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Emodin, a Phytotherapeutic Anthraquinone, Potently Attenuates Synaptic Redox Pathology in Aged Rats

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Background: Aging is associated with increased oxidative stress, which leads to synaptic vulnerability and psychiatric and cognitive deficits. Maintaining redox homeostasis is crucial for synaptic health. However, age-related alterations in synapse-specific antioxidant capacity remain poorly understood. Moreover, effective therapeutic strategies to counteract these changes are lacking. This study aimed to assess redox parameters in ex vivo synaptic terminals from young and old rat brains and evaluate the modulatory effects of the phytotherapeutic compound emodin.

Methods: Brain synaptosomes were isolated from young and old male Wistar rats. The antioxidant capacities were determined using 2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) and ferric-reducing antioxidant power (FRAP) assays. Oxidative stress and damage were assessed by quantifying reactive oxygen species (ROS) and nitrogen species (RNS) and examining oxidative modifications of proteins and lipids. The antioxidant effects of emodin were investigated in mitigating synaptic oxidative stress and damage.

Results: A significant decline in antioxidant capacity and increase in ROS levels were observed in the synaptosomes of aged animals. Oxidative damage was also evident as increased protein carbonylation, thiol oxidation, and lipid peroxidation. Emodin treatment improved redox balance by reducing ROS levels, decreasing oxidative damage markers, and enhancing antioxidant defenses, particularly in older animals.

Conclusion: Aging disrupts synaptic redox homeostasis and increases the susceptibility to oxidative damage. Emodin exerts protective antioxidant effects by mitigating oxidative stress and enhancing the redox capacity of the synaptosomes. These findings suggest that emodin may have therapeutic potential in preserving synaptic function under conditions of age-related oxidative stress, although further functional and molecular studies are warranted to validate its neuroprotective efficacy.

Keywords: ageing, oxidative stress, antioxidants, synaptosomes, emodin

Introduction

Ageing is an inescapable biological phenomenon that leads to a continual deterioration in physiological and molecular functions, significantly affecting various organ systems, including the brain.[1] The ageing brain undergoes structural, functional, and biochemical changes that compromise its ability to perform complex cognitive tasks and have detrimental effects on psycho-behavioral attributes.[2] With ageing, the brain becomes more prone to oxidative stress, leading to an aberrant accumulation of reactive oxygen (ROS) and nitrogen species (RNS) and oxidative damage, key factors in neuronal pathogeneses.[3] The imbalances between heightened generation of free radicals and diminished neuronal antioxidant defense mechanisms result in cellular damage, affecting biomolecules such as lipids, proteins, and DNA.[4] Previous work has implicated redox homeostasis and loss of endogenous antioxidant capacity in several brain pathologies.[5] Neurons are particularly vulnerable to oxidative damage because of their unique physiological characteristics.[6] Despite accounting for only a very small mass compared to the body's weight, the brain consumes relatively higher quantities of the body's oxygen, reflecting its high metabolic demand to support neuronal activity.[7] This elevated oxygen consumption makes the brain a significant site for ROS production, which can lead to oxidative damage

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if not adequately neutralized by antioxidant defenses.[8] Studies have shown that various markers of oxidative damage, including protein thiol oxidation and carbonylation and lipid peroxidation, and elevated levels of ROS, are significantly elevated in aged brain tissue.[9,10] In conclusion, the intricate interplay of high energy demand, ROS generation, protein carbonyl formation, oxidation of free thiols, and lipid peroxidation underscores the brain's heightened sensitivity to disruptions in redox balance, contributing to ageing-related pathologies of psychiatric and cognitive decline.[11]

Synaptic connections appear to be primary targets of redox-associated neuropathological mechanisms, including neurodegenerative and neuropsychological conditions. The functional loss of synapses disrupts inter-neuronal communication, highlighting the importance of synaptic redox homeostasis in maintaining neuronal communication and plasticity.[12] Disparities in synaptic ROS/RNS generation and their endogenous antioxidant capabilities may be critical factors driving ageing-associated neuronal dysfunction. However, the status of the endogenous antioxidant capacity of synapses during ageing remains obscure. In this regard, "synaptosomes," biochemically isolated ex vivo preparations of nerve terminals, have been routinely used to evaluate synaptic pathophysiology in conditions such as Alzheimer's disease (AD), early life stress, and lead (Pb) neurotoxicity.[13–16] Evaluation of synapse-specific aspects of free radical generation, oxidative damage, and changes in antioxidant status have also been undertaken in synaptosomal studies.[17,18] Conclusively, these preparations are potent tools for studying ageing-associated redox changes at the synapses, as well as for evaluation of therapeutic strategies.

In search of alternative, safe, and viable therapeutic strategies, several studies have highlighted the potential utilities of plant-derived bioactives, particularly polyphenols and flavonoids such as curcumin, quercetin, and epigallocatechin gallate, in exerting potent neuroprotective effects by enhancing antioxidant defenses and mitigating oxidative stress associated with brain ageing.[19,20] One such phytotherapeutic is emodin, a natural anthraquinone known for its multimodal antioxidant and anti-inflammatory properties.[21,22] Importantly, neuroprotective effects of emodin have been evidenced by several research groups, with its antioxidant functions appearing to be a key feature.[23–26] Given its potent antioxidant and redox-modulating properties, emodin is a promising therapeutic candidate for AD;[27] however, its role in synaptic redox homeostasis during ageing remains unclear. This study aimed to evaluate ageing-related changes in endogenous neuronal antioxidant capacity in a synapse-specific manner, using synaptosomes isolated from young and old Wistar rats. Furthermore, given the antioxidant potency of emodin, we tested its beneficial effects in promoting endogenous synaptosomal antioxidant capacity and mitigating oxidative stress and damage during aging.

Materials and Methods

All chemicals and reagents used for the completion of this study were of laboratory grade. 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) and emodin were procured from Sigma-Aldrich. 2,2′-Azinobis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), 2,4-dinitrophenylhydrazine (DNPH), and 2,4,6-Tripyridyl-s-triazine (TPTZ) were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., India. The other lab-grade reagents used in this study were purchased from local suppliers. All spectrophotometric assays were performed on a multimode reader (Tecan Infinite 200 Pro, Grödig, Austria).

Animals and Experimental Paradigm

All animal experiments were conducted after approval and in accordance with the institutional guidelines for animal care with efforts to minimize pain and discomfort. The Institutional Animal Ethics Committee (IAEC) of the Vellore Institute of Technology (VIT), Vellore, India, approved the study protocol (approval no. VIT/IAEC/23/May23/04; dated 22 May, 2023). Two age-based experimental groups were used: young (3–4 months old) and old (22–24 months old) rats. Terminal anesthesia in all rats was induced using 5% isoflurane in O_2 gas at a flow rate of 0.5-1.0 L/min for 5-6 min. After confirming absence of response to toe pinch and loss of respiratory functions, the animals were sacrificed by thoracotomy. The procedure was in consistent with the American Veterinary Medical Association (AVMA, 2020) Guidelines for the Euthanasia of Animals. Subsequently, brain cortical tissues were collected immediately, flash-frozen in liquid nitrogen, and stored at $-80^{\circ}C$. Cortical synaptosomes (see below) from young and old rats were randomly assigned to the untreated and emodin-treated groups, resulting in a total of four experimental groups. There were five biological replicates for each group, and each replicate was derived from a different animal as an average of two duplicates. The sample size was determined based on previous studies involving synaptosomal oxidative stress analysis

in aging models.[28,29] The number of replicates was sufficient to detect statistically significant differences across treatment groups, as confirmed by consistent effect sizes and post-hoc statistical testing.

Isolation of Synaptosomes

Synaptosomes were isolated from brain cerebral cortical tissues of young and old animals and were designated as young (YCS) and old cortical synaptosomes (OCS), respectively. The protocol was adapted from a previous study[30] with slight modifications. Cortex tissues were weighed and homogenized in 10 volumes of ice-cold 1X homogenization buffer (5 mM HEPES, 0.32 M sucrose, pH 7.4) using a Potter-Elvehjem glass tube and pestle. The homogenate was centrifuged at 1,500 × g at 4°C for 10 min to pellet cellular debris and nuclei. The supernatant, collected into fresh tubes, was further centrifuged at 12,000 × g at 4°C for 15 min. The resulting pellet, representing the crude synaptosome fraction, was resuspended in an appropriate volume of ice-cold homogenization buffer based on the pellet size and used for future analysis. The resuspended synaptosomes were aliquoted into 50 µL portions, with one smaller aliquot (10 µL) reserved for protein concentration estimation using the Lowry method. Estimation of synaptosomal protein concentrations allowed normalization of the protein amounts for all subsequent treatment and analytical steps.

Emodin Treatment

Synaptosomes (YCS and OCS) were treated with 20 µM emodin for 120 min at 37°C to evaluate its effects on synaptosomal redox homeostasis. The choice of this specific emodin concentration was based on a previously published study,[31] as well as our preliminary optimization experiments. Emodin stock solutions (200 µM) were prepared in methanol and stored at 4°C in amber vials to prevent photodegradation. Subsequently they were diluted in synaptosomes suspended in the homogenization buffer (200 µL) to achieve the final working concentration of 20 µM. Vehicle untreated control groups comprised of YCS or OCS samples incubated with 20 µL methanol under identical conditions (120 min at 37°C). Following incubation, samples were centrifuged at 12,000 × g for 5 min. The supernatant was discarded, and the synaptosomal pellet was washed twice with 500 µL of PBS buffer at 12,000 × g for 5 min before being processed according to the experimental protocols described below.

Antioxidant Assays

ABTS radical scavenging assay[32] was conducted to assess the endogenous antioxidant activity of emodin-treated and untreated YCS and OCS. Generation of $ABTS^{\bullet+}$ radicals was performed by mixing 7 mM ABTS with 2.45 mM potassium persulfate in a 1:1 (v/v) ratio and incubating the mixture in dark at 4°C for 12–16 hours.[32] The resulting radical solution remained stable for over two days under these conditions.[33] Prior to assessment of synaptosomal radical scavenging capabilities, the $ABTS^{\bullet+}$ solution was diluted to achieve an absorbance value of ca. 0.7 at 734 nm. Neutralization of the radicals was followed by a decrease in absorbance upon addition of synaptosomal samples (200 µg of protein sample). The synaptosomal free radical scavenging capacity was estimated as the percentage inhibition of $ABTS^{\bullet+}$ radicals, according to the following formula:

$$\%Inhibition = \frac{A(0) - A(t)}{A(0)} * 100$$

where A(0) and A(t) represent the absorbance at times 0 and 30 min, respectively, after adding the synaptosomal sample or the positive control ascorbate. An ascorbic acid (0–100 µg/mL) standard curve was used as a reference in each of the sets.[33] Further, a blank sample with only $ABTS^{\bullet+}$ was used to confirm the negligible degradation of the radicals in the absence of ascorbic acid or synaptosomes in each experimental set.

The ferric-reducing antioxidant power (FRAP) assay was performed to further evaluate the antioxidant activity of YCS and OCS, and to evaluate the beneficial effects of emodin. The assay was used to evaluate the ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion-reducing capacity of the biological samples at 593 nm. Briefly, FRAP reagent was formulated using 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \bullet 6H_2O$ in a 10:1:1 (v/v/v) ratio.[34] Following baseline absorbance recording of 100 µL of FRAP reagent, 200 µg protein of the synaptosomal sample in 100 µL of PBS was added to it. After incubating the mixture at room temperature for 30 min, spectrophotometric measurements were again performed at 593 nm. The % inhibition of ferric reduction by the sample was calculated using

the same formula as that used for the ABTS assay. Ascorbic acid standards (0–100 μg/mL) and blanks were used in each experimental set to confirm the quantitative robustness of the assay.

ROS and RNS Assay

The levels of ROS in synaptosomal samples were measured using a fluorometric method based on DCFH-DA, a non-fluorescent dye that, upon reaction with oxidizing species, is converted into the highly fluorescent dichlorofluorescein (DCF).[35] Synaptosomal samples (200 μg protein in PBS; final volume 200 μL) were co-incubated with DCFH-DA (10 μM in methanol) at 30°C, and fluorescence was measured after 15 min (excitation: 495 nm, emission: 530 nm). A DCF standard curve (0–0.15 μM in PBS) was used to establish the quantitative nature of the assay.

Additionally, reactive nitrogen species (RNS) levels were quantified using the Griess reagent assay, which detects total nitrites (NO_x), a stable end product of NO metabolism. A freshly prepared home-made Griess reagent, composed of equal parts of component A (0.2% (w/v) N-napthyl ethylene diamine dihydrochloride in water) and component B (2% (w/v) sulphanilamide in 5% H_3PO_4) was used for the assays. Synaptosomal samples (200 μg protein in 100 μL of PBS) were mixed with the Griess reagent (100 μL) and incubated at room temperature for 15 min in the dark. Subsequently, the absorbance was recorded at 540 nm. NO_x concentrations were calculated based on the sodium nitrite standards (0–100 μM) in each experimental set.

Assay for Oxidative Damage

Oxidative damage markers in the cortical synaptosomal samples were measured using well-established protocols for protein thiol oxidation, lipid peroxidation, and protein carbonylation. These assays were based on Elman's reagent (DTNB; 5,5′-dithiobis-[2-nitrobenzoic acid]),[36] thiobarbituric acid (TBA),[37] and dinitrophenyl hydrazine (DNPH),[38] respectively.

Protein Thiol Oxidation

Protein thiol content was measured using DTNB, which reacts with free thiols to form 5-thio-2-nitrobenzoate ion (TNB^-), a yellow-colored product which can be detected spectrophotometrically at 412 nm. Synaptosomal samples (200 μg protein in PBS) were treated with 10% (w/v) TCA, incubated for 10 min, and centrifuged (14,000 × g for 3 min). After washing twice with 5% TCA, the protein pellet was resuspended in 100 mM Tris (pH 8.8), supplemented with 5% (w/v) SDS. The mixture was then incubated with 10 mM DTNB for 30 min in the dark. The absorbance of the samples was recorded at 412 nm, and the results were depicted as μmol TNB per μg protein (ε_{TNB} at 412 was taken as 14150 M^{-1} cm^{-1}). A blank sample containing DTNB was also used.

Lipid Peroxidation

Synaptosome samples (200 μg protein in PBS) in a final volume of 100 μL were mixed with 100 μL TBA-TCA-HCl reagent consisting of 0.375% (w/v) thiobarbituric acid, 15% (w/v) trichloroacetic acid, and 0.25M HCL. The samples were vigorously vortexed and incubated in boiling water for 15 min. After allowing the samples to cool to room temperature, they were centrifuged at 12,000 × g for 10 min and the supernatant was retrieved. Subsequently, thiobarbituric acid-reactive substances (TBARS) were quantified at 532 nm wavelength. The blank control was used to account for background absorbance in each experimental set. TBARS levels were calculated and expressed as pmol TBARS per μg of protein (ε_{TBARS} at 532 nm was taken as 1.56×10^5 M^{-1} cm^{-1}), with higher values indicating greater lipid peroxidation damage.

Protein Carbonylation

Protein carbonyl content was measured using DNPH, which forms a hydrazone product that is detectable at 370 nm. Briefly, synaptosome samples (200 μg protein) were suspended in PBS to a final volume of 100 μL. A blank control containing PBS only was used for each experimental set. The samples or controls were incubated with 10 mM DNPH in 2 N HCl (final volume, 100 μL) at room temperature for 1 h in the dark, with intermittent mixing every 10 min. Next, 10% TCA was used to precipitate synaptosomes at 12,000 × g for 2 min. After discarding the supernatant, the denatured protein pellet was washed three times with 500 μL acetone. Thereafter, the pellet was dissolved in 200 μL of 8 M urea in 20 mM potassium dihydrogen phosphate buffer (pH 2.3), using a vortex mixer. Synaptosomal levels of protein

carbonylation were monitored at 370 nm using an ε of 22 $mM^{-1}cm^{-1}$, and expressed as pmol carbonyl content per µg protein. A blank for the background correction was included in each experimental set.

Statistical Analyses

Graphical representation and statistical analyses were accomplished on the GraphPad Prism software (version 8.02). Data were expressed as mean ± standard error of mean (SEM). Comparisons among the four experimental groups were made using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test for pair-wise comparison of all groups. Statistical significance was determined at $p<0.05$.

Results

Emodin Enhances Endogenous Synaptic Antioxidant Capacity

The endogenous antioxidant capacities of YCS and OCS were evaluated using two widely used assays. Ascorbate control at different concentrations established the quantitative validation of both the ABTS radical scavenging (Figure 1A) and FRAP (Figure 1B) assays. With regards to synapse-specific antioxidant capacities, Significant differences were observed in the ABTS radical scavenging ($F(3,16)=314.4$, $p<0.0001$; Figure 1C) and ferric reducing capacities ($F(3,16)=304.8$, $p<0.0001$; Figure 1D) of the four groups of synaptosomal samples. Specifically, OCS showed a marked decline in scavenging and reduction capacities compared to young controls ($p<0.0001$), indicating significant compromises in the endogenous synaptic antioxidant capacities with age. Furthermore, emodin treatment significantly restored the

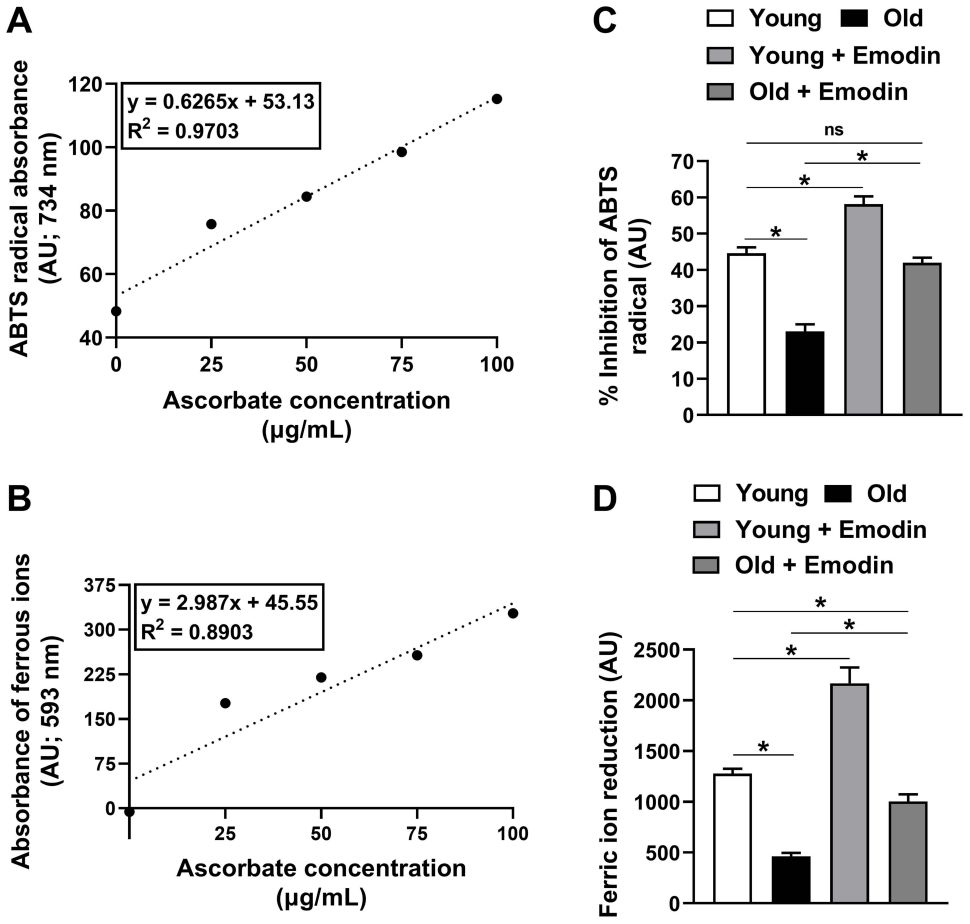

Figure 1 Emodin enhances synaptosomal antioxidant capacity cortical tissues of aged rats. (**A** and **B**) depict a representative ascorbic acid standard curve used as quantitative positive control in the ABTS and FRAP assays, respectively. Ageing-induced decline in ABTS radical scavenging (**C**) and ferric reducing (**D**) activities in the brain synaptosomes was mitigated by emodin treatment. Data are presented as the mean±SEM. "*" represents statistical significance, while "ns" denotes non-significant differences.

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antioxidant capacity of synaptosomes of the aged brains (p<0.0001). However, while emodin treatment recovered the ABST scavenging activity of OCS to the levels of YCS (p=0.1582), this was not the case for FRAP reducing power, in which the improvement did not reach the levels observed in untreated young controls (p=0.0011). Interestingly, emodin was also found to significantly increased the antioxidant capacity of synaptosomes isolated from young rat brains.

Emodin Depletes Oxidative and Nitrosative Species at the Synapses

ROS and RNS levels in the synaptosomes were measured using DCFH-DA and Griess assays. First, spectrophotometric assays were validated using standard curves of DCF (Figure 2A) and sodium nitrite (Figure 2B). Next, we evaluated the ROS ($F(3,16)=995.8$, $p<0.0001$; Figure 2C) and RNS ($F(3,10)=5.725$, $p=0.0152$; Figure 2D) levels in the four groups of synaptosomal samples and found statistically significant differences. As expected, post-hoc analyses indicated that both ROS ($p<0.0001$) and RNS ($p=0.0243$) levels were significantly elevated in OCS compared with those in YCS samples, indicating significantly higher levels of ageing-induced oxidative stress. Emodin treatment markedly reduced the levels of both these damaging species in synaptosomal preparations isolated from aged rats; however, the effects were more pronounced for ROS ($p<0.0001$ for ROS vs $p=0.0315$ for RNS). Indeed, emodin treatment resulted in a decrease in ROS levels in OCS samples, even below those in untreated YCS samples ($p<0.0001$). Regarding RNS, emodin caused a reduction in RNS levels in OCS to levels comparable to those in untreated YCS ($p=0.9996$). Interestingly, while emodin reduced ROS levels in YCS samples ($p<0.0001$), it failed to have a similar effect on RNS levels ($p=0.9919$).

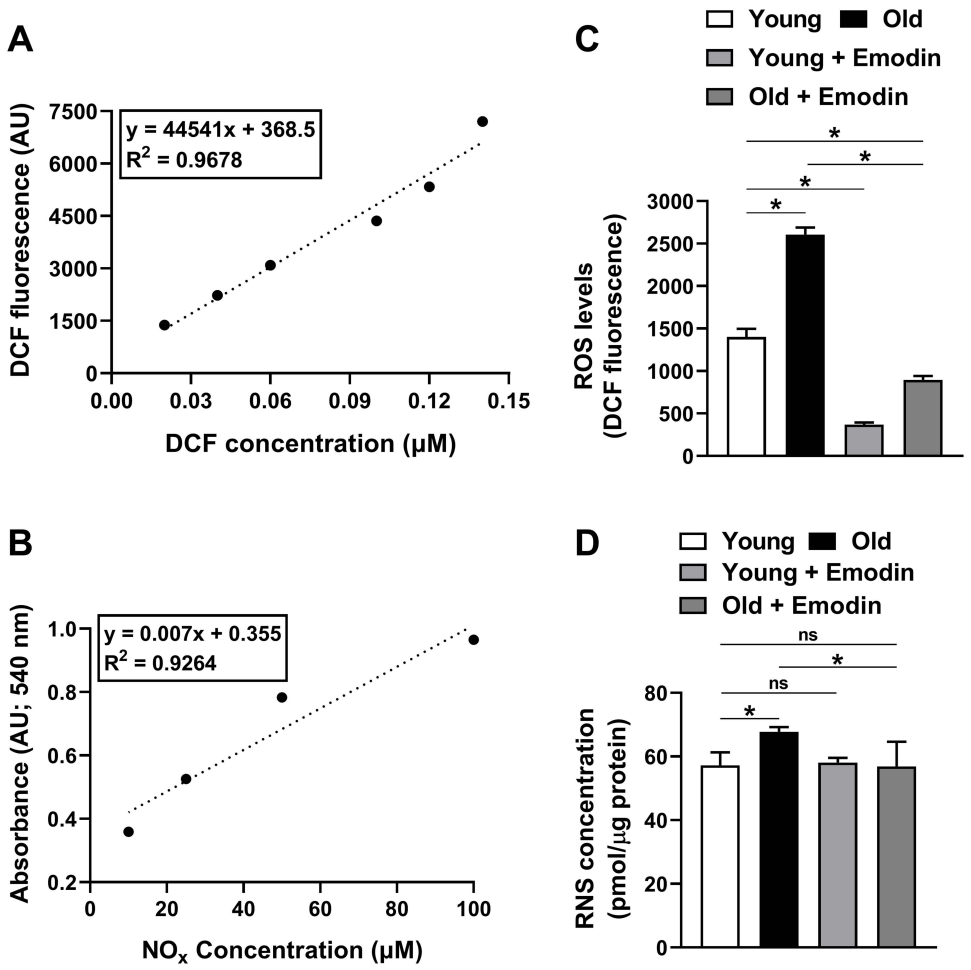

Figure 2 Emodin attenuates oxidative and nitrosative stress in cortical synaptosomes of aged Rats. (**A** and **B**) show representative standard curves of DCF and sodium nitrite for ROS and RNS estimation, respectively. Emodin treatment resulted in robust decreases in ROS (**C**) and RNS (**D**) levels in synaptosomes isolated from aged rat brains. Data are presented as the mean±SEM. "*" represents statistical significance, while "ns" denotes non-significant differences.

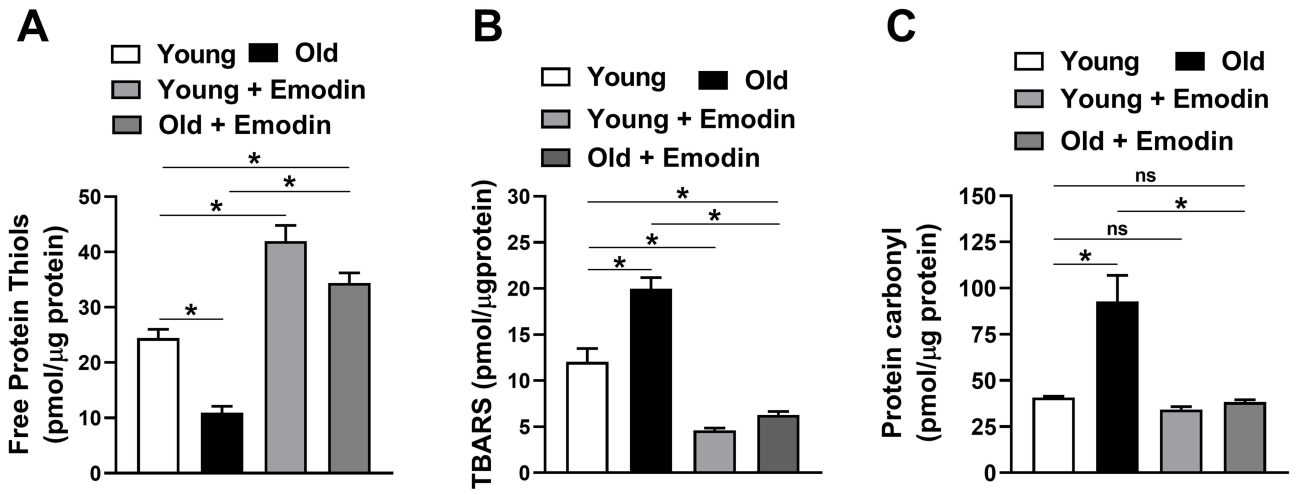

Figure 3 Emodin mitigates ageing-induced oxidative damage to synaptosomal proteins and lipids. Emodin treatment under ex vivo conditions resulted in significant recovery of the levels of oxidative damage markers, viz., oxidized protein thiols (**A**), TBARS (**B**), and protein carbonyls (**C**). Data are presented as the mean±SEM. "*" represents statistical significance, while "ns" denotes non-significant differences.

Emodin Mitigates Oxidative Damage to Synaptic Proteins and Lipids

The four groups of synaptosomal samples (YCS-untreated, YCS-emodin-treated, OCS-untreated, and OCS-emodin treated) showed significantly different levels of oxidative damage, as assessed by protein thiol oxidation ($F(3,16)=237.1$, $p<0.0001$; Figure 3A), lipid peroxidation ($F(3,16)=248.4$, $p<0.0001$; Figure 3B), and protein carbonylation ($F(3,16)=74.95$, $p<0.0001$; Figure 3C) assays. Post-hoc assessments indicated a significant reduction in free protein thiol content in OCS versus YCS samples ($p<0.0001$), indicating ageing-induced increased oxidative damage to the thiol groups of synaptic proteins. Similar results of elevated levels of lipid peroxidation ($p<0.0001$) and protein carbonylation ($p<0.0001$), the other two markers of oxidative damage, were observed in OCS compared to YCS. Consistent with its potent antioxidant functions, emodin significantly restored free protein thiol levels in OCS ($p<0.0001$), which were even greater than those observed in the untreated YCS group ($p<0.0001$). Furthermore, emodin treatment was found to be significantly associated with the reversal of enhanced levels of synaptosomal TBARS in aged rats ($p<0.0001$), which were below the levels observed in untreated YCS ($p<0.0001$). Lastly, protein carbonyl levels were significantly diminished upon emodin treatment in OCS ($p<0.0001$) but not in YCS ($p=0.4773$) samples. This may be because emodin had no additional effect on samples with low basal carbonylation levels.

Discussion

Aging enhances susceptibility to degenerative and psychiatric neuropathologies. One of the primary mechanisms is the progressive decline in redox homeostatic abilities of the brain, leading to increased oxidative stress and subsequent damage to cellular biomolecules, lipids, proteins, and nucleic acids.[39–41] Synapses, the fundamental units of neuronal communication, are highly vulnerable to oxidative damage because of their extraordinarily high energy demands and reliance on mitochondrial bioenergetic activity, and the presence of peroxidation-sensitive polyunsaturated fatty acids (PUFAs) in synaptic membranes.[42,43] Oxidative stress at synapses disrupts neurotransmission, impairs synaptic plasticity, and contributes to the cognitive and motor deficits observed during aging.[44,45] Our study aimed to assess the redox imbalance in the cortical synaptosomes of aged rats and to investigate whether emodin, a potential neuroprotective phytochemical, could mitigate these age-associated impairments.

To this end, we used crude synaptosomal preparations isolated from young and old rat brains. Previous studies have shown the utility of synaptosomes as ex vivo models to test the antioxidant actions of a variety of plant-based bioactives. For instance, berry fruit wines have been reported to mitigate H_2O_2-induced oxidative stress by stimulating endogenous antioxidant enzyme systems,[46,47] while vinpocetine was found to prevent ascorbate-Fe^{2+}-induced ROS generation and TBARS accumulation.[48] Similarly, curcumin has been proposed to alleviate induced oxidative stress in ex vivo preparations

of rodent synaptosomes.[49,50] Synaptosomal studies also support the antioxidant properties of quercetin[51] and epigallocatechin gallate.[52] In the light of these success stories, we used synaptosomal preparations for the evaluation of oxidative damage parameters as functions of age and emodin treatment.

Among the primary contributors to oxidative stress is the accumulation of ROS, which can disrupt cellular homeostasis by damaging biomolecules.[53,54] Our results indicate significantly elevated ROS levels in the synaptosomes of aged rats, indicating an increased oxidative burden. This aligns with previous reports showing that aging is characterized by excessive ROS production due to mitochondrial dysfunction and impaired antioxidant defense mechanisms.[55] Notably, emodin treatment significantly reduced ROS levels, suggesting that it acts as a potent free radical scavenger. This protective effect could be attributed to the ability of emodin to enhance endogenous antioxidant enzyme activity or directly neutralize ROS, as demonstrated in prior studies.[56] The changes observed in RNS levels were comparatively less pronounced than those observed for ROS. Unlike ROS, which showed a clear increase with age and a reduction following emodin treatment, RNS levels remained relatively stable. Indeed, while aging was associated with a modest elevation in RNS levels and emodin treatment reduced RNS in aged synaptosomes, these effects were weaker and less consistent than the robust ROS modulation. This suggests that, under the present experimental conditions, oxidative stress rather than nitrosative stress may represent the dominant contributor to synaptic redox imbalance during aging. Accordingly, the protective effects of emodin appear to be more strongly mediated through modulation of ROS. Overall, this indicates that oxidative stress, rather than nitrosative stress, may be the predominant contributor to synaptic redox dysregulation in this model, and that the protective effects of emodin are likely mediated by the modulation of ROS rather than RNS.

In addition to ROS and RNS accumulation, aging resulted in a marked decline in antioxidant capacity, as indicated by decreased ABTS and FRAP values in OCS. These assays measure the overall ability of synaptosomes to neutralize free radicals and reduce oxidative stress. The decline in antioxidant potential suggests an impaired endogenous defense system, rendering synapses more vulnerable to oxidative damage. However, emodin treatment significantly restored endogenous synaptic antioxidant capacity, indicating its role in reinforcing the redox balance. While assessment of the mechanistic details of emodin's antioxidant actions was beyond the scope of this preliminary study, these findings may lend support to the hypothesis that emodin upregulates antioxidant defense pathways such as the Nrf2/Keap1 signaling pathway,[23,57] which governs the expression of several downstream antioxidant enzymes.

Finally, we evaluated the extent of age-associated oxidative damage to synaptosomal proteins and lipids, and emodin's ability to prevent them. Of note, previous studies have shown that synaptosomes can be used to study the ex vivo reversal of these damages by antioxidant therapeutics.[37,58] We observed a significant depletion of free (reduced) protein sulfhydryl groups in the OCS, signifying increased oxidative modifications. However, emodin treatment effectively preserved reduced thiol levels, further supporting its role in maintaining protein integrity and synaptic function. Lipid peroxidation, a hallmark of oxidative damage in biological membranes,[59] was also found to be significantly elevated in the OCS samples, indicating enhanced oxidative damage to membranes. This increase in lipid peroxidation could compromise synaptic integrity, contributing to impaired neurotransmission and neuronal dysfunction observed during brain aging. However, emodin supplementation significantly reduced TBARS levels, suggesting a protective role of emodin in maintaining membrane stability. Another key indicator of oxidative damage evaluated in our study was protein carbonylation, an irreversible modification of proteins caused by the ROS-mediated oxidation of amino acid side chains. OCS exhibit significantly elevated protein carbonyl levels, indicating extensive oxidative protein damage, which could lead to impaired synaptic function. This observation is in line with previous reports suggesting that oxidative protein modifications contribute to neuronal dysfunction in aging and neurodegeneration.[60] However, emodin treatment markedly reduced protein carbonylation, suggesting its potential role in preventing oxidative modifications and preserving protein functionality. The ability of emodin to reduce protein carbonylation can be attributed to its direct scavenging of ROS and/or activation of cellular antioxidant or proteolytic defenses.

Collectively, these findings suggest that emodin mitigates age-related oxidative stress by restoring synaptic antioxidant defenses and by reducing oxidative damage. Given the crucial role of oxidative stress in aging-associated neurodegenerative and neuropsychiatric conditions, our results highlight the potential therapeutic value of emodin in these conditions. However, there are two limitations of the study which should be acknowledged. First, synaptic profiling of neurotransmitter release, bioenergetic function, and chemically induced long-term potentiation, processes whose dysfunction potentially contribute to

pathogenic mechanisms in brain ageing, were not examined in the present study. Further, the absence of direct evaluation of antioxidant pathways, such as Nrf2/Keap1, SIRT1–FOXO, and NF-κB, and their downstream antioxidant effectors, limits the mechanistic interpretation of the findings of emodin's neuroprotective functions. These are important considerations which are a focus of our ongoing studies. Second, rather than relying on time-consuming and cost-intensive sucrose gradient based ultracentrifugation methods, we used a cruder preparation of synaptic fractions in our study, which may be contaminated with extrasynaptic mitochondrial components. Although this limits the ability to extricate synapse-specific effects, it concurrently provides insights into the redox status of neuronal mitochondria. The validity of our findings is not necessarily undermined; rather, in light of the tight pathophysiological interdependence of synaptic and mitochondrial dysfunctions underlying ageing-related oxidative stress and neuronal deficits, this approach strengthens the biological relevance of the observed effects. Interestingly, a similar approach based upon crude synaptosomal preparations was used by Elinos-Calderón and coworkers to investigate the protective effects of S-allylcysteine on synaptic and mitochondrial dysfunction induced by prooxidant toxicant, quinolinic acid and 3-nitropropionic acid.[18]

Conclusion

Our study demonstrated that aging disrupts synaptic redox homeostasis, as indicated by elevated ROS levels, diminished antioxidant capacity, and increased oxidative damage in synaptosomes, hallmarks associated with synaptic dysfunction, and consequently, neurodegenerative and neuropsychiatric ailments. Emodin supplementation effectively mitigated these alterations by enhancing antioxidant defences, reducing ROS levels, and decreasing lipid and protein oxidation levels. These findings underscore the potential of emodin to preserve the synaptic redox balance and attenuate oxidative stress during aging. Further studies using detailed functional and molecular analyses are however warranted to comprehensively assess its neuroprotective efficacy.

Abbreviations

AA(t), absorbance after adding the sample; ABTS, 2,2′-azinobis-[3-ethylbenzothiazoline-6-sulfonic acid]; AC (0), absorbance of the control; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5′-dithiobis -[2-nitrobenzoic acid]; Fe^{2+}, ferrous; Fe^{3+}, ferric; FRAP, ferric reducing antioxidant power; IAEC, Institutional Animal Ethics Committee; MDA, malondialdehyde; OCS, old cortical synaptosomes; Pb, lead; RNS, reactive nitrogen species; ROS, reactive oxygen species; SD, standard deviation; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; TBA-TCA-HCl, thiobarbituric acid, trichloroacetic acid, hydrochloric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; TNB, 5-thio-2-nitrobenzoic acid; TPTZ, 2,4,6-tripyridyl-s-triazine; t, time; YCS, young cortical synaptosomes.

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Disclosure

The authors report no conflicts of interest in this work.

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