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Protective Roles of Kaempferol and Quercetin against Cisplatin-Induced Oxidative Damage and DNA Toxicity in Human Peripheral Blood Lymphocytes

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ABSTRACT

Cisplatin ranks among the most commonly employed chemotherapeutic agents. Certain lesser-recognized adverse effects, such as genetic toxicity, are associated with its extensive application. Kaempferol and quercetin represent two flavonoids known for their antioxidant capabilities. The purpose of this research was to examine cisplatin's genetic toxicity in human peripheral blood lymphocytes and to explore the potential safeguarding roles of quercetin and kaempferol. Lymphocytes were divided into several categories: a group exposed to cisplatin (0.8 µg/mL); group 1 treated with cisplatin (0.8 µg/mL) plus kaempferol (25 µM); group 2 treated with cisplatin (0.8 µg/mL) plus quercetin (25 µM); and a negative control group. Genetic damage was assessed via the alkaline single-cell gel electrophoresis (comet) assay and the cytokinesis-block micronucleus assay. Markers of oxidative stress, including glutathione levels, malondialdehyde content, superoxide dismutase activity, and catalase activity, were also determined. Findings indicated that cisplatin triggers oxidative stress through depletion of glutathione, reduced catalase and superoxide dismutase activities, and elevated lipid peroxidation. Genotoxicity tests revealed that cisplatin increased the frequency of micronuclei as well as the tail DNA percentage and tail moment in the comet assay. Both quercetin and kaempferol reduced the micronucleus frequency, tail DNA percentage, tail moment, and lipid peroxidation levels. Each compound enhanced superoxide dismutase activity; however, only quercetin markedly elevated glutathione levels and catalase activity relative to the cisplatin-exposed group. Oxidative stress appears to play a central role in the genetic toxicity caused by cisplatin. Additionally, quercetin or kaempferol supplementation could offer protection against cisplatin-related DNA damage in human peripheral blood lymphocytes.

Keywords: Cisplatin, Comet assay, Kaempferol, Micronuclei, Quercetin

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Introduction

Cis-diamminedichloroplatinum(II), commonly referred to as cisplatin, is a highly effective chemotherapeutic agent widely utilized [1]. It is applied in the management of numerous solid tumors, such as those affecting the head and neck, lungs, testes, ovaries, and breasts. Although it remains one of the most powerful options for treating malignancies, cisplatin is associated with various limitations, including genetic toxicity, liver damage, nerve toxicity, and particularly kidney toxicity, many of which are related to oxidative stress mechanisms [2].

Oxidative stress arises from a disruption in the equilibrium between antioxidant systems and the generation of oxidants, leading to harm in proteins, fats, and DNA (both nuclear and mitochondrial) due to reactive oxygen species (ROS) [3, 4]. ROS can cause various DNA lesions, including strand breaks (single and double), loss of purines (creating apurinic sites), cross-links between DNA and proteins, sugar moiety breakdown in deoxyribose, and impairment of DNA repair processes [4]. Research has shown that cisplatin generates ROS in rodent liver tissues, resulting in altered oxidative stress indicators, such as reduced glutathione (GSH) and diminished activities of catalase (CAT) and superoxide dismutase (SOD) [5, 6]. Cisplatin also elevates malondialdehyde

(MDA) concentrations, a marker of lipid peroxidation [7]. Byproducts of lipid peroxidation that react with aldehydes have been linked to DNA alterations [8].

The involvement of oxidative stress in cisplatin's DNA-damaging effects has been repeatedly demonstrated. For instance, Nazari *et al*. (2021) reported marked genetic toxicity in the cisplatin-treated group, evidenced by higher counts of micronucleated polychromatic erythrocytes (MNPCE), along with increased ROS in bone marrow [9, 10]. Similarly, Ali *et al*. (2019) observed that cisplatin substantially raised average micronucleated reticulocyte and colonic epithelial cell numbers in Sprague-Dawley rats, with accompanying rises in MDA highlighting the contribution of oxidative stress to this toxicity [11].

Flavonoids constitute a prominent class of plant-derived compounds with diverse health benefits, including antitumor, antiviral, anti-inflammatory, and antioxidant effects [12]. Evidence suggests that flavonoids can mitigate oxidative stress [13]. Moreover, combining these compounds with chemotherapeutic agents has shown promise in alleviating side effects from chemotherapy, an area that has gained significant interest in recent years [14]. Kaempferol and quercetin are two prominent flavonoids noted for their strong antioxidant activities.

Kaempferol, present in sources like Crocus sativus (saffron) and Capparis spinosa (capers), belongs to the flavonol subgroup and is sometimes called kaempferol-3 or kaempferide [15]. It has demonstrated multiple beneficial actions, such as antioxidant, anti-inflammatory, and nerve-protective effects [16]. Kaempferol boosts antioxidant defenses by upregulating heme oxygenase (HO)-1 expression, thereby improving cellular tolerance to oxidative injury [17]. It has been shown to counteract declines in CAT, glutathione peroxidase (GSH-Px), glutathione-S-transferase, and GSH levels in rat liver exposed to alcohol- and polyunsaturated fatty acid-induced stress (ΔPUFA). Studies also indicate that kaempferol elevates mRNA levels of antioxidant enzymes (including glutathione-S-transferase, GSH-Px, and CAT) that were suppressed by diethylnitrosamine in Chang liver cells [18].

Quercetin (3,3',4',5,7-pentahydroxyflavone), abundant in foods like onions, berries, and apples, provides extensive biological benefits, encompassing antioxidant, antitumor, anti-inflammatory, and antimicrobial properties [19, 20]. It is particularly effective at neutralizing free radicals. Quercetin has reduced oxidative stress in various cell models, including human gastric epithelial (GES-1), pheochromocytoma (PC-12), and hepatoma (HepG2) lines [21]. Multiple investigations confirm its strong protective action against oxidative DNA harm, shown by lowered micronucleus rates and comet assay parameters in lymphocytes from nicotine-exposed rats, as well as decreased single-strand breaks in hydrogen peroxide-treated Caco-2 cells [22-25].

The primary goal of this investigation was to evaluate oxidative stress's involvement in cisplatin-triggered genetic toxicity within human peripheral blood lymphocytes and to determine whether kaempferol and quercetin, as natural antioxidants, could provide mitigation. Outcomes from this work may contribute to strategies for reducing genetic toxicity and associated complications from cisplatin therapy.

Materials and Methods

Reagents

Cisplatin, quercetin, cytochalasin B, disodium hydrogen phosphate, EDTA, kaempferol, Giemsa stain, and Triton X-100 were sourced from Sigma (United States). Fetal bovine serum (FBS) and phytohaemagglutinin (PHA) came from Gibco (United States). Phosphate-buffered saline (PBS) and DMEM medium were acquired from Bio-Idea (Iran). Methanol, glacial acetic acid, normal melting agarose, potassium chloride, sodium chloride, Na2EDTA, dimethyl sulfoxide (DMSO), and sodium hydroxide were supplied by Merck (Germany). Low melting point agarose was obtained from Biobasic (Canada). Assay kits for GSH (CAT No. ZB-GSH-48A), MDA (CAT No. ZB-MDA-48-A), CAT (CAT No. ZB-CAT-48A), and SOD (CAT No. ZB-SOD-48A) were provided by ZellBio (Germany).

Blood collection and experimental treatments

Peripheral blood was collected from a healthy young male volunteer who neither smoked nor consumed alcohol. The donor reported no contact with potentially confounding agents, such as chemicals or ionizing radiation, in the six months preceding sampling. The blood was immediately anticoagulated with heparin. Whole blood was diluted in DMEM supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. The cultures were then allocated to the following experimental conditions:

Negative control: phosphate-buffered saline (PBS) plus dimethyl sulfoxide (DMSO)

1. Cisplatin-only: 0.8 µg/mL cisplatin [26]
2. Treatment group 1: 0.8 µg/mL cisplatin combined with 7.15 µg/mL (25 µM) kaempferol [27]
3. Treatment group 2: 0.8 µg/mL cisplatin combined with 7.355 µg/mL (25 µM) quercetin [28]

All cultures were maintained for 24 hours in a humidified incubator at 37 °C with 5% CO_2.

Genotoxicity assessment

Cytokinesis-block micronucleus (CBMN) assay

The cytokinesis-block micronucleus assay was selected as the primary method for detecting chromosomal damage and was performed following the procedure described by Sommer *et al*. [29]. Phytohemagglutinin (PHA) was added to each culture to stimulate lymphocyte proliferation. Cultures were incubated at 37 °C under 5% CO_2. At the 44-hour mark, cytochalasin B (3 µL of 300 µg/mL stock) was introduced to arrest cytokinesis. Cells were harvested after an additional 28 hours of incubation. Following centrifugation at 146 × g and removal of supernatants, cells were subjected to hypotonic treatment in a potassium chloride solution (methanol:acetic acid, 3:5). A pre-fixation step used glacial acetic acid:methanol (5:3), followed by centrifugation at 146 × g for 10 minutes. Cells were then fixed in ice-cold methanol (−20 °C), centrifuged at 228 × g for 10 minutes, and finally fixed in methanol:acetic acid (3:1) with another centrifugation at 228 × g for 10 minutes. Cell suspensions were dropped onto pre-chilled slides, air-dried, stained with 5% Giemsa solution, rinsed, and stored protected from light. Micronuclei were scored in 1000 binucleated lymphocytes per group under 400× magnification.

Alkaline single-cell gel electrophoresis (Comet) assay

DNA strand breaks were evaluated using the alkaline comet assay, adapted from the method of Singh *et al*. with slight modifications [30]. After centrifugation at 200 × g, cell pellets were resuspended in 1% low-melting-point (LMP) agarose and layered onto frosted slides pre-coated with 1% normal-melting-point agarose. Slides were covered and cooled at 4 °C in the dark for 10 minutes to allow solidification. Lysis was performed for 24 hours at 4 °C in the dark using a standard lysis buffer. Post-lysis, slides were placed in alkaline electrophoresis buffer for 20 minutes at 4 °C to permit DNA unwinding, followed by electrophoresis at 4 °C (1 V/cm, 300 mA) for 20 minutes. Slides were neutralized with Tris buffer, dehydrated twice with ethanol, stained with SYBR® Gold for 15 minutes in the dark, and rinsed with deionized water. One hundred nucleoids per slide were analyzed at 200× magnification using a fluorescence inverted microscope. DNA migration parameters (head and tail intensity) were quantified with CASPLab® software (version 1.2.3 beta2), and tail moment was computed as an indicator of DNA damage extent.

Assessment of oxidative stress markers

Samples were homogenized and centrifuged at 300 × g for 5 minutes prior to biochemical analyses.

Glutathione (GSH)

Reduced glutathione, a key non-enzymatic cellular antioxidant [6], was quantified using a commercial ZellBio kit. The method relies on the reaction of GSH with DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)), yielding the yellow-colored 2-nitro-5-thiobenzoic acid. Proteins were precipitated with 5-sulfosalicylic acid (SSA) to prevent GSH oxidation and inhibit γ-glutamyl transpeptidase activity. Absorbance of the resulting chromophore was measured at 412 nm on a microplate/ELISA reader to determine GSH concentration.

Malondialdehyde (MDA)

Malondialdehyde, an end product of lipid peroxidation, was determined with a ZellBio kit. The assay involves formation of an MDA-thiobarbituric acid (TBA) adduct at elevated temperature (100 °C), producing a pink chromogen. Following the manufacturer's protocol, absorbance was recorded at 535 nm using a microplate/ELISA reader.

Catalase (CAT)

Catalase activity, essential for hydrogen peroxide and reactive nitrogen species detoxification, was assessed via a ZellBio kit employing a two-step reaction. In the first step, catalase in the sample decomposes a defined amount

of H_2O_2 to water and oxygen in proportion to its activity. After precisely one minute, residual H_2O_2 reacts with a chromogen upon addition of a quencher that stops catalase activity. The resulting color was measured at 405 nm on a microplate/ELISA reader, and catalase activity (U/mL) was calculated using the formula provided in the kit instructions.

Superoxide dismutase (SOD)

Superoxide dismutase represents a primary enzymatic antioxidant defense in the organism. SOD activity was quantified using a commercial ZellBio assay kit. The method relies on the enzymatic conversion of superoxide anions into hydrogen peroxide and molecular oxygen. Samples were processed according to the manufacturer's protocol, and the resulting colored product was measured colorimetrically at 420 nm with a microplate/ELISA reader. SOD activity (expressed in U/mL) was subsequently calculated based on the formula supplied in the kit guidelines.

Statistical analysis

Data are presented as mean ± standard deviation from a minimum of three independent experiments. All statistical evaluations were performed using GraphPad Prism® software (version 6). Differences among groups were assessed by one-way analysis of variance (ANOVA), with Tukey's post hoc test applied for multiple comparisons. A p-value less than 0.05 was regarded as indicative of statistical significance.

Results and Discussion

Micronucleus formation was quantified via the CBMN assay to determine the extent of genetic damage **(Figure 1).** Exposure to cisplatin led to a marked elevation in the mean micronucleus frequency relative to the control group ($p < 0.001$). In contrast, co-treatment with either kaempferol or quercetin significantly lowered the mean micronucleus percentage when compared to the cisplatin-alone group ($p < 0.05$). Furthermore, quercetin demonstrated a more pronounced protective effect, achieving a greater reduction in micronucleus frequency than kaempferol when both were evaluated against the cisplatin group ($p < 0.001$) **(Figure 2).**

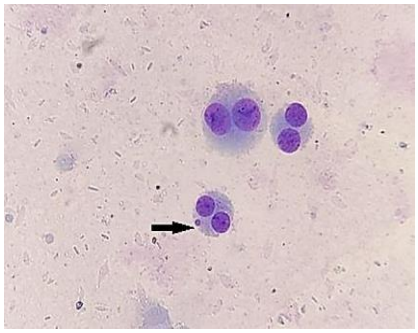

Figure 1. Illustration of a Giemsa-stained human blood lymphocyte showing two nuclei (400X), with the micronucleus indicated by an arrow.

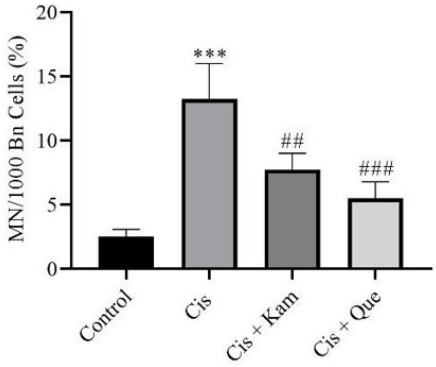

Figure 2. Illustration of the protective roles of kaempferol and quercetin against cisplatin-induced genotoxicity, measured by micronucleus (MN) frequency in human lymphocytes; data are expressed as mean ± SD; Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) denote cisplatin, kaempferol, and

quercetin, respectively; *** indicates a significant difference from the control group ($p<0.001$); ## and ### indicate significant differences from the cisplatin group ($p<0.01$ and $p<0.001$, respectively).

Figure 3 presents the analysis of DNA percentage in the comet tail. Cisplatin treatment caused a marked increase in DNA in the tail compared to the control ($p<0.001$), while kaempferol significantly reduced this percentage, and quercetin produced an even greater reduction ($p<0.01$) as shown in **Figure 4**. Evaluation of the tail moment demonstrated that cisplatin significantly elevated the tail moment relative to the control ($p<0.001$), whereas both kaempferol and quercetin treatments significantly decreased the tail moment compared to the cisplatin group ($p<0.05$), with both compounds exhibiting comparable protective effects **(Figure 5).**

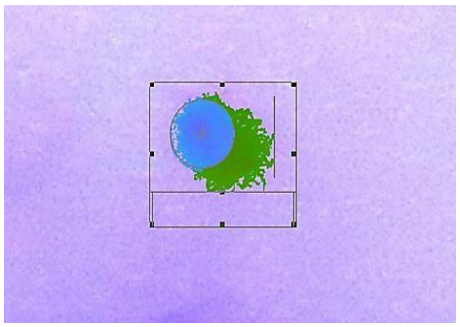

Figure 3. Fluorescent image of a human blood lymphocyte stained with SYBR® Gold (200X), showing DNA in the tail highlighted in green.

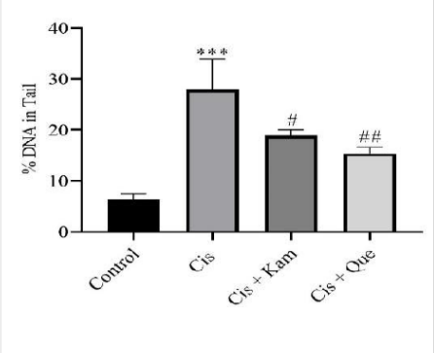

Figure 4. Percentage of DNA in the tail across the different study groups; data are presented as mean ± SD; Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) represent cisplatin, kaempferol, and quercetin, respectively; *** indicates a significant difference from the control group ($p<0.001$); # and ## indicate significant differences from the cisplatin group ($p<0.05$ and $p<0.01$, respectively).

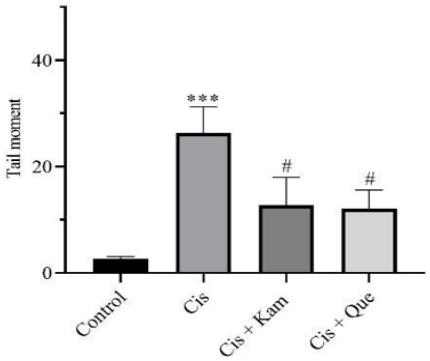

Figure 5. Variations in tail moment across the different experimental groups, with results presented as mean ± standard deviation. Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) represent cisplatin, kaempferol, and quercetin, respectively. *** indicates a highly significant difference compared to the control group ($p<0.001$); # denotes a significant difference relative to the cisplatin group ($p<0.05$).

In this investigation, glutathione concentrations were assessed as a marker of oxidative stress. Treatment with cisplatin markedly reduced glutathione levels in the cells relative to the control group ($p<0.05$). When kaempferol was combined with cisplatin, no notable elevation in glutathione levels was observed compared to the cisplatin-only group. However, the addition of quercetin (at an equivalent concentration to kaempferol) to cisplatin-treated lymphocytes led to a statistically significant rise in glutathione concentrations versus the cisplatin-alone group ($p<0.05$) **(Figure 6).**

Malondialdehyde (MDA), a key byproduct of lipid peroxidation, was also measured. As shown in **Figure 7**, cisplatin exposure substantially increased MDA levels compared to the control group. In contrast, co-treatment with either quercetin or kaempferol markedly lowered MDA concentrations ($p<0.001$). Notably, no significant distinction was found between the effects of kaempferol and quercetin on MDA levels **(Figure 7).**

Catalase (CAT) activity served as an additional indicator of oxidative stress. **Figure 8** reveals that cisplatin at 0.8 µg/mL caused a profound reduction in CAT activity relative to the control group ($p<0.001$). Co-administration of quercetin with cisplatin, however, produced a substantial restoration of CAT activity ($p<0.001$). In comparison, combining kaempferol with cisplatin did not yield a statistically significant improvement in CAT activity **(Figure 8).**

Superoxide dismutase (SOD) activity was examined as another measure of oxidative stress. The group treated with cisplatin (0.8 µg/mL) exhibited a clear decline in SOD activity versus the control group ($p<0.01$). Conversely, the inclusion of either quercetin or kaempferol with cisplatin effectively countered this reduction, resulting in a significant elevation of SOD activity ($p<0.01$). No meaningful difference was detected between the impacts of quercetin and kaempferol on SOD activity **(Figure 9).** Specifically, both flavonoids, when given with cisplatin, significantly enhanced SOD activity compared to the cisplatin-only treatment ($p<0.01$), with comparable efficacy between quercetin and kaempferol **(Figure 9).**

Cisplatin remains among the most widely used platinum-containing chemotherapeutic agents for managing diverse malignancies [31]. Although it provides substantial therapeutic advantages in cancer treatment, research has highlighted its various toxicities, chiefly inducing DNA damage via oxidative stress mechanisms [32].

The main objective of this research was to provide further support for the potential involvement of cisplatin in inducing genotoxicity mediated by oxidative stress, while investigating the protective properties of kaempferol and quercetin against such damage.

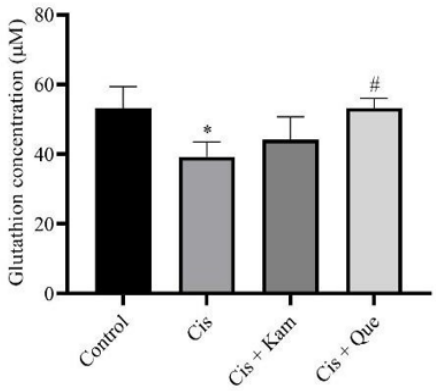

Figure 6. Safeguarding influence of kaempferol and quercetin against cisplatin-induced alterations in glutathione concentrations within human lymphocytes, with values expressed as mean ± standard deviation. Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) correspond to cisplatin, kaempferol, and quercetin, respectively. * denotes a significant difference relative to the control group ($p<0.05$); # indicates a significant difference compared to the cisplatin group ($p<0.05$).

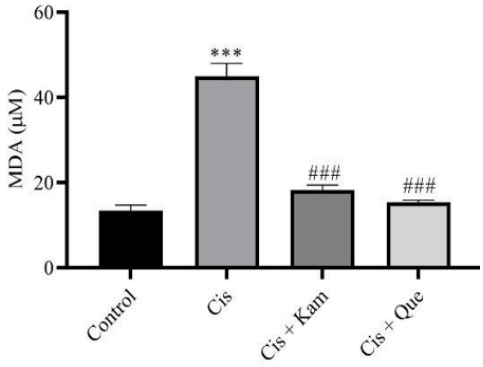

Figure 7. The antioxidant protection provided by kaempferol and quercetin against lipid peroxidation induced by cisplatin in human peripheral blood lymphocytes; values are presented as mean ± standard deviation; Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) represent cisplatin, kaempferol, and quercetin, respectively; *** denotes a statistically significant difference compared to the control group ($p < 0.001$); ### denotes a statistically significant difference compared to the cisplatin-treated group ($p < 0.001$).

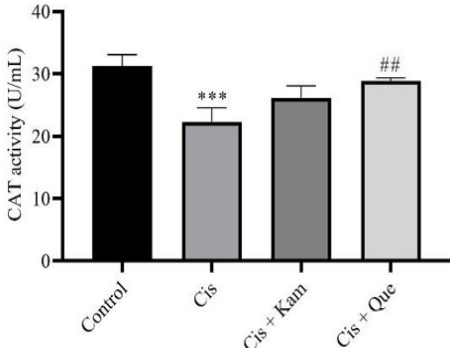

Figure 8. The protective role of kaempferol and quercetin in preventing the cisplatin-induced reduction in catalase (CAT) activity in human peripheral blood lymphocytes; results are expressed as mean ± standard deviation; Cis (0.8 µg/mL), Kam (7.15 µg/mL), and Que (7.35 µg/mL) denote cisplatin, kaempferol, and quercetin, respectively; *** represents a statistically significant difference from the control group ($p < 0.001$); ## represents a statistically significant difference from the cisplatin group ($p < 0.01$).

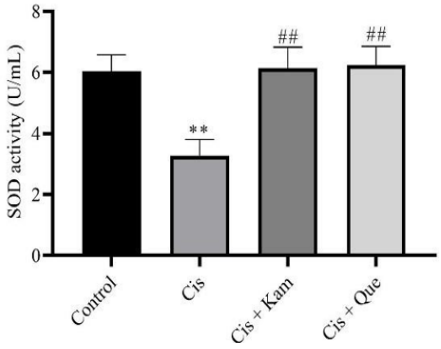

Figure 9. The protective role of kaempferol and quercetin in preventing the cisplatin-triggered reduction in superoxide dismutase (SOD) levels within human peripheral blood lymphocytes. Results are presented as averages with standard deviations. Abbreviations include Cis (0.8 µg/mL) for cisplatin, Kam (7.15 µg/mL) for kaempferol, and Que (7.35 µg/mL) for quercetin. ** denotes a statistically significant deviation from the control group ($p<0.01$), while ## signifies a notable difference compared to the cisplatin-treated group ($p<0.01$).

Both the alkaline comet assay and the micronucleus test were employed to demonstrate the DNA-damaging potential of cisplatin [33, 34]. In the present investigation, exposure to cisplatin led to elevated micronucleus

formation in the micronucleus test, along with increased tail DNA percentage and extended tail moment in the alkaline comet assay, indicating DNA injury. These observations align with prior research [35]. An earlier in vitro experiment on human lymphocytes detected cisplatin-related DNA breakage via the alkaline comet method [36]. Additionally, the cytokine-induced micronucleus (CBMN) assay revealed that alkylating chemotherapeutic agents, such as cisplatin, raised micronucleus rates in a human monocytic cell line [37].

As noted in various reports, oxidative stress is a key contributor to major cisplatin-associated toxicities, including nephrotoxicity, which causes kidney injury and cell death [38, 39]. The main pathways involved in cisplatin-induced kidney damage are thought to involve reactive oxygen species (ROS) production, accumulation of lipid peroxidation byproducts in renal tissue, and diminished antioxidant capacities. Within cells, cisplatin transforms into a highly reactive species that rapidly binds to thiol-based antioxidants like glutathione, resulting in glutathione depletion and heightened cellular oxidative burden [40].

In terms of oxidative markers, cisplatin exposure markedly lowered glutathione (GSH) concentrations ($p<0.05$), catalase (CAT) activity ($p<0.001$), and SOD activity ($p<0.01$), while substantially elevating malondialdehyde (MDA) levels ($p<0.001$). These patterns match findings from previous investigations [41-43]. Research involving Wistar rats administered a single intraperitoneal cisplatin injection documented reduced GSH content and SOD function, coupled with heightened lipid peroxidation in brain cells, suggesting that cisplatin-provoked oxidative stress may contribute to its neurotoxic effects [44].

In recent times, natural compounds with antioxidant properties have gained attention for alleviating drug-induced oxidative damage [45]. Multiple studies indicate that supplementation with such antioxidants can counteract cisplatin-related oxidative stress [46-48]. Here, the protective capacities of two flavonoid antioxidants from natural sources, quercetin and kaempferol, were evaluated and compared. Kaempferol, a flavonol-class flavonoid, has been linked to benefits such as reducing vascular endothelial inflammation, safeguarding liver function, combating obesity and diabetes, supporting cardiovascular health, and aiding in the management of fibroproliferative conditions and hypertrophic scars [15]. Both quercetin and kaempferol have demonstrated properties including blood pressure reduction, anti-arrhythmic actions, cholesterol-lowering effects, liver protection, antiviral activity, anti-ulcer effects, anti-thrombotic potential, anti-ischemic benefits, anti-allergic responses, and neuroprotection [49]. The antioxidant mechanisms of flavonoids involve metal ion chelation and direct neutralization of reactive oxygen species. Numerous reports highlight quercetin's ability to neutralize free radicals, inhibit lipid peroxidation, and enhance antioxidant systems both in living organisms and cell cultures [50, 51]. Similar capabilities have been attributed to kaempferol in earlier work [52]. In this work, quercetin and kaempferol exhibited protective antioxidant actions against cisplatin-triggered genotoxicity in peripheral blood lymphocytes. Quercetin proved superior in elevating GSH content and CAT activity, as well as in reducing micronucleus formation; nevertheless, both compounds performed equivalently in lowering lipid peroxidation and restoring SOD activity. Furthermore, quercetin more effectively reduced tail DNA percentage, though both equally diminished comet tail moment. These outcomes are in agreement with comet assay data reported by Noroozi *et al*. [53].

The data imply that combining kaempferol or quercetin with cisplatin treatment might represent a valuable approach to lessen its adverse impacts. Nonetheless, additional investigations, especially in vivo and at the molecular scale, are required to elucidate the mechanisms by which these antioxidants block cisplatin's genotoxic actions in healthy cells while preserving its anti-tumor effectiveness.

Conclusion

This investigation highlights oxidative stress as a central pathway in the genotoxicity induced by cisplatin, a notable adverse effect of this chemotherapeutic agent. At the same time, the mitigating roles of two naturally derived compounds, kaempferol and quercetin, were examined in relation to cisplatin-associated genotoxicity. The findings revealed that both flavonoids provided substantial antioxidant protection; however, quercetin generally displayed greater potency compared to kaempferol.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: The research protocol received approval from the ethics committee at Guilan University of Medical Sciences prior to initiation (IR.GUMS.REC.1403.017). All procedures aligned with the principles outlined in the Declaration of Helsinki. Participants provided written informed consent.

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