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The effects of baicalein alone and in combination with losartan on DOX-induced nephrotoxicity in rats

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Abstract: The goal of this research was to determine whether the combination of baicalein (BL) and losartan (LT) would provide greater protection against DOX-induced nephrotoxicity. There were five groups of male rats in the experiment: the 1) control, 2) DOX, 3) DOX+LT, 4) DOX+BL and 5) DOX+LT+BL groups. A dose of DOX was administered following two weeks of LT and BL therapy. In the DOX-affected group, serum renal indicators, including creatinine and urea, rose considerably compared to those in the control groups ($p<0.01$). Further, there was a statistically significant increase ($p<0.001$) in the levels of the cytokines that promote inflammation in renal tissue, including tumor necrosis factor-α, interleukin (IL)-1 and IL-6. In addition, the level of the anti-inflammatory cytokine IL-10 decreased significantly ($p<0.001$) in the DOX-challenged group compared to the control groups. In addition, renal cell indicators of oxidative stress ($p<0.001$) and enzymatic activity ($p<0.01$) reduced dramatically in the DOX-challenged group, whereas renal cell thiobarbituric acid retroactive materials rose greatly ($p<0.001$). Finally, the DOX group had higher kidney protein expression and inflammatory activity than the control groups ($p<0.001$). The combination of BL and LT therapy protected DOX-challenged rats via antioxidant and anti-inflammatory activities.

Keywords: Baicalein, losartan, oxidative stress, nephrotoxicity, doxorubicin

INTRODUCTION

Carcinoid tumors are traditionally treated with chemotherapy, one of the most prevalent treatment methods. However, it is harmful to cells and can lead to cytotoxicity, which is caused by renal cells' attempts to remove poisons created by catabolic processes outside the body. Cytotoxicity also affects the filtration and reabsorption processes that occur in the kidneys. Free radicals in the cells are prone to the development of harmful lipid peroxidation molecules that cause apoptosis (Pizzino *et al*., 2017). In addition, nitric oxide (NO) can be modified and can accumulate in kidney tissue, which is another way in which kidney function can be affected (Carlstrom, M. 2021). There may be an indirect link between these substances and acute nephropathy, as they cause damage to vital organs, including the heart and liver (Petejova *et al*., 2019). Doxorubicin (DOX) is a powerful anticancer medication that is frequently prescribed to treat a variety of cancers (Micallef, I., & Baron, B. 2020). However, it is known to have a detrimental effect on many bodily tissues and it may lead to cardiotoxicity (Rawat *et al*., 2021), hepatotoxicity (Al-Oanzi *et al*., 2020) and nephrotoxicity (Soltani *et al*., 2021). As a result, it is necessary to investigate and identify potential avenues toward the reduction and eventual elimination of DOX-induced nephrotoxicity. In this paper, preventive treatment strategies are investigated with the goal of decreasing the adverse effects of DOX.

Flavonoids and other phenolic compounds found in medicinal plants are among the most potent antioxidants available and they help reduce pharmacological toxicity (Panche *et al*., 2016; Tungmunnithum *et al*., 2018). Recent research has shown promising results in extracting medicinal herbs as a possible defense mechanism against the harmful effects of cancer therapies (De Giffoni de Carvalho *et al*., 2019). During this experiment, the antioxidant properties of both baicalein (BL) and losartan (LT) were exploited. BL, an essential component of *Scutellaria baicalensis*, has been used as a traditional herbal treatment in China for about two decades (Zhao *et al*., 2016). In addition, it has been discovered that BL may minimize the damage produced by oxidative stress, which can be the result of inflammation (Patwardhan *et al*., 2016), diabetes (Dong *et al*., 2020), or hepatotoxicity (Al-Oanzi *et al*., 2020) and it is safe to use in the detection, treatment and prevention of a broad variety of disorders (Zhong *et al*., 2019).

LT is one of the primary angiotensin II (Ang-II) receptor antagonists (type AT1) responsible for efficiently controlling hypertension (Olschewski *et al*., 2018). In addition to being an effective treatment for hypertension, LT may inhibit the creation and stimulation of the protein known as transforming growth factor-1 (TGF-1) (Miguel-Carrasco *et al*., 2017). It has been shown that the medication known as LT, which is used to treat cirrhosis, may also inhibit the formation of fibrosis in the renal and cardiac tissues (Habashi *et al*., 2006; Salama *et al*., 2016). A relationship between the creation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) associated with Ang-II and the mitochondrial system was discovered by a study that demonstrated that antioxidants impair the regulation of Ang-II on the activator protein 1

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(AP-1) signal pathway. This interaction was discovered due to a study that demonstrated that antioxidants inhibit the regulatory effects of Ang-II. The findings of the research also show that antioxidants can block the consequences of Ang-II on the AP-1 signal pathway (Ratliff *et al*., 2016). It was hypothesized that because AP-1, the activity that drives the cytochrome c expression in response to oxidation/reduction, is responsible for driving changes in cytochrome c content for mitochondria, Ang-II would be responsible for causing these fluctuations. This is because AP-1 is accountable for regulating the expression of cytochrome c (Takada *et al*., 2008), while Ang-II can increase the generation of cytokines, such as alpha tumor necrosis factor (TNF-α) by activating the Ang-II type 1 receptor (AT1R) on single cells, macrophages and smooth muscle cells (Satou *et al*., 2018).

It has been demonstrated that DOX may exacerbate the oxidative stress encountered by the kidneys, which in turn can cause renal dysfunction. DOX-induced nephrotoxicity is caused mostly by anthracycline iron-free radicals that have been proven to increase oxidative stress, according to recent studies (El-Sayed *et al*., 2017; Refaie *et al*., 2016; Rafiee *et al*., 2020). Although it is efficient as a chemotherapeutic treatment, precautions must be taken to avoid potentially harmful side effects, such as nephrotoxicity. In this study, we will investigate the impact of treatment with BL alone and in combination with LT on renal oxidation and DOX-induced inflammation in experimental animals.

MATERIALS AND METHODS

Animals

Experiment Animal Care Centre-supplied male albino white Wistar rats weighing 200 to 300 g were used in this investigation (College of Pharmacy at King Saud University in Riyadh, Saudi Arabia). They were kept in a 22°C, 50-55% humidity and 12-h light/dark cycle-controlled environment. Before the trial began, the animals were given 7 d to become accustomed to the lab, during which time they received unlimited access to Purina rat food and water. Following the National Institutes of Health's (NIH) Publication No. 80-23; 1996, "Guidance for the Treatment and Usage of Laboratory Animals," all procedures, including euthanasia, were approved by the Ethical Committee of the Experiment Animal Care Centre (621-EACC-2016 dated 02-03-2016).

Chemicals and kits

In this study, the following chemicals were used: DOX from EBEWE Pharma in Austria; San Francisco-based Hoefer Inc. provided phosphate-buffered saline (PBS); baicalein (BL) was provided from Aldrich Chemicals in the United Kingdom (UK); LT was available from Toronto, Ontario, through Toronto Research Chemicals Inc.; and Human Diagnostics supplied us with diagnostic kits for measuring creatinine and urea levels (Wiesbaden, Germany). ELISA kits, from R&D Systems (Minneapolis, Minnesota, USA), were used to analyze NO, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), nuclear factor kappa-B (NF-B) p65, caspase-3, TNF-α, interleukin (IL)-1 beta, IL-1, IL-6, IL-10, superoxide dismutase (SOD), glutathione S transferase (GST), catalase (CAT) and glutathione peroxidase (Gpx). Cayman Chemical supplied both the thiobarbituric acid (TBARS) and glutathione (GSH) ELISA reactants (Ann Arbor, MI, USA) and the main nNOS, iNOS and eNOS antibodies were purchased from Abcam, UK (Catalog Numbers ab1376, ab3523 and ab76198). Finally, Santa Cruz Biotechnology Inc. supplied the primary NF-kB p65 (Catalog # Sc-8008) and B-Actin (Sc-47777) antibodies utilized in this investigation (USA), while Goat anti-Mouse Poly HRP (Catalog Number 32230) was acquired from Invitrogen (USA).

Study design

After being exposed to the new environment for a week, the rats were divided into five groups using a random selection process: a control group (vehicle), DOX group (vehicle), DOX with LT (0.7 mg/kg/day orally) group, DOX with BL (10 mg/kg/day orally) group and DOX with BL+LT group (combined therapy). Due to the significant death rate previously observed when DOX was delivered alone, only six animals were utilized as controls, whereas 12 were used in the DOX-injection groups (Siveski-Iliskovic *et al*., 1994). A single intraperitoneal dose (15 mg/kg) of DOX was given to the rats in the DOX-injection groups following two weeks of LT and BL therapy. Rats were weighed and sedated with a ratio of 94 mg/kg ketamine to 14 mg/kg xylazine. The heart puncture method was used to obtain blood samples in transparent tubes and to extract the serum, the tubes were centrifuged for 10 min at a speed of 4,000 rpm. After being dissected, measured and preserved, the kidneys were examined biochemically via a portion of the kidney that had been individually wrapped in foil and frozen at -80°C. At the same time, another section was preserved in formalin at a concentration of 10% for further histological analysis.

Serum analysis

The levels of the biomarkers renal creatinine and urea in the serum were analyzed using diagnostic kits according to the manufacturer's directions.

Tissue analysis

IL-6, IL-1, IL-10, nNOS, iNOS, eNOS, TNF-α, NF-Bp65, caspase-3, TBARS and GSH levels in kidney homogenates were determined using ELISA kits, while the SOD, CAT, GPx and GST concentrations in renal cell supernatant (PMS) were determined using an ELISA kit.

Western blot analysis

The extraction kit (Cat# 163-2086, Bio-Rad Inc., USA) provides a 30-mg sample of each kidney tissue homogenized in an ultrasound to check for protein extraction. We utilized Bio Specific Inc.'s SK 3041 Bradford protein check kit to determine the amount of total protein in the sample, which was done by electrophoresis of the protein bands using sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). At 25 volts, the restored protein bonds were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane and the primary antibody solution was incubated with the membrane overnight at 4°C. Following four washes with Tris-buffered saline with Tween 20 (TBST) buffer, the secondary antibody (HRP-conjugated solution) was incubated with the membrane at room temperature for 1 h. An enhanced chemiluminescence assay was used to detect immunoreactive bands on the membrane after being washed four times with TBST buffer (Bio-Rad Laboratories, Inc., California, USA) and image analysis software (ChemiDocTM MP imaging system, Bio-Rad) was used to determine the target protein's band intensity.

Histology

After being preserved in a 10% buffered formalin solution, the kidneys of both the experimental and control groups were encased in paraffin blocks. H&E-stained slices with a thickness of five microns were cut using an American microtome manufactured by Leica Camera AG (Wetzlar, Germany).

STATISTICAL ANALYSIS

Graph pad prisms were used to analyze the data (V.5). A one-way analysis of variance (ANOVA), followed by a multiple reference study students-Newman-Keuls test (n = 6), was performed. a Control vs DOX group; b DOX vs DOX with BL or DOX vs DOX with LT or DOX vs DOX with BL+LT. Data are presented as mean ± standard deviation (SD). Significant p-values are denoted as follows: *p<0.05, **p<0.01 and ***p<0.001.

RESULTS

We have already demonstrated that the average body weight of rats treated with DOX decreased considerably (p<0.001) compared to the control group (Al-Oanzi *et al*., 2020). Compared to the DOX group, the body weight of animals treated with BL+LT and a single dose of DOX for three weeks increased (p<0.05). Researchers have not, however, changed the importance of organs, such as the kidneys.

The impact of BL and LT therapy on DOX-induced kidney toxicity markers

In this work, we investigated the influence of BL and LT treatment on the DOX-induced alterations in blood creatinine and urea levels, which are markers of renal disease. Our findings were significant (p<0.001) in the DOX-challenged group compared to the control group. Serum levels of creatinine and urea were considerably decreased (p<0.05) in the BL group, but the greatest effect occurred in the BL + LT group (p<0.001; fig. 1: A, B).

The impact of BL and LT therapy on DOX-induced alterations in oxidative stress indicators and enzymatic activity

Compared to the control group, we discovered that DOX had a substantially (p<0.001) enhanced impact with TBARS and a reduced effect with GSH (fig. 1: C, D). When compared to the DOX group, the TBARS levels were lower in the DOX treated with LT (p<0.05), both BL and the BL+LT (p<0.001) groups. A significant reduction in renal cell enzyme activity (P<0.01) was observed in rats exposed to DOX compared to the control group (fig. 1: E-I). In addition, the DOX with BL and BL+LT (p<0.01, p<0.001, respectively) groups showed a substantial increase in renal SOD activity compared to the DOX group. All three DOX groups treated with LT, BL and BL+LT showed significant increases in CAT activity compared to the control group (p<0.05, BL, BL+LT). Further, DOX treatment with LT or BL or BL+LT (p<0.05, p<0.01, p<0.001, respectively) substantially improved renal cell GPx activity. In comparison to the DOX-challenged group, the levels of GST enzymatic activity in the renal cells were considerably elevated in the DOX treated with LT and BL (both p<0.01) and the DOX treated with BL+ LT groups (p<0.001).

The impact of BL and LT therapy on DOX-induced alterations in pro-inflammatory an anti-Inflammatory cytokines

The production of TNF-, IL-1 and IL-6 rose substantially (P<0.001) in the DOX-treated group compared to the control group. On the other hand, the production of IL-10 dropped dramatically (P<0.001) in the DOX-treated group compared to the controls. The DOX treated with LT, BL and BL+LT groups showed lowered levels of pro-inflammatory cytokines by an order of magnitude in comparison with the DOX-challenged groups (LT and BL: p<0.05, BL+LT: p<0.01). Although the individual therapy groups had a lower cytokine inhibition, the BL+LT treatment groups had substantially more potent cytokine inhibition. The anti-inflammatory (IL-10) marker rose considerably (P<0.01) in the LT+BL group compared to the DOX group, but in contrast to the DOX group, individual therapy with LT and BL failed to produce substantial benefits (fig. 2).

The impact of BL and LT therapy on DOX-induced alterations in inflammatory activities and protein expression

Compared to the control groups, the levels of the inflammatory activities represented by nNOS, iNOS and eNOS, as well as Caspase-3 and NF-κB p65, were significantly (P <0.001) higher in the renal cells of DOX-challenged individuals, as shown in fig. 3. Compared to the DOX-challenged group, the DOX groups treated with LT, BL and BL+LT exhibited a significant reduction in Caspase-3 activation ($p<0.05$ for BL and BL+LT). Compared to the DOX group, the DOX treated with BL and BL+LT groups ($p<0.05$, $p<0.01$, respectively) exhibited substantially reduced levels of NF-κB p65 activity in renal cells. Finally, in comparison to the DOX group, the DOX treated with LT or BL or with BL+LT ($p<0.05$, $p<0.01$, $p<0.001$, respectively) groups' renal cells demonstrated considerable reductions in nNOS, iNOS and eNOS.

According to fig. 4, the DOX-challenged group exhibited considerably elevated amounts of nNOS, iNOS, eNOS and NF-B p65 compared to the control group, with a p-value of 0.001. Meanwhile, the LT-treated group had lower levels of nNOS, iNOS and NF-κB p65 proteins ($p<0.05$) than the DOX group. According to this study, the BL group had significantly lower expressions of nNOS, INOS, ENOS and NF-κB p65 ($p<0.05$) compared to the DOX group, while the nNOS, iNOS, eNOS and NF-κB p65 proteins were significantly reduced in rats exposed to DOX after treatment with BL+LT compared to the control group ($p<0.001$).

The impact of BL and LT therapy on DOX-induced histopathological changes

The histopathological changes in the renal tissue of the control, BL- and LT-treated DOX-challenged rats are shown in fig. 5. BL and LT reduced the effect on DOX-induced histopathologic changes in renal tissue. The renal cortices from control animals had normal glomerular and renal tubular structures (fig. 5-A), while the DOX-challenged treated rats had highly concentrated protein casts (arrow), glomerular hemorrhage and degenerated tubes (fig. 5-B). Meanwhile, renal tissue from LT-treated rats had minor partial pathologic injuries (fig. 5-C), the renal tissue of rats treated with BL showed mild degenerative renal cells (fig. 5-D) and the renal tubules and glomeruli of BL- and LT-treated rats showed complete regeneration (fig. 5-E).

DISCUSSION

Renal toxicity is caused by a series of events involving oxygen molecules that culminate in ROS creation (Kaushal *et al*., 2019). DOX is responsible for generating free radicals, including hydroxyl, hydrogen peroxide (H_2O^2) and superoxide, which is evidence that lipid peroxidation occurs rapidly with lipids (Fujii *et al*., 2022). The most reliable nephritis indicators, such as urea and creatinine serum concentrations, are elevated when oxidative stress affects the kidneys (Gyurászová *et al*., 2020). DOX treatment has a renal impact, resulting in increased serum urea and creatinine concentrations in the blood (Afsar *et al*., 2020). According to the findings of several studies conducted on rats, malondialdehyde (MDA) levels rose because of the DOX injection, which in turn caused an increase in lipid oxidation and, as a consequence of the enhanced oxidation, a more substantial degree of lipid peroxidation (Refaie *et al*., 2016). Antioxidants, such as SOD, CAT, GPx and GST, in addition to such non-enzymes as GSH, are enzymes that function within the cell to combat the consequences of oxidative stress (Vona *et al*., 2021). In earlier investigations, we showed that treatment with DOX results in low levels of these enzymes or non-enzymatic antioxidants. Thus, rats treated with DOX exhibited lower levels of SOD and CAT than the untreated controls, according to Oguz *et al*. (2016). In addition, when free radicals grow, SOD and CAT activity, which protect the cell membrane and its contents, decreases significantly and oxidative stress results in low levels of SOD and CAT, increasing the amount of H_2O_2 in cells (Ighodaro & Akinloye, 2018). Toxins are mostly eliminated and reduced in cells affected by GSH and low levels of GSH weaken the body's ability to fight against ROS (Oguz *et al*., 2016). It has been hypothesized that there is a relationship between GPx and GSH, because earlier studies have shown that decreased GPx activity could have played a role in determining the availability of GSH in comparison to the DOX-treated group and control groups (Shabalala *et al*., 2017).

Increases in TNF- α, IL-1, NF-κB p65 and NO are seen after DOX treatment, as DOX may have a direct or indirect function in NO metabolism, depending on the number of free radicals generated. The toxicity of DOX is exacerbated by the formation of free radicals and NO released from DOX (Afsar *et al*., 2020). According to several research findings, adequate levels of NO in the bloodstream are necessary for healthy kidney function (Carlstrom, M. 2021). When NO production is too high, it causes oxidative stress in the body and tissue damage, which may lead to acute kidney failure (Ratliff *et al*., 2016).

We used the flavonoid antioxidants BA and LT to prevent the toxic symptoms caused by DOX. We showed that BL and/or LT reduced the serum concentrations of creatinine and urea in DOX-challenged rats, suggesting a protective effect on renal cells. Another indicator of oxidative stress, TBARS, decreased and returned to normal in the groups treated with BL and/or LT. Antioxidants, whether catalytic or noncatalytic, produced by enzymes or without enzymes, are found in low concentrations in DOX-challenged rats given LT, BL, or LT+BL therapy.

Cytokines that promote inflammation play a role in the early stages of acute inflammation. According to other studies' findings, BA can decrease inflammation by stopping its synthesis via NF-kB-controlled COX-2 and iNOS (Kim *et al*., 2013).

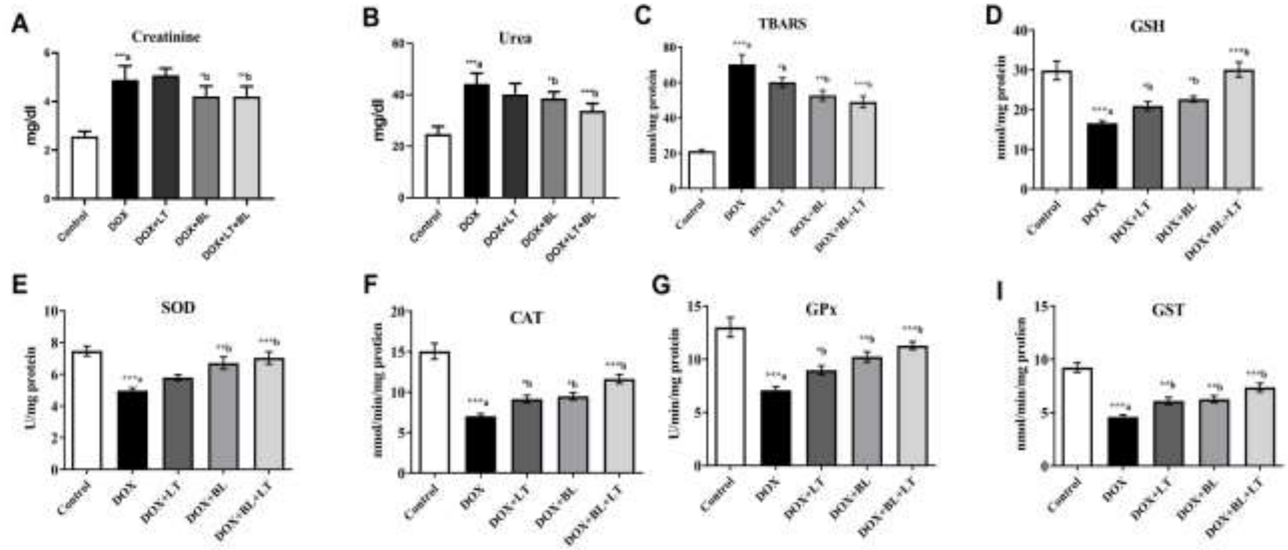

Fig. 1: Effect of baicalein (BL) and/or losartan (LT) on doxorubicin (DOX)-induced changes in serum levels of creatinine and urea, oxidative stress biomarkers including thiobarbituric acid reaction substances (TBARS) and glutathione (GSH) levels, enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in renal cells. Data were expressed as Mean±S.D., (n=6) and analyzed using one-way ANOVA followed by Student Newman-Keuls as post hoc test. [a] Control vs DOX group; [b] DOX vs DOX+BL or DOX vs DOX+LT or DOX vs DOX+BL+LT. P values consider significant when $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

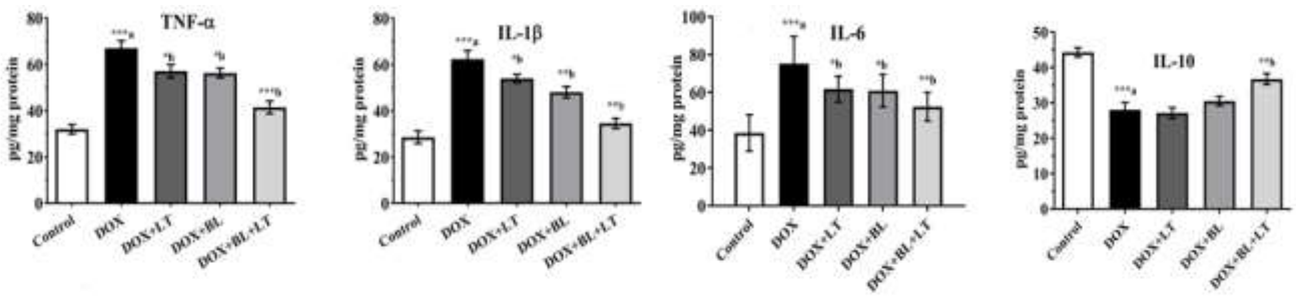

Fig. 2: Effect of baicalein (BL) and/or losartan (LT) on doxorubicin (DOX)-induced changes in renal levels of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) and inyrtleukin-10 (IL-10). Data were expressed as Mean±S.D., (n=6) and analyzed using one-way ANOVA followed by Student Newman-Keuls as post hoc test. [a] Control vs DOX group; [b] DOX vs DOX+BL or DOX vs DOX+LT or DOX vs DOX+BL+LT. P values consider significant when $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

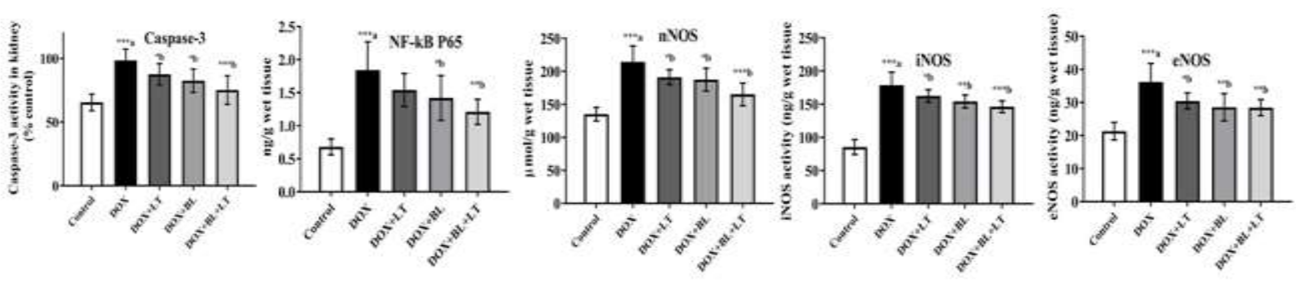

Fig. 3: Effect of baicalein (BL) and/or losartan (LT) on doxorubicin (DOX)-induced changes in renal activities of inflammatory biomarkers including caspase-3, nuclear factor Kappa-B (NF-kB p65), n-nitric oxide syntheses (nNOS), inducible nitric oxide synthase (iNOS) and endothelialnitric oxide synthase (eNOS) were measured by ELISA system. Data were expressed as Mean±S.D., (n=6) and analyzed using one-way ANOVA followed by Student Newman-Keuls as post hoc test. [a] Control vs DOX group; [b] DOX vs DOX+BL or DOX vs DOX+LT or DOX vs DOX+BL+LT. P values consider significant when $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

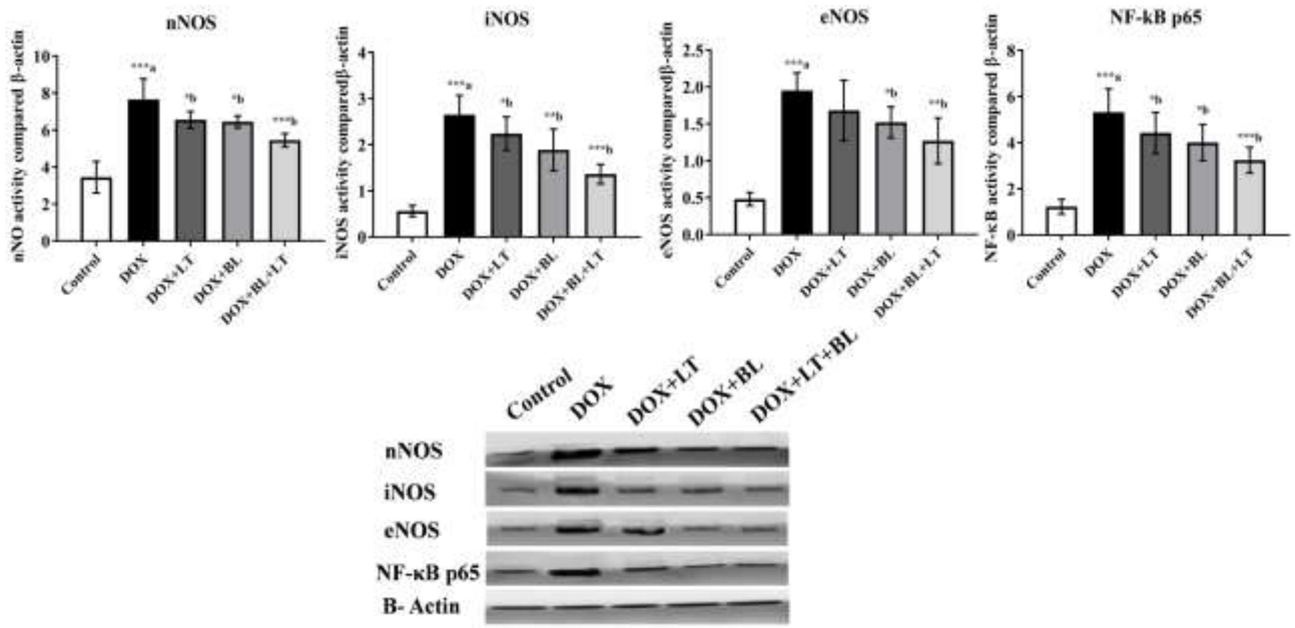

Fig. 4: Effect of baicalein (BL) and/or losartan (LT) on doxorubicin (DOX)-induced changes in renal protein expressions ofn-nitric oxide syntheses (nNOS), inducible nitric oxide synthase (iNOS), endothelialnitric oxide synthase (eNOS) and nuclear factor Kappa-B (NF-kB p65)measured by western blots. Data were expressed as Mean ± S.D., (n=6) and analyzed using one-way ANOVA followed by Student Newman-Keuls as post hoc test. [a]Control vs DOX group; [b] DOX vs DOX+BL or DOX vs DOX+LT or DOX vs DOX+BL+LT. P values consider significant when $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$.

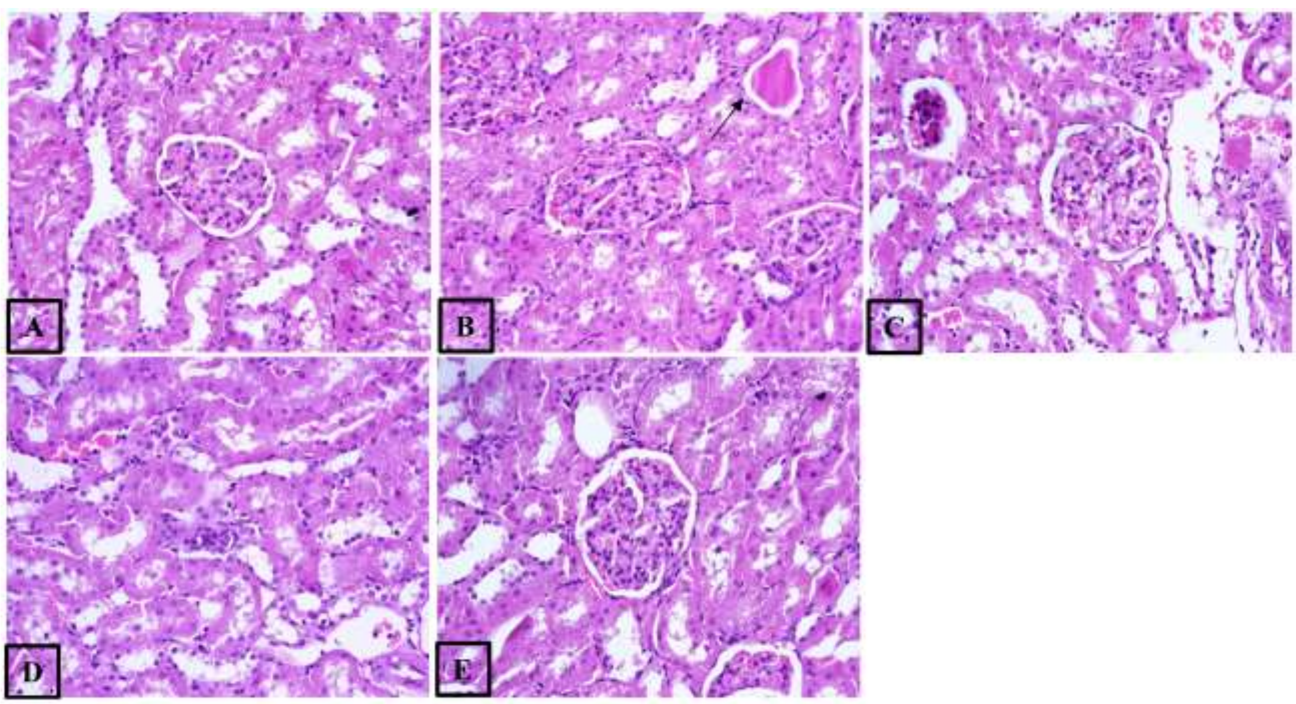

Fig. 5: Effect of baicalein (BL) and/or losartan (LT) on doxorubicin (DOX)-induced histopathological changes in renal tissue. (A) Section from cortex control showing normal architecture of glomeruli and renal tubules. (B) Section from DOX showing marked congestion, glomerular hemorrhage and degenerative tubules with abundant protein casts (arrow). (C) Section from LT showing partial attenuation in the severity pathological damage. (D) Section from BL showing mild degenerative renal cells. (E)Section from LT and BL showing marked improvement in the architecture of glomeruli and renal tubules. Scale bar = 50 μm.

According to the findings of our investigation, treatment with DOX led to an increase in the levels of three essential cytokines that contribute to inflammation: TNF-, IL-1 and IL-6. In addition, we found that inflammation activates NO, iNOS, eNOS, NF-κB p65 and caspase-3, all of which were increased due to the harmful effects of DOX. BL and LT individually inhibited both inflammatory progression and oxidative stress; however, the maximum effect was found in the BL+LT group, while a highly antioxidative effect was observed in the group that received a combination of BL and LT. A limitation of this study was that the gene expressions of inflammatory activities were not measured to confirm the results of the protein expressions in renal tissues.

CONCLUSION

The findings of this study show that BL and LT demonstrated considerable therapeutic efficacy in terms of anti-oxidant and anti-inflammatory effects in rats treated with DOX and combined administration of these compounds significantly enhanced their protective effects. We suggest that the synergistic action of the combined therapy was significant in restoring the systemic histological features of tissues and it may contribute to reducing renal oxidation injuries by inhibiting ROS formation and promoting antioxidant enzymatic imbalances and anti-inflammatory mechanisms.

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