



## ORIGINAL ARTICLE

# Beneficial effect of garlic on D-galactosamine and lipopolysaccharide-induced acute hepatic failure in male albino rats

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IL-4;  
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### Abstract

**Background and aims:** Activation of the pro-inflammatory and anti-inflammatory cytokine cascade, including tumour necrosis factor (TNF)-alpha and interleukin (IL)-4, is considered to play an important role in severe liver injury. Kupffer cells, resident macrophages of the liver, activated with lipopolysaccharide (LPS) release pro-inflammatory cytokine. D-Galactosamine (D-GalN), a hepatocyte-specific inhibitor of RNA synthesis, is known to sensitise animals to the lethal effects of LPS. In the present study we seek to reverse some altered parameters, immunological and histopathological, to normal values of rats pre-treated with garlic.

**Methods:** Acute hepatic failure was induced in male albino rats by the intraperitoneal injection of 500 mg D-GalN and 50 µg LPS/kg body weight. Expression levels of TNF-α and IL-4 were detected by ELISA. Leukocytes proliferation was carried out by differential count. For histopathology, liver sections were stained with haematoxylin and eosin. Data were analysed by SPSS program version 13.0.

**Results:** The data showed significant increase in the numbers of granulocytes, but with significant decreases in lymphocyte and monocytes proliferation and the TNF-alpha and IL-4 levels in D-GalN/LPS-induced group. Garlic pre-treatment of liver-injured rats induced significant amelioration in the numbers of monocytes and lymphocytes, with significant increase in granulocytes numbers, TNF-α level and IL-4 level.

**Conclusions:** Results of this study revealed that garlic could afford a significant protection in the alleviation of D-GalN/LPS-induced hepatocellular injury.

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## Introduction

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is generally considered to be a major pathogenic element in Gram-negative bacterial

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infection, and can eventually cause systemic inflammatory response syndrome.<sup>1</sup>

The innate immune system copes with infection by producing pro-inflammatory mediators such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ); however, overproduction of these mediators eventually leads to systemic inflammatory response syndrome.<sup>2</sup> LPS stimulates monocytes/macrophages via Toll-like receptor 4 (TLR4), which belongs to the TLR family.<sup>3</sup> LPS is recognised by TLR4 together with accessory molecules such as myeloid differentiation protein-2 and CD14, and TLR4 then transduces LPS signalling via both myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways, each of which activates nuclear factor- $\kappa$ B (NF- $\kappa$ B).<sup>4</sup>

D-Galactosamine (D-GalN) increases the susceptibility of mice to LPS-induced shock by impairing liver metabolism.<sup>5</sup> In contrast to high dose LPS-induced shock which induces a systemic disorder including multiple organ failures,<sup>6</sup> liver is a major target organ after challenge with low doses of LPS in conjunction with D-GalN.<sup>7</sup> Similarly to high dose LPS-induced shock, TNF- $\alpha$  plays a central role in low dose LPS-induced shock/liver injury.<sup>8</sup>

Endotoxin makes macrophages, neutrophils, and other inflammatory cells release cytokines, such as TNF- $\alpha$ , in great amounts, which may render inflammatory reactions out of control, ultimately resulting in sepsis, septic shock, or multiple organ failure syndromes.<sup>2,9</sup> The release of TNF- $\alpha$  is the hallmark of the cellular response to the activation of the innate immune system.<sup>10</sup> The essential role of TNF in control of several infections was documented.<sup>11</sup>

A critical degree of liver cell death due to apoptosis or necrosis is fundamental to the development of fulminant hepatic failure.<sup>12</sup> Activation of the pro-inflammatory cytokine cascade, including TNF- $\alpha$  is considered to play an important role in the pathophysiology and clinical outcome of this severe liver injury.<sup>13</sup> Kupffer cells, resident macrophages of the liver, have a transmembrane protein TLR-4, which recognises LPS and mediates macrophage activation and pro-inflammatory cytokine release.<sup>14,15</sup> Upregulated TLR4 expression and function was observed in various situations of experimental liver injury.<sup>15–18</sup> Liver macrophages are reported to be increased in number and considered to be closely related to the upregulation of cytokines and chemokines in fulminant hepatic failure.<sup>19</sup>

Garlic is a sulphur-rich phytochemistry with a long history of therapeutic use. It has been shown to be useful as an antiplatelet,<sup>20</sup> antihypertensive,<sup>21</sup> antifungal,<sup>22</sup> anticancer,<sup>23</sup> antioxidant,<sup>24</sup> hypolipidaemic<sup>25</sup> and hypoglycaemic.<sup>26</sup> Its variety of therapeutic actions probably relates to the complex nature of the chemistry of garlic. It contains many sulphur-rich derivatives of the amino acid cysteine. The immunomodulatory effects of garlic have been demonstrated on a small variety of cells and tissues. The effects of garlic on human and murine peripheral blood mononuclear cells (PMBCs) and neutrophils have been investigated. Although not investigating the effect of garlic on cytokine production. Therefore, the aim of the present study is to examine the *in vivo* effect of garlic on normal and D-GalN/LPS treated liver tissues. We directed our efforts at the effect of garlic on the inflammatory cytokine balance of the liver tissue. We wished to

examine garlic's potential to increase anti-inflammatory, and decrease inflammatory mediator production, and thus explore its use as a substance with the potential to enhance or correct liver functions.

## Materials and methods

### Reagents

D-GalN and LPS were obtained from Sigma (St. Louis, MO, USA). ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals were obtained commercially as reagent-grade products.

### Experimental animals

Male albino rats, *Rattus norvegicus* (6–8 weeks old, weight 100–120 g) were purchased from the Biological Supply Center, Theodore Bilharz Research Institute, TBRI, Cairo, Egypt and housed under specific pathogen-free conditions and maintained on a 12-h light–dark cycle, with food and water *ad libitum*. Animals were classified into 12 groups (ten animals each). After rats were fasted for 18 h, the first group (negative control) was untreated; the second group (first positive control) was injected with the same amount of saline intraperitoneally; the third group (second positive control) was allowed free to feed on a crude garlic used as a food supplement (5 g) daily for one week before D-GalN/LPS injection. The groups from four to eleven were injected intraperitoneally with D-GalN at 500 mg/kg and LPS at 50  $\mu$ g/kg (dissolved in 500 ml of saline) to induce liver injury; the fifth, seventh, ninth and twelfth groups were allowed free to feed on garlic daily for one week after D-GalN/LPS injection. Blood samples were collected from tail at 6, 12, and 24 h after D-GalN/LPS injection and at one week before and one week after D-GalN/LPS injection.

### Cytokines in serum

Seventy-two hours after the last treating, 10 animals from each group were sacrificed under chloroform anaesthesia. Blood samples were taken and centrifuged at 3000 rpm for 30 min. Sera were removed and kept at  $-20^{\circ}\text{C}$  for the estimation of TNF- $\alpha$  and IL-4 levels.

### Leukocytes differential count

Freshly collected blood samples of about 20  $\mu$ l were spread on clean slides into a thin film using another smooth-edged glass slide. Each blood smear was left to air dry before being fixed with methanol for 2–3 min and then labelled by the number of the animal. Blood smears were stained with 10% Giemsa's stain (Aldrich) in buffered distilled water containing 0.021 M  $\text{Na}_2\text{HPO}_4/0.015$  M  $\text{KH}_2\text{PO}_4$ . pH 7–7.2 for 30 min far from sun light. After that, the stain was removed by gentle washing with distilled water and the slides were air-dried at room temperature. Using light microscopy at 400 $\times$  magnification, different types of blood leukocytes were recorded. At least double smears for each blood samples were counted.

## Histopathological examination

Liver samples were dissected after DGalN/LPS injection and garlic treating, fixed in 10% formal saline, embedded in paraffin, and cut into 5  $\mu$ m thickness in microtome. These sections were collected in slides and then stained with haematoxylin and eosin. The stained sections were examined under low and high power by using an Olympus microscope.

## Statistical analysis

Data were analysed using SPSS program version 13.0. Statistical analysis of the obtained data was performed using one way analysis of variance (ANOVA) test followed by least square differences (LSD) analysis for comparison between means. Results were expressed as mean  $\pm$  standard error (SE). Values of  $P < 0.05$  were considered statistically significant, while value of  $P > 0.05$  were considered statistically non-significant.

## Results

### Estimation of TNF- $\alpha$

Serum TNF- $\alpha$  levels were demonstrated in Fig. 1, where the negative control group (1405.26  $\pm$  5.252 pg/ml), the first PBS positive control (1431.59  $\pm$  10.692 pg/ml) and the second positive control pre-treated with garlic one week before D-GalN/LPS injection (1361.48  $\pm$  8.302 pg/ml) is higher than the D-GalN/LPS treated group after 6 h (172.16  $\pm$  2.049 pg/ml), 12 h (880.87  $\pm$  1.717 pg/ml) and 24 h (969.73  $\pm$  3.114 pg/ml) which is lower than the D-GalN/LPS group treated with garlic (1129.73  $\pm$  3.650 pg/ml, 1726.02  $\pm$  4.376 pg/ml and 1561.90  $\pm$  6.574 pg/ml at 6, 12 and 24 h, respectively). In contrast, the garlic treated group after one week of D-GalN/LPS injection (1466.51  $\pm$  1.451 pg/ml) is higher than the D-GalN/LPS injected groups at 6, 12 and 24 h (172.16  $\pm$  2.049 pg/ml, 880.87  $\pm$  1.717 pg/ml and 969.73  $\pm$  3.114 pg/ml, respectively). These results showed

a significant difference in TNF- $\alpha$  level between all groups ( $P < 0.05$ ).

### Estimation of IL-4

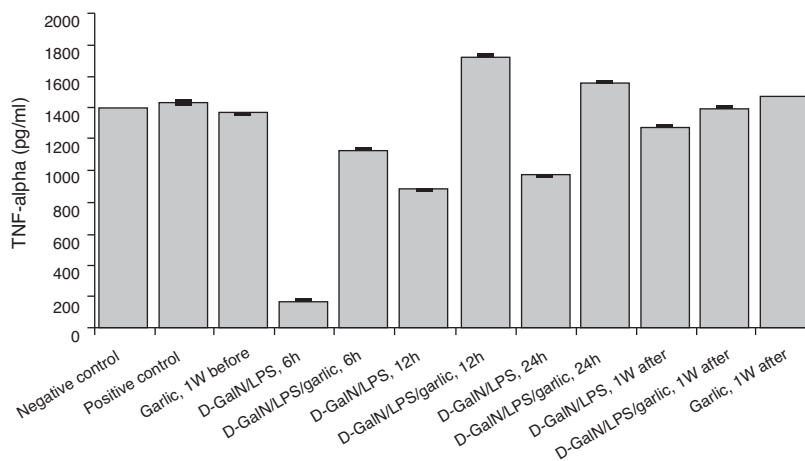
Results of ELISA in Fig. 2 showed that IL-4 recorded 667.72  $\pm$  7.270 pg/ml in negative control group (untreated), 640.09  $\pm$  3.176 pg/ml in the first PBS positive control and 687.00  $\pm$  4.078 pg/ml in the second positive control. Liver injury group injected with D-GalN/LPS counted low levels of IL-4 at 6 h (283.50  $\pm$  1.417 pg/ml), 12 h (368.10  $\pm$  3.607 pg/ml) and 24 h (454.20  $\pm$  1.994 pg/ml) than the D-GalN/LPS injected groups treated with garlic (555.75  $\pm$  1.864 pg/ml, 813.59  $\pm$  2.081 pg/ml and 769.63  $\pm$  3.421 pg/ml, at the same time, respectively). In addition, the D-GalN/LPS injected groups at 6, 12 and 24 h are lower than the garlic treated group after one week of D-GalN/LPS injection (751.01  $\pm$  1.789 pg/ml). Statistical analysis showed that there is a significant difference in IL-4 between the liver injury groups and either the control group or the D-GalN/LPS injected groups treated with garlic,  $P < 0.05$ .

### Leukocyte differential count

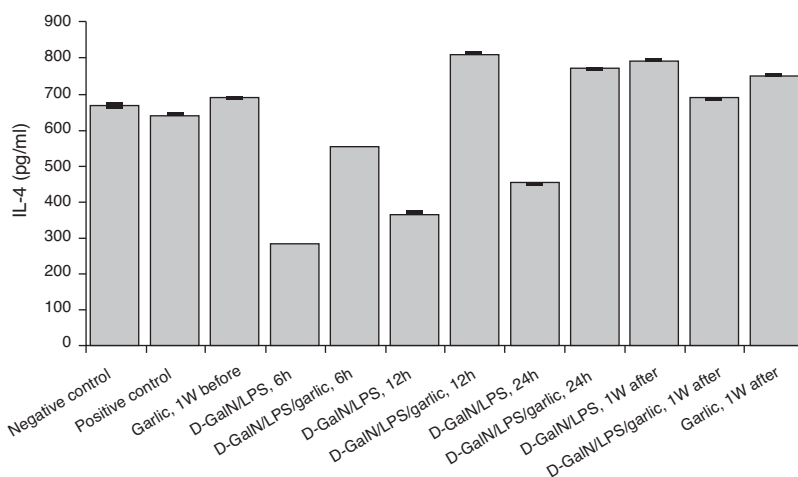
Table 1 shows that granulocytes percentage in the garlic treated groups before (57.60  $\pm$  0.509 pg/ml) and after (57.60  $\pm$  0.509 pg/ml) the D-GalN/LPS injection, the negative control group (57.20  $\pm$  0.374 pg/ml), the first positive control group treated saline (57.20  $\pm$  0.374 pg/ml) are nearly similar in population and higher than the other groups ranged from 53.80  $\pm$  0.374 pg/ml to 55.80  $\pm$  0.583 pg/ml, except for the D-GalN/LPS injected group after 12 h which showed the highest population (63.80  $\pm$  0.663 pg/ml).

In contrast to granulocytes, the lymphocytes and monocytes percentages showed the lowest population in the D-GalN/LPS injected group after 12 h (30.20  $\pm$  0.663 pg/ml and 6.00  $\pm$  0.316 pg/ml, respectively), Table 1.

Statistical analysis showed that there is a significant difference in leukocytes counts between the liver injury groups



**Figure 1** Changes in TNF- $\alpha$  level in D-GalN/LPS injected groups versus control group and garlic treated groups. Significant differences between all groups,  $P < 0.05$ , were monitored.



**Figure 2** Changes in IL-4 levels in  $\alpha$ -GalN/LPS injected groups versus control group and garlic treated groups. Significant differences between all groups,  $P < 0.05$ , were monitored.

**Table 1** Changes of granulocytes, lymphocytes and monocytes percent in  $\alpha$ -GalN/LPS injected group versus control groups and garlic treated groups. Statistical analysis showed a significant difference,  $p < 0.05$ .

Groups	Granulocytes	Lymphocytes	Monocytes
1. Negative control, untreated	57.20 $\pm$ 0.374	35.40 $\pm$ 0.244	7.40 $\pm$ 0.244
2. Positive control, PBS	57.20 $\pm$ 0.374	35.80 $\pm$ 0.374	7.20 $\pm$ 0.200
3. Garlic 1 W before	57.60 $\pm$ 0.509	35.20 $\pm$ 0.374	7.20 $\pm$ 0.200
4. $\alpha$ -GalN/LPS, 6 h	55.20 $\pm$ 0.374	35.20 $\pm$ 0.374	9.60 $\pm$ 0.678
5. $\alpha$ -GalN/LPS/garlic, 6 h	55.00 $\pm$ 0.707	35.00 $\pm$ 0.316	10.00 $\pm$ 0.447
6. $\alpha$ -GalN/LPS, 12 h	63.80 $\pm$ 0.663*	30.20 $\pm$ 0.663*	6.00 $\pm$ 0.316*
7. $\alpha$ -GalN/LPS/garlic, 12 h	53.20 $\pm$ 0.663	32.20 $\pm$ 0.374	14.60 $\pm$ 0.400
8. $\alpha$ -GalN/LPS, 24 h	55.00 $\pm$ 0.707	34.60 $\pm$ 0.509	10.40 $\pm$ 0.600
9. $\alpha$ -GalN/LPS/garlic, 24 h	53.80 $\pm$ 0.374	29.20 $\pm$ 0.374	17.00 $\pm$ 0.447
10. $\alpha$ -GalN/LPS, 1 W after	55.80 $\pm$ 0.583	32.80 $\pm$ 0.374	11.40 $\pm$ 0.244
11. $\alpha$ -GalN/LPS/garlic, 1 W after	53.80 $\pm$ 0.374	29.20 $\pm$ 0.374	17.00 $\pm$ 0.447
12. Garlic, 1 W after	57.60 $\pm$ 0.509	35.40 $\pm$ 0.244	7.00 $\pm$ 0.316

Values expressed are mean  $\pm$  SE of five samples.

\* Statistical significance ( $P < 0.05$ ) is shown on comparing infected or treated samples versus the control ones.

and either the control group or the groups treated with garlic,  $P < 0.05$ .

### Histopathological findings

Histopathological examination of liver sections from control animals revealed normal architecture, Fig. 3A.  $\alpha$ -GalN/LPS-intoxicated rat liver section showed loss of architecture and prominent inflammatory collections in the central rather than around central vein, Fig. 3B. In contrast, a slight inflammatory reaction was found in liver section of rat pre-treated with garlic prior to  $\alpha$ -GalN/LPS challenge, Fig. 3C.

### Discussion

Many approaches to suppress the effects of inflammatory mediators have been unsatisfactory for the effective treatment of Gram-negative bacterial.<sup>2</sup> This promoted us to use a medicinal plant, garlic, to treat bacterial infection causing liver injury.

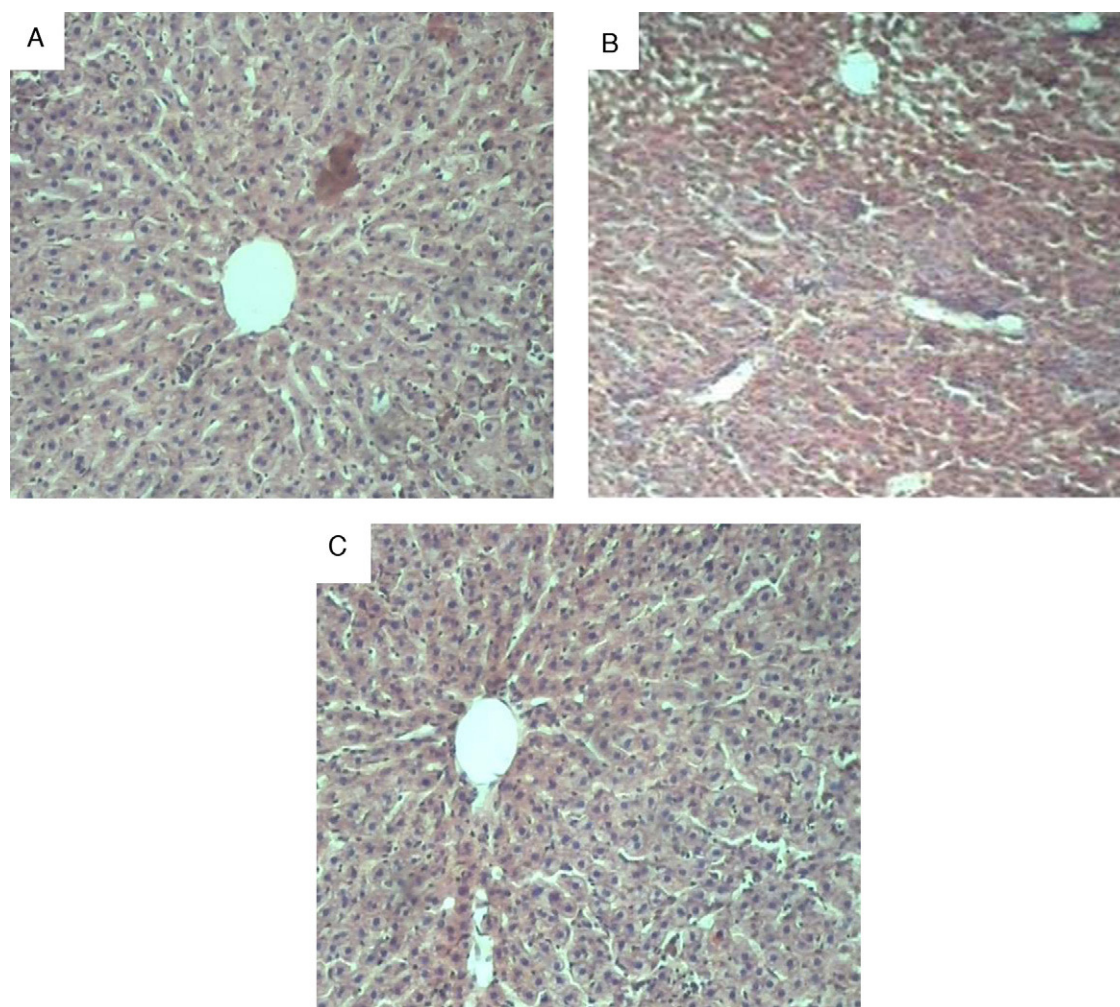
This paper describes participation of  $\alpha$ -GalN and LPS in liver injury and points to garlic as a critical medicinal plant of resistance to this injury. Histological disorder is accompanied with the level of some cytokines, such as the pro-inflammatory TNF- $\alpha$  and the anti-inflammatory IL-4, and leukocytes proliferation.

The liver is an important organ within the body with a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage, and redistribution of nutrients, carbohydrates, fats, and vitamins. Hepatitis is the best-known liver disease.

Acute liver injury was induced by  $\alpha$ -GalN/LPS.<sup>7</sup> The advantage of this induction is that  $\alpha$ -GalN can potentiate the toxic effects of LPS and produce fulminant hepatitis within a few hours.<sup>27</sup> In the present study, we demonstrated that pre-treatment with garlic attenuated liver injury caused by  $\alpha$ -GalN/LPS, where the administration of  $\alpha$ -GalN/LPS significantly down-regulated the expression of both serum TNF- $\alpha$  and IL-4 that are up-regulated with garlic.

NF- $\kappa$ B plays a key role in the regulation of TNF- $\alpha$  transcription stimulated by LPS.<sup>28</sup> In the latent





**Figure 3** Light photomicrographs of rat liver (haematoxylin and eosin stains, magnification 100 $\times$ ): (A) Control rat liver section (B)  $\text{D-GalN/LPS}$ -intoxicated rat liver section; (C) liver section of rat pre-treated with garlic.

state, NF-kappa B is found in cytoplasm. After exposure to LPS, NF-kappa B is translocated to the nucleus and binds to NF-kappa B promoter sites on DNA, activating gene transcription of cytokines such as TNF-alpha.<sup>29</sup>

Taken together, our results revealed that the up-regulation of TNF-alpha of garlic against  $\text{D-GalN/LPS}$ -induced liver injury in rat could be related to inhibiting the activation of NF-kappa B. In addition, the down-regulation of the TNF-alpha might also be due to the death of the Kupffer cells, resident macrophages of the liver, where they have a transmembrane protein Toll-like receptor 4 (TLR4), which recognises endotoxin (LPS) and mediates macrophage activation and pro-inflammatory cytokine release.<sup>14</sup>

The histological injuries such as inflammation induced by  $\text{D-GalN/LPS}$  were markedly improved by garlic, which showed a protective effect as a control. Previous studies showed that LPS exerted the toxic effects mainly through the generation of endogenous inflammatory cytokines secreted from macrophages. Among these cytokines, TNF-alpha is a key factor that contributes to the triggering of an inflammatory cascade involving the induction of cytokines including interferon-gamma, nitric oxide, IL-1beta and cell adhesion molecules, etc.<sup>30</sup>

A proper balance between pro- and anti-inflammatory mediators is necessary for modulating an adequate immune response toward LPS. IL-4 is known to be an anti-inflammatory cytokine which can suppress serum TNF-alpha levels and reduce the mortality of mice exposed to  $\text{D-GalN/LPS}$ .<sup>31</sup> In the present study, there was a massive induction of the inflammatory cytokines TNF-alpha in hepatic failure caused by  $\text{D-GalN/LPS}$ , which were not counterbalanced by the anti-inflammatory cytokine IL-4. This imbalance may promote inflammatory responses to liver damage. After administration of garlic, serum IL-4 maintained at a higher level, which indicated that the imbalance was reversed by garlic to a certain extent.

Interaction between T-cells and hepatocytes that was regulated by adhesion molecules is dysregulated in harmful inflammatory processes. During the  $\text{D-GalN/LPS}$  intoxication, the expressions of ICAM-1 and LFA-1 in hepatocytes and non-parenchymal cells were enhanced. The elevation of these molecules was essential to trigger the extravasations of neutrophils into the liver parenchyma, which produced cytotoxic damage to hepatocytes.<sup>32</sup> This is in accordance with the high percentage of neutrophils monitored in our results. Previous studies suggested that TNF-alpha could induce the

transcription of adhesion molecules.<sup>32</sup> The effect of garlic on the adhesion molecules may partially relate to the inhibition of cytokines.

In conclusion, the present data demonstrates that garlic had a protective effect in the imbalance of serum TNF- $\alpha$  and IL-4 levels, leukocytes proliferation and liver histological architecture.

## Conflict of interest

The authors have no conflict of interest to declare.

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