

Screening impacts of Tilmicosin induced-hepatic and renal toxicity in rats: Protection by Rhodiola Rosea extract through the involvement of oxidative stress, antioxidants, and inflammatory cytokines biomarkers

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Abstract

Tilmicosin (TIL) is a semisynthetic macrolide antibiotic with a broad spectrum of activity derived from tylosin. TIL is effective in the treatment of bovine and ovine respiratory diseases caused by different microbes. In parallel, *Rhodiola rosea* (RHO) is a popular herbal remedy because of its anti-inflammatory and antioxidant qualities. Rats received saline or RHO for sequential 12 days. TIL was injected as a single dose subcutaneously (75 mg/kg BW) on day 6 of experiment. The protective group received RHO daily for sequential 12 days, TIL was injected as a single dose 1 hour after RHO administration on day 6 of experiment and continued for extra 6 successive days with RHO only. Samples and blood were collected for serum analysis, gene expression and immunohistochemistry screening at liver and kidney levels. TIL injection increased serum levels of hepatic and renal markers (ALP, ALT, AST, TC, TG, creatinine and urea) with a decrease in total proteins. In parallel, TIL induced hepatic and renal oxidative stress as there was an increase in malondialdehyde levels, with a decrease in catalase and reduced glutathione activities. Of interest, pre-administration of RHO inhibited TIL-induced increase in hepato-renal markers and decrease the oxidative stress and increased antioxidant activities of both liver and kidney. Quantitative RT-PCR showed that TIL increased HSP70 (heat shock protein), NF κ B and TNF- α mRNA expression in liver. Moreover, TIL upregulated the expression of desmin, nestin, and vimentin expression in kidney. The upregulated genes were decreased significantly in the protective group received RHO. Serum inflammatory cytokines, together with genes of inflammatory markers in liver tissues (HSP70, NF κ B and TNF- α) and in kidney tissues (desmin, nestin, and vimentin) were all affected. TIL induced hepatic vacuolation and congestion together with glomerular atrophy. The immunoreactivity of PCNA and HMGB1 were examined immunohistochemically. At cellular levels, PCNA was decreased while HMGB1 immunoreactivity was increased in TIL injected rats and was improved by pre administration of RHO. RHO administration protected the altered changes in liver and renal histology. Current findings support the possible use of RHO to shield the liver and kidney from the negative effects of tilmicosin.

Introduction

Tilmicosin (TIL) is an extensively used macrolide antibiotic (semi-synthetic) used in a wide range in veterinary purposes. TIL inhibits the protein synthesis of most microbes through the binding of 50S ribosome subunit. TIL is widely known for pneumonia treatment caused by several microbes and mycoplasma in sheep, ruminants, and swine [1, 2]. In addition, TIL prevent and treat mastitis in ruminants [3]. TIL's efficacy is a result of its low inhibitory concentrations, and extended elimination half-life [4]. Previous research has shown that improper administration of TIL therapy may result in organ harm, including hepatotoxicity [5] and cardio-toxicity [6]. Even though, the mechanisms underlying TIL-induced hepatotoxicity and nephrotoxicity remain unknown, it has been reported that TIL induces protein, DNA, and lipid damages that result in apoptosis [7]. Additionally, TIL may induce oxidative stress and deplete hepatic, renal and cardiac antioxidant defense mechanisms of treated animals [5, 8, 9].

It is widely accepted that the human liver and kidneys are the two most important organs for maintaining health. The kidneys regulate acid-base balance, blood pressure, and electrolyte balance in the body. The

kidney and liver both act as natural blood filters, eliminating toxins from the body [10]. The hematopoietic system relies on both organs. Drugs and their metabolites undergo metabolism in the liver and are detoxified and excreted through the kidneys. This results in frequent injury to them [11].

The most important kidney component for maintaining glomerular structure is the podocyte. As the final component of the ultrafiltration barrier, podocytes are located at the basement of glomeruli and control the flow of most of the filtrate through the holes between cells [12]. Chronic renal disorders and focal segmental glomerulosclerosis are brought on by damage to podocytes [13]. For visceral epithelial cells, podocytes express unique intermediate filament proteins (IFs) named vimentin, nestin, and desmin. Changes in IFs' gene expression are frequently linked to tissue damage [14]. Podocytes boost a cell's mechanical resistance by attaching to many capillaries via their foot and main processes [15].

TIL-induced hepatic and renal tissue damage may be mediated in part by inflammation since oxidative stress and inflammation mutually reinforce one another, leading to a self-perpetuating loop of inflammatory response and oxidative injury [16]. As a result, there is a greater need to identify new ingredients from natural sources as safe therapeutic agents against different toxicants [17].

Rhodiola rosea (RHO), named "golden root" or "roseroot," is a valuable phytochemistry that is native to the frigid, mountainous regions of Tibet and China. RHO is mostly recognized as an adaptogen [18]. Both the cardiovascular and central nervous systems can benefit from RHO's effects. Several physiologically active chemicals found in RHO have been hypothesized to induce its actions [19]. Glycoside compound rhodioloside (salidroside) and the family of rosavins, which includes the predominantly rhizome-derived rosavin, rosarin, and rosin, have shown medicinal efficacy [20]. Salidroside has been hypothesized to be RHO's primary active component in several investigations [21]. RHO's pharmacological impacts and therapeutic usefulness for a variety of cardiovascular disorders [22] are dependent on the composition of its many chemical components.

Many biological functions, such as antioxidant, proliferation inhibition of cancer cells, and immune regulation have been attributed to RHO in both clinical practice and experimental studies [18]. It was also shown that RHO extract protected muscular tissue during exercise and had anti-inflammatory actions [23]. Anti-aging, anti-cancer, cardio-protective, anti-diabetic, and neuro-protective benefits are only few of the many beneficial effects it possesses [18]. When given to rats, RHO reduced the negative effects of stress [24, 25].

Therefore, this research describes RHO's potential protective mechanisms that reduce TIL's liver and kidney toxicity by regulating inflammation, oxidative stress, and antioxidant markers at molecular, biochemical, and cellular signaling pathways.

Materials and methods

Reagents, chemicals and kits

Elanco Animal Health Company, Australia provided a commercial formula of tilmicosin (Micotil® 300 solution; AH0230). Rhodiola root extract was supplied in capsules each containing 250 mg and were purchased from the Puritan's Pride® Company (New York, USA). Test kits for alanine amino-transferase (ALT), total proteins, urea, aspartate amino-transferase (AST), creatinine, and alkaline phosphatases (ALP) were purchased from Bio-diagnostics Laboratory (Giza, Egypt). The malondialdehyde (MDA) used was manufactured by the North American branch of the Life Span Biosciences Company, USA. The reduced glutathione (GSH) and catalase (CAT) were from Biodiagnostic Company, Egypt. Sigma Aldrich (Memphis MO, USA) supplied the H&E (hematoxylin and eosin) stain. *Rhodiola rosea* was bought from Shijiazhuang Zangnuo Bio-Tech Co., Ltd. Qiazol, and PCR Master Mix of SYBR Green type were from QIAGEN Company, Valencia, USA. Antibodies for PCNA (Anti-PCNA Mouse mAb GB12010) and HMGB1 (Anti-HMGB1 Rabbit pAb GB11103) immunohistochemistry were from Wuhan Servicebio technology co., LTD.China 430000. The secondary antibodies were from HRP Goat Anti-Rabbit IgG (GB23303) and were from same company.

Rhodiola rosea preparation for HPLC Analysis for phenols and flavonoids compounds

For the total phenols and flavonoids, the *R. rosea* capsules were dissolved in methanol and then used for HPLC analysis according to the protocol [26]. An Agilent 1260 Infinity HPLC (Agilent, USA) was utilized, equipped with a quaternary pump. A Kinetex® 5 µm EVO C18 100 mm × 4.6 mm (Phenomenex, USA) column was used and operated at 30°C. The separation was done using a ternary linear elution gradient with (A) HPLC-grade water and 0.2% H₃PO₄ (v/v), (B) methanol, and (C) acetonitrile. Thereafter, 20 µL of the sample was injected. A variable wavelength detector set (284 nm) was used for the recognition of phenols and flavonoids. The analysis was conducted at Cairo University, Faculty of Agriculture, Food Safety and Quality Control Laboratory (Reference Number: FSQC0007-21).

Animals design, treatments, and sampling

Twenty-four male Wister rats were from experimental breeding farm at Helwan, Cairo, Egypt. These rats were 60 days old and weighed between 180 and 200 gm. Kafer Elsheikh University ethical committee approved sampling, procedures and treatments used in this study (Ethical code: KFS-IACUC/132/2023). Fifteen days before onset of experiment, animals were maintained at 12 hrs a day and/night cycle without limitation in feed and water (El-Nasr Co., Cairo, Egypt) at room temperature (25 ± 5°C). Animals were acclimatized and stressed-out during therapy and administration by being physically handled. After the rats had adjusted to their new environment, they were allocated into four groups of six. Each day for 12 days, rats in Group I (control) were given 1 mL of distilled water through stomach tube. Group II (Rhodiola; RHO), rats were given 1 mL of RHO orally at a concentration of 400 mg/kg BW daily [27] for a total of 12 days. Rats in Group III (Tilmicosin-treated) (TIL) were injected subcutaneously (SC) once with 75 mg/kg body weight (BW) [28, 29] on day six of the trial. Rats in Group IV (RHO + TIL) were given Rhodiola for a total of 12 days; on day 6, they received a single SC injection of TIL 1 hour after the RHO administration, and for the next 6 days, they received only RHO. On day six of the trial, all rats in the normal and RHO groups got a single subcutaneous (SC) injection of the vehicle (NaCl 0.9%), whereas those in the other two groups received a single SC injection of TIL. Previous dosage-dependent studies [23, 24] shown that

injections of TIL caused considerable tissue damage through oxidative stress and apoptosis, and this information was used to establish the dose used in the current investigation. [28, 29].

On day 13 of experiment, rats were fasted for 12 hours after last treatment [9]. The rats were decapitated after being sedated for 2–5 minutes in a desiccator using diethyl-ether. Serum was separated from blood samples by centrifuging at 5000 rpm for 8 minutes after obtaining them through cardiac puncture. For biochemical evaluations, serum was kept at 30°C. Parts of kidney and liver were kept at 70°C in Qiazol for future gene expression analysis and qRT-PCR. Second some parts from the kidney and liver were homogenized and used for measurement of oxidative stress markers. For immunohistochemistry and histology, the third part of tissues preserved in Bowman's solution.

Hepatic and Renal biomarkers measurements

Bio-diagnostic Company, Dokki, Giza, Egypt, supplied the kits used to measure blood levels of ALT, AST, ALP, creatinine, and urea. BIO-RAD spectrophotometer was used to measure biochemical markers in the serum following the instructions supplied with kits [30, 31]. Both cholesterol (TC) and triglyceride (TG) levels were measured spectrophotometrically using commercial assay kits (Sigma Chemical Co., India). Lowry et al.'s technique [32] was used to quantify total proteins in the serum.

Oxidative stress assessment in kidney and liver homogenate

Tissue (liver and kidney) was weighted out at 0.1 g homogenized at a homogenate yield of 10%, and centrifuged with 4000 rpm, 4°C, for 8 minutes for collection of clear upper layer. Using Ellman's method [33], the GSH concentrations were determined spectrophotometrically at 410 nm in the homogenate. The obtained information was expressed as U/g of tissue. MDA was measured as previously described [34]. The obtained values were expressed as nmol/g tissue. Catalase was measure as state before [35].

Assessment of cytokines levels

Serum levels of IL-1 β , and TNF- α were assayed commercially using available ELISA kits based on the instruction's guidelines for each kit (ab255730 and ab46070, respectively). The levels of IL-10 were determined with the use of a commercial kit (Rat IL-10, ab100765) imported from Abcam (Memphis, LA, USA). Data obtained from ELISA reader was analyzed based on specific steps described in the kit manual.

Gene Expressions analysis in liver and kidney tissues

Quantitative real time-PCR (qRT-PCR) was used to express and quantify different genes in liver and kidney. Briefly, Qiazol reagents were used for RNA extraction from approximately 100 μ g liver or kidney sample. cDNA was done using Multi-Scribe reverse transcription-enzyme kit. Reactions of qRT-PCR were carried out in 7500 RT-PCR machine using PCR Master Mix of SYBR Green stain type. Examined genes were quantified using $2^{-\Delta\Delta CT}$ method. The standard internal gene was β -actin was used to normalize altered genes. Changes in genes' intensity was analyzed using the comparative cycle threshold values (CT) [36].

Hepatic and renal histopathology and Immunohistochemistry

Liver and kidney slices were embedded in paraffin after sectioning and dehydration. The liver and kidney slides were stained by H&E and seen with an optical microscope. For immunohistochemistry, sections were cut to be 3 μ m thick. The endogenous peroxidase activity in the sections was inhibited by dewaxing, rehydrating, and treating with a 3% hydrogen peroxide in methanol solution. The sections were submerged in 10 mM citric acid solution (pH = 6.0) for ten minutes before being incubated with specific antibodies. For PCNA immunoreactivity, we first used a primary anti serum specific to PCNA (DAKO, Denmark). Next, we used secondary horse anti-mouse antiserum (a biotinylated avidin-biotin), and diaminobenzidine. Finally, hematoxylin solution was used to counterstain the slices before they were dried and mounted in Canada balsam. The nuclei of liver and tubular cells turned a dark brown color, indicating a good response [37, 38].

High mobility group box-1 (HMGB1) expression and immunoreactivity was examined immunohistochemistry (IHC) in successive 4- μ m slices for liver and kidney. The slides were rehydrated with ethanol after being deparaffinized in xylene. To facilitate better visibility of extra nuclear HMGB1, antigen retrieval treatment was eliminated prior to staining. Sections were incubated in 3% hydrogen peroxide, followed with human sera (2%) for half an hour to block nonspecific reactants, and finally in an avidin/biotin-blocking process (Vector Laboratories Inc., USA) to eliminate any remaining endogenous peroxidase activity. Next, we used a mouse IgG2b anti-HMGB1 antibody for overnight (Critical Therapeutics Inc., USA). For detection, we employed a anti-mouse antibody of horse type tagged with biotin (Vector Laboratories Inc. USA), which was diluted in 2% normal horse serum. The staining was created using DAB kit. Mayer's hematoxylin was used as a counterstain on tissue sections. Specificity of HMGB1 staining was evaluated using specific primary isotype-matched immunoglobulin (Dako, Glostrup, Denmark) in parallel staining studies in which the primary antibody was omitted [39].

The number of immune-reactive cells for PCNA and HMGB1 were determined by the percentage of cells that stained positively compared to the total examined analyzed number of cells. ANOVA tests were used to examine the significance between 3 slides from 6 rats in each group.

Statistical analysis

Data were analyzed and presented by SPSS software (SPSS Inc., Chicago, IL) to create tables and different correlations on the collected data. Statistics were reported using a mean and standard deviation. The Shapiro-Wilks test was used to ensure normality with the assumption that $t p < 0.05$. ANOVA analyzed the significance between examined groups of normally distributed variables. Post hoc Turkey test was used to identify statistically significant pairings following a significant analysis of variance. In this study, a significant level of $p < 0.05$ was considered significant.

Results

HPLC components of RHO extract

Table 2 and Fig. 1 shows the different phenols and flavonoids peaks and contents detected in the rhodiola rosea extract, respectively. The extract shows major contents of silymarin (27.2 mg/l), rutin (2.7 mg/l), vanillic acid (0.1 mg/l), syringic acid (0.016mg/l), gallic acid (0.0037mg/l), para-amino benzoic acid (0.002mg/l), ferulic acid (0.00010 mg/l) and apeinine in a concentration of 0.00006 mg/l.

Table 1
The primers of gene expression by qRT-PCR

Gene	Direction	Primer sequence	Annealing temp.	(bp)	Accession number
HSP70	For:	GCTGCTGGTGCACGATTCTT	58°C	214	NM_001329896.1
	Rev:	TGGCAGTGCTGAGGTGTTC			
NF-kB	For:	TTCAACATGGCAGACGACGA	57°C	133	NM_001415012.1
	Rev:	GGTATGGGCCATCTGCTGTT			
IL-1β	For:	TTGAGTCTGCACAGTTCCCC	57°C	161	NM_031512.2
	Rev:	GTCCTGGGGAAGGCATTAGG			
TNF-α	For:	ACTGAACTTCGGGGTGATCG	57°C	153	NM_012675.3
	Rev:	GCTTGGTGGTTTGCTACGAC			
GAPDH	For:	AGGTGGAAGAATGGGAGTTG	55°C	197	NM_017008.4
	Rev:	GCTTTCTCCAACCTCTCCTACTACA			
Desmin	For:	CCAACTGAGAGAAGAAGCAGAG	56°C	223	NM_022531.2
	Rev:	CTTATTGGCTGCCTGAGTCAAG			
Nestin	For:	GTCCTGGTTCCTGAACTTGTC	58°C	161	AF538924
	Rev:	GCTTCTTTCTCTACCAGTTCCC			
Vimentin	For:	CTGCTGGAAGGGGAGGAGAG	58°C	226	M84481.1
	Rev:	GGTCATCGTGGTGCTGAGAAG			

Table 2
Total phenolic compounds of Rhodiola roesa extract.

Compound Name	Response	Retention time per minute	Amount [mg/l]
Gallic Acid	176.123	1.848	0.003710
Trans Ferulic Acid	450.865	3.293	0.000010
Para Amino Benzoic Acid	646.512	4.327	0.002021
Vanillic Acid	2493.633	8.023	0.105928
Syringic Acid	1019.886	8.210	0.016094
Apeinine	4047.686	9.195	0.000059
Rutin	22727.052	15.742	2.035400
Sylimarine	47287.849	21.527	27.692959
All Signals Total 29.856171			

Impacts of RHO on TIL-induced alteration in liver and kidney biomarkers

The TIL-treated group showed significant elevations in the ALT, AST, and ALP and a decrease in the total proteins. In same time TIL induced significant increase in renal biomarker urea and creatinine in TIL injected rats. TC and TG levels were increased in TIL injected rats. Oral pre-administration of RHO then TIL protected rats from such alteration in liver and kidney activity markers (Table 3). RHO retrieved the changes in ALT, ALP, AST, and total proteins, TC, TG urea and creatinine.

Table 3
Ameliorative impacts of RHO against TIL-induced changes in kidney and liver biomarkers.

		Control	RHO	TIL	TIL + RHO
Liver	AST (IU/L)	78.75 ± 2.84 ^a	80.75 ± 3.35 ^a	178.66 ± 4.32 ^b	125.83 ± 3.97 ^c
	ALT (IU/L)	29.75 ± 1.69 ^a	31.25 ± 1.96 ^a	130.16 ± 2.85 ^b	62 ± 2.36 ^c
	ALP (IU/L)	129 ± 2.82 ^a	132 ± 3.34 ^a	207.08 ± 5.27 ^b	174.16 ± 4.70 ^c
	Total Protein (g/dL)	7.12 ± 0.91 ^a	7.02 ± 0.85 ^a	3.1 ± 0.60 ^b	5.32 ± 0.74 ^c
	TC (mg/dL)	75.83 ± 3.71 ^a	70.83 ± 3.18 ^a	240.25 ± 4.37 ^b	184.5 ± 3.93 ^c
	TG (mg/dL)	64.5 ± 3.40 ^a	62.58 ± 3.35 ^a	200.16 ± 5.94 ^b	103.83 ± 4.16 ^c
Kidney	Urea (mg/dL)	70.58 ± 2.17 ^a	73.75 ± 2.25 ^a	150 ± 3.03 ^b	120.41 ± 2.47 ^c
	Creatinine(mg/dL)	1.64 ± 0.40 ^a	1.72 ± 0.43 ^a	3.79 ± 0.66 ^b	2.73 ± 0.58 ^c
Values are means ± SD. Values with different letters are significantly different at P < 0.05. SD, standard deviation					

Impacts of RHO on TIL-induced changes in hepatic and renal antioxidants

As seen in Table 4, TIL increases hepatic and renal tissue oxidative markers as represented by the significant increase in MDA levels. In same time TIL decrease the antioxidant activity of liver and kidney, as TIL decreased significantly ($p < 0.5$) CAT and GSH in liver and kidney homogenate. The protective group that received RHO, showed ameliorative anti-oxidative effects as it increased CAT and GSH, and decreased MDA levels, as shown in Table 4.

Table 4

Ameliorative impacts of RHO against TIL- induced oxidative stress in kidney and liver homogenates

		Control	RHO	TIL	TIL + RHO
Liver	MDA(nmol/g protein)	80.08 ± 2.88 ^a	85.5 ± 3.09 ^a	151.5 ± 4.18 ^b	106.83 ± 3.81 ^c
	CAT (U/g protein)	520 ± 9.54 ^a	516.66 ± 9.053 ^a	306.33 ± 7.52773 ^b	420.83 ± 8.75 ^c
	GSH (U/g protein)	17.66 ± 1.75 ^a	17.33 ± 1.63 ^a	10.16 ± 1.08 ^b	14.91 ± 1.15 ^c
Kidney	MDA (nmol/g protein)	61.33 ± 1.75 ^a	63.33 ± 2.16 ^a	129 ± 3.74 ^b	90 ± 3.40 ^c
	CAT (U/g protein)	419.66 ± 7.68 ^a	414.5 ± 7.39 ^a	206.08 ± 6.42 ^b	315.66 ± 6.68 ^c
	GSH (U/g protein)	12.16 ± 1.29 ^a	11.25 ± 1.21 ^a	5.06 ± 0.48 ^b	9.5 ± 0.81 ^c
Values are means ± SD. Values with different letters are significantly different at P < 0.05. SD, standard deviation					

Impacts of RHO on TIL-induced changes in cytokines levels

The TIL injected rats showed significant increase in the IL-1 β and TNF- α cytokines. In contrast, TIL lowered serum IL-10 levels, that is widely known as anti-inflammatory cytokine (Fig. 2). The prior administration of RHO to TIL administered rats showed an ameliorative protective effect as it decreased the levels of IL-1 β and TNF- α , while a clear increase in IL10 levels was reported compared to other groups (Fig. 1).

Impacts of RHO against TIL-induced alteration in hepatic genes

TIL induced a systemic state of inflammation in rats. As shown in Fig. 1, TIL upregulated HSP70, NFkB, IL-1 β and TNF- α expression of at least 2 folds increase relative to control and RHO receiving groups. Prior administration of RHO ameliorated rats received TIL, as it significantly decreased the upregulation of HSP70, NFkB, IL-1 β and TNF- α genes (Fig. 3).

Impacts of RHO against TIL-induced alteration in renal genes

TIL induced a systemic state of inflammation in the kidney. TIL increased and up regulated the expression of intra filament proteins vimentin desmin, and nestin (Fig. 4). TIL increased the expression of nestin, desmin, and vimentin 3 folds compared with that in the control and RHO groups. Prior administration of

RHO ameliorated rats received TIL, as it normalized the up regulation occurred in renal vimentin desmin, and nestin genes (Fig. 4).

Effects of RHO on TIL-induced changes in histology of liver and kidney

In Fig. 5A left side, liver of control and RHO treated rats (A&B) showed regular arranged hepatocytes forming hepatic cords around the central vein. The hepatic cords are separated by blood sinusoids and were lined by von kupffer cells. TIL treated rats showed highly disturbed irregular pattern with irregular dilated congested central vein surrounded by edema. Hepatocytes are disturbed with pyknotic nuclei separated with irregular dilated blood sinusoid, some areas of focal necrosis and others with vacuolated cytoplasm (C). In the protective group RHO administration controlled TIL induced changes in liver cells as there are arranged hepatocytes forming hepatic cords around less congested central vein (D).

In Fig. 5 on the right side, kidney cortex of control and RHO treated rats showed normal glomerular tuft of capillaries surrounded by Bowman's capsule with normal proximal (blue arrow) and distal renal tubules (E and F). TIL injected rats showed atrophy and destruction of glomeruli, widening of the capsular space, degenerated proximal and distal tubules, nuclear pyknosis and coagulative necrosis with thickened congested blood vessels (G). In the protective group, RHO inhibited the TIL effects on renal histology. RHO showed nearly normal glomeruli, proximal and distal tubules like control rats, but still there are few areas of interstitial hemorrhage (H).

Effects of RHO on TIL-induced changes in PCNA immunoreactivity in liver and kidney

In Fig. 6 the hepatic and renal PCNA immunoreactivity was examined. Both control and RHO administered rats clarified normal expression of PCNA with few positive stained nuclei of hepatocytes (A and B) and few positive stained nuclei of the renal tubules (F and G). In C and H, TIL treated rats showed marked decrease in PCNA expression in hepatic and renal tubules. RHO plus TIL-injected rats (D and I) showed high PCNA reactivity in hepatic and renal tubules (strong positive stained nuclei). Positive PCNA immune reactivity and intensity in liver and kidney are shown in down histograms. Both down histograms in Fig. 6E and J, clarified significant decreases in PCNA reactivity that were increased by RHO administration.

Effects of RHO on TIL-induced changes in HMGB1 immunoreactivity in liver and kidney

Figure 7 shows the HMGB1 immunostaining in liver and kidney tissues. Both control and RHO treated rats showed normal reactivity for HMGB1 in hepatic and renal tubules (A and B for liver, and F and G for kidney). In C and H, TIL receiving rats showed marked increase in HMGB1 expression in hepatic and renal tubules (strong positive stained nuclei). In D and I, RHO and TIL treated rats showed marked decrease in HMGB1 expression in hepatic and renal tubules (few positive staining nuclei). Positive HMGB1 immune reactivity and intensity in liver and kidney are presented as histograms in E and J. The area percent (%) of

HMGB1 immunohistochemical staining was high in TIL injected rats and were restored in the protective group as seen down in histogram in Fig. 7E and J. The collective role of rhodiola rosea extract against TIL induced renal and hepatic toxicity are shown in Fig. 8.

Discussion

Current research verified RHO's protective benefits against TIL-induced hepatic and renal impairment. Total serum protein levels dropped while those of aminotransferases (AST, ALT, ALP), triglycerides (TC), and urea and creatinine all rose sharply. Here and in other reports [5, 9, 40, 41] with minor adjustments reports confirmed that TIL-administered rats had genetic, histological, and immunohistochemical alterations in liver and kidneys. When RHO was given before TIL was given to rats, all the negative effects were reduced. Previous studies [27, 42, 43] that analyzed the ameliorative effects of RHO in lead-induced hepato-renal toxicity and CCL-4-induced hepatotoxicity in rats with RHO treatment are consistent with ours in some respects.

Oxidative stress is the cause for impairments in RNA processing, transcription, and translation, together with, cellular membrane function and structure [44]. Heat shock protein70 (HSP70) is a molecular chaperon that protects cells from different form of stresses, including heat [45], oxidative stress, hypoxic conditions [46] and irradiations [47]. ROS has the potential to regulate transcriptional factors responsible for antioxidant response of body cells. NF κ B is a transcriptional factor that becomes active in times of adversity. It helps in the detoxification of external toxins and plays a critical role in boosting antioxidant defense system of different cells [48].

Increased ROS production and decreased GSH and CAT activity were both strongly linked to the oxidative tissue damage generated by TIL [49]. They are free radical scavengers for ROS and serve as the initial defensive line in the liver and kidneys against oxidative injury [50]. Current findings demonstrated significant reductions in CAT, GSH, and the induction of lipid peroxidation in homogenate of liver and kidney in TIL-injected rats that were ameliorated by ROH administration. Lipid peroxidation and redox dysfunction are two mechanisms through which TIL is known to impair liver and kidney function, as demonstrated in previous works [9, 41] that mostly coincided with our reported findings. Pre-administration of RHO increased antioxidants activity in liver and kidney homogenate and decreased oxidative stress biomarkers to confirm the crucial impact of RHO in the control of hepatic and renal oxidative stress. Similar protective role for RHO was confirmed in previous reports using lead and acetaminophen as hepatic and renal oxidative stress toxicants [27, 51, 52].

NF κ B is one of numerous transcriptional factors linked to a variety of inflammatory reactions [53]. It regulates gene expression of cytokines and chemokines (inflammatory mediators) [54]. Upon DNA binding, NF κ B activates the response of pro-inflammatory genes [55]. As a result, inhibiting NF κ B pathways may help to control inflammation [56] as NF κ B expression is activated by ROS-derived oxidative stress markers [57]. TIL causes cellular damage through ROS production and promote the release of certain cytokines like TNF- α [5]. TNF- α increases the induction of IL-1 β and interferon- γ [58]. In our study, TIL injection elevated TNF- α and IL-1 β significantly [5, 9], up-regulated HSP70, NF κ B, and TNF- α mRNA

expression in liver [5] with significant reduction in serum IL-10 [59]. Pre-administration of RHO and TIL mitigated the changes in HSP70. Up-regulation in NF κ B and TNF- α expression in liver with elevation in IL-10 is the result of powerful anti-inflammatory impact for RHO. Earlier reports showed the anti-inflammatory role for RHO in non-alcoholic fatty liver disease [56, 60], and in acute lung injury [61].

The active ingredients of RHO (rosarin and salidroside) showed anti-oxidation and anti-inflammation effect through multiple inflammatory signaling pathways [62, 63]. NF κ B signaling pathway is linked to down regulation in the expression of JAK2, STAT3, and PI3K/Akt pathways [18, 56, 64]. Salidroside maintains mitochondrial integrity and function by preventing ROS biosynthesis. Salidroside has antioxidant and liver-protective role, as it inhibited all genes incorporated in the activation of Mapk7 pathway that induce oxidative stress [43].

In podocytes, intermediate filaments stand out among the other cytoskeletal proteins. The intermediate filaments create a seamless structural network from cell nucleus to cell membrane. Their roles in signal transduction, cellular shape and motility determination, and many other cellular processes are crucial [65]. Acute podocyte injury can be detected by an increase in the expression of desmin (a type III IF protein), which correlates well with albumin/creatinine ratio, glomerular damage and TGF- β 1 [9]. Nestin, is a type VI intra-filament protein, and a marker for fibrosis of tubule-interstitial cells and peritubular endothelial cell injury [66]. Nestin is closely associated with vimentin, a marker of renal tubular damage [9]. Here, our results showed up-regulation of mRNA expression of IFs proteins in TIL-treated rats due to glomerular podocyte injuries that is associated with cellular repair and proliferation [9]. Desmin, vimentin and nestin expression have been down regulated in kidney rats pre-treated with RHO, confirming that it has anti-inflammatory and anti-fibrotic effects [67]. RHO suppressed IFs expression after it had been elevated by TIL, which preserved the renal barrier and normal renal function. IFs overexpression promotes podocyte cell hypertrophy, which is favorable for glomerular expansion.

PCNA, a nuclear protein incorporated in DNA synthesis, cell-cycle control, and DNA repair, is a sign of cell proliferation and division [68]. Our results revealed that TIL treated rats showed down regulation of PCNA immune reactivity of liver and kidney tissues, resulting in decreased cell proliferation. RHO pre-administration renovated the altered parameters to counteract the decrease in cellular proliferation induced by TIL. Previous study documented RHO has cellular proliferative effect, up-regulates PCNA expression in indomethacin induced gastro-toxicity in rats [69], demonstrating that RHO can reduce TIL-induced damage to the liver and kidneys in rats by modulating gene expression related with cellular proliferation.

DNA-binding protein HMGB1 is known to translocate to the cytosol or be secreted extra-cellular in response to cellular stress or death. HMGB1 is required for DNA structural maintenance, autophagy induction and gene expression. HMGB1 acts as a damage-associated molecule, alerting the host to damage by activating immunological responses [70]. HMGB1 also is secreted from necrotic or damaged cells to serve as initiator for inflammation and systemic inflammation [71]. Consequently, HMGB1 activates the innate immune system and the release of inflammatory mediators (TNF- α , NF κ B, and IL-1 β),

causing severe hepatic and renal injuries [72]. Activation of HMGB1 binding receptors initiated the inflammatory responses [70, 73]. In our study, TIL injection upregulated HMGB1 immune reactivity in hepatic and renal tissues, and pre-administration of RHO produced significant decrease in HMGB1 expression to counteract inflammation and necrosis induced by TIL. Salidroside has anti- HMGB1 effect causing down-regulating NF κ B and HMGB1 pathways in acute lung injury in rats [74]. These results show that RHO can reduce the gene expression of mediators involved in inflammation and necrosis, protecting the liver and kidneys from the damage caused by TIL in rats. The collective ameliorative impacts of rhodiola against tilmicosin induced hepatorenal dysfunction are illustrated in Fig. 8.

Conclusions

The current study verified the protective impacts of RHO against the hepatocellular and nephron damage caused by tilmicosin. Tilmicosin changed the expression of many genes in the liver and kidneys, raised serum inflammatory cytokines, and lowered antioxidant activities. All the biochemical, cellular, and genetic indicators that had been changed prior to RHO treatment had been restored. These findings provide credence to the idea that RHO might be used to prevent the adverse effects of tilmicosin on liver and kidney and pave the door for future research into the possibility that additional signaling pathways are involved in such regulation.

Declarations

Conflict of interest

Authors declare that there is no reported conflict of interest for current study.

Ethical Statement

All the experiments were done according to the National Institutes of Health's guidelines for the care and use of lab animals, and all of the steps meant to keep the animals from suffering were taken.

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Author Contribution

Salwa Elgendy, Heba Elnoury and Mostafa Shukry: Methodology, Investigation, Formal analysis, Visualization, Writing—original draft preparation. Saed Althobaiti, Lina Mohammed, Hend Nasr and Khalid Alotaibi: Validation. Investigation, Formal analysis. Daklallah Almalki: Investigation, Formal analysis, and

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Data Availability Statement

Upon request present data are available

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Figures

Figure 1 . Phenolic peaks identified by HPLC

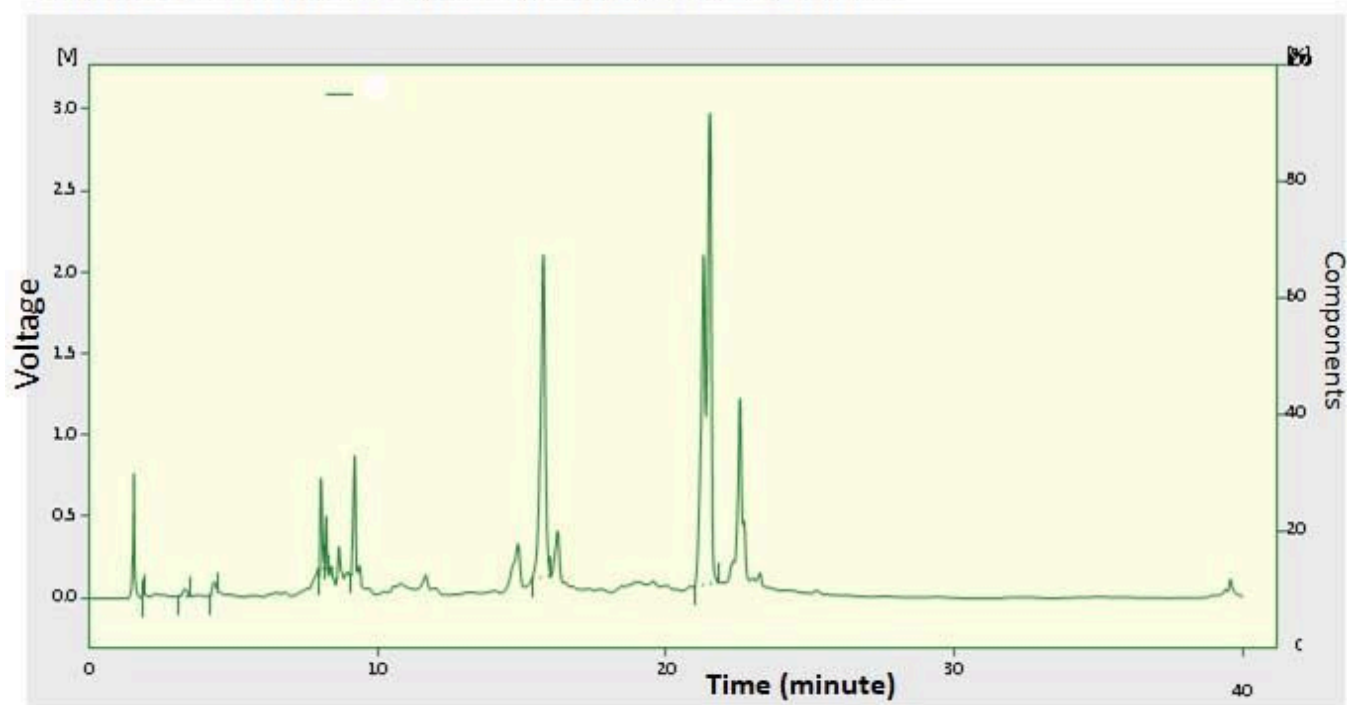


Figure 1

See image above for figure legend

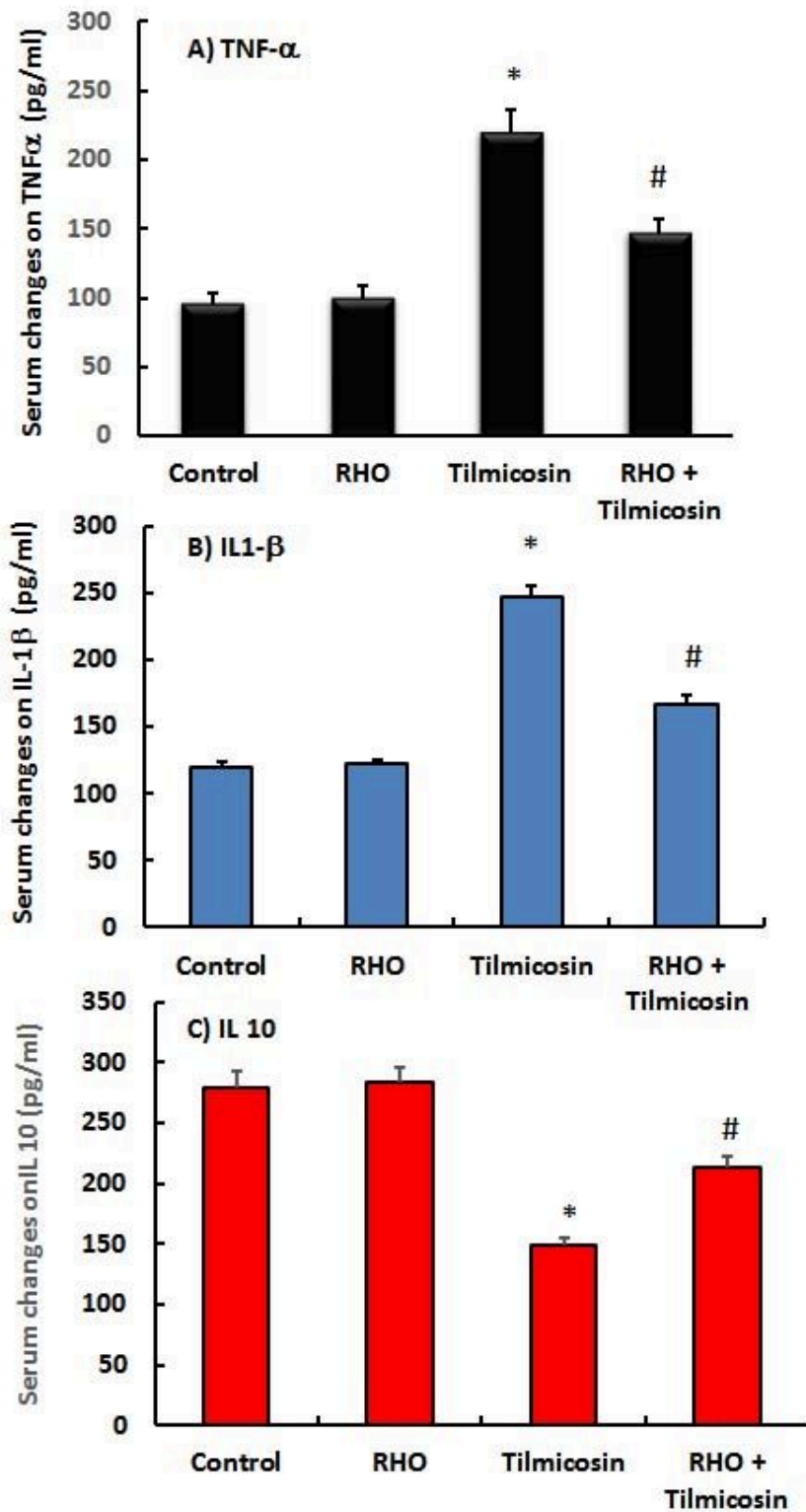


Figure 2

Impacts of RHO on tilmicosin induced changes on pro- and anti-inflammatory cytokines. Changes on IL-1 β (A), TNF- α (B) and IL-10 (C) in 6 different rats per each treatment. Values are significantly at * $p < 0.05$ compared to control and RHO groups and # $p < 0.05$ Vs. TIL group.

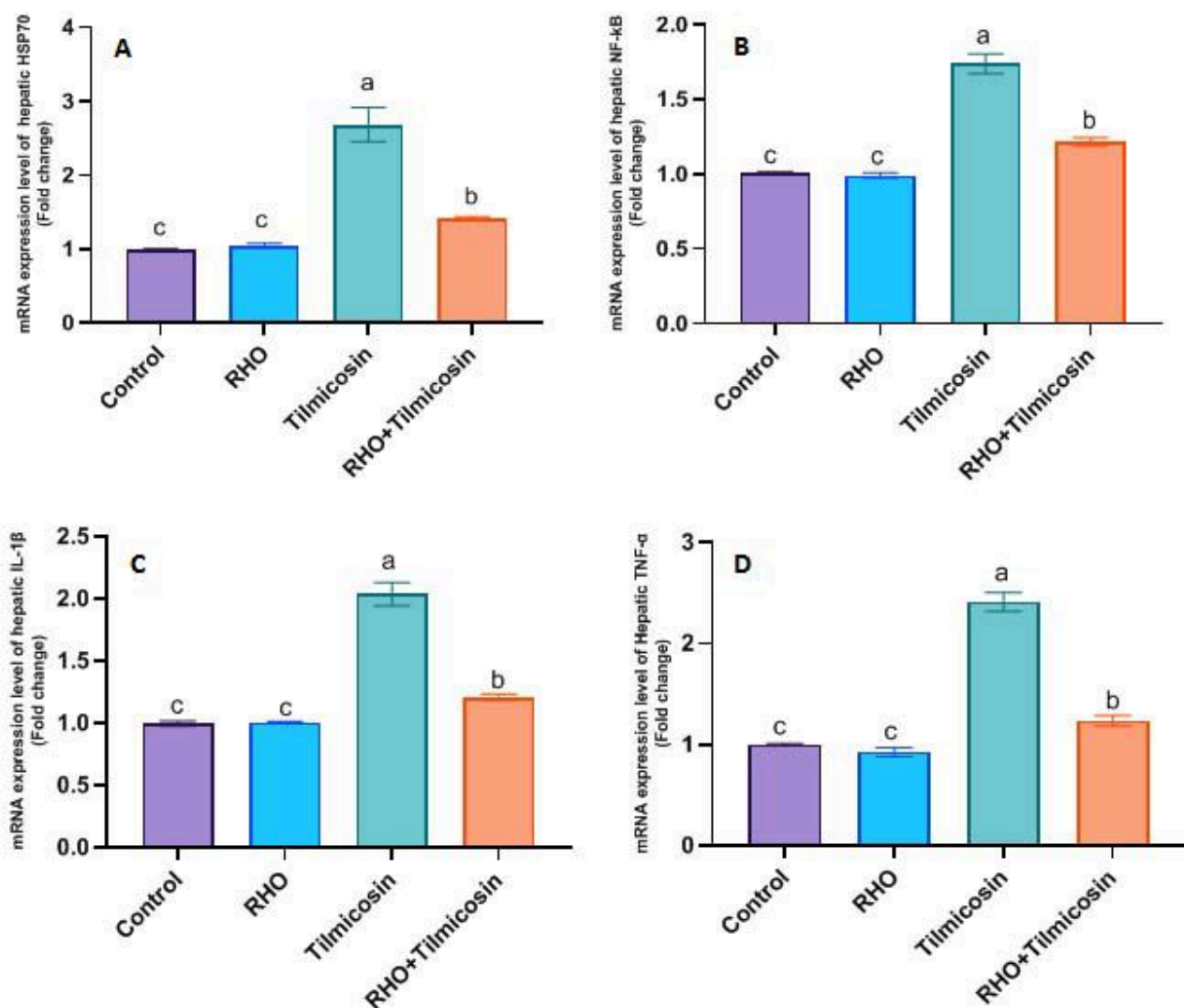


Figure 3. Quantification of mRNA expression of HSP70 (A), NFkB (B), IL-1 β (C) and TNF- α (D) genes. The graphic presentations of examined genes were based on qRT-PCR analysis for HSP70, NFkB, IL-1 and TNF- α after normalization with GAPDH in different groups. * $p < 0.05$ versus the control and RHO groups; $^{\#}p < 0.05$ versus TIL group.

Figure 3

See image above for figure legend

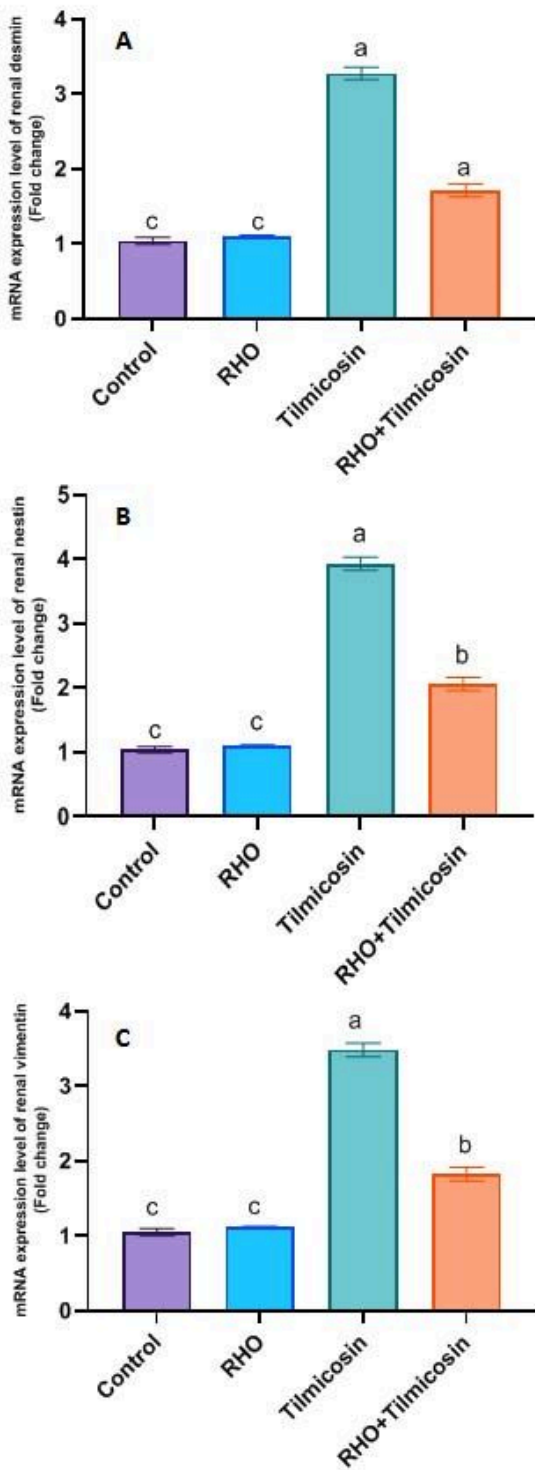


Figure 4

Quantification of mRNA expression of desmin (A), nestin (B) and vimentin (C) genes. The graphic presentations of examined genes were based on qRT-PCR analysis for desmin, nestin and vimentin after normalization with GAPDH in different groups. * $p < 0.05$ versus the control and RHO groups; # $p < 0.05$ versus TIL group.

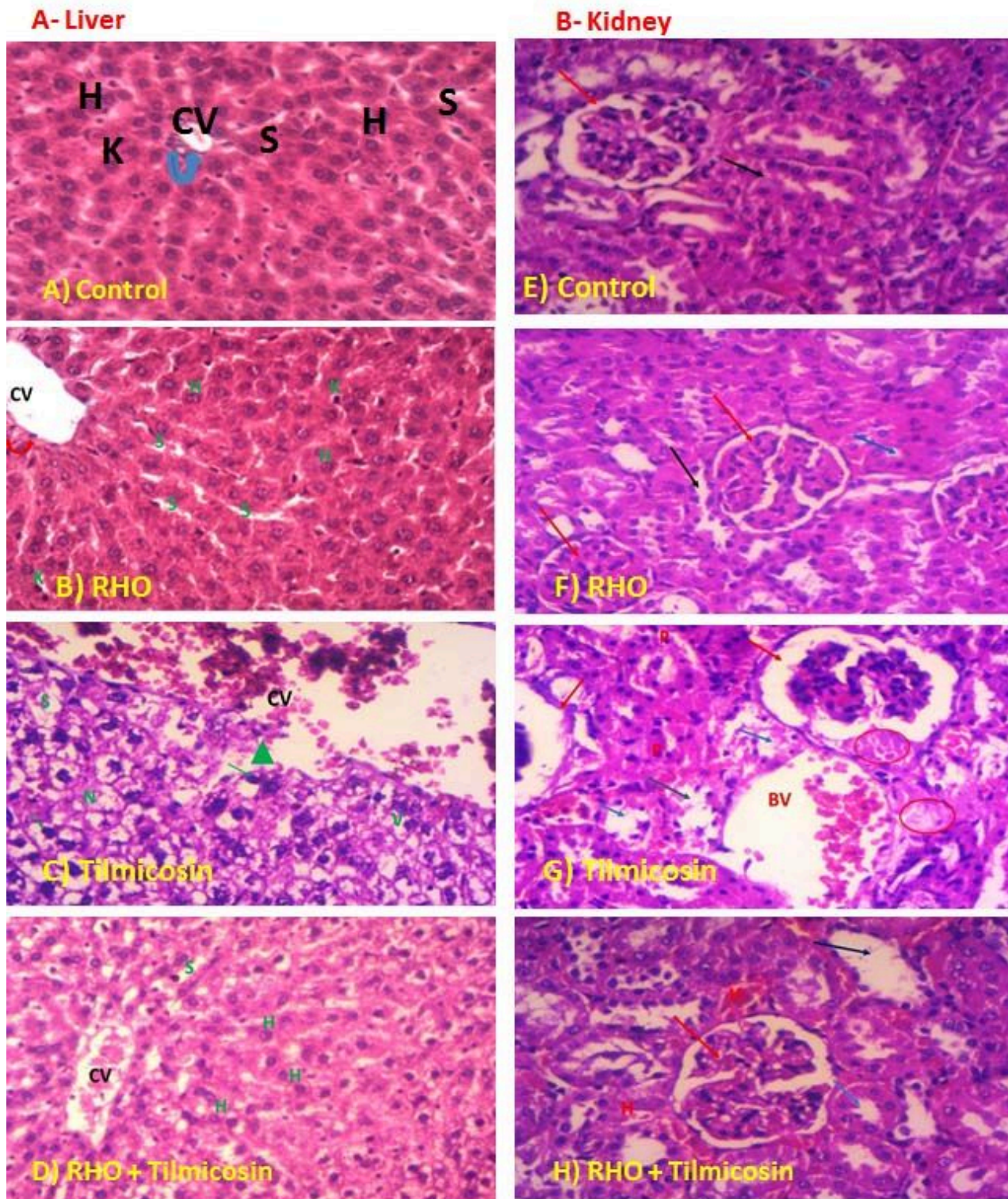


Figure 5

Protective effect of RHO against TIL-induced changes kidney and liver histology. In Liver Photomicrographs of the liver sections (A), control and RHO treated rats (B) showed regularly arranged hepatocytes (H) forming hepatic cords around a central vein (CV) lined with flat endothelium (curved arrow), these cords are separated by blood sinusoid (S) lined by von kupffer cells (K). TIL treated rats (C) showed highly disturbed irregular pattern with irregular dilated congested central vein (CV) surrounded by

oedema (arrow head). Hepatocytes are disturbed with pyknotic nuclei (arrow) separated with irregular dilated blood sinusoid (S), some areas of focal necrosis (N) and others with vacuolated cytoplasm (V). RHO plus TIL treated rats (D) showed regularly arranged hepatocytes (H) forming hepatic cords around congested central vein (CV), these cords separated by nearly normal blood sinusoid (S) (**H&E, X400**).

In Kidney photomicrograph of kidney cortex of control and RHO treated rats (F and F) showing normal glomerular tuft of capillaries (red arrow) surrounded by Bowman's capsule with normal proximal (blue arrow) and distal (black arrow) renal tubules. TIL treated rats(G) showing atrophy and destruction of glomeruli, widening of the capsular space (red arrow), degenerated proximal (blue arrow) and distal tubules (black arrow), nuclear pyknosis (P) and coagulative necrosis (circle) with thickened congested blood vessels (BV). RHO+TIL (H) showed nearly normal glomeruli (red arrow), proximal (blue arrow) and distal tubules (black arrow) more or less like control rats, but still there are areas of interstitial hemorrhage (H) (**H&E, X 400**).

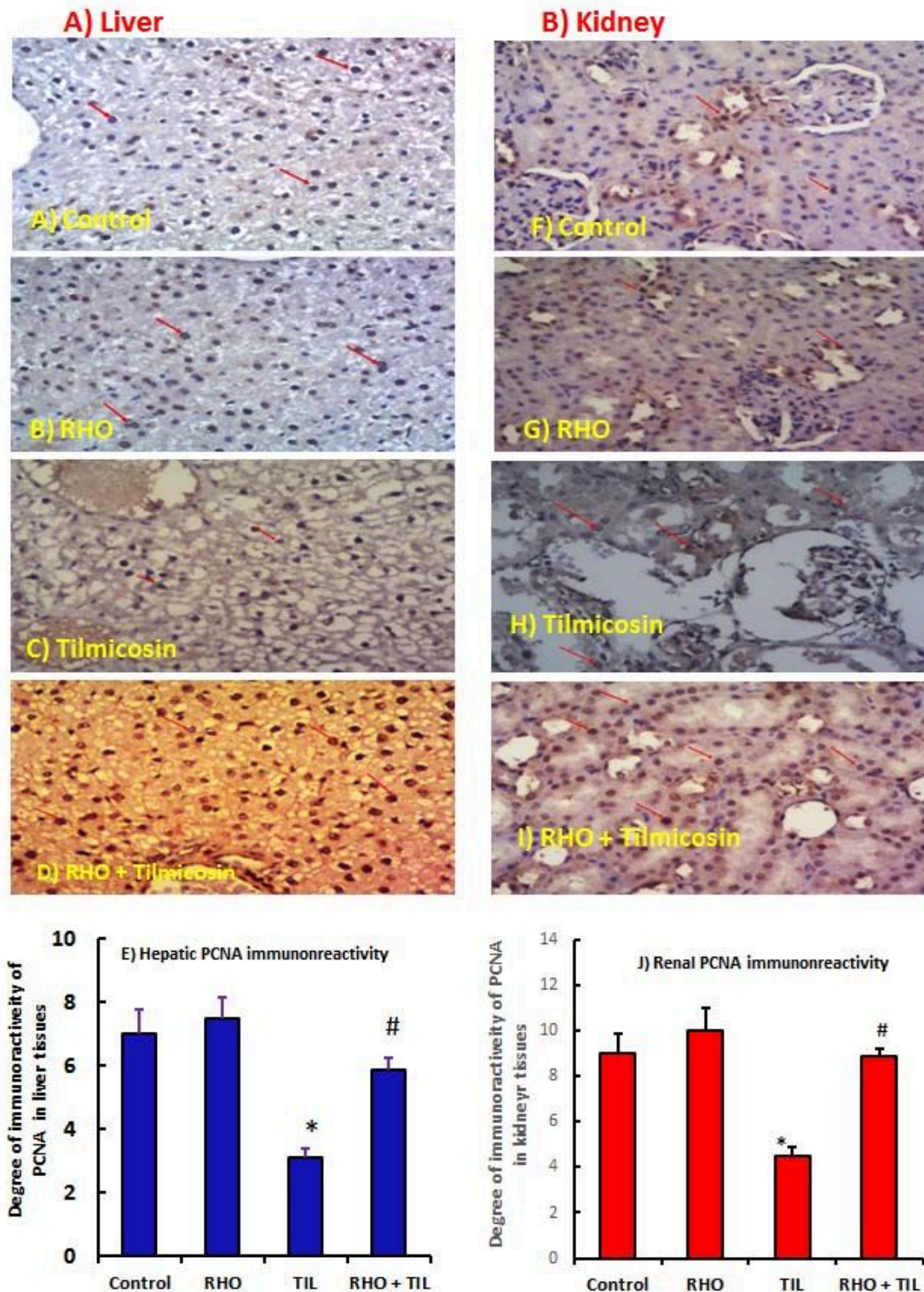


Figure 6

Immunohistochemical staining of PCNA expression in the experimental rats' hepatic and renal tissues (IHC, $\times 400$). Both control and RHO rats showing normal expression of PCNA with few positive stained nuclei of hepatocytes (brown coloration) (red arrow) and few positive stained nuclei of the renal tubules (red arrow) as seen in A and B for liver, and F and G for kidney. TIL treated rats showing marked decreases in PCNA expression in hepatic (C) and renal tubules (H) as seen as red arrow. RHO + TIL treated rats

showing high expression of PCNA in hepatic (D) and renal tubules (I) (strong positive stained nuclei) (red arrow). Bar scale for all photos = 50 μ m. All data are presented as means + SD, and the significance level for symbol * was compared to control and RHO, while symbol # was compared to TIL injected group. The level of significance is $p < 0.05$.

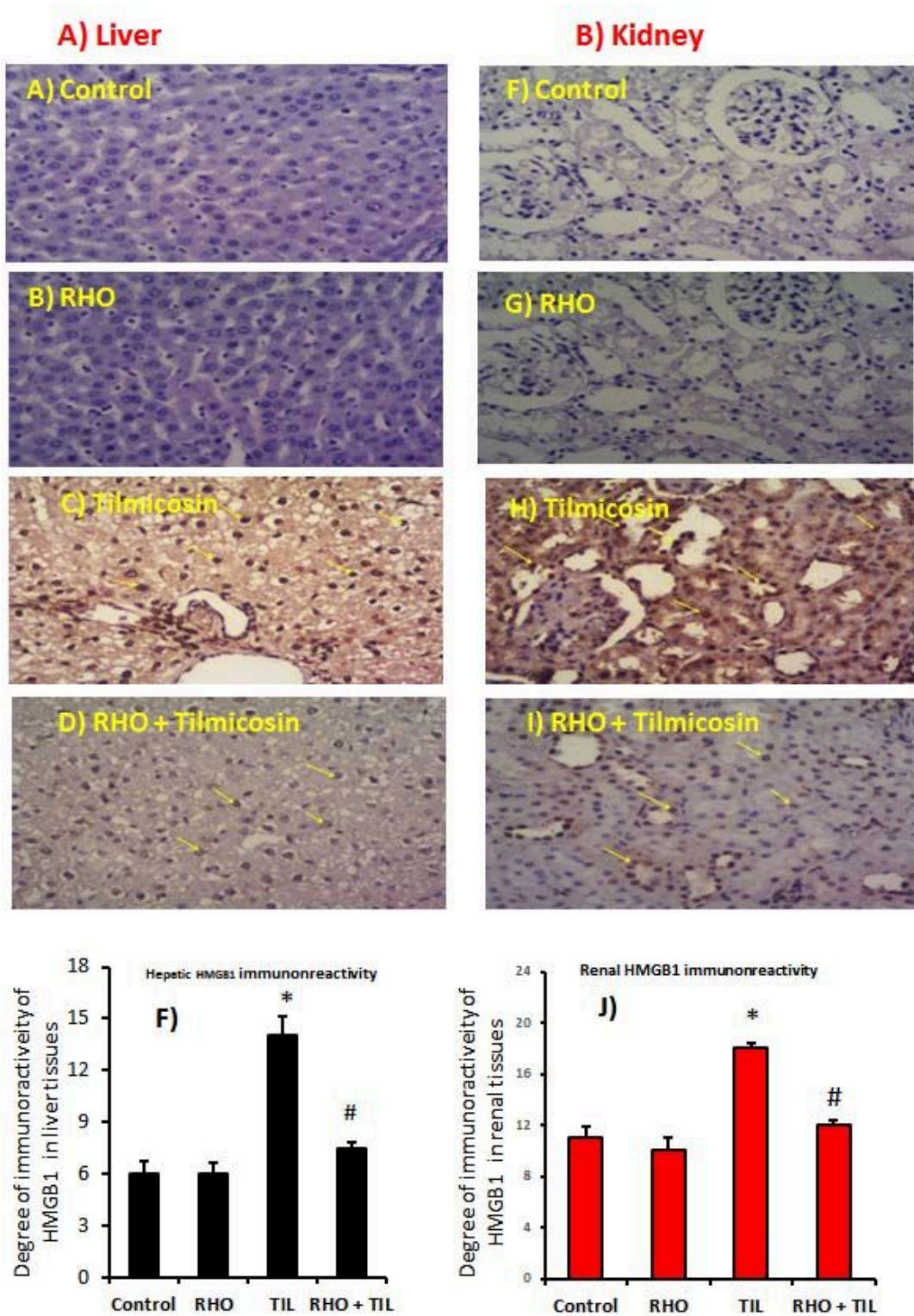


Figure 7

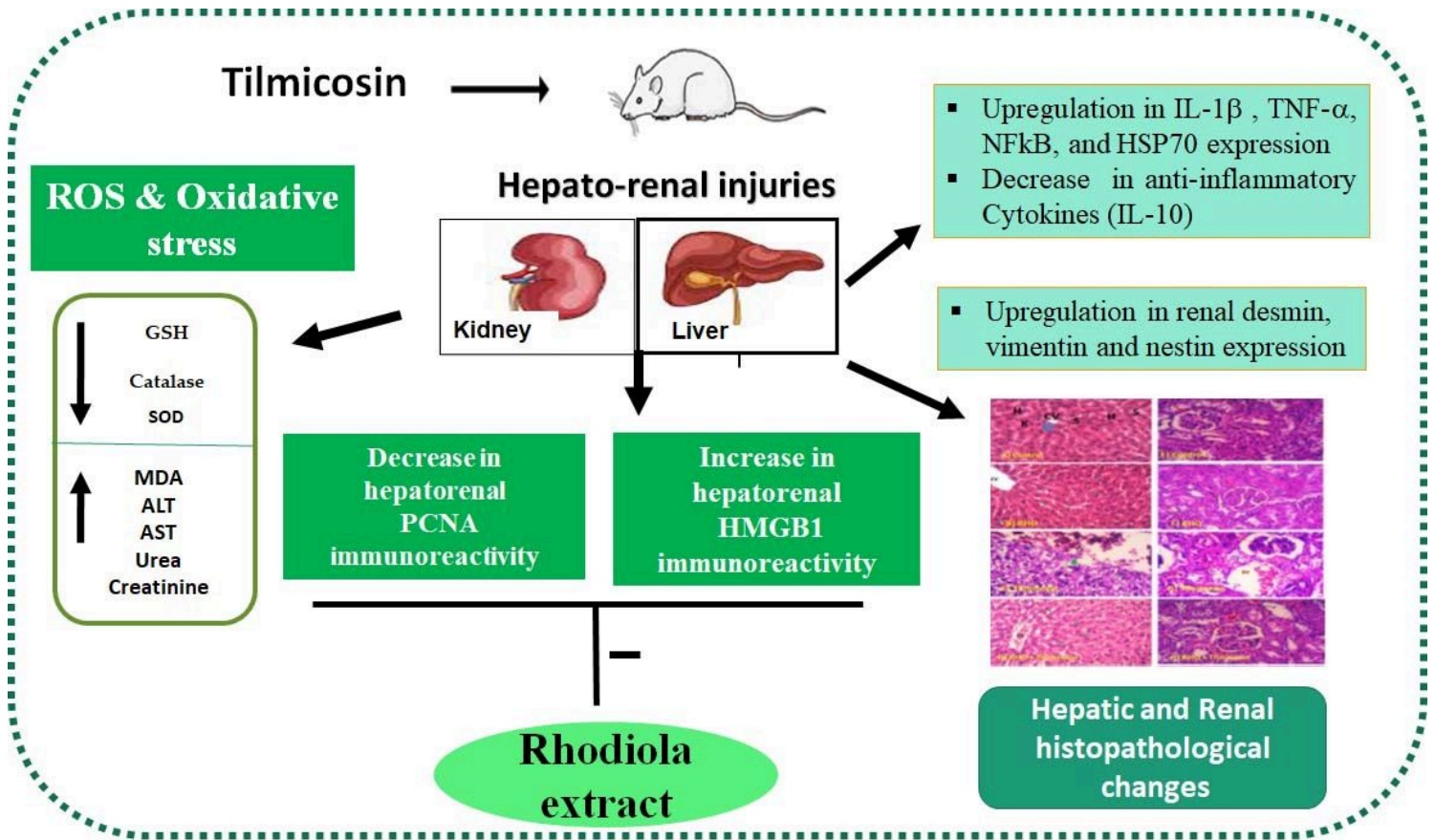


Figure 8

Collective impacts of *Rhodiola rosea* extract against hepatorenal dysfunction induced by tilmicosin. (-) means stimulations.