

In Vitro Callus Cultured of *Epimedium Brevicornum* as Sustainable Source of Icariin with Demonstrated Hepatic Protective Activity Against Cadmium Toxicity

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Abstract

In vitro experiment was conducted to develop callus cultures of *Epimedium brevicornum* for sustainable icariin (ICA) production. Callus development was enhanced on Murashige and Skoog (MS) basal medium supported by selected growth regulators. HPLC results showed that callus cultures yielded high ICA content, suggesting tissue culture as an efficient alternative for controlling ICA production. Icariin (ICA) is a naturally plant flavonoid product used for its diverse biological and pharmacological uses. Cadmium (Cd) is a widespread and highly hazardous heavy metal that poses severe toxicity risks to both human and animal health. However, the shielding impact of ICA on hepatic damage caused by Cd has not yet been explored. For this purpose, 40 mice were randomly assigned into four investigational groups: CON, ICA, Cd, and ICA+Cd. Experimental mice were gavage with either ICA (100 mg/kg BW), Cd (5 mg/kg BW), and co-treatment via intragastric administration for 2 weeks. Results indicated that Cd-alone treated mice induced hepatic injury as evidenced by increased liver function (ALP, ALT, AST, and GGT) enzymes and induced histopathological alteration in mice. Moreover, Cd exposure induced oxidative stress by reducing activities and expression of antioxidant-related genes (GSH-Px, T-AOC, GSH, T-SOD, and CAT) and accumulation of MDA content in the liver of mice. The Cd treated group showed increased inflammatory response as evidenced by increased pro-inflammatory (TNF- α , IL-1 β , IL-6) and a decrease in anti-inflammatory (IL-10) mediator. Interestingly, ICA treatment restored the abovementioned alterations and alleviated hepatic injury by reducing oxidative stress and inflammation that was initiated by Cd. Eventually, the present finding indicates that the protective effect of ICA against Cd-inflicted hepatic damage is associated with inhibiting inflammatory response and oxidative stress in mice.

Keywords: Callus, Cadmium chloride, Icariin, Liver, Oxidative stress, Inflammation

1. Introduction

Traditional Chinese Medicine (TCM) has gained popularity and medical importance due to its low toxic effects and multi-targeted treatments (Ma *et al.*, 2019). Within TCM, identifying active medicinal compounds is a major research focus. The herb *Epimedium* (commonly known as Horny Goat Weed or *Yin Yang Huo*) has been used in China since ancient times for the treatment of various disease (Ma *et al.*, 2011). Icariin (ICA), a biologically active flavonoid glycoside extracted from the *Epimedium brevicornum*, is widely recognized for its pharmacological activity. Previous studies have demonstrated that ICA alleviates oxidative stress and lipid peroxidation in multiple models of toxicant-induced injury (Shaukat *et al.*, 2022, Chung *et al.*, 2008, el-Shitany & Eid, 2019). Moreover, ICA enhances antioxidant enzyme activity in organs such as liver, kidney, spleen, and testes (Ma *et al.*, 2015, Zhao *et al.*, 2020, Sai *et al.*, 2022, Ma *et al.*, 2023). In addition, ICA exhibits anti-inflammatory (Bi *et al.*, 2022), anti-apoptotic (Jia *et al.*, 2019), anti-osteoporosis (Liu *et al.*, 2024), and anti-tumor effects (Zhou *et al.*, 2011). Collectively, these findings suggest

that ICA may offer protection against cadmium (Cd)-induced hepatic damage.

Plant tissue culture has emerged as an effective and environmentally friendly approach for producing secondary metabolites (Prashant & Bhawana, 2024). Through callus induction, plants can biosynthesize flavonoids and phenolics in controlled conditions, independent of season and geography (Yamamoto, *et al.*, 1992). In *Epimedium* species, *in vitro* callus cultures have been shown to enhance icariin yield due to optimized hormonal and nutritional conditions (Mihaljevic, 2009). The application of Plant tissue culture has expanded across numerous medicinal species, serving purposes such as rapid multiplication, germplasm preservation, secondary metabolite production, and genetic advancement (Shatnawi, *et al.* 2011). *In vitro* conservation provides an alternative approach to traditional field conservation (Shahrour, *et al.* 2024). Plant tissue culture, particularly callus culture, offers a sustainable and controlled approach for producing pharmacologically important secondary metabolites with consistent quality and yield. Unlike field-grown plants, callus cultures enable continuous production independent of environmental and seasonal variability, ensuring reproducibility for downstream biological studies

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(Murthy *et al.*, 2014; Espinosa-Leal *et al.*, 2018). In this study, callus culture was optimized to obtain icariin as a standardized source, which was subsequently used to evaluate its hepatoprotective effects in a cadmium-induced toxicity model. Sustainable production of icariin is critical due to increasing demand and ecological concerns related to overharvesting of *Epimedium* species. Tissue culture-based systems provide an environmentally friendly alternative that supports scalability and batch-to-batch consistency, which are essential for pharmacological and toxicological investigations (Verpoorte *et al.*, 2002; Cragg & Newman, 2013).

Cd is a prevalent heavy metal emitted by industrial activities or naturally occurring in the environment. It has significant toxicity and represents a risk to both human and animal health (Mishra *et al.*, 2019). The primary ways that Cd enters the human body are through cigarette smoke, polluted water and food, and industrial exposure (Suhani *et al.*, 2021). Studies have shown that Cd accumulates in the body, leading to pathological damage in the kidneys, liver, testes, spleen, bones, lungs, and neurological system (Fang *et al.*, 2021, Genchi *et al.*, 2020, Davidova *et al.*, 2024). The International Agency for Research on Cancer has classified Cd as a potent carcinogen because of its widespread environment occurrence and cumulative toxic effect in humans (Joseph, 2009). The liver is among the most severely affected organs and is particularly vulnerable both acute and chronic Cd-induced injury (Yang *et al.*, 2021). Epidemiological and experimental investigations have established that Cd exposure leads to hepatic oxidative damage, inflammation, fibrosis, and impaired liver function (Yang *et al.*, 2021, Fang *et al.*, 2021). Oxidative stress has been identified as a key contributor to Cd-induced liver injury. Cd may indirectly cause cellular damage towards oxidative conditions by producing free radicals, reactive oxygen species (ROS) and reduces the concentration of primary antioxidant compound inside cells through deactivation of enzymes and other antioxidant compounds, thus leading to lipid peroxidation (LPO) (Matovic *et al.*, 2015, Cuypers *et al.*, 2010). Numerous research has shown that Cd exposure could disrupt cell-mediated oxidative stress and lead to the impairment of cell membrane, potentially causing secondary liver injuries such as inflammatory response, cell death, and liver failure (Yang *et al.*, 2021, Zhang *et al.*, 2019). Enhancing antioxidant capacity in organisms is crucial for alleviating the pathogen alterations induced by Cd exposure through the reduction of oxidative stress. Current knowledge indicates that exposure to Cd may induce oxidative damage and led to hepatotoxicity.

ICA is known for its potential antioxidant and anti-inflammatory properties, yet its protective role against Cd-induced hepatic injury has not yet been fully explored. This study was conducted to evaluate whether ICA supplementation can attenuate Cd-induced oxidative stress and inflammation in the liver of mice. Additionally, an *in vitro* component was included to establish a callus culture system of *E. brevicornum* for sustainable ICA production. The findings of this research may provide a scientific basis for developing novel therapeutic and biotechnological strategies to manage heavy metal-induced hepatic toxicity. Despite extensive reports on the antioxidant and anti-inflammatory properties of icariin, limited information is available on the efficacy of callus-derived icariin against

heavy metal-induced liver injury. Therefore, this study aimed to optimize callus culture for sustainable icariin production and to assess its protective effects against cadmium-induced hepatic toxicity. We hypothesized that callus-derived icariin would mitigate cadmium-induced liver damage by reducing oxidative stress and inflammatory responses.

2. Materials and Methods

2.1. Chemicals and reagents

Cadmium chloride (CdCl_2) was obtained from Sigma Aldrich (city, country). *Epimedium brevicornum*, and measurement kits for IL-10, IL-1 β , IL-6, TNF- α , T-AOC, GST, GSH-Px, T-SOD, CAT, and MDA were obtained from Novus Biologicals, Thermofisher, and sigma Aldrich. Commercial suppliers were used to obtain additional chemicals and analytical grades.

2.2. Plant material, extraction, and analysis of ICA

2.2.1. Plant materials and culture conditions:

Epimedium brevicornum plants and seeds, *in vitro* cultured, followed a previously published procedure (Al Zoubi, 2019). Micro-shoot segments (2-3mm) were cultured in 9 cm Petri dishes containing 15 mL of MS (Murashige & Skoog, 1962) supplement with serial concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹) of 6-Benzyladenine (BA), and 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA). Cultures were kept in darkness inside a growth chamber adjusted at 25 $\pm$ 2°C to evaluate the most effective Auxin/Cytokinin combination for callus induction. Each treatment included ten replicates arranged in a completely randomized design. After weeks the percentage of callus induction, color and texture were documented. Fresh weights were determined from three randomly selected samples, which were subsequently dried in oven at 80°C until the weight was constant to get the dry weight measurement.

2.2.2. Extraction, and analysis of ICA

The extraction method was followed as a previously published procedure (De Naeyer *et al.*, 2005). High-Performance Liquid Chromatography determined icariin (ICA) following a previously published method (Shaukat *et al.*, 2022). The purity of ICA was determined using HPLC (Waldbronn, Germany) connected to a Hypersil C18 column.

2.3. Animals, experimental design and treatment

Male C57BL/6 mice were obtained from animal house and maintained under controlled conditions at 23 $\pm$ 2 °C in a twelve-hour light/ twelve-hour dark cycle, with water and food supplies provided upon request *ad libitum*. Euthanasia and all the procedures used in this study were done based on the standards for Animal Ethical and Welfare Committee. Following a 7-day acclimation period, the animals were randomly allotted to four experimental treatment groups (12 mice per group): 1) Control, received corn oil; 2) ICA, received 100 mg/kg BW icariin; 3) Cd, received 5 mg/kg BW cadmium chloride, and 4) ICA + Cd, received 100 mg/kg BW icariin and 5 mg/kg BW cadmium chloride. All mice received a daily oral gavage of either ICA dissolved in corn oil or Cd dissolved in fresh distilled water. The dosages, administration routes, and

duration for ICA and Cd were calculated from prior studies (Li *et al.*, 2021, Yang *et al.*, 2021). The liver coefficient was estimated depending on this equation:

$$\text{Liver index (\%)} = \text{Liver weight (g)} / \text{body weight (g)} \times 100\%$$

2.4. Liver function enzymes analysis

The blood was obtained via the ocular regions of mice. Following serum collection, the activity of the enzymes AST, ALT, GGT, and ALP was assessed using an automatic biochemistry analyzer based on the manufacturer's protocol.

2.5. Histopathological observation

Livers of experimental animals were collected and then kept in paraformaldehyde at a concentration of 4%. Subsequently, for the dehydration of liver tissues, alcohol was used and then embedded in paraffin, segmented to a 5 μm , and finally prepared for staining with hematoxylin and eosin. The liver section from all groups was microscopically examined.

2.6. Analysis of inflammatory cytokines

Liver samples were homogenized, centrifuged and supernatants were stored and kept at -20°C for later investigation. The inflammatory response was influenced by measuring the levels of IL-6, IL-1 β , IL-10, and TNF- α in the supernatants of mice. The cytokines were assessed by employing an ELISA kit following the procedure recommendations.

2.7. Assessment of oxidative stress in the liver

According to a prior study, the liver's tissue homogenates were produced (Rajput *et al.*, 2021b). The liver tissues were briefly homogenized at 4°C with a Potter homogenizer in an ice-cold 0.9% NaCl solution. Following the centrifugation of homogenates, the supernatants were collected. GSH-Px, GSH, CAT, T-AOC, T-SOD, MDA, and total protein in the liver tissue were determined based on the manufacturer-recommended procedure.

2.8. Analysis of quantitative real-time PCR (qRT-PCR)

The whole RNA was isolated from liver tissue by Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following a previously published method (Rajput *et al.*, 2021a). PrimeScript™ RT reagent Kit was utilized to perform reverse transcription for cDNA synthesis based on the manufacturer's instructions. Successively, quantitative real-time PCR (qRT-PCR) was achieved on a real-time PCR system by SYBR Green PCR Master Mix (Applied Biosynthesis, Waltham, MA, USA) (Rajput *et al.*, 2019). In the current research, the sequence of primers is listed in Table S1. The β -actin was used as a standard gene. The data was quantified using the $2^{-\Delta\Delta\text{CT}}$ method as previously described (Gao *et al.*, 2018).

2.9. Statistical analysis

Data was analyzed by SPSS software V. 22 (IBM Corporation, Armonk, NY, USA). Statistically Significant ($P < 0.05$) was determined using one-way ANOVA followed by an LSD test. Data were displayed as mean $\pm$ SD.

3. Results

3.1. The role of Plant Growth Regulators on Callus Induction from *E. brevicornum*

Callus initiation started within 10–12 days, forming friable yellow-green tissue. The effect of different concentrations of BA, NAA, 2,4-D and their mixtures were assessed for their impact on callus induction, fresh and dry weight *in vitro* after twelve weeks were summarized in Tables 1–4.

Table 1: *In vitro* growth response of *E. brevicornum* to different BA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) BA	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	687 $\pm$ 152 ^b	52.71 $\pm$ 6.70 ^b
0.5	2891 $\pm$ 136 ^a	125.3 $\pm$ 54.6 ^a
1	3011 $\pm$ 557 ^a	161.5 $\pm$ 62.9 ^a
1.5	3452 $\pm$ 323 ^a	185.3 $\pm$ 53.3 ^a
2	3558 $\pm$ 254 ^a	196.6 $\pm$ 56.7 ^a
2.5	3803 $\pm$ 341 ^a	222.4 $\pm$ 76.2 ^a

*Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 2: *In vitro* growth response of *E. brevicornum* to different 2,4D concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹)) 2,4D	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	889 $\pm$ 255.0 ^c	53.6 $\pm$ 8.90 ^c
0.5	2542 $\pm$ 389.0 ^b	141.3 $\pm$ 35.4 ^{ba}
1	3245 $\pm$ 335.0 ^b	157.4 $\pm$ 44.29 ^{ba}
1.5	4001 $\pm$ 846.0 ^a	223.6 $\pm$ 61.23 ^{ba}
2	4178 $\pm$ 876.1 ^a	243.4 $\pm$ 82.09 ^{ba}
2.5	4298 $\pm$ 897.0 ^a	266.7 $\pm$ 81.96 ^a

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 3: *In vitro* growth response of *E. brevicornum* to different NAA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) NAA	Fresh weight (mg) $\pm$ SD	Dry weight (mg) $\pm$ SD
0.0	816 $\pm$ 215.0 ^c	54.7 $\pm$ 10.70 ^c
0.5	2265 $\pm$ 145.3 ^b	181.7 $\pm$ 35.76 ^b
1	2654 $\pm$ 791.2 ^{ba}	197.8 $\pm$ 46.50 ^{ba}
1.5	3212 $\pm$ 1028.3 ^a	264.3 $\pm$ 56.10 ^{ba}
2	3819 $\pm$ 657.1 ^a	287.9 $\pm$ 76.90 ^a
2.5	3936 $\pm$ 787.6 ^a	309.3 $\pm$ 73.40 ^a

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

Table 4: *In vitro* growth response of *E. brevicornum* to different combinations of 2,4D and BA concentrations in terms of callus formation and biomass yield after 12 weeks.

Concentration (mg L ⁻¹) 2,4D	Concentration (mg L ⁻¹) BA	Fresh weight (mg) ±SD	Dry weight (mg) ±SD
0.0	0.0	823±226 ^c	72.7±19.11 ^d
2	0.5	5357±123 ^a	357.8±54.8 ^a
2	1	2315±406.82 ^{bc}	143.4±41.05 ^{bc}
2	1.5	2534±326.3 ^b	146.6±32.71 ^{bc}
2	2	2650±276.1 ^b	149.8±42.3 ^{bc}
2.5	0.5	2101±204 ^c	131.6±31.7 ^{bc}
2.5	1	1667±251 ^d	103.1±34.4 ^d
2.5	1.5	1611±263 ^d	102.6±24.8 ^d
2.5	2	1247±233 ^d	74.7±44.3 ^d

* Means sharing identical letters within column are not significantly different according to the LSD test ($p \leq 0.05$).

All growth regulators significantly enhanced callus induction compared with the control. Increasing BA concentrations led to a steady rise in callus biomass, with the highest fresh (3803 mg) and dry (222.4 mg) weights obtained at 2.5 mg L⁻¹ BA. Similarly, 2,4-D treatments showed a marked stimulatory effect, where callus mass increased from 898 mg (control) to 4298 mg at 2.5 mg L⁻¹ 2,4-D, accompanied by a dry weight of 266.7 mg. NAA also promoted callus growth in a concentration-dependent manner, reaching maximum values of 3936 mg fresh and 309.3 mg dry weight at 2.5 mg L⁻¹ NAA. Among the combinations, the interaction between auxin and cytokinin significantly influenced callus proliferation. The mixture of 2 mg L⁻¹ 2,4-D with 0.5 mg L⁻¹ BA resulted in the highest callus biomass (5357 mg fresh and 357.8 mg dry weight), while higher BA concentrations caused a gradual decline in growth. Overall, auxin-based treatments, particularly 2,4-D and NAA, were more effective than BA alone, and the combination of 2 mg L⁻¹ 2,4-D with a low BA concentration provided the most favorable conditions for callus induction and growth in *E. brevicornum*.

3.2. ICA alleviates liver injury induced by Cd

As shown in Fig. 1, the ALP, AST, ALT, and GGT concentrations were markedly raised after Cd exposure in comparison to the untreated-CON group. However, in comparing the Cd alone treated group, the ALP, AST, ALT, and GGT levels decreased in the liver of the ICA + Cd group ($P < 0.05$). Compared to untreated group (CON), the liver index was increased under Cd exposure while recovering to the normal values under the co-treatment of ICA and Cd ($P < 0.05$) (Fig. 2B).

Based on the histopathological observations from the experimental groups presented in Fig. 2A, the CON and ICA groups showed normal liver architecture with intact hepatic cords and hepatic sinusoids. Moreover, the central vein has intact endothelium and the hepatic triad consisting of portal vein, bile duct, and hepatic artery were also normal. There were no obvious microscopic changes in the hepatocytic nucleus in the CON and ICA-treated groups. Contrastingly, mice exposed with Cd revealed significant pathological changes including, central vein was filled with proteinaceous fluid along with inflammatory cells. The endothelium of central vein was distorted.

Degenerative changes such as ballooning of hepatocytes with peripheral nucleus due to fatty change were detected. Furthermore, necrotic changes including karyolysis were observed in hepatocytes which indicate mild to moderate coagulative necrosis of liver. Strikingly, ICA treatment significantly ameliorated and restored liver injury caused by Cd (Fig. 3A).

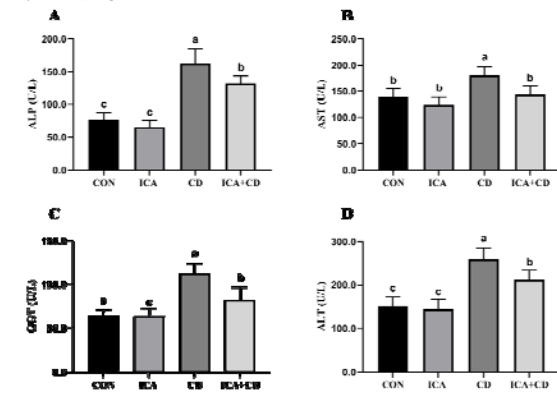


Figure 1. Effect of ICA on liver function enzymes under Cd-exposed mice. The results are stated as mean ± standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. AST: Aspartate aminotransferase; ALP: Alkaline phosphate; ALT: Alanine aminotransferase and GGT: Gamma-glutamyl transferase.

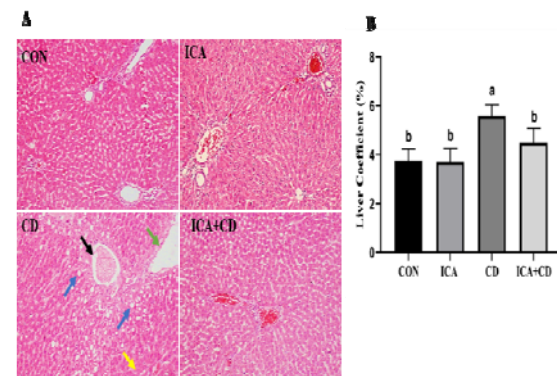


Figure 2. (A) Effect of ICA and Cd on Histopathological observations. (B) Liver coefficient. The results are shown as mean ± standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns.

3.3. ICA alleviates inflammatory response induced by Cd

The effects of ICA and Cd on the inflammatory cytokines are presented in Fig. 3A, B. Compared with the experimental groups, the TNF- α , IL-1 β , and IL-6 levels, as well as gene expression were enhanced in response to Cd treatment ($P < 0.05$). On the other hand, co-treatment of ICA and Cd exhibited significantly decreased expressions of these pro-inflammatory cytokines in comparison to the Cd group. However, the ICA + Cd administered group showed an up-regulation in the concentration and transcriptional abundance of anti-inflammatory cytokines (IL-10) in comparison to the Cd alone treated group.

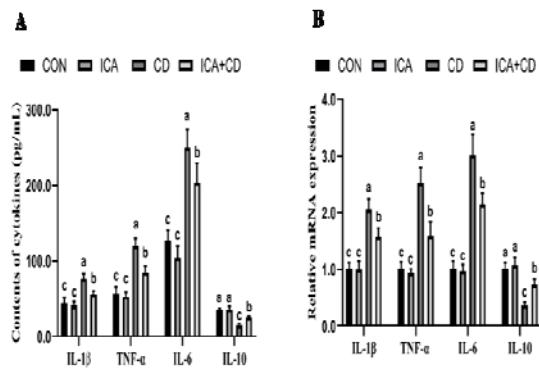


Figure 3. Effect of ICA on inflammatory response under Cd-exposed mice. (A) Levels of inflammatory cytokines. (B) The transcriptional expression of inflammatory cytokines. The results are presented as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. IL-10: Interleukin-10; TNF- α : Tumor necrosis factor-alpha; IL-6: Interleukin-6, and IL-1 β : Interleukin 1 beta.

3.4. ICA mitigates hepatic oxidative damage induced by Cd

To examine the mitigation effect of ICA on oxidative stress caused by Cd in the liver of mice, T-AOC, CAT, T-SOD, GSH-Px, GSH, and MDA content or activities were determined. As shown in Fig 4, the activities of T-AOC, CAT, T-SOD, GSH-Px, and GSH markedly reduced in the Cd alone treated group as compared with the other experimental groups. However, Cd-exposed displayed a significant increase in MDA levels when compared to the experimental groups. Contrastingly, the antioxidant and oxidative stress markers alterations were ($P < 0.05$) markedly attenuated by ICA treatment under Cd exposure.

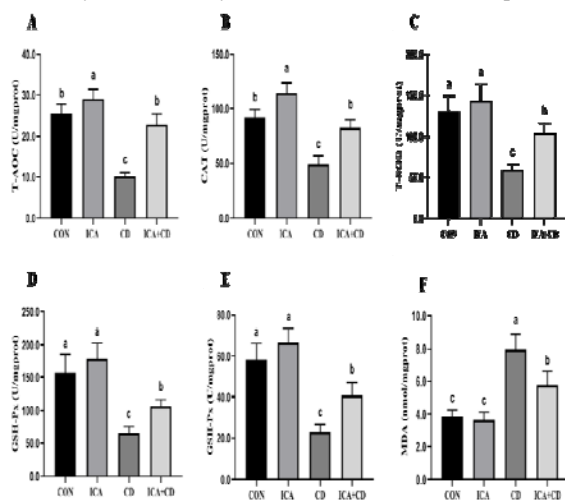


Figure 4. Effect of ICA on oxidative stress under Cd-exposed mice. Activities of antioxidant enzymes. The results are shown as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. MDA: Malondialdehyde; CAT: Catalase; T-SOD: Total superoxide dismutase; GSH-Px: Glutathione peroxidase; GSH: Glutathione.

The transcriptional expression of antioxidants-related genes was further detected to confirm the antioxidant capability of ICA. Compared with experimental groups, the transcriptional levels of antioxidant genes were

markedly ($P < 0.05$) down-regulated in the Cd-treated group. However, the expression of these genes was significantly regulated in the ICA + Cd group (Fig. 5).

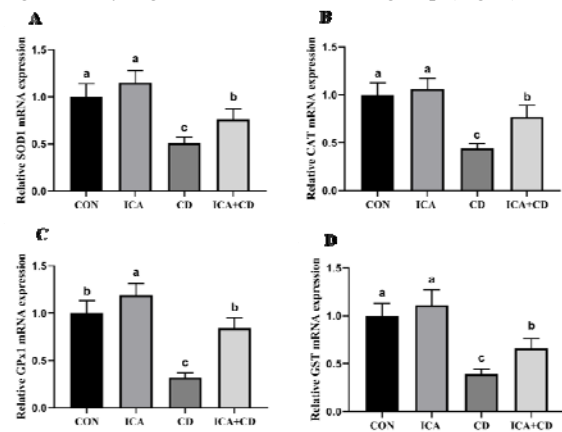


Figure 5. Effect of ICA on antioxidant-related transcriptional levels in Cd-exposed mice. The results are shown as mean $\pm$ standard deviation SD (n=6). Statistically significant was considered at ($P < 0.05$) were observed, followed by an LSD test by using one-way ANOVA. ^{abc}, Significant differences between the comparing groups are indicated by distinct lowercase letters within columns. SOD1: Superoxidase dismutase 1; CAT: Catalase; GPx1: Glutathione peroxidase 1; and GST: Glutathione S-transferase.

4. Discussion

The present study shows that plant growth regulators (PGRs) strongly influenced callus induction and biomass accumulation in *E. brevicornum* after 12 weeks. Cytokinin (BA) promoted callus formation in a concentration-dependent manner, with fresh and dry weights increasing steadily from the control to 2.5 mg L⁻¹ BA. Auxins (2,4-D and NAA) produced even greater biomass accumulation than BA alone at higher concentrations, indicating that callogenesis in *E. brevicornum* is largely auxin-responsive. Notably, the combination treatment 2 mg L⁻¹ 2,4-D + 0.5 mg L⁻¹ BA gave the highest biomass (fresh weight 5357 mg, dry weight 357.8 mg), showing a synergistic auxin-cytokinin interaction where moderate auxin with low cytokinin favored maximal callus proliferation; higher BA levels in the presence of auxin reduced biomass, consistent with cytokinin-induced shift away from unorganized proliferation toward differentiation.

Previous studies have confirmed that *Epimedium* callus cultures can produce characteristic flavonoid glycosides (Yamamoto *et al.*, 1992). The high biomass obtained under auxin-dominant conditions, which may mimic mild stress states appears advantageous for secondary metabolites biosynthesis. However, since both drying and PGR regimes can affect metabolite composition, a trade-off exists between maximizing callus biomass and optimizing the yield of specific secondary metabolites such as icariin and epimedesides. According to our findings, the following recommendations can be made for practical applications-metabolite production or mid-term storage:- (1) use auxin-rich media (e.g., 2–2.5 mg L⁻¹ 2,4-D or NAA) to maximize callus biomass; (2) maintain low BA levels (≈ 0.5 mg L⁻¹) in auxin-cytokinin combination with sustain proliferation; and (3) carefully control drying and storage condition, as temperature and drying rate strongly influence flavonoid stability. These recommendations are

consistent with micropropagation, and callus-culture protocols previously developed for other *Epimedium* species (Al-Matar *et al.*, 1999) and support the potential of callus-based systems for controlled flavonoid production in the genus.

The improved icariin (ICA) yield in callus cultures indicates that *in vitro* conditions stimulate secondary metabolism, likely through activation of phenylpropanoid pathway regulated by 2,4-D and BAP (Niazian & Sabbatini, 2021). Such culture systems provide a sustainable and controlled alternative to field cultivation, ensuring consistent ICA production irrespective of environmental fluctuations (Alamgir, 2017). Integrating tissue culture-based biosynthesis with pharmacological investigation, as demonstrated in this study, could therefore advance commercial-scale icariin production for hepatoprotective and therapeutic applications (Kiran & Tiwari, 2024).

Cadmium (Cd) is considered one of the most noxious heavy metals in the environment. The liver is known to be a major target of Cd toxicity and accumulation, leading to liver damage (Imed *et al.*, 2009, He *et al.*, 2022). The current findings demonstrated that Cd exposure significantly raised liver index in mice. The increased liver weight can be explained by the fact that Cd can accumulate lipids in the liver, resulting in hepatomegaly. The present study results agreed with the earlier studies, which reported that Cd could cause alterations in liver index in experimental animals (Chen *et al.*, 2023). The detection of AST, ALT, GGT, and AST, can directly indicate the damage and dysfunction of liver caused by Cd. Histopathological changes are a direct parameter used to judge the impairment of tissue structure (Fang *et al.*, 2021). The current observations revealed that Cd caused pathological and clinical symptoms of hepatotoxicity in mice. This is evidenced by increased ALT, AST, GGT, and ALP concentration along with degenerative changes in the liver of mice. The current outcomes indicate that Cd caused liver injury, in agreement with the previous studies (He *et al.*, 2022, Souza-Arroyo *et al.*, 2022, Yang *et al.*, 2021). ICA administration ameliorated Cd-induced growth retardation and mitigated harmful impacts on liver function enzymes and pathological alterations in the liver of mice. The findings align with previous studies that revealed ICA-protected thioacetamide-induced liver fibrosis (Algandaby *et al.*, 2017) and CCl₄-induced acute liver injury in mice (Xiao *et al.*, 2024). The current results suggested that ICA could alleviate liver injury in Cd-induced mice.

Inflammation is a well-established area of Cd-induced poisonousness (Yang *et al.*, 2021). After the initiation of Cd toxicity, macrophages and Kupffer cells are activated, generating an intricate system of inflammatory mediators and releasing detrimental cytokines, including IL-6, IL-1 β , and TNF- α (Renu *et al.*, 2021). These cytokines are essential in the progression of inflammation. TNF- α secreted by T-lymphocytes, macrophages, and monocytes as well as proved to have a crucial function in releasing and helping the other pro-inflammatory cytokines and activating neutrophils (Burger & DAYER, 2002). Moreover, IL-6 is released during infections and stress, and its major function is maintaining tissue injury induced by internal and external stimuli. Furthermore, IL-1 β secreted by macrophages and modulates the inflammatory

response caused by toxicants (Megha *et al.*, 2021). Therefore, pro- and anti-inflammatory mediators by ELISA and qRT-PCR techniques were detected. The results showed that ICA treatment decreased all the validated pro-inflammatory cytokines and raised the anti-inflammatory cytokines induced by Cd. These results are consistent with earlier research (Luo *et al.*, 2022, Shaikat *et al.*, 2022, Yang *et al.*, 2021) and, therefore, highlighted the mitigation impact of ICA on Cd-induced liver inflammation.

Oxidative damage is categorized by the accumulation of free radicals and reactive oxygen species, which cause lipid peroxidation and the breakdown of cell membranes (Recknagel *et al.*, 2020). Various antioxidant defense reactions reduce oxidative damage and regulated the status of free radicals in the body, such as (GSH, CAT, T-SOD, and GSH-Px) (Davies, 2000). Furthermore, MDA is a hallmark of lipid peroxidation and is frequently used to detect the state of endogenous oxidative damage (Rajput *et al.*, 2017). Therefore, detecting MDA, CAT, T-SOD, GSH-Px and GSH can measure an organism's oxidative status. The present study findings revealed that Cd exposure increased MDA content and decreased the GSH, GSH-Px, CAT, T-AOC, and T-SOD activities. The results showed that Cd could induce oxidative stress in the liver of mice, which aligns with prior studies (Fang *et al.*, 2021, Cuypers *et al.*, 2010). Notably, the administration of ICA restored the antioxidant defense capacity, which was reduced by Cd. Our results are in agreement with studies that showed supplementation of ICA alleviates oxidative damage in the liver induced by Cd (Shaikat *et al.*, 2022, Wang *et al.*, 2019). These results verified that ICA has the capability to rescue Cd-induced hepatic oxidative stress.

In summary, this study demonstrates: (1) optimized *in vitro* callus culture provides a reliable strategy for sustainable icariin production from *E. brevicornum*, and (2) icariin exerts a potent hepatoprotective effect against Cd-induced injury by enhancing antioxidant defense and suppressing inflammatory responses. These findings bridge plant biotechnology and pharmacological application, offering a promising framework for future studies and commercial-icariin-based therapeutics.

5. Conclusion

Auxin-dominated media and an optimized auxin-cytokinin balance produce the highest callus biomass in *E. brevicornum*. Future studies should focus on quantifying key flavonoids such as icariin and epimedinol under the most effective PGR combination and standardized drying condition to determine the optimal balance between biomass yield and metabolite quality.

Additionally, this study demonstrated that ICA exerts marked hepatoprotective effects against cadmium (Cd) – induced liver injury by restoring liver function enzymes, improving histopathological integrity, reducing oxidative stress, and suppressing inflammatory response. These findings provide new insights into the therapeutic potential of ICA in mitigation Cd-induced hepatic toxicity and establish a novel knowledge for its future development as natural histopathological agents.

In conclusion, the present study demonstrates that callus-derived icariin effectively attenuates cadmium-induced hepatic injury in mice. The protective effects are

associated with the restoration of antioxidant gene expression and the reduction of pro-inflammatory cytokines. These findings support the potential of sustainably produced icariin as a hepatoprotective agent against heavy metal-induced toxicity. Further studies are warranted to investigate protein-level mechanisms and to confirm these effects in additional experimental models.

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Supplementary material:**Table S1.** Sequence of the primers used for quantitative real-time PCR assay.

Target gene	Primer	Primer sequence (5'→3')	Accession No.
β-Actin	Forward	GTTGGAGCAAACATCCCCCA	NM_007397.5
	Reverse	ACGCGACCATCCTCCTCTTA	
SOD1	Forward	GGTCTCCAACATGCCTCTCT	NM_011434.2
	Reverse	AACCATCCACTTCGAGCAGA	
CAT	Forward	CACTGACGAGATGGCACACT	NM_009804.2
	Reverse	TGTGGAGAATCGAACGGCAA	
GPx1	Forward	GTACTTGGGGTCGGTCATGA	NM_001329527.1
	Reverse	GGTTTCCCGTGCAATCAGTT	
GST	Forward	TGGCCGGAAGCACAAACCTG	BC031818
	Reverse	CCCCACCAACACCGGCAC	