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Research Article | Araştırma Makalesi

DOES LUTEOLIN PROTECT THE THYROID FROM DOXORUBICIN-INDUCED TOXICTY? EXPERIMENTAL EVIDENCE SUGGEST DOSE LIMITATIONS

LUTEOLİN DOKSORUBİSİN KAYNAKLI TİROİT TOKSİSİTESİNE KARŞI KORUYUCU MUDUR? DENEYSEL BULGULAR DOZ SINIRLAMALARINA İŞARET EDİYOR

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ABSTRACT

Objective: Doxorubicin (DOX) is a chemotherapeutic agent known to cause multi-organ toxicity, including thyroid dysfunction. While biochemical evidence of DOX-induced hypothyroidism exists, histopathological changes and apoptosis regulation in the thyroid remain poorly understood. In addition; although luteolin (LUT) shows protective effects in other tissues, its role against DOX-induced thyroid injury has not yet been studied in vivo. This study aimed to evaluate the protective effects of LUT on DOX-induced thyroid damage in rats, focusing on histopathology, apoptosis, and inflammation.

Methods: Adult male Sprague Dawley rats were divided into four groups: control, DOX (5 mg/kg, i.p.), LUT (administered at 20 µg/kg), and DOX+LUT (combined treatment). On day 29 thyroid tissues were harvested. Histopathological evaluation included hematoxylin-eosin (H&E), Periodic-acid Shiff (PAS) and Toluidine Blue (TB) staining and immunofluorescence analysis for Bax and Bcl2 expression. The Bax/Bcl2 ratio was calculated to quantify apoptosis.

Results: DOX administration induced marked apoptotic changes in thyroid tissue, reflected by an increased Bax/Bcl2 ratio, without evidence of mast cell infiltration. LUT treatment alone produced no morphological alterations, including follicular damage or colloid deposition. Although co-treatment with LUT enhanced Bcl2 expression, it did not significantly reduce the Bax/Bcl2 ratio. Chronic DOX exposure induces marked apoptosis and histopathological alterations in thyroid tissues of rats.

Conclusion: LUT at the tested dose partially enhanced Bcl2 expression but did not reduce apoptosis or prevent histopathological damage, indicating limited protective efficacy. These findings suggest the need for dose optimization and further investigation in future studies.

Keywords: Apoptosis, doxorubicin, luteolin, thyroid

ÖZ

Amaç: Doksorubisin (DOX), tiroit fonksiyon bozukluğu dahil olmak üzere çoklu organ toksisitesine neden olduğu bilinen bir kemoterapötik ajandır. DOX'un neden olduğu hipotiroidizmin biyokimyasal kanıtları mevcut olmakla birlikte, tiroitteki histopatolojik değişiklikler ve apoptoz düzenlemesi hala tam olarak anlaşılamamıştır. Ayrıca, luteolin (LUT) diğer dokularda koruyucu etkiler gösterse de DOX'un neden olduğu tiroit hasarına karşı rolü henüz in vivo olarak çalışılmamıştır. Bu çalışma, histopatoloji, apoptoz ve inflamasyona odaklanarak, sıçanlarda DOX kaynaklı tiroit hasarına karşı LUT'nin koruyucu etkilerini değerlendirmeyi amaçlamıştır.

Yöntem: Yetişkin erkek Sprague Dawley sıçanları dört gruba ayrılmıştır: kontrol, DOX (5 mg/kg, i.p.), LUT (20 µg/kg olarak uygulanan) ve DOX+LUT (kombine tedavi). 29. günde tiroit dokuları alındı. Histopatolojik değerlendirme, hematoksilen-eozin (H&E), periyodik asit Shiff (PAS) ve toluidin mavisi (TB) boyama ve Bax ve Bcl2 ekspresyonu için immünofloresan analizini içeriyordu. Apoptozu ölçmek için Bax/Bcl2 oranı hesaplandı.

Bulgular: DOX uygulaması, mast hücre infiltrasyonu belirtisi olmaksızın, Bax/Bcl2 oranındaki artışla yansıtılan tiroit dokusunda belirgin apoptoz değişikliklerine neden oldu. LUT tedavisi tek başına foliküler hasar veya kolloid birikimi dahil olmak üzere morfolojik değişikliklere neden olmadı. LUT ile birlikte tedavi Bcl2 ekspresyonunu artırsa da, Bax/Bcl2 oranını önemli ölçüde azaltmadı. Kronik DOX maruziyeti, sıçanların tiroit dokularında belirgin apoptoz ve histopatolojik değişikliklere neden olur.

Sonuç: Test edilen dozda LUT, Bcl2 ekspresyonunu kısmen artırdı, ancak apoptozu azaltmadı veya histopatolojik hasarı önlemedi, bu da koruyucu etkinliğinin sınırlı olduğunu gösterdi. Bu bulgular, gelecekteki çalışmalarda doz optimizasyonu ve daha fazla araştırma yapılması gerektiğini göstermektedir.

Anahtar Kelimeler: Apoptoz, doksorubisin, luteolin, tiroit

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Introduction

Doxorubicin (DOX) is an anthracycline chemotherapy agent widely used in the treatment of various cancers. Despite its efficacy, its clinical application is constrained by cumulative, dose-dependent toxicities affecting multiple organs, including the cardiovascular, gastrointestinal, and endocrine systems.[1] Recent studies suggest that DOX may also disrupt endocrine functions, particularly by affecting thyroid hormone levels, though the exact histopathological impact on the thyroid gland remains poorly characterized.[2]

Accumulating evidence indicate that apoptosis plays a key role in the DOX-induced toxicity.[3] DOX induces oxidative stress and mitochondrial damage, leading to activation of the intrinsic apoptotic pathway. A key event is the upregulation and mitochondrial translocation of pro-apoptotic Bax, which promotes outer membrane permeabilization and cytochrome c release. Simultaneously, DOX downregulates anti-apoptotic proteins like Bcl2 and Bcl-XL, shifting the Bax/Bcl2 balance toward apoptosis.[4] This imbalance is further driven by p53 activation and GATA4 suppression.[5] This apoptotic shift can be histologically detected in various organs, but little is known about its presence in the thyroid tissues of DOX-treated rats.

In parallel, DOX has been shown to disrupt thyroid function in preclinical models. Several studies have demonstrated biochemical hypothyroidism, characterized by reduced serum thyroid hormones (T3, T4, FT3, and FT4) with elevated Thyroid-Stimulating Hormone (TSH) levels following DOX administration.[2,6] While studies have shown DOX-induced alterations in thyroid hormone profiles in patients and animal models, few have examined the corresponding structural or cellular damage within the thyroid tissue itself, leaving a significant gap in understanding the underlying mechanisms.[7] The hypothesized mechanisms of DOX-induced thyroid injury include oxidative stress, inflammation, and apoptosis—all of which can contribute to thyroid dysfunction and potentially lead to hypothyroidism.[8] Despite this, detailed histopathological assessment of the thyroid gland under DOX exposure has been largely overlooked, creating a significant gap in the literature.[9]

Luteolin (3′,4′,5,7-Tetrahydroxyflavone; LUT), a naturally occurring flavonoid found in celery, parsley, and chamomile, has attracted considerable interest due to its antioxidant**,** anti-inflammatory, antidiabetic, antiapoptotic, autophagic-regulatory, antimicrobial, and neuroprotective properties.[10,13] Multiple studies have demonstrated LUT's protective effects against DOX-induced organ damage, particularly in the heart and nervous system, through mechanisms that involve suppression of oxidative stress, inhibition of proinflammatory cytokines, and modulation of apoptosis-related pathways, including the downregulation of Bax and caspase-3 and upregulation of Bcl2.[14] These mechanisms are especially relevant to thyroid tissue, which is vulnerable to oxidative damage and apoptosis-mediated follicular cell injury. Importantly, LUT has also shown efficacy in autoimmune thyroiditis models by preserving follicular structure, reducing lymphocytic infiltration, and inhibiting phosphorylation of STAT3 (Signal transducer and activator of transcription 3) which is crucial transcription factor of T cell-mediated immunity.[15] Additionally, in thyroid cancer cells, LUT inhibited growth and promoted apoptosis modulation via the TSH receptor pathways, suggesting its capacity to influence thyroid-specific signaling cascades.[16]

Despite promising evidence of LUT's protective properties, no in vivo study to date has evaluated its potential against DOX-induced injury in the thyroid particularly concerning histopathological changes and apoptosis regulation at a low dose (20 µg/kg). LUT has been shown to exert strong antioxidant and anti-inflammatory effects even at low doses by reducing reactive oxygen species (ROS) accumulation.[17] However, flavonoids may display dose-dependent effects, with high doses potentially inducing pro-oxidant activity and disrupting cellular homeostasis.[18] Given the thyroid gland's reliance on hydrogen peroxide (H_2O_2) for hormone synthesis and its sensitivity to oxidative balance, excessive antioxidant exposure may impair thyroid function.[19] Therefore, the present study was designed to investigate the protective effects of low dose LUT against DOX-induced damage in the thyroid of rats.

Methods

Animals and Experimental Design

Male Sprague-Dawley rats were obtained from the Hamidiye Research Laboratory at the University of Health Sciences, following approval by the Institutional Ethics Committee (Approval No: 2020-06/13). Animals were maintained under standard laboratory conditions, including a 12-hour light/dark cycle, with daily monitoring. Standard rodent chow and water were available ad libitum.

A total of 34 adult male rats, aged 8 weeks and weighing between 90–150 g, were randomly assigned to four experimental groups. The control (CONT) group (n = 7) received 0.5 mL/day of intraperitoneal (i.p.) normal saline on days 1, 7, 14, 21, and 28. The LUT group (n = 9) was administered luteolin (LUT; Cayman Chemicals, USA) at a dose of 20 µg/kg i.p. daily for 28 days, following the protocol of Yahyazadeh and Altunkaynak

(2019). The DOX group (n = 9) received doxorubicin hydrochloride (DOX; Cayman Chemicals) at 5 mg/kg i.p. on days 1, 7, 14, 21, and 28. The DOX+LUT group (n = 9) was treated with both DOX (5 mg/kg i.p. on the same schedule) and LUT (20 µg/kg i.p. daily for 28 days).

Twenty-four hours after the final injection, the animals were euthanized by cervical dislocation under anesthesia induced with ketamine (75 mg/kg) and xylazine (10 mg/kg). To avoid surgical damage to the rats' thyroid tissue, the gland was excised together with the adjacent trachea. Thyroid tissues were harvested and fixed in formalin for subsequent histopathological analysis.

Cryoprotection and Embedding

The fixed tissue samples were preserved via cryopreservation and standard tissue processing. Fixed tissues were gradually equilibrated in increasing concentrations of sucrose solutions (10%, 20%, and 30% w/v in PBS) at 4 °C for 24 hours each, or until the samples sank, to ensure effective cryoprotection. Subsequently, the tissues were embedded in optimal cutting temperature (OCT) compound (Thermo Fisher Scientific, USA), placed into molds, and properly oriented for sectioning.

Sectioning and Staining

Frozen tissue blocks were sectioned at 5 µm thickness using a Leica cryostat (Leica Microsystems, Germany) and mounted on positively charged glass slides. Sections were air-dried, and the OCT compound was removed prior to staining. Hematoxylin & Eosin (H&E) was used to evaluate general tissue morphology, Periodic Acid–Schiff (PAS) to assess glycogen content, and Toluidine Blue (TB) to detect mast cell infiltration. Stained sections were examined using a Zeiss Scope. A1 light microscope (Germany) equipped with a Zeiss Axiocam 105 Color digital camera (Germany).

Semi-quantitative Analysis

Quantitative evaluation was restricted to central lobular follicles, representing the metabolically active population. Thyroid injury was graded on a four-tier scale: 0 = no damage; 1 = mild degeneration involving few follicles; 2 = moderate degeneration involving a moderate number of follicles; and 3 = severe degeneration involving most follicles.[20] Colloid content was expressed as the percentage area in PAS-stained sections. [21] For each section, 10 randomly selected, non-overlapping fields were analyzed. Image quantification was performed using ImageJ (NIH, USA). Toluidine blue–positive mast cells were counted in 10 randomly selected, non-overlapping fields, and results were expressed as the number of mast cells per high-power field (HPF) at ×40 magnification.

Immunofluorescence Analysis

Immunofluorescence staining was performed to evaluate the expression of apoptotic markers Bax (pro-apoptotic) and Bcl2 (anti-apoptotic). Sections were first incubated in 0.1% Triton X-100 for 30 min for permeabilization, followed by washing with phosphate-buffered saline containing 0.05% Tween-20 (PBS-T). Non-specific binding was blocked with blocking buffer consisting of 4% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in PBS for 1 h at room temperature in a humidified chamber. Subsequently, sections were incubated overnight at 4°C with the following primary antibodies of anti-Bcl2 (GTX100064, GeneTex) and anti-Bax (MA5-42750, Thermo Scientific). After PBS-T washes, sections were incubated for 2 h at room temperature with Alexa Fluor 488–conjugated goat anti-rabbit IgG (1:1000, Abcam) secondary antibody. Nuclei were counterstained with Hoechst and mounted with PBS: Glycerol (1:20000). Fluorescent images were acquired using a fluorescence microscope and Bax and Bcl2 expression levels were quantified using ImageJ software (NIH, USA). Quantification of Bax and Bcl2 expression was based on mean fluorescence intensity (MFI) from each histological section, with corrected total cell fluorescence calculated by normalizing fluorescence intensity to mean background fluorescence. The Bax/Bcl2 ratio was then calculated as an indicator of apoptotic activity.

Statistical Analysis

Sample size was estimated using power analysis with a significance level of 0.05 and a statistical power of 0.80. Data analysis was performed with GraphPad Prism software (Version 8.1.0; GraphPad Inc., USA). The Shapiro–Wilk test was employed to assess normality of data distribution. Group differences were analyzed using one-way analysis of variance (ANOVA), and post hoc pairwise comparisons were conducted with Bonferroni adjustment to account for multiple testing. Considering six pairwise comparisons among the four groups, the adjusted threshold for statistical significance was set at $p<0.0083$.

Results

DOX-induced Thyroid Histopathology and Lut effects

H&E staining revealed normal follicular architecture in control and LUT-treated groups, with cuboidal follicular cells, spherical nuclei, and homogeneous colloid. DOX administration caused marked follicular degeneration, including disrupted epithelial lining, reduced colloid content, and partially collapsed follicles. Co-treatment with LUT preserved overall follicular morphology, with only occasional degenerative changes, while LUT alone had no detectable histopathological effects (Figure 1a).

PAS staining showed uniform colloid in control and LUT groups, whereas DOX-exposed follicles exhibited irregular or reduced PAS-positive colloid, reflecting impaired thyroglobulin synthesis. Semi-quantitative scoring and colloid area measurements confirmed significant follicular damage in the DOX group ($p < 0.0083$), which was not substantially reversed by LUT co-treatment (Figure 1b, c, e) ($p > 0.0083$). TB staining demonstrated no significant mast cell infiltration in any group (Figure 1d, f) ($p > 0.0083$).

Apoptotic Regulation in Thyroid Tissue

Immunofluorescence analysis revealed increased Bax expression and reduced Bcl2 in the DOX group, resulting in a significantly elevated Bax/Bcl2 ratio (Figure 2a–e) ($p < 0.0083$). Although co-treatment with LUT further increased Bcl2 immunoreactivity ($p < 0.0083$), it did not significantly lower the elevated Bax/Bcl2 ratio induced by DOX ($p > 0.0083$).

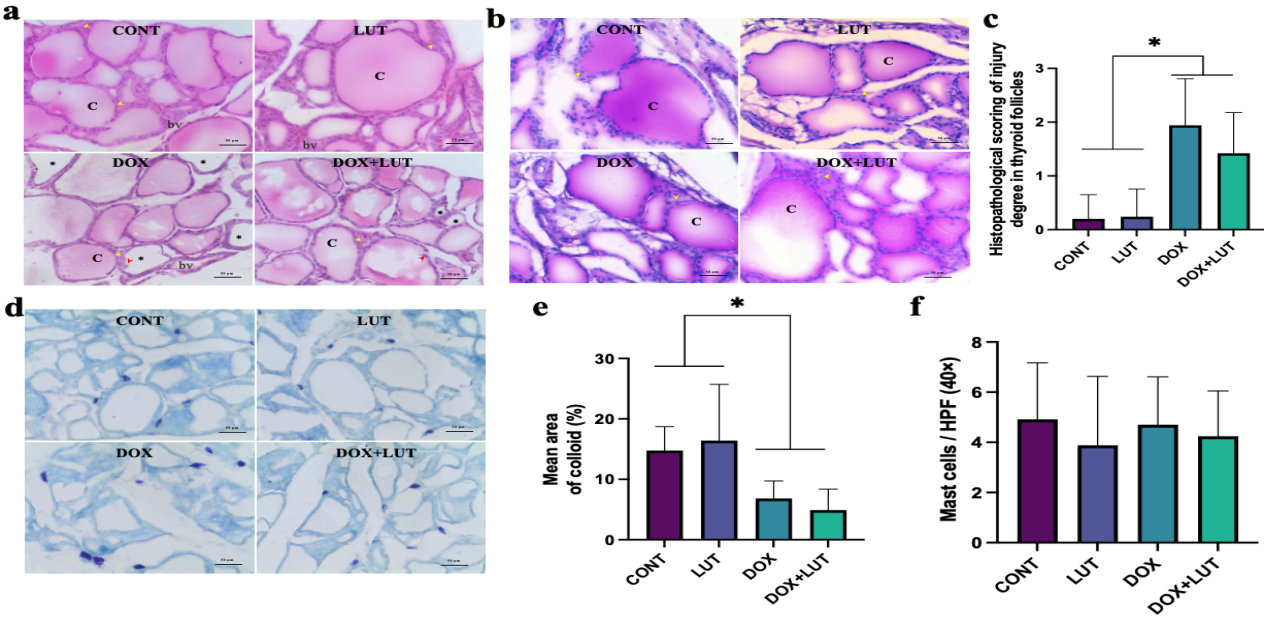

Figure 1. (a) Representative H&E-stained photomicrographs of thyroid gland tissue. Control (CONT) group shows normal thyroid follicles lined by a single layer of cuboidal follicular cells with spherical nuclei, filled with homogeneous acidophilic colloid (C), and surrounded by blood capillaries (bv). Parafollicular cells appear as larger cells with pale cytoplasm and large pale nuclei that do not reach the follicular lumen (yellow arrowhead). DOX group exhibits degenerated thyroid follicles with disrupted follicular lining (red arrowhead), reduced colloid content (C), or follicles that are nearly devoid of colloid/partially collapsed (star). DOX+LUT group shows occasional degenerated follicles among predominantly normal follicles. (b) PAS-stained photomicrographs showing thyroglobulin glycoprotein content in the colloid. CONT group displays normal follicles with a single layer of cuboidal follicular cells and homogeneous colloid of variable densities (C). Parafollicular cells (yellow arrows) are identifiable as in panel (a). DOX group shows multiple follicles with reduced or irregular PAS-positive colloid content (C), indicating impaired colloid synthesis. (c) Semi-quantitative scoring of thyroid follicle damage (n = 5). (d) Representative Toluidine Blue (TB) staining of mast cells with coarse metachromatic granules in thyroid tissue. (e) Mean colloid area in PAS-stained sections (×100) measured using ImageJ (n = 5). (f) Mast cell counts per high-power field (HPF, ×40) in different groups (n = 5). Scale bar = 50 µm (×400). CONT: Control; LUT: Luteolin; DOX: Doxorubicin; DOX+LUT: Doxorubicin + Luteolin; H&E: Hematoxylin & Eosin; PAS&H: Periodic Acid–Schiff & Hemalum; TB: Toluidine Blue. *p<0.0083.

Discussion

This study aimed to evaluate the potential protective effect of luteolin (LUT) against doxorubicin (DOX)-induced thyroid toxicity. To achieve this, histopathological alterations were assessed using H&E, PAS and TB staining, while apoptosis-related changes were examined via immunofluorescence detection of Bax and Bcl2 proteins. The findings indicate that DOX causes thyroid damage via apoptotic mechanisms, as evidenced by follicular degeneration and increased Bax/Bcl2 ratio. In addition, although LUT at 20 µg/kg partially supports anti-apoptotic signaling, it cannot completely prevent the colloid loss and apoptosis induced by DOX. To date, the absence of detailed histological examination of the thyroid following DOX exposure represents a significant gap in understanding the full extent of its endocrine toxicity.

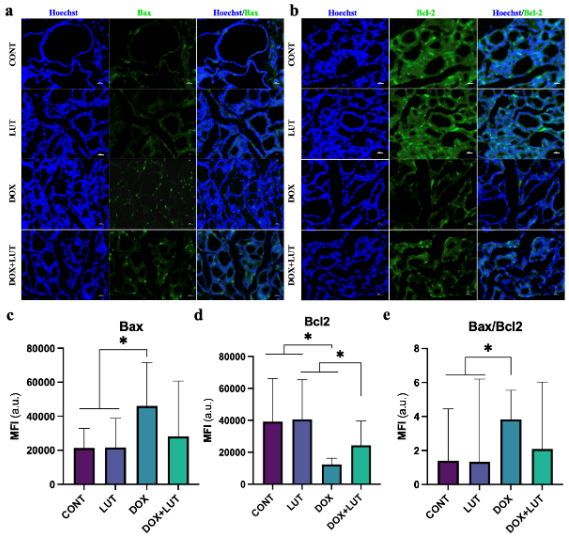

Figure 2. Immunofluorescence analysis of Bax and Bcl2 expression. (a) Histological assessment of Bax immunolocalization. (b) Histological assessment of Bcl2 immunolocalization. Images were captured at 20× magnification using a CCD camera attached to a Zeiss microscope. Apoptosis-inducing Bax was predominantly detected in large follicles of the control group, whereas apoptosis-inhibiting Bcl2 displayed strong immunolabelling in the LUT-treated group. (c, d) Quantification of Bax and Bcl2 expression based on mean fluorescence intensity (MFI) from each histological section. Corrected total cell fluorescence was calculated by normalizing fluorescence intensity to mean background fluorescence. (e) Bax/Bcl2 ratio calculated from average MFI per group. The Bax/Bcl2 ratio was significantly increased in the DOX group ($p < 0.0083$). Data are presented as mean ± SD, with statistical significance between groups indicated ($p < 0.0083$; $n = 5$). Bax, Bcl2: green; Hoechst: blue. Light microscopy, 20× magnification.

The present study, by demonstrating structural and apoptotic changes in thyroid follicles, addresses this gap and emphasizes the importance of thyroid-targeted evaluation in DOX toxicity. Even research into the systemic oxidative stress potentiated by hypothyroidism during DOX treatment has focused primarily on cardiac tissue, overlooking the thyroid gland itself.[9] It has been demonstrated that DOX induces biochemical hypothyroidism in rats and suggested a protective role of pioglitazone, yet no thyroid histopathology was presented to support tissue-level damage.[6] Similarly, another study investigating the cognitive and inflammatory effects of DOX also reported significant thyroid hormone suppression but did not assess thyroid tissue morphology.[2] In this study; histopathological evaluations revealed that DOX exposure led to pronounced colloid depletion and follicular degeneration indicating reduced thyroid activity.[22] However, dose of 20 µg/kg LUT was failed to recover the colloid production and follicular damage at the histopathological level that could be related to suboptimal dose or possible antithyroid potential of LUT. Although LUT at comparable doses has shown protective effects in other models[23,24], its anti-apoptotic impact on the thyroid was limited, likely due to tissue-specific differences in DOX sensitivity and stress response. Moreover, the well-documented thyroid peroxidase (TPO)-inhibition and antithyroid potential of several dietary flavonoids may also have hindered the histological recovery of follicular structure.[25,26] Future work might test higher or formulated LUT (e.g., nanoemulsions or vesicles) and/or combinations that directly target mitochondrial ROS to restore follicular morphology and colloid synthesis.[27]

The antineoplastic effects of DOX are mainly attributed to topoisomerase II inhibition, increased ROS production, and mitochondrial dysfunction.[28] Recently, toxic effects on the thyroid, including altered hormone levels and inflammation, have also been reported.[29,30] Consistent with these findings, DOX promotes thyroid injury through mitochondrial-dependent apoptotic mechanisms, consistent with previous reports showing DOX-induced ROS generation, mitochondrial dysfunction, and apoptotic signaling activation.[31] However; in this study although LUT increased the Bcl2 level in the tissue, it was failed to recover the apoptotic index demonstrating a possible mechanistic mismatch with the dominant injury pathways in DOX-treated thyroids. Recently, LUT (50–100 mg/kg) has been shown to protect against DOX-induced cardiotoxicity and renal injury by upregulating Bcl2 and downregulating Bax and caspase-3, thereby reducing apoptosis and enhancing cell survival. These effects are mediated through activation of the Akt/Bcl2 pathway, inhibition of phlpp1, and suppression of inflammatory cytokines.[32,33] In the present study, LUT treatment partially attenuated DOX-induced apoptosis, as indicated by increased Bcl2 expression; however, it was insufficient to completely prevent follicular degeneration or restore colloid content. Although follicular degeneration and colloid depletion were not fully prevented, LUT treatment partially attenuated DOX-induced apoptosis, as indicated by increased Bcl2 expression. This observed protective trend suggests that LUT has promising therapeutic potential, which may be further optimized with adjusted dosing or combinatorial strategies. In addition; LUT has notoriously poor oral bioavailability and very low aqueous solubility; without formulation enhancement, systemic and tissue levels remain low, so 20 µg/kg may remain sub-therapeutic for reversing tissue-level damage.[34] Multiple pharmacokinetic and formulation studies emphasize that native LUT is poorly absorbed and that nano/micro-delivery systems are often required to reach effective concentrations.[35]

This study has some limitations that should be considered. First, the experimental design primarily focused on histopathological and immunofluorescence evaluations, without assessment of circulating thyroid hormone levels (T3, T4, FT3, FT4, TSH), which could have provided additional functional insight into the structural changes observed. Second, only a single dose of LUT was tested; exploring different doses and longer treatment durations may further clarify its protective potential. Third, as this is an animal study, translation of these results to human thyroid physiology should be made with

caution. Studies incorporating biochemical, molecular, and functional assays, together with varied dosing regimens, will be valuable to more comprehensively define the protective role of LUT against DOX-induced thyroid toxicity.

In conclusion, chronic DOX exposure led to significant apoptotic and structural damage in rat thyroid tissue, while low dose LUT conferred only partial protection, reflected by increased Bcl-2 expression without substantial prevention of apoptosis or histological injury. These results highlight the thyroid as a vulnerable target of DOX toxicity and suggest that the protective capacity of LUT may depend on dose, duration, and possibly tissue-specific factors. Importantly, the partial benefits observed point to its potential as a candidate for mitigating chemotherapy-induced thyroid injury, warranting further studies with optimized dosing, combinatorial approaches, and comprehensive functional assessments.

Compliance with Ethical Standards

The study was conducted at the Hamidiye Experimental Animal Production and Research Laboratory, University of Health Sciences, following approval by the Institutional Ethics Committee (Approval No: 2020-06/13).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Author Contributions

NET; Surgical and medical practices, NET, AKV; Concept, Design, YC, NET, KS; Data Collection or processing, KS, AKV; Analysis or interpretation, KS, YC; Literature search, writing.

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