



# Icariin inhibits cisplatin-induced ovarian toxicity via modulating NF- $\kappa$ B and PTEN/AKT/mTOR/AMPK axis

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## Abstract

Cisplatin (CP) is a highly effective broad-spectrum chemotherapeutic agent for several solid tumors. However, its clinical use is associated with ovarian toxicity. Icariin (ICA) is a bioactive flavonoid of *Epimedium brevicornum* with reported protective activities against inflammation, oxidative stress and ovarian failure. This study aimed to explore the protective effects of ICA against CP-associated ovarian toxicity in rats. Rats were randomized into five groups and treated for 17 days: control, ICA (10 mg/kg/day, for 17 days, p.o.), CP (6 mg/kg, i.p. on days 7 and 14), CP+ICA (CP 6 mg/kg i.p. on days 7 and 14 and ICA 5 mg/kg p.o. daily), and CP+ICA (CP 6 mg/kg i.p. on days 7 and 14 and ICA 10 mg/kg p.o. daily). Our results indicated that ICA effectively improved ovarian reserve as indicated by attenuating CP-induced histopathological changes and enhancing serum anti-müllerian hormone (AMH). Furthermore, co-administration of ICA with CP showed restoration of the oxidant-anti-oxidant balance in ovarian tissues, evidenced by decreased malondialdehyde (MDA) concentrations and elevated superoxide dismutase (SOD) and catalase (CAT) activities. Also, ICA suppressed ovarian inflammation as evidenced by down-regulation of the expression of interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and nuclear factor kappa B (NF- $\kappa$ B). ICA inhibited ovarian apoptosis in CP-treated rats by down-regulation of CASP3 and Bax and up-regulation of Bcl-2 mRNA expression. Further, ICA enhanced PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  expression. In conclusion, ICA possesses a protective activity against CP-induced ovarian toxicity in rats by exhibiting antioxidant, antiinflammatory, anti-apoptotic activities and modulating NF- $\kappa$ B expression and PTEN/AKT/mTOR/AMPK axis in ovarian tissues.

**Keywords** Icariin · Cisplatin · Ovaries · Inflammation · Apoptosis · NF- $\kappa$ B · PTEN/AKT/mTOR/AMPK axis

## Introduction

Adverse effects of cancer chemotherapy on women of reproductive ages (15–44 years) are well-documented (Vo and Kawamura 2021; Hoefgen et al. 2023). The ovaries possess a predetermined number of follicular reserves, which diminish progressively from birth until menopause (Broekmans et al. 2009). Therefore, exposure to chemotherapeutic regimens can negatively influence the depletion rate of these follicular reserves (Spears et al. 2019). In particular,

treatments involving platinum complexes, such as cisplatin (CP), alkylating agents, and taxanes, have been identified to cause significant ovarian toxicity (van Dorp et al. 2018; van denBoogaard et al. 2022). Consequently, the likelihood of pregnancy among women post-cancer diagnosis and treatment is 38% lower compared to the general female population (Anderson et al. 2018).

CP, a potent broad-spectrum chemotherapeutic agent, is efficaciously employed against numerous solid tumors and gynecological cancers (Ozols et al. 2003; Jang et al. 2016; Kumar et al. 2017). Once inside the cell, CP binds to DNA bases, disrupting DNA replication and inducing apoptosis in cancer cells (Forgie et al. 2022). Despite its clinical efficacy, CP is associated with a significant risk of ovarian failure (Blumenfeld 2012). Several studies have consistently shown that CP leads to depletion of the ovarian primordial follicle reserve, leading to premature ovarian failure (POF) and infertility (Chang et al. 2015; Jang et al. 2016). The condition of POF is marked by reduced levels of gonadal hormones, notably the

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anti-müllerian hormone (AMH) (Pouresmaeili and Fazeli 2014; Bedenk et al. 2020). Studies have demonstrated that CP ovarian toxicity involves the induction of oxidative stress (Altuner et al. 2013; Yan et al. 2022), inflammation (Ibrahim et al. 2021; Algandaby 2021), and apoptosis (kaygusuzoglu et al. 2018; Algandaby 2021; Yan et al. 2022; Xing et al. 2022). Further, adverse ovarian effects of CIS were reported to involve modulated phosphorylation and expression of key proteins including PTEN and Akt, eventually lead to loss of primordial follicles, accelerated activation of primordial follicles, follicular atresia, and damage to the vasculature (Spears et al. 2019).

*Epimedium brevicornum* is a Chinese medicine that is commonly used for treatment of sexual dysfunction and osteoporosis (Agrawal et al. 2024). Also, it is also a component of modern proprietary traditional Chinese medicine products for the treatment of several ailments including arthritis, amnesia, infertility, and impotence (Ma et al. 2011). The plant belongs to the genus *Epimedium* that has been reported to contain flavonoids, lignans, ionones, phenol glycosides, phenethyl alcohol glycosides and sesquiterpenes (Ma et al. 2011; Zhuang et al. 2023). Icaritin (ICA) was found to be the major constituent of *Epimedium brevicornum* and responsible for almost every pharmacological activity (Agrawal et al. 2024). Also, ICA possesses a rich a wide range of applications in traditional medicine (He et al. 2020). It is commonly used as an aphrodisiac agent and for the treatment of erectile dysfunction through its ability to increase blood flow and enhance sexual function (Chen et al. 2014). Beyond its traditional uses, ICA has been extensively reported to offer various pharmacological properties. These include protective effects on the cardiovascular and immune systems and anti-depressant and anti-tumor properties (Li et al. 2015a). Moreover, ICA protects against experimentally-induced ovarian failure (Wang et al. 2019; Li et al. 2024) and ovarian insufficiency (Chen et al. 2014). It exhibits potent antioxidant activities, contributing further to its therapeutic profile (El-Shitany and Eid 2019; Xia et al. 2022). Additionally, ICA has been shown to exert significant anti-inflammatory effects by inhibiting nuclear factor kappa B (NF- $\kappa$ B) signaling pathways (Deng et al. 2017; El-Shitany and Eid 2019; Zhang et al. 2021). It also demonstrates anti-apoptotic activities, highlighting its potential for cellular protection (Deng et al. 2017). Thus, the current study aimed to investigate the protective potential of ICA against CP-induced ovarian toxicity in rats as well as its impact on expression of key proteins ovarian injury.

## Materials and methods

### Chemicals

ICA (> 97% purity) was acquired from Royal Pharm (Hangzhou, China). CP was sourced from EBEWE Pharma

G.m.b.H. Nfg. KG (Unterach Am Attersee, Austria). All other chemicals used were of the highest commercial analytical quality.

### Animals

Female Wistar rats (12–14-week-old, 200–220 g) were kept in our animal facility for two weeks to acclimate to laboratory conditions. The humidity and temperature in the animal house were maintained at 50% and  $22 \pm 2$  °C, respectively. A 12-h cycle of darkness and light was provided. There were plenty of standard food pellets and drinking water. The study was ethically approved by the Research Ethics Committee, the Faculty of Pharmacy, King Abdulaziz University (PH-1442–53).

### Experimental design

A total of 30 female Wistar rats were allocated randomly into five groups (6 rats per group) as follows: Group I (Control) was the control group and received normal saline daily p.o. for 17 days and i.p. on the 7th and 14th days. Group II (ICA 10 mg) received ICA (10 mg/kg/day p.o.) by oral gavage once daily for 17 days. Group III (CP group) was injected by CP only (6 mg/kg/day; i.p.) as a single dose on the 7th and 14th days. Group IV (ICA 5 mg + CP) received ICA (5 mg/kg/day, p.o.) by gavage once daily for 17 days and was injected with CP 6 mg/kg/day i.p. one hour after ICA oral administration on the 7th and 14th days. Group V (ICA 10 mg + CP) received ICA (10 mg/kg/day, p.o.) by gavage once daily for 17 days and was injected with CP 6 mg/kg/day i.p. one hour after ICA oral administration on the 7th and 14th days (Algandaby 2021). The chosen doses were based on a pilot experiment.

At the end of the study, animals were anesthetized using ketamine (80 mg/kg, i.p.), and blood was collected from the retro-orbital plexus and centrifuged for 15 min at 3000 rpm to separate the sera, which were subsequently stored at -80°C for future analyses. Next, animals were sacrificed by decapitation, and the ovaries were carefully removed, cleansed with normal saline, and weighed. The right ovaries were homogenized in phosphate buffered saline (0.1 M, pH 7.4) to yield a 10% w/v homogenate for the biochemical tests. The homogenate was kept at 4 °C for 15 min before being centrifuged at 10,000 rpm, and the supernatant was kept at -80 °C until the assays were performed. Part of the left ovaries were kept in 10% neutral formalin for histological and immunohistochemical examinations. The other part of the left ovary was flash-frozen in liquid nitrogen and kept in RNA later for molecular biomarker investigations.

## Determination of the serum levels of AMH and estradiol

According to the vendor's instructions, the serum levels of AMH and Estradiol were measured using an ELISA kit (MBS726534, MyBioSource, USA) and (K4266-100, Biovision, USA), respectively, following the manufacturer's instructions.

## Histological assessments

Portions of the left ovaries that were fixed in formalin were dehydrated using ascending concentrations of alcohol. This was followed by paraffinization of the ovarian tissues and sectioning at a thickness of 5  $\mu$ m. Xylene was used to deparaffinize the sections, after which they were rehydrated and stained with hematoxylin and eosin (H&E). The slides were studied and photographed by light microscopy (Carl Zeiss Axiostar plus, Oberkochen, Germany). A pathologist blindly examined the slides and classified the follicles as primordial, pre-antral, antral, or atretic. Then, the percentage of healthy follicles was calculated as follows (Algardaby 2021):

$$\frac{\text{Number of primordial} + \text{pre antral} + \text{antral follicles}}{\text{Total number of follicles}} \times 100$$

## Assessment of ovarian oxidative stress biomarkers

Oxidative injury markers such as content of malondialdehyde (MDA) (Cat. No. MD 25 29, Biodiagnostic, Giza, Egypt) and activities of the enzymes superoxide dismutase (SOD) (Cat. No. SD 25 21, Biodiagnostic, Giza, Egypt) and catalase (CAT) (Cat. No. CA 25 17, Biodiagnostic, Giza, Egypt) in the homogenates of the ovary were assessed using commercial kits.

## Immunohistochemical analysis

Paraffin-embedded blocks were deparaffinized by immersion of the tissue sections twice in 100% xylene for 15 min each time. Graded ethanol was used to complete a rehydration

stage. Tissues were rehydrated and then rinsed with phosphate buffered saline (PBS) for 5 min. For antigen retrieval, sections were then incubated in boiling citrate buffer for 5 min. Endogenous peroxidases were quenched by incubation in 3% H<sub>2</sub>O<sub>2</sub> for 10 min. The sections were then exposed to one of the following primary antibodies: TNF- $\alpha$  (Catalog # ab307164, ABCAM), IL-6 (Catalog # ab9324, ABCAM), NF- $\kappa$ B (Catalog # 8242, CST), PTEN (Catalog # ab170941, ABCAM), p-AKT (Catalog # 4060, CST), p-mTOR (Catalog # 2976, CST), and p-AMPK $\alpha$  (Catalog # 2535, CST) for overnight incubation at 4 °C, followed by a 20-min incubation with the secondary antibody at room temperature. Hematoxylin was used to counteract staining caused by peroxidase activity using DAB. Light microscopy was employed to assess the immunostaining. Image J software (1.46a, NIH, Bethesda, MD, USA) was used to quantify the immunostaining.

## qRT-PCR analysis

To determine the effect of ICA on CP-induced ovarian apoptosis, qRT-PCR was used to evaluate the mRNA expression levels of CASP3, Bax, and Bcl-2. Direct-zol RNA Miniprep Plus kit (R2072, ZYMO RESEARCH CORP., USA) was used to extract the total RNA from the ovary homogenate. A spectrophotometer was used to determine the total RNA, which was then reverse-transcribed to cDNA using an appropriate kit (12594100, Thermo Fisher Scientific, Waltham, MA, USA). This was followed by amplification using the SYBR Green Master Mix Kit (204143, Qiagen, MD, USA). In the analysis of qRT-PCR data using the 2<sup>- $\Delta\Delta$ Ct</sup> method,  $\beta$ -actin was used as a housekeeping gene. The primers are listed in Table 1 (Wang et al. 2015; Hareeri et al. 2023).

## Statistical analysis

GraphPad Prism version 8 (GraphPad, La Jolla, CA, USA) was used for the statistical analysis. Mean  $\pm$  standard deviation (SD) was used to present the results. One-way ANOVA and the Tukey-post hoc test were used to determine the statistical significance between groups for each biomarker. Significance was taken at P < 0.05.

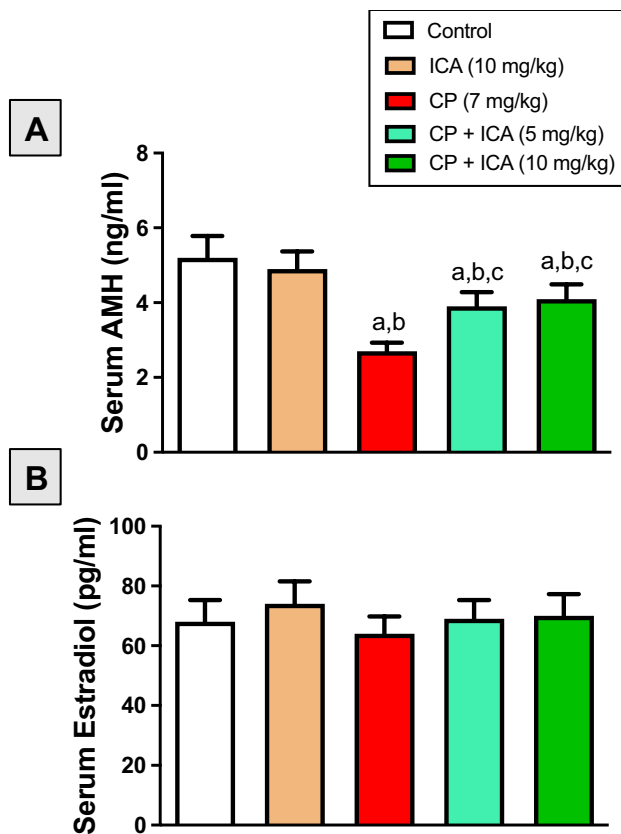
**Table 1** Primers sequences

	Forward	Reverse
Bax	5'- CCTGAGCTGACCTTGGAGCA-3'	5'-GGTGGTTGCCCTTTTCTACT-3'
Bcl-2	5'-TGATAACCGGGAGATCGTGA-3'	5'-AAAGCACATCCAATAAAAAGC-3'
CASP3	5'-AGCTGGACTGCGGTATTGAG-3'	5'-GGGTGCGGTAGAGTAAGCAT-3'
$\beta$ -actin	5'- TCCGTCGCCGGTCCACACCC-3'	5'-TCACCAACTGGGACGATATG-3'

## Results

### Assessment of serum estradiol and AMH levels

Serum AMH and estradiol levels were evaluated as indicators of ovarian function to determine the protective effects of ICA against CP-induced ovarian damage. As shown in Fig. 1, administration of ICA (10 mg/kg) alone had no impact on serum AMH levels. Cisplatin (7 mg/kg) injection significantly reduced serum AMH by 49% compared to the control group. Conversely, co-administration of ICA with CP mitigated this effect and enhanced AMH levels by 44 and 52% as compared to CP-alone group. There were no significant differences in serum estradiol levels between all treatment groups.



**Fig. 1** Effect of ICA administration on serum hormones level in CP-induced ovarian impairment in rats. (A) AMH (ng/ml) level and (B) estradiol (pg/mg) level in different experimental groups. Data are expressed as mean  $\pm$  SD (n=6). <sup>a, b, c</sup> indicate significant differences from control, ICA (10 mg/kg), or CP (7 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

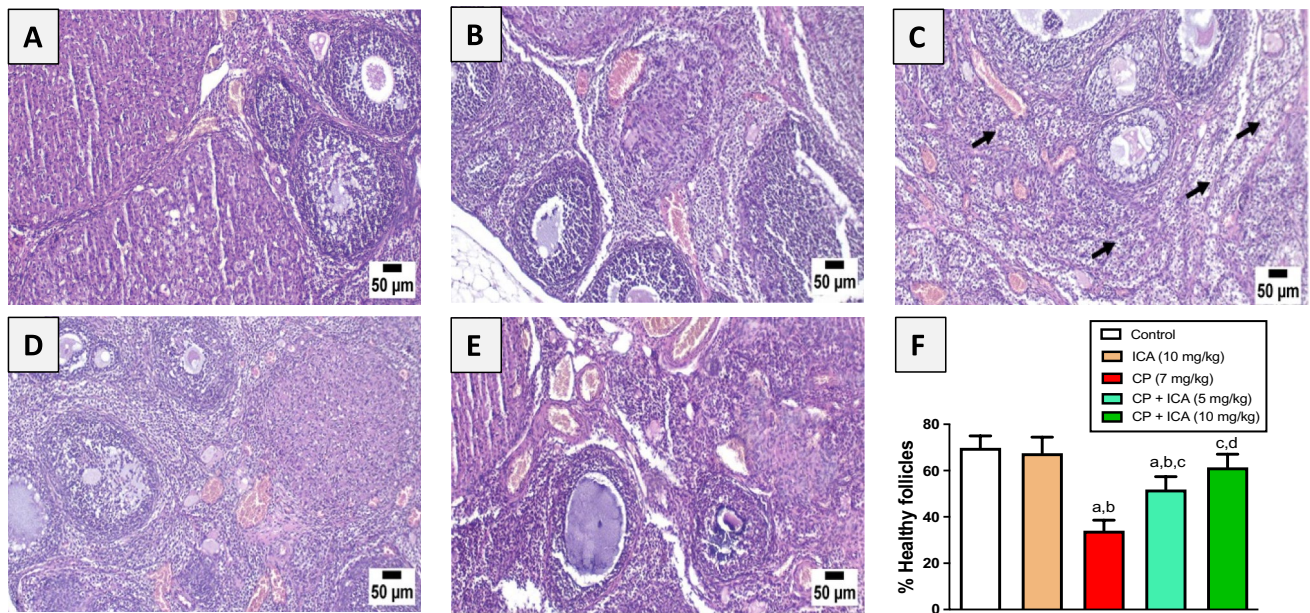
### Histopathological examination

Microscopic examination of ovaries from the control group (Fig. 2A) revealed normal histology of rat ovaries that exhibited numerous corpora lutea with growing follicles in different stages of maturation and a few interstitial tissue cells. Likewise, the ovaries of rats from the ICA alone (10 mg/kg) group (Fig. 2B) were also histologically normal. Regarding the CP (7 mg/kg) treated group (Fig. 2C), the examined sections showed marked histopathological alterations; interstitial cell hyperplasia was noticed, manifested by large polygonal pale vacuolated cells in between growing follicles. Ovarian fibroplasia and fibrous strands in the ovarian tissue were also seen. Moderate improvement was noticed in the CP + ICA (5 mg/kg) co-treated group (Fig. 2D), as the examined sections exhibited mild and limited interstitial cell hyperplasia. The best protective action was detected in the CP + ICA (10 mg/kg) co-treated group (Fig. 2E), as all examined sections showed normal architecture.

Figure 2F demonstrates the effects of the four treatments on the ovarian tissues of rats as a % of healthy follicles. The combined administration of CP and ICA helped in retaining the health of the follicles. The group that was administered ICA (10 mg/kg) did not show a change in the percentage of healthy follicles compared with the control group. Cisplatin (7 mg/kg) reduced the follicle count by 52% compared to the control group. In contrast, the groups treated with CP + ICA (5 mg/kg) and CP + ICA (10 mg/kg) had significantly more healthy follicles (52% and 80%, respectively) than the CP group.

### Assessment of oxidative stress biomarkers

To assess the effect of ICA on CP-induced ovarian oxidative damage, the lipid peroxidation biomarker MDA, along with antioxidant enzymes SOD and CAT, were measured in ovarian tissues using ELISA kits. Table 2 demonstrates that ICA treatment (10 mg/kg) did not significantly alter oxidative stress markers. However, the ovarian MDA content was significantly increased by 55%, while the levels of ovarian SOD and CAT were markedly reduced by 48% and 47% respectively as compared to control values following CP administration (7 mg/kg). Notably, the oxidant/antioxidant balance in the ovaries of CP-intoxicated rats was effectively restored by ICA co-treatment (5 and 10 mg/kg), as shown by a significant decrease in MDA levels (approximately 27% and 38% respectively) as compared to CP-alone group. This was paralleled by enhancements of SOD (37% & 63%) and CAT (43% & 65%) activities in the ovarian tissues by ICA (5 or 10 mg/kg, respectively) as compared to CP-alone values.



**Fig. 2** Representative photomicrographs of ovarian sections showing the effect of ICA administration on CP-induced ovarian impairment in rats. The ovarian sections stained with hematoxylin and eosin (H&E) are shown from the following groups: (A) The control group has a normal architecture of the ovaries. (B) ICA 10 mg/kg group with no observable histological changes. (C) CP 7 mg/kg group showing inflammation in the ovarian follicles. Arrows point to marked interstitial cells hyperplasia as cords and clusters of vacuolated cells occupying the ovarian tissue with increasing numbers of

atretic follicles. (D) CP group co-treated with ICA 5 mg/kg showing almost normal follicles and corpora lutea with mild interstitial cells hyperplasia. (E) CP group co-treated with ICA 10 mg/kg showing healthier development. (F) Effect of ICA on the percentage of healthy follicles in CP-induced ovarian impairment in rats. Data are expressed as mean  $\pm$  SD (n=6). a, b, c, d indicate significant differences from control, ICA (10 mg/kg), CP (7 mg/kg), or CP + ICA (5 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

**Table 2** Effect of ICA administration on ovarian oxidative stress biomarkers in CP-induced ovarian impairment in rats

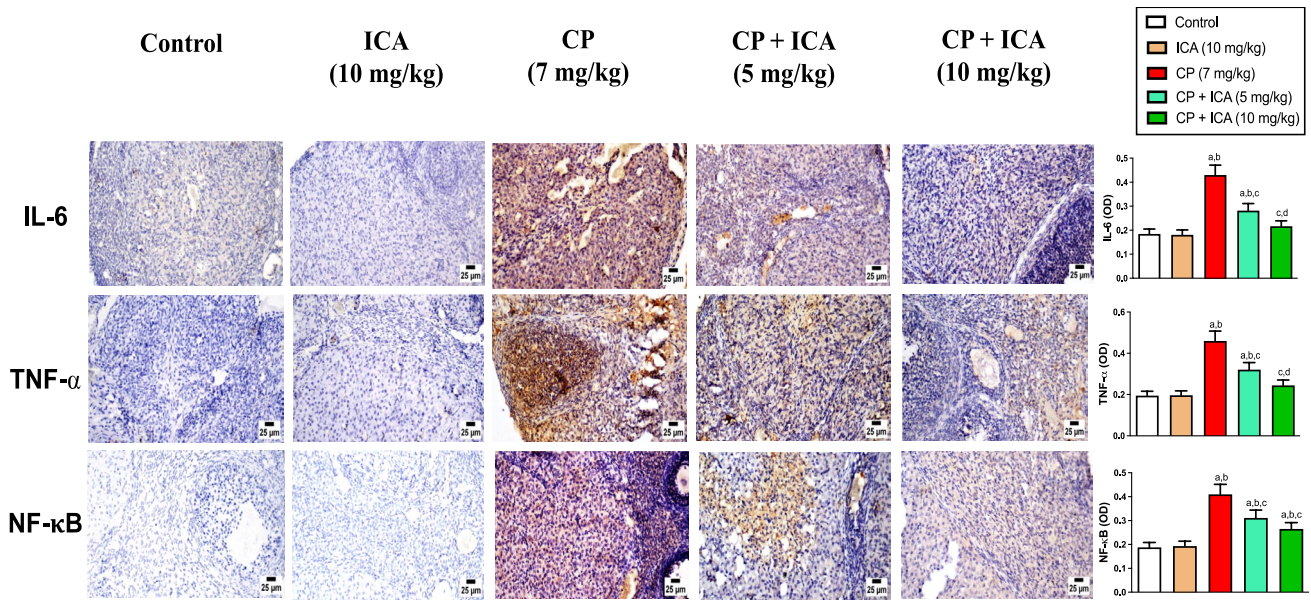
Group	MDA (nmol/mg Protein)	SOD (U/mg Protein)	CAT (U/mg Protein)
Control	2.90 $\pm$ 0.30	6.60 $\pm$ 0.70	51.30 $\pm$ 5.60
ICA (10 mg/kg)	2.65 $\pm$ 0.25	6.50 $\pm$ 0.68	53.00 $\pm$ 6.10
CP (7 mg/kg)	4.50 $\pm$ 0.44 <sup>a,b</sup>	3.50 $\pm$ 0.37 <sup>a,b</sup>	27.20 $\pm$ 3.10 <sup>a,b</sup>
CP+ICA (5 mg/kg)	3.30 $\pm$ 0.36 <sup>c</sup>	4.80 $\pm$ 0.50 <sup>a,b,c</sup>	39.00 $\pm$ 4.00 <sup>a,b,c</sup>
CP+ICA (10 mg/kg)	2.80 $\pm$ 0.29 <sup>c</sup>	5.70 $\pm$ 0.58 <sup>c</sup>	45.00 $\pm$ 4.70 <sup>c</sup>

Data are expressed as mean  $\pm$  SD (n=6). a, b, c indicate significant differences from control, ICA (10 mg/kg), or CP (7 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

**Immunohistochemical determination of IL-6, TNF- $\alpha$  and NF $\kappa$ B (p65)**

The anti-inflammatory effects of ICA against CP-induced ovarian inflammation were investigated. Figure 3 demonstrates that the control group displayed only slight immunopositivity for IL-6, TNF- $\alpha$ , and NF- $\kappa$ B. The expression levels of these inflammatory markers were unchanged when rats were treated with ICA alone (10 mg/kg). However, CP injection (7 mg/kg) significantly increased the expression of inflammatory markers IL-6, TNF- $\alpha$ , and NF- $\kappa$ B (in the evaluated nuclei), highlighted by strong

brown staining. Quantification of optical densities showed significant increases in IL-6, TNF- $\alpha$ , and NF- $\kappa$ B (p65) expression by 132%, 135%, and 118%, respectively, compared to respective control values. Co-administration of ICA (5 mg/kg) to CP treatment significantly reduced the immunostaining intensity for IL-6, TNF- $\alpha$ , and NF- $\kappa$ B (p65), which dropped by 35%, 31% and 25%, respectively, compared to the CP group. Increasing the ICA dose to 10 mg/kg further decreased the immunostaining for IL-6, TNF- $\alpha$ , and NF- $\kappa$ B (p65), which declined by 50%, 47%, and 36%, respectively, compared to the CP group.



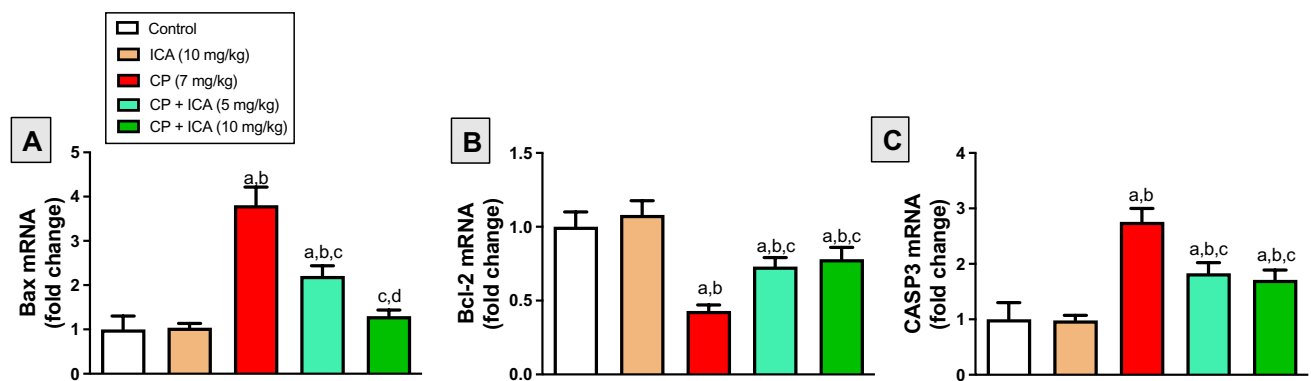
**Fig. 3** Effect of ICA administration on the expression of inflammatory markers in ovarian sections. This figure presents photomicrographs of IL-6 (upper panel), TNF- $\alpha$  (middle panel), and NF- $\kappa$ B (lower panel) immune reactions in different experimental groups. Bar charts represent the semi-quantification of the expression of corre-

sponding proteins. Data are expressed as mean  $\pm$  SD (n=6). a, b, c, d indicate significant differences from control, ICA (10 mg/kg), CP (7 mg/kg), or CP + ICA (5 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

**Assessment of mRNA expression of Bax, Bcl-2 and CASP3**

To assess the effect of ICA on apoptosis, the mRNA expression levels of the apoptotic markers Bax, Bcl-2, and CASP3 were evaluated by RT-PCR in ovarian tissues (Fig. 4). Treating rats with ICA alone (10 mg/kg) did not affect the expression level of these apoptosis markers relative to the control. Conversely, the administration of CP (7 mg/kg) resulted in an increased mRNA expression of Bax (a pro-apoptotic protein) by 280% in comparison to

the control (Fig. 4A). Interestingly, co-treatment of the CP group with ICA at doses of 5 and 10 mg/kg significantly reduced this increase in Bax mRNA expression by 42% and 66%, respectively, compared to the CP group. Moreover, the CP (7 mg/kg) group exhibited a significant decrease in the mRNA expression level of Bcl-2 (an anti-apoptotic protein) by 57% compared to the control (Fig. 4B). In contrast, ICA co-administration at 5 and 10 mg/kg doses markedly enhanced the Bcl-2 mRNA expression by 69% and 81%, respectively, compared to the CP group.



**Fig. 4** Effect of ICA administration on mRNA expression of apoptotic markers in CP-induced ovarian impairment in rats. (A) Bax, (B) Bcl-2, and (C) CASP3 in different experimental groups. Data are expressed as mean  $\pm$  SD (n=6). a, b, c, d indicate significant differences

from control, ICA (10 mg/kg), CP (7 mg/kg), or CP + ICA (5 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

Additionally, CP (7 mg/kg) injection led to a significant elevation in CASP3 mRNA expression by 176% relative to the control (Fig. 4C). As expected, co-administration with ICA at 5 and 10 mg/kg provided considerable protection against this increase by 34% and 39%, respectively, compared to the CP group.

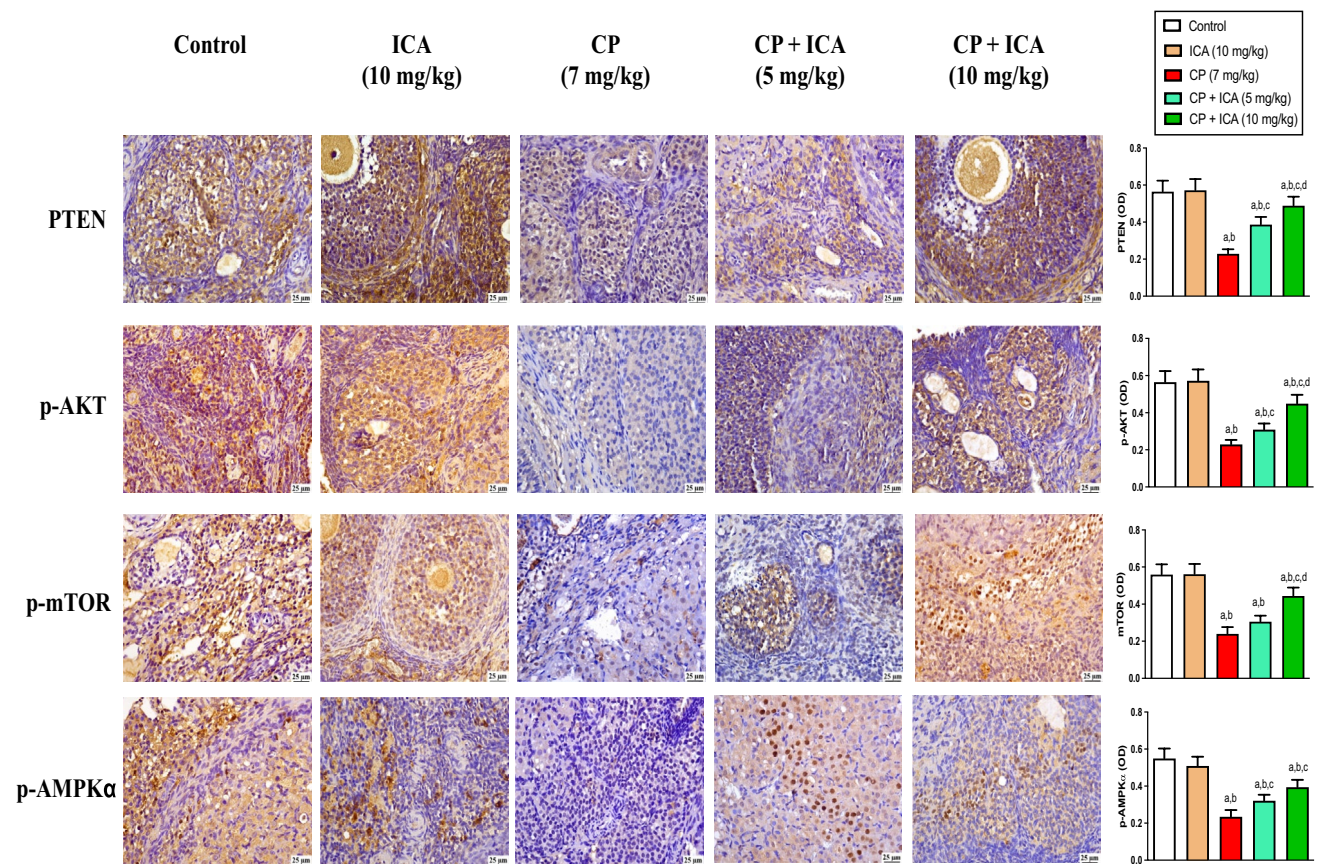
**Immunohistochemical determination of PTEN, AKT, mTOR and AMPK**

Figure 5 demonstrates no notable difference between the ICA and the control groups. However, rats that received CP (7 mg/kg) exhibited a significant decrease in immunostaining for PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  with reductions of 60%, 60%, 57%, and 58%, respectively, when compared to the control group. When ICA (5 mg/kg) was co-administered, there was a marked increase in the levels of PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  increasing by 68%, 34%, 26%, and 37%, respectively, in comparison to the CP treated group. Importantly, using a higher dose of ICA (10 mg/kg) with CP led to even more significant

enhancements in PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  expression by 113%, 95%, 84%, and 68%, respectively, compared to the CP treated group.

**Discussion**

The advent of chemotherapeutic agents like CP has markedly improved cancer survival rates. However, the resultant off-target effects, particularly ovarian toxicity, pose significant challenges, especially for female patients of reproductive age (Chow et al. 2016). This has driven a search for mitigating strategies and increased interest in phytochemicals known for their extensive therapeutic potential and minimal side effects. Our study focused on ICA, a flavonoid known for its multifaceted pharmacological activities, including anti-oxidative and anti-inflammatory effects (Li et al. 2010; He et al. 2020; Seyedi et al. 2023). Here, we demonstrated ICA's effectiveness in alleviating CP-induced ovarian damage by reducing histological damage and improving ovarian



**Fig. 5** Effect of ICA administration on ovarian PTEN/Akt/mTOR/AMPK signaling pathway in CP-induced ovarian impairment in rats. Bar charts represent the semi-quantification of the expression of corresponding proteins. Data are expressed as mean  $\pm$  SD (n = 6). a, b, c, d

indicate significant differences from control, ICA (10 mg/kg), CP (7 mg/kg), or CP + ICA (5 mg/kg), respectively, at  $p < 0.05$  using one-way ANOVA followed by Tukey post-analysis

function markers such as AMH while modulating key signaling pathways involved in cellular survival and inflammation.

In evaluating the protective effects of ICA against CP-induced ovarian toxicity, this study assessed biochemical markers and histological changes in ovarian tissues. It was observed that serum AMH levels, indicative of the quantity of primordial ovarian follicles, were significantly declined following CP administration (Kevenaar et al. 2006). ICA treatment restored serum AMH levels to almost normal values, highlighting its capacity to shield against CP-induced ovarian damage—a finding supported by earlier reports (Li et al. 2015b; Jiang et al. 2019). Despite the observed changes in AMH levels, serum estradiol levels remained stable across all groups. This suggests that the protective mechanism of ICA may specifically target the preservation of ovarian reserve without altering the overall hormonal balance. Histological analysis revealed substantial ovarian damage in response to CP exposure, notably a reduced count of healthy primordial ovarian follicles. This observation aligns with previous findings indicating CP's impact on ovarian primordial follicle numbers (Chang et al. 2015). However, our data revealed that ICA treatment effectively mitigated these histopathological changes. The administration of ICA, particularly at higher doses, significantly preserved the count of healthy follicles in a dose-dependent manner. These findings are in line with previous studies marking the ability of ICA to protect against experimentally-induced ovarian injury induced by D-galactose (Li et al. 2019) and CP (Li et al. 2024).

Oxidative stress is a critical factor in CP-induced toxicities, substantially impairing the antioxidant defense system and raising the production of ROS, which in turn triggers apoptosis through increased release of pro-apoptotic factors (Santos et al. 2007; Hassanein et al. 2022; Ali et al. 2023). Specifically, CP has been shown to elevate MDA levels while decreasing the activities of critical free radical scavenging enzymes such as SOD and CAT in ovarian tissues, confirming its role in ovarian toxicities in rat models (Altuner et al. 2013). This insight builds on our prior findings, where ICA was shown to mitigate carrageenan-induced acute inflammation by enhancing both enzymatic and non-enzymatic antioxidants (El-Shitany and Eid 2019). Additionally, previous reports emphasize ICA's effectiveness in countering CP-induced oxidative stress and cell damage (Zhou et al. 2019). ICA has been shown to explicitly attenuate oxidative stress by modulating antioxidants such as glutathione peroxidase (GSH-Px), CAT, and SOD and lowering MDA levels (Yoon et al. 2021), thereby diminishing the detrimental effects of CP (Zhou et al. 2019; Xia et al. 2022). Our current study extends these observations by confirming ICA's capacity to alleviate oxidative damage, as evidenced by its impact on enhancing antioxidant enzyme activities (SOD and CAT) and reducing MDA levels in the ovaries of CP-treated rats.

CP-induced release of oxidative stress has been linked to inflammatory pathways, notably activating NF- $\kappa$ B, which is implicated in organ toxicities (Abdel-Wahab et al. 2021; Ali et al. 2021; Sami et al. 2022; Ramadan et al. 2023). In line with these findings, a previous study showed that CP injection elevated expression of pro-inflammatory cytokines like IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in ovarian tissues (Algendaby 2021). Interestingly, our findings showed that ICA effectively reduces NF- $\kappa$ B expression along with diminishing the expression of IL-6 and TNF- $\alpha$  in ovarian tissues in a dose-related fashion. This suggests ICA's effectiveness in reducing the pro-inflammatory impacts triggered by CP. Such findings align with prior research recognizing ICA's anti-inflammatory effects, further establishing its role as an effective countermeasure to CP-induced inflammatory harm (Deng et al. 2017; El-Shitany and Eid 2019; Zhang et al. 2021).

This study explored ICA's critical function in protecting ovarian tissues from CP-induced apoptosis, specifically through its interactions with the Bcl-2 family of proteins essential for regulating apoptosis. This family includes pro-apoptotic proteins such as Bax, which initiate apoptosis via caspase activation, and anti-apoptotic proteins like Bcl-2, which block apoptosis by preventing Bax translocation to mitochondria (Deng et al. 2017). Our findings revealed that CP significantly altered the critical balance within ovarian tissues, enhancing Bax and decreasing Bcl-2 mRNA expression, amplifying apoptotic activities. This highlights the complex regulation of apoptosis essential for cellular health, reflecting similar outcomes observed in previous research (Hengartner 2000; Cory and Adams 2002; Deng et al. 2017). Further, a rise in CASP3 mRNA expression was observed following CP insult, confirming apoptosis progression. However, these observations were significantly mitigated by ICA treatment. This aligns with previous studies, reinforcing ICA's anti-apoptotic efficacy (Shao et al. 2022; Wu et al. 2023). The ability of ICA to reverse CP-induced changes in gene expression related to apoptosis suggests a complex mechanism of action.

PTEN/AKT/mTOR/AMPK signaling pathway plays a vital role in cellular defenses against chemotherapy's oxidative and inflammatory impacts, influencing crucial cellular processes such as proliferation, survival, and metabolism (Zhou et al. 2017). Previous studies highlighted CP's detrimental effects on this pathway, mainly showing that a significant reduction in the mRNA expression levels of AKT, mTOR, and PTEN in ovarian tissues, contributes to ovarian toxicity (Al-Shahat et al. 2022). Our immunohistochemical analysis corroborates these findings, revealing a substantial decrease in the immunostaining of PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  in CP-treated rats. This disruption in signaling pathways is likely pivotal in reducing ovarian primordial follicles and the subsequent onset



of premature ovarian failure (Chang et al. 2015). In contrast, the co-administration of ICA with CP effectively counteracted the negative impacts of CP, showing a dose-dependent upregulation of PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$ . Data on ICA's impact on PTEN expression are controversial (Zou et al. 2020; Al-Shahat et al. 2022). Notably, ICA has been shown to enhance the expression of the Akt/mTOR signaling pathway and reduce autophagy and apoptosis in cisplatin-resistant ovarian cancer cells (Jiang et al. 2019). Additionally, ICA influences the proliferation and apoptosis of human ovarian cancer cells by increasing the expression of targeting proteins such as PTEN and Bcl-2 (Li et al. 2015b). These findings suggest that ICA's modulation of this signaling pathway could be a promising strategy for protecting against CP-induced ovarian toxicity, highlighting its potential therapeutic benefits in enhancing the efficacy and safety of chemotherapy treatments.

## Conclusion

In conclusion, our study highlights the potential of ICA to mitigate the adverse effects of CP on ovarian tissues, demonstrating its potential as a viable adjunct therapy in chemotherapy. Administration of ICA was associated with attenuation of the histopathological changes, enhancing serum AMH levels, and restoring the oxidant-anti-oxidant balance in ovarian tissues. Furthermore, ICA effectively exhibited anti-inflammatory and anti-apoptotic activities. Additionally, ICA enhanced PTEN, p-AKT, p-mTOR, and p-AMPK $\alpha$  expression. These multifaceted protective effects justify further substantiation of ICA's efficacy in preserving ovarian reserve in CP-treated patients.

**Author contributions** B.E.: Project administration, Data curation, Methodology, Formal analysis, Funding acquisition. L.B.: Formal analysis, Data curation, Methodology. R.S.: Methodology, Investigation, Validation. A.B.: Data analysis, Investigation, Methodology, Software. A.S.: Data curation, Investigation, Methodology. A.A.: Funding acquisition, Methodology, Investigation. All authors reviewed and revised the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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**Data availability** The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Ethical approval** The study received ethical approval from the Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (PH-1442–53).

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