Effect of camphor on biochemical factors and gene expression of antioxidant enzymes, inflammatory and apoptotic factors against gentamicin-induced nephrotoxicity in rats

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ABSTRACT

Introduction: Camphor is a natural antioxidant with anti-inflammatory and tissue repair properties. Nephrotoxicity is the most important side effect of gentamicin (GEM) administration. Therefore, investigating the effect of natural antioxidants can resolve this complication.

Objectives: We aimed to assay the effect of camphor on biochemical factors and gene expression of antioxidant enzymes (catalase [CAT], glutathione peroxidase [GPX]) and inflammatory markers (tumor necrosis factor-alpha [TNF-α], nuclear factor kappa-B [NF-κB], interleukine-6 [IL-6]), and apoptotic indices (BCL2-associated X protein [Bax], B-cell lymphoma 2 [Bcl-2], caspase-3], against GEM-induced nephrotoxicity in rats.

Materials and Methods: Thirty adult male Wistar rats were allocated to five groups. Positive control and treatment groups were given GEM to induce nephrotoxicity. Animal treatment groups were treated with camphor in olive oil for 12 days. Renal biopsies, serum, extraction of renal tissue and urine of rats were taken after the twelfth day. Biopsies were examined for structural changes using a light microscope, moreover, apoptosis, desired biochemical and inflammatory factors, were investigated by suitable methods.

Results: Camphor had no effect on biochemical factors, including malondialdehyde (MDA), glutathione (GSH), nitric oxide (NO), urea, creatinine and urine protein. However, it reduced the gene expression of TNF-α, NF-κB, IL-6, and caspase-3 and increased the gene expression of GPX and CAT and Bcl-2. Moreover, camphor improved kidney histopathological changes in the camphor groups in comparison with the GEM group.

Conclusion: Camphor can be useful in the attenuation of GEM-induced nephrotoxicity based on expression levels of examined enzymes and factors and improving kidney histopathological changes.

Implication for health policy/practice/research/medical education: It has been proposed that nephrotoxicity is the most important side effect of gentamicin use, in addition, camphor is a natural antioxidant. The present study aimed to evaluate the effect of camphor on biochemical factors and gene expression of antioxidant enzymes, inflammatory and apoptotic factors against gentamicin- induced nephrotoxicity in rats. Camphor can be useful in the attenuation of gentamicin-induced nephrotoxicity. However, more studies with more experimental groups and longer follow-up are necessary to confirm the results of this study.

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Introduction

The kidney is an essential organ of the body, which performs several vital functions, such as maintaining the body's homeostasis and organizing the extracellular environment (detoxification, excretion of toxins and drugs) (1). The kidneys constitute less than 0.5% of the body's weight and they receive 20% to 25% of cardiac output; thus, they are more prone to toxic damage (2). The kidneys have a significant role in excreting most drugs; since some drugs may have side effects on kidney function (3).

Aminoglycoside antibiotics including amikacin, neomycin, streptomycin, gentamicin (GEM) and tobramycin are frequently used to treat infections caused by gram-negative bacteria (4). The main complication induced by these antibiotics is nephrotoxicity (5). Nephrotoxicity is defined as the poisonous effect of substances on renal function (6). GEM, the most commonly used aminoglycoside, is associated with inducing the production of free radicals, decrease of antioxidant defenses and acute tubular necrosis which consequently motivates reducing glomerular filtration rate (GFR) and renal impairment (7).

Mechanisms of GEM-induced nephrotoxicity are associated with induction of transforming growth factor-beta 1 (TGF-β), endothelin-1, macrophages or monocyte infiltration into the renal cortex and medulla, oxidative stress and necrosis, and apoptosis (8). GEM is capable of increasing the number of reactive oxygen species (ROS) such as superoxide anions (O₂⁻), hydroxyl radicals (-OH), and hydrogen peroxides (H₂O₂). Moreover, GEM is able to raise reactive nitrogen species (RNS) in the renal cortex, which may lead to impaired kidney function and structure (9).

Numerous studies have been conducted to investigate the effect of antioxidants on GEM-induced nephrotoxicity (10). Camphor is a natural antioxidant and a ketone terpenoid with the chemical formula C₁₀H₁⁰O₂ that is found in the Cinnamomum camphora tree (11). Researches have shown that camphor effectively reduces pain, removes warts, treats hemorrhoids and osteoarthritis. Furthermore, it was suggested that it has anti-inflammatory, antioxidant and antimicrobial properties (12).

Rats are similar to humans in terms of anatomy, genetics and physiology; therefore, they are used as a preferred animal in many experimental studies (13). Although the pathogenesis of renal is complex, rats were employed in several studies as experimental animals to investigate kidney function and treatment in human (14).

Objectives

The primary object of the present study was to evaluate the effect of camphor on biochemical factors and gene expression of antioxidant enzymes (catalase [CAT], glutathione peroxidase [GPX]), inflammatory (tumor necrosis factor-alpha [TNF-α], nuclear factor kappa-B [NF-κB], interleukine-6 [IL-6]) and apoptotic (Bax, Bcl-2, and caspase-3) factors against GEM-induced nephrotoxicity in rats. The secondary objectives were to (i) compare the serum urea and creatinine levels between the case and control groups, and (ii) compare levels of GPX, CAT, glutathione (GSH), malondialdehyde (MDA), and nitric oxide (NO) between the case and control groups.

Materials and Methods

Study design

In this experimental study, we investigated renal histology and function in the male adult Wistar albino rats, with GEM-induced nephrotoxicity.

Animal models and housing conditions

Thirty adult male Wistar rats (in the age range of 10 to 12 weeks and an average weight of 180 ± 20 g) were used in this study. The rats were housed under a 12-12 hour light-dark cycle, at a constant temperature/humidity (temperature of 23 ± 2°C and air humidity of 50% to 55%) and free access to water and food. Two weeks before the exposure, the animals were allowed to adapt to the new environment. The rats were randomly allocated to five groups (6 rats per group) and all animals were injected intra-peritoneally (IP).

Preparation of camphor solution

Camphor (C₉H₁⁰O₂, Sigma-Aldrich, Germany) was dissolved in olive oil. Treatment groups were treated with different dosages of camphor in olive oil (per kilogram of rat body weight) by oral gavage once a day in the morning time (Table 1).

| Group | Treatment | Dosage |
|-------|-----------|--------|
| 1     | Negative  | 100 mg/kg of saline |
| 2     | Positive  | 100 mg/kg of saline and 100 mg/kg of GEM |
| 3     | Treated 1 | 100 mg/kg of GEM and 50 mg/kg of camphor |
| 4     | Treated 2 | 100 mg/kg of GEM and 150 mg/kg of camphor |
| 5     | Treated 3 | 100 mg/kg of GEM and 300 mg/kg of camphor |

Histological studies

The kidney was divided into two equal sections, then a section of the tissue was fixed in 10% buffered formaldehyde solution. Prepared tissues were cut at 4-μm thick using a microtome. Hematoxylin and eosin day was used to stain the tissue. The tissue sections were carefully examined for the presence of abnormalities using a light microscope with ×400 magnification. Then, the method by Caramelo et al was applied to measure leukocyte infiltration, eosinophilic casts and tubular necrosis (Table 2) (15).
Effect of camphor on nephrotoxicity in rats

Table 1. Properties of injection material and its dosages in the animal groups

| Groups                  | Injection material                                      | Dosages (mg/kg) | Duration  |
|-------------------------|---------------------------------------------------------|-----------------|-----------|
| Negative control        | Saline                                                  | 100             | For 12 days |
| Positive control        | Gentamicin + Saline (one hour after the injection of gentamicin) | 100 + 100       | For 12 days |
| Treated 1               | Gentamicin + (Camphor + Olive oil) (one hour after the injection of gentamicin) | 100 + 50        | For 12 days |
| Treated 2               | Gentamicin + (Camphor + Olive oil) (one hour after the injection of gentamicin) | 100 + 150       | For 12 days |
| Treated 3               | Gentamicin + (Camphor + Olive oil) (one hour after the injection of gentamicin) | 100 + 300       | For 12 days |

Glomerular destruction and changes such as Bowman's space narrowing, glomerular adhesion into Bowman's capsule and glomerular collapse were distinguished by Çakir et al method (16). In this method, 0: no damage; 1: less than 25% of glomeruli was affected; 2: 25-50% of glomeruli was affected; 3: More than 50% of glomeruli was affected.

Tissue extract preparation
A slice of tissue sample was weighted and 10 mL of phosphate buffered saline (PBS) was added for each gram of tissue. The tissue was homogenized in ice-cold PBS buffer and used for biochemical tests.

Assessment of biochemical factors
All the rats were euthanized at the end of the twelfth day; for this purpose, the combination of ketamine–xylazine (KX) (13 mg/kg and 87 mg/kg, respectively) was used for anesthesia in rats. Animal blood samples were collected from their hearts using a 5 cc syringe. The samples were incubated at room temperature for 15 minutes. The blood samples were centrifuged at 3000 × g for 10 minutes. Serum samples were collected in microtubes and stored at −80°C before the examination. Serum and renal tissue activity of GPX and glutathione (GSH) was determined by the modified methods of Rotruck et al and Rahman et al, respectively (17,18). The CAT enzyme activity of renal tissue was assessed by the modified method of Aebi (19). Thiobarbituric acid (TBA) assay was used to measure MDA (20). Serum NO levels were tested using a commercially available kit (YTA, Yekta Tajhiz Azma, Iran) according to the manufacturer's instruction. Multiplex real-time polymerase chain reactions (PCR) were carried out. The reaction mixture contained 2 x SYBR Green qPCR Mix (1 X), 0.2 µL of primers for the desired gene (Table 3), 1 µL of cDNA template and 8.2 µL of RNAse free water (DEPC water). The ultimate volume of the reaction mixture (20 µL) was examined for proliferation under these conditions: Initial denaturation step at 95°C for 3 minutes; then 40 cycles of denaturation for 5 seconds at 95°C, and annealing for 30 seconds at 60°C, followed by 5 minutes final extension step at 50-99°C in a Real-Time PCR System thermocycler. The products were loaded on 1% agarose gel in 1X TBE solution and PCR bands were observed with an UV transilluminator (Table 3).

Ethical issues
This study was confirmed by Local Ethics Committee of Lorestan University of Medical Sciences. As an experiment, the protocols were approved to be in accordance with the guidelines of Animal Ethics Committee of Lorestan University of Medical Sciences, Khorrambad, Iran (IR. LUMS.REC.1399.092). This study was extracted from the MSc thesis of Ali Valibeik at this University (Thesis #1397-1-99-1528).

Statistical analysis
The analysis of all data was done by SPSS version 22.0 statistical software. One-way analysis of variance (ANOVA) and Tukey's post tests were used to compare the data. For non-parametric data, the Kruskal Wallis test and the Mann-Whitney U test together with the Bonferroni correction were used. The delta- delta Ct (ΔΔCt) method, also known as the 2−ΔΔCT method, was used to analyze gene concentration of urine (22).
expression. The probability of $P < 0.05$ was considered statistically significant. The data were reported as means ±SD in tables, and ±95% confidence intervals on graphs.

**Results**

**Biochemical parameters in the urine, serum, and extracted renal tissue**

Table 4 presents the results of biochemical parameters in urine, serum and renal tissue. The CAT enzyme, GPX, MDA and GSH activity levels in the renal tissue were significantly lower in the GEM group in comparison with the other groups ($P<0.001$). However, there were no significant differences between the control group and the treatment groups. The GSH and GPX activity levels in the serum were significantly lower in the GEM group in comparison with the other groups ($P<0.001$), however, there were no significant differences between the control group and the treatment groups. The observed

**Table 4. Biochemical factors and antioxidant enzymes in the urine, serum and extracted renal tissue**

| Parameters                            | Control          | Gentamicin       | Camphor (50 mg/kg) | Camphor (150 mg/kg) | Camphor (300 mg/kg) |
|---------------------------------------|------------------|------------------|--------------------|---------------------|---------------------|
| Renal tissue MDA (μmol/mg of proteins) | 45.7227±3.60246a-b | 50.8081±5.84877a | 45.5615±6.16445a-b | 44.8032±7.31203a-b | 37.7130±7.19601a    |
| Serum MDA (μmol/mg of proteins)       | 0.9861±0.41862a  | 1.6030±0.22541b  | 1.4619±0.909a      | 1.4708±0.21151a     | 1.4476±0.26916a     |
| Renal tissue GSH (μmol/mg of proteins)| 3.0532±0.10245a  | 2.3257±0.22628b  | 2.4387±0.32439a    | 2.5337±0.24721a-b   | 2.3358±0.55557a     |
| Serum GSH (μmol/mg of proteins)       | 0.2389±0.2218b   | 0.1873±0.2242a   | 0.2713±0.3545a     | 0.2247±0.6848b      | 0.2422±0.2569b      |
| Renal tissue GPX (μmol/mg of proteins)| 254.4852±10.82917a-b | 229.4864±23.37117a | 298.5246±27.06573b | 288.0397±35.56474b | 303.3239±31.70671b  |
| Serum GPX (μmol/mg of proteins)       | 41.6115±4.99075a | 32.4124±4.31588a | 42.3743±1.36766a   | 42.8975±4.2373a     | 43.4986±5.28237a    |
| Renal tissue catalase (U/mg of protein)| 5.9123±0.58203a  | 3.3883±0.37932a  | 5.6323±0.58964a    | 5.8771±0.71047a     | 5.5169±0.52477a     |
| Serum NO (μmol/mg of proteins)        | 42.4190±2.5345a  | 46.4773±1.54674a | 46.9857±2.44111a   | 48.3440±2.53129a    | 47.6357±3.12218a    |
| Serum urea (mmol/L)                   | 35.3333±4.08248a | 90.3333±14.45914a| 88±49.27a          | 68±25.069a          | 58±10.2127a         |
| Urine urea (mmol/L)                   | 37.006±45.98b    | 1716.666±730605b | 1783.333±645.47a   | 1700±673.79a        | 1837.5±301039a      |
| Urine protein (mg/day)                | 293.6250±110.062a | 493.3125±61.077b | 336.1250±80.071a-b | 383±86.658b         | 312.3750±197.427a   |
| Serum Creatinine (mg/dL)              | 0.665±04278a     | 0853±19.9937a    | 0.9767±0.35303a    | 0.72±0.19501a       | 0.728±0.8377        |
| Urine Creatinine (mg/dL)              | 37.1667±2.2286   | 24.5±8.3373      | 21.25±7.8022       | 29.9167±33.34       | 17.5±5.8236        |

* Statistically significant differences between the groups are shown with different letters above the data (for example, a group with the letter a, is significantly different from groups with the letters b, c, and bc, however, is not significantly different from groups with the letters a, ab, and abc).
concentrations of MDA, urea and NO in the serum were significantly higher in the GEM group in comparison with the other groups \((P<0.001)\) of rats. The creatinine concentration of urine and serum was not significantly different between the groups. The protein concentration of urine was significantly higher in the GEM group compared with the other groups \((P<0.001)\). Despite this, it did not differ significantly between the control group and the treatment groups. The concentration of urea in urine was significantly lower in the GEM group in comparison with the other groups \((P<0.001)\). However, it did not differ significantly between the control group and the treatment groups (Table 4).

**Histological studies**
Kidney tissue in the control group revealed a healthy histological structure of the renal tubular (Figure 1A). Kidney sections of the GEM group showed renal epithelial degeneration and tubular necrosis with leukocytes infiltration in the interstitial area and the epithelial cast of renal (Figure 1B). Kidney tissue of rats treated with camphor50 showed a typical histological structure with leukocytes infiltration in the interstitial area (Figure 1C). Kidney tissue from those treated with the camphor150 group showed a typical histological structure with leukocytes infiltration in the interstitial area, epithelial degeneration and tubular necrosis (Figure 1D). Kidney sections treated with camphor300 showed similar findings to the camphor150 group (Figure 1E).

The rate of tubular necrosis in the GEM group had shown a significant increase compared to the control group. Camphor intake at three different doses was able to significantly reduce the rate of tubular necrosis compared to the GEM group, of course, camphor 50 had further reduced the rate of tubular necrosis compared to camphor 150 and group 300. However, the rate of tubular necrosis in none of the camphor-receiving groups was as low as in the control group (Table 5).

Leukocyte infiltration in the GEM group had shown a significant increase compared to the control group. Camphor intake at two doses of 50 and 150 was able to reduce the rate of lymphocyte infiltration compared to the GEM group. Of course, the mean lymphocyte infiltration in these two groups was different from the control group (Table 5).

The rate of eosinophilic cast in the GEM group had shown a significant increase compared to the control group. Camphor intake at three different doses was able to reduce the eosinophilic cast rate in a promising manner compared to the GEM group. The comparison of the mean of these three groups with the control group shows that the amount of eosinophilic cast in these groups was reduced as much as the control group (Table 5).

The rate of glomerular damage in the GEM group had shown a significant increase compared to the control group. Camphor intake at three different doses was able to reduce the rate of glomerular degradation compared to the GEM group and the mean decrease in the three groups was slightly different from the mean of the control group (Table 5).

**Gene expression of GPX, Catalase, TNF-α, NF-κB, IL-6, Bax, Bcl-2, and caspase-3 receptors**
The gene expression of TNF-α, NF-κB, IL-6, Bax, and caspase-3 factors in the renal tissue was significantly lower in the camphor group in comparison with the GEM group. However, the gene expression of GPX and CAT enzymes and Bcl-2 apoptotic factor was significantly higher in the camphor group in comparison with the GEM group (Figures 2 and 3).

**Discussion**
GEM is widely used to treat bacterial infections, however...
this antibiotic may cause severe renal impairment (24). Previous studies suggest that the interference of ROS with the renal is an impact of GEM. ROS, principally O$_2^-$ and ·OH, induce cellular destruction and separation by different mechanisms, including repression of electron transport chain, destruction of respiration and ATP generation. Similar to humans and experimental animals, the increase of GEM in the renal leads to renal morphological transformations. Therefore, we chose this animal model to study biochemistry, histology and molecular assessment of GEM-induced nephrotoxicity (25). Various researchers have studied on the antioxidant properties of camphor as natural therapeutic agent for numerous human diseases (26). Camphor leaf is used to treat rheumatoid arthritis, muscular tension, intestinal disorder, rheumatic disease and pulmonary disorders (27). Hence, we did an experimental study to evaluate the effect of camphor on the change of renal function and histology in rats with GEM-induced nephrotoxicity.

Our study explained that the administration of GEM (100 mg/kg/d) for 12 days, strongly caused nephrotoxicity in all treated rats. These results were supported by some previous studies (28, 29). GEM reduces the level of glomerular filtration that induces a rise in urea and creatinine of serum (9). Our study revealed that nephrotoxicity induced by GEM showed a marked decrease in the CAT, GPX, MDA and GSH activity levels of renal tissue and the GSH and GPX activity of serum, however, presented an increase in MDA, urea and NO activity in the serum, and did not show changes in creatinine concentration of serum and urine. The insignificant levels of biochemical factors in the serum and renal tissue of camphor treated groups, as compared with the GEM group, indicated that the camphor may have no effect on biochemical reactions.

GEM does not generate morphological alterations in glomerulus. However, studies have revealed that high doses of GEM can generate moderate growth of glomeruli and circular transformation (30). Our study revealed that camphor 50 could improve the histological alterations induced by GEM.

Researches have shown that nephrotoxicity induced by GEM is related to high expression of p38-mitogen activated protein kinase (p38MAPK) and NF-κB pathways (31). Furthermore, oxidative stress that can be induced by GEM leads to the release of pro-inflammatory mediators such as TNF-α and interleukins such as IL-1, IL-4, IL-6 (32). Treatment by GEM, up-regulates the expression of Bax protein and down-regulates the Bcl-2 expression (9). Our study showed a decrease in the gene expression of TNF-α, NF-κB, IL-6, Bax, and caspase-3 factors and an increase in the gene expression of GPX and CAT enzymes and Bcl-2 apoptotic factor. Interpretation of decreased gene expression of TNF-α, NF-κB, IL-6, and caspase-3 factors is not supported by other studies.

**Conclusion**

This was the first study to explain that camphor could attenuate GEM-induced nephrotoxicity. As mentioned above, the results of this study demonstrated that camphor had no notable effect on biochemical factors like MDA, GSH, NO, urea, creatinine and urine protein. It considerably reduced the gene expression of inflammatory factors like NF-κB, TNF-α and IL-6 and some apoptotic...
factors such as Bax and caspase-3. However, it significantly increased the gene expression of Bcl-2 apoptotic factor and antioxidant enzymes such as GPX and CAT. In addition, improvement in histopathological changes was observed in the camphor-treated groups compared with the positive control group (GEM group).

In conclusion, camphor, due to its antioxidant properties, can be effective in improving kidney damage in some diseases and used as nephroprotective agent against kidney injury induced by nephrotoxins like GEM. However, more extensive studies with more experimental groups and longer follow-up are necessary to confirm the results of this study.

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Authors’ contribution
AV and HA were the principal investigators of the study. NN, AA, NTD, SRM and SV were the project collaborators. MB participated as a statistical consultant, ANS as a genetic consultant and LJP as a tissue collaborator. All authors participated in preparing the final draft of the manuscript, revised the manuscript and critically evaluated the intellectual contents. All authors read and approved the content of the manuscript and confirmed the accuracy or integrity of any part of the work.

Conflicts of interest
The authors declare that they have no conflict of interest.

Ethical considerations
Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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