NARINGIN AND RUTIN PREVENT D-GALACTOSAMINE-INDUCED HEPATIC INJURY IN RATS VIA ATTENUATION OF THE INFLAMMATORY CASCADE AND OXIDATIVE STRESS

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Abstract
The dried stems and leaves of Citrus jambhiri Lush. (Rutaceae) were extracted with aqueous methanol and the extracts were fractionated using light petroleum, chloroform and ethyl acetate. Column chromatography of the ethyl acetate fractions resulted in the isolation of naringin, rutin, hesperidin, and neohesperidin. Their structures were identified by MS and different NMR techniques. Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of these fractions allowed the identification of 7 flavonoid glycosides. Hepatoprotective properties of naringin and rutin were evaluated in d-galactosamine (d-GalN)-induced hepatic injury in rats. d-GalN increased serum aminotransferase activity, total bilirubin, liver tumor necrosis factor-α level (TNF-α), hepatic lipid peroxidation, nuclear factor κB and decreased hepatic glutathione content, IL-10 levels and the IL-10/TNF-α ratio. These changes were attenuated in rats pretreated with rutin and naringin (40 mg/kg body weight). They increased liver IL-10 levels and the IL-10/TNF-α ratio. Rutin but not naringin down-regulated NF-κB gene expression and decreased gamma-glutamyltransferase (GGT) activity.
Keywords: Citrus jambhiri, naringin, rutin, d-galactosamine, TNF-α, IL-10

Introduction
Liver diseases are a major medical problem worldwide and affect a large population of people especially in Egypt (Farghali et al., 2009). Inflammation and the generation of reactive oxygen species (ROS) play a key role in pathological changes in the liver (Stehbens, 2003; Wu et al., 2006). In acute liver injury, the apparent cell damage is not only due to direct effects of the involved drug, toxin, viral or other cause, but also due to a secondary release of pro-inflammatory mediators from activated Kupffer, stellate, and sinusoidal endothelial cells, thus creating a vicious circle. One of the most important pro-inflammatory cytokines stimulated during liver injury is TNF-α. It can induce necrosis of hepatocytes in vivo or in vitro and is elevated in acute and chronic liver diseases. Apparently TNF-α plays a major role in liver damage which is associated with a ischemia reperfusion (I/R) injury in rats. Using infliximab, a monoclonal antibody against TNF-α, can partially protect against liver I/R injury (Mahmoud et al., 2012). IL-10 is an important anti-inflammatory cytokine (Pestka et al., 2004) which can protect mice from death during septic peritonitis (van der Poll et al., 1995).

Flavonoids are a widespread group of naturally occurring antioxidants and anti-inflammatory agents in plants. They widely occur in vegetables, fruits, nuts, seeds, leaves, flowers and barks of plants (Middleton et al., 2000) and are important active components of many medicinal plants (van Wyk and Wink, 2004). Medicinally used flavonoids include rutin and naringin.

Citrus species are a rich source for flavonoids. Rough lemon (Citrus jambhiri Lush.) is one of the commercially used Citrus species. It is a large tree which derived as a hybrid between mandarin (Citrus reticulata) and lemon (Citrus medica). Chemical composition and biological activities of the fruit peel of C. jambhiri grown in Egypt have been reported previously by our research team (El-Readi et al., 2010; Hamdan et al., 2010, 2011). Recently, the fruit peel of a Sudanese variety was investigated for its minerals content, essential oil composition and physicochemical properties (Mohammed et al. 2013).

In this study, the flavonoid composition of an ethyl acetate fractions of an aqueous methanol extract from stems and leaves of C. jambhiri was investigated. In addition, a potential hepatoprotective effect and the possible underlying mode of action of the major flavonoids (naringin and rutin) on d-galactosamine-induced liver injury (similar to viral hepatitis) were evaluated in rats.
Material and methods

Plant material

Stems and leaves of rough lemon (*C. jambhiri* Lush.; Rutaceae), were collected from the Research Station of the Faculty of Agriculture, Benha University, Egypt in March 2010. The identity of the plants was confirmed by Prof. Dr. B. M. Houlyel, Dept. of Pomology, Faculty of Agriculture, Benha University, Egypt. A voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt (accession no. P-80 -1).

Instruments

Electron impact mass spectra (EIMS) were recorded on a MAT 8200 (Bremen, Germany) instrument with an electron energy of 70eV. In case of FAB-MS, 3-nitrobenzyl alcohol was used as a matrix. NMR spectra (*¹H and *¹³C*) were recorded on a Mercury 300 and VARIAN 500 instrument (Darmstadt, Germany) at 300 and 500 MHz for *¹H* measurements and 75 and 125 MHz for *¹³C* measurements, respectively. CD₃OD, and DMSO-*d₆* were used as solvents. Chemical shifts are given in ppm with TMS as internal standard. APT, 2D-NMR, H-H COSY and HMPQC experiments were carried out to obtain reliable assignments. Experimental data were processed using MestRe-C software. Qualitative and quantitative determination of the flavonoids in the ethyl acetate fractions were carried out by LC-ESI/MS (negative ion mode) as described by Hamdan *et al.* (2011).

Extraction and fractionation

Dried stems (2.5kg) and leaves (700g) of *C. jambhiri* were exhaustively extracted with 80% aqueous methanol (3×9 L and 3×3 L, respectively) at room temperature. The methanol extracts were filtered, concentrated under reduced pressure to yield 500g of a brownish-green residue and 101g of viscous dark green residue, respectively. The residues were suspended in methanol–water (1:9) and partitioned against light petroleum (b.p. 60–80°C), dichloromethane and ethyl acetate. The organic solvents of stems were evaporated under vacuum using rotary evaporator at lower temperature to yield 18, 17 and 11g of a final residue; the leaf extract yielded 14, 6.5 and 5g of a final residue, respectively.

Isolation of flavonoids

The dried ethyl acetate fraction of the leaves (4g) and stems (8g) were individually applied on the top of silica gel columns (60 to 230 mesh; Fluka, Buchs, Switzerland) at room temperature. The columns were packed in dichloromethane and eluted with a gradient using a mixture of dichloromethane-methanol as a mobile phase. Fractions were collected,
concentrated under reduced pressure and monitored on pre-coated TLC plates (Silica gel 60 F254, Merck, Darmstadt, Germany) and a mixture of chloroform-methanol-water (6: 4: 0.5 v/v/v) and ethyl acetate - formic acid-acetic acid-water (10:1.1:1.1:2.6 v/v/v/v) for TLC development. Spots were visualized by UV or by spraying with 10% (v/v) NH4OH or H2SO4 in water followed by heating at 105 ºC for 5 min. Rutin, naringin, hesperidin and neohesperidin were isolated and purified. The purity of compounds was confirmed by TLC and HPLC and NMR analysis.

Animals

Twenty four adult male Wistar rats (180–200 g; Zagazig University, Zagazig, Egypt) were used in the present study. All the animals were maintained under standard husbandry condition with food and water ad libitum. The experimental procedures were approved by the Institutional Animal Ethics Committee of the Faculty of Pharmacy, Zagazig University (approval number P1/3/2013) and animals were handled following the International Animal Ethics Guidelines, ensuring minimum animal suffering.

Study protocol

Rats were randomly divided into four experimental groups (six animals each): control, d-GalN, naringin (40mg/kg) and rutin (40mg/kg). Control group received only the vehicle (normal saline). The d-GalN group received 800 mg/kg d-GalN, dissolved in normal saline and given i.p as a single dose. Naringin and rutin (suspended in gum acacia solution, 10mg/ml saline w/v) were applied by orogastric gavage. One hour later, rats of the flavonoid groups were injected i.p with d-GalN. Twenty later rats were anaesthetized with a subcutaneous injection of 1.2 g/kg urethane (Sigma, St. Louis, MO, USA); blood was collected from the retro-orbital plexus and centrifuged (3000x g, 4°C, 20 min) for the separation of plasma. The obtained plasma was used to analyze liver enzymes and total bilirubin. Thereafter, animals were sacrificed by cervical dislocation and livers were dissected and washed with cold saline. Livers were divided into two parts: one part was immediately flash-frozen in liquid nitrogen and kept at -80 °C for measurement of tissue parameters and the other part was kept in 10% formalin for histopathological examination.

Pharmacological study

Biochemical analysis

Liver enzyme tests. The plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) activities and total bilirubin level were measured using commercially available analytical kits (Biodiagnostic Co, Egypt).
Determination of oxidative stress. The generation of reactive oxygen species (ROS) in response to d-GalN-induced liver injury was evaluated in liver tissues by measuring the lipid peroxidation product malondialdehyde (MDA) and reduced glutathione content (GSH). MDA and GSH were determined photometrically (spectrophotometer, Jenway®, England, UK) according to Wills (1987) and Ellman (1959), respectively.

Inflammation markers

Tumor necrosis factor alpha (TNF-α). TNF-α levels in liver homogenate were measured by sandwich ELISA according to the method of Maskos et al. (1998) using an ELISA kit (Quantikine, R&D system USA).

Interleukin-10 (IL-10). IL-10 levels in liver homogenate were determined by sandwich ELISA according to the method of Marconi et al. (1998) using an ELISA kit (Quantikine, R&D system USA). The IL-10 to TNF-α ratio was calculated.

Detection of the expression of nuclear factor kappaB (NF-κB) by RT-PCR. For the detection of NF-κB, total RNA was extracted from liver tissue homogenates using RNeasy purification reagent (Qiagen, Valencia, CA) according to the instructions of the manufacturer. RNA was photometrically quantified at 260/280 nm. cDNA was generated from 5 µg of total RNA using 20 pmol oligo-dT and superscript AMV reverse transcriptase for 60 min at 37 °C. For PCR, cDNA was incubated with dNTPs (10mM), 10 x PCR buffer, 2.5 U Taq DNA polymerase, and 10 pmol of each primer. The reaction mixture was subjected to 40 cycles of PCR as follows: Denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. 10 µl of the PCR mixture was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by ultraviolet light. For semiquantitative analysis a gel documentation system was used (BioDO, Analyser, Biometra, Göttingen, Germany). According to the amplification procedure, relative expression of each studied gene (R) was calculated according to the following formula: densitometrical units of each studied gene/densitometrical units of beta actin (a house keeping gene).

Primer sequence was as follows:

Forward 5′-GCG CAT CCA GAC CAA CAA TAA C-3′
Reverse 5′-GCC GAA GCT GCA TGG ACA CT- 3′

Histopathological study. Liver tissues were fixed in 10% formalin and embedded in paraffin. Sections of 5 µm thickness were stained with hematoxylin and eosin (H & E) then examined under the light microscope to determine of histopathological changes.
Bioassay materials and reagents

D-galactosamine was supplied by Sigma Chemicals (ST. Louis, Mo, USA). Authentic compounds such as rutin, eriocitrin, neoeriocitrin, diosmetin-6-C-glucoside, narirutin, naringin, hesperidin, and neohesperidin were obtained from the Institute of Pharmacy and Molecular Biotechnology (Heidelberg University, Germany). In addition, methanol, dichloromethane, chloroform, petroleum ether, benzene, ethylacetate, formic acid, acetonitrile and other solvents used for extraction, separation and/or detection, which were of analytical grade, were obtained from Merck (Germany).

Statistical analysis. Differences between groups were determined by one-way ANOVA using a multiple comparison procedure (Tukey post hoc test). A P-value of less than 0.05 was considered significant. Data are given as mean ± SEM. All statistical analyses were performed employing GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

Results and discussion

Identification of flavonoidal compounds

The ethyl acetate fractions of stems and leaves were analyzed by LC-ESI/MS which is a rapid, powerful and efficient tool to identify flavonoid glycosides (Zhou, et al., 2006; Ding, et al., 2007). A total of 7 flavonoid glycosides (Table 1) were identified in the leaf fraction including eriocitrin, diosmetin-6-C-glucoside, narirutin, naringin, hesperidin and neohesperidin with rutin being the most abundant followed by hesperidin and neohesperidin (Fig.1A). Analysis of the stem fraction revealed the presence of 6 compounds. The prominent component is hesperidin followed by eriocirin and naringin (Fig. 1B). However, there is no evidence for the presence of neohesperidin in the stem. The identification of eriocirin, narirutin, and diosmetin-6-C-glucoside was based on MS data and elution order described in the literature (Dugo, et al., 2005), while the four other compounds (rutin, hesperidin and neohesperidin and naringin) were isolated (using silica gel column) and identified by MS, 1H- and 13C NMR and 2D-NMR analyses (Maltese et al., 2007; Hassan et al., 2012). Quantification of compounds in the ethyl acetate fractions were performed using standard calibration plots (Table 1). Naringin and rutin were used for following pharmacological experiments. To our knowledge, the isolated and identified compounds from this study have not been reported before for stems and leaves of C. jambhiri.

Pharmacological study

Effect of naringin and rutin on markers of d-GalN-induced hepatic injury

In order to assess a potential hepatic injury induced by a d-GalN injection, serum transferase activities (AST, ALT and GGT)were measured
as markers of necrosis and total bilirubin. After 24 h of d-GalN injection, AST, ALT, GGT activities and total bilirubin were dramatically increased as compared to non-treated controls (P< 0.05; Table 2). Pretreatment of rats with naringin attenuated the increase in AST and ALT activities and total bilirubin level but had no effect on serum GGT activity. Rutin exerted a more potent hepatoprotective effect as evidenced by a reduction of all liver enzyme activities and of total bilirubin level as compared with the d-GalN group (P< 0.05; Table 2).

**Histopathological changes in hepatic tissue after d-GalN administration**

Liver sections from d-GalN treated rats revealed areas of portal cell infiltration with fibroblasts adjacent to congested portal vein and edematous hyalinized hepatic arterioles (Fig. 2B) as compared to normal rats (Fig.2A). Naringin treated rats showed random areas of coagulative necrosis infiltrated with eosinophiles (Fig. 2C). In rutin treated rats (Fig. 2D), the majority of hepatocytes were normal with mild interstitial aggregation and no portal reaction.

**Local inflammatory changes and hepatic neutrophiles accumulation**

D-GalN significantly increased the level of the pro-inflammatory cytokine TNF-α in liver tissues, reduced the anti-inflammatory cytokine and IL-10 levels and the IL-10/ TNF-α ratio as compared with the control group (P< 0.05; Fig.3). Hepatic TNF-α levels were markedly reduced in the both naringin and rutin-treated animals, as compared to the d-GalN control group. IL-10 and IL-10/ TNF-α ratio were significantly elevated in naringin and rutin-gavaged animals as compared with the d-GalN control group (P< 0.05; Fig.3).

Hepatic neutrophiles infiltration increased 24 h after d-GalN administration (Fig. 2B) especially in the portal region, compared with values of control rats (Fig. 2A). Naringin reduced neutrophiles infiltration to a lesser extent as compared to the d-GalN control group (Fig. 2C). Rutin markedly diminished hepatic neutrophils infiltration as compared with the d-GalN control group (Fig. 2D). There was no portal reaction in rutin-gavaged animals.

**Effect of naringin and rutin on the hepatic gene expression of NF-κB**

The expression of NF-κB, as revealed by RT-PCR, increased markedly 24h after d-GalN administration as compared to the non-treated control (P< 0.05; Fig.4). Pre-treatment with naringin failed to attenuate the increased expression of NF-κB as compared to the d-GalN control group (P> 0.05; Fig.4). However, hepatic NF-κB expression was markedly reduced in rutin group as compared with d-GalN control group (P< 0.05; Figure 4).
Effect of naringin and rutin on ROS production by d-GalN treatment

In liver tissues d-GalN administration caused an increased ROS production which was manifested by the depletion of GSH and the increase of the lipid peroxidation product, MDA as compared to control animals. When rats were pretreated with naringin or rutin, the GSH levels was elevated and MDA levels significantly reduced (P< 0.05, Table 2).

Our results clearly demonstrated that both naringin and rutin could suppress hepatic injury from d-GalN application, with rutin as a more potent drug. Oxidative stress is considered as one of the major causes of d-GalN-induced liver damage (Han et al., 2006). The increased production of free radicals resulting from oxidative stress can damage macromolecules such as lipids, proteins and nucleic acids. The present study demonstrated that d-GalN application caused a hepatic lipid peroxidation manifested by the production of MDA and depletion of hepatic GSH. The decreased concentration of GSH in liver which plays a key role in protective processes after d-GalN administration may be due to NADPH reduction or GSH utilization in the elimination of peroxides (Yadav et al., 1997). Treatment with rutin or naringin significantly decreased MDA, and elevated hepatic GSH content. These results suggest that both naringin and rutin might have a direct scavenging activity or stimulating effect on the antioxidative enzyme system. It was reported previously that rutin exerted a similar effect in CCl4 treated rats (Khan et al., 2012). Naringin and rutin also have a direct antioxidant effects as they belong to flavonoids which contain one or more phenolic hydroxyl groups in their moiety, which are responsible for the antioxidant activity of flavonoids (Van Akre et al., 2000).

It seems that not the free radical generation in hepatic tissues but a marked inflammation is the main player in d-GalN–induced hepatotoxicity. Both rutin and naringin inhibited an inflammatory cell infiltration of the liver, TNF-α production, increased IL-10 levels and IL-10/TNF-α ratio. Only rutin administration resulted in down-regulation of NF-κB gene expression.

NF-κB is a major mediator of the inflammatory process and a key participant in innate and adaptive immune responses (Hayden and Ghosh, 2012; DiDonato et al., 2012). NF-κB is confined to the cytoplasm as a complex with a special inhibitor, IκBα, under the non-stimulated conditions. When Kupffer cells (KCs) are activated through TLR4, phosphorylation, a degradation of IκBα takes place (Sun et al., 2005). We reported previously that d-GalN increased TLR4 expression and TNF-α production (unpublished data). The inactive NF-κB-IκBα complex is dissociated and the free NF-κB translocates to the nucleus (Dyson and Komives, 2012). Once in the nucleus, NF-κB binds to target DNA sequences and causes expression of various inflammatory cytokine genes, such as those of TNF-α, IL-1β, IL-6 and IFN-γ.
(Deng et al., 2012; Ren et al., 2012). This leads to the development of an acute inflammatory response. The present study showed that rutin administration improved the morphological changes and reduced inflammatory cell infiltration in hepatic tissues more than naringin. This may be attributed to a down-regulation of NF-κB gene expression rather than a rutin effect on TNF-α production or IL-10 levels. Naringin could not modulate NF-κB gene expression and thus it only moderately reduced the deleterious effects of d-GalN administration.

In the present study d-GalN-induced lipid peroxidation and inflammation; it interfered with liver cell membrane integrity and caused leakage of intracellular enzymes like ALT, AST and GGT. The observed elevation in the concentration of serum bilirubin after d-GalN treatment is also in agreement with a hepatic damage (Zhu et al., 2013). In this study, we could demonstrate that rutin significantly reduced ALT, AST and GGT activity and total bilirubin levels whereas naringin only moderately reduced ALT and AST activity and total bilirubin but had no effect on GGT activity. These results are in accordance with the previous finding that naringin possesses weaker anti-inflammatory effect than rutin (Manthey et al., 2001; Tripoli et al., 2007; Akondi et al., 2011; Sandhar et al., 2011).

**Conclusion**

Our data demonstrate that oxidative stress is not the main player in d-GalN-induced hepatic injury. The potential role of NF-κB in d-GalN-induced hepatic injury is discussed and our experiments provide evidence that especially rutin rather than naringin could serve for treatment for liver diseases because of its more potent anti-inflammatory effect. The efficacy of rutin which is already used in phytotherapy to treat inflammations of veins (van Wyk and Wink, 2004) for liver inflammatory disorders needs to be established in clinical trials.

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References:


Table 1
Qualitative and quantitative analysis of flavonoid in the leaf and stem of Citrus jambhiri using LC-ESI/MS

<table>
<thead>
<tr>
<th>Compound</th>
<th>RRt*</th>
<th>RRt**</th>
<th>m/z***</th>
<th>Leaf (w/w%)</th>
<th>Stem (w/w%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eriocitrin</td>
<td>1.26</td>
<td>1.25</td>
<td>595/287</td>
<td>0.54</td>
<td>1.09</td>
</tr>
<tr>
<td>Rutin</td>
<td>1.36</td>
<td>1.40</td>
<td>609/301</td>
<td>0.73</td>
<td>0.45</td>
</tr>
<tr>
<td>Diosmetin-6-C-glucoside</td>
<td>1.41</td>
<td>1.42</td>
<td>461</td>
<td>0.21</td>
<td>0.28</td>
</tr>
<tr>
<td>Narirutin</td>
<td>1.45</td>
<td>1.45</td>
<td>579/271</td>
<td>0.09</td>
<td>0.05</td>
</tr>
<tr>
<td>Naringin</td>
<td>1.52</td>
<td>1.53</td>
<td>579</td>
<td>0.27</td>
<td>0.66</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>1.56</td>
<td>1.56</td>
<td>609/301</td>
<td>0.61</td>
<td>2.10</td>
</tr>
<tr>
<td>Neohesperidin</td>
<td>1.63</td>
<td>1.67</td>
<td>609</td>
<td>0.09</td>
<td>-</td>
</tr>
</tbody>
</table>

* Maesured relative retention time
** Reported relative retention time  (Dugo, et al., 2005).
*** Glycoside/aglycone

Table 2
Effect of d-galactosamine-induced liver injury (d-GalN, 800mg/kg, single IP dose) and oral administration of naringin and rutin (40mg/kg) on liver enzymes (serum ALT, AST and GGT activities), total bilirubin, liver GSH and MDA (pg/mg. tissue) levels.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>d-GalN</th>
<th>Naringin</th>
<th>Rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>52 ± 6</td>
<td>6524* ± 549.4</td>
<td>1180^@± 73.34</td>
<td>458.3^@± 28.42</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>135.8 ± 5.7</td>
<td>6859^* ± 790.7</td>
<td>1176^@± 70.33</td>
<td>545.3^@± 30.32</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>0.67 ± 0.21</td>
<td>8.0^* ± 1.4</td>
<td>5.58± 0.20</td>
<td>1.58@± 0.20</td>
</tr>
<tr>
<td>T. bilirubin (mg/dL)</td>
<td>0.02 ± 0.002</td>
<td>0.37* ± 0.07</td>
<td>0.06^@± 0.01</td>
<td>0.20^@#± 0.016</td>
</tr>
<tr>
<td>Liver GSH</td>
<td>51.83±2.7</td>
<td>31.97*±2.25</td>
<td>46.13^@±2.55</td>
<td>46.87^@±2.54</td>
</tr>
<tr>
<td>Liver MDA</td>
<td>3.1±0.38</td>
<td>6.65*±0.43</td>
<td>3.39^@±0.39</td>
<td>4.73^@±0.60</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. (*) significant difference compared to normal control group (@) significant difference compared to d-GalN treated group and (#) significant difference from naringin 100 at p < 0.05. n = 6; by One Way ANOVA and Tukey post hoc test.
Figure 1: LC–ESI/MS (negative ion mode) of the ethyl acetate fraction from *C. jambhiri*. A: Leaves, B: Stems. Eriocitrin (1), rutin (2), diosmetin-6-C-glucoside (3), narirutin (4), naringin (5), hesperidin (6), and neohesperidin (7).

Figure 2: Histopathological changes occurring in rutin or naringin pre-treatment groups after d-GalN intoxication (staining with hematoxylin and eosin, 200×). (A) untreated control; (B) d-GalN group, 800 mg/kg i.p.; (C) naringin (40 mg/kg) + d-GalN; (D) rutin (40 mg/kg) + d-GalN.
**Figure 3:** Effect of both naringin and rutin on the liver levels of interleukin 10 (IL-10), tumor necrosis factor-alpha (TNF-α) and IL-10/ TNF-α ratio. Each value is the mean ±standard error; * & †@ indicate significant difference at p < 0.05.

**Figure 4:** Immunoblotting of nuclear factor kappa b (NF-κB). Densitometric results are expressed as arbitrary units corresponding to signal intensity normalized to β-actin. Values are means ±SE of three experiments performed in duplicate.*P < 0.05.