

Thymol exerts antioxidant, anti-inflammatory, and anti-apoptotic protective effects against gentamicin nephrotoxicity in rats

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Abstract

The renoprotective effect of thymol (TML) was investigated in rats challenged with gentamicin (GN). Rats received TML (20 mg/kg/day, p.o.) for 15 days, and GN (80 mg/kg/day, i.p.) starting from the 8th day. TML significantly lowered serum creatinine and neutrophil gelatinase-associated lipocalin, and renal malondialdehyde, nitric oxide, tumor necrosis factor- α , interleukin-18, Bax, caspase-3, and caspase-9 in GN-challenged rats. In addition, TML caused a significant increment of renal total antioxidant capacity in rats received GN. Moreover, TML significantly ameliorated GN-induced histopathological kidney tissue injury, and significantly decreased nuclear factor- κ B p65 and kidney injury molecule-1 expressions in kidneys of GN-challenged rats. It was concluded that TML guarded against GN-induced nephrotoxicity in rats via inhibition of oxidative stress, inflammation, and apoptosis.

Keywords

thymol, gentamicin, kidney, rats

Introduction

Gentamicin (GN), an antibiotic related to aminoglycosides, is commonly used to treat serious infections caused by aerobic Gram-negative bacilli. Despite its efficacy in bacterial eradication, GN-induced nephrotoxicity is considered a major adverse effect which limits its usefulness (Hayward et al. 2018; Srisung et al. 2017). Acute kidney injury (AKI) and dysfunction is a well-known dose-dependent adverse impact of GN, and can even occur after

a single dose administration (Hayward et al. 2018). GN accumulates in the cells of renal proximal tubules and causes direct toxic effect. The precise procedures contributing to GN nephrotoxicity are not fully uncovered. However, a well-built evidence proposes that increased manufacturing of reactive oxygen species (ROS) and reactive nitrogen species (RNS), are implicated in GN-induced AKI. This leads to unbalance in the oxidant/antioxidant status, devours endogenous antioxidants with subsequent increment of lipid peroxidation of cell biomembranes and

increased malondialdehyde (MDA) generation (Galal and Abd El-Rady 2019; Famurewa et al. 2020). Oxidative stress upregulates the inflammatory cascades, including nuclear factor- κ B (NF- κ B) pathway, with subsequent increment in the generation of inflammatory cytokines, as tumor necrosis factor- α (TNF- α) and interleukin-18 (IL-18) (Abdelrahman and Abdelmageed 2020). Besides, the use of antioxidants and anti-inflammatory compounds proved effective as renoprotective strategy against GN-induced AKI and dysfunction in prior investigations (Cao et al. 2019; Abdelrahman and Abdelmageed 2020; Aziz et al. 2020; Mahi-Birjand et al. 2020).

Thymol (TML), 2-isopropyl-5-methylphenol, is a monoterpenoid phenolic compound found mainly in the volatile oil of thyme. Previous investigations demonstrated that TML possessed a wide range of biological activities, including antioxidant, anti-inflammatory, antiapoptotic, antimicrobial, antidiabetic, anticancer, and cardioprotective effects (Kumari et al. 2019). It was also reported in the literature that TML impeded kidney injury and dysfunction induced by cisplatin in rats and mice (El-Sayed et al. 2015; Hosseinimehr et al. 2015), prevented the deleterious effects of high fat diet-induced diabetes mellitus on the kidneys of mice (Saravanan and Pari 2016), and inhibited streptozotocin-induced diabetic nephropathy in rats (Oskouei et al. 2019). Therefore, the current investigation was performed to demonstrate the possible protective effect of TML in GN-challenged rats, and to explore the probable mechanisms underlying this effect.

Materials and methods

Drugs and chemicals

GN and TML were purchased from Sigma-Aldrich, USA. GN was dissolved in physiological saline, and TML was dissolved in corn oil. The utilized doses of GN and TML in the current study were selected from prior investigations (El-Sayed et al. 2015; Abdelsameea et al. 2016).

Laboratory animals and study scheme

Twenty-eight male Sprague-Dawley rats (230 ± 10 g, weight) were obtained from the National Research Centre, Giza, Egypt. They were housed at 24 °C, 45% humidity, and 12 h light/dark cycle. They were fed ordinary chew, supplied with tap water *ad libitum*, and were adapted for one week. The study protocol was approved by the Research Ethics Committee, Faculty of Medicine, Minia University (approval number: 227–42019). The international guidelines for care and use of laboratory animals were considered. Rats were randomly allocated to the following groups:

Group 1 ($n = 6$) was the control and received corn oil, *p.o.*, daily for 15 days, and physiological saline, *i.p.*, daily starting from the 8th day.

Group 2 ($n = 8$) received corn oil, *p.o.*, daily for 15 days, and GN (80 mg/kg/day, *i.p.*) starting from the 8th day.

Group 3 ($n = 8$) received TML (20 mg/kg/day, *p.o.*) for 15 days, and GN (80 mg/kg/day, *i.p.*) starting from the 8th day.

Group 4 ($n = 6$) received only TML (20 mg/kg/day, *p.o.*) for 15 days.

Sampling and biochemical studies

Rats were euthanized 24 h after the last GN injection by urethane (1 g/kg, *i.p.*). Blood samples were withdrawn via cardiac punctures, and centrifuged for 10 min at 4000 rpm after clotting. Thereafter, creatinine was determined by a colorimetric kit (Biodiagnostic, Egypt), and neutrophil gelatinase-associated lipocalin (NGAL) was determined by an ELISA kit (R&D Systems, USA) in the obtained serum samples.

The kidneys were dissected out, and their dry weights were determined. Right kidneys were homogenized for 15 min at 5000 rpm in cold potassium phosphate buffer (pH 7.3, 0.05 M). Colorimetric kits were used to assess the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), and nitric oxide (NO) (Biodiagnostic, Egypt), and caspase-9 and caspase-3 (R&D Systems, USA). Additionally, ELISA kits were used to assess TNF- α and IL-18 (RayBiotech, USA), and Bax (LifeSpan Biosciences, USA).

Histopathological studies

Left kidneys were kept in 10% formalin solution, dehydrated in alcohol, and embedded in paraffin. Sections at 5 μ m were cut, and stained with hematoxylin and eosin. The pathologist who visualized the slides under light microscope was unaware of slide identity. Renal tubular injury was assessed by a semi-quantitative score using a scale 0–4, where 0 = normal, 1 = < 10%, 2 = 10–25%, 3 = 25–75%, and 4 = > 75% (Ramesh and Reeves 2005).

Immunohistochemical studies

Paraffin blocks were cut into 4 μ m-thick sections, which were deparaffinised, rehydrated and treated with 3% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. Antigen retrieval was done by boiling the slides in 10 mM citrate buffer (pH 6.0) for 10 min and then cooled at room temperature for 20 min. Sections were incubated with rabbit polyclonal antibodies against rat nuclear factor- κ B p65 (NF- κ B p65) (Thermo Scientific, USA, 1:100), and kidney injury molecule-1 (KIM-1) (Thermo Scientific, USA, 1:200) for 30 min. After washing with phosphate buffer solution (PBS), the slides were incubated with biotinylated secondary antibody for 10 min, streptavidin peroxidase complex for 5 min, and finally with DAB, as chromogen for 3 min. The slides were counterstained by hematoxylin for 1 min. Negative controls were done, in which the sections were stained by the same technique but using PBS instead of the primary antibodies.

The slides were inspected by an image analyzer computer system using software Leica Qwin 500 (Leica Microsystems

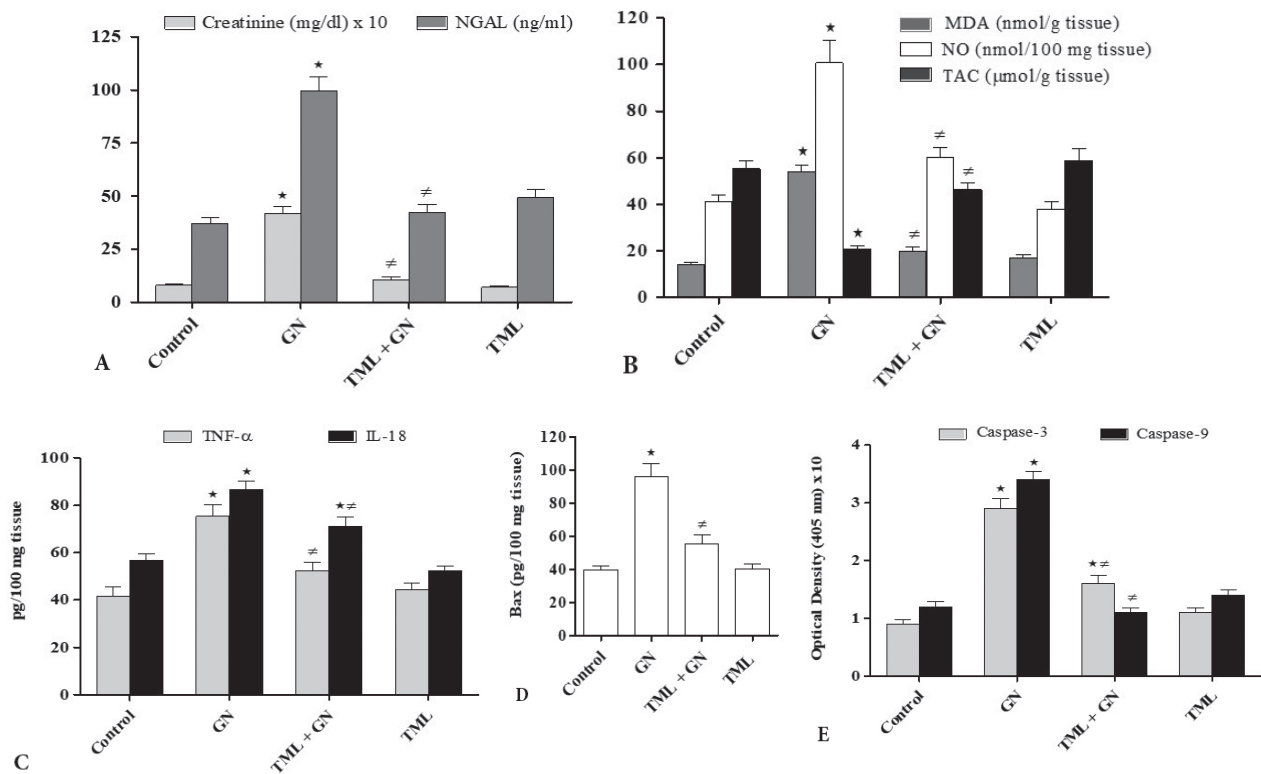


Figure 1. Results of thymol (TML) on: **A.** Serum creatinine and neutrophil gelatinase-associated lipocalin (NGAL); **B.** Renal malondialdehyde (MDA), nitric oxide (NO), and total antioxidant capacity (TAC); **C.** Renal tumor necrosis factor- α (TNF- α) and interleukin-18 (IL-18); **D.** Renal Bax; **E.** Renal caspase-3, and caspase-9 in gentamicin (GN)-challenged rats. Results are mean \pm S.E.M., * $p < 0.05$ vs. control, # $p < 0.05$ vs. GN.

Imaging Solutions Ltd, UK). The immunopositive cells were calculated in 10 non-overlapping fields of the tissue sections of each rat in all groups.

Statistical analysis

GraphPad Prism Software Program (version 6.01) was applied for data analysis using one-way ANOVA test followed by Tukey test for *post hoc* comparisons. Results expressed as mean \pm S.E.M., and significance level was at $p < 0.05$.

Results

Biochemistry sequels

Administration of GN (80 mg/kg/day, i.p.) for 8 days resulted in significant increases of serum creatinine and NGAL ($p < 0.05$) in comparison with the control values (Fig. 1A). Treatment with TML (20 mg/kg/day, p.o.) for 15 days, starting 7 days before GN administration, significantly reduced serum creatinine and NGAL ($p < 0.05$) in GN-challenged rats (Fig. 1A). Additionally, GN caused significant increments of MDA, NO, TNF- α , IL-18, Bax, and caspases-3 and 9 ($p < 0.05$), and a significant decrement of TAC ($p < 0.05$) in kidney tissue as compared to the control (Fig. 1B–E). Contrarily, TML significantly reduced renal MDA, NO,

TNF- α , IL-18, Bax, and caspases-3 and 9 ($p < 0.05$), and significantly increased renal TAC ($p < 0.05$) in rats received GN (Fig. 1B–E).

Histopathology sequels

Figure 2 shows that GN caused marked distortion of the normal kidney architecture, necrosis and desquamation of renal tubular epithelium, tubular dilatation, inflammatory cell infiltration, and coagulative necrosis. Contrarily, TML minimized kidney tissue damage, maintained normal renal histology, and significantly decreased renal tubular injury score in GN-challenged rats (Fig. 2).

Immunohistochemistry sequels

Significant increments of NF- κ B p65 and KIM-1 expressions ($p < 0.05$) were observed in the kidneys of rats received GN in comparison with the control rats (Figs 3, 4). On the other hand, TML caused significant decrements of renal NF- κ B p65 and KIM-1 expressions ($p < 0.05$) in rats challenged with GN (Figs 3, 4).

Discussion

Previous investigations, in consistence with the present one, showed that GN caused AKI in rats due to oxidative/

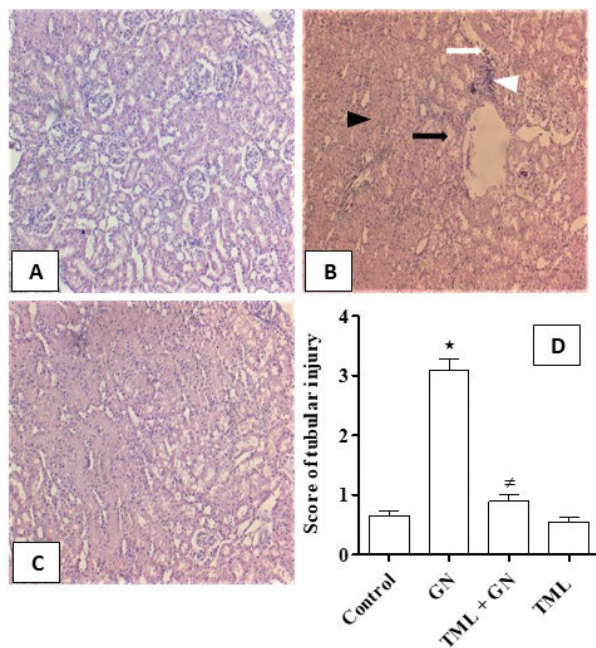


Figure 2. H&E (200 \times) of rat kidneys of: **A.** Control showing normal renal architecture; **B.** Gentamicin (GN) group demonstrating marked distortion of kidney architecture, renal tubular necrosis and dilatation, desquamation of lining epithelium (white arrow), cytoplasmic vacuolization (black arrow), interstitial edema, coagulative necrosis (black head), and inflammatory cell infiltration (white head); **C.** Thymol (TML) + GN showing preservation of the normal kidney histological picture; **D.** Score of tubular injury. Results are mean \pm S.E.M., * $p < 0.05$ vs. control, # $p < 0.05$ vs. GN.

nitrosative stress (Galal and Abd El-Rady 2019; Famurewa et al. 2020). This was evidenced by the significant increase of kidney tissue MDA, the indicator of lipid peroxidation of biomembranes, and significant depletion of renal TAC. In addition, increased production of NO, the nitrosative stress biomarker, in kidney tissue denoted further injury due to nitration of cellular macromolecules. Prior studies also revealed that GN upgraded inflammatory cascades with increased production of inflammatory cytokines. This is in agreement with the current investigation, which revealed that GN activated NF- κ B pathway and resulted in increased level of the active subunit, NF- κ B p65 in rat kidneys. Nuclear translocation of NF- κ B p65 activates gene transcription of inflammatory cytokines, as TNF- α and IL-18, leading to augmentation of inflammatory cascades and further damage of kidney tissue (Ansari et al. 2016; Abdelrahman and Abdelmageed 2020).

Similar to the current study, prior investigations revealed that TML, the active ingredient of thyme, performed as ROS scavenger, provided significant antioxidant effect, prevented biomembrane lipid peroxidation, and maintained endogenous antioxidant capacity (El-Sayed et al. 2015; Hosseinimehr et al. 2015). This was attributed to the inhibitory effect of TML on NADPH oxidase, the major source of generation of ROS, as hydrogen peroxide and superoxide anion (Kavoosi et al. 2012). Additionally, TML enhanced the biosynthesis

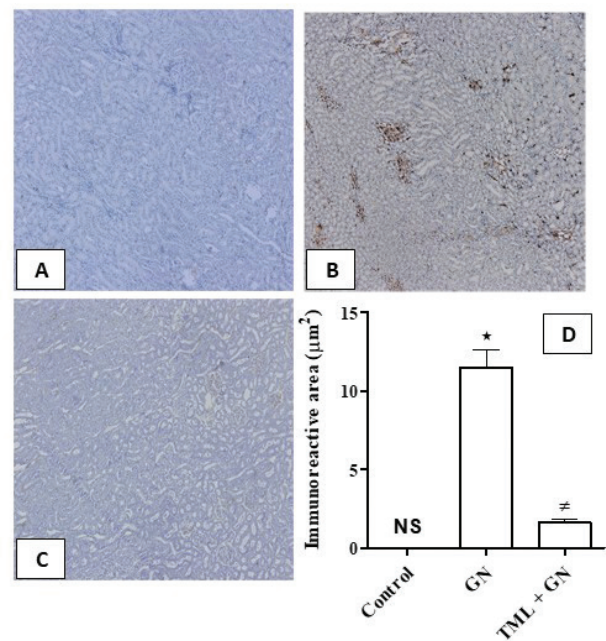


Figure 3. Immunohistochemistry (200 \times) of nuclear factor- κ B p65 (NF- κ B p65) of rat kidneys of: **A.** Control showing no staining (NS); **B.** Gentamicin (GN) group demonstrating a significant increment of NF- κ B p65 immunopositivity in brown color; **C.** Thymol (TML) + GN showing a significant decrement of NF- κ B p65 immunoreactivity; **D.** Immunoreactive area (μm^2). Results are mean \pm S.E.M., * $p < 0.05$ vs. control, # $p < 0.05$ vs. GN.

of reduced glutathione (GSH), the major endogenous ROS scavenger, by upregulating the gene expression of γ -glutamylcysteine synthetase 1 enzyme, essential for GSH production (Ye et al. 2016). It was also reported in the literature that TML exerted antinitrosative effect by decreasing the production of RNS, as NO, through inhibition of inducible nitric oxide synthase enzyme (Wu et al. 2017). Besides, TML inhibited the NF- κ B-dependent inflammatory responses and decreased the generation of TNF- α and other inflammatory cytokines through down-regulation of NF- κ B p65 protein production (Chamanara et al. 2019).

Moreover, previous studies, similar to the present one, showed that GN activated the mitochondrial apoptotic pathway in the kidney causing increased release of Bax, the pro-apoptotic protein. This resulted in increased mitochondrial membrane permeability and the release of cytochrome C in the cytosol. Subsequent activation of apoptotic signals lead to up-regulation of cleaved caspase-9 activity, and finally cleaved caspase-3 activity, which resulted in execution of cell apoptosis (Sahu et al. 2014; Cao et al. 2019). It was also mentioned in the literature that TML inhibited the mitochondrial apoptotic pathway, and downregulated the activity of Bax and caspase family of proteases (El-Sayed et al. 2015; Fizur et al. 2015). This is in accordance with the current investigation, which revealed that TML significantly reduced the level of apoptotic biomarkers, Bax, caspase-9, and caspase-3, in the kidneys of GN-challenged rats.

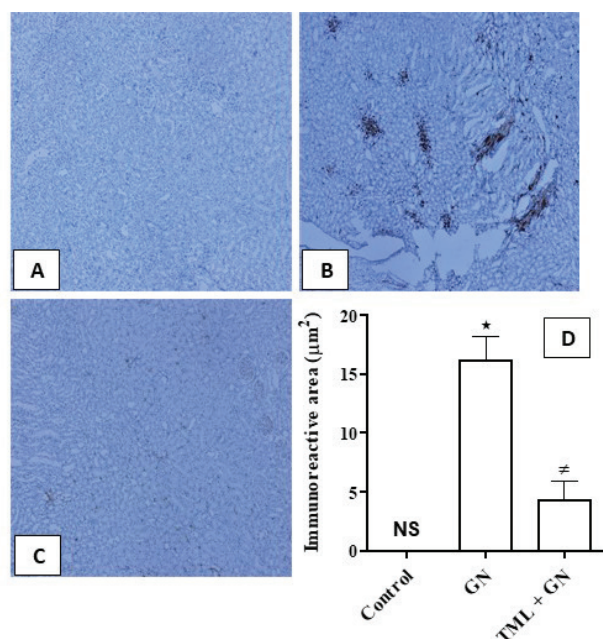


Figure 4. Immunohistochemistry (200×) of kidney injury molecule-1 (KIM-1) of rat kidneys of: **A.** Control showing no staining (NS); **B.** Gentamicin (GN) group demonstrating a significant increment of KIM-1 immunopositivity in brown color; **C.** Thymol (TML) + GN showing a significant decrement of KIM-1 immunoreactivity; **D.** Immunoreactive area (µm²). Results are mean ± S.E.M., **p* < 0.05 vs. control, #*p* < 0.05 vs. GN.

In addition, NGAL, IL-18, and KIM-1 are considered novel sensitive diagnostic and prognostic biomarkers of AKI (Koza 2016; Beker et al. 2018). NGAL is produced by renal tubular cells as self-defensive mechanism upon exposure to nephrotoxic agents. IL-18 is a chemokine derived from the endothelial cells, and it is important in neutrophil recruitment and chemotaxis to the injured kidneys. KIM-1 is a proximal tubular cell transmembrane

glycoprotein expressed upon exposure to harmful kidney stimuli, which may be involved in cleaning debris from injured renal cells (Koza 2016; Beker et al. 2018). The present work showed that TML significantly inhibited the GN-induced increments of serum NGAL, and renal IL-18 and KIM-1 in rats. Besides, the current investigation showed that TML prevented the histopathological damage induced by GN in rat kidneys.

Conclusions

TML significantly preserved kidney structure and function against GN insult in rats. The antioxidant, anti-inflammatory, and antiapoptotic properties of TML are the most probable contributing factors for this nephroprotective effect.

Conflict of interest

All the authors declare that there are no conflicts of interest.

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