Traditional Chinese Medicinal Herb (*Epimedium brevicornum*) Rescued Aflatoxin B_1-Induced Liver Damage via Suppressing Inflammatory Responses and Oxidative Stress in Mice

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***Abstract*-** Icariin (ICA) is a major bioactive constituent isolated from Traditional Chinese Medicine *Epimedium brevicornum*. A solid-phase extraction polyphenolic extract of *Epimedium brevicornum* leaves was tested for ICA efficacy against AFB_1-induced hepatotoxicity in mice. Recently, the ability of bioactive compounds to regulate oxidative stress and inflammatory response has received much attention. Aflatoxin B_1 (AFB_1) is one of the utmost prevalent agricultural contaminants and has devastating effects on the health of animals and humans. In this regard, forty-eight mice were indiscriminately assigned to the following groups (n = 12): 1) Control (CON), 2) 50 mg/kg BW icariin (ICA), 3) 1 mg/kg BW AFB_1 (AFB_1), 4) 1 mg/kg AFB_1 + 50 mg/kg BW icariin (AFB_1 + ICA). The groups received treatments with AFB_1 or ICA for 15 days. The findings indicated that AFB_1 markedly reduced average daily feed intake, average daily gain and provoked liver damage as evidenced by both pathological and biochemical changes. In contrast, oral supplementation of ICA significantly rescued these changes. Furthermore, ICA dramatically decreased oxidative stress inflicted by AFB_1 by neutralizing MDA and ROS formation and by promoting the antioxidant enzymes (T-SOD, T-AOC, CAT, and GSH-Px) activities. Additionally, ICA demonstrated a notable modulation of pro- and anti-inflammatory cytokines in mice exposed to AFB_1. In summary, ICA could act as a shielding agent against hepatic injury caused by AFB_1 and could contribute to the advancement of new therapies for treating liver conditions in both humans and animals.

***Index Terms*-** Aflatoxin B_1; *Epimedium brevicornum*; Icariin, Oxidative stress; Inflammation; Mice

I. INTRODUCTION

Epimedium brevicornum Maxim, a member of the *Berberidaceae* family, is both a decorative herb and a medicinal herb in Traditional Chinese Medicine (TCM). It is popular in Asia and the Mediterranean. As early as 400 AD, *E. brevicornum* had become in use as a medicinal herb. It has a history of use as a reproductive system tonic, boosting libido while tackling impotence. *E. brevicornum* extracts have been shown to contain a variety of components (Li et al., 2017). The genus *Epimedium* is comprised of perennial woodland herbs that are found in thickets or slopes at elevations ranging from 650 to 3000 meters. In the family *Berberidaceae*, the herbaceous genus *Epimedium* is the most extensive. It was determined that the genus *Epimedium* contains around 62 different species. It is found in the Old World and is disjunctively and extremely unevenly distributed in woodlands or scrubs in the Mediterranean region, western Asia, and Eastern Asia. About 51 species are found in central southeastern China, five in Algeria and Caucasus, six in Japan, Korea, north-eastern China, and Far Eastern Russia. China serves as *Epimedium's* distribution and diversification hub (He et al., 2020). Icariin (ICA) is a bioactive flavonoid compound found in *Epimedium brevicornum* Maxim, also known as goat weed. ICA is a powerful natural antioxidant owing to its capability to assimilate free oxygen radicals (Xia et al., 2022). ICA has protective impacts on various ailments including, cardiovascular disorders (Zeng et al., 2022), neurotoxicity (Khezri and Ghasemnejad-Berenji, 2022), hepatic injury (Algandaby et al., 2017) nephrotoxicity (Xie et al., 2018), as an antioxidant, anti-inflammatory, anti-apoptotic (Jia et al., 2019), and anticancer agent (Tan et al., 2016). Previously, it was found that ICA potentially protected mice from LPS-induced endometritis via decreasing inflammatory response and oxidative stress (Shaukat et al., 2022).

Food security is a global issue and is essential to meeting the urgent human for safe and healthy food (King et al., 2017). One of the most significant challenges to food safety is the contamination caused by mycotoxins, which are toxic secondary metabolites produced by fungi (Adegbeye et al., 2020). Mycotoxins affect 25% of global crop production and pose a significant health risk to humans and animals, causing acute and chronic toxicity (Abidin et al., 2013; Marroquín-Cardona et al., 2014). Aflatoxins are widely recognized among the most hazardous types of mycotoxins, predominantly produced by *Aspergillus parasiticus* and *Aspergillus flavus*, which are commonly present in food products and agricultural environments (Rahman et al., 2024). By virtue of its established carcinogenic effects, The International Agency for Research on Cancer classifies Aflatoxin B1 (AFB_1) as a Group 1 carcinogenic (Ahmadi et al., 2024). AFB_1 has been linked to critical health issues, such as growth inhibition, liver damage, nerve damage, mutagenesis, teratogenesis, and immune system suppression in both humans and animals (Cheng et al., 2001; Dai et al., 2024; Jalili et al., 2024; Saleemi et al., 2023). Novel studies show that AFB_1 toxicity is a major risk factor for oxidative stress in the liver tissue (Ye et al., 2024; Zhang et al., 2024). Previously, another revealed that AFB_1 has the ability to cause oxidative damage in the hepatic tissue (Rajput et al., 2017; Saleemi, et al., 2023). Oxidative stress is a well-known phenomenon defined by variations within the formation of ROS and antioxidant mechanisms. This imbalance is linked to a variety of diseases and results in cellular injury (Khater et al., 2020; Sies et al., 2017). Multiple investigations have shown that AFB_1 could

augment ROS generation, which in turn can cause oxidative stress, apoptosis, and liver damage (Saleemi, et al., 2023; Theumer et al., 2010).

Functional foods and nutraceuticals have gained popularity among scientists and the public due to their low toxicity and therapeutic benefits (Subhi and Al-Okaily, 2023; Zang et al., 2023). However, the preventive effects of ICA against AFB_1-induced hepatic injury have not been explored yet. The present research hypothesized that ICA could exert a shielding impact against inflammation and oxidative damage inflicted by AFB_1 in mice. Presumably, this is the first research to explore the ameliorative effect of ICA against liver damage in mice induced by AFB_1.

II. Material and Methods

Plant Material and Extraction of Icariin

Epimedium brevicornum leaves were acquired from Aktin Biotechnology Chemicals Co., Ltd. (Guangan, China) (Figure 1). The extraction was conducted following the previously published method (De et al., 2005). Briefly, After the *Epimedium brevicornum* leaves had been dried and pulverized, 10 grams were extracted with isooctane in a nitrogen-rich atmosphere and the residue was extracted with MeOH/water in a ratio of three parts by volume. At the same time, the mixture was recycled in nitrogen. An initial step in the preparation of stock solutions consisted of dissolving 10 mg of the polyphenolic fraction in 1 ml of MeOH, which was then gradually modified. A 0.2-Am Millipore filter was used to filter a small volume (about 1 ml) for high-performance liquid chromatography (HPLC).

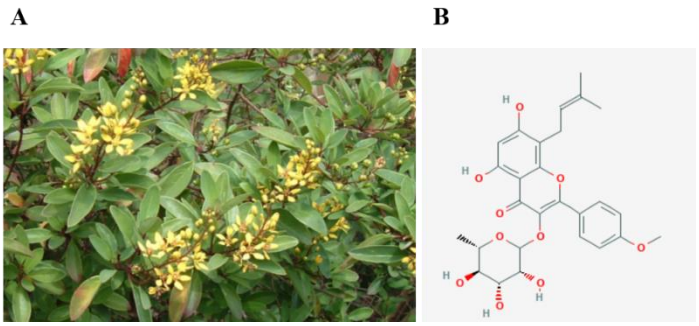

Figure 1. (A) *Epimedium brevicornum:* a Chinese herb from which icariin is extracted. (B) Chemical structure of icariin.

HPLC analysis

The HPLC analysis was determined as the previously described method (De al., 2005). The purity of icariin was analyzed using an Agilent 1260 series HPLC (Waldbronn, Germany) equipped with the Hypersil C18 column (250 × 4.6 mm, 5 µm, Dimka Technologies, USA). The experiment employed a mobile phase with a flow rate of 0.9 mL/min that was established with MeOH and acetonitrile at a ratio of 50:50. A 280 nm-wavelength fluorescence detector was used for detection. There was a retention time of 10.3 minutes for icariin, and its purity was 98%.

Animals

For this investigation, male C57BL/6 mice that were four weeks old were provided by Wuhan University, China. Mice were individually caged and provided an appropriate climate for one week for adaptation purposes. Each animal was provided with normal feed pellets and fresh water *ad libitum*. All mice were kept in a controlled laboratory condition with a constant 12-hour light-dark cycle, a relative humidity ranging from 45% to 60%, and a temperature of 22 ± 2°C. Throughout the whole trial, the welfare of the experimental animals was carefully observed, and required actions were taken to ensure that they experienced the greatest possible degree of comfort.

Experimental Design and Treatments

A total of forty-eight four weeks-old experimental mice were indiscriminately assigned 4 groups, each group comprising 3 replicates of 12 mice in each replicate. Treatments were (1) physiological saline (CON); (2) 1 mg/kg body weight (WB) aflatoxin B_1 (AFB_1); (3) 50 mg/kg BW icariin (ICA); (4) 1 mg/kg BW aflatoxin B_1 + 50 mg/kg BW icariin (AFB_1+ICA). Mice were orally administered icariin (ICA) and aflatoxin B_1 (AFB_1) dissolved in phosphate buffer saline. The concentrations of AFB_1 and ICA were selected predicated on the basis of prior experimental findings (Rajput et al., 2021; Shaukat, et al., 2022). The experiment lasted 15 days, during which all groups received oral administration of the treatments every day at 9:00 a.m. Feed intake, body weight, and water consumption were continuously observed during the experiment.

Sample collection

The 15-day experiment concluded with an overnight fasting period followed by euthanasia of all mice by CO_2 inhalation. Samples of blood were obtained from the retro-orbital plexus and then placed in centrifuge tubes to separate the serum. The serum was isolated by centrifugation and kept at a temperature of -20 °C. The liver specimens were rapidly detached and washed with ice-cold phosphate-buffered saline. After that, samples were dipped in liquid nitrogen and kept at -80 °C. All measures were performed under sterile conditions.

Serum biochemical analysis

The content of globulin, albumin, and the GGT, ALP, ALT, and AST activities in serum was validated using a biochemistry analyzer according to the manufacturer's protocol.

Analysis of antioxidants enzymes and indicators of oxidative stress markers

The preparation of liver tissue homogenates to determine the oxidative stress and antioxidant markers was performed following the instructions provided by the respective kits and previously described method (Rajput et al., 2021). BCA kit was utilized in order to determine the amount of protein contained within the sample. The T-AOC, ROS, T-SOD, MDA, CAT, and GSH-Px levels were validated by using ELISA kits. All the procedures are in accordance with the kit protocols.

Detection of cytokines

The contents of pro- and anti-inflammatory mediators (IL-10, TNF-α, IL-2, and IL-6) were quantified in mouse serum samples using specialized kits acquired from R&D Systems (Minneapolis, MN, USA), in accordance with the recommended protocol supplied by the manufacturer.

RNA extraction and qRT-PCR analysis

The methods of total RNA extraction and quantitative real-time PCR were followed by the previously described method (Rajput et al., 2021). In brief, following the RNA extraction reagent Trizol® (Invitrogen, Carlsbad, CA, USA) instructions, tissue was lysed and RNA was extracted. This section prepared all solutions in a safety hood free of contaminants with DEPC-treated pipette tips and tubes. RNA concentration and purity were assessed using a Nanodrop (Waltham, MA, USA) to ensure quality. Removal of genomic DNA (gDNA) contamination from isolated RNA and reverse transcription was performed for complementary-DNA (cDNA) synthesis using 1µg of total RNA as the template using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara RR047A) with oligodT primer in accordance with manufacturer's method. Quantitative real-time PCR (qRT-PCR) was performed by using PowerUp™ SYBR™ Green Master Mix (Waltham, MA, USA) on a real-time PCR system (Bio-Rad CFX384, CA, USA). Table 1 displays the primers that were tested in this research. β-actin was used as an internal gene and the results were calculated using the 2-$^{\triangle\triangle}$Ct method. The final results were presented as relative mRNA abundance.

Animal Ethics

The Laboratory Animals Care and Use Committee of Huazhong Agricultural University, China (HZAU) approved all the experimental procedures. All the measures were performed under HZAU's approved guidelines.

Statistical analysis

One-way analysis of variance with the Least Significant Difference ($p < 0.05$) was conducted to analyze all the data. Statistical significance among groups was estimated using SPSS software (SPSS 17.0, SPSS, Inc., USA). Results are reported as the mean ± SEM.

Table 1. Sequence of the primers used for quantitative real-time PCR assay.

Target gene	Primer	Primer sequence (5′→3′)	Accession No.
Nrf2	Forward Reverse	ATGACCATGAGTCGCTTGCC AATCAGTCATGGCTGCCTCC	NM_010902.4
HO-1	Forward Reverse	TTGCCAGTGCCACCAAGTTC TCAGCAGCTCCTGCAACTCC	NM_001014912.1
GCLC	Forward Reverse	AAGATGCGGAGGCATCAAA TCAAAGCCATAACAATTGGGCAG	NM_010295.2
SOD1	Forward Reverse	GGAACCATCCACTTCGAGCA CTGCACTGGTACAGCCTTGT	NM_011434.1
β-actin	Forward Reverse	GTTGGAGCAAACATCCCCCA ACGCGACCATCCTCCTCTTA	NM_007397.5

III. RESULTS

ICA ameliorates the growth retardation caused by AFB_1 in mice

Our results showed that the AFB_1-treated mice decreased in both ADFI and ADG compared to the CON, AFB_1+ICA, and ICA groups, respectively ($P < 0.05$). However, the group treated with AFB_1 + ICA significantly improved ADG and ADFI in comparison to AFB_1-challenged mice (Figure 2).

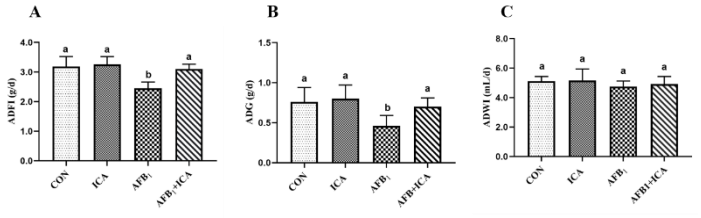

Figure 2. ICA ameliorates the growth retardation caused by AFB1 in mice. The results are shown as the means ±SD (n = 12). [a,b] Different superscript letters indicate that variables within columns differed significantly ($P < 0.05$).

ICA rescued AFB_1-induced liver damage in mice

Our findings showed that the group treated with AFB_1 alone had significantly higher ALP, AST, ALT, and GGT levels, as well as lower globulin and albumin levels when compared to the CON, AFB_1+ICA, and ICA groups (Figure 3). Conversely, treatment with ICA significantly reversed the alterations in these markers caused by AFB_1. Moreover, ICA treatment significantly decreased the liver function enzymes and increased the albumin and globulin contents altered by AFB_1.

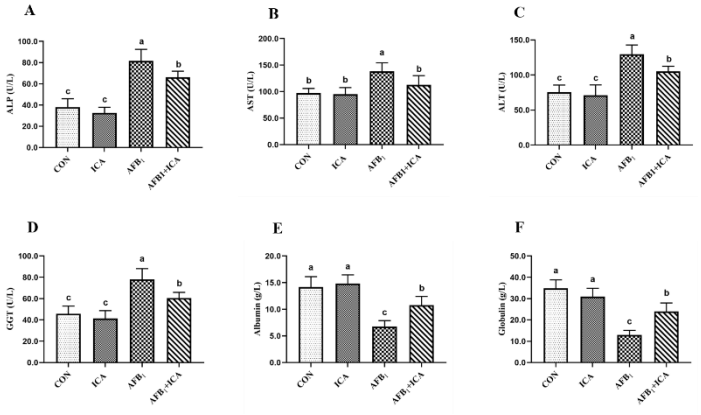

Figure 3. ICA rescued AFB_1-induced liver damage in mice. The results are shown as the means ±SD (n = 6). [a,b,c] Different superscript letters indicate that variables within columns differed significantly ($P < 0.05$).

ICA treatment alleviates AFB_1-induced inflammatory response

The contents of pro-inflammatory and anti-inflammatory mediators were assessed using ELISA and assay (Figure 4). The IL-1β, TNF-α, and IL-6 content caused by AFB_1 was suppressed after ICA administration ($p < 0.05$). In contrast, ICA exhibited a noteworthy ($p < 0.05$) enhancement in IL-10 content in the comparison of the AFB1-challenged group.

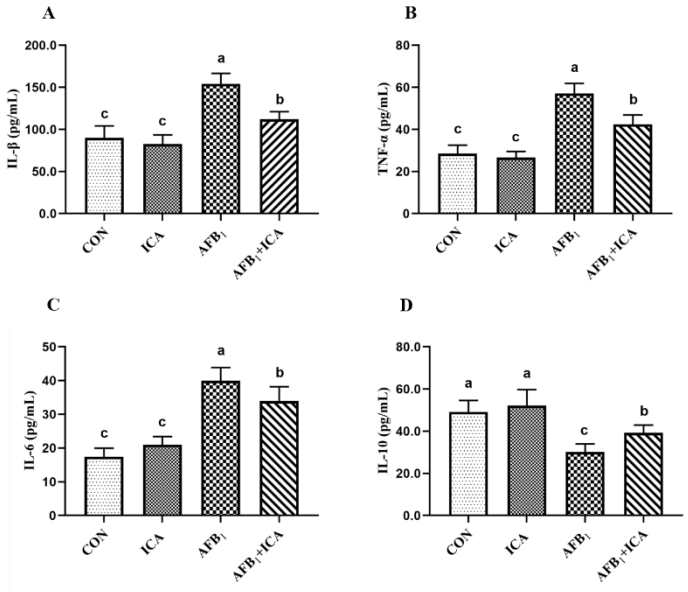

Figure 4. ICA treatment alleviates AFB_1-induced inflammatory response. The results are shown as the means ±SD (n = 6). [a,b,c] Different superscript letters indicate that variables within columns differed significantly (P < 0.05).

ICA treatment mitigated AFB_1-induced oxidative damage

Antioxidant enzymes (CAT, T-AOC, T-SOD, and GSH-Px) and oxidative stress markers (ROS and MDA) were assessed to evaluate the hepatic redox status of the mice. We found that the level of ROS and MDA in the mice treated with AFB_1 was markedly increased than in the CON group (Figure 5A and B). Nonetheless, the administration of ICA considerably decreased the contents of ROS and MDA in the comparison of the AFB_1-challenged mice ($p < 0.05$). Additionally, activities of CAT, T-AOC, T-SOD, and GSH-Px were ($p < 0.05$) reduced in the AFB_1-challenged group than in the CON group. However, as shown in Figure 5C-F, ICA therapy dramatically boosted CAT, T-AOC, T-SOD, and GSH-Px activities in comparison with AFB_1-challenged mice ($p < 0.05$).

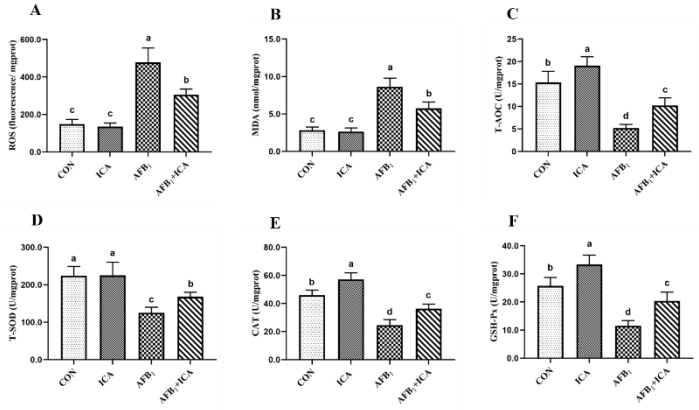

Figure 5. ICA treatment mitigated AFB_1-induced oxidative damage. The results are shown as the means ± SD (n = 6). [a,b,c,d] Different superscript letters indicate that variables within columns differed significantly (P < 0.05).

ICA restrains AFB_1-induced oxidative stress by the activation of Nrf2 signaling

We further explore the antioxidant capacity of ICA by using qRT-PCR detection. The relative mRNA abundance of Nrf2, GCLC, HO-1, and SOD1 in the liver tissue of mice was evaluated and presented in Figure 6. After the challenge with AFB1, the gene expression of Nrf2, GCLC, HO-1, and SOD1 were markedly down-regulated as compared with CON, ICA, and AFB_1+ICA groups. In addition, ICA significantly up-regulated the abundance of Nrf2, GCLC, HO-1, and SOD1 in the comparison of the AFB1-challenge group.

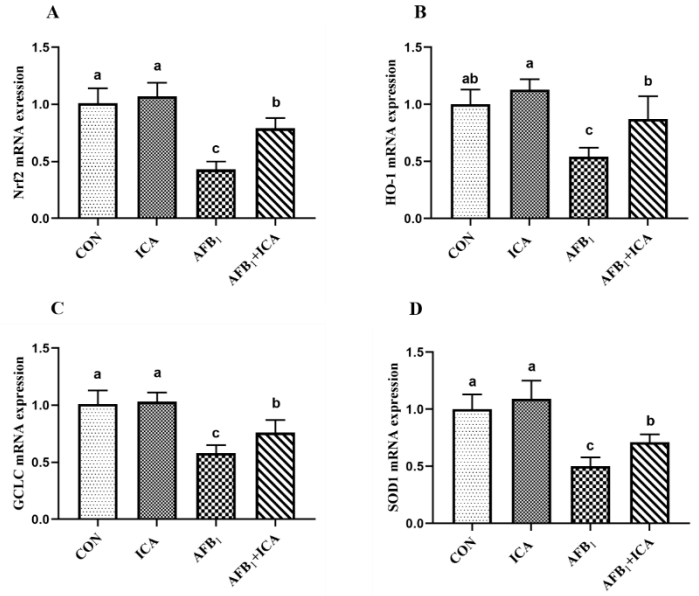

Figure 6. ICA restrains AFB_1-induced oxidative stress by the activation of Nrf2 signaling pathway. The results are shown as the means ±SD (n = 6). [a,b,c] Different superscript letters indicate that variables within columns differed significantly (P < 0.05).

IV. DISCUSSION

AFB_1, a frequent mycotoxin in food and feed, is dangerous to humans and animals, due to its potential to cause hepatotoxicity, neurotoxicity, mutagenicity, immunotoxicity, and carcinogenicity (Hamzah et al., 2019; Zhao et al., 2019). The liver is the primary target organ as it is enormously vulnerable to the toxic impact of AFB_1 exposure. Previous research has confirmed that AFB_1 exposure can cause persistent hepatotoxicity leading to liver cancer. Hence, it is imperious to discover an efficient bioactive agent to shield the liver and ameliorate AFB_1 toxicity. Plant-derived naturally occurring bioactive components received increasing interest in the last years due to low poisonousness (Hussain et al., 2023; Majeed et al., 2021; Muhammad et al., 2013). Icariin (ICA) is a naturally occurring plant-derived chemical compound. ICA has many chemoprotective, biological, therapeutic, and pharmacological properties against oxidative stress (Li et al., 2015; Zeng, et al., 2022). Furthermore, ICA has beneficial effects, as it has Cardioprotective, neuroprotective, and nephroprotective impacts (He et al., 2020; Khezri and Ghasemnejad-Berenji, 2022; Tan, et al., 2016; Xie, et al., 2018). However, the preventive effects of

ICA against AFB_1-induced hepatic injury have not been explored until now. Therefore, the present investigation aims to examine the shielding properties of ICA on AFB_1-inflicted hepatic damage.

Food refusal and growth retardation are among the most significant symptoms of AFB_1 poisoning in humans and livestock (Liu et al., 2019). Our study found that the group exposed to AFB_1 induced food refusal and growth retardation in mice, as recorded with the lowest ADFI and ADWG. These results agree with other studies (Liu, et al., 2019), which showed that oral supplementation of AFB_1 markedly reduced ADFI and ADWG. The growth retardation of mice might be attributed to the deleterious consequences of AFB_1, such as anorexia, and inhibiting lipid, protein, and carbohydrate metabolism (Sun et al., 2018). Interestingly, ICA treatment significantly attenuated AFB_1-induced growth retardation in mice. Our results align with a previously published study (Zheng et al., 2019), and indicate that ICA has the potential to alleviate the adverse impacts of AFB_1 on the growth performance of mice.

The liver is the major target of AFB_1 toxicity (Subhani et al., 2018). Moreover, liver function enzymes (ALT, AST, ALP, GGT) are crucial indicators for evaluating hepatic health status (Agrawal et al., 2016). Previously, it was reported that AFB_1 can alter the biochemical indicators (Rajput, et al., 2017); these were similar to the current observations, which revealed that AFB_1 induced liver injury evidenced by clinical symptoms such as decreased albumin and globulin content while increased ALP, ALT, GGT, and AST. The changes in liver enzymes might be due to the distraction of the hepatic cells, leading to necrosis or altering the cell membrane permeability. Multiple studies have shown that aflatoxins cause various adverse effects in humans and animals, such as pathology and changes in organ weights (Rajput, et al., 2017). Interestingly, oral supplementation of ICA alleviated the secretion of biochemical indicators that were induced by AFB_1. Hence, our outcomes established that ICA protects against adverse effects on the hepatic damage caused by AFB_1, as shown by pathological and physiological validation.

AFB_1 is known to induce inflammatory responses in various tissues and organs (Dai et al., 2024). Pro-inflammatory cytokines have a role in the development of liver inflammation and cancer (Hou et al., 2022; Qin et al., 2016). Our study discovered that AFB_1 therapy dramatically raised TNF-α, IL-6, and IL-1β contents. These cytokines are very crucial to initiate and enhance inflammatory processes (Dai et al., 2024). These findings were consistent with those previously observed in broiler chickens, stating that hepatotoxicity is linked with inflammation caused by AFB_1 (Hatipoglu and Keskin, 2022). It was stated that ICA could considerably modulate the inflammatory mediators (Bi et al., 2022). These observations were consistent with the present results, revealing that ICA mitigated IL-1β, TNF-α, and IL-6 induced by AFB_1 in mice. The anti-inflammatory cytokines (IL-10) help alleviate toxic effects and reduce inflammation and tissue regeneration caused by noxious agents (Opal and DePalo, 2000). Furthermore, our results demonstrated that mice treated with ICA significantly up-regulated the anti-inflammatory markers (IL-10) down-regulated by AFB_1. The present results showed that ICA has the potential to alleviate inflammatory reactions by regulating the release of pro- and anti-inflammatory mediators.

Oxidative stress originates once the reactive oxygen species (ROS) formation surpasses the antioxidative defense system's capacity (Bello at al., 2023; Sies et al., 2017). Multiple studies explored that oxidative damage is crucial in the pathological process of liver damage promoted by AFB_1 (Rajput et al., 2019; Zhang et al., 2016). Our analysis revealed that exposure to AFB_1 increased the formation of ROS and suppressed the antioxidant enzymes in the liver of experimental animals. Previous report indicated that an uncontrolled formation of ROS is a major factor in the hepatotoxicity caused by AFB_1 (Rajput et al., 2019), consistent with the present study. However, icariin administration dramatically reduced oxidative damage by decreasing MDA and ROS formation while increasing CAT, T-SOD, GSH-Px, and T-AOC activities exposed to AFB_1. Based on our findings, the antioxidative properties of icariin are among the underlying mechanisms that help to alleviate AFB_1-induced oxidative stress. This aligns with prior research indicating that icariin has the potential to mitigate oxidative stress induced by various toxic agents (Ma et al., 2014; Xia, et al., 2022). Taken together, our study suggests that icariin could reduce AFB_1-induced oxidative stress by eliminating free oxygen radicals and strengthening the antioxidant response mechanism.

Nrf2 signaling pathway plays a critical role in strengthening cell protection against oxidative stress. Multiple research projects reported that it is possible that oxidative damage caused by AFB1 is linked to malfunction in the Nrf2 signaling pathway (Rajput et al., 2021; Shaukat et al., 2022). Our experimental results showed that there was an increase in the expression of genes for Nrf2, HO-1, GCLC, and SOD1 in the ICA group when they were exposed to AFB1. This suggests that ICA could exert an antioxidant impact via activating the Nrf2 signaling pathway. According to the findings of a various studies, ICA possesses antioxidant and anti-inflammatory properties and it may exhibit potential benefits in the treatment of disorders that are associated with oxidative stress and inflammation (Bi et al., 2022; Xia et al., 2022). Therefore, the findings suggest that ICA is crucial in mitigating oxidative damage caused by AFB_1 via increasing the activation Nrf2 signaling pathway.

V. CONCLUSION

In summary, AFB_1 could provoke oxidative damage and inflammatory response in mice. However, ICA inhibited the inflammatory response by modulating pro- and anti-inflammatory cytokine secretion. Additionally, ICA ameliorates AFB_1-induced oxidative damage in mice by reducing ROS formation and increasing antioxidant enzyme capacity, the mechanism was associated with Nrf2 signaling activation.

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