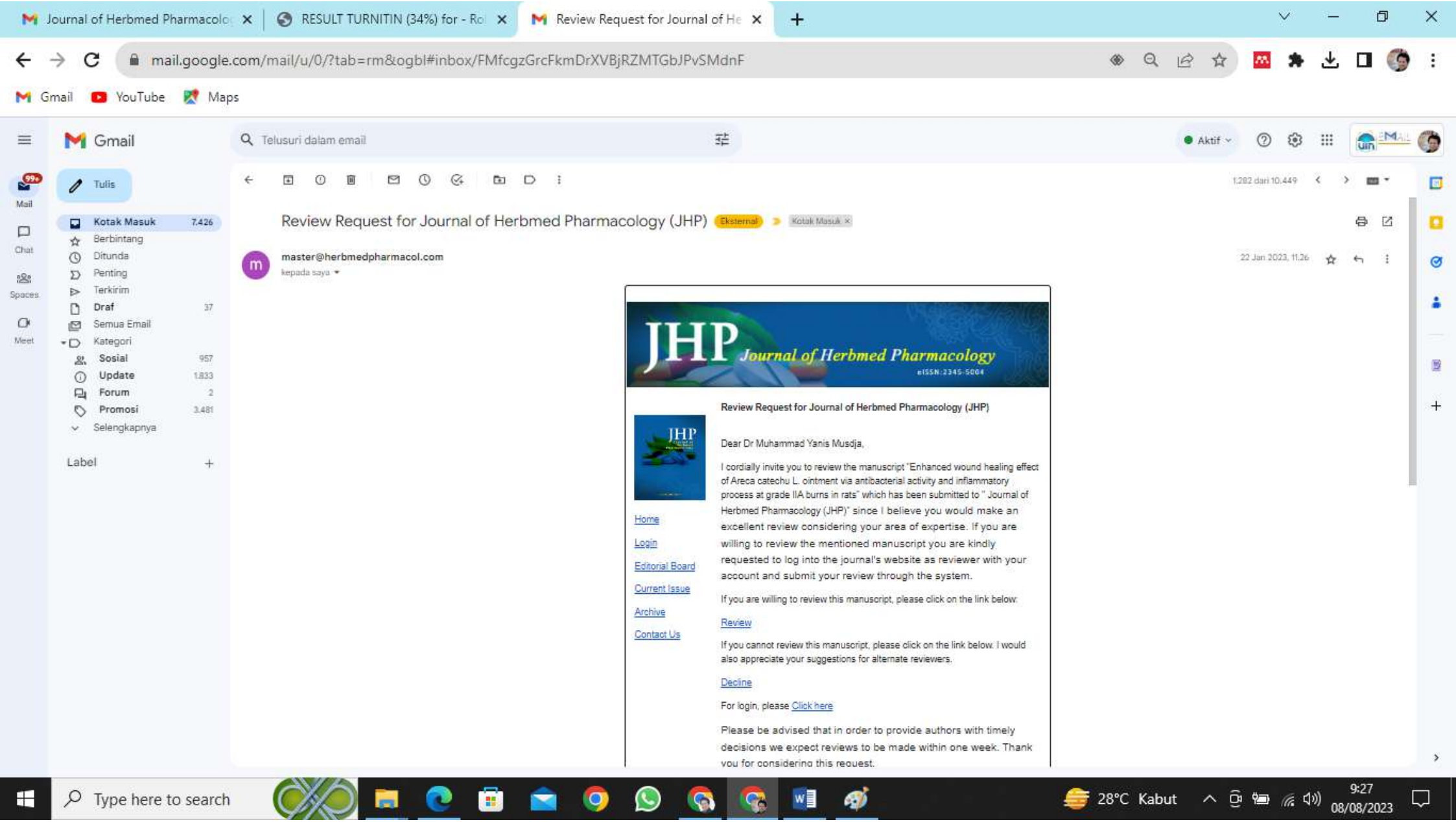
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1. The chemical compounds in date seeds should be written in the Introduction and in the discussion section, the possible chemical compounds involved in RXR-α and receptors activated by protease 2 (PAR-2) by chemical compounds in the date seeds should be written.
2. Write down the age of the date palm seeds, how many days are used for research
3. Date seeds, how are they dried?
4. The body weight of 8 rats cannot be the same, write down the weight of the rats from how many .... grams to how many ..... grams and write how many days old the mouse is
5. Why is date seed extract only given 100 mg/kg, why not try it at a dose of 50 mg/kg and 200 mg/kg maybe this dose is better, please explain
6. What is the basis for choosing dosing once a day and for 10 days, please explain
7. In this histopathological study, whose method was used should be stated.
8. Mention for Bouin's trichrome staining, whose method was used
9. For immunofluorescence of tissue sections, whose method was used, should be stated
10. In the discussion section, it is necessary to write down what chemical compounds may be involved in RXR-α and protease 2 (PAR-2) activated receptors by chemical compounds in the date seeds.

Best Regards

**Dr. Muhammad Yanis Musdja**

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# Role of PAR-2 and RXR- α in mitigating gentamicin-induced renal injury in mice by *Phoenix dactylifera L.* seed extract and herbal mixture


## ABSTRACT

**Introduction**: Retinoid receptors, including retinoid X receptor (RXR), are vitamin A receptors expressed in the kidney and control several physiological functions by regulating different genes. Previously we demonstrated a coagulation system-dependent loss of RXR-α in monocrotaline/lipopolysaccharide-induced renal toxicity. Herein, we examined the involvement of RXR-α and protease-activated receptor 2 (PAR-2) in the protective effect of date palm seed extract (DPSE) and one nephroprotective herbal mixture (HM) against Gentamycin (Gen)-induced renal toxicity in mice.

**Methods**: Thirty-two mice divided randomly into four groups were either treated with saline, Gen (100 mg/kg/IP, daily for 10 days starting from the third day of the experiment), Gen and DPSE (100 mg/kg/P.O, daily for 10 days), or Gen and HM (100 mg/kg/P.O, daily for 10 days). Mice were sacrificed 24 h after the last dose administration, and kidney tissues were dissected out, weighed, and subjected to histological, immunofluorescence, and biochemical assays.

**Results**: The Gen-induced renal toxicity group demonstrated a significant decrease in RXR-α and a significant increase in PAR-2 protein expression. Treatment with DPSE or HM significantly improved Gen-induced effects on serum creatinine, BUN, WBCs, Platelets, RXR-α extracellular matrix deposition and PAR-2.

**Conclusion**: The present study revealed, for the first time, that retinoid receptors and PAR-2 might play an important role in Gen-induced renal toxicity. Furthermore, the nephroprotective effects of DPSE and HM were confirmed.

**Key words:** PAR-2; RXR-α; Gentamicin; Renal toxicity; Date palm seed, Herbal mixture.

**Abbreviations:** hepatic stellate cells (HSCs), retinoic acid (RA), retinoic acid receptors (RAR-α), retinoid X receptors (RXR-α), Monocrotalin/Lipopolysaccharide (MCT/LPS), retinoid X response element (RXRE), tissue factor (TF), platelet-derived growth factor (PDGF), transforming growth factor- β (TGF-β), reactive oxygen species (ROS) production, protease-activated receptors (PARs), Date palm seed extract (DPSE), Herbal mixture (HM).

## 1. INTRODUCTION

The kidneys are the body organ responsible for health maintaining functions such as preserving stable levels of important molecules in the blood as well as toxin excretion. Although kidneys are efficient in clearing many toxins from the blood, some chemicals are difficult to eliminate resulting in their accumulation with subsequent kidney damage (1).

Gentamicin (Gen) is an aminoglycoside antibiotic with broad spectrum activity against Gram-negative and Gram-positive bacteria. Though, it was reported that, in up to 30% of Gen-treated patients some signs of renal toxicity are observed with the drug treatment for more than 7 days (2).

Retinoids, found in the liver or other parts of the body, are stored mainly in hepatic stellate cells (HSCs), contributing significantly to cell proliferation and differentiation (3). In mice, hepatic retinoids are converted to retinyl ester and stored as lipid droplets in HSCs (4). In response to liver toxicity, HSCs activate with structural change, leading to loss of lipid droplets and stored retinoids (4). These lipid droplets are composed of retinoic acid (RA), retinyl ester, triglycerides, cholesteryl ester, cholesterol, phospholipid, and free fatty acids (5). Recently we reported the presence of retinoic acid receptors (RAR-α), and retinoid X receptors (RXR-α) in the released lipid droplets from HSCs in a new Monocrotalin/Lipopolysaccharide (MCT/LPS) idiosyncratic hepatotoxic

model and confirmed them as early and sensitive markers for HSCs activation. Signaling of RA is mediated *via* its occupancy of RAR and RXR, with subsequent DNA binding of a RAR–RXR heterodimer or an RXR–RXR homodimer to RA response element (RARE) or retinoid X response element (RXRE), respectively (6), thereby, regulating the transcription of target genes that control cellular proliferation, differentiation, and apoptosis (7). RAR-selective agonists are used clinically as anti-cancers, acne, and psoriasis treatments, whereas RXR agonists show potential for treating hyperglycemia in animal models of type II diabetes (8). RARs regulate the transcription of responsive genes as heterodimers with RXRs. In contrast, RXRs play a central role in nuclear receptor signaling by forming homodimers or acting as obligatory heterodimerization partners for various nuclear receptors (e.g., RARs, peroxisome proliferator-activated receptors, vitamin D receptors). Cytosolic speckled RAR-α distribution has been observed in activated HSC *in vitro* (9) and *in vivo* in our previous publication (10).

Many renal diseases such as membranous glomerulopathy, diabetic nephropathy, minimal change nephritis, and IgA nephritis are still difficult to diagnose without patient biopsy, thus complicating the treatment. The pathogenesis of the above-mentioned renal diseases has to be clarified to find preventive and suitable management. There is an urgent need for an accurate and sensitive biomarker to differentiate among the various renal diseases mentioned. In this regard, insufficient studies have discussed RXR-α expression level and its role in renal diseases. The RXR-α is predominantly found in renal tubules while it lacks the glomeruli expression (11). Our preliminary data revealed a translocation of RXR-α from basolateral into the apical site of distal tubules and collecting duct after MCT/LPS co-treatment, indicating a large probability to find RXR-α in the urine of these mice. In addition, RXR-α translocation, was a tissue factor (TF) dependent response.

The coagulation system's main role is to control the hemostasis and balance thrombus formation (12), in addition to its critical role in inflammation and angiogenesis (13). Various proteins play a part in the coagulation cascade; TF proteins' expression initiates the coagulation cascade till the accumulation of fibrin and clot formation (14). Thrombin does not only play a critical role in the initiation of coagulation cascade through platelet activation and conversion of fibrinogen to fibrin, but also potentiates in the movement of platelet-derived growth factor (PDGF), thromboxane A2 and transforming growth factor- β (TGF-β) from the platelets as well demonstrating its inflammatory effect through increased numbers of adhesion molecules, chemokines cytokines, and stimulating reactive oxygen species (ROS) production (15).

Thrombin acts as a recruiter for neutrophils and monocytes at the inflammatory site, thereby taking the inflammatory reaction forward like multiple sclerosis endotoxemia or sepsis (13). Similarly, thrombin's potential pathogen property is described in brain or liver injury and neurotoxicity (16,17).

Coagulation factors have a pleiotropic effect by activating protease-activated receptors (PARs), a G protein-coupled receptor family (16), like the activation of PAR-2 by expression of tissue factor/VIIa complex or factor Xa in the kidney (16,17). There are various reports of exacerbation of glomerular injury by PAR-2 in diabetic kidney disease (DKD) or glomerulonephritis (17), including preeclampsia antiphospholipid syndrome kidney injury models, while its role in Gen-induced kidney injury remains controversial. Conversely, PAR-2 signaling has demonstrated its role in endothelial proliferation/migration (18), including pro-angiogenic roles on limb ischemia and retinal neovascularization (19).

Herbal medicines have demonstrated their potential in the treatment of various ailments (20,21). Several plants are used in traditional systems of treatments for their nephroprotective activity e.g.

ginger, pomegranate seed oil, garlic, etc., however, most of these herbals are noted for nephroprotective effect on basis of old-age practices. Accordingly, extensive scientific studies are required in order to evaluate their pharmacological profile (22). Date palm seed extracts, *Phoenix dactylifera L.* (Family Arecaceae), is an industrial by-product of date processing, commonly used in some countries as an animal feed or coffee substitute. Although considered a waste product, its high content of polyphenolic compounds suggests its biological potential. Several studies reported its antimicrobial (23), antioxidant (24), and hepatoprotective activity (25). Other reports have demonstrated the nephroprotective effect of date palm`s fruits and pits extracts through their significant reduction in plasma creatinine and urea concentrations and amelioration of the proximal tubules' damage (2).

Also, previous studies have demonstrated the effect of poly herbal formulations like Sairie-to and BNO 2103 against Gen- or chromate-induced nephrotoxicity, respectively (26,27). In this context, one herbal mixture composed of *Tribulus terrestris, Aerva lanata, Andrographis paniculata and Raphanus sativus* in the ratio of (3:3:3:1) is claimed to have protective effect against kidney impairment, in India. *T. terrestris*, also known as “Qutiba” or “Darisa,” belonging to the family Zygophyllaceae, grows in tropical zones and survives in the desert with low-nutrient soil in Saudi Arabia, southern Europe, southern Asia, and Africa. In addition to the various medicinal uses like an aphrodisiac, analgesic, antihypertensive, diuretic, urinary anti-septic, cardiotonic, hepatoprotective, anti-cancer properties (28), the plant is used locally in Saudi Arabia for urinary infections treatment (29). Moreover, the ethanol extract of *A. lanata* and aqueous extracts of *A. paniculata* and *R. sativus* demonstrated nephroprotection in Gen-induced nephrotoxicity in rats (30–32). *A. paniculata* plant is used in Ayurveda for various ailments and has demonstrated immunological, antibacterial, anti-inflammatory, antithrombotic, and hepatoprotective properties.

Accordingly, this study aims to shed light on the role of PAR-2 and RXR-α in Gen-induced renal injury. The study also clarifies the possible protective role of DPSE and the HM (*T. terrestris, A. lanata, A. paniculata,* and *R. sativus)* in renal toxicity induced model.

## 2. MATERIALS AND METHODS

### 2.1 Animal

Thirty-two male albino mice (22 ± 2 weeks old, weighing 30±2 g) were used. Animals were obtained from Qassim University Animal Facility, Qassim, Saudi Arabia, housed at temperature (25˚C ± 0.5), relative humidity with free access to standard forage and drinking water *ad libitum*. The animals were kept in a pathogen-controlled and air-conditioned room in the animal house. The institutional Research Ethics Committee, College of Pharmacy, Qassim University, Saudi Arabia, approved the animal experimental procedure and care (Approval ID 2020 - CP- 2). All practical experiments were carried out according to NIH Guidelines for the Care and Use of Laboratory Animals.

### 2.2 Chemicals, antibodies, and diagnostic kits

Gentamicin (Gen) was purchased from Mylan (IL, USA). Bovine serum albumin (BSA), DAPI (4, 6-diamidino-2- phenylindole), and Fluoromount were obtained from BIOMARK laboratories (India), horse serum was obtained from Sigma-Aldrich Co. (MI, USA), Dako solution was purchased from Dako (CA, USA). All other chemicals and solvents used were of analytical grade. Mouse monoclonal antibodies against PAR-2 (sc-514363) and RXR-□ (sc-28358) were purchased from Santa Cruz Biotechnology (TX, USA). Total protein in the urine colorimetric kit was obtained from Spinreact (Barcelona, Spain). Blood urea nitrogen (BUN) and creatinine diagnostic kits were obtained from Crescent Diagnostic Tests (KSA). Goat anti-rabbit Alexa fluor 488 was

purchased from Invitrogen (TX, United States). Cy3- conjugated Goat anti-rabbit antibody was obtained from Jackson Immunoresearch (PA, USA).

### 2.3 Plant material

The herbal mixture was obtained from HABBA Herbal Pvt. Ltd. (Bangalore, India). The herbal mixture consisted *of Tribulus terrestris, Aerva lanata, Andrographis paniculata and Raphanus sativus* in the ratio of (3:3:3:1).

Date palm (*Phoenix dactylifera* L.), var. Khodary fruit samples were collected from the Qassim region, Saudi Arabia, and the Ministry of Agriculture verified the sample identity. Date seeds were removed from the fruits, adequately washed with water, dried, and powdered. The powder was extracted exhaustively using aqueous methanol (80%), and the extract was dried at 40 °C using rotatory evaporator to give reddish brown residue.

### 2.4 Experimental design

Mice were randomly classified into four weight-matched groups, each of 8 mice. Group 1: received saline only (control group). Group 2: received Gen only (225 mg/kg, i.p., Gen group). Gen dose was chosen based on our preliminary experiments. Group 3: mice were treated with date palm seed extract (100 mg/kg, P.O) daily for ten days and Gen (225 mg/kg, i.p.) starting from the third day of the experiment and continued for seven days. Group 4: mice were treated with Herbal mixture extract (100 mg/kg, P.O) daily for ten days and Gen (225 mg/kg, i.p.) starting from the third day of the experiment and continued for seven days.

### 2.5 Serum preparation

Mice were anesthetized using Thiopental (40mg/kg, i.p), and blood was taken using a retro-orbital route with a non-heparinized capillary tube into EDTA tubes. Immediate estimation of the total leucocytes count and platelets was performed. Kidney function parameters: serum creatinine (Sr. Cr.) and BUN were determined in plasma obtained upon blood samples' centrifugation at 4000 rpm for 20 min.

### 2.6 Assessment of hematological parameters (WBCs and Platelets).

According to the manufacturer's instructions, white blood cells (WBCs) and platelet count were performed on whole blood using VABIO360 Auto Hematology Analyzer (BIOTA, Istanbul, Turkey).

### 2.7 Calculation of relative kidney weight

Animals' body weight was determined prior to the sacrifice. The whole kidney tissues were carefully isolated and washed with 0.9% sterile ice-cooled saline to remove any blood from the tissues and then gently pressed between 2 filter papers to absorb the excess saline solution. Afterward, each kidney was weighed, and the relative kidney weight calculated according to the following equation:

$$\textbf{Relative kidney weight} = \frac{\text{Weight of kidney (gm)}}{\text{Bodyweight of mice (gm)}} \times 100$$

### 2.8 Histopathological study

The kidneys were fixed in Davidson`s solution, followed by paraffin embedding, and tissue sections (4 µm) were stained with Hematoxylin and Eosin (H&E) and observed under a light microscope.

### 2.9 Bouin's trichrome staining

Bouin Trichrome Stain Kit was used to identify the extracellular matrix deposition in mice kidney tissues according to the manufacturer's instructions. Kidney tissues were immersed in tap water and transferred to warm Bouin's media (56°C) for 1 hr, then cooled to room temperature for 30 min. Prepared sections were then washed with tap water. Tissues were stained with Weigert's iron hematoxylin solution for 15 min then again washed with water. Finally, sections were stained with Trichrome for 20 minutes, placed in 0.5% acetic acid, and mounted with a mounting solution.

### 2.10 Photography

All photomicrographs were taken utilizing an Olympus (U.TV0.5XC-3) light microscope and digital camera.

### 2.11 Morphometric study

Using Image J 22 software (Version 1.52), the following items were detected in 10 non-overlapping fields in each mouse (×400) for each studied group:

1- The mean area percentage of collagen fibers deposition (%).
2- Maximal diameter of glomeruli in the mid-cortical region.

### 2.12 Immunofluorescence of tissue sections

Paraffin tissue sections of 4 µm thickness were deparaffinized by incubation of slides in xylene, 2 times for 15 minutes, then rehydrated through a graded ethanol series (2 x 100%, 95%, 70%, 50%, 30%, and distilled water) for 5 minutes each and washed in 10 mM phosphate-buffer 150 mM saline, pH 7.4. Antigen retrieval was performed by incubating the tissue sections in DAKO Target Retrieval Solution (10 mM Na-Citrate pH 6.0) for 20 min in a microwave oven (500W). After being cooled to RT, tissue sections were treated with methanol (100%) for 30 min at RT then washed twice with a washing solution (0.05% tween 20/ PBS). After blocking the sections with PBS containing 10% horse serum, 1% BSA in PBS for 1 hour, the slides were incubated with the primary antibody for 2 hours at 37ºC and then overnight at 4ºC. The slides were washed three times for 3 minutes in the washing solution and incubated directly with the fluorescence conjugated secondary antibody for 30 minutes at RT. The slides were washed two times for 3 minutes in the washing solution and incubated with DAPI (diluted 1:5000 in PBS) for 3 minutes. Slides were extensively washed three times for 10 minutes in the washing solution, and the excess washing solution was gently removed. Finally, tissue sections were mounted with fluoromount G. The slides were kept at a 37ºC in the oven for 2 hours, and the evaluation was performed by a Zeiss microscope coupled to a 12-bit digital image camera

### 2.13 Statistical analysis

Data analysis was executed by unpaired two-tailed t-test or one-way ANOVA with Tukey- Kramer test for multiple comparisons using Graph Pad Instsat-2. A p-value of less than 0.05 was considered significant.

## 3 RESULTS

### 3.1 Effect of Gen alone or with DPSE or HM on WBCs and platelet counts:

Our results showed that Gen significantly altered WBCs and platelet counts in mice. In the current study, we observed that the injection of Gen significantly elevated WBCs count and significantly decreased platelet count compared to normal mice (Table 1).

Pre-treatment of animals with DPSE or HM significantly decreased WBCs count compared to Gen-induced renal toxicity group. Furthermore, both treatments renormalized platelet count compared to the Gen-induced renal toxicity mice group (Table 1).

**Table 1. Effect of Date palm seed extract (DSE) or Herbal mixture (HM) on hematological parameters in Gen-induced renal toxicity of mice**

| Groups | WBCs ($10^3$/ml) | Platelets ($10^3$/ml) |
|---|---|---|
| **Control** | 5.38 ± 0.301 | 768 ± 17.9 |
| **Gen** | 11.03 ± 0.72[a] | 381.06 ± 29.87[a] |
| **Gen + (DSE)** | 8.44 ± 0.56[a,b] | 512.04 ± 17.26[a] |
| **Gen + (HM)** | 6.64 ± 0.46[b] | 598.03 ± 19.05[b] |

Values are expressed as means ± SEM (n=6). WBCs, White blood cells. **[a]**: Significantly different from control group using one-way ANOVA followed by Tukey-Kramer post-test for multiple comparisons ($P < 0.05$). **[b]**: Significantly different from Gen treated group using one-way ANOVA followed by Tukey-Kramer post-test for multiple comparisons ($P < 0.05$).

### 3.2 Effect of Gen in the presence or absence of DPSE or HM on renal function parameters

In the current study, mice treated with gentamycin alone demonstrated significant elevation of Cr, and BUN levels in addition to kidney relative weight while a significant reduction in the % change

in the weight relative to the initial weight was observed compared to the normal mice (Figure 1: A-D).

Mice pre-treated with DPSE or HM (100 mg/kg) displayed a significant decrease in serum level of Cr and BUN as well as kidney relative weight. On the other hand, increased % change in the weight, was observed compared to the Gen-induced renal toxicity group. In addition, no significant difference was observed between mice treated with either DPSE and those treated with HM (Figure 1: A-D).

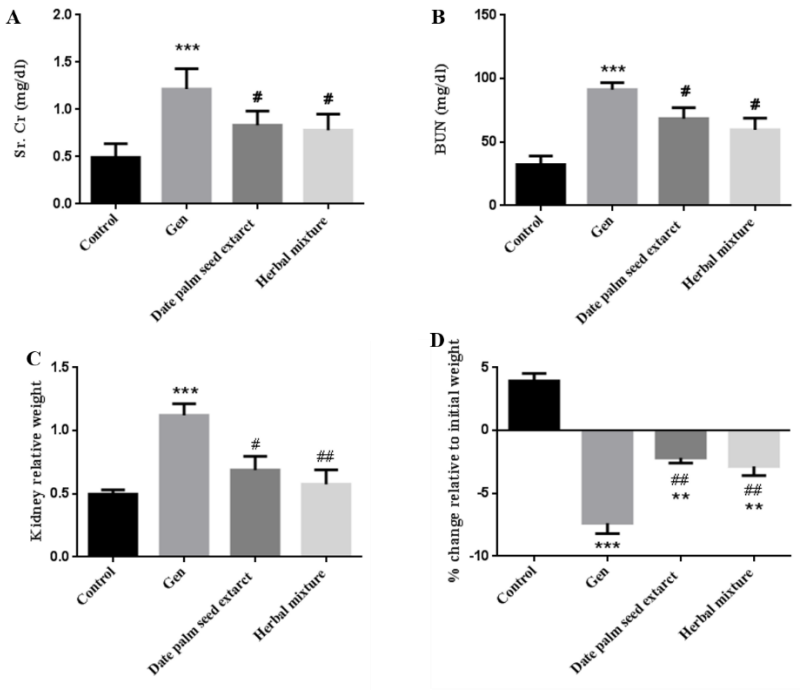


Figure 1: Effect of GEN injection in the presence or absence of Date palm seed extract or Herbal mixture on serum Creatinine (A); serum blood urea nitrogen parameters (B); kidney relative weight (C); and % change relative to initial weight (D). Data are expressed as mean + SEM. *: significantly different compared to control group, #: significantly different compared to Gen-treated group, at $p < 0.05$.

### 3.3 Effect of Gen with or without DPSE or HM in kidney histological examination

The control group showed normal renal tissues, including proximal and distal convoluted tubules. Renal glomeruli comprise renal corpuscles surrounding Bowman's space and capsules that look normal size and normal structure. On the other hand, the Gen-treated group's histopathological structures showed distorted tubules with apoptotic cells lining these tubules. Furthermore, the Gen-treated group exhibited inter-tubular inflammatory cells, widening the inter-tubular spaces with apoptotic cells in renal glomeruli. Additionally, obliterated Bowman's space in renal glomeruli and marked atrophic glomeruli were seen among the sections. The tubules appeared with cytoplasmic vacuolations and apparent dilated tubules with flatted cells and dilated renal blood vessels (Figure 2A, 2B). In contrast, tissues obtained from Gen + DPSE and Gen + HM (100 mg/kg) treated mice demonstrated moderate structural changes; fewer cytoplasmic vacuolations, few atrophic glomerular cells, mild inter-tubular inflammatory cells and widening of the inter-tubular spaces (Figure 2A, 2B).

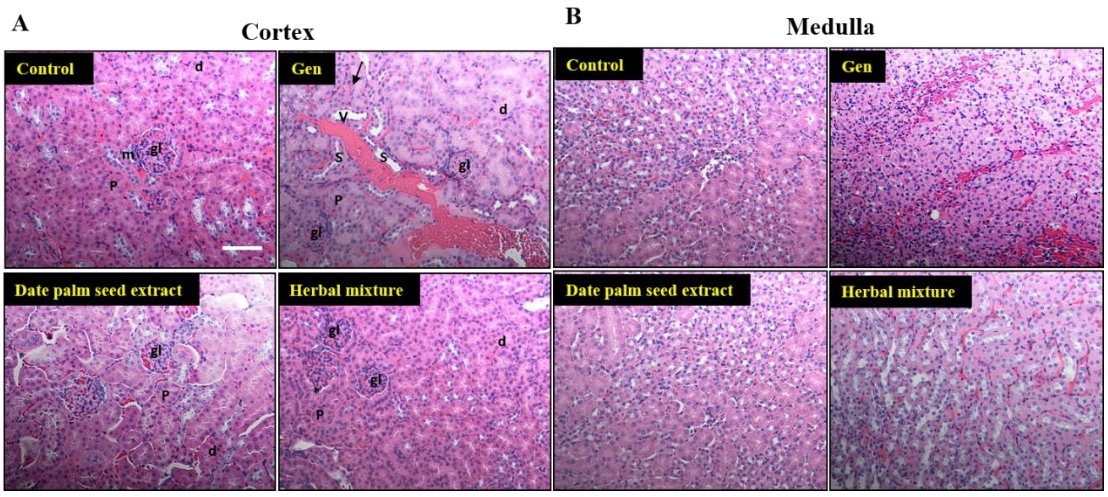


Figure 2: Histopathological examination of the control group's renal tissues, Gen-treated group, Gen + Date palm seed extract group, and Gen + Herbal mixture group. Photomicrographs of tissues in the renal cortex (A) and Medullary tubules (B) from control showing renal glomeruli (gl), proximal convoluted tubules (p), distal convoluted tubules (d), Bowman's spaces (star), and macula densa (m). Gen treated mice showing atrophic glomerulus (gl). The tubule appears with cytoplasmic vacuolations (v), and the dilated tubule appears with flatted cells (arrow). Notice the widening of inter-tubular space (s). The Gen + Date palm seed extract group shows less distorted glomerulus (gl) and tubule (d).

Less apoptotic cells are shown, and little cytoplasmic vacuolations are seen. Gen + Herbal mixture group showing less distorted tubule (d) and glomerulus (gl); but with a slight narrowing of renal Bowman's space, fewer apoptotic cells are shown, and little cytoplasmic vacuolations are seen (H&E × 200).

### 3.4 Effect of Gen and DPSE or HM on kidney fibrogenesis

Sections in the renal cortex from control group showed fewer collagen fibers surrounding the renal tubules, Bowman's capsules, and the capillary tuft of glomeruli, while gentamycin injection increased collagen deposition around the dilated blood vessels. On the other hand, Gen + DPSE or Gen + HM (100 mg/kg) treated mice showed fewer collagen fibers around a capillary tuft of glomeruli and blood vessels (Figure 3A, 3B).

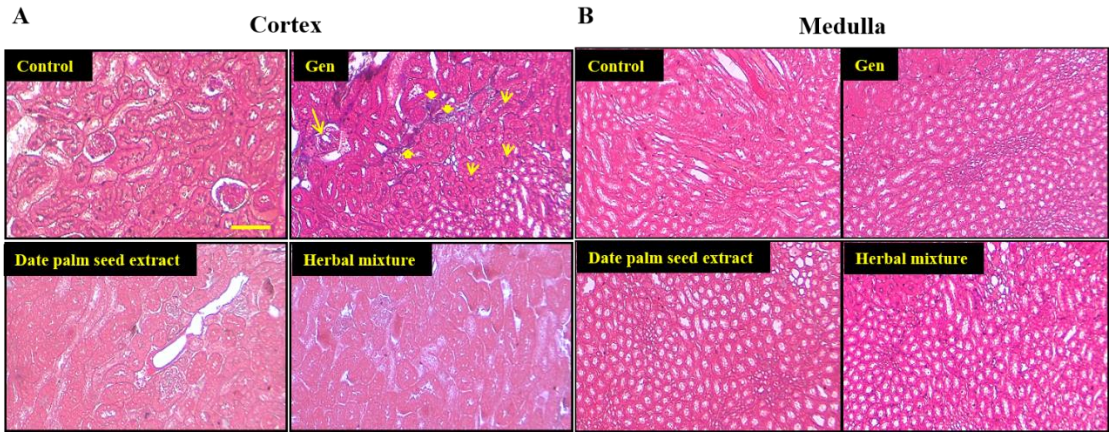


Figure 3: Gomori's trichrome stain renal tissues from control, Gen, Gen + Date palm seed extract and Gen + Herbal mixture treated mice in the cortex (A) and medulla (B) showing collagen deposition in the tubular interstitial spaces (arrowhead), apical part of the distal, proximal tubules (short arrows) and glomeruli (long arrow) in Gen-treated animals compared to control mice. Mice treated with Date palm seed extract or Herbal mixture in Gen's presence showing less collagen deposition than Gen treated mice. The pictures were taken under 200 X magnification.

### 3.5 Effect of Gen with or without DPSE or HM pre-treatment on RXR-α proteins expression

Immunofluorescence staining showed a constitutive protein expression of RXR-α in the glomeruli, distal tubules, proximal tubule, collecting ducts, and early part of Henle's loops in the control group. On the other hand, mice injected with gentamycin alone showed reduced expression of RXR-α in the glomeruli, distal tubules, proximal tubule, collecting ducts, and early part of Henle's loops compared to control mice. Mice treated with Gen + DPSE or Gen + HM displayed significant restoration in RXR-α protein expression in all renal parts types compared to Gen treated mice (Figure 4 A, B, C).

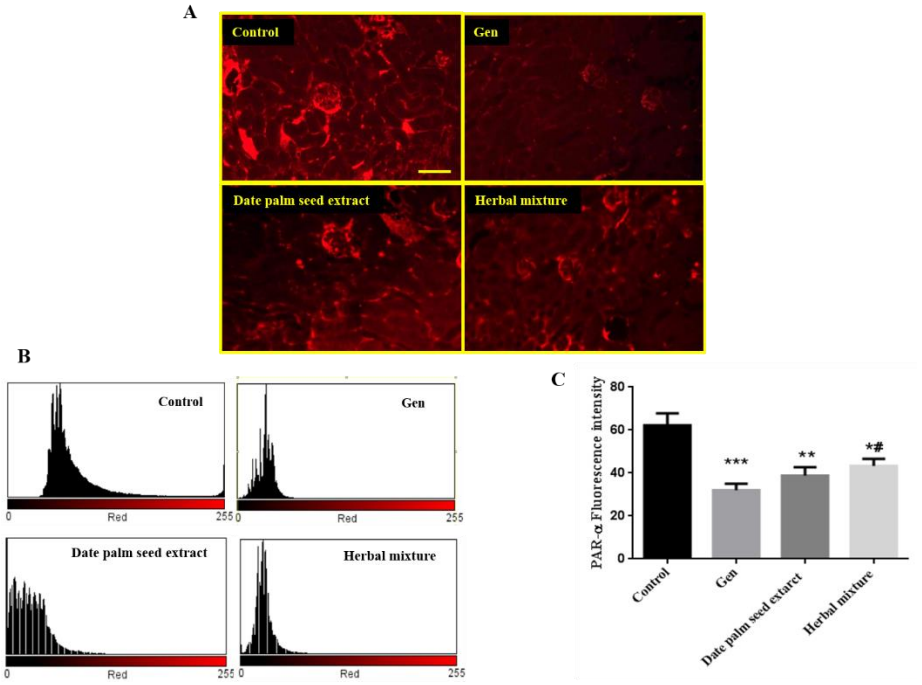


Figure 4: Photomicrographs of kidney sections showing the effect of Gen or Date palm seed extract or Herbal mixture on renal cortex using immunofluorescence analysis showing RXR-a expression (A), histogram showing the effect of treatments on different treated groups (B), and quantitative analysis of immunofluorescence staining of RXR-a (C). The intensity of fluorescence was quantified utilizing Image-J/ NIH software. Values are expressed as mean + SEM. *: significantly different compared to control mice and Gen in the presence or absence of Date palm seed extract or Herbal mixture, at $p < 0.05$.

### 3.6 Effect of Gen alone or with DPSE or HM on the expression of PAR-2

We identified negative or basal expression of PAR-2 proteins in renal tubules and glomeruli in control animals (Figure 5 A, B, C). Gen-treated mice displayed a significant increase in the protein expression of PAR-2 in the luminal part of renal tissue tubules. Pre-treating the mice with DPSE or HM decreased PAR-2 protein expression in the basolateral and apical sites of mice kidney sections (Figure 5 A, B, C).

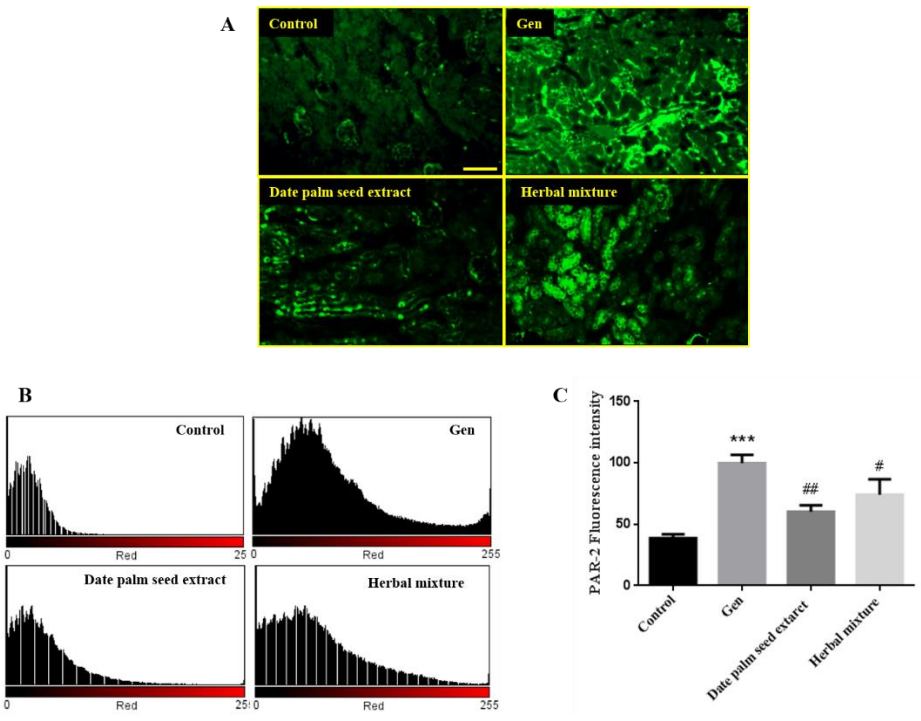


Figure 5: Photomicrographs of kidney sections showing the effect of Gen or Date palm seed extract or Herbal mixture on renal cortex using immunofluorescence analysis showing PAR-2 expression (A), histogram showing the effect of treatments on different treated groups (B), and quantitative analysis of immunofluorescence staining of PAR-2 (C). The intensity of fluorescence was quantified utilizing Image-J/ NIH software. Values are expressed as mean + SEM. *: significantly different compared to control mice and Gen in the presence or absence of Date palm seed extract or Herbal mixture, $p < 0.05$.

## 4 DISCUSSION

311 The current work is considered the first *in-vivo* study that clarifies the role of PAR-2 and RXR-α
312 and their effects in Gen-mediated renal toxicity. Moreover, the study evaluated the possible
313 protective effect of DPSE and HM against Gen- induced nephrotoxicity, as a confirmatory
314 evidence of our hypothesis.

315 Although Gen is considered as a low-cost and effective antibacterial drug, its clinical use is limited
316 due to its nephrotoxicity (33) demonstrated in the form of cortical and medullary tubular toxicities
317 as well as a reduction in glomerular filtration. A single dose of Gen caused nephrotoxicity in 10-
318 25% of patients (34). The kidney's main role is blood filtration as well as water and electrolytes
319 balance. One of the renal dysfunction results is its inability to filter the blood by the glomeruli or
320 the renal tubules' inability to keep water and electrolytes balanced. Increased reactive oxygen
321 species (ROS) production and oxidative stress are important mediators in renal injury. Gen
322 administration increases renal toxicity markers, including serum levels of BUN and Cr (35,36). In
323 the current work, injection of mice with Gen (100 mg/kg, i.p.) for seven days significantly
324 increased the plasma levels of BUN, Cr, and total WBCs, concomitant with a significant decrease
325 in platelets count compared to the control group. These findings confirmed the tubular dysfunction
326 of the kidney after Gen administration. On the other hand, Gen pre-treated mice with DPSE or HM
327 significantly decreased BUN, Cr, and total WBCs, and significantly elevated platelet counts
328 compared with Gen administration. These results come in great accordance with the previous study
329 which reported the significant effects of date palm`s flesh and seed extracts on reducing the
330 elevated plasma levels of urea and Cr in Gen-induced nephrotoxicity. This effect was suggested to
331 be attributed to the antioxidant phytoconstituents of date palm flesh and seed (2). *T. terrestris*
332 which is one of the herbal mixture components was previously reported to ameliorate Gen-induced
333 nephrotoxicity (37). The second component in the HM, *A. lanata*, is commonly employed in

Siddha system of medicine and is stated to exhibit a marked protective effect against Gen-induced nephrotoxicity in rat (38,39). Furthermore, the nephroprotective potential of *A. paniculata* and *R. sativus* was confirmed by several studies (31,32,40,41).

The current results demonstrated that, Gen caused distorted tubules with apoptotic cells lining these tubules. Furthermore, the Gen group exhibited inter-tubular inflammatory cells, widening the inter-tubular spaces with apoptotic cells in the renal glomeruli. Additionally, obliterated Bowman's space in the renal glomeruli and marked atrophic glomeruli were observed. The tubules appeared with cytoplasmic vacuolations and apparent dilated tubules with flatted cells and dilated renal blood vessels. These findings agree with previous results (42) in which Gen treatment (100mg/kg, i.p.) daily for seven days caused a massive tubular injury, necrosis, infiltration of inflammatory cells, and intraluminal hyaline casts. Another study reported that single intraperitoneal administration of Gen 200 mg/kg daily for 8 days significantly elevated renal toxicity biomarkers level and caused remarkable damage to glomerular and tubular structure (43). On the other hand, mice pre-treated with DPSE or HM markedly improved the obliterated histopathological features distorted by Gen alone. These findings add more evident for the possible nephroprotective effect of DPSE and HM. Few previous studies concluded similar findings, of *T. terrestris* and *A. lanata* on histopathological features (37,38).

The current work found a significant increase in PAR-2 protein expression in Gen-injected mice compared to the control group. To our best knowledge, this is the first report showing the involvement of PAR-2 in Gen-induced renal toxicity in mice. The coagulation system physiologically controls the hemostasis and thrombus development (12). Conversely, previous studies demonstrated coagulation factors involved in inflammatory responses and tissue repair (13,44). Many factors are involved in the coagulation system activation, and they have an essential

role in the activation, amplification, or stabilization of clotting (45). One of these factors is thrombin, an important key factor in activating the coagulation system through platelet activation and fibrinogen conversion to fibrin (46). Thrombin activates coagulation cascades and stimulates the release of various mediators like platelet-derived growth factor (PDGF), thromboxane A2, and transforming growth factor- β (TGF-β) from the platelets. Our results found an increase in the extracellular matrix accumulation after Gen treatment and reduced in mice pre-treated with DPSE or HM in the presence of Gen. Moreover, thrombin has significant pro-inflammatory effects by increasing numerous adhesion molecules, chemokines cytokines, and stimulating ROS production (15).

Thrombin also works as a chemo-attractant for neutrophils, monocytes, and macrophages to the area of inflammation. For these reasons, thrombin is a prospective driver of inflammatory reactions in diverse animal models like multiple sclerosis endotoxemia or sepsis (13). The pathogenic role of thrombin protein was previously reported in cerebral injury (6), liver injury (47), liver fibrosis (48), and neurotoxicity (49). It is noteworthy that this is the first report showing the coagulation system activation due to a significant elevation of PAR-2 compared to its basal expression in the control mice's renal tissues. On the other hand, pre-treatment of mice with DPSE or HM showed a significant decrease of PAR-2 protein expression compared to the Gen-treated group.

Retinoids are important in keeping the cell's regular function, such as a healthy immune system, differentiation, proliferation, normal male and female reproduction (3). Retinoic acid (RA) mediates these activities by binding to a family of nuclear receptors, the retinoid X receptors (RXRs), which involve three isotypes (α, β and γ) which affect transcription of several genes during vertebrate development (50). In the kidney, RXRs are cell-specific in expression; thus, they are practically responsible for activating both receptors by their respective agonists (51). No data

were found concerning the expression of RXR-α in the kidney and their role in renal disease progression in Gen-renal toxicity. In addition, the possible relation between PAR-2 and the retinoid receptor (RXR- α) needs to be clarified. Herein, results showed a significant reduction in RXR-α protein expression compared to its constitutive expression in the cortical and medullary tubules and the glomeruli of the control mice. On the other hand, Gen in the presence of DPSE or HM significantly increases RXR-α protein expression compared to Gen only treated group.

In conclusion, the current work displayed that the elevation of PAR-2 and reduction of RXR- α protein expression have an essential role in developing nephrotoxicity after Gen administration. Moreover, DPSE or HM reduced Gen-induced nephrotoxicity in mice mediated by reducing the coagulation system activation and prevention of RXR-α protein loss.

**Declaration of competing interest**

The authors declare no conflict of interest.

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# Role of PAR-2 and RXR- α in mitigating gentamicin-induced renal injury in 1 mice by Phoenix dactylifera L. seed extract and herbal mixture

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# Role of PAR-2 and RXR- α in mitigating gentamicin-induced renal injury in mice by *Phoenix dactylifera L.* seed extract and herbal mixture


## ABSTRACT

**Introduction**: Retinoid receptors, including retinoid X receptor (RXR), are vitamin A receptors expressed in the kidney and control several physiological functions by regulating different genes. Previously we demonstrated a coagulation system-dependent loss of RXR-α in monocrotaline/lipopolysaccharide-induced renal toxicity. Herein, we examined the involvement of RXR-α and protease-activated receptor 2 (PAR-2) in the protective effect of date palm seed extract (DPSE) and one nephroprotective herbal mixture (HM) against Gentamycin (Gen)-induced renal toxicity in mice.

**Methods**: Thirty-two mice divided randomly into four groups were either treated with saline, Gen (100 mg/kg/IP, daily for 10 days starting from the third day of the experiment), Gen and DPSE (100 mg/kg/P.O, daily for 10 days), or Gen and HM (100 mg/kg/P.O, daily for 10 days). Mice were sacrificed 24 h after the last dose administration, and kidney tissues were dissected out, weighed, and subjected to histological, immunofluorescence, and biochemical assays.

**Results**: The Gen-induced renal toxicity group demonstrated a significant decrease in RXR-α and a significant increase in PAR-2 protein expression. Treatment with DPSE or HM significantly improved Gen-induced effects on serum creatinine, BUN, WBCs, Platelets, RXR-α extracellular matrix deposition and PAR-2.

**Conclusion**: The present study revealed, for the first time, that retinoid receptors and PAR-2 might play an important role in Gen-induced renal toxicity. Furthermore, the nephroprotective effects of DPSE and HM were confirmed.

**Key words:** PAR-2; RXR-α; Gentamicin; Renal toxicity; Date palm seed, Herbal mixture.

**Abbreviations:** hepatic stellate cells (HSCs), retinoic acid (RA), retinoic acid receptors (RAR-α), retinoid X receptors (RXR-α), Monocrotalin/Lipopolysaccharide (MCT/LPS), retinoid X response element (RXRE), tissue factor (TF), platelet-derived growth factor (PDGF), transforming growth factor- β (TGF-β), reactive oxygen species (ROS) production, protease-activated receptors (PARs), Date palm seed extract (DPSE), Herbal mixture (HM).

## 1. INTRODUCTION

The kidneys are the body organ responsible for health maintaining functions such as preserving stable levels of important molecules in the blood as well as toxin excretion. Although kidneys are efficient in clearing many toxins from the blood, some chemicals are difficult to eliminate resulting in their accumulation with subsequent kidney damage (1).

Gentamicin (Gen) is an aminoglycoside antibiotic with broad spectrum activity against Gram-negative and Gram-positive bacteria. Though, it was reported that, in up to 30% of Gen-treated patients some signs of renal toxicity are observed with the drug treatment for more than 7 days (2).

Retinoids, found in the liver or other parts of the body, are stored mainly in hepatic stellate cells (HSCs), contributing significantly to cell proliferation and differentiation (3). In mice, hepatic retinoids are converted to retinyl ester and stored as lipid droplets in HSCs (4). In response to liver toxicity, HSCs activate with structural change, leading to loss of lipid droplets and stored retinoids (4). These lipid droplets are composed of retinoic acid (RA), retinyl ester, triglycerides, cholesteryl ester, cholesterol, phospholipid, and free fatty acids (5). Recently we reported the presence of retinoic acid receptors (RAR-α), and retinoid X receptors (RXR-α) in the released lipid droplets from HSCs in a new Monocrotalin/Lipopolysaccharide (MCT/LPS) idiosyncratic hepatotoxic

model and confirmed them as early and sensitive markers for HSCs activation. Signaling of RA is mediated *via* its occupancy of RAR and RXR, with subsequent DNA binding of a RAR–RXR heterodimer or an RXR–RXR homodimer to RA response element (RARE) or retinoid X response element (RXRE), respectively (6), thereby, regulating the transcription of target genes that control cellular proliferation, differentiation, and apoptosis (7). RAR-selective agonists are used clinically as anti-cancers, acne, and psoriasis treatments, whereas RXR agonists show potential for treating hyperglycemia in animal models of type II diabetes (8). RARs regulate the transcription of responsive genes as heterodimers with RXRs. In contrast, RXRs play a central role in nuclear receptor signaling by forming homodimers or acting as obligatory heterodimerization partners for various nuclear receptors (e.g., RARs, peroxisome proliferator-activated receptors, vitamin D receptors). Cytosolic speckled RAR-α distribution has been observed in activated HSC *in vitro* (9) and *in vivo* in our previous publication (10).

Many renal diseases such as membranous glomerulopathy, diabetic nephropathy, minimal change nephritis, and IgA nephritis are still difficult to diagnose without patient biopsy, thus complicating the treatment. The pathogenesis of the above-mentioned renal diseases has to be clarified to find preventive and suitable management. There is an urgent need for an accurate and sensitive biomarker to differentiate among the various renal diseases mentioned. In this regard, insufficient studies have discussed RXR-α expression level and its role in renal diseases. The RXR-α is predominantly found in renal tubules while it lacks the glomeruli expression (11). Our preliminary data revealed a translocation of RXR-α from basolateral into the apical site of distal tubules and collecting duct after MCT/LPS co-treatment, indicating a large probability to find RXR-α in the urine of these mice. In addition, RXR-α translocation, was a tissue factor (TF) dependent response.

The coagulation system's main role is to control the hemostasis and balance thrombus formation (12), in addition to its critical role in inflammation and angiogenesis (13). Various proteins play a part in the coagulation cascade; TF proteins' expression initiates the coagulation cascade till the accumulation of fibrin and clot formation (14). Thrombin does not only play a critical role in the initiation of coagulation cascade through platelet activation and conversion of fibrinogen to fibrin, but also potentiates in the movement of platelet-derived growth factor (PDGF), thromboxane A2 and transforming growth factor- β (TGF-β) from the platelets as well demonstrating its inflammatory effect through increased numbers of adhesion molecules, chemokines cytokines, and stimulating reactive oxygen species (ROS) production (15).

Thrombin acts as a recruiter for neutrophils and monocytes at the inflammatory site, thereby taking the inflammatory reaction forward like multiple sclerosis endotoxemia or sepsis (13). Similarly, thrombin's potential pathogen property is described in brain or liver injury and neurotoxicity (16,17).

Coagulation factors have a pleiotropic effect by activating protease-activated receptors (PARs), a G protein-coupled receptor family (16), like the activation of PAR-2 by expression of tissue factor/VIIa complex or factor Xa in the kidney (16,17). There are various reports of exacerbation of glomerular injury by PAR-2 in diabetic kidney disease (DKD) or glomerulonephritis (17), including preeclampsia antiphospholipid syndrome kidney injury models, while its role in Gen-induced kidney injury remains controversial. Conversely, PAR-2 signaling has demonstrated its role in endothelial proliferation/migration (18), including pro-angiogenic roles on limb ischemia and retinal neovascularization (19).

Herbal medicines have demonstrated their potential in the treatment of various ailments (20,21). Several plants are used in traditional systems of treatments for their nephroprotective activity e.g.

ginger, pomegranate seed oil, garlic, etc., however, most of these herbals are noted for nephroprotective effect on basis of old-age practices. Accordingly, extensive scientific studies are required in order to evaluate their pharmacological profile (22). Date palm seed extracts, *Phoenix dactylifera L.* (Family Arecaceae), is an industrial by-product of date processing, commonly used in some countries as an animal feed or coffee substitute. Although considered a waste product, its high content of polyphenolic compounds suggests its biological potential. Several studies reported its antimicrobial (23), antioxidant (24), and hepatoprotective activity (25). Other reports have demonstrated the nephroprotective effect of date palm`s fruits and pits extracts through their significant reduction in plasma creatinine and urea concentrations and amelioration of the proximal tubules' damage (2).

Also, previous studies have demonstrated the effect of poly herbal formulations like Sairie-to and BNO 2103 against Gen- or chromate-induced nephrotoxicity, respectively (26,27). In this context, one herbal mixture composed of *Tribulus terrestris, Aerva lanata, Andrographis paniculata and Raphanus sativus* in the ratio of (3:3:3:1) is claimed to have protective effect against kidney impairment, in India. *T. terrestris*, also known as "Qutiba" or "Darisa," belonging to the family Zygophyllaceae, grows in tropical zones and survives in the desert with low-nutrient soil in Saudi Arabia, southern Europe, southern Asia, and Africa. In addition to the various medicinal uses like an aphrodisiac, analgesic, antihypertensive, diuretic, urinary anti-septic, cardiotonic, hepatoprotective, anti-cancer properties (28), the plant is used locally in Saudi Arabia for urinary infections treatment (29). Moreover, the ethanol extract of *A. lanata* and aqueous extracts of *A. paniculata* and *R. sativus* demonstrated nephroprotection in Gen-induced nephrotoxicity in rats (30–32). *A. paniculata* plant is used in Ayurveda for various ailments and has demonstrated immunological, antibacterial, anti-inflammatory, antithrombotic, and hepatoprotective properties.

Accordingly, this study aims to shed light on the role of PAR-2 and RXR-α in Gen-induced renal injury. The study also clarifies the possible protective role of DPSE and the HM (*T. terrestris, A. lanata, A. paniculata,* and *R. sativus)* in renal toxicity induced model.

## 2. MATERIALS AND METHODS

### 2.1 Animal

Thirty-two male albino mice (22 ± 2 weeks old, weighing 30±2 g) were used. Animals were obtained from Qassim University Animal Facility, Qassim, Saudi Arabia, housed at temperature (25˚C ± 0.5), relative humidity with free access to standard forage and drinking water *ad libitum*. The animals were kept in a pathogen-controlled and air-conditioned room in the animal house. The institutional Research Ethics Committee, College of Pharmacy, Qassim University, Saudi Arabia, approved the animal experimental procedure and care (Approval ID 2020 - CP- 2). All practical experiments were carried out according to NIH Guidelines for the Care and Use of Laboratory Animals.

### 2.2 Chemicals, antibodies, and diagnostic kits

Gentamicin (Gen) was purchased from Mylan (IL, USA). Bovine serum albumin (BSA), DAPI (4, 6-diamidino-2- phenylindole), and Fluoromount were obtained from BIOMARK laboratories (India), horse serum was obtained from Sigma-Aldrich Co. (MI, USA), Dako solution was purchased from Dako (CA, USA). All other chemicals and solvents used were of analytical grade. Mouse monoclonal antibodies against PAR-2 (sc-514363) and RXR-□ (sc-28358) were purchased from Santa Cruz Biotechnology (TX, USA). Total protein in the urine colorimetric kit was obtained from Spinreact (Barcelona, Spain). Blood urea nitrogen (BUN) and creatinine diagnostic kits were obtained from Crescent Diagnostic Tests (KSA). Goat anti-rabbit Alexa fluor 488 was

purchased from Invitrogen (TX, United States). Cy3- conjugated Goat anti-rabbit antibody was obtained from Jackson Immunoresearch (PA, USA).

### 2.3 Plant material

The herbal mixture was obtained from HABBA Herbal Pvt. Ltd. (Bangalore, India). The herbal mixture consisted *of Tribulus terrestris, Aerva lanata, Andrographis paniculata and Raphanus sativus* in the ratio of (3:3:3:1).

Date palm (*Phoenix dactylifera* L.), var. Khodary fruit samples were collected from the Qassim region, Saudi Arabia, and the Ministry of Agriculture verified the sample identity. Date seeds were removed from the fruits, adequately washed with water, dried, and powdered. The powder was extracted exhaustively using aqueous methanol (80%), and the extract was dried at 40 °C using rotatory evaporator to give reddish brown residue.

### 2.4 Experimental design

Mice were randomly classified into four weight-matched groups, each of 8 mice. Group 1: received saline only (control group). Group 2: received Gen only (225 mg/kg, i.p., Gen group). Gen dose was chosen based on our preliminary experiments. Group 3: mice were treated with date palm seed extract (100 mg/kg, P.O) daily for ten days and Gen (225 mg/kg, i.p.) starting from the third day of the experiment and continued for seven days. Group 4: mice were treated with Herbal mixture extract (100 mg/kg, P.O) daily for ten days and Gen (225 mg/kg, i.p.) starting from the third day of the experiment and continued for seven days.

### 2.5 Serum preparation

Mice were anesthetized using Thiopental (40mg/kg, i.p), and blood was taken using a retro-orbital route with a non-heparinized capillary tube into EDTA tubes. Immediate estimation of the total leucocytes count and platelets was performed. Kidney function parameters: serum creatinine (Sr. Cr.) and BUN were determined in plasma obtained upon blood samples' centrifugation at 4000 rpm for 20 min.

### 2.6 Assessment of hematological parameters (WBCs and Platelets).

According to the manufacturer's instructions, white blood cells (WBCs) and platelet count were performed on whole blood using VABIO360 Auto Hematology Analyzer (BIOTA, Istanbul, Turkey).

### 2.7 Calculation of relative kidney weight

Animals' body weight was determined prior to the sacrifice. The whole kidney tissues were carefully isolated and washed with 0.9% sterile ice-cooled saline to remove any blood from the tissues and then gently pressed between 2 filter papers to absorb the excess saline solution. Afterward, each kidney was weighed, and the relative kidney weight calculated according to the following equation:

$$\textbf{Relative kidney weight} = \frac{\text{Weight of kidney (gm)}}{\text{Bodyweight of mice (gm)}} \times 100$$

### 2.8 Histopathological study

The kidneys were fixed in Davidson`s solution, followed by paraffin embedding, and tissue sections (4 $\mu$m) were stained with Hematoxylin and Eosin (H&E) and observed under a light microscope.

### 2.9 Bouin's trichrome staining

Bouin Trichrome Stain Kit was used to identify the extracellular matrix deposition in mice kidney tissues according to the manufacturer's instructions. Kidney tissues were immersed in tap water and transferred to warm Bouin's media (56°C) for 1 hr, then cooled to room temperature for 30 min. Prepared sections were then washed with tap water. Tissues were stained with Weigert's iron hematoxylin solution for 15 min then again washed with water. Finally, sections were stained with Trichrome for 20 minutes, placed in 0.5% acetic acid, and mounted with a mounting solution.

### 2.10 Photography

All photomicrographs were taken utilizing an Olympus (U.TV0.5XC-3) light microscope and digital camera.

### 2.11 Morphometric study

Using Image J 22 software (Version 1.52), the following items were detected in 10 non-overlapping fields in each mouse (×400) for each studied group:

1- The mean area percentage of collagen fibers deposition (%).

2- Maximal diameter of glomeruli in the mid-cortical region.

### 2.12 Immunofluorescence of tissue sections

Paraffin tissue sections of 4 $\mu$m thickness were deparaffinized by incubation of slides in xylene, 2 times for 15 minutes, then rehydrated through a graded ethanol series (2 x 100%, 95%, 70%, 50%, 30%, and distilled water) for 5 minutes each and washed in 10 mM phosphate-buffer 150 mM saline, pH 7.4. Antigen retrieval was performed by incubating the tissue sections in DAKO Target Retrieval Solution (10 mM Na-Citrate pH 6.0) for 20 min in a microwave oven (500W). After being cooled to RT, tissue sections were treated with methanol (100%) for 30 min at RT then washed twice with a washing solution (0.05% tween 20/ PBS). After blocking the sections with PBS containing 10% horse serum, 1% BSA in PBS for 1 hour, the slides were incubated with the primary antibody for 2 hours at 37°C and then overnight at 4°C. The slides were washed three times for 3 minutes in the washing solution and incubated directly with the fluorescence conjugated secondary antibody for 30 minutes at RT. The slides were washed two times for 3 minutes in the washing solution and incubated with DAPI (diluted 1:5000 in PBS) for 3 minutes. Slides were extensively washed three times for 10 minutes in the washing solution, and the excess washing solution was gently removed. Finally, tissue sections were mounted with fluoromount G. The slides were kept at a 37°C in the oven for 2 hours, and the evaluation was performed by a Zeiss microscope coupled to a 12-bit digital image camera

### 2.13 Statistical analysis

Data analysis was executed by unpaired two-tailed t-test or one-way ANOVA with Tukey- Kramer test for multiple comparisons using Graph Pad Instsat-2. A p-value of less than 0.05 was considered significant.

## 3 RESULTS

### 3.1 Effect of Gen alone or with DPSE or HM on WBCs and platelet counts:

Our results showed that Gen significantly altered WBCs and platelet counts in mice. In the current study, we observed that the injection of Gen significantly elevated WBCs count and significantly decreased platelet count compared to normal mice (Table 1).

Pre-treatment of animals with DPSE or HM significantly decreased WBCs count compared to Gen-induced renal toxicity group. Furthermore, both treatments renormalized platelet count compared to the Gen-induced renal toxicity mice group (Table 1).

**Table 1. Effect of Date palm seed extract (DSE) or Herbal mixture (HM) on hematological parameters in Gen-induced renal toxicity of mice**

| Groups | WBCs ($10^3$/ml) | Platelets ($10^3$/ml) |
|---|---|---|
| **Control** | 5.38 ± 0.301 | 768 ± 17.9 |
| **Gen** | 11.03 ± 0.72[a] | 381.06 ± 29.87[a] |
| **Gen + (DSE)** | 8.44 ± 0.56[a,b] | 512.04 ± 17.26[a] |
| **Gen + (HM)** | 6.64 ± 0.46[b] | 598.03 ± 19.05[b] |

Values are expressed as means ± SEM (n=6). WBCs, White blood cells. [a]: Significantly different from control group using one-way ANOVA followed by Tukey-Kramer post-test for multiple comparisons ($P < 0.05$). [b]: Significantly different from Gen treated group using one-way ANOVA followed by Tukey-Kramer post-test for multiple comparisons ($P < 0.05$).

### 3.2 Effect of Gen in the presence or absence of DPSE or HM on renal function parameters

In the current study, mice treated with gentamycin alone demonstrated significant elevation of Cr, and BUN levels in addition to kidney relative weight while a significant reduction in the % change

in the weight relative to the initial weight was observed compared to the normal mice (Figure 1: A-D).

Mice pre-treated with DPSE or HM (100 mg/kg) displayed a significant decrease in serum level of Cr and BUN as well as kidney relative weight. On the other hand, increased % change in the weight, was observed compared to the Gen-induced renal toxicity group. In addition, no significant difference was observed between mice treated with either DPSE and those treated with HM (Figure 1: A-D).

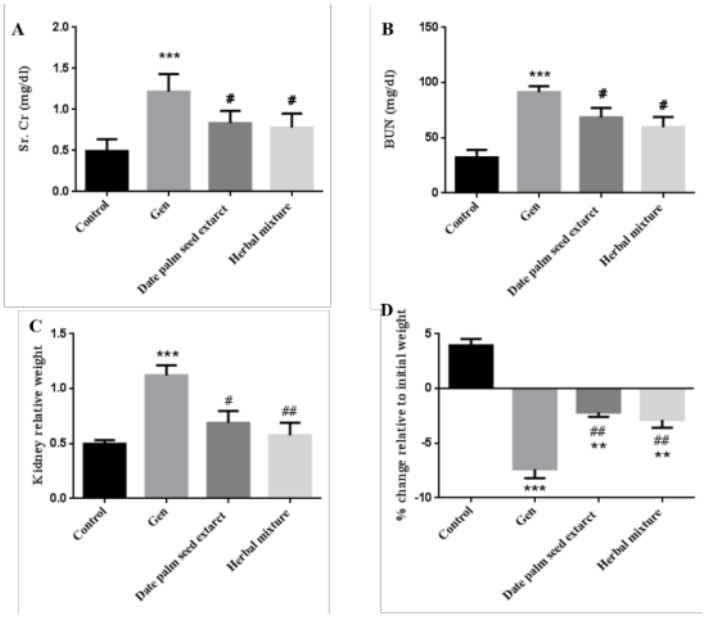


Figure 1: Effect of GEN injection in the presence or absence of Date palm seed extract or Herbal mixture on serum Creatinine (A); serum blood urea nitrogen parameters (B); kidney relative weight (C); and % change relative to initial weight (D). Data are expressed as mean + SEM. *: significantly different compared to control group, #: significantly different compared to Gen-treated group, at $p < 0.05$.

### 3.3 Effect of Gen with or without DPSE or HM in kidney histological examination

The control group showed normal renal tissues, including proximal and distal convoluted tubules. Renal glomeruli comprise renal corpuscles surrounding Bowman's space and capsules that look normal size and normal structure. On the other hand, the Gen-treated group's histopathological structures showed distorted tubules with apoptotic cells lining these tubules. Furthermore, the Gen-treated group exhibited inter-tubular inflammatory cells, widening the inter-tubular spaces with apoptotic cells in renal glomeruli. Additionally, obliterated Bowman's space in renal glomeruli and marked atrophic glomeruli were seen among the sections. The tubules appeared with cytoplasmic vacuolations and apparent dilated tubules with flatted cells and dilated renal blood vessels (Figure 2A, 2B). In contrast, tissues obtained from Gen + DPSE and Gen + HM (100 mg/kg) treated mice demonstrated moderate structural changes; fewer cytoplasmic vacuolations, few atrophic glomerular cells, mild inter-tubular inflammatory cells and widening of the inter-tubular spaces (Figure 2A, 2B).

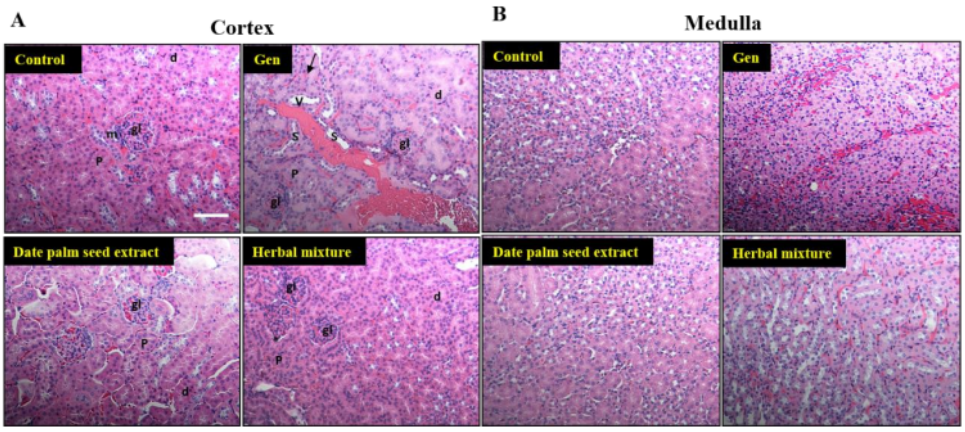


Figure 2: Histopathological examination of the control group's renal tissues, Gen-treated group, Gen + Date palm seed extract group, and Gen + Herbal mixture group. Photomicrographs of tissues in the renal cortex (A) and Medullary tubules (B) from control showing renal glomeruli (gl), proximal convoluted tubules (p), distal convoluted tubules (d), Bowman's spaces (star), and macula densa (m). Gen treated mice showing atrophic glomerulus (gl). The tubule appears with cytoplasmic vacuolations (v), and the dilated tubule appears with flatted cells (arrow). Notice the widening of inter-tubular space (s). The Gen + Date palm seed extract group shows less distorted glomerulus (gl) and tubule (d).

Less apoptotic cells are shown, and little cytoplasmic vacuolations are seen. Gen + Herbal mixture group showing less distorted tubule (d) and glomerulus (gl); but with a slight narrowing of renal Bowman's space, fewer apoptotic cells are shown, and little cytoplasmic vacuolations are seen (H&E × 200).

### 3.4 Effect of Gen and DPSE or HM on kidney fibrogenesis

Sections in the renal cortex from control group showed fewer collagen fibers surrounding the renal tubules, Bowman's capsules, and the capillary tuft of glomeruli, while gentamycin injection increased collagen deposition around the dilated blood vessels. On the other hand, Gen + DPSE or Gen + HM (100 mg/kg) treated mice showed fewer collagen fibers around a capillary tuft of glomeruli and blood vessels (Figure 3A, 3B).

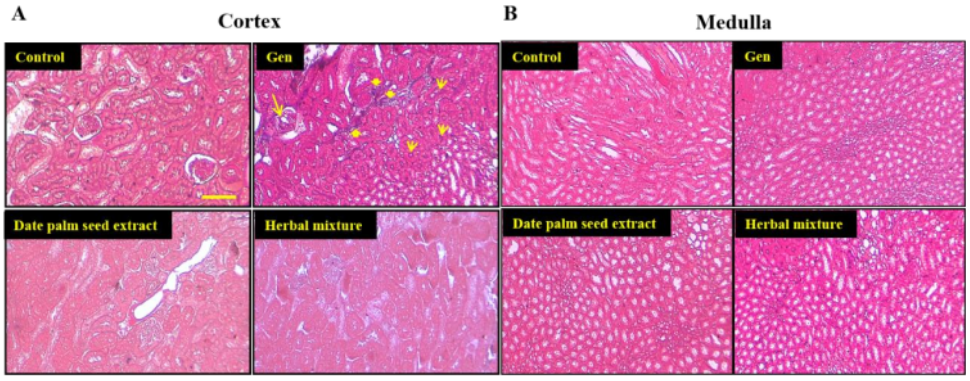


Figure 3: Gomori's trichrome stain renal tissues from control, Gen, Gen + Date palm seed extract and Gen + Herbal mixture treated mice in the cortex (A) and medulla (B) showing collagen deposition in the tubular interstitial spaces (arrowhead), apical part of the distal, proximal tubules (short arrows) and glomeruli (long arrow) in Gen-treated animals compared to control mice. Mice treated with Date palm seed extract or Herbal mixture in Gen's presence showing less collagen deposition than Gen treated mice. The pictures were taken under 200 X magnification.

### 3.5 Effect of Gen with or without DPSE or HM pre-treatment on RXR-α proteins expression

Immunofluorescence staining showed a constitutive protein expression of RXR-α in the glomeruli, distal tubules, proximal tubule, collecting ducts, and early part of Henle's loops in the control group. On the other hand, mice injected with gentamycin alone showed reduced expression of RXR-α in the glomeruli, distal tubules, proximal tubule, collecting ducts, and early part of Henle's loops compared to control mice. Mice treated with Gen + DPSE or Gen + HM displayed significant restoration in RXR-α protein expression in all renal parts types compared to Gen treated mice (Figure 4 A, B, C).

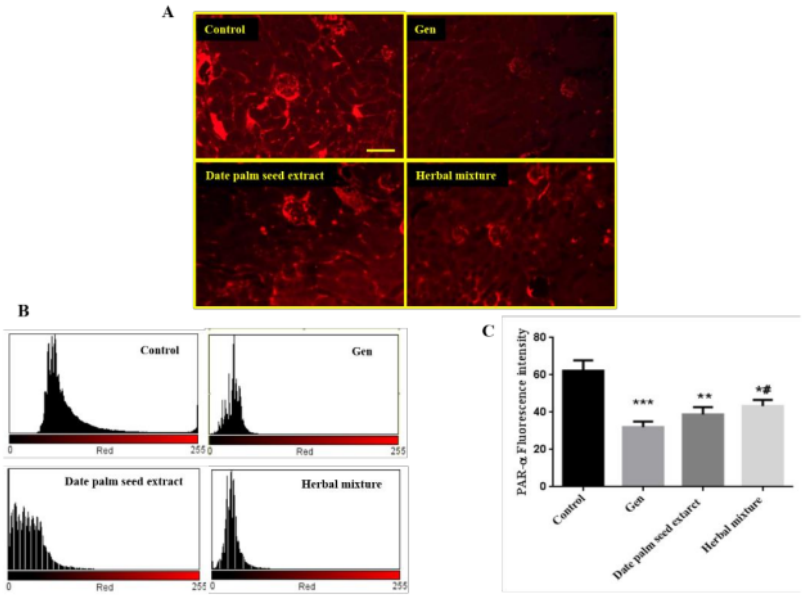


Figure 4: Photomicrographs of kidney sections showing the effect of Gen or Date palm seed extract or Herbal mixture on renal cortex using immunofluorescence analysis showing RXR-a expression (A), histogram showing the effect of treatments on different treated groups (B), and quantitative analysis of immunofluorescence staining of RXR-a (C). The intensity of fluorescence was quantified utilizing Image-J/ NIH software. Values are expressed as mean + SEM. *: significantly different compared to control mice and Gen in the presence or absence of Date palm seed extract or Herbal mixture, at $p < 0.05$.

### 3.6 Effect of Gen alone or with DPSE or HM on the expression of PAR-2

We identified negative or basal expression of PAR-2 proteins in renal tubules and glomeruli in control animals (Figure 5 A, B, C). Gen-treated mice displayed a significant increase in the protein expression of PAR-2 in the luminal part of renal tissue tubules. Pre-treating the mice with DPSE or HM decreased PAR-2 protein expression in the basolateral and apical sites of mice kidney sections (Figure 5 A, B, C).

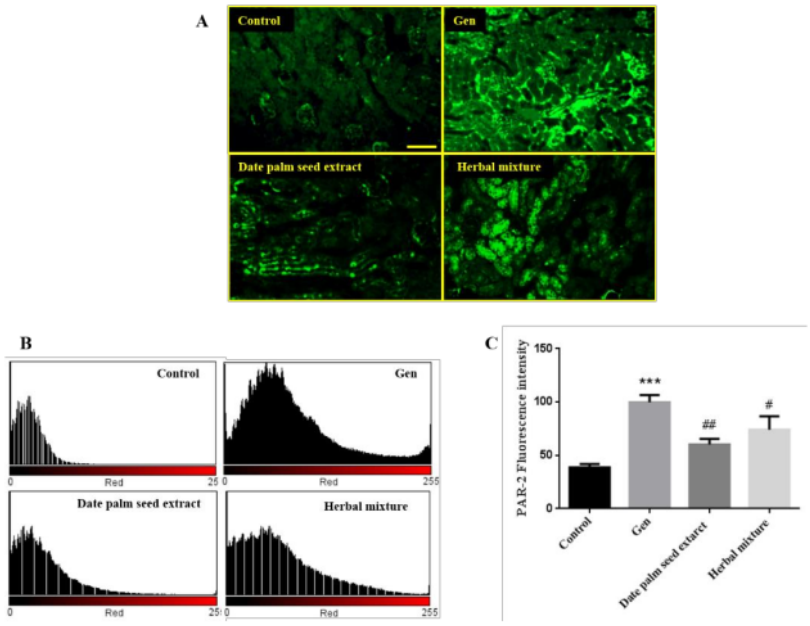


Figure 5: Photomicrographs of kidney sections showing the effect of Gen or Date palm seed extract or Herbal mixture on renal cortex using immunofluorescence analysis showing PAR-2 expression (A), histogram showing the effect of treatments on different treated groups (B), and quantitative analysis of immunofluorescence staining of PAR-2 (C). The intensity of fluorescence was quantified utilizing Image-J/ NIH software. Values are expressed as mean + SEM. *: significantly different compared to control mice and Gen in the presence or absence of Date palm seed extract or Herbal mixture, $p < 0.05$.

## 4 DISCUSSION

The current work is considered the first *in-vivo* study that clarifies the role of PAR-2 and RXR-α and their effects in Gen-mediated renal toxicity. Moreover, the study evaluated the possible protective effect of DPSE and HM against Gen- induced nephrotoxicity, as a confirmatory evidence of our hypothesis.

Although Gen is considered as a low-cost and effective antibacterial drug, its clinical use is limited due to its nephrotoxicity (33) demonstrated in the form of cortical and medullary tubular toxicities as well as a reduction in glomerular filtration. A single dose of Gen caused nephrotoxicity in 10-25% of patients (34). The kidney's main role is blood filtration as well as water and electrolytes balance. One of the renal dysfunction results is its inability to filter the blood by the glomeruli or the renal tubules' inability to keep water and electrolytes balanced. Increased reactive oxygen species (ROS) production and oxidative stress are important mediators in renal injury. Gen administration increases renal toxicity markers, including serum levels of BUN and Cr (35,36). In the current work, injection of mice with Gen (100 mg/kg, i.p.) for seven days significantly increased the plasma levels of BUN, Cr, and total WBCs, concomitant with a significant decrease in platelets count compared to the control group. These findings confirmed the tubular dysfunction of the kidney after Gen administration. On the other hand, Gen pre-treated mice with DPSE or HM significantly decreased BUN, Cr, and total WBCs, and significantly elevated platelet counts compared with Gen administration. These results come in great accordance with the previous study which reported the significant effects of date palm`s flesh and seed extracts on reducing the elevated plasma levels of urea and Cr in Gen-induced nephrotoxicity. This effect was suggested to be attributed to the antioxidant phytoconstituents of date palm flesh and seed (2). *T. terrestris* which is one of the herbal mixture components was previously reported to ameliorate Gen-induced nephrotoxicity (37). The second component in the HM, *A. lanata*, is commonly employed in

Siddha system of medicine and is stated to exhibit a marked protective effect against Gen-induced nephrotoxicity in rat (38,39). Furthermore, the nephroprotective potential of *A. paniculata* and *R. sativus* was confirmed by several studies (31,32,40,41).

The current results demonstrated that, Gen caused distorted tubules with apoptotic cells lining these tubules. Furthermore, the Gen group exhibited inter-tubular inflammatory cells, widening the inter-tubular spaces with apoptotic cells in the renal glomeruli. Additionally, obliterated Bowman's space in the renal glomeruli and marked atrophic glomeruli were observed. The tubules appeared with cytoplasmic vacuolations and apparent dilated tubules with flatted cells and dilated renal blood vessels. These findings agree with previous results (42) in which Gen treatment (100mg/kg, i.p.) daily for seven days caused a massive tubular injury, necrosis, infiltration of inflammatory cells, and intraluminal hyaline casts. Another study reported that single intraperitoneal administration of Gen 200 mg/kg daily for 8 days significantly elevated renal toxicity biomarkers level and caused remarkable damage to glomerular and tubular structure (43). On the other hand, mice pre-treated with DPSE or HM markedly improved the obliterated histopathological features distorted by Gen alone. These findings add more evident for the possible nephroprotective effect of DPSE and HM. Few previous studies concluded similar findings, of *T. terrestris* and *A. lanata* on histopathological features (37,38).

The current work found a significant increase in PAR-2 protein expression in Gen-injected mice compared to the control group. To our best knowledge, this is the first report showing the involvement of PAR-2 in Gen-induced renal toxicity in mice. The coagulation system physiologically controls the hemostasis and thrombus development (12). Conversely, previous studies demonstrated coagulation factors involved in inflammatory responses and tissue repair (13,44). Many factors are involved in the coagulation system activation, and they have an essential

role in the activation, amplification, or stabilization of clotting (45). One of these factors is thrombin, an important key factor in activating the coagulation system through platelet activation and fibrinogen conversion to fibrin (46). Thrombin activates coagulation cascades and stimulates the release of various mediators like platelet-derived growth factor (PDGF), thromboxane A2, and transforming growth factor- β (TGF-β) from the platelets. Our results found an increase in the extracellular matrix accumulation after Gen treatment and reduced in mice pre-treated with DPSE or HM in the presence of Gen. Moreover, thrombin has significant pro-inflammatory effects by increasing numerous adhesion molecules, chemokines cytokines, and stimulating ROS production (15).

Thrombin also works as a chemo-attractant for neutrophils, monocytes, and macrophages to the area of inflammation. For these reasons, thrombin is a prospective driver of inflammatory reactions in diverse animal models like multiple sclerosis endotoxemia or sepsis (13). The pathogenic role of thrombin protein was previously reported in cerebral injury (6), liver injury (47), liver fibrosis (48), and neurotoxicity (49). It is noteworthy that this is the first report showing the coagulation system activation due to a significant elevation of PAR-2 compared to its basal expression in the control mice's renal tissues. On the other hand, pre-treatment of mice with DPSE or HM showed a significant decrease of PAR-2 protein expression compared to the Gen-treated group.

Retinoids are important in keeping the cell's regular function, such as a healthy immune system, differentiation, proliferation, normal male and female reproduction (3). Retinoic acid (RA) mediates these activities by binding to a family of nuclear receptors, the retinoid X receptors (RXRs), which involve three isotypes (α, β and γ) which affect transcription of several genes during vertebrate development (50). In the kidney, RXRs are cell-specific in expression; thus, they are practically responsible for activating both receptors by their respective agonists (51). No data

were found concerning the expression of RXR-α in the kidney and their role in renal disease progression in Gen-renal toxicity. In addition, the possible relation between PAR-2 and the retinoid receptor (RXR- α) needs to be clarified. Herein, results showed a significant reduction in RXR-α protein expression compared to its constitutive expression in the cortical and medullary tubules and the glomeruli of the control mice. On the other hand, Gen in the presence of DPSE or HM significantly increases RXR-α protein expression compared to Gen only treated group.

In conclusion, the current work displayed that the elevation of PAR-2 and reduction of RXR- α protein expression have an essential role in developing nephrotoxicity after Gen administration. Moreover, DPSE or HM reduced Gen-induced nephrotoxicity in mice mediated by reducing the coagulation system activation and prevention of RXR-α protein loss.

**Declaration of competing interest**

The authors declare no conflict of interest.

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