

## Biochemical, histological and ultrastructural studies on the ameliorative effects of chrysin on the hepatotoxicity of clonazepam in developing male albino rats

Sahar A. Sabry<sup>1\*</sup>, Marwan A. Ibrahim<sup>2</sup> and Rehab M. Mosaad<sup>2,3</sup>

1- Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Egypt

2- Department of Biology, College of Science, Majmaah University, Majmaah 11952, Saudi Arabia,

3-Department of Zoology, Women's College, Ain Shams University, Egypt

Corresponding author Email: [sahar\\_ahmed@edu.asu.edu.eg](mailto:sahar_ahmed@edu.asu.edu.eg)

Co-authors E-mail: [m.ab.ibrahim@mu.edu.sa](mailto:m.ab.ibrahim@mu.edu.sa) , [rehab.m@mu.edu.sa](mailto:rehab.m@mu.edu.sa)

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### ABSTRACT

Chrysin is bioactive flavonoids that has numerous pharmacological activities and known to have hepatoprotective effect. The present study aimed to study the potential ameliorative effects of Chrysin (50 mg/kg b.wt./day) on Clonazepam (2 mg/kg b.wt./day) induced liver toxicity. Animals were divided into 4 groups, ten rats in each. Group 1 (Control group received a vehicle 1% w/v Tween 80), group 2 (received 2 mg/kg b.wt./day Clonazepam (CZP) suspended in 1% w/v Tween 80, group 3, (received 50 mg/kg b.wt./day Chrysin suspended in 1% w/v Tween 80, group 4 (received 2 mg/kg b.wt./day CZP, and Chrysin, 50 mg/kg b.wt./day). All animal groups were treated by oral gavage daily for 6 weeks starting at the first day of the experiment. The results indicated that in contrast to the control group, liver transaminases (ALT and AST), malondialdehyde (MDA) and cytochrome P450 (Cyp3A4) were increased after the Clonazepam treatment, while the liver protein and the total antioxidant activity (TAA), glutathione reduced (GSH) contents and glutathione S-transferase (GSTs) activity were decreased. Also, the histological examination demonstrated that liver sections of CZP treated developing rats showed cytoplasmic vacuolation and fatty degeneration of some hepatocytes and their nuclei exhibited pyknosis and karyolysis. In addition to conspicuous distortions in the vasculatures, Kupffer cells proliferation and infiltration of lymphocyte were observed. Electron microscopic investigation of hepatocytes of CZP treated animals' revealed clear changes as mitochondrial dysfunction with loss of their cristae and compressed matrices. Also, dilatation and fragmentation of the rough endoplasmic reticulum into smaller stacks have been observed.

On the other hand, marked improvement in the liver tissue against the damage displayed by CZP was recorded in biochemical, histological and ultrastructural screening of the hepatic animal sections treated with CZP+ Chrysin.

**Keywords:** Chrysin, hepatotoxicity, Clonazepam, developing rats.

### INTRODUCTION

Benzodiazepines (BZDs) are sedative-hypnotic agents that are the most specific group of psychotropic drugs. BZDs, Clonazepam is an antiepileptic drug which has a therapeutic effect in psychiatric disease (Nardi and Perna, 2006; Mendonca *et al.*, 2015). Clonazepam

decreases the level of anxiety, tension, and agitation, besides manage aggression in schizophrenia disease and targets GABA-A receptors. Clonazepam potentiates the effect of GABA in the brain by increasing the GABAergic inhibition. Clonazepam is metabolized by nitro-reduction and then N-acetylated to 7-amino-clonazepam and 7-

acetamido-clonazepam, respectively. The Nitro-reduction of clonazepam was catalyzed by cytochrome P450 (CYP3A) enzymes (Tóth *et al.*, 2016; Badawia *et al.*, 2016). As benzodiazepines, the clonazepam misuse increased and it was used as a street drug (Frauger *et al.*, 2009; Frauger *et al.*, 2013). Benzodiazepines are known by its association with hepatic encephalopathy in cirrhosis when treated for 3-10 days (Johnson and Hayward, 2021). As other benzodiazepines, clonazepam may induce anxiety symptoms, as the increase of dependence and tolerance (Mowla *et al.*, 2007; Kacirova *et al.*, 2016; Dokkedal-Silva *et al.*, 2020 a&b).

All drugs have side effects on liver and cause hepatotoxicity due to the important role of the liver in medication metabolism. Liver converts drugs into byproducts that are easily excreted. These byproducts are metabolites that have higher toxicity than the original drug itself (Atici *et al.*, 2005).

Cultivated and wild plants are rich in their natural healthy compounds that have a good potential in treatment and prevention of diseases (El-Zayat *et al.*, 2021). Chrysin is a bioactive flavonoid existing in plants related to the genus *Passiflora* and bee propolis (Mani and Natesan 2018; Ignat *et al.*, 2020; Temel *et al.*, 2020). It has many pharmacological properties such as protection of neurons, heart and kidney diseases. Also, it has antibacterial, anticancer, antiarthritic and antiasthmatic (Mani and Natesan 2018; Pai *et al.*, 2019; Ignat *et al.*, 2020). Moreover, it has hepatoprotective effects against hepatotoxins such as ethanol (C<sub>2</sub>H<sub>5</sub>OH) (Tahir and Sultana, 2011), carbon tetrachloride (CCl<sub>4</sub>) (Anand *et al.* 2011), ammonia (NH<sub>3</sub>) (Renuka *et al.* 2016), cisplatin (Rehman *et al.*, 2014), d-galactose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) (Anand *et al.* 2012), D-galactosamine (Pushpavalli *et al.*, 2010), 2,3,7,8 - tetrachlorodibenzo - p - dioxin (TCDD) (Ciftci *et al.*, 2011), paracetamol (Eldutar *et al.*, 2017), methotrexate

(C<sub>20</sub>H<sub>22</sub>N<sub>8</sub>O<sub>5</sub>) (Ali *et al.*, 2014), and chemotherapy by doxorubicin (Rashid *et al.*, 2013). On the other hand, Chrysin has hypolipidemic, anti-inflammatory, antidiabetic activities (Zarzecki *et al.*, 2014; Feng *et al.*, 2014; Samarghandian *et al.*, 2016). Moreover, it has anti-fibrotic effect in hepatic fibrosis which confirmed in recent studies (Ignat *et al.*, 2020) and anti-allergic, anti-apoptotic, and antioxidant effects (Eldutar *et al.*, 2017).

The current study was prepared to determine the ameliorative effects of Chrysin versus Clonazepam -induced liver toxicity, especially its role in decreasing oxidative stress, and apoptosis.

## MATERIALS AND METHODS

### 1. The experimental animals

Forty Wister developing male rats four weeks old and weighing (60 ± 5 g) were utilized in this study. The rats were purchased from the animal house of the National Organization for Drug Control and Research (NODCAR) and were kept in plastic cages, ten rats in each cage, at a constant temperature of 25±2 °C and 12 hours of light/12 dark period throughout the experiment. A pellet diet billboard was used during the experiment. The animals were given one week to adjust to the laboratory settings before the investigation started. In compliance with the rules of the Ethics Committee of Scientific Research, Ain Shams University, Cairo, Egypt, the experimental procedures were carried out.

### 2. The drugs

Chrysin, 5,7-Dihydroxyflavone, 98% was manufactured by Alfa Aesar Germany. Clonazepam (CZP) 2 mg oral tablets are available as generic drug and brand name drug is Apetryl. Apetryl tablets are produced by Multi-Apex for medicinal drugs industries S.A.E- Badr City -Egypt.

### 3. The experimental design

Animals re wdivided into 4 groups, ten rats in each, as the following:

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- Group 1 (Control group received a vehicle 1% w/v Tween 80) according to (El Khashab *et al.*, 2019).
- Group 2 (received 2 mg/kg b.wt./day Clonazepam (CZP) (Mohamed *et al.*, 2015) suspended in 1% w/v Tween 80 (Socala *et al.*, 2018).
- Group3, (received 50 mg/kg b.wt./day of Chrysin (Mehri *et al.*, 2014) suspended in 1% w/v Tween 80 according to (El Khashab *et al.*, 2019).
- Group 4 (received 2 mg/kg b.wt./day CZP and Chrysin, 50 mg/kg b.wt./day).

All animal groups were treated by oral gavage daily for 6 weeks starting at the first day of the experiment. The used doses are human equivalent pharmaceutical doses and were calculated as stated by Reagan-Shaw *et al.* (2008).

### 4. Biochemical assays:

Liver transaminases (ALT) and (AST) activities were determined in liver tissue by colorimetric method of Young (1990). Glutathione reduced concentration was estimated by colorimetric method of Beutler *et al.* (1963). Malondialdehyde was determined as the thiobarbituric acid (TBA) according to Ohkawa *et al.* (1979). Hepatic cytochrome P450 (Cyp3A4), total antioxidant capacity (TAC) Glutathione S-transferase (GSTs) activity, and protein content were calculated by ELISA assay.

### 5. Histological and Ultrastructural Preparations

In aqueous Bouin's fixative, small pieces of the liver of control and treated rats were fixed for 24 hours for light microscopic examination. Then, all specimens were dehydrated, purified in terpeneol and embedded in paraffin wax. The hematoxylin and eosin stain were used for sections of 5  $\mu$ m thickness, microscopical examination and photomicrographs were made. Fresh very small pieces of liver were fixed

immediately for 24 hours in cold 4FIG (4% formalin+1% glutaraldehyde at pH 2.2) for ultrastructural preparation by transmission electron microscopy (Dykstra *et al.*, 2002). Then, they were postfixed in buffered (0.1 M phosphate buffer) 1% osmium tetroxide, dried in a chain of ethanolic culminating in 100% acetone, and penetrated with epoxide resin. After keeping the semi-thin sections (0.5 $\mu$ m) overnight at 60 °C for polymerization, they were stained with 1% toluidine blue and investigated by the light microscope. The ultrathin sections mounted on 200 mesh copper grids, were stained, examined and photographed by a JEOL –JEM-1400 EX- electron microscope at the Regional Center for Mycology and Biotechnology, Al-Azhar University.

## RESULTS

### 1. Biochemical study

There was a significant increase in liver enzymes ALT and AST activities in Clonazepam (2mg/kg b.wt.) group and clonazepam+chrysin treated group as compared to the treated control one (Table 1). The group received chrysin with clonazepam exerted a significant ( $P<0.05$ ) ameliorative effect on liver function parameters. Rats injected with clonazepam displayed a significant decline in liver protein content (TP) as compared to the treated control group and there was a valued decrease in chrysin group (50mg/kg) with clonazepam as compared to the clonazepam treated group. Chrysin (50mg/kg) group showed significantly decreased malondialdehyde (MDA) ( $P<0.05$ ) content in liver of clonazepam treated group. Additionally, chrysin caused a significant increment in the total antioxidant activity (TAA), glutathione reduced (GSH) concentration and glutathione S-transferase (GSTs) activity in liver tissue when injected with clonazepam as compared to clonazepam alone.

In Clonazepam treated group (2mg/kg), there was a significant ( $P<0.05$ ) elevation in liver cytochrome P450 (Cyp3A4) as compared to the treated control group and a valued decrease was

reported in group injected with clonazepam and chrysin (Table 1). Further, there was no significant changes in all parameters of chrysin treated group.

**Table 1:** The values of alanine aminotransferase (ALT), aspartate aminotransferase (AST) contents in the liver (u/ml), protein content (ng/ml), malondialdehyde (MDA) contents (n mol/mg), total antioxidant activities (TAA) (ng/mg/ protein), Glutathione reduced (GSH) concentration (m. mol/mg), Glutathione S-transferase (GSTs) activity (ng/mg protein) and cytochrome Cyp3A4 (ng/mg) of various treated groups of developing male albino rats.

| Parameters Groups  | ALT              | AST              | TP             | MDA            | TAA             | GSH             | GST             | CYT P           |
|--------------------|------------------|------------------|----------------|----------------|-----------------|-----------------|-----------------|-----------------|
| CONT               | 33.49<br>±0.42   | 47.38<br>±0.82   | 2.60<br>±0.19  | 0.74<br>±0.03  | 6.03<br>±0.33   | 7.23<br>±0.21   | 5.19<br>±0.22   | 4.63<br>±0.15   |
| CHRY               | 31.82<br>±0.92   | 46.43<br>±2.14   | 2.89<br>±0.03  | 0.73<br>±0.07  | 6.37<br>±0.11   | 7.24<br>±0.18   | 4.86<br>±0.26   | 4.77<br>±0.10   |
| CLONZ              | 74.38<br>±0.90a  | 87.69<br>±1.27a  | 1.60<br>±0.05a | 3.20<br>±0.19a | 1.63<br>±0.10a  | 1.21<br>±0.07a  | 0.89<br>±0.06a  | 11.03<br>±0.30a |
| CLONZ<br>+<br>CHRY | 45.98<br>±1.25ab | 55.95<br>±1.41ab | 2.70<br>±0.04b | 0.93<br>±0.03b | 3.95<br>±0.28ab | 4.80<br>±0.24ab | 3.79<br>±0.09ab | 4.44<br>±0.39b  |

Values are means of 8 rats  $\pm$  SE at  $p<0.05$ . 'a' represents significant change from control group, 'b' represents significant change from Clonazepam group.

## 2. Histological and histopathological examination

### Group I: Control- animals

Characteristic lobular organization of the mammalian liver was apparent in the livers of developing rats of the control group, each lobule is formed by hepatocyte cords extending from a central vein. The liver strands are distinct by blood sinusoids, which are interspersed with Kupffer cells and covered by endothelial cells (Fig. 1 a.).

Liver lobules divided by loose connective tissue that contains the portal triads, which include a thin bile ductile, portal vein branches and hepatic artery branches (Fig. 1 b).

### Group II: Chrysin-treated animals

Study of liver sections from developing rats given 50 mg/kg/day of Chrysin indicated a typical pattern of

hepatic lobules and well-organized hepatic threads (Fig.2 a). The appearance of the hepatic cells was nearly normal. (Figs. 2 a &b.) The portal space appeared with approximately similar appearance to the control group (Fig. 2 b).

### Group III: Clonazepam-treated rats

The hepatic tissues of developing rats received 2mg/kg/day of Clonazepam (CZP) revealed marked changes. Some hepatocytes showed signs of cytoplasmic vacuolation and fatty degeneration (Figs. 3 a & b). Pyknosis and karyolysis were also seen in the nuclei of many hepatocytes (Figs. 3 a&b). There were a liver sinusoidal dilatation and congestion with swelling of Kupffer cells (Fig. 3a). Congestion of blood vessels was indicated by the dilation of the central vein which contained stagnant hemolyzed blood cells (Fig.3 a). Inflammatory lymphocytes invaded all portal areas (Fig. 3 b).

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### **Group IV: CZP+ Chrysin -treated animals**

Liver histological structure of most developing animals injected with 2 mg/kg b.wt./day CZP + 50 mg/kg b.wt./day Chrysin, manifested remarkable improvements for the damage effects of CZP only. The hepatic cords were well organized and the cytoplasmic vacuolation fade (Figures.4 a &b). Most nuclei presented in normal shape, spherical and located centrally (Figure. 4 a). The portal space demonstrated that it was possible to recover effectively (Fig. 4 b).

### **3. Ultrastructural study**

#### **Group I: Control- animals**

The livers of the control animals showed a normal ultrastructural aspect (Figures 5a &b). Hepatocytes have many mitochondria dispersed in the cytoplasm. The mitochondria possess well-developed cristae and are ovoid or spherical in shape (Fig. 5a). The rough endoplasmic reticulum is made up of flattened, parallel cisternae studded with ribosomes (Fig.5 a). Hepatocyte nuclei are spherical, present its own nuclear membrane, and the euchromatin and heterochromatin materials aggregations in nucleoplasm (Fig. 5a). Kupffer cells border the hepatic sinusoids, which are formed between hepatocytes (Fig. 5 b).

#### **Group II: Chrysin- treated animals**

There were no observed cytopathological changes in liver cells of Chrysin treated group as compared to the control group. The rough endoplasmic reticulum clumped together, and the mitochondrial appearance in a typical configuration (Fig. 6 a). Hepatocyte nuclei

revealed moderate heterochromatin aggregation on the inner surface of the nuclear membrane (Fig.6 a). Kupffer cell nuclear envelope is irregular in Figure (6 b).

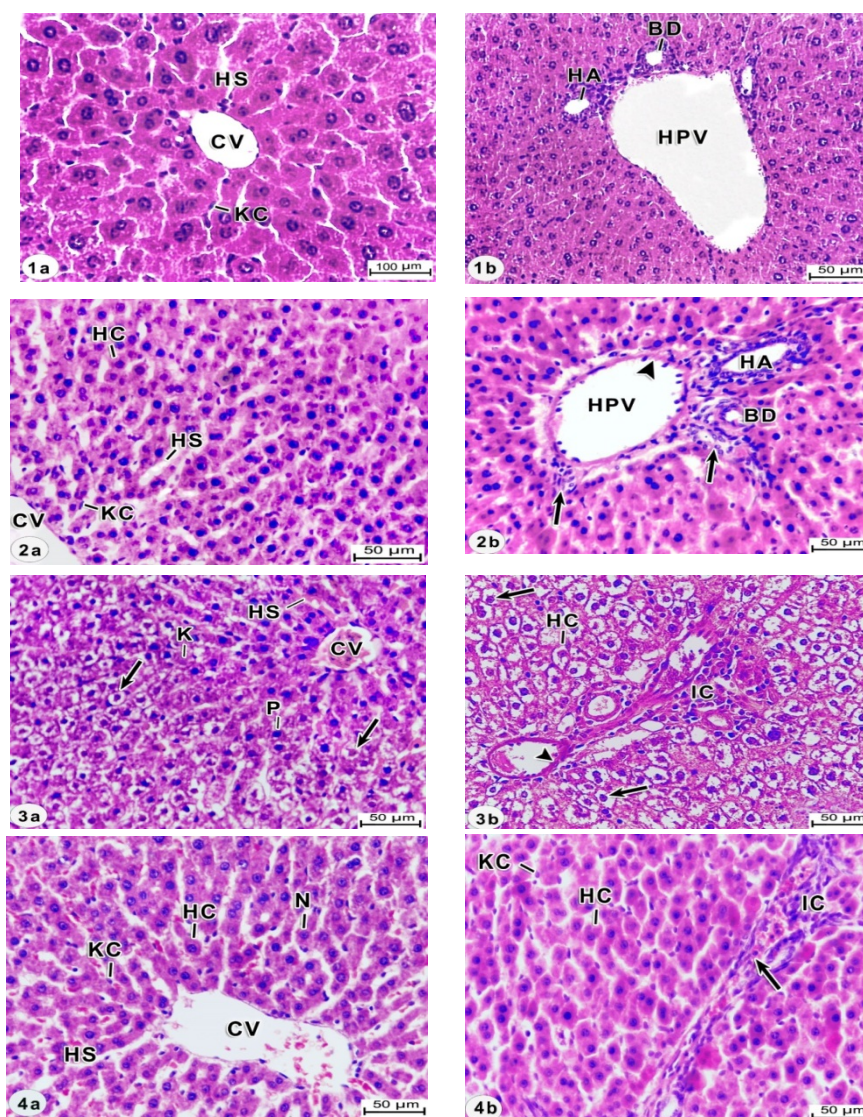
#### **Group III: Clonazepam-treated rats**

Clonazepam treatment elicited devastating effects in the ultrastructure of developing rats' hepatocytes. The cytoplasm of the hepatocytes was visibly degraded and vacuolized (Fig.7 a). With the condensation of their matrices, the mitochondria appeared swollen, and some lost their internal cristae. Rough endoplasmic reticulum fragmentation into smaller stacks is also appeared (Fig. 7 a). Moreover, the nuclei showed shrinkage and condensation of the chromatin material as seen in Figure (7a). Kupffer cell hypertrophy was also seen, the nucleoplasm displayed clusters of heterochromatin adhering to the nuclear envelope, and irregular nuclear membrane (Fig. 7b).

#### **Group IV: CZP+ Chrysin-treated rats**

Clonazepam +Chrysin treated animal group had remarkable improvement. The hepatocyte cytoplasm showed more or less nuclei damage with little chromatin concentration as seen in the hepatocytes electron micrographs (Fig. 8a), regaining the condition of almost normal appearance (Fig. 8a). Numerous mitochondria with a nearly typical configuration were seen in the hepatocytes, as well as a fully developed rough endoplasmic reticulum in the shape of parallel and flattened cisternae (Fig. 8a). Kupffer cells with lysosomes and an uneven nuclear membrane also emerged (Fig. 8b).





**Figures (1-4):** Photomicrographs of hepatic sections of the control and treated developing rats stained with HX-E.

**Figs. (1 a & b). Control developing rats (group 1).** **Fig. (1a):** A web of liver strands around the central vein (CV), the hepatic sinusoids (HS) between the rows of hepatocytes which limited by flattened endothelial cells interfere by large phagocytic Kupffer cells. **Fig. (1b):** The portal space, which includes a branch of the liver portal vein (HPV), the liver artery (HA), and the tiny bile ductule (BD).

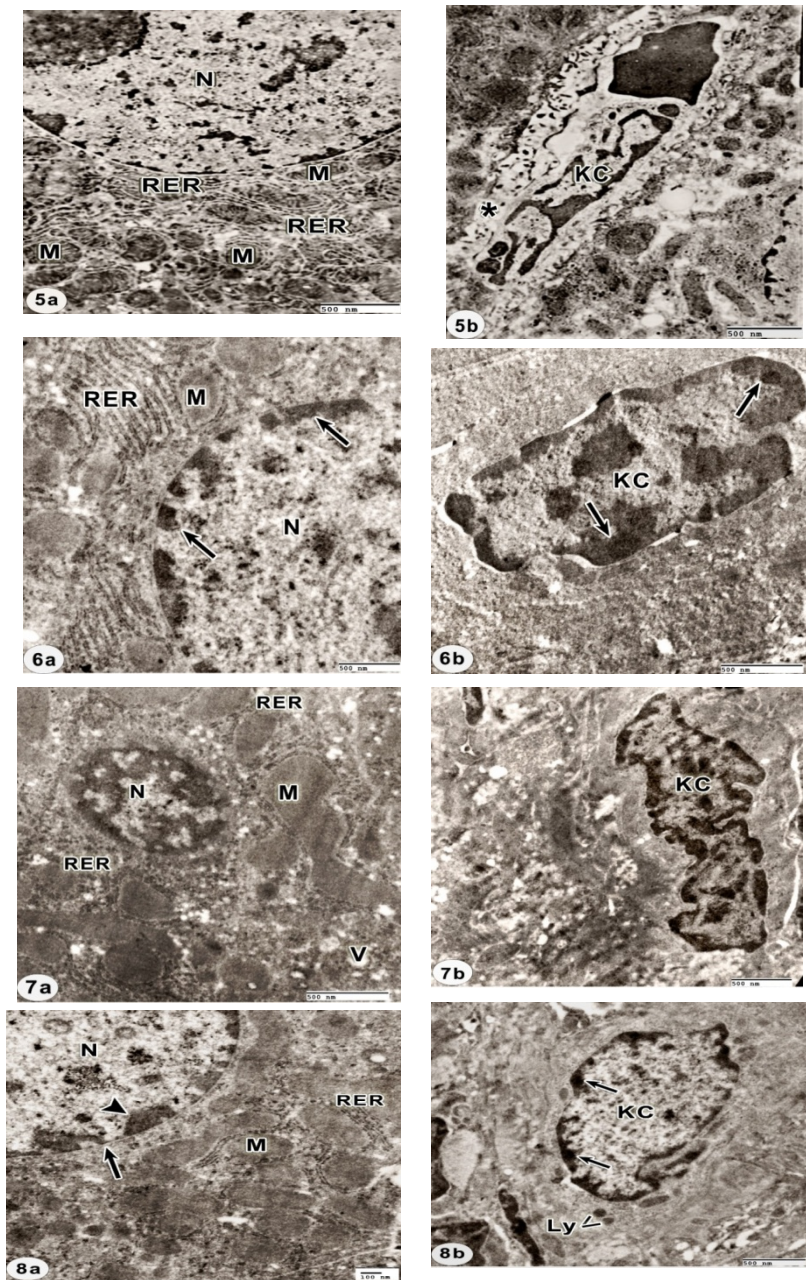
**Figs (2 a & b). Rats of group (2) received chrysin identify:** **Fig. (2a).** Well appearance hepatic strands with frequently normal hepatocytes (HC), central vein (CV), and some hepatic sinusoids engaged by red blood cells (arrows). Also swollen Kupffer cells (KC) is also appeared. **Fig. (2 b).** Nearly normal appearance for the portal space, with few inflammatory cells (arrows) around the hepatic portal vein (HPV) which have thickening of the endothelial lining (arrow head), the hepatic artery (HA), and the bile ductule (BD).

**Figs. (3 a & b). Rats (group 3) injected with Clonazepam.** **Fig. (3a).** Representing dilated and engorged central vein and liver sinusoids (HS), extensive vacuolar and fatty degenerations of hepatocytes (arrows) with clear karyolysis (K) and pyknosis (P) of some liver cells nuclei **Fig. (3 b).** Showing fatty changes in hepatic cells (HC) with karyolysed and karyorrhixed their nuclei (arrows). There was a thickness in the endothelial lining of the liver portal vein (IC) and the portal space (\*) infiltrated by Inflammatory cells.

**Figs. (4 a & b). Rats (group 4) injected with Clonazepam + Chrysin.** **Fig. (4a).** Central vein (CV) surrounded by the hepatocytes (HC) partly repaired their normal figure and their nuclei (N) appeared nearly normal. Hepatic sinusoids (HS) dilatation with swollen Kupffer cells (KC) were noticed. **Fig. (4b).** Affected portal space surrounded by inflammatory cells (IC), engaged by RBCs (arrows) with good configuration of hepatocytes (HC) and well developed Kupffer cells (KC).



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**Figures (5-8). Electron micrographs of liver sections of control and treated developing rats.**

**Figs. (5 a & b). Rats of control.** **Fig. (5a).** The cytoplasm of liver cell has mitochondria (M) of various shapes, well developed rough endoplasmic reticulum (RER) and a Part of nucleus (N) with its nuclear membrane. **Fig. (5 b).** Liver sinusoid is detached from the neighboring liver cell(\*). The sinusoid is bounded by a notable Kupffer cell (KC).

**Figs. (6 a & b). Rats of group (2) received Chrysin.** **Fig. (6a).** Mitochondria (M) with normal appearance, obvious aggregation of rough endoplasmic reticulum (RER) and heterochromatin aggregation on the inner surface of nuclear envelope (arrows). **Fig. (6 b).** Activated Kupffer cell (KC) in dilated hepatic sinusoid was appeared. Condensed heterochromatin presented on the nuclear inner membrane (arrows).

**Figs. (7 a & b). Rats (group 3) injected with Clonazepam.** **Fig. (7a).** The hepatocyte cytoplasm contains many vacuoles (V), clear swelling of mitochondria (M) with no internal ridges and rough endoplasmic reticulum fragmentation. Shrinkage and pyknotic nucleus (N) is also seen. **Fig. (7b).** Kupffer cell has Pyknotic nucleus.

**Figs. (8 a & b). Rats (group 4) injected with Clonazepam + Chrysin.** **(Fig. 8 a).** Partly restore of normal hepatocytes configuration with spherical mitochondria, rough endoplasmic reticulum (RER) found near the nuclear membrane. Distinct nuclear envelope (arrow) and nucleoplasm with euchromatin and heterochromatin (arrowheads). **Fig. (8b).** Heterochromatin clumps presented in Kupffer cell nucleus (arrows) and lysosomes (Ly) vary in size in the cytoplasm.

## DISCUSSION

Liver is the organ that responsible for drugs and xenobiotics detoxification and metabolism. Drugs and xenobiotics up uses can damage hepatocytes and release ALT and AST into blood circulation and cause many hepatic diseases as fatty liver, hepatic fibrosis, and cirrhosis (Chiang, 2014). In the present study, liver ALT and AST activities were increased significantly in clonazepam group.

Drug-induced liver injury is realized by abnormal changes in liver biochemistries, which will increase the assessment of the specific causes of liver toxicity and eliminate false positives. This led to early detection and prediction of drug-induced liver injury. The modern Roussel Uclaf Causality Assessment Method (RUCAM) uses ALT more than five-times the upper limit of normal to identify liver damage. Moreover, the liver cell injury suggests an increment in ALT and AST (Aithal *et al.*, 2011; Danan and Teschke, 2018&2019; Sandhu and Navarro, 2020). Also, it was recorded by Hyman Zimmerman who examined 114 patients taking isoniazid that there is an elevation in ALT >3-times ULN (Sandhu and Navarro, 2020).

In hepatocytes, most lipophilic drugs undergo phase 1 reaction and mediated by the cytochrome P450 system. Then, the resulted new intermediate reactive metabolites are unoperated by phase 2 reactions as glutathione or sulfate conjugation. If coupling pathways are saturated by toxic reactive metabolites, the binding of the reactive metabolites to mitochondrial proteins results. when coupling paths are saturated with more toxic reactive metabolites, the reactive metabolites bind to mitochondrial proteins. In addition, this leads to manufacture of ROS and ATP depletion, resulting in attacking of macromolecules like lipids, carbohydrates, proteins, nucleic acids and cellular organelle dysfunction and then causes liver dysfunction, necrosis, and cell

death (David and Hamilton, 2010; Mosaad and Sabry, 2015; Mosaad and Sabry, 2017; Iorga *et al.*, 2017; Nouredin and Kaplowitz, 2018; Sandhu and Navarro, 2020).

Lipid peroxidation produced by ROS attack to lipid membrane, resulting in large amounts of reactive substances. Polyunsaturated fatty acids peroxidation produced MDA. To control the reactive oxygen species, cellular antioxidant system includes enzymatic and non-enzymatic compounds have developed (Wayhs *et al.*, 2010; Ibrahim and Saleh, 2012; Heibash *et al.*, 2014; Ibrahim and Salah-Eldin, 2019; Ibrahim *et al.*, 2020). The data in table (1) illustrated that clonazepam recorded a significant decrement in protein content, total antioxidant activity, glutathione reduced content and glutathione S-transferase activity in hepatocytes. We are referring these results to the elevation of hepatocyte malondialdehyde as a result of the reactive oxygen species (ROS). This may expose the liver to reactive metabolites which can covalently bind to hepatocyte and mitochondrial proteins and causing overwhelming of total antioxidant and GSH content. (Ibrahim, 2017; Ibrahim and Mosaad, 2021) attributed the reduction in GSH and GST to the cellular protection of glutathione from oxidative stress and its Sulphydryl group plays essential role in this action. Also, Glutathione-S-transferase accelerate the reduced glutathione conjugation reactions with drugs. In our previous studies, we attributed the decrement in TAA and GSH to the decrement of antioxidant pool and oxidative stress elevation (Mosaad and Sabry, 2017; Ali *et al.*, 2015).

Cytochrome P450 (CYP450) responsible for the biotransformation of drugs and classified into families and subfamilies depending on amino acid sequences. Most drugs in the United States stated to be metabolized by cytochrome P450 (CYP1, CYP2 and CYP3) families. The enzymes responsible for oxidation of 79 percentage



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of these drugs are CYP2C9, CYP2D6, CYP2C19 and CYP3A4/5 (Patel *et al.*, 2020).

In experiments carried out by Seree (1993) on 14 human liver microsomal preparations, there was a correlation ( $r=0.70$ ) between benzphetamine and clonazepam metabolism and P450 3A4 was involved in benzphetamine metabolism and clonazepam was likely nitroreduced by the same isozyme. Elbekai *et al.* (2004) indicated that CYP3A4 is the most important drug metabolizing enzyme because it catalyzes the oxidation of over 50% of actually used drugs. In the current study, there was a significant ( $P<0.05$ ) elevation in liver cytochrome P450 (Cyp3A4) followed the injection of clonazepam. This elevation may be attributed to the response of hepatocytes to metabolize the clonazepam drug.

Using of Chrysin treatment in the present study gave a significant decreasing in liver transaminases activity compared to the clonazepam group. This result agrees with that of Temel *et al.* (2020). Hermenean *et al.* (2017) reported that serum AST and ALT activities decreased in CCl<sub>4</sub>-treated mice administered chrysin (50mg/kg) and they attributed this to the protective effect of chrysin on membranes integrity. Also, Pai *et al.* (2019) recorded that 100 mg/kg b.wt. chrysin improved liver enzymes significantly due to its highly beneficial effects as antioxidant and anti-inflammatory agent. In general, Antioxidants decrease the oxidative stress by binding dangerous oxidants and repairing tissue damage (Ibrahim, 2017).

Administration of Chrysin in the current investigation caused liver MDA reduction as compared to clonazepam treated group since chrysin has a significant amelioration in hepatic MDA and upregulated fatty acid oxidation (Pai *et al.* (2019) and Temel *et al.* (2020) .

Moreover, in the present study the administration of 50mg/kg chrysin significantly increased TP, TAA, GSH

contents and GST activity as compared to clonazepam injected rats. Chrysin has ameliorative effects on lipid peroxidation by increasing liver SOD, catalase, GPx activities and GSH content (Saarghandian *et al.*, 2016; Eldutar *et al.*, 2017; Pai *et al.*, 2019; Song *et al.*, 2019; Temel *et al.*, 2020). Also, chrysin has a positive effect on liver proteins by decreasing in the carbonyl content of liver (Pai *et al.*, 2019). Additionally, Temel *et al.* (2020) attributed the ability of chrysin to reduce free radicals to its hydroxyl groups at 5<sup>th</sup> and 7<sup>th</sup> positions.

Marked histological changes were observed in the liver of clonazepam (CLZ) treated rats in this study which including disorganized hepatic cords, vacuolated cytoplasm and nucleic damage. An abundance of infiltrative inflammatory cells near the portal space, as well as remarkable congestion and dilatation of the central, hepatic sinusoids, and portal veins, associated with corrosion of their endothelial lining cells and hyperplasia of phagocytic Kupffer cells. These findings were in agreement with the studies performed by Atici *et al.* (2005) on a long-time use of opioids on liver and Badawia *et al.* (2016) on the liver of rats treated with diazepam, tramadol, and their combination.

At the ultrastructural level, the hepatocytes cytoplasm displayed noticeable deterioration and vacuolation in CZP-treated rats. In the hepatocytes of this group, the mitochondria swollen with the condensation of their matrices, and fragmented stacks rough endoplasmic reticulum were appeared. These changes influenced protein synthesis, keeping in consideration that the SER and RER have a detoxifying effect on toxins reaching any cell in the body (Fawcett, 1994). The heterochromatin clumps and adherent to the nuclear envelope in the nucleoplasm. There was also hypertrophy of Kupffer cells. And these alterations may be due to CLZ oxidative stress in the liver cells. On these regard Musavi and Kakkar (2003)

stated that diazepam has effective role in developing of oxidative stress in rat livers. In addition, there were marked devastation of mitochondria and pyknosis of the nuclei. This might be due to a defense mechanism exerted by the hepatic cells against the toxic effect of clonazepam, mitochondria act as gate keepers of cell life and so its disturbances involving both apoptotic and necrotic cell death (Michael *et al.*, 2010).

Cell stress, mitochondrial dysfunction, and particular immunological responses are all involved in drug-caused liver damage (DILI) and hepatic apoptosis. DILI involves hepatocytes, Kupffer cells, and endothelial cells (Yin *et al.*, 2006). Also, Hypertrophied Kupffer cells were present, ultrastructural of Kupffer cells are usually associated with hepatocellular diseases (Haschek and Rousseaux 1991). This result is in agree with those stated by Sabry *et al.* (2017) who recorded such changes of Kupffer cells in the hepatocytes of Haloperidol treated rat application.

On the other side, the current findings from the histology of the treated rat liver with CLZ+Chrysin indicated a remarkable improvement in CLZ damage. The liver cords were well organized; the vacuolation and fatty degeneration disappeared in cytoplasm and most nuclei exhibit normal structure. Also, on the ultrastructural level, the results revealed remarkable improvements in the most rat hepatocytes in CLZ+ Chrysin treated group. The hepatocytes represented a state of nearly normal appearance, i.e. their cytoplasm has abundant mitochondria and well developed rER. This can be attributed to the biological and pharmacological actions of chrysin, such as antioxidative agent, decreasing inflammation and cancer formation (Mani and Natesan, 2017). These findings agree with the results obtained by Pai *et al.* (2019) who noticed that Chrysin reduced liver enzymes and the amount of free fatty acids, triglycerides, and cholesterol besides inhibited carbonyl oxidation, advanced glycation end products, and collagen synthesis in the

liver. The authors also found that in the HFD (a high fructose diet) control group, the histology of the livers of rats given chrysin demonstrated a substantial reduction in steatosis, ballooning, and lobular inflammation. As a result, chrysin exhibited anti-steatotic, anti-glycating, antioxidant, anti-inflammatory, and antifibrotic properties, making it a potential treatment of fatty liver disease.

In isolated cardiomyocytes, Khezri *et al.* (2020) found that the injection of chrysin (up to 10M) effectively reduced cardiotoxicity, oxidative stress and lysosomal and mitochondrial disfunctions produced by AIP (aluminum phosphide). Talebi *et al.* (2020) found that honey's anti-inflammatory and antioxidant properties are primarily attributed to flavonoids such chrysin, increasing cells antioxidant and reducing inflammation occurred by down regulating NF-KB, NLR3 inflammasome, MAPK signaling and upregulating AMPK, Nrf2/ARE/HO-1, and IL-10 signaling. In this regard Eldutar *et al.* (2017) found that chrysin (25 and 50 mg/kg, IP) reduced acetaminophen-induced hepatotoxicity in rats. Chrysin reduced lipid peroxidation, resulting in an increase in antioxidant enzyme activity. TNF and IL-1 levels are elevated by chrysin, which modulates inflammatory responses.

### Conclusion:

In conclusion, Chrysin reduced apoptosis and autophagy via lowering caspase-3 activity, and the amount of LC3B. Chrysin appears to protect against Clonazepam -induced hepatotoxicity via lowering oxidation, apoptosis, inflammation, and autophagy.

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دراسات بيوكيميائية ونسجية وتركيبية دقيقة حول التأثيرات المحسنة لمادة الكريسين على السمية الكبدية الناجمة عن عقار الكلونازيبام في ذكور الجرذان المهقاء النامية

سحر أحمد صبرى\*<sup>1</sup>، مروان عبد اللطيف ابراهيم<sup>2</sup>، رحاب محمد مسعد<sup>3</sup>

١- قسم العلوم البيولوجية والبيولوجية، كلية التربية، جامعة عين شمس

٢- قسم الأحياء، كلية العلوم، جامعة المجمعة، المملكة العربية السعودية

٣- قسم علم الحيوان، كلية البنات، جامعة عين شمس

\* البريد الإلكتروني للباحث الرئيسي: [sahar\\_ahmed@edu.asu.edu.eg](mailto:sahar_ahmed@edu.asu.edu.eg)

### المستخلص

الكريسين هو احدى مركبات الفلافونويد النشطة بيولوجياً الموجودة في النباتات المتعلقة بجنس نبات الباسيفلورا وصمغ النحل وله العديد من الأنشطة الدوائية كما أن له تأثير وقائي على انسجة الكبد. تهدف هذه الدراسة الى اظهار تأثير المعالجة بالكريسين (٥٠ مجم / كجم من وزن الجسم / اليوم) على سمية المستحقة بعقار الكلونازيبام (٢ مجم / كجم من وزن الجسم / اليوم) على نسيج الكبد. استخدم في هذه الدراسة ٤٠ من ذكور الفئران المهقاء والتي تم تقسمها بالتساوى إلى ٤ مجموعات، المجموعة الاولى وهى المجموعة الضابطة وتم حقنها بالمذيب (1% w/v Tween 80). المجموعة الثانية تم حقنها بعقار الكلونازيبام (2 mg/kg b.wt./day Clonazepam)، المجموعة الثالثة تم حقنها بالكريسين (50 mg/kg b. wt./day of Chrysin)، اما المجموعة الرابعة فتلقى الحيوانات التابعة لها (2 mg/kg b.wt./day CZP + 50 mg/kg b.wt./day Chrysin) تم معاملة جميع المجموعات الحيوانية عن طريق الفم يوميا لمدة ٦ أسابيع ابتداء من اليوم الأول من التجربة. أشارت نتائج الدراسة بالمقارنة بالمجموعة الضابطة، الى زيادة انزيم ناقل أمين الالانين بالكبد (ALT و AST) ومالونديالدهيد (MDA) والسيستوكروم P450 بعد المعاملة بعقار الكلونازيبام بينما حدث نقص في بروتين الكبد ومضادات الأوكسدة الكلية (TAA). وانخفضت محتويات الجلوتاثيون (GSH). كما أظهر الفحص النسيجي لقطاعات الكبد في الفئران النامية المعاملة ب CZP تدهم واضح في الخلايا الكبدية علي شكل تحلل فجوي ودهنى للستوبلازم وكذلك حدوث حالات مختلفة من البكنزة والتحلل النووي، فضلا عن حدوث إحتقان وإتساع للوريد المركزي و الجيوب الدموية المحيطة به وفقدان للخلايا المبطنة لهم وازدياد نشاط خلايا كبر البعمية. كما أظهرت الفحوص التركيبية الدقيقة بالمجهر الإلكتروني النافذ للخلايا الكبدية حدوث تغييرات في بعض عضيات الخلايا تمثلت في الميتوكوندريا والشبكة الأندوبلازمية الخشنة. حيث أظهرت الميتوكوندريا إنتفاخ مع فقدان أعرافها، مع تفتت للشبكة الأندوبلازمية الخشنة الي أجزاء صغيرة. من ناحية أخرى، تم تسجيل تحسن ملحوظ في أنسجة الكبد مقابل الضرر الذي أظهره CZP في الفحص البيوكيميائي والنسيجي والتركيبى الدقيق وذلك فى المجموعة المعالجة ب CZP + Chrysin.