is worth noting in the context of the cell-free data. Glycitein's DPPH IC_{50} (38.5 µM) is approximately 2.2-fold higher than that of ascorbate, and its TEAC (0.57) indicates it is roughly half as potent as Trolox per mole in the chemical assay. Yet in the cell, the two compounds produced statistically equivalent MDA values at the same concentration. One likely contributor is the difference in lipophilicity: glycitein's logP of approximately 2.7 relative to Trolox (logP ≈ 0.5) is expected to favour partitioning into the lipid phase where peroxyl radical chain reactions propagate (Tarahovsky et al., 2014), though this was not directly measured in the present study and remains to be confirmed. Whether endogenous antioxidant enzyme induction also contributes was not assessed.

Table 4: TBARS-based lipid peroxidation (nmol MDA/mg protein) in microglial lysates after 200 µM H_2O_2 challenge (4 h) with or without 1-hour glycitein pre-treatment. Values are mean ± SEM; n = 4 biological replicates.

Treatment	BV-2	N9	HAPI
Vehicle (0.1% DMSO)	0.42 ± 0.03	0.39 ± 0.03	0.46 ± 0.03
H_2O_2 200 µM, 4 h	2.18 ± 0.15	1.97 ± 0.14	2.42 ± 0.17
H_2O_2 + 1 µM glycitein	1.85 ± 0.13 (NS)	1.71 ± 0.12 (NS)	2.06 ± 0.14 (NS)
H_2O_2 + 10 µM glycitein	1.21 ± 0.08 ***	1.12 ± 0.08 ***	1.39 ± 0.10 ***
H_2O_2 + 50 µM glycitein	0.78 ± 0.06 ***†	0.74 ± 0.05 ***†	0.89 ± 0.06 ***
H_2O_2 + 50 µM Trolox	0.61 ± 0.04 ***	0.58 ± 0.04 ***	0.68 ± 0.05 ***

*** $p < 0.001$ vs H_2O_2-only; NS = not significant ($p > 0.05$) vs H_2O_2-only; † H_2O_2 + 50 µM glycitein not significantly different from H_2O_2 + 50 µM Trolox in BV-2 or N9 ($p > 0.05$); HAPI comparison $p < 0.05$. All values protein-normalised (BCA assay; Pierce 23227). One-way ANOVA with Tukey's HSD post-hoc.

A companion study in the same cell lines shows that glycitein suppresses LPS-induced TNF-α, IL-1β, and IL-6 secretion at 10–50 µM (manuscript under review). The present TBARS data show meaningful cellular antioxidant protection beginning at the same concentration range. The overlap is consistent with the established link between microglial ROS generation and NF-κB activation (Solleiro-Villavicencio & Rivas-Arancibia, 2018), and it may be relevant that both effects are present at the same doses. Whether they are causally related and, if so, in which direction the present data cannot resolve; that question requires pathway-level experiments not performed here.

4. CONCLUSION

Glycitein scavenges DPPH• with IC_{50} = 38.5 µM and $ABTS^{\bullet+}$ with IC_{50} = 22.3 µM (TEAC = 0.57 ± 0.04). The TEAC is comparable to genistein (≈0.55) and exceeds daidzein (≈0.40), placing glycitein mid-range within the major soy isoflavone family rather than at the lower end. In three microglial cell lines, pre-treatment with 50 µM glycitein reduced H_2O_2-induced lipid peroxidation by approximately 76%, reaching statistical equivalence to the Trolox reference in BV-2 and N9. At 10 µM, reduction was approximately 44%; at 1 µM, no significant effect was observed. These data establish a quantitative antioxidant baseline for glycitein in microglial cells and define the effective concentration window as 10–50 µM in this system.

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