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Protective impact of hesperidin on aluminum chloride splenic toxicity in rats, halting oxidative insult, histopathological changes, necroptosis, and apoptosis

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Abstract

Aluminum (Al) is a toxic heavy metal that stores in the spleen, where it induces structural damage and disrupts immune function. Hesperidin, a citrus derived flavonoid, is known for its strong antioxidant activities. The present study evaluated the protective role of hesperidin against aluminum chloride (AlCl3) induced splenic damage. Forty Male Wistar rats were classified to 4 groups: control, hesperidin alone (100 mg/kg), $AlCl_3$ alone (128 mg/kg), and combined $AlCl_3$ plus hesperidin, all administered orally for 30 days. Splenic tissues were assessed for oxidative stress biomarkers (MDA, GSH, SOD, CAT), Nrf2/HO 1 antioxidant pathway, necroptosis and apoptosis markers (RIPK1, RIPK3, MLKL, caspase 3, Bax, Bcl 2), TNF α expression, and histopathological alterations. $AlCl_3$ exposure significantly increased (MDA) and suppressed antioxidant defenses (SOD, CAT, GSH). It also downregulated Nrf2/HO 1 signaling, elevated TNF α, activated necroptotic mediators (RIPK1, RIPK3, MLKL), and triggered apoptosis, by increased caspase 3 and Bax with reduced Bcl 2. Histologically, $AlCl_3$ caused marked lymphocyte depletion and expansion of the red pulp. Co administration of hesperidin markedly ameliorated these changes by enhancing Nrf2/HO 1 activation, restoring antioxidant enzyme levels, reducing TNF α expression, suppressing necroptotic and apoptotic pathways, and improving splenic architecture. In conclusion, hesperidin protects against $AlCl_3$ induced splenic injury through dual mechanisms: activation of the Nrf2/HO 1 antioxidant axis and inhibition of TNF α driven necroptosis and apoptosis. These findings highlight hesperidin as a promising dietary supplement for mitigating aluminum related immunotoxicity.

Keywords: Hesperidin, aluminum chloride, spleen, necroptosis, inflammation, oxidative stress

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1. INTRODUCTION

Aluminum (Al) is a widely distributed heavy metal that accumulates in biological systems and poses significant health risks to both humans and animals. Environmental factors such as acid rain and intensive bauxite extraction have accelerated the mobilization of Al from mountainous regions into freshwater ecosystems. Moreover, Al is extensively used in industrial and consumer applications, including water purification systems and food preservatives (Kopáček et al., 2009; Krewski et al., 2007). Elevated exposure to Al has been associated with toxic effects in several organs, involving the brain, liver, kidneys, lungs, ovaries, skeletal tissues, and the immune system (Sun et al., 2015). Among immune organs, the spleen is particularly susceptible to Al accumulation, which leads to structural alterations and impaired immune function (Hu et al., 2013; Zhu et al., 2012).

Oxidative damage is considered a central mechanism underlying Al-induced immunotoxicity. Aluminum exposure enhances the ROS while diminishing the activity of endogenous antioxidant enzymes, resulting in oxidative damage within splenic tissue (Luo et al., 2014). Excessive ROS can compromise mitochondrial membrane integrity, triggering the release of pro-apoptotic factors that activate caspases and initiate programmed cell death. This apoptosis-driven loss of lymphocytes contributes to immune suppression (Allagui et al., 2015; Chen et al., 2016). Therefore, strategies aimed at reducing oxidative stress may offer therapeutic potential against Al related immune dysfunction (Sivakumar et al., 2014; Zhuang et al., 2018).

Hesperidin, a citrus-derived polyphenolic flavonoid, and its aglycone form, hesperidin, are abundant in oranges and other citrus fruits (Garg et al., 2001). Hesperidin has been reported to exert diverse biological properties, including antihyperlipidemic, antihyperglycemic, antihypertensive, and neuroprotective effects (Ashafaq et al., 2014; Dokumacioglu et al., 2018; Iskender et al., 2017; Justin Thenmozhi et al., 2018; Kakadiya et al., 2010). Its antioxidant potential has been demonstrated in several experimental models; for example, hesperidin reduced hepatic ROS and enhanced CAT and superoxide dismutase in the pleurisy rat model (Adefegha et al., 2017). Similarly, hesperidin improved antioxidant defenses in aged rats by increasing catalase and glutathione reductase in hepatic tissue (Miler et al., 2016). In addition, hesperidin has been shown to attenuate hyperglycemia-induced apoptosis by reducing oxidation and inflammatory mediators in ganglion cells in the retina (Liu et al., 2017). Although previous works have highlighted the immunomodulatory properties of hesperidin (Camps Bossacoma et al., 2017; Estruel Amades et al., 2019), its effect on oxidative balance within lymphoid tissues under trace element-induced oxidative stress remains unexplored.

2. MATERIALS AND METHODS

2.1. Animals

Forty male Wistar rats (190–220 g; 11–14 weeks old) were housed in standard controlled laboratory conditions. Animals had unrestricted access to water and a pellet diet. A stable light–dark cycle was maintained, and the room temperature was regulated at 21 ± 2 °C. Throughout the experimental time, the rats were kept in group housing.

2.2. Study plan

A total of forty healthy adult male Wistar rats were allocated into four experimental groups, with ten animals in each group. Group I (control) took normal saline orally throughout the study period. Group II (hesperidin-treated group) was administered hesperidin at a dose of 100 mg/kg b.w per day via oral gavage (hesperidin obtained from Sigma-Aldrich, company), accompanying the protocol by Hanchang et al. (2022). Group III (aluminum group, AG) received aluminum chloride ($AlCl_3$) at 128 mg/kg/day by oral gavage for 30 days, according to the method of Fu et al. (2014) ($AlCl_3$ purchased from Sigma-Aldrich). This dosing regimen has previously been shown to induce splenic injury and immunosuppression in rats (Zhuang et al., 2016). Group IV ($AlCl_3$ + hesperidin) received both $AlCl_3$ (128 mg/kg/day) and hesperidin (100 mg/kg/day) concurrently for the same duration.

At the end of the 30-days, all animals were euthanized by i.p of sodium pentobarbital at a dose of 100 mg/kg b.w. The spleens were immediately excised, rinsed, and weighed. Each spleen was then divided into two parts: one half was fixed in ten percentage of formalin for histopathological evaluation, while the remaining half was homogenized and stored for biochemical and molecular analyses.

2.3. Measurement of splenic MDA, GSH, SOD, and CAT

The concentrations of MDA, as well as GSH levels and the enzymatic activities of SOD and CAT in splenic tissue, were quantified using established analytical procedures. MDA was measured according to the Ohkawa et al. (1979) method, GSH content was determined following the protocol of Beutler et al. (1963), SOD activity was assessed using the technique described by Nishikimi (1972), and CAT activity was evaluated based on the procedure outlined by Gross et al. (1967).

2.4. Histological examination

Spleen samples fixed in 10% formalin were processed and embedded in paraffin, sectioned at a five-micrometer thickness, and stained with H&E. The stained sections were examined for histopathological alterations using an Olympus light microscope (Japan).

2.5. ELISA assessment of necroptosis, apoptotic, and Nrf2/HO-1 markers

Splenic tissue homogenates were used to measure necroptosis markers (RIPK1, RIPK3, and MLKL) and apoptosis markers (caspase-3, Bax, and Bcl-2) by ELISA. The following kits were employed: RIPK1 (LS-F56081-1, 0.156–10 ng/mL), RIPK3 (LS-F35895-1, 0.156–10 ng/mL), MLKL (MBS2707293, 0.156–10 ng/mL), caspase-3 (E1648Ra, 0.05–10 ng/mL), Bax (ER0512, 0.313–20 ng/mL), Bcl-2 (abx155248, 78–5000 pg/mL), Nrf2 (NBP3-08161, 15.63 - 1000 pg/mL), and HO-1 (orb2567593, 0.313-20ng/ml). Manufacturer instructions were followed for all measurements.

2.6. mRNA quantification

Total RNA of splenic tissues was extracted by using TRIzol reagent in accordance with the manufacturer's instructions. The isolated RNA was further purified with the RNeasy Mini Kit supplemented with an RNase-Free DNase treatment. Complementary DNA was synthesized from purified RNA using the Omniscript® Reverse Transcription Kit and Oligo-dT primers (Takara). The QuantiTect™ SYBR Green® PCR Kit performed quantitative real-time PCR. B-actin served as the internal reference gene for normalization. The sequences of primers used were as follows: TNF-α F: TTCCGTCCCTCTCATACACTG; R: AGACACCGCCTGGAGTTCT (accession no. NM_012675.3) β-actin F: AGCCATGTACGTAGCCATCC; R: CTCTCAGCTGTGGTGGTGAA (accession no. NM_031144.3). Relative mRNA expression levels were measured using the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical analysis

Data were analysed using GraphPad Prism version 8.0 and are presented as mean ± SD. Differences between groups were analysed by using one-way ANOVA accompanied by Tukey's post hoc test. A p-value < 0.05 is significant.

3. RESULTS

3.1. Hesperidin activates Nrf2 signaling and mitigates AlCl3-induced splenic oxidative injury

Oral administration of AlCl3 (128 mg/kg/day) produced a pronounced oxidative burden in splenic tissue, as reflected by a significant elevation in MDA levels (mean ± SD: 192.9 ± 2.0 nmol/mg) compared with the control group (100.8 ± 1.0 nmol/mg). This oxidative shift was accompanied by a marked suppression of endogenous antioxidant defenses, including reductions in CAT (11.8 ± 1.4 vs. 31.8 ± 2.0 U/mg), SOD (27.0 ± 4.7 vs. 74.0 ± 2.4 U/mg), and GSH (0.88 ± 0.16 vs. 2.60 ± 0.40 µmol/g) relative to control animals.

Consistent with this redox imbalance, AlCl3 markedly downregulated the Nrf2/ARE antioxidant pathway. Splenic Nrf2 protein levels declined to 24.0 ± 6.0 pg/mg compared with 287.7 ± 52.0 pg/mg in controls, while HO-1 expression decreased to 5.41 ± 1.4 ng/mg versus 17.3 ± 1.3 ng/mg in control rats.

Co-administration of hesperidin (100 mg/kg) with AlCl3 significantly counteracted these alterations. Hesperidin reduced lipid peroxidation (MDA: 151.9 ± 10.0 nmol/mg) and restored antioxidant capacity, evidenced by increases in CAT (21.6 ± 3.3 U/mg), SOD (44.7 ± 7.0 U/mg), and GSH (1.82 ± 0.25 µmol/g) compared with the AlCl3 group. Importantly, hesperidin markedly reactivated the Nrf2/HO-1 axis, elevating Nrf2 to 101.7 ± 29.0 pg/mg and HO-1 to 11.0 ± 2.3 ng/mg, confirming its ability to restore ARE-driven antioxidant signaling.

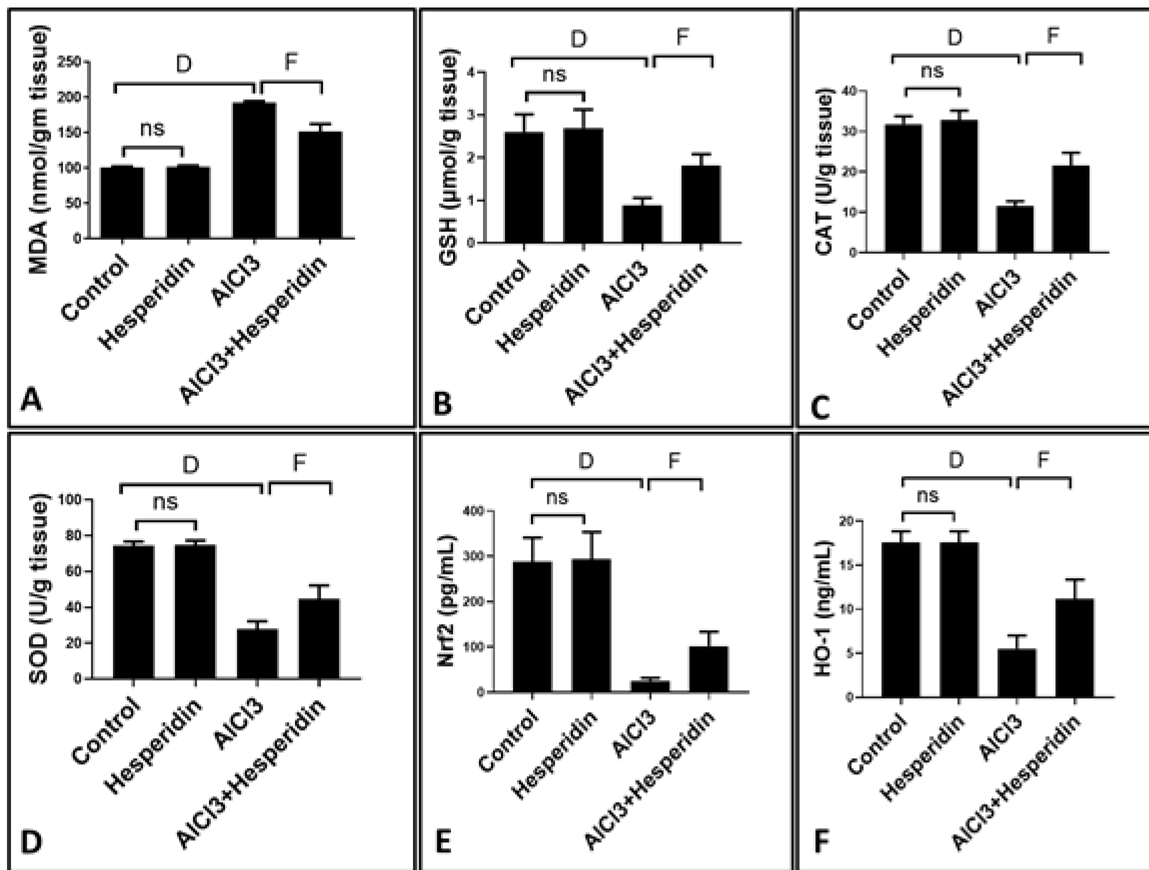

Fig.1. (A-F) Protein levels of MDA, GSH, CAT, SOD, Nrf2, and HO-1 in the splenic supernatant of all groups. ns means non-significant, D significant to control, F significant to AlCl3.

3.2. Histological findings

Histological examination of control spleens revealed the expected architecture with well-defined white pulp rich in lymphocytes and normally arranged periarteriolar lymphoid sheaths, surrounded by intact red pulp containing abundant sinusoids and erythrocytes. In contrast, AlCl3-treated rats exhibited marked distortion of splenic morphology. The white pulp appeared depleted of lymphocytes and poorly demarcated, while the red pulp showed conspicuous expansion and congestion, indicating severe structural disruption.

Co-administration of hesperidin with AlCl3 produced a notable improvement in splenic histoarchitecture. Sections from the AlCl3 + hesperidin group demonstrated partial restoration of white pulp boundaries with only mild lymphocytic necrosis, along with reduced red pulp expansion compared with the AlCl3 group. These findings collectively confirm the protective effect of hesperidin against AlCl3-induced splenic injury, supporting its role in preserving tissue integrity under oxidative stress.

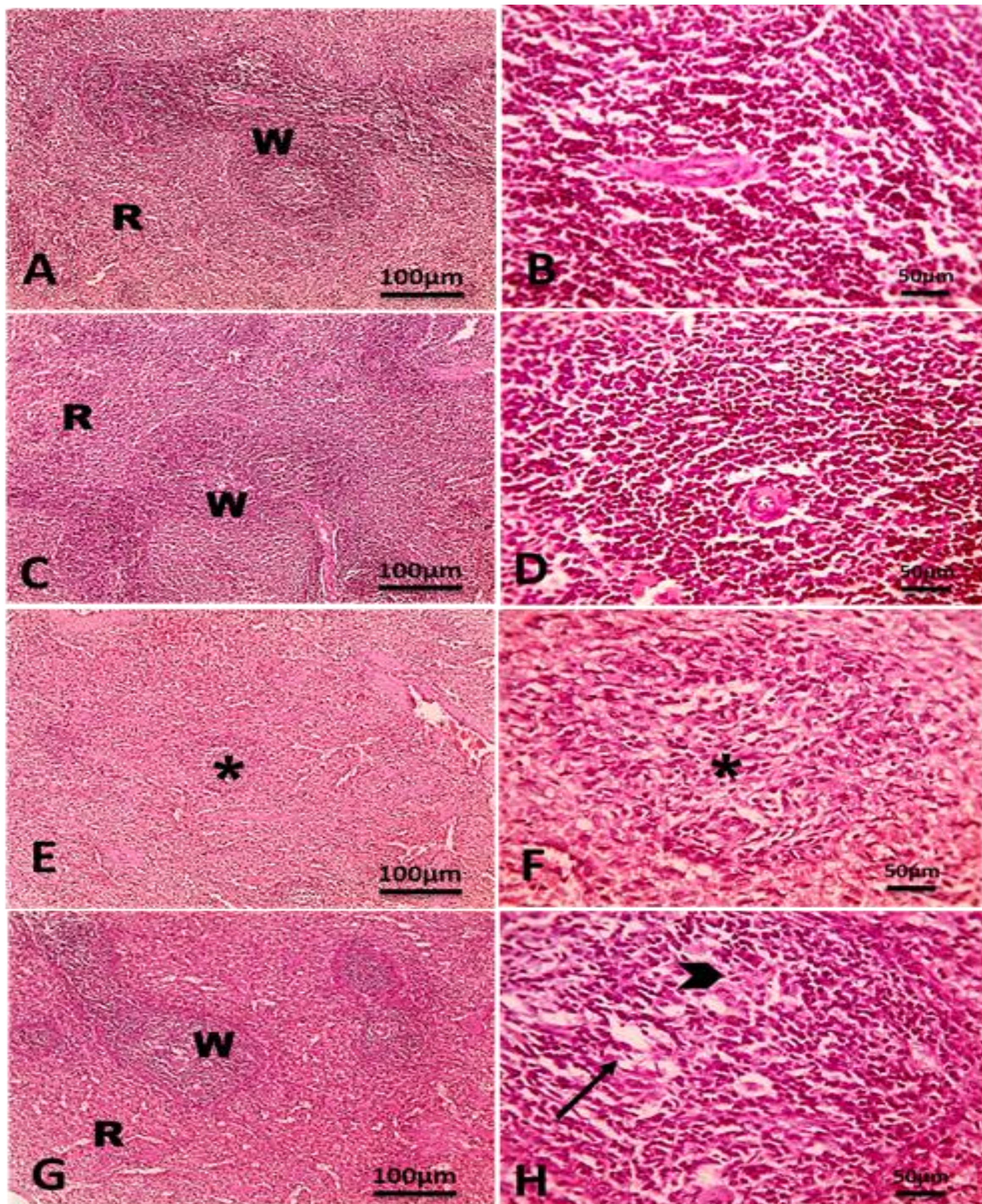

Fig. 2. A-D. Spleen sections showing normal large well-defined white pulp (W) and red pulp (R) containing RBCs, lymphocytes, and sinusoids in the control normal group and Hesperidin groups. E, F. Spleen sections of $AlCl_3$ group showing small, ill-defined white pulp (W) due to severe depletion of lymphocytes (*) and expanded red pulp (R). G, H. Spleen sections of $AlCl_3$+hesperidin group showing increased size of well-defined white pulp (W) with normal red pulp (R). Mild depletion (arrow) and necrosis (arrowhead) of lymphocytes are additionally seen in the white pulp (W). Magnifications: (100 bar 100, x400 bar 50).

3.3. Anti-inflammatory and anti-necroptotic effects of hesperidin

As depicted in Figure 3, $AlCl_3$ administration produced a marked pro-inflammatory and necroptotic response in splenic tissue. ELISA analysis showed a substantial elevation in TNF-α levels in the $AlCl_3$ group (6.82 ± 0.82 pg/mg) compared with the control (1.00 ± 0.00 pg/mg). This inflammatory surge was accompanied by a pronounced activation of the necroptotic machinery, as indicated by significant increases

in RIPK1 (8.48 ± 0.93 vs. 1.13 ± 0.25 ng/mg), RIPK3 (6.48 ± 0.70 vs. 2.01 ± 0.36 ng/mg), and MLKL (4.04 ± 0.34 vs. 0.57 ± 0.23 ng/mg) relative to control rats.

Co-treatment with hesperidin markedly attenuated these AlCl3-induced alterations. TNF-α levels were reduced to 3.09 ± 0.52 pg/mg, while necroptotic markers showed substantial downregulation: RIPK1 decreased to 4.03 ± 1.15 ng/mg, RIPK3 to 4.02 ± 0.56 ng/mg, and MLKL to 2.14 ± 0.61 ng/mg compared with the AlCl3 group. Although these values did not fully return to baseline, the reductions represent a clear suppression of necroptotic signaling.

These biochemical findings align with the previously described antioxidant effects of hesperidin and correlate with the improved splenic morphology observed histologically. Together, the data confirm that hesperidin exerts a dual protective action, anti-inflammatory and anti-necroptotic, thereby mitigating AlCl3-induced splenic injury.

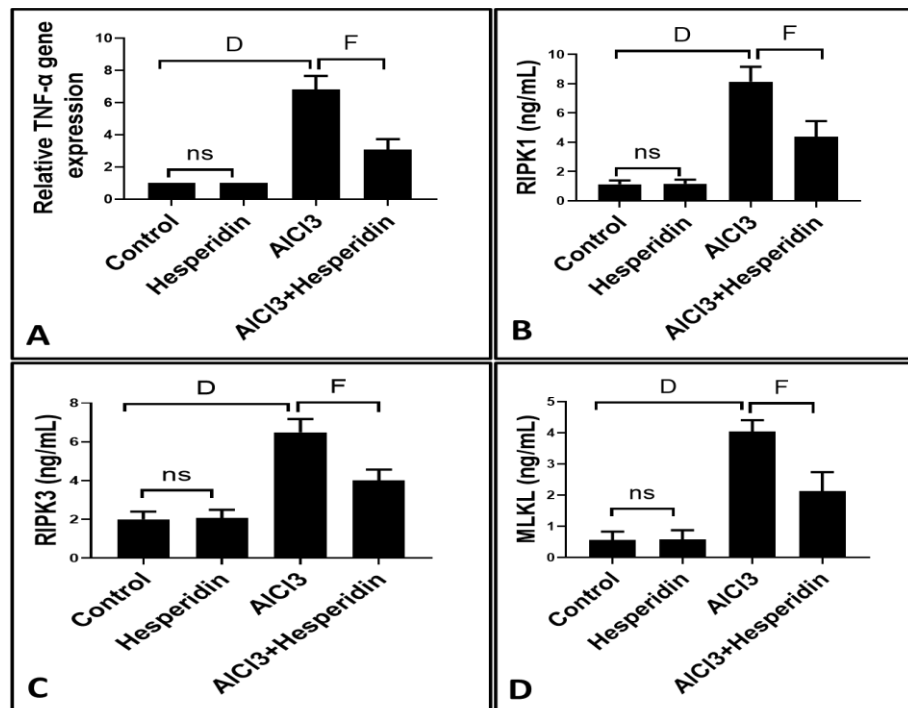

Fig. 3. ELISA measurement of (A) TNF-α, (B-D) RIPK1, RIPK3, MLKL in different groups. All values are expressed in means and standard deviations.

3.4. Anti-apoptotic effect of hesperidin against AlCl3-induced splenic apoptosis

AlCl3 intake triggered a pronounced apoptotic response in splenic tissue. ELISA analysis revealed a significant elevation in caspase-3 levels in the AlCl3 group (5.83 ± 0.64 ng/mg) compared with the control (1.25 ± 0.20 ng/mg). Similarly, the pro-apoptotic protein Bax increased markedly (3.65 ± 0.33 vs. 0.42 ± 0.07 ng/mg), indicating strong activation of the intrinsic apoptotic pathway. In contrast, the anti-apoptotic marker Bcl-2 showed a substantial decline (366.3 ± 96.0 vs. 1120.5 ± 118.0 ng/mg), further confirming severe AlCl3-induced apoptotic injury.

Co-administration of hesperidin significantly mitigated these apoptotic alterations. Caspase-3 levels were reduced to 2.65 ± 0.47 ng/mg, while Bax expression decreased to 2.04 ± 0.48 ng/mg compared with the AlCl3 group. Concurrently, Bcl-2 content increased markedly to 627.7 ± 157.0 ng/mg, reflecting a strong shift toward cell survival signaling (Figure 4).

These biochemical improvements align with the histological findings, which demonstrated better preservation of lymphocytic architecture in the hesperidin-treated group. Collectively, the data confirm that hesperidin exerts a robust anti-apoptotic effect, counteracting AlCl3-induced splenic cellular damage and supporting overall tissue integrity.

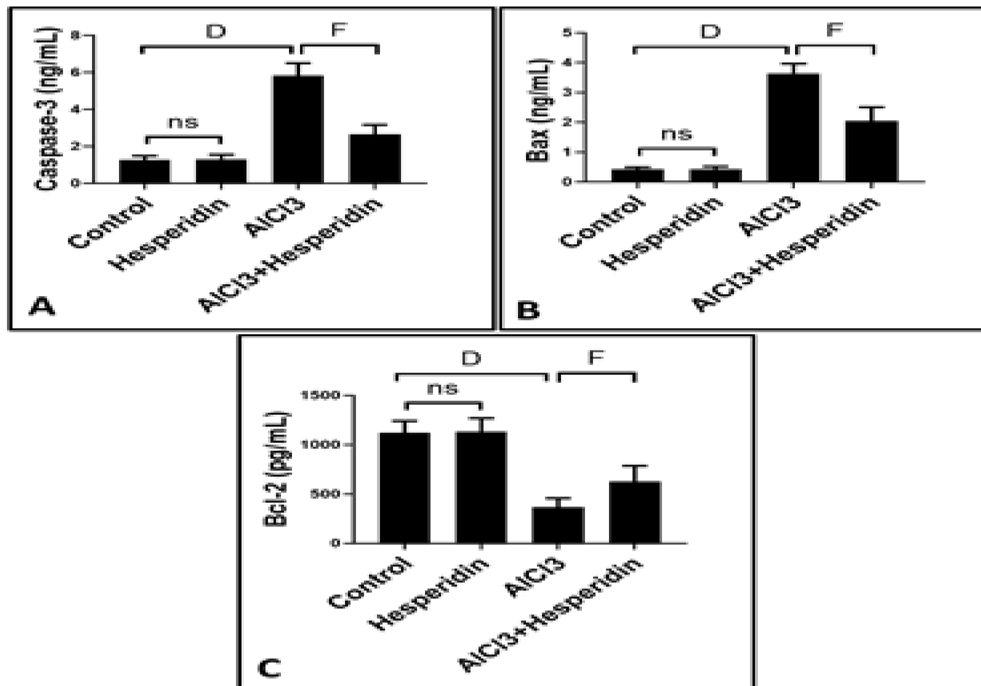

Fig. 4 illustrates the impact of AlCl3 and hesperidin on splenic apoptotic markers. (A) Caspase-3 levels. (B) Bax expression. (C) Bcl-2 content.

Data are presented as M± SD. D denotes a significant difference vs the control group; F denotes a significant difference vs the AlCl3 group.

4. DISCUSSION

Aluminium, a toxic metal, tends to accumulate in the spleen, leading to structural damage, reduced cytokine expression, and a decline in T lymphocyte counts (Hewitt et al., 1990; Zhu et al., 2012b). In the present study, hesperidin improved the histological architecture of the spleen in AlCl3-exposed rats. This protective effect was linked to a reduction in oxidative degeneration in splenic tissue, accompanied by upregulation of Nrf2/HO-1. Additionally, hesperidin suppressed TNF-α associated with the activation of necroptosis, thereby halting this cell death pathway. These changes collectively reduced apoptosis in splenic lymphocytes. Taken together, the findings support the attenuative role of hesperidin against experimental splenic toxicity induced by aluminium chloride.

Oxidative stress develops when pro-oxidant activity exceeds the antioxidant defence system capacity. In the spleen, this imbalance promotes lipid peroxidation and suppresses key antioxidant enzymes, contributing to tissue injury (Luo et al., 2014). The accumulation of ROS can damage cellular macromolecules, including DNA, membrane lipids, and proteins, ultimately impairing cell viability. MDA, a major end product of lipid peroxidation (Jing et al., 2013). Enzymes such as SOD and CAT form the first line of antioxidant defense by directly detoxifying ROS and maintaining redox balance (Yu, 1994). The Nrf2/HO-1 signaling pathway is a key antioxidant mechanism that is triggered by oxidative stress or exposure to toxic substances, thereby strengthening cellular defense through its protective and detoxifying roles (Khadrawy et al., 2021). In our experiment, treatment with AlCl3 led to a marked rise in lipid oxidation, a decline in antioxidant enzyme activity, and reduced expression of the Nrf2/HO-1 signaling relative to the control rodents. These findings are in line with those reported by Yu et al. (2019), who found that aluminum compounds induce oxidative degeneration in the spleen, characterized by elevated ROS and MDA levels along with decreased SOD and CAT activities. Furthermore, our results corroborate the work of Sedik et al. (2023), who observed that aluminum chloride suppresses Nrf2 signaling and the downstream ARE-driven HO-1 antioxidant response in a rodent model of AlCl3-induced liver and kidney toxicity. In contrast to the

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oxidative damage induced by $AlCl_3$, co-administration of hesperidin markedly attenuated redox imbalance by enhancing Nrf2 signaling and restoring the antioxidant defense system. This was marked by the elevation of GSH, SOD, and CAT, accompanied by a pronounced reduction in MDA. These findings are consistent with Hegazy et al. (2023), who demonstrated that hesperidin activates the Nrf2/HO-1 pathway in a lung model of hypothyroidism, as shown by increased Nrf2 and HO-1 protein expression and elevated tissue levels of GSH, SOD, and CAT. Similar antioxidant and Nrf2-enhancing effects of hesperidin have been stated in models of nephrotoxicity and hepatotoxicity by Subramanian et al. (2015) and Karabekir et al. (2025), respectively. Together, these studies support our results, indicating that hesperidin mitigates $AlCl_3$-induced OS through Nrf2 activation and reinforcement of endogenous antioxidant defenses. Hesperidin exerts potent antioxidant activity primarily through Nrf2/HO-1 stimulation, a master regulator of cellular redox homeostasis. Under oxidative stress, hesperidin facilitates the dissociation of Nrf2 from its cytoplasmic inhibitor Keap1, enabling its translocation into the nucleus, where it binds to AREs. This interaction enhances the transcription of HO-1, NQO1, and enzymes involved in glutathione synthesis. Through this mechanism, hesperidin boosts endogenous antioxidant capacity by elevating GSH levels and restoring the activities of SOD and CAT, while simultaneously reducing lipid peroxidation products such as MDA. Collectively, these actions counteract ROS accumulation.

The present findings indicate that hesperidin induces a robust inhibitory effect on necroptosis in the spleen following $AlCl_3$ exposure. Aluminum chloride markedly activated the necroptotic machinery, as evidenced by the pronounced elevation of RIPK1, RIPK3, and MLKL—core components of the necrosome complex responsible for executing regulated necrotic cell death. This pathway is typically initiated when TNF-α signaling proceeds under conditions favoring RIPK1–RIPK3 interaction and subsequent MLKL phosphorylation, ultimately compromising plasma membrane integrity and promoting inflammatory cell death. Hesperidin co-administration significantly attenuated this cascade by reducing TNF-α levels and suppressing the expression of RIPK1, RIPK3, and MLKL, suggesting that hesperidin interferes with both the upstream inflammatory trigger and the downstream necroptotic execution pathway. These results are consistent with previous observations by Li et al. (2025), who reported that hesperidin mitigated necroptosis in a non-alcoholic fatty liver model through downregulation of p-MLKL, RIPK1, and RIPK3. Importantly, our study is the first to demonstrate that aluminum chloride induces necroptosis in splenic tissue and that hesperidin effectively counteracts this form of programmed necrotic cell death, highlighting its potential as a protective agent against aluminum-related immunotoxicity. Hesperidin likely suppresses necroptosis through a combination of anti-inflammatory, antioxidant, and kinase-modulating actions that interfere with the RIPK1–RIPK3–MLKL pathway.

Oxidative stress has the capacity to damage essential biomolecules, including mitochondrial components, and can initiate apoptosis—a central mechanism contributing to aluminum-induced immunotoxicity (Fang et al.; Zhuang et al., 2018). Consistent with previous reports, our findings show that $AlCl_3$ administration markedly decreased the Bcl-2 while elevating Bax and caspase-3, thereby promoting lymphocytic apoptosis in agreement with Yu et al. (2019). In contrast, hesperidin effectively counteracted this apoptotic shift by modulating these key apoptotic parameters. This protective anti-apoptotic action aligns with the work of Khamis et al. (2023), who demonstrated that hesperidin mitigated cyclophosphamide-induced reproductive injury.

5. CONCLUSIONS

This study demonstrates that hesperidin effectively mitigates aluminum -induced splenic toxicity in rats. The protective mechanism involves activation of the Nrf2/HO-1 antioxidant signaling pathway, which restores endogenous antioxidants and reduces lipid peroxidation. Additionally, hesperidin suppresses TNF-α-driven necroptosis by downregulating RIPK1, RIPK3, and MLKL, while also inhibiting the mitochondrial apoptotic pathway through modulation of Bax, Bcl-2, and caspase-3. These molecular improvements are reflected in the preservation of splenic histoarchitecture. Collectively, hesperidin exerts a triple protective action—antioxidant, anti-necroptotic, and anti-apoptotic—making it a promising natural agent for counteracting aluminum-related immunotoxicity. Dietary supplementation with hesperidin-rich citrus products may offer a feasible strategy to protect individuals at risk of chronic aluminum exposure from environmental or occupational sources.

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