

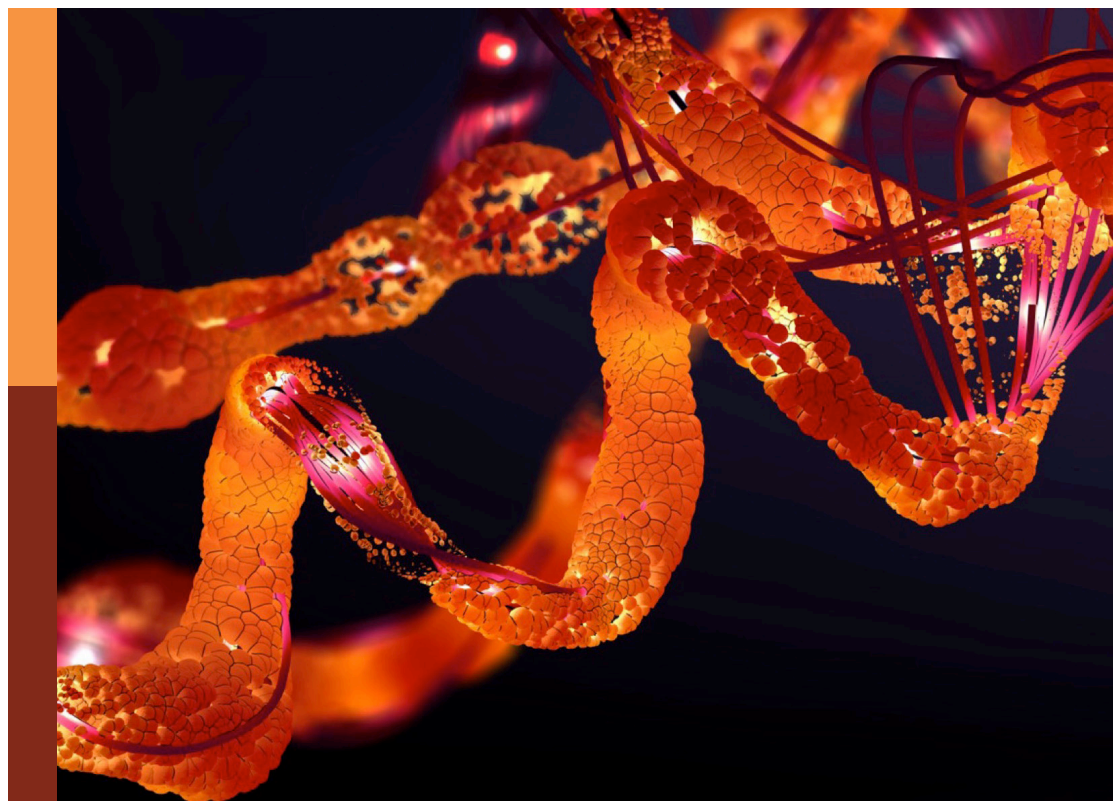
The role of natural and synthetic antioxidants in the therapeutic targeting of oxidative stress

Edited by

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The role of natural and synthetic antioxidants in the therapeutic targeting of oxidative stress

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Editorial: The role of natural and synthetic antioxidants in the therapeutic targeting of oxidative stress

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KEYWORDS

ROS, plant-derived antioxidants, synthetic antioxidants, oxidative stress, therapeutic agents

Editorial on the Research Topic

The role of natural and synthetic antioxidants in the therapeutic targeting of oxidative stress

Oxidative stress is involved in a wide range of diseases, including neurological (Pizzino et al., 2017), cardiovascular (Valaitienė and Laučytė-Cibulskienė, 2024), and pulmonary diseases, diabetes and cancer (Forman and Zhang, 2021). However, attempts at the prevention and treatment of such diseases using small-molecule antioxidants have often been unsuccessful. It was then suggested that the focus should be shifted towards disease-specific and target-directed therapy (Firuzi et al., 2011), leading to antioxidant compounds being accepted for clinical use. This Research Topic includes studies exploring the role of natural and synthetic antioxidants in the therapeutic targeting of oxidative stress, assessing the efficacy and the mechanisms by which antioxidants exert their effects.

Plant-derived antioxidants are a large group of natural compounds possessing ROS-scavenging activity (Remigante et al., 2023; Pirtskhalava et al., 2024). Despite the accumulated data on antioxidant structures and properties, the bioactivity of many natural compounds remains unknown. The action mechanism and therapeutic potential of licochalcone B, a plant-derived chalcone, were examined in the extensive review by Shaikh et al. Licochalcone B has antioxidant properties, possessing anti-inflammatory, anti-cancer, hepatoprotective, and neuroprotective effects. These diverse pharmacological activities were linked to autophagy, apoptosis, inflammation, and oxidative stress. The signalling mechanisms that modulated these responses were identified, and the potential of licochalcone B as a lead compound for drug discovery was demonstrated.

The review of Ghosh et al. discusses the biological activities of *Vaccinium*-derived anthocyanins (quercetin, myricetin and their glycosidic derivatives), which demonstrated cardioprotective, antidiabetic, anticancer, neuroprotective, anti-inflammatory, antimicrobial and antiviral effects. The review discusses the experimental

results, which indicate that total anthocyanin, phenolic and antioxidant contents may be associated with the berry's effectiveness. However, challenges regarding *in vitro* and *in vivo* studies for elucidating the mechanism of action and health benefits for various groups of patients remain.

The action of another natural antioxidant, flavonoid chrysin, against the effects of organophosphorus pesticide methidathion on ovaries was demonstrated in the study of [Hamed et al.](#)

Chrysin efficiently alleviated pesticide-induced ovarian damage, as was demonstrated by significantly improved lipid peroxidation and the content of non-enzymatic and enzymatic antioxidants, preventing hormonal imbalance and histopathological changes in methidathion-treated rats.

For the first time, the antimalarial potential of bioactive constituents derived from the bark of *Schleichera oleosa* was reported in the study of [Vanaja et al.](#) The novel compounds scillarenin, possessing antioxidant properties and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside were identified, using GC-MS and LC-MS. Molecular docking confirmed the strong binding affinities of these compounds to malaria target proteins 1CEQ and 4ZL4, surpassing some standard drugs in efficacy.

Since oxidative damage is strongly implicated in the pathogenesis of neurodegenerative ([Kamat et al., 2008](#)) and neuropsychiatric diseases ([Pandya et al., 2013](#)), the mechanism of oxidative damage and the role of antioxidants in the prevention and treatment of these diseases were explored in several studies.

Thus, the review by [Yao et al.](#) discusses the role of potential oxidative biomarkers in the prognosis of intracerebral haemorrhage (ICH) and the development of antioxidative treatments. The higher levels of oxidative stress indicators (malondialdehyde, hydroxy-2-nonenal, F2 isoprostanes, and ischemia-modified albumin) were detected in ICH patients. ICH also increased the activity of antioxidant enzymes (SOD, CAT, GPX) and non-enzymatic antioxidants (vitamins A, E and C). However, the results of ROS-targeted therapy of ICH did not demonstrate high effectiveness. It was suggested that the successful treatment of ICH requires an increase in the degradation and neutralisation of ROS, which could be achieved via modulation of several signalling pathways (Nrf2/ARE, Wnt/ β).

[Liu et al.](#) demonstrated the role of oxidative stress in the pathogenesis of schizophrenia, major depressive disorder, anxiety and bipolar disorder and explored the role of antioxidants in the prevention of these diseases. The functions of such antioxidants as N-acetylcysteine, sulforaphane, alpha-lipoic acid, L-carnitine, ascorbic acid and flavones in the alleviation of symptoms of neuropsychiatric disorders via the provision of antidepressant, neurotropic and anti-inflammatory effects were discussed. The authors concluded that antioxidants maintain the normal functioning of the nervous system by regulating the balance of neurotransmitters, inhibiting inflammatory responses and improving the efficiency of the mitochondrial energy metabolism.

[Micucci et al.](#) assessed the toxicity of Q10-diacetate, a derivative of coenzyme Q10, on the mouse hippocampal neuronal cell line under rotenone-induced oxidative stress. Q10-diacetate possesses antioxidant properties and is not toxic under normal conditions. Q10-diacetate reduced superoxide production, enhanced the

expression of mitochondrial biogenesis genes, and contributed to sustained ATP synthesis under oxidative stress conditions. These findings indicate the need for an *in vivo* investigation into the pharmacokinetics of Q10-diacetate for therapeutic application.

Several studies revealed the association between the reduced expression and activity of antioxidant enzymes and the development of vascular diseases ([Radovanovic et al., 2021](#)), but the data on the use of antioxidant enzymes as markers of such disease are contradictory. The clinical study by [Chung et al.](#) performed on randomly selected immunosuppressive drug-naïve patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) demonstrated for the first time that the activity of the one of first-line antioxidant defence enzyme, serum glutathione peroxidase isoenzyme (GPX-3) correlates with vasculitis activity and was significantly lower in patients with general AAV manifestation in comparison with those without. The study suggests using serum GPX-3 as a complementary biomarker for assessing AAV activity in clinical practice.

The magnetically targeted nanocomposite for the delivery of hydrophobic antioxidant curcumin and a calcitonin gene-related peptide (CGRP) antagonist (CGRP_i) for the treatment of neuropathic pain was obtained in the study of [Zhu et al.](#) Curcumin provides an analgesic effect, and CGRP expression levels increase after nerve injury. An *in vivo* study demonstrated that the drug-delivery system can effectively enter the site of sciatic nerve injury in mice, alleviating the injury, inflammatory and neuropathic pain response. The introduction of nanocomposites also inhibited oxidative stress and ROS production, indicating the feasibility of the application in the treatment of neuropathic pain.

The studies presented in this Research Topic provide important insights into the structure and mode of action of synthetic and natural antioxidants. We are grateful to all authors, reviewers, and contributors who helped to make this Research Topic a success. Their studies have increased our comprehension of the role of antioxidants as therapeutic agents and enabled future developments in this field.

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Therapeutic potential and action mechanisms of licochalcone B: a mini review

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Licochalcone B (LicB), a chalcone derived from *Glycyrrhiza uralensis* and *Glycyrrhiza glabra*, has received considerable attention due to its diverse pharmacological properties. Accumulated data indicates that LicB has pharmacological effects that include anti-cancer, hepatoprotective, anti-inflammatory, and neuroprotective properties. The action mechanism of LicB has been linked to several molecular targets, such as phosphoinositide 3-kinase/Akt/mammalian target of rapamycin, p53, nuclear factor- κ B, and p38, and the involvements of caspases, apoptosis, mitogen-activated protein kinase-associated inflammatory pathways, and anti-inflammatory nuclear factor erythroid 2-related factor 2 signaling pathways highlight the multifaceted therapeutic potential of LicB. This review systematically updates recent findings regarding the pharmacological effects of LicB, and the mechanistic pathways involved, and highlights the potential use of LicB as a promising lead compound for drug discovery.

KEYWORDS

flavonoid, licochalcone B, anti-cancer, anti-inflammatory, hepatoprotective

1 Introduction

The pharmacological properties of various herbal preparations and natural compounds have been the subjects of increasing research in recent years (Atanasov et al., 2021; Shaikh et al., 2021; Ahmad et al., 2023). *Glycyrrhiza* spp. (belonging to Fabaceae family), also called licorice, sweet wood, or mulaithi, is a perennial herb found globally. The name combines the Greek words “glykys” (sweet) and “rhiza” (root) (El-Saber Batiha et al., 2020). *Glycyrrhiza* spp. contains a variety of bioactive compounds including chalcones with various biological properties that include anti-inflammatory, antiviral, antimicrobial, antioxidant, anticancer, immunomodulatory, hepatoprotective, and cardioprotective effects (Asl and Hosseinzadeh, 2008; Yang et al., 2015). Chalcones and their derivatives have attracted considerable interest in the medical field due to their wide-ranging pharmacological activities and clinical potential. At the molecular level, chalcones contain two aromatic rings linked by an

Abbreviations: LicB, Licochalcone B; SM, Skeletal muscle; NLRP3, NLR family pyrin domain containing 3; LPS, Lipopolysaccharide; NO, Nitric oxide; 15-LOX, 15-lipoxygenase; MDA, Malondialdehyde; OS, Osteosarcoma; DR, Death receptor; FnC60, Fullerene C60; LDH, Lactate dehydrogenase; CCl₄, Carbon tetrachloride; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; Nrf2, Nuclear factor erythroid 2-related factor 2; HCK, Hemopoietic cell kinase; STING, stimulator of interferon genes.

α , β -unsaturated α -carbon ketone moiety, which forms the core structure (Zhuang et al., 2017). Pharmacologically, licochalcone A and licochalcone B (LicB), which are components of the roots of *Glycyrrhiza* spp., have emerged as noteworthy bioactive compounds. The pharmacological effects of licochalcone A have recently been reviewed extensively elsewhere (Li M. T. et al., 2022), and thus, here we focus on the pharmacology of LicB.

LicB has a variety of pharmacological effects; for example, it is cardioprotective (Han et al., 2014), has anti-Alzheimer's disease effects (Cao et al., 2020), possesses anti-oxidant and radical scavenging properties (Fu et al., 2013), and the ability to induce cancer cell apoptosis (Yuan et al., 2014; Yu et al., 2016). Our research group continues to search for naturally occurring compounds that target proteins involved in skeletal muscle (SM) and associated disorders (Baig et al., 2017; Ahmad et al., 2021; Lee et al., 2021; Ali et al., 2022; Ali et al., 2023a; Ali et al., 2023b). Recently, we demonstrated that LicB binds strongly to the catalytic site of DPP4 and concentration-dependently inhibits its activity, which suggests LicB has potential use as an antidiabetic agent (Shaikh et al., 2022). The promising therapeutic potential of LicB for the management and prevention of clinical diseases emphasizes the need for an updated review of its pharmacology. Thus, this review was undertaken to summarize recent pharmacologic advances, investigate the potential applications of LicB for the management of various diseases, and provide critical insight into the clinical use of LicB. In addition, we discuss the toxicity profile of LicB.

2 Pharmacological effects

2.1 Effects on skeletal muscle/myogenesis

SM is the most abundant body tissue, and in addition to body movements and posture, it regulates temperature and physically protects internal organs and soft tissues. In addition, SM can regenerate in response to injury or disease and is an important marker of diabetes, obesity, and age-related diseases. Recently, we found that *Glycyrrhiza uralensis* inhibits MSTN, a key (negative) regulator of muscle growth, and promotes myogenesis and that LicB is a primary promoter of myoblast differentiation and proliferation (Lee et al., 2021). This study provided a basis for a more comprehensive investigation of the LicB's biologic mechanisms of LicB, especially on its ability to regulate MSTN and atrogen1 and MuRF1 (markers of muscle atrophy) employing *in silico*, *in vitro*, and *in vivo* approaches. A subsequent study revealed how LicB inhibits MSTN and promotes muscle proliferation and differentiation, which are both required for muscle regeneration. These studies improved our understanding of the therapeutic mechanisms of LicB, its anti-aging properties, and its ability to ameliorate muscle-wasting conditions. These findings enhanced the therapeutic narrative and established LicB as a clinical candidate (Ahmad et al., 2024).

2.2 Anti-inflammatory effects

The abnormal activation of NLRP3 (NLR family pyrin domain containing 3) inflammasome has been linked to a

variety of inflammatory pathologies, and LicB inhibits NLRP3 inflammasome activation in macrophages. The mechanism involves LicB binding directly to NEK7, thus disrupting the interaction between NLRP3 and NEK7 and effectively suppressing the activation of NLRP3 inflammasome. Additionally, in murine models, LicB protected against NLRP3-mediated diseases such as non-alcoholic steatohepatitis, lipopolysaccharide (LPS) and monosodium urate stimulated septic shock, and peritonitis (Li Q. et al., 2022). Collectively, LicB appears to be a selective NLRP3 inhibitor with therapeutic potential for the treatment of NLRP3 inflammasome-related disorders.

LicB has been demonstrated to suppress PKA activation effectively, and thus, to inhibit LPS-stimulated NF- κ B phosphorylation at serine 276 and activation in RAW264.7 cells. Furthermore, LicB significantly reduced LPS-stimulated nitric oxide (NO), TNF- α , and MCP-1 generation in RAW264.7 cells (Furusawa et al., 2009). Similarly, in another study, LicB exhibited potent inhibitory activity against LPS-induced NO production in RAW 264.7 cells with an IC₅₀ of 8.78 μ M (Lin et al., 2017b). In addition, LicB attenuated BDE-47-stimulated oxidative damage in RAW264.7 cells by activating the Nrf2 pathway and inhibiting the NF- κ B pathway, thereby preventing immunological dysfunction and apoptosis (Dong M. et al., 2024).

LicB also potently scavenged ABTS(+) radicals and inhibited lipid peroxidation in rat liver microsomes, dose-dependently suppressed ROS generation in RAW264.7 cells, and effectively inhibited NO, IL-6, and prostaglandin E2 generation in LPS-stimulated macrophages (Fu et al., 2013).

The enzyme 15-lipoxygenase (15-LOX) is a critical target for treating various inflammatory disorders, and LicB inhibits 15-LOX with an IC₅₀ of 9.67 μ M (Li et al., 2021). Furthermore, LicB inhibits TNF- α release by LPS-induced RAW264.7 cells. Computational analysis demonstrated that the targeting of Thr412, Arg415, Val420, Thr429, Ile602, and Trp606 of 15-LOX by LicB was largely responsible for 15-LOX inhibition (Li et al., 2021). This study identified LicB as a 15-LOX inhibitor and laid the groundwork for future 15-LOX inhibitor research.

LicB reduced inflammation and oxidative stress in LPS-stimulated periodontal ligament cells. LicB inhibited the NF- κ B/NLRP3 signaling pathway and reversed LPS-induced inhibition of osteogenic differentiation. These findings suggest that LicB has the potential to be a periodontal therapeutic agent (Du et al., 2024).

2.3 Neuroprotective effects

Interestingly, LicB was also found to ameliorate memory impairments and neurological deficits in a mouse Middle Cerebral Artery Occlusion (MCAO) model of stroke. Histological examinations revealed that LicB-mediated improvements in neuronal injury and apoptosis were accompanied by the downregulation of apoptosis-related proteins. Furthermore, LicB attenuated post-MCAO reductions in brain-derived neurotrophic and nerve growth factor levels.

Furthermore, LicB suppressed oxidative stress and inflammation, as evidenced by increased SOD and GSH levels, elevated IL-4 levels, and reduced malondialdehyde (MDA), iNOS (inducible nitric oxide synthase), and TNF- α levels (Zhou et al., 2021).

Qu et al. (2022) investigated the neuroprotective abilities of LicB on oxidative stress-stimulated damage in neural cells. H₂O₂ was used to simulate ROS-induced cell apoptosis in PC-12 cells, and in these stimulated cells, LicB reduced apoptosis and cell cytotoxic and enhanced the levels of apoptosis-related proteins such as caspase-3 and cleaved caspase-3. Notably, LicB effectively reduced MDA, SOD, and ROS in H₂O₂-induced cells. In the same study, LicB inhibited apoptosis by activating ATG7-dependent autophagy and the SIRT1/AMPK signaling pathway, as demonstrated by an increase in LC3-II levels and a reduction in p62 levels in neural cells and *Caenorhabditis elegans* models. Altogether, LicB has exhibited promising neuroprotective effects in models of oxidative damage and has been found to employ a variety of pharmacological mechanisms.

LicB has also been shown to inhibit the self-aggregation of A β ₄₂ and to disassemble pre-formed A β ₄₂ fibrils. Furthermore, LicB reduces chelating metal ion-induced A β ₄₂ aggregation. In silico analysis showed that LicB inhibits A β ₄₂ self-aggregation by forming two H-bonds with Lys28 and obstructing the salt bridge interaction at the C-terminus of A β ₄₂. Furthermore, LicB exhibits neuroprotective properties in SH-SY5Y cells against H₂O₂-induced cell death (Cao et al., 2020).

2.4 Anti-cancer effects

LicB had a dose-dependent inhibitory effect on the growth of osteosarcoma (OS) cells, specifically MG-63 and U2OS cells. In these cells, LicB induced autophagy and apoptosis and, at the molecular level, reduced Bcl-2, p62, caspase-3, and Ki67 protein levels but increased cleaved caspase-3, Beclin1, Bax, Atg7, and LC3B levels, and thus, promoted apoptosis. Furthermore, LicB inhibited the PI3K/AKT/mTOR pathway and induced autophagy in MG-63 and U2OS cells (Huang and Jin, 2022). These findings shed light on the intricate molecular mechanisms underlying the anti-cancer effects of LicB in osteosarcoma.

Wang et al. reported LicB inhibited HepG2 cell growth (IC₅₀ = 110.15 μ M), causing morphological distortions, cell cycle arrest at the G2/M phase, apoptosis, and accompanying intracellular ROS generation. LicB also significantly altered mRNA and protein expressions of the cell cycle-related genes. Notably, the inhibitions of caspase 8 and 9 reduced LicB-stimulated apoptosis (Wang et al., 2019).

In addition, the transcriptome and small RNA levels of LicB-responsive mRNAs and miRNAs in HepG2 cells have been investigated. A thorough examination revealed 85 differentially expressed miRNAs (DEMs) and 763 differentially expressed genes (DEGs) and that DEGs in LicB-treated cells were significantly enriched in components of the MAPK signaling pathway, a key regulator of hepatocellular carcinoma. Two key DEMs, namely, hsa-miR-29b-3p and has-miR-96-5p, were

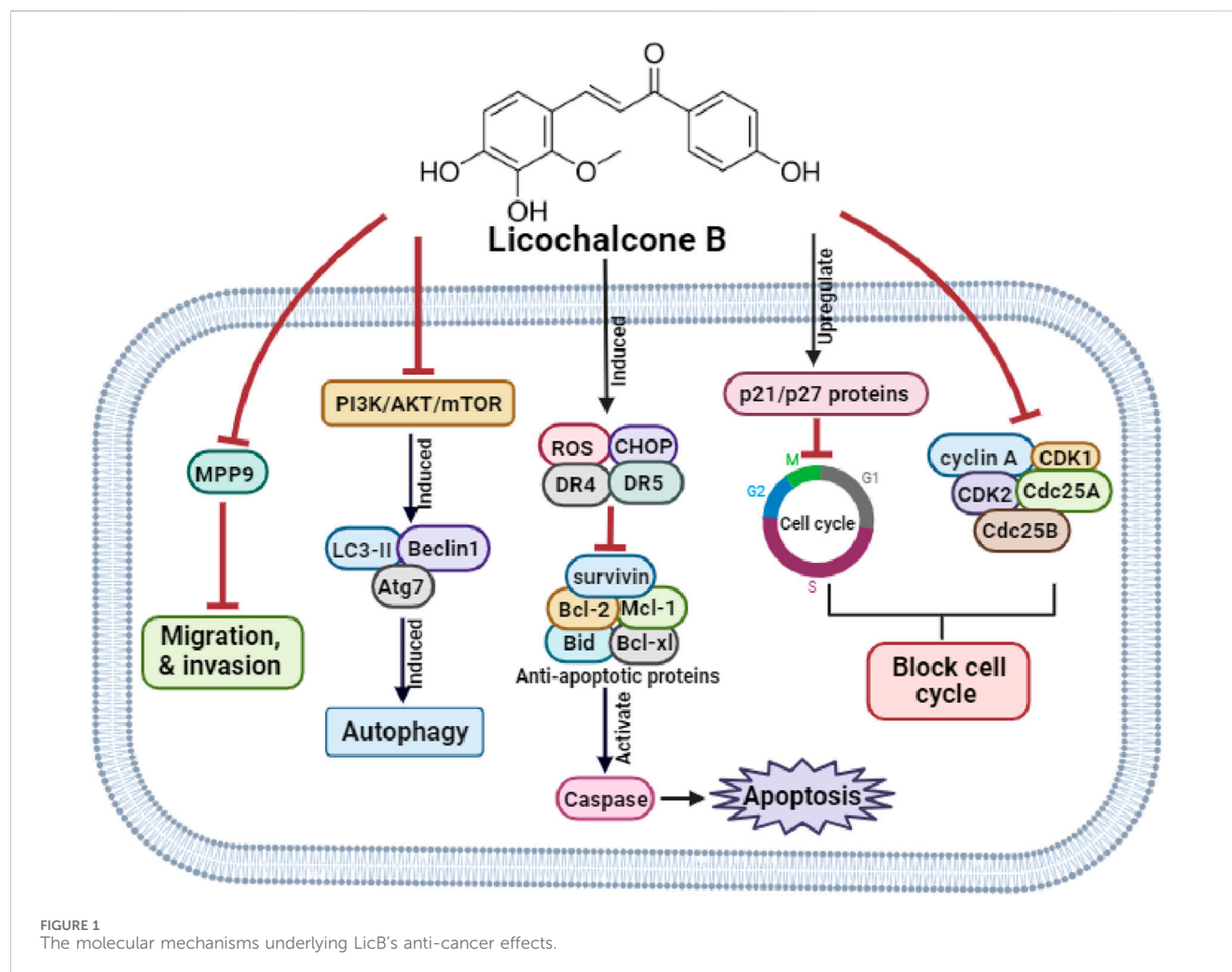
identified. These findings emphasized the importance of understanding the regulatory mechanisms of LicB in HepG2 cells and confirmed its potential as a functional dietary ingredient in liver cancer therapy. Understanding of the potential anti-tumor effects of LicB was also improved by omics data suggesting that miRNA and mRNA expression patterns might serve as promising biomarkers for identifying novel therapeutic targets in liver cancer (Wang and Wang, 2021).

LicB significantly inhibited oral squamous cell carcinoma (SCC) cell proliferation, particularly HN22 and HSC4 cells. LicB arrested the cell cycle at G1, downregulating cyclin D1 while upregulating the expression of p21 and p27 proteins. Additionally, LicB translocated phospholipid phosphatidylserine from the inner to outer leaflets of the plasma membrane, causing chromatin condensation, DNA fragmentation, and sub-G1 cell accumulation. LicB also increased ROS production, which induced CHOP, death receptor (DR) 4, and DR5. Furthermore, anti-apoptotic proteins (Bid, Bcl-xl, and Mcl-1) were downregulated, while the pro-apoptotic protein (Bax) was upregulated by LicB, and LicB-induced MMP loss resulted in the release of cytochrome c. Apoptotic protease activating factor-1 and survivin expression were also altered to promote apoptosis (Oh et al., 2016). Altogether, LicB shows significant promise as a therapeutic agent for managing human oral cancer by inducing apoptotic cell death.

In hepatocellular carcinoma cells, LicB inhibited proliferation and induced DNA damage, cell cycle arrest, and apoptosis. LicB inhibited the AKT/mTOR pathway and simultaneously activated ER stress and the MAPK signaling pathway. Furthermore, in these cells, co-treatment with LicB and TRAIL (TNF-related apoptosis-inducing ligand) significantly enhanced LicB-induced viability reduction and apoptosis. LicB also increased the protein expressions of DR4 and DR5. On the other hand, treatment with PD98059 or SP600125 (ERK inhibitor and JNK inhibitors, respectively) significantly decreased the LicB-stimulated upregulation of DR5 expression and attenuated LicB-mediated TRAIL sensitization (Zhang et al., 2022).

In a recent study, Kwak et al. (2023) investigated the anti-tumor efficacy of LicB against human colorectal cancer (CRC) and the underlying molecular mechanisms involved. LicB treatment significantly decreased cell viability, increased ROS generation, mitochondrial membrane potential (MMP) depolarization, caspase activation, and JNK/p38 MAPK signaling pathway activations, and induced apoptosis and G2/M cell cycle arrest. However, pretreatment with SB203580 or SP600125 (p38 and JNK inhibitors, respectively) suppressed LicB-stimulated reductions in cell viability. In addition, the ROS scavenger N-acetylcysteine effectively prevented the effects of LicB on tumor cell viability, apoptosis, ROS production, cell cycle arrest, MMP depolarization, and upregulations of the activities of caspases and JNK/p38 MAPK. This study demonstrated LicB has therapeutic potential in CRC.

Sadek et al. (2020) investigated the combined effects of LicB and fullerene C₆₀ (FnC₆₀) nanoparticles on diethylnitrosamine-induced hepatocarcinoma *in vivo*. Treatment with LicB alone or



in combination with F_nC₆₀ reduced diethylnitrosamine-induced DNA fragmentation and oxidative DNA damage and downregulated the mRNA expressions of APE1/Ref-1, CDK-4, retinoblastoma, Bcl-2, B-cell lymphoma-xL, and β -arrestin-2 and the protein expressions of APE1/Ref-1 and CDK-4. Furthermore, these LicB-induced changes increased the protein expressions of p53 protein, Bcl-2-associated X, and caspase-3.

In addition, LicB inhibited esophageal SCC growth by directly inhibiting the activity of JAK2 and its subsequent signaling pathway. LicB also inhibited the growth of KYSE450 and KYSE510 esophageal SCC cells by arresting the cell cycle at the G2/M phase and increasing apoptosis. Notably, the effect of AZD1480 (a JAK2 inhibitor) on esophageal SCC cells was comparable to that of LicB. Furthermore, LicB reduced the phosphorylation of STAT3 at Y705 and S727 and the expression of Mcl-1 (a target of STAT3) (Song et al., 2020). These findings point to the potential therapeutic role of LicB for managing esophageal SCC based on targeting the JAK2 signaling pathway.

LicB was also found to inhibit the proliferation of SCC and human melanoma cells by causing apoptotic cell death through specificity protein 1 (transcription factor) and other apoptosis-

related proteins, including CCAAT/enhancer-binding protein homologous protein, DRs, and poly (ADP-ribose) polymerase (PARP) (Kang et al., 2017).

In human non-small cell lung cancer (NSCLC) cells, Oh et al. (2019) found that LicB inhibited the activities of EGFR and MET kinase and cell viability in HCC827 and HCC827GR cells. In addition, LicB stimulated G2/M cell-cycle arrest and apoptosis, caused ER stress, ROS production, and MMP loss, and activated caspases. Also, the inhibitions of EGFR and MET reduced activation of the ERBB3/AKT axis. These observations indicate LicB is a promising, novel therapeutic candidate for the treatment of NSCLC.

LicB inhibited proliferation, caused S phase arrest, reduced cyclin A, CDK1, and CDK2 levels and the expression of cell division cycle 25 (Cdc25A and Cdc25B) in T24 EJ human bladder cancer cells, upregulated the expressions of Bcl-2, survivin, and Bax expression, and caused PARP cleavage. LicB also markedly reduced the tumorigenicity of LicB-treated MB49 cells *in vitro*, and this finding was supported by *in vivo* studies on an MB49 mouse model of bladder cancer (Yuan et al., 2014). Similarly, in another study, LicB inhibited the cell migration, adhesion, and invasion of T24 cells, inhibited the expressions of MMP-9 mRNA and protein, reduced NF- κ B

p65 levels, and impeded the nuclear translocation of NF- κ B (Zhao et al., 2014).

Yu et al. (2016) reported that LicB inhibited the proliferation of MCF-7 breast cancer cells using a mechanism involving S phase arrest attributed to decreases in Cyclin A, Cdk2, and Cdc25 A levels and an increase in p21 protein levels and that it induced apoptosis, as evidenced by morphological changes including phosphatidylserine externalization and DNA fragmentation. LicB also disrupted MMP causing cytochrome C release, and molecular analysis revealed that LicB increased the expressions of Caspase-3, Caspase-9, and Bax expression but reduced Bcl-2 expression. Furthermore, these effects were attributed to an increased p53 protein level in LicB-treated cells. Collectively, LicB has potential use as a therapeutic agent for the management of breast cancer. The anti-cancer molecular mechanisms of LicB are depicted in Figure 1.

2.5 Cardioprotective effects

ROS production is a major contributor to ischemia-reperfusion-induced cardiac injury. In a study by Han et al. (2014), pretreatment with LicB improved heart rate, left ventricular developed pressure, and reduced creatine kinase and LDH levels in coronary flow in Sprague-Dawley rat model. LicB treatment also increased SOD levels and GSH/GSSG ratios and reduced IL-8 and IL-6 activities and MDA, TNF- α , and C-reactive protein (CRP) levels. Furthermore, LicB-treated group had smaller infarct size and exhibited less apoptosis than ischemia/reperfusion control group. The authors suggested that its cardioprotective effects were probably due to its antioxidative, anti-apoptotic, and anti-inflammatory properties. In another study, LicB potentially inhibited angiotensin-converting enzyme (a critical target in cardiovascular disease) with an IC₅₀ value of 0.24 μ M (Li et al., 2023).

2.6 Hepatoprotective effects

LicB also exhibits hepatoprotective effects against carbon tetrachloride (CCl₄)-induced injury, which was characterized by elevated ALT, AST, MDA, IL-6, CRP, and TNF- α levels and a reduction in serum SOD and GSH/GSSG ratio. A histopathologic examination of CCl₄-treated animal liver sections revealed inflammation and necrosis. However, LicB pretreatment prevented these CCl₄-induced effects, reduced ALT, AST, MDA, GSSG, IL-6, CRP, and TNF- α levels and the protein expressions of p38 and NF- κ B but enhanced SOD and GSH levels (Teng et al., 2016). Similarly, in another study, LicB protected against CCl₄ and acetaminophen-induced hepatocellular injury in HepG2 cells and increased cell viability by more than 80% (Kuang et al., 2017).

Nrf2 protein is a basic-region leucine zipper transcription factor that protects against endogenous and exogenous stressors. Nrf2 increases cellular sensitivity to oxidants and electrophiles by inducing the expressions of various cytoprotective and detoxifying genes. Interestingly, transient Nrf2 activation, facilitated by specific activators, protects against cancer development (Pouremamali et al.,

2022), and notably, LicB activation of Nrf2 in HepG2 cells significantly reduced CCl₄-stimulated acute liver injury in animal model (Lin et al., 2017a).

Oxidative stress is a major contributor to alcohol-induced hepatotoxicity, and LicB reduced ethanol-induced cellular damage and inhibited apoptosis in BRL cells, suggesting a hepatoprotective effect on alcohol-induced cell injury. In addition, LicB markedly reduced ethanol-induced intracellular ROS levels, increased p-Erk expression, and induced the nuclear translocation of Nrf2 (Gao et al., 2017). These results indicate LicB has the potential to treat liver disorders caused by alcohol consumption.

2.7 Protective effects on lung injury

Acute lung injury (ALI) is a severe and often fatal pulmonary disorder. Huang et al. recently explored the effects of LicB on human pulmonary microvascular endothelial cells and LPS-challenged mice. LicB promoted cell viability, had no cytotoxic effect, suppressed apoptosis, and reduced ROS levels. In addition, LicB reduced lung tissue weights, oxidative stress, and inflammatory markers in the ALI murine model. LicB also increased the expressions of Nrf2, HO-1, and NQO1 but decreased the expression of Keap1 (Huang et al., 2023). Collectively, LicB has potential as a therapeutic strategy for alleviating LPS-induced ALI. A summary of the pharmacological properties of LicB is provided in Figure 2.

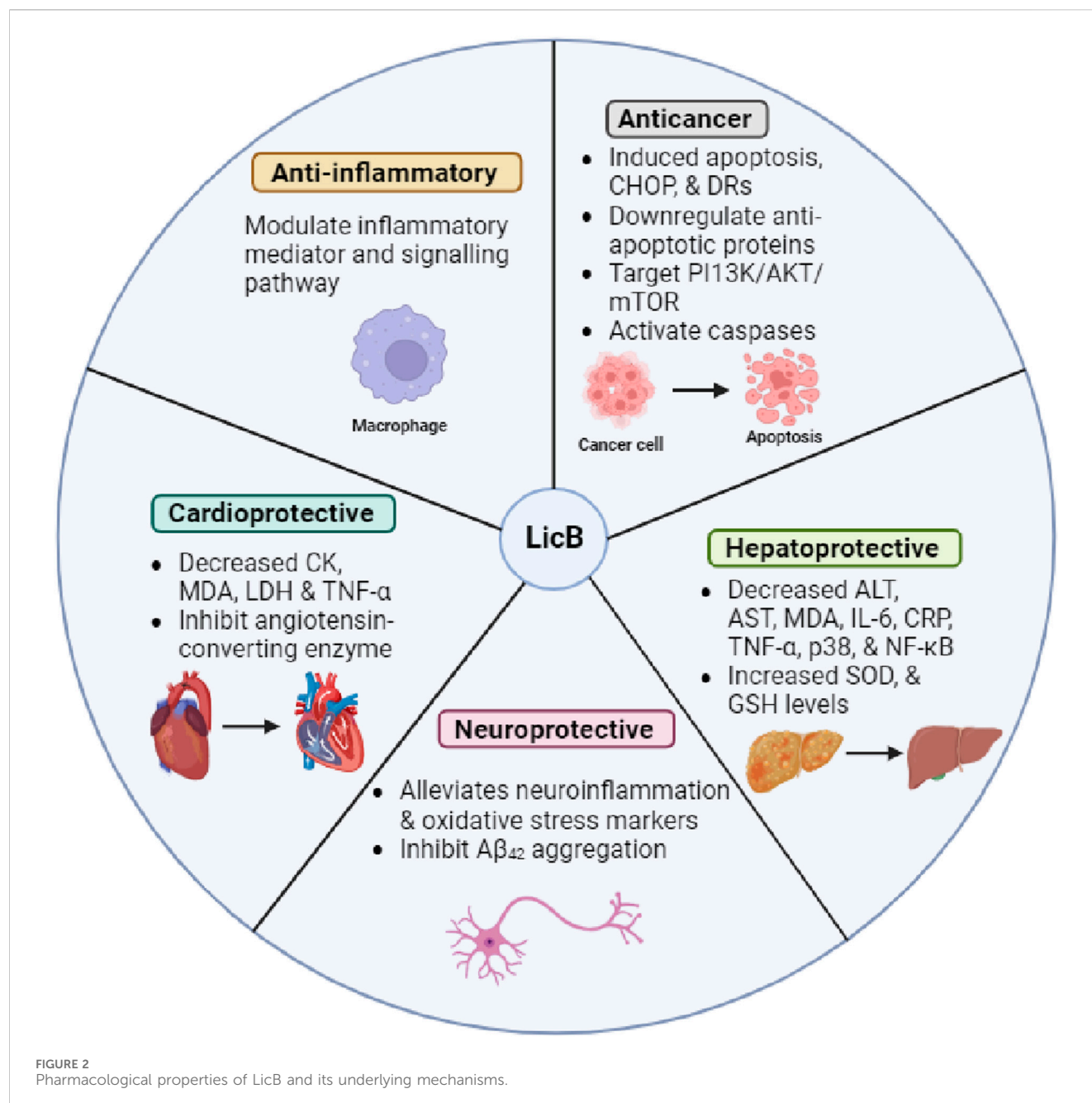
The optimal combination of LicB and liquiritin showed increased anti-inflammatory, antioxidant, and anti-fibrotic activity. The hemopoietic cell kinase inhibitor (iHCK-37) confirmed that LicB and liquiritin prevent chronic obstructive pulmonary disease (COPD) by inhibiting HCK, resulting in antioxidative, anti-inflammatory, and anti-fibrotic effects. A 1:1 ratio of LicB and liquiritin demonstrated synergistic efficacy in treating COPD by inhibiting HCK activity (Dong S. et al., 2024).

2.8 Antiviral effects

LicB has been shown to deactivate herpes simplex virus two particles and prevent viral multiplication via inhibiting Akt phosphorylation and downstream targets. *In vivo* investigations revealed that LicB dramatically lowered viral titers, delayed weight loss, and reduced pathological alterations in vaginal tissues in mouse models of vaginal infection (Li et al., 2024).

2.9 Effect in autoimmune diseases

Abnormal activation of the cGAS-stimulator of interferon genes (STING) pathway by abnormal DNA has been linked to autoimmune diseases (Liu and Pu, 2023). LicB inhibited the STING signaling cascade in macrophages by disrupting the STING-TBK1-IRF3 axis and preventing downstream activation. In addition, LicB lowered type I interferon levels in



mice treated with the STING agonist CMA and dramatically reduced systemic inflammation in *Trex1*^{-/-} animals (Luo et al., 2024).

3 Toxicity and safety

The toxicological properties of LicB are poorly understood. The enzyme 11 β -HSD2 regulates cortisol levels by converting cortisol, an active glucocorticoid hormone, to cortisone, its inactive form (Chapman et al., 2013; Adamidis et al., 2019), and a recent study demonstrated that LicB inhibits human and rat 11 β -HSD2 with IC₅₀ values of 31.85 μ M and 56.56 μ M, respectively (Lin et al., 2024).

4 Conclusion and future prospective

As described above, LicB has wide-ranging pharmacological activities, which include anti-inflammatory, hepatoprotective, anti-cancer, and neuroprotective effects. These activities are closely related to cellular responses involving inflammation, autophagy, apoptosis, and oxidative stress, which are modulated by signaling pathways such as NF- κ B, SIRT1/AMPK, Nrf2, PI3K/Akt/mTOR, p53, CHOP, DRs, p38, and iNOS, and involve multiple targets such as TNF- α , PI3K, AKT, Bid, Bcl-xL, Mcl-1, and caspases. Hence, we believe that LicB has great promise as a lead compound for drug discovery. Nonetheless, it is essential to remember that most reported LicB findings are based on cellular experiments. Therefore, more clinical research is required to confirm its

pharmacological efficacy. Furthermore, its toxicological profile is less well characterized and requires further investigation.

Author contributions

SS: Conceptualization, Writing—original draft. EL: Writing—review and editing. KA: Writing—original draft. IC: Conceptualization, Writing—review and editing.

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The ameliorative effect of chrysin on ovarian toxicity caused by methidathion in female rats

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Introduction: Methidathion (MD) is commonly used in agriculture and has adverse effects on reproduction. Chrysin (CHR) has several advantageous properties, such as anti-inflammatory, anti-cancer, and antioxidant properties. The purpose of the current investigation was to assess CHR's therapeutic efficacy in reducing ovarian toxicity brought on by MD.

Methods: Twenty-four female rats were divided into four groups of six animals each. Group 1 served as a control, while group 2 rats received MD (5 mg/kg). Rats in Group 3 received CHR at a dose of 50 mg/kg. Rats in group 4 received treatment with CHR after MD intoxication.

Results and Discussion: Our research revealed that MD significantly ($p < 0.001$) increased the levels of MDA, caspase-3, FSH, LH, CA-125, and TNF- α but significantly ($p < 0.001$) decreased the levels of SOD, GSH, E2, and progesterone when compared to the control and CHR groups. After receiving CHR therapy, damage induced by MD was significantly ($p < 0.001$) repaired.

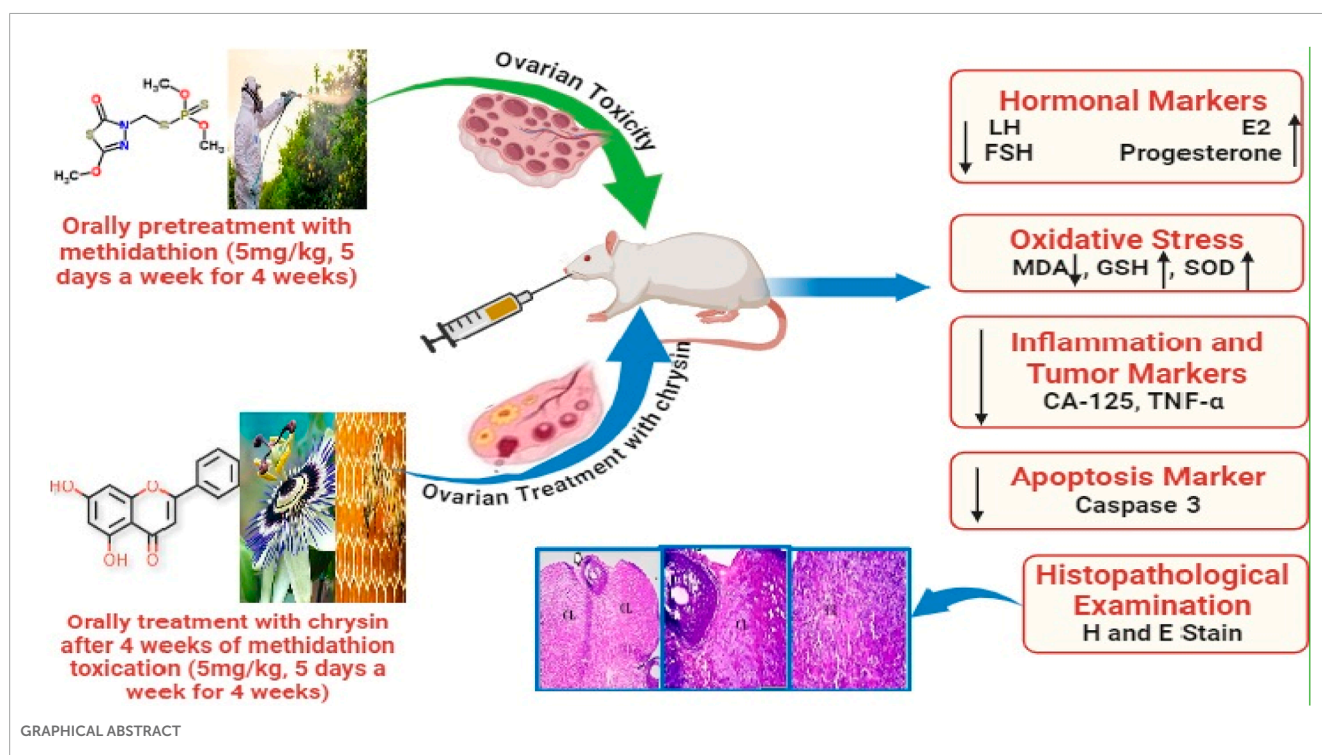
Conclusion: This study showed that CHR could mitigate the adverse effects that MD causes to the ovaries by decreasing oxidative stress, inflammation, and apoptosis; improving antioxidant status; and restoring the correct ratio of sex hormones.

KEYWORDS

methidathion, chrysin, inflammation, ovarian damage, oxidative stress

1 Introduction

Since organophosphorous pesticides (OPPs) are used for eliminating insects that harm crops in the field, they fall under the category of insecticides. Nevertheless, other data indicates that these substances may also be employed as herbicides by influencing the soil biota population and subsequently changing the biomass of plants, leading to their deterioration (Ajiboye et al., 2022). OPPs, one of the main chemicals used to control pests, have been widely utilized in agriculture since the ban on organochlorine pesticides (Costa, 2018). Acetylcholinesterase (AChE), a vital and essential enzyme to produce nerve impulses, is inhibited by the chemical organophosphate. Acetylcholine accumulates as a result of AChE inhibition, permanently depolarizing



the organism and causing tremors, respiratory arrest, and eventually death. Owing to the widespread application of these chemicals on agricultural products, they have infiltrated groundwater through seepage, entered rivers via agricultural runoff, and appeared on the surfaces of sprayed plants. Thus, food, inhalation, and skin adsorption have been the primary routes by which people have been exposed to lower concentrations of these pesticides (Costa, 2018; González-Alzaga et al., 2014). There are 220,000 deaths and one million cases of severe poisoning each year; most of these poisonings and 99% of the deaths they cause happen in developing countries (Abdulrahman et al., 2023).

OPPs toxicological effects are classified as mild, moderate, or high depending on when the person is exposed to them and what proportion of their enzymes are inhibited afterward. Due to the rising levels of pesticides in the environment, established and developing nations that rely heavily on agriculture for their economies now have virtually no choice but to expose living things to them (Georgiadis et al., 2018). OPPs have been demonstrated to trigger biochemical and histological alterations in various organs, including the kidney, immune system, pancreas, liver, heart, and vascular walls. The ovary is one of the organs discussed, and it has a crucial function by generating oocytes and synthesizing hormones in a normal reproductive process (Güney et al., 2007). Moreover, additional studies indicate that oxidative stress can play a major role in the toxicity mechanism of OPPs. These pesticides can cause oxidative stress, which can result in the generation of free radicals and changes to antioxidants or the enzymes that scavenge reactive oxygen species (ROS) (Sharma et al., 2014), which coexist in a healthy body and are balanced with antioxidants. The process that results in an imbalance that generates an excess of ROS is known as oxidative stress. A woman is affected by

oxidative stress throughout her whole reproductive life, even after menopause. An imbalance between the body's ability to scavenge free radical species and antioxidants results in oxidative stress. In addition to being crucial signaling molecules in physiological processes, ROS are involved in pathological processes that impact the female reproductive system. ROS influences a wide range of physiological processes, including oocyte maturation, fertilization, embryo development, and pregnancy. Both DNA damage to the ovarian epithelium and oxidative base damage caused by ovulation can be prevented by antioxidants (Agarwal et al., 2005).

Methidathion [S-(5-methoxy-2-oxo-2,3-dihydro-1,3,4-thiadiazol-3-yl)methyl O,O-dimethyl phosphorodithioate] (Kim et al., 2011) is one of the OPPs that is most frequently employed in public health and agriculture initiatives (Bhattu et al., 2022).

A flavonoid called chrysin (5, 7-dihydroxyflavone) is taken from propolis, passion flowers, and honey (Naz et al., 2019). Chrysin is used to treat liver, nervous system, and reproductive disorders (Mentese et al., 2022; Souza et al., 2015). Numerous biological characteristics of chrysin, such as its antioxidant, anti-apoptotic, anti-inflammatory, and anti-cancer capabilities, have been demonstrated by previous studies (He et al., 2012; Rashno et al., 2019). Moreover, the protective benefits of chrysin against oxidative stress in rats have been determined. According to the study, chrysin therapy lowers MDA levels and increases antioxidant enzyme activity (Anand et al., 2012). Additionally, it has been demonstrated to regulate the level of sexual hormones, protect ovarian tissues from oxidative stress and tissue damage, and reduce apoptosis (Mohammadi et al., 2022). Therefore, this study aimed to investigate the ameliorative effect of chrysin against MD-induced ovarian damage in female rats by evaluating serum biochemical antioxidative markers, sex hormones, and inflammatory and

apoptotic markers, in addition to histopathological examinations of ovarian tissue.

2 Materials and methods

2.1 Chemicals

Methidathion (CAS No. 950-37-8), Chrysin (CAS No. 480-40-0), and other reagents were supplied by Sigma-Aldrich (St. Louis, MO, United States). The selection of MD and CHR doses was based on previously published studies by [Sulak et al. \(2005\)](#) and [Mantawy et al. \(2017\)](#) respectively.

2.2 Ethical considerations

The housing accommodations and experimental protocols followed the European Union Council's (2010/63/EU) guidelines for the use of laboratory animals. The animal study and all experimental procedures have been approved by the veterinary medical research ethics committee, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt, with protocol number Soh. un.vet/00066R. Every precaution was taken to prevent pain and suffering for the animals.

2.3 Animals

Female Wistar albino rats, weighing between 180 and 200 g and aged 10–12 weeks, were obtained from the animal house at Sohag University in Sohag, Egypt. The rats were maintained at a constant temperature of $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a 12-hour light/dark cycle and $45\% \pm 5\%$ humidity. They were acclimated to their new environment for 1 week before the experiment. Throughout the study, water and food were provided *ad libitum*.

2.4 Experimental design

Animals were randomly divided into four groups (6 rats/group) as follows: Group 1 (vehicle control): healthy rats that received DMSO orally only, Group 2 (MD): ovarian toxicity was induced by methidathion (5 mg/kg.b.w), Group 3 (CHR): healthy rats that received chrysin (50 mg/kg), and Group 4 (MD + CHR): ovarian toxicity was induced by MD the same as in group 2, then rats received chrysin at the same dose mentioned in group 3. Treatment with CHR started 4 weeks after ovarian toxicity induction by gavage 5 days a week and continued for 5 days a week for 4 weeks by gavage administration. MD and CHR were dissolved in DMSO, and doses of MD and CHR were determined from previous studies of [Sulak et al. \(2005\)](#), [Mentese et al. \(2022\)](#), [Mantawy et al. \(2017\)](#). At the end of the treatment, animals were sacrificed and dissected.

2.5 Blood sampling for hormonal and biochemical analyses

Blood samples were collected and centrifuged for 10 min at 3,000 rpm to obtain a clear serum and stored at -20°C for hormonal

and biochemical analyses. Ovarian tissue samples were dissected and processed for biochemical analysis, and histopathological examination.

2.6 Determination of sex hormone

Rats' serum levels of progesterone, estrogen (E2), follicular stimulating hormone (FSH), and luteinizing hormone (LH) were quantitatively assessed by ELISA using kits [PerkinElmer Company, Hayward, CA 94545 for progesterone (Catalogue Number: 10005), and BIOS Company, South San Francisco, CA 94080, United States for LH (Catalogue Number: 1004), FSH (Catalogue Number: 10001), and (E2 (Catalogue Number:1009)]. The experiment was carried out according to the manufacturer's guidelines.

2.7 Determination of oxidative stress

All the rats' ovaries were removed immediately and weighed. In a glass homogenizer, each rat's right ovary was homogenized in cold phosphate-buffered saline (1:4) (pH 7, 0.01 mol/L). The homogenate that was created was centrifuged at $5,000 \times g$ for 5 min, filtered, and utilized to measure markers of oxidative stress. The experiments were repeated three times.

2.7.1 Determination of SOD

The body's antioxidant capacity and oxidative balance can be determined by measuring the activity of SOD, an enzyme that scavenges superoxide anion-free liquid ($\text{O}_2 - \bullet$) to protect cells from damage ([Liang et al., 2022](#)). The total SOD assay kit (MyBioSource, China, Catalogue Number: MBS036924) was used in this experiment to measure SOD activity (U/mg protein) in ovarian homogenates of each group, according to the instructions.

2.7.2 Determination of MDA

Through the action of its enzyme system, which can target polyunsaturated fats in biological membranes, initiate lipid peroxidation, harm cells, and generate lipid peroxides like MDA, the body produces oxygen-free fluid. As a result, the body's measured MDA concentration may indicate the level of lipid peroxidation and, in consequence, indicate the degree of cell damage ([Yang et al., 2022](#)). Using the TBA method MDA detection kit (MyBioSource, China, Catalogue Number: MBS268427), the researchers measured the amount of MDA in each group's ovarian tissue. Following the instructions provided in the kit, the ovarian tissues were homogenized and the concentration of MDA per mg protein (nmol/mg protein) was determined.

2.7.3 Determination of GSH

GSH is an essential enzyme that is extensively distributed throughout the body and catalyzes the breakdown of H_2O_2 . Its action may contribute to maintaining the cell membrane's structural and functional integrity. Consequently, assessing the GSH activity in tissues may provide information regarding the body's oxidative equilibrium and antioxidant potential ([Yang et al., 2022](#)). In this work, the reduced GSH activity in ovarian homogenates of each group was measured according to the instructions using a GSH

test kit (Shanghai BlueGene Biotech Co., Ltd., Shanghai, China, Catalogue Number: E02G0367).

2.8 Detection of ovarian apoptosis

One important protease in the pathway of mitochondria-mediated cell death is caspase 3. This factor is released into the cytoplasm in response to apoptotic stimuli, where it sets off a series of processes that lead to cell death. Using an ELISA kit (United States of America, from Elbscience Biotechnology Company, Catalogue Number: E-EL-R0160), we determined the concentration of Caspase3 in each group's serum by the manufacturer's instructions.

2.9 Detection of tumor marker

A significant percentage of epithelial ovarian tumors exhibit the high molecular weight glycoprotein known as carcinoembryonic antigen (CA) 125 (Moss et al., 2005). Serum level was quantitatively assessed by ELISA using a kit from BIOS Company, South San Francisco, CA 94080, United States of America, Catalogue Number: 10,103. The experiment was carried out according to the manufacturer's guidelines.

2.10 Detection of cytokine

The oocytes and macrophages of the neonatal rat ovary contain the multifunctional cytokine known as tumor necrosis factor α (TNF α). TNF α may play a role in follicle construction or oocyte atresia, as evidenced by the presence of both the TNF α and its receptors in the ovary of a newborn rat (Morrison and Marcinkiewicz, 2002). The serum level was quantitatively assessed by ELISA using a kit from R&D Systems in Minneapolis, MS, United States of America, Catalog Number QK210. The experiment was carried out by the manufacturer's guidelines.

2.11 Histopathological examination

Animals were sacrificed after the experimental duration, and tissue samples from left ovaries were collected, dissected, and immediately fixed in 10% formalin for 24 h, dehydrated in a succession of graded alcohols, clarified in xylene, and encapsulated in paraffin (Suvana and Layton, 2013). Tissue sectioning was done at 3–5 μ m thickness and stained with hematoxylin and eosin (H&E) (Bancroft et al., 1996) for histological evaluation. All sections were inspected and photographed using an OLYMPUS CX43 microscope and a microscope-adapted OLYMPUS SC52 camera.

2.12 Morphometric study

Each animal was assigned a score based on histopathological examination of the tissue samples (Gibson-Corley et al., 2013). The section samples were scored semi-quantitatively, depending on the

visual field inspection of 10 sections from each group. Photographs of ovarian tissues were taken, and the cellular alterations were counted in 10 random areas (each 1 mm²) at a magnification of 20 $\times$. The degree of follicular degeneration, stromal degeneration, and stromal fibrosis was scored between 0 and 3 according to the severity of the damage. A value of 0 means no pathological damage, +1 value is less than 33% of ovarian damage, +2 is damage between 33% and 66%, and +3 is more than 66% (Pala et al., 2014; Kaplan et al., 2021).

2.13 Statistical analysis

The Statistical Package for the Social Science (S.P.S.S. version 27) was used to analyze the data. The mean $\pm$ SD was used to express the results. To test differences between groups, statistical analysis was performed using analysis of one-way variance (ANOVA) and Tukey's post-hoc multiple comparison test. Each group's results were characterized by identical variance and a normal distribution. A value of p of <0.05 means that differences between all groups are statistically significant.

3 Results

3.1 Clinical observation

Following MD administration, the rats displayed intense, uncontrolled behaviors that lasted for nearly 2 hours, after which they showed signs of exhaustion. In the subsequent days, the severity of these behaviors persisted but gradually diminished. No mortality was observed in any group.

3.2 Biochemical findings

3.2.1 Chrysin regulate serum hormonal markers in methidathion-treated rats

The hormone values of experimental groups are displayed in (Figure 1). In the current investigation, the FSH and LH hormone serum levels were considerably higher ($p < 0.001$) in the MD group than in the control and CHR groups. There was no significant difference ($p > 0.05$) in the serum levels of LH and FSH hormones between the CHR and control groups. In contrast to the MD group, the LH and FSH hormone levels were considerably lower in the MD + CHR treated group ($p < 0.001$). (Figures 1A, B).

Comparing the MD group to the control and CHR groups, the MD group's serum levels of progesterone hormone and E2 were significantly lower ($p < 0.001$). Progesterone hormone and E2 levels in the MD + CHR group's blood increased significantly ($p < 0.001$) following CHR treatment. There was no discernible difference in progesterone hormone and E2 serum levels between the CHR and control groups. (Figures 1C, D).

3.2.2 Chrysin improved ovarian tissue antioxidants and oxidative stress markers in methidathion-treated rats

When comparing the MD untreated group to the control group, (Table 1), the levels of MDA were substantially

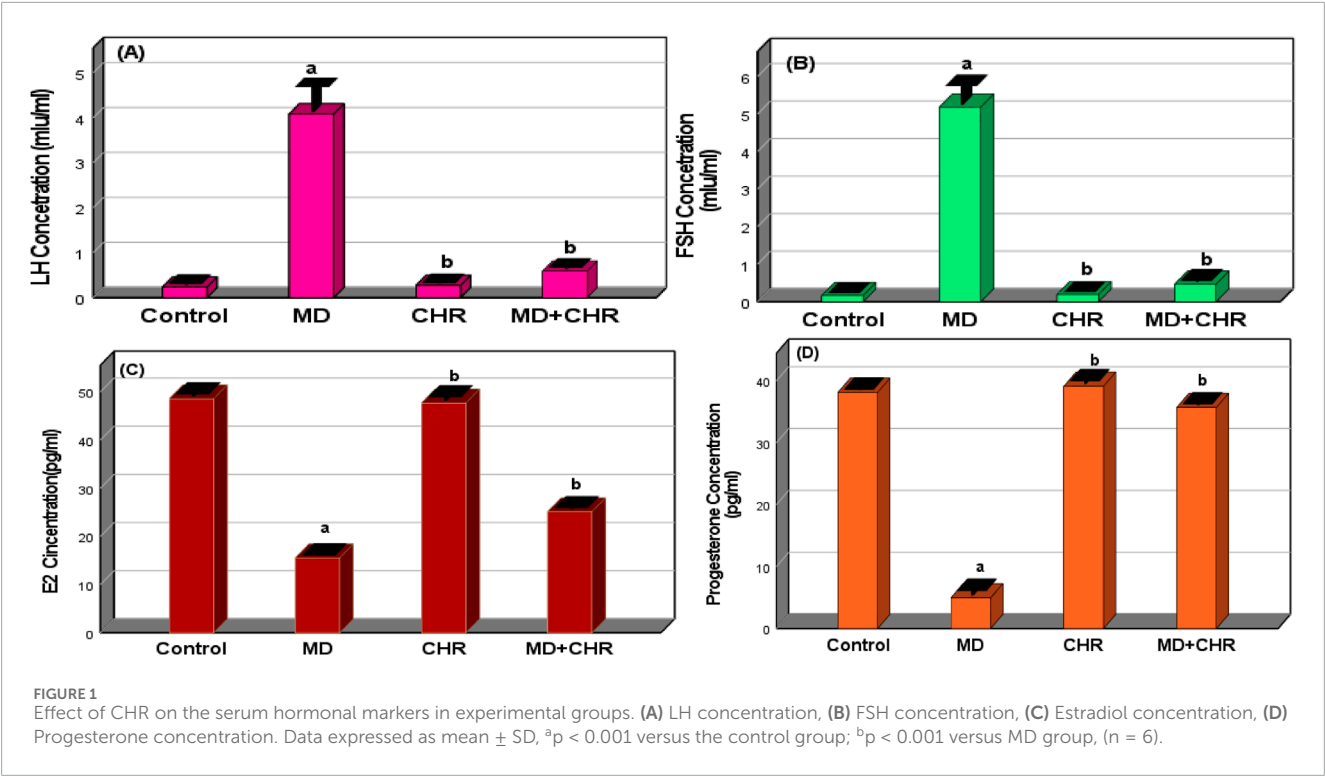


TABLE 1 Effect of CHR on the ovarian tissue antioxidants and oxidative stress markers in experimental groups.

Groups	MDA nmol/mg	GSH pg/g	SOD U/mg
Control	0.34 $\pm$ 0.05	2.79 $\pm$ 0.03	6.19 $\pm$ 0.05
MD	5.05 $\pm$ 0.08 ^a	0.48 $\pm$ 0.03 ^a	2.20 $\pm$ 0.16 ^a
CHR	1.14 $\pm$ 0.09 ^b	3.55 $\pm$ 0.04 ^b	7.38 $\pm$ 0.09 ^b
MD + CHR	0.80 $\pm$ 0.01 ^b	2.19 $\pm$ 0.034 ^b	5.64 $\pm$ 0.06 ^b

Values are presented as mean $\pm$ SD; n = 6 rats in each group; values with different superscripts (a, b) among experimental groups are significantly different ($p < 0.001$).
^aVersus the control group.
^bVersus MD, group using ANOVA, and *post hoc* test; SD, standard deviation.

elevated ($p < 0.001$) along with a significant decline in GSH level and SOD activity. Surprisingly, the CHR treatments improved GSH and SOD activity and dramatically downregulated ($p < 0.001$), MDA levels in the MD + CHR group in comparison with the MD group. These findings indicated that CHR mitigated the oxidative stress induced by MD treatment in the rats that received it.

3.2.3 Effect of chrysin on the serum ovarian inflammation and tumor markers in methidathion-treated rats

The levels of TNF- α and CA-125 in the MD rats were greatly higher ($p < 0.001$) than those in the control rats. However, after CHR treatment, the values in the MD + CHR group were notably lower ($p < 0.001$). Additionally, there was no significant

TABLE 2 Effect of CHR on the ovarian inflammation and tumor markers in experimental groups.

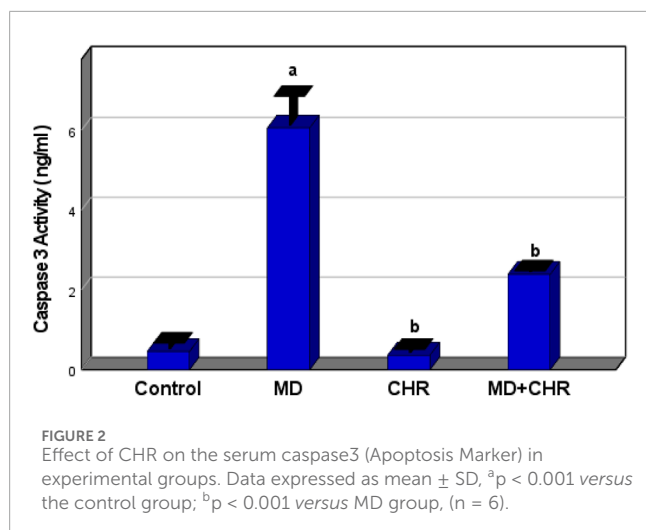
Groups	CA-125 (U/mL)	TNF- α (pg/mL)
Control	22.35 $\pm$ 0.68	52.30 $\pm$ 0.38
MD	54.32 $\pm$ 0.57 ^a	234.79 $\pm$ 0.78 ^a
CHR	30.54 $\pm$ 0.63 ^b	61.83 $\pm$ 0.61 ^b
MD + CHR	27.28 $\pm$ 0.33 ^b	49.63 $\pm$ 0.51 ^b

Values are presented as mean $\pm$ SD; n = 6 rats in each group; values with different superscripts (a, b) among experimental groups are significantly different ($p < 0.001$).
^aVersus the control group.
^bVersus MD, group using ANOVA, and *post hoc* test; SD, standard deviation.

difference ($p > 0.05$) in the levels of TNF- α and CA-125 between the control and CHR groups. These findings suggest a strong association between the cytokine system and the tumor marker CA-125. (Table 2). Consequently, it can be concluded that CHR treatment effectively reduces the inflammation caused by MD treatment in rats.

3.2.4 Effect of chrysin on the serum caspase 3 (Apoptosis Marker) of methidathion-treated rats

There was a significant increase ($p < 0.001$) in the serum Caspase-3 activity level in the MD group compared to the control group. In contrast, the MD + CHR group receiving CHR therapy saw a significant drop in the mean value of Caspase-3 activity. Serum Caspase-3 levels showed no noticeable variations between the control and CHR groups. (Figure 2).



3.3 Histopathological assessment

The histological examination of ovarian tissue samples revealed distinctive morphological differences between the treated groups and the control as shown in (Figure 3). In the control group, normal ovarian tissue exhibited various stages of ovarian follicles, including a Graafian (secondary) follicle with a well-defined follicular antrum housing an eccentrically positioned secondary oocyte, surrounded by the zona pellucida and corona radiata. This follicle was enveloped by layers of granulosa and theca cells. Furthermore, the corpus luteum maintained a typical size and structure (Figures 3A–C). Contrarily, the MD group displayed numerous atretic follicles characterized by degenerated follicular cells and compromised corpora bodies. Aberrant secondary follicles lacking oocytes were observed alongside the corpus luteum exhibiting luteal cell vacuolation. Degenerative changes, including cellular loss of features and vacuolation, were evident in the corpora, accompanied by an increase in inter-corpora stromal fibrous connective tissue (Figures 3D–F).

In comparison, the CHR group showed a comparable number of normal ovarian follicles and corpus luteum structures to the control (Figures 3G–I). Remarkably, the MD + CHR group exhibited a notable improvement in ovarian structures. Healthy growing follicles containing intact oocytes surrounded by normal granulosa cells were observed, alongside more or less normal corpus luteum structures with polyhedral luteinized cells (Figures 3J–L).

Histomorphometric evaluation of ovarian lesions such as follicular degeneration, stromal degeneration, and stromal fibrosis recorded in the examined tissue sections exhibits a significant ($p \leq 0.001$) increase in the MD group compared with the control group. Interestingly administration of CHR dramatically decreased those recorded lesions ($p \leq 0.001$) compared with the MD untreated group. The histological picture in MD + CHR showed non-significant change compared with the control group (Figure 4).

4 Discussion

The present study investigated the therapeutic potential of CHR against MD-induced ovarian damage in female rats.

Concerning hormonal balance, the MD group in this study had considerably higher serum levels of FSH and LH and significantly lower serum levels of progesterone and E2. Severe toxicity to the follicular ovarian reserve and function is another explanation for this effect. The presence of these hormonal alterations is utilized as a sign of ovarian failure (Afifi and Reyad, 2013). Our findings match up with a previous study (Güney et al., 2007), which reported that MD induced ovarian toxicity in female rats. Additionally, compared to the MD group, the group treated with CHR after intoxication with MD showed a significant decrease in FSH and LH levels along with a significant increase in estradiol and progesterone levels. The beneficial effects of CHR on the reproductive processes of female rats may have contributed to our findings (Mentese et al., 2022). Our findings are consistent with those of Mentese et al. (2022), who displayed that CHR prevention female rats from ovarian toxicity.

Additionally, our results suggested that administering MD to normal rats resulted in a substantial drop in GSH and SOD levels that were comparable to those of normal rats as well as a significant increase in MDA levels. This may be because MD poisoning causes a variety of metabolic disturbances (Kose et al., 2009). Oxidative stress is caused by antioxidant enzyme deactivation (Mobasher and Valverde, 2014). This finding is consistent with the results of Wang et al. (2018), which associated increased MDA levels and decreased GSH and SOD levels with acrolein's interference in antioxidant defense mechanisms. Oxidative stress can lead to ovarian failure by impairing CYP450 in two ways: disrupting normal oocyte development and inducing apoptosis. Since oxidative stress hinders both nuclear and cytoplasmic maturation of oocytes while promoting cell death, it disturbs the intraovarian environment by creating an imbalance between ROS production and elimination. In the present study, elevated ovarian MDA levels may be linked to increased reactive oxygen species induced by MD (Güney et al., 2007). These observations support the hypothesis that MD-induced ovarian damage results from lipid peroxidation (LPO), a biochemical mechanism. Developing effective antidotes, particularly those with strong antioxidative properties, is crucial to counteracting MD toxicity. Chrysin, a flavonoid with potent antioxidant activity, represents a promising candidate in this regard (Alsawaf et al., 2022).

According to the author's knowledge, there is limited information about the potential benefits of CHR in combating MD toxicity. Chrysin is a potential naturally occurring flavonoid that is usually found in propolis and honey. Because of its anti-inflammatory and antioxidative features, it has a protective effect (Farkhondeh et al., 2019). Chrysin's mode of action involves reducing inflammation, inducing apoptosis in cells, and decreasing cell proliferation without harming healthy cells or having any unfavorable side effects (Xue et al., 2016; Kasala et al., 2015; Samarghandian et al., 2011). In comparison to the MD group, treating MD rats with CHR resulted in significant increases in ovarian levels of GSH and SOD and significant decreases in ovarian MDA levels. These results are consistent with those of Ye et al. (2022), who discovered that rats receiving CHR therapy showed a significant return of these parameters to normal. Additionally, CHR demonstrated improved antioxidant status, a decrease in oxidative stress, and the prevention of the generation of free radicals

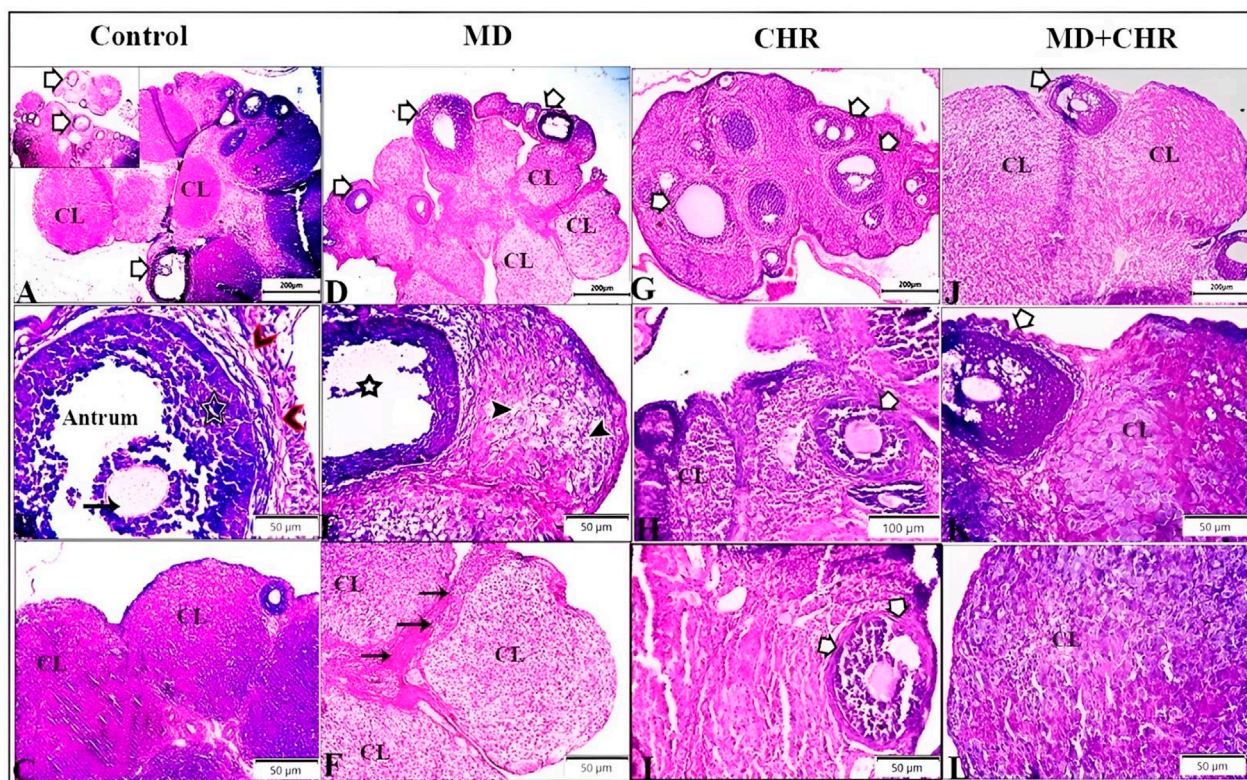


FIGURE 3

Representative photomicrographs of ovarian tissue samples from treated groups stained by hematoxylin and eosin (H, E) demonstrate the control group: (A): different stages of ovarian follicles (arrows), and corpus luteum (CL). (B): Graafian (secondary) follicle with a large follicular antrum. Secondary oocyte located eccentrically and surrounded by the zona pellucida and a layer of several cells known as the corona radiata (arrow), surrounded by multiple cellular layers of granulosa cells (star), and theca cell layers (arrowheads). (C): normal size and structure of corpus luteum (CL). MD group (D–F): (D): Number of atretic follicles with degenerated follicular cells (arrows), degenerated corpora bodies (CL). (E): Abnormal secondary follicles without oocyte (star), Corpus luteum showing deteriorating luteal cells with vacuolation (arrowheads). (F): Corpora showed degenerative changes marked by loss of cellular features and cellular vacuolation (CL) with increased inter-corpora stromal fibrous connective tissue (arrows). CHR group (G–I): Number of normal ovarian follicles (arrows), and corpus luteum (CL). MD + CHR group (J–L): the presence of growing healthy follicles containing an oocyte with an intact zona pellucida and surrounded normal granulosa cells (arrows), more or less corpus luteum containing polyhedral luteinized cells (CL).

(Temel et al., 2020). It has been found that phytochemicals, especially flavonoids, can shield biological macromolecules and membranes against damage caused by free radicals (Karak, 2019). Consistent with these outcomes, the current research demonstrated that flavonoid compounds found in CHR may reduce the oxidative stress that MD causes in MD rats by increasing antioxidant status and lowering lipid peroxidation levels (Alsawaf et al., 2022). According to earlier research, the compound's antioxidant qualities and ability to eliminate free radicals are assumed to be attributed to the hydroxyl groups at positions 5 and 7 in the CHR structures (Eldutar et al., 2017). CHR therapy has been shown by some other authors (Rehman et al., 2013) to enhance antioxidant enzyme activity and protect tissues from oxidative damage.

The cytokine TNF- α was first discovered to play a part in inflammatory processes. This factor activates by attaching itself to one of its two receptors, a type 1 receptor (TNFR1) and a type 2 receptor (TNFR2). TNF- α stimulates CA-125 in breast, endometrial, and ovarian cancers through nuclear factor kappa B (NF- κ B) (Morgado et al., 2016). Moreover, TNF- α plays a role in

controlling physiological processes like corpus luteum function, steroidogenesis, ovulation, and follicular growth. Additionally, it has been documented that TNF- α may control granulosa cell differentiation and death according to the embryonic stage (Silva et al., 2020). According to certain research, serum TNF- α receptor 1 binds to CA-125 more frequently than receptor 2 (Rzymiski, 2005). TNF has been linked to elevated serum CA 125 levels, according to a prior study. These findings imply that cytokines and CA-125 might be related (Kosar et al., 2006). Furthermore, ovarian follicular loss is significantly impacted by the inflammatory response (Mantawy et al., 2019). An increasing number of research has shown that abnormal inflammation can change the dynamics of the ovarian follicles in a way that can lead to infertility (Boots and Jungheim, 2015; Urieli-Shoval et al., 2013). In rats exposed to Organophosphate-Pesticide, serum level of TNF- α was increased (Alam et al., 2019). Our study revealed that CHR administration significantly reduced the rise in TNF- α levels in MD-treated rats, suggesting that CHR alleviated the overexpression of inflammatory markers in their ovaries. Concurring with the results of the present

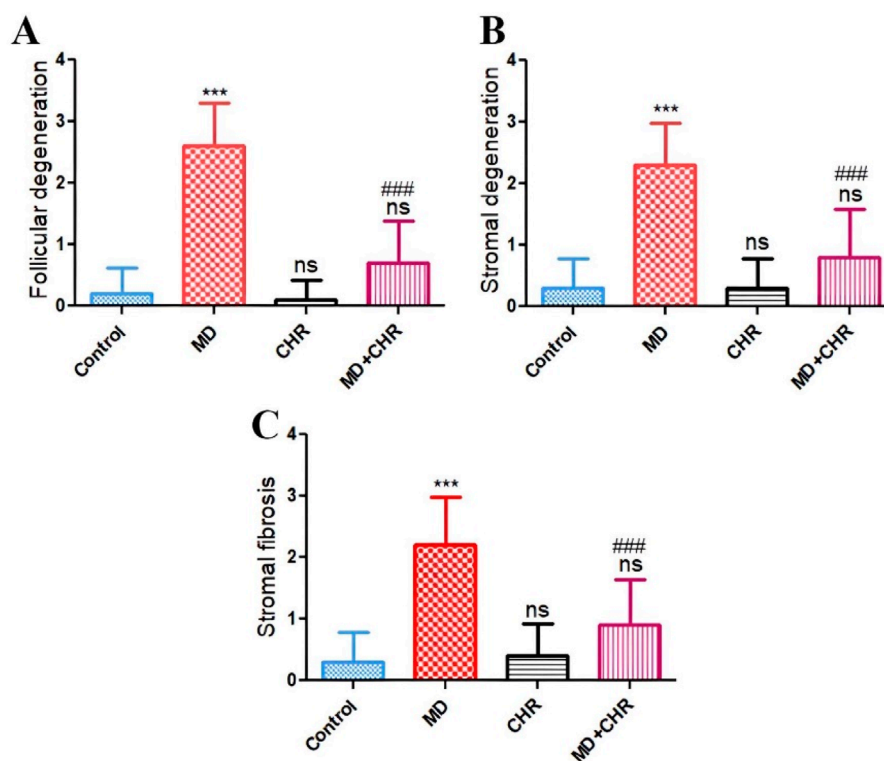


FIGURE 4

Histomorphometry graph showing semiquantitative measurements of lesion scores recorded in ovarian tissue sections among the experimental groups (A): Follicular degeneration, (B): Stromal degeneration, and (C): Stromal fibrosis. Results were analyzed using one-way ANOVA and Tukey's *post hoc* tests. Results are shown in mean $\pm$ SD. ***, ### $p \leq 0.001$ compared to control, and MD groups respectively. ns = $p > 0.05$ (non-significant vs. Control).

investigation, Ai et al. (2013) discovered possible suppression of the pro-inflammatory TNF- α pathway by additional flavonoids.

In the current study, feeding rats MD resulted in a considerable increase in CA-125 levels compared to the control group. CA-125 is a protein produced by various cell types, including ovarian cancer cells (Rao et al., 2021). CA-125 is commonly referred to as a tumor marker or biomarker for ovarian cancer because it provides information about the history of the disease. Measuring CA-125 is the most frequently used test to assist in the diagnosis and follow-up of ovarian cancer (Nossov et al., 2008). According to our findings, the MD-induced increase in CA-125 may indicate a heightened risk of ovarian cancer. However, CHR treatment reduced the elevated CA-125 levels. As per prior reports, oxidative damage and the ROS it produces are thought to be one of the primary initiators of cell death (apoptosis) (Matés et al., 2008; Newsholme et al., 2016; Sifuentes-Franco et al., 2018). The Caspase family of proteases mediates apoptosis, a type of controlled cell death characterized by particular structural alterations (Elmore, 2007). MD treatment caused a state of cell death (Wu et al., 2023). Our findings suggested that marked activation of caspase-3 induced by MD caused inflammation and fibrosis of the ovary. In contrast, CHR treatment inhibited the elevation of caspase-3 level. Thus, the results of the current study suggested that inhibition of caspase-3 activation by CHR results in the prevention of ovarian fibrosis with a significant impact on the production of pro-inflammatory cytokines such as TNF- α and IL-6.

Chemicals that destroy oocytes in primordial follicles might exhibit a delayed effect on the estrous cycle or prolonged reproductive dysfunction, persisting until the recruitment of growing and antral follicles can no longer be sustained (Pocar et al., 2003). Consequently, an increase in ROS in the ovaries leads to fast corpus luteum deterioration, granulosa cell mortality, and a loss of oocyte quality which was present in our histopathological findings in the presence of atretic follicles with degenerated cells and altered corpus luteal size and structure (Peters et al., 2020; Khirallah et al., 2022). Surprisingly, treatment with CHR could restore ovarian tissue damage.

Our research indicates that chrysin, a polyphenol compound with a variety of health-promoting properties, particularly flavonoids, the most prevalent chemical class of phytochemicals, is a promising compound for use in the prevention of ovarian toxicity against toxic agents. In nature, flavonoids can be found everywhere. They can be found in food as well, which makes the connection between nutrition and illness prevention crucial.

5 Conclusion

Our study provides novel findings, that exposure to MD in female rats could diminish fertility by inducing oxidative stress, disrupting hormonal balance during reproduction, causing

histopathological changes, triggering ovarian inflammation, altering tumor marker levels, and affecting ovarian cell apoptosis. Furthermore, our study illustrated that CHR could mitigate the ovarian damage induced by MD. These findings shed light on the reproductive health risks associated with MD exposure. Further research is needed to fully understand the potential benefits of CHR in preventing MD-induced ovarian damage.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by veterinary medical research ethics committee, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt, which was carried out by protocol no. Soh. un. vet/00066R. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AH: Conceptualization, Data curation, Investigation, Methodology, Writing–original draft, Writing–review and editing. FA: Data curation, Methodology, Writing–review and editing. AM:

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antioxidants in neuropsychiatric disorder prevention: neuroprotection, synaptic regulation, microglia modulation, and neurotrophic effects

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Oxidative stress, caused by an imbalance between the generation of reactive oxygen species (ROS) and the body's intrinsic antioxidant defenses, plays a critical role in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's. Beyond these conditions, recent evidence indicates that dysregulated redox balance is implicated in neuropsychiatric disorders, including schizophrenia, major depressive disorder, and anxiety disorders. Preclinical and clinical studies have demonstrated the potential of antioxidants, such as N-acetylcysteine, sulforaphane, alpha-lipoic acid, L-carnitine, ascorbic acid, selenocompounds, flavones and zinc, in alleviating neuropsychiatric symptoms by mitigating excitotoxicity, enhancing synaptic plasticity, reducing microglial overactivation and promoting synaptogenesis. This review explores the role of oxidative stress in the pathogenesis of neuropsychiatric disorders. It provides an overview of the current evidence on antioxidant therapy's pharmacological effects, as demonstrated in animal models and clinical studies. It also discusses the underlying mechanisms and future directions for developing antioxidant-based adjuvant therapies. Given the limitations and side effects of existing treatments for neuropsychiatric disorders, antioxidant therapy presents a promising, safer alternative. Further research is essential to deepen our understanding and investigate the clinical efficacy and mechanisms underlying these therapies.

KEYWORDS

oxidative stress, neuropsychiatric disorders, antioxidants, neuroprotection, synaptic regulation, microglia modulation, neurotrophic effects

1 Oxidative stress and brain disorders

The brain relies heavily on oxygen to generate the energy required for cognitive function. The release of neurotransmitter-loaded vesicles at synapses demands substantial energy, with approximately 1.64×10^5 ATP molecules needed per vesicle released (Alle et al., 2009; Magistretti and Allaman, 2015). Consequently, neuronal mitochondria must consume oxygen (O_2) at a disproportionately high rate to meet the brain's energy needs (Alle et al., 2009). The brain depends on O_2 for aerobic respiration to sustain its high metabolic activity; however, this process produces reactive oxygen species (ROS) as byproducts, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), alongside the complete reduction of oxygen to water (Lennicke and Cocheme, 2021). Under normal physiological

conditions, ROS participates in cellular signaling, regulating cell growth and maintaining homeostasis. For instance, low concentrations of O_2^- and H_2O_2 can stimulate the proliferation of adult hippocampal progenitor cells (Dickinson et al., 2011). However, when oxidative stress (OS) overwhelms the body's antioxidant defenses, excessive ROS can damage neurons, contributing to the development of neurodegenerative (Shadfar et al., 2023) and neuropsychiatric diseases (Rossetti et al., 2020).

While the body's antioxidant enzymes typically neutralize peroxidation products, ROS have evolved to fulfill critical physiological roles, especially within the central nervous system. Consequently, the brain's antioxidant system must make certain compromises (Murphy et al., 2011). For instance, neurons contain significantly lower levels of catalase (CAT) (approximately 50 times less than liver cells) (Ren et al., 2017) and approximately half the amount of cytoplasmic glutathione (GSH) compared to liver cells (Paul et al., 2018; Cobley et al., 2018). This relatively weak endogenous antioxidant defense makes the brain particularly vulnerable to OS (Cobley et al., 2018).

Moreover, the brain's neuronal membranes are rich in unsaturated fatty acids, making them susceptible to oxidative damage, which can produce reactive aldehydes (Maiorino et al., 2018). During immune responses, microglia release substances such as O_2^- and ROS (Block et al., 2007). Furthermore, H_2O_2 is produced during the metabolism of neurotransmitters (Ren et al., 2017). Mitochondrial dysfunction further exacerbates OS by increasing ROS production, creating a vicious cycle in neuronal cells reliant on mitochondrial activity (Rizzuto et al., 2012; Slimen et al., 2014).

The brain's vulnerability to OS stems from several factors, including its high metabolic demands, relatively weak antioxidant defenses, and abundant unsaturated fatty acids in neuronal membranes. These characteristics suggest that OS plays a pivotal role in the pathogenesis of neurological and psychiatric disorders.

2 The role of oxidative stress in neuropsychiatric disorders

The brain is particularly susceptible to OS, and its role in the pathogenesis of neuropsychiatric disorders has gained increasing attention in recent years (Rossetti et al., 2020). Therefore, this section reviews the evidence linking OS to conditions such as schizophrenia (SZ), anxiety disorders, major depressive disorder (MDD) and bipolar disorder (BD).

2.1 Oxidative stress in schizophrenia

SZ is a severe mental disorder affecting approximately 0.3 to 0.66% of the population, significantly impairing quality of life and imposing a substantial socio-economic burden (Maas et al., 2017).

While traditional models of SZ pathogenesis emphasize neurotransmitter dysfunction, particularly involving dopamine, emerging research points to OS as an additional underlying mechanism (Miljevic et al., 2018). This hypothesis is supported by numerous studies and meta-analyses (Goh et al., 2021; Goh et al., 2022). For instance, research by Li et al. (2024) and Chien et al. (2020) has shown significantly elevated levels of malondialdehyde (MDA), a marker of lipid

peroxidation (LP), in the blood samples of patients with SZ. Similarly, a study by Jia et al. (2023) indicated that OS contributes to hippocampal damage in patients with first-episode SZ, leading to cognitive impairment. Raffa et al. (2011) identified reduced activity of antioxidant defense systems, such as GSH and CAT, in individuals with SZ. Further evidence from Al-Amin et al. (2016) suggests that the antioxidant astaxanthin can ameliorate behavioral deficits in SZ mice. Concurrently, MacDowell et al. (2016) suggested that the antipsychotic drug paliperidone may mitigate OS by upregulating nuclear factor erythroid 2-related factor 2 (Nrf2) in the Phosphoinositide 3-kinase/Protein kinase B (PI3K/AKT) pathway. Kulak et al. (2013) observed heightened OS in the anterior cingulate cortex during early development in GSH synthesis-deficient (gclm $-/-$) mice accompanied by microglial activation and redox-sensitive matrix metalloproteinase 9 (MMP9) upregulation. Inhibiting MMP9 activation can normalize parvalbumin-expressing interneurons (PVI)/ perineuronal nets (PNN) maturation and alleviate SZ-related psychopathology (Dwir et al., 2020).

Furthermore, extensive research suggests that OS may impact cognitive function through various pathways, such as directly damaging parvalbumin-expressing interneurons (PVIs) (Schiavone et al., 2009), hindering oligodendrocyte precursor cell (OPC) proliferation and myelin formation in the prefrontal cortex (PFC) (Maas et al., 2021), disrupting the blood-brain barrier (BBB) (Geng et al., 2023), and inducing mitochondrial dysfunction (Fizikova et al., 2023). Therefore, targeting OS may be crucial for SZ prevention and treatment.

2.2 Oxidative stress in major depressive disorder

According to the World Health Organization (WHO), MDD was the fourth leading cause of disability worldwide and was predicted to rise to second by 2020. Nearly half of those affected may not receive timely diagnosis and treatment, underscoring the urgent public health challenge of managing depression (Lolak et al., 2014).

Traditional models attribute depression to disruptions in monoamine and glutamate neurotransmission. However, emerging evidence suggests that OS and pro-inflammatory signaling may also contribute to MDD (Bader et al., 2024; Tuon et al., 2021). Jiménez and Chung et al. found significantly elevated levels of MDA in the plasma of patients with MDD (Jimenez-Fernandez et al., 2022; Chung et al., 2013). Similarly, Maas et al. (2019) reported increased levels of superoxide dismutase 1 (SOD1), nitric oxide (NO), ROS, and lipid peroxides in patients with depressive symptoms. Conversely, Kotan et al. (2011) identified decreased activity of antioxidant enzymes, such as SOD and CAT, in the serum of patients with MDD. Szebeni et al. (2014) reported significantly reduced mRNA levels of SOD, CAT, and glutathione peroxidase (GPX) in oligodendrocytes from the white matter of patients with MDD in post-mortem analysis. Moreover, Moreno et al. (2013) found elevated platelet NO and platelet mitochondrial membrane potential (PMMP) in patients with MDD, suggesting that mitochondrial bioenergetic alterations may contribute to the onset and progression of depression via OS. This evidence is further supported in animal models of depression (Tuon et al., 2021). Moreover, knockout (KO) mice lacking the antioxidant transcription factor Nrf2 displayed depression-like behaviors in various tests (Dang et al., 2022; Zeng et al., 2023).

OS may disrupt neurotransmitter metabolism, such as that of serotonin (Ding et al., 2020), impair neurogenesis and synaptic plasticity (Hou et al., 2017), and induce DNA and RNA hypermethylation (Wu et al., 2021; Han et al., 2022), all of which may contribute to depression. These findings underscore the therapeutic potential of antioxidants in treating depression.

2.3 Oxidative stress in anxiety disorders

Anxiety, an essential evolutionary mechanism for alertness and self-protection, can become maladaptive when excessive, leading to anxiety disorders. The lifetime prevalence of pathological anxiety exceeds 20% (Filiou and Sandi, 2019; Koskinen and Hovatta, 2023).

Anxiety disorders, including generalized anxiety disorder (GAD) and phobias, are not fully understood. However, emerging research hints at a potential role for impaired antioxidant defense and oxidative damage in their development (Kaya et al., 2013; Oktay et al., 2024). Oktay et al. (2024) clinical study revealed significantly increased levels of LP markers, such as MDA and F2-isoprostanes, in patients with severe anxiety. Bellisario et al. (2014) demonstrated that deleting the p66Shc gene, a key regulator of mitochondrial ROS production, reduced anxiety behaviors by reducing OS. Furthermore, Bersuker et al. (2019) discovered that *Lactobacillus plantarum* guanidinoacetate (LbGp), an OS regulator, alleviated anxiety-like behavior by enhancing glutathione peroxidase 4 (GPX4) activity and preventing ferroptosis. Conversely, the deletion of the GPX4 gene in dopaminergic neurons increased anxiety behaviors (Dang et al., 2022). Moreover, overexpression of genes such as glutathione reductase 1 (GSR1) and glyoxalase enzyme 1 (GLO1) has been strongly correlated with anxiety phenotypes (Hovatta et al., 2005), with GLO1 inhibitors showing potential in alleviating anxiety (Distler et al., 2012). Moreover, OS may exacerbate anxiety by depleting reduced GSH (Nisar et al., 2023) and promoting N-methyl-D-aspartate (NMDA) receptor-mediated synaptic inhibition in the basolateral amygdala (BLA) (Wu et al., 2022).

Despite inconsistent findings across studies, a general pattern of oxidative imbalance has been observed in patients with anxiety, suggesting that targeting OS may offer a promising therapeutic avenue for anxiety disorders.

2.4 Oxidative stress in bipolar disorder

Bipolar disorder (BD) is a chronic mental illness characterized by an alternation between mania or hypomania and depression. It is often associated with impaired functionality (Munkholm et al., 2024).

Several lines of evidence point to the presence of low-grade inflammation and oxidative stress in patients with bipolar disorder (Rosenblat and McIntyre, 2016), while findings to some extent are inconsistent and have been limited by methodological issues (Garcia-Gutierrez et al., 2020; Kirkpatrick et al., 2021; Munkholm et al., 2024). Increased lipid peroxidation has been observed in the prefrontal cortex and anterior cingulate cortex of patients with BD (Wang et al., 2009). Moreover, One study conducted with 94 BD patients and 41 healthy controls reported higher OS index levels in the BD patients compared with the controls (Yumru et al., 2009). It also found decreased antioxidant and OS markers; however, many other studies

have reported the opposite finding. For example, some studies corroborated this finding of increased serum TBARS levels in BD patients during mania, depression, and euthymia (Andreazza et al., 2007). Moreover, Sowa-Kucma et al. (2018) found a significant positive association between higher TBARS level and severity of BD, including the risk of suicidality. Additionally, studies have found that serum copper concentrations may be higher in certain subgroups, such as patients in the early stages of the disease. Furthermore, serum copper concentrations may be associated with certain pathophysiological processes of bipolar disorder, such as oxidative stress. Although this study suggests that there are differences in serum copper concentrations among bipolar disorder patients at different stages of the disease, these differences did not reach statistical significance (Siwek et al., 2017).

BD is becoming increasingly understood as a condition of aberrant neuroplasticity. Multiple factors, such as OS, imbalance of neurotransmitters, and genetics, are associated with the pathophysiology of BD.

3 The role of antioxidants in treating neuropsychiatric disorders

The antioxidant system of cells is mainly composed of two parts: the enzymatic antioxidant system and the non-enzymatic antioxidant system. These two systems are not isolated but form an integral whole. The enzymatic antioxidant system includes a series of active enzymes with antioxidant properties, such as superoxide dismutase (SOD, including Cu-Zn SOD and Mn-SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxin (Trx), and others (Wen et al., 2022; Chen et al., 2023). These enzymes can catalyze antioxidant reactions, converting free radicals into harmless substances, thereby maintaining redox balance within organisms. The non-enzymatic antioxidant system, on the other hand, is primarily composed of small molecular antioxidant substances. Numerous preclinical and clinical studies highlight the potential of antioxidants (Rossetti et al., 2020) such as N-acetylcysteine (NAC), sulforaphane (SFN), alpha-lipoic acid (ALA), L-carnitine (L-Car), ascorbic acid, selenocompounds, and flavones. Beyond their direct radical-scavenging properties, these compounds have demonstrated an ability to modulate endogenous antioxidant systems. (Figure 1).

3.1 N-acetylcysteine

N-acetylcysteine (NAC), an essential precursor for GSH synthesis, is a critical brain antioxidant (Raghu et al., 2021). Its antioxidant mechanisms primarily encompass: serving as a reductant to reduce oxidized molecules by donating electrons; activating the glutathione (GSH) cycle to restore glutathione to its reduced form; directly scavenging free radicals, including superoxide anions, hydroxyl radicals, and hydrogen peroxide; and curbing inflammation by suppressing oxidative stress and inflammatory cytokine production (Raghu et al., 2021). Several studies have suggested that NAC can ameliorate clinical symptoms in patients with SZ, AN, and MDD (Sceneay et al., 2013; Hoepner et al., 2021). For instance, NAC modulates GSH and glutamate levels, potentially reducing the negative symptoms and cognitive impairments associated with SZ (Yang et al.,

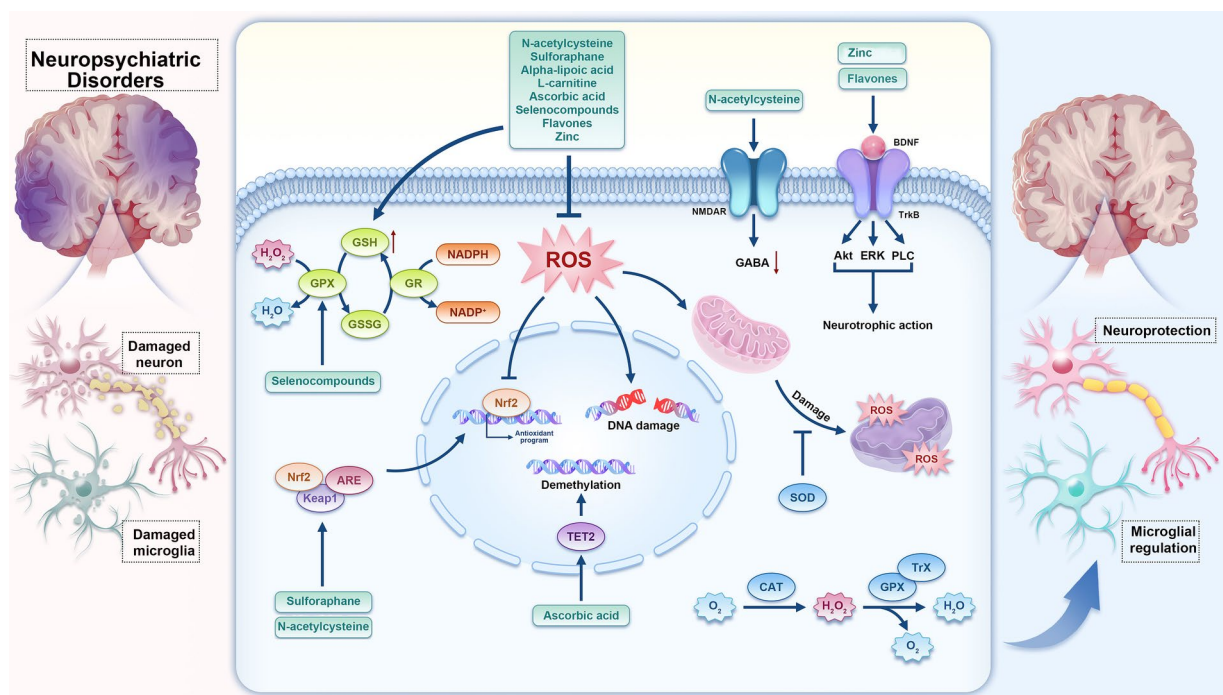


FIGURE 1

The hypothesis map illustrates the interplay between natural antioxidant compounds, the body's innate antioxidant defenses, reactive oxygen species (ROS), microglial inflammatory responses, and the management of neuropsychiatric disorders. Antioxidants such as N-acetylcysteine, sulforaphane, alpha-lipoic acid, L-carnitine, ascorbic acid, selenocompounds, flavones, and zinc, etc., not only function by scavenging ROS, enhancing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and thioredoxin (Trx), and promoting the expression of antioxidant genes such as nuclear factor erythroid 2-related factor 2 (Nrf2), but they also exert effects on specific molecular targets. For instance, N-acetylcysteine (NAC) and zinc can directly interact with N-methyl-D-aspartate (NMDA) receptors, ascorbic acid can directly affect the activity of Tet Methylcytosine Dioxygenase 2 (TET2) enzymes, and Flavonoid compounds have the ability to directly interact with tyrosine kinase receptor B (TrkB) receptors and subsequently activate downstream signaling pathways such as Phospholipase C (PLC), Extracellular Signal-Regulated Kinase (ERK), and Protein kinase B (AKT).

2022). However, while evidence supports NAC's therapeutic effect in stable patients with SZ, its efficacy in patients with refractory SZ on clozapine remains inconclusive (Fornaro et al., 2024). Furthermore, animal experiments indicate that NAC can mitigate elevated glutamate levels in the cerebral cortex, reduce ROS levels in interneurons (Neill et al., 2022; Buhner et al., 2022), and upregulate brain-derived neurotrophic factor (BDNF) mRNA and protein, leading to improved behavioral and cognitive outcomes in SZ animal models (Phensy et al., 2017; Aslanlar et al., 2024). Similarly, NAC has been shown to alleviate moderate depressive symptoms (Liang et al., 2022) by curbing ROS production in microglia (Lehmann et al., 2019) and regulating the glutamatergic system in the PFC (Nery et al., 2022). However, a meta-analysis of randomized controlled trials found that NAC was not significantly better than placebo in treating severe depression or bipolar disorder (Andrade, 2021).

Overall, NAC exhibits multiple biological activities, demonstrating promise as a treatment for SZ, MDD, and AN; however, further research is warranted.

3.2 Sulforaphane

Sulforaphane (SFN) is a naturally occurring organic sulfur compound found in cruciferous vegetables such as broccoli, cauliflower, and mustard greens, characterized by its unique

isothiocyanate group (Kamal et al., 2020). As an indirect antioxidant, SFN activates the Nrf2/Kelch-like ECH-associated protein 1 (Keap1)/Antioxidant response element (ARE) signaling pathway. When cells are stimulated by oxidative stress or other stressors, SFN binds to specific sites on Keap1, causing a conformational change in Keap1. This change frees Nrf2 from its binding with Keap1, allowing it to translocate to the nucleus. In the nucleus, Nrf2 binds to ARE, initiating the transcription of a series of antioxidant enzymes and proteins, thereby preserving cellular redox balance and homeostasis (Mangla et al., 2021). Additionally, by activating the Nrf2/Keap1/ARE signaling pathway, SFN upregulates the activity of multiple antioxidant enzymes, protecting cells from oxidative damage (Ma et al., 2023). Beyond its antioxidant effects, SFN exhibits potent anti-inflammatory properties (Kiser et al., 2021).

Some clinical studies indicate that SFN can prevent cognitive impairment in SZ through its anti-inflammatory (Zeng et al., 2024) and antioxidant effects (Shirai et al., 2015). However, other trials have not consistently replicated these findings (Dickerson et al., 2021). In animal models, SFN appears to be a promising adjunct therapy for SZ, mitigating side effects such as metabolic defects, biochemical imbalances, and liver histological abnormalities associated with olanzapine (OLA) (El-Shoura et al., 2024). Concurrently, SFN has been shown to improve anxiety and depression symptoms in mice by activating the Nrf2/ heme oxygenase-1 (HO-1) signaling pathway (Ferreira-Chamorro et al., 2018) and inhibiting the

hypothalamic–pituitary–adrenal (HPA) axis and stress response (Wu et al., 2016). Furthermore, SFN may activate Nrf2 by initiating the transcription of trigger receptor expressed on myeloid cells-2 (TREM2) in the medial PFC (mPFC), increasing the expression of the M2 microglial marker arginase 1 (ARG1), which may alleviate depressive phenotypes through its anti-inflammatory and neuroprotective functions (He et al., 2022).

In summary, SFN has demonstrated potential as a treatment for SZ, MDD, and AN. However, further high-quality clinical and animal studies are necessary to confirm its therapeutic efficacy and mechanisms of action.

3.3 Alpha-lipoic acid

Alpha-lipoic acid (ALA) is a natural compound commonly found in the diet, serving as a crucial cofactor for mitochondrial respiratory enzymes and playing a vital role in maintaining cellular oxidative metabolism (Holmquist et al., 2007). ALA can directly scavenge ROS, promote the regeneration of vitamins C and E, and upregulate the activity of antioxidant enzymes like superoxide dismutase and catalase (El-Houseiny et al., 2023). Evidence suggests that ALA may alleviate symptoms associated with SZ and reduce OS (Emsley et al., 2014; Vasconcelos et al., 2015). Furthermore, ALA supplementation has been shown to improve the psychopathology of patients with treatment-resistant SZ (TRS) by decreasing OS (Sanders et al., 2017; Mishra et al., 2022). However, these promising findings were not confirmed in a subsequent double-blinded, placebo-controlled trial conducted by Emsley et al. (2014), warranting caution due to potential side effects, including a decrease in blood cell count associated with ALA treatment (De Lima et al., 2023). Iannuzzo et al. (2022) investigated ALA's potential for treating depression, particularly in combination with other therapies, as it can effectively mitigate drug-related side effects such as the risk of diabetes and liver dysfunction. Moreover, ALA has been demonstrated to regulate the neuropathology of BDNF in mice model (Vasconcelos et al., 2015; Aliomrani et al., 2022). Furthermore, ALA alleviates methamphetamine-induced memory deficits and anxiety-like behavior in rats by enhancing the activity of antioxidant enzymes, including SOD and CAT (Kargar and Noshiri, 2024).

These findings underscore ALA's potential to enhance cognitive function and emotional well-being while highlighting the necessity for further clinical validation in human populations.

3.4 L-carnitine

L-Carnitine (L-Car) is an essential nutrient in human tissues, including the brain. The antioxidant mechanism of L-Car primarily involves facilitating fatty acid entry into mitochondria for oxidative breakdown, reducing intracellular fatty acid accumulation, stabilizing mitochondrial membrane potential, scavenging free radicals, upregulating the expression of antioxidant enzyme genes, and enhancing antioxidant enzyme activity (Da Silva et al., 2023). These actions collectively protect cells from damage caused by oxidative stress. Specifically, acetyl-L-Car (ALCAR), as a critical form of L-Car, has been substantially linked to several mental health disorders (Cao et al., 2019). Previous studies indicate that low levels of ALCAR are closely associated with conditions such as depression

and SZ (Cao et al., 2020). L-Car has been shown to improve psychiatric scores in a mouse model of SZ through anti-inflammatory and antioxidant pathways (Ebrahimi et al., 2023). Meanwhile, clozapine can disrupt lipid metabolism in the liver by affecting L-Car reabsorption, and concurrent L-Car supplementation is an effective strategy to mitigate these metabolic disturbances (Bruno et al., 2016; Wang et al., 2018). Moreover, metabolomic analyses of serum from patients with severe depression suggest that L-Car and ALCAR may serve as potential biomarkers for this condition (Nie et al., 2021). Supplementation with L-Car may serve as an effective adjuvant therapy for patients with refractory depression. The Canadian Emotion and Anxiety Treatment Network has established clinical guidelines recommending ALCAR monotherapy as a third-line treatment option for mild to moderate depression based on existing research evidence (Yatham et al., 2018). A recent meta-analysis showed that ALCAR supplementation as a standalone intervention significantly alleviated depressive symptoms compared to placebo or no intervention (Veronese et al., 2018). Animal studies suggest that ALCAR may exert antidepressant effects through the PI3K/AKT/BDNF signaling pathway (Wang et al., 2015).

Although ALCAR's potential in treating mental illness has been preliminarily validated, further high-quality research is necessary to explore its specific mechanisms and optimize treatment dosages and regimens. Moreover, attention must be paid to the interactions between ALCAR and other medications and their potential adverse reactions.

3.5 Ascorbic acid

Ascorbic acid, or vitamin C, is a widely recognized antioxidant that plays a crucial protective role in the body (Conklin et al., 2024). Ascorbic acid directly scavenges superoxide anions, hydroxyl radicals, and other free radicals, and regenerates antioxidants such as vitamin E and GSH. It also modulates the expression of antioxidant enzymes like SOD and CAT, enhancing cellular antioxidant capacity and chelating metal ions to remove harmful ions such as iron and copper from the body (Chen et al., 2021). Systematic reviews indicate that ascorbic acid promotes neuronal differentiation of precursor cells, enhances adult hippocampal neurogenesis, and facilitates synaptic plasticity, thereby improving behavioral and biochemical changes in psychiatric disorders such as SZ, anxiety, MDD, and bipolar disorder (Moretti and Rodrigues, 2022). Evidence indicates that patients with SZ exhibit lower vitamin C levels (Myken et al., 2022). Research has shown that ascorbic acid can alleviate phenotypic symptoms of SZ by restoring the balance between ROS and antioxidant defenses (Dakhale et al., 2005; Damazio et al., 2017), reducing inflammatory factor levels, and employing other mechanisms (Supp et al., 2021). Similarly, ascorbic acid may exert antidepressant effects by restoring antioxidant enzyme activity (Moretti et al., 2013), activating the opioid receptor system (particularly the μ -opioid receptor), inhibiting NMDA receptors, or both (Moretti et al., 2018; Moretti et al., 2019). Furthermore, a recent study indicated that ascorbic acid can alleviate anxiety symptoms by upregulating synaptic proteins, increasing dendritic spine density, and promoting the maturation of the ventral dentate gyrus (DG) (Fraga et al., 2018; Fraga et al., 2020). Ascorbic acid can also directly enhance the catalytic activity of Tet

methylcytosine dioxygenase 2 (TET2) in the oxidation of 5-methylcytosine (5mC), promote the folding and/or recycling of the cofactor Fe (2⁺) for TET2, and improve symptoms of depression (Ma et al., 2024; Yin et al., 2013).

These findings collectively highlight the therapeutic potential of ascorbic acid in treating mental illnesses.

3.6 Selenocompounds

Selenium is the active center of GPX, and recent advancements have led to the development of various mimetics designed to replicate GPX functions (Ferreira et al., 2021). The antioxidant mechanism of selenocompounds primarily involves the direct reaction of selenium atoms with free radicals generated by oxidative stress, thereby reducing the number of free radicals (Bartolini et al., 2017).

Serum selenium levels are considerably lower in patients with SZ compared to healthy controls (Li et al., 2018), suggesting a protective role for selenium in SZ and AN (Guo et al., 2023). Moreover, GPX activity is generally reported to be reduced by approximately 20% in patients with SZ. Supplementation with selenium has been shown to enhance cognitive function and improve clinical symptoms such as appetite and memory (Alsharif et al., 2023). Furthermore, dietary selenium appears to mitigate stress-induced depression symptoms, with epidemiological studies linking low selenium intake to an increased risk of severe depression (Pasco et al., 2012). However, this association has faced scrutiny from other studies (Guo et al., 2023; Bot et al., 2019). Animal studies have demonstrated the antidepressant and anti-anxiety properties of selenium compounds. For instance, F-DPS [2,5-diphenyl-3-(4-fluorobenzeneselenyl) selenophenyl] alleviates depression symptoms by restoring glutamate uptake in the PFC of mice (Gai et al., 2014a) and activating Extracellular Signal-Regulated Kinase (ERK) signaling (Gai et al., 2014b) pathways. MFSeI [1-methyl-3-(phenylselenyl)-1H indole] exerts antidepressant and anti-anxiety effects by reducing OS, regulating neurotransmitter balance, and affecting glucocorticoid receptor expression (Bampi et al., 2020). Diphenyl diselenide (DPDS) shows anti-anxiety effects by modulating Gamma-Aminobutyric Acid Type A (GABAA) and 5-Hydroxytryptamine (5HT) receptors (Ghisleni et al., 2008). Similarly, ebselen reduces impulsivity in rodent models and has been suggested as an alternative to lithium in the treatment of bipolar disorder and other mood disorders (Singh et al., 2016). Liquiritigenin display neuroprotection through exerting anti-oxidative and anti-inflammatory activities to suppress neuronal apoptosis (Chiu et al., 2018).

Selenium and its compounds show considerable potential in regulating nervous system functions, alleviating stress responses, and preventing mental illnesses. However, further research is necessary to confirm these findings.

3.7 Flavones

Flavonoids are low-molecular-weight compounds that belong to a class of plant secondary metabolites characterized by a polyphenolic structure. Flavonoids primarily exhibit their antioxidant mechanism by directly scavenging free radicals such as reactive oxygen species (ROS). Through specific functional groups, they react with free

radicals to halt radical chain reactions. Moreover, flavonoids can also upregulate the activity of antioxidant enzymes, thereby enhancing the antioxidant defense system (Calis et al., 2020). They are categorized into six subcategories based on the carbon atoms connected to the C ring by the B ring, as well as the degree of unsaturation and oxidation of the C ring: flavanones, flavones, isoflavones, flavonols, chalcones, and anthocyanins (Hostetler et al., 2017).

Research has shown that 7,8-dihydroxyflavone (7,8-DHF) can alleviate SZ-like symptoms by effectively mimicking the effect of brain-derived neurotrophic factor (BDNF) in the brain (Jaehne et al., 2021) to selectively activate tyrosine kinase receptor B (TrkB) (Emili et al., 2022) and downstream Phospholipase C (PLC), AKT, and ERK1/2 signaling pathways. Similarly, the natural flavonoid 4',5,7-trihydroxyflavone boosts the neurotrophic effects of BDNF by strengthening TrkB receptor signaling (Gao et al., 2023). Meanwhile, Deng et al. (2024) suggest that flavonoids have a protective role against depression, a finding supported by various animal and epidemiological studies (Amin et al., 2020; Zhang et al., 2015; Gui et al., 2023). Moreover, 7,8-DHF improves anxiety-like behavior in mice subjected to chronic alcohol exposure by regulating TrkB signaling in the amygdala (Wang et al., 2021). Natural flavonoids, such as chrysin, have demonstrated anxiolytic effects in animal models through mechanisms including interaction with the GABAA/benzodiazepine receptor complex and free radical scavenging (Karim et al., 2012; Gadotti and Zamponi, 2019; German-Ponciano et al., 2020). In summary, flavonoids possess significant therapeutic potential in treating SZ, depression, and AN due to their diverse biological activities and effects.

3.8 Zinc

Zinc, as an essential trace element, possesses the ability to modulate intracellular redox levels, preventing oxidative damage to biomembrane systems and reducing the formation of reactive oxygen species. Deficiency in zinc can increase the susceptibility of the body to oxidative stress, and appropriate supplementation can alleviate the resulting damage (Chasapis et al., 2020).

In the exploration of zinc's potential in treating depression, a series of literature reviews have delved into the role of zinc in depression, including its potential mechanisms in regulating neurotransmitter, endocrine, and neurogenesis pathways, and have emphasized the reported antidepressant-like and mood-enhancing activities of zinc in both human and rodent intervention studies (Wang et al., 2019). Furthermore, a systematic review and meta-analysis found that zinc supplementation can alleviate depressive symptoms in patients undergoing antidepressant treatment (Da Silva et al., 2021). Another review has discussed the role of zinc in regulating brain-derived neurotrophic factor (BDNF) and its impact on neural function, suggesting that the combination of zinc supplementation with antidepressants can effectively treat major depressive disorder (Mlyniec, 2021). A preliminary study showed that individuals with anxiety have significantly elevated plasma copper levels and very low zinc levels, and supplementation with zinc significantly improved anxiety symptoms (Russo, 2011). However, the exact molecular mechanisms underlying the potential relevance of zinc have not been fully elucidated. Relevant animal studies have shown that

zinc can regulate 5-HT receptors, exerting antidepressant effects (Satala et al., 2018). Additionally, zinc can block NMDA receptors, preventing glutamate from entering cells, thus producing an anxiolytic effect (Dou et al., 2018). It is noteworthy that the G protein-coupled receptor 39 (GPR39) is abundantly distributed in brain regions related to anxiety, and zinc, being a natural ligand for GPR39, is involved in the regulation of anxiety (Laitakari et al., 2021). These findings collectively highlight the therapeutic potential of zinc in treating mental illnesses.

3.9 Gut microbial biotransformation

Microbiota, particularly the gut microbiota, has been confirmed to play a significant role in neuropsychiatric health (Xiong et al., 2023). In the small intestine, the absorption of polyphenolic compounds is limited, hence the majority of these compounds reach the colon where they interact with the gut microbiota, exerting their oxidative activity (Ozdal et al., 2016; Wang et al., 2022).

Studies have shown that the gut microbiota can convert dietary polyphenols into low molecular weight bioactive metabolites, such as short-chain fatty acids (SCFAs) and phenolic acids, which may exert their antioxidant and anti-inflammatory effects through signaling pathways like Nrf2 and NF- κ B (Balkrishna et al., 2024). Not only that, but polyphenols can utilize the structural characteristics of hydroxyl groups on their benzene rings to scavenge free radicals through H atom transfer (Papuc et al., 2017). Furthermore, polyphenols provide electrons to free radicals, stabilizing them and terminating the reaction. Epigallocatechin

gallate (EGCG) from green tea can stimulate the nuclear translocation of Nrf2 in HepG2 cells, modulating the expression of antioxidant genes (Mi et al., 2018). Concurrently, polyphenolic compounds can exert neuroprotective effects by regulating adult neurogenesis, synaptogenesis, and neuroplasticity, as well as the activation of microglia (Godos et al., 2020). These studies provide in-depth insights into the relationship between gut microbiota and its metabolic components with mental health and offer directions for the development of dietary natural products for the prevention and treatment of psychiatric disorders.

4 Mechanisms underlying the effect of antioxidants on neuropsychiatric disorders

The mechanisms through which antioxidants impact neuropsychiatric disorders can be summarized into several key areas: neuroprotection, synaptic regulation, modulation of microglial activity, and neurotrophic effects (as depicted in Figure 2).

4.1 Neuroprotection

Antioxidants are crucial in promoting the proliferation and differentiation of neural stem cells, enhancing neurons' number and functionality, and ultimately improving functional recovery in the nervous system. For instance, SFN protects neurons from inflammation-mediated damage by lowering inflammatory

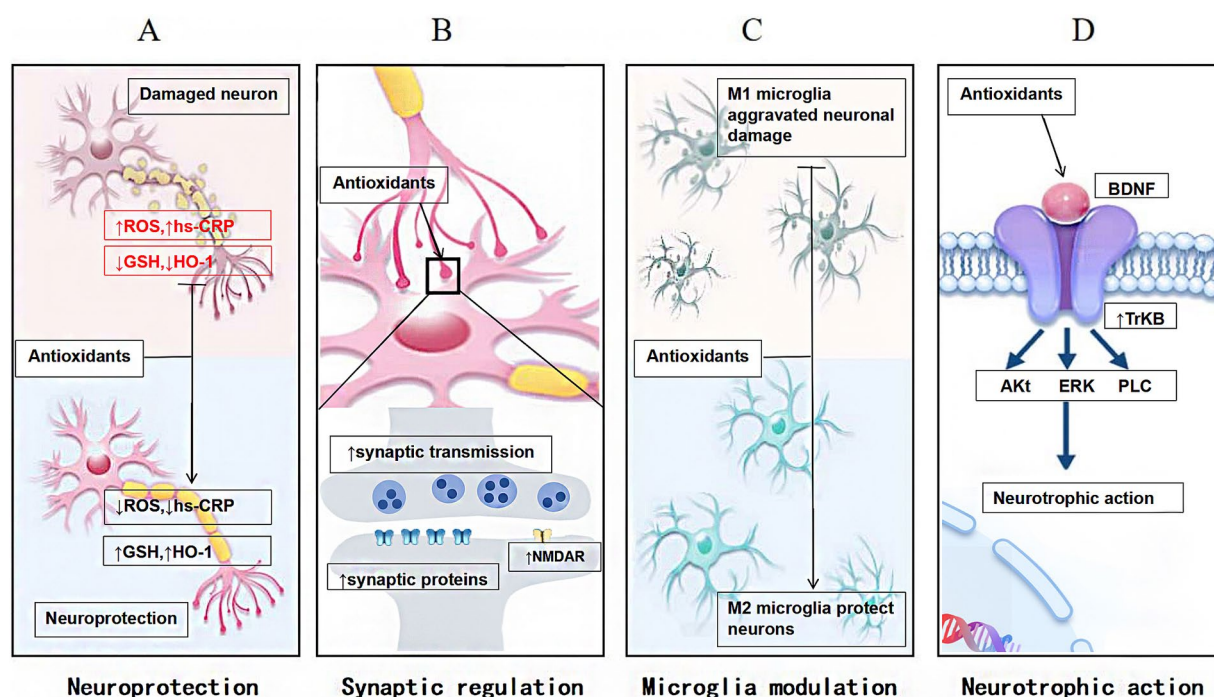


FIGURE 2

The mechanisms through which antioxidants impact neuropsychiatric disorders, including: (A) neuroprotection, which shields neurons from damage; (B) synaptic regulation, which modulates the transmission of signals between neurons; (C) modulation of microglial activity, which influences microglia polarization; and (D) neurotrophic effects, which support the growth and survival of neural cells.

markers such as high-sensitivity C-reactive protein (hs-CRP), restoring antioxidant enzyme expressions such as HO-1 and GSH, and reducing OS (Zeng et al., 2024). Similarly, ascorbic acid mitigates the production of ROS triggered by antipsychotic medications, thereby providing neuroprotective benefits (Dakhale et al., 2005; Damazio et al., 2017). Selenium supplementation can enhance the activity of GPX, thereby reducing OS damage to neurons (Bampi et al., 2020). Furthermore, flavonoids promote neuronal survival and repair through their antioxidant properties (Harvey, 2022).

4.2 Synaptic regulation

Antioxidants primarily function by mitigating OS effects on synaptic structure and function. Synaptic plasticity, characterized by dynamic synapse morphology, structure, and function changes, is essential for higher cognitive functions such as learning and memory (Magee and Grienberger, 2020). Antioxidants regulate synaptic plasticity by modulating neurons' metabolic and signaling processes at pre- and postsynaptic levels.

For instance, NAC enhances synaptic transmission efficiency by promoting NMDA receptor activation and depolarizing the postsynaptic membrane (Phensy et al., 2017). This mechanism has been shown to alleviate long-term behavioral deficits associated with ketamine treatment in a preclinical SZ model during the perinatal period (Neill et al., 2022; Buhner et al., 2022). Selenium compounds can normalize glutamate uptake in the PFC, a process frequently disrupted in neuropsychiatric disorders (Gai et al., 2014a). Similarly, ascorbic acid can improve SZ symptoms by upregulating synaptic proteins, increasing dendritic spine density, and facilitating the maturation of ventral DG (Fraga et al., 2020).

4.3 Microglia modulation

Neuropsychiatric disorders are frequently characterized by increased OS and inflammatory responses, with abnormal activation and dysfunction of microglia playing a significant role (Lehmann et al., 2019). Antioxidants can slow the progression of these disorders by modulating microglial function and activity. For instance, SFN alleviates depressive symptoms by activating the Nrf2/HO-1 signaling, reducing microglial activation, and facilitating a transition to the M2 phenotype (Ferreira-Chamorro et al., 2018; He et al., 2022). Concurrently, NAC prevents behavioral deficits in mice by inhibiting microglial activation (Lehmann et al., 2019).

4.4 Neurotrophic action

Neurotrophic action refers to the effects of specific substances that promote neuron growth, development, maintenance, and regeneration (Castren and Monteggia, 2021). For instance, N-acetylcysteine ameliorates chemotherapy-induced impaired anxiety and depression-like behaviors by regulating BDNF release (Aslanlar et al., 2024). Furthermore, ALA can reverse ketamine-induced SZ-like symptoms in mice, potentially through its influence on BDNF in the PFC, as well as in a mouse model of

depression (Vasconcelos et al., 2015; Aliomrani et al., 2022). Flavones enhance the neurotrophic effects of BDNF by reinforcing TrkB receptor signaling (Wang et al., 2021; Emili et al., 2022; Gao et al., 2023). Moreover, flavones significantly regulate neurotransmitter balance and improve the neuronal microenvironment, promoting neuronal nutrition and metabolic activity (Jaehne et al., 2021).

5 Conclusion

After a thorough review and analysis of existing literature, we have drawn the following conclusion: Antioxidants play a pivotal role in preventing neuropsychiatric disorders by effectively scavenging free radicals and mitigating oxidative stress, thereby forming a protective barrier for brain neural tissue. Specifically, antioxidants can efficiently neutralize reactive oxygen and nitrogen species, significantly reducing the damage these harmful molecules cause to brain neurons, and ensuring the preservation of neuronal structural and functional integrity. Furthermore, by regulating the synthesis, release, and reuptake of neurotransmitters, antioxidants maintain the normal functioning of the nervous system, providing robust support for the prevention of neuropsychiatric disorders. Additionally, antioxidants exhibit notable anti-inflammatory effects, inhibiting inflammatory responses and mitigating the damage caused by inflammatory mediators to neural tissue, thereby protecting the nervous system from inflammatory diseases. Lastly, antioxidants improve mitochondrial energy metabolism efficiency and antioxidant capacity, reducing the production of free radicals and further alleviating the potential damage caused by oxidative stress to neuronal cells.

While early studies suggest potential therapeutic effects of antioxidants in certain conditions, many of these studies are limited by small sample sizes, raising concerns about the reliability and reproducibility of the findings. Furthermore, the heterogeneity among patients remains a significant challenge in clinical trials. Factors such as physiological status, genetics, and lifestyle can significantly influence the effectiveness of antioxidant treatments. Moreover, the potential side effects of antioxidants may limit their therapeutic value. Therefore, a comprehensive evaluation of safety and efficacy is essential during drug development. Despite the numerous challenges and limitations associated with targeting OS for disease treatment, advances in science and technology, coupled with continued research, offer hope for overcoming these barriers. Future breakthroughs may provide novel approaches to disease prevention and treatment.

Author contributions

FL: Writing – original draft. QB: Writing – original draft. WT: Writing – original draft. SZ: Writing – review & editing. YG: Writing – review & editing. SP: Writing – review & editing. XM: Writing – review & editing. YY: Funding acquisition, Writing – review & editing. HF: Funding acquisition, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of *Vaccinium*-derived antioxidants on human health: the past, present and future

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Dietary intake of *Vaccinium* berries has demonstrated significant potential in preventing many risk factors associated with metabolic syndromes in the human population. In recent years, a multitude of research has shown the role of antioxidants derived from *Vaccinium* berries on chronic diseases such as cardiovascular disorders, diabetes, obesity, and cancer. Several studies have also investigated the effect of *Vaccinium* berry consumption on their ability to modulate the risk factors associated with oxidative stress, vascular function, inflammation, and lipid metabolism. Regarding cancer, studies showed that the consumption of berries reduces inflammation, inhibits angiogenesis, protects against DNA damage within the cell, and controls apoptosis and proliferation rates in malignant tumours. However, which components are responsible for the health benefits is still unclear. Reports show that whole berry consumption usually confers positive effects on human health, and the health-promoting potentials are likely due to the presence of polyphenols with antioxidant activities. Among these polyphenols, various *Vaccinium* berry species have been reported to contain anthocyanins and flavonoids. These two polyphenolic compounds are known to have higher antioxidant activity and are beneficial for human health. There are now several studies and human clinical trials documenting the beneficial effects of *Vaccinium* berries, and these findings suggest that they may be promising for preventing and treating neurodegenerative diseases. This review focuses primarily on dietary *Vaccinium* berries consumption effects on human health and their potential role as therapeutic agents.

KEYWORDS

Vaccinium, berries, antioxidants, phenolics, anthocyanin, flavonoids

1 Introduction

The eternal pursuit of finding and identifying health-promoting agents has changed how we view our food sources. We have introduced superfoods, food supplements, and nutraceuticals in developed countries, reinforcing the food industry's further growth (European Commission, 2006). Berries represent a large group of functional foods, also popularly known as “superfoods” due to their high content of disease-preventing and health-boosting chemicals (Ferlemi and Lamari, 2016). The genus *Vaccinium* L. (Ericaceae) includes approximately 450 diverse species, including these main commercial crops such as highbush blueberry (*V. corymbosum* L.), rabbiteye blueberry (*V. virgatum* Aiton, formerly known as *V. ashei* J.M.Reade), lowbush blueberry (*V. angustifolium* Aiton), bilberry (*V. myrtillus* L.), cranberry (*V. macrocarpon* Aiton), and lingonberry (*V. vitis-idaea* L.).

(Retamales and Hancock, 2018). In general, berry fruit consumption has increased in recent years. Several research papers show that the increased consumption of berries is associated with a reduced risk of disorders linked with reactive oxygen species (ROS), such as cardiovascular disorders, cancer, and other inflammatory processes (Gomes-Rochette et al., 2016). A large number of scientific research cites the effect of berry consumption in three major groups: (a) physical and mental health maintenance; (b) reduction of the rate of obesity; and (c) decreased rate of chronic diet-related diseases, e.g., cardiovascular and metabolic disorders, type II diabetes (Giampieri et al., 2015). *Vaccinium* berries contain high concentrations of beneficial nutrients and other bioactive phytochemicals, which has led them to become the center of attraction for researchers working on the potential role of these phytochemicals in preventing chronic diseases (Colak et al., 2016). Many research papers have shown that these active compounds, which are present in phenolic form, are associated with high antioxidant activity. Additionally, there are several studies suggesting that wild *Vaccinium* Berries contain higher phenolic content and antioxidant activity than cultivated berries (Braga et al., 2013; Dinstel et al., 2013; Kanget al., 2015). Phenolic compounds present in *Vaccinium* berries are classified into diverse groups, which include phenolic acids such as hydroxybenzoic and hydroxycinnamic acids and their derivatives, flavonoids (flavonols, flavanols, and anthocyanins), and tannins. Tannins are further sub-grouped into condensed tannins like proanthocyanidins and hydrolysable tannins (Ferlemi and Lamari, 2016). Blueberries (*Vaccinium* spp.) contain the highest amount of *p*-coumaric acid, chlorogenic acid, and other caffeic acid derivatives, which are types of hydroxycinnamic acids (Mattila et al., 2006; Määttä-Riihinen et al., 2004a; Kylli, 2010; Häkkinen et al., 1999; Taruscio et al., 2004). When it comes to flavonoids among the *Vaccinium* berries, lingonberries (*V. vitis-idaea* L.), highbush blueberries, and American cranberries (*V. macrocarpon*) are known to be the richest source of flavonols such as quercetin and myricetin derivatives and aglycones (Määttä-Riihinen et al., 2004b; Määttä-Riihinen et al., 2004a; Kylli, 2010; Häkkinen et al., 1999; Taruscio et al., 2004). These chemical compounds possess high antioxidant activity (Wilms et al., 2005) and play a major role in preventing many chronic diseases (Castañeda-Ovando et al., 2009; Duthie et al., 2006). However, the concentration of these compounds depends on the species, genotype, growing condition and their post-harvesting techniques (Manganaris et al., 2014; Kresty et al., 2006).

Anthocyanins are important secondary plant metabolites, primarily occurring as glycosides of their aglycone anthocyanidins. The contents of the small edible berries are responsible for their bright colours as this pigment is evenly distributed in the epidermal tissues of the berries (Del Bo et al., 2015). The pigment in anthocyanins is water-soluble and responsible for orange, red, purple, and blue in fruits and vegetables (Delgado-Vargas et al., 2000). Anthocyanins are present in substantial quantities in glycosylated and various other forms in European cranberries (*V. oxycoccus*) and blueberries (Del Bo et al., 2015). European blueberries or bilberries contain fifteen anthocyanins, such as delphinidin and cyanidin monoglycosides, malvidin glycosides, petunidin, and peonidin. In the case of American cranberries, the principal anthocyanins are cyanidins, while in the case of European

cranberries, it is peonidins (Norberto et al., 2013; Kaume et al., 2012; Gopalan et al., 2012; Seeram, 2008; Paredes-López et al., 2010). Similar to American cranberries, lingonberries also mainly contain cyanidin monoglycosides. Besides cyanidins, high and lowbush blueberries, lingonberries, and American cranberries contain procyanidins such as catechin and epicatechin polymers (Norberto et al., 2013; Zafra-Stone et al., 2007).

Commonly consumed *Vaccinium* berries have been studied for their effects on human health, but the nature and extent of their impact on humans remain vague. Hence, this article aims to provide a comprehensive overview of human clinical trials investigating the acute and chronic effects of *Vaccinium* berry polyphenols derived from fruits, their extracts and their derived products on inflammation, gut microbiota, diabetes, heart health, cancer, and brain activities.

2 Bioactive compounds

Plants produce numerous bioactive compounds, which belong to different classes of secondary metabolites including polyphenols, phytosterols, lipoates, carotenoids, etc. (Acquaviva et al., 2021; He et al., 2024). In berries, the most abundant bioactive compounds are phenolics, which are mostly found in leaves, fruits, and seeds but can also be present in other parts of plants. The chemical structure of the phenolic compounds carries one or more aromatic rings with one or more hydroxyl groups (Szajdek and Borowska, 2008; Nile and Park, 2014; Del Bo et al., 2015; Skrovankova et al., 2015). Phenolics are either present in free or conjugated forms with water or fat-soluble compounds (Figure 1). Conjugated forms of phenolics are predominantly present as conjugated hydroxycinnamic acids, flavonol glycosides, and anthocyanins (Määttä-Riihinen et al., 2004a). These phenolics are not species-specific but shared across the genera. Among the berry polyphenols, anthocyanins constitute a large percentage. They have a characteristic C6–C3–C6 carbon structure and are glycosylated polyhydroxy and polymethoxy derivatives of flavylum salts (Wallace, 2011). A study has reported that anthocyanins have a glycosidic structure containing more than two sugar molecules, such as galactose, arabinose, xylose, and glucose, that effectively connect with aglycon and form through the phenylpropanoid pathway. Anthocyanins have more than 600 compounds and more than 30 anthocyanidin compounds (Bilawal et al., 2021). The major phenolic compounds (anthocyanins) found in *Vaccinium* berries are listed in Table 1. Anthocyanins are uniquely characterized by an oxonium ion on the C ring and are highly pigmented (Pandey and Rizvi, 2009). Among these anthocyanin compounds, quercetin, myricetin and their glycosidic derivatives reach up to 30%. Anthocyanins, including procyanidins and anthocyanidins such as cyanidin, malvidin, peonidin, delphinidin, and petunidin, can account for up to 24% of all polyphenolic compounds. Phenolic acids primarily include *p*-coumaric acid, chlorogenic acid, caffeic acid, ferulic acid and vanillic acid. They account for up to 12% of the total polyphenols (Nemzer et al., 2022).

Plant phenolics were regarded as antinutritional and toxic for a long time as these compounds' chemical nature of functioning as an inhibitor to proteolytic, lipolytic and glycolytic enzymes reduces their ability to absorb nutrients (Olas, 2018). However,

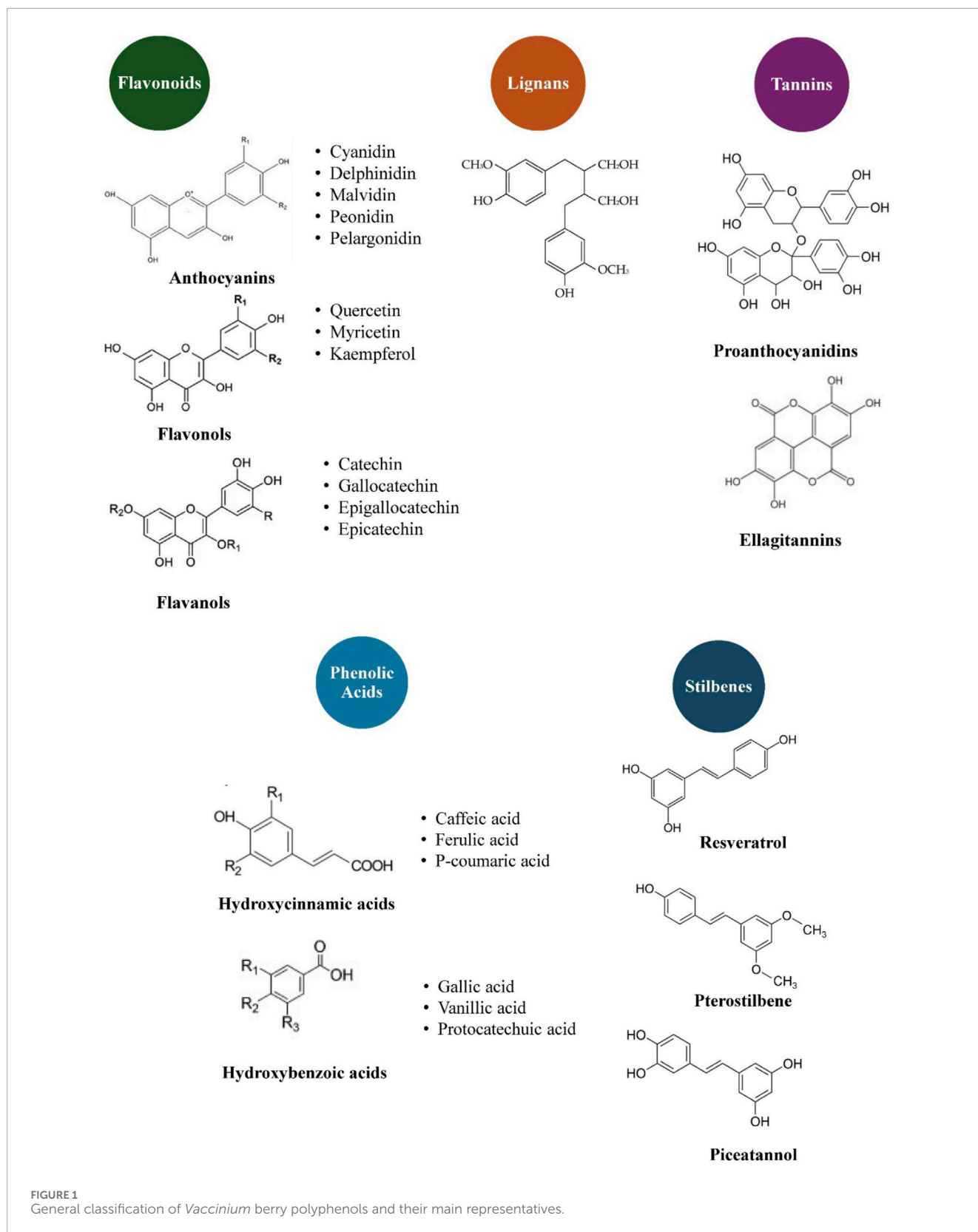


FIGURE 1
General classification of *Vaccinium* berry polyphenols and their main representatives.

the toxicity of the phenolic compounds derived from berries was generally unnoticed in the previous studies, while the benefits were observed. Apart from being a source of non-nutritive compounds

such as phenolics (Singh and Basu, 2012), *Vaccinium* berries also contain a wide range of nutritive compounds such as simple sugars like glucose and fructose, minerals, for example, phosphorus,

TABLE 1 Major polyphenols found in *Vaccinium* berries.

Type of berry	Scientific name	Types of polyphenols	References
Bilberry	<i>Vaccinium myrtillus</i>	delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, malvidin-3-O-glucoside	Määttä-Riihinen et al. (2004a)
Blueberry	<i>Vaccinium</i> spp.	delphinidin 3-O-galactoside, -arabinoside; malvidin 3-O-galactoside, petunidin 3-O-galactoside	Cho et al., 2004, Määttä-Riihinen et al., 2004a
Cranberry	<i>V. macrocarpon</i>	peonidin 3-O-galactoside, -arabinoside; cyanidin 3-O-galactoside	Zheng and Wang (2003)
Lingonberry	<i>V. vitis-idaea</i>	cyanidin 3-O-galactoside, peonidin 3-O-galactoside	Määttä-Riihinen et al. (2004a)

calcium, iron, potassium, magnesium, manganese, sodium and copper, etc. (Del Bo et al., 2015; Szajdek and Borowska, 2008). Iron and manganese are essential components of antioxidant enzymes among these above-mentioned minerals. Moreover, berries contain vitamins A and E, reducing inflammation and acting as antioxidants (Skrovankova et al., 2015). Apart from that, berries contain high concentrations of dietary fibres and low concentrations of lipids. These fibers reduce the concentration of low-density lipoproteins (LDL) in blood serum and reduce the chances of occurrence of cardiovascular and neurodegenerative diseases and cancer. All these nutritive, non-nutritive compounds, vitamins, and minerals in the *Vaccinium* berries synergistically affect human health (Olas et al., 2016).

Apart from the polyphenolic compounds, *Vaccinium* berries also contain major lipid groups such as unsaturated fatty acids, sterols, terpenoids, and others that have high biological activity. These lipids are different from those found in mammals, which is why consuming these lipids has a significant role in human metabolism (Klavins et al., 2015). The first report of the presence of lipids was investigated in cranberries (Croteau and Fagerson, 1969). Klavins et al. (2015) studied the lipid profile of blueberry, bilberry, lingonberry and cranberry grown in the wild in Latvian forests and bogs. The lipid profile revealed 111 different types of lipid fractions, including fatty acids, sterols, triterpenoids, alkanes, phenolic and carboxylic acids and carotenoids. Since then, this group of compounds has been studied in many berry species. However, there is no detailed report on lipids found in *Vaccinium* berries.

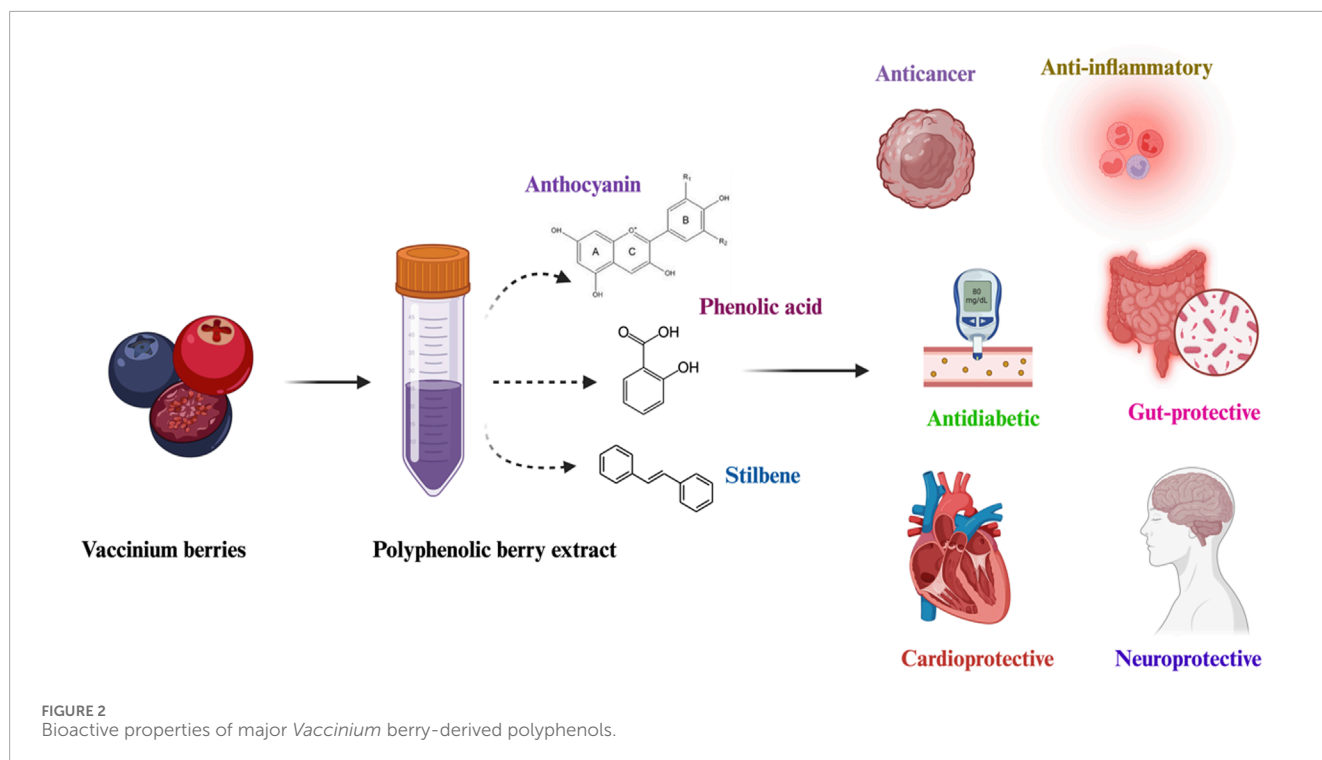
3 Biological activities

3.1 Overview of biological activities

Due to the response to the biotic and abiotic stresses, plants produce phytochemicals. They are also known as secondary metabolites. Like other fruits and vegetables, berries were found to be a great source of bioactive phytochemical components. Berry phytochemicals comprise bioactive components such as tannins, polysaccharides, alkaloids, vitamins, flavonoids, and other trace elements. They also contain sugar and fiber, which increases fruit taste and possesses many biological properties. In berries, these bioactive properties are directly related to the concentrations of

the various phytochemicals in these fruits. Berry research has always been generally focused on their antioxidant properties. The antioxidant properties of these phytochemicals reduce oxidative damage to DNA, RNA, proteins and lipids at the cellular level by scavenging reactive oxygen species (ROS) (Bilawal et al., 2021). ROS are responsible for triggering aging and several inflammatory conditions, as well as cancer (Sosa et al., 2013; Nemzer et al., 2022). They also promote the regeneration of other antioxidants and endogenous antioxidant defense systems (Bujor et al., 2019). The imbalance between oxidants and antioxidants results in abnormalities. It produces significant ROS, including $O_2^{\cdot-}$, $HO^{\cdot}$, NO, and $RO^{\cdot}$, which interferes with the cellular processes (Bujor et al., 2019; Maya-Cano et al., 2021). These superoxide ions can convert into hydrogen peroxide (H_2O_2), which can further convert into the highly reactive hydroxyl radical ($OH^{\cdot}$). Hydroxyl radicals, due to their high reactivity, cause oxidative damage, including lipid peroxidation in membranes, oxidative modification of proteins, and oxidative damage to DNA (Sosa et al., 2013).

Various methods have been used to determine the antioxidant activity of *Vaccinium* berries. Among them, the Folin-Ciocalteu method, the copper ion reducibility assay (CUPRAC), the ferric ion reducibility assay (FRAP), the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging method, and the ABTS method were mostly used in the scientific literature (Bujor et al., 2019). Goyali et al. (2013) examined the oxidative capacity of lowbush blueberry (*V. angustifolium*). It was found that the total phenolic content (TPC) value ranged from 34.2 to 42.7 mg GAE/g FW, total flavonoid content (TFC) from 12.7 to 22.3 mg CE/g FW, and proanthocyanidin content (PAC) from 4.7 to 6.5 mg CE/g FW in the greenhouse-grown and their cutting counterparts. In another study on half-high blueberries, results of the biochemical assays of the greenhouse-grown and somatic embryogenesis-derived plants revealed that TPC varied from 0.26 to 0.46 GAE/g lw, TFC varied from 7.93 to 11.65 CE/g lw, and antioxidant activity (AA) varied from 0.08 to 14.85 GAE/g lw. The results showed that the propagation method and genotype impact the phenols and flavonoids in the leaves (Ghosh et al., 2018). A study on *V. oxycoccus* and *V. macrocarpon* compared the AA by the Folin-Ciocalteu method. It was found that polyphenol quantity in *V. macrocarpon* was 296.3 mg/100 g fresh weight while in *V. oxycoccus*, it was 288.5 mg/100 g fresh weight. However, DPPH revealed that *V. oxycoccus* had a stronger antioxidant potential (16.4 μ mol TE/g FW) than *V. macrocarpon* varieties



(13.08 $\mu\text{mol TE/g FW}$), which led to the inference that the concentration of resveratrol in the analyzed samples of both the species that may have an impact on the AA of the varieties (Borowska et al., 2009). The antioxidant capacity of the *Vaccinium* species has been tested in *in-vivo* studies as well. A study was conducted on male *Drosophila melanogaster* to analyze the anti-aging effect of anthocyanins derived from the bilberry extracts. It was reported that the administration of anthocyanin extracts at the concentrations of 2.5, 5.0 and 10.0 mg/mL extended the life of the flies by 9.16%, 11.90% and 6.88%, respectively, compared to the control sample (Zhang and Dai, 2022). Several pieces of research show that the effect of phytochemicals derived from *Vaccinium* berries is directly associated with their anticancerous activities (Juranić and Žižak, 2005). Not only anticancerous properties, but numerous studies have shown that all these components are believed to hold a broad spectrum of biomedical functions, including anti-inflammatory, antimicrobial, antiviral and antioxidant properties (Figure 2) (Puupponen-Pimia et al., 2004; Seeram, 2008; Skrovankova et al., 2015). Due to their health-promoting activities, these berries are highly recommended for the human diet, as their antioxidant effects have been explored in several *in vitro* and *in vivo* studies (Juranić and Žižak, 2005; Skrovankova et al., 2015) (Table 2).

Studies have reported that adding fruits to our diet reduces the risk of chronic diseases like cancer, type II diabetes, obesity, and cardiovascular disorders (Skrovankova et al., 2015). It was revealed that dietary intake of flavonoids is associated with a lowered risk of all-cause mortality (Liu et al., 2017), including CVD. However, it is important to understand that all subclasses of flavonoids are not equally involved with cardioprotection, as there is a huge gap between flavonoids' bioavailability and bioactivity. Anthocyanins, a subclass of flavonoids, are one of the main

phytochemicals present in *Vaccinium* berries. It was found that anthocyanins have a greater bioavailability than was previously estimated (Czank et al., 2013). Several *in-vitro* studies have shown that anthocyanins are as bioactive as their parent compounds and sometimes even more (Amin et al., 2015; Keane et al., 2016). Anthocyanins are known to stimulate anti-inflammatory, anti-atherogenic, antioxidant and vasodilatory actions (Castaneda-Ovando et al., 2009; Edwards et al., 2015; Wang et al., 1999). Not only that but there is also a growing body of evidence revealing that dietary inclusion of anthocyanins improves *in vivo* vascular health (Fairlie-Jones et al., 2017; Jennings et al., 2012) as they have a potential underlying mechanism of augmenting endothelial-derived nitric oxide (NO) bioavailability. It was found that anthocyanins can directly or indirectly increase NO availability either by upregulating endothelial nitric oxide synthase and L-arginine pathways or via optimizing nitrate-nitrite-NO pathway and reducing NO degradation by their antioxidant activities (Edwards et al., 2015; Rocha et al., 2014). NO is an important molecule as it regulates endothelial homeostasis. Anthocyanin displays strong antioxidant activities, and foods high in anthocyanin have been shown to improve endothelial function (Rodriguez-Mateos et al., 2013). Research on the effect of flavonoid intake on mortality showed that intake of anthocyanidins is positively correlated with decreased risk of CVD mortality (Summary relative risk = 0.89, 95% CI: 0.83, 0.95) when tested amongst 5 cohorts (Grosso et al., 2017). All the available research done *in vitro* and *in vivo* on antioxidant and anticancerous properties of *Vaccinium* berries has advanced our understanding of their effects on human health and diseases. Therefore, the current review comprehensively summarizes what is currently known about the medicinal potential of these berries.

TABLE 2 Effect of *Vaccinium* berry consumption on metabolic syndrome and risk factors modulation.

Type of berry	Intervention	Results	References
Blueberry	8-week randomized (R), single bound (SB), placebo-controlled (PC), parallel intervention (PI)	Reduced systolic and diastolic blood pressure	Basu et al. (2010)
	Diabetic C57b1/6J mice model with acute feeding of blueberry extracts with Labrasol (Gavage- 500 mg/kg body wt.)	Lowered elevated blood glucose levels	Grace et al. (2009)
	8-week, R, double-blind (DB), PC, PI	Reduced systolic and diastolic blood pressure, increased NO plasma levels and superoxide dismutase activity	Johnson et al. (2015)
	<i>In vitro</i> -TC-tet model	2.8-fold increase in cell proliferation	Martineau et al. (2006)
	6-week R, DB, PC, crossover intervention (CI)	Reduced endogenous and oxidatively induced DNA damage	Riso et al. (2013)
	6-week R, DB, PC, PI	Higher insulin sensitivity	Stull et al. (2010)
	6-week R, DB, PC, PI	Increased blood pressure and insulin sensitivity	Stull et al. (2015)
Bilberry	4-week, R, C, PI	Decreased serum levels of CRP, IL-6, IL-15, TNF- α , MIG	Karlsen et al. (2010)
	8-week R, Controlled (C), PI	Reduced serum levels of hs-CRP, IL-6, IL-12 and inflammation score and decreased expression of MMD and CCR2 transcripts	Kolehmainen et al. (2012)
	5-week, R, CI	Decreased body weight, waist circumference, increased insulin sensitivity	Lehtonen et al. (2011)
	R, C, 2 arm, chronic feeding (24 weeks) in type II diabetic patients	Significantly lowered fasting plasma glucose and homeostasis model assessment for insulin resistance index	Li et al. (2015)
	Diabetic Mice model with chronic feeding of bilberry extract for 5 weeks	Improved hyperglycemia and insulin sensitivity	Takikawa et al. (2010)
Cranberry	8-week, R, DB, PC, PI	Reduced ox-LDL, MDA and HNE plasma/serum levels	Basu et al. (2011)
	12-week R, C, PI	Increased insulin levels after placebo treatment	Chambers and Camire (2003)
	Post-prandial (PP) 4-week R, DB, CI	Increased flow mediated dilation and reduced carotid-femoral pulse wave velocity (a measure of central aortic stiffness) and HDL-cholesterol	Dohadwala et al. (2011)
	12-week, R, PC, DB, PI	Lower total cholesterol	Lee et al. (2008)
	60 days, PI	Decreased serum homocysteine levels, lipoperoxidation, and protein oxidation, increased serum folic acid levels	Lozovoy et al. (2013)
	2-week intervention	Reduced BMI, plasma ox-LDL levels, and higher Total plasma antioxidant capacity	Ruel et al. (2005)

(Continued on the following page)

TABLE 2 (Continued) Effect of *Vaccinium* berry consumption on metabolic syndrome and risk factors modulation.

Type of berry	Intervention	Results	References
	12-week intervention (3x 4-week intervention with 125, 250, and 500 mL/day cranberry juice)	Decreased body weight, BMI, waist circumference, waist-to-hip ratio, total HDL cholesterol apo B, after intervention with 250 and 500 mL cranberry juice Increased plasma nitrite/nitrate following intervention with 500 mL, and higher plasma antioxidant capacity following 250- and 500-mL cranberry juice. Higher HDL cholesterol following 250 mL cranberry juice	Ruel et al. (2006)
	12-week intervention (3x 4-week intervention with 125, 250 and 500 mL/day cranberry juice)	Reduced ox-LDL following 250 and 500 mL cranberry juice and decreased systolic blood pressure, s-VCAM, ICAM plasma levels following 500 mL cranberry juice. Decreased ox-LDL, ICAM plasma levels in subjects With previous history of metabolic syndrome following 12-week intervention Higher HDL cholesterol following 250 and 500 mL cranberry juice	Ruel et al. (2008)
	4-week, PC, DB, CI	Reduced arterial stiffness and global blood pressure	Ruel et al. (2013)
	12-week, R, DB, PI	Reduced glucose level	Shidfar et al. (2012)
	PP intervention	Decreased plasma insulin and glycemic response	Wilson et al. (2008)
	PP cross-over Intervention	Decreased glycemic and insulinemic response following SDC-LS	Wilson et al. (2010)
Lingonberry	Administration of Quercetin-rich extract from lingonberry to C2C12 myoblasts	Increased insulin-independent glucose uptake and stimulated AMPK	Eid et al. (2010)
	RCT, 4 arm, PP crossover	Reduced sucrose-induced PP glucose and insulin concentrations during the first half an hour post-intake Prevented sucrose-induced late PP hypoglycemic response	Torronen et al. (2012)

3.2 Anti-inflammatory effect

Inflammation is known as the first line of defense in animals in response to the attack of pathogens, allergens, or any kind of tissue injury. As a result of the inflammatory response, macrophages, which are part of our immunity system, release inflammatory mediators such as interleukins, nitric oxide (NO), tumour necrosis factor- α (TNF- α), and prostaglandin (PGE2) (Joseph et al., 2014). Usually, overexpression of such mediators is associated with a response to type II diabetes, cancer, and cardiovascular diseases (CVDs) (Joseph et al., 2016). Over the years, much-accumulated data on pre-clinical studies of mice shows that *Vaccinium* berries, such as blueberries, reduce adiposity while increasing insulin sensitivity and decreasing inflammatory responses (Land Lail et al., 2021). A study on blueberries and blackberries disclosed that a daily dietary intake of 9–18.9 mg/kg BW of phenolic extract reduced cholesterol in blood plasma and metabolic dysfunctions induced by

high-fat diet (HFD) in C57BL/6J mice (Joseph et al., 2016). Another study showed that the continuous intake of Nordic wild blueberries with HFD (45% fat) for 12 weeks slows down weight gain because of obesity-induced inflammation in C57BL/6 (Mykkänen et al., 2014). Similarly, HFD mixed with blueberry powder for 12 weeks helped to restore innate immune response and T-cell proliferation in HFD-induced obese mice (Lewis et al., 2018). This also shows that consuming an adequate quantity of blueberry with an HFD may target the crucial factors in immune response and inflammation. A study in macrophages (RAW 264.7) revealed that blueberry anthocyanin reduced the expressions of two cytokines, such as Tumor necrosis factor α (TNF α) and Interleukin 1 β (IL-1 β), after 3 h of treatment by suppressing the NF- κ B pathway expression (Lee et al., 2014). A study on *V. floribundum* revealed that phenolic extract of the berries reduced lipid accumulation and inhibited the production of anti-inflammatory response-inducing enzymes such as PGE2, NO, COX-2, and iNOS in macrophages (LPS-RAW 264.7)

(Schreckinger et al., 2010). The antioxidant and anti-inflammatory effects correlate with phenolic content in berries being dependent on the species, varieties, and geographical location (Grace et al., 2014).

3.3 Maintenance of gut microbiota and antimicrobial and antiviral activities

Dietary consumption of berries is also known to help grow bacteria in the gut. Berries contain polyphenols, which promote symbiotic bacteria, reducing dysbiosis disorders (Pap et al., 2021). Phenolics and flavonoids present in the berries are also found to be effective against pathogenic bacteria and fungi (Kranz et al., 2020). The human gut microbiota consists of viruses, bacteria, fungi, protozoa, and archaeobacteria (Burgos-Edwards et al., 2020). A berry diet can promote the growth of beneficial microbiota and inhibit negative bacterial populations in the gut. Studies have shown that black raspberries and *Vaccinium* berries, such as blueberries and lingonberries, help increase the growth of *Lactobacillus* and some of their subspecies, the population of *Bifidobacterium*, and help in slowing down obesity-related problems (Pap et al., 2021). A study revealed that cranberry juice has a strong activity against co-adhesion and co-aggregation of oral plaque bacteria (Gupta et al., 2015). In another study, several types of berry extracts, including lingonberry (*V. vitis-idaea*), bilberry (*V. myrtillus*), were found to have antibacterial effects against commonly found pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* (Tian et al., 2018). Berry and leaf extracts of lingonberry have shown maximum anti-microbial effects against *S. aureus* (strain ATCC-25923) and MRSA (clinical) oral cavity isolates (Kryvtsova et al., 2020). Research done on Romanian blueberry (var. Elliot) showed that minimum inhibitory concentration of leaf extract was discovered to be highly effective against bacterial strains such as *S. aureus*, *Escherichia faecalis*, *Rhodococcus equi*, *E. coli*, and *Klebsiella pneumoniae* and a few *Candida* fungal strains, such as *C. albicans*, *C. zeylanoides*, and *C. parapsilosis* (Ştefanescu et al., 2020).

3.4 Antidiabetic effect

Diabetes mellitus (DM) is a chronic disease which is associated with other lethal diseases, including hypertension, obesity, cardiovascular diseases, and hyperlipidemia. DM is classified into type I and type II; among these, type II contributes to more than 90% of all diabetes globally (Batool et al., 2021; Skyler et al., 2017). According to the World Health Organization (WHO), 108 million in 1980 and 422 million in 2014 were living with diabetes. A report done by Cho and co-workers in 2018 revealed that 451 million adults were diagnosed globally with diabetes, and it was predicted to reach up to 693 million by 2045 (Cho et al., 2018). Either form of DM is known to increase the risk of serious chronic illnesses such as blocking heart and blood vessels and affecting kidneys, eyes and nerve functions. This is due to the high blood sugar level, which affects and damages the nerves and the blood vessels controlling these organs. Blockage of the heart and blood vessels may also cause complications like CAD and stroke (Edirisinghe and Burton-Freeman, 2016). Not only CAD but CVD is also known to

be the prime cause of death in patients with DM (Grundy et al., 1999). Several researchers have investigated and supported the idea that berry polyphenols have an antidiabetic effect, which is usually associated with glucose homeostasis. Glucose homeostasis can be regulated in an insulin-dependent and independent manner. Polyphenols derived from berries have been studied for years for their effects on insulin-dependent glucose metabolism. This can be achieved by regulating insulin secretion via modulating pancreatic-cell function and peripheral tissue sensitivity (Edirisinghe and Burton-Freeman, 2016). It was found that Canadian blueberry extracts increased 3H-thymidine incorporation in TC-tet cells and increased cell proliferation by 2.8-fold (Martineau et al., 2006). In another study, it was seen that dietary supplementation of freeze-dried whole blueberry powder in a double-blinded and placebo-controlled sensitivity had antidiabetic effects in obese, nondiabetic, and insulin-resistant human participants ($p < 0.05$) when administered for over 6 weeks and reported to improve insulin sensitivity (Stull et al., 2010). In diabetic C57b1/6J mice, feeding them a blueberry diet also displayed antidiabetic activity. Blueberry fraction enriched with phenolics and anthocyanin, in addition to Labrasol (a pharmaceutically acceptable self-micro emulsifying drug delivery system), was reported to lower raised blood glucose levels when fed to diabetic C57b1/6J mice. The hypoglycemic effect of the concoction was equivalent to that of metformin, a well-known antidiabetic drug (Grace et al., 2009). A study on postprandial healthy women showed that the administration of either whole lingonberries or extracts reduced sucrose-induced postprandial glucose and insulin concentrations throughout the first 30 min of consumption. Furthermore, it was seen that during the second-hour post-intake, the concentration declined slowly but improved the overall glycemic profile ($p < 0.05$). Additionally, the investigation showed that whole berries and extracts stopped the sucrose-induced late postprandial hypoglycemic response and the compensatory free fatty acid recovery (Torrönen et al., 2012). Schell et al. (2017) experimented with the anti-diabetic activities of cranberry extract in T2D and revealed that administration of dried cranberry significantly improved the postprandial glucose excursion. These situations revealed that the increasing incidence of DM and its associated diseases can be controlled with a *Vaccinium* berry-rich diet.

3.5 Cardioprotective effect

Several studies have shown a strong connection between berry-derived anthocyanins and cardiovascular health. Clinical studies such as the Kuopio Ischemic Heart Disease Risk Factor Study for a follow-up of around 13 years revealed a considerably lowered risk of CVD-associated death among men who had a significantly higher quartile of berry intake (>408 g/day) than men with the lowest intake (<133 g/day) (Basu et al., 2010). Although these results positively impacted CVD risk factors, the models also showed an inverse correlation between the intake of fruits, berries, and vegetables and serum haptoglobin in blood, an inflammation marker (Rissanen et al., 2003). A large group of postmenopausal women ($n = 34,489$) contributing to a CVD mortality study associated with blueberry intake for a 16-year follow-up period at Iowa Women's Health Study found that consumption of blueberries once a week

significantly decreased coronary heart disease mortality using an age- and energy-adjusted model (Mink et al., 2007). The most significant conclusion drawn from all these clinical studies was that dietary inclusion of berries in everyday diet might decrease LDL oxidation and lipid peroxidation, reduce plasma glucose or total cholesterol, and increase HDL cholesterol and plasma or urinary antioxidant capacity (Basu et al., 2010). As a high content of plasma glucose, lipids, and lipid oxidation have been associated with coronary artery disease (CAD), these researchers suggested that edible berries, including berries from the *Vaccinium* family, can be consumed to reduce the risk factors of CAD significantly (Krentz, 2003; Gupta et al., 2009). It was further shown that the regular inclusion of berries in the diet also reduces the postprandial metabolic and oxidative stresses, which are also associated with CAD (O'Keefe et al., 2008). Cranberry has been proven to be very effective against several health issues, including the management of systolic blood pressure in healthy men (Ruel et al., 2008). Another study showed that cranberry extract positively affects lipid profiles in subjects with type I or II DM (Lee et al., 2008). Various other studies showed that consuming blueberries and cranberries significantly decreases postprandial oxidative stress, specifically lipid peroxidation (Pedersen et al., 2000; Kay and Holub, 2002; Mazza et al., 2002; Ruel et al., 2005; Ruel et al., 2006). Many studies suggest the inverse correlation of flavonoid (specifically anthocyanin) intake with the occurrence of CVD and the elevated risk factors involved with CVD (Cassidy et al., 2011; Jennings et al., 2012; McCullough et al., 2012). Berry polyphenols positively affect the lipid profile and endothelial function of the blood vessels by reducing blood pressure and platelet accumulation (Pojer et al., 2013; Rodriguez-Mateos et al., 2014a; Rodriguez-Mateos et al., 2014c). Not only that, but their antioxidant and anti-inflammatory activities also support cardiovascular health (Rodriguez-Mateos et al., 2014a; Pojer et al., 2013). Not only the berry extracts from fresh or frozen fruits but baked goods with lowbush blueberries (*V. angustifolium*) exhibited similar effects on endothelial function (FMV) as with the drink made with freeze-dried blueberry powder (Rodriguez-Mateos et al., 2014b). All this available research suggests that dietary inclusion of berries can potentially be used as a therapy for pre-hypertension and hypertension management (Basu et al., 2010). However, none of these clinical trials were found to interfere with biomarkers responsible for inflammation, except in one case, it was found that cranberry juice supplementation substantially decreases adhesion molecules in healthy volunteers (Ruel et al., 2008).

3.6 Anticancerous effect

Among all the other healthy eating habits, including berries in the everyday diet is one of the most promising ways to prevent cancer (Baby et al., 2018). Phytochemicals present in the berry extracts influence genome stability at several stages, such as malignant transformation, initiation modulation, promotion and progression of cancer (Duthie, 2007). In general, berry extracts combat carcinogenesis in animal models. However, when exposed to chemical carcinogens, blueberry extracts did not protect animal models. DNA damage was noticed in the tumours, and there was no evidence of a reduction in the proliferation rate of the

cancerous cells or size of the tumours when pre- or co-treated with blueberry extract. These results further suggested that despite having higher antioxidant capacity than other berry species, blueberries are deficient in one or more cryoprotective phytochemicals, preventing chemically induced cancer in the animal model (Aziz et al., 2002).

It was reported that the growth of the HT-29 cell lines in colon cancer was significantly inhibited using phenolics, such as anthocyanins and flavonols extracted from cranberry juice (Desrouillères et al., 2020). In another study, lingonberry-derived quercetin and procyanidin-A2 displayed anticancerous activity against colon (HT-29), melanoma (IGR39), and renal (CaKi-1) cancer. It was also observed that quercetin demonstrated the best anticancerous activity against renal cell carcinoma (CaKi-1) (Vilkickyte et al., 2020). Fermented catechol extracted from fermented Rabbiteye blueberry (*V. virgatum*) extract along with *Lactiplantibacillus plantarum* (CK10) resulted in inducing apoptosis and inhibiting HeLa cell multiplication after 24–72 h of administration (Ryu et al., 2019). Various flavonol compounds such as kaempferol, quercetin, and genistein acid extracted from bilberry or European blueberry (*V. myrtillus*) demonstrated cytotoxic effects against HCT-116 colon cancer cells. This study further showed that kaempferol had better anticancerous activity than other flavonols, inducing apoptosis by preventing apoptosis proteins (IAPs) inhibitors (Sezer et al., 2019). Extracts from *Vaccinium*-berries such as blueberry, bilberry, cranberry, and lingonberry contain anthocyanins and ellagic acid, which are known to exhibit anticarcinogenic activities (Seeram, 2008). Other reports suggest that cranberry extracts and press cake can significantly inhibit cell growth in breast, prostate, skin, brain and liver cancer cases by stopping the G1 stage of the cell cycle and initiating apoptosis (Sun et al., 2002; Sun and Liu, 2006). Additionally, bilberry extracts were found to induce programmed cell death in patients with leukemia (Katsube et al., 2003). Extracts of several fruits, including blueberries, blackcurrant, black chokeberries, and raspberries, showed a strong antagonistic effect on the proliferation of breast cancer cell line MCF-7 and the colon cancer cell line HT29 and reduced their growth by up to 74% (Olsson et al., 2004).

3.7 Neuroprotective activity

There is much evidence supporting that oxidative stress of reactive oxygen species (ROS) in cells is responsible for the progression of neurodegenerative diseases such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (Lim et al., 2024), and berry-derived antioxidants were effective against neurodegenerative diseases (Nile and Park, 2014). Berry antioxidants also demonstrate neuroprotective activities, and several studies have shown that phenolic components from the *Vaccinium* species have anti-inflammatory and neuroprotective effects. In a study done with blueberry and lingonberry, brain-derived cell cultures from rats were found to be significantly tolerant against glutamate excitotoxicity when treated with blueberry extracts for 24 h. However, lingonberry (*V. vitis-idaea* L.) extracts failed to provide any protection against it. Additionally, leaf extracts of blueberry and lingonberry displayed significant neuroprotective effects, while among the fruits, only blueberry fruits showed neuroprotection on the same brain cells

(Vyas et al., 2013). That is why further work has been done to investigate the neuroprotective activity of blueberry leaf extract in microglial cells derived from mice. Microglial cells are the brain's first line of defence cells; glutamate or α -synuclein was administered to microglial cells to induce an inflammatory response. The cells were treated with blueberry fruit and leaf extracts, which decreased cell death and reduced inflammation after 24 h. This result further points to the fact that a blueberry-rich diet, with leaves or fruits, can protect against neurodegenerative disorders (Debnath-Canning et al., 2020). Over the years, studies have suggested that the inclusion of blueberries in a regular diet may help with age-related and oxidative stress, which are responsible for declined brain function (Wang et al., 2005; Krikorian et al., 2010). Another report suggested that a blueberry-supplemented diet can improve behavioural deficits associated with age or a high-fat diet (Carey et al., 2014). Overproducing ROS and reactive nitrogen species (RNS) free radicals cause aging and neurodegenerative diseases. Cortical cell cultures derived from neonatal rat pups were intoxicated with glutamate for 24 h, and it was seen that glutamate was responsible for morphological disruptions such as increased formation of dark punctae and disruption of cell bodies. Glutamate-treated cells were administered with lingonberry and blueberry leaf and fruit extract. They were found that, while lingonberry fruits failed to provide any protection from glutamate toxicity, the leaf extracts from both the berries and blueberry fruit extract displayed no cell death in the presence of glutamate (Kalidindi, 2014).

It was found that aged rats fed with blueberries have shown a reduction of ischemia-induced apoptosis in brain cells which is due to their capability of interacting with ROS and RNS, which accumulated during the ischemic phase in the central nervous system (Wang et al., 2005; Andres-Lacueva et al., 2005). Krikorian et al. (2010) reported in older humans that improved memory capabilities were detected by increased synaptic plasticity as a result of microglial modulation of the microglia-neuron crosstalk through the increase of the expression of CX3CR1 receptor was associated with a blueberry rich diet (Meireles et al., 2016). Memory loss is often associated with oxidative damage to lipids, proteins, and nucleic acids. Oxidative damage to all three can disrupt neural function. It was seen that bilberry extract was significantly effective against oxidative damage by decreasing lipid peroxides and increasing superoxide dismutase activity in the brain. Additionally, it was found that long-term supplementation of bilberry extract in the diet of the OXYS rats prevented learning and memory deficits (Rahman, 2007; Uttara et al., 2009). These findings specifically signify the effect of *Vaccinium* berry antioxidants on the neuroprotection of brain function.

4 Conclusion

Several *in vitro* and *in vivo* studies now indicate that berries positively impact human health by acting as strong anticancer and antioxidant agents. They are an ideal dietary source of bioactive components and could play a role in reducing cancer risk. The unique phytochemical constituents in berries act individually or synergistically to provide protection against several health disorders, including cancer and CADs. It is evident from this review that a

lot has been done in this direction, but much more needs to be done to pinpoint the molecular mechanisms associated with the most beneficial phytochemicals that make up these nutritious fruits. The review summarizes the effects of bioactive compounds present in *Vaccinium* berries and their function against cardiovascular and neurodegenerative diseases. It was seen that measurable criteria like total anthocyanin or total phenolic content and total antioxidant content may also be associated with the effectiveness of health benefits. Overall, more *in vivo* data are required to understand the mechanisms of action, while more human clinical trials using different parameters such as gender, age, and any pre-existing condition should be performed such new information on the bioactive components of berries can be revealed, and the existing information could be validated. Also, using berry phenolic compounds as antimicrobial agents provides many possibilities for use in the food and medical industry. It will also be a very interesting topic for future research priority by developing new ways for berry compounds to avoid and manage antibiotic-resistant infections. In addition to the phenolic compounds, phytosterols are well-known for their antioxidant activities. Epidemiological and experimental reports suggest that they help reduce cholesterol and potentially protect against several types of cancer. Furthermore, berry lipids are also used in many commercial products. These ignited a general interest in studying these compounds in depth to understand their potential application in cosmetics, pharmacy and the food industry.

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Conflict of interest

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The role of potential oxidative biomarkers in the prognosis of intracerebral hemorrhage and the exploration antioxidants as possible preventive and treatment options

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Intracerebral hemorrhage (ICH) is a non traumatic hemorrhage that occurs in a certain part of the brain. It usually leads to brain cell damage. According to a large number of experimental research, oxidative stress is an important pathophysiological processes of cerebral hemorrhage. In this paper, we aim to determine how changes in oxidative stress biomarkers indicate the damage degree of cerebral hemorrhage, and to explore and summarize potential treatments or interventions. We found that patients with cerebral hemorrhage are characterized by increased levels of oxidative stress markers, such as total malondialdehyde (MDA), F2 isoprostaglandin, hydroxynonenal, myeloperoxidase and protein hydroxyl. Therefore, the changes of oxidative stress caused by ICH on these markers can be used to evaluate and diagnose ICH, predict its prognosis, and guide preventive treatment to turn to antioxidant based treatment as a new treatment alternative.

KEYWORDS

oxidative stress, intracerebral hemorrhage, oxidative stress biomarkers, antioxidant therapy, stroke

1 Introduction

Intracerebral hemorrhage (ICH) is a serious neurological disease with high morbidity and mortality worldwide (GBD 2019 Stroke Collaborators, 2021). The global economic burden of stroke is estimated to exceed 721 billion US dollars, representing approximately 0.66% of the global GDP (Feigin et al., 2022). On a global scale, the absolute incidence of stroke has risen by 37%, with hemorrhagic stroke accounting for 47% of all cases (GBD 2016 Neurology Collaborators, 2019). As a destructive form of stroke, ICH has a higher disability rate (Gong et al., 2021).

The risk factors of ICH are usually divided into unalterable risks (previous history of ICH, age, gender, race, cerebral amyloid angiopathy, chronic kidney disease, congenital

coagulation dysfunction, etc.) and changeable risk factors (hypertension, coagulation dysfunction, current smoking, excessive drinking, diabetes, sympathetic nerve/illegal drugs, etc.) (Larsson et al., 2024). According to statistics, only 6 out of every 10 ICH patients can survive 1 month after the onset (Stokum et al., 2021), and two-thirds of the survivors are moderately or severely disabled (Chen et al., 2021a). Survivors also have motor, sensory or cognitive impairment, which ultimately affects cognitive, work and social abilities (Shi et al., 2021).

Although many preclinical and clinical trials have been completed in the past decades, and these trials have clarified the potential causes and mechanisms of cerebral hemorrhage injury, including the correlation between edema, apoptosis and oxidative stress (Li Y. et al., 2021; Li et al., 2020; Bobinger et al., 2018; Helmuth et al., 2023). However, no treatment has been proved to significantly improve the mortality and neurological prognosis after ICH. ICH surgical treatment focuses on risk factor management and prevention of deterioration after initial bleeding (Hanley, 2009; Mendelow, 2015). Many trials investigated the best drug and surgical management of ICH, but did not significantly improve the survival rate and functional outcome (Magid-Bernstein et al., 2022a; Chen P. et al., 2022; Kellner et al., 2021). Therefore, the prospect of ICH treatment is more based on primary prevention, ultra early hemostasis treatment and injury protection by pathobiology and prognostic biomarkers.

Oxidative stress (OS) is a serious imbalance between the generation of large amount of oxidative active substances and the antioxidant system in organisms. Common oxidative active substances include reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Yoshikawa and You, 2024). Following ICH, the metabolism of hemoglobin released from the hematoma results in substantial iron accumulation, disrupting cellular iron homeostasis. This disruption further impairs mitochondrial function and promotes the generation of ROS (Yan et al., 2023a). The pathological injury mechanism after ICH mainly includes primary injury and secondary injury. The term refers to the initial physical damage to brain structures caused by rupture of small arteries and hemorrhage, along with the rise in intracranial pressure resulting from the mass effect of the hematoma. Secondary injury refers to the damage of toxic substances released by hematoma to the tissues around hematoma, which will further aggravate the tissue injury and neurological function defect after ICH (Hilkens et al., 2024). Oxidative stress induced neuronal damage is a major factor in the secondary injury process after ICH (Magid-Bernstein et al., 2022b). After ICH, hematoma can lead to rapid and continuous increase of intracranial ROS, leading to neuronal death, and finally secondary neurological dysfunction (Zhang et al., 2022). ROS can directly attack lipids, proteins, nucleic acids and other macromolecules to cause corresponding damage, and can also aggravate tissue damage by activating inflammatory response (Jomova et al., 2023). Due to the high demand for oxygen by neuronal cells to produce ATP via the mitochondrial respiratory chain in order to sustain normal cellular function, this process also generates substantial precursor molecules for the production of ROS (Feng et al., 2024a). Furthermore, brain tissue is particularly susceptible to oxidative damage due to its high content of polyunsaturated fatty acids, which are prone to ROS-induced oxidation. This makes the brain less equipped with robust antioxidant defense

mechanisms (Yan et al., 2023b). The brain's high oxidative metabolic rate, coupled with its low antioxidant capacity, makes it highly susceptible to ROS-induced damage. This leads to structural and functional abnormalities in mitochondria, thereby initiating the mitochondrial apoptosis pathway (Xie et al., 2020a; Zhao et al., 2024; Wang M. et al., 2022).

The main source of ROS is the mitochondrial electron transport chain and enzymatic reactions catalyzed by various enzymes (Cheung and Vousden, 2022). The superoxide anion produced by mitochondria is a by-product of the electron leakage of mitochondrial respiratory chain complex. Both superoxide anion and its product hydrogen peroxide are considered to be mitochondrial ROS (mtROS) (Okoye et al., 2023). During the pathogenesis of ICH, impaired energy supply leads to a partial loss of ion channel function in maintaining the electrochemical gradient across the cell membrane. This results in an influx of calcium ions, a reduction in the selective permeability of the mitochondrial permeability transition pore, and the influx of ions, ROS, and other small molecules into the cell. Consequently, mitochondrial dysfunction occurs, accompanied by excessive accumulation of oxidative free radicals, contributing to oxidative damage (Di et al., 2023).

Beyond mitochondria, NADPH oxidase (NOX) and myeloperoxidase (MPO) are also critical sources of superoxide anions, which serve as key reactive free radicals following ICH. NOX is a specialized enzyme that regulates the generation of ROS. ROS generated by NOX function as secondary messengers, participating in the regulation of cell differentiation, proliferation, and apoptosis. The NOX2 and NOX4 isoforms are particularly important in modulating both the physiological and pathological processes in brain tissue (Mamelak, 2024). In an experimental model of ICH in rats, upregulation of NOX4 expression was observed, which may contribute to the disruption of oxidative stress balance in brain cells (Xie et al., 2020b). Other studies have confirmed that the increase of NOX2 aggravates the oxidative damage after ICH (Wang et al., 2018).

As another important source of ROS in ICH, MPO enhances the reactivity of H_2O_2 by producing hypochlorite, free radicals and RNS (Lin et al., 2024). When MPO breaks out in neutrophil respiration, heme is required as a cofactor and hypochlorite is produced from the reaction of H_2O_2 and chloride (Marcinkiewicz and Walczewska, 2020). MPO tends to aggravate brain injury (Manoharan et al., 2024). Animal experiments found that the expression of MPO protein in rat brain tissue increased after ICH, and inhibition of MPO can alleviate ICH induced brain injury (Zuo et al., 2022a).

Other major enzyme pathways that catalyze ROS release are endoplasmic reticulum, hemoglobin, ferrous and ferritin. The metabolism of hemoglobin released from hematoma after ICH will cause serious iron ion overload. Excessive free iron release generates superoxide anion and highly active hydroxyl radical through Fenton and Haber Weiss reaction, leading to imbalance of brain iron homeostasis and promoting ROS (Zhu et al., 2021). In addition, a large number of free radicals are produced under the induction of products after the decomposition of blood cells, such as thrombin and heme. These reactions produce ROS, such as O_2^- and HO^- (Xu et al., 2023). ICH can cause endoplasmic reticulum stress (ERS), and decreased expression of c/eip homologous protein (CHOP) will

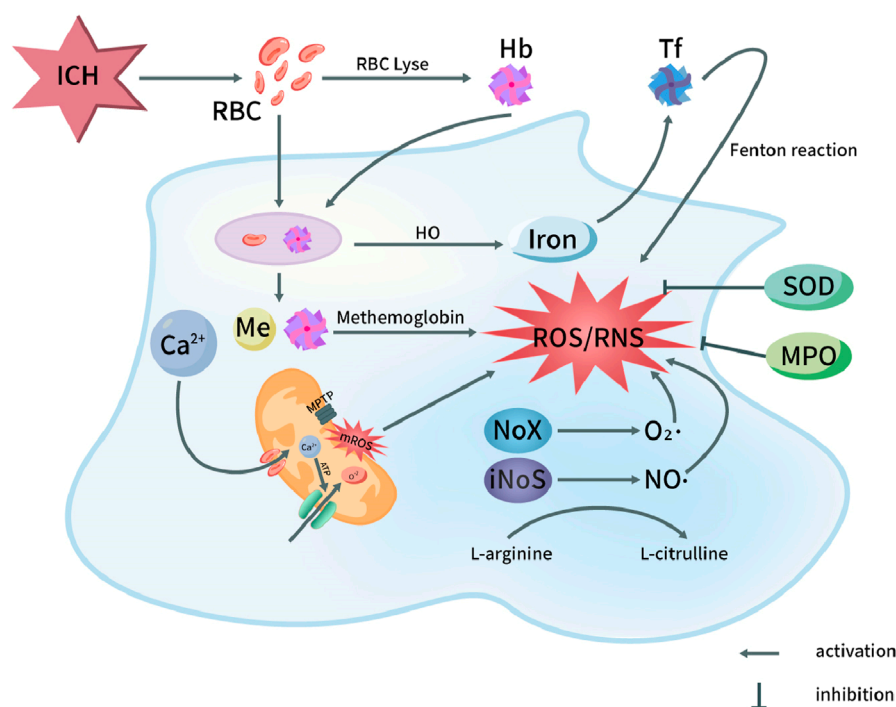


FIGURE 1

Oxidative stress pathways in pathophysiology of ICH. After ICH, the destruction of red blood cells in the hematoma releases hemoglobin and heme. Both red blood cells and degraded heme components are phagocytosed, leading to the oxidation of hemoglobin into methemoglobin, which subsequently generates reactive oxygen species (ROS). Iron is transported into the nervous system, ultimately triggering the Fenton reaction and producing ROS. On the other hand, the opening of the mitochondrial permeability transition pore leads to the release of ROS, with Ca^{2+} influx playing a role in this process. Additionally, the activation of NOX and NOS in microglial cells results in the production of superoxide (O_2^-) by NOX and nitric oxide (NO) by NOS through the conversion of L-arginine to L-citrulline. This activation contributes to the generation of ROS and reactive nitrogen species (RNS). Furthermore, myeloperoxidase (MPO) and superoxide dismutase (SOD) exert inhibitory effects on the production of ROS/RNS. Hb, Hemoglobin; HO, Heme oxygenase; NOS, Nitric oxide synthase; ICH, Intracerebral hemorrhage; MPTP, Mitochondrial permeability transition pore; RBC, Red blood cell; Tf, Transferrin; NO , Nitric oxide; O_2^- , Superoxide anion radical; ROS, Reactive oxygen species; NOX, NADPH oxidase; MPO, Myeloperoxidase; mROS, Mitochondrial reactive oxygen species; RNS, Reactive nitrogen species; SOD, Superoxide dismutase.

lead to the secondary increase of ROS level (Chen et al., 2021b). Other factors, in the inflammatory reaction process after ICH, some inflammatory cells are stimulated and activated, resulting in the release of a large number of ROS, no and other substances, and the consumption of a large number of superoxide dismutase (SOD) and lipid peroxide (Cheng et al., 2023) (Figure 1).

Recent studies indicate that a single episode of ICH can result in varying degrees of disability, including motor impairments, language deficits, and, in some cases death (Sembill et al., 2021). ICH is still a disease with high morbidity and mortality (Gómez-González et al., 2021). Therefore, understanding the underlying mechanisms of oxidative damage following ICH is crucial for reducing associated mortality. However, it is important to acknowledge that changes in the levels of redox biomarkers are observed in a variety of diseases, making it challenging to specifically associate any single biomarker with ICH. Furthermore, alterations in the body's redox balance can be assessed both qualitatively and quantitatively through biomarkers, which provide valuable information for disease prognosis. Consequently, redox biomarkers are currently considered vital tools for monitoring the progression of ICH and its prognosis (Shao et al., 2019).

At the same time, the specific correlation between various oxidative biomarkers and ICH has been successfully confirmed (Diao et al., 2024). However, a major challenge lies in the direct detection of ROS due to their high reactivity and extremely short half-life. In other neurological disorders, OS levels have been inferred from the measurement of oxidation products in DNA, proteins, and lipids as indirect indicators (Wang L. et al., 2024). Recent study showed anti OS is an important measure to improve the neurological function of ICH and affect the prognosis (Tang et al., 2023a; Shirzad et al., 2023; Liao et al., 2024). The detection of oxidative biomarkers is not only important for the diagnosis and prognosis of ICH, but also essential for the treatment of ICH. Therefore, to explore effective laboratory indicators for evaluating the severity of oxidative stress after ICH can provide an effective means for early diagnosis or efficacy evaluation of secondary brain injury.

Biomarkers are measurable indicators used to objectively and accurately distinguish between normal biological status and pathological status, and to respond to specific treatment interventions. This review examines the potential role of oxidative stress biomarkers in the diagnosis and prognosis of ICH, as well as their utility in predicting disease outcomes. The aim is to

improve diagnostic accuracy, optimize treatment strategies, enhance health outcomes.

2 Lipid peroxidation

DNA, Oxidation products of proteins and lipids were recorded as indirect readings of OS levels. Lipid peroxides are key products resulting from brain injury. They primarily originate from the secondary peroxidation of unsaturated fatty acids in membrane phospholipids (Zhou SY. et al., 2020). As one of the harmful products of oxidative damage, like $\cdot\text{OH}$ and other free radicals, it can extract hydrogen ions on fatty acids to further generate new lipid peroxides, so once the reaction is started, it will be cascaded and amplified to produce chain reaction (Lee et al., 2023). Oxidative damage to lipids was evaluated using both quantitative and qualitative assessments of several biomarkers, including MDA, 4-hydroxy-2-nonenal (HNE), oxidized LDL, and F2 isoprostaglandin, measured in blood, cerebrospinal fluid, or other relevant bodily fluids. At present, lipid peroxides in peripheral blood are widely regarded as a reliable marker for assessing oxidative stress.

2.1 Malondialdehyde

MDA is the main end product of lipid peroxide and has cytotoxicity. It can react with proteins and enzymes to destroy the structure of brain tissue and dysfunction (Tsikas, 2023). Its formation arises from the destruction of brain cells and the oxidation of polyunsaturated fatty acids into peroxides, followed by their degradation (Wei et al., 2024). Due to its involvement in lipid peroxidation, MDA is the most commonly used biomarker for assessing oxidative damage and is widely recognized as a key indicator of oxidative stress (Thakkar et al., 2024).

MDA is easy to react with primary ammonia of biological macromolecules, so it exists in free state and bound state *in vivo* (Moldogazieva et al., 2023). The conventional method for the determination of free MDA or bound MDA is thiobarbituric acid (TBA) colorimetric analysis; Although TBA can also react with other unsaturated aldehydes, more than 85% of the products produced by TBA reaction (TBARS) come from the addition polymerization of MDA and TBA molecules (Jiménez-Jiménez et al., 2024). Other methods for determining MDA include high performance liquid chromatography and immunological methods (Mas-Bargues et al., 2021) (Table 1).

Regarding the relationship between MDA and ICH, recent studies indicate that serum levels of MDA may have a positive correlation with changes in neurological function associated with ICH (Akyol et al., 2024).

The formation of MDA and TBARS is not solely attributed to oxidative stress, but also results from the degradation of endogenous epoxides. Therefore, MDA assessment extends beyond blood samples and is often used in conjunction with routine measurements of serum total antioxidant status (TAS) as a more comprehensive indicator of oxidative balance. Masomi et al. Suggested that markers with increased OS could also be detected in cerebrospinal fluid (CSF) and plasma, and higher MDA levels were detected in

cerebrospinal fluid of a large number of ICH patients, leading to adverse results after 6 months (Masomi-Bornwasser et al., 2021a).

2.2 4-Hydroxy-2-nonenal (4-HNE)

Protein oxidation induced by ROS can result in the formation of carbonyl derivatives and nitrated tyrosine residues, with 4-HNE being a key byproduct. Among lipid aldehydes, 4-HNE is considered one of the most biologically active (Jaganjac et al., 2022). 4-HNE is an advanced lipid peroxidation end product (Lamb et al., 2024). 4-HNE is directly involved in cytotoxic processes and is strongly associated with oxidative damage (Yamashima et al., 2024; Lee et al., 2024). Like MDA, it can react with various cell components and can also be used as an indicator of lipid peroxide *in vivo*, and is considered to be one of the most powerful active aldehydes (Zhang Y. et al., 2024). Animal studies indicated that the accumulation of 4-HNE progressively increased over the 3–18 days following ICH (Wang et al., 2022b). It is reported that Chen et al. Used rats as experimental models to inhibit 4-hne-related oxidative stress through edaravone, thus providing neuroprotective effect after ICH (Chen Q. et al., 2022). A clinical study, in comparison with animal model findings, demonstrated that levels of 4-HNE in blood samples were significantly elevated in all ICH patients compared to non-ICH subjects, providing valuable insights into the oxidative stress-related consequences following ICH (Jarocka-Karpowicz et al., 2020a).

2.3 F2-isoprostanes and 8-iso-Prostaglandin F2 alpha

F2 isoprostanes are a series of prostaglandin F2 like compounds produced by lipid peroxidation reaction of arachidonic acid on cell membrane by ROS (Mottola et al., 2024). From the varying impacts of reactive oxygen species on arachidonic acid locations, a total of 64 isomers of F2 isoprostane may be produced (Cioffi et al., 2021). 15-F2t-isoprostane is the most studied and most representative isomer of F2 isoprostane (Simantiris et al., 2023). In recent years, F2 isoprostaglandin has emerged as a reliable biomarker for oxidative stress and lipid peroxidation *in vivo*, attributed to the following characteristics: ① F2 isoprostaglandin is a specific byproduct of lipid peroxidation that does not require cyclooxygenase for its formation, and it possesses relatively stable chemical properties and content (Katsiouna et al., 2023); ② In some animal models of oxidative damage, its level increased significantly; ③ Its level is not affected by eating lipids (Tares et al., 2022). Currently, F2 isoprostaglandin is recognized as among the most dependable markers for assessing oxidative stress *in vivo* (de Mello Barros Pimentel et al., 2023). Recent studies have shown that the level of F2 isoprostaglandin has a certain correlation with free radicals and oxidative damage in many human diseases (including cardiovascular diseases, lung diseases, nervous system diseases, kidney and liver diseases, etc.) (Menzel et al., 2021). In their investigation of F2 isoprostaglandins for prognostic purposes in ICH, Gomes et al. reported that specific markers of *in vivo* lipid peroxidation, namely, F2 isoprostaglandins (F2 IsoPs) and isoprostaglandins (isof), were elevated and influenced prognosis following SAH (Gomes et al., 2022). Another study also showed

TABLE 1 Approaches for detecting biomarkers of oxidative stress.

Oxidative stress biomarkers	Detection strategy examples	References
Malondialdehyde	SERS TBARS Test Fluorescence UV-Vis GC-MS Electrochemistry	Mas-Bargues et al. (2021), Toto et al. (2022)
4-Hydroxy-2-Nonenal	MALDI-TOF-MS HPLC DNPH Derivatization 2-AP LC-MSMS Western blot 32P-PostlabelingGC-MS Sandwich ELISA FT-ICR MS	Fuloria et al. (2020), Žarković et al. (2024), Zhou et al. (2020b)
F2-isoprostanes	SPE-HPLC-MS/MS ELISA GC-MS HPLC-MS/MS GC-NICI-MS	Fuloria et al. (2020), Zhou et al. (2022), Zhou et al. (2023)
8-Iso-Prostaglandin F2α	HPLC-ED	Steffensen et al. (2020)
LOX-1	Western blot ELISA Flow cytometry analysis Fluorescence	Deng et al. (2024), Sánchez-León et al. (2024), Ashok et al. (2024), Bing et al. (2024)
8-oxo-7	Fluorescence HPLC-ED ELISA	Dong et al. (2024a), Nian et al. (2024), Fujikawa et al. (2024)
Protein carbonyls	Levine spectrophotometric method In-gel fluorophoric tagging ELISA Western blot HPLC	Ladouce et al. (2023), Zhang et al. (2025), Nocera et al. (2024)
RAGE	Western blot ELISA	Han et al. (2024), Vega-Cárdenas et al. (2023), Kanikowska et al. (2024)
Homocysteine	Immunonephelometric method HPLC-ED EIA LC-MS-MS Immunoassay HPLC with fluorometric detection Fluorescence polarization	Alam et al. (2019)
Gluthatione	DTNB/GR enzyme recycling method HPLC	Added et al. (2023), Carrão Dantas et al. (2022)
GFAP	Spectroscopy Fluorescence polarization	Probert et al. (2022), Pereira et al. (2021)
HMGB1	Western blot LC-MS-MS ELISA	Held et al. (2024)
IMA	Western blot LC-MS-MS	Li et al. (2022), Karakılıç et al. (2023)
S100B	ELISA HPLC-MS/MS Fluorescence	Bjurstén et al. (2024)

(Continued on the following page)

TABLE 1 (Continued) Approaches for detecting biomarkers of oxidative stress.

Oxidative stress biomarkers	Detection strategy examples	References
AOPP	ELISA Fluorescence HPLC-MS/MS	Li et al. (2024), Wybranowski et al. (2023)
Myeloperoxidase	BLI CRET ADHP MPO-Gd MR imaging	Gramstad et al. (2023), Majid et al. (2023)

SPE, polymeric weak anion-exchange solid-phase extraction; TBARS, thiobarbituric Acid Reactive Substances; GC-MS, gas chromatography-mass spectrometry; UHPLC-MS/MS, isotope-dilution ultrahigh performance liquid chromatography electrospray ionization–tandem mass spectrometry; NICI-MS, gas chromatography-negative-ion chemical ionization mass spectrometry; HPLC, high performance liquid chromatography; HPLC-ED, high performance liquid chromatography with electrochemical detection; ELA, enzyme-linked immunoassay; ADHP, 10-acetyl-3, 7-dihydroxyphenoxazine; 2-AP, fluorescent probe 2-aminopyridine; CRET, chemiluminescence resonance energy transfer; DNPH, 2,4-dinitrophenylhydrazine; BLI, bioluminescence imaging; MPO-Gd, bis-5-hydroxytryptamide-diethylenetriaminepentaacetate-gadolinium; measurement detection spectroscopy.

that F2 isoprostaglandin can be used as a non-invasive prognostic biochemical marker for ICH patients (Wiśniewski et al., 2017). Nevertheless, while F2 isoprostaglandins are generally reliable biomarkers for forecasting stroke outcomes, they do possess certain limitations. Because the formation of F2 isoprostaglandin is disturbed under high oxygen tension, F2 isoprostaglandin is not sensitive enough as an indicator of lipid peroxidation under high oxygen tension (Thatcher and Peters-Golden, 2022).

2.3.1 8-Iso-prostaglandin F2α

8-Isoprostaglandin F2α is widely present in the body, and its formation occurs independently of cyclooxygenase catalysis, which is why it is named for its structural similarity to prostaglandins. As the most representative isomer of isoprostaglandin, both side chains are CIS structure, which is the most widely studied isoprostaglandin (Boldeanu et al., 2023). Due to the stable structure *in vivo*, the content of isoprostaglandin is not affected by the lipid in food, with good sensitivity and specificity, and is closely related to oxidative stress injury, it is currently considered to be an ideal index for evaluating the oxidative stress state of the body (Zhang M. et al., 2021). Recent studies indicate that the levels of 8-Isoprostaglandin F2α increase in response to oxidative stress following ICH (Yang et al., 2021). In their clinical investigation of the prognosis and outcomes of ICH, Du et al. observed a increase in plasma levels of 8-Isoprostaglandin F2α in ICH patients. Furthermore, these elevated levels were positively correlated with hematoma volume and associated with unfavorable clinical outcomes in ICH (Du et al., 2014).

2.4 Lectin-like oxidation of the LDL receptor-1 (LOX-1)

LOX-1 is widely expressed in endothelial cells, where it can activate multiple cell death pathways, elevate reactive oxygen species, and contribute to endothelial dysfunction (Akhmedov et al., 2021). LOX-1 is expressed not only on the surface of cells but also exists as soluble molecules within the bloodstream (Pyrpyris et al., 2024). There are case reports that the expression of LOX-1 and matrix metalloproteinase can be significantly increased in the intima of ruptured and unruptured middle cerebral artery, and the expression

level of s LOX-1 in patients with acute aortic dissection is extremely increased, which is speculated to be related to the rapid and severe damage of vascular endothelium (Kobayashi et al., 2013). ICH, LOX-1 binds to red blood cells, leading to the upregulation of both LOX-1 and sLOX-1 expressions. Inoue et al. reported that serum levels of LOX-1 were elevated in ICH patients, based on analysis of blood samples, suggesting that LOX-1 could serve as a potential biomarker for ICH (Inoue et al., 2019). Similarly, a clinical study conducted by Yokota et al. found that, compared to healthy individuals, the levels of soluble LOX-1 increased in patients following the onset of ICH. This finding supports the use of elevated sLOX-1 levels as a potential biomarker for ICH (Yokota et al., 2016).

3 DNA oxidation

In biological macromolecules, proteins, lipids, and RNA typically undergo degradation and recycling following oxidative damage, whereas DNA requires repair to preserve genomic integrity (Draxler et al., 2023). Oxidative damage to DNA includes various forms such as base oxidation and deoxyribose oxidation, with base oxidation being the predominant type of damage (Andrés et al., 2023). Among the four nucleobases, guanine exhibits the lowest redox potential, making it particularly susceptible to oxidative damage (Gupta and Imlay, 2023). As an oxidative adduct, 7,8-dihydro-8-oxoguanine (8-oxoG) is stable within the body. Once formed, it is not further metabolized and is not influenced by dietary intake or other external factors. It can be excised and removed by specific DNA repair enzymes and subsequently excreted in urine via the kidneys. (Dong et al., 2024b). Therefore, 8-oxo-7 may be a sensitive biomarker of brain DNA damage driven by oxidative stress (Shkirkova et al., 2024).

In previous preclinical and clinical studies on central and peripheral nerve disorders and mental disorders, it was found that its content increased with the increase of brain injury (Schiaivone et al., 2017). The study found that after ICH, the expression of 8-oxo-7 in brain tissue increased significantly (Liu et al., 2020). In a recent observational prospective study by Lorente et al., the average urinary levels of 8-oxo-7 in 100 stroke patients on day 7 were higher than those in the control group. Furthermore, the authors found a positive correlation between 8-oxo-7 levels and mortality, based on

comparisons of serum samples from patients with ICH and their 30-day mortality outcomes (Lorente et al., 2020).

4 Oxidative protein modifications

The oxidation of amino acids in protein composition usually has reversible and irreversible effects on the function and structure of the affected proteins, which makes protein oxidation products a potential biomarker of ICH oxidative damage. This section will discuss the relationship between common protein oxidation products and ICH.

4.1 Protein carbonyls

Protein carbonylation is one of the oxidative damage of proteins. It is an irreversible chemical modification in oxidative stress, which means that the side chain of amino acid residues is attacked by oxygen free radicals and finally transformed into carbonyl products (Nègre-Salvayre and Salvayre, 2024). There are two major pathways for the formation of carbonyl proteins: (1) ROS directly oxidizes the side chain amino acids of proteins to form carbonyl proteins (i.e., free radical oxidation); (2) proteins generate carbonyl proteins (i.e., glycosylation); after lipid oxidation and nonenzymatic glycosylation (Wang et al., 2023). Utilizing an *in vivo* metal ion catalytic oxidation system, ROS can directly oxidize protein side chain residues, including lysine, arginine, proline, and threonine, leading to the formation of carbonyl groups (Gulyak et al., 2023). Therefore, protein carbonylation as a necessary marker of OS is of great significance because of its sensitivity and specificity (Chen et al., 2023).

There are relatively few studies on protein carbonyl as an oxidative marker, and there is a lack of sufficient scientific data. One common reason is that although lc-ms/ms is an established method for detecting and quantifying oxidative markers (including carbonylated proteins), its huge cost and the demand for a large amount of expertise limit its wide adoption (Martínez-Orgado et al., 2023a). Martínez-Orgado et al. proposed a potential correlation between plasma protein carbonyl levels and brain injury, suggesting that these carbonyl compounds could serve as biomarkers for the extent of brain damage induced by oxidative stress (Martínez-Orgado et al., 2023b). A recent clinical study on the risk factors of aneurysm size, multiple, previous SAH history and oxidative stress showed that when SAH and aneurysm rupture occurred, myeloperoxidase activity, malondialdehyde and carbonyl levels increased significantly (Šćepanović et al., 2018). It is suggested that carbonyl determination may be a potential marker of oxidative stress after ICH.

4.2 Receptors for advanced glycation end products (RAGE)

In recent years, RAGE and its soluble form (sRAGE) have increasingly been recognized as biomarkers for ICH (Balança et al., 2021). The RAGE interacts with various ligands, including HMGB-1, advanced glycation end-products (AGEs), and S100 proteins.

The expression of RAGE is associated with oxidative stress induction, while sRAGE exerts a protective effect by competing with RAGE for ligand binding (Reddy, 2023). Lei et al. Carried out an interesting study, revealing the relationship between the soluble RAGE level and the severity of ICH, as well as the significant increase in adverse functional outcomes (Lei et al., 2020). Another study found that the level of sRAGE in SAH patients increased early and then changed dynamically, which may be a potential biomarker of poor prognosis, providing more accurate prognostic information for sRAGE quantification after ICH (Chu et al., 2023).

4.3 Homocysteine

Homocysteine is produced by adenosine transfer, demethylation and hydrolysis under the catalysis of enzyme. It is metabolized by re methylation or trans sulfur under the action of folic acid and vitamin B group (Grande et al., 2023). Homocysteine exists in the form of the plasma in four different forms: about 1% in plasma in free disulfide homocysteine, 70%–80% is keeping disulfide and plasma protein binding (mainly albumin), 20%–30% is disulfide bond form dimer, and a few in the form of other thiol-containing group in plasma. The concentration of plasma homocysteine that we usually measured refers to the total concentration of (Zhilyaeva et al., 2023). Homocysteine in plasma predominantly exists in its oxidized state, with only a minor fraction present in its reduced form. The auto-oxidation of homocysteine is a well-established pathway for ROS generation (An et al., 2024). Homocysteine possesses a reactive thiol group, which is readily subject to auto-oxidation, leading to the generation of various reactive oxygen species (Gawel et al., 2024). The sulfhydryl group of homocysteine can also undergo one electron oxidation reaction to generate sulfur free radical or disulfide anion free radical with another compound containing sulfhydryl group, and these two free radicals are more likely to cause protein oxidation (Zarembaska et al., 2023). As a cell injury factor, the main mechanism of homocysteine is to induce the occurrence of oxidative stress (Guo et al., 2024). A clinical study involving 551 patients with ICH found that elevated homocysteine levels were present in 284 patients (51.5%). Significant differences in the percentage of males, smoking and drinking behaviors, and triglyceride levels were observed among the different homocysteine level groups. In ICH patients, higher homocysteine levels were associated with reduced survival rates and worse prognoses (Wang D. et al., 2020). A recent multicenter, hospital-based study conducted nationwide in China by the China Stroke Center Alliance (CSCA) assessed 705 patients with ICH. The results revealed a significant association between elevated homocysteine levels and both the severity of ICH at admission and unfavorable functional outcomes at discharge. These findings suggest that homocysteine may be a useful biomarker for predicting the severity of ICH and functional prognosis at discharge (Wang et al., 2022c). Li et al. Carried out a 6-month clinical experiment involving 84 subjects with peripheral blood NLRP3 mRNA and Hcy as observation indicators, suggesting that serum Hcy, blood loss and ventricular system permeability are independent risk factors for poor prognosis in ICH patients (Li Q. et al., 2021).

4.4 Glutathione

Glutathione is condensed from glutamic acid, cysteine and glycine (Lapenna, 2023). Glutathione plays a crucial role as an intracellular regulatory metabolite by activating various enzymes, thereby influencing cellular metabolic processes (Ferreira et al., 2023). Glutathione participates in *in vivo* oxidation-reduction processes and can effectively interact with peroxides and free radicals, protecting sulfhydryl groups in antioxidants. It safeguards sulfhydryl-containing proteins and enzymes in cell membranes from damage and mitigates the harmful effects of free radicals on vital organs (Ichihara et al., 2023). Under the influence of oxidants, glutathione (GSH) and its oxidized form (GSSG) can interconvert through enzymatic action, establishing a dynamic equilibrium that contributes to an effective antioxidant system (Fernández-Lázaro et al., 2024). As iron death has become a recent research hotspot, the oxidative damage mechanism of GSH has been repeatedly verified in animal experiments (Nie et al., 2024). In the human study, Jarocka et al. (Jarocka-Karpowicz et al., 2020b) reported that the plasma GSH level of 30 subjects increased within 6–8 days of SAH compared with the matched control group. Similarly, Akyol et al. conducted a clinical study involving 200 subjects and observed that, compared to the control group, GSH levels increased following ICH. Moreover, the GSH levels in ICH patients who underwent decompression surgery were found to be comparable to those of patients who did not receive surgery (Akyol et al., 2024). These results underscore the feasibility of GSH as a biomarker for oxidative damage in ICH.

4.5 Glial fibrillary acidic protein (GFAP)

GFAP is a cytoskeletal protein existing in astrocytes, which has two forms of soluble protein and intermediate filament protein. It is used to maintain the morphology and function of astrocytes, but also to protect and supply neurons. It is very sensitive to oxidative stress damage, and has been used as a clinical oxidative stress related molecule for monitoring (Passos et al., 2022). Bhatia et al. reported that collecting blood samples within 24 h of ICH episode revealed significantly elevated serum content of GFAP in ICH patients (Bhatia et al., 2020). Gyldenholm et al. also reported on a study involving 156 participants, in which GFAP levels were measured using ultra-sensitive single molecule array and ELISA, while clinical data, including mortality and functional outcomes, were recorded. The study found that serum GFAP levels were elevated in patients with ICH compared to healthy controls, and that the increase in GFAP levels was predictive of both mortality and poor prognosis in ICH patients (Gyldenholm et al., 2022).

4.6 High mobility group box1 (HMGB1)

HMGB1 is mainly involved in cell differentiation, stabilizing chromatin structure, regulating gene transcription and translation, and steroid hormone regulation and other life activities (Chen R. et al., 2024; Zhu et al., 2024). Nuclear HMGB1 is nonspecifically bound to DNA with low affinity, and is involved in cell differentiation, DNA repair, DNA recombination, steroid

hormone regulation and other life activities in the nucleus (Tang D. et al., 2023). RAGE mentioned above is a receptor in the traditional signal transduction pathway of HMGB1, which belongs to the immunoglobulin superfamily and is expressed on a variety of cell surfaces (Lu Z. et al., 2023). A recent study on acetaminophen toxicity suggests that HMGB1 undergoes oxidative modification and can be used as pathological specific biomarkers and drug targets (Pirnie et al., 2023). A clinical experiment showed that with the increase of HMGB1 and age, the adverse functional outcome rate of ICH patients increased significantly, and the quantification of HMGB1 provided more accurate prognosis information after ICH (Lei et al., 2020). Hemmer et al. reported on a prospective, single-blind observational study designed to investigate the role of HMGB1 in aSAH. The study found that serum HMGB1 can serve as an independent biomarker for predicting delayed cerebral ischemia and elucidated its potential role in the sequelae of aSAH (Hemmer et al., 2022).

4.7 Ischemia-modified albumin (IMA)

IMA is more sensitive to ischemia and hypoxia in tissues and organs of the body, and we can generally detect it a few minutes after ischemia (Mangoni and Zinellu, 2024). Generally, IMA was initially recognized as a useful biomarker in context of ischemic diseases (Jena et al., 2017). Several studies have confirmed that free radicals increase in patients with cerebrovascular disease during episodes of local brain tissue ischemia, hypoxia, or local vascular reperfusion injury (Elshony et al., 2021). In the context of acute cerebrovascular disease, oxidative stress reactions occur progressively. The production of IMA can be partially attributed to the concurrent generation of reactive oxygen species and the disruption of the blood-brain barrier (Ayca et al., 2024). In a study, Gad et al. confirmed the expression significance of IMA in ICH through clinical experiments, and also confirmed that it can be used to distinguish ICH from is (Gad et al., 2019). Mangoni et al. reported that in 17 previous clinical studies, the IMA concentration gradually increased in patients with SAH, ICH and acute ischemic stroke through meta-analysis. In the sensitivity analysis, when individual studies were deleted in turn, the merged SMD did not change. It is suggested that IMA concentration may help to diagnose stroke and distinguish acute ischemic stroke, ICH and SAH (Mangoni and Zinellu, 2022).

4.8 Calcium binding protein S100

S100 protein is a kind of neural tissue protein. At present, nearly 20 kinds of S100 proteins have been found in different tissues, which can regulate intracellular and extracellular calcium ions with similar structure and function (Gayger-Dias et al., 2023). S100B is specifically found in astrocytes and glial cells within the central nervous system (Hernández-Ortega et al., 2024). Its content in brain tissue is much higher than that in other tissues, so it is considered to be brain specific protein (Santing et al., 2024). Generally, after brain injury, the expression of S100B increases with the occurrence of oxidative damage and neuroinflammation (Chong, 2016). Kellermann et al. reported that in patients with traumatic brain injury (TBI) and SAH, the concentrations of S100B in cerebrospinal

fluid and serum were significantly higher in those with poor prognoses compared to those with favorable outcomes. Additionally, an initial serum S100B level greater than 0.7 µg/dl was associated with a 100% mortality rate, suggesting its potential utility in guiding treatment strategies for severe trauma (Kellermann et al., 2016).

4.9 Advanced oxidized protein (AOPP)

The principal component of AOPP is albumin that has been oxidized by free radicals. This compound can mediate lipid peroxidation, activate monocytes and NADPH oxidase, and promote the release of intracellular reactive oxygen species (de Brum and Bochi, 2024). It is one of the specific markers of protein oxidation (Lievykh et al., 2023). Rendeviski et al. reported that AOPP may serve as a significant prognostic biomarker for ICH through modeling analyses (Rendeviski et al., 2023). In another study, the expression of AOPPs in cerebrospinal fluid of 50 patients with aSAH at different time after hemorrhage was measured. The level of CSF AOPP at each time point after hemorrhage in patients with aSAH was independently correlated with the poor prognosis of 90 days follow-up, suggesting that AOPPs can be used as a potential biomarker for evaluating the prognosis of aSAH (Shen et al., 2022).

5 Haem peroxidase-cyclooxygenase superfamily

5.1 Myeloperoxidase (MPO)

MPO mainly exists in aniline blue granules of myeloid cells and is a specific indicator reflecting the activation of neutrophils and macrophages (Quinn et al., 2024). MPO especially reflects the function and activity of granulocytes (Tran et al., 2024). MPO itself is not inherently oxidative; however, under specific conditions, it catalyzes the production of reactive species such as 3-chlorotyrosine, which can induce oxidative damage (Wu TJ. et al., 2024). Zuo et al. performed experimental investigations to explore the involvement of MPO in oxidative stress after ICH. They examined the effects of an MPO inhibitor on neurobehavior in a rodent model of ICH and reported that inhibiting MPO can alleviate secondary injury after cerebral hemorrhage (Zuo et al., 2022b). Witsch et al. Conducted clinical observation on 100 patients with SAH (Witsch et al., 2022), and pointed out the diagnostic potential of mpo-dna complex and its possibility as a potential therapeutic target of aSAH. More specifically, Zheng et al. (Zheng et al., 2018) reported that by measuring serum myeloperoxidase (MPO) concentrations in 128 patients with cerebral hemorrhage and 128 controls, they assessed dependent variables such as neurological function, mortality, and adverse outcomes at various time points. The results indicated a significant increase in serum MPO concentration among patients with ICH. The study concluded that MPO concentration in ICH patients was positively correlated with hematoma volume and the NIHSS score. Furthermore, serum MPO detection significantly enhanced the ability to differentiate neurological function and prognosis related to hematoma in ICH patients.

Although the role of oxidative damage as a secondary injury in the pathophysiology of ICH is well established, the use of

oxidative biomarkers for ICH diagnosis remains challenging due to the widespread occurrence of oxidative damage in various other diseases. Nevertheless, the correlation of oxidative biomarkers with the monitoring of ICH severity and prognosis is still acknowledged.

Although the role of oxidative stress as a mechanism of secondary injury in the pathophysiology of ICH is well established, the use of oxidative stress biomarkers for diagnosing ICH remains a significant challenge. This is because oxidative stress also occurs in various other diseases associated with energy metabolism dysfunction, neuroinflammation, tissue damage, and cell loss (Chen X. et al., 2024; Santos et al., 2024; Park et al., 2024). Additionally, several biomarkers mentioned above, which are metabolites or end products, may exhibit fluctuations in their levels due to the impact of other diseases on liver or kidney metabolic functions, further limiting the accuracy of these biomarkers. Therefore, improvements in methodology and the reliability of biomarkers are essential prerequisites for ensuring the reliability of biomarker detection. Moreover, a comprehensive analysis of various oxidative stress biomarkers following ICH may provide a more accurate reflection of the collective oxidative stress level. Currently, oxidative stress biomarkers are increasingly recognized as important tools for monitoring the severity and prognosis of ICH (Hu et al., 2016).

6 Assessment of antioxidant defense modifications as potential indirect markers of oxidative stress

Because reactive oxygen species are extremely unstable, they will react with cellular components or be catabolized rapidly, so it is difficult for existing clinical test methods to truly evaluate the content of ROS (Hosoki et al., 2023). Therefore, oxidative homeostasis is more indirectly assessed by evaluating oxidative stress products. To gain insights into the mechanisms of these changes, it is essential to examine the actual participants involved in the regulation of oxidants and antioxidants (Jomova et al., 2024). Some studies have attempted to utilize antioxidant defense to assess the extent of oxidative damage, which is considered a feasible oxidative biomarker (García-Giménez et al., 2024). The antioxidant system serves as an effective protective mechanism against potentially harmful oxidative damage. Antioxidant systems are primarily categorized into two types: enzymatic antioxidant systems, which include SOD, glutathione peroxidase, and catalase (CAT), and non-enzymatic antioxidant systems. The latter is further divided into water-soluble antioxidants, such as vitamin C (VC), and fat-soluble antioxidants, including carotene and vitamin E (VE) (Houldsworth, 2024).

6.1 Antioxidant enzymes

SOD is a key antioxidant enzyme in the body. It can cooperate with CAT and other enzymes to transform and decompose harmful superoxide free radicals into water that is harmless to the body, effectively reduce superoxide free radicals, and repair damaged cells and tissues in time, so as to improve the oxidative stress response (Militello et al., 2024). CAT is broadly present in animals, plants,

and microorganisms (Afzal et al., 2023). Glutathione peroxidase (GPX) is a selenium containing antioxidant enzyme (Ikeda and Fujii, 2023). SOD, CAT, and GPX have distinct roles in both physiological and pathological processes. SOD degrades superoxide ions by converting them into ROS, while GPX and CAT are involved in the neutralization of these ROS, facilitating their conversion into oxygen and water (Grzeszczak et al., 2023).

A clinical study on ICH evaluated the oxidative stress marker SOD in cerebrospinal fluid and recorded the admission status, treatment outcomes, and degree of neurological impairment in ICH patients. The study found that SOD levels in the cerebrospinal fluid increased 24 h after the onset of ICH and were associated with poorer neurological outcomes (Krenzlin et al., 2020). Another clinical study involving 200 participants further validated the differences in serum levels of SOD and CAT between patients with ICH and healthy controls (Akyol et al., 2024). The study showed that GPX was measured by spectrophotometry and high performance liquid chromatography, respectively. It was found that SAH was accompanied by the change of antioxidant capacity in plasma, including the increase of GPX activity in the first day, and then its decrease, suggesting that the level of plasma GPX can support the monitoring of patients' clinical status (Jarocka-Karpowicz et al., 2020b). Zhang et al. reported on a clinical study involving 116 patients with severe ICH admitted to the ICU from June 2018 to June 2020. The study observed significantly elevated levels of SOD and GPX in the plasma of ICH patients, indicating the occurrence of oxidative damage *in vivo* following ICH (Zhang J. et al., 2021).

6.2 Non-enzymatic antioxidants

Compared to enzymatic antioxidants that are directly involved in the metabolism of ROS, non-enzymatic antioxidants primarily function as ROS scavengers (Rudenko et al., 2023). These antioxidants terminate the redox reaction by eliminating the intermediates of free radicals, and also generate new free radicals (Dyachenko and Bel'skaya, 2023). Vitamin E can be converted into tocopherol carboxyl radical, which has relatively low activity. It also needs to be antioxidant and scavenged by another kind, such as ascorbic acid (vitamin C) (Feng et al., 2023). If the clearance of tocopherol carboxyl radical is delayed, it will promote lipid peroxidation (Lian et al., 2023). Vitamin C is an important antioxidant because ascorbic acid free radicals are usually disproportionately converted to ascorbic acid and dehydroascorbic acid (Fiorentino et al., 2024). In recent years, the antioxidant effect of natural antioxidants has become a research hotspot. The study measured the plasma levels of vitamins A, E, and C in patients with ICH using spectrophotometry. Compared to healthy controls, patients with ICH exhibited elevated levels of these vitamins, suggesting an association with oxidative stress injury and potentially reflecting the prognostic outcomes for individuals affected by ICH (Jarocka-Karpowicz et al., 2020b).

6.3 Total antioxidant capacity (T-AOC)

T-AOC is a comprehensive index to measure the functional status of antioxidant system, and its content can reflect the

compensatory ability of antioxidant system against external stimuli and the status of free radical metabolism (Hu W. et al., 2023). T-AOC is defined as the total antioxidant capacity of all antioxidants in the sample to be tested. It is a clinical biochemical examination index, which is measured by detecting the total amount and activity of antioxidants in blood, urine or other body fluids (Mei et al., 2021). Animal research models show that ICH is usually accompanied by the increase of total antioxidant capacity. It is observed that the T-AOC in the serum of ICH group increases at 24 and 72 h, which may be the physiological mechanism against the increase of ROS production (Mazhar et al., 2023). In a study by Zhang et al., it was reported that the T-AOC and other indicators of oxidative damage in the experimental group were elevated above the average values. This finding suggests an enhancement in oxidative stress processes within this group (Zhang J. et al., 2021). However, an increase in serum antioxidant capacity may not represent optimal state, as it could indicate the presence of ongoing pathological processes in the body. Additionally, a subsequent decrease in these levels may reflect a response to the reduction of active substances (Morimoto et al., 2019). Consequently, new insights have emerged regarding the survival strategies for patients with ICH and the management of oxidative stress: specifically, the importance of restoring oxidative balance while avoiding excessive activation of the immune system.

6.4 Total antioxidant status (TAS)

TAS is an indicator of the reserve state of reduced complex, which can more accurately assess the body's oxidative stress (Oktay et al., 2024). The general significance of TAS assessment is to better understand the ability to resist ROS attack on cells (Ghasemi et al., 2023). In a few published studies using TAS to measure the antioxidant potential of diseases, animal models showed that the occurrence of SAH was often accompanied by the increase of TAS, suggesting that this may be a response to the increase of ROS (Senol et al., 2021). Masomi et al. conducted a clinical study involving 48 patients, which revealed that the TAS of cerebrospinal fluid increased further by the seventh day following ICH. The plasma TAS levels were found to be independently associated with poor prognostic outcomes in patients with ICH (Masomi-Bornwasser et al., 2021b).

7 Future treatment directions

Currently, the primary treatment options available in clinical practice include hematoma evacuation and edema management. While numerous clinical trials have been conducted to assess these treatments, there is no conclusive evidence suggesting that surgical interventions can improve neurological deficits following ICH (Hanley et al., 2019). Vascular edema induced by ICH has also attracted the attention of researchers. For instance, there have been efforts to investigate the use of glibenclamide for edema treatment, with some progress observed in clinical trials (Zhao et al., 2022). However, it is interesting to note that preclinical studies on the efficacy of glibenclamide in treating ICH seem to present conflicting results (Kung et al., 2021). Additionally, emerging therapeutic

strategies such as miRNA-based treatments and exosome therapies have gained attention. The use of miRNA mimics or antagonists to improve outcomes after experimental brain hemorrhage has been validated in numerous studies (Wu X. et al., 2024; Hou et al., 2024). This suggests that, with proper delivery methods, miRNA-based therapy may hold potential for improving clinical outcomes in ICH. Exosome therapy, a more recent approach, has been shown in preclinical studies to mitigate inflammatory damage following ICH, thereby offering neuroprotective effects, and its potential has been substantiated (Jiang et al., 2024; Nan et al., 2025). However, both miRNA and exosome therapies have yet to be evaluated in clinical trials. In summary, while hematoma evacuation and edema management have uncertain clinical efficacy, emerging therapies remain in the early stages of research and face challenges such as high costs, indicating that treatment strategies for ICH still require further optimization.

Numerous studies have demonstrated that oxidative damage is a critical pathological mechanism involved in ICH injury. Consequently, oxidative stress has emerged as a significant area of research in the treatment and prevention of ICH.

7.1 Targeted oxidative stress may be a potential treatment for ICH

The existing treatment strategies for ROS to treat ICH can be divided into “upstream” (reducing reactive oxygen species) or “downstream” (reactive oxygen species neutralization) strategies. Although a large number of studies in animal and cell models have shown the positive effect of antioxidants on ICH (Tang et al., 2023c; Zhang Z. et al., 2024; Liu et al., 2024; Endo et al., 2024), the same sufficient data support has not been obtained in clinical trials, which may be due to the unclear understanding of the complex mechanism of secondary injury after ICH. Recent studies on practical drugs for targeted treatment of oxidative stress in ICH treatment mainly include rosiglitazone, edaravone, atorvastatin, minocycline and deferoxamine (Luo et al., 2021; Wang Z. et al., 2024; Sun et al., 2023; Gan et al., 2023; Daou et al., 2019).

Rosiglitazone, by activating PPAR γ , reduces the expression of NF- κ B and MMP9, thereby alleviating oxidative stress damage following ICH. However, this research is currently limited to animal studies, and no clinical trial results are available. Edaravone, a free radical scavenger, has demonstrated certain antioxidant effects. Animal experiments have shown that edaravone can attenuate oxidative damage to brain tissue in mice following ICH through the OS/MMP9/ β -DG pathway (Shan et al., 2021). On the other hand, significant progress has been made in clinical trials involving edaravone. In a clinical trial, Shan et al. reported that edaravone inhibits lipid peroxidation during the process of free radical scavenging, leading to anti-inflammatory protection of neural cells, reducing neuronal damage, and improving neurological function and prognosis after ICH (Munakata et al., 2009). Deferoxamine (DFX) works by inhibiting redox reactions through competitive binding with iron ions, thereby reducing free radical production following ICH (Wan et al., 2025). A phase II clinical trial involving 324 patients with spontaneous ICH demonstrated that high-dose deferoxamine effectively reduces oxidative stress-induced

damage and improves clinical outcomes (Yeatts et al., 2013). Minocycline, an MMP inhibitor, has notable antioxidant properties, lowering ROS levels in brain tissue after ICH, possibly through the clearance of free radicals (Zheng et al., 2022a). Some studies suggest that minocycline has significant neuroprotective effects in acute stroke and may be a promising therapeutic agent (Malhotra et al., 2018). Previous animal studies have found that atorvastatin protects neurons in ICH rats by reducing MMP-9-induced brain injury (Cui et al., 2012). Interestingly, however, the effectiveness of this protective effect in clinical trials has not yet been confirmed (Lei et al., 2014) (Table 2).

A possible explanation for the poor effectiveness of ROS targeted therapy in ICH has been proposed, because it is easier to inhibit the production of ROS than to degrade and neutralize ROS, and oxidative damage and inflammatory damage are often accompanied in the secondary injury of ICH. Upstream strategies targeting the inhibition of MPO, COX-2, and MMP-9 have been proposed. Hua et al. reported that Y-2, a drug currently undergoing Phase III clinical trials for ischemic stroke in China, may also exert therapeutic effects in ICH. Specifically, Y-2 appears to reduce the levels of MPO, pro-inflammatory mediators, and oxidative products in the brain tissue surrounding the hematoma core (Hua et al., 2021). Tang et al. investigated the pathological mechanisms underlying ICH and highlighted the critical role of Nrf2 within the Nrf2/ARE signaling pathway (Yan et al., 2023c). This pathway has been shown to attenuate the levels of serum superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) following ICH, suggesting that targeting Nrf2 may represent a promising strategy to mitigate oxidative stress. In addition to the Nrf2 pathway, recent studies have highlighted the significant role of the Wnt signaling pathway in oxidative stress following ICH. Low-density lipoprotein receptor-related protein 6 (LRP6), a transmembrane cell surface protein that induces the canonical Wnt signaling pathway, plays a significant role in the development and metabolism of the nervous system. It is involved in regulating neuronal processes such as differentiation and synaptic plasticity, and is particularly important in the context of oxidative damage (Clark-Corrigall et al., 2023; Wang Y. et al., 2020). Jin et al. evaluated the pathological processes of oxidative damage after ICH and reported the critical involvement of the Wnt signaling pathway in this process (Jin et al., 2022). Furthermore, intervention targeting LRP6 alleviated oxidative damage following ICH, suggesting that modulating LRP6 and the Wnt pathway may be an effective strategy for mitigating oxidative stress. Additionally, Riitano et al. reported the effects of methyl- β -cyclodextrin on the Wnt/ β -catenin signaling pathway, demonstrating that it inhibited the phosphorylation of β -catenin (Riitano et al., 2020). This finding suggests that methyl- β -cyclodextrin, in combination with statins, could represent a potential therapeutic strategy for intervention. Although the effects of this drug have not been explored in the context of oxidative damage, it provides a potential avenue for further investigation. Recent studies have also confirmed the role of the Notch signaling pathway (Notch1, Notch4) in the development of the vascular system, as well as in neurovascular formation and antioxidant effects (Jin et al., 2011; Muley et al., 2022). Although the role of Notch signaling in oxidative stress after ICH has not been extensively studied, targeting the Notch pathway may offer new insights for future research into therapies for oxidative stress following ICH.

TABLE 2 The development for antioxidative treatment of ICH.

Drug name	Different types of intracerebral hemorrhage	Action mechanism	Research progress	References
Rosiglitazone	ICH	Activation of PPAR γ reduces the expression of NF- κ B and MMP9	Animal test phase	Luo et al. (2021)
Edaravone	ICH	Free radical scavenger	Preliminary clinical trial	Munakata et al. (2009)
Deferoxamine mesylate	ICH	Iron-chelator	Phase II clinical trial	Yeatts et al. (2013)
Minocycline	ICH	Free radical scavenger	Preliminary clinical trial	Malhotra et al. (2018)
Atorvastatin	ICH	Inhibition of MMP-9 expression	Animal test phase	Lei et al. (2014)

7.2 Potential of antioxidants to prevent ICH

Antioxidants have been widely recognized and used to improve the body's immunity (Halliwell, 2024; Yang et al., 2024). In addition, the application of antioxidant therapy in non communicable diseases has also achieved exact curative effect (Gao et al., 2023; Lu X. et al., 2023; Feng et al., 2024b; Hu Y. et al., 2023). In animal model experiments, antioxidants are often chemically synthesized. Because of their potential harmful effects, they are rarely used in clinical practice. Therefore, antioxidants from dietary sources may be a key factor in the prevention of ICH. Food sources of oxidants usually include fruits, vegetables and some herbs. Zheng et al. identified several medicinal plants with potential therapeutic benefits, categorizing them into groups such as polyphenols and phenolic compounds, terpenoids, and alkaloids. Examples include garlic, *Ligusticum chuanxiong*, *Cordyceps sinensis*, and bitter melon. These plants have been reported to exert significant effects in animal models of ICH, primarily through their antioxidant properties (Zheng et al., 2022b). Several studies have demonstrated that cordycepin improves brain hemorrhage-induced neurological and cognitive impairments in mouse models by reducing oxidative stress (An et al., 2022). Allicin has been shown to alleviate oxidative stress after ICH by inhibiting MDA expression and increasing SOD levels (Atef et al., 2023). Gastrodin, a phenolic glycoside derived from the plant *Gastrodia elata*, has been reported by Liu et al. to reduce oxidative damage caused by ICH by suppressing levels of ROS, 8-OHdG, 3-nitrotyrosine, and MDA, while simultaneously increasing the activities of GSH-Px and SOD (Iu et al., 2020). Puerarin, an extract from the plant *Pueraria lobata*, has been shown to reduce oxidative damage caused by ICH by lowering the activity of 8-OHdG and ROS (Zeng et al., 2021). Naringin, a flavonoid found in fruits such as grapefruit, has been found to effectively reverse oxidative damage and elevate endogenous antioxidant levels when administered to ICH rats (Singh et al., 2017). Artemisinin, extracted from the plant *Artemisia annua*, not only combats malaria but also exerts a neuroprotective effect by upregulating the expression of the neural cell adhesion molecule L1, contributing to its antioxidant properties and brain protection (Wang J. et al., 2022). Similarly, baicalin, derived from the herb *Scutellaria baicalensis*, has been shown to reduce oxidative stress levels in ICH rats, with its mechanism involving the miR-106a-5p/PHLPP2 axis to activate

the Nrf2/ARE pathway (Tang et al., 2023d). Ursolic acid, found in plants like hawthorn and gardenia, has been demonstrated in animal studies to effectively enhance the activity of GSH, CAT, and SOD, thereby mitigating oxidative stress following SAH (Zhang et al., 2014). Tetramethylpyrazine nitrate, extracted from *Chuanxiong* (*Ligusticum chuanxiong*), possesses antioxidant properties. Wu et al. reported that it can alleviate oxidative damage after SAH, likely through the modulation of the Nrf2/HO-1 pathway (Wu et al., 2019). Isorhynchophylline (IRN), a compound found in the herb *Uncaria rhynchophylla*, has been shown to exert neuroprotective effects in ICH rats by modulating oxidative stress levels (Zhao et al., 2021). Polydatin, primarily extracted from grapes, red wine, and *Crocus sativus*, has been found in a recent study to exert antioxidant effects in SAH rats by downregulating NO and MDA while upregulating SOD, GSSG, and GSH (Zhao et al., 2020). Phillyrin, a natural extract, increases Nrf2, HO-1, and SOD-1 levels in the brain tissue of ICH mice, likely through the modulation of the Nrf2 signaling pathway (Guo et al., 2021). Epigallocatechin gallate (EGCG), a major catechin in green tea, has been confirmed to suppress oxidative stress in ICH rats by activating the Keap1/P62/Nrf2 pathway (Hao et al., 2024). Sosa et al. reported similar antioxidant effects for black tea (Sosa et al., 2018). Duan et al. found that *Momordica charantia* polysaccharide (MCP) can scavenge ROS in the brain tissue of ICH rats and reduce neuronal death, possibly through the inhibition of the JNK3 signaling pathway (Duan et al., 2015) (Table 3).

While these natural products demonstrate promising antioxidant activity in experimental studies, further research is needed to validate their properties. In the next phase, integrating genomics and proteomics approaches could help elucidate the specific molecular targets of these natural products and explore their potential for future therapeutic applications.

The antioxidant potential of many foods is also shown under pathological conditions, such as nuts, goat milk, blueberries, etc. (Lorenzon Dos Santos et al., 2020; Feng and Wang, 2020; Martini et al., 2023). However, the added ingredients of foods tend to be cytotoxic, and most evidence points out that oxidative stress is the main mechanism of toxicity (Medina-Reyes et al., 2020; Martínez-Leo et al., 2021). Therefore, the intake of naturally grown products without chemicals, which show additional antioxidant properties, may be a potential prevention method.

TABLE 3 Antioxidant activity of nature product in ICH related studies.

Food sources	Chemical composition	Different types of intracerebral hemorrhage	Action mechanism	References
Cordyceps sinensis	Cordycepin	ICH	Increase endogenous antioxidant levels	An et al. (2022)
Garlic	Allicin	ICH	Inhibiting MDA expression and increasing SOD levels	Atef et al. (2023)
Rhizoma Gastrodiae	Gastrodin	ICH	Increase GSH-Px and SOD activity	Iu et al. (2020)
Pueraria lobata	Puerarin	ICH	Reduce the activity of 8-OHdG and ROS	Zeng et al. (2021)
Grapes, cherries, tomatoes, beans	Naringin	ICH	Enhance endogenous antioxidant levels	Singh et al. (2017)
Artemisia annua	Artemisinin		Upregulation of the L1CAM	Wang et al. (2022d)
Scutellaria baicalensis	Baicalein	ICH	Mir-106a-5p/phlpp2 axis activates Nrf2/are pathway	Tang et al. (2023d)
Hawthorn, cranberry, gardenia	Ursolic Acid	SAH	Increase GSH,CAT and SOD activity	Zhang et al. (2014)
Chuanxiong	Tetramethylpyrazine nitron	SAH	Upregulation of the Nrf2/HO-1 pathway	Wu et al. (2019)
Uncaria rhynchophylla	IRN	ICH	Reduction in the production of ROS, 4-HNE, and MDA	Zhao et al. (2021)
Grapes, red wine, Crocus sativus	Polydatin	ICH	Downregulation of NO and MDA, and upregulation of SOD, GSSG, and GSH	Zhao et al. (2020)
Forsythia suspensa	Phillyrin	ICH	Activation of the Nrf2 signaling pathway	Guo et al. (2021)
Green tea, black tea	EGCG	ICH	Activation of the Nrf2 signaling pathway	Hao et al. (2024), Sosa et al. (2018)
Balsam pear	Momordica charantia polysaccharide	ICH	Inhibition of the JNK3 signaling pathway	Duan et al. (2015)

8 Conclusion

The primary damage resulting from ICH is largely attributable to secondary injury processes, which include local tissue damage and programmed cell death. Experimental evidence strongly supports the involvement of oxidative stress, stemming from an imbalance in redox homeostasis, in the pathophysiology of ICH. Consequently, the assessment of oxidative stress biomarkers holds promise as a means to elucidate the underlying mechanisms of ICH, offer diagnostic and prognostic insights, and guide the identification of potential therapeutic targets for antioxidant-based interventions. However, despite the recognized potential of oxidative damage in ICH prediction and treatment, clinical studies have shown limited reproducibility in oxidative stress marker measurements. This inconsistency is largely due to variability in disease subtypes, as well as limitations in sample collection, storage, and pretreatment protocols. Furthermore, the complex interplay of other mechanisms contributing to ICH-induced injury remains incompletely understood,

leading to conflicting findings and inconclusive results. While comprehensive analysis of various oxidative stress biomarkers after ICH may provide an indication of oxidative damage levels, significant improvements in methodology are required to enhance the reliability and accuracy of these measures.

Author contributions

JY: Conceptualization, Formal Analysis, Investigation, Methodology, Supervision, Writing–original draft, Writing–review and editing. XD: Investigation, Writing–review and editing. XY: Conceptualization, Writing–original draft. LZ: Writing–review and editing. JZ: Writing–review and editing. BK: Writing–review and editing. WT: Writing–review and editing. WY: Writing–review and editing. ML: Formal Analysis, Writing–original draft. HC: Writing–original draft. WZ: Funding acquisition, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Serum glutathione peroxidase-3 concentration at diagnosis as a biomarker for assessing disease activity and damage of antineutrophil cytoplasmic antibody-associated vasculitis at diagnosis

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Background: In this study, we investigated whether serum glutathione peroxidase-1 (GPX-3) concentration at diagnosis could be used to assess vasculitis activity and damage at diagnosis in immunosuppressive drug-naïve patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).

Methods: We included 71 immunosuppressive drug-naïve patients newly diagnosed with AAV. Medical records were retrospectively reviewed and serum GPX-3 concentration was measured using serum samples collected and stored at diagnosis. The degree of vascular activity and extent of damage were assessed using the Birmingham vasculitis activity score (BVAS) and vasculitis damage index (VDI), respectively. Poor outcomes including all-cause mortality, end-stage kidney disease, and cerebrovascular and cardiovascular diseases were also investigated.

Results: The median age of the study subjects was 63.0 years, 26 and 45 patients were males and females, respectively. The median GPX-3 concentration was measured as 82.8 ng/mL. Serum GPX-3 concentration at diagnosis was inversely correlated with BVAS ($r = -0.280$), VDI ($r = -0.263$), and C-reactive protein ($r = -0.261$) at diagnosis, whereas, it was positively correlated with haemoglobin ($r = 0.255$), and serum albumin ($r = 0.240$) at diagnosis, respectively. However, serum GPX-3 concentration at diagnosis was not significantly associated with poor outcomes during follow-up in patients with AAV.

Conclusion: In this study, we demonstrated for the first time that serum GPX-3 concentration at diagnosis correlates with vasculitis activity

and damage at diagnosis in patients with AAV, suggesting a possible role of serum GPX-3 as a complementary biomarker for assessing AAV activity in real clinical practice.

KEYWORDS

glutathione peroxidase-3, activity, inflammation, antineutrophil cytoplasmic antibody, vasculitis

1 Introduction

Glutathione peroxidases (GPXs) are a group of enzymes that can detoxify hydrogen peroxide to produce other free radicals (Handy and Loscalzo, 2022). Glutathione peroxidase-3 (GPX-3) is one of the 8 isozymes of GPX and particularly, as plasma GPX because it is mainly distributed extracellularly unlike other GPX isozymes (Rush and Sandiford, 2003). Several studies were measuring serum GPX-3 concentrations and investigating the clinical significance in patients with various inflammatory or cancerous diseases. Low serum GPX-3 concentration is considered a biomarker to assess a high degree of inflammation or poor prognosis (Bierl et al., 2004; Manzanares et al., 2009; Agnani et al., 2011), which might be attributed to be due to the clinical properties of GPX-3 as an antioxidant enzyme (Chang et al., 2020). Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a systemic vasculitis that invades the smallest units of arteries, veins, and capillaries and may potentially affect almost all major organs (Jennette et al., 2013; Watts et al., 2007). According to the clinical, laboratory, radiological, and histological features, AAV is divided into three subtypes: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic GPA (EGPA) (Suppiah et al., 2022; Robson et al., 2022; Grayson et al., 2022). Although three subtypes are essentially characterized by each subtype's predominant features, given the affected vessel system, they may share the common clinical manifestations related to the three typical major organs with the largest surface area of capillaries such as the kidneys, lungs, and skin (Kronbichler et al., 2024). On the other hand, the pathogenesis of AAV involves many immunological processes and responses, and particularly, the role of reactive oxygen species (ROS) is known to contribute significantly to vascular inflammation and damage (Kitching et al., 2020; Choi et al., 2019). Therefore, it can be reasonably assumed that if the expression of GPX isozymes with a peroxidase function to remove ROS is reduced, the degree of vascular inflammation and damage may increase. Moreover, it could be speculated that serum GPX-3 concentration may be inversely associated with the current degree of vascular inflammation and damage in patients with AAV. However, no study has elucidated the clinical role of serum GPX-3 concentration in assessing the current degree of vascular inflammation and damage in patients with AAV. Hence, in this study, we investigated whether serum GPX-3 concentration at diagnosis could be used to assess vasculitis activity and damage at diagnosis in immunosuppressive drug-naïve patients with AAV.

2 Materials and methods

2.1 Patients

We randomly selected 71 immunosuppressive drug-naïve patients newly diagnosed with AAV from an observational single-centre cohort of Korean patients with AAV at the University Tertiary Hospital and included them in this study. The inclusion criteria were i) fulfilment of the 2007 European Medicine Agency algorithm for AAV, the 2022 revised Chapel Hill Consensus Conference nomenclature of vasculitides, and the 2022 American College of Rheumatology and European Alliance of Associations for Rheumatology classification criteria for MPA, GPA, and EGPA (Handy and Loscalzo, 2022; Rush and Sandiford, 2003; Suppiah et al., 2022; Robson et al., 2022; Grayson et al., 2022); ii) first diagnosis of AAV by the specialised Rheumatologists in this tertiary hospital; iii) sufficiently documented medical records for collecting clinical, laboratory, radiological, and histological data; iv) well-prepared sera obtained on patients' consent and stored at diagnosis; v) follow-up duration for 6 months or greater after diagnosis; vii) no serious concomitant medical conditions mimicking AAV such as malignancies, severe infectious diseases requiring hospitalization, and autoantibody-mediated autoimmune diseases, or inducing ANCA false positive at diagnosis; vii) no exposure to immunosuppressive drugs for AAV treatment at least within 4 weeks before diagnosis. This study was approved by the Institutional Review Board (IRB) of Severance Hospital, Seoul, Republic of Korea (IRB number 4-2016-0901) and, when required, written informed consent was obtained from patients at the time of blood sampling. The IRB waived the need for written informed consent when it was previously obtained at entry into the SHAVE cohort.

2.2 Blood sampling and consent form

On the day of AAV diagnosis, informed consent was obtained, AAV-specific indices were assessed, and whole blood was collected from patients with AAV. Sera were immediately isolated from whole blood and stored at -80°C on the day of AAV diagnosis.

2.3 Clinical data at diagnosis

Age, sex, smoking history, and body mass index (BMI) were recorded. Data on AAV-subtype, ANCA type and positivity, and

AAV-specific indices were also collected. In the present study, myeloperoxidase (MPO)-ANCA and proteinase 3 (PR3)-ANCA measured using an immunoassay as well as perinuclear (P)-ANCA and cytoplasmic (C)-ANCA detected using an indirect immunofluorescence assay with ethanol fixation were all recognised as ANCA test results according to the 2022 criteria (Suppiah et al., 2022; Robson et al., 2022; Grayson et al., 2022). AAV-specific indices included the Birmingham vasculitis activity score (BVAS), the five-factor score (FFS), the 36-Item Short Form Survey (SF-36) physical and mental component summaries (PCS and MCS), and the vasculitis damage index (VDI) (Mukhtyar et al., 2009; Guillevin et al., 2011; Flossmann et al., 2007; Han et al., 2004). In this study, the degrees of vascular activity and the extent of damage were expressed using BVAS and VDI, respectively. Routinely performed laboratory test results, including those of acute-phase reactants such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) tests, were recorded.

2.4 Measurement of serum GPX-3 concentration at diagnosis

Serum GPX-3 concentration was assessed from stored sera at diagnosis using enzyme-linked immunosorbent assay kits (Mybiosource, San Diego, CA, United States) according to the manufacturer's instructions. In this study, a continuous variable of serum GPX-3 concentration was used for statistical analyses.

2.5 Clinical data during follow-up

All-cause mortality, end-stage kidney disease (ESKD), cerebrovascular accident (CVA), and acute coronary syndrome (ACS) were considered poor AAV outcomes during follow-up. The follow-up duration based on each poor outcome was defined as the period from diagnosis to its occurrence in patients with a corresponding poor outcome, whereas the duration from diagnosis to the last visit was defined for those without. Medications administered after AAV diagnosis and during the disease course, including glucocorticoids and immunosuppressive drugs, were also assessed. Accordingly, ESKD, CVA, and ACS that occurred before diagnosis were not considered poor AAV outcomes in the present study.

2.6 Statistical analyses

All statistical analyses were performed using the SPSS version 26 (IBM Corporation, Armonk, NY, United States) for Windows (Microsoft Corporation, Redmond, WA, United States). Continuous and categorical variables are expressed as medians (interquartile range, Q1–Q3), and numbers (percentages). The Mann-Whitney U test was used to compare significant differences between continuous variables. The correlation coefficients (r) between the two variables were determined using Pearson's correlation analysis. Cox proportional hazards model analysis was performed to obtain

the hazard ratio (HR) of serum GPX-3 concentration at diagnosis for each poor outcome during follow-up. A significant area under the curve (AUC) was determined using receiver operating characteristic (ROC) curve analysis. A comparison of the cumulative survival rates between the two groups was performed using Kaplan Meier survival analysis with the log-rank test. Statistical significance was set at $P < 0.05$.

3 Results

3.1 Characteristics of patients with AAV at diagnosis

The median age of the study subjects was 63.0 (51.0–74.0) years, and 26 and 45 patients were males and females, respectively. Two patients were ex-smokers and the median BMI was 22.4 (21.1–24.8) kg/m^2 . Of these 71 patients, 35, 23, and 13 were diagnosed with MPA, GPA, and EGPA, respectively. The median BVAS, FFS, SF-36 PCS and MCS, and VDI values were 5.0 (3.0–17.0), 0 (0–1.0), 52.5 (34.4–68.1), 58.4 (40.0–73.4), and 3.0 (2.0–4.0), respectively. The median ESR and CRP were 21.0 (7.0–85.3) mm/h and 3.8 (0.9–29.1) mg/L, respectively. The median GPX-3 concentration was measured as 82.8 (44.3–156.0) ng/mL (Table 1).

3.2 Correlation analysis of serum GPX-3 concentration and continuous variables at diagnosis in patients with AAV

Serum GPX-3 concentration at diagnosis was inversely correlated with BVAS ($r = -0.280$, $P = 0.018$), VDI ($r = -0.263$, $P = 0.029$), and CRP ($r = -0.261$, $P = 0.028$) at diagnosis, whereas, it was positively correlated with haemoglobin ($r = 0.255$, $P = 0.032$), and serum albumin ($r = 0.240$, $P = 0.045$) at diagnosis, respectively. Additionally, both SF-36 PCS and ESR tended to correlate with serum GPX-3 concentration simultaneously but the correlation was not statistically significant (Table 2).

3.3 Comparison of serum GPX-3 concentration between the two groups

At the time of diagnosis, serum GPX-3 concentrations according to sex, AAV subtype, MPO-ANCA (or P-ANCA) positivity, PR3-ANCA (or C-ANCA) positivity, and comorbidities were compared but no significant differences between the two groups were observed (Figure 1). Additionally, since serum GPX-3 concentration was inversely correlated with BVAS, we divided the study participants into two groups according to each of the nine systemic items of BVAS and compared serum GPX-3 concentrations between the two groups (Mukhtyar et al., 2009). We found that only serum GPX-3 concentrations, according to general manifestation, were significantly different between the two groups (55.8 ng/mL for patients with general manifestation vs. 84.8 ng/mL for those without, $P = 0.021$) (Table 3).

TABLE 1 Characteristics of patients with AAV at diagnosis (N = 71).

Variables	Values
Demographic data	
Age (years)	63.0 (51.0–74.0)
Male sex [N, (%)]	26 (36.6)
Female sex [N, (%)]	45 (63.4)
Ex-smoker [N, (%)]	2 (2.8)
Body mass index (kg/m ²)	22.4 (21.1–24.8)
AAV subtype [N, (%)]	
MPA	35 (49.3)
GPA	23 (32.4)
EGPA	13 (18.3)
ANCA type and positivity [N, (%)]	
MPO-ANCA (or P-ANCA) positive	41 (57.7)
PR3-ANCA (or C-ANCA) positive	11 (15.5)
Both ANCA positive	3 (4.2)
AAV-specific indices	
BVAS	5.0 (3.0–17.0)
FFS	0 (0–1.0)
SF-36 PCS	52.5 (34.4–68.1)
SF-36 MCS	58.4 (40.0–73.4)
VDI	3.0 (2.0–4.0)
Comorbidities [N, (%)]	
Type 2 diabetes mellitus	15 (21.1)
Hypertension	23 (32.4)
Dyslipidaemia	13 (18.3)
Routinely performed laboratory test results	
ESR (mm/hr)	21.0 (7.0–85.3)
CRP (mg/L)	3.8 (0.9–29.1)
White blood cell count (/mm ³)	7,500.0 (5,930.0–9,640.0)
Haemoglobin (g/dL)	12.4 (10.4–13.6)
Platelet count (x 1,000/mm ³)	243.5 (193.0–354.8)
Fasting glucose (mg/dL)	94.0 (88.0–109.0)
Blood urea nitrogen (mg/dL)	19.2 (13.1–28.7)

(Continued on the following page)

TABLE 1 (Continued) Characteristics of patients with AAV at diagnosis (N = 71).

Variables	Values
Serum creatinine (mg/dL)	0.8 (0.6–1.5)
Total serum protein (g/dL)	6.8 (6.4–7.4)
Serum albumin (g/dL)	4.2 (3.7–4.4)
Alkaline phosphatase (IU/L)	70.0 (58.0–92.0)
Aspartate transaminase (IU/L)	20.5 (16.0–25.8)
Alanine transaminase (IU/L)	16.5 (11.0–26.0)
Serum GPX-3 (ng/mL)	82.8 (44.3–156.0)

Values are expressed as a median (25–75 percentile) or N (%). ANCA, antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic GPA; MPO, myeloperoxidase; P, perinuclear; PR3, proteinase 3; C, cytoplasmic; BVAS, the Birmingham vasculitis activity score; FFS, the five-factor score; SF-36, the 36-Item Short Form Survey; PCS, physical component summary; MCS, mental component summary; VDI, the vasculitis damage index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; GPX-3, glutathione peroxidase-3.

3.4 Poor outcomes and immunosuppressive drugs administered during follow-up in patients with AAV

During follow-up after diagnosis, among the 71 patients, 6 (8.5%) died, and 17, four, and one had ever experienced and suffered from ESKD, CVA, and ACS, respectively. Glucocorticoids were administered to 70 (98.6%) patients. Among the intravenous induction therapeutic regimens, cyclophosphamide, and rituximab were administered to 47 (66.2%), and 13 (18.3%) patients, respectively. Among oral immunosuppressive drugs, the most frequently administered was azathioprine (57.7%), followed by mycophenolate mofetil (26.8%) (Table 4).

3.5 Cox analysis of serum GPX-3 concentration for each poor outcome

Serum GPX-3 concentration at diagnosis was not significantly associated with all-cause mortality, ESKD, CVA, or ACS during follow-up in patients with AAV (Table 5).

4 Discussion

Considering the clinical utility of low serum GPX-3 concentration (or GPX-3 deficiency) in various diseases, in this study we investigated whether serum GPX-3 concentration at diagnosis could be used to assess vasculitis activity and damage at diagnosis in immunosuppressive drug-naïve patients with AAV, and obtained some interesting findings. First, significant correlations were observed between serum GPX-3 concentration at diagnosis and both BVAS and VDI at diagnosis, indicating that serum GPX-3

TABLE 2 Correlation analysis of serum GPX-3 concentration and continuous variables at diagnosis in patients with AAV.

Variables	Correlation coefficient (r)	P-value
Demographic data		
Age (years)	−0.054	0.652
Body mass index (kg/m ²)	0.013	0.917
AAV-specific indices		
BVAS	−0.280	0.018
FFS	−0.131	0.277
SF-36 PCS	0.219	0.067
SF-36 MCS	0.197	0.100
VDI	−0.263	0.029
Acute-phase reactants		
ESR (mm/hr)	−0.217	0.071
CRP (mg/L)	−0.261	0.028
Routinely performed laboratory test results		
White blood cell count (/mm ³)	−0.107	0.374
Haemoglobin (g/dL)	0.255	0.032
Platelet count (x 1,000/mm ³)	−0.120	0.322
Fasting glucose (mg/dL)	−0.132	0.303
Blood urea nitrogen (mg/dL)	0.021	0.862
Serum creatinine (mg/dL)	−0.051	0.675
Total serum protein (g/dL)	0.168	0.166
Serum albumin (g/dL)	0.240	0.045
Alkaline phosphatase (IU/L)	−0.048	0.691
Aspartate transaminase (IU/L)	0.047	0.703
Alanine transaminase (IU/L)	0.129	0.288

GPX-3, glutathione peroxidase-3; ANCA: antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis; BVAS: the Birmingham vasculitis activity score; FFS, the five-factor score; SF-36, the 36-Item Short Form Survey; PCS, physical component summary; MCS, mental component summary; VDI, the vasculitis damage index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

can be a potential biomarker to assess vasculitis activity and damage at diagnosis in patients newly diagnosed with AAV. Additionally, serum GPX-3 had another potential to reflect the inflammatory burden as much as traditional acute-phase reactants such as CRP and serum albumin. However, serum GPX-3 did not predict poor

AAV outcomes during follow-up. Therefore, in the present study, we demonstrated that serum GPX-3 concentration at diagnosis has the potential to be a novel biomarker that can assess and estimate activity and damage caused by AAV, although it is confined to the time of diagnosis.

ROS are well-known mediators of the pathophysiological signal transduction of inflammation processes (Forrester et al., 2018). At the site of inflammation, the increased ROS production by polymorphonuclear cells may induce endothelial cell dysfunction and tissue damage. Furthermore, the combination of endothelial impairment and oxidative stress may loosen the endothelial junction of the blood-tissue barrier and accelerate the extravascular migration of inflammatory immune cells to the inflamed tissue (Mittal et al., 2014). Similarly, in the pathophysiology of AAV, ROS is also known to play an important role in the development and exacerbation of AAV (Kitching et al., 2020; Choi et al., 2019). GPX-3 can reduce oxidative stress by scavenging ROS, which may reduce the nuclear translocation of transcription factors such as the p65 unit of nuclear factor kappa-light-chain-enhancer of activated B cells, ultimately suppressing the expression of inflammatory mediators located downstream of the promoter sites (An et al., 2018; Nirgude and Choudhary, 2021). Based on these findings, it is reasonable to infer that decreased GPX-3 expression and production may increase vascular activity and damage owing to a diminished effect in suppressing the transcription of inflammatory mediators. This inference supports the key finding of this study that serum GPX-3 concentration at diagnosis was inversely correlated with BVAS, VDI, and CRP at diagnosis.

We investigated which item among the nine systemic items of BVAS based on major organ involvement contributed to the inverse correlation with serum GPX-3 concentration. We divided the study participants into two groups according to each of the nine systemic items of BVAS and compared serum GPX-3 concentrations between the two groups (Mukhtyar et al., 2009). Only serum GPX-3 concentrations, according to general manifestation, was significantly different between the two groups. Additionally, otorhinolaryngologic (68.9 ng/mL vs. 88.6 ng/mL, $P = 0.054$) and pulmonary (71.3 ng/mL vs. 86.5 ng/mL, $P = 0.070$) manifestations showed significant differences between the two groups but they did not reach statistical significance. Although GPX-3 has been reported to be associated with renal pathological findings (Burk et al., 2011), this study was not able to reveal an association between serum GPX-3 concentration and kidney involvement of AAV (Table 3). As per the general manifestation, since its minor items were closer to constitutional symptoms, such as myalgia, arthralgia/arthritis, fever, and weight loss, rather than those limited to a specific organ (Mukhtyar et al., 2009), this subgroup analysis was not sufficient to explain the inverse correlation between serum GPX-3 concentration and BVAS. Therefore, we conclude that the correlation between serum GPX-3 concentration and BVAS may be based on the sum of the cumulative contributions of several organ involvements, such as the ear, nose, throat or lungs, rather than that of specific organ invasion by AAV.

In the present study, serum GPX-3 concentration at diagnosis could not predict poor outcomes during follow-up in patients with AAV. Given that the risk factors for all-cause mortality among the four poor outcomes were relatively well established, to verify the methodological error of the Cox analysis for the results

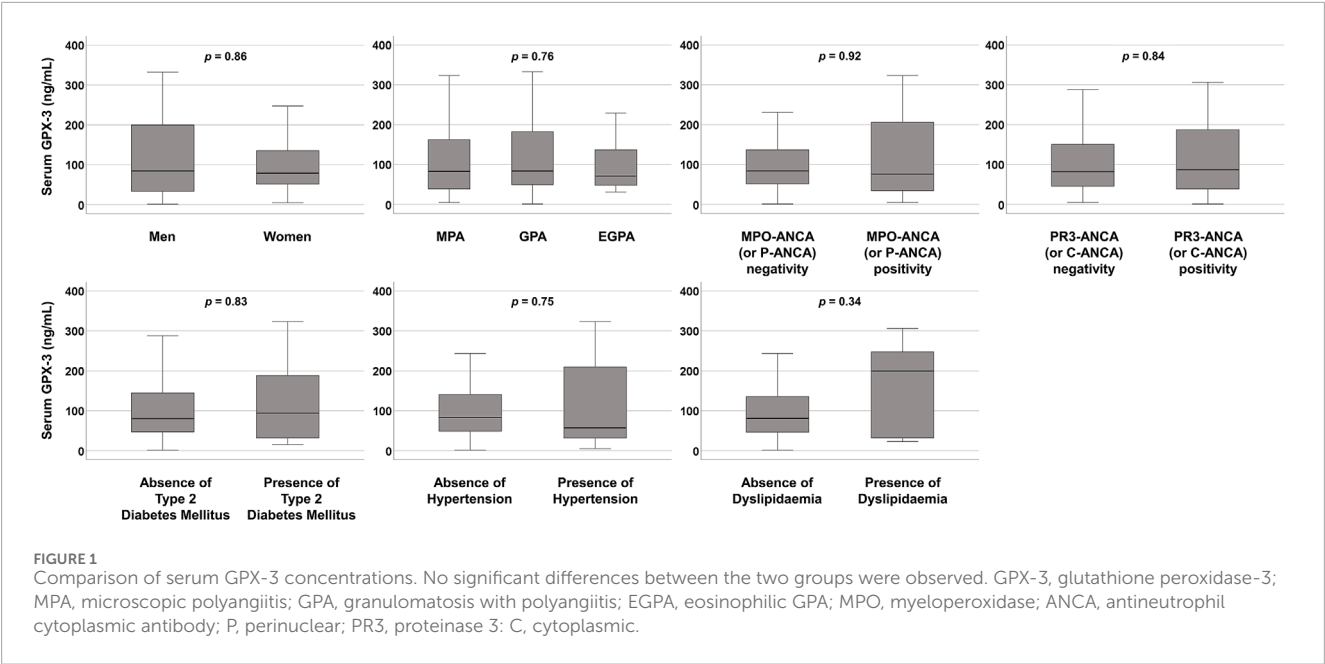


FIGURE 1 Comparison of serum GPX-3 concentrations. No significant differences between the two groups were observed. GPX-3, glutathione peroxidase-3; MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis; EGPA, eosinophilic GPA; MPO, myeloperoxidase; ANCA, antineutrophil cytoplasmic antibody; P, perinuclear; PR3, proteinase 3; C, cytoplasmic.

TABLE 3 Comparative analysis of serum GPX-3 according to the presence of each systemic item of BVAS in patients with AAV.

Systemic item of BVAS	Median serum GPX-3		P-value
	Absence	Presence	
General manifestation	84.8 ng/mL	55.8 ng/mL	0.021
Cutaneous manifestation	83.9 ng/mL	63.2 ng/mL	0.247
Mucous and ocular manifestation	81.3 ng/mL	83.4 ng/mL	0.694
Otorhinolaryngologic manifestation	88.6 ng/mL	68.9 ng/mL	0.054
Pulmonary manifestation	86.5 ng/mL	71.3 ng/mL	0.070
Cardiovascular manifestation	83.9 ng/mL	57.6 ng/mL	0.283
Gastrointestinal manifestation	N/A	N/A	N/A
Renal manifestation	80.1 ng/mL	84.3 ng/mL	0.972
Nervous systemic manifestation	84.3 ng/mL	82.0 ng/mL	0.568

GPX-3, glutathione peroxidase-3; BVAS, the Birmingham vasculitis activity score; ANCA, antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis; N/A, not applicable.

in Table 4 results, we performed univariable and multivariable Cox proportional hazards analyses including traditional, AAV-specific, and inflammation-related risk factors along with GPX-3 concentration at diagnosis for all-cause mortality during follow-up (Murray et al., 2013; Tan et al., 2017; Mukhtyar et al., 2008). In univariable Cox analysis, dyslipidaemia, VDI, white blood cell count, haemoglobin, and serum albumin were significantly

associated with all-cause mortality in patients with AAV; however, serum GPX-3 concentration was not. Nevertheless, to investigate the predictive and independent potential of serum GPX-3 concentration for all-cause mortality, we included serum GPX-3 concentration in multivariable Cox analysis. However, in multivariable Cox analysis, only dyslipidaemia was independently associated with all-cause mortality (Supplementary Table S1). Therefore, we verified that there was no methodological error in the Cox analysis, and demonstrated that the independent predictive potential of serum GPX-3 concentration for all-cause mortality was not notable.

Additionally, although a continuous value of serum GPX-3 concentration did not show significant predictive potential for poor outcomes, we wondered whether a categorical value of serum GPX-3 concentration according to its cut-offs of poor outcomes such as all-cause mortality, ESKD, CVA, and ACS could be a significant predictor of those poor outcomes. We attempted to obtain each optimal cut-off of serum GPX-3 concentration for each poor outcome using the receiver operating characteristic ROC curve analyses; however, we found no significant areas under the curve (AUC) of serum GPX-3 concentration for poor outcomes. Therefore, we failed to obtain the optimal cut-offs of serum GPX-3 concentration for all-cause mortality, ESKD, CVA, or ACS in this study (Supplementary Figure S1). Meanwhile, among the four poor outcomes, despite no statistical significance, all-cause mortality showed a relatively higher AUC compared to the remaining ESKD, CVA, and ACS. When the patients were divided into two groups such as the higher and lower groups according to the median value of GPX-3 concentration of 82.8 ng/mL, in Kaplan Meier survival analysis, however, no significant difference in cumulative patients' survival rates between patients in the higher and lower groups was observed (Supplementary Figure S2). We concluded that serum GPX-3 can be useful in estimating the current activity and damage of vasculitis but not anticipating future poor outcomes in patients with AAV. This study has the advantage that this is the first to demonstrate the significant correlations between serum GPX-3

TABLE 4 Poor outcomes and immunosuppressive drugs administered during follow-up in patients with AAV (N = 71).

Variables	Values
Poor outcomes [N, (%)]	
All-cause mortality	6 (8.5)
ESKD	17 (23.9)
CVA	4 (5.6)
ACS	1 (1.4)
Follow-up periods based on each poor outcomes (months)	
All-cause mortality	26.1 (10.8–45.7)
ESKD	24.3 (8.3–45.7)
CVA	25.8 (8.5–42.2)
ACS	25.8 (9.1–42.2)
Immunosuppressive drugs administered [N, (%)]	
Glucocorticoids	70 (98.6)
Cyclophosphamide	47 (66.2)
Rituximab	13 (18.3)
Mycophenolate mofetil	19 (26.8)
Azathioprine	41 (57.7)
Tacrolimus	6 (8.5)
Methotrexate	3 (4.2)

Values are expressed as a median (25–75 percentile) or N (%).

ANCA, antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis; ESKD, end-stage kidney disease; CVA, cerebrovascular accident; ACS, acute coronary syndrome.

TABLE 5 Univariable Cox proportional hazards analysis of serum GPX-3 concentration at diagnosis for each poor outcome during follow-up in patients with AAV.

Poor outcomes	HR	95% CI	P-value
All-cause mortality	0.998	0.988–1.008	0.694
ESKD	0.999	0.993–1.005	0.751
CVA	0.997	0.984–1.010	0.640
ACS	1.014	0.992–1.038	0.218

GPX-3, glutathione peroxidase-3; ANCA, antineutrophil cytoplasmic antibody; AAV, ANCA-associated vasculitis; ESKD, end-stage kidney disease; CVA, cerebrovascular accident; ACS, acute coronary syndrome.

concentration with activity (BVAS) and damage (VDI) at diagnosis in patients with AAV.

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombotic, non-thrombotic, and obstetric manifestations in individuals with persistent antiphospholipid

antibodies (aPL). These antibodies are commonly detected through immunoassays for anticardiolipin (aCL) and anti- β 2-glycoprotein-I (a β 2GPI) antibodies, or lupus anticoagulant tests. The interaction between aPL and targets like β 2GPI triggers the activation of endothelial cells, platelets, monocytes, and neutrophils, as well as the complement and coagulation systems, forming a key molecular mechanism underlying APS pathogenesis (Knight and Erkan, 2024). This interaction occurs at specific microdomains of the cell plasma membrane known as lipid rafts, which are rich in glycosphingolipids and cholesterol (Capozzi et al., 2023). Recent reviews highlight that Mitogen-Activated Protein Kinases (MAPKs) play a crucial role in transmitting signals from the cell surface to the nucleus, regulating gene activity. Among the four main branches of the MAPK pathway, p38MAPK is a key regulator in APS. Additionally, aPL stimulates the production of ROS in endothelial cells. These ROS act as secondary messengers, activating the p38MAPK pathway, which is involved in the pathogenesis of APS (Feng et al., 2024). GPX-3 is an antioxidant enzyme that plays a critical role in removing ROS, and thus, APS patients may exhibit a decrease in serum GPX-3 concentration. However, this study did not include patients with aPL antibodies, and therefore, subgroup analysis related to this aspect could not be performed.

5 Limitations

This study has several limitations. The most critical limitation was that the number of participating patients was not sufficiently large to generalize these results and apply them to real clinical practice and that clinical data and sera samples from the time of diagnosis were used retrospectively. Additionally, although this study focused on the clinical implications of serum GPX-3 concentration among patients with AAV only, this study could not provide the results of the comparative analysis of serum GPX-3 concentration between AAV patients and age- and sex-matched healthy controls. However, given that this is the first pilot study to explore the clinical utility of serum GPX-3 concentration in patients with AAV, we believe that this study has clinical implications in that this identified a novel biomarker that can assess and estimate activity and damage caused by AAV at diagnosis, although confined to the time of diagnosis. A prospective future study enrolling more patients and equipping serial clinical data and paired serum samples will provide more reliable and dynamic information on the clinical significance of serum GPX-3 concentration for not only assessing activity and damage at diagnosis but also predicting and monitoring the prognosis during follow-up in patients with AAV.

6 Conclusion

In this study, we demonstrated for the first time that serum GPX-3 concentration at diagnosis correlates with vasculitis activity and damage at diagnosis in patients with AAV, suggesting a possible role of serum GPX-3 as a complementary biomarker for assessing AAV activity in real clinical practice.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was approved by the Institutional Review Board (IRB) of Severance Hospital, Seoul, Republic of Korea (IRB number 4-2016-0901) and, when required, written informed consent was obtained from patients at the time of blood sampling. The IRB waived the need for written informed consent when it was previously obtained at entry into the SHAVE cohort.

Author contributions

JC: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing—original draft, Writing—review and editing. JH: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Visualization, Writing—original draft, Writing—review and editing. Y-BP: Conceptualization, Data curation, Methodology, Project administration, Validation, Writing—original draft, Writing—review and editing. S-WL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing—original draft, Writing—review and editing.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Metabolite profiling, antimalarial potentials of *Schleichera oleosa* using LC-MS and GC-MS: *in vitro*, molecular docking and molecular dynamics

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Purpose: To explore the phytochemical composition of *Schleichera oleosa* bark and evaluate its potential antimalarial activity through *in vitro* and *in silico* analyses.

Methods: The bark of *S. oleosa* was subjected to Soxhlet extraction using petroleum ether, chloroform, and methanol. The quantitative analysis of the extracts was performed to determine total phenolic, flavonoid, and tannin contents. Advanced analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) were employed to identify 175 phytoconstituents from the methanolic extract. *In-vitro* antimalarial activity was assessed against *Plasmodium falciparum* using the candle jar method, measuring parasite growth inhibition. The inhibitory concentration (IC₅₀) values were calculated and compared with standard antimalarial drugs, chloroquine and quinine. Furthermore, computational analyses, including molecular docking and molecular dynamics simulations, were conducted to evaluate the interactions of identified phytochemicals with key malarial targets (1CEQ and 4ZL4). The efficacy of these compounds was compared with standard drugs like artesunate and chloroquine. Additionally, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling and drug-likeness assessments were performed.

Results: The methanolic extract of *S. oleosa* exhibited promising *in-vitro* antimalarial activity with an average IC₅₀ value of 0.780 µg/mL, which, while higher than chloroquine (0.020 µg/mL) and quinine (0.268 µg/mL), still demonstrated significant efficacy. GC-MS and LC-MS analyses identified 175 phytoconstituents, among which two novel lead compounds, scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, exhibited the highest docking scores and favorable ADMET profiles. Molecular docking and dynamics simulations confirmed strong binding affinities to malarial targets, surpassing some standard drugs in efficacy.

Conclusion: This study reports, for the first time, the antimalarial potential of bioactive constituents derived from the bark of *S. oleosa*. The identified

compounds, scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, demonstrated promising antiplasmodial activity, validating traditional medicinal claims. The findings highlight the potential of *S. oleosa* as a source of novel antimalarial agents with fewer side effects compared to existing therapies. Further *in vivo* studies are warranted to confirm these results and support the development of new antimalarial drugs. This groundbreaking discovery contributes to the growing evidence supporting the role of medicinal plants in drug discovery.

KEYWORDS

antimalarial, *in silico*, *in vitro*, phytoconstituents, *Schleichera oleosa*

1 Introduction

For thousands of years, plants have been used as medicines and have played a significant part in traditional medical practices across many countries. In response, scientists are investigating different phytoconstituents to discover possible treatments. Among these, plants have geared attention due to their rich diversity of bioactive substances. These include flavonoids, alkaloids, and terpenoids, which possess therapeutic properties. Flavonoids, known for their antioxidant effects, can help in reducing inflammation and boosting the immune response. Alkaloids, with their broad spectrum of pharmacological activities, may inhibit the growth of the malaria parasite. Terpenoids, often noted for their antimicrobial properties, offer potential in disrupting the lifecycle of the parasite. Herbal remedies have been used to address a variety of health issues since time immemorial, ranging from common colds to chronic diseases, and play a crucial role in complementary and alternative medicinal practices. Furthermore, about 50% of modern-day medications originate from plant sources., thus highlighting the ongoing importance of plants in the process of drug discovery and development (Raj and Jhariya, 2023).

S. oleosa (Merr.) Oken. Commonly known as Kusum, which contains 1,858 species. India has 72 plant species of the Sapindaceae family. It's a big, almost evergreen, deciduous tree with a somewhat short fluted trunk and can reach a height of 40 m. *S. oleosa*, commonly known as Kusum, is recognized for its distinctive bark, which plays a crucial role in its identification and usage. The bark has been traditionally utilized in various cultures for its medicinal properties. It is typically rough and dark grey, often fissured with vertical cracks that give it a rugged appearance. This texture not only provides protection to the tree but also serves as a habitat for various microorganisms (Karthikeyan et al., 2023). This plant also has many reported pharmacological activities like anti-inflammatory activity, analgesic activity, anthelmintic activity, antibacterial activity, antioxidant activity, and reproductive activity (Goswami and Singh, 2017). According to phytochemical investigations, the bark of this plant contains scopoletin, beta-sitosterol, betulin, betulinic acid, lupeol and lupeol acetate (Bhatia et al., 2013). Additionally, another study has demonstrated that the outer bark of the *S. oleosa* plant contains tricadenic acid A and taraxerone (Ghosh et al., 2011). In addition to its approximately 10% tannin content, the bark is a source of significant anticancer agents, including betulin and betulinic acid.

These compounds have geared an interest in its phytochemical research due to their potential therapeutic benefits.

Malaria, a potentially deadly disease caused by parasites from infected mosquitoes, affects nearly half of the world's population, with approximately 3.3 billion people at risk of contracting the disease, according to the World Health Organization (Patel et al., 2024). It is predominantly affecting sub-Saharan Africa, also Asia, Latin America, and parts of Europe and the Middle East. Efforts to tackle malaria have made strides through various strategies, including mosquito nets treated with insecticides, indoor spraying, and effective antimalarial drugs. Research is ongoing to develop an effective malaria vaccine, which could play a crucial role in reducing the disease's global burden. The pathogens that cause malaria have several enzymes and active pathways that serve as targets for antimalarial drugs. The treatment of malaria has been done using chloroquine and derivatives. Quinine, the first antimalarial medication, was extracted from the bark of the cinchona tree in the 1940s. Due to its affordability, effectiveness, and lower toxicity, chloroquine was the preferred treatment for malaria for many years (Unnissa et al., 2015). However, in modern malaria therapy, the use of chloroquine has been restricted due to the resistance developed in the malaria parasite. Instead of chloroquine, other novel derivatives like amodiaquine, mefloquine, and artemisinin were developed to fight against the plasmodium parasites to cure malaria.

The term anti-malarial activity describes a substance's ability to prevent or cure malaria, a parasitic illness spread by the bites of infected mosquitoes. Antimalarial drugs work by targeting specific stages of the parasite's life cycle inside the human body, particularly concentrating on the erythrocytic phase, which is responsible for the symptoms of malaria (Na-Bangchang and Karbwang, 2019). The WHO presently advises using artemisinin-based combination therapies (ACTs) as the primary treatment option for uncomplicated malaria caused by *Plasmodium falciparum*. However, the advent of ACTs has largely replaced the use of quinine as the first-line treatment due to their superior efficacy and reduced side effects. The parasite responsible for this disease, primarily *Plasmodium falciparum*, has shown an alarming capacity to develop resistance to existing antimalarial drugs such as chloroquine and ACTs. This resistance not only complicates treatment efforts, but also increases the risk of transmission and severe outcomes. Consequently, the scientific community is actively engaged in the discovery and development of novel therapeutic agents. Research has focused on identifying novel drug targets within the lifecycle of the

parasite, leveraging advanced technologies, such as genomics and bioinformatics, to accelerate drug discovery. Additionally, alternative strategies, such as combination therapies and adjunctive treatments, are being explored to enhance the efficacy and prevent resistance. As the fight against malaria continues, innovation and dedication in the field of antimalarial research remain crucial in the quest to eradicate this debilitating disease.

In-silico methods are computational techniques and simulations used to model, analyze, and predict biological and chemical processes. This approach leverages the power of computer models to predict the behavior of molecules, interactions, and reactions, often before any physical experiments are conducted. In the process of drug discovery and development of new drugs, *in silico* techniques are frequently employed to identify promising therapeutic candidates, forecast their effectiveness, and evaluate their safety profiles. The primary advantage of *in silico* methods is their ability to rapidly analyze large datasets and generate hypotheses that can be tested *in vitro* and *in vivo*, thereby saving time and resources. As computational power continues to grow, *in silico* methodologies are becoming increasingly integral to modern scientific research and development.

In vitro studies allow researchers to examine the efficacy of plant extracts or compounds against the malaria parasite in a controlled laboratory environment. This method is crucial for identifying active ingredients that can potentially inhibit the growth and survival of the parasite. Additionally, *in silico* methods, which involve computer-based simulations and modeling, provide an efficient way to predict the interaction between plant-derived compounds and the molecular targets of the malaria pathogen. These approaches not only accelerate the identification of promising antimalarial agents but also help in understanding the underlying mechanisms of action (Boniface et al., 2015). Malarial proteins were selected based on previous reports like 1CEQ (*P. falciparum* lactate dehydrogenase with NADH binding site) (Kalani et al., 2013), 4ZL4 (plasmeprin V from *P. vivax* bound to a transition state mimetic) (Onguéné et al., 2014). The study focused on a comprehensive analysis of chemical constituents in the bark of *S. oleosa*, specifically phenols, flavonoids, and tannins, utilizing advanced techniques like GC-MS, LC-MS and molecular dynamics. GC-MS and LC-MS are critical tools for identifying and quantifying compounds in complex mixtures and provide detailed molecular information that is essential for understanding the pharmacokinetics and metabolism of potential drug candidates. GC-MS is particularly useful for volatile compounds, whereas LC-MS excels in the analysis of larger, non-volatile molecules, offering high sensitivity and specificity (Kumar et al., 2024). Molecular dynamics simulations complement these techniques by providing insights into the structural and functional dynamics of biological molecules at the atomic level (Huggins et al., 2019). This computational approach helps to predict how potential drugs interact with their targets, allowing for the optimization of binding affinities and the identification of promising therapeutic candidates. Together, these technologies form a powerful toolkit for drug discovery, enabling researchers to efficiently screen, identify, and refine new drug candidates with greater precision and speed (Duarte et al., 2019).

Malaria remains a global health challenge that requires the discovery of novel therapeutic agents to combat drug-resistant

Plasmodium strains (Okombo and Fidock, 2024). In the present study, we have investigated the potential of secondary metabolites from the bark of *Schleichera oleosa* as anti-malarial agents. We hypothesized that these compounds exhibit significant therapeutic properties through their interaction with specific malaria-related molecular targets. To test this hypothesis, a combination of *in vitro* and *in silico* approaches were used. Molecular docking was performed to evaluate the binding efficiency and intermolecular interactions, followed by ADMET prediction to assess the pharmacokinetic properties of the compounds. Additionally, molecular dynamics simulations were conducted to observe the stability and dynamics of these interactions over time. By addressing these aspects, this study aimed to elucidate the therapeutic potential of *S. oleosa* constituents and contribute to the development of novel antimalarial drugs.

2 Results and discussion

2.1 Yield of extraction

The extract was placed in a container that had already been weighed, and its weight was documented. To find the net weight of the extract, the weight of the empty container was subtracted from this measurement. The percentage yield was then determined by dividing the extract's weight by the initial sample's weight. For *S. oleosa*, the extractive values in petroleum ether, chloroform, and methanol were found to be 0.1618 g, 0.115 g, and 4.1385 g, respectively. Because methanol is highly polar, it was able to extract more components, resulting in higher extractive values with methanol compared to the other solvents.

2.2 Quantitative analysis

To evaluate these components, quantitative analyses were performed on three distinct extracts to determine their total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC). The findings for the TPC, TFC, and TTC of *S. oleosa* bark are detailed in Table 1, with standard graphs displayed in Figure 1. The methanolic extract of *S. oleosa* bark contained higher levels of tannin, flavonoid, and phenol compared to the other two other extracts like petroleum and chloroform (Kyaw et al., 2019; Thind et al., 2011). In this study, we report for the first time a quantitative analysis of the total flavonoid and tannin content of the methanolic extract of the bark of *S. oleosa*. Reddy et al. (2007) highlighted the anti-malarial properties of tannins extracted from *Punica granatum*, which is commonly known as pomegranate. Tannins are polyphenolic compounds known for their astringent properties and have been recognized in various traditional medicines for their therapeutic benefits (Pizzi, 2021). These compounds may inhibit the growth of Plasmodium species, the parasites responsible for malaria, by interfering with their lifecycle. This discovery opens new avenues for further exploration of how natural compounds can be harnessed to combat malaria, a disease that continues to affect millions worldwide.

TABLE 1 Quantitative tests with quantity of phytoconstituent present in each solvent.

S. No	Quantitative test	% Of PE	% Of CE	% Of ME
1	Total Phenolic Content	—	10.26%	64.09%
2	Total Flavonoid Content	24.68%	21.22%	60.37%
3	Total Tanin Content	—	6.03%	67.06%

2.3 GC-MS and LC-MS analysis

The analysis of *S. oleosa* bark's methanolic extract using GC-MS and LC-MS provides a comprehensive understanding of its phytoconstituents. The GC-MS analysis identified 151 compounds within the extract, demonstrating the technique's capability to separate and identify a wide range of volatile and semi-volatile compounds. Similarly, through the LC-MS analysis identified 24 compounds, focusing on those with sharp peaks, which often represent more stable, non-volatile compounds that are better suited for liquid chromatography. Figures 2, 3 likely illustrate the chromatograms obtained from these analyses, showing peaks that correspond to different phytoconstituents. Supplementary Table S1 appears to provide detailed information of identified phytoconstituents from GCMS and LCMS. Both methodologies utilize mass spectroscopy to identify compounds through their fragmentation patterns. This process involves comparing the observed patterns with existing spectral databases to accurately deduce the structure and identity of the compounds present. The exploration of phytoconstituents from plants such as *Hibiscus cannabinus*, *Corchorus capsularis*, and *Tetrapleura tetraptera* has shown promising results against malaria through both *in silico* and *in vitro* methods. Studies by Brahma et al. (2024) and Hamidu et al. (2023) have highlighted the potential of these plants as sources of antimalarial compounds. These investigations used computational models to predict interactions with malarial targets, followed by laboratory experiments to validate these findings.

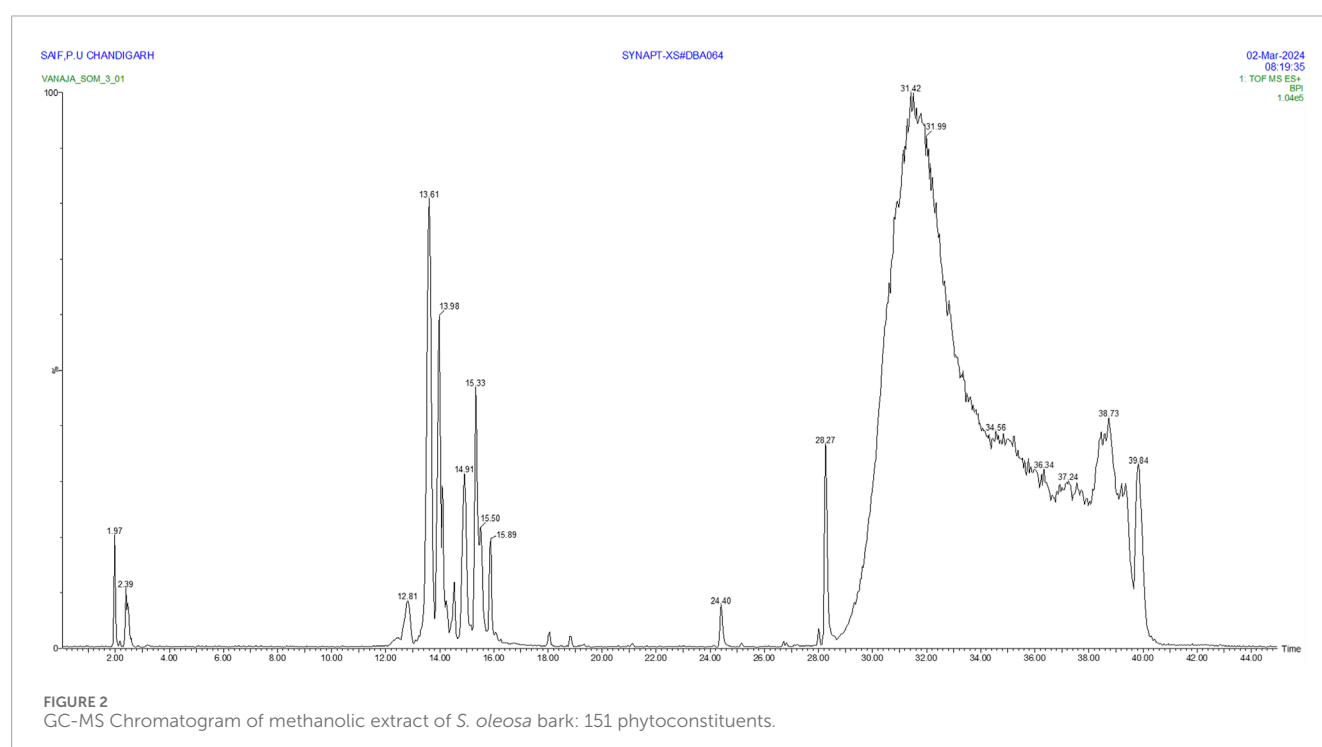
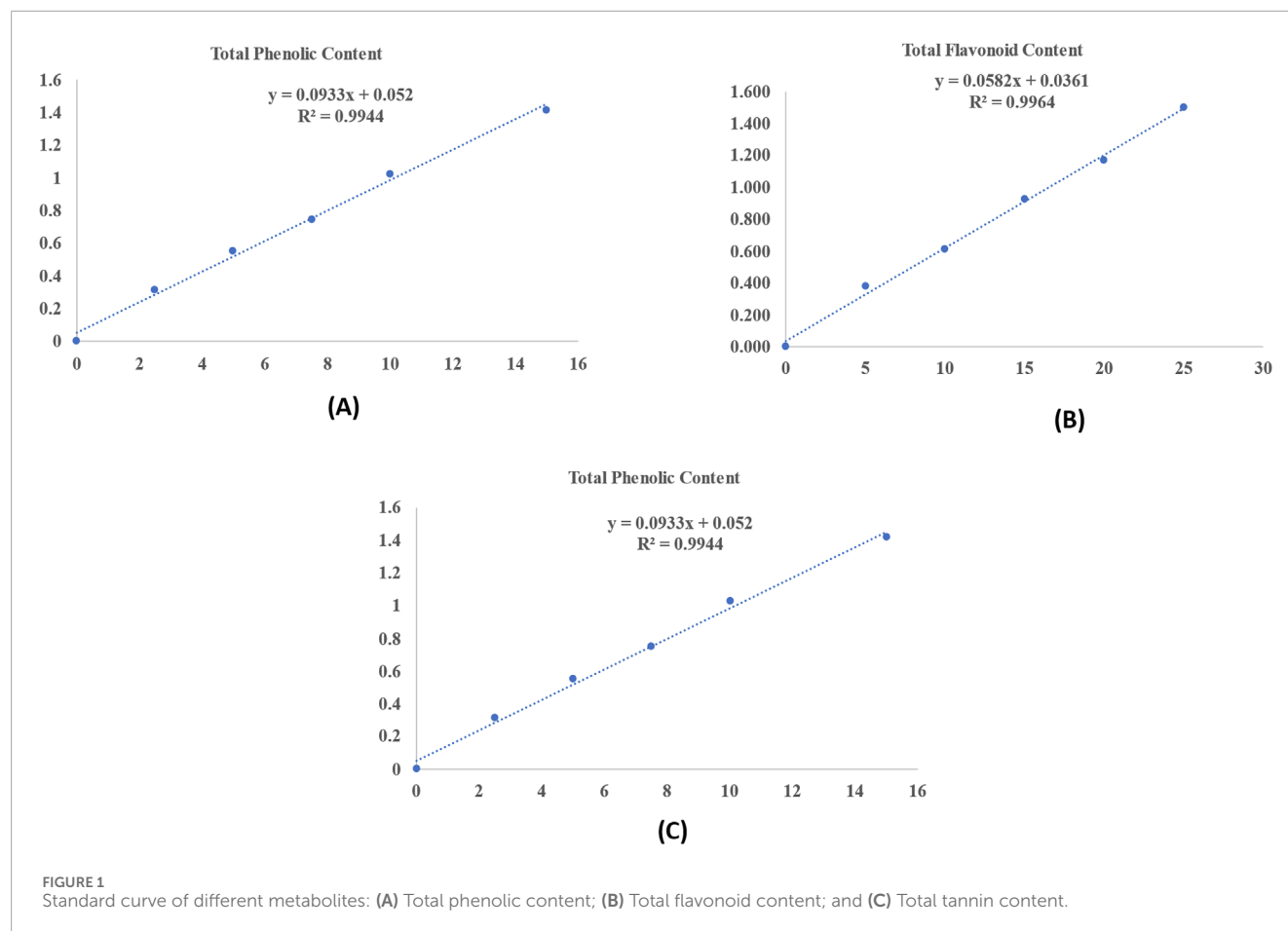
This dual approach of using GC-MS and LC-MS allows for a more robust and detailed characterization of the phytochemical profile of *S. oleosa*, highlighting its potential pharmacological and therapeutic applications. The compounds scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside have shown promising potential in combating malaria, as evidenced by their high binding affinity to malaria targets in molecular docking studies. Following this discovery, these compounds underwent comprehensive analyses to evaluate their drug-likeness, which involved assessing their physicochemical properties and compliance with criteria such as Lipinski's Rule of Five, indicating their potential as orally active drugs. Additionally, ADMET studies were also conducted to predict the pharmacokinetic profiles and safety of these compounds to ensure that they possessed favorable characteristics for therapeutic

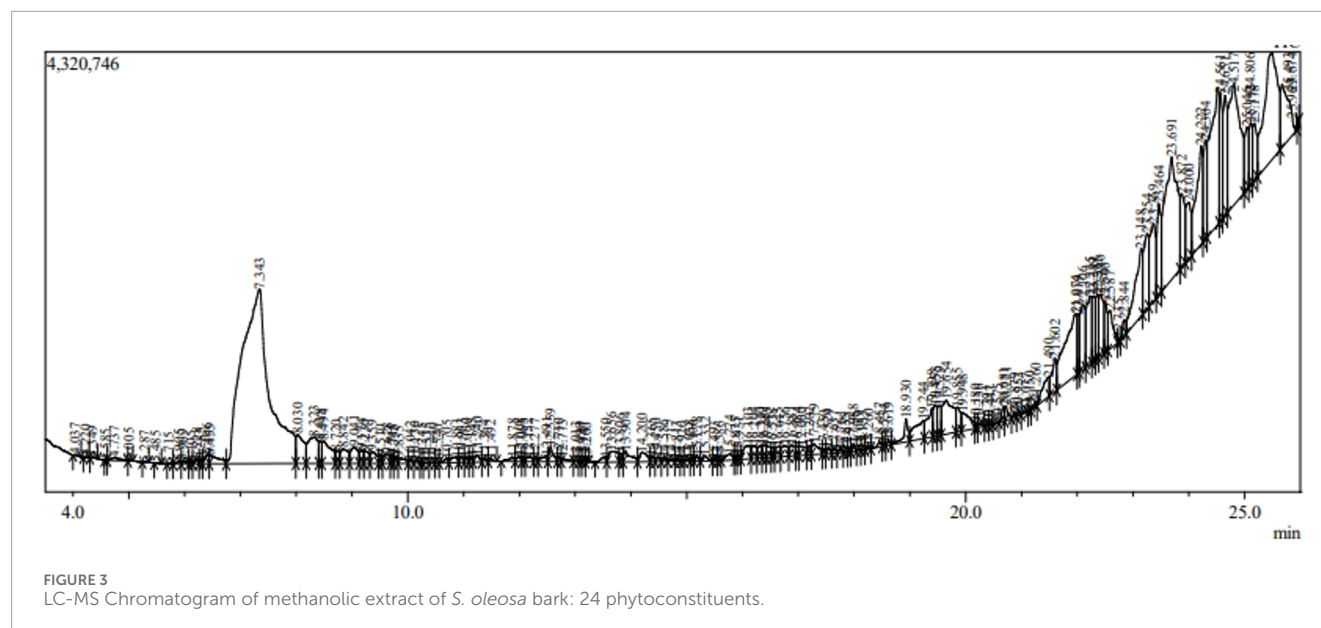
use. Furthermore, their stability was examined under molecular dynamics conditions to verify their structural integrity and performance under physiological conditions. These analyses provide a robust foundation for developing an effective anti-malarial agents from *S. oleosa*.

2.4 In vitro antimalarial activity

The study aimed to assess the antimalarial properties of a methanolic plant extract against *Plasmodium falciparum* using the Rieckmann micro-assay method (Rieckmann et al., 1968). After a 38-h incubation period, researchers counted the average number of rings, trophozoites, and schizonts per 100 parasites from duplicate wells. The effectiveness of the plant extract was measured by comparing the percentage of maturation inhibition to a control group. Remarkably, the extract demonstrated a minimum inhibitory concentration (MIC) of 0.78 µg/mL, with an IC₅₀ value matching this MIC, indicating strong inhibitory potential against the malaria-causing parasite. This finding suggests that the plant extract could be a promising candidate for developing new antimalarial treatments, offering a potential alternative to existing therapies. *In vitro* systems, which are invaluable in scientific research, have notable limitations that must be considered when interpreting their results. The primary drawback is the inability to fully mimic intricate interactions within living organisms. These systems typically focus on 1 cell type or a limited combination of cell types, which restricts their ability to replicate complex cellular interactions and physiological processes *in vivo*. This limitation can affect their utility in comprehensive pharmacokinetic or toxicokinetic studies, as they cannot accurately simulate the distribution, metabolism, or excretion of substances within an entire organism. Despite these constraints, *in vitro* systems remain valuable for examining specific cellular responses, such as changes in morphology, particle uptake, cell signalling, gene expression, and protein production, which often correlate with *in vivo* outcomes (Stone et al., 2009).

In the present study, the average IC₅₀ value of the methanolic extract (0.780 µg/mL), which was found to be higher than that of the standard drugs, chloroquine (0.020 µg/mL) and quinine (0.268 µg/mL), however, confirming its antimalarial efficacy. This diminished efficacy could be attributed to the concentration of phytoconstituent(s) which is/are responsible for antimalarial property in the crude extract may be very low or the net antimalarial property of the extract may not be due to a single phytoconstituent, rather it may be due to a mixture of phytoconstituents present in low concentration in the crude extract or the bioavailability of active compounds in the extract may very be low. The potential antimalarial properties of bark extract may be linked to the presence of phenols, flavonoids, and tannins (Goswami and Singh, 2017). These phytoconstituents are known for their antioxidant and antimicrobial activities, which could contribute to combating malaria (Khandekar et al., 2015). However, due to the complex nature of malaria treatment and the need for precise action against the parasite, the extract may not achieve the same level of effectiveness as established pharmaceuticals. Further research could explore optimizing extraction methods or combining plant-based compounds with standard treatments to enhance their efficacy.





2.5 Molecular docking

AutoDock Vina is a widely-used molecular docking software that facilitates the study of interactions between proteins and various compounds, aiding in drug discovery and design. In this context, the proteins 1CEQ and 4ZL4 were sourced from the Protein Data Bank to serve as targets for docking studies. The docking process involves evaluating 175 phytoconstituents, with their scores derived from GCMS and LCMS analyses illustrated in [Supplementary Table S1](#). These scores reflect the stability and affinity of the compounds at the protein binding sites. Typically, compounds with lower docking scores are considered more stable and potentially more effective due to their stronger binding affinity. Conversely, compounds that exhibit higher docking scores than the standards are highlighted in bold, indicating a potential deviation in binding efficiency. In molecular docking studies, identifying the most favorable docking pose is crucial for understanding the interactions between a ligand and its target protein. The lowest binding energy conformation is often considered the most stable and thus most likely to occur in nature. This energy was calculated as the sum of the total intermolecular energy, total internal energy, and torsional free energy, with the energy of the unbound system subtracted, providing an accurate estimate of the binding affinity. In this process, tools such as AutoDock are used to generate multiple conformations of the ligand, typically selecting the top ones based on their energy values ([Hasan et al., 2015](#)). The top 16 ligand conformations were generated based on the binding energy value using AutoDock. The conformation with the lowest binding energy is typically considered to be the most favorable because it suggests a more stable interaction ([Chaniad et al., 2016](#)). This conformation is often found in the most populated cluster, indicating that it is not only energetically favorable but also statistically significant. Analyzing this conformation allows researchers to gain insights into key interactions, such as hydrogen bonds, hydrophobic interactions, and electrostatic forces, which contribute to binding affinity and specificity. Structural biology has seen significant advancements,

particularly through the integration of computational methods, such as structure-based virtual high-throughput screening, which plays a critical role in drug discovery and development. This approach allows researchers to efficiently identify potential drug candidates by simulating their interactions with target proteins. However, despite these advances, current docking methodologies have several limitations. For instance, accurately modeling the flexibility of ligands and proteins remains challenging, which can affect the precision of binding predictions. Additionally, entropic effects, solvation/desolvation processes, and the presence of water molecules and ions further complicate the simulations. The existence of tautomers, allosteric effects, and the molecular context of the binding sites also add layers of complexity. Achieving binding and pharmacokinetic effects is crucial for developing effective therapeutics. Addressing these challenges requires the continual refinement of computational models and methodologies to improve drug discovery accuracy and reliability.

2.5.1 Phytoconstituents with top docking score for 1CEQ protein target

The methanolic extract of *S. oleosa* bark has demonstrated promising potential in terms of binding affinity when compared to the standard reference compound, chloroquine, which has a binding affinity of -8 . This suggests that ten compounds might have a stronger interaction with the target site, indicating potential therapeutic benefits such as Isorhamnetin 3-glucoside (-8.8), (+)-epicatechin-3-O-gallate (-8.8), (–)-Epicatechin-3-O-gallate (-8.7), Scillarenin (-8.6), (3beta, 17xi)-Stigmast-5-en-3-yl D-glucopyranoside (-8.6), (1Z, 2E)-1-([[(2R,3S,4S,5R, 6S)-6-[[5,7-Dihydroxy-2-(4-oxo-2,5-cyclohexadien-1-ylidene)-2H-chromen-3-yl] oxy]-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl] methyl] oxonio)-3-(4-hydroxyphenyl)-2-propen-1-olate (-8.6), 5-(beta-D-Glucopyranosyloxy)-2-(4-hydroxyphenyl)-7-chromeniumolate (8.2), (1S)-1,5-Anhydro-1-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromen-8-yl)-D-glucitol (-8.1), 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside (-8.1) and dUDP (-8.1).

2.5.2 Phytoconstituents with top docking score for 4ZL4 protein target

The study on the methanolic extract of *S. oleosa* bark reveals that several phytoconstituents demonstrate a higher binding affinity than chloroquine, which has a binding affinity of -8.1 . This suggests that fifteen compounds might have a stronger interaction with the target site, indicating potential therapeutic benefits such as (1Z, 2E)-1-(((2R,3S,4S,5R, 6S)-6-[[5,7-Dihydroxy-2-(4-oxo-2,5-cyclohexadien-1-ylidene)-2H-chromen-3-yl] oxy}-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl] methyl} oxonio)-3-(4-hydroxyphenyl)-2-propen-1-olate (-9.9), 5-(beta-D-Glucopyranosyloxy)-2-(4-hydroxyphenyl)-7-chromeniumolate (-9.2), Isorhamnetin 3-glucoside (-9), (+)-epicatechin-3-O-gallate (-9), 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside (-9), Glutathione (-9), (3beta, 17xi)-Stigmast-5-en-3-yl D-glucopyranoside (-8.6), Paromomycin (-8.6), 2-Oxazamine, 4,5-dihydro-5-(phenoxy methyl)-N-[(phenylamino)carbonyl]- (-8.4), Scillarenin (-8.3), (1S)-1,5-Anhydro-1-(5,7-dihydroxy-4-oxo-2-phenyl-4H-chromen-8-yl)-D-glucitol (-8.3), aloesin (-8.2), tetrapentacontane, 1,54-dibromo- (-8.2), magnesium 3-[8-(1-hydroxy-3-methoxy-3-oxopropyl)-2,7,13,17-tetramethyl-3,18-divinyl-21H-porphin-24-id-12-yl] propanoate (-8.2) and (-)-epicatechin-3-O-gallate (-8.2). These findings suggest that these phytoconstituents could potentially offer more effective binding interactions compared to chloroquine, indicating their potential as candidates for further investigation in the development of therapeutic agents.

2.6 Drug likeness and ADMET studies

Drug likeness and ADMET studies are crucial in the drug development process as they assess the Absorption, Distribution, Metabolism, Excretion, and Toxicity of potential compounds illustrated in Table 2. In this study, based on the higher docking score of ligands than the standard chloroquine, sixteen ligands with high-binding affinity were evaluated using the ADMET study and drug likeness characters. The evaluation of ADMET properties plays a crucial role in drug development, influencing decisions at every stage, from hit selection to preclinical development. Despite significant advances in the assessment of ADMET properties, several limitations hinder this process. The major challenge is the discrepancy between the *in vitro* and *in vivo* results. Although *in vitro* models provide valuable insights, they often fail to fully replicate the complex biological environments of living organisms. Algorithm limitations also pose a challenge, as the computational models used to predict ADMET properties may not always accurately reflect real-life scenarios. Furthermore, the physiological relevance of *in vitro* models is sometimes limited because they do not account for factors such as human-specific metabolic pathways. Lastly, variability in absorption can complicate predictions because individual differences in physiology can lead to significant variability in drug adsorption (Chandrasekaran et al., 2018). These factors are vital in determining the most suitable route of administration for drug formulations. ADMET properties help to predict how a drug behaves in the body, thereby influencing its efficacy and

safety. Absorption determines how well a drug is taken up into the bloodstream; distribution assesses how it spreads through tissues; metabolism examines how the body processes it, often into active or inactive forms; excretion focuses on how the drug and its metabolites are eliminated; and toxicity evaluates the potential for adverse effects (Chandrasekaran et al., 2018). Among them sixteen phytoconstituents, nine phytoconstituents were common for both the targets. Only two compounds identified as scillarenin and 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, exhibited the highest docking scores for the selected targets. This suggests that these two phytoconstituents have significant potential for further development as therapeutic agents, due to their strong binding affinity, good ADMET properties and favorable drug-likeness properties. As a result, these two phytoconstituents have been selected for further investigation due to their promising ADMET profiles and drug-likeness properties from the methanolic extract of *S. oleosa* bark.

2.7 Intermolecular interactions

In this study, to examine intermolecular interactions, Discovery Studio and LigPlot were employed to analyze ligand 100, standard chloroquine, and ligand 165. These tools are instrumental in visualizing and understanding how ligands interact within their target environments, often highlighting key bonds and interactions such as hydrogen bonds and hydrophobic contacts. The detailed findings of this analysis were systematically presented in Supplementary Table S2 and visually illustrated in binding site prediction through the Discovery studio (Figure 4), 2D interactions (Figure 5) and Intermolecular interactions through the LigPlot analysis (Figure 6). These figures and tables likely provide a comparative visual representation of the interaction patterns of each ligand, offering insights into their binding affinities and potential efficacy. Such analyses are crucial in drug discovery and development, aiding in the identification of promising lead compounds based on their molecular interactions. In this study of intermolecular interactions, the comparison of ligand interactions with the 1CEQ and 4ZL4 protein complexes reveals interesting insights. For the 1CEQ complex, scillarenin (ligand 100) formed 8 hydrophobic contacts and 1 hydrogen bond, whereas standard chloroquine showed higher hydrophobic interactions with 12 contacts. For the 1CEQ complex, 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside (ligand 165) demonstrated a significant affinity by establishing 8 hydrophobic contacts and 6 hydrogen bonds, indicating a robust interaction profile. Common interaction sites for ligand 100 and chloroquine included Gly29, Met30, Ile31, Thr97, Ala236, and Pro246, while ligand 165 and chloroquine also have common interactions like Asn140, His195, and Gly196. Similarly, for the 4ZL4 complex, scillarenin formed 7 hydrophobic contacts, chloroquine complex with 4ZL4 had 11, and 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside (ligand 165) complex with 4ZL4 exhibits 12 hydrophobic contacts and 2 hydrogen bonds. These findings highlight the variability in ligand-protein interactions depending

TABLE 2 Drug likeness and ADMET property of the selected top ligands from methanolic bark extract of *S. oleosa* and reference standard drugs.

Ligand No	Lead hit for target (s)	Drug likeness						ADMET property			
		MR 40–130	MW <500	HBD <5	HBA <10	LogP <5	RO5	BBB	PPB (%)	WS log (mol/L)	Carcinogenicity
4	4ZL4	87.7083	311	2	6	2.641	100%	96.70%	82.00%	−4.038	36.10%
58	4ZL4	136.3873	615	18	19	−8.861	0%	10.09%	0.00%	−0.515	23.80%
100	1CEQ, 4ZL4	105.7045	384	2	4	3.964	100%	89.90%	81.59%	−4.953	35.50%
134	4ZL4	267.672	914	0	0	22.061	20%	93.60%	95.30%	−8.535	51.58%
152	1CEQ	72.7088	388	5	13	−3.045	80%	18.30%	62.109	0.632	24.10%
153	1CEQ, 4ZL4	145.9851	580	7	12	2.101	20%	4.90%	98.10%	−3.568	52.00%
157	4ZL4	71.2743	307	6	9	−2.206	80%	15.50%	9.50%	−0.568	46.43%
159	4ZL4	93.9034	394	5	9	−0.346	100%	44.20%	32.50%	−2.295	82.25%
163	1CEQ, 4ZL4	101.8692	416	6	9	0.228	80%	11.60%	77.40%	−3.409	65.78%
164	1CEQ, 4ZL4	0	416	5	9	0	80%	56.80%	55.10%	−2.396	57.29%
165	1CEQ, 4ZL4	100.9284	416	5	9	0.1	100%	69.49%	65.80%	−3.152	79.43%
167	4ZL4	166.3394	604	3	8	2.983	60%	40.10%	98.50%	−5.787	58.20%
168	1CEQ, 4ZL4	111.161	478	7	12	−0.427	60%	39.80%	47.30%	−2.309	44.18%
169	1CEQ, 4ZL4	107.256	442	7	10	2.527	80%	6.20%	90.00%	−2.922	47.80%
170	1CEQ, 4ZL4	107.256	442	7	10	2.527	80%	6.20%	90.00%	−2.922	47.80%
171	1CEQ, 4ZL4	160.8503	576	4	6	5.849	40%	50.90%	98.00%	−6.711	67.63%
Chloroquine		96.8596	319.5	1	3	4.81	100%	95.80%	85.10%	−4.426	59.12%
Artemisinin		77.2728	300	3	6	2.608	100%	22.60%	100.00%	−3.63	76.90%

MR, Molar refractivity; MW, molecular weight; HBD, Hydrogen-bond donor; HBA, Hydrogen-bond acceptor; LogP, Partition coefficient; RO5, Lipinski's rule of five; PPB, Plasma Protein Binding; WS, Water solubility. Bold values indicate 100% drug-likeness and common for both the selected targets.

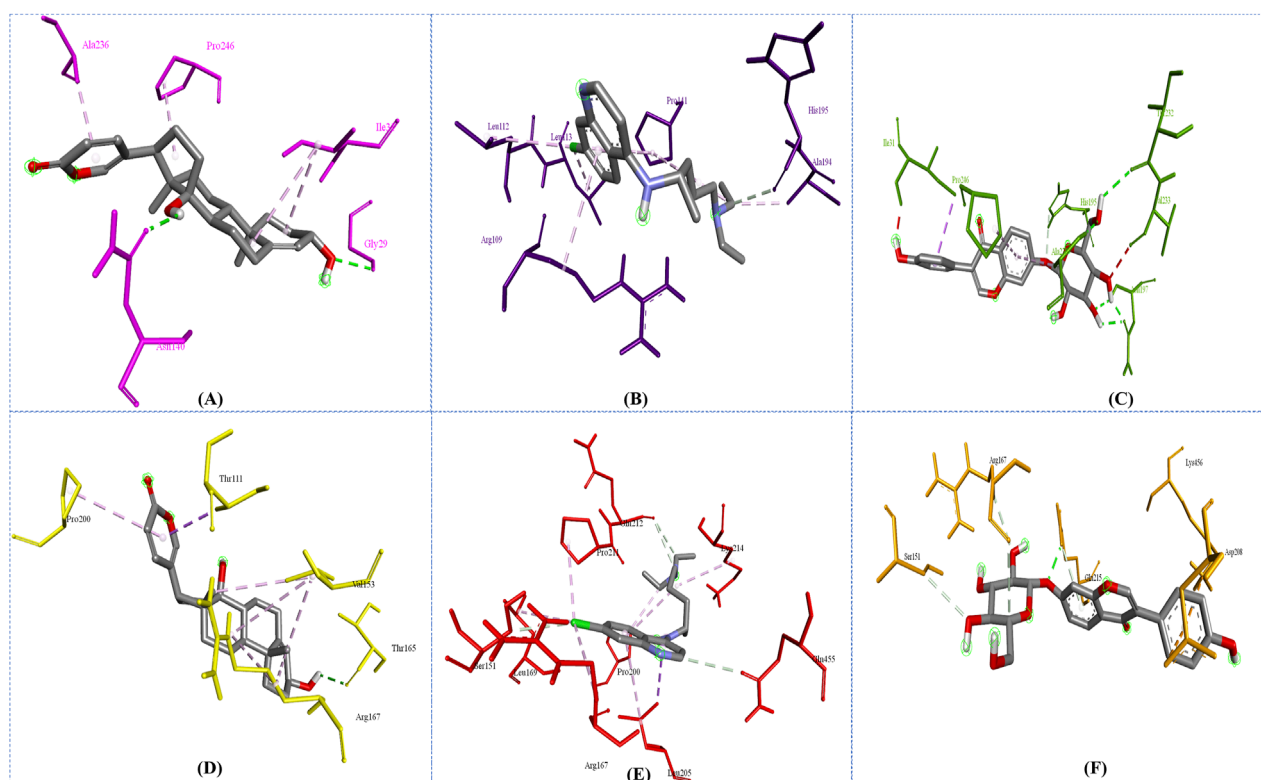


FIGURE 4
Binding site prediction through the Discovery studio: 1CEQ–Ligand 100 (A), 1CEQ–standard chloroquine (B), 1CEQ–Ligand 165 (C), 4ZL4 – Ligand 100 (D), 4ZL4 – standard chloroquine (E) and 4ZL4 – Ligand 165 (F).

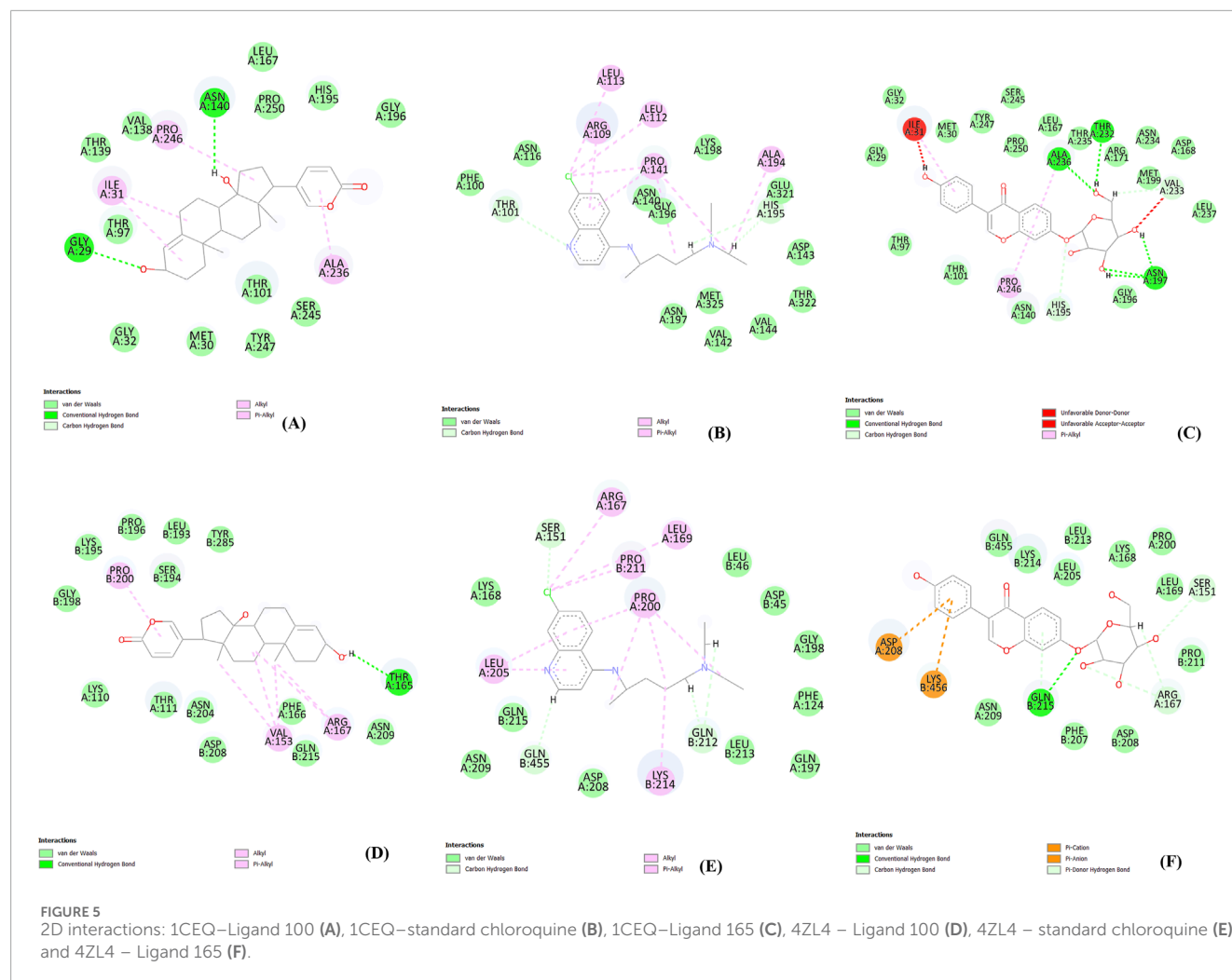
on the specific protein structure and the chemical nature of the ligands involved.

The observed anti-malarial activity of lead compounds is often influenced by a combination of structural features such as hydrogen bond donors and acceptors, aromatic rings, and hydrophobic regions. Hydrogen bond donors and acceptors play a crucial role in forming strong interactions with the active sites of target proteins in malarial parasites, potentially inhibiting their function. Aromatic rings contribute to the ability of the molecule to engage in π - π stacking interactions, which enhances its binding affinity and specificity to the target. Furthermore, the hydrophobic regions in the compound facilitate interactions with nonpolar residues in parasite proteins, thereby increasing the overall binding strength and efficacy of the compound (Chen et al., 2018; Silva-Álvarez et al., 2015). The interplay of these structural elements allows the optimization of the pharmacodynamic and pharmacokinetic properties, ultimately driving the antimalarial activity of lead compounds. The hydrophobic regions in compounds play a crucial role in enhancing their interactions with nonpolar residues in parasite proteins, as demonstrated by the data. For instance, ligand 100 engages with hydrophobic residues, such as ALA236, PRO246, and ILE31, in the 1CEQ target protein through alkyl and π -alkyl interactions. Similarly, in the 4ZL4 target protein, interactions are predominantly hydrophobic, involving residues such as VAL153, ARG167, and PRO200, highlighting the significance of nonpolar interactions in stabilizing the ligand-receptor complex.

These hydrophobic contacts are essential for binding affinity and specificity because they fortify the noncovalent interactions essential for molecular recognition. This underscores the importance of designing ligands with strategically positioned hydrophobic groups to optimize binding to parasite target proteins, thereby enhancing the effectiveness of therapeutic interventions.

2.8 MD simulations

The study aimed to evaluate the stability and interaction dynamics of protein-ligand complexes using molecular dynamics (MD) simulations, focusing on standard (chloroquine) and lead compounds scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside against the targets - 1CEQ and 4ZL4. The simulations, carried out over a 50-nanosecond production phase, provided insights into the structural and dynamic characteristics of these complexes. An important part of the analysis focused on evaluating the backbone root-mean-squared deviation (RMSD) and Root Mean Square Fluctuation (RMSF), which offer insights into the stability and flexibility of protein-ligand interactions. Figures 7, 8 present the RMSF plots for ligands and standard chloroquine protein complexes, revealing detailed information about the flexibility and dynamic behavior of these interactions during molecular dynamics simulations. These plots pinpoint areas of the protein with



notable fluctuations, suggesting regions of flexibility or instability in the complex. Furthermore, Table 3, which illustrates the average RMSD and RMSF values, provided a quantitative overview of the stability and conformational changes experienced by the protein ligand and standard chloroquine complexes throughout the simulation. The study of ligand-protein complexes through molecular dynamics simulations provides valuable insights into their binding modes and stability over time. Snapshots were taken at intervals of 10, 30, and 50 ns to observe interactions within the different complexes. For the 1CEQ protein, three different ligands were evaluated: Ligand 100, standard chloroquine, and ligand 165. The 4ZL4 protein was analyzed using the same set of ligands. The cartoon representations of these complexes reveal distinct binding interactions and conformational changes over time. In the case of 1CEQ, ligand 100 showed a stable interaction pattern, whereas standard chloroquine and ligand 165 exhibited unique binding characteristics that could influence their efficacy. For the 4ZL4 protein, ligand 100 and standard chloroquine exhibited different binding dynamics, with ligand 165 showing potential for alternative interaction pathways. Snapshots of the ligand-protein complexes were created at specific time points (10, 30, and 50 ns) to visualize the position and binding modes of the ligands over a 50-ns MD

simulation, as illustrated in Figures 9A–F. These observations will inform further research on optimizing ligand design for targeted therapeutic applications.

Monitoring the conformational and structural changes of malarial targets complexed with lead compounds such as scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene)] using RMSD and RMSF analyses provides valuable insights into their stability and dynamics. The RMSD analysis, conducted over a 50 ns trajectory, revealed that the 1CEQ – ligand 165 complex exhibited the lowest average RMSD value of 0.281 nm, indicating high stability. Comparatively, the standard chloroquine and ligand 100 showed slightly higher RMSD values of 0.293 nm and 0.306 nm, respectively. In another set of analyses, the 4ZL4 – ligand 165 complex similarly demonstrated the lowest RMSD at 0.606 nm, suggesting better stability compared to ligand 100 and standard chloroquine, which had RMSD values of 0.647 nm and 0.690 nm respectively. These findings suggest that ligand 165 might have a more stable binding conformation with both targets compared to the other compounds tested.

The RMSF analysis provides valuable insights into the dynamic behavior of proteins during molecular simulations, particularly in understanding protein-ligand interactions. By

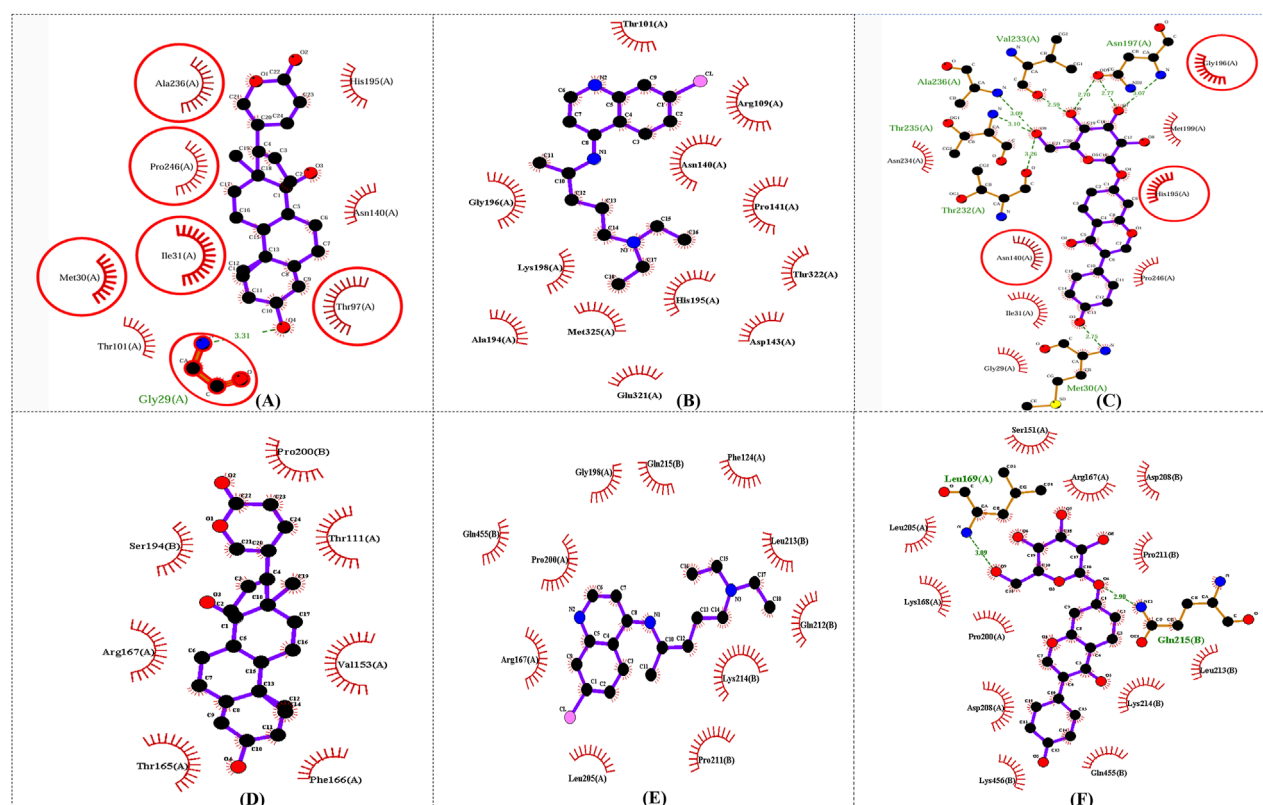


FIGURE 6

Intermolecular interactions through the LigPlot analysis: 1CEQ–Ligand 100 (A), 1CEQ–standard chloroquine (B), 1CEQ–Ligand 165 (C), 4ZL4 – Ligand 100 (D), 4ZL4 – standard chloroquine (E) and 4ZL4 – Ligand 165 (F).

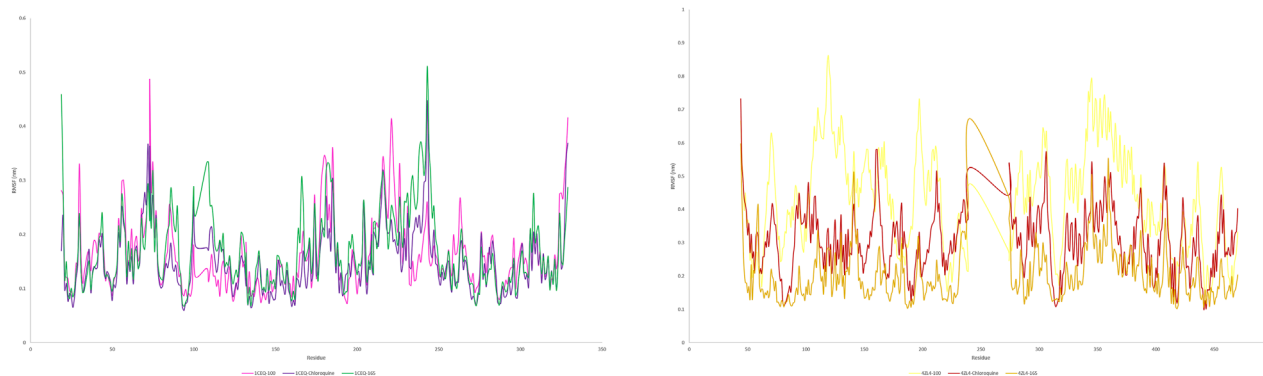


FIGURE 7

RMSD plot of protein(s) complex with standard chloroquine and lead phytoconstituents from methanolic extract of *S. oleosa* bark.

examining RMSF values, researchers can discern flexible regions within protein structures, such as 1CEQ and 4ZL4, and compare their interactions with various ligands, including lead compounds and standard chloroquine. Regions with higher RMSF values exhibit more flexibility, whereas lower values indicate stability in secondary structures. This information is crucial for assessing the energy and stability of ligand-binding interactions, which can influence the efficacy of potential

drug compounds. In the specific cases of 1CEQ, significant RMSF values were observed with different ligands: ligand 165 (0.171 nm), standard chloroquine (0.149 nm), and ligand 100 (0.613 nm). Similarly, for 4ZL4, the RMSF values with ligand 165 (0.203 nm), standard chloroquine (0.283 nm), and ligand 100 (0.394 nm) reveal variations in flexibility and interaction dynamics. These findings are pivotal for designing and optimizing therapeutic agents targeting such proteins.

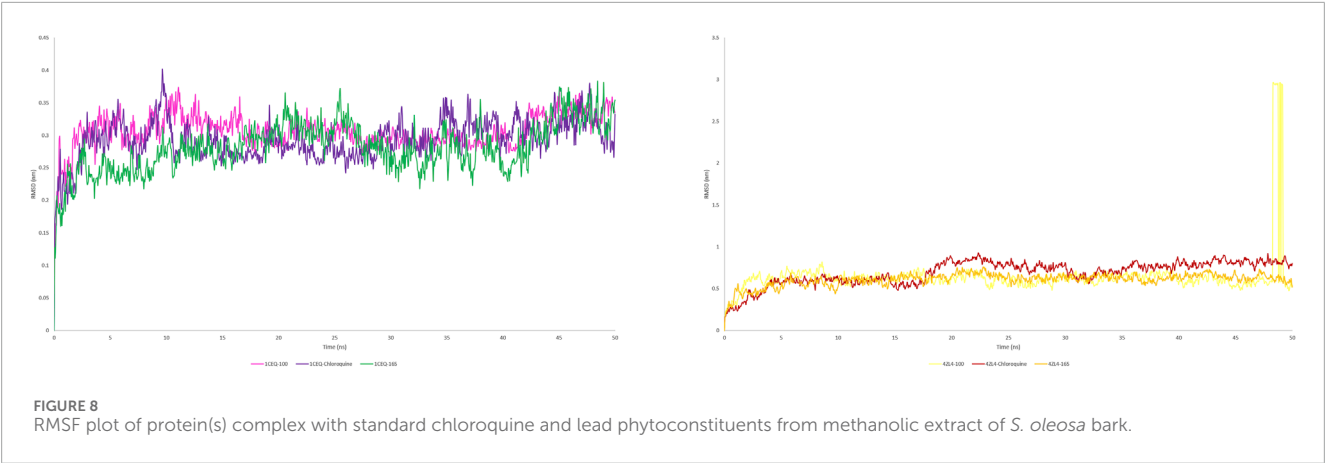


TABLE 3 The average values of RMSD and RMSF of lead hit from the bark of *S. oleosa* and standard chloroquine.

S. No	Target	Ligand or Std	RMSD (nm) Average $\pm$ SD	RMSF (nm) Average $\pm$ SD
1	1CEQ	Ligand 100	0.306 $\pm$ 0.027	0.613 $\pm$ 0.068
		Std Chloroquine	0.293 $\pm$ 0.031	0.149 $\pm$ 0.059
		Ligand 165	0.281 $\pm$ 0.038	0.171 $\pm$ 0.071
2	4ZL4	Ligand 100	0.647 $\pm$ 0.294	0.394 $\pm$ 0.146
		Std Chloroquine	0.690 $\pm$ 0.143	0.281 $\pm$ 0.098
		Ligand 165	0.606 $\pm$ 0.078	0.203 $\pm$ 0.081

In comparing the results of our MD simulations of scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside with those of chloroquine, it is essential to contextualize our findings within the broader landscape of existing literature. MD simulations revealed that ligand 165 exhibited a lower average RMSD, indicating greater stability than chloroquine, which aligns with findings from other studies that have reported similar stability profiles for phytochemicals targeting the same protein receptors. For instance, previous MD studies have shown that certain natural compounds maintain stable interactions through robust hydrogen bonding and hydrophobic contact, similar to those observed in our simulations. However, our results also highlight unique interaction patterns, such as the additional hydrogen bonds formed by ligand 165, which may enhance its binding affinity and therapeutic potential. This comparative analysis not only underscores the promising nature of our lead compounds but also suggests that they could offer advantages over chloroquine in terms of efficacy and safety. Future research should focus on the experimental validation of these computational findings and explore additional ligands to further elucidate the mechanisms underlying their interactions with target proteins. By situating our results within the context of existing studies, we can better understand the implications of drug design and the potential for developing new antimalarial therapies.

Hydrogen bonds offer precise and directional connections between the polar groups of the ligand and complementary polar residues of the protein, thereby enhancing binding affinity through a network of stable interactions. These bonds ensure that the ligand is maintained in the optimal orientation, which is crucial for effective binding. MD simulations revealed that ligand 165 formed multiple hydrogen bonds with key residues, demonstrating increased stability compared to chloroquine. On the other hand, hydrophobic contacts occur between the non-polar regions of the ligand and hydrophobic residues of the protein, driven by the principle of minimizing exposure to water. These contacts generate a favorable entropic effect by bearing non-polar regions within the interior of the protein. In our study, the combination of significant hydrophobic contacts and hydrogen bonds in ligand 165 resulted in a synergistic effect, further stabilizing the protein-ligand complex.

Molecular simulations have witnessed a transformative shift because these techniques are closer to practical applications in drug discovery, particulate generation, and optimization. The dynamic nature of target-ligand interactions poses significant challenges, especially in the accurate prediction of the thermodynamics and kinetics of bonding. MD-based dynamic docking is a more nuanced approach than traditional static docking because it considers the flexibility and electronic structure of such interactions. However, the complexity of these simulations implies that advancing them to a point at which they can replace static docking remains a

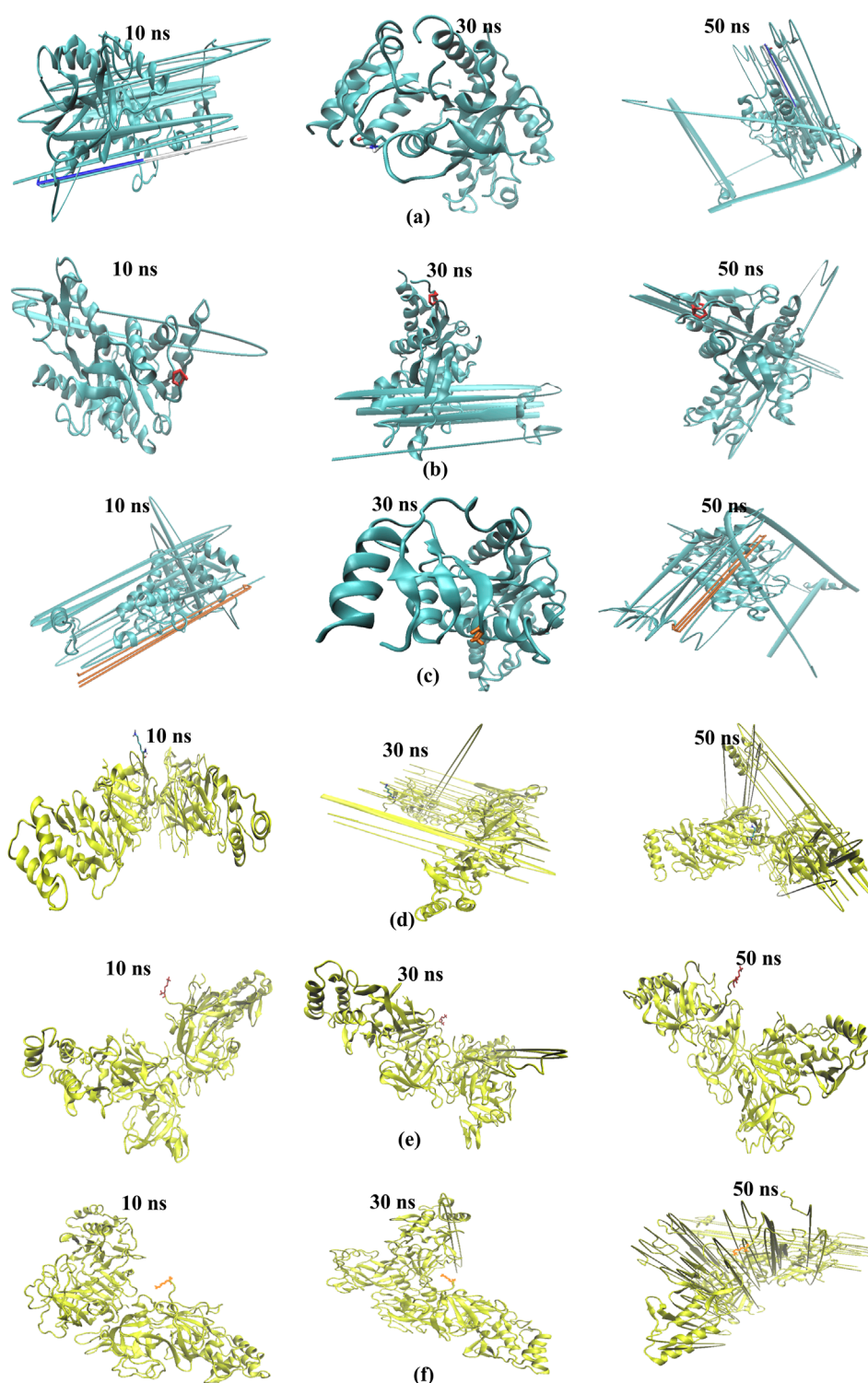


FIGURE 9

Snapshots at different intervals (time step = 10, 30, 50 ns) for the binding modes of the ligand-protein complexes. The cartoon representation shows 1CEQ-Ligand 100 (A), 1CEQ-standard chloroquine (B), 1CEQ-Ligand 165 (C), 4ZL4 – Ligand 100 (D), 4ZL4 – standard chloroquine (E) and 4ZL4 – Ligand 165 (F).

critical hurdle. Overcoming this challenge would mark a significant leap forward, enabling more accurate and efficient drug discovery processes that can better mimic real-world biological environments.

Researchers are actively refining these techniques to enhance their speed and precision, thereby promising a new era of pharmaceutical development (Riccardi et al., 2018).

Molecular dynamics simulations serve as a powerful tool for evaluating the potential of lead compounds like scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, particularly in comparison with standard antimalarial drugs such as artesunate and chloroquine. These simulations provide a dynamic view of how these compounds behave in a physiological setting, offering valuable insights into their stability and flexibility. The stability of a compound in a simulated physiological environment is a crucial factor in assessing its potential as a therapeutic agent, particularly for treating diseases like malaria. When compounds such as scillarenin exhibit stable interactions with target proteins during simulations, they exhibit strong binding affinity. This stability suggests that the compound can effectively maintain its inhibitory action against the malaria parasite over time, thereby enhancing its potential efficacy as a treatment.

The flexibility of lead compounds is crucial for determining their efficacy, particularly in the context of drug design and development. When a compound can adapt its conformation to more precisely fit the binding site of a target protein, it often results in more robust and specific binding interactions. This adaptability can enhance the ability of a compound to effectively inhibit or modulate a target protein's activity, which is essential for therapeutic applications. In the case of antimalarial agents like scillarenin, their ability to undergo conformational changes while maintaining favorable interactions with the target protein can significantly contribute to their effectiveness. This flexibility allows patients to better navigate the complex environment of the human body and adapt to the dynamic nature of biological systems, ultimately improving therapeutic outcomes.

Malaria remains a significant global health challenge, particularly in regions with tropical climates where severe cases contribute to higher mortality rates. The disease not only affects the health of individuals but also imposes substantial social and economic burdens on affected communities. Although numerous natural and synthetic anti-malarial agents have been developed, the continued evolution of drug-resistant strains of the malaria parasite has hindered effective disease control. This resistance underscores the urgent need for novel and innovative therapeutic approaches that can successfully overcome these challenges (White et al., 2014). Research into new drugs, including those targeting different stages of the parasite's life cycle or employing unique mechanisms of action, is crucial. Multidisciplinary efforts combining genomics, drug discovery, and public health strategies are essential to develop effective solutions and ultimately reduce the impact of malaria worldwide.

Herbal medicines have long been recognized as a rich source of bioactive compounds, which have the potential to be developed into new pharmaceutical drugs. The diverse chemical structures found in plants can lead to the discovery of unique mechanisms of action that traditional synthetic drugs might not offer. Moreover, the study of herbal medicines can inspire the synthesis of new compounds that mimic the beneficial properties of these phytoconstituents, leading to innovative treatments for various health conditions. As research in ethnopharmacology and phytochemistry advances, the potential for herbal medicines to contribute to modern drug development continues to grow, offering promising avenues for both prevention and treatment of diseases (Mustafa et al., 2017).

The study on the bark of *S. oleosa* provides significant insights into its potential medicinal properties, particularly its antimalarial effects. By employing advanced analytical techniques such as GC-MS and LC-MS, researchers identified 175 phytoconstituents in the bark. Among these, phenolics, flavonoids, and tannins were quantitatively analyzed, as they are known for their health benefits, including antimicrobial and antioxidant properties. The methanolic extract of the bark demonstrated promising *in-vitro* antimalarial activity, particularly against the malaria-causing *Plasmodium* species. The candle jar method is a simple and effective technique for cultivating microorganisms, particularly in anti-malarial *in vitro* studies. This method involves placing Petri dishes or culture flasks containing the parasite and erythrocytes inside a sealed jar with a light candle. The burning candle consumes oxygen and raises carbon dioxide levels, creating a low-oxygen, high-carbon dioxide environment that mimics the conditions found in human blood. This environment is conducive to the growth and replication of *P. falciparum*, allowing researchers to study its life cycle and test its potential as an antimalarial drug (Hanifan et al., 2022). These findings suggest that *S. oleosa* could be a valuable source for developing new antimalarial treatments, warranting further investigation and development. The methanolic extract's average IC₅₀ value of 0.780 µg/mL, though higher than that of standard antimalarial drugs chloroquine (0.020 µg/mL) and quinine (0.268 µg/mL), still demonstrates promising antimalarial efficacy. While a higher IC₅₀ value generally indicates a lower potency, the extract's ability to inhibit malaria parasites suggests it could serve as a potential alternative or complementary treatment. The bark extract, which exhibits anti-malarial properties, is generally less effective than the standard treatments of chloroquine and quinine. This diminished efficacy could be attributed to the concentration and bioavailability of active compounds in the extract.

The presence of tannins in the bark of *S. oleosa* is a significant contributor to its antimalarial properties. These phytochemicals, known for their pharmacological activity, have been extensively studied for their ability to combat malaria parasites. Research, such as that by Bhatia et al. (2013), confirms the presence of tannins in *S. oleosa*, supporting the notion that these compounds play a crucial role in the medicinal efficacy of the plant. Furthermore, recent studies, such as Paes et al. (2024), emphasize the potential of tannins as effective agents against malaria, highlighting the importance of these compounds in developing alternative therapeutic strategies. The high tannin content in *S. oleosa* may thus offer valuable insights into natural antimalarial solutions, highlighting the potential of the plant for medical and pharmacological applications.

Further research could explore its active compounds, optimize its formulation, and assess its safety and efficacy in clinical settings, potentially leading to new therapeutic options in the fight against malaria. The study of computational analyses involving molecular docking and dynamics has revealed promising insights into the potential antimalarial properties of the bark of *S. oleosa*. By targeting malaria-related proteins such as 1CEQ and 4ZL4, the research aimed to compare the effectiveness of these compounds against established antimalarial drugs like quinine and chloroquine. The results highlighted those compounds derived from *S. oleosa* exhibited the highest docking scores, suggesting a strong potential for binding and inhibition of the target proteins. Furthermore, comprehensive ADMET profiling and evaluation of drug likeness

characters demonstrated the feasibility of these compounds as viable therapeutic agents. *In silico* ADMET profiling involves the use of computer-based simulations and models to evaluate the pharmacokinetic and safety profiles of compounds before they reach the costly and time-intensive stages of *in vitro* and *in vivo* testing. By employing advanced algorithms, machine learning, and molecular modeling techniques, scientists can efficiently screen large libraries of compounds and identify those with favorable ADMET characteristics. This not only accelerates the development of new pharmaceuticals and reduces the likelihood of late-stage failures, making the drug development process more cost-effective and streamlined. *In silico* ADMET profiling is particularly valuable for identifying potential safety concerns, optimizing lead compounds, and guiding experimental design.

The evaluation of compounds as potential drug candidates for malaria involves more than simply analyzing numerical docking scores. Although these scores provide preliminary insights into the binding affinities of compounds with malaria receptor targets, they are insufficient as standalone indicators of drug viability. To fully assess a compound's drug candidacy, it is crucial to consider a range of factors beyond its binding affinity. ADMET properties play pivotal roles in determining the safety and efficacy of a compound in a biological context. Additionally, understanding a compound's pharmacokinetics and pharmacodynamics, potential off-target effects, and synthetic feasibility is essential for comprehensive evaluation. Validation through rigorous *in vitro* and *in vivo* studies is necessary to confirm initial hypotheses regarding biological activity and specificity. Benchmarking these new compounds against established antimalarial drugs, such as chloroquine and artemisinin, can provide valuable information regarding their therapeutic potential. By integrating these dimensions, researchers can identify viable drug candidates and advance their development toward effective malaria treatment.

This study highlights the promising potential of phytoconstituents derived from the bark of *S. oleosa* as therapeutic agents against malaria, with a focus on docking scores. Two compounds, 4-[(Z)-(6-Hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside and scillarenin, demonstrated the lowest binding energies (high docking scores) compared with the standard chloroquine, indicating a strong affinity for the malaria target proteins 1CEQ and 4ZL4. The dual targets of these proteins demonstrate their potential effectiveness. In addition, the favorable drug-likeness properties of the compounds and their ADMET profiles indicated promising safety and efficacy profiles, which are essential for drug development. The promising characteristics of the compounds prompted further investigation through MD simulations, which helped to evaluate their stability and behavior within a simulated biological setting, yielding more in-depth information about their therapeutic potential against malaria. The exploration of natural products with antimalarial activity has a rich history, with compounds such as artemisinin from *Artemisia annua* and chloroquine from *Cinchona calisaya* being notable successes in the fight against malaria (Dahnum et al., 2012; Carroll et al., 2012). In particular, artemisinin has revolutionized malaria treatment because of its rapid action against Plasmodium parasites. Similarly, chloroquine has been a cornerstone treatment for decades, until resistance issues emerged. These successes underscore the potential of harnessing natural

phytoconstituents to combat malaria. The bark of *Schleichera oleosa*, a lesser-known botanical source, has been investigated for its similar properties. Research on *Schleichera oleosa* has identified novel compounds that could enhance existing anti-malarial regimens or provide entirely new therapeutic options. This study would benefit from including references to similar *in vitro* antimalarial studies, as this would help position the findings within the broader context of phytoconstituents research (Deshpande et al., 2014). This research highlights the significance of their findings, demonstrates how their work contributes to existing knowledge, and identifies novel aspects of their study. The recent study on the bark of *S. oleosa* has led to the groundbreaking identification of two key phytoconstituents, scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, which demonstrate promising antimalarial activity. Recent *in silico* studies have identified compounds such as 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside, which were previously unreported for such activity, exhibit promising antimalarial properties. Although scillarenin has already been noted for its antioxidant activity (Kakouri et al., 2019), this finding expands the potential therapeutic applications of these compounds. This study highlights the power of computational methods in drug discovery and provides a foundation for further experimental studies to explore the efficacy and safety of these compounds in treating malaria. This discovery marks a significant step forward in the search for new antimalarial drugs, as these compounds have shown potential in initial testing for their ability to combat the malaria parasite. The study not only highlights the potential of these bioactive constituents but also supports the traditional medicinal use of *S. oleosa*. Further *in vivo* testing is recommended to fully assess the therapeutic potential of these compounds and to develop them into effective treatments for malaria, a disease that continues to have a devastating impact worldwide.

3 Conclusion

The present study highlights the promising anti-malarial potential of bioactive phytoconstituents from *S. oleosa*, revealed through advanced GC-MS and LC-MS analyses. By employing *in silico* molecular docking studies, researchers identified compounds with strong binding affinities to malarial targets 1CEQ and 4ZL4, suggesting that these phytoconstituents could serve as lead molecules in drug discovery. The virtual screening methods, including ligand-based drug design and MD simulations, demonstrated that these phytoconstituents not only possess drug-like properties but also exhibit favorable pharmacokinetic profiles, reducing potential side effects and the likelihood of failure in clinical trials. The investigation into the potential antimalarial properties of chloroquine and identified phytoconstituents from the methanolic extract of *S. oleosa* bark has yielded promising results. The analysis of drug likeness, ADMET, and molecular dynamics simulations, particularly looking at RMSD and RMSE, has demonstrated the effectiveness of these compounds as potential drug candidates. The study highlighted the phytoconstituents such as scillarenin and 4-[(Z)-(6-hydroxy-3-oxo-1-benzofuran-2(3H)-ylidene) methyl] phenyl beta-D-glucopyranoside standing out as strong inhibitors

against malaria targets. These results highlight the promise of these compounds in malaria treatment, indicating that further research is necessary to confirm their therapeutic potential and determine the optimal dosage for clinical use. This research is a promising step towards developing new plant-based antimalarial therapies, which could provide more effective strategies in the fight against this global health issue. The discovery of these lead compounds represents a significant advancement in the creation of new malaria medications.

4 Experimental

4.1 Chemicals

Petroleum ether, chloroform, methanol, gallic acid, hydrochloric acid (Molychem, Mumbai); folin-ciocalteu reagent, rutin, tannic acid, quercetin, sodium nitrite, sodium hydroxide (CDH, Delhi); aluminum chloride (Qualikems, Vadodara); HEPES, 1% D-glucose, 0.23% sodium bicarbonate, 10% heat-inactivated human serum, 5% D-sorbitol, 3% hematocrit and JSB stain.

4.2 Collection, authentication, and preparation of plant material

The process of identifying *S. oleosa* began with the meticulous collection of plant material from its natural habitat. This was followed by the preparation of a herbarium voucher specimen, cataloged as IGNTU/DOB/2024/Sap/S0/01, to ensure accurate documentation and future reference. The taxonomical features of *S. oleosa* were thoroughly examined against descriptions found in local flora, as outlined in published sources. Photographs and voucher specimens were checked against trusted botanical databases such as the Plant List, the Flora of India websites, and the International Plant Names Index (IPNI) to guarantee accurate identification. Furthermore, the taxonomy of the species was corroborated using the Germplasm Resources Information Network and other specialized databases like IMPPAT. This comprehensive approach allowed for the confirmation of the species, genera, and family classifications of *S. oleosa*, as documented by Rao et al. (2024). The final step in this identification process involved validation by a taxonomist at Indira Gandhi National Tribal University, located in Amarkantak, Madhya Pradesh, India.

The fresh bark of the *S. oleosa* plant was collected from the nursery in Bhadi, district Mandla, Madhya Pradesh, India. Drying, grinding, and storing plant material as a powder before extraction are crucial steps to ensure efficient and high-quality extraction. Drying removes moisture content, prevents the microbial growth and degradation of active compounds. This step helps to preserve the plant material and extend its shelf life. The grinding of dried plant material into a fine powder increases the surface area, enhancing the ability of the solvent to penetrate plant cells and extract desired compounds more effectively. This process also ensures more uniform extraction, which improves the yield and consistency of the active ingredients. Proper storage of the powdered material is essential to protect it from environmental factors such as light, air, and humidity, which can further degrade the compound (Krakowska-Sieprawska et al., 2022). The recently

collected bark was cut into small pieces and allowed to air dry under shade for 10 days and was pulverized into a coarse powder using an electric blender. Unwanted debris was removed and weighed on a scale balance to know the initial weight of the powder. For later use, the pulverized plant material was then stored in a dry and clean bag.

4.3 Extraction of *S. oleosa* bark powder

Soxhlet extraction is a widely favored technique for extracting compounds from solid materials because of its efficiency, simplicity, and ability to handle various sample types. One of the primary reasons for selecting Soxhlet extraction is its ability to repeatedly wash samples with fresh solvents, thereby enhancing the extraction process without manual intervention. This method is particularly effective for extracting non-volatile and semi-volatile compounds because it allows for the continuous recycling of solvents, which ensures thorough extraction. In addition, Soxhlet extraction is compatible with various solvents, enabling researchers to select the most appropriate solvent for specific compounds (Bhadange et al., 2024). A 100 g sample of powdered *S. oleosa* bark underwent sequential extraction using petroleum ether (60°C–80°C), chloroform, and methanol with the help of a Soxhlet apparatus, resulting in their respective extracts. The process continued until the siphoning tube of the Soxhlet apparatus showed no further coloration. The obtained extracts were then processed with a rotary evaporator to eliminate the solvent from each extract. The final weight of each extract was measured, and stored under 4°C. The percentage yield was determined based on the initial weight of the powder, as demonstrated below:

$$\% \text{ Yield} = \frac{\text{Final weight of extract}}{\text{Initial weight of the powdered plant bark}} \times 100$$

4.4 Quantitative analysis

Quantitative analysis involves measuring the total quantity of active compounds, also known as secondary metabolites, in a plant sample. Each type of secondary metabolite requires a specific reference standard for accurate quantification (Phong et al., 2022).

4.4.1 Determination of total phenolic content

With some adjustments, the FC reagent method was employed to measure the total phenolic content (Georgé et al., 2005a). A 100 mL volumetric flask was filled with 10 mg of accurately measured standard gallic acid to prepare a working standard (WS) solution with a concentration of 100 µg/mL. Methanol was used as the solvent to fill the flask to the mark. Specific volumes of this WS solution were then transferred into several 10-mL volumetric flasks to achieve final gallic acid concentrations ranging from 0 to 15 µg/mL, which were used to construct a calibration curve. Each flask containing different standard concentrations was supplemented with 2 mL of FC reagent, gently mixed, and allowed to sit for 2 minutes. Following this, 2 mL of a 20% Na₂CO₃ (w/v) solution was added, and the volume was adjusted with distilled water. The samples were then incubated in the dark for 30 min or until a blue color developed. The absorption peaks of these solutions

were measured against a blank solution at 755 nm using UV-visible spectrophotometry with the UV-Probe on a UV-1900, Shimadzu (Shimadzu, Japan). This method was similarly used on samples prepared in triplicate by taking suitable portions of the sample solutions to measure their concentration. The TPC is quantified by comparing the sample data with a standard known as gallic acid, a common benchmark in phenolic studies. The results are expressed in terms of milligrams of gallic acid equivalents (GAE) per gram of the dried sample, using a gallic acid standard curve (Ahmed et al., 2017; Assefa and Keum, 2017; Georgé et al., 2005b; Rao et al., 2024).

$$\text{Total phenolic content (mg GAE/g)} = c. \frac{V}{M}$$

Where C – is the regression equation's predicted amount of gallic acid (mg/mL); V – volume of extract used for analysis (mL); M – Weight of extract used for analysis (g).

4.4.2 Determination of total flavonoid content

With only slight adjustments, the colorimetric technique was employed to assess the total flavonoid content (TFC) of the extract using aluminum chloride (AlCl₃) (Matvieieva et al., 2019a). A 100 mL volumetric flask was used to prepare a working standard (WS) solution by adding 10 mg of precisely measured standard rutin, resulting in a concentration of 100 µg/mL. Methanol served as the solvent to fill the flask to the required volume. The described procedure outlines a method for constructing a calibration curve for rutin concentration determination using spectrophotometry. To begin, various aliquots of a working solution were precisely transferred into 10 mL volumetric flasks to achieve rutin concentrations varying from 0 to 25 µg/mL. This range enables the creation of a detailed calibration curve. Each flask was supplemented with 0.3 mL of a 5% sodium nitrite (NaNO₂) solution, followed by a gentle shake and a 10-min incubation to allow for any necessary reactions. Subsequently, 0.3 mL of a 10% AlCl₃ solution was added to each flask, and another incubation period was observed. To complete the process, 2 mL of a 1 M sodium hydroxide (NaOH) solution was added, and the final volume was adjusted with distilled water. The absorbance of each prepared sample was measured at a wavelength of 419 nm using a Shimadzu UV/Probe 1900 UV/visible spectrophotometer. For sample analysis, appropriate aliquots of the extracts were prepared, and the procedure was consistently applied to all sample solutions in triplicate. The Total Flavonoid Content (TFC) was determined as outlined in the Total Phenolic Content (TPC) section, with results expressed in milligrams of Rutin Equivalents (mg RE) per gram of sample extract (Faujdar et al., 2019; Matvieieva et al., 2019b; Rao et al., 2024).

4.4.3 Determination of total tannin content

The plant extract's total tannin content was assessed with tannic acid serving as the reference standard (Hagerman and Butler, 1978). A 100 mL volumetric flask was carefully filled with 10 mg of accurately measured standard tannic acid to prepare a working standard (WS) solution with a concentration of 100 µg/mL. Methanol served as the solvent to adjust the volume to the desired level. Specific aliquots of this WS solution were transferred into several 10-mL volumetric flasks, creating final tannic acid concentrations between 0 and 25 µg/mL, intended for a calibration

curve. To each 10 mL flask containing these different standard concentrations, 0.5 mL of FC reagent was added. Following this, 1 mL of a 35% Na₂CO₃ solution was introduced into each flask and gently mixed for 5 minutes. The solution was then placed in a dark setting for 30 min. The plant extract was processed similarly to produce the sample solution. The absorbance of each standard solution was measured at 419 nm using the Shimadzu UV/Probe 1900 UV-visible spectrophotometer, with a blank reference. This procedure quantified the TTC as milligrams of Tannic Acid Equivalents (TTE) per gram of dried plant extract. (Muddathir and Mitsunaga, 2013; Rao et al., 2024).

4.5 GC-MS and LC-MS instrument and chromatographic conditions

Gas chromatography-mass spectrometry (GC-MS) is a powerful analytical technique for the identification and quantification of compounds in complex mixtures. One of the primary reasons for selecting GC-MS is its ability to provide both qualitative and quantitative data with high sensitivity and specificity. The combination of gas chromatography, which separates volatile and semi-volatile compounds, with mass spectrometry, which identifies them based on their mass-to-charge ratio, allows for the precise analysis of diverse samples. GC-MS is particularly advantageous in fields such as environmental analysis, forensic science, and pharmaceuticals because of its capacity to detect trace levels of substances and its robustness in handling complex matrices (Beale et al., 2018). GC-MS analysis of the samples was meticulously conducted using the GC-MS-QP2010 Plus system. This GCMS instrument was equipped with a 5-ms VF-fused silica capillary column of length 30 m, diameter 0.25 mm, and film thickness. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1.5 mL/min. Initially, the oven temperature was maintained at 50°C for 1 minute, then methodically increased to 300°C over a period of 10 min at a rate of 7.5°C/min. The sample injector was precisely controlled at 280°C, while a 2.0 mL volume of the analytical solution was injected with a split ratio set to 1:3. The system utilized highly pure helium as the carrier gas to ensure optimal performance of the GC apparatus. The interface was consistently kept at 300°C, while the ion source was maintained at a slightly lower temperature of 250°C, optimizing conditions for efficient ionization of the analytes. The mass spectrometer operated in acquisition mode, which facilitated a comprehensive scan across a mass-to-charge (m/z) range of 50–1000. This wide range was crucial for capturing the complete fragmentation patterns of the target analytes, enabling detailed quantitative analysis. By utilizing a full scan, the process ensured that all relevant data points were collected, allowing for precise identification and quantification of the compounds under study (Patel et al., 2010). The detection was performed using the National Institute of Standards and Technology (NIST) mass spectral library. The NIST mass spectral library is a comprehensive tool for identifying chemical compounds using mass spectrometry. This library contains an extensive collection of mass spectra, which are unique fingerprints of compounds based on their mass-to-charge ratios. Mass spectrometry analysis of a sample yields a spectrum that can be matched against NIST spectra. Peak alignment is a crucial step in this process because it ensures that the observed

peaks in a sample spectrum are correctly aligned with the reference spectra for accurate identification. Deconvolution processes were employed to separate overlapping peaks, which can occur when multiple compounds are present in a sample, making it possible to discern individual components. The identification criteria typically involve comparing the experimental spectrum to the library spectra using metrics such as spectral similarity scores to determine the best match.

Liquid Chromatography-Mass Spectrometry (LCMS) is a powerful analytical technique that is widely used because of its ability to separate, identify, and quantify complex mixtures with high precision and sensitivity. One primary reason for choosing LCMS is its versatility in handling a vast range of chemical compounds, from small to large biomolecules, making it invaluable in fields such as pharmaceuticals, environmental analysis, and proteomics. Additionally, LC-MS offers excellent spectrometry because of its dual-phase operation: liquid chromatography effectively separates compounds, whereas mass spectrometry provides detailed molecular information through mass-to-charge ratio analysis. This combination allows the accurate identification and quantification of compounds in trace amounts (Bhole et al., 2020). During liquid chromatography, compounds are separated by their interactions with a stationary phase and a mobile phase, allowing for the isolation of individual components within a mixture. The Waters Alliance HT 2795 system, paired with the Micro mass Quattro Micro, offers a robust solution for analyzing complex mixtures. LC-MS was coupled with a Micromass QTOF Micro Mass Spectrometer (Waters, Milford, MA, United States). Separation was performed on an XBridge C18 column (130 Å, 3.5 µm, 4.6 mm × 150 mm; Waters). The mobile phase was composed of 80% methanol and 20% water and was run in isocratic mode. The flow rate was set at 0.7 mL/min. The system's sensitivity and precision arise from its ability to operate in positive ion mode with an electrospray ionization (ESI) source. This configuration allows for the effective ionization of analytes, which are then channelled through the mass spectrometer for analysis. The following changes have been made to the settings: the source temperature is maintained at 100°C to facilitate the efficient evaporation of solvents, while the temperature drop is set to 400°C to promote desolvation. The airflow settings, with a reduced airflow of 500 L/h and cone airflow of 50 L/h, are fine-tuned to stabilize the ion stream. The choice of argon as the collision gas ensures effective fragmentation of ions, critical for detailed mass analysis. MassLynx 4.1 is an advanced software tool that facilitates data processing and interpretation in mass spectrometry. The MassLynx 4.1 software provides a comprehensive suite of tools for accurate and efficient analysis of complex datasets. Intuitive data processing and visualization capabilities allow users to effectively interpret mass spectra and chromatograms. This process ensures that the peaks are accurately matched across the datasets, accounting for shifts that may occur due to experimental variations. In addition, MassLynx 4.1 offers robust deconvolution processes, which enable users to resolve complex spectra into individual components. This software is particularly useful for identifying and quantifying overlapping peaks, thereby enhancing the clarity and accuracy of analysis. In addition, the software provides comprehensive identification criteria, allowing users to set specific parameters for peak identification, such as retention time, mass accuracy, and intensity thresholds.

This software integrates seamlessly with both Liquid Chromatography (LC) and Mass Spectrometry (MS), creating a powerful hyphenated technique for comprehensive chemical analysis. The LC component of this system plays a critical role in isolating and separating chemical substances within a complex mixture. This separation is achieved by exploiting the differing affinities of the compounds to the stationary phase and the solvent system, allowing for precise analysis of individual components. MS component provides an in-depth examination of the ionized particles by measuring their mass-to-charge (m/z) ratios. This detailed analysis generates mass spectra, which are essentially fingerprints of the molecular entities present in the sample. The mass spectra not only reveal the molecular weights but also offer insights into the structural characteristics of the sample's constituents, thus enabling researchers to identify unknown compounds, determine the purity of a sample, and understand the molecular structure of complex substances. Data collection and processing were performed using Masslynx software (Mas-sLynx 4.1, Waters). It is a hyphenated technique, such as LC and MS, where LC is a separation technique that separates compounds in a mixture based on their affinity for stationary and solvent (mobile phase) systems. MS is a technique that analyzes the mass-to-charge (m/z) ratio of ions. MS spectra show the molecular weights and structural properties of the various components of the samples.

4.6 *In vitro* testing of extract for antimalarial activity

The *in vitro* antimalarial test described utilized 96-well microtiter plates, a widely recognized technique for assessing the efficacy of antimalarial compounds. This method, pioneered by Rieckmann and colleagues at Microcare Laboratory and TRC, provides a standardized approach for evaluating the growth inhibition of *Plasmodium falciparum*, a parasite that causes malaria. The 3D7 strain of this parasite was cultured in a carefully prepared RPMI-1640 medium. This medium was enriched with 10% heat-inactivated human serum to provide essential growth factors, 1% D-glucose as an energy source, 0.23% sodium bicarbonate for maintaining pH balance, and 25 mM HEPES as a buffering agent. The parasites of *P. falciparum*, initially in an asynchronous state, were synchronized to produce only ring-stage infected cells after treatment with 5% D-sorbitol.

The use of Jaswant Singh Bhattacharya (JSB) staining in this assay is crucial for accurately measuring the initial ring stage parasitemia, which ranges from 0.8% to 1.5% at a hematocrit of 3% within a 200 µL volume of RPMI-1640 medium. This method ensures that parasitemia is consistently sustained by incorporating 50% O+ red blood cells into the culture. To evaluate the efficacy of different test samples, stock solutions were initially prepared at a concentration of 5 mg/mL in DMSO. These solutions were subsequently diluted with the culture medium to achieve the desired concentrations for testing. To obtain final concentrations ranging from 0.4 µg/mL to 100 µg/mL after a fivefold dilution, 20 µL of the diluted samples were introduced into duplicate wells that contained parasitized cells. The culture plates were then incubated at 37°C in a candle jar to create the ideal conditions for parasite proliferation. After incubation, thin blood smears were made and stained with JSB

stain to allow for detailed microscopic analysis. Results are given as mean value of triplicate analyses of each sample and the MIC was determined as the lowest concentration that completely inhibited schizont development. Chloroquine was used as the reference drug.

4.7 *In silico* experiment

4.7.1 Hardware specification

The study leveraged the computational power of an AMD Ryzen 5 5500U processor, complemented by a Radeon graphics processor, and operated on the Windows 11 to ensure efficient handling of tasks and data-intensive processes.

4.7.2 Software specification

The receptor protein's structure can be downloaded from the RCSB website. Receptor screening was conducted using AutoDock software (MGL Tools 1.5.7), which was obtained from the Scripps Center for Chemical Biology's site. The structures of the compounds identified by GC-MS were created using Chem Draw 19.1, developed by Perkin Elmer in Waltham, Massachusetts. Molecular docking was carried out through Auto Dock Vina, which is available at the Scripps Vina website. To validate the molecular docking results, the Discovery Studio Visualizer from [2dsbiovia.com](https://www.2dsbiovia.com) was used. SMILES codes were converted online via the NCI's Cactus server. Admet SAR, available at the East China University of Science and Technology's website, was utilized for *in silico* studies, and LigPlot analysis was performed using LigPlot + V 2.1, received academic license from the European Bioinformatics Institute in Hinxton, United Kingdom. By using the CHARMM-GUI web server, researchers can effectively solvate these complexes, preparing them for molecular dynamics (MD) simulations. CHARMM-GUI streamlines the process, allowing for the generation of input files tailored for various simulation environments. Once the protein-ligand complexes are solvated, MD simulations can be performed using software like VMD and NAMD. VMD (Visual Molecular Dynamics) provides a robust interface for visualizing and analyzing molecular systems, while NAMD (Nanoscale Molecular Dynamics) is renowned for its high-performance simulation capabilities.

4.8 Preparation of the receptor

The Protein Data Bank (PDB) supplied the three-dimensional crystal structures for two enzymes that combat malaria. These proteins were visualized using Discovery Studio 2021. All water molecules were stripped from the protein, and Kollman charges along with polar hydrogens were incorporated. AutoDock was utilized to develop the grid map and identify the protein's active site. The grid centers, specified by coordinates (x, y, and z), along with their respective receptors, are as follows: 1CEQ at (25.585, 27.077, 9.299) and 4ZL4 at (3.020, 71.923, 36.935) ([Sazed et al., 2021](#))

4.9 Preparation of ligand

All the identified 175 phytoconstituents were considered for the ligand preparation process, involved several steps, such as structural

modifications and adjustments, lead optimization and removal, and variations in structures. ChemDraw was utilized to create the ligand's structure, which was then converted into a 3D mol format ([Suri and Naik, 2012](#)). The ligand preparation process is a critical step in computational chemistry and drug design, involving a series of meticulous transformations to ready a molecule for simulation or analysis. This process begins with the addition of hydrogen atoms, ensuring that all potential binding sites are satisfied, followed by the removal of heteroatoms that may interfere with the model's accuracy. Neutralization of charges is essential to maintain a balanced system, while the formation of ionization states and tautomers allows for the exploration of different chemical possibilities. Filtration is employed to refine the dataset by removing irrelevant or redundant entries, and consideration of alternative chiralities ensures that all stereochemical configurations are accounted for. Optimization of geometries and identification of low energy ring conformers is crucial for achieving the most stable molecular structure ([Usha et al., 2013](#)). Docking studies utilize scoring algorithms to implicitly screen a chemical database, enabling the prediction of the most effective binders.

4.10 Molecular docking studies

Molecular docking analysis is a computational method employed to anticipate the interaction between a protein receptor and potential ligands, typically small molecules or drug candidates. This method is crucial for understanding how these molecules fit together in three dimensions, providing insights into their binding affinity and potential efficacy as therapeutic agents. In the described study, Dock Vina, a popular docking software, was employed to analyze 175 phytoconstituents derived from *S. oleosa*, identified through GC-MS/LC-MS analysis. These compounds were docked against specific protein targets, namely, 1CEQ and 4ZL4, to assess their potential as inhibitors or binders. For comparison, known antimalarial drugs, Artesunate and Chloroquine, were also docked under identical conditions. The primary aim was to evaluate the binding energy and molecular interactions between the phytoconstituents and the receptors to identify promising candidates for further biological testing. Through this computational approach, researchers can efficiently screen large compound libraries and focus on the most promising candidates for subsequent experimental validation ([Faujdar et al., 2019](#); [Maurya et al., 2023](#)).

4.11 *In silico* ADMET analysis of selected phytochemicals

The SMILES representations of the finalized ligands were generated through the Chemdraw, and were submitted to druglikeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction programs, such as Druglikeness (<http://scfbio-iitd.res.in>) and AdmetSAR (<https://lmmd.ecust.edu.cn>). These computational tools are essential in the early stages of drug discovery because they provide insights into the pharmacokinetic and pharmacodynamic properties of compounds. In the pursuit of discovering new antimalarial therapies, researchers

are increasingly focusing on phytochemicals and potential efficacy. When a phytochemical demonstrates a higher docking score than standard antimalarial drugs, it becomes a candidate for further ADMET analysis. This comprehensive evaluation considers several factors critical to drug development. MR (Molar refractivity) provides insight into the molecular volume and polarizability, while MW (Molecular weight) is crucial for ensuring the compound can be efficiently absorbed by the body. HBD and HBA (Hydrogen-bond donors and acceptors) are vital for understanding the compound's ability to form hydrogen bonds, influencing its interaction with biological targets. LogP, the partition coefficient, indicates the compound's hydrophilicity or lipophilicity, which affects absorption and distribution. Adherence to RO5 (Lipinski's rule of five) ensures the compound has properties consistent with oral bioavailability. PPB (Plasma Protein Binding) assesses how much of the drug will be available in the bloodstream, and WS (Water solubility) is essential for absorption and bioavailability. Together, these analyses help identify phytochemicals with promising drug-like properties for further development as potential antimalarial agents (Ganesan, 2008; Subash and Kareti, 2021). The *in silico* predictions are followed by successful *in vivo* experiments, it underscores the reliability and accuracy of the ADMETSAR model used (Abdou et al., 2024).

4.12 Intermolecular interactions

The methanolic extract of the bark of *S. oleosa* has been studied for its lead phytoconstituents using advanced computational tools like Discovery Studio Visualizer and LigPlot+. These tools are instrumental in understanding molecular interactions, especially in the context of protein binding sites. By utilizing Discovery Studio Visualizer, scientists can identify important contact points and interactions that play a critical role in the binding affinity and specificity of the protein-ligand complex. This visualization facilitates a deeper understanding of the molecular mechanisms involved, aiding in the design and optimization of new compounds for drug discovery or other biochemical applications. LigPlot+, on the other hand, provides 2D schematic diagrams that highlight hydrogen bonds and hydrophobic interactions, complementing the 3D visualizations from Discovery Studio to give a comprehensive view of the binding interactions (Jain et al., 2022; Saliu et al., 2021). LigPlot analysis is an essential tool in computational biology for visualizing the interactions between ligands and target macromolecules, such as proteins. By transforming intricate three-dimensional structures into clear two-dimensional diagrams, LigPlot simplifies the understanding of how ligands—small organic molecules, peptides, or biologically active compounds—interact with their target proteins. This visualization is particularly valuable to biochemists, pharmacologists, and drug designers as it reveals critical information about the binding properties and affinities of these compounds. The graphical maps produced by LigPlot highlight different types of interactions, such as hydrogen bonds, ionic interactions, and hydrophobic contacts, with the ligand centrally positioned. These maps not only elucidate the spatial arrangement of functional groups crucial for binding but also facilitate comparative analysis across various ligand-target complexes. This understanding is fundamental to optimizing the

therapeutic efficacy of these compounds, advancing drug design, and enhancing biochemical research (Subash and Kareti, 2021)

4.13 MD simulation

NAMD is a powerful tool for conducting molecular dynamics (MD) simulations, allowing researchers to study the complex movements and interactions of biomolecules over time. By leveraging the CHARMM-GUI online platform, scientists can streamline the process of preparing simulation inputs, particularly in generating ligand topology files. This platform offers a user-friendly interface and a suite of tools designed to facilitate the integration of NAMD's input generator. The collaboration between these resources provides a robust framework for evaluating the optimal binding energy of ligand-protein complexes (Thandivel et al., 2024; Le et al., 2016). The CHARMM-GUI server is an invaluable tool for researchers conducting MD simulations, particularly when it comes to constructing ligand and standard topologies. In this specific study, K⁺ and Cl⁻ ions were incorporated to neutralize the systems, which were then solvated in water. The systems underwent a rigorous equilibration process using the standard CHARMM-GUI Membrane Builder protocol, starting with energy minimization through the steepest descent method for 1000 steps. This was followed by equilibration steps under NVT and NPT ensembles for 100 ps each, leading up to a comprehensive 50-nanosecond MD simulation. The relatively short timescale of 50 ns and idealized simulation conditions often used in computational studies can pose significant limitations on the accuracy and applicability of the results. In molecular dynamics simulations, for example, a 50 ns timescale may only capture transient phenomena or initial stages of molecular interactions, potentially missing slower, more complex processes that occur over longer periods. Idealized conditions, such as simplified molecular models or perfect environmental settings, can further limit the realism of the simulation because they might not account for variables such as temperature fluctuations, impurities, or real-world constraints. Consequently, while these simulations can provide valuable insights and theoretical frameworks, it is crucial to complement them with longer-timescale studies and experimental data to ensure comprehensive understanding and applicability to real-world scenarios (Huggins et al., 2019). The subsequent analysis of the docked ligand-target complex yielded valuable insights into the molecular interactions between the enzyme and its target. This approach not only enhances the accuracy of docking results but also provides a detailed assessment of the system's stability, contributing to a deeper understanding of the underlying molecular mechanics (Eastman et al., 2017). To determine the best configuration, a high-throughput dynamic simulation approach is necessary for examining the ligand-target receptor binding process during differentiation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

PV: Investigation, Validation, Visualization, Writing–original draft, Writing–review and editing. NM: Data curation, Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. VR: Conceptualization, Investigation, Validation, Writing–original draft, Writing–review and editing. HR: Investigation, Visualization, Writing–original draft, Writing–review and editing. RG: Data curation, Validation, Writing–original draft, Writing–review and editing. PS: Investigation, Software, Supervision, Validation, Writing–original draft, Writing–review and editing. SuK: Investigation, Validation, Visualization, Writing–original draft, Writing–review and editing. KR: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2025.1543939/full#supplementary-material>

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Q-Der: a next-generation CoQ10 analogue supercharging neuroprotection by combating oxidative stress and enhancing mitochondrial function

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Background: Mitochondrial dysfunction and oxidative stress are central mechanisms in the progression of neurodegenerative diseases. This study first evaluated the toxicity of Q-Der (Q10-diacetate), a derivative of Coenzyme Q10, in HT22 hippocampal neurons under normal and oxidative stress conditions.

Methods: HT22 cells were treated with Q-Der at 2.5, 5 and 10 μ M with and without rotenone. Mitochondrial superoxide production (Mitosox), gene expression (via qRT-PCR), and protein levels (via Western blot) were measured. Morphological analyses were performed using transmission (TEM) and scanning (SEM) electron microscopes.

Results: Q-Der significantly reduced mitochondrial superoxide levels, particularly at 5 μ M, and upregulated key mitochondrial biogenesis genes, including PGC-1 α and TFAM. Additionally, it restored the expression of MT-ND1 and MT-COI, which were downregulated by rotenone. Western blot results showed a significant recovery in CV-ATP5A (complex V) expression ($p < 0.05$), preserving mitochondrial ATP production. Morphological analyses further confirmed Q-Der's ability to maintain cellular and mitochondrial structure under stress conditions.

Conclusion: These findings suggest that Q-Der is non-toxic under normal conditions and protects against oxidative stress, supporting its potential as a therapeutic agent for neurodegenerative diseases.

KEYWORDS

coenzyme Q10, Q-Der, oxidative stress, mitochondrial dysfunction, rotenone, neuroprotection, HT22 cells, ATP synthesis

1 Introduction

Neurodegeneration is an inflammatory response in the central nervous system (CNS). It represents a complex constellation of conditions characterized by the progressive decline in neurons' structure, function, and viability. This pathological phenomenon is central to various disorders that collectively are known as neurodegenerative diseases (NDs). These conditions—such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and multiple sclerosis (MS)—are unified by their insidious onset and the inexorable deterioration of cognitive, motor, or sensory functions they inflict upon individuals.

These diseases share a gradual onset and a relentless progression of cognitive, motor, or sensory impairments, making them a leading cause of disability worldwide, particularly among older populations. As society ages, the prevalence of NDs is expected to increase, highlighting the critical need for enhanced understanding and development of innovative preventive strategies.

At the cellular level, neurodegeneration is characterised by a series of events including synaptic dysfunction, neuronal loss, and brain atrophy. These processes are driven by the neurons' inability to sustain necessary biochemical and cellular operations, leading to apoptosis or other forms of cell death (Granatiero et al., 2019; Angelova and Abramov, 2018; Wilson et al., 2023). This results in the breakdown of neural networks and neurotransmission disruption, manifesting in symptoms depending on the affected brain regions. The molecular mechanisms at play, such as mitochondrial dysfunction, oxidative stress, and low-grade inflammation, are pivotal to understanding the pathology of NDs. Mitochondrial dysfunction compromises cellular energy metabolism, contributing to the cell death seen in NDs. Oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, further damages neurons (Lin and Beal, 2006). Key molecular players in these processes include oxidative stress induction and molecular networks related to neuroinflammation and cellular dysfunction, exacerbating neuronal damage (Sivandzade et al., 2019).

The environmental and lifestyle factors, including diet, physical activity, and exposure to toxins, further modulate the risk and progression of neurodegenerative diseases. Here, the role of diet emerges as both a potential risk factor and a protective ally against neurodegeneration (Solfrizzi et al., 2017; Zhang et al., 2021; Bianchi et al., 2021; Yang et al., 2021; Godos et al., 2024). Nutritional patterns rich in polyphenols and healthy fats can mitigate the risk of neurodegenerative diseases by influencing the molecular mechanisms of neurodegeneration. Diets that reduce oxidative stress, dampen low-grade inflammation, and improve mitochondrial function can significantly impact the progression of these diseases. Additionally, consistent consumption of fruits like berries and cherries, which are high in polyphenols, may contribute to maintaining cognitive health (Wang et al., 2023). Investigating phytocomplexes used in folk medicine could lead to the discovery of compounds with diverse preventive benefits across various diseases, including cancer (Micucci et al., 2024), cardiovascular issues (Micucci et al., 2020), and neurological disorders (Lapi et al., 2020).

The exploration of phytocomplexes derived from plants such as *Acacia catechu* (Chiaino et al., 2021), *Castanea sativa* (Brizi et al., 2016), *Olea europea* (Chiaino et al., 2020), and phytochemicals like flavonoids and curcumin have unveiled their potential to modulate critical molecular pathways involved in neurodegeneration. These substances can inhibit the activation of NF- κ B, thereby reducing the production of pro-inflammatory cytokines such as IL-1 and IL-6. They also promote the activation of Nrf2, enhancing the cellular antioxidant response and protecting against oxidative damage (Abrahams et al., 2019; Calis et al., 2020; Farzaei et al., 2019). Indeed, generally, flavonoids lead to a reduction in ROS, regardless of their source (endogenous: mitochondria, peroxisomes, xanthine oxidase, Fenton reaction, NADPH oxidase, lipoxygenases, cytochrome P450 or exogenous: visible, UV and ionising radiation, chemotherapeutics) (Kicinska and Jarmuszkiewicz, 2020). Additionally, compounds naturally occurring in the human body, such as melatonin and Coenzyme Q10 (CoQ10), have also been studied and extracted, leading to the development of novel agents to mitigate oxidative stress and support mitochondrial function in neurodegenerative conditions (Areti et al., 2017; Bagheri et al., 2023).

Amidst these complex interactions, CoQ10 and its derivatives stand out for their dual role in mitochondrial function and antioxidant defence. Coenzyme q10 provides a promising avenue for neurodegenerative disorders, acting on oxidative phosphorylation, which occurs in the mitochondria via the electron transport chain, the primary process responsible for ATP production (Kang et al., 2020). However, challenges in bioavailability and optimal dosing underscore the need for further research to harness CoQ10's full potential in combating NDs.

This study introduces a pioneering *in vitro* comparison of a novel CoQ10 analog with CoQ10, focusing on their neuroprotective effects and underlying mechanisms in HT22 neuronal cells. Our investigation delves into mitochondrial ROS modulation, regulation of genes implicated in mitochondrial biogenesis and function (*PGC-1 α* , *TFAM*, *MT-ND1*, *MT-COI*, *MT-COX5B*), and OXPHOS subunit protein expression, aiming to illuminate the novel analog's superior efficacy and mechanism profiles. Finally, SEM and TEM analyses suggest neuroprotective effects evidence by preserved mitochondrial integrity, reduced apoptotic cells, autophagic vacuoles, and cellular morphology in the treatment group compared to controls. This work represents a significant stride in overcoming CoQ10's dosing challenges, potentially setting a new benchmark in neuroprotective strategies.

2 Materials and methods

2.1 Chemicals and reagents

CoQ10 was obtained from Sigma-Aldrich (C9538). CoQ10 derivative (Q-Der) was kindly provided by Umolsystem Srl. Rotenone was used as the neurotoxin at a concentration of 5 μ M. Dimethyl sulfoxide (DMSO) was used as a vehicle control. Other reagents included fetal bovine serum (FBS), L-glutamine (100 mM), and antibiotics (penicillin and streptomycin). All chemicals were sourced from Sigma-Aldrich and prepared according to the manufacturer's instructions.

2.2 Cell cultures

Mouse hippocampal neuronal cell line (HT22) was maintained in DMEM-Ham's F12, supplemented with 10% fetal bovine serum, L-glutamine (100 mM), and 1% antibiotics (penicillin, streptomycin), and incubated in humidified 5% CO₂ atmosphere at 37°C. At 80% confluence, cells were detached with trypsin-EDTA, washed, and sub cultivated in new flasks for 1–2 days before the experimental procedures. Neuroprotective effects were evaluated by pre-incubating cells with varying concentrations of Q10 and Q-Der for 3 or 24 h, followed by a 24-h exposure to 5 μ M rotenone to induce neurotoxicity.

2.3 Experimental design

Cells were divided into six experimental groups, as reported in [Figure 1](#):

- Control: cells treated with the vehicle (DMSO).
- Q10: cells treated with 5 μ M Q10 for 24 h.
- Q-Der Low, Medium, High: cells treated with 2.5, 5, or 10 μ M Q-Der for 24 h.
- Rotenone: cells exposed to 5 μ M rotenone for 24 h to induce neurotoxicity.
- Q10 + rotenone: cells pre-treated with 5 μ M Q10 for 3 or 24 h, followed by 24-h rotenone exposure.
- Q-Der + rotenone: cells pre-treated with 5 μ M Q-Der for 3 or 24 h, followed by 24-h rotenone exposure.

2.4 Cell viability assay (Propidium iodide staining)

Propidium Iodide (PI) staining was performed to assess cell viability. PI is a fluorescent dye that penetrates only cells with compromised membranes, indicating cell death. Control and rotenone-challenged cells treated with 2.5, 5, and 10 μ M Q-Der at 24 h post-treatment, were incubated with PI for 10 minutes. PI fluorescence was measured using a flow cytometer (FACSCanto II). The percentage of PI-positive cells, representing dead or necrotic cells, was calculated for each experimental group.

2.5 Mitochondrial ROS measurement (MitoSOX red assay)

Mitochondrial ROS production was measured using MitoSOX Red (Thermo Fischer, M36008), a dye specifically oxidized by mitochondrial superoxide. Control and rotenone-challenged HT22 cells treated with 2.5, 5, and 10 μ M Q-Der for 24 h were incubated with 5 μ M MitoSOX Red for 10 min at 37°C. Flow cytometry measured fluorescence intensity (excitation wavelength 561 nm, emission wavelength 610 nm). The results were expressed as percentages of MitoSOX positive cells.

2.6 qRT-PCR analysis

Total RNA was isolated from treated and control cells using the Omega Bio-Tek E.Z.N.A.[™] Total RNA Kit following the manufacturer's protocol. RNA concentrations were measured using the SpectraMax[®] QuickDrop[™] Micro-Volume Spectrophotometer. cDNA was synthesized from 500 ng of total RNA using the Takara PrimeScript[™] RT Master Mix. Quantitative real-time PCR (qRT-PCR) was performed using the StepOnePlus[™] Real-Time PCR System (Applied Biosystems) and PowerUp SYBR Green Master Mix. Primers used for PGC-1 α , TFAM, MT-ND1, MT-COI, COX5B, and S16 were designed based on previous studies (see [Table 1](#) for primer sequences). Relative gene expression was normalized to S16 as the internal control, and data were analyzed using the $2^{-\Delta\Delta CT}$ method.

The target genes and the corresponding primer sequences used in qRT-PCR quantification are provided in [Table 1](#).

2.7 Western blot analysis

Total protein extracts were obtained from the organic phase following the QIAzol protocol, solubilised in ISOT lysis buffer containing 8 M urea, 4% CHAPS, 65 mM DTE, 40 mM Tris base, 1 mM NaF, 1 mM Na₃VO₄, 1 $\times$ complete protease inhibitor cocktail (Roche Diagnostics), then sonicated three times for 5 s on ice. After centrifugation at 12,000 $\times$ g for 10 min, protein concentration was determined using Bradford colorimetric assay (Bio-Rad Laboratories) ([Bradford, 1976](#)). Equal amounts (30 μ g) of total proteins were resolved in precast stain-free 4%–15% SDS polyacrylamide gels (Bio-Rad Laboratories, 4568084) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, 1620177) using the Power Blotter System semi-dry transfer device (ThermoFisher Scientific). Western blot analyses were performed using total oxidative phosphorylation (OXPHOS) cocktail from Invitrogen (458099) diluted 1:1,000 to detect individual complexes of the electron transport chain: CI-NDUFB8 (20 kDa), CII-SDHB (30 kDa), CIII-UQCRC2 (48 kDa), CIV-MT-COI1 (40 kDa) and CV-ATP5A (55 kDa) ([Welinder and Ekblad, 2011](#)).

2.8 Morphological analyses environmental scanning electron microscopy (ESEM) and transmission electron microscopy (TEM)

Control and treated HT22 hippocampal cells were directly processed on coverslips in Petri dishes. After careful washing with 0.1 M phosphate buffer, monolayers were fixed with 2.5% glutaraldehyde in the same buffer for 1 h. All the specimens were post-fixed with 1% OsO₄ in 0.1 M phosphate buffer for 1 h. After alcohol dehydration, they were critical point dried, gold-sputtered, and observed with an ESEM scanning electron microscope (UMKC, Kansas City, MO, United States). For TEM analysis, HT22 hippocampal cells, growing adherent in flasks, were washed, immediately fixed “*in situ*” with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min, gently scraped, and centrifuged at 1,200 rpm. The pellets were fixed in

TABLE 1 Primers used in real-time RT-PCR quantification.

Gene	Primer forward (5'-3')	Primer reverse (5'-3')
<i>PGC-1α</i>	ACTGAGCTACCCTTGGGATG	TAAGGATTTTCGGTGGTGACA
<i>TFAM</i>	GGGAGCTACCAGAAGCAGAA	CTTTGTATGCTTTCCACTCAGC
<i>MT-ND1</i>	AGGCCCTAACATTGTTGGTCC	TGTGAGTGATAGGGTAGGTGC
<i>MT-COI</i>	TCTACTATTTCGGAGCCTGAGC	CAAAAGCATGGGCAGTTACG
<i>COX5B</i>	CCGTCCATCAGCAACAAGAGAA	GCCAAAACCAGATGACAGTACAGT
<i>S16</i>	TGAAGGGTGGTGGACATGTG	ATAAGCTACCAGGCCTTTGA

Note: *PGC-1α*, Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha; *TFAM*, Transcription Factor A, mitochondrial; *MT-ND1*, Mitochondrially Encoded NADH: Ubiquinone Oxidoreductase Core Subunit 1; *MT-COI*, Cytochrome C Oxidase Subunit I; *COX5B*, Cytochrome C Oxidase Subunit 5B; *S16*, Mitochondrial Ribosomal Protein S16.

2.5% glutaraldehyde for an additional 30 min. All the specimens were post-fixed in 1% OsO₄ for 1 h, alcohol dehydrated, and embedded in araldite (Salucci et al., 2015). Ultrathin sections of the embedded samples were cut using a Leica UC7 ultramicrotome, and thin sections were stained with uranyl acetate and lead citrate and then analyzed with a Philips CM10 transmission electron microscope.

2.9 Comprehensive statistical analysis

For a holistic analysis of data derived from various assays discussed—including cell viability, mitochondrial functionality, and qRT-PCR for *PGC-1α*, *TFAM*, *MT-ND1*, *MT-COI*, and *COX5B* expression—statistical evaluation was meticulously designed to ensure the accuracy and reliability of the findings. Initially, all data were subjected to the Shapiro-Wilk test to assess normality, a critical step in determining the appropriate statistical methods for analysis. For data conforming to a normal distribution, analysis of variance (ANOVA) was employed to compare the mean values across different experimental and control groups. Subsequent *post hoc* analysis, utilizing Tukey's and Sidak's tests, helped identify specific groups showing statistically significant differences. In contrast, for datasets not meeting normal distribution criteria, the Kruskal-Wallis test was applied as a non-parametric alternative to ANOVA for comparing means across groups. Dunn's *post hoc* test was then used for detailed pairwise comparisons to pinpoint where significant differences lay. The statistical analyses were performed using advanced software tools like GraphPad Prism, ensuring high precision and reliability. The significance threshold was consistently set at $p < 0.05$ across all tests. Results were reported as mean $\pm$ SD for parametric data sets or median with interquartile range for non-parametric datasets, appropriately representing the data's central tendency and dispersion, from at least three independent experiments. Statistical significance was indicated as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

3 Results

3.1 Cell viability assessment (Propidium iodide staining)

The results from the PI staining assay reveal distinct effects of Q-Der, both in the absence and presence of rotenone, on HT22 cell viability (Figure 2). Under baseline conditions, healthy cells treated with Q-Der at 5 μ M show a reduction in PI-positive cells compared to the control. However, this decrease was not significant at 2.5 μ M. These data suggest the *in vitro* safety profile of Q-Der under non-stressful conditions.

The neurotoxic effects of rotenone were evident, as indicated by a marked increase in PI-positive cells relative to the control. Pre-treatment with Q-Der at both concentrations significantly attenuates this rotenone-induced effect. Notably, the reduction in PI-positive cells in the Q-Der pre-treated groups approaches levels observed in control cells, indicating a robust protective effect against rotenone-mediated cytotoxicity.

These findings underscore the *in vitro* efficacy of Q-Der in preserving cell viability under oxidative stress conditions, with its protective effects becoming more pronounced in the presence of rotenone compared to the modest impact observed in the absence of the neurotoxin.

3.2 Mitochondrial superoxide production (MitoSOX red analysis)

The results of the MitoSOX Red assay, used to quantify mitochondrial superoxide production, demonstrate the efficacy of Q-Der in mitigating oxidative stress induced by rotenone. In the control group, mitochondrial superoxide levels remain at baseline, suggesting physiological mitochondrial function. Mitochondrial ROS levels were not modified in healthy cells treated with Q-Der at all concentrations. On the contrary, rotenone-induced a substantial elevation in superoxide production. Pre-treatment with Q-Der

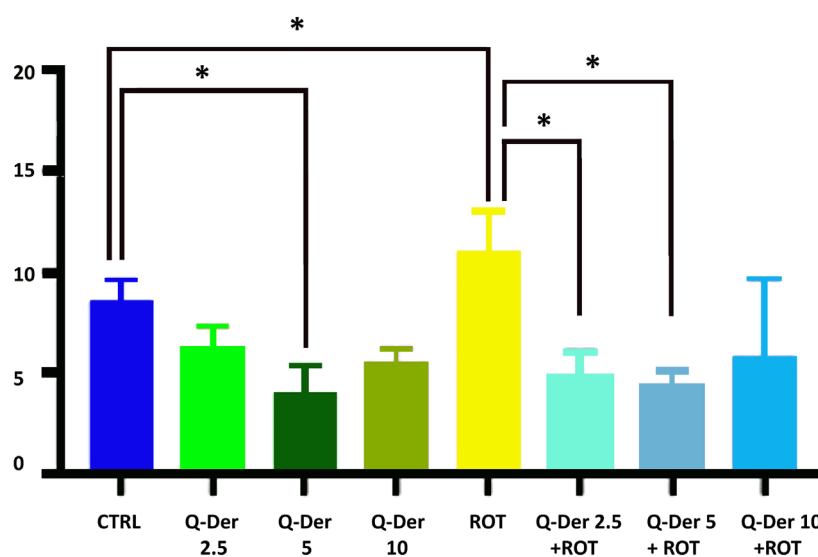
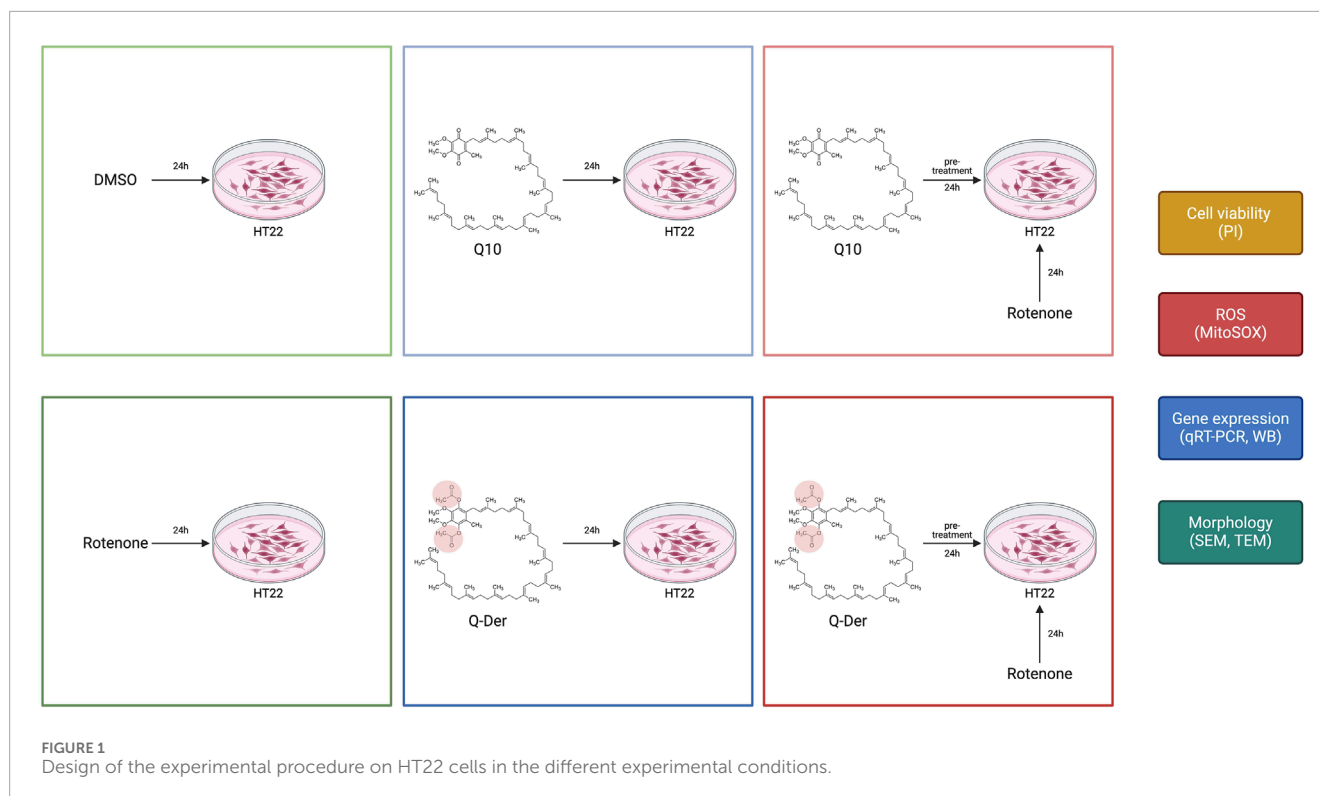


FIGURE 2
Q-Der effects on HT22 viability in healthy and rotenone-treated cells. The panel shows the percentage of PI positive cells, indicating cell death. Ordinary one-way ANOVA, Šidák's multiple comparison, *P value <0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

at 2.5 μ M and 5 μ M reduced the rotenone-induced increase of mitochondrial ROS levels, nearly restoring them to baseline. Q-Der at 10 μ M reduces mitochondrial superoxide levels to a lesser extent than the lower doses, suggesting a potential dose-dependent plateau. These findings indicate that Q-Der effectively counters oxidative stress at optimal concentrations, with a strong statistical significance in the protective effects observed at 2.5 μ M and 5 μ M. This underscores its potential to preserve mitochondrial superoxide levels under induced oxidative stress (Figure 3).

3.3 Gene expression analysis (qRT-PCR)

The qRT-PCR results reveal significant insights into the impact of Q-Der on the expression of key mitochondrial genes under basal and rotenone-induced conditions. The genes analyzed include *PGC-1 α* , *TFAM*, *MT-ND1*, *MT-COI*, and *COX5B*, integral to mitochondrial biogenesis, respiratory function, and oxidative phosphorylation.

In the absence of rotenone, Q-Der at 5 μ M induced a noticeable upregulation of *PGC-1 α* expression, while CoQ10 at the same

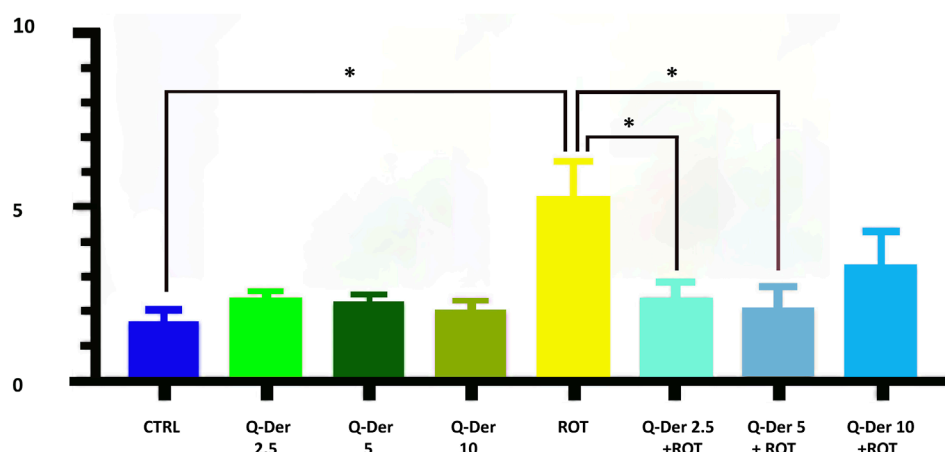


FIGURE 3

Evaluation of mitochondrial superoxide levels in control and rotenone-treated cells upon the treatment of 2.5, 5, and 10 μ M Q-Der. Ordinary one-way ANOVA, Šidák's multiple comparison, *P value <0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

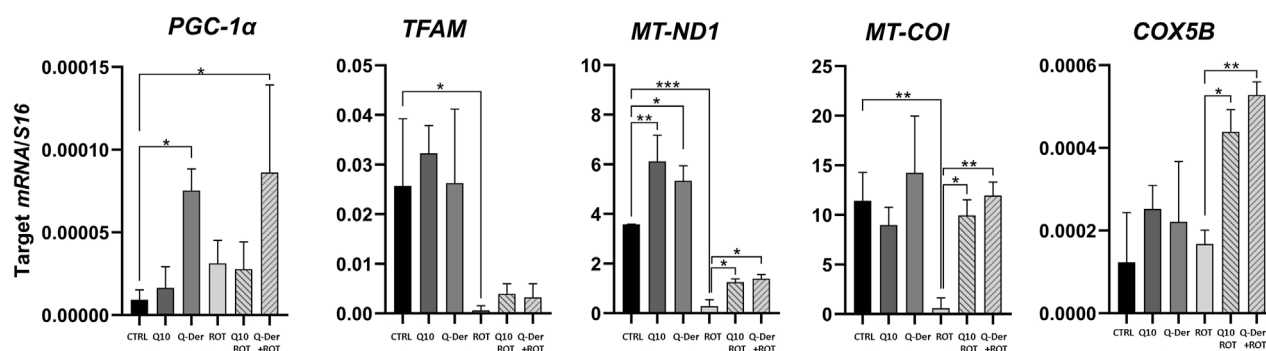


FIGURE 4

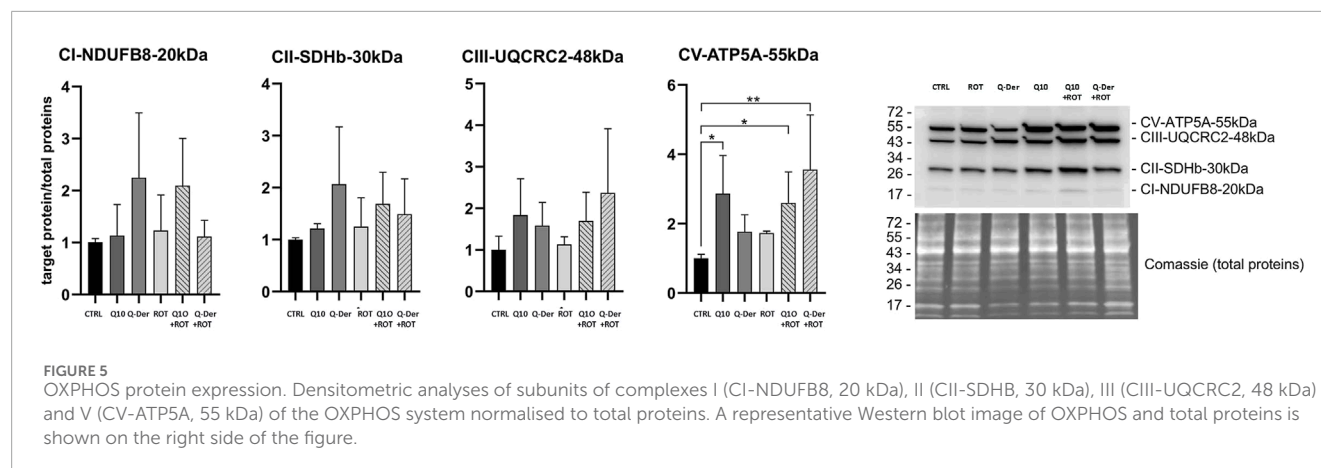
Mitochondrial-Related Gene expression. HT22 mRNA expression levels of *PGC-1α*, *TFAM*, *MT-ND1*, *MT-COI* and *COX5B* in healthy and rotenone-treated cells challenged with CoQ10 and Q-Der at 5 μ M*P < 0.05, **P < 0.01, ***P < 0.001.

concentration did not affect this parameter. The expression of *TFAM*, a gene essential for mitochondrial DNA maintenance, was not modified by CoQ10 or Q-Der treatment. As expected, rotenone markedly reduced *TFAM* expression, both CoQ10 and Q-Der partially antagonized this effect. *MT-ND1* and *MT-COI*, which encode subunits of the mitochondrial respiratory chain complexes I and IV, showed a similar pattern: the active compounds partially restored their rotenone-decreased expression. Finally, *COX5B*, a nuclear-encoded Cytochrome C oxidase subunit (complex IV), increased its expression only in rotenone-challenged cells treated with CoQ10 and Q-Der, with Q-Der showing a higher potency.

The qRT-PCR analysis reveals that Q-Der is critical in promoting mitochondrial gene expression, particularly under oxidative stress conditions. The significant upregulation of *PGC-1α*, partial recovery of *TFAM*, and restoration of *MT-ND1* and *MT-COI* expression highlight Q-Der's efficacy in maintaining mitochondrial function and biogenesis (Figure 4).

3.4 Western blot analysis of OXPHOS complexes

The Western blot analysis of the OXPHOS complexes reveals key findings, particularly in relation to the expression of CI-NDUFB8 (complex I), CII-SDHB (complex II), CIII-UQCRC2 (complex III), and CV-ATP5A (complex V). Despite the range of experimental conditions, the analysis shows that the expression of CI-NDUFB8 remains unaffected by rotenone treatment. There is no statistically significant alteration in CI-NDUFB8 levels across all groups, including both Q-Der and CoQ10 pre-treatments. This stability indicates that rotenone does not exert a detectable impact on complex I subunit expression under these experimental conditions, and neither Q-Der nor Q10 significantly modifies CI-NDUFB8 levels. Similarly, the expression of CII-SDHB and CIII-UQCRC2 follows a comparable trend, with no statistical significant differences were observed between the control, rotenone, and pre-treatment groups. Although there is a slight trend toward



recovery in the pre-treated groups, these changes do not meet the threshold for statistical significance. These observations suggest that while a minor biological effect may exist, it is not strong enough to be conclusive in terms of altering the expression of complex II and complex III subunits. The only subunit showing a statistically significant change is CV-ATP5A (complex V). Q-Der increased this subunit expression only in rotenone-treated cells, while CoQ10 determined a similar effect both in control and rotenone-treated cells (Figure 5).

3.5 Morphological analysis

3.5.1 Scanning electron microscopy observation

In the control condition at 24 h, cell morphology appears well preserved, HT22 cells appear elongated, and sometimes, some show a slightly rounded shape (Figures 6A–C). The cells appear confluent and generate a carpet by forming extracellular membrane protrusions that create bridges between adjacent cells.

24 h of rotenone treatment induces a deep change in cell morphology; the HT22 appears predominantly rounded, probably due to a rearrangement of the cytoskeletal structure (Figures 6D–F).

The cells show fewer cytoplasmic protrusions, and a loss of extracellular junctions reduces cell carpet formation. Numerous blebs on the cellular membrane and the apoptotic bodies demonstrate the presence of numerous apoptotic cells.

After pre-treatment with Q10, the morphology is restored to the control's; new cell junctions (Figures 6G–I) can be formed. Finally, pre-treatment with Q-Der shows the cells all flattened, more confluent, and with characteristic microvilli on the surface (Figures 6J–L). Both Q10 and Q-Der can reduce the percentage of apoptotic cells and induce the percentage of apoptotic cells and the preservation of cell communication.

3.5.2 Transmission electron microscopy observation

For all experimental conditions we evaluate three grids. Every grid consisted of ten thin sections. Therefore, we performed the evaluation of the ultrastructural changes in 200–300 cells. The ultrastructural evaluation evidence good cell viability in control conditions (Figures 7A–C). HT22 cell morphology appeared

elongated and confluent. After rotenone treatment, several rounded cells with blebs and apoptotic bodies appeared (Figures 7D–F). We can observe numerous autophagic vacuoles (G), with different sizes ranging from 200 to 500 nm. In the nucleus, we can observe chromatin condensation and a diffuse detachment of the nuclear membrane. Mitochondria appear swollen and heavily damaged; their maintained membrane is well preserved but appears well preserved while create are completely disrupted.

After Q10 treatment, the cells appeared confluent and showed well-preserved mitochondria (Figures 8A–C) comparable to the control conditions. A better mitochondria morphology appeared when cells were treated with Q10-der (Figures 8D–F). We can see well-preserved mitochondria that appear more numerous if compared pre-treatment to control or rotenone treatment. Mitochondria are present in high number but smaller than control condition, suggesting a mitochondrial biogenesis.

4 Discussion

The findings from this study highlight the significant protective effects of Q-Der on HT22 neuronal cells, particularly under conditions of oxidative stress induced by rotenone. This condition offers a targeted model for studying mitochondrial dysfunction, as it selectively inhibits Complex I of the electron transport chain, disrupting ATP synthesis and causing localized ROS generation (Menke et al., 2003; Moon et al., 2005). This inhibition leads to mitochondrial membrane depolarization, increased expression of mitochondrial fission markers and activation of caspase-3-dependent apoptotic pathways. These effects are highly relevant to neurodegenerative diseases like Parkinson's disease, where mitochondrial dysfunction and selective dopaminergic neuronal death are central features (Moon et al., 2005; Li et al., 2017). Several stressors act on neurons through the affection of the same pathways targeted by rotenone. For example, H₂O₂ induces oxidative stress generating ROS across multiple cellular compartments, leading to generalized oxidative damage that is not specific to mitochondria. Despite this distinction, both rotenone and H₂O₂ share some key effects, including ROS generation, mTOR pathway suppression, apoptotic activation involving caspase-3 cleavage and PARP fragmentation. These shared mechanisms provide

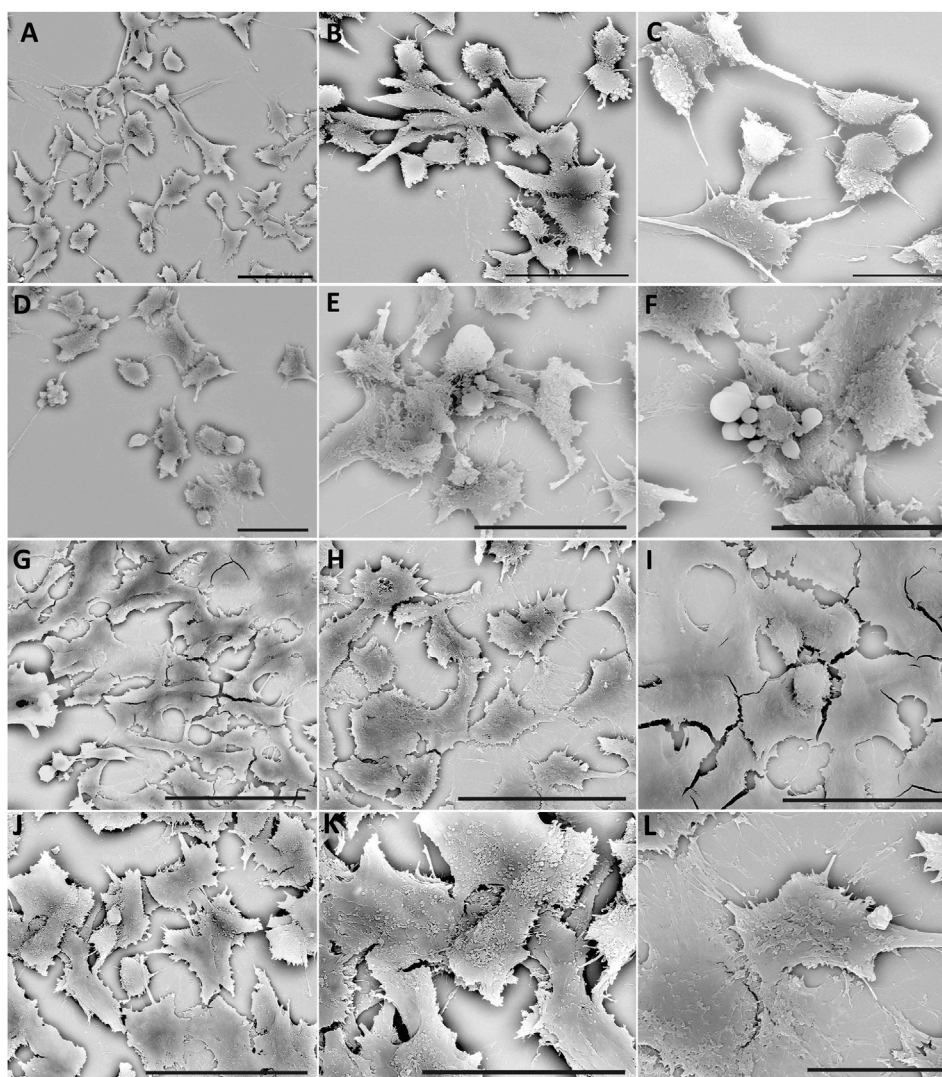


FIGURE 6

SEM of HT22 hippocampal cells. Control cells (A–C); cells treated with rotenone (5 μ M) for 24 h (D–F); rotenone-treated cells pretreated with Q10 (5 μ M) for 24 h (G–I), rotenone-treated cells pretreated with Q-Der (5 μ M) for 24 h (J–L). (A, B, D–I), Bar = 20 μ m; (C, J, K), Bar = 10 μ m.

complementary insights into oxidative stress and neuroprotection, with rotenone being a specific tool for the study of mitochondria-dependent neurodegenerative pathologies (Panee et al., 2007; Zhou et al., 2015; Giraldo-Berrio et al., 2024; Roy et al., 2023; Millichap et al., 2024).

Our study focuses on rotenone-induced oxidative stress due to its mitochondria-specific effects. Our results add to a growing body of evidence that underscores the importance of targeting mitochondrial dysfunction in neurodegenerative diseases such as Parkinson's and Alzheimer's, where oxidative stress plays a pivotal role. Q-Der demonstrated substantial efficacy in reducing cell death, restoring mitochondrial superoxide levels, and modulating key gene expressions involved in mitochondrial biogenesis and respiration. While the protective effects of Q10 have been well-documented in similar models (Bhagavan and Chopra, 2006), the current data suggest that Q-Der may offer superior benefits, particularly in maintaining mitochondrial homeostasis under stress conditions.

Chemically, Q-Der differs from Coenzyme Q10 in that it carries two acetyl groups on its quinone ring, increasing its lipophilicity and potentially enhancing its stability against oxidation. While CoQ10 functions through rapid cycling between oxidized and reduced forms to neutralize reactive oxygen species, the addition of acetate groups in Q-Der is theorized to confer a protective effect on the molecule itself, reducing its reactivity to oxidative agents. This structural modification may support a prolonged antioxidant presence within the mitochondrial membrane, thus maintaining cellular protection over extended periods of oxidative stress. These properties underscore Q-Der's potential as a neuroprotective agent, particularly in environments where mitochondrial stability and sustained antioxidant effects are essential.

A key aspect of this study is the ability of Q-Der to preserve mitochondrial function and ROS levels. Compared to Q10, Q-Der consistently showed a trend toward better protection against oxidative stress markers such as mitochondrial superoxide,

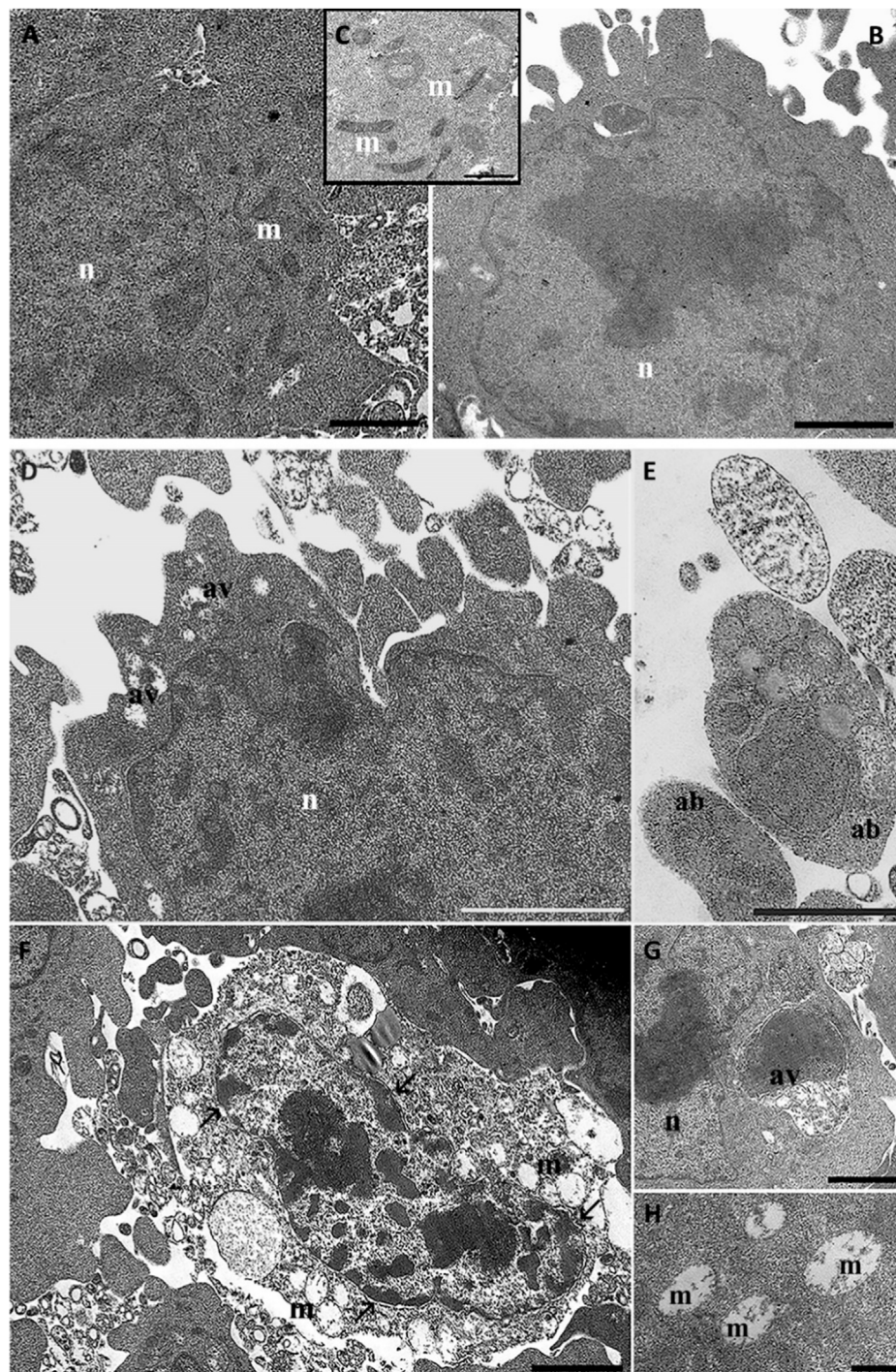


FIGURE 7
HT22 hippocampal cells at TEM. Control (A, B), cells treated with rotenone (5 μ M) for 24 h (C–F, G). m: mitochondria; n: nucleus; ab: apoptotic bodies; av: autophagic vacuole; $\rightarrow$: chromatin condensation. (A, B), Bar = 1 μ m; (C, F), Bar = 500 nm; (D, E), Bar = 2 μ m.

although this trend did not reach statistical significance. This mirrors findings in other studies where derivatives of Q10, such as MitoQ and Ubidecarenone, have been shown to exhibit enhanced mitochondrial targeting and antioxidant properties compared to the parent compound (Smith and Murphy, 2010). MitoQ, a mitochondria-targeted form of CoQ10, has demonstrated strong efficacy in reducing mitochondrial ROS

and improving bioenergetics by selectively accumulating in the mitochondrial membrane, facilitating more effective protection against oxidative damage (Cochemé et al., 2007). Although MitoQ and Q-Der share similarities in their mechanisms of action—both aim to enhance mitochondrial function by modulating ROS levels—the differences in their molecular structures likely contribute to their varying degrees of efficacy. For instance, MitoQ's

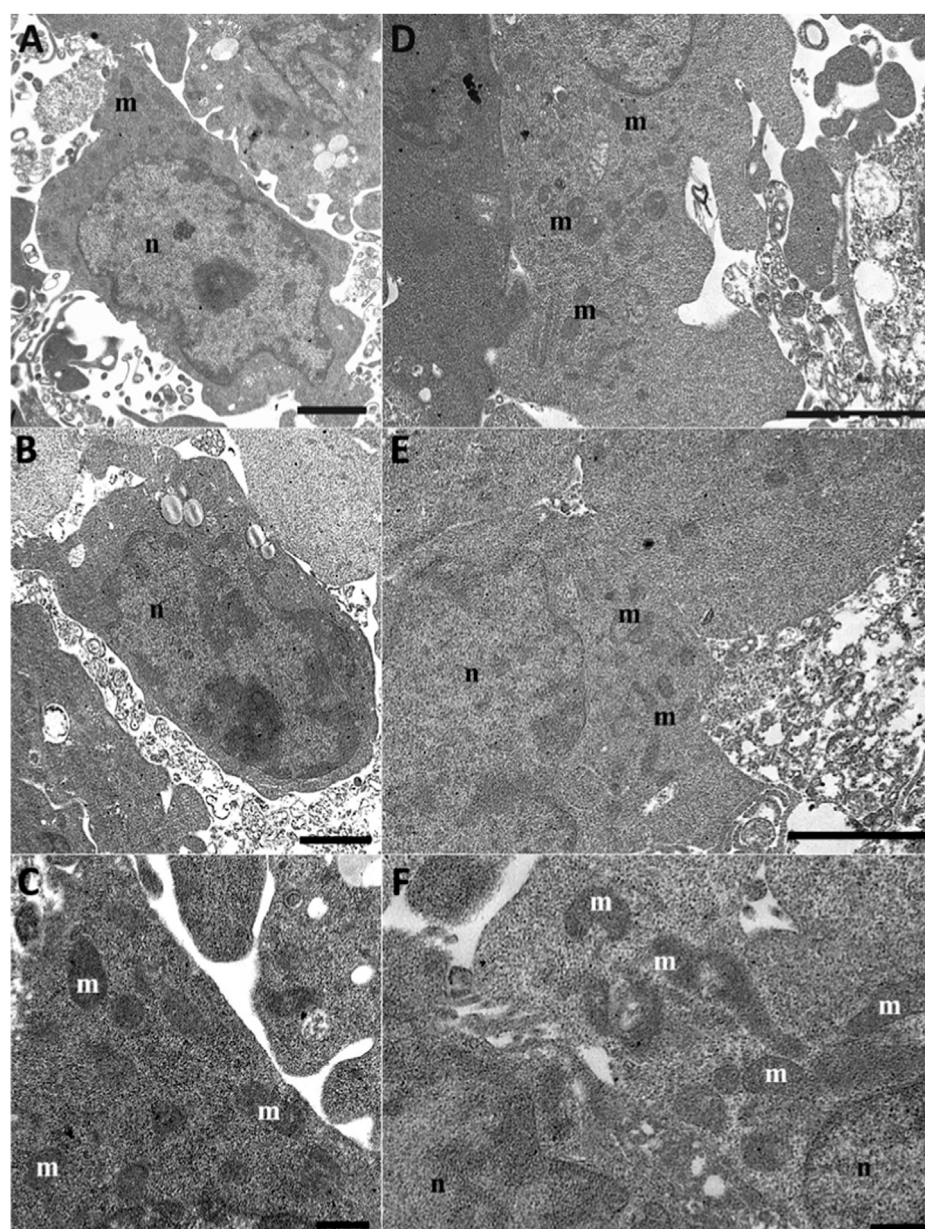


FIGURE 8
HT22 hippocampal cells at TEM. Rotenone-treated cells pretreated with Q10 (5 μ M) for 24 h Q10 (A–C) and rotenone-treated cells pretreated with Q-Der (5 μ M) for 24 h Q-der (D–F). m: mitochondria; n: nucleus. (A–C, E), Bar = 2 μ m; (C, F), Bar = 500 nm.

triphenylphosphonium cation facilitates its direct accumulation in the mitochondria, while Q-Der may rely more on its metabolic conversion within cells.

The qRT-PCR analysis further underscores the potential of Q-Der, particularly in its ability to upregulate key genes involved in mitochondrial biogenesis, such as *PGC-1 α* and *TFAM*. This is consistent with literature indicating that compounds enhancing *PGC-1 α* activity could promote mitochondrial biogenesis and repair, thereby offering neuroprotection (Wenz, 2009). The upregulation of *PGC-1 α* and partial rescue of *TFAM* under rotenone-induced oxidative stress suggest that Q-Der helps restore mitochondrial integrity and function, which is crucial for maintaining ATP

production and preventing cellular apoptosis. Comparatively, studies on other Q10 derivatives like Q10-hydroxydecyl benzoate (IDE) have shown similar mitochondrial-protective effects, particularly in preventing the loss of complex I and complex III activity, further highlighting the importance of preserving mitochondrial bioenergetics in disease models (Villalain, 2004). However, Q-Der's ability to modulate both mitochondrial biogenesis and complex V expression distinguishes it as a promising candidate for further exploration.

The Western blot analysis revealed that while there were no significant changes in the expression of CI-NDUFB8, CII-SDHB, or CIII-UQCRC2 across the treatment groups, Q-Der significantly

increased CV-ATP5A (complex V) expression in rotenone-treated cells. This result is particularly noteworthy, as complex V is essential for ATP synthesis, and its dysfunction is a critical contributor to energy deficits observed in neurodegenerative diseases (Nicholls and Budd, 2000). The statistically significant recovery of CV-ATP5A in Q-Der-treated cells indicates that this novel compound effectively preserves mitochondrial ATP production capacity under oxidative stress conditions. This protective effect on ATP synthase has also been observed in studies using idebenone, a synthetic analog of CoQ10, which has shown promise in clinical trials for conditions such as Friedreich's ataxia and Leber's hereditary optic neuropathy (Schiff and Rustin, 2016; Lyseng-Williamson, 2016). While idebenone primarily targets complex I deficiency, the broader effect of Q-Der on many mitochondrial parameters suggests a more comprehensive mitochondrial support mechanism.

An interesting finding in our study is that, despite QDer improving numerous parameters of mitochondrial function, it does not appear to increase the levels of UQCRC2, a key component of Complex III of the electron transport chain. This result may seem to contrast with well-established CoQ10-mediated regulation of other mitochondrial markers (Yousef et al., 2019). One possible explanation is that QDer exerts its effects primarily on Complex I or V, leaving Complex III relatively unaffected. Additionally, UQCRC2 levels might already be saturated under basal conditions, leaving little room for further regulation. Studies on PARL-deficient models demonstrate selective defects in Complex III mediated by TTC19 instability without impacting other complexes of the ETC (Spinazzi et al., 2019).

Furthermore, the partial increase in Complex III activity observed in idebenone-treated models suggests that increased protein expression does not always correlate with functional recovery (Llewellyn et al., 2015). Similarly, the unchanged GSSG:GSH ratio in the cerebellum suggests that certain oxidative stress markers may resist modulation even under treatment.

Morphologically, the structural preservation observed through SEM and TEM further supports the molecular data. Even after rotenone exposure, cells pre-treated with Q-Der exhibited well-preserved cellular morphology and mitochondrial structure. This contrasts with the significant cytoskeletal and mitochondrial damage seen in rotenone-treated cells, aligning with the concept that maintaining mitochondrial integrity is crucial for cellular survival under oxidative stress. Studies on other derivatives, such as SkQ1 (a plastoquinone derivative), have also demonstrated the importance of targeting mitochondrial health to prevent structural damage in neurons, supporting the idea that mitochondrial-targeted therapies can significantly affect disease progression (Fedorov et al., 2022; Sacks et al., 2021). However, while SkQ1 is known for its anti-apoptotic properties, Q-Der offers a broader protective mechanism by reducing oxidative stress and enhancing mitochondrial gene expression, further solidifying its role as a potential neuroprotective agent.

In conclusion, the results of this study place Q-Der in a promising position as a neuroprotective agent aimed at preserving mitochondrial function, particularly in NDs. While other derivatives of Q10, such as MitoQ, idebenone, and SkQ1, have shown efficacy in specific mitochondrial pathways, Q-Der appears to provide a multifaceted approach, combining antioxidant effects, mitochondrial biogenesis and enhanced ATP production. The

statistical significance observed in the restoration of CV-ATP5A expression and the trends toward improvement in mitochondrial superoxide levels and gene expression positions Q-Der as a strong candidate for further *in vivo* studies and eventual clinical translation. Future research should aim to explore the exact molecular pathways through which Q-Der exerts its effects and to compare its efficacy *in vivo* against other Q10 derivatives in models of neurodegeneration. Additionally, a deeper understanding of the pharmacokinetics and bioavailability of Q-Der relative to other mitochondrial-targeted therapies will be crucial in determining its therapeutic potential.

5 Conclusion

The present study provides compelling evidence of Q-Der's neuroprotective efficacy in HT22 cells, highlighting its ability to attenuate rotenone-induced oxidative stress and preserve mitochondrial function. Q-Der's effects were evident through significant reductions in mitochondrial superoxide production, enhanced expression of mitochondrial biogenesis genes (PGC-1 α and TFAM), and the upregulation of complex V ATP synthase, contributing to sustained ATP synthesis under stress conditions. These findings support Q-Der as a possible candidate for further investigation in neurodegenerative disease models, particularly those characterized by mitochondrial dysfunction and oxidative stress.

While this study establishes Q-Der's protective effects *in vitro*, its translational potential requires further examination. The exclusive use of HT22 cells limits applicability across broader neuronal models, and *in vivo* studies are essential to confirm bioavailability and metabolic stability. Future research should expand to diverse cellular models and focus on Q-Der's pharmacokinetics to substantiate its therapeutic promise.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

MMi: Conceptualization, Funding acquisition, Writing - review and editing. FG: Investigation, Writing - original draft. SD: Investigation, Writing - original draft. GA: Investigation, Writing - original draft. BC: Investigation, Writing - original draft. FF: Investigation, Writing - original draft. RS: Investigation, Writing - original draft. RO: Investigation, Writing - original draft. MMo: Investigation, Writing - original draft. DL: Investigation, Writing - original draft. GN: Investigation, Writing - original draft. GP: Investigation, Writing - original draft. EB: Investigation, Writing - original draft. FL: Investigation, Writing - original draft. MR: Investigation, Writing - original draft. MMA: Investigation, Writing - original draft. GZ: Supervision, Writing - original draft. VS: Visualization, Writing - original draft. AB: Visualization, Writing - original draft. SB: Investigation, Writing - original draft. MB:

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Conflict of interest

Author ZG was employed by Umolsystem Srl.

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Supplementary material

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Mesoporous silica loaded with calcitonin gene-related peptide antagonist and *curcumin* alleviate oxidative stress and inflammation in the sciatic nerve

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Background: Neuropathic pain (NP) is a kind of chronic pain that can lead to neurasthenia. The effectiveness of current drug treatment for NP is still unsatisfactory due to its side effects, addiction and withdrawal. In recent years, researchers have begun to develop nano-drug delivery systems for the diagnosis and treatment of NP diseases.

Methods: We developed a disulfide-bonded magnetic mesoporous silica dual-drug delivery system consisting of *curcumin* (Cur) and a calcitonin gene-related peptide (CGRP) antagonist (CGRPi), and characterized by electron microscopy, Dynamic Light Scattering (DLS), Zeta, specific surface area and pore size detection. At the cellular level, the biocompatibility of CGRPi@Cur@Fe₃O₄@mSiO₂-PEG (FMCC) nanoparticles were tested by CCK-8 and dead/alive staining kit in BV2 cells; Inflammation levels and oxidative stress were measured by enzyme linked immunosorbent assay (ELISA) in lipopolysaccharide (LPS)-induced BV2 neuroinflammation model. *In vivo*, chronic constriction injury (CCI) model was constructed, and the effect of FMCC on pain behavior of CCI mice was detected by von Frey filaments test and thermal hyperalgesia; The effects of FMCC on the anti-inflammatory and oxidative stress of CCI were determined by pathological tests (HE and ROS staining), RT-PCR and ELISA.

Results: FMCC had good biocompatibility and could be taken up by BV2 cells. At the cellular level, FMCC could effectively reverse oxidative stress, inflammation and CGRP expression in LPS-induced neuroinflammation model *in vitro*. At the animal level, the mice with CCI were administered with FMCC, which effectively reduced oxidative stress and inflammation and sustained relief of neuropathic pain.

Conclusion: This study provides a new approach for the treatment of neuropathic pain.

KEYWORDS

CGRP antagonist, curcumin, mesoporous materials, nanoparticles, neuropathic pain

1 Introduction

Neuropathic pain is pain caused by damage or disease of the sensory system, which is often manifested as spontaneous persistent pain and hyperalgesia (Rosenberger et al., 2020). Epidemiological studies have shown that the incidence of neuropathic pain in the general population is about 3 %–17% (Cavalli et al., 2019), which brings serious economic and social burden to people and society. At present, the exact pathogenesis of neuropathic pain is still unclear, and the clinical treatment of neuropathic pain is mainly through drug intervention (Finnerup et al., 2021). However, pharmacologic treatment of neuropathic pain, such as nonsteroidal anti-inflammatory drugs and opioids, often has unsatisfactory results (Attal and Bouhassira, 2021). Most patients do not receive satisfactory treatment, and some drugs are often accompanied by various adverse reactions (Macone and Otis, 2018). Therefore, the search for drugs with better efficacy, fewer side effects and affordability in the treatment of neuropathic pain has become one of the hot spots in the research of neuropathic pain.

Curcumin (*Cur*) is a hydrophobic acidic compound extracted from the herb *Curcuma longa* (Hassanzadeh et al., 2020). *Cur* plays an important role in the treatment of a variety of diseases, such as nerve injury, atherosclerosis, malignant tumors, diabetes, autoimmune diseases and neuropathic pain (Kotha and Luthria, 2019). The analgesic effect of *Cur* depends on the comprehensive action of a variety of drug mechanisms, such as: reducing the damage caused by oxidative stress, inhibiting the release of inflammatory cytokines and adhesion molecules, activating glial cells, and inhibiting the activation of protein kinases (Uddin et al., 2021).

The neuropeptide calcitonin gene-related peptide (CGRP) is the main neurotransmitter of C-nerve fibers, which is produced in the central and peripheral nervous system, especially in the spinal cord, dorsal root ganglion and trigeminal ganglion, and plays an important role in the initiation and maintenance of neuropathic pain (Kang and Govindarajan, 2021). Studies have reported that the expression of CGRP is increased after nerve injury and plays an important role in the generation and maintenance of pain behavior (Hegazy et al., 2020). Inhibiting the expression of CGRP can significantly improve the pain behavior after peripheral nerve injury (Paige et al., 2022). *Cur* is a polyphenol compound extracted from the root of turmeric, which has anti-oxidative stress effect. It has been reported to reduce the level of CGRP in DRG neurons and reduce lumbar (Xiao et al., 2017). Although the above studies suggest that *Cur* can be used as an alternative to conventional drugs for the treatment of neuropathic pain, the poor water solubility, poor targeting and poor bioavailability of *Cur* have become difficulties in the development of *curcumin* drugs.

Currently, magnetic targeting offers tantalizing possibilities (Polyak and Friedman, 2009). It was originally developed to optimize chemotherapy procedures for *in vivo* targeting with the assistance of magnetic fields, which would allow a greater proportion of the magnetic nanomaterials to reach the injury site quickly (Bhattacharya et al., 2022). The Fe_3O_4 form of iron oxide nanoparticle is approved for clinical applications due to their remarkable biocompatibility (Zhao et al., 2020). As one of the most popular magnetic resonance imaging reagents, superparamagnetic Fe_3O_4 nanoparticles are widely used for disease diagnosis (Park et al., 2020). Mesoporous silica nanoparticles

(MSNs) have also been shown to deliver drug molecules to target cells and tissues due to their plasticity in varying sizes, shapes, and surface modifications (García-Fernández et al., 2021). Due to its large surface area and mesoporous structure, mesoporous silica can make it possible to deliver different drugs in a multi-stage manner, which may become an ideal method for targeted treatment of chronic pain.

In recent years, MSNs have attracted the interest of many researchers in therapeutic applications as a promising drug carrier due to their unique properties. Uribe Madrid et al. synthesized core-shell nanostructures $\text{Fe}_3\text{O}_4@\text{mSiO}_2$. The nanostructure has reasonable biocompatibility and good drug release performance. After loading ibuprofen, the drug release amount can reach 81% (Uribe Madrid et al., 2015). Elbially et al. synthesized MSNs with pH responsive properties, which exhibited high sustained release in the tumor microenvironment after loading *Cur* (Elbially et al., 2020). Kong et al. encapsulated *Cur* in MSN pores to enhance its antioxidant activity, biocompatibility, and anticancer activity (Kong et al., 2019). Due to the better bioavailability and solubility of *Cur* encapsulated in the pores of MSNs compared to *Cur*, the therapeutic effect of MSNs loaded with *curcumin* is greater than that of *Cur* (Yadav et al., 2019). The polyethylene glycol functionalized MSNs enhances the biocompatibility, stability, and permeability of *curcumin*, prolonging the circulation time of the nanomedicine delivery system in the bloodstream (Park et al., 2009). Chronic constriction injury (CCI) model of the sciatic nerve was first proposed by Bennett and Xie as a model to study neuropathic pain (Bennett and Xie, 1988). It has since been widely used as one of the most commonly used animal models of neuropathic pain.

In this study, we fabricated a disulfide-bonded magnetic mesoporous silica dual drug delivery system ($\text{Fe}_3\text{O}_4@\text{mSiO}_2$), which allows efficient encapsulation of *Cur* and CGRPi due to the high specific surface area and mesoporous channels of mesoporous silica (Supplementary Figure S1). The safety and efficacy of CGRPi@*Cur*@ $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ -PEG (FMCC) nanocomposites in reducing pathological pain, neuroinflammation and oxidative stress *in vitro* and *in vivo* were investigated.

2 Materials and methods

2.1 Materials

$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (cat: 220299), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (cat: 236489), $\text{NH}_3 \cdot \text{H}_2\text{O}$ (28 wt%, cat: 105423), chloroform, cetyltrimethylammonium bromide (CTAB, cat: H6269), ethyl orthosilicate (cat: 131903), ethyl acetate (cat: 650528), methanol (cat: 34860), (3-Mercaptopropyl) trimethoxysilane (MPTMS, cat: 175617), acetone (cat: 179124), cysteamine (cat: 30070), *Curcumin* (cat: C1386), hydrazine (cat: 225819), buffered saline (PBS, cat: P2272), DAPI (cat: D9542), lipopolysaccharide (LPS, cat: L4391) and BSA (cat: V900933) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CGRPi (BIBN4096), dimethyl sulfoxide (DMSO, cat: HY-Y0320), fluorescein 5-isothiocyanate (FITC, cat: HY-66019) and SYBR Green qPCR Master Mix (cat: HY-K0501A) were obtained from MCE (USA). 0.25% pancreatic enzyme (cat: 25200072) and MEM medium (cat: 11095080) were supplied by Gibco (USA). CCK-8

reagent (cat: E-CK-A362) was purchased from Elabscience (China). Calcein/PI solution (cat: C2015S) was obtained from Beyotime Biotechnology (China). Enzyme linked immunosorbent assay (ELISA) kit (cat: mIC50536-1, mIC50274-1, mIC50300-1 and IC50325-1) was from Shanghai Enzyme-linked Biotechnology (China). DCFH-DA (cat: S0033S) was supplied by Beyotime Biotechnology (China). TRIzol Reagent (cat: 15596018CN) was purchased from Invitrogen (Carlsbad, CA, USA). iScript™ cDNA Synthesis Kit (cat: 1708891) was obtained from Bio-Rad Laboratories (USA).

2.2 Synthesis of CGRPi@Cur@Fe₃O₄@mSiO₂-PEG (FMCC)

Firstly, Fe₃O₄ nanoparticles are synthesized. They were prepared by coprecipitation according to the previous research (Albalawi et al., 2021). Briefly, 5.56 g of FeCl₂·4H₂O and 10.8 g of FeCl₃·6H₂O were dissolved into aqueous solution (100 mL). Then 20 mL of NH₃·H₂O was injected into this solution and continued stirring for 1 h at 85°C. Next, the hydrophilic magnetic nanocrystals were collected with magnets and washed with ultra-pure water three times. Finally, the precipitate obtained were dried to obtain magnetic Fe₃O₄ nanoparticles.

Then Fe₃O₄@mSiO₂ was synthesized with ferric oxide as substrate. 75 mg of magnetic Fe₃O₄ nanoparticles were dispersed into chloroform solution (1 mL), and 0.25 g of CTAB was dissolved in DI water solution (12 mL), followed by mixing and heating to 70°C. Then 250 mL of DI water solution was immediately added to the mixture and mechanically stirred at 40°C for 1 h, followed by rapid addition of 7.5 mL NH₃·H₂O, 1.25 mL of ethyl orthosilicate, and 12.5 mL of ethyl acetate and stirring at 40°C at 80 rpm for 6 h. The mixture was washed by centrifugation with water and ethanol for five times, and magnetic Fe₃O₄@mSiO₂ nanoparticles was obtained.

Then, disulfide bond modified Fe₃O₄@mSiO₂ nanoparticles were synthesized on the basis of Fe₃O₄@mSiO₂. Firstly, 250 mg of Fe₃O₄@mSiO₂ nanoparticles was dispersed in 80 mL of methanol, and then 0.7 mL of MPTMS was added into the mixture. The reaction mixtures were stirred for 20 h at room temperature under N₂ protection, and immediately acetone was added. The Fe₃O₄@mSiO₂-SH nanoparticles was obtained after centrifugation for five times. 0.1 g of Fe₃O₄@mSiO₂-SH was ultrasonic-dispersed in 10 mL methanol and 0.2 g of cysteamine was added, followed by stirring at room temperature for 24 h. The disulfide bond modified Fe₃O₄@mSiO₂ nanoparticles were collected by washing for three times and drying in vacuum oven for 24 h.

Then CGRPi@Fe₃O₄@mSiO₂-PEG (CGRPi@FM) nanoparticles were synthesized on the basis of disulfide bond modified Fe₃O₄@mSiO₂. 10 mg of disulfide bond modified Fe₃O₄@mSiO₂ and 2 mg CGRP antagonist were added to 1 mL of DMSO. The mixture was stirred in dark place at room temperature overnight. The CGRPi@Fe₃O₄@mSiO₂ nanoparticles were obtained after centrifugation and drying.

10 mg of CGRPi@Fe₃O₄@mSiO₂ nanoparticles were ultrasonic-dispersed into 10 mL of DI water solution. Subsequently, 10 mg DCC, 5 mg DMAP and 25 mg of PEG were added and stirred at room temperature for 24 h. Then 1 mL hydrazine phosphate was

added into the above solution and stirred at room temperature for 24 h. The resulting mixture was centrifugally (10,000 rpm, 10 min) separated and then vacuum dried to obtain CGRPi@Fe₃O₄@mSiO₂-PEG (CGRPi@FM-PEG).

Cur@Fe₃O₄@mSiO₂-PEG (Cur@FM) were synthesized on the basis of disulfide bond modified Fe₃O₄@mSiO₂. 10 mg of Fe₃O₄@mSiO₂ nanoparticles were dispersed in 2 mL of DMSO solution containing 2.5 mg of *curcumin*. Then the mixture was stirred in dark place at room temperature for 12 h. Afterwards, the precipitation after centrifugation was Cur@Fe₃O₄@mSiO₂. 10 mg of Cur@Fe₃O₄@mSiO₂ nanoparticles were ultrasonic-dispersed into 10 mL of DI water solution. Subsequently, 10 mg DCC, 5 mg DMAP and 25 mg of PEG were added and stirred at room temperature for 24 h. Then 1 mL hydrazine phosphate was added into the above solution and stirred at room temperature for 24 h. The resulting mixture was centrifugally (10,000 rpm, 10 min) separated and then vacuum dried to obtain Cur@Fe₃O₄@mSiO₂-PEG (Cur@FM).

Finally, the FMCC nanoparticles were synthesized on the basis of disulfide bond modified Fe₃O₄@mSiO₂. 10 mg of Fe₃O₄@mSiO₂, 2 mg of CGRPi and 2.5 mg of Cur dissolved in 1 mL of DMSO solution. The mixture was stirred in dark place for 24 h. After washed with DI water solution three times, CGRPi@Cur@Fe₃O₄@mSiO₂ was obtained. 10 mg of CGRPi@Cur@Fe₃O₄@mSiO₂ nanoparticles were dispersed ultrasonically into 10 mL of DI water solution. Subsequently, 10 mg DCC, 5 mg DMAP and 25 mg of PEG were added and stirred at room temperature for 24 h. Then 1 mL hydrazine phosphate was added into the above solution and stirred at room temperature for 24 h. The resulting mixture was centrifugally (10,000 rpm, 10 min) separated and then vacuum dried to obtain FMCC.

2.3 Drug loading and release of FMCC

The FMCC nanocomposites were prepared as described above. The resulting mixture was centrifugally (10,000 rpm, 10 min) separated and then vacuum dried to obtain FMCC. The supernatant was detected at 426 and 285 nm by spectrophotometer (SpectraMax iD3, Molecular Devices, USA). The concentrations of Cur and CGRPi in the supernatant were calculated using the standard curve. The amount of drug encapsulated was obtained by the difference between the initial amount of drug added and the amount of drug remaining in the supernatant. Drug loading efficiency (%) was calculated using the following equation. Drug loading efficiency (%) = Amount of drug encapsulated (mg)/(weight of FMCC) × 100.

For Cur and CGRPi release studies, 1 mg of FMCC was dispersed in 50 mL of PBS solution. The resulting mixture was stirred regularly at 100 rpm and 37°C. While mixing was in progress, samples were taken from the drug release medium at regular intervals to determine the amount of drug released. For this, the sample was first centrifuged at 12,000 rpm for 15 min. The sample was detected at 426 nm by spectrophotometer. The concentrations of Cur in the sample were calculated using the standard curve. After reading, the sample was returned to the release medium. The amount of drug released was calculated with the help of a standard calibration curve. The same method given above was followed for the CGRPi release from FMCC. The absorbance at 285 nm was detected by spectrophotometer and corrected by the standard curve method.

The concentrations of CGRPi in the sample were calculated using the standard curve.

2.4 Characterization

SEM images were captured by scanning electron microscopy (JEM100CXII, Japan) at 1.0 kV. TEM images were captured by electron microscopy (LVEM5, USA) at 5 kV. Sample preparation: add 5 mg nanoparticles to ethanol, ultrasonic for 5 min. Then they were added to the copper mesh and dried for testing.

The measurement of particle sizes and zeta potential were performed by a nanometer particle size analyzer (Nanotrac Flex, Germany).

The specific surface area and pore size distribution of the samples were detected by an automatic gas adsorption instrument (BELSORP HP, Japan) and analyzed by Brunauer-Enmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods.

2.5 BV2 cell culture

BV2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in MEM supplemented with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C.

2.6 CCK-8 assay

5×10^3 BV2 cells were inoculated on 96-well plates and incubated overnight in an incubator at 37°C and 5% CO₂. The cells were treated with different concentrations of FMCC (0, 1, 5, 10, 20 µg/mL) for 24 h in the presence or absence of LPS. 10 µL CCK8 reagent was added to incubate in the incubator for 2 h, and the absorbance value was detected at 450 nm.

2.7 Dead-living cell staining

2×10^5 BV2 cells were inoculated on 12-well plates and incubated overnight in an incubator at 37°C and 5% CO₂. The cells were treated with 20 µg/mL Fe₃O₄@mSiO₂ and FMCC for 6 h. Then Calcein/PI solution were added to incubate at 37°C in dark place, washed with PBS for three times and observed with microscope (Leica DMi8, Germany). Fluorescence intensities were measured using a fluorescence microscope, with calcein detected at excitation/emission wavelengths of 494/517 nm (blue filter) and propidium iodide (PI) detected at 535/617 nm (green filter).

2.8 Cellular uptake assay

1×10^5 BV2 cells were inoculated on 24-well plates and incubated overnight in an incubator at 37°C and 5% CO₂. The cells were treated with 20 µg/mL Fe₃O₄@mSiO₂-FITC. Fe₃O₄@mSiO₂-FITC was obtained by reacting Fe₃O₄@mSiO₂ with FITC under dark condition for 12 h. At 2 and 6 h, DAPI was added to stain

10 min, washed with PBS for three times and observed with microscope. Fluorescence intensities were measured using a white light laser confocal microscope (Leica STELLARIS, Germany), with FITC detected at excitation/emission wavelengths of 490/520 nm and DAPI at 358/461 nm. The ImageJ software was used for quantitative analysis (Jensen, 2013).

2.9 *In vitro* neuroinflammatory model

BV2 cells were treated with 100 ng mL⁻¹ LPS for 24 h to establish an *in vitro* neuroinflammatory model. Then, BV2 cells were divided into five groups: 1) Control group; 2) LPS group; 3) LPS + CGRPi@FM group; 4) LPS + Cur@FM group; 5) LPS + FMCC group.

2.10 Chronic constriction injury (CCI) model

6–8 weeks-old, 18–22 g healthy C57BL/6 mice were purchased by SPF Biotechnology Co., Ltd. CCI surgery was performed in accordance with previous study (Zhang et al., 2019). The mice were anesthetized by inhaling 4% isoflurane. The right thigh hair of the mouse was scraped and the left biceps femoris was bluntly separated to expose the sciatic nerve. The sciatic nerve was partially ligated at four points using 4-0 colored sutures, with each ligature placed approximately 1 mm apart proximal to the trifurcation site. After that, the muscles and skin were treated with iodine solution. In the sham operation group, the operation method was the same except that the sciatic nerve was not lapped. After CCI surgery, a small magnet was sterilized and fixed to the skin at the surgical site.

2.11 *In vivo* experiment design

25 C57BL/6 mice were divided into five groups 7 days after CCI surgery: 1) Control group: the sham operation group; 2) CCI group; 3) CCI + CGRPi@FM group; 4) CCI + Cur@FM group; 5) CCI + FMCC group. In the treatment group, 120 mg/kg nanoparticles were injected through the tail vein on day 1 and day 6. After treatment, the threshold of paw withdrawal threshold was detected by using the mesh to stimulate the right plantar of mice with von Frey fibers. And the paw withdrawal latency was detected by stimulating the right plantar of mice with heat. Then sciatic nerve was taken and the therapeutic effect of each group was observed by H&E staining.

2.12 Enzyme linked immunosorbent (ELISA) assay

BV2 cells were treated with LPS for 24 h and then treated with 20 µg/mL CGRPi@FM, Cur@FM, FMCC, or PBS for 24 h. The cell supernatant of each group was collected. *In vivo*, blood was collected from the orbital vein after treatment. The samples were allowed to clot for 30 min at room temperature and then centrifuged to isolate the supernatant. Then the content of TNF-α, IL-1β, IL-6 and IL-10 content was detected by Mouse TNF-α ELISA kit, Mouse IL-1β ELISA kit, Mouse IL-6 ELISA kit and Mouse IL-10 ELISA kit.

TABLE 1 The primer sequences of TNF- α , IL-1 β , IL-6, IL-10 and β -actin.

Gene name	Forward 5'-3'	Reverse 5'-3'
TNF- α	CACAGAAAGCATGATCCGCG	GTGCCCTCTGTGCTTGATCT
IL-1 β	CCCATGTTGTAGTGACCCCC	CATGGTTGGGCTTGGGAGTG
IL-6	TGAGAGAGGAGTGTGAGGCA	ACAGAGAATGGCCCACTGTG
IL-10	CAGAGTGTGGCAGTGGGAAT	TGCTTCAAACCCCAACCT
β -actin	CTTCGCTCTCTCGTGGCTAG	AAGAGGGGGAGAGGAAGAGC

2.13 Detection of oxidative stress indexes

BV2 cells were treated with LPS for 24 h and then treated with 20 μ g/mL CGRPi@FM, Cur@FM, FMCC, or PBS for 24 h. The cell of each group was collected and malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GSH-Px) content was detected by ELISA kit.

2.14 Reactive oxygen species (ROS) detection

BV2 cells were treated with LPS for 24 h and then treated with 20 μ g/mL CGRPi@FM, Cur@FM, FMCC, or PBS for 24 h. Then the DCFH-DA were added to incubate for 30 min. And the ROS signals were observed with microscope. Fluorescence intensities were measured using a fluorescence microscope at excitation/emission wavelengths of 488/525 nm (blue filter). The ImageJ software was used for quantitative analysis.

The mice in each group were euthanized after treatment. The sciatic nerve was quickly extracted and frozen with liquid nitrogen. The ROS content was detected by DHE probe method and photographed by microscope. Fluorescence intensities were measured using a fluorescence microscope at excitation/emission wavelengths of 535/610 nm (green filter).

2.15 RT-PCR assay

The total RNA was extracted from sciatic nerve using TRIzol Reagent. The cDNA was synthesized using iScript™ cDNA Synthesis Kit. The qPCR was performed on ABI 7500-Fast Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) using SYBR Green qPCR Master Mix and the β -actin was used as an internal control. The primer sequence is shown in Table 1.

2.16 Biosafety assay

6–8 weeks-old, 18–22 g of healthy C57BL/6 mice were given a single dose of FMCC 120 mg/kg via the tail vein injection. On 0 day, 3 days and 30 days, blood was then taken from the ocular vein for routine blood test and biochemical analysis. Routine blood test and biochemical analysis were performed by a fully automated

biochemical analyzer (Indiko™ Plus, Thermo Scientific, USA). The heart, liver, spleen, lung and kidney were extracted to H&E staining.

2.17 Statistical analysis

Data are presented as the mean $\pm$ standard error of mean (SEM) from at least three separate experiments. Statistical analysis was performed using GraphPad Prism statistical software. Comparisons between two groups were performed using Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA, with Bonferroni correction applied. The differences were deemed statistically significant at $p < 0.05$.

3 Results and discussion

3.1 Characterization of FMCC drug delivery system

As reported the core-shell nanostructures Fe₃O₄@mSiO₂ has been shown reasonable biocompatibility and good drug release performance (Zhang et al., 2021). Fe₃O₄@mSiO₂ is a suitable drug delivery carrier, and it also possesses functionalities such as magnetic targeting, magnetic resonance imaging. Curcumin has been reported to produce a healing analgesic effect in chronic neuropathic pain (Caillaud et al., 2020). In addition, CGRP expression levels are elevated after nerve injury and play an important role in generating and maintaining pain behavior, and inhibiting CGRP expression can significantly improve pain behavior after peripheral nerve injury (Benarroch, 2011). Therefore, in this study, we have synthesized Fe₃O₄@mSiO₂ with novel structure as the combined delivery carrier for curcumin and CGRPi. Firstly, Fe₃O₄ nanoparticles synthesized by coprecipitation method were extensively characterized. The SEM images showed that it was spherical, with a particle size of about 110 nm (Figure 1A). TEM images showed that Fe₃O₄ nanoparticles were round, well dispersed, and relatively uniform in particle size, mainly about 110 nm (Figure 1B). Dynamic light scattering (DLS) results (Supplementary Figure S2A) showed an increase in the hydrodynamic size of Fe₃O₄ nanoparticles to 122.4 nm.

Further, mesoporous silicon dioxide (mSiO₂) was grown on the surface of Fe₃O₄ nanoparticles to synthesize the shell karyotype nanoparticles Fe₃O₄@mSiO₂. Furthermore, since disulfide bonds

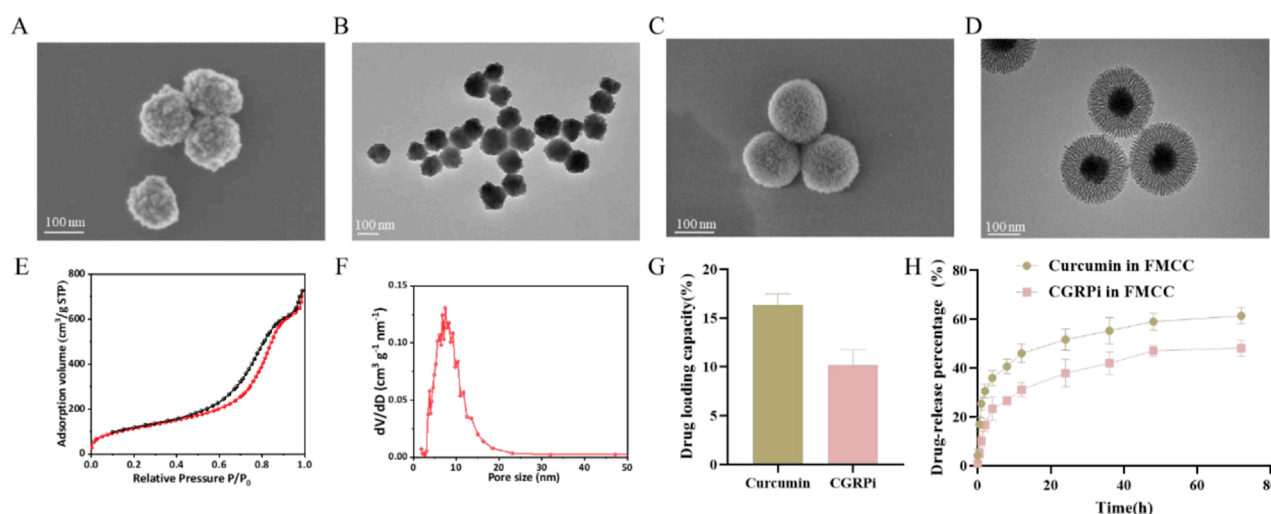
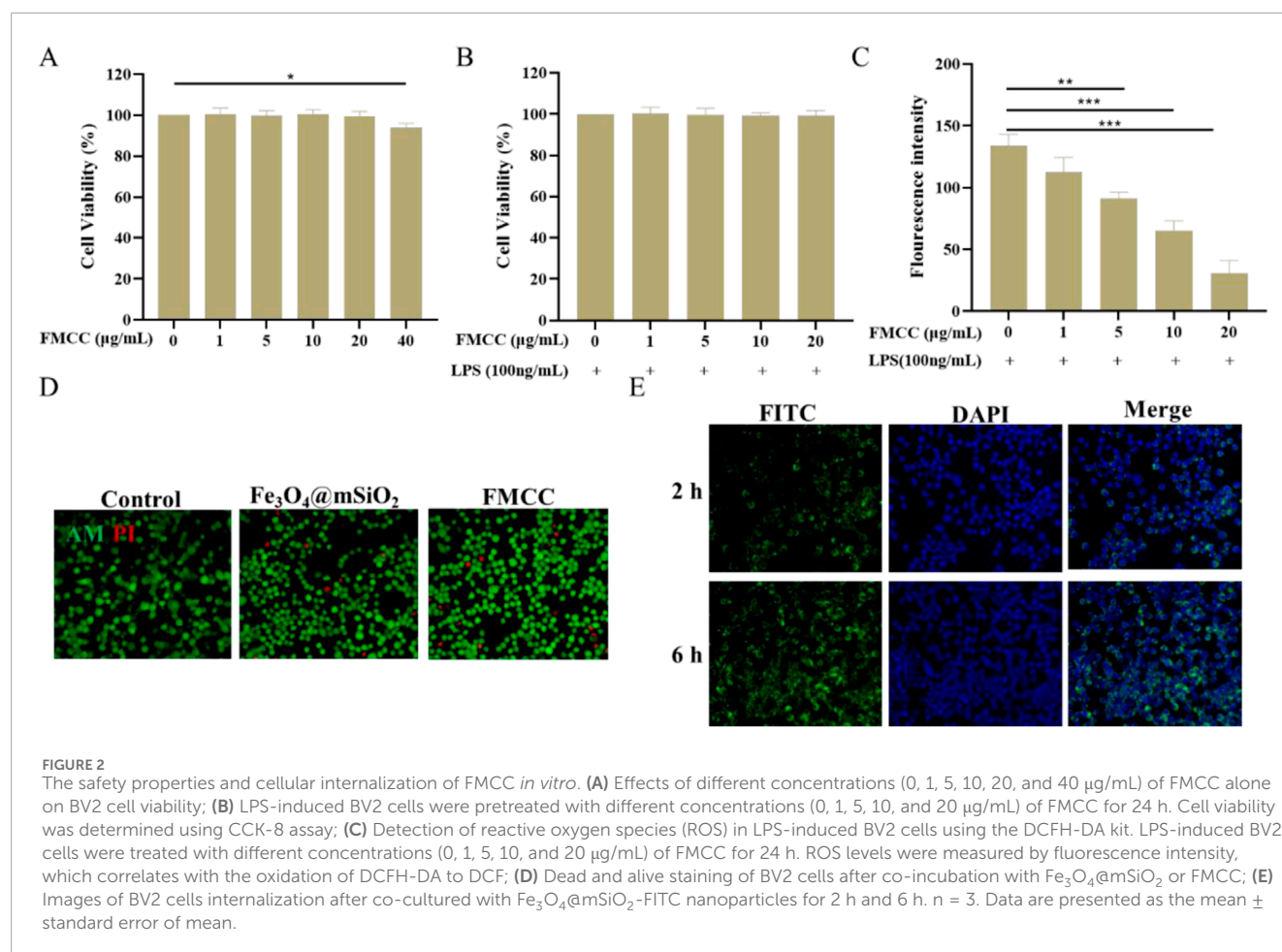


FIGURE 1

Characterization of Fe₃O₄, Fe₃O₄@mSiO₂ and FMCC nanoparticles. (A) SEM images of Fe₃O₄ nanoparticles; (B) TEM images of Fe₃O₄ nanoparticles. (C) SEM images of Fe₃O₄@mSiO₂ nanoparticles; (D) TEM images of Fe₃O₄@mSiO₂ nanoparticles; (E) Nitrogen adsorption-desorption isotherm of Fe₃O₄@mSiO₂ nanoparticles. The adsorption volume (cm³/g STP) of N₂ gas was measured as a function of relative pressure (P/P₀, where P₀ is the saturation pressure of N₂ at 77 K). The specific surface area (427 m²/g) was calculated from the linear region of the BET plot based on the BET theory; (F) Pore size distribution (dV/dD) of Fe₃O₄@mSiO₂ nanoparticles was analyzed using the BJH method, showing a predominant pore size of ~7.4 nm. (G) The drug loading capacity of *Cur* and CGRP antagonist on Fe₃O₄@mSiO₂-PEG; (H) Plots of the release curves of the drugs *Cur* and CGRP antagonist from the FMCC nanocomplex in PBS buffer.

in the nanocore framework are one of the most efficient cleavable joints in biology, they can be degraded and cleaved in the presence of glutathione (Fu et al., 2022). Therefore, cysteamine is used to make mSiO₂ doped with disulfide bonds. Then, the Fe₃O₄@mSiO₂ nanoparticles were characterized by electron microscopy, DLS, Zeta potential analysis and nitrogen isothermal adsorption-desorption curves. It can be observed from SEM (Figure 1C) and TEM (Figure 1D) that the length of mSiO₂ nanorods is about 60 nm, and mesoporous pores are obvious and uniform, and they grow evenly on the surface of Fe₃O₄, completely enveloping Fe₃O₄ nanospheres. The morphology of Fe₃O₄ nanospheres remained intact. High specific surface area and large pore volume can effectively adsorb and load drugs. Therefore, the specific surface area and pore size of Fe₃O₄@mSiO₂ were measured by nitrogen isothermal adsorption-desorption curve. The synthesized Fe₃O₄@mSiO₂ showed a typical type IV isotherm and hysteresis ring. This is caused by the ordered mesopore in the mSiO₂ nanorods (Figure 1E). Fe₃O₄@mSiO₂ has a Brunauer-Emmett-Teller (BET) surface area and a Barrett-Joyner-Halenda (BJH) pore size of 427 m²/g and ~7.4 nm, respectively (Figure 1F). In addition, the hydrated particle size of Fe₃O₄@mSiO₂ measured by DLS was 220.2 nm (Supplementary Figure S2B), and the hydrated particle size of FMCC was 240.2 nm (Supplementary Figure S2C). The structure of the nanoparticles loaded with *Cur* and CGRPi was not altered as observed by TEM (Supplementary Figure S2D), and the BJH pore size was reduced to ~6.3 nm (Supplementary Figure S2E). The Zeta surface potentials of Fe₃O₄, Fe₃O₄@mSiO₂, Fe₃O₄@mSiO₂ loaded with *Cur* and CGRP antagonists, and FMCC were -19.2, -16.5, -15.5, and -22.9 mV, respectively (Supplementary Figure S2F).

The loading capacities of *Cur* and CGRPi on FMCC were 16.4% and 10.2%, respectively (Figure 1G). To investigate the kinetics of drug release from the nanocomposites in detail, we tracked the release of the drugs *Cur* and CGRPi from FMCC in PBS solution over time. Under the experimental conditions (PBS solution, pH7.4), both drugs demonstrated slow release (Figure 1H). In this regime, the rate of *Cur* release increased slightly compared to CGRPi. Although the release rates of *Cur* were only slightly higher than those of CGRPi, the actual release amounts of *Cur* were approximately twice that of CGRPi, due to the higher initial loading of *Cur* in the nanoparticles. The differences in release efficiency and drug loading between *Cur* and CGRPi can be attributed to several factors. First, the molecular properties of the drugs, such as solubility, hydrophobicity, and molecular size, significantly influence their interaction with the nanocarrier and subsequent release behavior. *Cur* is a hydrophobic molecule with a relatively small molecular size, which facilitates its encapsulation and sustained release from the hydrophobic core of the nanoparticle (Wang et al., 2020). In contrast, CGRPi, being a peptide, has higher hydrophilicity and larger molecular size, which may limit its loading efficiency and result in slower release kinetic (Luo et al., 2023). Second, the chemical interactions between the drugs and the nanocarrier material play a crucial role. *Cur* can form strong π - π interactions and hydrogen bonds with the silica matrix of the nanoparticles, leading to higher loading capacity and controlled release (Lin et al., 2022). On the other hand, CGRPi may rely more on electrostatic interactions or physical entrapment, which are less stable and result in lower loading efficiency and faster initial release (Liang et al., 2020).



3.2 The safety properties and cellular internalization of FMCC *in vitro*

Current studies have shown that CGRP content increases in inflammatory pain and neuropathic pain, and the upregulation of CGRP level contributes to the enhancement of pain perception, which is the target of analgesic treatment (Johanes et al., 2020). Prior to this, the toxicity of FMCC on BV2 cells was first investigated using CCK-8 assay for a period of 24 h at different concentrations. As shown in Figure 2A, the concentrations of FMCC less than 20 µg/mL did not induce any detectable cytotoxicity, whereas cytotoxicity was induced at concentrations of 40 µg/mL. Next, we treated LPS-induced BV2 cells with different concentrations of FMCC for their cytotoxic effects. Different concentrations of FMCC up to 20 µg/mL had no significant toxic effect on BV2 cell viability (Figure 2B). The ROS fluorescence was used to further detect the effect of different concentrations of FMCC on the ROS level of BV2 cells induced by LPS. With the increase of FMCC concentration, the intracellular ROS level in BV2 cells gradually decreased and reached the lowest level at 20 µg/mL (Figure 2C). These results indicate that the incubation of FMCC significantly reduces LPS-induced ROS production in BV2 cells without cytotoxicity, which would potentially alleviate inflammatory pain and neuropathic pain. In subsequent experiments, we chose FMCC at a concentration of 20 µg/mL for subsequent cell experiments.

Based on this, cell viabilities and cellular internalization of FMCC were studied BV2 cells. As shown in Figure 2D, even the incubated concentration set as high as 20 µg/mL and the co-culture time extended as long as 24 h, the cell viabilities of BV2 cells remained over 90%, pronouncedly confirming that the FMCC nanocomposites possessed perfect biocompatibility. Further, Figure 2E and Supplementary Figure S3 showed the images and quantitative fluorescence intensities of BV2 cells after incubated with FMCC for 2 h and 6 h, respectively. This nanocomposite material showed a green light, and the intensity of the fluorescence becomes stronger with the increase of time. These results indicate that FMCC can effectively carry drugs into glial cells, consistent with previous reports that Fe₃O₄@mSiO₂ promotes drug uptake by cells (Sun et al., 2020). Efficient cellular uptake and drug release may be critical for pain relief by FMCC.

3.3 Effects of FMCC on inflammatory factors, oxidative stress and reactive oxygen species (ROS) in LPS-induced BV2 cells *in vitro*

It is well known that neuropathic pain induced by chronic constriction injury (CCI) elicits a persistent inflammatory response (Liu P. et al., 2023). Tumor Necrosis Factor-α (TNF-α) appears as

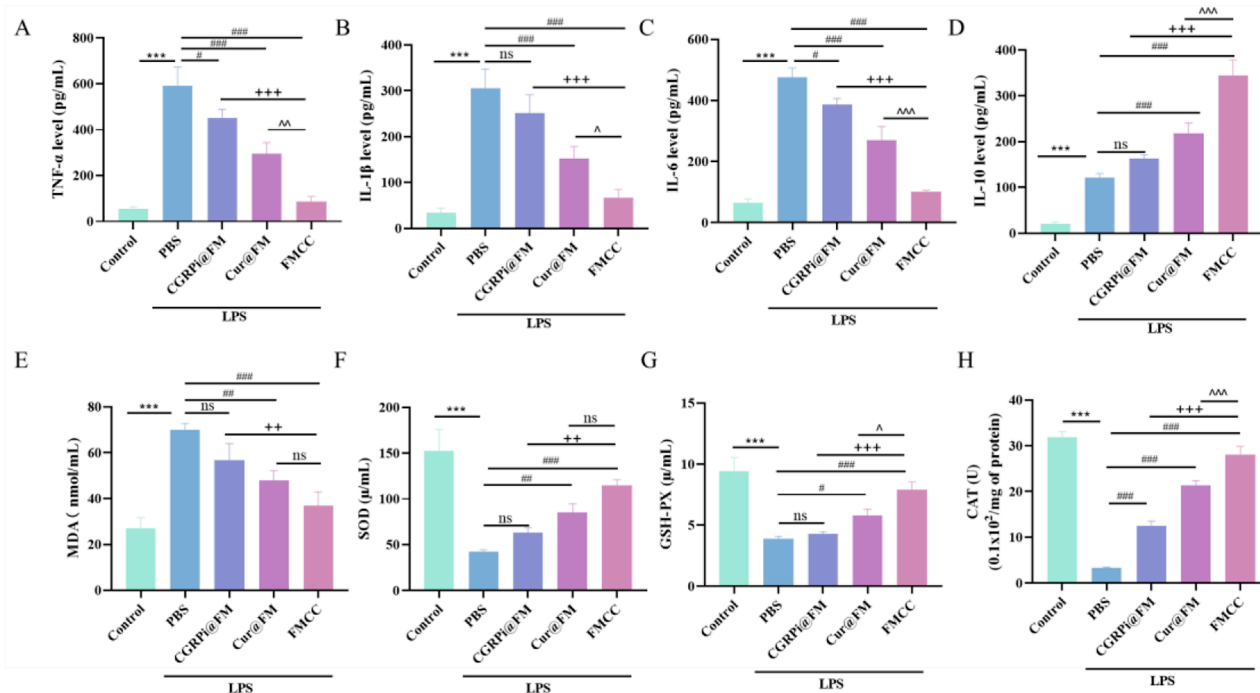


FIGURE 3

Effects of FMCC on inflammatory factors and oxidative stress in LPS-induced BV2 cells *in vitro*. (A–D) TNF- α , IL-1 β , IL-6 and IL-10 content in the supernatant of BV2 cells treated with LPS, CGRPi@FM, Cur@FM, and FMCC were detected by ELISA analysis; (E–H) MDA, SOD, GSH-Px, and CAT content in BV2 cells treated with LPS, CGRPi@FM, Cur@FM, and FMCC were detected by ELISA analysis. ns $P > 0.05$ no significant difference. $n = 3$. Data are presented as the mean $\pm$ standard error of mean.

the earliest cytokine in the inflammatory cascade and reaches its peak at an early stage (Jang et al., 2021). TNF- α and IL-1 β , as early proinflammatory factors, have been well validated in peripheral and central sensitization of neuropathic pain (Boakye et al., 2021). To determine whether FMCC nanocomposites could protect BV2 cells from inflammatory injury, we used LPS treatment of BV2 cells as an *in vitro* model of neuroinflammation and compared the effects of nanoparticles loading two drugs or a single drug. Upon LPS stimulation, cells increased the production of pro-inflammatory cytokines, including TNF- α , IL-1 β , and Interleukin-6 (IL-6), and the anti-inflammatory cytokine Interleukin-10 (IL-10). Direct treatment with Cur@FM or CGRPi@FM decreased TNF- α , IL-1 β , and IL-6 secretion (Figures 3A–C) and increased IL-10 production (Figure 3D). Notably, a more potent effect was observed in cells treated with FMCC nanocomplexes, suggesting additive or synergistic pharmacological effects of the two loaded drugs. The intracellular CGRP mRNA expression was further detected by RT-PCR assay (Supplementary Figure S4). Consistent with the expected results, LPS treatment enhanced the expression level of CGRP, which was significantly downregulated by FMCC nanocomposites.

The neuroinflammatory process after nerve injury releases a variety of cytokines, which in turn promotes the occurrence of oxidative stress and the increase of reactive oxygen species, which is also considered to be an important factor in stimulating inflammation (Fakhri et al., 2022). Therefore, research on inhibiting

oxidative stress and ROS after nerve injury can play a role in the treatment of neuropathic pain. To explore the effects of LPS induction and FMCC treatment on oxidative stress level and ROS in BV2 cells, we used MDA, SOD, CAT and GSH-Px assay kits to determine changes in the activities of MDA, SOD, GSH-Px, and CAT in BV2 cells after LPS induction and different treatments. Figures 3E–H showed that LPS induced significant increases in MDA and decreases in SOD, GSH-Px and CAT in BV2 cells. CGRPi@FM and Cur@FM were able to inhibit the increase of MDA and the decrease of SOD, GSH-Px and CAT, and the inhibitory effect of FMCC was more significant. DCFH-DA kit results showed that LPS induced a significant increase in ROS production in BV2 cells, which was inhibited by CGRPi@FM and Cur@FM, and the inhibitory effect of FMCC was more significant (Figures 4A, B). The above results confirmed that FMCC could inhibit LPS-induced oxidative stress and ROS production in BV2 cells.

The above results indicate that inhibiting CGRP activity can effectively suppress the inflammatory response of glial cells and the increase of ROS caused by inflammation. The anti ROS generation and anti-inflammatory effects of curcumin may come from its regulatory role in validating signaling pathways and the inhibitory activity of CGRP (Liu et al., 2023b), but the latter effect still needs further investigation. The above results also indicate that the dual drug FMCC of curcumin and CGRPi can exert the combined anti-inflammatory and ROS effects of the two drugs, enhancing the therapeutic effect.

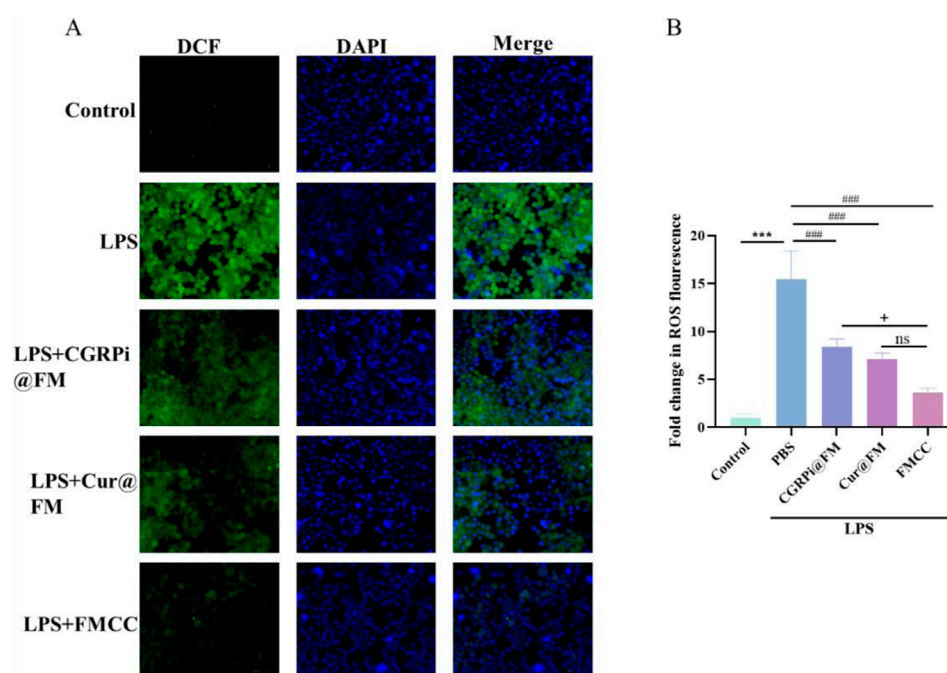


FIGURE 4

Effects of FMCC on ROS in LPS-induced BV2 cells *in vitro*. DCFH-DA kit detection of ROS in BV2 cells treated with LPS, CGRPi@FM, Cur@FM, and FMCC; (B) Fluorescence semi-quantitative statistics of (A). ns $P > 0.05$ no significant difference. $n = 3$. Data are presented as the mean $\pm$ standard error of mean.

3.4 FMCC nanocomposites alleviated CCI-induced neuropathic pain, inflammation and oxidative stress *in vivo*

To elucidate the *in vivo* effects of the FMCC drug delivery system, we generated a mouse model of chronic constriction injury (CCI) of the sciatic nerve (Fonseca-Rodrigues et al., 2021). CGRPi@FM, Cur@FM and FMCC were injected into the tail vein of CCI mice on the first day and the sixth day after successful modeling. At the same time, in order to make the nanocomposite magnetic targeting, a strong neodymium magnet was placed at the sciatic nerve injury site. Pain behavior was examined within 2 weeks after surgery (Figures 5A, B). CCI resulted in chronic pain in mice as indicated by increased thermal hyperalgesia and mechanical hyperalgesia compared to the sham-operated group. In contrast, CGRPi@FM, Cur@FM and FMCC nanocomposites significantly attenuated neuropathic pain responses, with FMCC having the most significant effect. RT-PCR assay was used to detect the mRNA expression of CGRP in the injured sciatic nerve tissue. The results were consistent with the cellular level (Figure 5C). The cross-sectional sections of sciatic nerve tissue in each group were further stained with hematoxylin-eosin to examine whether FMCC alleviated the histological changes in the sciatic nerve. Compared with the sham group, in the CCI group, the intercellular space was enlarged and the cell arrangement was disordered, which were effectively alleviated by FMCC (Figure 5D). In addition, ROS was significantly increased in the CCI group, while FMCC significantly abolished ROS production (Figure 5E).

Both RT-PCR and ELISA results showed the ability of FMCC nanocomposites to inhibit inflammation *in vivo*. CCI stimulated

the expression of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6), on the other hand, the CCI-induced increase in the anti-inflammatory cytokine IL-10 was further enhanced by CGRPi@FM and Cur@FM. The FMCC nanocomposite had the most pronounced effect, as shown by the greatest decrease in proinflammatory cytokines and the greatest increase in anti-inflammatory cytokine (Figures 6A–H). Finally, the oxidative stress indicators (MDA, SOD, GSH-Px and CAT) were detected by the kit. Compared with the sham operation group, MDA in the CCI group was significantly increased, and SOD, GSH-Px and CAT were significantly decreased; CGRPi@FM and Cur@FM alleviated this phenomenon, with FMCC having the most significant effect (Figures 6I–L).

The pathogenesis of neuropathic pain is complex. In addition to the inflammatory responses are involved in the pathogenesis of neuropathic pain, the dorsal root ganglion (DRG) neurons sense pain and transmit it to the central nervous system, which is involved in the occurrence and maintenance of neuropathic pain (Martin et al., 2019). Lim et al. found that japonica rice leaf extract may relieve CCI-induced neuropathic pain by activating MAPK in DRG and microglia in the spinal cord (Lim et al., 2022). Chu et al. found that atorvastatin may inhibit neuroinflammation in rats with chronic systolic injury by down-regulating dorsal root ganglia and spinal cord nuclear NF- κ B (Chu et al., 2015). It was found that electroacupuncture alleviated neuropathic pain by promoting autophagy of dorsal root ganglion macrophages mediated by AMPK/MTOR (Xu et al., 2022). In addition, montelukast can effectively reduce neuropathic pain in CCI rats by inhibiting the activation of p38MAPK and NF- κ B signaling pathways in spinal microglia (Zhou et al., 2014).

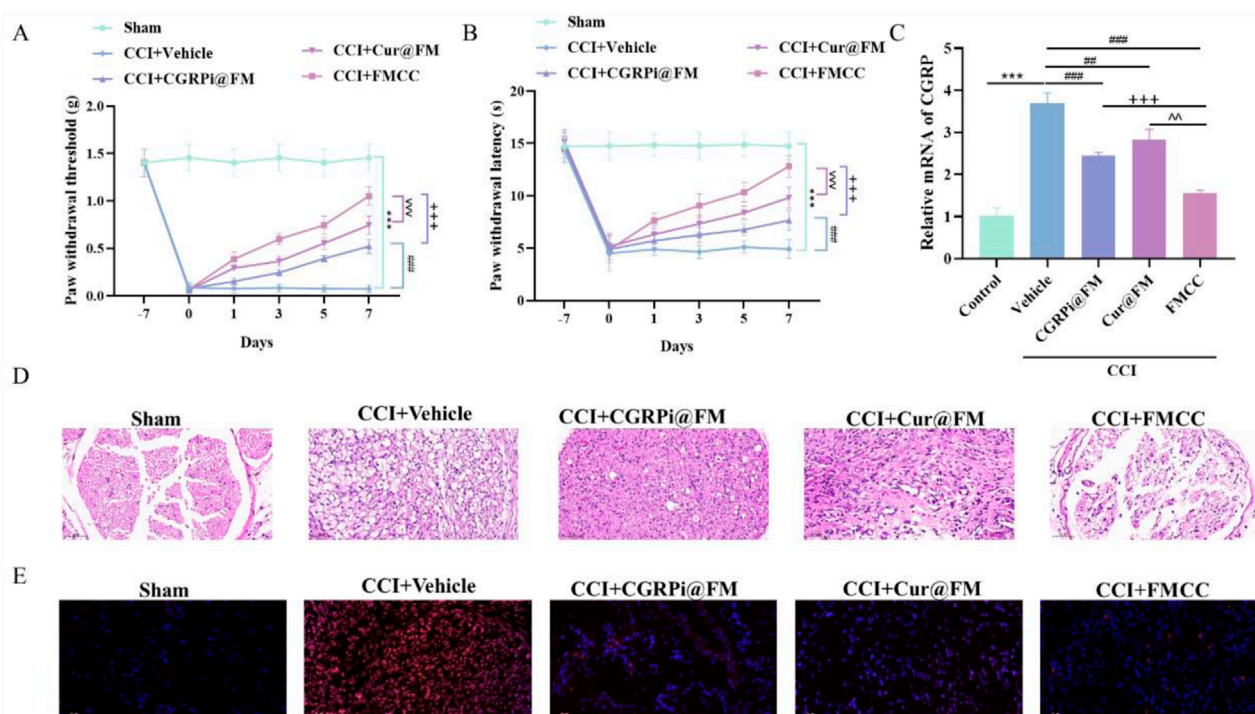


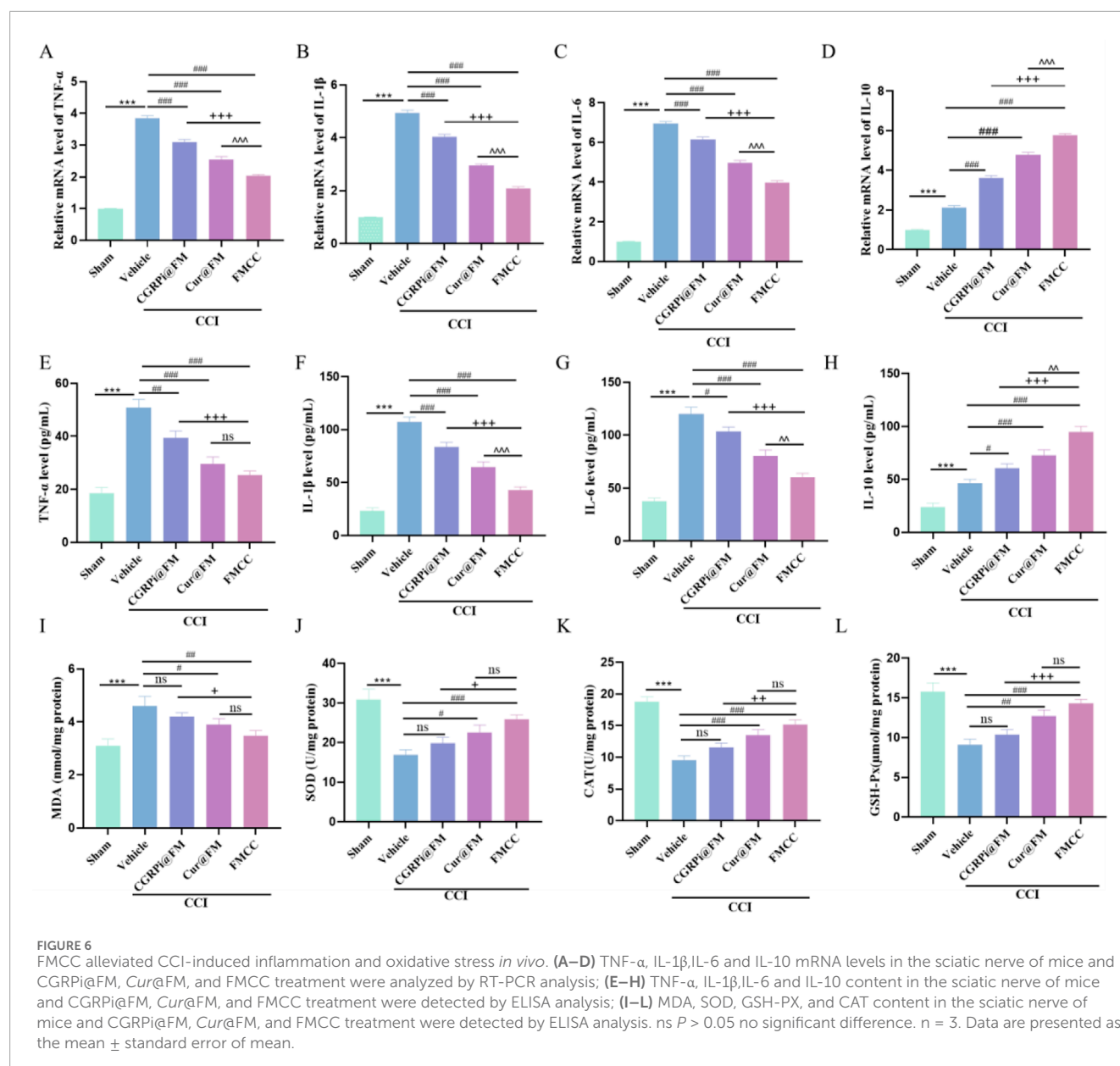
FIGURE 5 FMCC alleviated CCI-induced neuropathic pain *in vivo*. (A) Neuropathic pain was assessed by von Frey filaments test; (B) Thermal hyperalgesia was assessed by a radiant heat test; (C) RT-PCR detection of CGRP mRNA expression levels in CCI model mice after CGRPi@FM, Cur@FM, and FMCC treatment; (D) Sciatic nerve sections were stained with hematoxylin and eosin; (E) sciatic nerve sections were stained with DHE kit. $n = 5$. Data are presented as the mean $\pm$ standard error of mean.

These results indicate that FMCC can effectively enter the site of sciatic nerve injury in mice, delivering the loaded curcumin and CGRPi to damaged nerve cells, alleviating chronic sciatic nerve injury, inflammatory response, and neuropathic pain response in mice. This is because FMCC can leverage the magnetic targeting effect of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ to targeted delivery the curcumin and CGRPi to the damaged nerve area under the influence of an external magnetic field, and treats sciatic nerve pain through the combined action of curcumin and CGRPi. Liang et al. (Liang et al., 2025) synthesized mesoporous silica nanoparticles (MSNs) coated with Fe_3O_4 as a drug carrier, which is similar to our nanoparticle design. They loaded the nanoparticles with RADA16-I/RAD-RGI peptide (PD) to construct a neurotrophic microenvironment for the treatment of peripheral nerve defects. Additionally, they functionalized the MSNs with the neurotargeting peptide HLNILSTLWKYR (PT) to enhance targeting efficiency. While their study focused on peripheral nerve regeneration, our work explores the application of $\text{Fe}_3\text{O}_4@\text{mSiO}_2$ for drug delivery in neuropathic pain, highlighting the versatility of this nanoparticle platform in different biomedical contexts.

3.5 Biocompatibility assessment of FMCC *in vivo*

Additionally, *in vivo* biocompatibility of FMCC was determined. After intravenous injection of our nanoparticles

for 3 days and 30 days, blood samples and major organ tissue were collected. Blood bio-chemical tests and H&E staining studies were conducted for exploring the biosafety of FMCC *in vivo*. Here, the serum bio-chemistry factors include alanine transaminase (ALT, Supplementary Figure S5A), aspartate transaminase (AST, Supplementary Figure S5B), serum creatinine (CRE, Supplementary Figure S5C), blood urea nitrogen (BUN, Supplementary Figure S5D), white blood cell (WBC, Supplementary Figure S5E), and Platelet (PLT, Supplementary Figure S5F). Collectively, in comparison with control group (0 day), there were insignificant discrepancy in these indexes of the FMCC based groups at 3 days and 30 days post-administration. In the bargain, the H&E-stained-images of the main normal organ tissues (including heart, liver, spleen, lung and kidney) revealed no apparent pathological injury or normal cellular damage were discovered (Supplementary Figure S5G), preliminarily illustrating the excellent *in vivo* histocompatibility of FMCC as well as its potent bio-application for neuropathic pain patients. These data indicate that FMCC is a nanomedicine with good biocompatibility and *in vivo* targeting. It can simultaneously load two or more drugs and deliver them to the damaged nerve site under the action of an external magnetic field, exerting a combined therapeutic effect. At the same time, it also indicates that the combination of curcumin and CGRPi has a significantly better therapeutic effect on neuropathic pain than the use of the two drugs alone. Through *in vitro* and *in vivo* experiments, we have provided evidence for its feasibility in treating neuropathic pain,



which may help promote the development of effective treatments for chronic pain.

4 Conclusion

In this study, we successfully designed and synthesized a magnetically targeted nanocomposite that can target the injured area of the sciatic nerve and achieve multifunctional analgesia for the delivery of a single or two analgesic drugs. Our results show that this disulfide bonded magnetic mSiO₂ nanoparticles can effectively load Cur and CGRPi and deliver them to the sciatic nerve injury area. Under conditions of elevated GSH, the disulfide bonds are cleaved, and PEG modification facilitates the complete release of the therapeutic payloads. Through *in vitro* and *in vivo* experiments, we provided evidence for the feasibility of its application in the treatment of neuropathic pain, which may help facilitate the development of effective treatments for chronic pain.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Ethics Committee of Nanjing Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YZ: Writing–original draft. ZZ: Writing–original draft. LG: Writing–review and editing. YT: Writing–review and editing. XL:

Writing–review and editing. YJ: Writing–review and editing. HS: Writing–review and editing. CG: Writing–review and editing. CS: Writing–review and editing. LW: Conceptualization, Funding acquisition, Supervision, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2025.1510141/full#supplementary-material>

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