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Investigating the Potential Hepatoprotective Effect of Quercetin in Male Rats Following Acute Exposure to Cyclophosphamide

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A B S T R A C T

This study aimed to assess the hepatoprotective efficacy of quercetin against hepatotoxicity induced by cyclophosphamide in a rat model. A total of 28 male Wister albino rats (*Rattus norvegicus*), with body weights ranging from 195.5 to 198.2 g and approximately three months of age, were randomized into four different groups: the untreated Control group received no interventions; the CYP group was treated with an intraperitoneal injection of cyclophosphamide at a dose of 200 mg/BW; the Qt group received an oral administration of quercetin at 100 mg/kg BW daily for ten days; and the combined (Qt+CYP) group received quercetin orally for ten days, followed by a cyclophosphamide injection on the tenth day. Various biochemical markers, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and liver glutathione (GSH), and malondialdehyde (MDA), were analyzed, in addition to body weight and prothrombin time. The Untreated Control group exhibited baseline levels for all assessed markers. In contrast, the CYP group showed elevated levels of ALT, AST, ALP, and MDA, coupled with a decrease in GSH. Notably, the Qt+CYP group demonstrated a statistically significant reduction ($P<0.05$) in ALT, AST, ALP, and MDA levels, as well as an increase in GSH and prothrombin time, when compared to the CYP group. No significant differences in body weight were observed across all groups ($P<0.05$). The results of the study indicate that quercetin has the potential to be used as a hepatoprotective agent, protecting liver tissues from the cytotoxic effects of cyclophosphamide.

Keywords: quercetin, cyclophosphamide, hepatoprotection, MDA, antioxidants

INTRODUCTION

Liver injury due to toxic substances, including pharmaceuticals, poses a significant risk to public health (1). Various types of liver damage—ranging from acute and chronic forms of hepatitis to conditions like microvesicular steatosis and cholestasis—fall under the broader category of hepatotoxicity (2, 3). Elevated enzymatic levels often accompany these liver ailments, particularly when exposed to higher concentrations of the offending substances (4).

Cancer therapy relies on chemotherapy, which, however, is not without disadvantages. It can enhance patient well-being but may also negatively impact healthy cells and tissues (5). The alkylating agent cyclophosphamide (CYP), which is often utilized in chemotherapy and immunotherapy, is an example. CYP is processed in the liver by the cytochrome P450 enzyme, resulting in two toxic byproducts: phosphoramide and

acrolein, which trigger the oxidative stress (6). While acrolein causes necrosis, apoptosis, oncosis, and cell death, phosphoramide makes cyclophosphamide antineoplastic (7, 8).

The application of CYP is limited by a multitude of adverse effects, not just hepatotoxicity but also neurotoxicity, and cardiotoxicity (9, 10). Both the liver and kidneys are critical for the metabolism and removal of CYP, making toxicity in these organs among the most severe side effects (11, 12).

The shortcomings of synthetic drugs like CYP have led to increased focus on herbal remedies as adjunct treatments (13). Quercetin (Qt), a prominent flavonoid found in a variety of foods such as leafy vegetables, fruits, and red wine, has shown promise due to its multiple health advantages, including liver protection (14-16). It exhibits a broad spectrum of biological functions, such as anti-inflammatory, anti-oxidative, and anti-apoptotic activities (17, 18). Its antioxidant capabilities are especially significant, largely owing to its chemical composition that includes numerous hydroxyl groups and double bonds (20, 21).

Recent trends highlight the use of dietary antioxidants such as Qt in combination with chemotherapeutic drugs to alleviate their negative side effects (19). Nevertheless, there is a scarcity of research exploring the potential of Qt to counteract liver injury induced by CYP. This study is designed to address this knowledge gap by assessing the effectiveness of Qt in reducing hepatotoxicity triggered by CYP.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the local Animal Care and Use Committee at the College of Veterinary Medicine, University of Baghdad, with Approval Number 1418 P.G., dated 6th May 2023.

Animals

A total of 28 male Wistar albino rats (*Rattus norvegicus*), with body weights (BW) ranging between 195.5-198.2 g and approximately three months of age, were obtained from the animal house, College of Veterinary Medicine, University of Baghdad. Rats were kept in plastic cages 20×50×75 cm^3 in dimensions with stainless-steel wire mesh lids. The animals were allowed for two weeks adaptation period. Standard rodent diet (Commercial feed pellets) and tap water were freely available. The housing conditions were maintained at a temperature between 20-25 °C, with a light/dark cycle of 14/10 h. Ventilation vacuums were employed for air circulation, and cage litter was replaced weekly.

Experimental Design

The rats were divided randomly into four groups (7 rats in each group). The Control group was administered only distilled water orally for ten days, without any additional treatment. The cyclophosphamide (CYP) group received a single intraperitoneal injection of cyclophosphamide at a dosage of 200 mg/kg BW on the tenth day, without any preceding treatment (23). The quercetin (Qt) group was treated with quercetin orally at a dose of 100 mg/kg BW (24) once daily for a duration of ten days. Finally, the Combined (Qt+CYP) group, received oral administration of quercetin at the same dosage as the Qt group for ten days, followed by a single injection of cyclophosphamide on the tenth day at the same dosage as the CYP group.

Preparation of Stock Solutions and Doses

The stock solution of cyclophosphamide (Baxter, Germany) was prepared at a concentration of 500 mg, diluted with 10 mL of distilled water (DW), for a final dosage of 0.4 ml per 100 g of rat body weight (23). Similarly, the quercetin (Natural Factor, Canada) stock solution had a concentration of 500 mg, diluted with 20 mL of DW, for a final dosage of 0.4 mL per 100 g of rat BW (24).

Blood Sample

At the end of experimental period, all rats were initially weighed and then anesthetized using chloroform, administered via inhalation under a controlled environment to minimize stress and discomfort. Blood samples were carefully collected directly from the heart using a sterile 5 mL syringe equipped with a 23-gauge needle. Immediately after collection, each blood sample was divided into two aliquots: one for plasma separation and another for serum isolation. The aliquot intended for serum preparation was allowed to clot at room temperature for 30 min and subsequently centrifuged at 3000 rpm for 15 min. The supernatant, which constitutes the serum, was carefully pipetted out and transferred to sterile microcentrifuge tubes. These serum samples were then frozen at -20 °C for future biochemical analyses focusing on liver enzyme assays (ALT, ALP, and AST). The aliquot for plasma was mixed with an anticoagulant (EDTA) and immediately centrifuged at 3000 rpm for 10 min. The resulting plasma was separated and stored in sterile microcentrifuge tubes at -20 °C until further use for prothrombin time tests.

Liver Tissue Collection

Post-blood collection, the rats were euthanized humanely with an overdose of a ketamine-xylazine cocktail (95 mg/kg of ketamine and 5 mg/kg of xylazine). The abdominal cavity was opened with a midline incision, and liver samples were rapidly excised.

Liver Enzyme Assays

Biochemical assays for liver enzymes (ALT, ALP, and AST) were conducted using colorimetric kits from Linear Company, USA.

Hepatic Oxidative Stress Markers

Immediately after euthanasia, A section of the liver was weighed and at once submerged in ice-cold homogenization buffer. It was then homogenized using a Brinkmann PT-10-35 High-Speed Mixer/Homogenizer. Homogenates were prepared using a Brinkmann PT-10-35 High-Speed Mixer/Homogenizer. The homogenization buffer contained PBS at pH 7.2, 4 °C, 0.05% sodium azide, 0.5% Triton ×-100, and a cocktail of protease inhibitors. The concentrations of malondialdehyde (MDA) and glutathione (GSH) were measured using ELISA kits from MyBioSource Company, USA, and expressed as pg/mg of total protein (24).

Prothrombin Time Estimation

Clotting time, denoted as Prothrombin Time (PT), was measured by mixing citrated plasma samples with a thromboplastin reagent. The procedure involved prewarming the HemoStat Thromboplastin-SI reagent at 37 °C and following a set of incubation and recording steps. The assay was conducted using reagents from Human company (Germany) (25).

Statistical Analysis

Data analysis was performed using the Statistical Analysis System (SAS) 2018 software. The Least Significant Difference (LSD) test was part of the Analysis of Variance (ANOVA) to identify significant differences between the means of the study parameters (26).

Results and Discussion

BW

All rats treated with CYP and Qt demonstrated a difference in BW, though this effect was not statistically significant ($P>0.05$) (Figure 1). No significant variations were observed in BW among the Control group, the Qt group, the CYP group, and the Qt+CYP group.

Qt did not significantly affect BW in either the Qt group or the combined Qt+CYP group, compared to the Control group. Furthermore, the CYP group did not exhibit any significant changes in BW. These findings are consistent with a previous study (27), which reported that Qt led to a non-significant reduction in BW. Similarly, the lack of BW alteration in rats treated with CYP aligns with the results of another study (28).

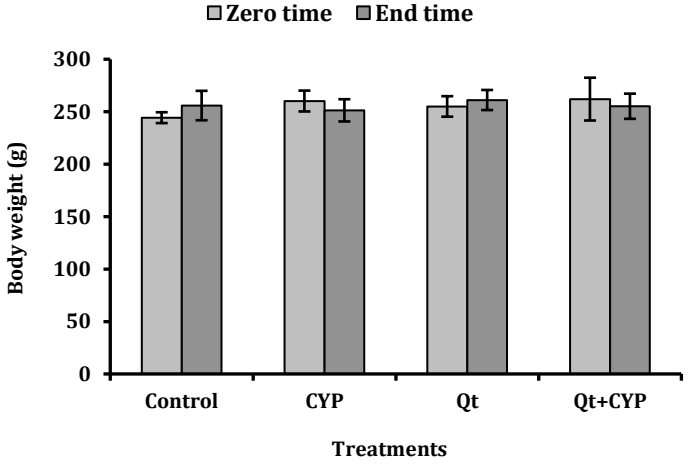

Figure 1. Effect of quercetin and cyclophosphamide on body weight (g) of male rats for acute study (10 days). Bars and error bars represent the mean and SEM, respectively, n=7. Different letters indicate statistical significance among treatment groups ($P\leq0.05$). Qt, quercetin; CYP, cyclophosphamide; Qt+CYP, quercetin+cyclophosphamide

Liver Enzymes

The effects of CYP and Qt treatments on serum levels of liver enzymes ALT, AST, and ALP are summarized in Figure 2 A-C). CYP treatment exhibited a marked elevation in serum ALT levels, registering a value of 33.5±2.44 U/L, which was statistically significant ($P<0.05$) when compared to the Control group. Such an increase is indicative of liver cellular damage, aligning with the known hepatotoxic effects of CYP. Conversely, the Qt group exhibited a decrease in ALT levels compared to the CYP group but showed no significant difference when compared to the Control group. The Combined group (CYP+Qt) showed a significant reduction in ALT levels (17.4±0.81 U/L) when compared to the CYP group. The levels were comparable to those in the negative Control and Qt groups, showing a potential hepatoprotective effect of Qt when administered alongside CYP.

A significant increase in AST levels (31.7±2.37 U/L) was observed in the CYP group, reinforcing the notion of hepatocellular injury induced by CYP. The Qt group showed AST levels were lower (22.8±1.42 U/L) compared to the CYP group but did not differ significantly from the Control group. The Combined group, however, showed AST levels in this group (26.6±2.09 U/L) were elevated compared to the negative control group but lower than the CYP group, indicating a potential ameliorative effect of Qt on CYP-induced liver injury.

A significant increase in ALP levels was observed (113±5.02 U/L) in the CYP group, indicative of possible biliary obstruction or cholestasis, a known side effect of CYP. The Qt group showed ALP levels in this group decreased in comparison to the CYP group (80.5±4.32 U/L), suggesting that Qt has protective effects. Interestingly, the combined group displayed a significant The ALP levels were reduced (95.7±3.79 U/L) in comparison to the CYP group but remained elevated compared to the Control group.

The observed effects of CYP on liver enzyme activity align with previous investigations (29). Hepatic enzymes such as AST, ALT, and ALP are commonly used as biochemical markers for hepatocellular necrosis and are considered indicators of liver dysfunction and damage (30, 31). These findings corroborate earlier research that reported CYP-induced hepatotoxicity (32, 33). consistent with previous studies (34). Hepatocyte dysfunction was seen as the procedure progressed after the administration of greater dosages of the medication(35, 36).

In contrast, the hepatoprotective effects of Qt observed in recent study agree with other research that used Qt extracts (37). The combination of Qt and CYP significantly attenuated the elevated levels of ALT, AST, and ALP, suggesting that Qt could mitigate CYP-induced liver damage by clarified that, pretreatment with FSP (orally at 2 g/rat/day) for 42 days significantly restored the elevated ALT, AST, ALP and LDH activities close to the control value in dieldrin intoxicated rats, This may be referred to, the fenugreek seeds are rich in polyphenolic flavonoids as Qt, that was able to protect rat hepatocytes against oxidative damage owing to its antiradical and antioxidant potential also could prevents TGF-β1 gene expression and signaling pathways as well as connective tissue proteins synthesis in activated hepatic stellate cells, These findings are in line with studies that reported the protective effects of fenugreek seeds, which are rich in polyphenolic flavonoids like Qt, against liver damage (38, 39).

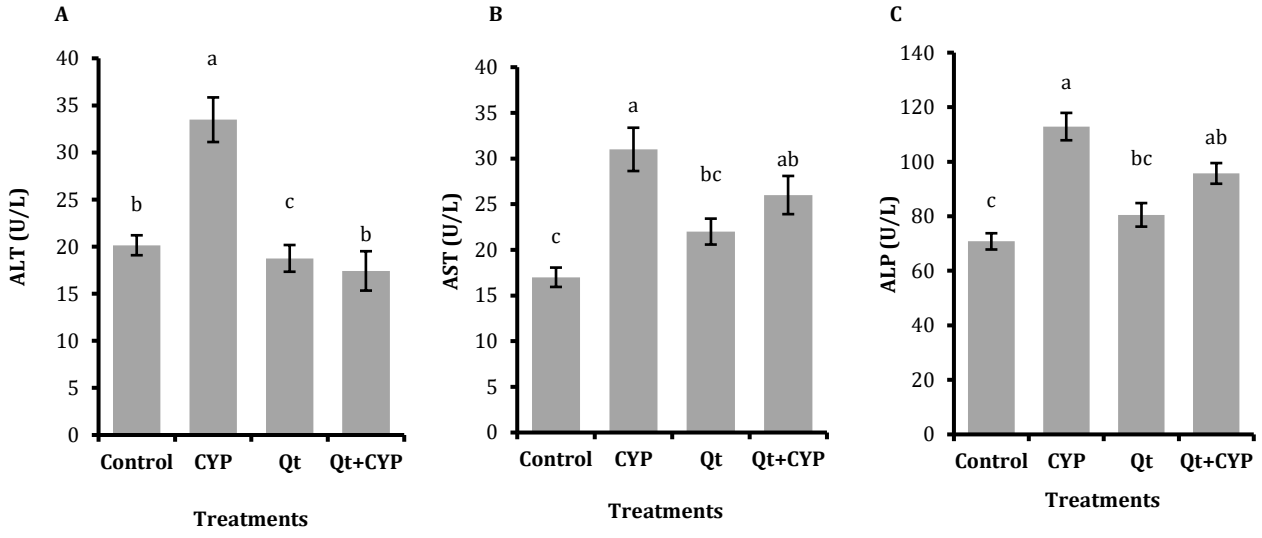

Figure 2. Effects of cyclophosphamide and quercetin on serum levels (U/L) of **(A)** alanine aminotransferase (ALT), **(B)** aspartate aminotransferase (AST), and **(C)** alkaline phosphatase (ALP) in male Wister albino rats in an acute study. Bars and error bars represent the mean and SEM, respectively, n=7. Different letters indicate statistical significance among treatment groups (P≤0.05). Qt, quercetin; CYP, cyclophosphamide; Qt+CYP, quercetin+cyclophosphamide

MDA and GSH in Hepatic Tissue

The impact of CYP and Qt on hepatic tissue levels of GSH and MDA during an acute study period (10 days) is elucidated in Figure 3 A, and B, respectively. These markers were chosen because of their importance in assessing the redox balance in liver tissue. The Control group showed the highest level of GSH (85.1±3.72 pg/mg), serving as the baseline against which other groups were compared. In the CYP group, a drastic reduction in GSH levels was observed (13.9±0.81 pg/mg), significantly (P<0.05) different from the control. This indicates severe oxidative stress induced by CYP. GSH levels in the Qt group were significantly (P<0.05) higher (25.0±1.58 pg/mg) compared to the CYP

group but still lower than the Control. Animals in the combined group showed GSH levels (29.2±2.06 pg/mg) that were higher than those in the CYP group but lower than the control, reinforcing the protective effect of Qt against CYP-induced oxidative damage.

The Control group exhibited the lowest level of MDA (1.58±0.16 pg/mg), which serves as the baseline for comparison. There was a significant (P<0.05) elevation in MDA levels (7.10±0.37 pg/mg) of the CYP group, indicating elevated oxidative stress and lipid peroxidation. In the Qt group, a moderate level of MDA (3.26±0.19 pg/mg) was observed, significantly (P<0.05) lower than in the CYP group. The MDA level in the combined group was 4.37±0.22 pg/mg, lower than the CYP group but higher than the Qt and Control groups, indicating a partial protective effect of Qt.

The findings align with existing literature indicating that CYP's cytotoxic action is largely due to its harmful metabolite, acrolein, which triggers ROS production (40, 41). Elevated MDA levels and depleted GSH levels suggest an imbalance between oxidant and antioxidant statuses, leading to hepatocellular damage (42, 43). Oxidative stress plays a significant role in CYP-induced hepatotoxicity (44). Similarly, GSH plays a critical role in cellular defense against drug toxicity, and its reduction indicates liver injury and oxidative stress (45). Previous studies have also shown that a single dose of CYP can significantly increase hepatic MDA levels while depleting GSH (41, 46-48).

On the other hand, Qt seems to exert a protective effect. This agrees with studies showing that Qt can restore hepatic thiol levels, potentially through its regulatory effect on glutamate cysteine ligase, the rate-limiting enzyme in thiol synthesis (49, 50). Qt's efficacy in scavenging hydroxy radicals and detoxifying H2O2 has been well-documented (51, 52). It also has anti-inflammatory properties and can suppress inflammatory signaling pathways while enhancing antioxidant defense (53, 54). This protective effect could be particularly important in conditions where the liver's ability to neutralize free radicals is compromised, possibly due to inhibition of the antioxidant enzyme activity of the Nrf2-ARE pathway (55-58).

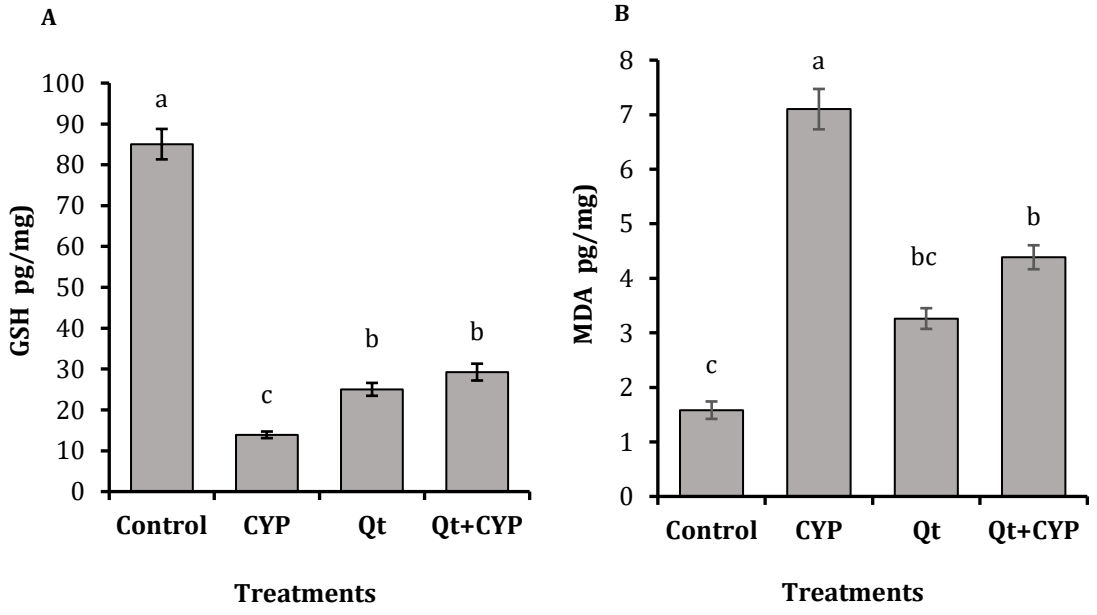

Figure 3. Effects of cyclophosphamide and quercetin on hepatic tissue concentrations (pg/mg) of **(A)** glutathione (GSH) and **(B)** malondialdehyde (MDA) in male Wister albino rats in an acute study. Bars and error bars represent mean values and SEM, respectively, n=7. Different letters indicate statistical significance among treatment groups (P≤0.05). Qt, quercetin; CYP, cyclophosphamide; Qt+CYP, quercetin+cyclophosphamid

Effects on Hemostasis: Prothrombin Time

The acute study reveals significant variations in prothrombin time across different treatment groups, as displayed in Figure 4. The baseline prothrombin time was 15.40±0.64 seconds in the Control group. In CYP group, a significant prolongation in prothrombin time was observed (20.392±1.07 seconds), when compared to the Control group (P<0.05). No significant change in prothrombin time (19.1±0.96 seconds) was observed in Qt group when compared to the Control, CYP, or Qt+CYP. There was a significant increase in prothrombin time (22.0±1.36

seconds) of Qt+CYP group when compared to the Control group ($P<0.05$). However, no significant change was observed when compared to the CYP and Qt groups.

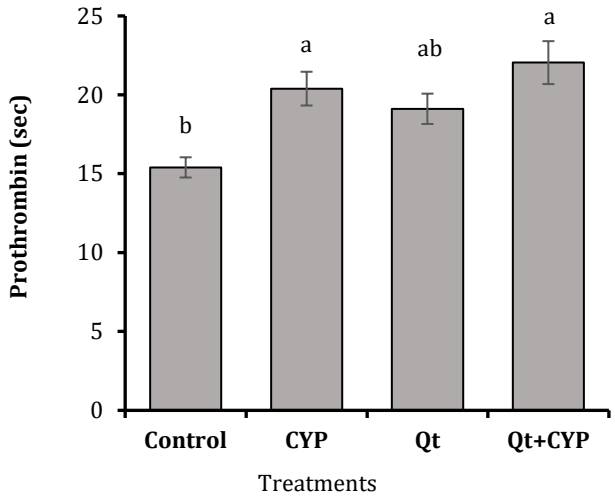

Figure 4. Effects of cyclophosphamide and quercetin on prothrombin time (seconds) in male Wister albino rats in an acute study. Bars and error bars represent mean values and SEM, n=7. Different letters indicate statistical significance among treatment groups ($P\leq0.05$). Qt, quercetin; CYP, cyclophosphamide; Qt+CYP, quercetin+cyclophosphamid

The findings of current study regarding the cytotoxic effects of CYP align with those reported in existing literature (59). The onset of chemotherapy-related drug-induced thrombocytopenia is primarily attributed to hepatotoxicity or myelotoxicity (60). It is important to note that chemotherapeutic agents exert their influence not only on rapidly proliferating cancer cells but also on normal cells, such as those in hematopoietic bone marrow, hair follicles, as well as oral and intestinal mucosa (61). Such an indiscriminate action often leads to thrombocytopenia, a condition precipitated by impaired platelet formation due to bone marrow toxicity (62, 63).

Thrombopoietin is identified as a critical cytokine in the regulation of platelet production during the process of megakaryopoiesis (64). In the context of Qt's effect on prothrombin time, results are congruent with both in vitro and in vivo studies (65). These studies indicate that a crude extract of *B. orthobotrys* (that contains Qt as extracted) led to a notable prolongation in prothrombin time relative to control groups. Such an extension in prothrombin time serves as an indicator of obstruction in the tissue factor pathway, a process that necessitates the presence of Factor VII and its subsequent activation by Vitamin K. Historically, Warfarin and other vitamin K antagonists have been associated with elongated prothrombin times. Moreover, previous research has demonstrated that polysaccharide-phenolic-protein complexes exert both procoagulant and anticoagulant activities within the hemostatic system (66

The present study has elucidated the multifaceted effects of CYP and Qt on liver function, oxidative stress markers, and hemostatic parameters in an acute setting. CYP exhibited a considerable impact on liver enzymes, oxidative stress, and prothrombin time, indicating its potential for inducing hepatotoxicity and affecting coagulation mechanisms. On the other hand, Qt demonstrated some protective effects, notably on oxidative stress markers. Given these findings, future research should delve into the mechanistic underpinnings of these effects, potentially exploring synergistic interactions between CYP and Qt. Further studies could also investigate other biomarkers related to liver function and coagulation, thereby providing a more comprehensive understanding of the drugs' impact on physiological systems.

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N/A

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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التأثير الوقائي المحتمل للكيرسيتين على تلف الكبد في ذكور الفئران بعد التعرض الحاد لسيكلوفوسفاميد

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الخلاصة

تمت دراسة البحث النشاط المحتمل للكويرسيتين فيما يتعلق بالقدرة على الحماية الكبدية ضد السمية التي تسببها السيكلوفوسفاميد في الجرذان. المجموعة (أ) هي المجموعة السلبية (غير مصابة ولم تعالج)، المجموعة (ب) هي المجموعة الإيجابية (تم حقنها بالسيكلوفوسفاميد بجرعة ٢٠٠ ملغ/كغ من وزن الجسم ولم تتلق علاجًا)، المجموعة (ج) تم علاجها بالكويرسيتين عن طريق الفم بجرعة ١٠٠ ملغ/كغ من وزن الجسم لمدة عشرة أيام، والمجموعة (د) تم علاجها بالكويرسيتين عن طريق الفم بجرعة ١٠٠ ملغ/كغ من وزن الجسم لمدة عشرة أيام مرة واحدة يوميًا وتم حقنها بالسيكلوفوسفاميد بجرعة ٢٠٠ ملغ/كغ من وزن الجسم في اليوم العاشر. البارامترات المستخدمة في هذا البحث كانت وزن الجسم، ومستويات إنزيمات الكبد (ALT، AST، ALP)، ومستوى الجلوتاثيون (GSH)، ومستوى مادة المالونديالدهيد (MDA)، وزمن التجلط البروثرومبيني. أظهرت الجرذان الصحية (المجموعة أ) مستويات طبيعية في هذه البارامترات، بينما أظهرت المجموعة الإيجابية السيطرية (المجموعة ب) زيادة في مستويات ALT، AST، ALP، MDA وزمن التجلط البروثرومبيني وانخفاضًا في مستوى GSH مقارنة بالمجموعة (أ). أما المجموعة (ج) فقد أظهرت مستويات طبيعية في ALT، AST، ALP، MDA، وزمن التجلط البروثرومبيني وانخفاضًا في مستوى GSH. أما المجموعة (د) فقد أظهرت انخفاضًا كبيرًا بمستويات ALT، AST، ALP، وMDA بالمقارنة مع المجموعة (ب) وزيادة في مستوى GSH وزمن التجلط البروثرومبيني. أما بالنسبة لوزن الجسم، فلم تظهر اختلافات ذات دلالة إحصائية بالمقارنة مع المجموعة (أ). تسلط الدراسة الضوء على الاستخدام السريري للكويرسيتين كوسيلة فعالة لحماية الكبد وتقليل سمية تأثير السيكلوفوسفاميد السيتوتوكسي على الكبد.

الكلمات المفاحية: السيكلوسبورين، أزاسيتيدين، نخاع العظم، فقر الدم الانتسجي، إناث الجرذان