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Yusuf Ensari Toraman

Manisa Celal Bayar University

Semra Tutçu Şahin

`semra.tutcu@cbu.edu.tr`

Manisa Celal Bayar University

Erdoğan Kocamaz

Manisa Celal Bayar University

Muhammet Burak Batır

Manisa Celal Bayar University

Mehmet İbrahim Tuğlu

Manisa Celal Bayar University

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Yusuf Ensari Toraman¹, Semra Tutçu Şahin^{1*}, Erdoğan Kocamaz², Muhammet Burak Batır³, Mehmet İbrahim Tuğlu²

¹ Department of General Surgery, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Türkiye

² Department of Histology and Embryology, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Türkiye

³ Department of Medical Biology, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Türkiye

* **Corresponding author:** Semra Tutçu Şahin, MD, Department of General Surgery, Faculty of Medicine, Manisa Celal Bayar University, Manisa, Türkiye. E-mail: semra.tutcu@cbu.edu.tr

ORCID: Y.E. Toraman 0009-0009-9244-2829; S. Tutçu Şahin 0000-0003-2141-1063; E. Kocamaz 0000-0002-4787-8561; M.B. Batır 0000-0002-8722-5055; M.İ. Tuğlu 0000-0002-0569-8415

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive malignancy that lacks targeted therapies and relies on cytotoxic chemotherapy. Doxorubicin (DX) is a cornerstone anthracycline for TNBC, but its clinical use is limited by dose-dependent toxicity. Boric acid (BA), a boron derivative abundant in mammalian tissues, possesses antioxidant, anti-inflammatory and anticancer properties. This study investigated whether BA potentiates the antitumoral and anti-inflammatory effects of DX in a 4T1 TNBC model. In vitro proliferation and scratch-migration assays were performed on 4T1 cells across six groups comprising BA (6 and 60 μ M), DX (1.2 μ M) and their combinations. For the in vivo phase, 40 female BALB/c mice bearing orthotopic 4T1 tumours were assigned to Tumour Control (TC), Doxorubicin (DX, 5 mg/kg/week i.p.), Boric Acid (BA, 2 mg/mL oral) and Combination (DB) groups. After 30 days, primary tumour, lung, liver and kidney tissues were examined histopathologically and immunohistochemically (Ki-67, eNOS, caspase-3, VEGF, TNF- α ; H-score). DX inhibited proliferation dose-dependently ($IC_{50} = 12.3 \pm 2.8$ nM), and BA + DX markedly enhanced migration inhibition. In vivo, the DB group showed the lowest tumour volume ($p < 0.0001$ versus TC) and better weight stabilisation. Ki-67, VEGF and NOS H-scores were most strongly suppressed and caspase-3 activity was highest in the DB group across all tissues ($p < 0.0001$), whereas TNF- α expression was systematically lowest and organ architecture best preserved. Boric acid synergistically enhanced the antitumoral and anti-inflammatory effects of doxorubicin and may attenuate systemic organ toxicity, supporting its potential as an adjuvant agent in TNBC chemotherapy.

Keywords Boric acid · Doxorubicin · Triple-negative breast cancer · 4T1 mouse model · Immunohistochemistry · Trace elements

Abbreviations

TNBC Triple-negative breast cancer; **DX** Doxorubicin; **BA** Boric acid; **DB** Doxorubicin + boric acid combination; **TC** Tumour control; **IHC** Immunohistochemistry; **eNOS** Endothelial nitric oxide synthase; **VEGF** Vascular endothelial growth factor; **TNF- α** Tumour necrosis factor-alpha; **ROS** Reactive oxygen species; **H&E** Haematoxylin–eosin; **ARRIVE** Animal Research: Reporting of In Vivo Experiments

Introduction

Breast cancer is the most frequently diagnosed malignancy among women worldwide, with approximately 2.26 million new cases reported in 2020 [1]. Triple-negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression, represents 15–20 % of all breast cancers and is characterised by a more aggressive clinical course, larger tumour size, higher lymph-node positivity and poorer survival rates [2, 3]. Chemotherapy remains the primary treatment modality in this subtype [4].

Doxorubicin (DX), an anthracycline antibiotic isolated from *Streptomyces peucetius*, is one of the cornerstone chemotherapeutic agents in TNBC management [5]. Despite its efficacy, its clinical use is constrained by a lack of tumour-cell specificity and dose-dependent toxicities including cardiotoxicity and oxidative stress [6]. DX exerts its antitumour effects primarily through topoisomerase-II inhibition, DNA intercalation and free-radical generation [5, 7].

Boric acid (BA), a boron derivative abundantly present in mammalian tissues, has gained increasing attention for its antioxidant, anti-inflammatory and anticancer properties [8]. BA modulates NF- κ B and PI3K/AKT signalling pathways, thereby suppressing the release of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and neutralising reactive oxygen species (ROS) while upregulating endogenous antioxidant enzyme activities [9, 10]. Previous studies have demonstrated that BA inhibits cancer-cell proliferation, induces apoptosis and suppresses cell migration [11–13].

The 4T1 cell line is the gold-standard in vivo TNBC model because, when injected orthotopically into BALB/c mice, it recapitulates human stage-IV breast cancer and spontaneously metastasises to the lung, liver and bone [14, 15]. A comprehensive review of the literature revealed no study examining the combined effect of BA and DX on TNBC using multi-organ immunohistochemical markers. Therefore, the present study aimed to evaluate the effects of BA combined with DX on primary tumour growth, lung, liver and kidney metastasis, and key markers of proliferation (Ki-67), endothelial nitric oxide synthase activity and vascular regulation (eNOS), apoptosis (caspase-3), angiogenesis (VEGF) and inflammation (TNF- α) in the 4T1 TNBC model.

Materials and Methods

Ethics Statement

This study was approved by the Manisa Celal Bayar University Faculty of Medicine Local Animal Experiments Ethics Committee (Approval No. E-77637435-050.04-752887). All

procedures were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Cell Line and Culture Conditions

4T1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM/F-12 (Thermo Fisher Scientific) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin at 37 °C in a 5 % CO₂ atmosphere. Subculturing was performed at 80–90 % confluency using 0.25 % trypsin–EDTA.

In Vitro Proliferation and Migration Assays

Proliferation analysis was performed in 48-well plates with six treatment groups (n = 3 per group): control (blank), BA 6 µM, BA 60 µM, DX 1.2 µM, BA 6 µM + DX 1.2 µM, and BA 60 µM + DX 1.2 µM. Cell proliferation was assessed 72 h after treatment (n = 8 per group; experiment repeated four times). The IC₅₀ value of DX was calculated from logarithmic concentration–response curves as $pD_2 = -\log(EC_{50})$. Migration analysis was performed using the scratch assay: confluent monolayers were pretreated with mitomycin-C (10 µg/mL, 2 h) to inhibit proliferation and isolate migratory capacity, scratched with a pipette tip, and wound closure was imaged at 24 h.

Experimental Animals and Grouping

Forty 8–10-week-old female BALB/c mice weighing 24–25 g were obtained from the Manisa Celal Bayar University DEHAM (Laboratory Animal Research and Application Centre) and housed under controlled conditions (22 ± 2 °C, 50–60 % humidity, 12 h/12 h light–dark cycle) with ad libitum access to food and water. All animal procedures were approved by the Manisa Celal Bayar University Faculty of Medicine Local Animal Experiments Ethics Committee (Approval No. E-77637435-050.04-752887) and reported in accordance with the ARRIVE guidelines. The study was approved by the ethics committee/IRB (MCBU/HADYEK). An orthotopic tumour model was established by injecting 2×10^5 4T1 cells in 50 µL PBS into the right inguinal fourth mammary fat pad under ketamine/xylazine anaesthesia (30/10 mg/kg, i.m.). Animals were allocated into four equal groups: Group 1 — Tumour Control (TC, n = 10): PBS injection, no treatment; Group 2 — Doxorubicin (DX, n = 10): 5 mg/kg DX intraperitoneally, once weekly (days 3, 10, 17 and 24 post-implantation; cumulative dose 20 mg/kg) [16]; Group 3 — Boric Acid (BA, n = 10): 2 mg/mL BA orally via drinking water; Group 4 — Combination (DB, n = 10): DX + BA at the same doses. Tumour dimensions were measured twice weekly with digital callipers; tumour volume was calculated as $\text{volume} = 0.5 \times \text{length} \times \text{width}^2$. After 30 days, all animals were euthanised with high-dose ketamine/xylazine (150/20 mg/kg).

Tissue Processing and Histopathology

Primary tumour, lung, liver and kidney specimens were fixed in 10 % neutral buffered formalin for 48 h, processed through graded alcohol and xylene series, and embedded in paraffin. Five-micrometre sections were stained with haematoxylin and eosin (H&E). Histopathological alterations were scored on a 0–4 scale by a blinded investigator.

Immunohistochemical Analysis

Five-micrometre paraffin sections were deparaffinised and subjected to antigen retrieval in citrate buffer. Endogenous peroxidase activity was blocked with 3 % H₂O₂, and non-specific binding was blocked with 2 % bovine serum albumin. Sections were incubated overnight at 4 °C with primary antibodies against Ki-67 (proliferation), eNOS (endothelial nitric oxide synthase; vascular tone and angiogenesis regulation), active caspase-3 (apoptosis), VEGF (angiogenesis) and TNF- α (inflammation). After incubation with an HRP-conjugated secondary antibody (30 min) and DAB-substrate application, counterstaining was performed with haematoxylin. Immunoreactivity was quantified by H-score: $H\text{-score} = \sum P_i(I + 1)$, where I is staining intensity (0 = none, 1 = mild, 2 = moderate, 3 = intense) and P_i is the percentage of cells at each intensity [17, 18].

Statistical Analysis

Data are expressed as mean $\pm$ SD. Differences between groups were assessed by one-way ANOVA followed by Tukey's multiple-comparison test; $p < 0.05$ was considered statistically significant. G-power analysis indicated that 10 animals per group provided 80 % power at a 95 % confidence level. Analyses were performed using GraphPad Prism 8.1 (GraphPad Software, San Diego, CA, USA) [19, 20]. Generative AI (Claude, Anthropic) was used for language and formatting assistance during manuscript preparation; all content was critically reviewed, edited and approved by the authors.

Results

In Vitro Proliferation and Migration

DX exhibited dose-dependent proliferation inhibition in 4T1 cells at all tested concentrations (1 nM–10 μ M) 72 h post-treatment; proliferation percentages in all DX groups were significantly lower than the tumour-control (TC) group (* $p < 0.05$). The IC₅₀ of DX for 4T1 cells was determined as 12.3 ± 2.8 nM ($pD_2 = 7.91 \pm 0.19$). The scratch assay demonstrated that both BA and DX individually inhibited cell migration, and their combination (particularly 60 μ M BA + 1.2 μ M DX) markedly enhanced this inhibitory effect (Fig. 1).

Macroscopic Tumour Evaluation

Tumour development was confirmed in all groups following 4T1 cell injection. In the TC group, tumours exhibited large, irregular masses with necrotic foci. In the DX group, tumour size was partially reduced with persistent necrosis and haemorrhage. The BA group showed a morphologically more compact tumour mass. In the DB group, tumour volume was the lowest among all groups and the tumour tissue had acquired a fibrotic character (Fig. 2).

Body Weight and Tumour Volume

Tukey's multiple-comparison test revealed no significant difference in body weight between TC and DX ($p = 0.0916$) or TC and BA ($p = 0.2685$). A highly significant difference was observed between TC and DB ($p < 0.0001$) and between BA and DB ($p = 0.0050$). Regarding tumour volume, the highest value was recorded in the TC group ($\sim 1.10 \text{ cm}^3$) and the lowest in the DB group ($\sim 0.75 \text{ cm}^3$). TC vs. DX ($p = 0.0001$), TC vs. DB ($p < 0.0001$) and BA vs. DB ($p < 0.0001$) comparisons were all statistically significant (Fig. 3).

Primary Tumour Histopathology and Immunohistochemistry

H&E staining showed widespread pleomorphic nuclear structures and irregular cell arrangement in the TC and BA groups, whereas pleomorphic changes were markedly reduced in the DB group (TC vs. DB: $p < 0.0001$; Fig. 4). Ki-67 H-score was lowest in the DB group (TC vs. DB: $p < 0.0001$; Fig. 5). NOS H-score was highest in TC (~ 175) and lowest in DB (~ 110) ($p < 0.0001$; Fig. 6). Caspase-3 activity was highest in the DB group ($p < 0.0001$; Fig. 7). VEGF expression followed the order TC > BA > DX > DB ($p < 0.0001$; Fig. 8). TNF- α positivity was systematically lowest in the DB group ($p < 0.0001$; Fig. 9).

Lung Tissue Findings

H&E evaluation demonstrated subpleural, peribronchial and diffuse tumour-cell infiltration with necrotic foci in the TC and BA groups, while the DB group exhibited preserved alveolar architecture with reduced infiltration (Fig. 10). Ki-67, NOS, VEGF and TNF- α H-scores in the DB group were systematically the lowest across all comparisons ($p < 0.0001$), and caspase-3 activity was consistent with primary tumour findings (Figs. 11–15).

Liver Tissue Findings

The DB group exhibited the most organised liver-tissue architecture, with normal hepatocyte cord arrangement, open sinusoids and minimal infiltration (Fig. 16). All five immunohistochemical markers (Ki-67, NOS, caspase-3, VEGF, TNF- α) in the DB group were at the most stabilised and protective levels among all groups ($p < 0.0001$; Figs. 17–21).

Kidney Tissue Findings

The DB group demonstrated the most regular renal-tissue architecture; glomeruli preserved normal morphology, tubular epithelial cells maintained regular arrangement, and no significant inflammation or necrotic areas were observed (Fig. 22). All immunohistochemical marker H-scores were lowest in the DB group ($p < 0.0001$; Figs. 23–27).

Discussion

In the present study, we demonstrated that the combination of BA and DX most effectively suppressed primary tumour volume, metastatic markers across multiple organs and systemic inflammation, while maximising apoptotic activity in the 4T1 TNBC model. To our knowledge, this is the first study to comprehensively evaluate the combined effects of BA and DX on TNBC using multi-organ immunohistochemical profiling, thereby addressing several hallmarks of cancer simultaneously [21].

In vitro, DX showed dose-dependent proliferation inhibition in 4T1 cells ($IC_{50} = 12.3 \pm 2.8$ nM; $pD_2 = 7.91 \pm 0.19$), consistent with its established topoisomerase-II inhibition and DNA intercalation mechanisms [5, 7]. The scratch-assay results indicated that BA and DX individually inhibited cell migration, and their combination produced a markedly enhanced anti-migratory effect. These in vitro findings align with the in vivo results, supporting the anti-migratory synergy of the combination treatment.

The 4T1 model was chosen because orthotopic injection into BALB/c mice results in spontaneous lung, liver and bone metastasis within two weeks of inoculation, closely mimicking human metastatic breast cancer [14, 22, 23]. In the in vivo phase, the DB group exhibited significantly reduced tumour volume versus TC ($p < 0.0001$). Body-weight loss observed in DX-treated groups may be attributable to direct toxic effects on the intestinal mucosa [24, 25]; the partial stabilisation of weight loss in the DB group is consistent with the antioxidant protective role of BA [9].

Apoptosis is a critical mechanism in cancer therapy [26]. Caspase-3, the key effector caspase in both intrinsic and extrinsic pathways [27], reached its highest H-score in the DB group, demonstrating that the combination strongly induced apoptosis. This finding is in line with previous reports showing that BA upregulates Bax and downregulates Bcl-2 to promote apoptosis [28, 29]. The absence of a marked change in Bcl-2 in our study suggests that the extrinsic pathway and/or other Bcl-2 family members (Bak, Bcl-xL) may also contribute to caspase-3 activation.

VEGF is the primary mediator of angiogenesis, and high tumour VEGF expression is associated with poor prognosis in breast cancer [30, 31]. The reduction in eNOS H-scores observed in the DB group warrants careful interpretation. eNOS is a constitutive enzyme primarily expressed in vascular endothelial cells and is responsible for physiological nitric-oxide production governing vascular tone and endothelial integrity, rather than being a direct marker of oxidative stress per se. The observed decrease in eNOS expression in the DB group is most likely reflective of reduced tumoral neovascularisation, consistent with the parallel reduction in VEGF, rather than a direct antioxidant effect. Future studies should employ iNOS (inducible nitric oxide synthase), 4-hydroxynonenal (4-HNE) or malondialdehyde (MDA) as direct markers of oxidative stress and lipid peroxidation to more accurately characterise the antioxidant effects of the combination [32]. Consistent with an in vitro study reporting that BA suppresses MMP-9 and VEGF expression [33], VEGF H-scores were most significantly reduced in the DB group across all tissues ($p < 0.0001$). This may be mediated through BA's antioxidant capacity reducing ROS-driven VEGF induction [34, 35]. The anti-angiogenic superiority of the combination over monotherapies further supports the clinical rationale for adjuvant BA use.

TNF- α activates NF- κ B signalling to promote tumour progression and metastatic spread [36, 37]. The systematic reduction of TNF- α positivity in the DB group across lung, liver and kidney is consistent with BA's established NF- κ B inhibitory mechanism [9]. The corresponding improvements in organ histopathological scores reinforce the organ-protective potential of the combination. Importantly, doxorubicin itself can paradoxically activate NF- κ B through intracellular ROS accumulation, triggering a pro-survival response that upregulates TNF- α secretion and contributes to chemoresistance. The synergistic benefit of BA in the combination may therefore stem, at least in part, from its capacity to interrupt this vicious cycle: by scavenging ROS and suppressing NF- κ B activation, BA may prevent the compensatory pro-survival signalling that doxorubicin monotherapy inadvertently induces, thereby enhancing net apoptotic output. This cross-talk mechanism warrants direct investigation in future studies through NF- κ B nuclear-translocation assays and phospho-I κ B α measurements.

The present study has several limitations. First, cardiac tissue was not included in histopathological and immunohistochemical analyses. As doxorubicin-induced cardiotoxicity represents the most clinically relevant dose-limiting toxicity of anthracyclines—mediated through NADPH-oxidase-driven ROS generation in cardiomyocytes—future studies should incorporate cardiac-tissue assessment (Masson's trichrome staining for fibrosis, caspase-3 for

cardiomyocyte apoptosis and serum troponin levels) to substantiate the organ-protective hypothesis. Second, metastatic protein levels including MMP-2, MMP-9 and CD31 could not be measured in lung tissue due to budget constraints; extrinsic apoptosis-pathway markers (caspase-8, FADD) and Bcl-2 family proteins were not evaluated. Third, H-score quantification was performed by manual microscopic grading; digital image-analysis software (e.g. QuPath or ImageJ/Fiji) should be employed in future studies to eliminate observer bias and improve inter-rater reproducibility. Fourth, a healthy non-tumour sham-control group was not included, precluding the establishment of baseline physiological levels for immunohistochemical markers. Future studies incorporating these parameters alongside molecular-pathway analyses will be essential to fully elucidate the mechanistic basis of the observed synergy.

Conclusions

Boric acid combined with doxorubicin showed superior antitumoral and anti-inflammatory activity compared with either agent alone in the 4T1 TNBC model, while demonstrating potential to attenuate systemic organ toxicity. These preclinical findings provide a rationale for further investigation of boric acid as an adjuvant to standard chemotherapy in TNBC, and warrant mechanistic and clinical validation.

Statements and Declarations

Funding This study received no external funding.

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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Ethics Approval All procedures involving animals were performed in accordance with the ARRIVE guidelines and were approved by the Manisa Celal Bayar University Faculty of Medicine Local Animal Experiments Ethics Committee (Approval No. E-77637435-050.04-752887).

Consent to Participate Not applicable (this study did not involve human participants).

Consent to Publish Not applicable.

Data Availability The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Use of AI Tools Generative AI (Claude, Anthropic) was used for language and formatting assistance during manuscript preparation, as documented in the Materials and Methods; all content was reviewed and approved by the authors.

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Figures

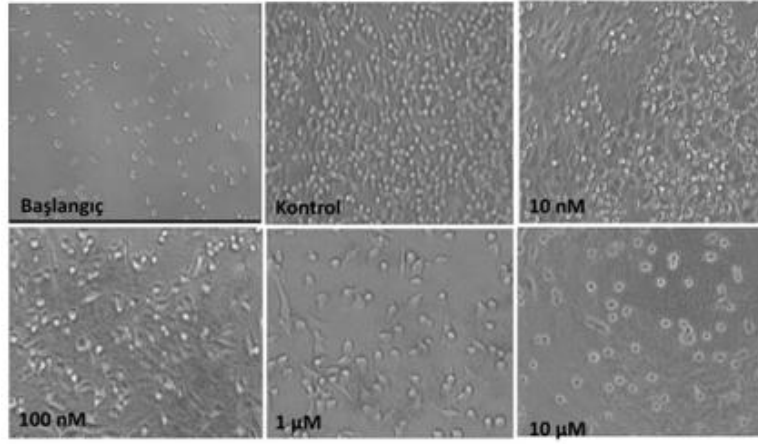
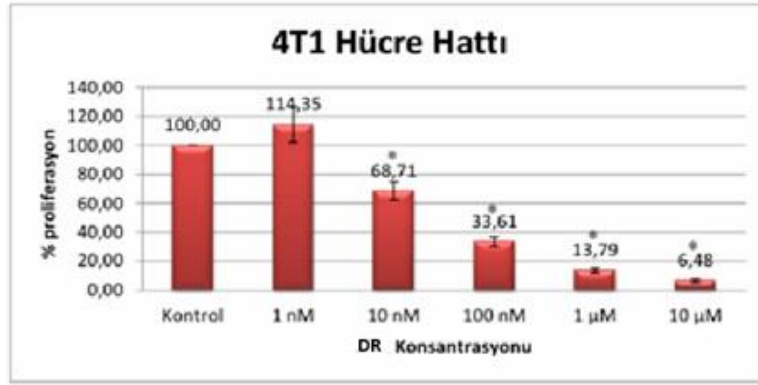
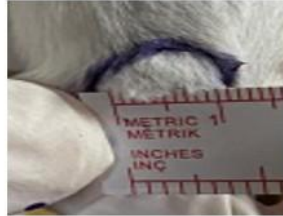
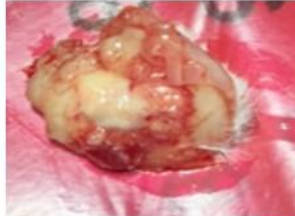


Fig. 1 Effect of doxorubicin on proliferation of 4T1 cells. Culture images and proliferation analysis of 4T1 cells treated with different doses of doxorubicin (DX). All DX groups showed significantly lower proliferation than the tumour-control group. IC_{50} of DX = 12.3 ± 2.8 nM ($pD_2 = 7.91 \pm 0.19$). Values $\pm$ SEM; n = 8; experiment repeated four times. * $p < 0.05$. TC tumour control, DX doxorubicin, BA boric acid, DB doxorubicin + boric acid combination

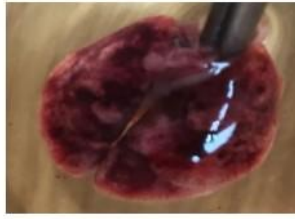
TK



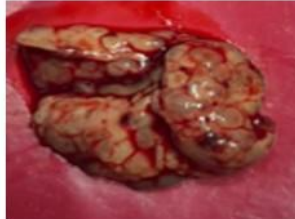
DX



BU



DB



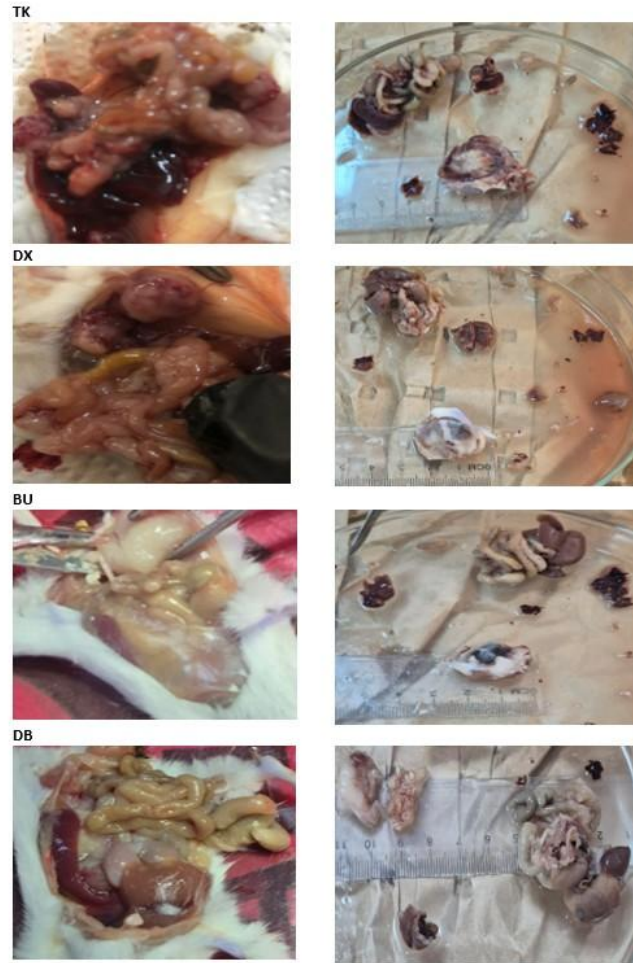


Fig. 2 Macroscopic tumour appearance in the 4T1 breast-tumour model. In situ (left) and excised (right) macroscopic images. The DB group showed smaller, more fibrotic tumour masses than other groups. Scale bar 1 cm

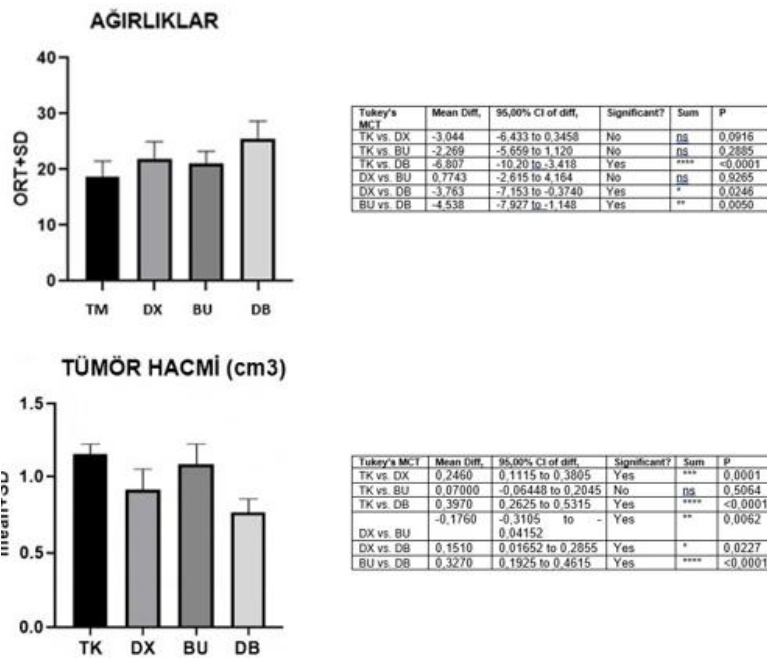


Fig. 3 Body weight and tumour volumes in the 4T1 breast-tumour model. a Mean body weights (mean $\pm$ SD, g). b Mean tumour volumes (mean $\pm$ SD, cm³). Tukey's multiple-comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant

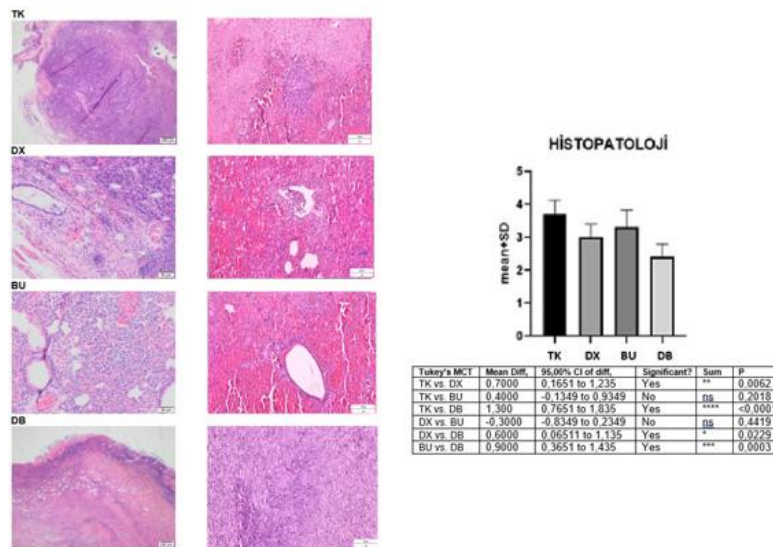
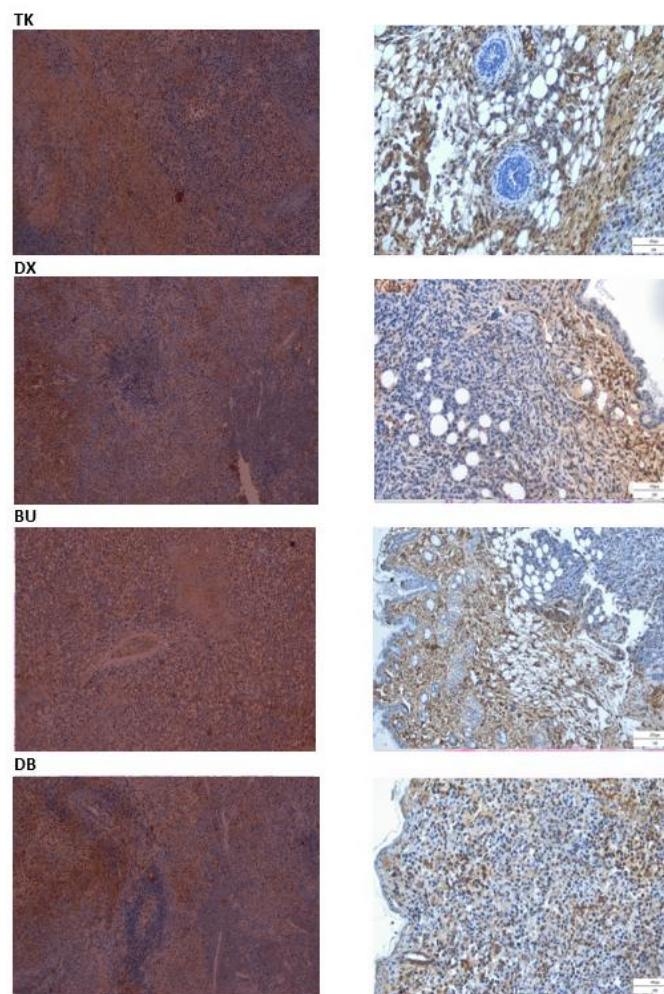


Fig. 4 Histopathological appearance of primary tumour tissue. Low- (left) and high-magnification (right) H&E-stained sections and histopathological scores (mean $\pm$ SD). Scale bar 50 μ m; $\times 20$



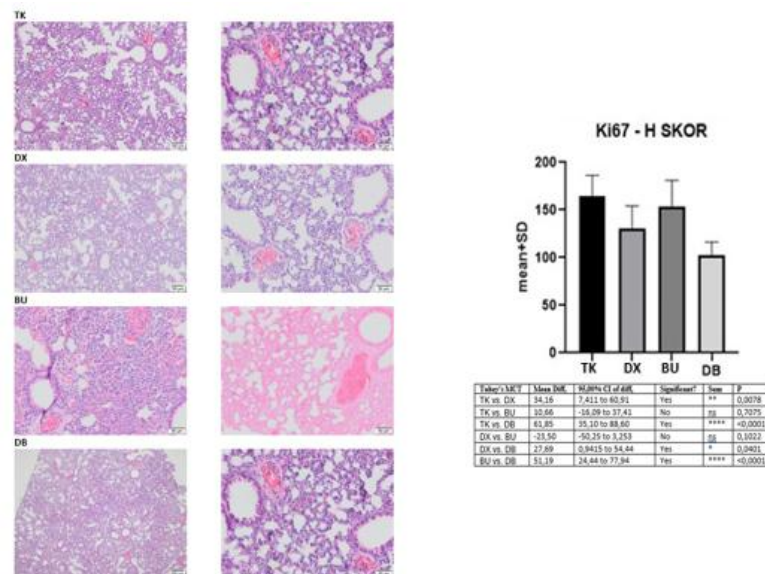


Fig. 5 Ki-67 immunohistochemical staining of primary tumour tissue with H-score analysis. Nuclear brown DAB positivity indicates proliferative activity. Scale bar 100 μ m; $\times 20$

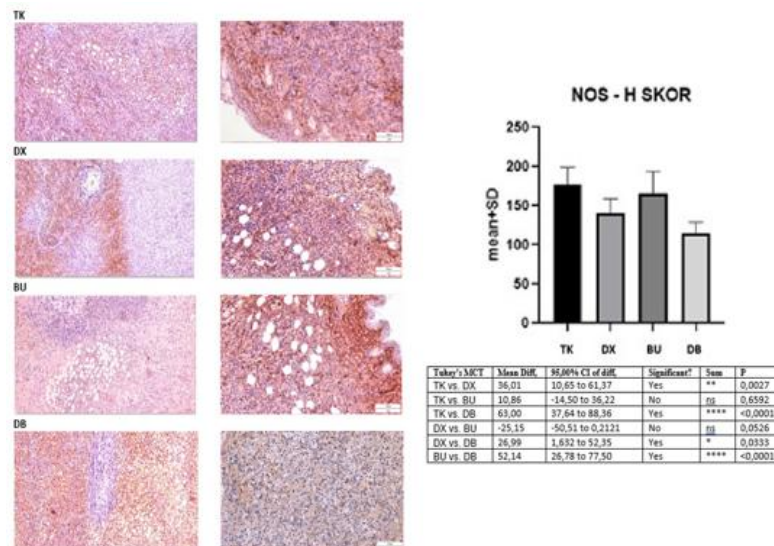
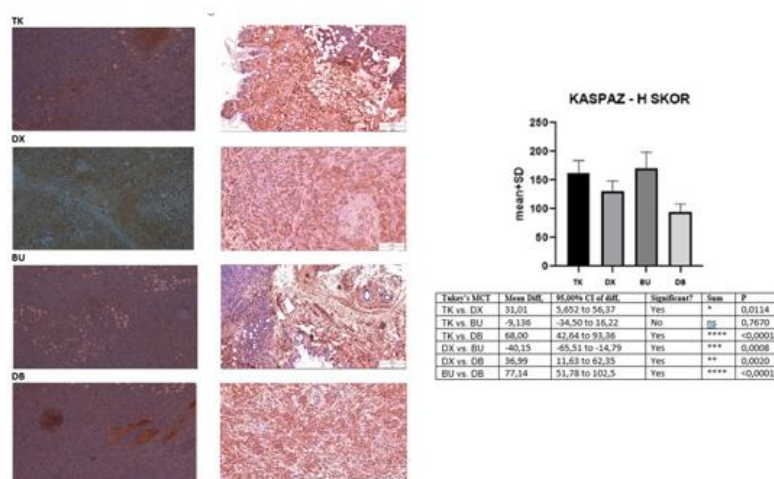
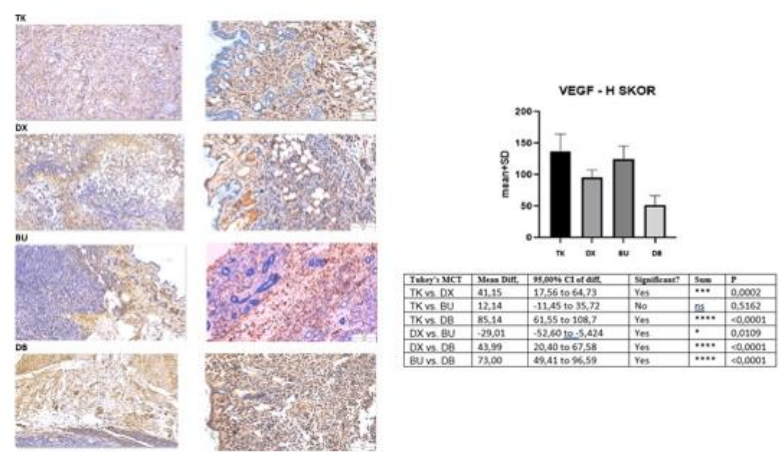


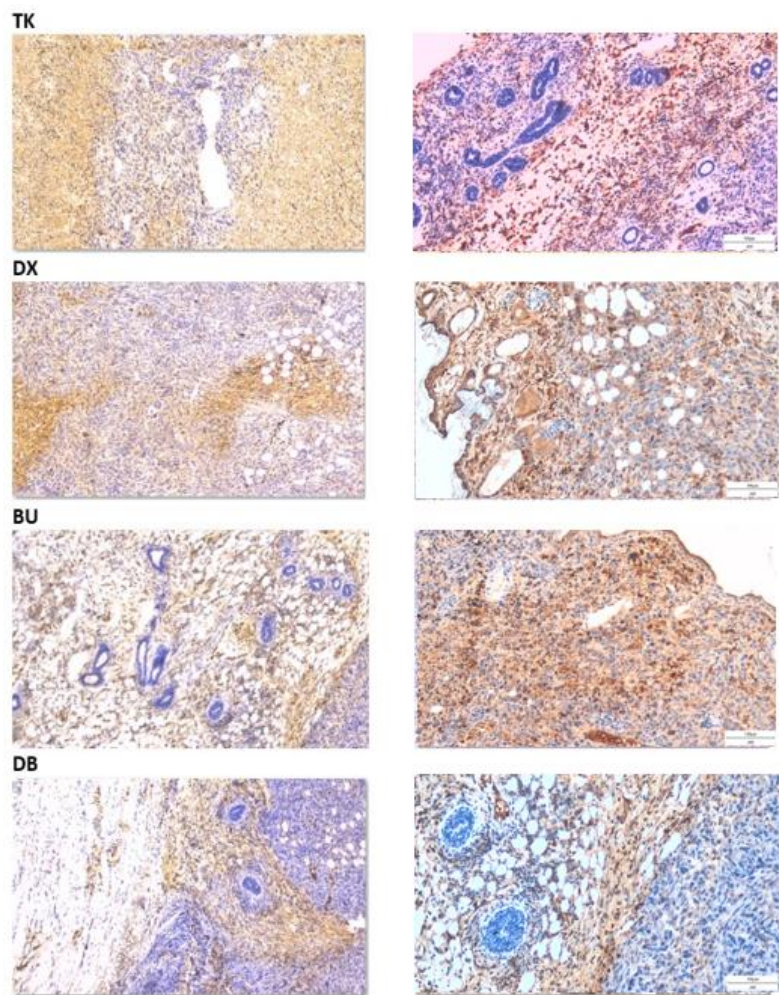
Fig. 6 NOS immunohistochemical staining and H-score analysis of primary tumour tissue. Brown immunoreactivity indicates NOS positivity. Scale bar 50 μ m; $\times 20$



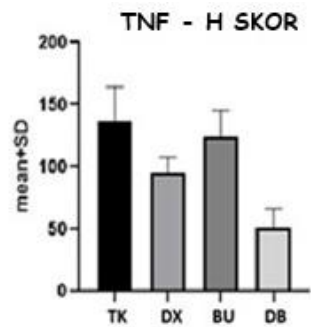
386 **Fig. 7** Caspase-3 immunohistochemical staining of primary tumour tissue. Brown staining represents
 387 apoptotic cells. Scale bar 100–200 μm ; $\times 10/\times 20$



388 **Fig. 8** VEGF immunohistochemical staining of primary tumour tissue at low (left) and high (right)
 389 magnification. Brown staining represents VEGF expression. Scale bar 100 μm ; $\times 20$
 390

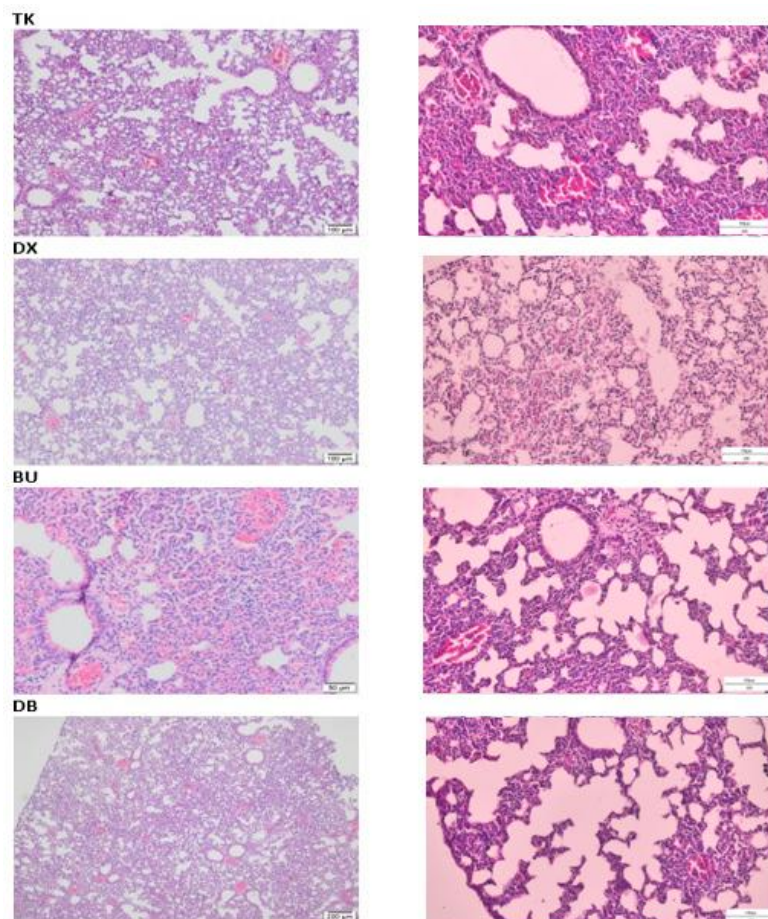


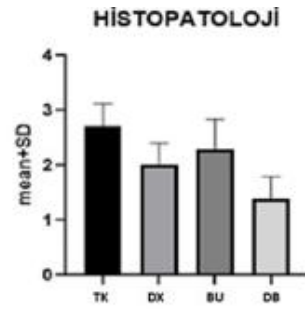
391



Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	41,15	17,56 to 64,73	Yes	***	0,0002
TK vs. BU	12,14	-11,45 to 35,72	No	ns	0,5162
TK vs. DB	85,14	61,55 to 108,7	Yes	****	<0,0001
DX vs. BU	-29,01	-52,60 to -5,424	Yes	*	0,0109
DX vs. DB	43,99	20,40 to 67,58	Yes	****	<0,0001
BU vs. DB	73,00	49,41 to 96,59	Yes	****	<0,0001

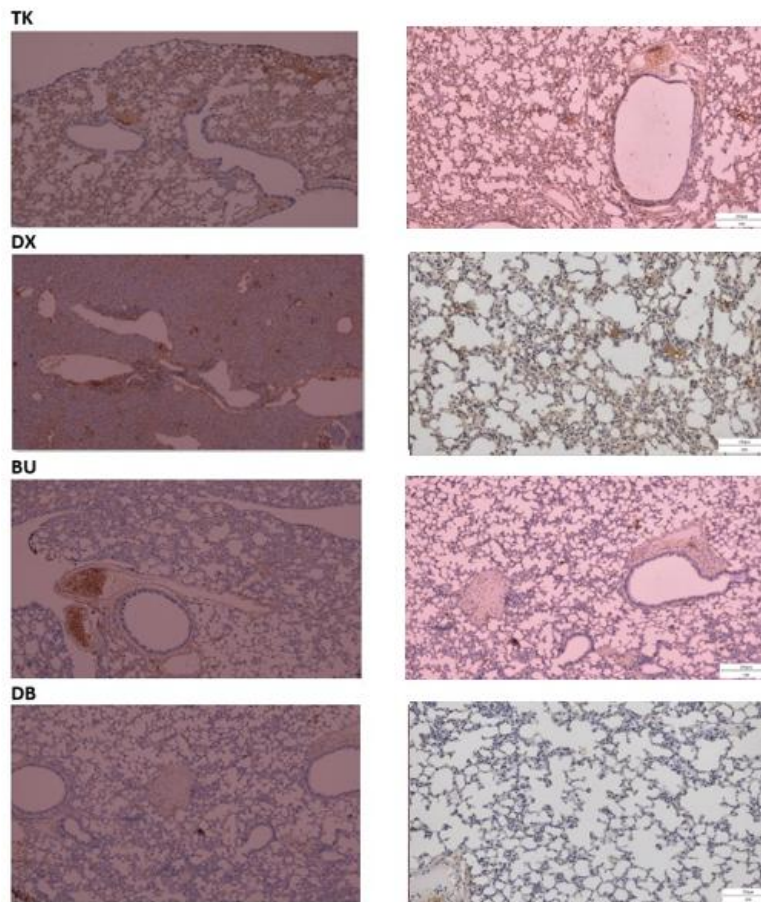
Fig. 9 TNF- α immunohistochemical staining of primary tumour tissue with H-score analysis. Brown staining indicates cytokine expression. Scale bar 100–200 μ m; $\times 10/\times 20$

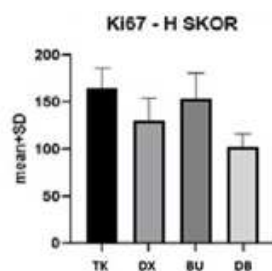




Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Sum	P
TK vs. DX	0,7000	0,1651 to 1,235	Yes	**	0,0062
TK vs. BU	0,4000	-0,1349 to 0,9349	No	<u>ns</u>	0,2018
TK vs. DB	1,300	0,7651 to 1,835	Yes	****	<0,0001
DX vs. BU	-0,3000	-0,8349 to 0,2349	No	<u>ns</u>	0,4419
DX vs. DB	0,6000	0,06511 to 1,135	Yes	*	0,0229
BU vs. DB	0,9000	0,3651 to 1,435	Yes	***	0,0003

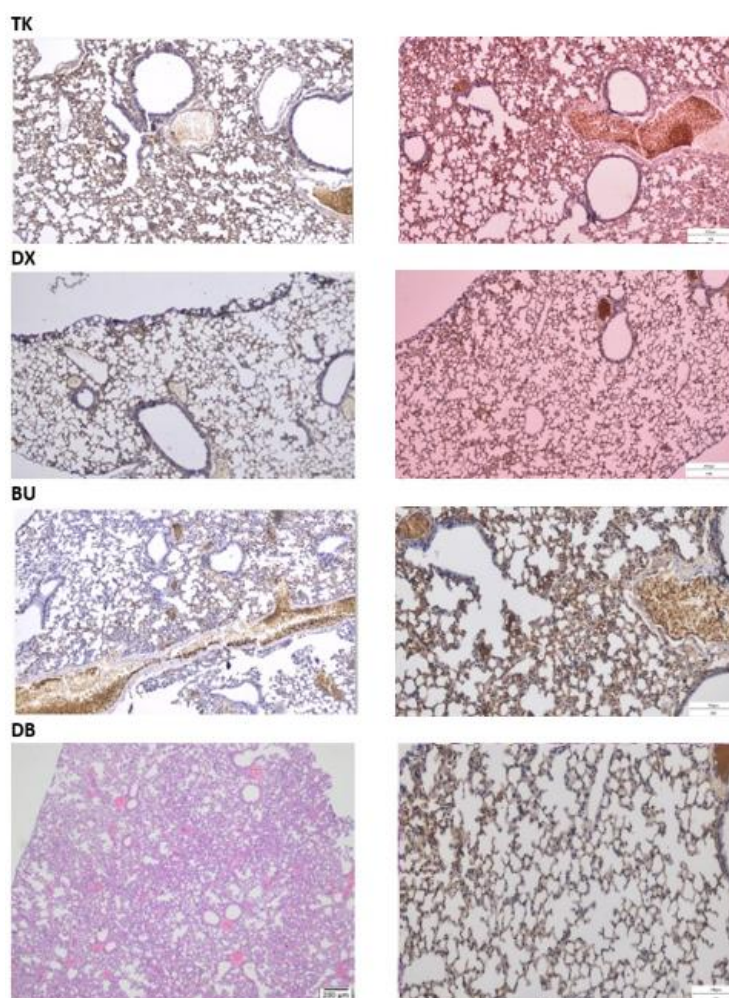
Fig. 10 Histopathological appearance of lung tissue. H&E-stained sections; the DB group shows preserved alveolar architecture with reduced infiltration. Scale bar 100 μ m; $\times 20$

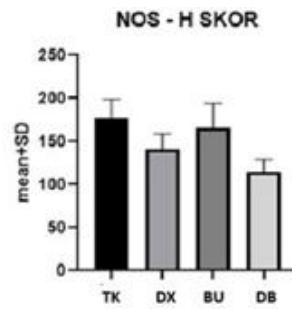




Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	34,16	7,411 to 60,91	Yes	**	0,0078
TK vs. BU	10,66	-16,09 to 37,41	No	ns	0,7075
TK vs. DB	61,85	35,10 to 88,60	Yes	****	<0,0001
DX vs. BU	-23,50	-50,25 to 3,253	No	ns	0,1022
DX vs. DB	27,69	0,9415 to 54,44	Yes	*	0,0401
BU vs. DB	51,19	24,44 to 77,94	Yes	****	<0,0001

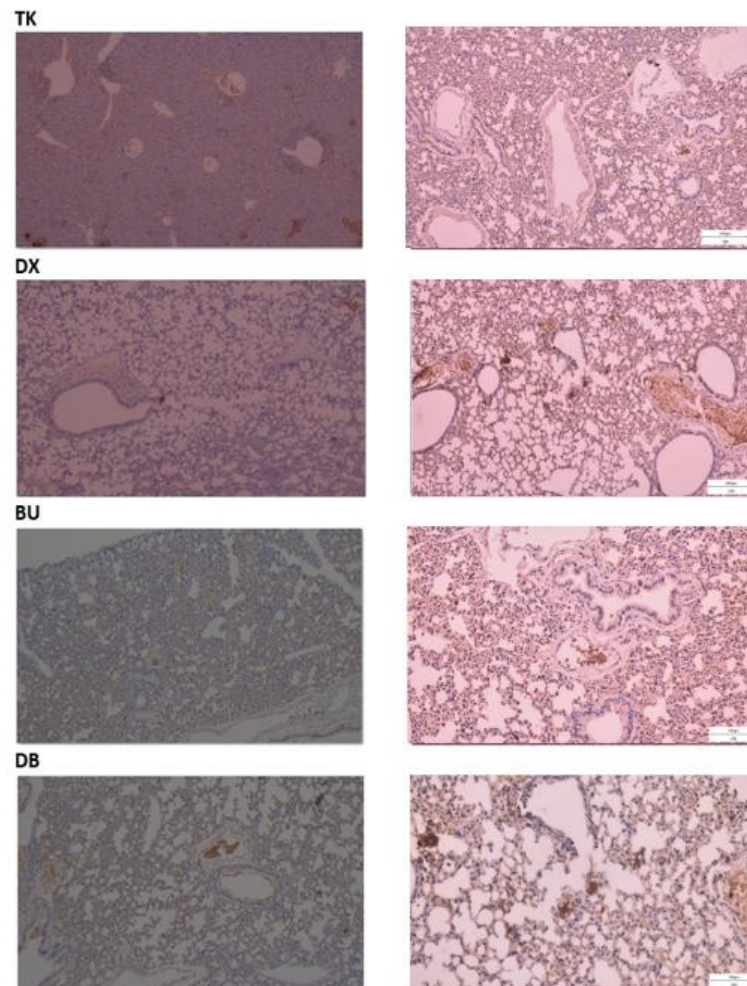
Fig. 11 Ki-67 immunohistochemical staining of lung tissue with H-score analysis. Positive nuclei appear brown; proliferation is markedly reduced in the DB group. Scale bar 100 μ m; $\times 20$





Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	36,01	10,65 to 61,37	Yes	**	0,0027
TK vs. BU	10,86	-14,50 to 36,22	No	ns	0,6592
TK vs. DB	63,00	37,64 to 88,36	Yes	****	<0,0001
DX vs. BU	-25,15	-50,51 to 0,2121	No	ns	0,0526
DX vs. DB	26,99	1,632 to 52,35	Yes	*	0,0333
BU vs. DB	52,14	26,78 to 77,50	Yes	****	<0,0001

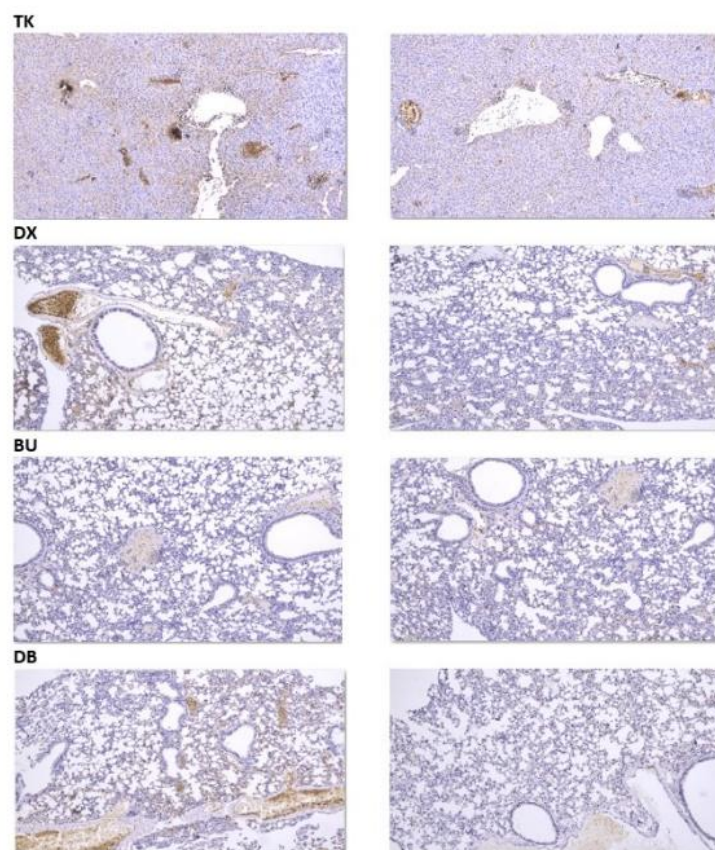
Fig. 12 NOS immunohistochemical staining of lung tissue with H-score analysis. Brown staining represents endothelial NOS expression. Scale bar 100 μ m; $\times 20$

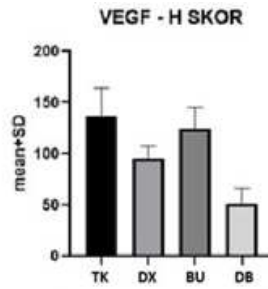




Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	31,01	5,652 to 56,37	Yes	*	0,0114
TK vs. BU	-9,136	-34,50 to 16,22	No	ns	0,7670
TK vs. DB	68,00	42,64 to 93,36	Yes	****	<0,0001
DX vs. BU	-40,15	-65,51 to -14,79	Yes	***	0,0008
DX vs. DB	36,99	11,63 to 62,35	Yes	**	0,0020
BU vs. DB	77,14	51,78 to 102,5	Yes	****	<0,0001

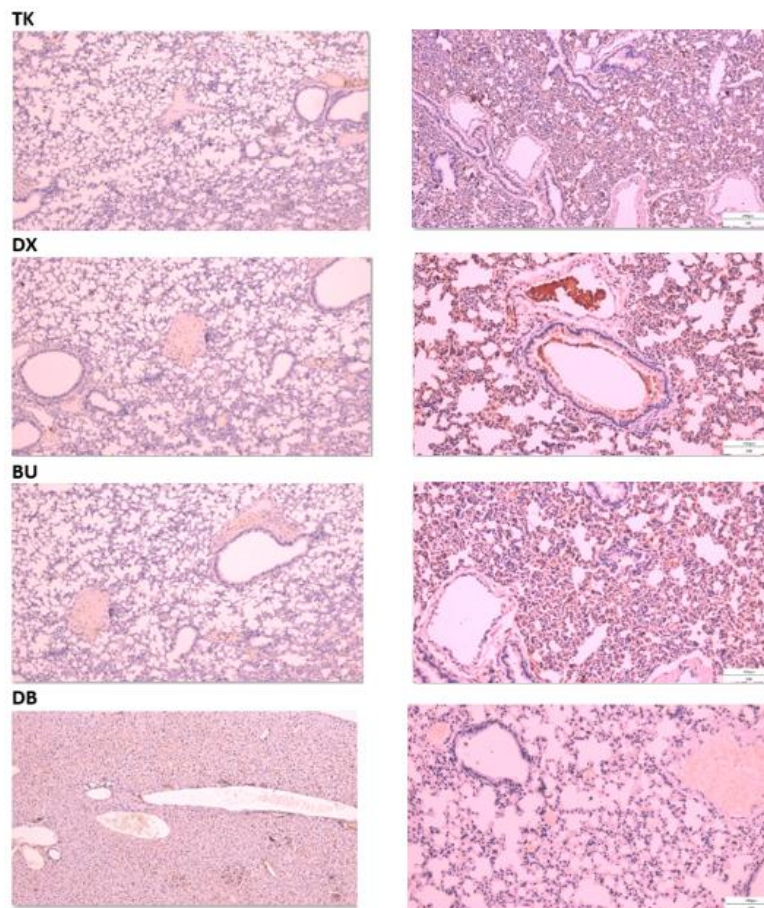
Fig. 13 Caspase-3 immunohistochemical staining of lung tissue with H-score analysis. Brown staining represents apoptotic cells. Scale bar 100–200 μm ; $\times 10/\times 20$

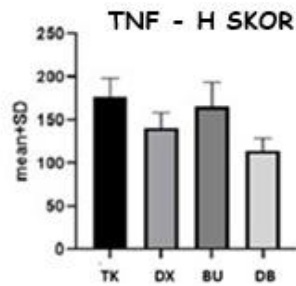




Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	41,15	17,56 to 64,73	Yes	***	0,0002
TK vs. BU	12,14	-11,45 to 35,72	No	ns	0,5162
TK vs. DB	85,14	61,55 to 108,7	Yes	****	<0,0001
DX vs. BU	-29,01	-52,60 to -5,424	Yes	*	0,0109
DX vs. DB	43,99	20,40 to 67,58	Yes	****	<0,0001
BU vs. DB	73,00	49,41 to 96,59	Yes	****	<0,0001

Fig. 14 VEGF immunohistochemical staining of lung tissue with H-score analysis. Brown staining represents VEGF expression. Scale bar 100 μ m; $\times 20$





Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	36,01	10,65 to 61,37	Yes	**	0,0027
TK vs. BU	10,86	-14,50 to 36,22	No	ns	0,6592
TK vs. DB	63,00	37,64 to 88,36	Yes	****	<0,0001
DX vs. BU	-25,15	-50,51 to 0,2121	No	ns	0,0526
DX vs. DB	26,99	1,632 to 52,35	Yes	*	0,0333
BU vs. DB	52,14	26,78 to 77,50	Yes	****	<0,0001

Fig. 15 TNF- α immunohistochemical staining of lung tissue with H-score analysis. Brown staining represents cytokine expression. Scale bar 100 μ m; $\times 20$

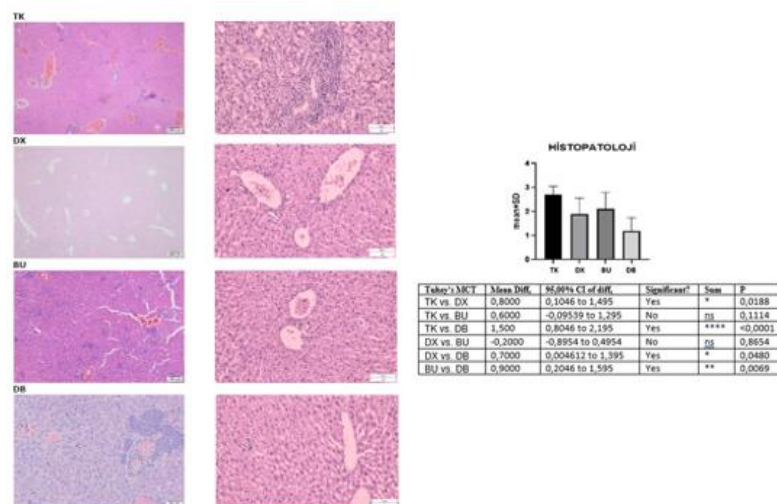
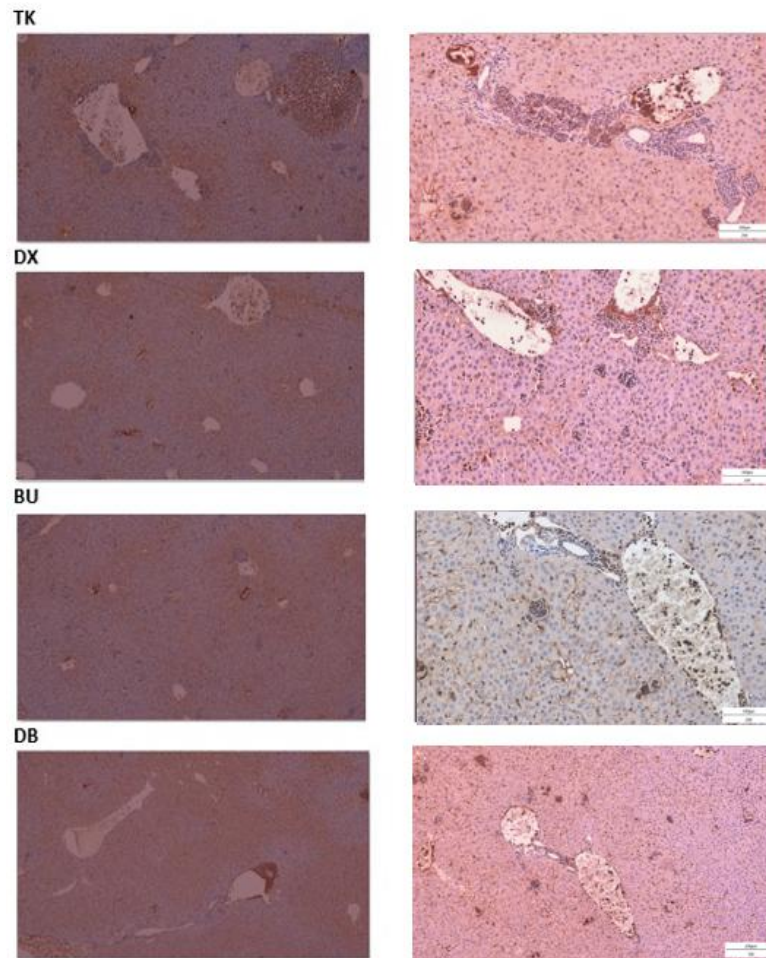
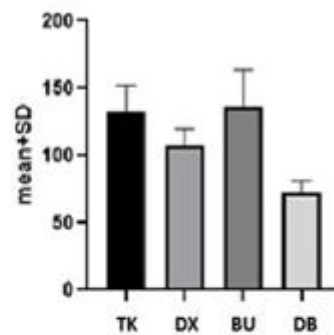


Fig. 16 Histopathological appearance of liver tissue. H&E-stained sections; the DB group shows the most organised architecture with minimal infiltration. Scale bar 100 μ m; $\times 20$

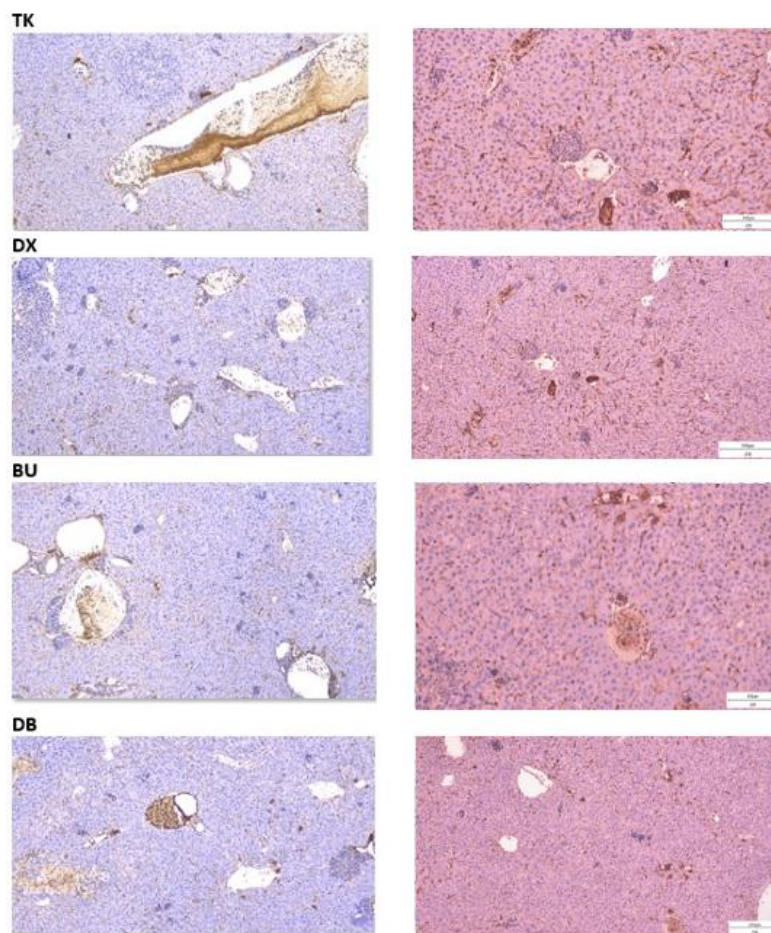
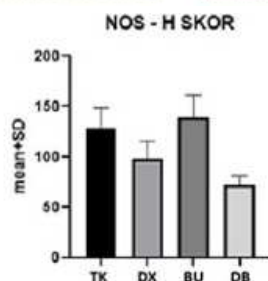


Ki67 IHC



Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	25,51	3,774 to 47,24	Yes	*	0,0161
TK vs. BU	-4,136	-25,87 to 17,60	No	ns	0,9556
TK vs. DB	60,00	38,27 to 81,74	Yes	****	<0,0001
DX vs. BU	-29,65	-51,38 to -7,910	Yes	**	0,0041
DX vs. DB	34,49	12,76 to 56,23	Yes	***	0,0007
BU vs. DB	64,14	42,40 to 85,87	Yes	****	<0,0001

Fig. 17 Ki-67 immunohistochemical staining of liver tissue with H-score analysis. Brown-stained nuclei represent proliferating cells. Scale bar 100 µm; ×20



Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Sum	P
TK vs. DX	30,51	9,501 to 51,52	Yes	**	0,0021
TK vs. BU	-11,14	-32,14 to 9,872	No	ns	0,4909
TK vs. DB	56,00	34,99 to 77,01	Yes	****	<0,0001
DX vs. BU	-41,65	-62,65 to -20,64	Yes	****	<0,0001
DX vs. DB	25,49	4,485 to 46,50	Yes	*	0,0122
BU vs. DB	67,14	46,13 to 88,15	Yes	****	<0,0001

Fig. 18 NOS immunohistochemical staining of liver tissue with H-score analysis. Brown staining represents endothelial NOS expression. Scale bar 100 µm; ×20

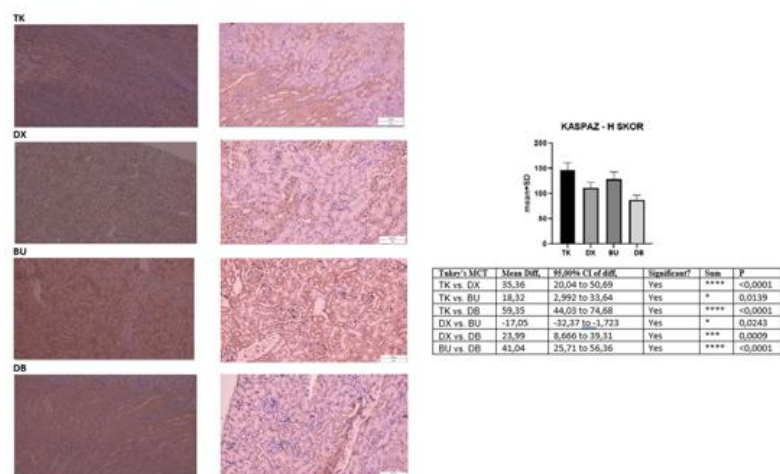
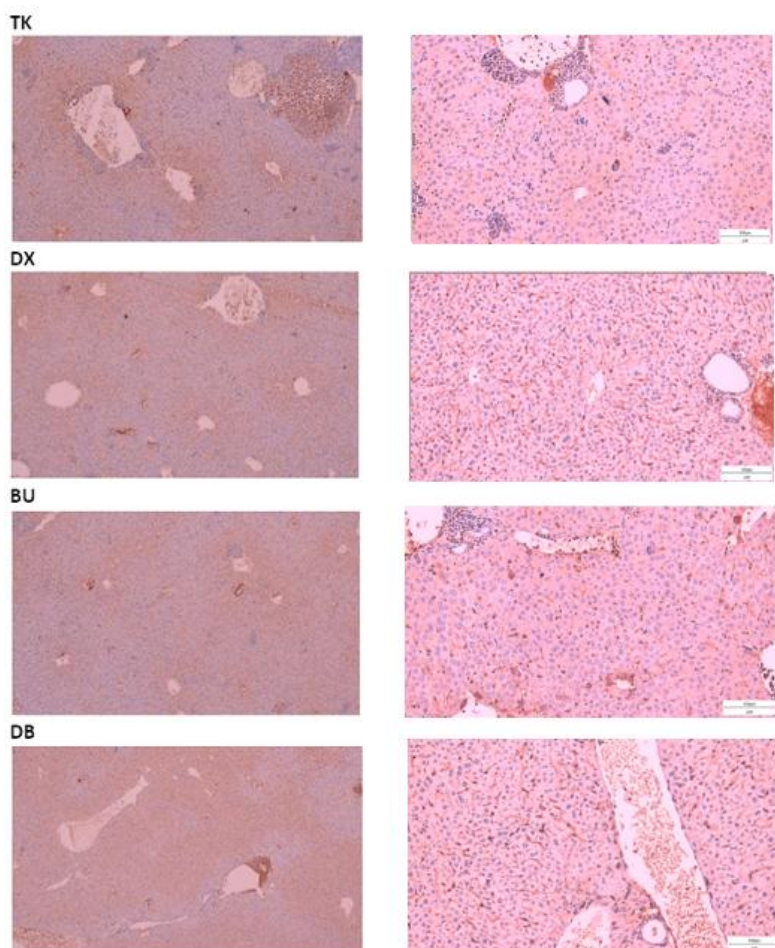
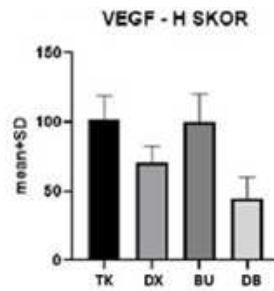


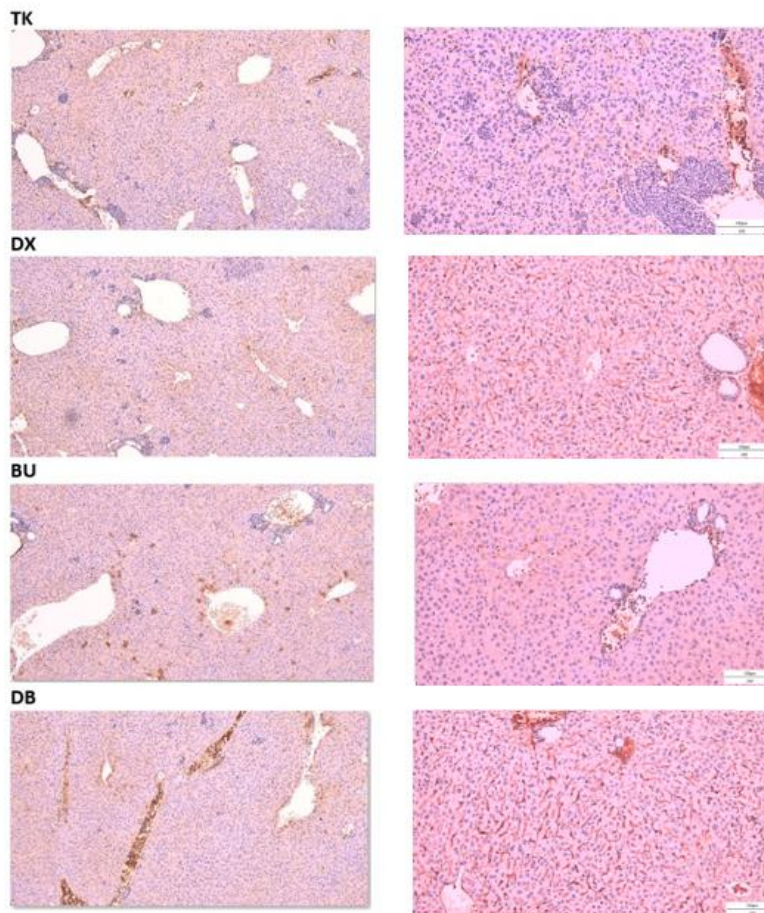
Fig. 19 Caspase-3 immunohistochemical staining of liver tissue. Brown-stained cells represent apoptotic hepatocytes. Scale bar 100 μ m; $\times 20$

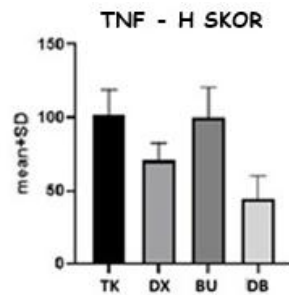




Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	31,15	11,35 to 50,95	Yes	***	0,0008
TK vs. BU	2,136	-17,66 to 21,93	No	ns	0,9913
TK vs. DB	57,14	37,34 to 76,94	Yes	****	<0,0001
DX vs. BU	-29,01	-48,81 to -9,212	Yes	**	0,0019
DX vs. DB	25,99	6,192 to 45,79	Yes	**	0,0060
BU vs. DB	55,00	35,20 to 74,80	Yes	****	<0,0001

Fig. 20 VEGF immunohistochemical staining of liver tissue with H-score analysis. Brown staining represents VEGF expression. Scale bar 100 μ m; $\times 20$





Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	31,15	11,35 to 50,95	Yes	***	0,0008
TK vs. BU	2,136	-17,66 to 21,93	No	ns	0,9913
TK vs. DB	57,14	37,34 to 76,94	Yes	****	<0,0001
DX vs. BU	-29,01	-48,81 to -9,212	Yes	**	0,0019
DX vs. DB	25,99	6,192 to 45,79	Yes	**	0,0060
BU vs. DB	55,00	35,20 to 74,80	Yes	****	<0,0001

Fig. 21 TNF- α immunohistochemical staining of liver tissue with H-score analysis. Brown staining represents pro-inflammatory cytokine expression. Scale bar 100 μ m; $\times 20$

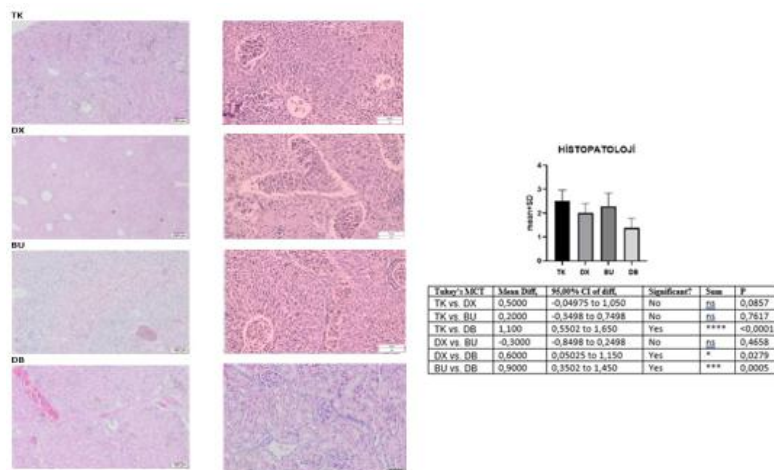
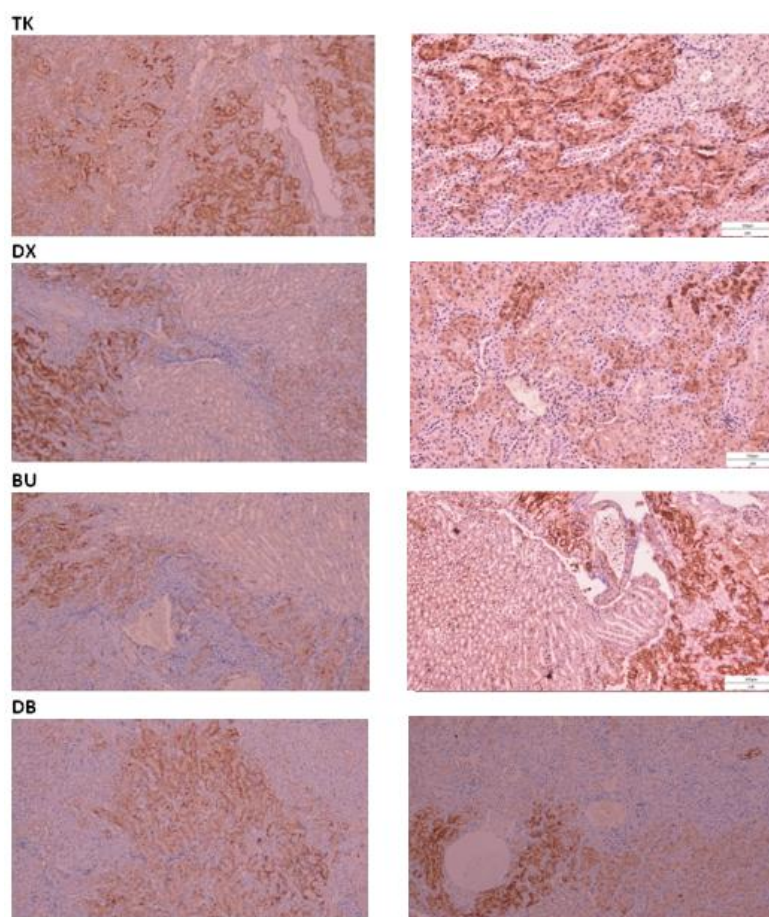
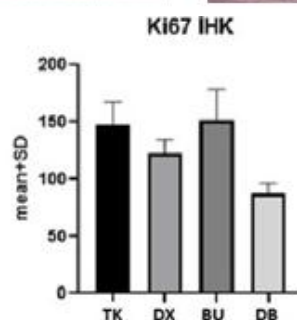
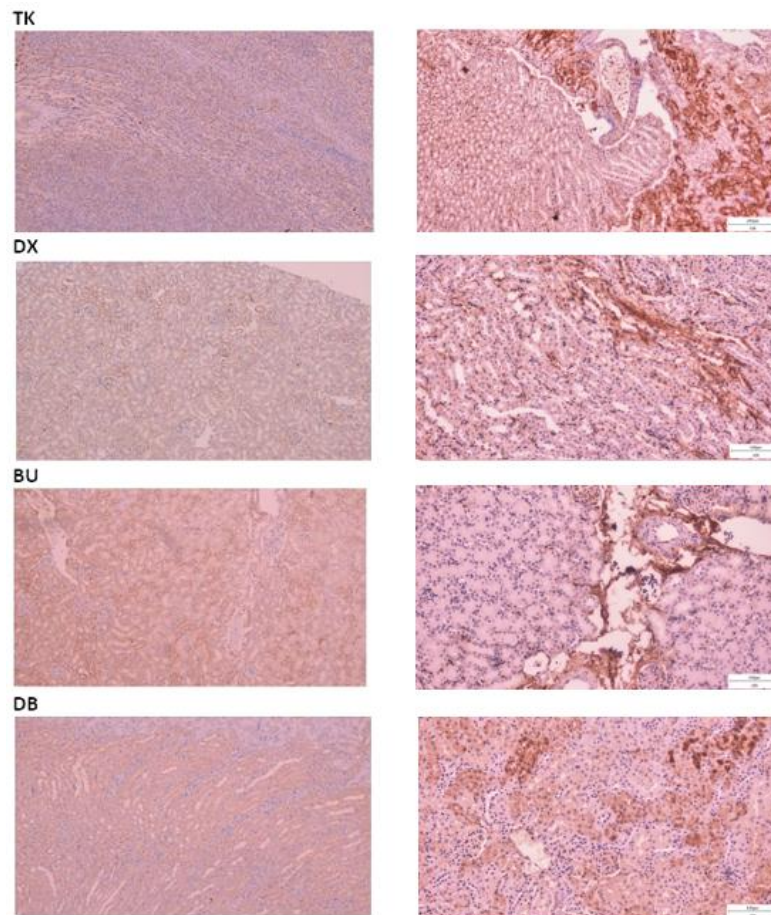
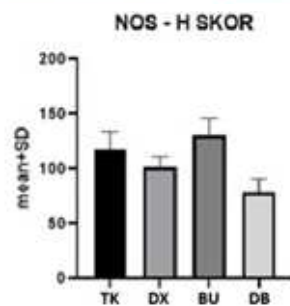


Fig. 22 Histopathological appearance of kidney tissue. H&E-stained sections; the DB group shows the most organised architecture with preserved glomerular morphology. Scale bar 100 μ m; $\times 20$



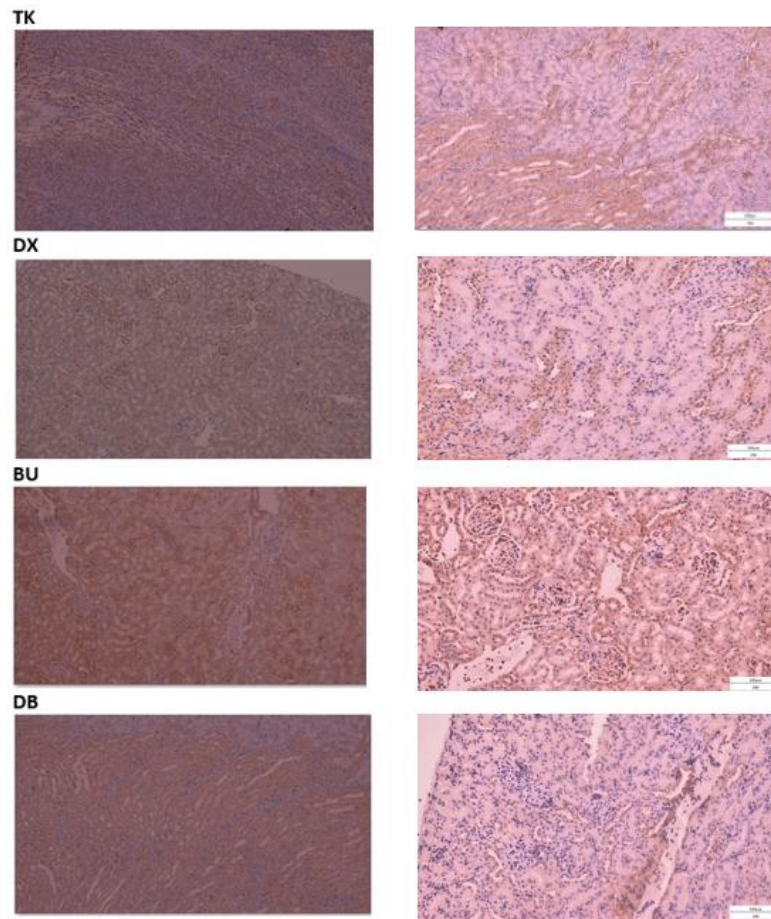
Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	25,51	3,774 to 47,24	Yes	*	0,0161
TK vs. BU	-4,136	-25,87 to 17,60	No	<u>ns</u>	0,9556
TK vs. DB	60,00	38,27 to 81,74	Yes	****	<0,0001
DX vs. BU	-29,65	-51,38 to -7,910	Yes	**	0,0041
DX vs. DB	34,49	12,76 to 56,23	Yes	***	0,0007
BU vs. DB	64,14	42,40 to 85,87	Yes	****	<0,0001

Fig. 23 Ki-67 immunohistochemical staining of kidney tissue with H-score analysis. Brown-stained nuclei represent proliferating cells. Scale bar 100 μ m; $\times 20$



Tukey's MCT	Mean Diff,	95,00% CI of diff,	Significant?	Sum	P
TK vs. DX	16,33	0,2495 to 32,40	Yes	*	0,0454
TK vs. BU	-13,00	-29,08 to 3,077	No	ns	0,1489
TK vs. DB	39,57	23,49 to 55,65	Yes	****	<0,0001
DX vs. BU	-29,33	-45,40 to -13,25	Yes	***	0,0001
DX vs. DB	23,24	7,166 to 39,32	Yes	**	0,0022
BU vs. DB	52,57	36,49 to 68,65	Yes	****	<0,0001

Fig. 24 NOS immunohistochemical staining of kidney tissue with H-score analysis. Brown staining represents endothelial NOS expression. Scale bar 100 μ m; $\times 20$



Tukey's MCT	Mean Diff.	95,00% CI of diff.	Significant?	Sum	P
TK vs. DX	35,36	20,04 to 50,69	Yes	****	<0,0001
TK vs. BU	18,32	2,992 to 33,64	Yes	*	0,0139
TK vs. DB	59,35	44,03 to 74,68	Yes	****	<0,0001
DX vs. BU	-17,05	-32,37 to -1,723	Yes	*	0,0243
DX vs. DB	23,99	8,666 to 39,31	Yes	***	0,0009
BU vs. DB	41,04	25,71 to 56,36	Yes	****	<0,0001

Fig. 25 Caspase-3 immunohistochemical staining of kidney tissue with H-score analysis. Brown-stained cells represent apoptotic renal epithelial cells. Scale bar 100 μ m; $\times 20$

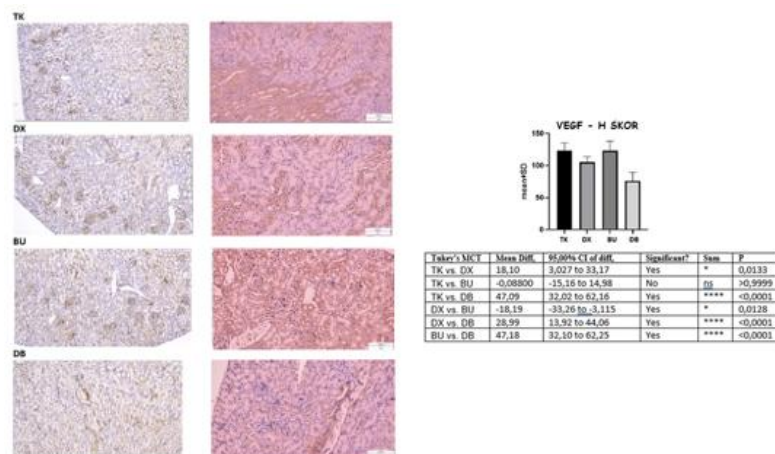


Fig. 26 VEGF immunohistochemical staining of kidney tissue. Brown immunoreactivity represents VEGF expression. Scale bar 100 μ m; $\times 20$

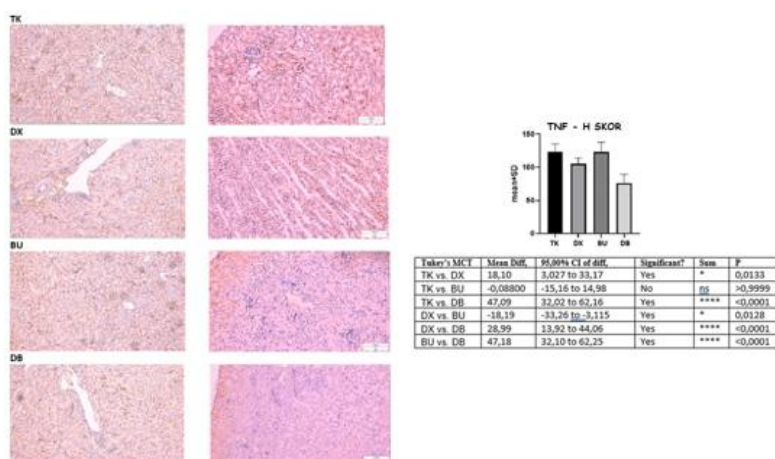


Fig. 27 TNF- α immunohistochemical staining of kidney tissue. Brown immunoreactivity represents TNF- α expression. Scale bar 50–100 μ m; $\times 20$