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# *Cuphea Ignea* Extract Relieved the Histological Changes and Activated the NF-κB Protein of Female Reproductive Organs and Stomach in EtOH-Treated Rats

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**Abstract**

Alcohol consumption poses adverse effects on humans and animals. There are different sources and several ways to exposure to alcohol toxicity. Approximately, 5.3% of human death have been attributed to alcohol toxicities. The experiment aimed to evaluate the protective effects of *C. Ignea* extract against EtOHtoxicities in rats. Histopathological and Immunohistochemical studies were applied to gastric and reproductive organs (ovaries, and uterus) following EtOH treatment. Moreover, the levels of Estradiol, FSH, and LH have been measured in serum samples. Rats were divided into six groups. Control group, EtOH-treated groups (1.5 ml/rat), *C. Ignea* treated groups (250 and 500 mg/kg BW), *C. Ignea* 250 + EtOH-treated group, and *C. Ignea* 500 mg/kg BW + EtOH-treated group. EtOH treatment caused severe negative histopathological events in tested organs. The *C. Ignea* extract at two tested doses before the dosing with EtOH relieved the histopathological changes in tested organs. Furthermore, immunohistochemically findings exhibited negative NF-κB immunoreaction in the tested tissues of EtOH-treated rats. The treatment with *C. Ignea* extracts for seven days at two mentioned doses before the dosing with EtOH showed NF-κB immunoreaction ranging from moderate to strong in the applied tissues. Also, the *C. Ignea* extract treatment at two tested doses succeeded to mitigate the serum estradiol, FSH, and LH levels in EtOH-treated rats. The *C. Ignea* plant extract enhances the synthesis of NF-κB and protects the rats from EtOH toxicity.

Keywords: *Cuphea-Ignea*, EtOH, Histopathology, Immunohistochemistry, NF-κB.

## 1. Introduction

Alcohol toxicity which occurred from different sources and ways is one of the main global public health challenges. The human deaths recorded in 2018 attributable to alcohol toxicity are 5.3% [1]. In humans, the adverse effects of alcohol toxicity on organs are the results of the alcohol metabolic by-products, acetaldehyde, and the generation of reactive oxygen species (ROS) [2]. In addition, the farm animals also suffer from EtOH toxicity that leads to generating alcohol-associated diseases and low production, and economic loss. Silage is the most common source of EtOH in ruminant feed, especially corn silage (12-15 g/kg from the total dry matter) where, EtOH is produced by a variety of microbes that existed in silage as yeasts, enterobacteria, and heterotactic acid bacteria [3]. The excessive alcohol concentration in silage (> 3-4%) causes many negative effects such as low

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fertility and an increase in the abortion rate in farm animals [4].

In humans and animals, EtOH is rapidly absorbed into the bloodstream from the gastrointestinal tract (GIT). Otherwise, in ruminants particularly in dairy cows, the dietary EtOH intake from silage alcohols is partly metabolized by the rumen microflora; however, EtOH is easily absorbed through ruminal epithelial cells [5] leading to the elevation of liver alcohol loads [3]. EtOH exposure resulted in the genotoxicity of sperm head formation and nuclear abnormalities in oocytes, suggesting that morphological abnormalities in germ cells and these effects were also observed in the first generation of mice, which may be related to EtOH-parent exposure [6].

The nuclear factor-kappa light chain B lymphocyte (NF-κB) is a transcription factor that regulates the expression of several genes involved in many physiological and pathological conditions, including immune response, apoptosis, carcinogenesis, and inflammatory processes [7]. The NF-κB protein exists in the cytoplasm in an inactivate form by entangling with an inhibitory protein family (IκB) such as IκBα [8]. Several diversified stimuli such as; stress factors, cytokines, microbial infections, and mitogens are working as activating factors to the IκB kinase enzyme complex (IκK) that leads to degradation of the IκBα through the ubiquitin-phosphorylation process. Therefore, the NF-κB protein was liberated and translocated from the cytoplasm to the nucleus, and that mainly occurred to the p50/c-Rel and p50/p65 dimers, where it induces its down-stream pro-inflammatory genes to generate the immunoreactive actions and inflammation response [8, 9].

*Cuphea Ignea* (*C. Ignea*) is one member of the flowering species in the Lythraceous family, it has been used in Brazilian medicine as a hypotensive, anti-inflammatory, and antiviral, as well as common use for treating both stomach disorders, cancer, syphilis, and gonorrhea [10-13]. The phytochemical analysis of *C. Ignea* extract was first recorded by Bate-Smith [14] who recorded the existence of flavonoid antioxidants such as kaempferol and quercetin, and glycosides in the *C. Ignea* plant. In the same regard, recent work reported stated the existence of. saponins, tannins, flavonoids, and triterpenoids [10].

This experiment aims to study the histopathological and reproductive hormonal changes that occurred in the EtOH-intoxicated Sprague Dawley female rat. Also studying the immune-suppressive effect of EtOH toxicity by the down-regulation of the NF-κB protein synthesis. On the contra; studying the protective role of the *Cuphea Ignea* plant extracts against EtOH toxicity and its up-regulating effect on the NF-κB protein as an immune-stimulant marker.

## 2. Experimental

### *2.1. C. Ignea* collection and extraction

The aerial parts of the *C. Ignea* fresh plant were gathered from local markets in northern Cairo. The plant authentication and voucher specimen were deposited at the herbarium of the NRC, EGYPT (voucher number C 182). The *C. Ignea* was dried in the shadow, mashed, and gruelingly extracted with 70% (v/v) aqueous EtOH under reflux. The acquired eluent was dried under a vacuum at 55-60 ºC and then dissolved in EtOH. The extract was stored for future use.

### *2.2.* Phytochemical screening

The phytochemical screening process for the *C. Ignea* extract was carried out as described by Sofowora [15], and the results were published in the previously published research paper as referred to [10].

### *2.3.* Estimation of the total phenolic content

The total phenolic contents were estimated according to the Folin-Ciocalteu method by Blainski, Lopes and De Mello [16]. In Brief, in attest tube, 100 µL of the plant extract was diluted with distal water and the total volume was adjusted up to 3.5 mL. Then the diluted plant extract was oxidized for 5 minutes by adding 250 µL of Folin-Ciocalteu reagent. This mixture was neutralized by adding 1.25 mL of aqueous sodium carbonate solution 20% for 40minute and at 725 nm against the blank, the absorbance was recorded. The previously prepared gallic acid-calibration curve was used to detect the total phenolic contents using the equation: y=0.024x+ 0.018 (R2 =0.998), which displayed the results as gallic acid equivalents.

### *2.4.* Estimation of the total flavonoids

The total flavonoid contents in the *C. Ignea* aqueous ethanolic plant extract were estimated using an aluminum chloride assay according to Lamien-Meda, Lamien, Compaoré, Meda, Kiendrebeogo, Zeba, Millogo and Nacoulma [17]. Concisely, 100 µL of *C. Ignea* extract was mixed with 300 µL of 5% sodium nitrite for 6 min. Then, 300 µL of a 10%

$AlCl_3$ solution was added and the total volume was adjusted by distilled water up to 2.5 mL. 7 min later, add 1.5 mL 1M NaOH, and centrifugate the mixture (5000 g/10 min). The supernatant absorbance was measured at 510 nm against the solvent blank. The flavonoid contents were estimated by the catechins calibration curve and using the equation:

y = 0.003x - 0.004 (R2 = 0.998).

### *2.5.* Determination of radical scavenging capacity

The quantitative scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was detected according to Brand-Williams, Cuvelier and Berset [18]. The plant extract was dissolved in a concentration of 1 mg/mL in EtOH. From this stock solution, different concentrations of regular dilutions were prepared. Then both 500 mL of sample, 375 ml EtOH, and 125 mL of 1 mmol/ L prepared to scavenge radical solution were mixed. The test was done in a triplicate manner. After 30 minutes of incubation at darkroom temperature, the absorbance was estimated at 517 nm on a UV–a vis spectrophotometer (Shimadzu, Duisburg, Germany). The reference standard ascorbic acid was utilized to detect the radical scavenging activity percentage (RSA):

$$\mathbf{RSA}\% = \frac{(\text{Control absorbance} - \text{Sample absorbance})}{(\text{Control absorbance})}\mathbf{x100.}$$

### *2.6.* Experimental animals and experimental design

Thirty-six adult female Sprague-Dawley rats, weighing (150-210 g) were kept under standard factors in the experimental animal house of the National Research Center (NRC), Egypt. The experimental rats were randomly divided into six groups (n=6) as fellow:

**Group 1**: control rats were treated with normal saline.

**Group 2**: rats were treated with absolute EtOH 1.5 ml/rat (equivalent to 1.2 g EtOH/rat) in one shoot as referred by Liu, Tian, Gou, Fu, Li, Lan and Yin [19].

**Group 3**: rats were treated only with *C. Ignea* extract 250 mg/kg BW for seven days according to [10].

**Group 4**: rats treated only with *C. Ignea* extract 500 mg/kg BW (double dose) for seven days.

**Group 5**: rats were pretreatment with *C. Ignea* extract (250 mg/kg BW) for seven days and followed by one dose of EtOH at a mentioned dose

**Group 6**: rats were pretreatment with *C. Ignea* extract (500 mg/kg BW) for seven days and followed by one dose of EtOH at mentioned dose.

The administration program for *C. Ignea* extract and EtOH was orally by intragastric gavage. The experimental rats were fasting for 24 h before EtOH administration with free access to water.

The experimental steps were carried out according to the ethical standards for the convenient care of the laboratory animals which were approved by the Research Ethical Committee of NRC, Egypt (No: 20191). All attempts were taken to reduce the animals' suffering.

### *2.7.* Samples collection

Blood samples were collected 24 hrs. after EtOH administration. Rats were anesthetized using a 1.9% diethyl ether-saturated cotton ball in a small chamber for 3–5 min and then euthanized by cervical dislocation. The blood samples were collected and centrifuged at 3000 rpm/10 min for serum separation. Sera were kept at −20 °C for hormonal analysis [20]. Postmortem inspection was done on all rats. Extirpation of the stomach, ovaries, and uterus from rats was carried out for histopathological and immunohistochemical examination.

### *2.8.* Histopathology protocol

After carrying out the postmortem examination, tissue specimens from the stomach, ovaries, and uterus were taken from experimental rats. These tissue specimens were fixed at 10% neutral buffered formalin (NBF) overnight, then transferred to EtOH 70% to maintain the antigenic structure of the NF-κB P65 protein from the effect of formalin. Routine tissue processing, dehydration, embedding in paraffin wax, and sectioning at 3-5 µm were performed. The formed paraffinized tissue blocks are convenient for histopathological and immunohistochemical studies. Tissue slides were stained with hematoxylin and eosin (H and E) for histopathological examination[21].

### *2.9.* Immunohistochemistry protocol (IHC)

The previously prepared paraffinized tissue blocks from the ovary, uterus, and stomach tissue specimens which are convenient for IHC protocol were sectioned at 3 µm and stuck on positive charge slides. Antigen retrieval for NF-κB P65 protein was performed by dipping the slides into sodium citrate buffer 10% as a retrieval solution, then autoclaved for 5 minutes at 20-25 PSI pressure (Pounds per Square Inch). Blocking of the nonspecific binding was performed on the tissue slides using the super-blocking reagent that existed in the kit (before the specific primary antibody was used). Tissue slides are

incubated with rabbit anti-NF-κB p65 IgG2a diluted in PBS buffer pH 7.4 by the rate of dilution 1: 100, containing 0.9% sodium azide (NaN3) and 0.2% bovine serum albumin (BSA) [22]. CRFTM anti-polyvalent polymer stain peroxidase detection kit imported from Scy-Tek Laboratories, USA, was used. The species specificity of the kit is anti-rabbit and anti-mouse. Control negative for the tested tissue was incubated with normal saline instead of the primary antibody. Also, a control positive tissue specimen which is rat stomach tissue previously showed strong positive results for the NF-κB p65 by IHC was inserted into the test. Tissue slides were counterstained with hematoxylin and examined under a light microscope. The positive findings range from light brown, golden brown to deep brown according to the antigenic intensity in the examined tissues) in comparison to the positive and negative control slides [23].

### *2.10.* Reproductive hormone assessments

The assessment of LH, FSH, and estradiol (E2) hormones in the collected serum samples was performed by ELISA assay kits (CUSABIO China Inc). The rat LH and FSH minimum measurable levels are < 0.5 and 0.35 U/mL respectively. The serum E2 hormone level was detected quantitatively by the quantitative ELISA assay kit with a sensitivity of 10 pg/mL, which was imported from (Diagnostics Biochem. Canada Inc) according to the procedure of Nna, Ujah, Mohamed, Etim, Igba, Augustine and Osim [24].

### Statistical Analysis

Experimental data presented as (mean ± SD) and $P < 0.05$ was considered statistically significant. Statistical tests were carried out using SPSS software. One-way analysis of variance followed by Duncan's multiple range test (DMRT) for several comparisons between the groups.

## 3. Results

### *3.1.* Phytochemical analysis and antioxidant activity

As referred to in previous work, the qualitative phytochemical analysis of an ethanolic extract of *C. Ignea* revealed that the extract enrichment with total phenolic equivalent 121.66 mg gallic acid/g extract and total flavonoids 105.33 mg catechin g extract. Furthermore, a DPPH assay is done to estimate the antioxidant activity of *C. Ignea* extract in vitro and the results revealed that *C. Ignea* extracts exhibited nearly similar scavenger activity to ascorbic acid at a concentration of 50 µg/ml, the extract completely inhibited DPPH absorbance and gave a potent radical scavenging activity with 98% at a concentration of 100 µg/ml [10].

### *3.2.* Pathological findings

### *3.3.* Gross gastric observations

Figure 1 illustrated the gross appearance of rat stomachs after a postmortem examination in the different experimental groups revealing that; the gastric mucosa of the control rats seemed completely normal. In EtOH-treated rats, the glandular part of the gastric tissue exhibited severe diffuse hemorrhagic sticks and patches (Fig1 A). One of the obvious obtained results of the rats treated with *C. Ignea* extract at a dose of 250 and 500 mg/kg BW respectively, showed normal coloration and thickness of the glandular mucosa (Fig. 1, B & C). On the other hand, the rats treated with the *C. Ignea* extract (250 mg/kg BW) and EtOH (1.5 ml/rat) illustrated a high protection degree against the hemorrhagic effect of EtOH, meanwhile, mild gastric mucosal congestion was still noticed (Fig. 1 D). In addition, the rats treated with *C. Ignea* extract (500 mg/kg BW) and EtOH (1.5 ml/rat) showed complete protection against the hemorrhagic effect of EtOH. Neither congestion nor hemorrhages in the gastric mucosa were observed (Fig.1 E).

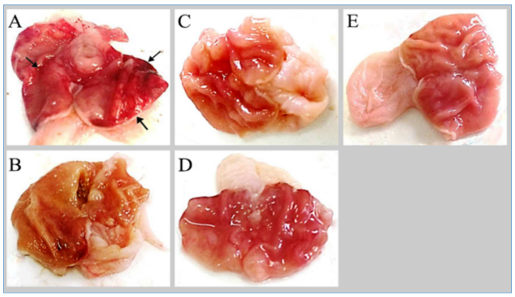


**Figure 1:** Gross stomach tissue findings of the different experimental rats. The photo of the control rats showed normal gastric tissues. A) The glandular part of the gastric tissue in ethanol-treated rats showed severe diffuse submucosal hemorrhages (black arrows). B) The *C. Ignea*-treated rats at low dose exhibited normal rosy color and normal thickening of the glandular gastric mucosa. C) The *C. Ignea*-treated rats at high dose illustrated also complete normal mucosal color and thickness. While D) The *C. Ignea* at low dose plus ethanol treatment displayed mild congestion in the gastric mucosa with complete disappearance of the hemorrhagic lesion. E) The *C. Ignea* at a high dose plus ethanol showed no congestion and no hemorrhages in the gastric mucosa of treated rats

### 3.4. Findings of H&E inspection in the gastric, ovarian, and uterine tissues

#### 3.4. 1. *Gastric histopathological findings*

First of all, the examined control rat tissues appeared normal histological structure of gastric tissues**.** On the other side, the histopathological findings of the stomach which are illustrated in figure 3 showed severe intervillous hemorrhages were observed among the gastric mucosal villi of the EtOH-treated rats, also severe desquamation of the parietal and chief cells lining the gastric villi was noticed (Fig. 2 A). The *C. Ignea* plant extract-treated rats at doses of 250 and 500 mg/kg BW exhibited no histopathological changes in the gastric tissue (Fig 2 B and C). otherwise, the groups treated with both *C. Ignea* extract (250 mg/kg BW and 500 mg/kg BW) seven days before the treatment with EtOH (1.5 ml/rat) showed that tightly intact the gastric mucosa lining epithelium and protected from the EtOH toxic effects where it illustrated no cellular desquamation from the gastric villi, also no submucosal hemorrhagic sticks were detected (Fig. 2 D and E).

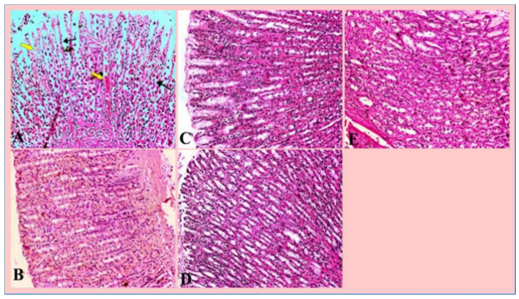


**Figure 2:** Histopathological changes in stomach tissues of different experimental rats (H&D). The control rats showed normal gastric. The gastric tissue of the ethanol-treated rat showed severe intervillous hemorrhage in the gastric mucosa (yellow arrows) associated with severe exfoliations and looseness of the parietal and chief cells of the gastric villi (black arrows) (A, X 200). While the *C. Ignea* extract at 250 and 500 mg/kg-BW revealed no histopathological changes occurred in the gastric tissue, respectively (B and C, X 100). Moreover, simultaneous treatments with the *C. Ignea* and ethanol at both the low and the high doses showed no negative effects of ethanol treatment alone in the lining epithelium of the gastric mucosa and no desquamation in the mucous cell, and also the submucosal hemorrhage was not detected (D and E, X 100)

#### 3.4.2. **Ovarian histopathological findings**

The control rats showed the normal histological structure of the ovarian tissues, also the slides of rats treated with *C. Ignea* presented no histopathological illustration in ovarian tissue (Fig. 3 B and C). While the slide of EtOH-treated rats displayed severe multifocal hemorrhages in the intercellular spaces between the lutein cells of the mature corpus luteum in the ovarian tissues, in addition, vacuolar degeneration in most of the lutein cells that surround an area of necrosis was also pronounced (Fig. 3 A). The protective effects of the *C. Ignea* toward EtOH have occurred in the treated rats with (250 and 500 mg/kg BW) and EtOH (Fig.3 D and E).

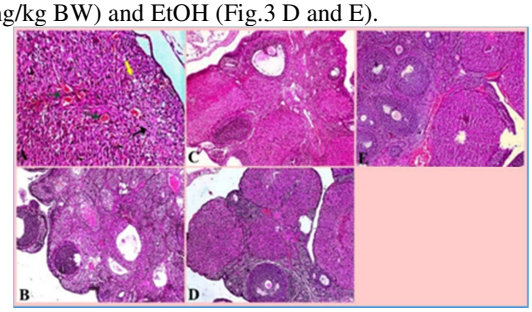


**Figure 3:** Histopathological changes in ovarian tissues of different experimental rats (H&D). The control rats showed normal ovarian histological structure. The Ovary of the ethanol-treated rats showed severe hemorrhage among the lutein cells of the mature (green arrows) associated with vacuolar degeneration in large numbers of the lutein cells (black arrow) surrounding an area of necrosis (yellow head) (A, X-200). The *C. Ignea* treatment at (250 and 500 mg/kg BW) illustrated completely normal ovarian tissue and no histopathological changes were found (B and C, X 40). The treatment of *C. Ignea* at (250 mg/kg and 500 mg/kg BW) in the ethanol-treated rats displayed tissue protection against ethanol toxicity and restored the ovarian tissue structure to the normal (D and E, X 40)

#### 3.4.3. **Histopathological findings in the uterus**

In the same trend, the histological inspection findings for uterus tissues exhibited normal histological structure with normal endometrial epithelium. Whereas the slides of EtOH-treated rats showed severe vacuolar degeneration and nuclear pyknosis in the uterine tissue, furthermore, to moderate cystic dilatation in some of the uterine glands, and moderate endometrial blood vessel congestion also noticed (Fig. 4 A). Contrariwise; the sides of rats treated with *C. Ignea* extract at doses of (250 or 500 mg/kg BW) unveiled complete safeness for the *C. Ignea* extract on the examined rat tissues where the uterine tissues were detected no histopathological changes (Fig. 4 B and C). In the fourth and fifth groups, the rats that were co-treated with *C. Ignea* at two mentioned doses for seven days and followed by one dose of EtOH illustrated the protection of the uterine tissue from the harmful effects of EtOH (Fig. 4 D and E).

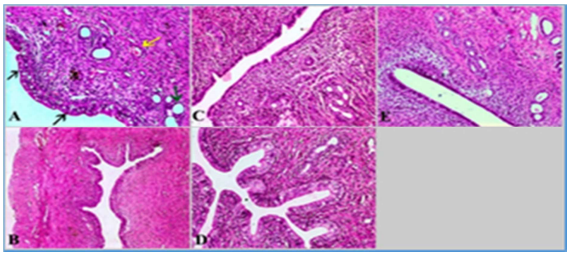

**Figure 4:** Histopathological changes in uterus tissues of different experimental rats (H&D). The control rats showed normal uterus histological structure. The histological inspection of uterus tissues of ethanol-treated rats displayed severe vacuolar degeneration and nuclear pyknosis in the endometrial epithelial cells in the uterus (black arrows) accompanied by moderate cystic dilatation in some of the uterine glands (yellow arrow). Moderate congestion in the endometrial blood vessels was also noticed (green arrow) (A, X-200). The dosing of the *C. Ignea* extract at (250 and 500 mg/kg BW) to rats, showed wholly normalized the histo-structure of uterine tissue without any detective pathological changes (B and C, X-100). The dosing of *C. Ignea* at (250 and 500 mg/kg BW) in ethanol-treated rats succeed to ameliorate the negative effects of ethanol in uterine tissue (D and E, X 200).

### 3.5. IHC results for detection of the NF-κB

The IHC protocol was carried out for the detection of the NF-κB protein (immune-stimulating factor) in gastric, ovarian, and uterine tissues of all different experimental rat groups. The control rats exhibited negative immunoreaction for detection of the NF-κB-P65 in all examined tissues.

#### 3.5.1. IHC findings in the stomach

Figure 5 illustrated a negative immunoreactive detection for the NF-κB antigen in the gastric mucosa and submucosa of the EtOH-treated rats (Fig.5 A). The obtained findings from gastric slides of both groups treated with the *C. Ignea* extract at (250 and or 500 mg/kg BW), revealed a mild positive intracytoplasmic golden-brown staining for the immunoreactive detection of the NF-κB antigen in the infiltrating macrophages in the gastric submucosa (Fig. 5 B and C). Otherwise, the gastric slides of the animals receivedwith*C. Ignea* extract 250 mg/kg BW and EtOH showed moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction against NF-κB antigen in the inflammatory cells of the gastric submucosa (Fig. 5 D). The highest IHC positivity was observed in the slides of rats treated with *C. Ignea* extract 500 mg/kg Bw and EtOH, which illustrated strong positive intracytoplasmic and intranuclear deep brown staining immunoreaction for the existence of NF-κB antigen in the diffusely infiltrated macrophages and lymphocytes in the gastric submucosa (Fig.5 E).

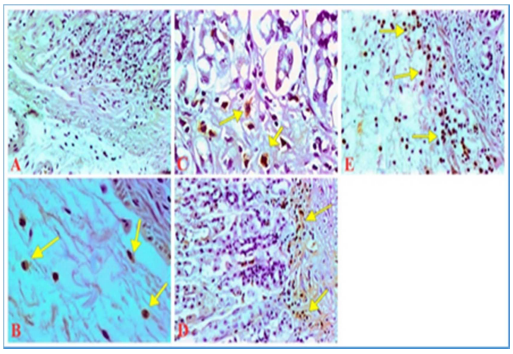


**Figure 5:** IHC findings of NF-κB-P65 in gastric tissue of different experimental rats. The IHC slide of gastric tissues of ethanol-treated rats exhibited negative immunoreactive stained for antigen (A, X 200). The *C. Ignea* -treated rats at the two doses (250 and 500 mg/kg BW) displayed mild positive intracytoplasmic golden brown staining immunoreaction (arrows) toward NF-κB P65 antigen in a few numbers of macrophages in the gastric submucosal layer (B and C, X 400). The gastric IHC slides of rats treated simultaneously with *C. Ignea* extract (250 mg/ kg BW) plus ethanol showed moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction (arrows) against NF-κB P65 antigen in the inflammatory cells of the gastric submucosal layer (D, X 200). In the same trend, the gastric IHC slides of rats treated with *C. Ignea* (500 mg/kg BW) plus ethanol illustrated strong positive intracytoplasmic and intranuclear deep brown staining immunoreaction (arrows) for the existence of NF-κB-P65 antigen in the diffused infiltrated cells mainly macrophages and lymphocytes in the submucosal layer (E, X 200)

#### 3.5.2. IHC findings in the ovary

Figure 6 The EtOH-treated rats, and also the rats treated with *C. Ignea* extract at both two tested doses (250 and 500 mg/kg BW), showed that; the ovarian follicles and the ovarian tissue stroma displayed negative immunoreaction for the existence of the NF-κB antigen (Fig. 6.A, B, and C). On the other hand, the rats treated by *C. Ignea* at 250 mg/kg BW and 500 mg/kg BW and followed by one dose of EtOH illustrated moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction against the NF-κB antigen in some of the primordial follicles of the ovarian tissue (Fig 6 D) and also detected in the infiltrated macrophages around the lutein cells of the corpus luteum (Fig. 6 E).

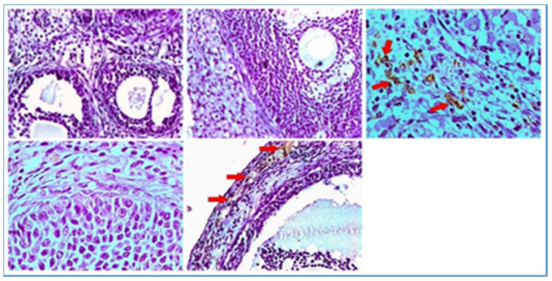

**Figure 6:** IHC findings of NF-κB-P65 in ovarian tissue of different experimental rats. The IHC slides of ethanol-treated rats (A, X 200), and *C. Ignea*-treated rats at two doses (250 and 500 mg/kg BW) exhibited displayed negative immunoreaction for the existence of the NF-κB P65 antigen in the ovarian follicles and ovarian tissue stroma, respectively (B, X 200 and C, X 400). The IHC Slide of the ovarian tissues of rats treated with *C. Ignea* (250 mg/kg BW) plus ethanol displayed moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction (arrows) toward NF-κB-P65 antigen in some of the primordial follicles found in the periphery of the ovarian tissue (D, X 200). A moderate positive intracytoplasmic golden brown staining immunoreaction (arrows) against the NF-κB-P65 antigen was noted in the infiltrated macrophages around the lutein cells of the corpus luteum in ovaries tissues of the rat treated with *C. Ignea*(500 mg/kg BW ) plus ethanol (E, X 400).

### 3.5.3. IHC findings in the uterus

The finding of detection of NF-κB in the uterus of EtOH-treated rats and the *C. Ignea* -treated rats at 250 and 500 mg/kg BW exhibited that the uterine tissue showed no existence of the NF-κB antigen (Fig 7 A, B & C). In contrast to that, the results of the detection of NF-κB in uterine tissues of *C. Ignea*-treated rats at two doses and EtOH showed moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction against the NF-κB antigen, which was detected in the infiltrated macrophages in the lamina propria submucosa and adjacent to the uterine glands (Fig 7 D &E).

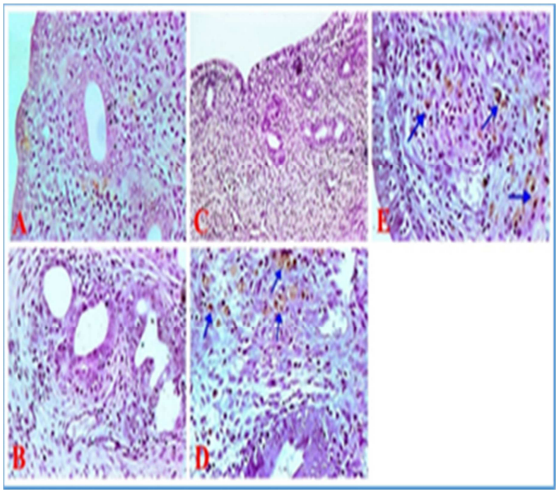


**Figure 7:** IHC findings of NF-κB-P65 in ovarian tissue of different experimental rats. The IHC slide of control rats showed no existence of the immunoreaction for the NF-κB-P65 antigen. (A, X 200) also the same results were noted in rats treated with *C. Ignea* at both low (250 mg/kg BW) and high (500 mg kg BW) doses (B, and C, X 100).A moderate positive intracytoplasmic and intranuclear golden brown stained immunoreaction (arrows) against the NF-κB P65 antigen was noticed in the infiltrated macrophages in the lamina propria submucosa and adjacent to the uterine glands in IHC slides of rats concurrently treated with ethanol and *C. Ignea* extract at tow stated doses ( D and E, X 200).

### *3.6.* The serum FSH, LH, and estradiol hormone levels

The serum female reproductive hormone levels in samples of all experimental rats have represented in figure 8. The serum level of hormones in the EtOH-treated group was significantly diminished when matched with their levels in the control rats. On the contrary, the pre-treatment of *C. Ignea* extract at two experimental doses led to significant improvement *($p < 0.05$)* in levels of FSH, LH, and estradiol in EtOH-treated rats (Fig. 8).

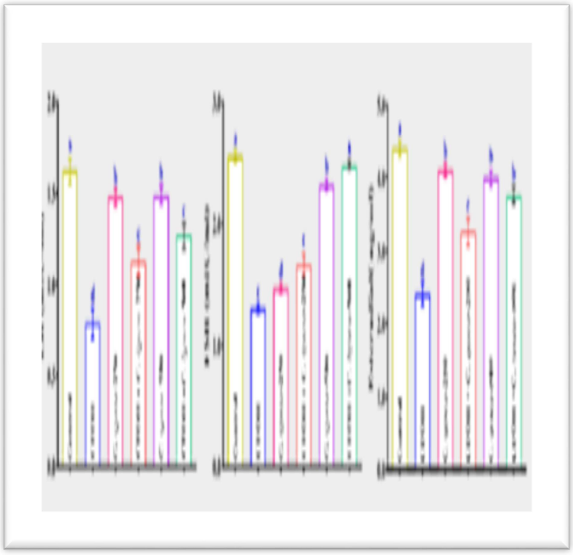

**Figure 8:** Hormonal assay in different experimental groups. Data represented as mean ± SD (n=6). EtOH = Ethanol; *C. Ignea* -250= Cuphea Ignea extract at dose (250 mg/kg BW); *C. Ignea* -500= *Cuphea Ignea* extract at dose (500 mg/kg BW). The different letters represent statistically significant differences ($p < 0.05$) between treatment and control.

## 4. Discussion

The EtOH toxicity occurred in farm animals through environmental intoxication and metabolic disturbance but occurred in humans via regular consumption [25]. Previous studies on EtOH metabolism reported that; In dairy cattle, the rumen fermentation in cows fed on a high-grain diet, promotes high rumen EtOH production as compared with the more hay-diet cows[26]. Alcohol dehydrogenase enzymes (ADH) in rumen microflora are shared in the alcohol interconversion, so prolonged alcohol production during fermentation and the lack of adaptation in ruminal ADH enzyme occurred leading to impairment in alcohol metabolism and toxicity occurred [27]. Moreover, sub-acute ruminal acidosis in calves induced substantial ruminal alcohol production [28].

EtOH intoxication in animals and humans has adverse effects on immune system activity. Excessive alcohol intake is associated with a decrease in the antigen-presenting capacity of monocytes and also

alters the production of the inflammatory cytokines and so raises the infection susceptibility [25]. Acute EtOH intake during the occurrence of microbial infection leads to suppression of the pro-inflammatory cytokine production of TNF-α, IL-6, and IL-1β [29]. Moreover, the decrease in TNFαin EtOH toxicity causes failure in the interaction between the endothelial and lymphoid cells, which leads to insufficient mobilization of the lymphoid cells to the inflammation sites [30]. In the liver, the alcoholic hepatotoxicity showed Kupffer cell depletion, which curbs the inflammatory response [31]. EtOH toxicity declines the natural killer (NK) cell cytotoxic activity which may explain the incidence of liver fibrosis in alcoholics, where the activated NK cells are responsible for killing the activated stellate cells, which produce collagen during liver fibrosis [32, 33].

In this study, the prominent macroscopical findings in the EtOH-treated rats are; severe gastric hemorrhage in the glandular part of the stomach and moderate congestion in the reproductive system. In addition, the main illustrated histopathological findings were severe hemorrhagic gastritis and oophoritis. On the other side, the *C. Ignea* extract (250 and 500 mg/kg BW) –treated rats displayed high tissue protection from the EtOH's toxic effects.

The hemorrhagic effect of EtOH toxicity could be attributed to the vascular impairment effect of EtOH, where the alcohol causes alterations in the function of the endothelial cells and nitric oxide availability that led to arterial plaque build-up, and arterial-vascular function disturbance [34]. Several reports indicated that alcohol causes adverse effects on the endothelial-nitric-oxide-generation system, where endothelial dysfunction is considered an early detector for blood vessel damage and atherosclerosis [35, 36]. Several research works in animals and humans revealed that the vascular wall oxidative stress is also a key way in EtOH toxicity where EtOH can increase the levels of the ROS leading to elevation of the redox-signaling pathways and diminishing the protective antioxidant levels [37].

Due to their pharmaceutical activities, medicinal plants and even herbs are being charmed targets by many researchers and drug manufacturers. the extracts of many medicinal plants and herbs succeed in protecting the organs and maintaining the physiological functions of the body against lots of environmental pollutants [38-42]. *C. Ignea* is one of the outstanding herbs, and it has a wide pharmaceutical activity. In the current study, *C. Ignea*extract y protected the EtOH -treated rats from hemorrhagic inflammation where *C. Ignea* is rich in coumarin [11] which beneficially acts as a vasodilator, antithrombotic, and anticoagulant, also coumarin can effectively decrease tissue edema via the inactivation of both cyclooxygenase and lipoxygenase enzymatic activities and prostaglandin synthesis [43].

The IHC findings in the gastric, ovarian, and uterine tissues displayed that; the control rats and the EtOH-treated rats showed negative immunoreactive detection for the NF-κB P65 antigen. While, a mild positive immunoreaction for the NF-κB P65 antigen was detected in the infiltrating macrophages of the gastric submucosa in the *C. Ignea* plant extract (250 and 500 mg/kg BW) –treated rats. These IHC findings come in the same line with several reports which recorded that, EtOH intoxication leads to a lowering of the NF-κB serum level, which is known as a central regulator for the gene transcription of the proinflammatory and immunostimulant mediators, and the inhibition of NF-κB by EtOH can lead to a reduction in both of IL-1, IL-6, TNF-α and IL-1β [44]. Several reports revealed that; acute EtOH toxicity leads to inhibition of the activation process of the NF-κB in the human monocyte inflammatory cells [45].

Moreover, in murine peritoneal macrophages, acute EtOH toxicity diminished the NF-κB activity [46]. The suppression in the NF-κB activity by EtOH toxicity was also causing a decrease in the TNF-α activity in the endothelial cells, which has a great role in the lymphoid cell recruitment toward the inflammation sites [47].

The IHC results of simultaneous treatment of the EtOH-treated and low dose of *C. Ignea* showed a moderate positive immunoreaction against the NF-κB antigen, which was detected in the inflammatory cells of the gastric submucosa and also found in the ovarian and uterine tissues. The great IHC positivity for NF-κB antigen was observed in *C. Ignea* plant extract 500 mg/kg + EtOH-treated rats. These findings could be attributed to the that; the recent phytochemical analysis recorded the existence of polyphenols like flavonoids, phenolic acids, and tannins in *C. Ignea* plant extracts [10, 48]. Several types of research on polyphenols have guided multiple insights concerning the effects of

polyphenols on immune function. Each polyphenol type targets and is joined with one or more immune cell receptors and therefore triggers the intracellular signaling pathways which eventually regulate the immune response [49]. One of these receptors is the tall-like receptor (TLR) on dendritic immune cells (DIC), where the polyphenol-TLR interaction induced activation of the NF-κB pathways, leading to DIC activation [44, 49]

The reproductive hormonal measurements in our study displayed a significant decrease in FSH, LH, and estradiol hormones in the EtOH-treated group. On the other hand, the *C. Ignea*-treated rats, and the *C. Ignea* + EtOH co-treated rats showed normal hormonal levels. The effects of EtOH toxicity on the reproductive hormones could clarify as; the alcohol intake is accompanied by reproductive function disorders where, alcohol has a deleterious effect on the hypothalamic, pituitary, and gonadal axis (HPG axis), and is associated with gonadal atrophy and infertility [50].

Another study recorded that, alcohol over-intake leads to alterations in the HPG axis activity during puberty via causing an alcohol-damaging action on the ovaries, and also joined with a significant decrease in LH and estrogen levels [51]. Moreover, the metabolic transformation of EtOH to acetaldehyde increases the oxidative stress of the rat ovarian tissue causing ovarian cell damage and dysfunction. In humans, acute alcohol toxicity in postmenopausal women treated with hormonal therapy causes a decrease in the conversion of estradiol to estrone [52].

In rats and monkeys, alcohol toxicity induced reproductive disturbances as those occurred in humans. It was reported that acute alcohol toxicity disrupts the normal cycle during the exposure period [50].

The phytochemical analysis for *C. Ignea* plant extract indicated its enrichment from polyphenols like flavonoids, phenolic acids, and tannins [10, 11]. Flavonoids are reported to have estrogenic and antiestrogenic properties and minimize gonadal tissue damage by oxidation [53, 54]. Phenolic compounds are considered potent chain-breaking antioxidants and have scavenging activity through their hydroxyl groups [55].

These antioxidants and scavenging activities have been reported to frustrate environmental toxicity in connection to the reproductive function where these phenols have androgenic properties [56]. Phenols and flavonoids are implicated in elevating sex hormone levels by inhibiting the cyclooxygenase-2 enzyme which is responsible for inhibiting the StAr gene and in turn inhibiting steroidogenesis [55].

## 5. Conclusion

The obtained results are; that EtOH toxicity in rats exhibited an immune-suppressive effect via inhibition of the NF-κB synthesis, which is considered an immune-stimulating factor responsible for several immune reactions and immune cell development, also the EtOH toxicity causes histopathological and hormonal changes in female reproductive organs. The *C. Ignea* plant extract showed an immune stimulant effect by enhancing the synthesis of NF-κB which is detected in different tissues by immunehistochemistry technique, and also the *C. Ignea* extract protects the rat organs from EtOH toxicity.

## 6. List of Abbreviations

ADH: alcohol dehydrogenase; $AlCl_3$: aluminum chloride;BW: body weight;BSA: bovine serum albumin; DIC: dendritic immune cells; DMRT: Duncan's multiple range test;DPPH-2,2 :diphenyl-1-picrylhydrazyl radical; E2: estradiol; ELISA: enzyme-linked immune-sorbent assay; EtOH: ethanol;Fig: figure;FSH: follicle stimulation hormone; g; Gram; GIT: gastrointestinal tract; h: hour;HPG axis: hypothalamic, pituitary, and gonadal axis; IHC: immunohistochemistry protocol; IL-1β: interlukin-1 betta;IL-6: interlukin-6; IκB: inhibitor of NF-kB; IκBα: inhibitor kinase of NF-kB; Kg: kilogram; LH: leutinizing hormone;M: molar:mg/kg: milligram per kilogram; min: miute;mL: milliliter;n: number; $NaN_3$: sodium azide; NBF: neutral buffered formalin; NF-kB-p65: nuclear factor kappa light chain b lymphocyte -heterodimeric p65; NF-κB: nuclear factor-kappa light chain b lymphocyte; NK: natural killer cell; nm: nanometer;NRC: National Research Centre, Egypt; ºC: Celsius degree;*p <0.05*: *p-value* less than 0.05; pg/ mL: picogram per milliliter; ROS:reactive oxygen species; rpm: revolutions per minute; RSA: radical scavenging activity; SD: standard deviation;TLR: tall-like receptor; TNF-α: tumor necrosis factor alfa; U/ mL: unit per milliliter; USA: United states of America;

UV: ultra-violate;v/v: volume/volume; µL: micro-litter; *C. Ignea*: Cuphea ignea; %: percent sign.

## 7. Conflicts of interest

There are no conflicts to declare.

## 8. Formatting of funding sources

Not applicable.

## 9. Authors Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by AAM, AMM, NME, SKH, MN, and MMS. The first draft of the manuscript was written by AAM and MMS reviewed and edited the last version of the manuscript. All authors read, commented, and approved the final versions of the manuscript.

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