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Research Article
Hypericum perforatum Extract Attennuates Gentamicin Induced Oxidative Stress, Apoptosis and Oedema in Kidney

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Abstract

Background and Objective: *Hypericum perforatum*, which have various names locally such as St. John Wort, is a plant which blooms between July and September at farms, borders of roads and woods, top of hills and grasslands, which is regulator of apoptotic mediators are shown in various studies. Also, it is known that gentamicin activates apoptotic mediators and causes necrosis in kidney. Due to this reason, a study was planned to examine the protective effects of Hypericum perforatum on nephrotoxicity caused by gentamicin. **Materials and Methods:** Mice were divided into three groups; control group, gentamicin group and perforatum extract group. About 100 mg kg^{-1} gentamicin and 70 mg kg^{-1} H. perforatum extract are administered to related groups for 9 days. Renal tissue samples and blood samples are obtained for biochemical analysis. caspase-3, bax and bcl-2 proteins are analyzed by using ELISA method. **Results:** Gentamicin administration increased caspase-3, bax while it decreased bcl-2. H. perforatum administration to mice that are administered gentamicin previously decreased the rate of caspase-3, bax caused by gentamicin while it increased bcl-2. Gentamicin also increased serum BUN, creatinine, TOS and TNF-α levels and renal MDA levels significantly and decreased catalase (CAT) and glutathione (GSH) significantly compared to other groups. Gentamicin+perforatum extract treatment reversed these factors compared to the gentamicin group ($p<0.05$). **Conclusion:** Histopathologic examination revealed severe edematous damage gentamicin group and reduced edematous damage in the gentamicin+perforatum extract group. Hypericım perforatum extract provides renal protection against gentamicin-induced nephrotoxicity through its antioxidant and anti-inflammatory effects.

Key words: Gentamicin, nephrotoxicity, *Hypericum perforatum*, edematous damage, apoptosis and apoptotic mediators

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hypericum perforatum is a herb flowering in the fields, on the roads near forests, hills and meadows from July to September and has different names such as St. John Wort and utilized through local people against certain diseases[1-3]. It has been reported in the previously conducted studies that this plant has very strong anti-inflammatory effects. A study has revealed that application of centaury has inhibited LSP induced cyclo-oxygenase-2 and iNOS enzymes[4]. It has also been shown that hyperforin, one of the bioactive agents of perforatum is a powerful cyclo-oxygenase-1 and 5-lipoxygenase inhibitor[5]. Furthermore, perforatum application also reduces the synthesis of prostaglandins associated with prostaglandin inflammation[6]. The perforatum extract used in this study has potential anti-oxidative properties due to the contained flavonoids and phenolic compounds[7,8]. It has been shown in many studies that cyclo-oxygenase pathway involved in inflammation accompanies in gentamicin nephrotoxicity. It has been reported in a study that perforatum reduces the gentamicin induced nephrotoxicity by selective cyclo-oxygenase-2 inhibition[9]. Furthermore, it has also been shown that gentamicin application leads to an increase in the activity of Phospholipase A2[10]. In another study, it has been revealed that nephrotoxicity created by gentamicin has led to increase in iNOS enzyme[11]. Increasing renal oxidative stress also has a role in nephrotoxicity caused by gentamicin. It leads to necrosis in nephrons by increasing oxidative stress, inflammatory mediators such as NF-kB, TNF-α and IL-6 and apoptotic proteins such as caspase-3, caspase-9, Bax and Bcl-2[12].

Studies have shown the fact that one of the main causes of acute intrinsic renal failure is aminoglycoside nephrotoxicity. Nephrotoxicity can be defined as nephrotoxic renal failure. The kidneys are susceptible to being affected toxic effects of drugs and other endogenous and exogenous toxins due to the excretion function arising from high rate of blood perfusion and metabolic activity. Active tubular secretion reabsorption and urine concentration mechanisms and renal tubular cells in the kidneys encounter high toxin concentrations more intensively than other body tissues. Therefore, they are direct targets of renal tubular nephrotoxicity. Evident feature of nephrotoxicity is tubular necrosis. As such, it is called as acute tubular necrosis[13].

Tubular necrosis is a programmed cell death which functions in the removal of unwanted tissues at apoptosis embryogenesis stage and reshaping of the tissue, in causing development and homeostasis and aging in subsequent years and in the elimination of all the infective tissues which have been damaged and transformed throughout the life process and healthy tissues which have completed their life expectancy. Bcl-2 family and caspases are major mediators in the apoptotic pathway. Caspase-3 is induced by intrinsic pathway by activation of caspase-8 and 10 and formation of death-inducing signaling complex and by extrinsic pathway by activation of caspase-9 and apoptosome complex. Caspase-3 causes typical DNA fragmentation in this last step. Bcl-2 family has both apoptotic and anti-apoptotic members. Proapoptotic or antiapoptotic signals affect mitochondria according to the balance between Bcl-2 proteins. If apoptotic signals are dominant pressure cytochrome c is released from mitochondria to create apoptosome complex. Inhibition of apoptosis causes hyperplasia and cancer while triggered apoptosis causes organ failure[14-16]. Apoptosis defects are important in developmental, autoimmune and neuro-degenerative diseases and in the development of cancer.

As such, this study was planned to find out if perforatum herb, which is shown to have powerful anti-inflammatory and anti-oxidative properties, will be effective on necrosis caused by apoptosis induced by gentamicin. For this purpose, gentamicin and gentamicin with perforatum extract are given to mice and the mediators of apoptosis in their kidneys which are caspase-3, bax and bcl-2 proteins are analyzed by using ELISA method.

MATERIALS AND METHODS

Eight weeks old balb\c albino mice were obtained from Cukurova University Animals Research Center and placed in a temperature (21±2°C) and humidity (60±5%) controlled room in which a 12:12 h light:dark cycle was maintained. The mice were fed with standard commercial pellets and water ad libitum. This study was approved by the Animal Care Committee and Ethics Committee of Çukurova University. All of the procedures were performed according to accepted standards of Guide for the Care and Use of Laboratory Animals.

Mice are divided into three groups which are; control group, gentamicin group and perforatum extract group. About 100 mg kg^{-1} gentamicin is applied intraperitoneally once a day to gentamicin group for 9 days. To perforatum group, 100 mg kg^{-1} gentamicin is applied intraperitoneally together with 70 mg kg^{-1} perforatum extract which is applied by using a gavage for 9 days. Physiological serum is applied intraperitoneally to control group under same experimental conditions for 9 days. After 24 h after the last dose,

the rats were anesthetized by ketamine (60 mg kg^{-1}, i.p.) and xylazine (10 mg kg^{-1}, i.p.) and the animals were decapitated after intracardiac blood sampling. Following a midline abdominal incision, the left kidney was removed for histopathologic examination and the right kidney was removed for biochemical analysis. The tissues were fixed in a 10% formalin solution and sent for pathological analysis. The blood samples and the renal tissues were stored at -80°C. Biochemical analysis included assessment of serum levels of blood urea nitrogen (BUN), creatinine, total antioxidant status (TAS), total oxidant status (TOS) and tumor necrosis factor alpha (TNF-α). In the renal tissues, malondialdehyde (MDA), catalase activity (CAT) and glutathione levels were measured.

Biochemical analysis

Measurement of BUN, creatinine, Na and K: Serum levels of BUN and creatinine were spectrophotometrically measured in an auto-analyzer (Architect c8000, Clinical Chemistry Analyzer, Abbott, USA).

Measurement of TAS and TOS: Serum TOS and TAS levels were measured by a novel colorimetric method proposed by Erel(Rel Assay Diagnostics kits, Mega Tip, Turkey)[17,18]. The TOS levels were expressed as micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L) and TAS levels were expressed as mmol Trolox Eq/L.

Measurement of TNF-α: Following blood collection, the blood samples were centrifuged at 1,500 rpm for 15 min. The serum samples were stored at -80°C for biochemical analysis. The TNF-α levels in the serum and peritoneal fluid samples were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Awareness Technology Inc., ChroMate Elisa Reader, USA) and were expressed as pg/mL protein.

Antioxidant enzyme activities

Measurement of catalase activity (CAT): Catalase activity was measured using the method proposed by Dolmanova and Ugarova[19]. Monitoring of the decomposition of the substrate H_2O_2 was achieved by using the spectrophotometric method at 240 nm (Shimadzu UV 1601, Japan). The calculation of CAT was based on the change in absorbance. The CAT was expressed as k mg^{-1} protein [k: rate constant of the first order reaction as defined by Aebi].

Measurement of glutathione peroxidase (GSH-Px) activity: GSH-Px activity was measured using the method proposed by Paglia[20]. The enzymatic reaction was initiated by adding H_2O_2 to the reaction mixture containing reduced glutathione, reduced nicotinamide adenine dinucleotide phosphate (NADP+) and glutathione reductase. The change in the absorbance at 340 nm was monitored spectrophotometrically. One unit of GSH-Px was defined as micromoles of NADPH oxidized per minute. Activity was given in units per mg protein.

Measurement of MDA levels: The MDA levels were measured using the double heating method proposed by Draper and Hadley[21]. This method is based on spectrophotometric measurement of the end product of lipid peroxidation with thiobarbituric acid at 532 nm. The calibration curve was formed by using commercially available MDA equivalents (1,1,3,3-tetramethoxypropane, Lot no, MKBP9901V, Sigma-Aldrich) and the MDA results were expressed as nmol mg^{-1} protein.

Plant extraction: Perforatum plant is grinded vigorously after being dried in an incubator. Then, it is mixed with 80% alcohol in 12:1 (alcohol:plant) ratio and put in shaker for 24 h at room temperature. After 24 h, it is filtered then alcohol is evaporated by using an evaporator and plant extract is obtained.

Quantitative analysis

Tissue homogenization: About 3 mL g^{-1} RIPA (Radio-immunoprecipitation Assay) buffer, 30 μL PMSF (phenylmethanesulfonyl fluoride), 30 μL sodium vanadate, 30 μL protease inhibitor is applied on frozen tissue samples that are stored in Eppendorf tubes then homogenates are obtained by using ultrasonication on those tubes on ice. Homogenates are then centrifuged at 10.000 rpm for 10 min and supernatants are taken and pellets are discarded.

Protein quantification: Bradford method is used to quantify the total protein in homogenized tissues. By using Bovine serum albumin (1 μg mL^{-1}), 1, 2, 3, 5, 7, 8, 10 (μg mL^{-1}) standards are prepared. About 10 μL is taken from every sample and completed to 100 μL by adding distilled water. One milliliter Bradford solution is added to standards and samples, vortexed and absorbances at 595 nm are measured manually. Protein quantification (μg μL^{-1}) is done according to the standard curve drawn in Prism software.

ELISA (enzyme linked immunosorbent assay) test: The ELISA test is used to examine the expression of caspase-3, bax and bcl-2.

Histopathology assessment: Isolated colon tissues for histologic examination were spread onto a plastic sheet, fixed in 3.7% formalin for 24 h and prepared for paraffin tissue slides. The paraffin sections were stained with hematoxylin and eosin.

Statistic analyzes: Results were expressed as Means±SEM. and n refers to the number of animals used for each experiments. Differences in results between tissues were tested by one way analysis of variance (one way ANOVA) corrected for multiple comparisons (Bonferroni corrections). The p-values less than 0.05 were considered to be significant.

RESULTS

While gentamicin application caused an increase in the activity of caspase-3 in kidneys, application of perforatum extract decreased this increase significantly. Means values of caspase-3 activity for control, gentamicin and gentamicin+extract groups are found to be 4.907(SEM 0.09), 8.711(SEM 0.19) 5.007(SEM 0.18) respectively (Fig. 1).

While gentamicin application caused an increase in the bax in kidneys, application of perforatum extract decreased this increase significantly. Means values of bax concentrations for control, gentamicin and gentamicin+extract groups are found to be 1.629 (SEM 0.23), 8.367 (SEM 0.24) 3.513 (SEM 0.54), respectively (Fig. 2).

While gentamicin application caused a decrease in the bcl-2 in kidneys, application of perforatum extract increased this decrease significantly. Means values of bcl-2 concentrations for control, gentamicin and gentamicin+extract groups are found to be 8.786 (SEM 0.33), 1.405 (SEM 0.35) 7.541 (SEM 0.69) respectively (Fig. 3).

In histopathological study while gentamicin application caused oedematous damage and lymph node application of perforatum extract attenuated edema (Fig. 4-6).

Data in Table 1 presented the effect of extract on oxidative stress in gentamicin-induced nephrotoxicity. No significant difference was found among the groups with regards to TAS levels ($p>0.05$). The TOS levels in the gentamicin group were significantly higher compared to other groups ($p<0.05$) and the TOS levels in the gentamicin+extract group were significantly lower compared to the gentamicin group ($p<0.05$) (Table 1).

Serum BUN levels were significantly higher in the gentamicin group compared to other groups ($p<0.05$) and were significantly lower in the gentamicin+extract group compared to the gentamicin group ($p<0.05$) (Table 2).

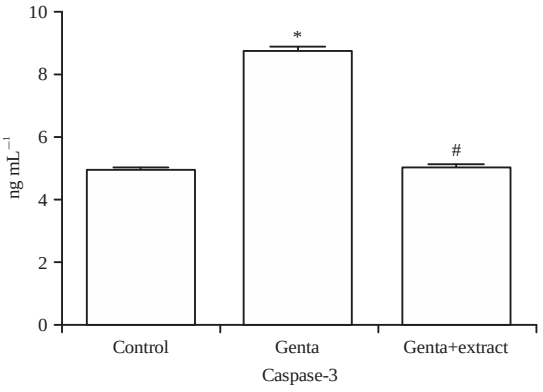

Fig. 1: Effect of perforatum extract on caspase-3 of the gentamicin applied mice (n = 6)
Statistical analysis: ANOVA. *Post hoc:* Bonferroni. (*For control $p<0.05$, #For gentamicin $p<0.05$)

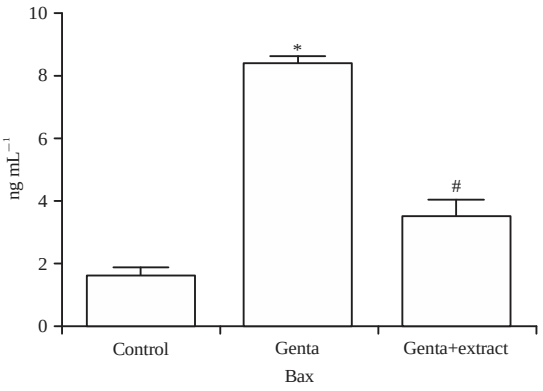

Fig. 2: Effect of perforatum extract on bax of the gentamicin applied mice (n = 6)
Statistical analysis: ANOVA. *Post hoc*. Bonferroni. (*For control $p<0.05$, #For gentamicin $p<0.05$)

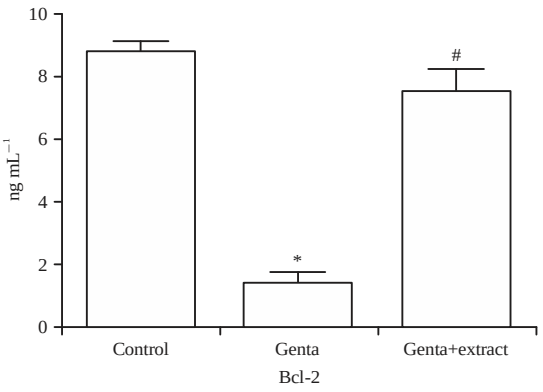

Fig. 3: Effect of perforatum extract on bcl-2 of the gentamicin applied mice (n = 6)
Statistical analysis: ANOVA. *Post hoc:* Bonferroni. (*For control $p<0.05$, #For gentamicin $p<0.05$)

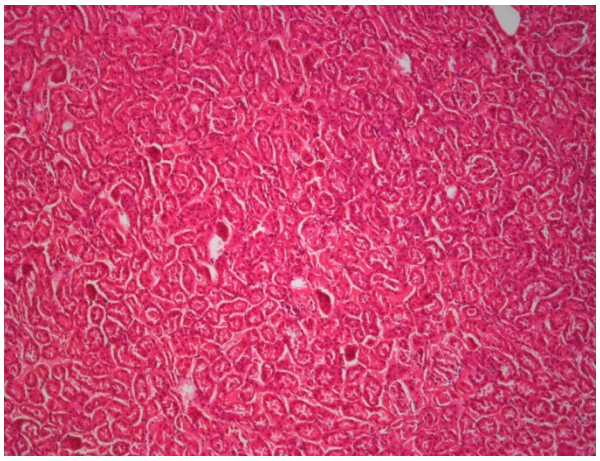

Fig. 4: Kidney of mice in control group were subjected to H and E

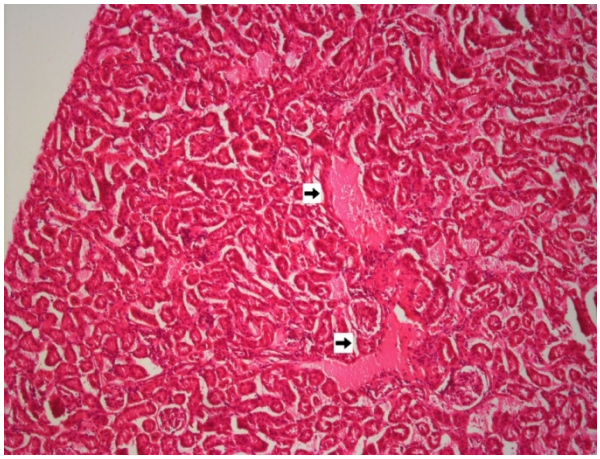

Fig. 5: Kidney of mice in gentamicin treated group were subjected to H and E

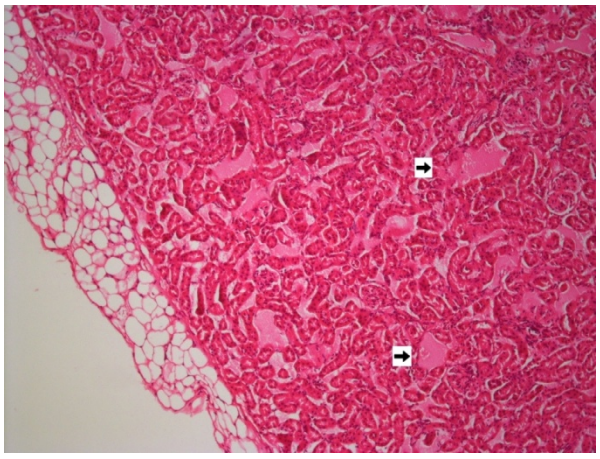

Fig. 6: kidney of mice in gentamicin together extract treated group were subjected to H and E

Table 1: Effect of *Hypericum perforatum* extract on oxidative stress in gentamicin-induced nephrotoxicity

Parameters	Control	Gentamicin	Genta+extract
TAS	0.88±0.05	0.87±0.02	0.92±0.07
TOS	10.50±2.00	28.00±4.00*	15.10±2.2#

Data were expressed as Mean±SE, *p<0.05 for the comparison between the gentamicin group and the control, extract and gentamicin+extract groups, #p<0.05 for the comparison between the gentamicin group and gentamicin+extract group. TAS: Total antioxidant status (μmol Trolox Eq/L), TOS: Total oxidant status (μmol H_2O_2 Eq/L)

Table 2: Serum BUN, creatinine, Na and K levels of the groups

Parameters	Control	Gentamicin	Genta+extract
BUN	17.2±1.80	105.80±8.0*	25.5±8.00#
Creatinine	0.5±0.08	2.33±0.5*	0.7±0.05#

Data were expressed as Mean±SE. *p<0.05 for the comparison between the gentamicin group and the other groups, #p<0.05 for the comparison between the gentamicin group and the gentamicin+extract group, BUN: mg dL^{-1}, Creatinine: mg dL^{-1}

Table 3: Serum TNF-α levels of the groups

Parameters	Control	Gentamicin	Genta+extract
TNFα	103±5	789.3±81*	255.3±35#

Data were expressed as Mean±SE, *p<0.05 for the comparison between the gentamicin group and the other groups, #p<0.05 for the comparison between the gentamicin group and the gentamicin+extract group, TNF-α: pg mL^{-1}

Table 4: MDA, catalase and GSH levels in renal tissue

Parameters	Control	Gentamicin	Genta+extract
MDA	0.84±0.12	2.9±0.05*	0.91±0.09#
Catalase	68.00±0.05	21.0±0.03*	58.00±4.00#
GSH	0.55±0.07	0.22±0.05*	0.48±0.01#

Data were expressed as Mean±SE, MDA: Malondialdehyde (nmol mg^{-1} protein), Catalase: (μ mg^{-1} protein), GSH: Reduced glutathione (U mg^{-1} protein), *p<0.05 for the comparison between the gentamicin group and other groups, #p<0.05 for the comparison between the gentamicin group and the gentamicin+extract group

Serum creatinine levels were significantly higher in the gentamicin group compared to other groups (p<0.05) and were significantly lower in the gentamicin+extract group compared to the gentamicin group (p<0.05) (Table 2).

Serum TNF-α levels were significantly higher in the gentamicin group compared to other groups and were significantly lower in the gentamicin+extract group compared to the gentamicin group (p<0.05) (Table 3).

The MDA levels in the renal tissues were significantly higher in the gentamicin group compared to other groups and were significantly lower in the gentamicin+extract group compared to the gentamicin group (p<0.05). The catalase levels in the renal tissues were significantly lower in the gentamicin group compared to other groups and were significantly higher in the gentamicin+extract group compared to the gentamicin group (p<0.05). Renal GSH levels were significantly higher in the gentamicin group compared to other groups and were significantly higher in the gentamicin+extract group compared to the gentamicin group (p<0.05) (Table 4).

DISCUSSION

Increasing renal oxidative stress plays a role in nephrotoxicity caused by gentamicin. Oxidative stress leads to necrosis in nephrons by increasing the rate of inflammatory mediators such as NF-kB, TNF-α and IL-6 and apoptotic proteins such as caspase 3, caspase 9, Bax and Bcl-2[12]. Gentamicin administration has increased the apoptotic proteins bax and caspase-3 activity similarly in this study while decreasing anti-apoptotic protein bcl-2. Findings of this study reveal the fact that gentamicin leads to necrosis in nephrons. Gentamicin application activates the rhoA/rho-kinase signaling pathway. On the other hand, there are studies which show that there is a strong correlation between oxidative stress and rhoA/rho-kinase[22,23]. In the previous studies, it was observed that rhoA/rho-kinase signaling pathway is effective in the regulation of apoptosis. It has been revealed that Rho-kinase and rho-kinase II are cut and activated in execution phase of apoptosis. Myosin light chain phosphorylation induced by active rho-kinase leads to formation of membrane buds. Furthermore, ROCK is known to be effective in cell lysis and other steps of apoptosis such as phagocytosis of apoptotic bodies[24]. It has been reported that rhoA regulates the JNK signaling pathway causing apoptosis[25]. Furthermore, it has been reported in another study that rhoA/rho-kinase signaling pathway also regulates PI3K/AKT/caspase-3 signal pathway[26]. Activation of rhoA/rho-kinase pathway which has association with apoptosis pathway by gentamicin also supports authors' findings.

Perforatum herb contains numerous active agents. Among these agents, substances such as apigenin, apigalactecin and hyperforin affects apoptosis. It has been shown in some cancer studies that perforatum extract induces apoptosis[27-29].

Epigallocatechin, an ingredient of perforatum, activates caspase-3 a proapoptotic mediator in some cancer cells[27-30]. Furthermore, application of apigenin, a substance which centaury contains, to human lung cancer cells has increased the expression of proapoptotic p53 and bax proteins and caused activation of caspase-3 and reduced the expression of anti-apoptotic BLC-2 protein. Hyperforin, another ingredient of perforatum, has increased apoptosis by causing activation of caspase-3 and inhibition of bcl-2 protein expression in human myeloid cancer cells[29]. Moreover, it has been shown that growth of breast cancer cells in mice are stopped by application of perforatum extract[31].

This study, in contrast to the studies mentioned above, has shown that perforatum protects against apoptosis. This means that perforatum extract is a regulator of apoptosis. In a previous study, authors have shown that perforatum extract decrease the expression of COX-2 and iNOS induced by gentamicin[32]. Furthermore, in another study, authors revealed that alpha-linolenic acid known as omega fatty acid and which had antioxidant properties is protective against gentamicin-induced nephrotoxicity[33]. It is thought that perforatum extract protects from apoptosis because flavonoids and phenolic compounds which it contains have strong anti-oxidative and anti-inflammatory features. In addition, because inhibition of inflammatory mediators will prevent migration of monocytes and macrophages it will also prevent renal damage.

The TNF-α have inflammatory, cytotoxic and angiogenic properties[34]. In this study, TNF-α levels were found significantly higher in the gentamicin group and were found significantly lower in the gentamicin+perforatum extract group compared to gentamicin groups. Also, anti-inflammatory effects of *Hypericum perforatum* extract in gentamicin induced renal inflammation is shown[32].

The serum creatinine concentration is higher than the serum BUN concentration in the early stages of renal disease. Serum BUN concentration starts to increase only after significant renal parenchymal injury[35]. In this study, gentamicin caused a significant increase in serum BUN and creatinine levels, whereas gentamicin+perforatum extract administration resulted in a significant decrease in serum BUN and creatinine levels. These results show that the reduced glomerular filtration associated with ROS leads to increased levels of BUN and creatinine and that the antioxidant effect of perforatum extract inhibits ROS production and thus leads to a decrease in BUN and creatinine concentrations.

This study also evaluated TAS and TOS levels. The results showed a significant increase in TOS levels, suggesting that gentamicin causes increased oxidative stress. Furthermore, the administration of gentamicin+perforatum extract led to a significant increase in TOS, indicating that perforatum extract decreased gentamicin induced oxidative stress.

Glutathione (GSH) acts as a radical scavenger that protects the cells against free radicals. Gentamicin has been shown to decrease GSH concentration either due to excessive production of free radicals including superoxide anion and hydrogen peroxide or excessive consumption of sulfhydryl (SH) groups of proteins[36].

Excessive production of ROS leads to the consumption of renal antioxidant enzymes such as catalase (CAT). In lots of studies, gentamicin has been shown to decrease expression of CAT[36]. In this study, GSH and CAT levels were also significantly decreased in the gentamicin group and were significantly increased in the gentamicin+perforatum extract group compared to other groups.

The MDA is a common indicator of peroxidative damage used in the determination of lipid peroxidation due to excessive ROS production[37]. In this study, as reported in previous studies, gentamicin led to a significant increase in MDA levels[37,38]. On the other hand, MDA levels were significantly decreased in the gentamicin+perforatum extract group compared to the gentamicin group.

CONCLUSION

This study has shown that perforatum herb which has anti-inflammatory and strong antioxidative properties has protective effect on necrosis caused gentamicin-induced apoptosis.

However, this study will be useful for the treatment of some toxicities which have similar pathology with gentamicin.

SIGNIFICANCE STATEMENT

Hypericum perforatum extract provides renal protection nephrotoxicity by decreasing caspase-3, bax and by increasing bcl-2. This study will provide information for future studies about the nature of nephrotoxicity and the antioxidant properties of Hypericum perforatum. The significance of use of gentamicin has decreased recently. Authors used gentamicin in this study because it constitutes a model in nephrotoxicity.

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