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COMPARATIVE EVALUATION OF ELLAGIC ACID AND 4-HYDROXYISOPHTHALIC ACID: ANTIOXIDANT, ANTI-INFLAMMATORY, AND NEUROPROTECTIVE ASSESSMENT AGAINST GLUTAMATE TOXICITY IN SH-SY5Y CELLS

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Abstract

Oxidative stress (OS) and inflammation play a substantial role in the progression of various chronic illnesses. Ellagic acid (EA) and 4-hydroxyisophthalic acid (4-HIA) are the natural phenolic compounds which possesses several therapeutic properties. This study comparatively assessed the antioxidant, anti-inflammatory, and neuro-protective properties of EA and 4-HIA using several assays like DPPH, ABTS scavenging, reducing power, and total antioxidant capacity, bovine serum albumin (BSA) denaturation assay. Further, the neuro-protective efficacy was determined in SH-SY5Y cells by MTT assay. Our results revealed that both EA and 4-HIA exhibited concentration-dependent scavenging activity in all the antioxidant assays performed. EA consistently exhibited superior antioxidant potential compared to 4-HIA and standard ascorbic acid. Further, the EA also demonstrated greater anti-inflammatory activity than 4-HIA. The findings of MTT assay revealed that EA offered superior protection against glutamate induced toxicity in SH-SY5Y cells. However, further mechanistic studies are needed to validate their therapeutic efficacy.

INTRODUCTION

Oxidative stress (OS) is a vital biochemical and physiological condition which have a critical role in the progression of various disorders including neurodegenerative disorders, cardiovascular diseases, cancer, and diabetes mellitus (Ma *et al*., 2025). Particularly, in the neurodegenerative diseases, the brain's extreme metabolic demands and comparatively low antioxidant defense capacity make it highly prone to oxidative injury (Hu *et al*., 2026). Moreover, several studies have indicated that severe inflammation leads to the onset of many disorders (Cervellati *et al*., 2020, Adamu *et al*., 2024). Both OS and inflammation leads to the extreme liberation of reactive species of oxygen and nitrogen (ROS, RNS) and which disturbs the redox homeostasis in cells,

resulting in oxidation of proteins and lipids. Further, the excessive presence of glutamate leads to hyper stimulation of NMDA receptors resulting in dysfunctional mitochondria, and neuronal apoptosis leading to cell death (Islam *et al*., 2017, Tiwari *et al*., 2023, Uremiş and Uremiş, 2025). Therefore, the natural bioactive compounds with strong therapeutic application holds a great potential in mitigating neurodegeneration related disorders.

Naturally occurring antioxidants have drawn immense attention as potential therapeutic agents (Oyovwi *et al*., 2025, Tauchen *et al*., 2025). Especially, the polyphenolic compounds show strong redox-modulation, metal-chelating, and free radical scavenging properties (Georgieva *et al*., 2021). Ellagic acid (EA), a natural polyphenol has been comprehensively explored for its potent anti-inflammatory, antioxidant, and neuroprotective effects. It has ability for efficient hydrogen atom donation and contribute to stabilize free radicals owing to its multiple hydroxyl groups (Zheng *et al*., 2020, Mohassel Yazdi *et al*., 2024). Further, the 4-hydroxyisophthalic acid (4-HIA), a structurally simpler polyphenol, also possess strong cytoprotective, cardioprotective, and neuroprotective properties (Ravikiran *et al*., 2021, Anupama *et al*., 2022, Niveditha and Shivanandappa, 2022). Our initial lab studies indicate that both EA and 4-HIA are abundantly found in the roots of *Decalepis hamiltonii* plant (Sowbhagya *et al*., 2016). However, the complexity of antioxidant pathways warrants the assessment through several *in vitro* assays, since a single assay cannot represent the antioxidant ability of any compounds adequately (Kotha *et al*., 2022). Furthermore, assessing the relevance of these biochemical activities with the cellular models is necessary to prove their biological significance. The SH-SY5Y (human neuroblastoma) cell line is extensively used to investigate the neurotoxicity and neuroprotective mechanism which is essential to determine the safety profile and therapeutic range of the test compounds (Ioghen *et al*., 2023, Pulkrabkova *et al*., 2023).

Therefore, this study aims for comparative estimation of the antioxidant and anti-inflammatory properties of EA and 4-HIA using multiple in vitro assays, including DPPH, ABTS, reducing power, total antioxidant capacity, lipid peroxidation and BSA denaturation assay. Furthermore, their neuroprotective activity was investigated in SH-SY5Y cells induced with glutamate toxicity. This combined approach

is essential for clear enumeration of these compounds as the potential candidates for mitigating neuronal damage.

MATERIALS AND METHODS

Chemicals and reagents

Ellagic acid (EA) was procured from Sigma Aldrich, USA. 4-Hydroxyisophthalic acid (4-HIA) was obtained from TCI Chemicals, Tokyo. 3(4,5-Dimethylthiazol-2)-2, 5-Diphenyl tetrazolium bromide (MTT), Bovine serum albumin (BSA), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Hi-media, Mumbai. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and other chemicals bought from Sisco Research Laboratories (SRL), Mumbai.

Antioxidant activities

DPPH radical scavenging assay

The protocol of Shon *et al*. (2003) was used to accomplish the DPPH scavenging assay. Varying concentrations of 0.5 mL of EA and 4-HIA were added with DPPH solution (1 mL, 0.1 mM) for 20 min at ambient temperature. The absorbance (517 nm) was recorded. Ascorbic acid was utilized as a standard reference.

The following equation was used for calculating the scavenging activity:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad \text{------Eq.1}$$

ABTS radical scavenging assay

The procedure of Ding *et al*. (2019) was followed to perform the ABTS scavenging assay. ABTS (7 mM) stock and 2.45 mM potassium persulfate were reacted (12-16 h) for the generation of ABTS radicals. The absorbance at 734 nm was adjusted (0.70 ± 0.02). Then 30 µL of various concentrations of EA, 4-HIA, and standard ascorbic acid was mixed with a diluted solution of ABTS (170 µL). The absorbance after 10 min was noted (734 nm). The above given Eq.1 was used for the calculation of ABTS radical scavenging activity.

Reducing power assay

Reducing power was accomplished following the procedure reported by Do *et al*. (2014). 0.5 mL of diverse concentrations of EA, 4-HIA and standard ascorbic acid was combined with 1% potassium ferricyanide and phosphate buffer (pH 6.6, 0.2 M) with subsequent incubation (50 °C, 20 min). Then the solution was centrifuged (3000 rpm, 10 min) after adding 10% trichloroacetic acid. 0.5 mL supernatant added with distilled water of an equal amount with subsequent addition of 0.1% freshly prepared ferric chloride.

After thorough mixing, a UV-Vis spectrophotometer (Shimadzu 1800) was used for reading the absorbance (700 nm). The increase in absorbance indicates greater reducing power.

Total antioxidant activity

The total antioxidant capacity of EA and 4-HIA along with std AA was performed by the phosphomolybdate method (Prieto *et al*., 1999). The samples (0.1 mL) were mixed with the reagent solution (sodium phosphate-28 mM, ammonium molybdate (4 mM), sulfuric acid (0.6 M) in equal ratio), incubated (95 ˚C, 90 min) and cooled. The mixture's absorbance (695 nm) was noted. The increased absorbance indicates a higher total antioxidant capacity.

Inhibition of lipid peroxidation activity

The Chaiklahan *et al*. (2025) procedure was utilized for analyzing the inhibition of lipid peroxidation (LPO) using thiobarbituric acid reactive substance (TBARS). The lipid peroxidation was induced by incubating (37 °C, 60 min) the homogenate of 10% v/v egg yolk with varying concentrations of EA, 4-HIA and standard Ascorbic along with ferrous sulphate (70 mM). Subsequently, trichloroacetic acid (2% w/v) was added to terminate the reaction and centrifuged. Then thiobarbituric acid (0.8% w/v) was combined with the supernatant and heated (95 °C, 30 min). The absorbance (532 nm) was noted after cooling down for calculating the percentage inhibition of LPO using Eq. 1.

Anti-inflammatory activity

BSA denaturation assay

The BSA denaturation assay was employed for the determination of the anti-inflammatory activities of EA and 4-HIA (Chahardoli *et al*., 2022). Diclofenac sodium served as a standard reference. 1% BSA solution was mixed with diverse concentrations of EA and 4-HIA, incubated (37°C, 20 min) followed by heating (57°C, 20 min) and subsequent measuring of absorbance at 660 nm to calculate percentage inhibition of BSA denaturation using Eq.1.

***In Vitro* Studies**

Cell culture and maintenance

SH-SY5Y cells were acquired from the National Centre for Cell Science (NCCS), Pune. The cells were cultured in DMEM and F12 (1:1) ratio, with FBS-10%, and antimycotic and antibiotic solution (1%). The CO_2 incubator (37 °C, 5% CO_2) was used for growing the cells and sub-cultured upon attaining the confluency of 70–80%.

Neuroprotective activity-*MTT assay*

The neuroprotective activity of EA and 4-HIA on SH-SY5Y cells were assessed

using MTT assay following the Mosmann, 1983 protocol. SH-SY5Y cells were plated for 24 h at a cell density (1×10^4 cells in 96-well plate). Then, different concentrations of EA, and 4-HIA were treated with the cells for the next 24 h. Subsequently, MTT (5 mg/ml, 10 µL) was added (37 °C, 4h). Then, DMSO (100 µL) added after discarding the medium. The absorbance was determined at 570 nm through multimode reader (Synergy LX, Agilent technologies, USA). Subsequently, the cells were induced with varying doses of glutamate to determine the IC_{50} value. Further, the non-toxic concentration of EA and 4-HIA were tested for their neuroprotective efficacy in glutamate induced toxicity in SH-SY5Y cells.

Statistical analysis

All the data was represented as mean ± SE. One-way ANOVA followed by Tukey's test was used for analyzing the statistical significance ($p < 0.05$).

RESULTS

Antioxidant activities

DPPH radical scavenging activity

The DPPH radical scavenging ability of EA and 4-HIA are represented in Fig. 1. Both EA and 4-HIA showed dose-dependent scavenging activity. However, EA (3.55±0.05 µg/mL) showed higher scavenging ability than the standard ascorbic acid (4.05±0.15 µg/mL) and 4-HIA (57.13±5.42 µg/mL) which is apparent with their respective IC_{50} values.

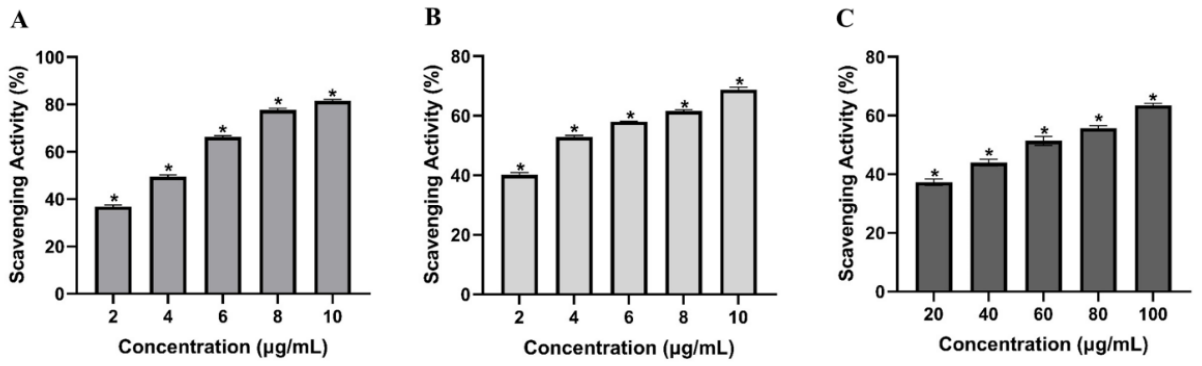

Fig. 1. DPPH radical scavenging activity of (A) Standard Ascorbic acid (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One- way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

ABTS radical scavenging activity

The inhibition of ABTS radical increases with the increasing doses of EA, 4-HIA, and standard ascorbic acid (Fig. 2). The IC_{50} values were found to be 29.34±6.90, 44.91±1.43, and 103.69±11.62 µg/mL in the increasing order of EA, standard ascorbic acid, and 4-HIA, respectively, revealing that EA has better scavenging activity.

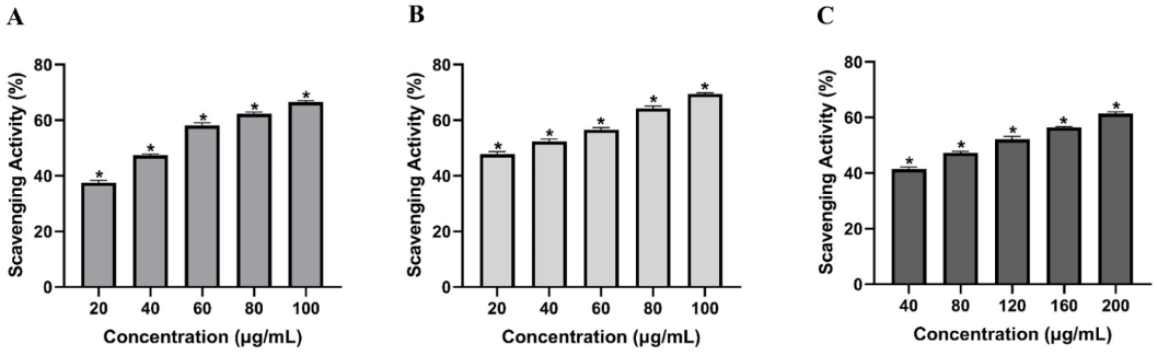

Fig. 2. ABTS radical scavenging activity of (A) Standard Ascorbic acid (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One- way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

Reducing power assay

Figure 3 depicts the reducing power of EA, and 4-HIA against the standard ascorbic acid. All the three compounds shows substantial increment in absorbance values with increasing concentrations which indicates its reducing capacity. While EA showed higher absorbance than the standard ascorbic acid at 10 µg/mL. However, much higher concentration of 4-HIA was required to show the noteworthy reducing capacity (100 µg/mL).

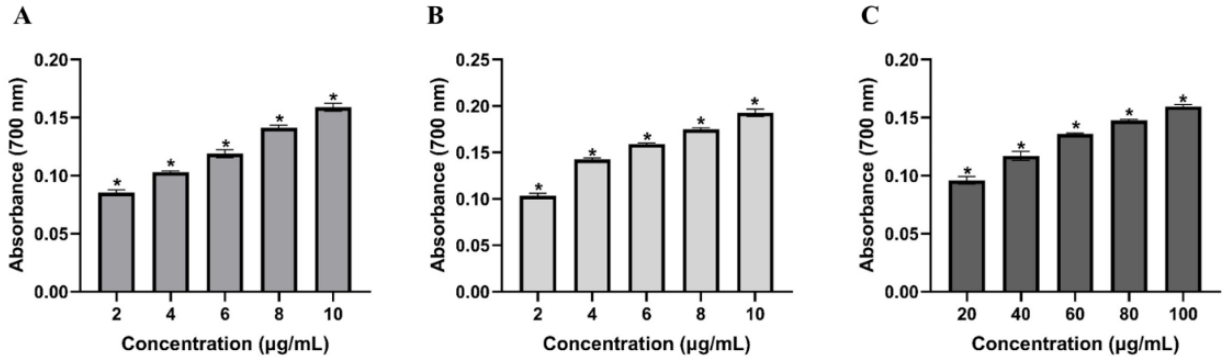

Fig. 3. Reducing power assay of (A) Standard Ascorbic acid (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One-way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

Total antioxidant assay

The clear dose-dependent correlation was seen for total antioxidant capacity of EA, 4-HIA, and standard ascorbic acid as indicated by its increasing absorbance (Fig. 4). Our results reveal that EA showed highest total antioxidant capacity than standard ascorbic acid. In contrast 4-HIA showed considerable total antioxidant capacity at much higher concentration in comparison with standard ascorbic acid.

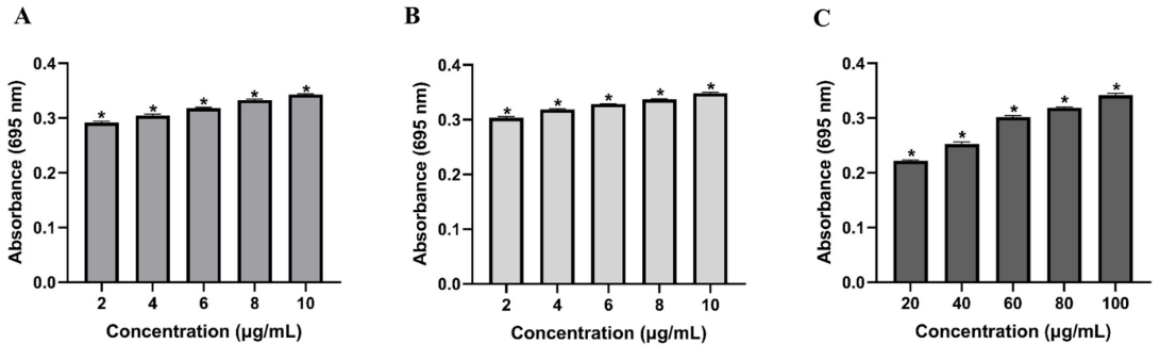

Fig. 4. Total antioxidant capacity of (A) Standard Ascorbic acid (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One- way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

Lipid peroxidation

The results of lipid peroxidation assay disclosed the concentration dependent antioxidant potential of EA and 4-HIA with ascorbic acid serving as the standard reference (Fig. 5). EA (IC_{50}-28.06±2.48 µg/mL) demonstrated slightly superior inhibition of lipid peroxidation with respect to the standard ascorbic acid (IC_{50}-32.79±4.47 µg/mL). While, the 4-HIA's capacity to inhibit lipid peroxidation was observed at much higher dose with an IC_{50} of 88.85 ± 5.39 µg/mL.

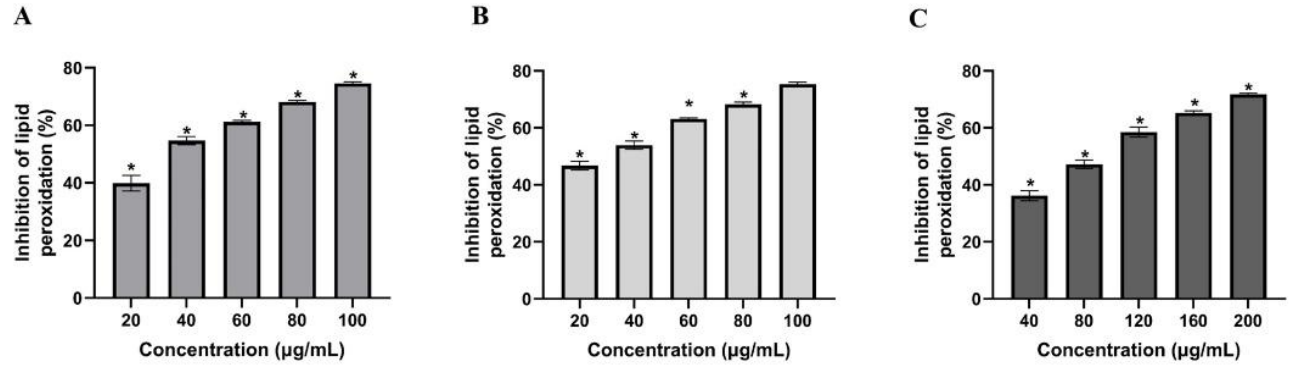

Fig. 5. Lipid peroxidation inhibitory activity of (A) Standard Ascorbic acid (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One- way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

Anti-inflammatory activity

BSA denaturation assay

The EA, 4-HIA, and standard diclofenac sodium showed dose dependent inhibition of heat-induced BSA protein denaturation as displayed in Fig. 6. Our results reveal that EA (IC_{50}- 368.17±29.91 µg/mL) demonstrated superior anti-inflammatory potential than 4-HIA (IC_{50}- 768.41±15.75 µg/mL). Nonetheless, standard diclofenac sodium displayed much lower IC_{50} of 169.15±21.61 µg/mL demonstrating its highest efficiency in preventing heat induced BSA denaturation over EA and 4-HIA.

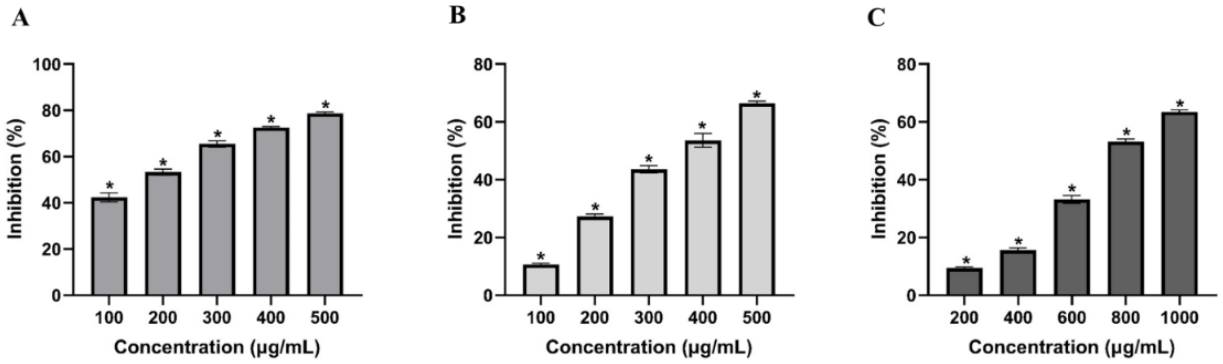

Fig. 6. Anti-inflammatory activity of (A) Standard Diclofenac sodium (B) Ellagic acid (C) 4-Hydroxyisophthalic acid. Data is represented as mean ± SE (n=3) and analyzed through One-way ANOVA followed by Tukey's test. *p <0.05 indicates significance between the concentrations.

Neuroprotective activity

Initially, various concentrations of EA and 4-HIA (7.813-250 µg/mL) were treated with the SH-SY5Y cells to determine their non-toxic concentrations. Our results indicate that both EA and 4-HIA showed dose-dependent reduction in cell viability with higher concentrations exhibiting pronounced toxicity in the SH-SY5Y cells (Fig. 7). However, the concentrations up to 31.25 µg/mL has retained more than 75% cell viability implying their better compatibility with SH-SY5Y cells. Therefore, three doses (7.813, 15.625 and 31.250 µg/mL) were selected for further neuroprotective investigation. Moreover, varying doses of L-glutamate exposure to the SH-SY5Y cells revealed an IC_{50} of 51.2±4.64 mM which was used for subsequent neuroprotective assessment. However, co-treatment of the SH-SY5Y cells induced with 51 mM L-glutamate toxicity with EA (15.625 µg/mL) and 4-HIA (31.250 µg/mL) led to the highest reduction in cell death confirming their neuroprotective efficacy which is further validated through phase contrast microscopic images (Fig. 7D).

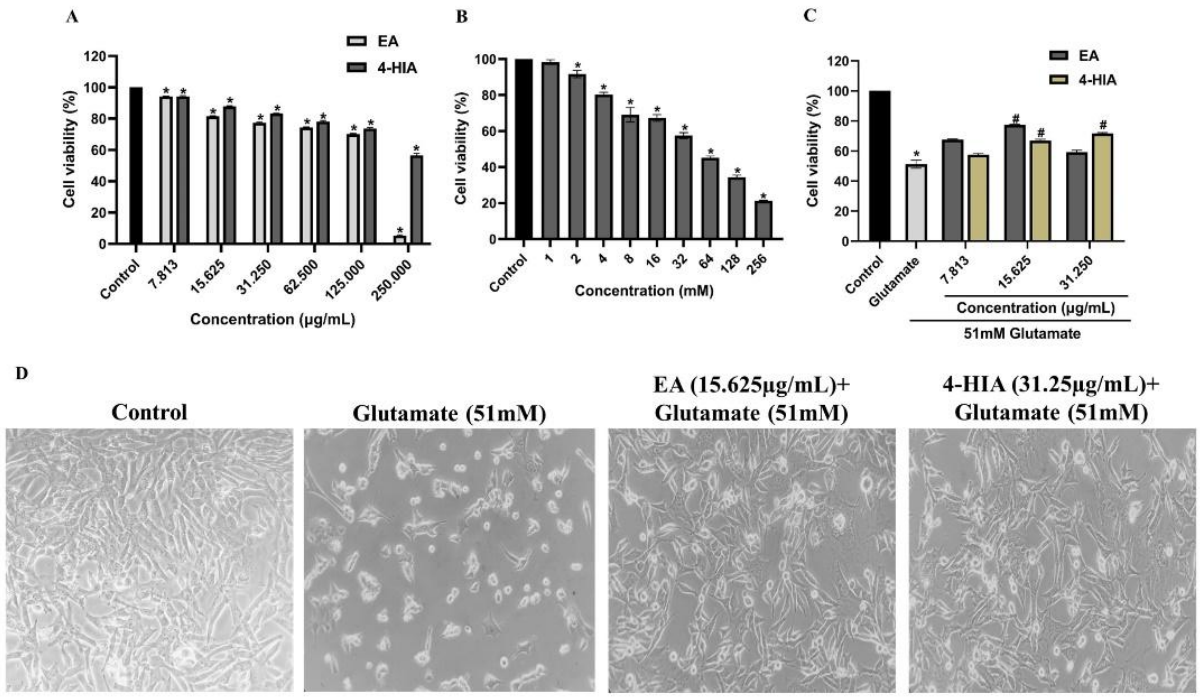

Fig. 7. Effect of (A) ellagic acid (EA) and 4-Hydroxyisophthalic acid (4-HIA), (B) Glutamate on SH-SY5Y cell viability, (C) Neuroprotective activity of EA and 4-HIA on glutamate induced toxicity in SH-SY5Y cells. Data is represented as mean ± SE (n=3) and analyzed through One-

way ANOVA followed by Tukey's test. *p <0.05 indicates significance compared to control. (D) Phase contrast microscopic images of SH-SY5Y cells depicting morphological changes (magnification:20X).

DISCUSSION

The naturally existing compounds with strong antioxidant potential may prove to offer promising therapeutic effects by alleviating ROS-mediated cellular damage. Hence our current study emphasized on the antioxidant, anti-inflammatory, and neuroprotective properties of EA and 4-HIA. Several assays such as DPPH, ABTS scavenging, reducing power, total antioxidant and lipid peroxidation assays were performed to assess the antioxidant activities of EA and 4-HIA. DPPH, and ABTS are the most common radical scavenging assays to estimate the antioxidant capacities of pure compounds and herbal extracts. The delocalization of the spare electron in DPPH molecule makes it a highly stable free radical (Gulcin and Alwasel, 2023). Whereas, the cationic ABTS radical gets liberated upon the ABTS oxidation in the potassium persulfate's presence. While, the reducing power assay signifies the compound's antioxidant potential based on the reduction of potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}) and forming ferric ferrous complex subsequently after combining with ferric chloride with an absorption maximum of 700 nm (Singhal *et al*., 2014). Whereas, the total antioxidant assay is centered on the creation of green phosphate (Mo (V)) complex due to the reduction of Mo (VI) to Mo (V) at acidic pH by the antioxidant compounds (Joshi *et al*., 2025). Further, Lipid peroxidation is consequently caused due to high ROS generation which in turn deteriorates biological systems. Incubating egg yolk homogenates with $FeSO_4$ leads to substantial increment in lipid peroxidation due to the formation of thiobarbituric acid reactive substances (TBARS). Therefore, the compounds ability to inhibit lipid peroxidation is directly proportional to the decrease in TBARS formation (Badmus *et al*., 2011). Both EA and 4-HIA demonstrated concentration-dependent inhibition in all the antioxidant assays performed. Our results imply that EA has higher potency to prevent radical chain reactions owing to the existence of many hydroxyl groups and extensive conjugation within the EA structure when compared to standard ascorbic acid and 4-HIA suggesting the superior electron-donating and reducing potential under the experimental conditions. Overall, our results

were similar to the studies of Yang *et al*. (2023) and Li *et al*. (2024) which also reported that EA exhibited better antioxidant activity with respect to standard ascorbic acid. In contrast, the comparatively lower activity of 4-HIA may be accredited to the presence of fewer hydroxyl groups influencing its radical scavenging and metal-chelating capacities.

Inflammation is a defense mechanism of the body that protects against various harmful agents, including bacteria, irritants, adverse stimuli, and damaged cells (Rehman *et al*., 2023). However, an excessive inflammation causes the generation of cytotoxic molecules, which in turn leads to cellular damage (Ramachandran *et al*., 2024). Therefore, those compounds that possess strong anti-inflammatory potential can act as promising therapeutic agents. Our results revealed the higher anti-inflammatory activity of EA in comparison with 4-HIA, which can be due to the modulation of inflammatory mediators. The results are in concordance with the reports of Gupta *et al*. (2021) which also disclosed higher anti-inflammatory activity of standard diclofenac sodium over EA. Furthermore, MTT assay is a most usual method for the assessment of cellular metabolic activity wherein living cells reduce MTT into insoluble formazan (Lai *et al*., 2026). Glutamate-induces toxicity through overstimulation of NMDA receptors and oxidative damage. Even though the SH-SY5Y cells lack NMDA receptor functions, oxidative damage is known as main reason for toxicity induction through glutamate (Palanivel *et al*., 2022). The MTT findings revealed that both EA and 4-HIA presented noteworthy protection against glutamate stress in the neuronal cells signifying their potential applications as neuroprotective agents.

CONCLUSION

Our study demonstrated the antioxidant, anti-inflammatory potential, and cyto-compatible nature of EA and 4-HIA through a series of *in vitro* assays. Both compounds displayed concentration-dependent antioxidant activity in all the assays. Nevertheless, EA consistently exhibited higher antioxidant efficacy over standard ascorbic acid and 4-HIA which may be due to EA's greater structural conjugation, facilitating efficient hydrogen donation and electron transfer mechanisms. Furthermore, both the compounds revealed considerable anti-inflammatory effects, with EA exhibiting greater activity than 4-HIA. The MTT analysis on SH-SY5Y cells confirmed the neuroprotective efficacy of both compounds at lower concentrations,

indicating their potential safety within an effective range for therapeutic applications. Although, additional studies are required to validate their mechanistic insights and therapeutic efficacy.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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