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ALLEVIATION OF DOXORUBICIN-INDUCED NEPHROTOXICITY IN BREAST CANCER MICE BY USING COMBINATION OF GREEN TEA AND MORINGA: FOCUS ON ANTIOXIDANT, APOPTOSIS, INFLAMMATION, AND HISTOPATHOLOGICAL INSIGHTS

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KEY WORDS:
doxorubicin, kidney toxicity, breast cancer, green tea, moringa, apoptosis, inflammation, antioxidant, histopathological

ABSTRACT

Doxorubicin (DXR)-induced nephrotoxicity remains a major concern in cancer treatment and calls for potential prevention of kidney injury. This study aims to evaluating the nephroprotective potentials of green tea and moringa used as 1% and 2% water extracts in DXR-induced kidney damage in female Balb/C mice with breast cancer. Thirty six female Balb/C mice were divided into six groups as follows: healthy control; 4T1 cells cancer-induced; healthy mice with DXR treatment; cancer-induced with DXR treatment; cancer-induced under DOX and treated with 1% green tea and moringa combination; cancer-induced under DOX and treated with 2% green tea and moringa combination. The variables of the experiment were body weight, tumor volume, antioxidant enzyme activities (CAT, GPx, SOD), oxidative stress markers (TOS, TAC, OSI), pro-inflammatory cytokines (IL-1, TNF-α), and apoptosis and inflammation-related genes (BAX, BCL2, NLRP3, NFKB). Histological analysis of the kidneys was also done to check for cellular changes. DXR treatment led to a decrease in the body weight and an increase in kidney enzymes, which is an indication of kidney damage. The levels of these enzymes were significantly lowered by the combination of herbal extracts, especially at 2%, indicating nephroprotective properties. The herbal extracts brought back the antioxidant enzyme activities to normal and reduced the oxidative stress markers in the kidney through raising the CAT, GPx, and SOD and decreasing the TOS and OSI levels. Furthermore, the herbal treatment also decreased the levels of pro-inflammatory cytokines and affected the apoptosis related gene expressions; the BAX was down-regulated and BCL2 was up-regulated, which helped in increasing the cell survival and decreasing inflammation. The extracts also reduced the NLRP3/NFKB in the kidneys of DXR-treated mice in a dose dependent manner. Based on these results, 1% and 2% mixture of green tea and moringa leaf aqueous extracts (1:1 ratio) can be considered an appropriate combination to reduce DOX-induced nephrotoxicity and kidney injury in cancer patients.

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ОСЛАБЛЕНИЕ ИНДУЦИРУЕМОЙ ДОКСОРУБИЦИНОМ НЕФРОТОКСИЧНОСТИ У МЫШЕЙ С РАКОМ МОЛОЧНОЙ ЖЕЛЕЗЫ ПУТЕМ ИСПОЛЬЗОВАНИЯ КОМБИНАЦИИ ЗЕЛЕНОГО ЧАЯ И МОРИНГИ: АКЦЕНТ НА АНТИОКСИДАНТНЫЕ, АПОПТИЧЕСКИЕ, ВОСПАЛИТЕЛЬНЫЕ И ГИСТОПАТОЛОГИЧЕСКИЕ АСПЕКТЫ

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КЛЮЧЕВЫЕ СЛОВА:
доксорубицин, рак молочной железы, зеленый чай, моринга, апоптоз, воспаление, антиоксидант, гистопатологический

АННОТАЦИЯ

Индуцируемая доксорубицином (DXR) нефротоксичность продолжает вызывать большую обеспокоенность при лечении рака и требует эффективного предотвращения повреждения почек. Целью данного исследования является оценка нефропротективного потенциала зеленого чая и моринги в 1% и 2% водных экстрактах при индуцированном доксорубицином повреждении почек у самок мышей Balb/C с раком молочной железы. Тридцать шесть самок мышей Balb/C были разделены на шесть групп следующим образом: здоровый контроль; мыши с раком молочной железы, индуцированным клетками 4T1; здоровые мыши, получавшие DXR; мыши с индуцированным раком молочной железы, получавшие DXR; мыши с индуцированным раком молочной железы, получавшие DXR и 1%-ю комбинацию зеленого чая и моринги; мыши с индуцированным раком молочной железы, получавшие DXR и 2%-ю комбинацию зеленого чая и моринги. Переменными эксперимента были масса тела, объем опухоли, активность антиоксидантных ферментов (CAT, GPx, SOD), маркеры окислительного стресса (TOS, TAC, OSI), провоспалительные цитокины (IL-1, TNF-α), и гены апоптоза и связанные с воспалением гены (BAX, BCL2, NLRP3, NFKB). Также был проведен гистологический анализ почек для анализа повреждений клеток. Обработка DXR приводила к снижению массы тела и увеличению ферментов почек, что указывало на повреждение почек. Уровни этих ферментов были значимо снижены в результате применения комбинации травяных экстрактов, особенно при концентрации 2%, что говорит от нефропротективных свойствах. Экстракты трав возвращали активности антиоксидантных ферментов к нормальному уровню и снижали

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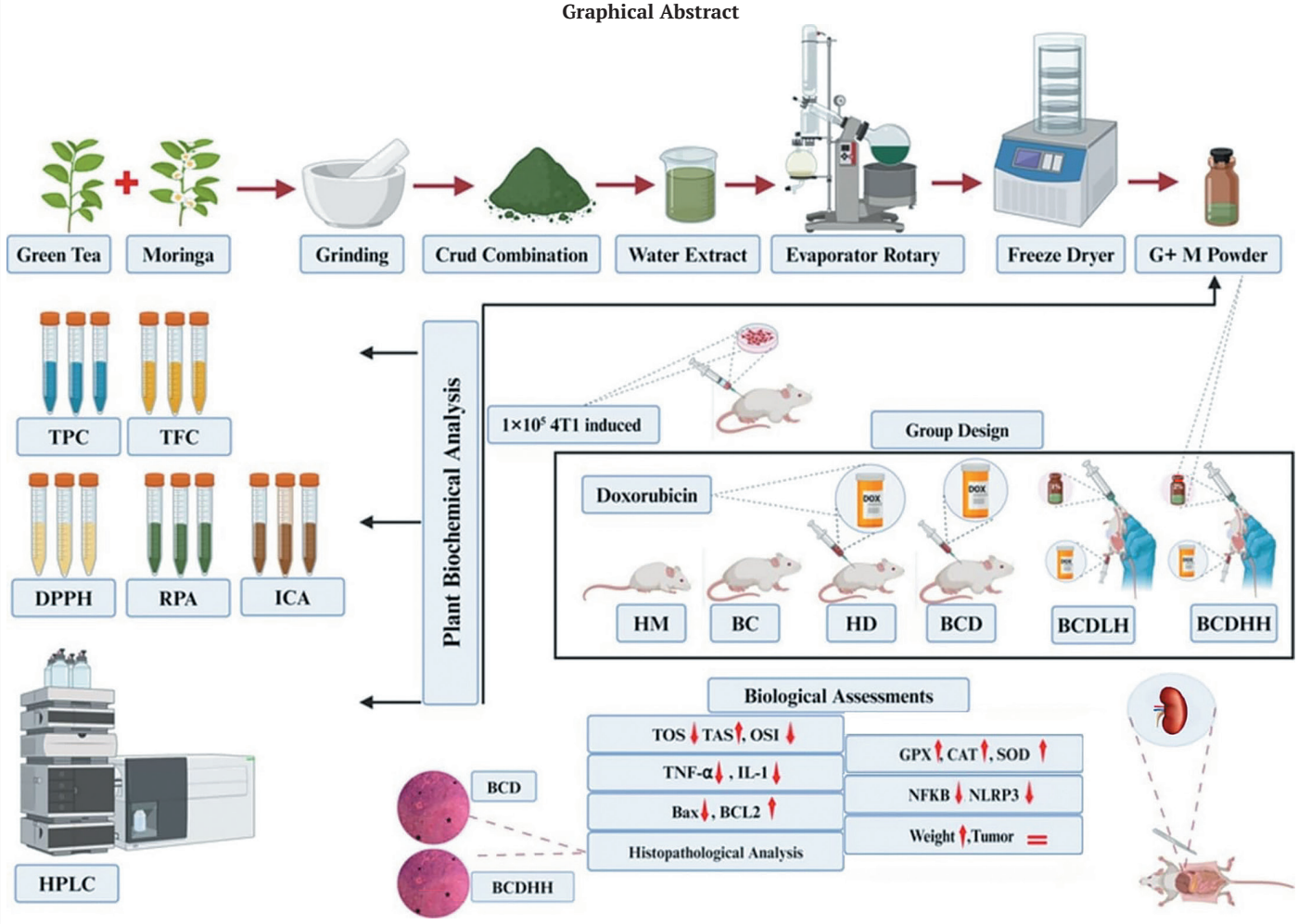
маркеры окислительного стресса в почках в результате повышения уровней CAT, GPx, и SOD, и снижения уровней TOS и OSI. Кроме того, обработка травами также снижала уровни провоспалительных цитокинов и влияла на экспрессию генов, связанных с апоптозом; BAX был негативно регулирован, BCL2 был позитивно регулирован, что способствовало увеличению выживаемости клеток и снижению воспаления. Экстракты также снижали NLRP3/NFKB в почках обработанных DXR мышей дозозависимым образом. На основании этих результатов сделан вывод, что 1% и 2% водные экстракты смеси листьев зеленого чая и моринги (соотношение 1:1) могут считаться пригодной комбинацией для снижения индуцируемой DOX нефоротоксичности и повреждения почек у пациентов с раком.

Graphical Abstract



1. Introduction

Doxorubicin is an anthracycline class of chemicals with the therapeutic role in chemotherapy used widely for treating the breast cancer and many other types of cancer. Although doxorubicin is effective in eliminating cancer cells in the body, its administration poses serious threats that include damaging the renal system. The fact has been clarified in several investigations showing that doxorubicin nephrotoxicity results in renal damage involving features such as glomerulosclerosis, interstitial fibrosis, albuminuria, a change in serum creatinine level, reduced glomerular filtration rate, and histological changes in the kidney [1]. Doxorubicin-induced nephrotoxicity is directly associated with oxidative stress and mitochondrial dysfunction, and these are crucial factors in renal toxicity [2]. The nephrotoxicity of doxorubicin is evidenced to be causing inflammatory changes that result in an increase in permeability of capillary walls and the shrinkage of the glomerulus [3]. Also, doxorubicin induces podocyte injury in the kidneys that leads to proteinuria and nephropathy, which shows that it has a toxic effect on the kidneys [4]. One of the processes affecting doxorubicin toxicity is the oxidation of cellular components due to the formation of reactive oxygen species, which is considered to be the main mechanism in the development of nephrotoxic effects. There is evidence that doxorubicin, causing kidney injury, activates oxidative stress, and mitochondrial dysfunction, along with inflammation [5]. The effect of the drug on the chain of mitochondrial electron transport stimulates the formation of reactive oxygen species and enhances the stress of renal cells in oxidation [1,6]. When doxorubicin is given repeatedly, this drug causes renal toxicity mainly due to its toxic effects on DNA and mitochondria [7].

Due to the presence of a number of bioactive compounds and plant-based nutrients, functional foods have emerged as the probable complementary therapies in cancer patients. Chemotherapy can be beneficial for cancer treatment, but it also has adverse side effects on the patient's body, and functional foods offer protective mechanisms that form a repertoire of defensive actions against those side effects. These compounds contain a wide range of health-enhancing attributes, primarily phytochemicals that are present in fruits, vegetables, legumes, seeds, whole grains, herbs, and spices including flaxseeds. These compounds have several advantages such as anti-cancer, antioxidant, and anti-inflammatory activities. Herbs and spices are vital ingredients that are incorporated into cooking meals in most parts of the world. Besides improving the taste of foods, they also possess some therapeutic properties and thus, cannot be overemphasized in functional foods [8,9,10]. Research has demonstrated that nephrotoxicity due to doxorubicin can be prevented by using natural compounds like crocin and luteolin, which possess antioxidant and anti-inflammatory effects [11,12]. The damage to the kidney by doxorubicin has been checked to occur through the formation of free radicals which causes oxidative stress, according to [1].

Camellia sinensis green tea contains polyphenols and other antioxidants that are known to have the nephroprotective property. Research has revealed that green tea polyphenols are effective in the protection of the kidney against toxins including heavy metals, through their action of fighting free radicals and inflammation [13]. Green tea extracts have been found to prevent tissue damage and inflammation caused by pollutants and toxins, which implies a potential in preventing organ damage [14]. It has been observed that green tea lowers oxidative stress and mitigates

renal toxicity in animal models, which makes it useful in the management of kidney diseases [15]. Previous studies have also indicated that the seed extract of moringa is protective for the liver and kidney tissues against toxic substances such as dichlorvos in rats [16]. Several antioxidant compounds were identified in the moringa extract. These antioxidant compounds have the potential to boost the activity of SOD, CAT, and GSH, reduce the levels of inflammatory cytokines, and thus, play a protective role for the kidneys [17].

Also, the effect of moringa water extract has been investigated for the prevention of gentamicin-induced hepatorenal toxicity in animal models. In the study done by Lukiswanto et al. [18], the oral administration of the moringa leaf extract has been established to alleviate the hepatotoxicity and nephrotoxicity effect of gentamicin, hence playing a protective role against drug-induced organ injury. The flavonoids and phenolic products present in the extract of *Moringa oleifera* seed inhibit the destruction of the renal tissue through cytoprotection and antioxidant activity [19].

Synergism can be defined as the situation where the total effect of chemicals is more than the effect of each of the chemicals taken independently [20]. The active substances used in the preparation of herbal medicines are not simple and come in many forms that make the medication diverse in its effects. Furthermore, all the active ingredients of all used herbs can interact and increase the therapeutic effect to treat various diseases. The coaction of two or more herbs during treatment has a better outcome because it affects multiple pathways of the disease development. This way, the probability of attaining better treatment results is higher compared to the probability of merely utilizing a single herb, particularly for ailments that have several causes [21]. The use of several herbs may reduce the side effects that are associated with high doses of a single herb [21,22,23]. A promising approach to the application of herbal medicines is their use with nanoscale drug delivery systems, which showed the potential of enhancing the antioxidant and anti-inflammatory activities and improving the protective mechanism in various diseases [24].

The 4T1 breast cancer model is well understood when it comes to the progression of breast cancer and the identification of potential treatment regimens. Thus, it would be most appropriate to use it in the assessment of hepatotoxicity resulting from chemotherapy drugs. From this animal model of breast cancer, it is possible to get many insights into how herbal therapies operate and the extent of their efficacy [25,26,27].

This paper aimed to assess whether combined green tea and moringa have the nephroprotective potential when used in doxorubicin-treated mice with breast cancer. In particular, this study aims at assessing the efficacy of green tea and moringa combination on apoptotic, inflammatory and oxidative pathways in the kidney with the purpose of widening the knowledge concerning the molecular mechanisms of the prevention of doxorubicin-induced nephrotoxicity.

2. Materials and methods

2.1. Collection, authentication and preparation of plants

The herbs were obtained from local vendors in Basra Governorate, situated in the southern area of Iraq. A specialist in medicinal plants from the Department of Horticulture at the University of Basra confirmed the authenticity of the plants. The samples exhibited no apparent contamination and were selected from dried leaves. The items received a comprehensive inspection to identify any contaminants and foreign objects. The herbs were ground into a fine powder using a manual grinder (DAMFOX, No: MK-Y599, Germany) and all samples were kept at room temperature until they were analyzed.

2.2. Preparation of water extracts of herbs

To make water extracts, 60 g of herb powder was mixed with 300 mL of boiling distilled water and stirred with a magnetic stirrer (Heidolph, Germany) for half an hour. The next step was to filter the samples under vacuum using a Whatman No. 1 filter paper and a Buchner funnel. After that, the solvent was removed and the extracts were dried using a rotary evaporator RE300 DB (Stuart, UK) set to 40 °C to concentrate the filtrate. Afterward, a freeze drier (CHRIST, Germany) was used to dry the samples. The resulting dry samples were then stored in amber glass tubes at a temperature of –18 °C until they were needed. A 1:1 ratio of green tea aqueous extract to moringa leaf aqueous extract was used.

2.3. HPLC Analysis

Following the methods described in the research by Mradu et al. [28], HPLC analysis was carried out. In brief, SYKAM S500 HPLC system series from Germany was used for the HPLC analysis, together with a PDA detector and a C18-ODS column from Zorbax Eclipse Plus with a particle size of 25 cm × 4.6 mm. The mobile phase consisted of DW (25%) with methanol (70%) and formic acid (5%). Using a constant flow rate of 1.0 mL/min, HPLC profiling was carried out at a temperature of 30 °C. For analysis, a wavelength of 280 nm was used, and 10 µL quantities were introduced into the system for each sample. The following acids and their standards were bought from Sigma-Aldrich in Germany: kaempferol, quercetin, catechin, ellagic acid, syringic acid, and hydrobenzoic acid (Figure 1).

2.4. Determination of total phenolic content (TPC)

The method for the assessment of TPC with the use of the Folin-Ciocalteu reagent, which was supplied by Avonchem of the United Kingdom, was applied as described by Kumar et al. [29] with slight modifications. Therefore, 100 µL of Folin-Ciocalteu reagent was diluted to 1/10 before adding it to 500 µL of each extract. The mixture was mixed thoroughly with 4.5 mL of distilled water and 0.5 mL of the 7.5% (w/v) sodium carbonate solution. After 5 minutes at room temperature, the samples were incubated. The mixture was mixed by vortexing and then was left at room temperature for 120 minutes in the dark. The optical density at 760 nm was recorded with a blank and a Metstar MUV-61PCS spectrophotometer from the Optima, England. A calibration curve for gallic acid, sourced from BHD in England, was used to determine the total phenolic content (TPC) at varying concentrations of 0, 100, 200, and 300 mg/g. Data obtained were presented in mg of gallic acid equivalent (GAE) per gram of dry weight.

2.5. Determination of total flavonoid content (TFC)

The $AlCI_3$ colorimetric assay was employed in the determination of TFC as indicated by Pandey et al. [30]. According to this method, 4 mL of double-distilled water and 0.3 ml of sodium nitrite (5% w/v) purchased from BHD England and 1 ml of the aqueous extract containing 1 mg/ml

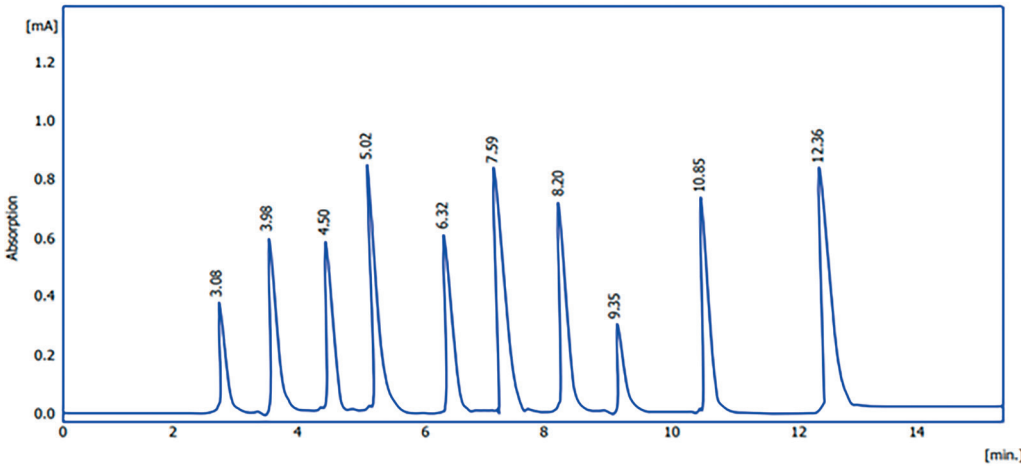


Figure 1. **Chromatogram of chemical compounds standards. Hydrobenzoic acid (3.08 min), kaempferol (3.98 min), isorhamnetin (4.50 min), syringic acid (5.02 min), quercetin (6.32 min), silybinin (7.59 min), epicatechin (8.20 min), caffeine (9.35 min), catechin (10.85 min), ellagic acid (12.36 min), under wavelenth of 280 nm**

Рисунок 1. **Хроматограмма стандартов химических соединений. Гидроксибензойная кислота (3,08 мин), кемпферол (3,98 мин), изорамнетин (4,50 мин), сиреневая кислота (5,02 мин), кверцетин (6,32 мин), силибинин (7,59 мин), эпикатехин (8,20 мин), кофеин (9,35 мин), катехин (10,85 мин), эллаговая кислота (12,36 мин), при длине волны 280 нм**

of the sample were added to a test tube. It was allowed to sit at room temperature for 5 minutes once solution was added. It was then supplemented with 10% (v/w) BHD $AlCl_3$ from England. Then, 2 mL of 1 M NaOH solution (VWR Chemicals, USA) was added to 10 mL of double distilled water until the volume was reached. The absorbance at 510 nm was determined using a double-beam UV–VIS spectrophotometer (model Optima-England) after the mixture had been well stirred. The concentrations of quercetin were prepared in the range of 0 to 1000 mg/mL to estimate the amount of flavonoids. The results were expressed in mg of quercetin equivalent per gram of dry weight.

2.6. Evaluation of the antioxidant potential

2.6.1. DPPH free radical scavenging assay

The antioxidant potential of the extracts was determined using the 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany) free radical scavenging activity method following the method described by Phuyal et al. [31]. An amount of 0.5 ml of the extracts (200 and 400 mg/g) was mixed with 1.0 ml DPPH solution and allowed to react at room temperature for 30 min in the dark. A control was prepared using 1 mL methanol and 1 mL DPPH solution. Lastly, the absorbance of the solutions was taken at 517 nm using a spectrophotometer made by England's company Optima. BHT from Samchun, Korea was used as a standard at concentrations of 200 and 400 mg/mL. Radical scavenging potential was determined by applying the following relationship formula:

$$\text{DPPH\%} = 1 - \frac{A_{sample}}{A_{control}} \times 100\%. \quad (1)$$

where $A_{control}$ is a control sample absorbance; A_{sample} is the extract absorbance.

2.6.2. Ferric reducing antioxidant power (FRAP) activity

FRAP activity was assessed according to the methodology described by Védékoi et al. [32]. Three milliliters of 1% potassium ferricyanide and one milliliter of a sodium phosphate buffer (0.2 M, pH 6.6) from Ino Lab pH7110 WTW, UK were combined in a test tube with one milliliter of the sample, which concentrations varied from 200 to 400 mg/g. The components were separated by centrifugation of the combination for 10 minutes at 3000 rpm in a Taiwanese-made Harmonic Series centrifuge (GEMMY, Taiwan). Prior to centrifugation, the mixture was heated in a water bath at 50 °C for 20 min. Two milliliters of 10% trichloroacetic acid were then added to the mixture. The mixture consisted of 2 milliliters of distilled water (DW), 0.5 milliliters of a recently prepared 1% ferric chloride ($FeCl_3$) solution from England (BHD), and 2 milliliters of the upper layer of the solution. Absorbance of the mixture was determined at 700 nm using a UV–VIS spectrophotometer (Optima England). The capacity of the reaction mixture to reduce ferric ions is strongly correlated with its absorbance. The total antioxidant content was determined and represented as absorbance values. Tocopherol served as a positive reference for comparative purposes.

2.6.3. Metal chelating ability

Chelation of ferrous ions was measured following the procedure described by Gulcin et al. [33]. In brief, several quantities (ranging from 200 to 400 mg/g) of the solution were made by mixing 0.25 mL of the extract with 0.05 mL of $FeCl_2$ (2mM). Sigma-Aldrich of Germany supplied ferrozine (2 mM), which was introduced to the mixture in an amount of 0.2 mL. After being mixed, the mixture was let to sit at 25 °C for 10 minutes. A wavelength of 562 nm was then used to measure the absorbance values of the solution. We utilized the component EDTA as a control and expressed the results of the total antioxidant content as a percentage. A percentage (%) representing the chelating effect was calculated using the following formula:

$$\text{Metal chelating effect \%} = Abs_{control} - \frac{Abs_{sample}}{Abs_{control}} \times 100\%. \quad (2)$$

where $Abs_{control}$ is a control sample absorbance; Abs_{sample} is the extract absorbance.

2.7. Animals

The Stemgene Biohealth laboratory in Ahvaz, Iran, supplied us with 36 female BALB/c wild-type mice that were 8 weeks old and weighed 20–22 g. The animals had free access to water and food (Parsfeed Co Iran) and were kept in a typical laboratory setting with a 12-hour light/dark cycle, standard laboratory condition of humidity, and temperature (22 ± 2 °C). The treatment that the animals received was strictly adhered to the protocols laid forth in the "Guide for the Care and Use of Laboratory Animals" (Washington, DC, USA).

2.7.1. 4T1 breast cancer mouse model

To create the 4T1 breast cancer mouse model, a group of twenty-four female BALB/c mice were used. The StemGene BioHealth laboratory in Ahvaz, Iran, provided the 4T1 cell line. A 10% fetal bovine serum (FBS) supplemented with 100 unit/ml of penicillin/streptomycin and a DMEM-HG medium (Bioidea, Iran) were added to the cell culture media. The serum came from Biosera in France. In a controlled environment of 37 °C, 5% CO_2, and 95% humidity, the cells were left to incubate for 36 hours. Isolated 4T1 cells (1×10^5) were injected subcutaneously in the 3rd mammary fat pad of BALB/c mice using 100 µL, following the previous description. The control groups of mice were given 100 µL of PBS at the same location [34]. Once weekly, we evaluated the weight of the mice, the size of the tumors, and the appearance. According to Sauter et al. [35], the width and length of the tumors were measured using calipers, and the volume was then determined using the following formula: 0.52 × width2 × diameter. Mouse behavior, food intake, and water intake were monitored every day.

2.8. Experimental design

At the point at which tumors became palpable and measured 100 mm in diameter, healthy and tumor-bearing mice were divided into six groups, with six mice in each group:

Control group (H): For five weeks, healthy mice were gavage-treated with 0.1 ml of normal saline once daily and received a single intraperitoneal injection of the same volume once per week.

Tumor group (BC): For four weeks, mice with tumors were gavage-treated with 0.1 ml of normal saline once daily and received a single intraperitoneal injection of the same volume once per week.

Healthy+DXR group (HD): Doxorubicin (5 mg/kg) (DOX–Cell, Germany) was administered intraperitoneally to healthy mice once weekly for five weeks.

Tumor + DXR (BCD) group: Mice with tumors were administered a single intraperitoneal dose of doxorubicin (5 mg/kg) once every week for a duration of five weeks.

Tumor +DXR+Formula 1 (BCDLH): Mice with tumors were given a single injection of doxorubicin (5 mg/kg) intraperitoneally once a week for five weeks, followed by a 1% dosage of green tea and moringa combination.

Tumor + DXR+Formula 2 (BCDHH): Mice with tumors were given a single injection of doxorubicin (5 mg/kg) intraperitoneally once a week for five weeks, followed by a 2% dosage of green tea and moringa combination.

2.9. Sampling

The rodents were anaesthetized with ketamine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) and decapitated at the end of the experiment. The kidney tissue was removed prior to histological examination and fixed in 10% formalin buffer. After being collected and kept at −70 °C, the kidney was used to analyze antioxidant indices and gene expression. For serum biochemical analysis, serum samples were extracted from the blood of each mouse via centrifugation at 2,700 g (GEMMY, Taiwan) for 15 minutes and stored at −20 °C.

2.10. Tissue preparation

Following the methods previously detailed by Amiri et al. [37], the tissues were homogenized prior to the measurement of inflammatory and antioxidant/oxidant components. In brief, the kidney sample was ground up in a 1:5 ratio using a homogenizer (Heidolph, Germany), in 1000 µl of RIPA lysis buffer (which contains 150 mM of sodium chloride, 0.1% SDS, 25 mM of tris, pH 7.4, 1 mM of NaF, 1 mM of phenylmethylsulphonyl fluoride, 50 mM of sodium fluoride, and a protease inhibitor cocktail; Sigma-USA). Homogenate tissues were centrifuged at 10,000 × RPM for 15 minutes at 4 °C. The clear supernatants were carefully collected, divided, and promptly frozen at −70 °C until use for analysis. A Bradford protein assay kit (made by KiaZist Co., Iran) was used to measure the concentration of proteins.

2.11. Antioxidant enzymes assay

Following the manufacturer's instructions, we used commercial kits to measure the activity of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD) (KiaZist Co, Iran). The antioxidant enzyme activities were measured in units per milligram of tissue protein.

2.12. Tissue cytokine measurement

The concentrations of IL1-β and TNF-α in the tissues were quantified using ELISA kits designed for mice (Sunlong Biotech, China) and reported as pg/mg of tissue protein.

2.13. Analysis of oxidative stress index

The oxidative stress index (OSI) was calculated by dividing the total oxidant status (TOS) by the total antioxidant capacity (TAC). A semi-automated microplate colorimetric technique was used to calculate the total oxidative status (TOS) using hydrogen peroxide (H_2O_2) as the standard of measurement. The test was conducted according to the method given by Erel in 2005 [38]. The ultimate outcomes were measured as millimoles

of hydrogen peroxide equivalent per milligram of protein. The measurement of total antioxidant capacity was conducted using the technique developed by Benzie and Strain [39]. In summary, a functional FRAP (ferric reducing antioxidant power) solution was prepared via mixing acetate buffer with TPTZ solution in HCl. Following the addition and mixing of $FeCl_3$, a mixture of 10 μL of tissue homogenate and 290 μL of the specified working solution was added and incubated for 10 minutes at room temperature. The samples were analyzed for optical density at a wavelength of 532 nm. Vitamin C was utilized as a standard. The expression of TAC was measured in micromoles of vitamin C equivalents per milligram of protein.

The OSI was determined using the following formula:

$$OSI = \left[\frac{TOS \text{ in } \mu mol\ H_2O_2\ Eq/mg\ protein}{TAS \text{ in } \mu mol\ Trolox\ Eq/mg\ protein}\right] \times 100\%. \quad (3)$$

2.14. RNA isolation and cDNA synthesis

The RNA from kidney tissues was isolated using the Tissue RNA isolation kit (ParsTous, Iran) according to the manufacturer's instructions. The RNA purity at the 260/280 OD ratio and the integrity of the RNA were assessed with an Eppendorf μCuvette G1.0 microvolume measurement cell (Eppendorf, Germany). The cDNA synthesis process used RNA of high purity, with an optical density ratio of 260/280 above 1.8. The cDNA was generated using 1 μg of RNA using the Easy™ cDNA Synthesis Kit (ParsTous, Iran) and random hexamer, following the instructions provided by the manufacturer.

2.14.1. Real-time quantitative polymerase chain reaction (qRT-PCR)

The qRT-PCR primers were generated using Primer3 software version 4.1.1 (Table 1), using the sequences of NLRP3, NFκβ, bax, bcl-2, and GAPDH genes obtained from the NCBI Gene bank (Table 1). The GAPDH gene was used for a reference gene in data analysis during this research. The SYBR® Green Real Time PCR Master mix from ParsTous in Iran was used for qRT-PCR analysis using the StepOnePlusTM Real-Time PCR detection System manufactured by Applied Biosystems in the United States. The PCR procedure included an initial denaturation step at 94 °C for 5 minutes, followed by 45 cycles of denaturation at 94 °C for 15 seconds and at 60 °C for 30 seconds. Two control reactions were taken into account, one being a negative control lacking cDNA and the other using RNA instead of DNA. The analysis included comparing the relative expression of the genes to the calibrator gene using the comparative 2−ΔΔCt technique. The amplification efficiency of the target genes relative to the reference gene (GAPDH) was assessed by creating several dilutions of cDNA and constructing an efficiency graph, using the methodology outlined in a recent study by Tabandeh et al. [40].

Table 1. **Primers characteristics used for real-time quantitative polymerase chain reaction**

Таблица 1. **Характеристики праймеров, использованных для количественной полимеразной цепной реакции в реальном времени**

Gene Bank	SEQUENCE	Size bp	GENE NAME
XM_017593963.1	F: AGTTCAACGGCACAGTCAAG	119	GAPDH
	R: TACTCAGCACCAGCATCACC		
NM_007527.4	F: AGGATGCGTCCACCAAGAAGCT	102	BAX
	R: TCCGTGTCCACGTCAGCAATCA		
NM_009741.5	F: CCTGTGGATGACTGAGTACCTG	122	BCL2
	R: GCCAGGAGAAATCAAACAGAGG		
NM_145827.4	F: TCACAACTCGCCCAAGGAGGAA	146	NLRP3
	R: AAGAGACCACGGCAGAAGCTAG		
NM_008689.3	F: GCTGCCAAAGAAGGACACGACA	130	NFKB-p65
	R: GGCAGGCTATTGCTCATCACAG		

2.15. Histopathological analysis

A 10% formalin buffer was used to fix the fresh kidney samples. Using the traditional approach, tissue microscopic slices were produced. Using a Histokinette machine (Leica, Germany), the necessary steps of tissue processing were carried out after fixation. These included dehydration with ethanol of progressively higher concentrations, clearing with xylene, and impregnation with paraffin. Slices with a thickness of 5–6 μm were made using a rotary hand microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Germany) after the samples were removed from the Histokinette device. Hematoxylin and eosin staining (H&E) was used on the sections. A digital camera (Microbin5, Microteb, Iran) attached to an optical microscope (Olympus Optical Co., Japan) was used to examine the slides. The stained sections of tissues from control and treated rats were scanned for the changes in the respective architectural pattern, portal triads, hepatocytes, sinusoids and the histopathological alterations in general.

2.16. Ethics statement

All animal procedures were approved by the University of Basrah — College of Veterinary Medicine Research Ethics Committee (Approval Number: 19/37/2024). The study complied with CIOMS and WHO guidelines, as well as OIE principles on animal ethics. Consent to participate is not applicable as this study did not involve human subjects.

2.17. Statistical analysis

Data analyses were conducted using Graph Pad Prism version 10 software (GraphPad software, San Diego, CA, USA). Statistical distinctions were ascertained by one-way analysis of variance (ANOVA). The Tukey test was used to assess the statistical significance of the findings. The data is shown as the mean value plus or minus a standard deviation of the mean (SD), and a P-value less than 0.05 was determined to be statistically significant.

3. Results and discussion

3.1. Charachteristics of herbal extracts

In the present study, we evaluated the ability of green tea and moringa extracts, individually and in combination, to mitigate DXR-induced nephrotoxicity in a murine model of breast cancer. It is shown that both green tea and moringa extracts can prevent kidney damage induced by DXR through the action of antioxidants and anti-inflammatory agents. Moringa and green tea extracts have high total phenolic and flavonoid contents related to their antioxidant activity. The total phenolic content in green tea, moringa, and their combination was 152 mg/g, 99.7 mg/g, and 89.4 mg/g, while the content of flavonoids was 302 mg/g, 140 mg/g and 190 mg/g, respectively (Table 2).

Table 2. **Total phenolic and flavonoid content in green tea, moringa and their mixture**

Таблица 2. **Общее содержание фенольных соединений и флавоноидов в зеленом чае, моринге и их смеси**

Formulations	Total phenolic content (TPC) mg/g	Total flavonoid content (TFC) mg/g
Green tea + moringa	89.4	190
Green tea	152	302
Moringa	99.7	140

The antioxidant activities of the extracts were determined by several methods, such as DPPH radical scavenging assay, reducing power, and iron chelating assay. As can be seen from Table 3, green tea had better DPPH radical scavenging activity compared to moringa at the concentration of 200 mg/g and 400 mg/g with inhibition rates of 82.2% and 83%, while moringa revealed inhibition rates of 80.2% and 81.5%. This was five percent lower than the control at the same concentrations. The combination of both extracts enhanced the activity where inhibition rates of 84.45% were recorded at 200 mg/g and 84.7% at 400 mg/g.

The activity of iron chelation is useful in the prevention of oxidative stress since it inhibits iron-dependent generation of reactive oxygen species. Green tea was found to have a modest capacity to chelate iron (42% and 43% at 200 and 400 mg/g, respectively). Moringa showed reduced chelating ability at levels of 30% and 36%, while the combination had even lower efficacy of 22% and 41% at 200 and 400 mg/g when compared to EDTA, a very effective chelator with efficiencies of 97.4% and 98% at the same concentrations. This finding implies that although the herb extracts used in this study have high antioxidant activities, their iron chelating abilities are much lower than synthetic chelators, such as EDTA.

The reducing power of the extracts was determined. Green tea had values of 0.7 nm at 200 mg/g and 1.2 nm at 400 mg/g, and moringa showed values of 0.5 nm and 0.8 nm at the same concentration. The combination presented a lower level of reducing power with values of 0.3 nm at 200 mg/g and 0.42 nm at 400 mg/g compared to tocopherol, which recorded a higher reducing ability of 0.8 nm and 1.3 nm.

The HPLC analysis pointed out the presence of various bioactive phytochemicals in each extract including gallic acid, chlorogenic acid, and caffeic acid. Those compounds are known for their antioxidant and anti-inflammatory effects, which also support the focused protecting effects of the extracts.

These outcomes are in agreement with previous studies that have revealed the high levels of antioxidants in green tea and moringa plants Senanayake [41], Musial et al. [42], Pękal et al. [43], Lorenzo and Munekata [44], Peñalver et al. [45], Ntshambiwa et al. [46], Sreelatha et al. [47].

Table 3. **Free radical scavenging by DPPH assay, reducing power by FRAP assay and metal chelating ability for green tea, moringa and their mixture**

Таблица 3. **Улавливание свободных радикалов, определенное методом DPPH, восстанавливающая способность, определенная методом FRAP, и способность хелирования металлов у зеленого чая, моринги и их смеси**

	DPPH, %		
	G + MO	**G**	**MO**
200 mg/g	**84.45**	**82.2**	**80.2**
400 mg/g	84.7	83.8	81.5
	Iron chelation, %		
	G + MO	**G**	**MO**
200 mg/g	22	42	30
400 mg/g	41	43.6	36
	Reducing power WA		
	G + MO	**G**	**MO**
200 mg/g	0.508	0.893	0.359
400 mg/g	0.779	1.378	0.621

G: green tea; M: moringa; G + MO: green tea+moringa.

3.2. HPLC analysis

HPLC chromatograms of green tea, moringa, and their combination are presented in Figure 2, Figure 3 and Figure 4.

Concentrations of the active compounds in green tea, moringa, and their combination are shown in Figure 4. The combination of green tea and moringa had a higher content of kaempferol (38.9±0.9 mg/kg; $p < 0.0001$) compared to green tea (20.1±0.1 mg/kg) and moringa (24.4±0.4 mg/kg), respectively, followed by quercetin with a significantly higher content in the combination (34.5±0.3 mg/kg; $p < 0.0001$) compared with green tea (18.7±0.4 mg/kg) and moringa (20.6±0.5 mg/kg). Moreover, the catechin content in the combination also showed a significant increase (18±0. mg/kg; $p < 0.0001$) compared with green tea and moringa (13.6±0.4 mg/kg and 9.8±0.3 mg/kg, respectively). Ellagic acid and syringic acid were detected at concentrations of 12.6±0.4 mg/kg and 13.6±1 mg/kg, respectively, in moringa and 11.6±0.4 mg/kg and 12.4±0.3 mg/kg, respectively, in the combination, whereas in green tea they were absent. The concentration of hydrobenzoic acid was 16.9±0.1 mg/kg in green tea and 16±0.2 mg/kg in the combination, while in moringa it was not found.

3.3. The impact of herbal extracts on the body weight and tumor growth in mice given DXR

The results demonstrated that DXR-treated healthy mice had a significantly reduced body weight compared to untreated animals ($P < 0.001$). Malignant mice in the BCD group had the same result as untreated malignant animals in the BC group after receiving DXR treatment ($P < 0.01$). No difference in body weight change was seen between the untreated and treated groups of mice when DXR was administered at 1% or 2% herb combinations (BCDLH and BCDHH) compared to the control group (Figure 5 (A)).

The results regarding tumor size are shown in Figure 5 (B). A significantly smaller tumor size was seen in the BCD group, which was treated with DXR, as compared to the untreated mice (BC) group ($P < 0.001$). Statistically significant changes in tumor size were not seen in the BCD group or in the groups treated with 1% (BCDLH) or 2% (BCDHH) herb extract.

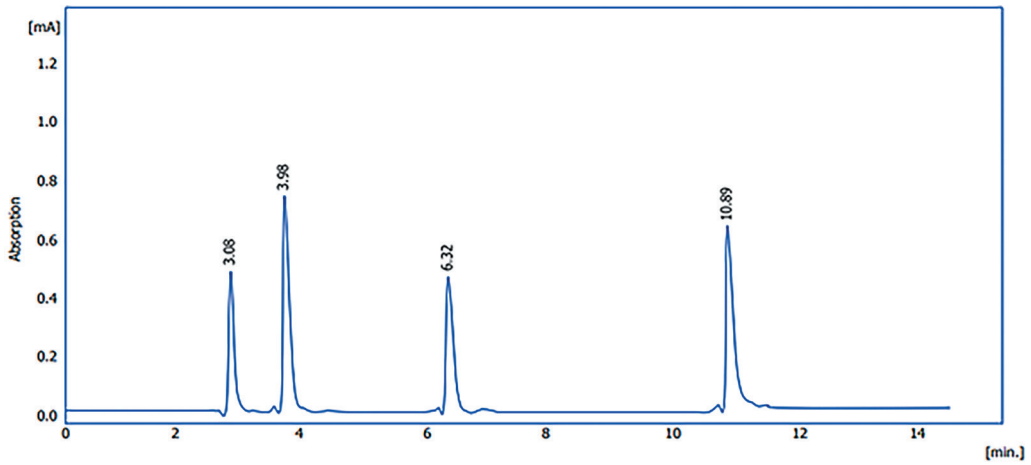


Figure 2. **Chromatogram of chemical compounds of green tea. Hydrobenzoic acid (3.08 min), kaempferol (3.98 min), syringic acid (5.02 min), quercetin (6.32 min), and catechin (10.85 min) under wavelength of 280 nm**

Рисунок 2. **Хроматограмма химических соединений зеленого чая. Гидроксибензойная кислота (3,08 мин), кемпферол (3,98 мин), сиреневая кислота (5,02 мин), кверцетин (6,32 мин) и катехин (10,85 мин) при длине волны 280 нм**

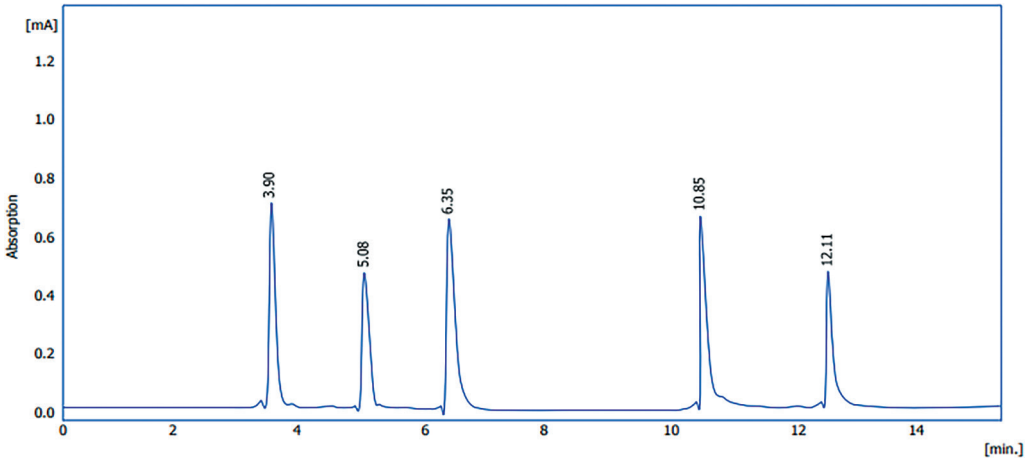


Figure 3. **Chromatogram of chemical compounds of moringa. Kaempferol (3.98 min), quercetin (6.32 min), catechin (10.85 min), and ellagic acid (12.36 min) under wavelenth of 280 nm**

Рисунок 3. Хроматограмма химических соединений моринги. Кемпферол (3,98 мин), кверцетин (6,32 мин), катехин (10,85 мин) и эллаговая кислота (12,36 мин) при длине волны 280 нм

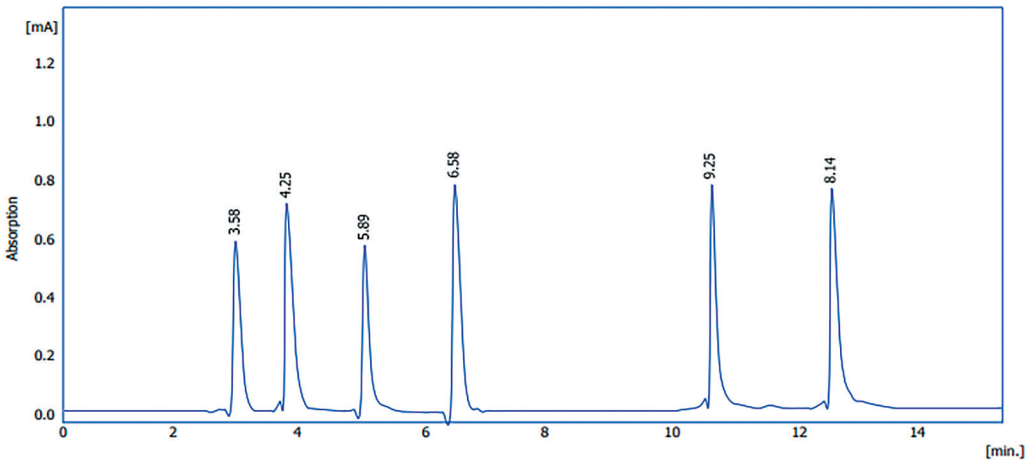


Figure 4. **Chromatogram of chemical compounds of green tea and moringa combination. Hydrobenzoic acid (3.08 min), kaempferol (3.98 min), syringic acid (5.02 min), quercetin (6.32 min), catechin (10.85 min), and ellagic acid (12.36 min) under wavelength of 280 nm**

Рисунок 4. **Хроматограмма химических соединений комбинации зеленого чая и моринги. Гидроксибензойная кислота (3,08 мин), кемпферол (3,98 мин), сиреневая кислота (5,02 мин), кверцетин (6,32 мин), катехин (10,85 мин) и эллаговая кислота (12,36 мин) при длине волны 280 nm**

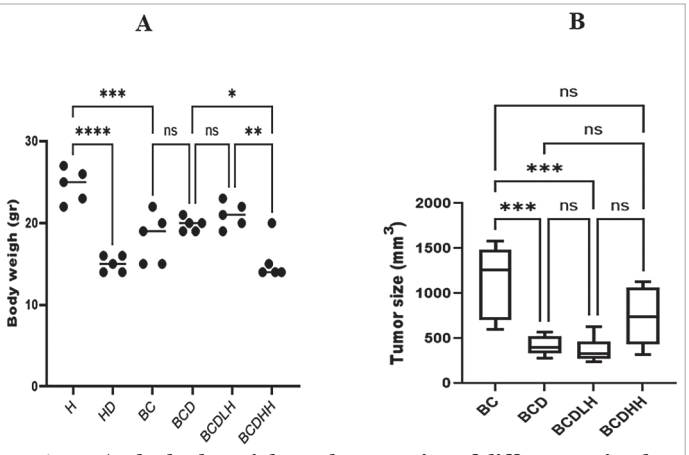


Figure 5. **The body weight and tumor size of different animal experimental groups. Results are means ± SD. *, ** and *** represent the significant difference between different experimental groups at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 5. **Масса тела и размер опухоли в различных группах экспериментальных животных. Результаты являются средними значениями ± стандартные отклонения. *, ** и *** представляют значимые различия между разными экспериментальными группами при $p < 0,05$, $p < 0,01$ и $p < 0,001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

The reduction in tumor size seen in mice given DXR is consistent with the shown anti-tumor efficacy of DXR. Conversely, the herbal extracts did not significantly impact the growth of tumors in mice treated with DXR. This implies that the extracts mitigate the toxic effects induced by DXR, without interfering with the therapeutic effects of doxorubicin. This result is crucial as it suggests that those herbal extracts can be utilized in combination with chemotherapy to reduce adverse effects without adversely affecting the anti-cancer properties of DXR.

3.4. Impact of herbal extracts with the nephroprotective activities on the oxidative stress index and antioxidant enzymes in mice given DXR

The TOS, TAC, and OSI values in the healthy mice (group H) were 1.139 ± 0.203, 2.69 ± 0.54, and 0.43± 0.08, respectively, according to our findings. There was no apparent change in the levels of TOS and OSI in cancerous mice (group BC) compared to healthy mice, while there was a drop in TAC. In comparison to untreated mice, healthy mice administered chemotherapy showed a substantial rise in TOS and OSI levels (6.47 ± 0.48 and 16.33 ± 6.93, respectively), and a significant drop in TAC levels (0.42 ± 0.08). When compared to cancer mice that were not treated with chemotherapy, mice with cancer plus chemotherapy (BCD) had significantly higher levels of TOS and OSI (TOS: 7.81 ± 0.51; OSI: 26.72 ± 7.248), as well as a significantly lower level of TAC (0.307 ± 0.08). Groups BCDHH and BCDLH showed a significant increase in TAC levels with p-values of less than 0.01, compared to group BCD, while mice with cancer under DXR with 1% herbal treatment (BCDLH) showed a significant decrease in TOS and OSI, respectively, with p-values of less than 0.01, 0.05 and 0.01 and 0.001 for BCDHH group. The ability of the herb mixture to reduce oxidative stress in the kidneys of cancer mice treated with DXR showed no significant difference between the 1% and 2% doses (Figures 6 (A, B, C)).

The effects of the herbal extracts on kidney oxidant/antioxidant indicators in breast cancer mice treated with DXR were studied. DXR was administered to the breast cancer mice. Figures 7 (A, B, C) displays the glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) levels, which are antioxidant enzymes. The levels of the CAT, GPX, and SOD enzymes in the healthy mice in group H were 68.93 ± 7.196, 64.14 ± 10.73, and 86.99 ± 10.26, respectively. The SOD activity of cancer mice (group BC) was shown to be significantly lower (56.69 ± 8.045, $p < 0.01$) in our investigation. Nevertheless, the development of cancer did not substantially impact the activities of CAT (60.09 ± 7.264) and GPX (59.12 ± 7.907) in comparison to healthy mice. The activities of CAT (25.11 ± 7.531, $p < 0.0001$), GPX (19.17 ± 2.366, $p < 0.0001$), and SOD (24.27 ± 6.042, $p < 0.0001$) were noticeably reduced in healthy mice (group HD) that were treated with DXR compared to animals that were not treated. DXR and 1% herb combination treatment (group BCDLH) of mice that had cancer did not seem to have any effect on GPX, SOD, and CAT functions. The administration of 2% herbal extracts (group HBDHH) caused a very significant increase in CAT ($p < 0.01$) and SOD ($p < 0.05$); however, there was no change in GPX when compared to the BCD group.

The oxidative stress produced in this study by DXR is evidenced by increased OSI and decreased activity of catalase, glutathione peroxidase and superoxide dismutase of kidney. The 2% herbal combinations significantly improved the activities of these enzymes. This implies that the extracts can improve the kidney's antioxidant capacity and protect it from damage due to DXR. The administration of the extract, particularly at 2%, lowered TOS and OSI levels while raising TAC. This can be regarded as evidence that the extracts can prevent oxidative stress by enhancing the kidneys' antioxidant capacity and, thus, decreasing the harm from ROS. The extracts possess several antioxidant compounds such as flavonoids (catechins and quercetin) and polyphenols (gallic, chlorogenic and caffeic acids) as was shown by the HPLC analysis. These substances are capable of directly scavenging ROS and have been found to have strong free radical scavenging ability. For instance, gallic acid and tannic acid can provide hydrogen atoms to the free radicals, thereby preventing the further chain reactions, which lead to the generation of free radicals. Moreover, chlorogenic and caffeic acids may raise the levels and activity of endogenous antioxidants, including catalase and superoxide dismutase. This improves the ability of breaking down hydrogen peroxide and superoxide anions

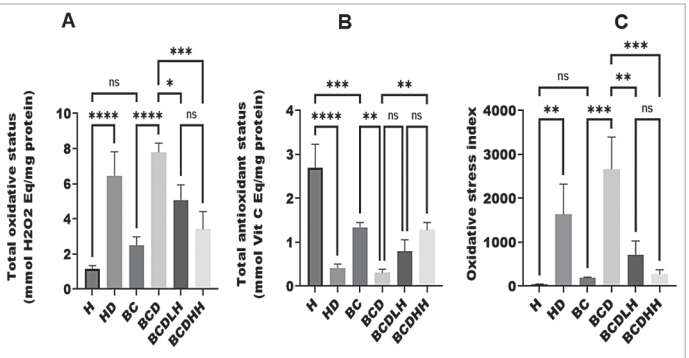


Figure 6. **Effect of the green tea and moringa mixture on renal levels of total oxidative status (TOS) (A), total antioxidant capacity (TAC) (B) and oxidative stress index (OSI) (C) in the kidney of the doxorubicin (DXR)-treated mice with cancer. Results are means ± SD. *, **, *** and **** represent the significant difference between different experimental groups at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 6. **Влияние смеси зеленого чая и моринги на ренальные уровни общего окислительного статуса (TOS) (A), общей антиоксидантной способности (TAC) (B) и индекса окислительного стресса (OSI) (C) в почках получавших доксорубицин (DXR) мышей с раком. Результаты являются средними значениями ± стандартные отклонения. *, **, *** и **** представляют значимые различия между разными экспериментальными группами при $p < 0{,}05$, $p < 0{,}01$, $p < 0{,}001$ и $p < 0{,}0001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

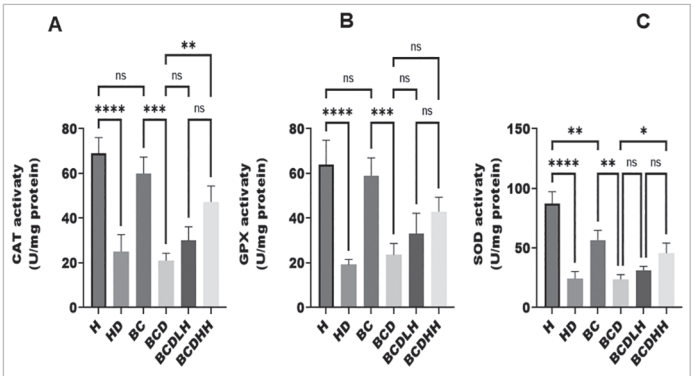


Figure 7. **Effect of the green tea and moringa mixture on the activities of renal catalase (A), GPX (B) and SOD (C) in the kidney of doxorubicin (DXR)-treated mice with cancer. Results are means ± SD. *, **, *** and **** represent the significant difference between different experimental groups at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 7. **Влияние смеси зеленого чая и моринги на активности ренальных каталазы (A), GPX (B) и SOD (C) в почках получавших доксорубицин (DXR) мышей с раком. Результаты являются средними значениями ± стандартные отклонения. *, **, *** и **** представляют значимые различия между разными экспериментальными группами при $p < 0{,}05$, $p < 0{,}01$, $p < 0{,}001$ и $p < 0{,}0001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

into less toxic products, thus decreasing oxidative stress [47,48,49,50]. Therefore, it can be deduced from the findings of this study that these bioactive compounds in combination with herbs aid in the restoration of the antioxidant defense system in the kidney through a dose-dependent manner to counter the effects of DOX-induced oxidative stress.

3.5. Impact of nephroprotective herbal extracts on the proinflammatory cytokine production in DXR- treated mice

Our findings showed that in healthy mice (group H), the levels of renal IL-1 and TNF-α were 30.93 ± 3.77 pg/mg proteins and 26.16 ± 8.42 pg/mg proteins, respectively. When compared to healthy mice, cancer animals (group BC) showed no significant changes in the levels of IL-1 and TNF-α, with levels of 51.13 ± 9.106 pg/mg proteins and 46.46 ± 10.42 pg/mg proteins, respectively. After healthy mice were given DXR (group HD), levels of IL1β and TNF-α were considerably higher than in the untreated animals (179.1 ± 33.02 pg/mg protein; $p < 0.0001$, 155.2 ± 29.45 pg/mg protein; $p < 0.0001$, respectively). Compared to the BC group, breast cancer mice in group BCD, who were administered DXR, displayed significantly higher levels of IL1β (209.2 ± 17.04 pg/mg protein; $p < 0.0001$) and TNF-α (168.4 ± 10.99 pg/mg protein; $p < 0.0001$). Our findings showed that compared to the BCD group, mice with cancer and DXR who also received the 1% herbal mixture (group BCDLH) showed a significant reduction in both IL1β (142.6 ± 17.31 pg/mg protein; $P < 0.01$) and TNF-α (116.9 ± 19.67 pg/mg protein; $P<0.05$) levels. Moreover, in comparison to the BCD group, mice given the 2% herbal extract (group BCDHH) demonstrated a significant decrease in IL1β (105.6 ± 13.31 pg/mg protein, $P < 0.001$) and TNF-α (80.83 ± 16.03 pg/mg protein, $P < 0.001$) levels, as shown in Figures 8 (A, B).

In our study, we were able to see that an increase in such factors as TNF-α and IL-1 in kidneys occurred in direct connection with the administration of DXR. All these inflammatory cytokines participate in the promotion of DXR-induced nephrotoxicity. The results predicate that these herbal extracts can be used as anti-inflammatory drugs as they effectively reduced the cytokines at 1% and 2% concentrations. Being an effective pro-inflammatory cytokine, IL-1β plays a role in inflammation and kidney damage. In response to this, signaling pathways are stimulated, leading to the synthesis of various inflammatory agents and enzymes, such as cyclooxygenase and nitric oxide synthase, which may further aggravate tissue damage [51,52]. Interleukin-1 (IL-1) induces apoptosis in kidney cells, raises production of pro-inflammatory cytokines and recruits inflammatory cells into renal tissue. This cytokine stimulates renal fibroblast cells to synthesize the extracellular matrix that enhances the scarring process and impairs renal function; it is also a factor in the progression of renal fibrosis [53].

One important cytokine linked to renal inflammation and damage is TNF-α. TNF-α binds to certain receptors in the kidney and other renal

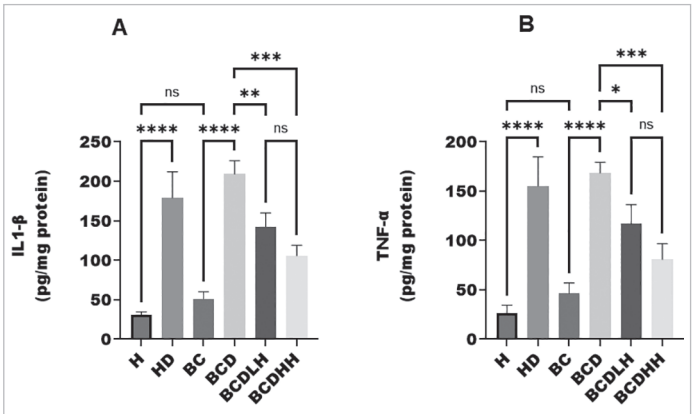


Figure 8. **Effect of the green tea and moringa mixture on renal levels of IL1β (A) and TNF-α (B) in the kidney of doxorubicin (DXR)-treated mice with cancer. Results are means ± SD. *, **, *** and **** represent the significant difference between different experimental groups at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 8. **Влияние смеси зеленого чая и моринги на ренальные уровни IL1β (A) и TNF-α (B) в почках получавших доксорубицин (DXR) мышей с раком. Результаты являются средними значениями ± стандартные отклонения. *, **, *** и **** представляют значимые различия между разными экспериментальными группами при $p < 0{,}05$, $p < 0{,}01$, $p < 0{,}001$ и $p < 0{,}0001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

cells, starting a cascade of internal reactions that might result in necrosis or apoptosis, two possible forms of cell death. TNF-α stimulates the production of ROS, leading to oxidative stress and cell damage. According to Kastl et al. [54], it also leads to fibrosis and cirrhosis by disrupting the amounts of cytokines that are normally anti-inflammatory and pro-inflammatory. Combining the moringa extract with green tea is thought to provide anti-inflammatory properties since both plants contain bioactive chemicals including antioxidants and flavonoids. Pro-inflammatory cytokine expression and activity have been shown to be inhibited by these substances [55]. Park et al. [56] and Wang et al. [57] stated that compounds found in green tea, such as quercetin and catechins, inhibit the pro-inflammatory gene activity (such as NF-kB) by obstructing crucial signaling pathways.

This is consistent with the hypothesis that green tea possesses anti-inflammatory properties. In addition, studies by Hamza [58] and Abd-Elnaby et al. [59] indicate that bioactive components of moringa, including moringin and other polyphenols, might reduce inflammation by preventing the generation of TNF-α and IL-1. These bioactive compounds do this by lowering the production of inflammatory cytokines and chemokines by preventing the activation of inflammatory cells such as macrophages. These findings highlight the preventative role of our herbal combination in minimizing kidney damage induced by DXR by lowering the production of inflammatory cytokines.

3.6. Impact of herbal extracts on the expression levels of BAX and BCL2 genes and of NLRP3 and NFKβ genes

Bax and Bcl2 gene expression levels in various experimental groups are shown in Figure 9 (A, B). While we found no statistically significant variations in BAX expression levels between healthy and malignant animals, we did find a statistically significant rise in BCL2 expression levels in the kidneys of both groups. In comparison to the untreated mice, there was a significant increase in Bax expression ($P<0.0001$ for the HD group; $P<0.001$ for the BCD group) and a significant decrease in BCL2 expression ($P<0.0001$ for the HD group; $P<0.01$ for BCD group) after the DXR administration to both healthy (group HD) and cancerous (group BCD) mice. A significant decrease in Bax expression ($P<0.01$) and a significant increase in Bcl2 gene expression ($P<0.05$) were seen in malignant mice treated with DXR and 1% herb extract (group BCDLH) compared to the BCD group, which did not receive herb therapy. The expression of the BAX ($p<0.01$) and BCL2 ($p<0.001$) genes in the kidney of the BCDHH group was found to be similar to that of the BCDLH group compared to the group that did not receive herb extract therapy.

Figure 10 (A, B) displays the renal expression of NLRP3 and NFKβ in different experimental groups. In the breast cancer groups of mice, there was a significant rise in NLRP3 ($P<0.05$), but in comparison with the healthy control group, there was no apparent effect on NFKβ expression. The levels of NLRP3 expression were not affected by the administration of DXR to either the healthy (group HD) or cancer (group BCD) mice, although they were shown to be higher in the BCD group ($P<0.001$) when compared to the untreated group. Obviously, in contrast to the control group, both the HD group and the BCD group exhibited elevated NFKβ gene levels ($P<0.01$ for the HD group and $P<0.0001$ for the BCD group). In comparison to the BCD group, malignant mice given 1% herb extract exhibited significantly reduced amounts of NLRP3 and NFKβ ($P<0.05$ and $P<0.0001$, respectively). Furthermore, group BCDHH, which received 2% herbal extract, showed a significant decrease in NLRP3 ($P<0.01$) and NFKβ ($P<0.0001$) in comparison to BCD. Regardless of the dose, there was no significant difference in the NLRP3 and NFKB expression levels between the BCDLH and BCDHH groups.

The activities of BAX and BCL2 were noticeably changed by DXR treatment. DXR increased apoptosis by lowering BCL2 levels and upregulating BAX, two genes that normally work together to prevent cell death. The expressions of the genes were altered by the herbal extracts; an upregulation of BCL2 and a downregulation of BAX were seen, indicating that the extracts help prevent cell death. Further, the extracts significantly reduced the expression of the inflammatory response-related genes that

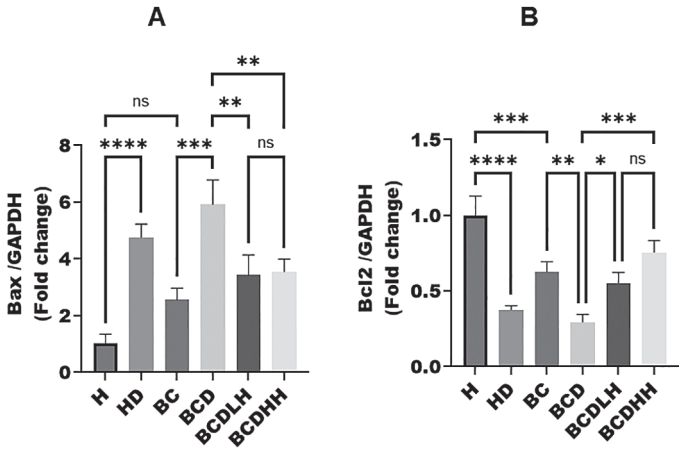


Figure 9. **Effect of the green tea and moringa mixture on expression levels of BAX (A) and BCL2 (B) genes in the kidney of doxorubicin (DXR)-treated mice with cancer. GAPDH was used as a housekeeping gene. Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method. Each reaction was run in triplicate. Results are means ± SD. *, **, *** and **** represent the significant difference between different experimental groups at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 9. **Влияние смеси зеленого чая и моринги на уровни экспрессии генов BAX (A) и BCL2 (B) в почках получавших доксорубицин (DXR) мышей с раком. GAPDH был использован в качестве гена домашнего хозяйства. Определение относительного количества было проведено в соответствии со сравнительным $2^{-\Delta\Delta Ct}$ методом. Каждая реакция была проведена в трехкратной повторности. Результаты являются средними значениями ± стандартные отклонения. *, **, *** и **** представляют значимые различия между разными экспериментальными группами при $p<0,05$, $p<0,01$, $p<0,001$ и $p<0,0001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

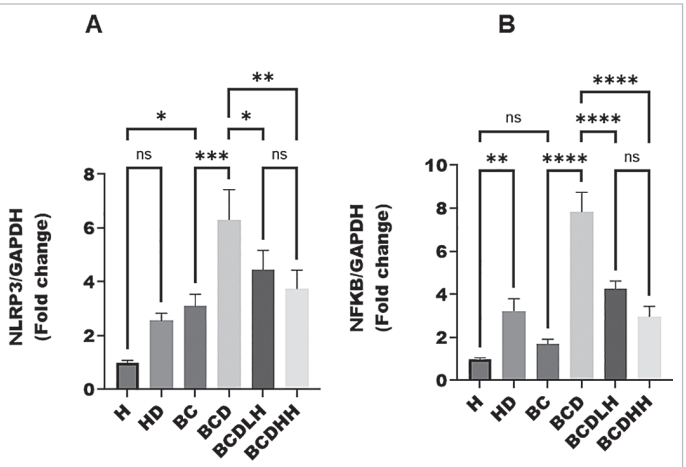


Figure 10. **Effect of the green tea and moringa mixture on expression levels of NLRP3 (A) and NFκβ (B) genes in the kidney of doxorubicin (DXR)-treated mice with cancer. GAPDH was used as a housekeeping gene. Relative quantification was performed according to the comparative $2^{-\Delta\Delta Ct}$ method. Each reaction was run in triplicate. Results are means ± SD. *, **, *** and **** represent the significant difference between different experimental groups at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively. H: healthy group, HD: healthy group treated with DXR, BC: mice with breast cancer, BCD: DXR-treated mice with cancer, BCDLH: cancer mice treated with DXR and 1% herbal extract, BCDHH: cancer mice treated with DXR and 2% herbal extract**

Рисунок 10. **Влияние смеси зеленого чая и моринги на уровни экспрессии генов NLRP3 (A) и NFκβ (B) в почках получавших доксорубицин (DXR) мышей с раком. GAPDH был использован в качестве гена домашнего хозяйства. Определение относительного количества было проведено в соответствии со сравнительным $2^{-\Delta\Delta Ct}$ методом. Каждая реакция была проведена в трехкратной повторности. Результаты являются средними значениями ± стандартные отклонения. *, **, *** и **** представляют значимые различия между разными экспериментальными группами при $p<0,05$, $p<0,01$, $p<0,001$ и $p<0,0001$, соответственно. H: здоровая группа, HD: здоровая группа, получавшая DXR, BC: мыши с раком молочной железы, BCD: мыши с раком молочной железы получавшие DXR, BCDLH: мыши с раком молочной железы получавшие DXR и 1% травяного экстракта, BCDHH: мыши с раком молочной железы получавшие DXR и 2% травяного экстракта**

promote inflammation NLRP3 and NFKB. This shows that the extracts' inflammatory and apoptotic control contributes to their nephroprotective effects. Research studies have clarified various types of useful compounds in green tea, including polyphenols, like epicatechin, kaempferol, epicatechin gallate (ECG), and epicatechin (EC).

Research indicates that epigallocatechin gallate (EGCG) and kaempferol may prevent NF-κB from activating and the NLRP3 inflammasome from developing. Consequently, they may decrease the production of pro-inflammatory cytokines and relieve inflammation [59,60]. Furthermore, EGCG has the ability to control the activity of BAX and BCL2, resulting in improved cell survival and decreased apoptosis [61,62]. In addition, moringa contains a substantial quantity of bioactive compounds such as flavonoids, phenolic acids, and glucosinolates. Abdel Fattah et al. [64] reported that these substances have potent antioxidant and anti-inflammatory properties. Quercetin, a flavonoid abundant in moringa, has shown the ability to inhibit the activation of pro-inflammatory genes by decreasing the NF-κB signalling pathway. Moreover, research has shown that quercetin may regulate the mechanism of cellular apoptosis by modulating the expression of BAX and BCL2, hence preventing cell death [65].

3.7. Histopathological findings

The histopathological changes in the renal cortex of mice receiving different treatments were examined in this study (Figure 11 (A, B)). Morphologically, the kidneys of group A, the healthy control mice, had intact structures of the distal convoluted tubules, proximal convoluted tubules, and glomeruli. Group B with breast cancer mice did not differ significantly from the healthy control group, which indicates that the breast cancer condition did not affect kidney histology at this stage. On the other hand, group C, which was the healthy mice treated with doxorubicin, had renal damage evidence. This was evidenced by mild cell swelling, glomerular atrophy and degeneration, and extensive necrosis, suggesting doxorubicin nephrotoxicity. The same observation was made in group D consisted of mice treated with doxorubicin and having breast cancer, which indicates that the presence of breast cancer did not decrease the nephrotoxicity of doxorubicin (Figure 11 (C, D)). The treatment groups E and F (Figure 11 (E, F)),

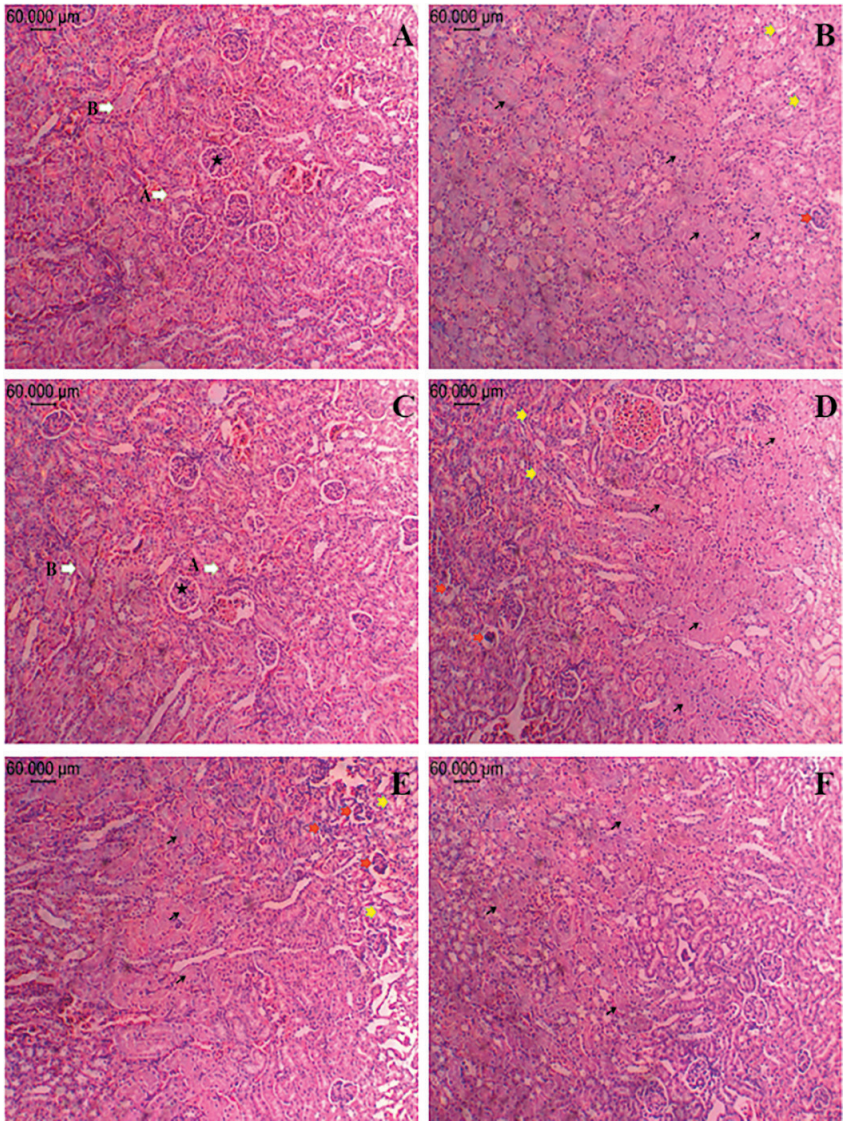


Figure 11. **Histopathological examination of mouse kidney (H&E staining, magnification 10X) in different experimental groups. Control group (H) and BC group (A, B): kidney cells show normal structure of distal convoluted (arrow A), the proximal convoluted tubule (arrow B), and the glomerulus (star sign) in the control group. HD (C) and BCD (D) groups: mild cell swelling (yellow arrows), glomerular atrophy and degeneration (red arrow), and extensive necrosis (black arrow). BCDLH group (E): mild cell swelling (yellow arrows), glomerular atrophy and degeneration (red arrow), and extensive necrosis (black arrow). BCDHH group (F): shows necrosis (black arrow). Control group (H); tumor group (BC); control + DXR group (HD); tumor + DXR group (BCD); tumor + DXR+ low dose of herb mixture (BCDLH); tumor + DXR + high dose of herb mixture (BCDHH)**

Рисунок 11. **Гистопатологичесое исследование почек мышей (окраска гематоксилином и эозином, увеличение 10X) в различных экспериментальных группах. Контрольная группа (H) и группа BC (A, B): клетки почки показали нормальную структуру дистального извитого (стрелка A), проксимального извитого канальца (стрелка B), и клубочка (звездочка) в контрольной группе. Группы HD (C) и BCD (D): умеренное набухание клеток (желтые стрелки), атрофия и дегенерация клубочков (красная стрелка) и обширный некроз (черная стрелка). Группа BCDLH (E): умеренное набухание клеток (желтые стрелки), атрофия и дегенерация клубочков (красная стрелка) и обширный некроз (черная стрелка). Группа BCDHH (F): показывает некроз (черная стрелка). Контрольная группа (H); группа с опухолью (BC); группа контроль + DXR (HD); группа с опухолью + DXR (BCD); группа с опухолью + DXR + низкая доза травяной смеси (BCDLH); группа с опухолью + DXR + высокая доза травяной смеси (BCDHH)**

administered doxorubicin in combination with the herb extract at concentrations of 1% and 2%, respectively, showed some protection against doxorubicin-induced renal damage. Group E had mild cell swelling, glomerular atrophy, and necrosis, suggesting some protection. Group F showed only necrosis, which may indicate that the 2% concentration of the herbs extract was more effective in the prevention of renal damage than the 1% concentration. Based on the findings of this study, it can be concluded that while doxorubicin results in severe renal toxicity, the addition of the herb extract may help in minimizing the degree of nephrotoxicity.

4. Conclusion

In conclusion, the green tea and moringa combination extracts show a significant protective effect against doxorubicin-induced renal toxicity due to their antioxidant, anti-inflammatory, and anti-apoptotic properties through the coordinated suppression of multiple molecular pathways involved in the nephrotoxicity. The combination of green tea and moringa can therefore be recommended as an adjuvant for the management of hepatotoxicity resulting from chemotherapy drugs.

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