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RESEARCH ARTICLE

Kaempferol Alleviates Copper Exposure-Induced Liver Injury in Chickens by Inhibiting Pyroptosis

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A B S T R A C T

Copper (Cu), an environmental metallic pollutant, is closely associated with hepatic injury and inflammation, yet its molecular mechanisms remain incompletely understood. Although kaempferol (KAE) exhibits anti-inflammatory properties, its role in Cu exposure-induced hepatotoxicity, which is not fully clarified. In this study, we integrated animal experiments with network pharmacology and molecular docking to explore KAE's protective effects in a chicken model of Cu exposure-induced liver injury. Histopathological analysis revealed that Cu exposure induced inflammatory infiltration, hemorrhage, and collagen deposition, which were alleviated by KAE. Network pharmacology identified insulin (INS) as a key target, supported by strong binding affinity in molecular docking. Consequently, Cu exposure elevated blood glucose and upregulated INS and p38-MAPK expression, effects reversed by KAE. Further, the results showed that copper activated the p38-MAPK/NF-κB pathway, promoting NLRP3 inflammasome assembly and caspase-1-dependent pyroptosis, as evidenced by increased levels of NLRP3, ASC, caspase-1, GSDMD, N-GSDMD, IL-1β, and IL-18. Immunostaining confirmed upregulation of NLRP3 and N-GSDMD after Cu exposure, which KAE treatment suppressed. These findings elucidate a novel mechanism of Cu exposure-induced liver injury and highlight KAE's potential as a therapeutic agent for metal overload-related hepatotoxicity.

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INTRODUCTION

Copper (Cu) is an essential trace mineral closely associated with numerous metabolic functions (Ramos-Zúiga *et al*., 2023). Although it participates in diverse enzymatic activities and metabolic processes, prolonged excessive exposure induces cellular death, resulting in a narrow threshold between its essentiality and toxicity. Nevertheless, current environmental copper exposure has become an unignorable issue. Anthropogenic activities, including smelting, mining, agricultural practices, industrial processes, and waste disposal, release excessive copper into ecosystems, leading to environmental accumulation that persistently threatens ecological safety. In avian species, copper serves as an indispensable micronutrient (Faghih-Mohammadi *et al*., 2022), with copper sulfate in poultry feed being its primary source. Consequently, excess copper exposure inflicts significant harm on poultry. Hepatic damage, currently under extensive investigation, encompasses structural impairment, oxidative stress, and dysregulated cholesterol metabolism (Chen *et al*., 2024). The synergistic effects of these mechanisms contribute to hepatic dysfunction and substantial health deterioration. Notably, the toxic accumulation of copper through food chain transmission poses potential risks to human health. Existing studies demonstrate that copper triggers inflammatory responses through multiple mechanisms. Research has shown that copper promotes inflammation by modulating immune cell activity and inflammatory mediators (Yang *et al*., 2020).

Furthermore, copper exacerbates inflammation via oxidative stress and mitochondrial dysfunction. Recent years have witnessed significant progress in targeted intervention strategies addressing core mechanisms of copper-induced inflammation (Zhong *et al*., 2023). Modulating inflammatory signaling pathways and suppressing inflammatory factor release to disrupt pathological cascades have led to breakthroughs in both mechanistic understanding and therapeutic development. Consequently, pharmacological interventions targeting inflammation associated with copper exposure represent a current research priority.

Kaempferol (KAE), one of the most widely recognized and extensively studied flavonoids, exhibits antibacterial, antifungal, antiprotozoal, anticancer, and anti-inflammatory properties (Periferakis *et al*., 2022). Supported by numerous studies, KAE is a natural flavonoid with notable anti-phlogistic effects (Zhuang *et al*., 2017). KAE also regulates critical trans-acting factors like NF-κB and AP-1, which serve pivotal roles in inflammatory reaction (Basu *et al*., 2017). Through these molecular interactions, KAE attenuates inflammation and has demonstrated efficacy in various inflammatory conditions, including cancer-related inflammation. Its potential to enhance conventional anti-inflammatory and anticancer therapies positions it as a promising natural compound for therapeutic applications (Al-Khayri *et al*., 2022). However, whether KAE can ameliorate Cu-induced hepatic inflammation and pyroptosis-related liver injury remains to be elucidated.

Pyroptosis, a recently characterized kind of predetermined cell death, has attracted increasing attention due to its involvement in inflammatory responses. In contrast to apoptosis, pyroptosis induces cell membrane disruption and the release of substantial pro-inflammatory cytokines, thereby eliciting robust inflammatory responses (Rao *et al*., 2022). Executed primarily by gasdermin family proteins, pyroptosis can be triggered through multiple signaling pathways, including the canonical inflammasome-mediated caspase-1 pathway and the non-canonical caspase-4/5/11 pathways. Pyroptosis plays a pivotal role in the development and progression of liver diseases (Mamun *et al*., 2020). While moderate pyroptosis aids in eliminating infected cells and combating bacterial infections, excessive pyroptosis provokes severe hepatic inflammation, damages healthy hepatocytes, and disrupts hepatic architecture and function. In the canonical pyroptosis pathway, inflammasome activation serves as the key trigger, wherein the NLRP3 inflammasome plays a central role, such as bacterial components, viral particles, toxins, ATP, and reactive oxygen species (ROS), via pattern recognition receptors (PRRs) like NLRP3 and AIM2 (Liu *et al*., 2024). The core components of the NLRP3 complex are the NLRP3 sensor protein, the ASC adaptor molecule, and an inflammatory caspase, including caspase-1. Upon stimulation by PAMPs or DAMPs, NLRP3 undergoes conformational changes, leading to inflammasome assembly. This process recruits pro-caspase-1 and facilitates its autoproteolytic cleavage into active caspase-1. Pore formation in the cell membrane is driven by the N-terminal segment of gasdermin D (N-GSDMD), which is released following caspase-1-mediated splitting of full-length GSDMD. These pores exacerbate inflammatory cascades by triggering cell swelling and membrane rupture and promoting the release of pro-inflammatory cytokines such as IL-1β and IL-18, thereby establishing a vicious cycle of inflammation (Liao *et al*., 2019). However, the correlation between pyroptosis and copper exposure-induced inflammation remains elusive. Furthermore, whether KAE can ameliorate copper overload-associated inflammation through pyroptosis inhibition has yet to be elucidated.

In this study, we confirmed a Cu-exposed chicken model to investigate the mechanisms underlying Cu-induced hepatotoxicity, with a focus on hepatic inflammation and pyroptosis. By integrating network toxicology and molecular docking analyses, we preliminarily identified the insulin (INS) and mitogen-activated protein kinase (MAPK) signaling pathways as critical targets in Cu-induced liver injury, revealing their central roles in inflammatory responses. Crucially, Cu exposure significantly induced hyperglycemia and upregulated INS and p38-MAPK, biomarkers indicative of INS resistance and inflammatory activation. This was followed by activation of the NF-κB/NLRP3 inflammasome axis, which triggered the maturation and release of pro-inflammatory cytokines (IL-1β, IL-18) and executed Caspase-1-dependent pyroptosis, as evidenced by GSDMD cleavage. Significantly, KAE effectively inhibited this cascade, alleviated pathological damage, suppressed the INS/p38-MAPK/NF-κB signaling module, and restrained NLRP3 inflammasome activation and pyroptosis. Collectively, our findings not only elucidate a novel mechanism by which Cu exposure induces hepatotoxicity via the INS/p38-MAPK-mediated pyroptotic pathways but also highlight KAE as a promising therapeutic candidate against Cu-induced inflammatory liver injury.

MATERIALS AND METHODS

Animal treatment: Before experimentation, ethical approval was obtained from the Institutional Animal Care and Use Committee of South China Agricultural University (approval number: 2022D113). A total of 240 one-day-old broiler chicks were randomly assigned to four groups: control (CON; 11 mg/kg $CuSO_4$), KAE control (KAE, 150 mg/kg KAE; 11 mg/kg $CuSO_4$), Cu treatment (Cu, 300 mg/kg $CuSO_4$), and KAE treatment (KAE+Cu, 150 mg/kg KAE; 300 mg/kg $CuSO_4$). Dosages were based on previous studies (Zhang *et al*., 2023; Zhao *et al*., 2023). Investigators were blinded to group allocation during the experiment, and all animals were housed under standardized conditions. After 42 days of feeding, the chickens were humanely sacrificed.

Histopathological analysis: Fresh liver tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 4 μm for H&E, Masson's trichrome, IHC, and IFA staining. H&E (Cat: C0105S; Beyotime, China) and Masson's trichrome (Cat: C0189S; Beyotime, China) were performed per manufacturer's instructions. IHC/IFA used antibodies against NLRP3, N-GSDMD, and Caspase1 (1:200, Wanleibio, China) with Mayer's hematoxylin counterstaining. Quantitative analysis of all sections was normalized to the control group.

Plasmid construction and transfection: Primary chicken hepatocytes were transfected with pFlag-CMV-3-INS (INS overexpression) and si-INS/negative control (NC, INS knockdown) via Lipo8000™ Reagent (Beyotime, China).

Western blotting: Protein extracts were obtained from liver tissues using RIPA lysis buffer (Cat: P0013; Beyotime, China). Western blotting was performed as previously described (Wu *et al*., 2023). This study summarizes all applied antibodies in Table 1.

RNA extraction and real-time fluorescent quantitative PCR (qPCR) assay: Total RNA was extracted from liver tissues using Trizol (TaKaRa, Japan). cDNA was synthesized with PrimeScript RT Master Mix (including Gdna Eraser; TaKaRa, Japan), per the manufacturer's instructions. Primers for Caspase-1, IL-1β, IL-18, NLRP3, NF-κB and GAPDH were designed with Primer 6.0 and optimized before qPCR, sequences (Table 2). In all qPCR analyses, the expression of the target gene was normalized to GAPDH.

Table 1: List of antibodies used in this study

Protein name	Manufacturers	Product code
INS	Selleck	F0436
p38-MAPK	Wanleibio	WL00764
NLRP3	Wanleibio	WL02635
N-GSDMD	Wanleibio	WL05411
GSDMD	Wanleibio	WL05686
Caspase1	Wanleibio	WL02996
IL-1β	Wanleibio	WL02557
ASC	Wanleibio	WL02467
NF-κB	ABclonal	AP1528
IL-18	ABclonal	A24057
GAPDH	Proteintech	60004-4-Ig

Table 2: Primer sequences used in this study

Gene name	Forward sequence (5'→3')	Reverse sequence (5'→3')
Gallus-INS	CGTGGCTTCTTCTACTCCCC	CCCTCGCTTGACTTTCTCG
Gallus-p38-MAPK	TGCCAAAAGGACCTACCGA	ATCCAGGATCTTCAGCTCACAG
Gallus-NF-κB	CGAACAGCAGATGGACCGTA	AGAAGCACCAGGAAGTCCAC
Gallus-NLRP3	GACCTACTGGAACACGAGGC	TTACCAGCAGGAAGAGCAGG
Gallus-Caspase1	CTGCGGGACCAAGAGTAATG	CAGTGTCAGGCGTGGAAGAAT
Gallus-IL-1β	CTGCCTGCAGAAGAAGCCT	TGTCAGCAAAGTCCCTGCTC
Gallus-IL-18	GAGCCCGTTCGGGGGA	AAAAGGCATCGCATTCCAGC
Gallus-GAPDH	AGAACATCATCCCAGCGTCC	CGGCAGGTCAGGTCAACAA

Network pharmacology: We predicted KAE's potential targets against copper-induced liver injury by combining the PharmMapper platform (http://www.lilabecust.cn/1000eg1000or-pper/) (Xiaofeng *et al*., 2010) and the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) (Daina *et al*., 2019). converted UniProt IDs to standardized gene names via UniProt database (https://www.uniprot.org/) (Consortium, 2020). and defined Venn-identified overlaps between merged KAE predicted targets and the chicken genome as its putative therapeutic targets in chickens (Hopkins and Andrew, 2008).

PPI network construction: As previously described by Han *et al*. (2025), the STRING database (http://stringdb.org) (Damian *et al*., 2011) was used to construct a protein-protein interaction (PPI) network with the species restricted to *Gallus gallus* and an interaction score threshold >0.9. Visualization was performed using Cytoscape (version 3.9.0) (Shannon *et al*., 2003), and hub genes were identified via the CytoHubba and MCODE plugins.

Functional enrichment analysis: GO (http://geneontology.org/) and KEGG (http://www.kegg.jp/) enrichment analyses were performed enrichment analyses were performed in R with *Gallus gallus* annotation datasets, with a dual significance threshold of $P<0.05$ and $q<0.05$. Results were visualized via R packages (circlize, ggsankey, ggplot2) from the bioinformatics platform (http://www.bioinformatics.com.cn/) (Tyanova *et al*., 2016).

Molecular docking: The 3D structure of KAE was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov) (Sunghwan *et al*., 2020), and core protein structures from AlphaFold (https://www.alphafold.eb-i.ac.uk) (Jumper *et al*., 2021). Molecular docking was performed with CB-DOCK2 (https://cadd.labsh-are.cn/cb-dock2/php/index.php) followed by interaction visualization using PyMOL (version 3.1.3).

Statistical analysis: Experiments were replicated at least three times. Data are presented as mean ± SD and analyzed with GraphPad Prism 9.0 and SPSS 22.0 (GraphPad Software, USA). Group allocation was blinded, and all data were normalized to the control group. Multi-group comparisons used one-way ANOVA with LSD post-hoc test; $P<0.05$ was considered significant (Sert *et al*., 2020).

RESULTS

KAE ameliorates copper exposure-induced pathological liver injury: Cu exposure resulted in ALT, AST, and LDH levels, indicating Cu-induced hepatic injury. However, KAE effectively attenuated the elevation of these enzymes (Fig. 1A-C) ($P<0.05$ or $P<0.01$). Furthermore, abnormal hepatic architecture, interstitial hemorrhage, and inflammatory infiltration were observed following Cu exposure. Compared to the Cu-exposed group, supplementation with KAE significantly alleviated these pathological changes (Fig. 1D). No notable abnormalities were detected in the hepatocytes of the CON and KAE-alone groups (Fig. 1D). Masson staining was further used to evaluate the extent of liver fibrosis. Results showed that Cu exposure led to a marked increase in collagen fiber deposition in liver tissues, whereas KAE supplementation attenuated such pathological damage (Fig. 1E and F). These findings suggest that KAE mitigates pathological liver injury induced by copper exposure.

Network pharmacological analysis of potential mechanisms underlying Cu-induced liver injury: To elucidate the mechanisms by which KAE alleviates Cu poisoning-induced inflammation, a network pharmacology analysis was conducted. By integrating data from the CTD,

GeneCards, and SwissTargetPrediction and identifying overlapping targets, 3,143 Cu-associated target genes, 6,905 liver injury-related targets, and 880 KAE-linked targets were screened. A Venn diagram revealed 183 potential hub genes (Fig. 2A). To identify core targets in Cu-induced liver injury, a PPI network was constructed for these 183 genes. Cytoscape was employed to extract central targets from the PPI network (Fig. 2B). KEGG and GO enrichment analyses demonstrated considerable enrichment of core targets in pathways related to inflammation and immune responses, including the MAPK signaling pathway, insulin signaling pathway (Fig. 2C-E). Further refinement using CytoHubba and MCODE identified INS and SRC as pivotal targets for KAE-mediated mitigation of Cu-induced liver injury (Fig. 2F-G). Molecular docking validated strong binding affinities between KAE and INS (Fig. 2H, binding energy: -6.7 kcal/mol) and SRC (Fig. 2I, binding energy: -8.0 kcal/mol). Given the established association between Cu exposure and blood glucose regulation, we focused on INS to further elucidate the mechanisms underlying KAE-mediated amelioration of hepatic injury (Yin *et al*., 2019).

KAE ameliorates Cu exposure-induced hepatic injury via INS and MAPK pathway regulation: INS was identified as a pivotal gene target, and the MAPK signaling pathway emerged as a critical mechanistic axis. To validate Cu exposure's impact on INS, blood glucose levels in chickens were monitored. Notably, Cu exposure triggered a significant elevation in blood glucose, which was markedly mitigated by KAE treatment (Fig. 3A). Concurrently, assessments of insulin resistance markers (IRS, phospho-IRS1 [Tyr612], phospho-IRS1 [Ser307]) confirmed that Cu exposure upregulated their expression levels compared to the control group, while KAE treatment effectively suppressed this upregulation. Concurrently, upregulated expression of INS and p38 MAPK under Cu exposure corroborated our hypothesis. Crucially, KAE effectively suppressed this protein upregulation, demonstrating its regulatory role in alleviating Cu-induced hepatotoxicity via INS and MAPK pathway modulation. Furthermore, hepatic mRNA expression levels of INS and p38 mirrored their protein-level changes, reinforcing the consistency of these findings (Fig. 3B-H). *indicates protein and mRNA expression levels in different treatment groups compared with the control group (*P<0.05, **P<0.01, ***P<0.001). This suggests that copper exposure induces an increase in blood glucose, INS, and MAPK signaling activity, potentially correlating with the observed inflammatory infiltration. To further establish a causal link between INS and p38 MAPK, we manipulated INS expression in chicken primary hepatocytes using an INS overexpression plasmid and si-INS. Overexpression of INS significantly increased p38 MAPK expression, whereas si-INS-mediated knockdown of INS markedly reduced p38 MAPK expression. Consistent with protein changes, mRNA levels of INS and p38 MAPK were similarly regulated upon INS manipulation (Fig. 3I-T). * indicates protein and mRNA expression levels in different treatment groups compared with the 110 μM Cu group (*P<0.05, **P<0.01, ***P<0.001). confirming a direct positive correlation between INS and p38 MAPK.

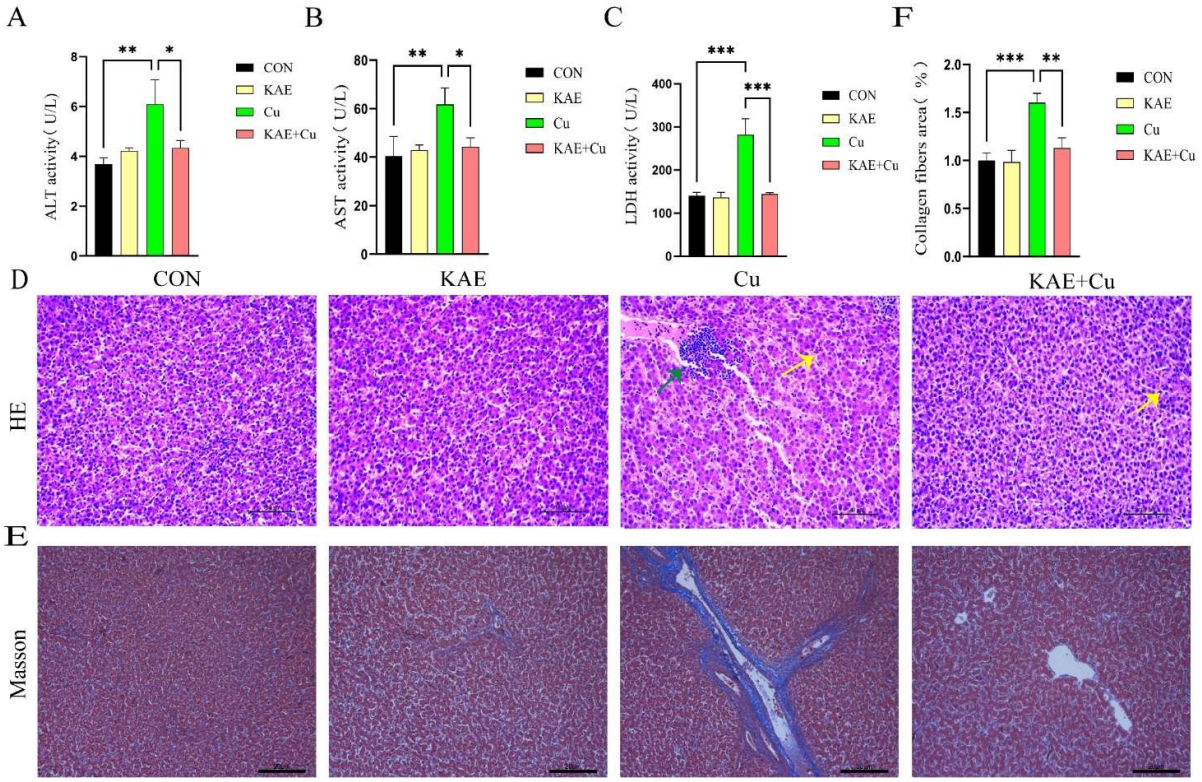

Fig. 1: Kaempferol (KAE) ameliorates copper (Cu) exposure-induced pathological liver injury. (A) LDH activity. (B) AST activity. (C) ALT activity. (D) Hematoxylin and eosin (H&E) staining (scale bar: 100μM). Green arrows represent inflammatory cell infiltration; Yellow arrows represent hemorrhage in hepatic interstitial spaces. (E) Masson's trichrome staining (scale bar: 100μM). (F) Quantitative analysis of (E). CON, control. All data are presented as mean ± SD, n=3, *P<0.05, **P<0.01, ***P<0.001.

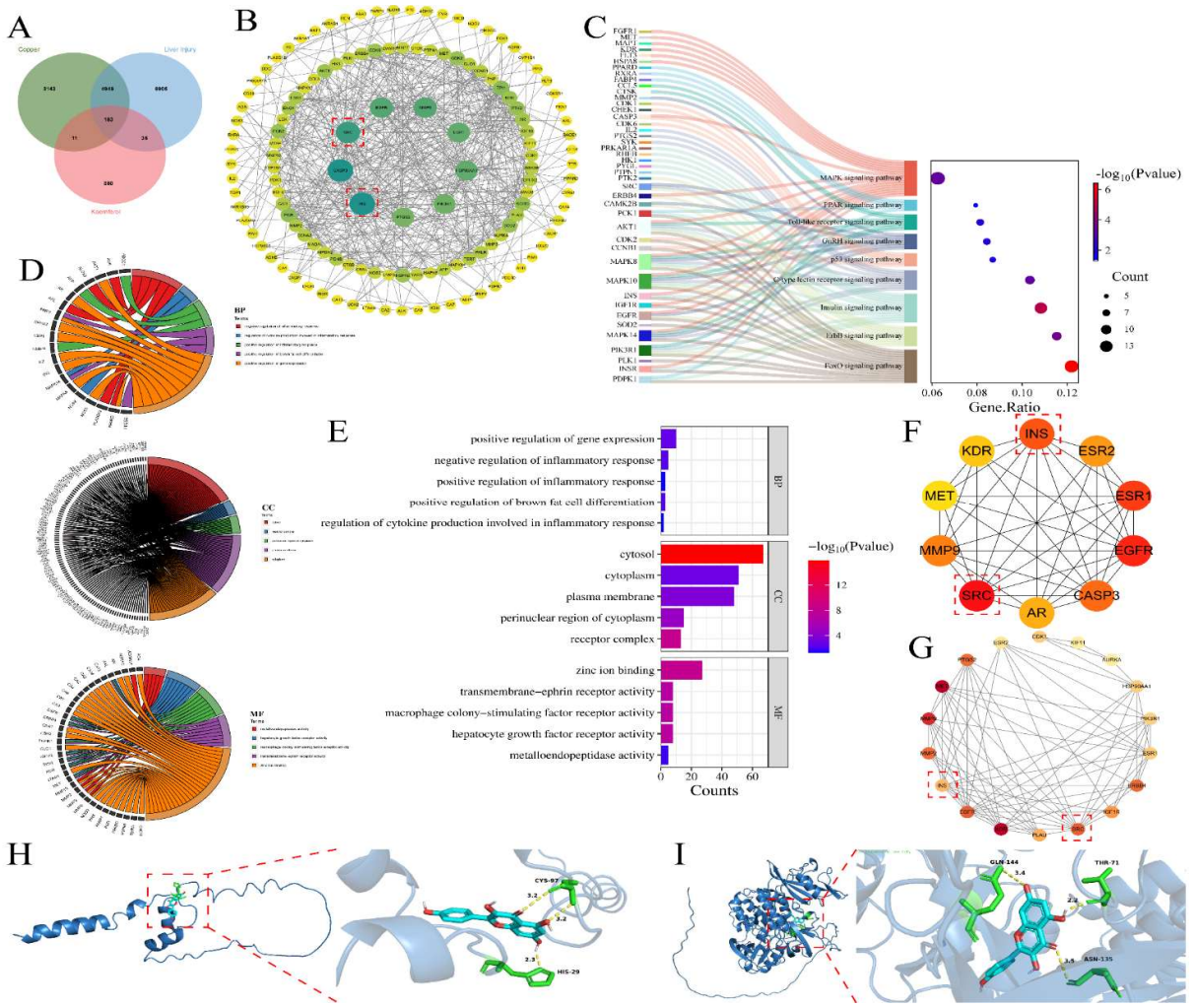

Fig. 2: INS serves as the hub gene underlying kaempferol (KAE)-mediated alleviation of copper exposure-induced hepatic injury. (A) Venn diagram illustrating overlapping therapeutic targets of KAE against copper exposure-induced hepatic injury. (B) Protein-protein interaction (PPI) network of candidate targets constructed using Cytoscape. (C) KEGG pathway enrichment analysis. (D and E) GO enrichment analysis. (F) Identification of hub genes via CytoHubba. (G) Cluster analysis of pivotal targets using MCODE. (H) Molecular docking validation of KAE-INS binding conformation. (I) Molecular docking validation of KAE-SRC binding conformation.

KAE alleviates copper exposure-induced liver injury by inhibiting the NF-κB-mediated NLRP3 inflammasome activation pathway: The p38-MAPK/NF-κB/NLRP3 pathway plays a pivotal role in coordinating inflammatory responses, and KAE exerts anti-inflammatory effects by suppressing this pathway. Additionally, levels of inflammatory mediators IL-18 and IL-1β, key indicators of inflammation, were demonstrated to be significantly downregulated by KAE treatment (Fig. 4A-E). Furthermore, the elevated hepatic mRNA expression of IL-18, IL-1β, and components of the p38-MAPK/NF-κB/NLRP3 pathway corroborated these findings (Fig. 4F-I). These findings demonstrate that copper exposure promotes hepatic inflammation, consistent with histopathological observations, and that this process is mechanistically linked to the MAPK/NF-κB/NLRP3 pathway. Notably, KAE effectively reversed the activation of this pathway, underscoring its therapeutic potential in mitigating copper-induced liver damage.

KAE alleviates Cu exposure-induced hepatic injury by suppressing pyroptosis: Our findings demonstrated that the p38 MAPK/NF-κB pathway was activated and synergistically promoted NLRP3 expression, wherein the NLRP3 inflammasome facilitated caspase-1-dependent pyroptosis. Immunohistochemical (IHC) analysis of NLRP3 (a key initiator of pyroptosis) and N-GSDMD (the terminal effector) revealed upregulated positive staining areas in Cu-exposed groups, while KAE supplementation significantly attenuated these alterations (Fig. 5A and B). Furthermore, western blot analysis showed elevated protein levels of pyroptosis-related markers, including NLRP3, ASC, Caspase-1, and the N-GSDMD/GSDMD ratio in Cu-treated groups, which were markedly reduced by KAE intervention (Fig. 5C-G). Immunofluorescence assay (IFA) further validated the Cu-induced upregulation of N-GSDMD and Caspase-1 expression, with notably suppressed activation observed in KAE-treated groups (Fig. 5H-K). These results collectively indicate that hypercupremia-induced hyperglycemia triggers insulin signaling dysregulation and MAPK pathway activation, subsequently initiating pyroptosis-mediated inflammatory responses that ultimately culminate in hepatic injury.

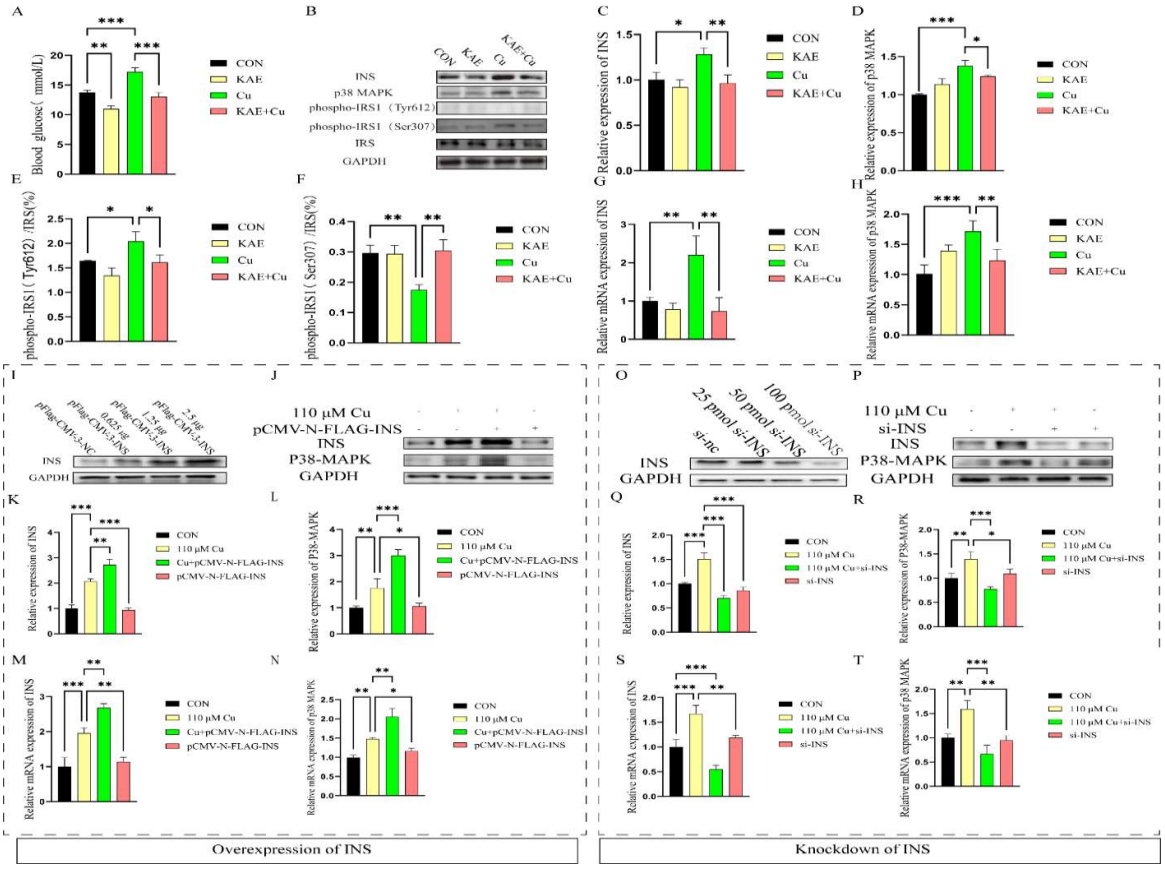

Fig. 3: Kaempferol (KAE) alleviates inflammatory responses by suppressing Cu-toxicity-induced upregulation of MAPK and insulin signaling. (A) Serum blood glucose levels. (B-F) Phosphorylation status of IRS-1 at Tyr612 and Ser307, protein expression levels of p38 MAPK, and INS. (G-H) Relative mRNA expression levels of p38 MAPK and INS. (I) The expression levels of INS. Based on the experimental results, a concentration of 2.5 μg/well was chosen for subsequent overexpression transfections. (J-L) The relative protein expression levels of INS and p38 MAPK in chicken hepatocytes following overexpression of INS. (M-N) mRNA expression of INS and p38 MAPK in chicken hepatocytes after INS overexpression. (O) The expression levels of INS. Based on the experimental results, a concentration of 100 pmol/well was chosen for subsequent knockdown transfections. (P-R) The relative protein expression levels of INS and p38 MAPK in chicken hepatocytes after INS knockdown. (S-T) mRNA expression of INS and p38 MAPK in chicken hepatocytes after INS knockdown. CON, control. Data are presented as mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.

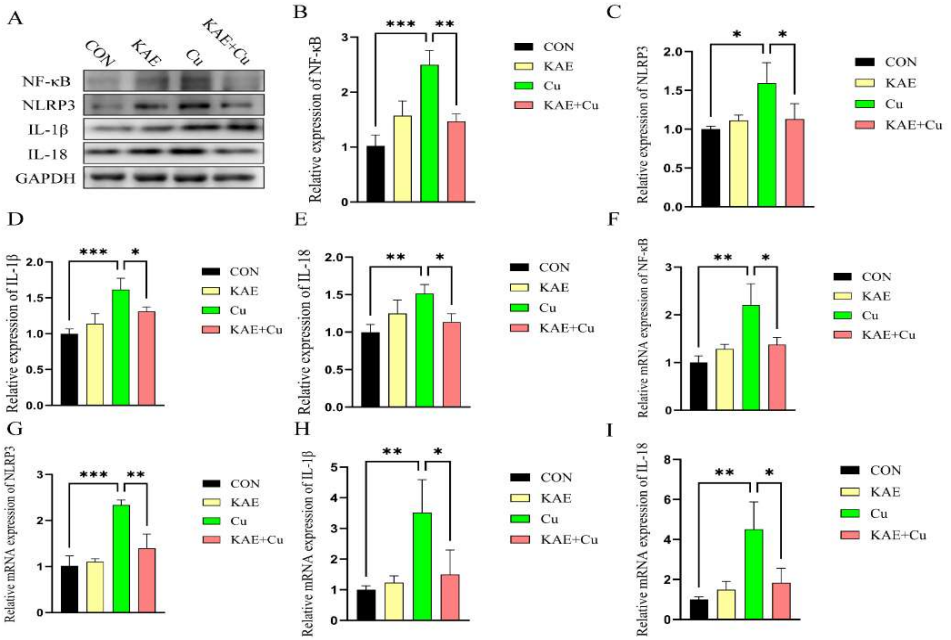

Fig. 4: KAE alleviates copper exposure-induced liver injury by inhibiting the NF-κB-mediated NLRP3 inflammasome activation pathway. (A–E) Expression levels of NF-κB, NLRP3, IL-1β, and IL-18 were analyzed by Western blot. (F–I) Relative mRNA expression levels of NF-κB, NLRP3, and the inflammation-related genes IL-1β and IL-18. CON, control. Data are presented as mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.

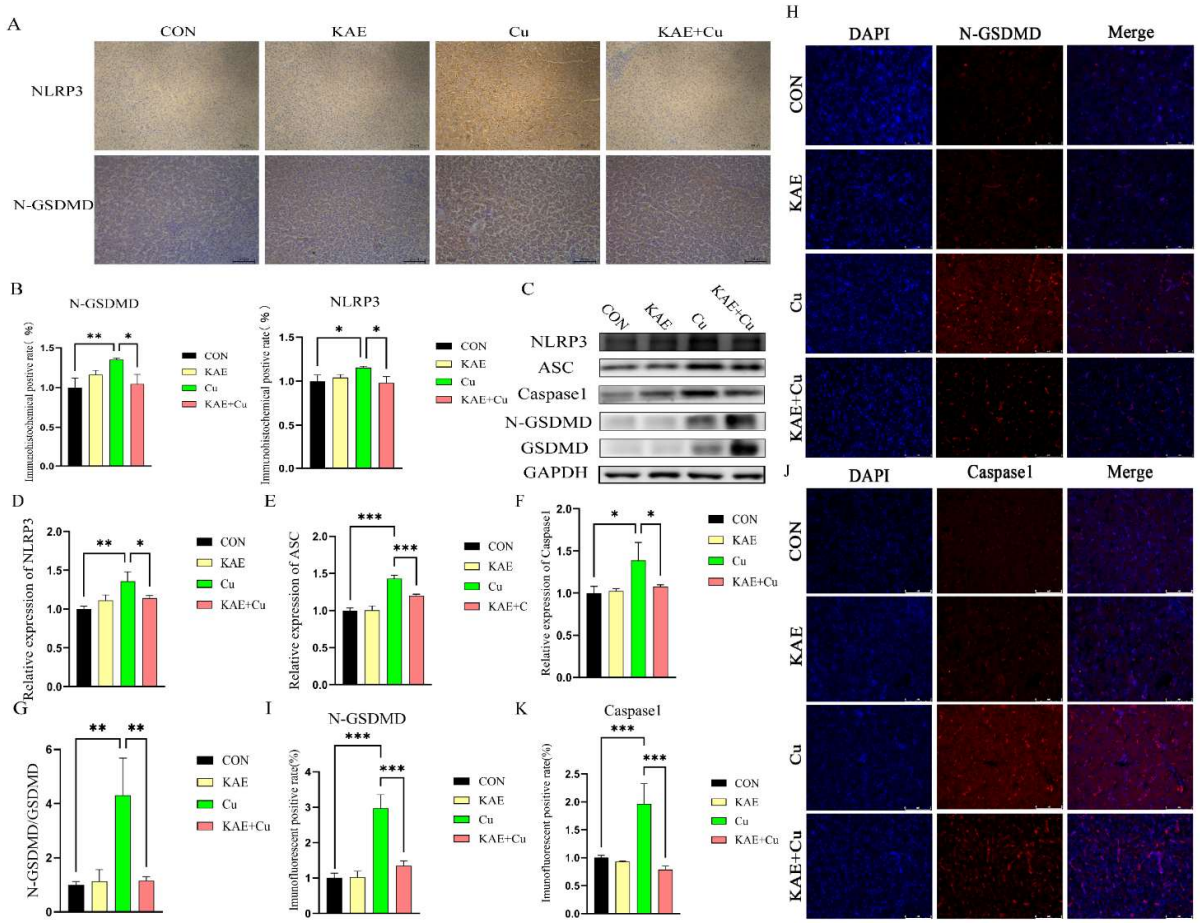

Fig. 5: Kaempferol (KAE) alleviates copper (Cu) exposure-induced hepatic injury by suppressing pyroptosis. (A and B) Immunohistochemical (IHC) images of NLRP3 and N-GSDMD with corresponding quantitative analysis (200× magnification). (C–G) Western blot analysis of pyroptosis-related proteins: NLRP3, ASC, Caspase-1, and the N-GSDMD/GSDMD ratio. (H-K) Immunofluorescence (IF) images of N-GSDMD and Caspase-1 with corresponding quantitative analysis (200× magnification). CON, control. Data are presented as mean ± SD (n = 3). *P<0.05, **P<0.01, ***P<0.001.

DISCUSSION

Copper is a micronutrient that participates in numerous critical physiological processes within the liver, including redox reactions, mitochondrial respiratory chain function, and the regulation of iron metabolism. These roles are vital for maintaining normal metabolic homeostasis and antioxidative balance in hepatic tissue (Chen *et al*., 2020). However, copper metabolism is precisely regulated and inherently delicate. Excessive accumulation of copper in the liver induces significant cytotoxic effects, disrupting cellular architecture and impairing hepatic function (Kahlson and Dixon, 2022). In recent years, pyroptosis has been recognized as an innovative form of programmed cell death, and its crucial role in inflammatory pathological processes has been increasingly elucidated (Wei *et al*., 2022). Pyroptosis is defined by inflammasome activation as its core molecular event, such as the NLRP3 inflammasome and pro-inflammatory signaling pathways, leading to membrane rupture, cellular swelling, and the massive release of pro-inflammatory mediators. This mode of cell death is mechanistically linked to the development and progression of multiple pathological states, including oxidative stress-related diseases and neurodegenerative disorders (Miao *et al*., 2010).

Kaempferol (KAE), a natural flavonoid, possesses anti-inflammatory, antioxidant, and anti-pyroptotic properties. Our results demonstrate that KAE attenuates copper-induced hepatocyte pyroptosis by suppressing INS/p38-MAPK activation, reducing NLRP3 inflammasome assembly, and blocking GSDMD cleavage. Nevertheless, the chosen dose (150 mg/kg), though supported by literature as optimal, has limitations. (Hoang *et al.*, 2015; Muhammad *et al*., 2020; Zhang *et al*., 2023). The absence of a dose-response study leaves the minimum effective dose and safety threshold unknown. Additionally, its applicability to other exposure durations or animal models remains unclear. Future research should include dose-graded designs to establish an optimal regimen.

Insulin typically exerts its hypoglycemic effects by activating insulin receptor substrates (IRS-1/2), which in turn stimulate the downstream PI3K/Akt signaling pathway, thereby promoting cellular glucose uptake (Yi *et al*., 2013). When exposed to copper (Cu), excessive Cu induces substantial in vivo reactive oxygen species (ROS), triggering elevated oxidative stress manifested by increased malondialdehyde (MDA) (Uriu-Adams and Keen, 2005). Pancreatic β-cells exhibit marked vulnerability to oxidative stress-induced damage, resulting in functional impairment and apoptosis (Lenzen and Sigurd, 2008). β-cell impairment results in the loss of core

physiological functions, leading to uncontrolled hyperglycemia. Insulin deficiency and subsequent metabolic disorders further aggravate persistent hyperglycemia. In early exposure, compensatory mechanisms (β-cell hyperplasia and hypersecretion) activate to counter Cu-induced insulin resistance, maintaining normoglycemia or mild hyperglycemia with hyperinsulinemia. With prolonged cumulative exposure, Cu toxicity damages β-cells via oxidative and endoplasmic reticulum stress in the decompensation phase, disrupting insulin synthesis and secretion; marked hyperglycemia is accompanied by dysregulated, reduced insulin secretion and loss of glycemic control. Finally, chronic Cu toxicity triggers extensive β-cell apoptosis and exhausted insulin synthesis in the late exhaustion phase, causing absolute insulin deficiency, persistent uncontrolled hyperglycemia, and eventual progression to diabetes. (Zheng *et al*., 2026). Our study revealed significant upregulation of insulin expression under copper (Cu) exposure. The upregulated p-IRS1 Ser307 and downregulated p-IRS1 Ser612 indicate insulin resistance in Cu-exposed poultry, which may represent a compensatory response to Cu-induced inflammatory and oxidative stress injury (Jian *et al*., 2020). To further investigate whether INS upregulation directly activates p38 MAPK, we manipulated INS expression in chicken primary hepatocytes using an INS overexpression plasmid and si-INS. INS overexpression significantly increased p38 MAPK expression, whereas si-INS-mediated knockdown of INS markedly reduced p38 MAPK expression. Consistent with the protein changes, mRNA levels of INS and p38 MAPK were similarly regulated upon INS manipulation (Fig. 3I-T). These results establish a causal link between INS and p38 MAPK activation. Thus, in the context of copper exposure, elevated INS levels are likely to contribute to p38 MAPK upregulation.

Functioning as a central mediator in stress-response signaling, p38-MAPK is induced under cellular stress and orchestrates key processes including oxidative stress management, inflammatory regulation, and cell death control (Alanazi *et al*., 2025). The p38 MAPK pathway constitutes a central stress-signaling hub in cellular responses to copper exposure. Under conditions of intracellular copper overload, copper catalyzes the formation of substantial reactive oxygen species (ROS) such as hydroxyl radicals, triggering intense oxidative stress (Yang *et al*., 2019)**.** This disruption of redox homeostasis is detected by cellular sensors, leading to activation of p38 MAPK through both direct and indirect mechanisms. In the indirect pathway, ROS triggers the oxidation-induced dimerization and autophosphorylation of upstream kinase ASK1, leading to the subsequent phosphorylation and activation of MKK3/MKK6. These activated kinases then phosphorylate threonine and tyrosine residues on p38 MAPK to achieve full activation. In the direct pathway, ROS may oxidize cysteine residues within p38 MAPK itself or modify regulatory proteins such as MAPK phosphatases, thereby enhancing its enzymatic activity and stability (Cuadrado and Nebreda, 2010). Upon activation, phosphorylated p38 MAPK orchestrates the expression regulation of multiple downstream effectors through specific phosphorylation events, including: 1) pro-inflammatory mediators, cytokines (e.g., IL-18 and IL-1β); 2) antioxidant defense systems (e.g., superoxide dismutase and glutathione synthase); 3) cell cycle regulators (e.g., p53); and 4) apoptotic signals (e.g., Bax and caspase family members) (Herlaar and Brown, 1999). Notably, the inflammatory cytokines IL-18 and IL-1β serve as key executioners of pyroptosis, establishing a critical mechanistic link between p38 MAPK activation and programmed inflammatory cell death processes. Through p38-MAPK-mediated nuclear translocation of NF-κB, the gene expression of the NLRP3 inflammasome and its cytokine precursors, pro-IL-1β and pro-IL-18, is significantly enhanced. Our data indicate a dose-dependent increase in the mRNA levels of Caspase-1, NLRP3, IL-18, and IL-1β in hepatocytes following copper exposure, indicating progressive activation of this signaling pathway. The assembly and activation of the NLRP3 inflammasome initiates the specific proteolytic splitting and activation of Caspase-1. Through cleavage of the pyroptosis executor GSDMD, this process drives pyroptosis and, in parallel, enables the maturation and secretion of IL-1β and IL-18, which intensifies the inflammatory cascade. The N-terminal fragment of GSDMD oligomerizes to form transmembrane pores in the plasma membrane, triggering pyroptotic cell death through osmotic lysis, uncontrolled leakage of cellular contents, and irreversible disruption of membrane integrity. KAE treatment significantly alleviates the upregulation of p38-MAPK and subsequently markedly reduces the occurrence of pyroptosis, thereby mitigating liver injury. It can thus be inferred that KAE ameliorates copper exposure-induced liver injury by suppressing pyroptosis.

In summary, we report that KAE modulates insulin expression levels under copper exposure conditions. Under complex scenarios such as insulin resistance, KAE influences the activation of p38-MAPK, which in turn affects the expression of key pyroptosis-related genes, including NLRP3, Caspase-1, GSDMD, N-GSDMD, IL-18, and IL-1β. Through this multifaceted mechanism, KAE alleviates copper-induced liver injury. The intricate interplay between copper homeostasis, insulin signaling, p38-MAPK activation, and the pyroptotic cascade underscores the complexity of metal-induced hepatotoxicity. This work demonstrates the therapeutic potential of KAE in preserving liver function under copper overload by modulating key interconnected pathways. These observations call for additional research to fully elucidate their mechanistic details and clinical applicability.

Conclusions: In summary, our findings confirm that high-level copper exposure triggers notable liver damage, marked by inflammatory infiltration, collagen deposition, and increased serum liver enzymes. Our integrated analysis, leveraging animal models, network pharmacology, and docking studies, revealed that the INS/p38-MAPK signaling axis plays a central role in the pathogenesis of copper-triggered hepatotoxicity. Copper exposure induced hyperglycemia, upregulated INS, and increased IRS-307/IRS-612 phosphorylation (indicative of insulin resistance), which in turn activated the downstream p38-MAPK/NF-κB pathway, with INS serving as a critical mediator in this process. This activation promoted NLRP3 inflammasome aggregation, Caspase-1 activation, and the

cleavage of GSDMD, ultimately executing pyroptosis. Crucially, KAE effectively mitigated copper-induced liver damage by targeting this signaling cascade. It normalized INS expression and consequently inhibited the p38-MAPK/NF-κB axis, which further blocked NLRP3 inflammasome formation and the release of IL-1β/IL-18, ultimately alleviating pyroptosis and safeguarding liver architecture. These findings not only elucidate a previously unexplored mechanism linking copper overload to pyroptosis via metabolic and MAPK signaling but also highlight KAE as a promising therapeutic candidate for preventing and treating metal-induced inflammatory liver injury.

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Availability of data and materials: The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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