شبكة المعلومات الجامعية

التوثيق الإلكتروني والميكروفيلم

بسم الله الرحمن الرحيم

HANAA ALY

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Investigating and Targeting Hepatic Steatosis Induced by Tamoxifen

by

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Master Degree in Pharmaceutical Sciences (Biochemistry)-Faculty of Pharmacy-Ain Shams University (2016)

For the fulfillment of Doctor of Philosophy Degree in Pharmaceutical Sciences (Biochemistry)

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بسم الله الرحمن الرحيم

"قُلْ سِيرُوا فِي الْأَرْضِ فَانظُرُوا كَيْفَ بَدَأَ الْخَلْقَ ثُمَّ اللَّهُ يُنشِئُ النَّشْأَةَ الْآخِرَةَ إِنَّ اللَّهَ عَلَىٰ كُلِّ شَيْءٍ قَدِير"

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Luteolin mitigates tamoxifen associated fatty liver and cognitive impairment in rats via modulating beta catenin

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Luteolin mitigates tamoxifen-associated fatty liver and cognitive impairment in rats by modulating beta-catenin

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ABSTRACT

Background and aim: Tamoxifen (TAM) therapy has been associated with fatty liver diseases. Recently, multiple reports have also shown that TAM is related to cognitive impairment in patients with breast cancer. Luteolin, a natural flavonoid, has been traditionally used to treat various inflammatory disorders, such as chronic liver diseases, cognitive impairments, and cancers. This study aimed to evaluate the potential protective effects of luteolin against the cognitive defects and liver steatosis induced by TAM in rats.

Experimental approach: The diseased group was subcutaneously (*s.c*) injected with TAM at a dose of 1 mg/kg daily for 7 days. The cotreated groups were given luteolin via oral gavage at a dose of 20 or 40 mg/kg concomitantly with *s.c* injection of TAM at a dose of 1 mg/kg for 7 days. All the groups were subjected to behavioral tests 24 h after the last TAM injection. Then, the rats were sacrificed 3 days after the last TAM injection.

Results: Luteolin cotreatment significantly alleviated the behavioral defects in rats with TAM-induced cognitive impairment. This finding was supported by the reversal of neurodegeneration in the cortex and in the hippocampal regions of the brain. Furthermore, luteolin attenuated hepatic steatosis and decreased the levels of serum aminotransferases and hypertriglyceridemia. As an anti-inflammatory agent, luteolin cotreatment similarly decreased the levels of hepatic inflammatory markers and increased the levels of hepatic β-catenin in TAM-induced fatty liver.

Conclusions: Luteolin improved the TAM-induced cognitive impairment and hepatic steatosis in rats by alleviating inflammation and modulating hepatic β-catenin levels.

1. Introduction

Tamoxifen (TAM), 2-[4-[(Z)-1,2-diphenylbut-1-enyl]phenoxy]-N,N-dimethylethanamine, is the oldest and most-prescribed selective estrogen receptor modulator. It is approved by the U.S. Food and Drug Administration for the treatment of patients with hormone-receptor-positive early-stage breast cancer (Nazarali and Narod, 2014). Moreover, TAM is considered an indispensable adjuvant hormonal therapy for reducing the recurrence rates of breast cancer (Davies et al., 2011).

Though TAM treatment reduces the mortality of patients with estrogen-positive breast cancer by 31%, its use has several side effects. One of the most common side effects of TAM treatment is fatty liver (Yang et al., 2016). Long-term TAM treatment may increase the risk of developing nonalcoholic fatty liver disease (NAFLD) (Chang et al., 2018; Pan et al., 2016). As a common progressive liver disease, NAFLD should be subjected to early intervention to decrease the risk of metabolic and liver associated comorbidities (Glass et al., 2019).

Moreover, patients suffering from cancer and taking TAM experience

Abbreviations: TAM, tamoxifen; NAFLD, nonalcoholic fatty liver disease; PPARγ, proliferator-activated receptor-gamma; SAP, spontaneous alternation percent; TAE, total number of arm entries; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; TGF-β, transforming growth factor-beta; GSK-3β, glycogen synthase kinase 3 beta; β-catenin, beta-catenin; ANOVA, analysis of variance; CA, cornu ammonis; DG, dentate gyrus.

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cognitive side effects, which involve memory domains, compared with patients receiving chemotherapy alone, i.e., without TAM (Bender et al., 2007; Boele et al., 2015a). TAM users also have a decrease in cognitive skills with low scores in verbal memory and executive functions (Boele et al., 2015b).

Luteolin, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxychromen-4-one, is a flavonoid with anticancer protective and treatment potentials (Lin et al., 2008). It is found in many fruits, vegetables, and medicinal herbs, such as parsley, thyme, peppermint, and celery (Birt et al., 2001). Interestingly, emerging evidence has shown that luteolin elicits anti-inflammatory, anti-apoptotic, and antioxidative effects (Lin et al., 2008). As an anticancer, luteolin significantly induces apoptosis and inhibits cell proliferation, metastasis, and angiogenesis in a cancer niche (Imran et al., 2019).

As a phosphodiesterase inhibitor, luteolin can effectively modulate cholesterol synthesis and triacylglycerol (TAG) accumulation (Kwon et al., 2015). In this context, luteolin ameliorates obesity induced by high-fat diets. Mechanistically, luteolin represses hepatic steatosis by regulating the sterol-regulatory element-binding protein-1c/liver X receptor signaling pathway (Yin et al., 2017a). It also decreases the expression of hepatic peroxisome proliferator-activated receptor--gamma (PPARγ) and increases the expression of adipocyte PPARγ (Yin et al., 2017a). Interestingly, luteolin upregulates β-catenin expression during stem cell proliferation (Wan et al., 2019). However, studies have demonstrated that luteolin diminishes β-catenin expression in cancer (Han et al., 2018). Furthermore, the effect of luteolin on β-catenin expression in TAM-induced fatty liver has yet to be identified.

Luteolin exhibits putative neuroprotective effects on other inflammation-related brain diseases by ameliorating learning and memory impairments (Yao et al., 2018). As such, luteolin has protective effects against Alzheimer's disease by regulating the cholinergic system and inhibiting oxidative stress injuries (Yu et al., 2015).

Accordingly, this study aimed to investigate the possible therapeutic effects of luteolin on fatty liver and cognitive impairments associated with TAM treatment in an experimental rat model.

2. Materials and methods

2.1. Animals

Eight-week-old male albino rats weighing about 150–200 g were obtained from an animal breeding facility (National Research Center, Giza, Egypt). They were housed in cages at a constant temperature of 25 °C under a 12 h/12 h day/night cycle. They were provided with free access to food pellets and water ad libitum. Then, they were acclimatized to the laboratory environment for 1 week before the experiment. All the experimental procedures were performed with approval from the Research Ethics Committee, Faculty of Pharmacy, Ain Shams University, Egypt under Memorandum No. 82.

2.2. Drugs and chemicals

The following substances were used in this study: luteolin (Inofine Chemical Company, Inc., Hillsborough, NJ, USA); hematoxylin and eosin (H&E), TAM, and dimethyl sulfoxide (DMSO; Sigma-Aldrich, Saint Louis, Missouri, USA); and neutral formalin and benzoyl alcohol (El-Nasr Chemical Co., Egypt). All other chemicals were of the highest purity grade commercially available.

2.3. Experimental design

Rats were divided into five groups (eight rats per group). In the first group (control group), the rats were subcutaneously (*s.c.*) injected with sesame oil containing 10% benzoyl alcohol daily for 7 days. In the second group (diseased group), the rats were exposed to *s.c* injections of TAM at a dose of 1 mg/kg (dissolved in sesame oil containing 10% benzoyl alcohol) daily for 7 days (Cole et al., 2010). The third and fourth groups were considered as cotreated groups. In the third group, the rats were administered with 40 mg/kg luteolin dissolved in 10% DMSO via oral gavage and concomitantly with *s.c* injection of TAM (1 mg/kg) daily for 7 days. In the fourth group, the rats were given an oral gavage of 20 mg/kg luteolin concomitantly with *s.c* injection of TAM (1 mg/kg) for 7 days. In the fifth group, the rats were treated with 40 mg/kg luteolin only via oral gavage daily for 7 days. The dose of luteolin was chosen in accordance with a previous study that investigated the antisteatotic effects of luteolin on NAFLD (Yin et al., 2017b).

All the groups were subjected to behavioral tests 24 h after the last TAM injection. The rats were sacrificed after 3 days from the last TAM injection. Then, blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by subjecting the blood to cold centrifugation at 4000 rpm for 20 min and stored at −80 °C for its subsequent use in biochemical tests. Liver and brain tissues were dissected and washed with ice-cold saline and either fixed in 10% formalin for histopathological examinations or stored at −80 °C for the subsequent molecular analysis.

2.4. Behavioral tests

2.4.1. Locomotor assessment test

The locomotor activity of animals was assessed using an activity monitor (Opto-Varimex-Mini Model B, Columbus Instruments, OH, USA) that consisted of an infrared (IR) photocell (68 × 68 × 45 cm) supplied with 15 IR beams (wavelength = 875 nm and diameter = 0.32 cm) spaced 2.65 cm apart. The scan rate was set to 160 Hz. The IR beams were interrupted by the rats' movement. Measurements were based on the emittance of IR beams, and the interruptions of these beams were sensed and counted. The locomotor activities of all the groups were presented as the number of movements every 5 min (El-Agamy et al., 2018).

2.4.2. Passive avoidance test

Memory defects and changes in cognition were evaluated via a step-through passive avoidance test (Ugo Basile, Italy; Abdel-Aziz et al., 2016). In this test, a Plexiglas device consisting of two chambers separated by an automatic sliding door was used. The first chamber was white and lit up by a 10 W bulb. The second chamber is black and had a grid floor that was programmed to provide an electric shock when a rat stepped on it. The experiment was performed on two subsequent days. During an acquisition (training) session on day 1, the rats were placed in the illuminated chamber. As they stepped in the dark chamber, the sliding door was closed, and an electric shock of 1 mA was delivered for 2 s. After 24 h, the rats were placed again in the illuminated chamber. Their latency to step-through the dark chamber was recorded to evaluate their memory acquisition after they were exposed to the electric shock in the training session.

2.4.3. Y maze test

Short-term memory was evaluated with a Y maze device that consists of three similar opaque arms (40 cm length, 15 cm height, and 8 cm width) intersected at 120° and labeled as arms A, B, or C (Nasr and Wahdan, 2019). The concept of this test is based on the recalling activities of rats, that is, rats must recall the arm that they entered to make an alternate choice in the next trial.

The rats were positioned in the middle of the device and allowed to move through the three arms for 5 min. A spontaneous alternation percent (SAP) was calculated using the following formula: SAP = the number of alternations/the total possible alternations. The total possible alternations are calculated as follows: (the total number of arm entries [TAE] − 2) × 100, i.e., SAP = [(number of alternations)/(TAE − 2)] × 100. Correlations between SAP and TAE were analyzed to exclude the influence of the hyper- or hypodynamic activity of each rat.

2.5. Histopathological examination

The liver and brain tissues of the rats in all the groups were dissected and fixed in 10% neutral formalin (pH 7.2) for 24 h. Paraffinized blocks were prepared and sectioned with a sledge microtome at a thickness of 4 μm. The tissue sections were mounted on glass slides and stained with H&E for histological analysis as previously described (Downie, 1990).

2.6. Assessment of serum transaminases

The serum concentrations of alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were determined via a colorimetric assay by using available commercial kits (Biodiagnostics, Cairo, Egypt).

2.7. Assessment of serum lipids

The serum concentrations of total cholesterol (TC) and TAG were measured via a colorimetric assay by using available commercial kits (Biodiagnostics, Cairo, Egypt).

2.8. Assessment of the hepatic inflammatory markers

Liver tissue homogenate was prepared by homogenizing with phosphate buffer saline (PBS; pH = 7.4). Total proteins were subsequently measured using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, USA). Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and transforming growth factor-beta (TGF-β) were measured via enzyme linked immunoassay (ELISA) by using bioassay kits (Biotech, Co., Ltd.) and a Hyprep automated ELISA system (Hyperion Inc., Miami, FL) in accordance with the manufacturer's instructions. TNF-α, IL-1β, and TGF-β levels were expressed per total protein.

2.9. Assessment of hepatic β-catenin and glycogen synthase kinase-3β

The hepatic levels of beta-catenin (β-catenin) was measured using an ELISA assay kit (Milpitas Blvd., Milpitas, CA., USA). The hepatic levels of glycogen synthase kinase 3 beta (GSK-3β) was measured using an ELISA assay kit (Wuhan Fine Biotech Co., Ltd.) and a Hyprep automated ELISA system (Hyperion Inc., Miami, FL) in accordance with the manufacturer's instructions.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Comparisons between parametric data in more than two groups were performed via ANOVA with a post hoc test (Tukey's test). Any skewed data were further analyzed with Kruskal–Wallis and Mann–Whitney U tests. Data with $P < 0.05$ were considered statistically significant. All analyses were performed using GraphPad.

3. Results

3.1. Effect of luteolin on the locomotor activity of rats with TAM-induced cognitive impairment

Locomotion activity was not affected by TAM. Our results showed no significant difference in the locomotor activities of the studied groups (Fig. 1A).

3.2. Effect of luteolin on passive avoidance in rats with TAM-induced cognitive impairment

On day 1 (training acquisition session), no significant difference was found in the step-through latency among different groups (Fig. 1B). On day 2 (test retention session), the step-through latency significantly

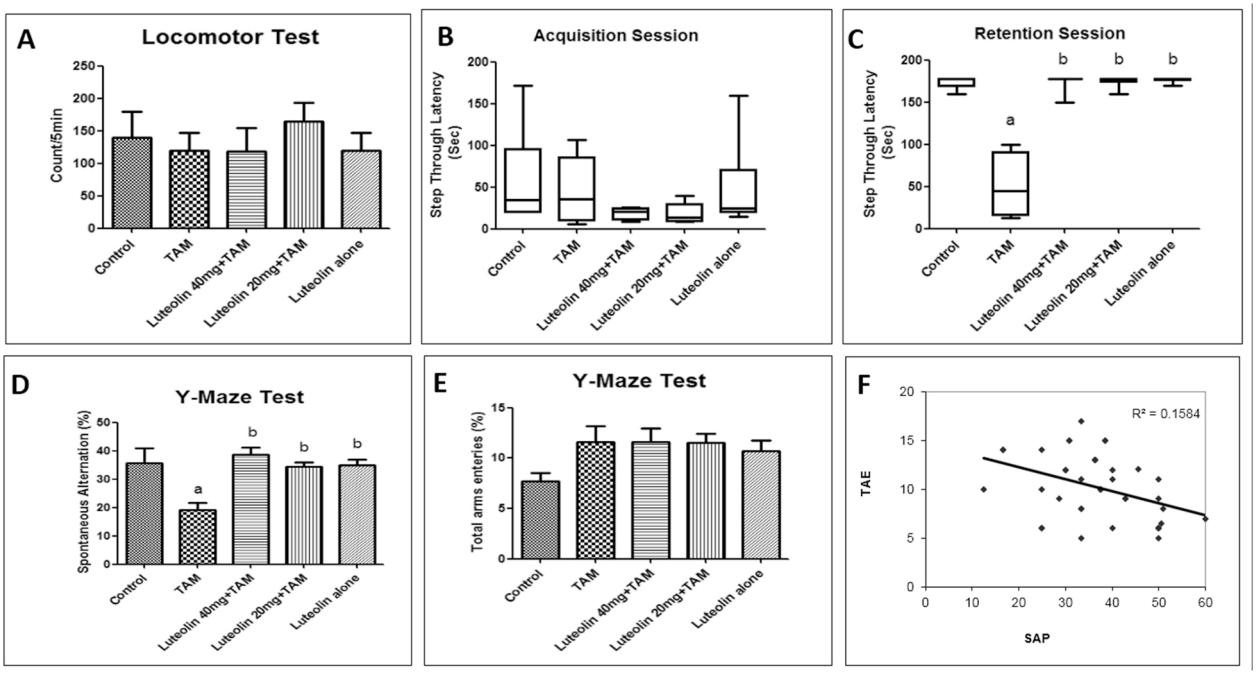

Fig. 1. Effect of luteolin treatment on the behavioral defects in rats with TAM-induced cognitive impairment. A) Locomotor activity, B) Step-through passive avoidance: Training session C) Step-through passive avoidance: Test session, D) Y-maze test: SAP E) Y-maze test: TAE and F) Correlation between SAP and TAE
a: Significantly different from the control group; b: Significantly different from TAM group.
Passive avoidance data are presented as medians and interquartile range (n = 8) and analyzed using Kruskal–Wallis test followed by Dunn's post hoc test at ($P < 0.05$).
Data for A, D and E are expressed as mean ± SEM (n = 8). Statistical analysis was carried out using one-way ANOVA followed by the Tukey post test at ($P < 0.05$). For (F) statistical analysis was performed using person's correlation.

decreased to 29% in the TAM group compared with that in the control group. Conversely, luteolin cotreatment reversed TAM-induced memory defects. This finding was evidenced by the significant increase in step-through latency to 304% in the cotreated group with 40 mg of luteolin and TAM compared with that in the TAM group. Similarly, the step-through latency significantly increased to 343% in the cotreated group with 20 mg of luteolin and TAM compared with that in the TAM group. Moreover, the step-through latency significantly increased to 347% in the group treated with luteolin alone compared with that in the TAM group (Fig. 1C).

3.3. Effect of luteolin on the recalling activity of Y maze in rats with TAM-induced cognitive impairment

The SAP of the TAM group significantly decreased to 53% compared with that of the control group. Conversely, the SAP of the cotreated group with 40 mg of luteolin and TAM significantly increased to 205% compared with that of the TAM group. In addition, the SAP of the cotreated group with 20 mg of luteolin and TAM significantly increased to 179% compared with that of the TAM group. Likewise, the SAP of the group treated with luteolin alone significantly increased to 184% compared with that of the TAM group (Fig. 1D). However, no significant difference was found in the TAE percent of all the studied groups (Fig. 1E), and no significant correlation was observed between SAP and TAE (Fig. 1F).

3.4. Effect of luteolin on histopathological changes in the brain of rats with TAM-induced cognitive impairment

The histopathological changes in the brain of the rats are shown in Fig. 2. The control group showed average meninges, average meningeal, and intracerebral blood vessels, average cerebral cortex with average neurons, and average astrocytes in a fibrillary background (**Lane 1**–Fig. 2A). The hippocampus had average cornu ammonis 1 (CA1), (CA2), and (CA3), average dentate gyrus (DG), average pyramidal neurons, average granular cells, average interneurons, and average blood vessels (**Lane 2**–Fig. 2A).

However, the TAM group showed mildly congested meningeal blood vessels and markedly congested intracerebral blood vessels with mild cortical edema, scattered degenerated neurons, astrogliosis, and eosinophilic plaque-like areas (Lane 1–Fig. 2B). The hippocampal region had indistinct CA1 and preserved CA2. Scattered and degenerated pyramidal neurons and granular cells were shown in CA3 and DG. Perivascular clefting with microcystification was also observed (**Lane 2**–Fig. 2B).

Luteolin cotreatment restored the normal brain histology (Fig. 2). By comparison, cotreatment with 40 mg of luteolin showed mildly congested meningeal blood vessels with mild cortical edema. The cerebral cortex had average neurons, and average astrocytes were found in the fibrillary background (Lane 1–Fig. 2C). The hippocampus had indistinct CA1; preserved CA2, CA3, and DG; few scattered and degenerated pyramidal neurons and granular cells in CA3; markedly congested blood vessels in DG with perivascular clefting; and microcystification (**Lane 2**–Fig. 2C).

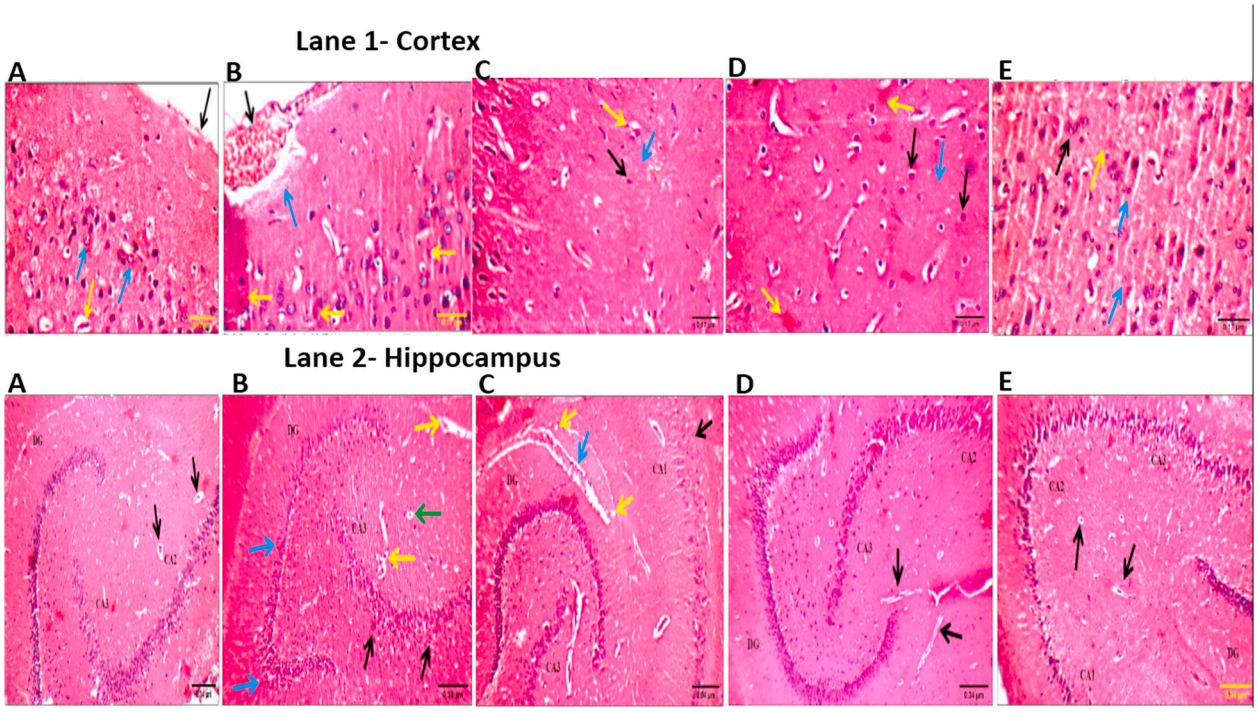

Fig. 2. Effect of luteolin treatment on rats with TAM-induced neurodegeneration. Photomicrographs of H&E-stained rat brain sections showing; Lane 1-Cortex (400x) (A) Control group cortex shows meninges with average size (black arrow), neurons with average structure (blue arrow), and blood vessels with average size (yellow arrow). (B) TAM group cortex shows showing mildly congested meningeal blood vessel (black arrow) with mild cortical edema (blue arrow) and scattered degenerated neurons (yellow arrows). (C) Luteolin 40 mg+TAM group shows average astrocytes (black arrow) in average fibrillary background (blue arrow), and average neurons (yellow arrow) (D) Luteolin 20 mg + TAM group shows average astrocytes (black arrows) in average fibrillary background (blue arrow), and eosinophilic plaque-like areas (yellow arrows) (E) Luteolin 40 mg alone group shows average neurons (black arrows), and average astrocytes (blue arrow) in average fibrillary background (yellow arrow).
Regarding Lane-2 Hippocampus (200x) (A) Control group hippocampus shows average CA1, CA2, CA3, average DG, and average blood vessels (black arrows). (B) TAM group hippocampus shows average CA3, average DG, scattered degenerated pyramidal neurons in CA3 (black arrows) and in DG (blue arrows), and peri-vascular clefting (yellow arrow). (C) Luteolin 40 mg+TAM group shows indistinct CA1 (black arrow), and markedly congested blood vessel in DG with peri-vascular clefting (blue arrow) with micro-cystification (yellow arrows). (D) Luteolin 20 mg + TAM group shows preserved CA2 and CA3, and peri-vascular clefting (black arrows). (E) Luteolin 40 mg alone group shows average CA1, CA2, CA3, average DG, and average blood vessels (black arrows).

The meninges are of average size, mildly congested meningeal and intracerebral blood vessels with mild cortical edema, and cerebral cortex showed scattered and degenerated neurons. Average astrocytes in the fibrillary background with eosinophilic plaque-like areas were detected in the cotreated group with 20 mg of luteolin and TAM (**Lane 1**–Fig. 2D). The hippocampal region had indistinct CA1 and preserved CA2, CA3, and DG. Besides, few scattered and degenerated pyramidal neurons, perivascular clefting, and microcystification were detected in the cotreated group with 20 mg of luteolin and TAM (Lane 2–Fig. 2D).

The group treated with luteolin alone showed average meninges, mildly congested meningeal blood vessels, average cerebral cortex with average neurons, and average astrocytes in the fibrillary background (Lane 1–Fig. 2E). The hippocampal region had average CA1, CA2, and CA3; average DG; average pyramidal neurons; average granular cells; mild interneuronal edema; and average blood vessels (Lane 2–Fig. 2E).

3.5. *Effect of luteolin on the histopathological changes in TAM-induced fatty liver*

The control group (Lanes 1 and 2–Fig. 3A) and the group treated with luteolin alone (Lanes 1 and 2–Fig. 3E) showed average portal tracts with average portal veins and average hepatocytes in the periportal area. These groups also had average central veins with average hepatocytes arranged in single-cell cords and average intervening blood sinusoids.

Liver steatosis was associated with the TAM group. This finding was confirmed by markedly edematous portal tracts with markedly dilated congested portal veins and average hepatocytes in the periportal area, markedly dilated congested central veins with detached lining and mild microvesicular steatosis of hepatocytes in the perivenular area, and few intralobular inflammatory infiltrate (Lanes 1 and 2–Fig. 3B).

Reversibly, the cotreatment of 40 mg of luteolin with TAM or 20 mg of luteolin with TAM showed mildly congested portal veins and portal tracts with average hepatocytes in the periportal area, average central veins, and few scattered intralobular inflammatory infiltrates with scattered apoptotic hepatocytes. Mildly edematous portal tracts with mildly congested portal veins and average hepatocytes in the periportal area, mildly dilated central vein, and moderate intralobular inflammatory infiltrate with scattered apoptotic hepatocytes were also detected (Lanes 1 and 2–Fig. 3C and 3D).

3.6. *Effect of luteolin on the hepatotoxicity of TAM-induced fatty liver*

Serum aminotransferases; ALT and AST in the TAM group obviously increased to 284% and 280% compared with those in the control group, respectively. Beneficially, serum AST and ALT levels in the cotreatment group with 40 mg of luteolin with TAM decreased to 60% and 36%, respectively, compared with those in the TAM group. Moreover, serum AST and ALT levels in the cotreatment group with 20 mg of luteolin with TAM significantly decreased to 65% and 39%, respectively, compared with those in the TAM group. AST and ALT levels in the group treated with luteolin alone significantly decreased to 60% and 28%, respectively, compared with those in the TAM group (Fig. 4A and B).

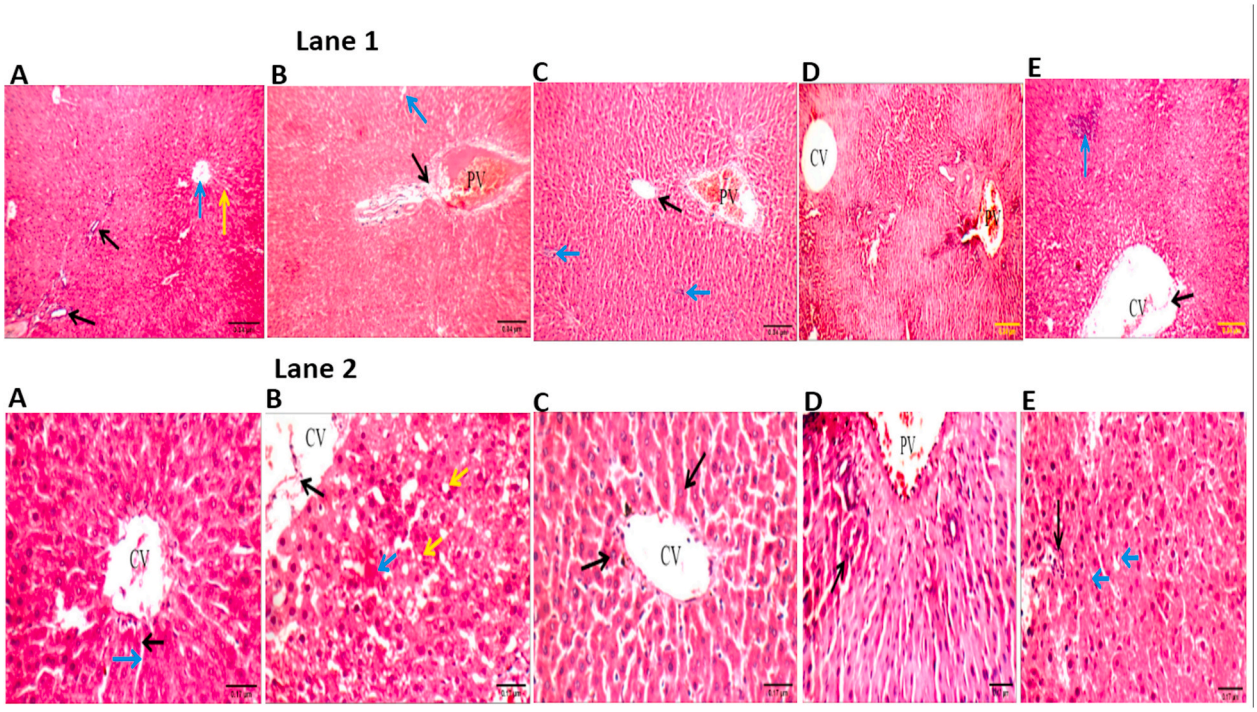

Fig. 3. Effects of luteolin treatment on the histopathological changes in TAM-induced fatty liver
Photomicrographs of H&E stained sections of liver depicting; Lane 1 (200x) (A) Control group shows average portal tracts (black arrows), average central vein (blue arrows) and average hepatocytes (yellow arrow) (B) TAM group shows markedly edematous portal tracts (black arrow) with markedly dilated congested portal vein, and average central vein (blue arrow) (C) Luteolin 40 mg + TAM group shows portal tract with mildly congested portal vein, average central vein (black arrow), and scattered intra-lobular inflammatory infiltrate (blue arrows) (D) Luteolin 20 mg + TAM group shows mildly edematous portal tract with mildly congested portal vein, and mildly dilated central vein. (E) Luteolin 40 mg alone group shows average dilated congested central vein with detached lining (black arrow), and intra-lobular inflammatory infiltrate, Photomicrographs of H&E stained sections of liver depicting Lane 2 (400x) (A) Control group shows average central vein, and average hepatocytes arranged in single cell cords (black arrow) with average intervening blood sinusoids (blue arrow) (B) TAM group shows markedly dilated central vein with detached lining (black arrow), scattered apoptosis (blue arrow), and moderate micro-vesicular steatosis of hepatocytes in peri-venular area (yellow arrows) (C) Luteolin 40 mg + TAM group shows average central vein, and average hepatocytes in peri-venular area (black arrows) (D) Luteolin 20 mg + TAM group shows mildly congested portal vein, and average hepatocytes in peri-portal area (black arrow) (E) Luteolin 40 mg alone group shows few intra-lobular inflammatory infiltrate (black arrow), very few micro-vesicular steatosis of hepatocytes (blue arrows).