

# Chrysin's dose-dependent effects on steroidogenesis in female BALB/c mice: *In vivo* study of adrenal, ovarian, and uterine hormone regulation

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## Abstract

**Background:** Chrysin is known for its pharmacological effects and structural resemblance to estrogen. The study explores the impact of chrysin on steroidogenesis focusing on adrenal and ovarian steroidogenic enzymes.

**Materials and methods:** Thirty female BALB/c mice were divided into three groups: a control group and two chrysin-treated groups (50 mg and 100 mg). Gene expression of key steroidogenic enzymes was assessed in adrenal glands and ovaries using RT-qPCR. Uterine expression of estrogen receptor alpha (ER $\alpha$ ) was also examined. Histological analysis of adrenal glands and ovaries was performed.

**Results:** High-dose chrysin downregulated CYP17A1 expression in adrenal glands compared to control and low-dose groups. In contrast, 3 $\beta$ -HSD was significantly downregulated in the high-dose group. In ovaries, high dose chrysin reduced aromatase expression.

**Conclusion:** Our findings revealed that chrysin's impact on steroidogenesis is dose-dependent. By downregulating CYP17A1 in adrenal glands, potentially affecting androgen and estrogen synthesis, and enhancing aromatase expression in ovaries at lower doses.

## Keywords

Adrenal gland, Chrysin, Endocrine disrupting compounds, Ovary, Steroidogenesis, Toxicity

## Introduction

The adrenal gland is the most frequently affected target organ in toxicological assessments of the endocrine system in living organisms, yet it remains overlooked in regulatory screening and testing for endocrine disruption (Harvey et al. 2007; Petruska et al. 2021). Harmful substances that impact the adrenal cortex encompass short-chain aliphatic compounds, inducers of lipidosis,

amphiphilic compounds, both natural and synthetic steroids, as well as compounds that disrupt hydroxylation processes. Assessing the morphology of cortical lesions offers valuable information regarding the specific points of inhibition in steroidogenesis (Rosol et al. 2001; Yilmaz et al. 2019).

Endocrine-disrupting chemicals (EDCs) are external substances that disrupt the normal functioning of hormones of the endocrine gland, leading to an elevated

risk of negative health consequences, such as cancer, reproductive problems, cognitive deficits, and obesity (La Merrill et al. 2019; Vieira et al. 2021). EDCs include some pesticides, fungicides, industrial chemicals, plasticizers, nonylphenols, metals, pharmaceutical agents and phytoestrogens (Yilmaz et al. 2019). The mechanisms by which EDCs are proposed to interfere with the endocrine system include direct interaction with the gland itself by altering hormone synthesis and/or metabolism. In addition to their inhibitory action affecting receptor-mediated hormones producing an antagonistic effect, or their stimulatory action by mimicking the hormone-producing an agonistic effect (Lee et al. 2013; Mimoto et al. 2017; Vieira et al. 2021).

Flavonoids and isoflavonoids, which belong to the category of polyphenolic compounds, are versatile natural substances. They constitute a significant portion of the secondary metabolites synthesized by higher plants and are abundant components in the human diet (Hodgson et al. 1996). Chrysin (5,7-dihydroxyflavone), is a naturally occurring polyphenol found in various plants, honey, and propolis, that exhibits a wide range of pharmacological effects, including but not limited to its potential as an anticarcinogenic, pro-apoptotic, antiangiogenic, antimetastatic, immunomodulatory, and antioxidant agent (Mani and Natesan 2018). Despite their beneficial effects, Isoflavones are classified as endocrine disruptors due to their structural resemblance to 17 $\beta$ -estradiol, which imparts them with potent estrogenic characteristics capable of influencing various organs, including the reproductive tract.

Some isoflavones, including chrysin, demonstrated the ability to hinder the conversion of androstenedione and testosterone into estrogens, which is catalyzed by human placental and ovarian microsomes. Their inhibitory potency, quantified by half maximal inhibitory concentration (IC<sub>50</sub>) values, fell within the micromolar range (Kellis and Vickery 1984). It has been reported that the administration of chrysin to rats during pregnancy caused damaging effects on the adrenocortical cells of their fetuses (Sabry 2023a).

However, the exact mechanism of adrenal toxicity and the effect of chrysin on adrenal gland steroidogenesis was not investigated. Therefore, in this study, we aim to investigate the effect of chrysin on key steroidogenic enzymes in both the adrenal gland and ovaries of BALB/c mice *in vivo*.

## Materials and methods

### Animal study

A total of thirty, sexually-mature female Bagg Albino (BALB/c) mice weighing 20–25 gm were acclimatized to the conditions of the animal room (23  $\pm$  2  $^{\circ}$ C, 12 hr dark/12 hr light). Chrysin (Sigma, USA) was administered intraperitoneally (i.p) after suspension in 1% sodium carboxymethylcellulose (CMC) (Sigma, USA). The i.p route was chosen due to poor intestinal absorption of chrysin. Animals received the following treatments for 30 days: Group 1: Vehicle (1% CMC), Group 2: 50 mg/kg chrysin, Group 3: 100 mg/kg chrysin. The choice of doses was based on previous studies. (Abbas et al. 2022) After 30 days mice were dissected, and their ovaries and adrenals were kept at -80  $^{\circ}$ C until used for gene expression study. All animal experiments were approved by the ethical committee for scientific research at Al-Ahliyya Amman University (Ethical approval number: 1/4/2019–2020).

### Gene expression of steroidogenic enzymes

RNA was extracted from adrenal, ovary, and uterus tissues using Quick-RNA MiniPrep Quick (ZYMO Research-R1054, China) following the manufacturer's instructions. cDNA was obtained using the PrimeScript RT Master Mix kit (TaKaRa-RR036A, Japan) as mentioned by the manufacturer's instructions. For gene expression, RT-qRT-PCR was used and performed using TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa-RR820L, Japan). Specific primers were designed to study the expression of the target genes (Table 1) and ordered from Macrogen, South Korea.

Before use, all the components were mixed evenly by gently inverting many times. TB Green Premix Ex Taq II (Tli RNaseH Plus) was gently handled and was allowed to stand protected from light. A total volume reaction of 20  $\mu$ L was prepared based on the manufacturer's instruction as follows: 6.32  $\mu$ L RNase/DNase free water, 10  $\mu$ L Green Premix Ex Taq II, 1  $\mu$ L PCR forward Primer, 1  $\mu$ L PCR reverse primer, 0.08  $\mu$ L ROX Reference Dye and 2  $\mu$ L template (cDNA) were added to each microplate well. The microplate was then inserted into Applied Biosystems 7500 and

**Table 1.** Sequences of primers used to examine gene expression.

Gene	Forward (5' $\rightarrow$ 3')	Reverse (5' $\rightarrow$ 3')
$\beta$ -actin	GGGCACAGTGTGGGTGAC	CTGGCACCACACCTTCTAC
P450scc	AGCCGTGACCAGAAAAGACA	ACCTGGGCAGGTAATCACAG
CYP17A1	CCAGGACCCAAGTGTGTTCT	CCTGATACGAAGCACTTCTCG
3 $\beta$ -HSD	CAGGAGAAAGAACTGCAGGAGGTC	GCACACTTGCTTGAACACAGGC
Aromatase	CATGGTCCCGAAACTGTGA	GTAGTAGTTGCAGGCACTTC
ER $\alpha$	TCTGCCAAGGAGACTCGCTACT	GGTGCATTGGTTTGTAGCTGGAC

\*P450scc, cholesterol side-chain cleavage enzyme; CYP17A1, cytochrome P450 family 17 subfamily A member 1; 3 $\beta$ -HSD, 3 $\beta$ -Hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase; ER $\alpha$ , Estrogen receptor alpha.

stepOnePlus Real-Time PCR System. The amplification protocol consisted of stage (1) initial denaturation which lasts for 1 repetition at 95 °C for 30 seconds, stage (2) PCR which lasts for 49 repetitions at 95 °C for 5 seconds and 60 °C for 34 seconds, and stage (3) the dissociation stage. All experiments were repeated at least twice for reproducibility.

## Histological study

Tissues were stored in a 10% buffered formalin solution for the preparation of histological slides. Formalin-fixed, paraffin-wax embedded serial sections (4 µm thick) of ovaries and adrenal glands were stained with hematoxylin and eosin (H & E) stain. After staining sections were examined using a Leica microscope and photographed using MC 170 HD Leica camera, Switzerland, and LAS EZ software.

## Statistical analysis

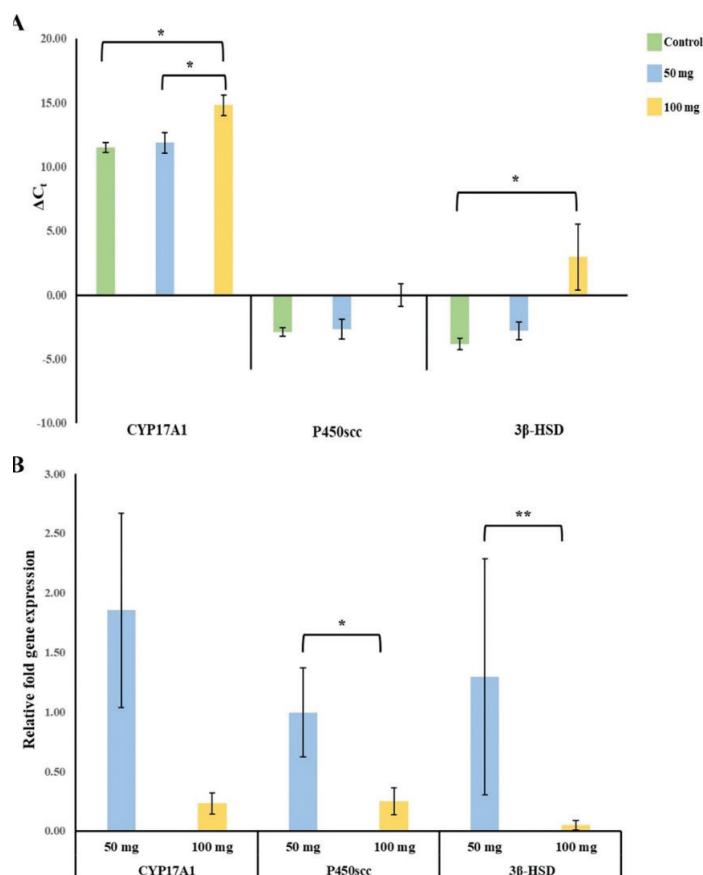
The statistical analyses were performed using GraphPad Prism 7 Software. The normality of the distribution of variables was checked by the Kolmogorov-Smirnov and Shapiro-Wilk tests. For normally distributed data, the significance of differences between groups was assessed using a one-way analysis of variance (ANOVA), followed by

Tukey's multiple comparisons test, and an unpaired t-test was used to compare the means of two groups. For the data that deviated from normal distribution, the Kruskal-Wallis test was used to analyze data between the groups followed by Dunn's multiple comparisons test, and the Mann-Whitney test for calculating the significant differences between the two groups. All the results were expressed as the mean and standard error of the mean (SEM), and a *P*-value less than 0.05 was considered statistically significant.

## Results

### Effect of Chrysin on steroidogenic enzymes' gene expression in the adrenal gland

In the adrenal gland, a high dose of chrysin (100 mg/kg) significantly decreased the expression of CYP17A1 compared to both the control group and the group treated with 50 mg chrysen (P < 0.05) (Fig. 1A). However, when CYP17A1 relative expression (Fig. 1B) was compared between the two treated groups, there was no significant difference with lower expression in the group treated with 100 mg chrysin (P = 0.065).



**Figure 1.** The expression of target genes in the adrenal gland of female mice is expressed as **A.** Delta cycle threshold ( $\Delta C_t$ ) and **B.** Relative fold gene expression.  $\beta$ -actin was used as a housekeeping gene for calculating  $\Delta C_t$  and relative fold gene expression. \*  $P \leq 0.05$ , \*\*  $P \leq 0.01$ . *CYP17A1*, cytochrome P450 family 17 subfamily A member 1; *P450scc*,<sup>1</sup>cholesterol side-chain cleavage enzyme; *3β-HSD*, 3β-Hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase.

Regarding P450scc, there was a significant difference in the expression of this enzyme when compared between all groups ( $P = 0.0327$ ) with the lowest expression in the 100 mg group (Fig. 1A). Furthermore, there was 4-fold less expression of P450scc in the 100 mg chrysin-treated group compared to the group treated with 50 mg chrysin ( $P = 0.04$ ) (Fig. 1B). Similarly, there was a significant difference in the expression of  $3\beta$ -HSD between all groups ( $P = 0.03$ ) with a high significant difference in the  $3\beta$ -HSD expression 27-fold higher in the 50 mg treated group compared to the 100 mg treated group (Fig. 1B).

### Effect of Chrysin on steroidogenic enzymes' gene expression in ovary

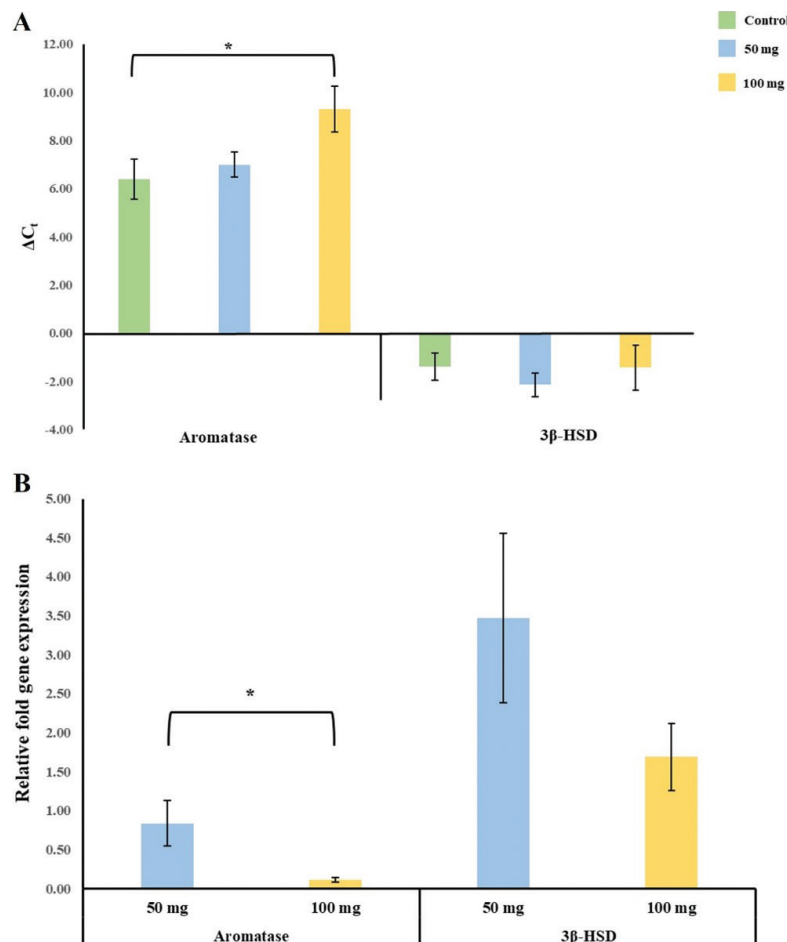
In the ovary, a significant difference in the expression of aromatase ( $p = 0.0193$ ) was found only in a high dose of chrysin (100 mg/kg) but not with the lower dose (50 mg/kg), while no effect on  $3\beta$ -HSD expression was found (Fig. 2A). Moreover, there was a significant 7-fold lower relative expression of aromatase in the group treated with the high dose of chrysin ( $P = 0.043$ ) (Fig. 2B).

### The effect of Chrysin treatment on the expression of er2 $\alpha$ in the uterus

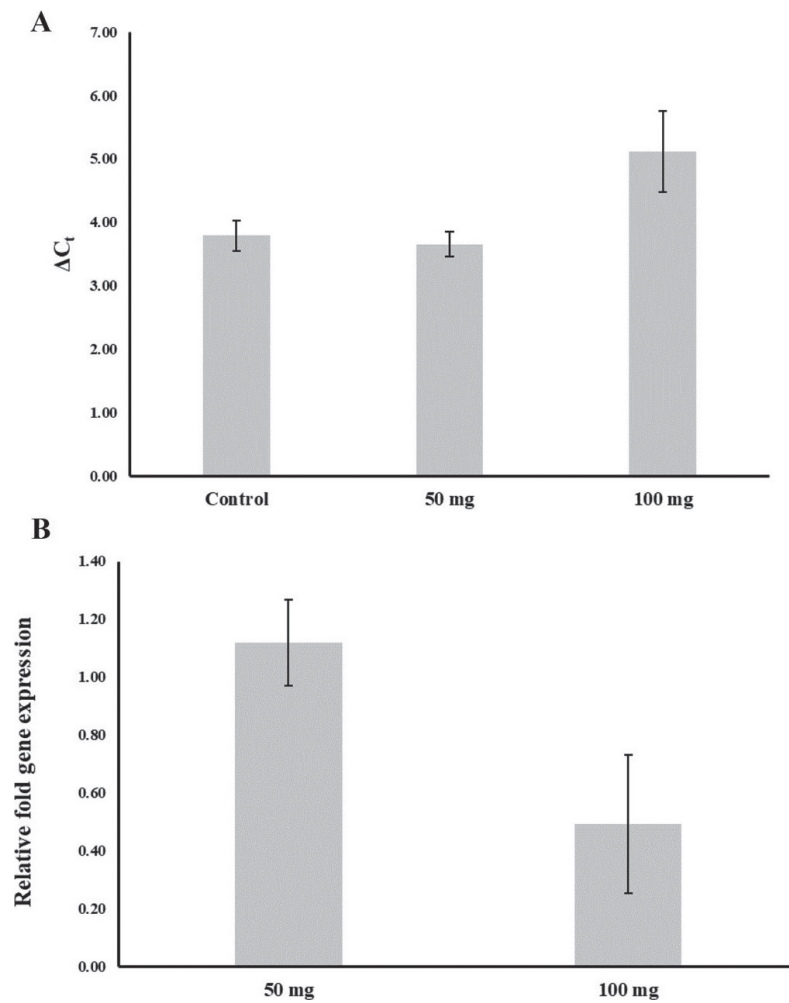
As shown in a previous study (Abbas et al. 2022), there was an effect of chrysin treatment on the histology of the uterus, therefore, the expression of ER2 $\alpha$  expression was examined in this study. There was no significant difference in the expression of this receptor in the treated groups compared with the control group ( $P = 0.34$ ). Nevertheless, the expression of this receptor was less in the high-treated group (100 mg chrysin) as shown in Fig. 3A, B.

### Effect of Chrysin on Adrenal Gland Histology

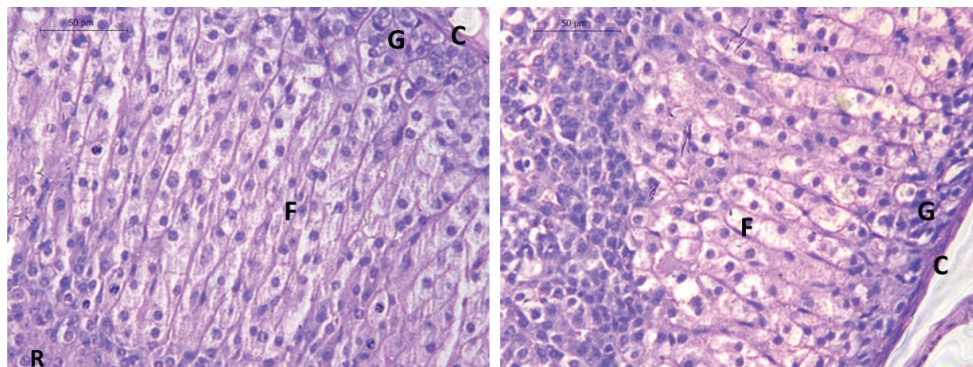
The capsule and underlying closely arranged cells of the zona glomerulosa with acidophilic cytoplasm and rounded densely stained nuclei are seen. Large polyhedral fasciculate cells are arranged in long straight columns. They have acidophilic, vacuolated cytoplasm and vesicular rounded nuclei with prominent nucleoli. Zona reticularis cells are small, closely packed with deeply stained nuclei arranged in anastomosing cords (Figs 4, 5).



**Figure 2.** The expression of target genes in the ovary of female mice is expressed as **A.** Delta cycle threshold ( $\Delta C_t$ ) and **B.** Relative fold gene expression.  $\beta$ -actin was used as a housekeeping gene for calculating  $\Delta C_t$  and relative fold gene expression. \*  $P \leq 0.05$ .  $3\beta$ -HSD,  $3\beta$ -Hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase.



**Figure 3.** The expression of estrogen receptor- $\alpha$  (ER $\alpha$ ) in the uterus of female mice is expressed as **A**. Delta cycle threshold ( $\Delta C_t$ ) and **B**. Relative fold gene expression.  $\beta$ -actin was used as a housekeeping gene for calculating of  $\Delta C_t$  and relative fold gene expression.

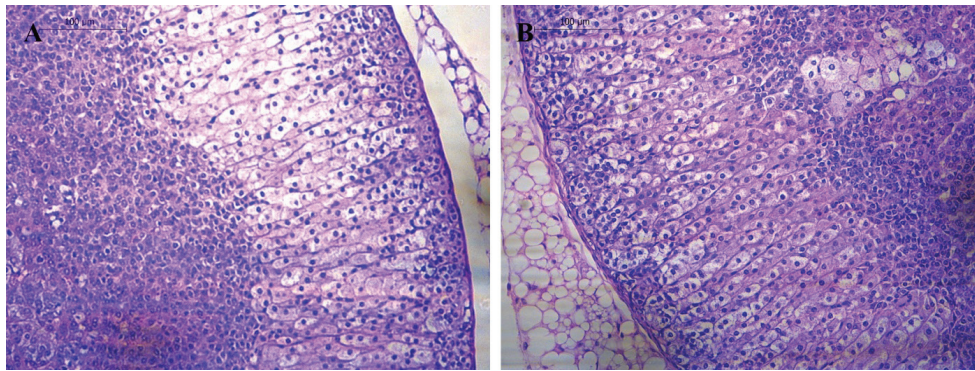


**Figure 4.** Effect of chrysin on adrenal gland histology. Stained section of the control adrenal cortex showing a thin capsule (C) with underlying zona glomerulosa (G) arranged in oval or curved clusters. Zona fasciculata cells (F) are arranged in parallel cords while cells of zona reticularis (R) with anastomosing cords (H & E stain).

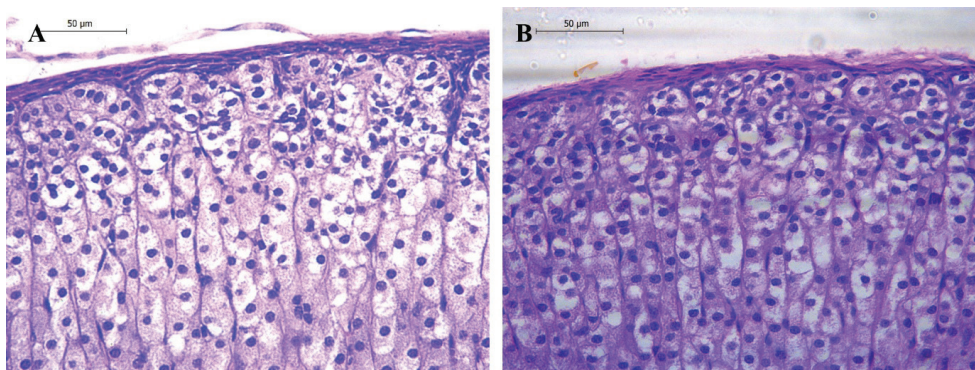
Histological analysis showed no visible changes in the adrenal cortex between controls and chrysin-treated groups (Fig. 6). In the adrenal medulla, many lesions were observed in the 100 mg chrysin-treated group compared to controls (Fig. 7). An increase in fat deposition in the abdominal cavity and around the kidney was seen in the 100 mg chrysin-treated group compared to controls.

## Discussion

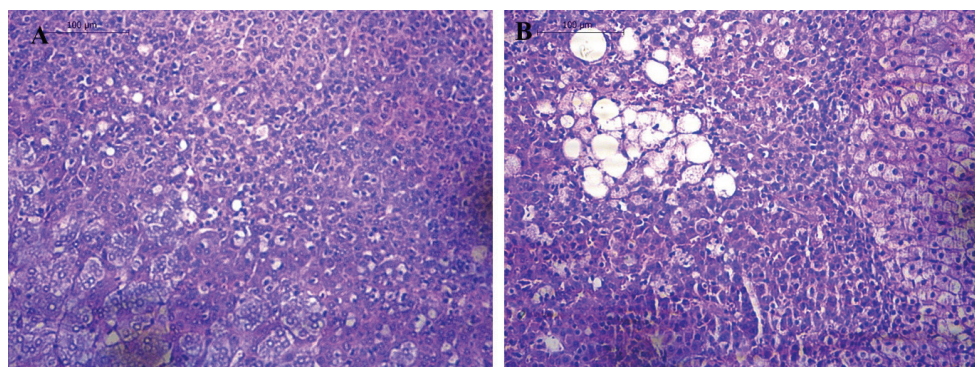
Chrysin, a naturally occurring flavonoid, is frequently employed for the treatment of organ toxicity. However, several studies demonstrated that chrysin interferes with steroid hormone synthesis (Soliman et al. 2022). To our knowledge, our study represents the first report that chrysin enhances steroidogenesis in adrenals and ovaries *in vivo*.



**Figure 5.** Histological analysis of the adrenal gland. **A.** Adrenal gland of control group; **B.** Adrenal gland of 100 mg chrysin-treated group.



**Figure 6.** Histological analysis of adrenal cortex. **A.** Adrenal cortex of the control group; **B.** Adrenal cortex of the 100 mg chrysin-treated group.



**Figure 7.** Histological analysis of the adrenal gland medulla. **A.** Adrenal medulla of control group; **B.** Adrenal medulla of 100 mg chrysin-treated group.

In this *in vivo* study, we explored the effects of low-dose (50 mg) and high-dose (100 mg) chrysin on the expression of key steroidogenic enzymes including CYP17A1, P450<sub>sc</sub>, 3 $\beta$ -HSD, in the adrenal glands of female mice, in addition to aromatase and 3 $\beta$ -HSD in the ovaries of female mice. Furthermore, we investigated the expression of ER $\alpha$  in the uterus of female mice. Our findings revealed that chrysin showed a dose-dependent downregulation of CYP17A1 expression, in which the high-dose chrysin significantly decreased the expression of CYP17A1 compared to both the control and low-dose chrysin groups. The CYP17A1 enzyme is of the cytochrome P450 family, This enzyme possesses both 17- $\alpha$ -hydroxylase and 17,20-lyase capabilities and plays a pivotal role in the steroidogenic pathway, which generates progestins,

mineralocorticoids, glucocorticoids, androgens, as well as estrogens (Hannah-Shmouni et al. 2017). When comparing the zone-specific CYP17A1 expression in the adrenals, the zona fasciculata and zona reticularis show the directed production of cortisol and dehydroepiandrosterone (DHEA) which is a precursor of testosterone and estrogen, whereas the zone glomerulosa is seen with an absence of CYP17A1 expression leading to aldosterone synthesis (Schiffer et al. 2019). While no previous studies have investigated the effect of chrysin on CYP17A1 expression in adrenal glands, findings from Hasegawa et al. study showed that apigenin (4',5,7-trihydroxyflavone), a type of flavonoids, inhibited the activity of CYP17A1 and 3 $\beta$ -HSD in an H295R human adrenal cells (Hasegawa et al. 2013). In male mice, chrysin treatment resulted in a

significant increase in sperm motility, sperm concentration, and serum testosterone levels, accompanied by a notable decrease in the rate of abnormal sperm (Ciftci et al. 2012). Additionally, exposure to chrysin during prepubertal development led to a disturbance in the regulation of androgenic and estrogen receptors in the prostate, enhancing the gland's responsiveness to the biological effects of endogenous steroids (Ribeiro et al. 2018).

However, chrysin did not reveal a significant effect on P450scc expression in our model, which was concordant with the findings of Jana et al. where they did not find a significant role of chrysin on P450scc expression in Leydig cells of a mouse model (Jana et al. 2008). Chrysin was observed to significantly elevate StAR protein levels in these cells, a factor responsible for enabling the transfer of cholesterol to the P450scc enzyme within the mitochondria. Importantly, this increase in StAR protein coincided with heightened steroid hormone production. Additionally, when protein kinase A (PKA) activity, which is involved in StAR protein expression, was inhibited, the enhanced steroidogenesis induced by chrysin in Leydig cells was reversed (Wang et al. 1998; Wang et al. 2002). Interestingly,  $\beta$ -hydroxysteroid dehydrogenase ( $\beta$ -HSD), which is well-known for its key role in steroid hormones synthesis and metabolism, was significantly downregulated by the high-dose chrysin in mice adrenals and significantly upregulated in the low-dose chrysin compared to the high-dose group with a 27-fold change difference.

Another pivotal steroidogenic enzyme is aromatase (CYP19A1), which participates in the catalytic transformation of adrenal androgens, such as testosterone and androstenedione, through a series of three consecutive hydroxylation reactions, resulting in the formation of aromatic estrogens, namely estradiol (E2) and estrone (E1) (Holschbach and Handa 2017; Lobo 2019). The effect of chrysin on aromatase was observed to significantly downregulate aromatase expression in the high-dose group compared to controls. In addition, chrysin showed a 7-fold change increase in relative expression in the low-dose group compared to the high-dose group, elucidating the chrysin dose-dependent effect on aromatase expression. It has been previously demonstrated that both genistein and daidzein (major types of isoflavones), also inhibit aromatase. This inhibition has been observed in various settings, including human placental microsomes, human preadipocyte cells, and primary cultures of human granulosa-luteal cells (Campbell and Kurzer 1993; Le Bail et al. 2000). Additionally, these isoflavones have been shown to reduce aromatase expression. However, it's worth noting that there is some controversy regarding the effectiveness of these isoflavones in inhibiting aromatase, as other studies have reported that daidzein or genistein had no impact on aromatase activity (Le Bail et al. 1998; Lacey et al. 2005). This could potentially suggest that chrysin can be thought of as an inducer of aromatase expression and enhancing estrogen biosynthesis in specific low-doses, while this action can be reversed by the administration of higher doses (Amaral et al. 2017).

Interestingly, both chrysin groups (low- and high-dose), did not show significant up- or downregulation in ER $\alpha$  in mice uterus compared to no chrysin group, however, it is worth noting that the low-dose group showed a 2-fold increase in ER $\alpha$  compared to high-dose chrysin. These results are in line with a previous immunohistochemistry study by Abbas and Alqaisi et al. of Wistar rats and high-dose chrysin, confirming our findings with no difference in aromatase expression in ovaries, nor ER $\alpha$  in the uterus (Abbas et al. 2022). This concludes that chrysin poses a controversial effect on steroidogenic pathways, by enhancing some enzyme expressions such as StAR, and downregulating others such as CYP17A1. Further preclinical studies are warranted to elucidate the effect of chrysin in each steroidogenic process and the downstream effect in enzyme regulation.

Furthermore, our histological analysis revealed an increase in fat deposition in the abdominal cavity around the kidney of the chrysin group with no noticeable changes in the adrenal cortex between the two groups. These findings are in line with a study by Choi et al. showing a dual role of chrysin by enhancing brown adipose tissue phenotype, in addition to modulating lipid metabolism (Choi and Yun 2016). These results can potentially be utilized in the development of obesity-related supplements and treatments. A recent study on Wistar male rats revealed that administering chrysin led to a notable improvement in histological alterations and a substantial, dose-dependent reduction in weight gain, hyperglycemia, and insulin resistance in the obese rats (Oriquat et al. 2023). This can be further used in reducing weight gain, improving the balance of glucose and lipid levels, influencing adipokines positively, enhancing the creation of liver mitochondria, and regulating the signaling pathways of AMPK/mTOR/SREBP-1c. In addition, our histological analysis showed noticeable lesions in the adrenal medulla of the chrysin-treated group. This is consistent with findings from Sabry et al. study showing histological changes of blood sinusoids enlargement between adrenal medullary cells and zona reticularis (Sabry 2023b).

In our investigation, chrysin was administered intraperitoneally due to the low intestinal absorption of chrysin. Several *in vivo* studies also showed that the intestinal absorption of chrysin is hindered by its low aqueous solubility, and various strategies aimed at improving chrysin's solubility have been explored in an effort to enhance its absorption in the intestines (Lee et al. 2019; Gao et al. 2021). Notably, the co-encapsulation of chrysin and quercetin, a flavonoid with MRP2 inhibiting properties, could potentially enhance chrysin absorption by facilitating a transcellular mechanism (Oh et al. 2019).

Our study has several strong points as follows. First, our study employs a well-structured experimental design involving *in vivo* animal models, gene expression analysis, and histological examination to assess the effects of chrysin on steroidogenic enzymes and hormone-related parameters. Second, it provided a dose-dependent analysis by exploring the impact of two different doses of

chrysin (50 mg/kg and 100 mg/kg) on various aspects of steroidogenesis, allowing for a dose-dependent assessment of its effects. In addition, this is the first study to our knowledge that investigated the effects of chrysin on key steroidogenic enzymes in mice models. However, our findings should be reported with caution in the context of a few limitations. First, the study focuses on female BALB/c mice, which may limit the applicability of the findings to other strains or genders. Second, the study uses a relatively small sample size (thirty mice), which may limit the generalizability of the findings. Future studies are needed to investigate the effects of chrysin in male mice and comparing them with female mice would provide a more comprehensive understanding of its gender-specific impacts. Furthermore, future translational research could assess the potential effect of chrysin on lipid and fat homeostasis and adrenal medullary function, considering factors like bioavailability and safety profiles of chrysin.

## Conclusion

In conclusion, this is the first study to investigate the overlooked impact of chrysin on steroidogenesis in the adrenal glands and ovaries of female BALB/c mice. Chrysin exhibited a dose-dependent effect on key steroidogenic enzymes, shedding light on its complex interactions within

the endocrine system. Our findings showed a downregulation of CYP17A1 by the high dose of chrysin. In addition, high-dose chrysin downregulated aromatase, the low dose led to an upregulation, emphasizing the complexity of chrysin's impact on estrogen biosynthesis. This finding aligns with existing controversies surrounding the effects of isoflavones on aromatase activity. Future investigations should explore chrysin's effects in different animal models and consider factors like bioavailability and safety profiles in humans.

## Conflict of interest

None of the authors declare any conflict of interest or financial disclosures.

## Authors contribution

Khalid M. Alqaisi: Study design, Animal dissection, Molecular Lab work, Data analysis, Manuscript Writing.

Manal A. Abbas: Study design, Animal treatment and blood collection, Histology lab work, Histology analysis, Manuscript writing.

Rand Alshawwreh: Animal dissection, Molecular lab work, Data analysis.

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