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Biochemical Study of The Ameliorative Influences of Fennel Oil against Cyproconazole-Induced Adrenal Gland Damage in Albino Rats

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ABSTRACT

Background: Cyproconazole (CPZ) is a triazole fungicide used to protect crops against a variety of fungal diseases.
Objective: This investigation aimed to evaluate the probable protective benefits of fennel oil, a natural oil extracted from the plant *Foeniculum Vulgare*, against CPZ-induced adrenal gland injury in rats.
Methods: Sixty male rats were allocated into: control, fennel oil, CPZ-low dose, CPZ-high dose, CPZ-low dose + fennel oil, and CPZ-high dose + fennel oil. After 15 days of daily treatment, serum levels of adrenocorticotropic hormone (ACTH), cortisol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), total cholesterol (TC), and free cholesterol (FC) were estimated. The adrenal gland tissues were also investigated to assess the amount of reduced glutathione (GSH), malondialdehyde (MDA), the efficacy of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), along with evaluating the status of DNA fragmentation using the Comet assay.
Results: CPZ substantially induced damage in the adrenal gland, as evidenced by elevated serum levels of ACTH, TC, FC, and LDL-c as well as reduced serum levels of cortisol and HDL-c. Also, high levels of MDA, decreased GSH levels, and diminished activities of SOD, CAT, and GPx in adrenal gland tissues in CPZ-treated groups. Furthermore, CPZ caused DNA damage in adrenal gland cells. Fennel oil co-treatment reversed CPZ hazardous effects on the biochemical parameters and DNA damage. **Conclusion:** This study claimed that fennel oil co-treatment could ameliorate CPZ-induced adrenal gland injury in male rats.
Keywords: Triazole, Fungicide, Cyproconazole, Fennel oil, Adrenal gland, Biochemistry.

INTRODUCTION

Conazoles fall into two main categories; triazole- and imidazole-containing conazoles. Cyproconazole (CPZ), one of the most popular triazole fungicides, is applied to safeguard against a vast range of fungal infections threaten fruits, vegetables, and field crops [(1)]. Due to their chemical durability, triazole fungicides are widely disseminated in the environment and enriched throughout the food chain. According to the literature, numerous triazole fungicides have good oral bioavailability, can cross the blood-brain barrier, impede hepatic cytochrome functioning, and have the potential to cause teratogenic impacts, cardiotoxicity, skin sensitization, and endocrine disturbance [(2)].

Conazoles work by inhibiting the enzyme lanosterol 14-α demethylase (CYP 51), an enzyme responsible for the synthesis of ergosterol which represents an essential component of the fungal cell membrane, causing alterations in membrane permeability and membrane-bound enzyme activity, and an increase in the saturation of fatty acids in the lipid bilayer [(3)]. Conazoles, in addition to blocking fungal enzymes, can interact with the mammalian cytochrome P450 (CYP450) system [(4)]. Therefore, they may have endocrine disruptive effects by blocking enzymes involved in the steroid production pathway.

Foeniculum Vulgare (fennel) is an aromatic member of the family Umbelliferaceae [(5)]. It has long been used as a culinary additive and as a source of folk medicine. Despite the fact that the entire plant can be used for medicinal purposes, the seeds of fennel are most often used for obtaining essential oils [(6)].

Trans-anethole, fenchone, estragol, and α-phellandrene have been identified as the major constituents of fennel seed essential oils [(7)]. Fennel oil is a potent antioxidant [(8)] in addition to having anti-inflammatory, anti-microbial, and anti-parasitic activities [(6)].

From the previous introductory remarks, the present investigation aimed to assess and evaluate the deleterious consequences evoked on the biochemical aspects of the adrenal glands of rats as a result of using CPZ besides the probable ameliorative influences of fennel oil.

MATERIAL AND METHODS

Materials

Cyproconazole of 96.8% Purity (CAS no. 94361-06-5 & Batch no. CHF1E00042) was obtained from Syngenta (Basel, Switzerland). The fennel oil (100% Pure & Natural, Authentic essential oil) was purchased from SVA, Amazon. The other compounds employed in this study were all of the highest analytical grade and purity.

Animals and dosing procedures

Sixty mature male Wistar rats (*Rattus norvegicus*) of approximately 200 to 250 g body weight (B.W.) were supplied by the closed colony of Theodor Bilharz Research Institute in El-Giza, Egypt. Prior to

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experimentation, they were left for one week to be adapted to the lab environment, where the animals were placed in clean plastic cages (4 rats/cage) with wood shavings as bedding under monitored environmental conditions (25 ± 2.0°C 12-h light/dark period). The rats were fed on a typical rodent pellet food and tap water *ad libitum*.

Six groups of ten rats each were formed using random selection. The following treatment was given to the rats every day at 9 a.m. for 15 days:

Control group: Normal rats were administered 10% DMSO [1 mL, intraperitoneally (i.p.)] as the vehicle for CPZ and 10% DMSO (1 mL, oral route) as the vehicle for fennel oil.

Fennel oil-treated group: Rats were administered 1 mL/kg B.W of fennel oil suspended in 10% DMSO orally. This dose was adjusted based on data from previous rat studies [9] and according to the body weight and body surface area.

CPZ-low dose-treated group: Rats were administered (i.p.) 20 mg/kg B.W (1/50 LD_{50}) of CPZ dissolved in 1 mL of 10% DMSO.

CPZ-high dose-treated group: Rats were administered (i.p.) 50 mg/kg B.W (1/20 LD_{50}) of CPZ dissolved in 1 mL of 10% DMSO.

The low and high dosages of CPZ were chosen in accordance with those employed in earlier studies [10].

CPZ-low dose-treated group+ Fennel oil: The animals received 1 mL/kg B.W of fennel oil suspended in 10% DMSO via oral gavage in conjunction with an i.p. injection of 20 mg/kg B.W of CPZ dissolved in 1 mL of 10% DMSO.

CPZ-high dose-treated group+ Fennel oil: The animals received 1 mL/kg B.W of fennel oil suspended in 10% DMSO via oral gavage in conjunction with an i.p. injection of 50 mg/kg B.W of CPZ dissolved in 1 mL of 10% DMSO.

Harvesting serum and tissue samples

Animals from all groups were fasted overnight after the treatment period, and in the following morning, they were anaesthetized using isoflurane. To obtain sera, blood samples were collected and centrifuged for 10 minutes at 1500×g and 4 °C, which were promptly frozen at -80°C till use. Adrenal glands of dissected animals were separated out from the surrounding adipose tissue and immediately flushed with 0.9% NaCl physiological saline and preserved in microtubes for biochemical analyses.

Preparation of adrenal gland tissue homogenates

The adrenal glands were homogenized in ice-cold (pH 7.4) phosphate buffer saline (PBS) using the tissue homogenizer (ultra turrax) to yield a 10% solution (w/v). To remove any erythrocytes and clots, heparin (0.16 mg/mL) was added to PBS. After centrifuging the homogenate for 15 minutes at 9000×g and 4 °C, the clear supernatant was collected and stored at -80 °C for further biochemical tests. The Lowry method [11] was used to determine protein content in tissue samples.

Biochemical assays

Adrenocorticotropic hormone (ACTH) and cortisol levels in the sera were determined utilizing enzyme-linked immunosorbent assay (ELISA) kits as instructed by the manufacturer (Cusabio Biotech Co., Ltd., Wuhan, China).

Using colorimetric assay kits (Biodiagnostic, Egypt) levels of total cholesterol (TC) and free cholesterol (FC) in sera were determined in accordance with the method of **Parakh and Jank** [12]. Serum concentrations of high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were determined using colorimetric test kits made by Biodiagnostic, Egypt, adhering to the method of **Warnick *et al.*** [13] and **Fruchart *et al.*** [14], respectively.

Using the **Buege and Aust** [15] technique, lipid peroxidation (LPO) levels in adrenal gland tissues were quantified using a spectrophotometric testing kit (Biodiagnostic, Egypt), depending on the development of thiobarbituric acid reactive substances (TBARS) and expressed as the amount of malondialdehyde (MDA) formation.

The amount of GSH and the efficacy of antioxidant enzymes (SOD, CAT, and GPx) in the tissues of the adrenal glands, were assessed utilizing spectrophotometric testing kits (Biodiagnostic, Egypt). GSH was estimated following a technique previously reported by **Moron *et al.*** [16], while the activity of SOD (EC 1.15.1.1) was evaluated following the technique of **Sun *et al.*** [17]. The **Aebi** method [18] was used to quantify CAT (EC 1.11.1.6) activity by monitoring H_2O_2 consumption. GPx (EC 1.11.1.9) efficacy was evaluated in accordance with **Paglia and Valentine** [19].

Comet test for measuring DNA damage

According to **Bandyopadhyaya *et al.*** [20], the alkaline comet test method was used to detect adrenal gland DNA damage.

Ethical approval:

The experimental methods were executed in strict accordance with the Faculty of Science, Ain Shams

University's Animal Care and Use Committee's rules and regulations (ASU-SCI/ZOOL/2022/11/2).

Statistical analysis

Windows-compatible IBM SPSS version (IBM Corp., Armonk, New York, United States) was used to tabulate and analyze the obtained biochemical and DNA damage data. The data were displayed as the mean ± SEM of six samples. To examine statistical differences among the groups, one-way ANOVA with Tukey's post-hoc test was utilized. Statistical significance was defined as *P*-values less than 0.05.

RESULTS AND OBSERVATIONS

Biochemical analyses

To assess adrenal gland injury, biochemical markers including ACTH, cortisol, TC, FC, HDL-c, and LDL-c levels in sera, as well as indicators of oxidative stress (SOD, GSH, CAT, GPx, and MDA) in adrenal gland tissues, were estimated.

Figure (1) depicts the levels of ACTH and cortisol in the sera of all animal groups. In comparison with the corresponding control animals, the results showed that providing fennel oil alone had no significant ($P > 0.05$) impact on the estimated hormonal levels. Meanwhile, rats given either a low or a high dose of CPZ showed a considerable rise ($P \leq 0.05$) in ACTH level (175.05% and 250.10%) and a sharp drop ($P \leq 0.05$) in cortisol level (-38.60% and -49.70%) when compared to the control values. In comparison with animals given CPZ alone, fennel oil supplementation resulted in adjustment of these estimated parameters, whereas the results remained substantially different ($P \leq 0.05$) relative to the corresponding control levels.

As shown in **figure (2)**, fennel oil supplementation alone showed insignificant ($P > 0.05$) impact on serum levels of TC, FC, HDL-c, and LDL-c. Meanwhile, rats treated with the low or high doses of CPZ had a significant increase ($P \leq 0.05$) in TC (16.60% and 24.10%), FC (30.97% and 56.12%), and LDL-c (86.56% and 134.91%) levels for low and high-dose CPZ-treated groups, respectively. On contrast, CPZ-treated rats (both the low and high-doses) exhibited noticeable ($P \leq 0.05$) diminished levels of HDL-c (-29.51% and -29.52%, respectively) in comparison with the corresponding control animals. The levels of TC, FC, and LDL-c in CPZ-treated rats, both in low and high doses, were markedly reduced ($P \leq 0.05$) by fennel oil supplementation while the levels of HDL-c were increased in comparison with those in rats treated with CPZ alone.

To track the oxidative stress status, the amounts of GSH and MDA, beside the efficacy of SOD, CAT, and GPx in the adrenal gland tissues of all groups, were examined **(Figures 3 & 4).** Fennel oil treatment solely had no distinguishable ($P > 0.05$) effect on these oxidative stress parameters. Both the low- and high-dose CPZ-treated rats encountered oxidative stress, as evidenced by a marked rise ($P \leq 0.05$) in MDA (58.08% and 118.98%, respectively) and a pronounced decrease ($P \leq 0.05$) in GSH (-26.91% and -48.29%, respectively), as well as SOD (-23.50% and -41.85%, respectively), CAT (-24.28% and -41.81%, respectively), and GPx (-27.88% and -82.65%, respectively) activities as compared to the corresponding control animals. The oxidative stress indicators tested were substantially enhanced ($P \leq 0.05$) in the fennel oil + low-dose CPZ -treated and fennel oil + high-dose CPZ -treated groups when compared to animals subjected to low- and high-dose CPZ alone.

Comet assay

The comet test (single-cell gel electrophoresis) detects DNA fragmentation in single cells.

Table (1) and **figure (5 A)** in this study showed the genotoxic potential of CPZ on adrenal gland cells, as well as the protective impact of fennel oil. This genotoxic impact was proved as an increase with statistical significance ($P \leq 0.05$) in the proportion of comet-tail DNA in the nuclei of adrenal gland cells. The fennel oil administration had no effect on DNA fragmentation as compared with the control group **[Table (1)** and **figure (5 B)].** CPZ treatment, whether at a low or high dose, resulted in a substantial rise ($P \leq 0.05$) in the number of tailed nuclei and breaks of DNA strands, which increased DNA diffusion from the nucleus towards the tail of the comet in the adrenal gland cells compared to the control group **[Table (1)** and **figure (5 C & D)].**

When fennel oil was concurrently given to rats receiving either a low or high dosage of CPZ, it significantly ($P \leq 0.05$) reduced the number of injured and severely damaged spots when compared to the rats receiving CPZ alone **[Table (1) and figure (5 E & 5 F)].**

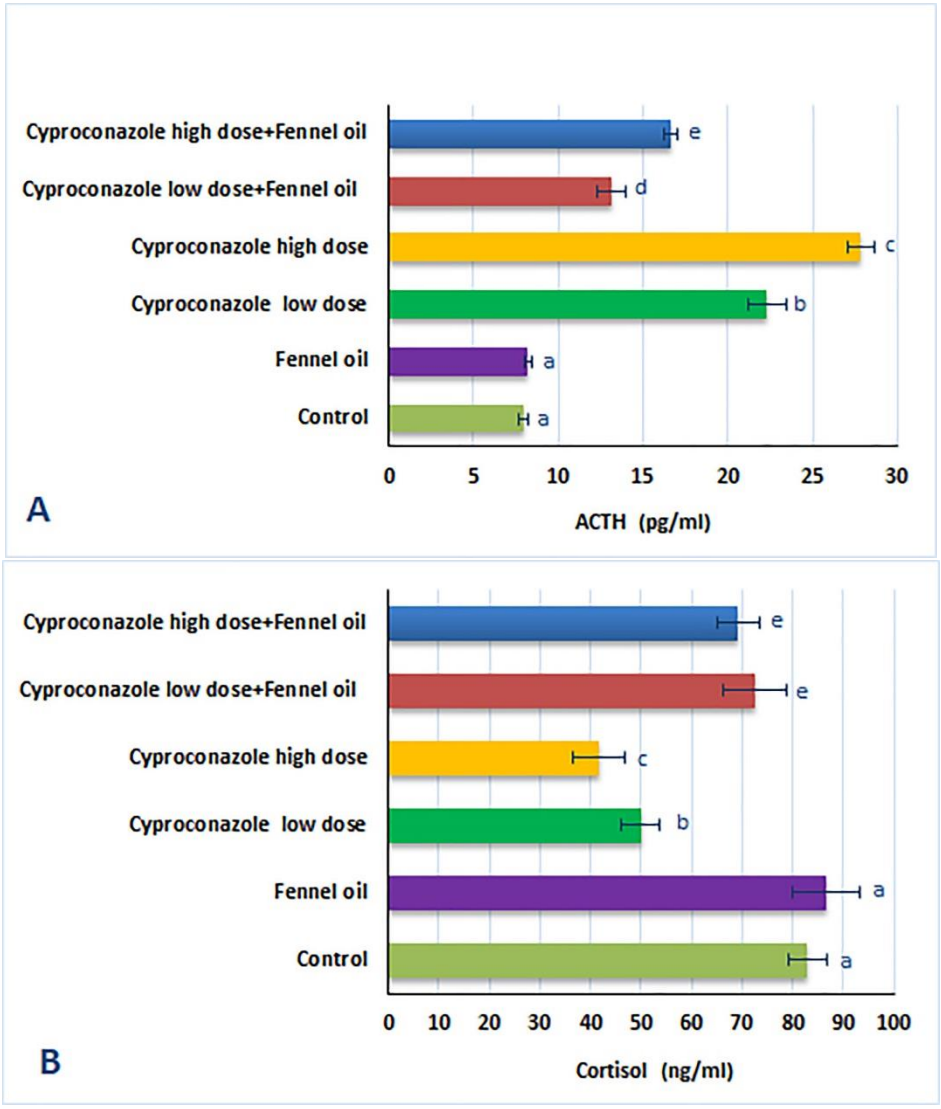
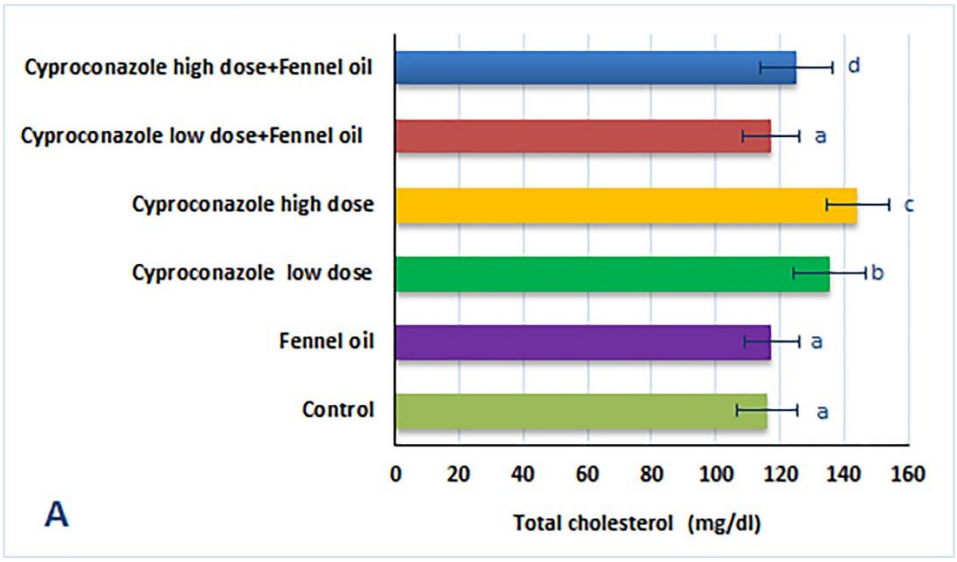


Figure (1): Levels of A: Adrenocorticotropic hormone (ACTH) and B: cortisol in the sera of control and treated animals. The data are displayed as Mean ± SEM (n = 6). According to the ANOVA and Tukey tests, horizontal bars with different superscript letters reveal a significant variance at the 5% ($P \leq 0.05$) threshold of significance.



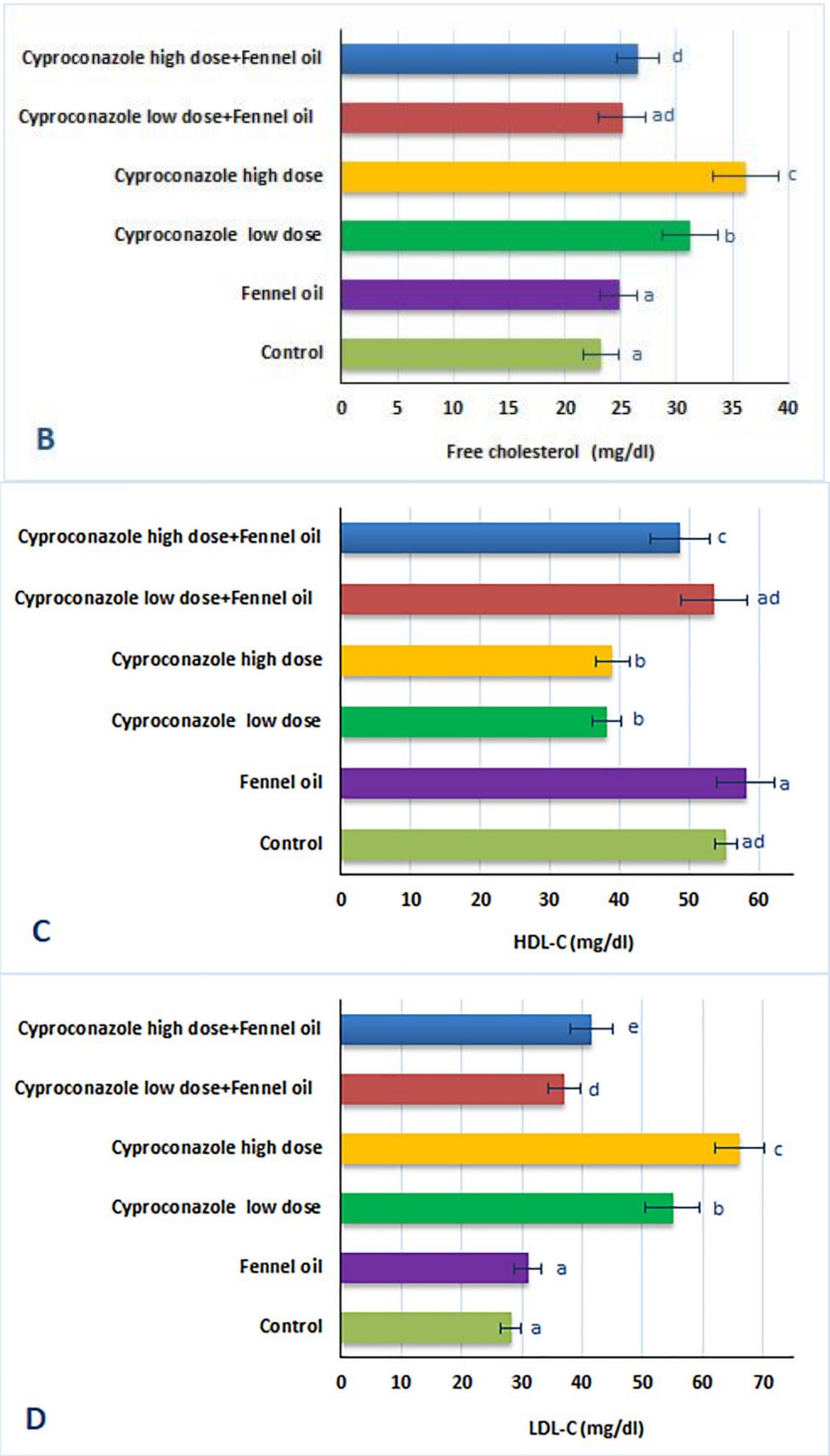
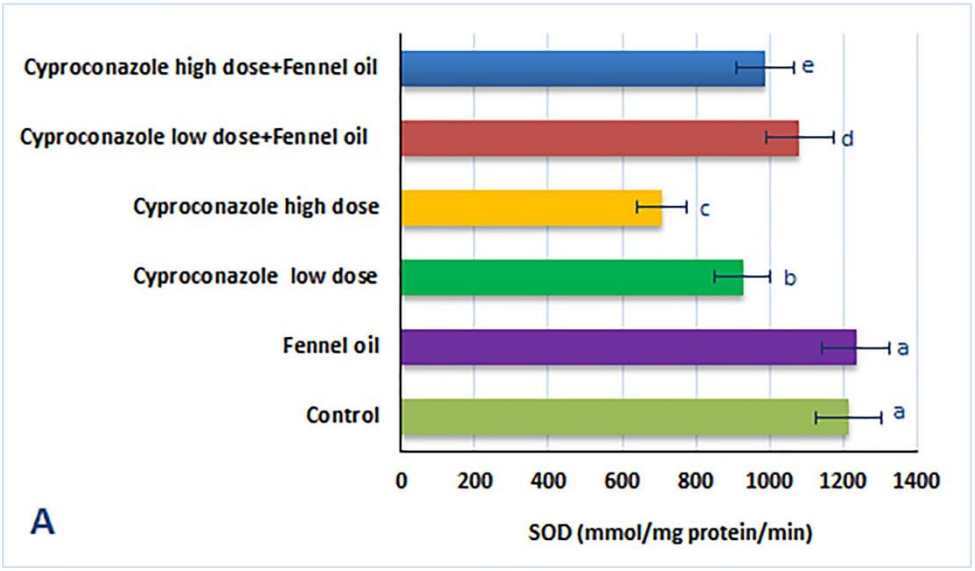


Figure (2): Levels of A: total cholesterol (TC), B: free cholesterol (FC), C: high-density lipoprotein cholesterol (HDL-c), and D: low-density lipoprotein cholesterol (LDL-c) in the sera of control and treated animals. The data are displayed as Mean ± SEM (n = 6). According to the ANOVA and Tukey tests, horizontal bars with different superscript letters reveal a significant variance at the 5% ($P \leq 0.05$) threshold of significance.


Cyproconazole high dose+Fennel oil
Cyproconazole low dose+Fennel oil
Cyproconazole high dose
Cyproconazole low dose
Fennel oil
Control
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400
600
800
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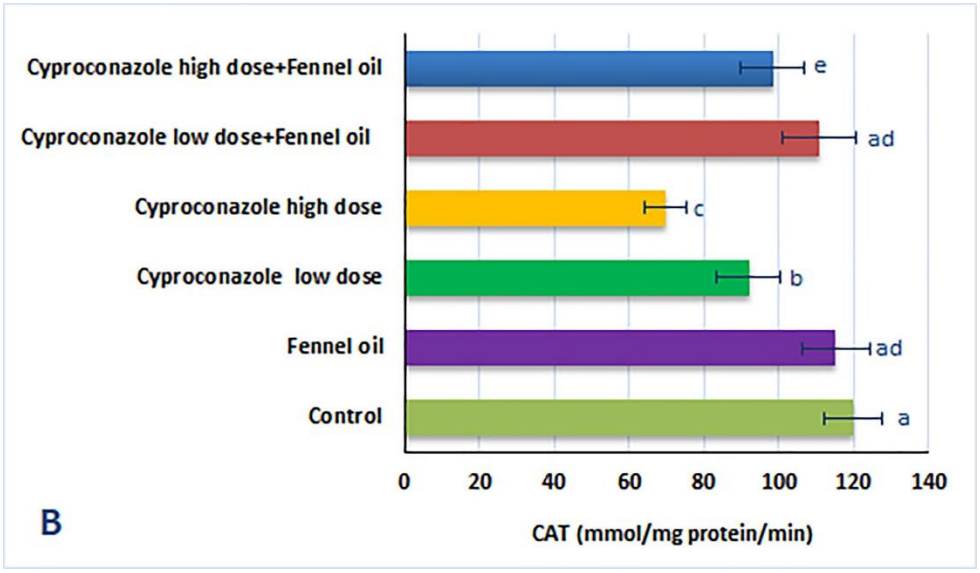
1200
1400
A
SOD (mmol/mg protein/min)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Cyproconazole low dose+Fennel oil
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Cyproconazole low dose
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100
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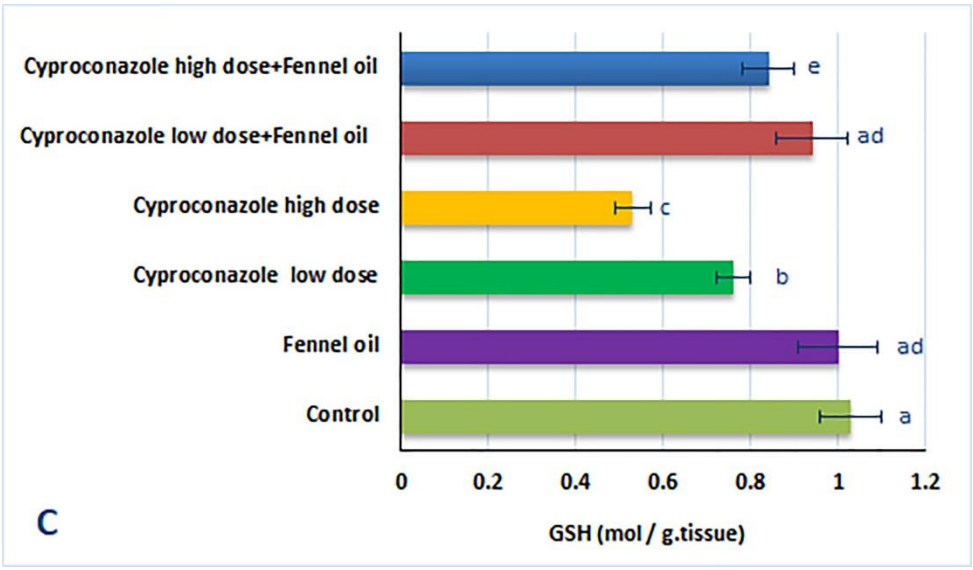
140
B
CAT (mmol/mg protein/min)


Cyproconazole high dose+Fennel oil
Cyproconazole low dose+Fennel oil
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Cyproconazole low dose
Fennel oil
Control
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GSH (mol / g.tissue)

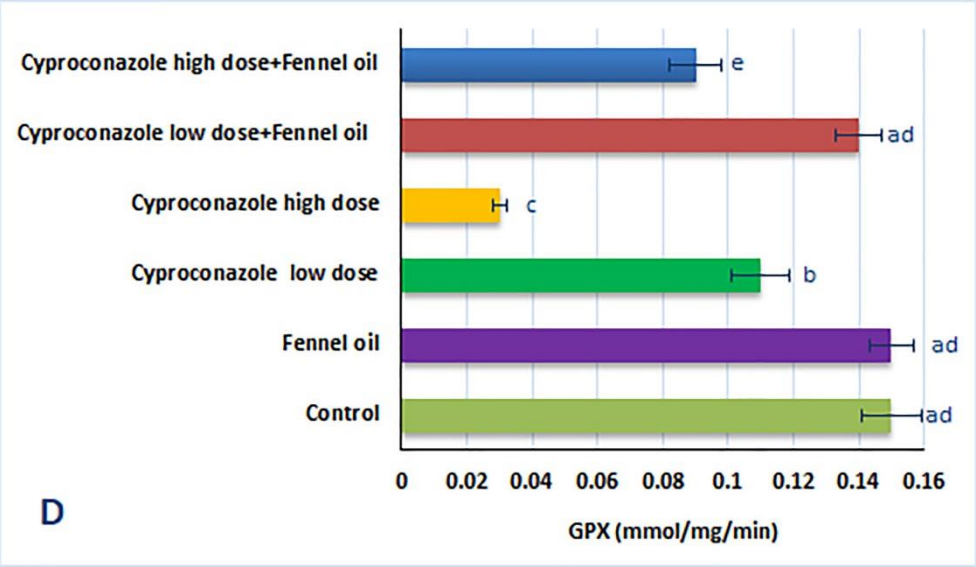

Figure (3): Antioxidant status in adrenal gland tissues of the control and treated animals: A: Superoxide Dismutase (SOD), B: Catalase (CAT), C: Reduced Glutathione (GSH), and D: Glutathione Peroxidase (GPx). The data are displayed as Mean ± SEM (n = 6). According to the ANOVA and Tukey tests, horizontal bars with different superscript letters reveal a significant variance at the 5% ($P \leq 0.05$) threshold of significance.

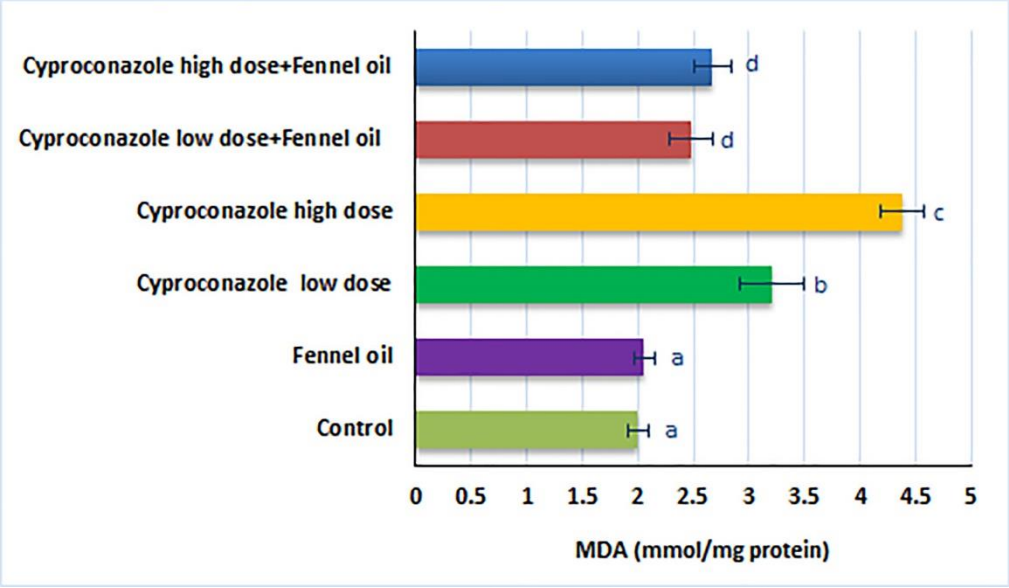

Figure (4): Levels of malondialdehyde (MDA) in adrenal gland tissues of the control and treated animals. The data are displayed as Mean±SEM (n = 6). According to the ANOVA and Tukey tests, horizontal bars with different superscript letters reveal a significant variance at the 5% ($P \leq 0.05$) threshold of significance

Table 1: levels of DNA fragmentation in adrenal gland tissues of the control and treated animal groups

Parameters	Animal Groups					
	Control	**Fennel oil**	**CPZ low dose**	**CPZ high dose**	**CPZ-low dose + Fennel oil**	**CPZ-high dose + Fennel oil**
%DNA in Head	88±5.2	84±4.1	62±3.7*	51±2.2*	73±3.7**	65±2.8#
%DNA in Tail	12±0.7	16±1.2	38±1.8*	49±3.2*	27±2.1**	35±1.6#
Tail Length (µm)	4.91±0.32	6.31±0.42	5.84±0.44*	11.21±1.12*	11.56±1.4**	15.43±1.01#
Tail Moment	0.40±0.05	0.29±0.04	0.73±0.07*	0.94±0.09*	0.53±0.04**	0.68±0.06#

The data are presented as Mean ± SEM (n = 6). * Significant change at P≤0.05 versus control group, ** Significant change at P≤0.05 versus CPZ low dose group, # Significant change at P≤0.05 versus CPZ high dose group according to ANOVA test and the Tukey test. CPZ, Cyproconazole.

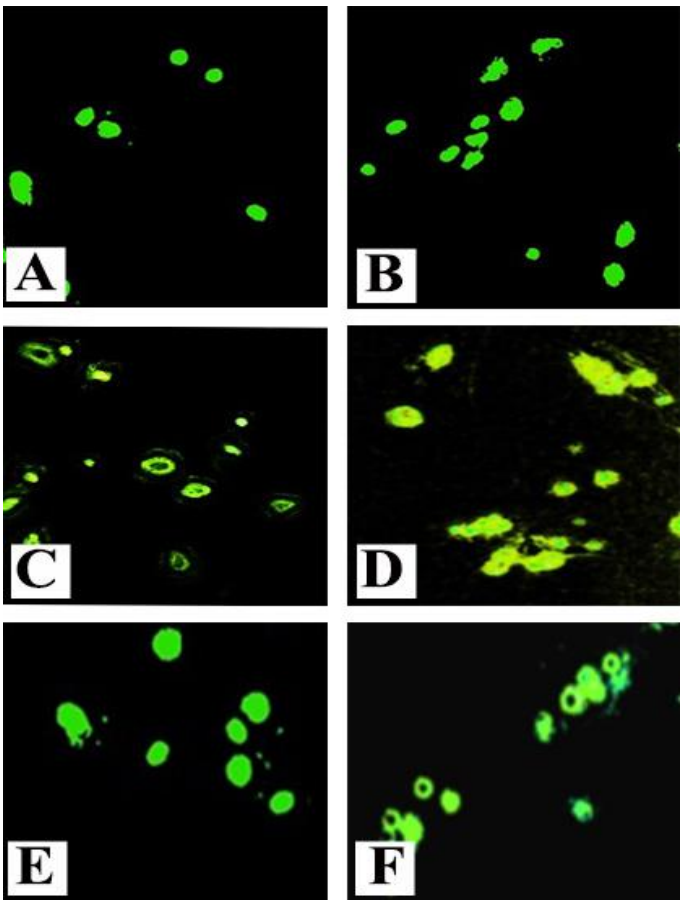

Figure (5): The comet assay images of adrenal gland cells. A: Control; B: Fennel oil; C: Cyproconazole (CPZ) low dose; D: CPZ high dose; E: Fennel oil + CPZ-low dose; and F: Fennel oil + CPZ-high dose.

DISCUSSION

Pesticides, including fungicides, are employed on a global scale by farmers to reduce damage from undesired organisms in order to increase crop quality and output. Triazoles have rapidly become some of the most profitable and commonly used fungicides in the world due to their efficacy in treating a variety of fungal infections in plant crops and vegetables. CPZ is one of these triazoles that is widely used across the world [1]. However, the experimental findings in non-target species demonstrate a variety of undesirable toxic effects of triazoles on organisms, including oxidative stress and endocrine disruption [2]. Consequently, a crucial topic for investigation is the possible toxicity of fungicides in various bodily organs. The adrenal gland is unique in this regard due to its biosynthetic potential, massive blood supply, and lipophilicity, which facilitates the accumulation of lipophilic substances, and as a result, it is the most prevalent endocrine target gland subjected to toxicity [21]. According to the literature a few previous studies have looked at the dangers of CPZ on the adrenal glands of mammals. Accordingly, the current study was intended to assess the toxicity of CPZ on the adrenal glands of rats in addition to the possible protective effects of fennel oil extract.

Cortisol is a key regulator of several physiological mechanisms that increase in response to stress. Our findings showed that CPZ exposure, at either low or high doses, caused a rise in ACTH levels in sera, as well as a decrease in cortisol level, when compared to control values, indicating that it can interfere with glucocorticoid production and release *in vivo*. Cholesterol is the source of all steroid hormones, including cortisol. In the mitochondria and endoplasmic reticula of all steroidogenic cells, a set of CYP450 enzymes and hydroxysteroid dehydrogenases catalyze a sequence of enzymatic events that convert cholesterol into steroid hormones [21]. In our study, the increased levels of ACTH recorded in sera of CPZ-intoxicated rats confirmed that the reduction in cortisol level may be attributed to the direct effect of CPZ on the steroidogenesis. **Solaiman and Seddik** [22] got similar findings after treating rats with 1 mg Tributyltin, antifouling paint, dissolved in 0.4 mL corn oil once daily via a gastric tube for one week. **Choi *et al.*** [23] revealed that azole compounds, such as ketoconazole and the fungicide fenarimol, inhibit various CYP450 isoforms and, as a result, can influence steroid synthesis. **Tully *et al.*** [24] previously conducted research on adult male rats in which four triazoles were shown to alter the normal function of many genes in the liver and testis, particularly the steroid metabolizing genes, which may be another explanation for the present study's cholesterol level disturbance. When fennel oil and CPZ were administered concurrently, the ACTH and cortisol levels in the sera were preserved. The precise mechanism of how the active ingredients of fennel oil affect plasma cortisol is still unknown [25].

According to the findings of this study, the administration of CPZ disrupted the blood lipid profile by raising the levels of TC, FC, and LDL-c while reducing the level of HDL-c, which was not found in fennel oil-treated rats. Our findings match with those of **Wolf *et al.*** [26], who discovered that rats exhibited an altered lipid profile in their sera after being exposed to Triazole fungicides. Fennel oil has previously been shown to lower cholesterol, while also decreasing peroxidative damage [27], decrease blood lipid levels [28], impede fat absorption, and improve beta oxidation [29]. Furthermore, **Fidèle *et al.*** [30] observed a pattern of lipid profile disruption similar to that shown in the current investigation in the sera of rats fed a diet enriched with cholesterol and subjected to an aqueous extract of *Cassia occidentalis* leaves. Reduced or inhibited intestinal cholesterol absorption and increased reverse cholesterol transport may serve as mediators for the hypolipidemic effect of fennel oil extract [30].

The results of the current investigation showed that rats given CPZ had elevated oxidative stress in the tissues of their adrenal glands as evidenced by higher levels of the lipid peroxidation end-product (MDA), decreased amounts of GSH, and reduced CAT, SOD, and GPx activity. In keeping with our findings, numerous investigations have shown that triazole fungicide causes oxidative stress in many rat tissues [10].

Many chemical agents, pesticides, xenobiotics, environmental contaminants, and drugs have been shown to be hazardous to cells, perhaps owing to the production of oxidative stress induced by an inequality between the formation of reactive oxygen species (ROS) and their deactivation by the antioxidant defense mechanisms [31]. The oxidative stress might be caused by CPZ inducing numerous forms of CYP450, which resulted in enhanced creation of ROS during CYP450's catalytic cycle and the synthesis of reactive metabolites during CPZ biotransformation. Increased ROS led to increased glutathione consumption, decreased antioxidant enzyme efficiency, and increased lipid peroxidation [10].

The mechanism looks to be linked to the reaction of the N-4 nitrogen atom of the five-membered aromatic ring of CPZ with the central iron atom of the CYP450 porphyrin complex [32]. Surprisingly, rats given fennel oil and CPZ at the same time improved in all of the adrenal gland oxidative stress indicators assessed. Fennel extract has been shown to contain a variety of polyphenolic chemicals. These polyphenolic compounds have high antioxidant activity, and it is possible that the action of fennel extract is related to these active components [27]. This effect is assumed to be mostly owing to their redox properties, which help in the adsorption and elimination

of ROS, the quenching of singlet and triplet oxygen, and the dissolution of peroxides [33].

Single-stranded DNA breaks are caused by xenobiotics and environmental pollutants. In the in vivo situation, DNA breakage and repair are more or less balanced. If the damage exceeds the cell's capacity to repair, the amount of breaks increases. The current study's comet assay results showed that CPZ has a genotoxic effect, as shown by the percentage increase in tail DNA and tail moment. Our findings agree with those of **Hamdi *et al.* [10],** who showed that rats exposed to epoxiconazole had increased levels of DNA damage in their liver and kidney in a dose-dependent manner. Excessive ROS generation overwhelms the antioxidant defense system, allowing oxidative DNA alterations such as strand breaks and base oxidations to occur [34]. Increased DNA degradation causes an increase in the rate of cell apoptosis that might justify the lower DNA content in CPZ-treated adrenal gland tissues. The enhanced rate of DNA breakage in adrenal gland tissues was considerably decreased in rats given fennel oil alongside either a low or high dosage of CPZ. Fennel oil's genoprotective effects may be attributed to its strong antioxidant activity, which stops oxidative damage including lipid peroxidation, potential to increase endogenous defence capacity, reduction of pro-inflammatory cytokines, preservation of nucleophilic sites of DNA, and avoidance or scavenging of free radicals, which can result in inhibition of endogenous mutagen creation [35].

CONCLUSION

The findings of the present investigation showed that when rats are exposed to CPZ they suffered from adrenal gland toxicity, as evidenced by oxidative stress, cholesterol and hormone disruption, and DNA damage. Fennel oil co-treatment reversed CPZ hazardous effects on biochemical parameters and DNA damage. Therefore, our findings shed new light on the modulating effect of fennel oil supplementation on CPZ-induced adrenal gland injury.

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