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Asiaticoside and Madecassoside from *Centella asiatica* Demethylate the Nrf2 Promoter and Enhance Neuroprotection *In Vitro*

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A B S T R A C T

Centella asiatica (L.) Urban (Apiaceae), known as pegagan in Indonesian Jamu and Brahmi in Ayurveda, contains triterpene saponins with reported neuroprotective properties, yet the epigenetic mechanisms remain poorly understood. This study investigated whether a triterpene-enriched fraction (TEF) standardized to asiaticoside (28.4%) and madecassoside (22.7%) modulates Nrf2/HO-1 pathway activation through promoter demethylation and histone acetylation in oxidatively stressed SH-SY5Y neuroblastoma cells. Cells were pre-treated with TEF (25, 50, 100 µg/mL) for 24 hours before H_2O_2 (200 µM) challenge. TEF at 100 µg/mL restored cell viability to 82.4 ± 4.3% versus 47.8 ± 4.1% in H_2O_2-only cells ($p < 0.001$, Cohen's $d = 8.22$), reduced ROS to 1.52 ± 0.15-fold ($p < 0.001$), and upregulated Nrf2 mRNA 2.83 ± 0.21-fold and HO-1 protein 3.14 ± 0.24-fold. Bisulfite sequencing revealed dose-dependent Nrf2 promoter demethylation from 67.8 ± 4.2% to 31.2 ± 2.6% ($p < 0.001$), paralleled by DNMT1 suppression (2.74 to 1.12-fold) and H3K27ac enrichment (0.34 to 1.43-fold) at the Nrf2 locus. A strong inverse correlation between methylation and Nrf2 expression ($r = -0.912$) and closely aligned IC_{50} values for ROS suppression (62.4 µg/mL) and demethylation (58.7 µg/mL) support a coordinated epigenetic-transcriptional mechanism. These findings provide the first evidence that *Centella asiatica* triterpenes activate neuroprotective pathways through dual epigenetic remodeling, offering a molecular rationale for the traditional cognitive-enhancing applications of this ethnopharmacologically significant herb.

1. Introduction

Neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) represent a growing global health burden affecting tens of millions of individuals worldwide, a figure projected to rise sharply with continued population aging.[1,2] Oxidative stress, characterized by excessive accumulation of reactive oxygen species (ROS) and depletion of antioxidant defenses, serves as a central pathomechanism underlying neuronal dysfunction and death.[3,4] Mitochondrial dysfunction, protein aggregation, and neuroinflammation cascade from the initial oxidative insult, yet conventional pharmacotherapies targeting single pathways demonstrate limited efficacy.[2] This therapeutic gap has motivated renewed interest in phytochemical approaches that modulate multiple neuroprotective pathways simultaneously.

Centella asiatica (L.) Urban, a perennial creeping herb of the Apiaceae family, has been employed for generations across two ethnopharmacological traditions. In Indonesian *Jamu, pegagan* functions as a cornerstone of cognitive tonics and wound-healing preparations,[5] documented in formulations spanning centuries of use.[6] In Ayurvedic medicine the herb is recognized as Brahmi and Gotu Kola, classified within the Medhya Rasayana category of cognitive-enhancing compounds.[5,7] These empirical claims have increasingly attracted modern scientific validation.[7,8]

The phytochemistry of *Centella asiatica* is dominated by pentacyclic triterpene saponins, principally asiaticoside and madecassoside, which together with

their aglycones constitute a substantial fraction of dried leaf material.[6] Hydrolysis yields the aglycones asiatic acid and madecassic acid, which penetrate cellular membranes more readily.[9] These triterpenes promote neuronal differentiation, suppress neuroinflammatory cytokines, and attenuate apoptosis in cultured neurons,[9,10] yet the epigenetic modulation of master antioxidant regulators remains incompletely characterized.

The nuclear factor erythroid 2-related factor 2 (Nrf2) is the paramount transcriptional regulator of cellular antioxidant defense.[11] Under basal conditions Nrf2 is sequestered by KEAP1 (Kelch-like ECH-associated protein 1) and directed toward proteasomal degradation.[11] Upon oxidative stress, Nrf2 accumulates in the nucleus and binds the antioxidant response element (ARE) of target genes including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and superoxide dismutase (SOD).[12,13] Nrf2 activation also attenuates neuroinflammatory NF-κB signaling and promotes mitochondrial resilience.[14,15]

Nrf2 expression is subject to tight epigenetic control. The Nrf2 promoter contains a CpG island whose methylation silences expression and renders cells vulnerable to oxidative assault.[16,17] DNA methyltransferase 1 (DNMT1) catalyzes this repressive methylation and is upregulated in stress and disease models,[18] whereas histone H3 lysine-27 acetylation (H3K27ac) marks active chromatin and promotes Nrf2 accessibility.[19] Nrf2 promoter hypermethylation contributes to impaired antioxidant responses,[16,20] and DNMT inhibitors and dietary epigenetic modulators can reverse this dysregulation.[16,19] However, synthetic inhibitors often display poor selectivity, prompting investigation of botanical dual-action epigenetic modulators.

To date, no study has examined whether triterpene saponins from *Centella asiatica* modulate Nrf2 through epigenetic remodeling; prior reports did not assess promoter methylation, DNMT activity, or histone modifications.[6,9] The present investigation therefore tests the hypothesis that a standardized TEF activates the Nrf2/HO-1 pathway in H_2O_2-challenged SH-SY5Y cells through dual Nrf2 promoter demethylation (DNMT1 inhibition) and H3K27 acetylation, and that the magnitude of demethylation correlates with Nrf2 activation and ROS suppression.

2. Methods

Ethical approval

This study was reviewed and approved by the CMHC Ethics Committee (Ref number CMHC/EC/2024/047). The investigation used a commercially sourced human neuroblastoma cell line and authenticated plant material; no human participants or live animals were involved. All procedures were performed in a private university research laboratory in Palembang, Indonesia, in accordance with institutional biosafety guidelines.

Plant material and TEF isolation

Centella asiatica (L.) Urban was sourced from a cultivated medicinal-plant collection in South Sumatra, Indonesia, in May 2024 during the vegetative phase, authenticated by a qualified botanist, and a voucher specimen (CA-2024-0892) was deposited at the institutional herbarium. Dried aerial parts (500 g) were macerated in 70% ethanol (3 × 5 L), concentrated, and lyophilized (crude yield 12.4% w/w). The crude extract was fractionated by silica-gel column chromatography (chloroform:methanol gradient) with TLC monitoring (R_f 0.35–0.55); triterpene-rich fractions were pooled and purified by preparative TLC to yield the TEF (≈8.2 g; 13.2% recovery).

HPLC standardization

The TEF was standardized for asiaticoside and madecassoside by validated reversed-phase HPLC (Shimadzu LC-20AT; Phenomenex Luna C18, 250 × 4.6 mm, 5 µm; acetonitrile–water gradient; 1.0 mL/min; 206 nm). Calibration curves were linear (asiaticoside R^2 = 0.9987; madecassoside R^2 = 0.9991), and the TEF was analyzed in triplicate.

Cell culture and experimental design

SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were cultured in DMEM/F-12 with 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO_2. Six groups (six replicates each, three independent experiments) were studied: Control; H_2O_2 200 µM; TEF 25, 50, and 100 µg/mL + H_2O_2; and vitamin E (VitE) 50 µM + H_2O_2 (positive control). Cells were seeded at 1×10^4 (96-well) or 5×10^5 (6-well), pre-treated with TEF or VitE for 24 h, then challenged with H_2O_2 (200 µM) for 24 h. Samples were coded to blind the analyst.

Assays and molecular analyses

Viability was measured by MTT assay (570 nm) as $[OD_{treatment}/OD_{control}] \times 100$, and ROS by DCFH-DA flow cytometry (BD FACSCalibur, 488 nm), expressed as $MFI_{treatment}/MFI_{control}$. Gene expression (Nrf2, HO-1, NQO1; GAPDH reference) was quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method. HO-1, DNMT1, and β-actin proteins were assessed by Western blot with ImageJ densitometry. Nrf2 promoter methylation (−518 to −276 relative to the TSS) was quantified by bisulfite sequencing of ten cloned CpG sites, and H3K27ac enrichment at the Nrf2 promoter by ChIP-qPCR (percent-input method).

Statistical analysis

Data are mean ± SD (SPSS v26.0). Normality and variance homogeneity were tested by Shapiro-Wilk and Levene tests; groups were compared by one-way ANOVA with Tukey HSD (or Kruskal-Wallis with Mann-Whitney *U* and Bonferroni correction). Effect sizes were Cohen's *d* and eta-squared (η^2); dose-response was assessed by linear regression with Pearson *r*, R^2, and IC_{50} estimation. A two-tailed $p < 0.05$ was significant.

3. Results and Discussion

HPLC standardization of the TEF

HPLC profiling revealed a triterpene-saponin composition dominated by asiaticoside (28.4 ± 1.2%, w/w; 28.2 min) and madecassoside (22.7 ± 0.9%; 31.9 min), with minor asiatic acid (14.3 ± 0.6%) and madecassic acid (11.8 ± 0.5%). Combined triterpene content reached 77.2%, confirming successful enrichment; the membrane-permeant aglycones may contribute to overall activity.[9]

Cell viability and dose-dependent cytoprotection

H_2O_2 challenge induced marked cell death, as detailed in Table 1 and illustrated in Figure 1A. Viability fell from 100% in controls to 47.8 ± 4.1% with H_2O_2 ($p < 0.001$, Cohen's $d = 15.93$). TEF conferred dose-dependent cytoprotection, restoring viability to 61.3 ± 3.8% (25 µg/mL), 72.6 ± 4.0% (50 µg/mL), and 82.4 ± 4.3% (100 µg/mL; all $p < 0.001$ vs. H_2O_2), comparable to vitamin E (78.9 ± 3.9%; $p = 0.247$ vs. TEF 100). One-way ANOVA confirmed strong effects [$F(5,30) = 142.7$, $\eta^2 = 0.960$], consistent with prior reports of *Centella asiatica* neuroprotection.[5,9]

Table 1. Cell viability after H_2O_2 challenge and TEF pre-treatment.

Treatment Group	Viability (%)	95% CI	vs. H_2O_2	*p*-value	Cohen's *d*
Control (untreated)	100.0 ± 3.2	96.2–103.8	—	—	—
H_2O_2 200 µM	47.8 ± 4.1	42.1–53.5	—	<0.001*	—
TEF 25 + H_2O_2	61.3 ± 3.8	55.8–66.8	+13.5%	<0.001*	3.39
TEF 50 + H_2O_2	72.6 ± 4.0	66.8–78.4	+24.8%	<0.001*	6.10
TEF 100 + H_2O_2	82.4 ± 4.3	76.1–88.7	+34.6%	<0.001*	8.22
Vitamin E 50 + H_2O_2	78.9 ± 3.9	72.6–85.2	+31.1%	<0.001*	7.64

*Notes: Data are mean ± SD (n = 6 per group, three independent experiments). Cohen's d is relative to the H_2O_2 group.*Significant at $p < 0.05$ (two-tailed).*

Reactive oxygen species suppression

As shown in Figure 1B, H_2O_2 elevated ROS to 3.87 ± 0.34-fold of control ($p < 0.001$, $d = 11.61$). TEF dose-dependently suppressed ROS to 2.54 ± 0.28-fold (25 µg/mL), 1.93 ± 0.22-fold (50 µg/mL), and 1.52 ± 0.15-fold (100 µg/mL; all $p < 0.001$ vs. H_2O_2), approaching baseline and comparable to vitamin E (1.68 ± 0.19-fold). Regression confirmed strong dose-dependence ($r = 0.958$, $R^2 = 0.918$), with an IC_{50} for ROS suppression of 62.4 µg/mL (95% CI 54.1–71.8; Table 4).

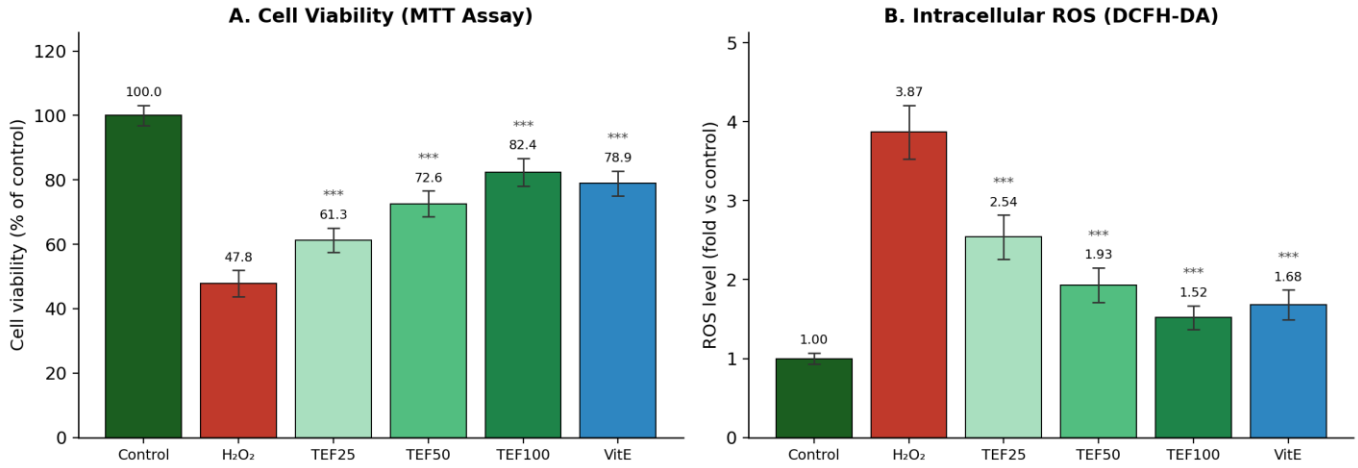

*Figure 1. Cell viability and ROS suppression. (A) Viability (MTT) and (B) intracellular ROS (DCFH-DA) in SH-SY5Y cells pre-treated with TEF (25, 50, 100 µg/mL) or vitamin E (50 µM), then exposed to H_2O_2 (200 µM). Mean ± SD (n = 6). ***p < 0.001 versus H_2O_2.*

Nrf2/HO-1 pathway activation

RT-qPCR and Western blotting confirmed Nrf2/HO-1 activation, as detailed in Table 2 and Figure 2A and 2B. H_2O_2 suppressed Nrf2 mRNA to 0.42 ± 0.07-fold, whereas TEF restored it to 1.21, 2.04, and 2.83 ± 0.21-fold across ascending doses (d = 14.30 at 100 µg/mL; vitamin E 2.61 ± 0.18-fold). HO-1 protein mirrored this pattern (0.38-fold with H_2O_2; 1.47, 2.34, and 3.14 ± 0.24-fold with TEF). ANOVA confirmed strong effects for Nrf2 mRNA [$F(5,30) = 127.3$] and HO-1 [$F(5,30) = 141.6$]. The 2.83-fold Nrf2 and 3.14-fold HO-1 induction by TEF 100 exceed values reported for sulforaphane (2.1–2.5-fold),[13] underscoring the potency of *Centella asiatica* triterpenes.

Table 2. Nrf2 mRNA (RT-qPCR, normalized to GAPDH) and HO-1 protein (Western blot, normalized to β-actin) expression.

Treatment Group	Nrf2 mRNA (fold)	HO-1 protein (fold)	*p* (Nrf2)	*p* (HO-1)
Control	1.00 ± 0.08	1.00 ± 0.06	—	—
H_2O_2 200 µM	0.42 ± 0.07	0.38 ± 0.06	<0.001*	<0.001*
TEF 25 + H_2O_2	1.21 ± 0.16	1.47 ± 0.18	<0.01*	<0.001*
TEF 50 + H_2O_2	2.04 ± 0.19	2.34 ± 0.21	<0.001*	<0.001*
TEF 100 + H_2O_2	2.83 ± 0.21	3.14 ± 0.24	<0.001*	<0.001*
Vitamin E 50 + H_2O_2	2.61 ± 0.18	2.89 ± 0.22	0.332	0.408

*Notes: Data are mean ± SD fold-change versus control (n = 6). p-values are versus the H_2O_2 group.*Significant at p < 0.05.*

Epigenetic remodeling: Nrf2 promoter demethylation

Bisulfite sequencing of ten CpG sites (Table 3; Figure 3A) showed control methylation of 22.4 ± 2.8%, rising to 67.8 ± 4.2% with H_2O_2 (p < 0.001, d = 12.48) and explaining the paradoxical Nrf2 suppression under oxidative stress. TEF reversed hypermethylation to 54.3 ± 3.9% (25 µg/mL; a 19.9% reduction relative to the H_2O_2 group), 41.8 ± 3.2% (50 µg/mL; 38.3% reduction), and 31.2 ± 2.6% (100 µg/mL; 54.0% reduction; all p < 0.001). Regression revealed a strong negative dose-response (r = −0.951, R^2 = 0.904), with an IC_{50} for demethylation of 58.7 µg/mL (95% CI 50.2–69.4) closely matching that for ROS suppression (62.4 µg/mL), consistent with reports that DNMT inhibition reverses NRF2 promoter hypermethylation.[16,20]

Table 3. Nrf2 promoter methylation (bisulfite sequencing) and DNMT1 protein (Western blot) expression.

Treatment Group	Methylation (%)	Reduction vs. H_2O_2	DNMT1 (fold)	*p* (Meth)	*p* (DNMT1)
Control	22.4 ± 2.8	—	1.00 ± 0.08	—	—
H_2O_2 200 µM	67.8 ± 4.2	—	2.74 ± 0.23	<0.001*	<0.001*
TEF 25 + H_2O_2	54.3 ± 3.9	19.9%	1.87 ± 0.19	<0.001*	<0.001*
TEF 50 + H_2O_2	41.8 ± 3.2	38.3%	1.43 ± 0.15	<0.001*	<0.001*
TEF 100 + H_2O_2	31.2 ± 2.6	54.0%	1.12 ± 0.11	<0.001*	<0.001*
Vitamin E 50 + H_2O_2	35.7 ± 3.1	47.3%	1.19 ± 0.12	<0.001*	0.697

*Notes: Data are mean ± SD (n = 6). Methylation reduction is relative to the H_2O_2 group.*Significant at p < 0.05.*

Epigenetic remodeling: DNMT1 suppression and H3K27ac enrichment

As summarized in Table 3 (DNMT1) and Figure 2C and 2D, H_2O_2 elevated DNMT1 to 2.74 ± 0.23-fold, providing a mechanism for Nrf2 hypermethylation,[18] while TEF suppressed DNMT1 to 1.87, 1.43, and 1.12 ± 0.11-fold across doses. H_2O_2 did not significantly alter H3K27ac (0.31 vs. 0.34-fold control; p = 0.621), indicating that promoter methylation, not histone deacetylation, is the primary oxidative dysregulation. TEF enriched H3K27ac to 0.67, 1.08, and 1.43 ± 0.14-fold (r = 0.958, R^2 = 0.918). The combined demethylation and H3K27ac enrichment constitute a dual epigenetic remodeling mechanism resetting the Nrf2 promoter to an active state.[19]

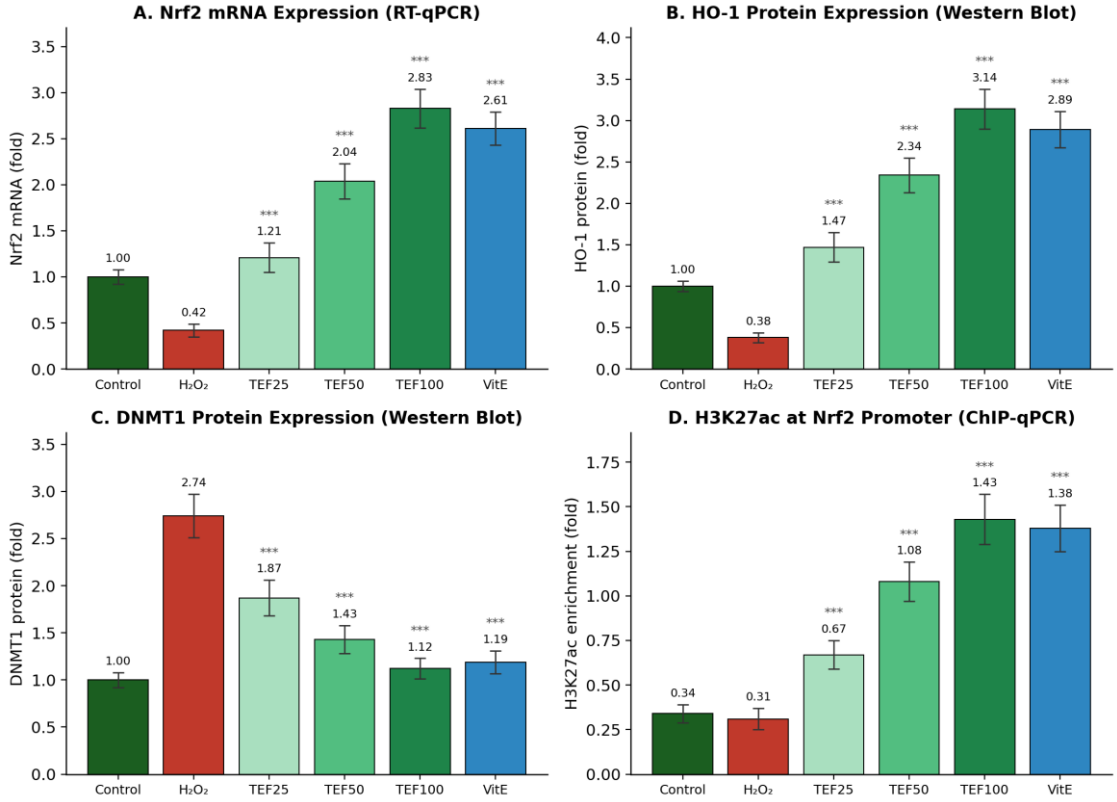

*Figure 2. (A) Nrf2 mRNA (RT-qPCR); (B) HO-1 protein (Western blot); (C) DNMT1 protein; (D) H3K27ac enrichment at the Nrf2 promoter (ChIP-qPCR). Mean ± SD (n = 6). ***p < 0.001 versus H_2O_2.*

Integrated and secondary analyses

Across all groups (n = 36; Figure 3B; Table 4), Nrf2 promoter methylation and Nrf2 mRNA were strongly inversely correlated (r = −0.912, p < 0.001; R^2 = 0.832). The IC_{50} values for ROS suppression (62.4 µg/mL) and demethylation (58.7 µg/mL) overlap considerably, indicating mechanistically linked processes.[17] Confirming broader ARE-pathway activation, H_2O_2 suppressed NQO1 to 0.51 ± 0.09-fold whereas TEF 100 restored it to 2.28 ± 0.25-fold (d = 7.08),[3,12] validating a coordinated antioxidant transcriptional program.

Table 4. Dose-response analysis and epigenetic endpoints.

Parameter	Correlation (*r*)	R^2	IC_{50} (µg/mL)	95% CI	*p*-value
ROS suppression	0.958	0.918	62.4	54.1–71.8	<0.001*
Nrf2 demethylation	−0.951	0.904	58.7	50.2–69.4	<0.001*
H3K27ac enrichment	0.958	0.918	61.2	52.6–72.1	<0.001*
Inverse correlation (all groups): methylation vs. Nrf2 mRNA — $r = -0.912$, $R^2 = 0.832$, $p < 0.001$*					

*Notes: Linear regression of TEF dose (25–100 µg/mL) versus ROS, methylation, and H3K27ac; the inverse correlation is computed across all groups (n = 36).*Significant at p < 0.05 (two-tailed).*

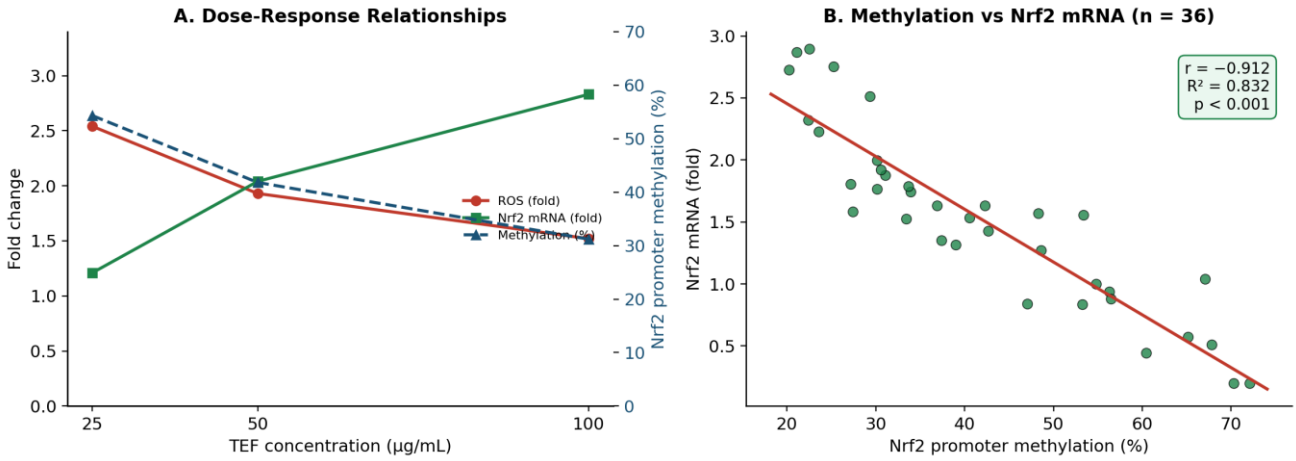

Figure 3. (A) Dose-response of TEF versus ROS and Nrf2 mRNA (left axis) and Nrf2 promoter methylation (right axis). (B) Inverse correlation between methylation and Nrf2 mRNA across all groups (n = 36; r = −0.912, p < 0.001, R^2 = 0.832).

Ethnopharmacological and mechanistic integration

These results provide the first molecular evidence linking traditional uses of *Centella asiatica* to epigenetic-transcriptional neuroprotection. TEF-mediated neuroprotection (82.4% viability at 100 µg/mL) equals or exceeds vitamin E, and the strong inverse methylation–mRNA correlation ($r = -0.912$) identifies epigenetic silencing as the dominant mechanism suppressing Nrf2 under oxidative stress. DNMT1 suppression is the proximal driver of demethylation, while H3K27ac enrichment installs active chromatin, together ensuring robust Nrf2 (2.83-fold) and HO-1 (3.14-fold) induction. Because Nrf2 hypermethylation characterizes Alzheimer's and Parkinson's disease,[1,8] botanical restoration of Nrf2 through epigenetic remodeling is a promising, multi-pathway neuroprotective strategy.[5,10,21-24]

Limitations include the use of a single cell line (SH-SY5Y), a single oxidative stressor (H_2O_2), a 24-hour timeframe, and a multicomponent TEF; future work should isolate individual triterpenes, assess apoptosis, and validate findings in animal models before clinical translation.

4. Conclusion

Triterpene saponins from *Centella asiatica*, standardized to asiaticoside and madecassoside, activate the neuroprotective Nrf2/HO-1 pathway in oxidatively stressed neuroblastoma cells through dual Nrf2 promoter demethylation and H3K27 acetylation. TEF at 100 µg/mL restored viability to 82.4% and reduced ROS to 1.52-fold—equivalent to vitamin E—via DNMT1 suppression that lowered promoter methylation from 67.8% to 31.2% and H3K27ac enrichment from 0.34- to 1.43-fold. The strong inverse methylation–expression correlation ($r = -0.912$) and aligned IC_{50} values reveal quantitative coupling of epigenetic remodeling and antioxidant activation, providing a molecular rationale for the traditional cognitive-enhancing use of pegagan and Brahmi.

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