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Nephroprotective and hepatoprotective effects of lemongrass essential oil and citral on diclofenac-induced toxicity in mice

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ABSTRACT

The present study was carried out to evaluate and compare the protective potential of two well-known antioxidants of herbal origin in a mouse model of acute DIC-induced nephro- and hepatotoxicity. The tested antioxidants included lemongrass essential oil (LO) and its predominant bioactive constituent citral (CIT). A third herbal product, silymarin (SILY), was used as a reference hepato-renal protective agent. DIC administration led to elevated serum urea and creatinine levels, and prompted oxidative stress along with histopathological changes in the kidney tissue. In parallel, DIC administration increased serum liver enzyme activity, decreased total protein, albumin, and globulin levels, and caused oxidative stress with associated histopathological changes in the liver tissue. Pre-treatment with LO or CIT mitigated DIC-induced alterations in all serum biochemical markers of kidney and liver health (except albumin). High-dose LO, like SILY, within kidney and liver tissues, counteracted DIC-induced oxidative stress and histomorphological alterations. By contrast, CIT failed to mitigate DIC-induced oxidative stress in the kidneys and provided only partial control of DIC-induced oxidative stress in the liver, resulting in less efficient preservation of kidney function and liver structural integrity than LO. Besides confirming the efficacy of SILY at protecting kidneys and liver against the toxicity of DIC in a rodent species different from the one tested so far (rat), this study demonstrated the preventive properties of LO and, to a lesser extent, of CIT against DIC-induced hepato-renal toxicity in mice, supporting their developmental potential as therapeutics.

1. Introduction

Herbal preparations have been used for centuries in traditional medicine practices [1]. Due to their (a) generally high safety margin for both patients and environment, (b) relatively low cost, and (c) broad range of pharmacological activities (including antibacterial, antiviral, analgesic, anti-inflammatory, anticancer, and antioxidant properties), modern medicine is increasingly recognizing their value. Herbal remedies and their bioactive compounds are now being explored as health aids for treating, preventing, and generally managing various ailments, including conditions arising from synthetic drugs [1–4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are a leading-cause of drug-related morbidity [5]. Their use, especially at high doses and/or in patients with risk factors (such as the elderly and patients with co-morbidities), can be complicated by several serious safety issues, including gastric, renal and liver injury [4–7]. To exploit the indisputable clinical benefits of these drugs (pain and inflammation control) in a safer way, various strategies have been considered for preventing or

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; DIC, diclofenac; ROS, reactive oxygen species; LO, lemongrass essential oil; CIT, citral; SILY, silymarin; GC-MS, gas chromatography-mass spectrometry; RI, retention indices; NIST, National Institute of Standards and Technology; ALT, alanine aminotransferase; ALP, alkaline phosphatase; H&E, hematoxylin and eosin; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

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mitigating NSAID-associated clinical complications [5,8–10]. One approach involves the use of herbal medicines, which, through their diverse biological actions, have the potential to counteract the multiple pathological pathways underlying NSAID toxicity [10].

Among NSAIDs, diclofenac (DIC), which is widely prescribed to humans worldwide for the symptomatic treatment of acute and chronic painful and inflammatory conditions such as osteoarthritis, rheumatoid arthritis, and musculoskeletal injury [11–15], is one of the top-ranking drugs implicated in causing kidney and liver toxicity [10,16–19].

The precise mechanisms of hepato-renal damage by DIC and its active metabolites are not fully elucidated [14]; however, there is substantial evidence that increased generation of reactive oxygen species (ROS) with consequent induction of oxidative stress in the target tissues could play a pivotal role [4,10,11,16,19,20]. The pathogenic involvement of oxidative stress in DIC-induced hepato-renal toxicity is also indirectly confirmed by the several pre-clinical studies conducted so far that report the ability of various antioxidant agents, including herbs, to confer protection against the adverse effects of DIC in kidneys and liver [3,4,13,14,16,19,21,22].

Among herbs with antioxidant properties there is *Cymbopogon citratus* (DC.) Stapf, commonly known as lemongrass [23]. While native to India, this grass is cultivated in numerous tropical and subtropical regions worldwide, due to its wide range of culinary and medicinal applications [24,25]. Indeed, lemongrass is known for its pleasant fragrance and taste; its essential oil is used in traditional medicine to treat various ailments, such as hypertension, gastric issues, and liver and kidney disorders [26]. Lemongrass essential oil (LO) consists of numerous bioactive constituents, with citral (CIT) being the predominant one [25,27,28]. CIT is an acyclic monoterpene aldehyde consisting of two racemic isomers: geranial (*trans*-citral) and neral (*cis*-citral). Studies have indicated that most of the beneficial properties possessed by LO (antioxidant, anti-inflammatory, analgesic, antimicrobial) [28] can also be documented for CIT when used alone [29–32].

Both lemongrass (either in the form of essential oil or aqueous extract) and CIT have been shown to be protective in rodent models of hepatic toxicity induced by acetaminophen [30,33,34]. Furthermore, protection against hepatic toxicity of anticancer drugs (cisplatin) and cytotoxicity caused by aspirin has been reported for LO [35] and CIT [29], respectively. However, to the best of our knowledge, there are no published data specifically addressing the efficacy of LO and CIT in counteracting the hepato-renal toxicity of DIC.

In order to at least partially fill this gap, the present study investigated and compared the hepato-renal protective potential of LO and CIT in a mouse model of acute DIC-induced nephro- and hepato-toxicity, using a biochemical and histological approach. Silymarin (SILY), a herbal preparation from milk thistle (*Silybum marianum*) [36] with well-documented protective activity against hepato- and nephrotoxic drugs [37–41], DIC included [10,42–44], was used as a standard reference treatment for further comparative purposes.

2. Materials and methods

2.1. Plant material and LO extraction

Fresh leaf segments of lemongrass were sourced from the Mirnia Botanical Garden (Babol, Mazandaran). Confirmation of the plant species was undertaken by the Sari Faculty of Agriculture and Natural Resources (Sari, Mazandaran). The essential oil was extracted using the hydro-distillation method employing a Clevenger-type apparatus. Chemical constituents of the LO were identified through gas chromatography-mass spectrometry (GC-MS) analysis. Chemical analysis was conducted using an Agilent 6890 GC-MS system, equipped with an HP-5 column (0.25 mm × 30 m × 0.25 μm) and an Agilent Technologies 5973 detector. The temperature program for the oven was as follows: an initial temperature of 50°C, ramped up at a rate of 15 °C/min to reach 240°C, and held steady for 30 minutes. The sample was dissolved in hexane. Helium was employed as the carrier gas, and the injector temperature was set at 300°C, with a split ratio of 20:1. The injector and detector temperatures were maintained at 280°C. Compound identification was accomplished by comparing their retention times and mass spectra with established standards or their retention indices (RI) in conjunction with published data, along with cross-referencing their mass spectra with the National Institute of Standards and Technology (NIST) library.

2.2. Chemicals

DIC was obtained from Caspiantamin pharmaceutical company (Tehran, Iran). CIT was purchased from Sigma (Sigma-Aldrich, Germany) and SILY was obtained from Goldaru (Isfahan, Iran). All other chemicals were analytical grade and commercially available.

2.3. Animals

Fifty-four male Swiss albino mice, weighing between 25 and 35 g on average, were obtained from the Pasteur Institute of Iran, North Research Center (Amol, Iran). The mice were housed in standard transparent plastic cages with unrestricted access to water and food, under a 12-hour light and 12-hour dark cycle and a controlled temperature environment. The experimental protocol received approval from the Institutional Animal Care and Use Committee of Amol University of Special Modern Technologies, under authorization number 45/7/01 (AUSMT, Amol, Iran), adhering to the ARRIVE guidelines for the ethical care and utilization of laboratory animals.

2.4. Experimental design

Following a one-week acclimatization period, the mice were allocated randomly into nine groups as outlined below:

CON: served as the control group; DIC: received DIC at a single dose of 200 mg/kg; LO10+DIC: received DIC (200 mg/kg) after pre-treatment with LO at the dose of 10 mg/kg; LO100+DIC: received DIC (200 mg/kg) after pre-treatment with LO at the dose of 100 mg/kg; CIT10+DIC: received DIC (200 mg/kg) after pre-treatment with CIT at the dose of 10 mg/kg; CIT100+DIC: received DIC (200 mg/kg) after pre-treatment with CIT at the dose of 100 mg/kg; SILY50+DIC: received DIC (200 mg/kg) after pre-treatment with SILY at the dose of 50 mg/kg; LO100: received LO at the dose of 100 mg/kg; CIT100: received CIT at the dose of 100 mg/kg.

DIC was administered via oral gavage, whereas LO, CIT, and SILY were administered via intraperitoneal (i.p.) injection 30 minutes before the DIC administration. The animals of the CON group received normal saline at both administrations. In contrast, the DIC group received normal saline in the pre-treatment injection, and both the LO100 and CIT100 groups received normal saline in the second administration. Twenty-four hours after the second administration (either of DIC or saline), blood samples were collected, and the mice were humanely euthanized for the retrieval of tissue samples. Samples were then subjected to subsequent biochemical and histomorphological assessments.

2.5. Evaluation of serum biochemical markers linked to kidney and liver health status

The impact of the treatments on the renal health of the mice was assessed by analyzing urea and creatinine levels in serum samples. In parallel, the effects of the treatments on liver health were evaluated through measurements of serum levels of hepatic functional markers (total proteins, albumin and globulins, the latter calculated by subtracting albumin from total proteins), as well as of hepatic enzyme activities (alanine aminotransferase - ALT, and alkaline phosphatase - ALP). These biochemical parameters were quantified using commercial kits (Pars Azmoon, Iran).

2.6. Evaluation of kidney and liver histomorphology

Some of the kidney and liver samples collected from the experimental groups were promptly placed in 10 % buffered formalin. Subsequent to fixation and embedding of the tissue samples in paraffin, sections with a thickness of 5 μm were meticulously crafted using a microtome. These sections were then stained with hematoxylin and eosin (H&E). The stained sections were examined under a light microscope at both ×100 and ×400 magnification (Olympus CX21, Japan). Photomicrographs were captured using a digital camera attached to the microscope (TrueChrome II, China). The assessment of liver and kidney sections hinged on evaluating the severity of pathological changes, encompassing factors like immune cell infiltration and fatty/hydropic degeneration. A grading system was employed, assigning scores based on the extent of histopathological lesions: 0 indicating none, 1 denoting mild, 2 signifying moderate, and 3 representing severe.

2.7. Evaluation of kidney and liver oxidative status

In kidney and liver homogenates, the levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), along with the content of the lipid peroxidation marker malondialdehyde (MDA), were determined. The measurements were conducted spectrophotometrically using commercial kits (Navand Salamat, Iran).

2.8. Statistical analysis

The obtained data were subjected to the Kolmogorov–Smirnov test to check normality. Serum biochemistry and tissue antioxidant data were expressed as mean ± standard error of the mean (SEM) and subjected to one-way analysis of variance (ANOVA) and Bonferroni's as the *post hoc* test. The semi-quantitative data of histopathological changes were expressed as median (first quartile, third quartile) and compared by the Kruskal-Wallis test and the post-hoc Dunn's multiple comparisons test. Values of $P < 0.05$ were considered statistically significant. The statistical analysis was performed by SPSS (version 26; Chicago, USA).

3. Results

3.1. LO chemical constituents

The results of the GC-MS analysis of LO are presented in Table 1. As expected, the primary components of LO were identified as geranial and neral, the two isomers of CIT. These constituents accounted for 49.38 % and 35.54 % of LO, respectively. These two isomers constituted approximately 85 % of LO and are collectively recognized as the CIT content of LO.

Table 1
Chemical composition of the lemongrass (*Cymbopogon citratus)* essential oil.

No	Component[a]	Calculated RI[b]	Abundance (%)[c]
1	Myrcene	991	10.64
2	(*Z*)-β-Ocimene	1037	0.52
3	(*Z*)-β-Ocimene	1056	0.26
4	Linalool	1099	0.92
5	Citronellal	1149	0.34
6	Citronellol	1225	0.61
7	Neral[d]	1238	35.54
8	Geranial[d]	1266	49.38
9	Geranyl acetate	1379	0.34
	Total identified (%)		98.55

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes.
[c] Relative percentage values are means of three determinations, with Relative Standard Deviation percentage (RSD%) in all cases below 10 %.
[d] Neral and Geranial are isomers of citral (when combined, they form approximately 85 % of the oil)

3.2. Effects of the treatments on serum biochemical markers of kidney health

In all of the groups receiving DIC, except for LO100+DIC (namely DIC, LO10+DIC, CIT10+DIC, CIT100+DIC, and SILY+DIC), serum urea levels were significantly elevated as compared to the CON group ($P < 0.01$) (Fig. 1A). Pre-treatment with the lowest dose of LO (10 mg/kg), as well as with CIT and SILY exhibited mitigating effects on the DIC-induced urea increase, as indicated by the lower urea levels observed in the LO10+DIC, CIT10+DIC, CIT100+DIC, and SILY+DIC groups in comparison to the DIC group ($P < 0.05$). Pre-treatment with LO at 100 mg/kg (LO100+DIC) resulted in urea levels akin to those of the CON group. In the absence of DIC treatment, the highest doses of LO and CIT did not induce any alterations in the urea levels of the mice.

Similarly, an elevation in serum creatinine was observed in most of the DIC-intoxicated groups (DIC, LO10+DIC, CIT10+DIC, and CIT100+DIC) when compared with the CON group ($P < 0.01$) (Fig. 1B). Pre-treatment with CIT (at both 10 and 100 mg/kg) displayed the potential to mitigate the nephrotoxic effects of DIC, resulting in creatinine levels lower than those measured in the DIC group ($P < 0.05$). Notably, pre-treatment with the highest dose of LO (100 mg/kg), as well as with SILY, showed an even more marked nephroprotective potential, as no significant difference was discerned in creatinine levels between the LO100+DIC and SILY+DIC groups when compared to the CON group. Administration of LO and CIT at the highest dose did not trigger any increases in creatinine levels in the treated mice.

3.3. Effects of the treatments on serum biochemical markers of liver health

In all DIC-treated groups, a considerable elevation in serum ALT activity was recorded as compared with the CON group ($P < 0.05$) (Fig. 2A). Pre-treatments with the highest dose of LO and CIT (in the LO100+DIC and CIT100+DIC groups), as well as with SILY (in the SILY+DIC group), resulted in ALT levels lower than those measured in the DIC group ($P < 0.05$). Notably, in the absence of DIC treatment, administering high doses of LO and CIT did not elicit changes in serum ALT activity.

As depicted in Fig. 2B, significantly increased serum ALP activity was recorded in the DIC, LO10+DIC, and CIT10+DIC groups compared with the CON group ($P < 0.01$). Pre-treatment with either LO or CIT at 10 mg/kg effectively mitigated the ALP activity compared to the DIC group ($P < 0.05$). Notably, ALP activity levels exhibited in L100+DIC, C100+DIC, and SILY+DIC groups did not differ significantly from those of the CON group, with LO100 pre-treatment causing the most notable reduction in ALP activity relative to all of the other pre-treatments ($P < 0.05$).

As shown in Fig. 3A, the total protein levels decreased similarly in the DIC, LO10+DIC, and CIT10+DIC groups compared to the CON group ($P < 0.001$). Pre-treatments with LO and CIT at the highest dose of 100 mg/kg, as well as pre-treatment with SILY, effectively maintained total protein levels at values not significantly different from those of the CON group.

Concerning albumin (Fig. 3B), the DIC, LO10+DIC, and CIT10+DIC groups exhibited similar lower levels compared with the CON group ($P < 0.01$). In LO100+DIC and CIT100+DIC groups, a trend towards higher albumin levels than in the other intoxicated groups was observed, leading to a lack of statistical significance in comparison with both the DIC and CON groups. In the SILY group, the albumin levels were significantly higher than those in the DIC group ($P < 0.05$), and not different from those recorded in the CON group.

The amount of globulin in the DIC-intoxicated mice showed a significant decrease compared with the CON mice ($P<0.01$) (Fig. 3C). On the other hand, all pre-treatments kept globulin levels at values that

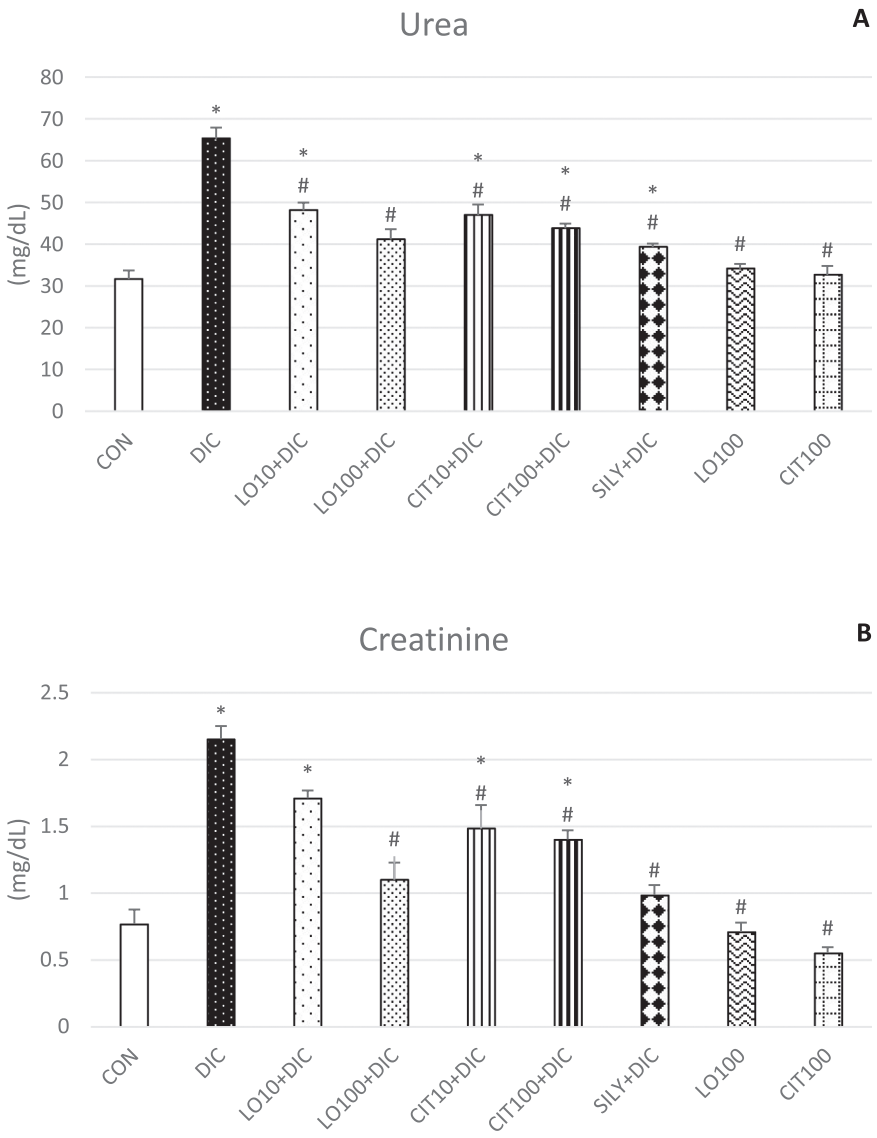


Fig. 1. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on serum biochemical markers of kidney function, urea (A) and creatinine (B), in diclofenac-intoxicated mice. Data are expressed as mean ± SEM (n=6 per group) and analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively. * $P < 0.01$ *Vs* CON, # $P < 0.05$ *Vs* DIC.

were not different from those of the CON group.

3.4. Effects of the treatments on kidney histomorphology

Fig. 4 displays the histological micrographs of the kidney tissue collected from the different experimental groups, while Fig. 5 provides the graphical representation of the scores assigned to the histopathological lesions detected. In the control group (CON), the normal architecture of kidney tissue was noticed (Fig. 4A). DIC administration to the animals in the DIC group resulted in hydropic degeneration and necrosis of tubular epithelial cells (Fig. 4B). Pre-treatment with the lower dose of either LO or CIT (in LO10+DIC and CIT10+DIC groups) resulted in slight but appreciable mitigation of the histopathologic changes induced by DIC in the kidney (Figs. 4C, 4E), even though the mild improvement of the scores assigned to the DIC-induced degenerative and infiltrative alterations did not achieve statistical significance (Fig. 5). The mitigating effect became significantly more pronounced ($P < 0.05$) when animals were pre-treated with either LO or CIT at the higher dose (i.e. in LO100+DIC and CIT100+DIC groups) (Figs. 4D, 4F and Fig. 5). Pre-treatment with SILY provided similar partial significant protection against the histopathological changes induced by DIC in the kidney (Fig. 4G and Fig. 5). LO and CIT, when administered *per se* to the animals at the highest dose, caused no remarkable changes in the kidney histological appearance (Figs. 4H, 4I and Fig. 5).

3.5. Effects of the treatments on liver histomorphology

Fig. 6 displays the histopathological micrographs of the liver tissue collected from the different experimental groups, while Fig. 7 provides the graphical representation of the scores assigned to the histopathological lesions detected. In the CON group, the liver tissue exhibited a normal architecture (Fig. 6A). Conversely, in the DIC group, pronounced immune cell infiltration and degenerative processes were observed (Fig. 6B).

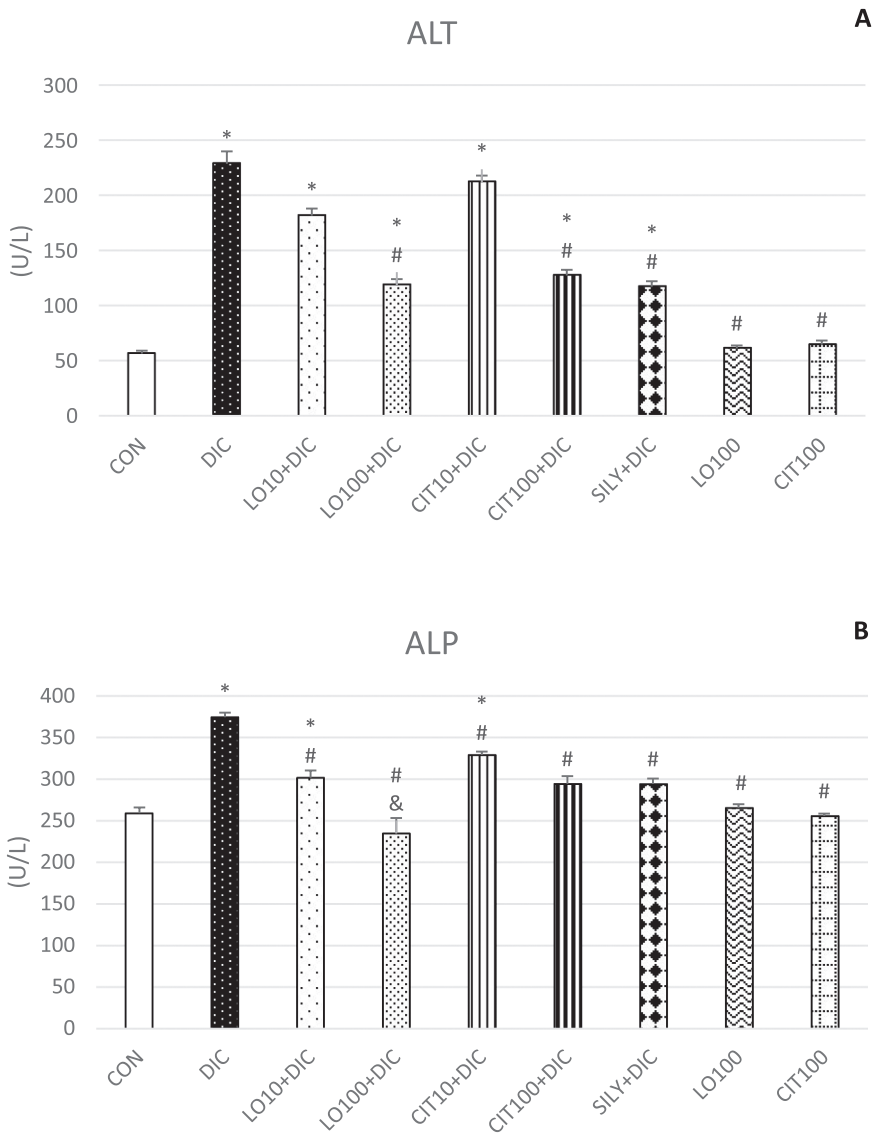


Fig. 2. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on serum biochemical markers of liver enzymes activity, alanine aminotransferase - ALT (A) and alkaline phosphatase - ALP (B), in diclofenac-intoxicated mice. Data are expressed as mean ± SEM (n=6 per group) and analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively. * $P < 0.01$ *Vs* CON, $^{\#}$ $P < 0.05$ *Vs* DIC, $^{\&}$ $p < 0.05$ *Vs* CIT100+DIC and SILY+DIC.

Pre-treatment with the lower dose of either LO or CIT (in LO10+DIC and CIT10+DIC groups) resulted in slight, but appreciable mitigation of the histopathologic changes induced by DIC in the liver (Fig. 6C, E), even though the mild improvement of the scores assigned to the DIC-induced degenerative and infiltrative alterations did not achieve statistical significance (Fig. 7). The mitigating effect became significantly more pronounced ($P < 0.05$) when the animals were pre-treated with either LO or CIT at the higher dose (i.e. in LO100+DIC and CIT100+DIC groups) (Figs. 6D, F and Fig. 7), with LO resulting more effective than CIT in exerting this protection.

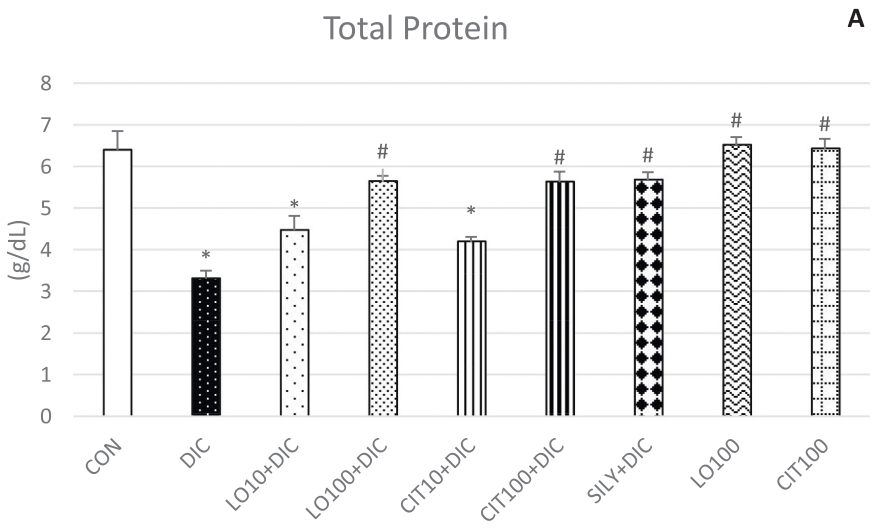
Pre-treatment with SILY provided partial significant protection against the histopathological changes induced by DIC in the liver to a similar extent as LO at 100 mg/kg (Fig. 6G and Fig. 7). The administration of LO and CIT on their own, at the highest dose, caused no remarkable changes in the histological appearance of the liver (Figs. 6H, I and Fig. 7).

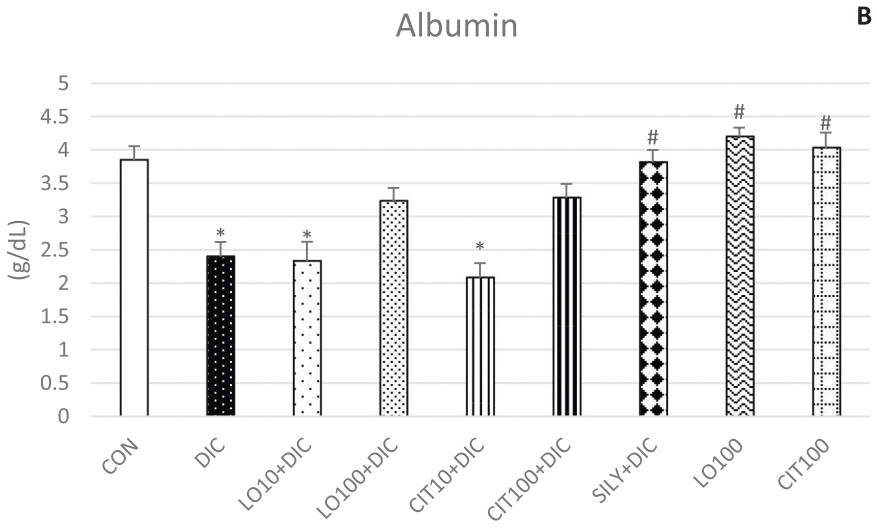
3.6. *Effects of the treatments on kidney and liver oxidative status*

Table 2 reports the levels of MDA and the two representative antioxidant enzymes (SOD and CAT), as measured in both kidney and liver samples from the experimental mice. In the DIC group, both kidney and liver SOD and CAT levels exhibited a noteworthy decrease in comparison with the CON group ($P < 0.01$). In parallel, the MDA content of both tissues increased significantly (about two-fold) in the DIC-treated mice relative to the CON group ($P < 0.01$).

The effects of DIC on kidney antioxidant enzymes were mitigated by pre-treatment with LO100 and SILY, resulting in levels only slightly lower than (SOD; $P < 0.05$) or even close to (CAT; $P > 0.05$) those measured in the CON group. The pre-treatments with LO100 and SILY also managed to substantially mitigate the DIC-induced increase in renal MDA levels, nearly keeping them at control values.

In the liver of the treated mice, all pre-treatments (except CIT10) resulted in a significant, though partial, mitigation of the DIC-induced





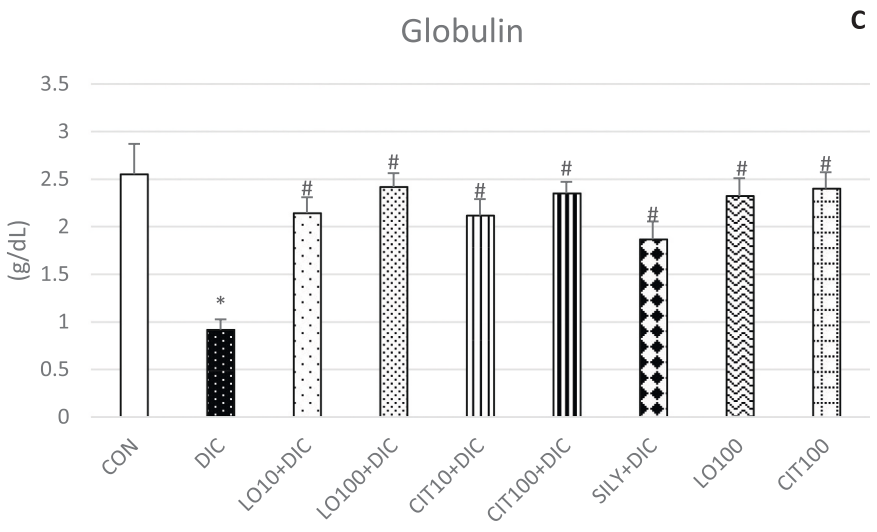


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Fig. 3. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on total protein (A), albumin (B), and globulin (C) in diclofenac-intoxicated mice. Data are expressed as mean ± SEM (n=6 per group) and analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively. * $P < 0.01$ *Vs* CON, # $P < 0.05$ *Vs* DIC.

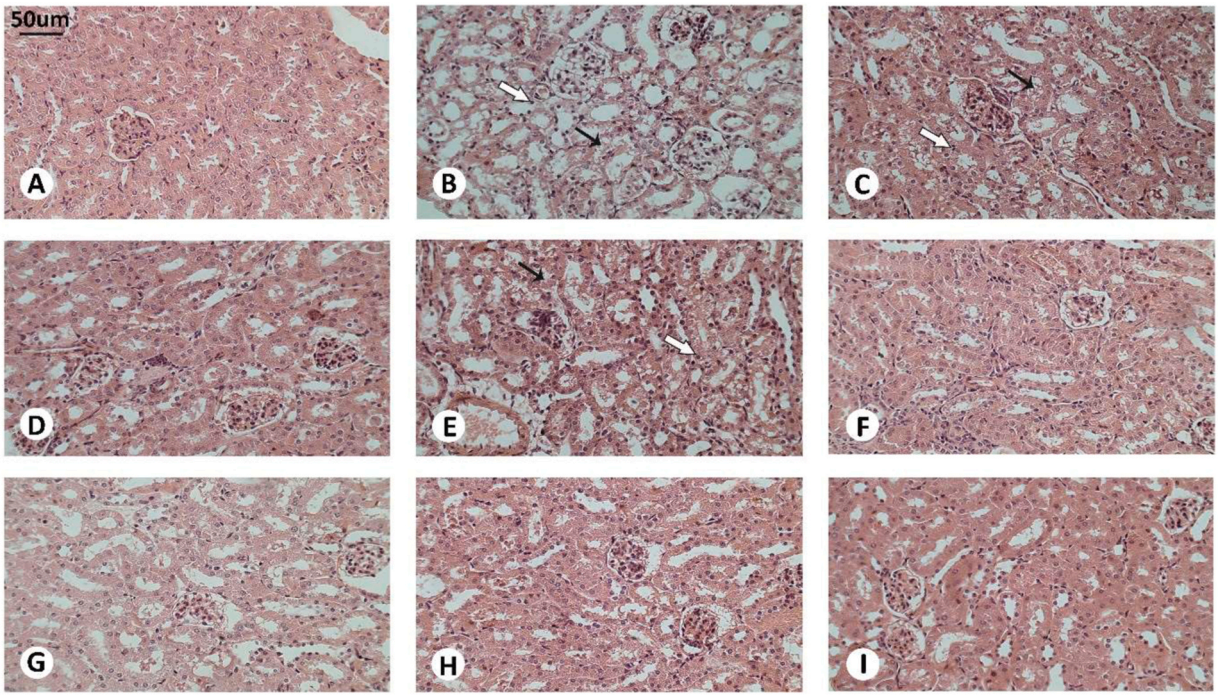

Fig. 4. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on kidney histomorphology in diclofenac-intoxicated mice. Representative pictures from n = 6 mice per group. **A- control group** (CON), shows the normal kidney tissue structure. **B- Diclofenac** (DIC) treated group exhibits severe hydropic degeneration of proximal tubules (black arrow) alongside necrotic tubular epithelial cells with pyknotic nuclei (white arrow). **C- Lemongrass Oil (Low dose) and Diclofenac** (LO10+DIC) treated group displays remarkable degenerative (black arrow) and necrotic processes of proximal epithelial cells (white arrow). **D- Lemongrass Oil (High dose) and Diclofenac** (LO100+DIC) treated group presents mild degeneration of proximal tubules epithelial cells. **E- Citral (Low dose) and Diclofenac** (CIT10+DIC) treated group shows notable hydropic degeneration of proximal tubules epithelial cells (black arrow) and necrotic cells with pyknotic nuclei (white arrow). **F- Citral (High dose) and Diclofenac** (CIT100+DIC) treated group reveals slight hydropic changes. **G- Silymarine and Diclofenac** (SILY+DIC) treated group indicates mild degenerative alterations of proximal tubules' epithelial cells. **H- Lemongrass Oil (High Dose)** (LO100) and **I- Citral (High Dose)** (CIT100) treated groups show nearly normal structure of kidney tissue. (H&E staining, ×400, scale bar represents 50 μm).

decrease in SOD levels, leading to higher SOD values than those measured in the DIC group ($P < 0.05$). As for the DIC-induced decrease in liver CAT levels, significant and even complete mitigation was only observed with pre-treatments with LO100 and SILY. Notably, pre-treatments with the higher dose of LO or CIT (LO100 and CIT100), as well as pre-treatment with SILY proved able to effectively mitigate the DIC-induced increase in liver MDA levels, leading to values not significantly different from those measured in the CON group.

4. Discussion

The present study used a mouse model of acute DIC-induced hepato-renal toxicity to assess and compare the ability of the herbal products LO, CIT and SILY (the latter used as a term of comparison) to provide effective hepato-renal protection.

4.1. Experimental model of acute DIC-induced hepato-renal toxicity

The protocol used in our study to induce acute DIC toxicity in mice (200 mg/kg PO) was similar to that adopted by Huo et al. [15] and Fattori et al. [16], and performed equally well at producing kidney injury. The successful induction of nephrotoxicity was revealed by increased serum levels of the renal functional markers urea and creatinine which were associated with histopathological alterations in the tissue.

In the aforementioned study by Huo et al. [15], the effect of DIC administration on the liver of the intoxicated mice was not examined. In contrast, the study by Fattori et al. [16] reported that DIC did not induce liver damage. However, in our research, DIC administration successfully co-induced liver injury, as evidenced by changes in serum biochemical markers of liver function, increased serum levels of liver enzyme activities, and altered liver tissue histology. The reason for this discrepancy is difficult to explain, especially considering that the same mouse strain (Swiss mice) and a similar DIC administration protocol were used in both studies. At any rate, our successful outcome in terms of DIC-induced hepatotoxicity seems less surprising than the failure reported by Fattori et al. [16], given the presence of several studies in the published literature that - although using different administration protocols - actually succeeded in observing hepatotoxic effects after experimental administration of DIC to either mice or rats [3,10,14,21,42,44].

Our findings of increased MDA levels and decreased SOD and CAT levels in both kidney and liver tissues of DIC-intoxicated mice provide further evidence in support of a role for oxidative stress in DIC-induced

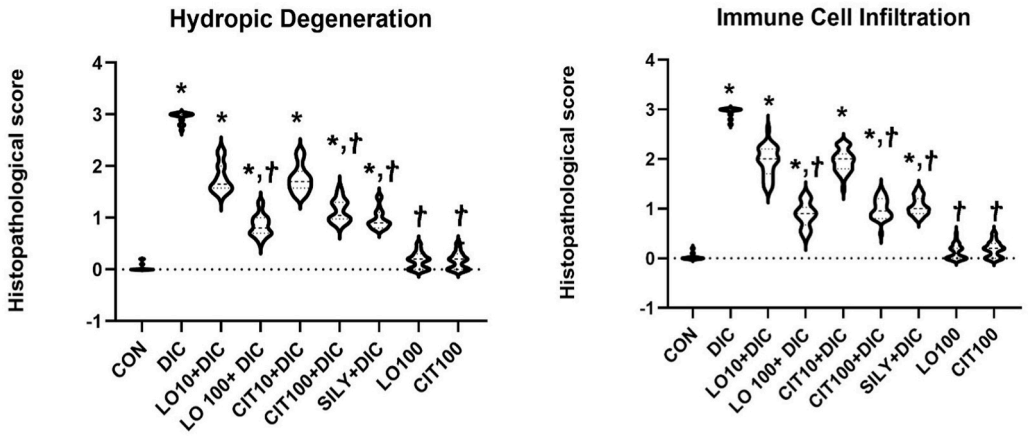

Fig. 5. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on diclofenac-induced histopathological lesions in kidney tissue of diclofenac-intoxicated mice. Data (n = 6 in each group) are presented as quartiles, showing the minimum value, first quartile, median, third quartile, and maximum value. Kruskal-Wallis test followed by Dunn's multiple comparison test were used for data analysis. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively. *P $<$ 0.05 *Vs* CON, † P $<$ 0.05 *Vs* DIC.

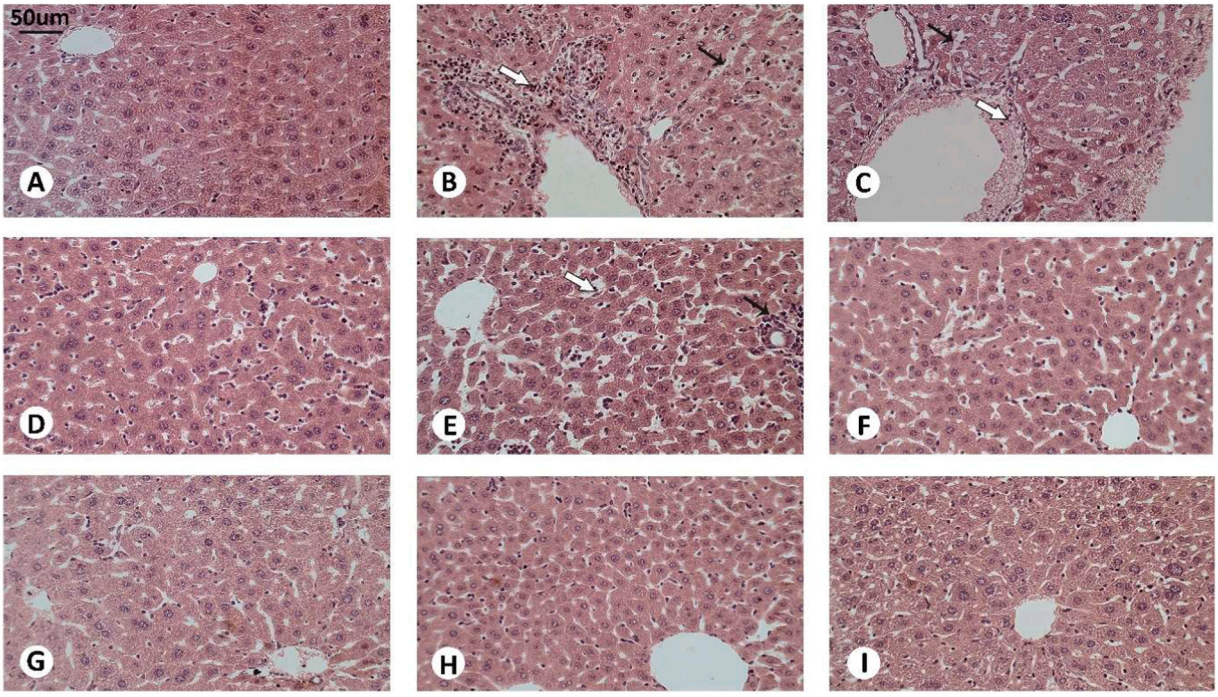

Fig. 6. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on liver histomorphology in diclofenac-intoxicated mice. Representative pictures from n = 6 mice per group. **A-control group** (CON) shows the normal liver tissue structure. **B- Diclofenac** (DIC) treated group displays severe immune cell infiltration (white arrow) alongside fatty degeneration of hepatocytes (black arrow). **C- Lemongrass Oil (Low dose) and Diclofenac** (LO10+DIC) treated group exhibits severe inflammatory cell infiltration of immune cells (white arrow) and remarkable fatty degeneration (black arrow). **D- Lemongrass Oil (High dose) and Diclofenac** (LO100+DIC) treated group presents mild inflammation and fatty degeneration in hepatocytes. **E- Citral (Low dose) and Diclofenac** (CIT10+DIC) treated group shows severe immune cell infiltration (white arrow) and hepatocyte degeneration (black arrow). **F- Citral (High dose) and Diclofenac** (CIT100+DIC) treated group reveals slight inflammatory cell infiltration along with mild fatty degeneration of hepatocytes (black arrow). **G- Silymarine and Diclofenac** (SILY+DIC) treated group indicates mild infiltrative and degenerative alterations. **H- Lemongrass Oil (High Dose)** (LO100) and **I- Citral (High Dose)** (CIT100) treated groups exhibit nearly normal structure of liver tissue. (H&E staining, ×400, scale bar represents 5 μm).

kidney and liver damage [4,10,11,16,19,20]. In all likelihood, the cellular damage produced by the condition of oxidative stress played a significant causative role in the loss of functional and structural integrity of kidneys and liver that we detected in the intoxicated mice [14,19].

4.2. Protective effects of pre-treatment with SILY on DIC-induced hepato-renal injury

In our study, pre-treatment with the reference herbal product SILY (administered i.p. at 50 mg/kg) demonstrated protective effects against DIC-induced nephro- and hepato-toxicity, maintaining most of the

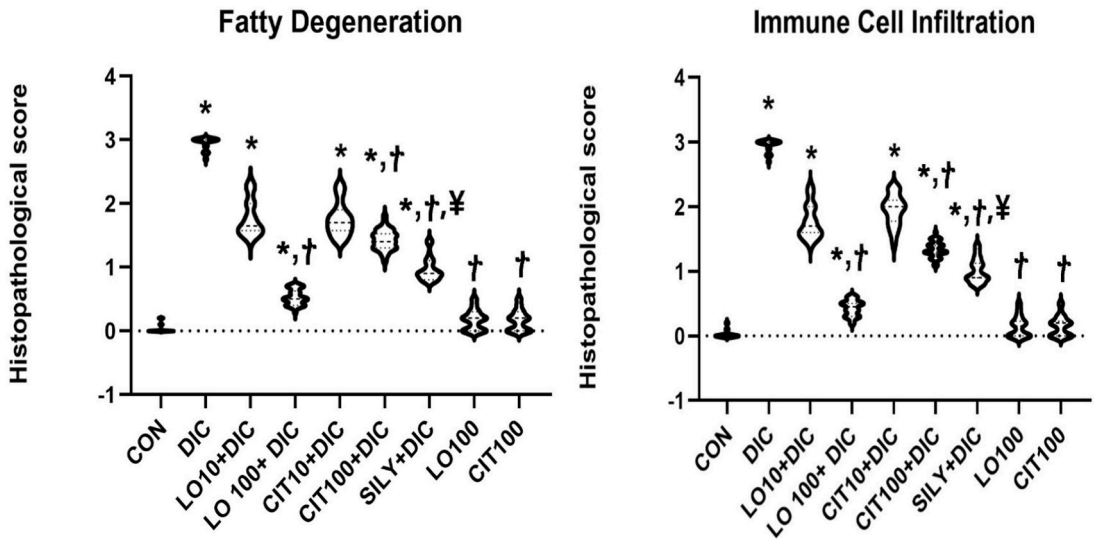

Fig. 7. Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on diclofenac-induced histopathological lesions in liver tissue of diclofenac-intoxicated mice. Data (n = 6 in each group) are presented as quartiles, showing the minimum value, first quartile, median, third quartile, and maximum value. Kruskal-Wallis test followed by Dunn's multiple comparison test were used for data analysis. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively. *P < 0.05 *Vs* CON, †P < 0.05 *Vs* DIC, ¥P < 0.05 Vs LO100 + DIC.

Table 2
Effect of pre-treatment with lemongrass (*Cymbopogon citratus*) essential oil, its main component citral, and silymarin on superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the liver and kidney tissues of diclofenac-intoxicated mice.

Parameters Groups	Kidney			Liver		
	SOD (Unit/mg protein)	CAT (Unit/mg protein)	MDA (nMol/g)	SOD (Unit/mg protein)	CAT (Unit/mg protein)	MDA (nMol/g)
CON	275.00±10.74	127.83±7.24	9.72±0.68	281.33±7.16	171.83±8.69	11.75±1.28
DIC	124.08±7.73[a]	57.40±11.09[a]	18.51±1.50[a]	136.50±7.59[a]	76.09±13.87[a]	21.42±2.67[a]
LO10+DIC	162.33±12.66[a]	67.72±2.74[a]	15.25±1.49[a]	165.33±8.42[a,b]	105.97±4.84[a,c]	18.86±0.92[a]
LO100+DIC	212.83±3.73[a,b]	110.73±4.21[b]	10.55±0.31[b]	208.16±6.42[a,b]	152.31±2.82[b]	13.79±0.49[b]
CIT10+DIC	147.83±5.36[a]	58.13±5.94[a]	17.26±1.78[a]	155.16±2.31[a]	105.16±12.46[a,c]	20.63±0.98[a]
CIT100+DIC	170.50±7.96[a]	86.73±6.50[a]	15.08±1.63[a]	179.50±4.01[a,b]	106.88±11.10[a,c]	17.05±1.38[b]
SILY+DIC	205.00±16.19[a,b]	118.96±1.63[b]	12.71±0.78[b]	197.66±7.78[a,b]	137.46±5.01[b]	14.85±1.38[b]
LO100	252.00±7.55[b]	131.56±7.13[b]	9.59±0.49[b]	253.00±5.40[b]	157.01±6.17[b]	12.16±0.83[b]
CIT100	264.00±21.01[b]	130.55±3.06[b]	10.63±0.62[b]	256.16±4.72[b]	168.20±7.16[b]	12.91±0.87[b]

Data are expressed as mean ± SEM (n=6 per group) and analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. CON: control; DIC: diclofenac 200 mg/kg p.o.; LO10+DIC: pre-treatment with lemongrass essential oil 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100+DIC: pre-treatment with lemongrass essential oil 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT10+DIC: pre-treatment with citral 10 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; CIT100+DIC: pre-treatment with citral 100 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; SILY+DIC: pre-treatment with silymarin 50 mg/kg i.p. followed by diclofenac 200 mg/kg p.o.; LO100 and CIT100: treatment with lemongrass essential oil and citral 100 mg/kg, respectively.

[a] P <0.01 *Vs* CON,

[b] P <0.05 *Vs* DIC,

[c] P <0.05 *Vs* LO100 + DIC.

parameters examined at normal levels. The mechanisms underlying this hepato-renal protective efficacy of SILY likely involved antioxidant activity, as suggested by the finding in the SILY-pretreated mice of tissue levels of MDA, SOD, and CAT that were not different (or only slightly different) from those measured in the control mice. Considering the several experimental studies that document the ability of SILY to counteract DIC toxicity [10,42–44], our results may appear somewhat expected, and primarily confirmatory of the suitability of SILY for being used as a reference hepato-renal protective agent in the preclinical evaluation of new candidate products. However, it is worth noting that all of the abovementioned studies were performed in rats. Therefore, to the best of our knowledge, the present report is the first to document the efficacy of SILY in counteracting DIC-induced hepato-renal toxicity in a murine model. This particular scientific outcome can be of a certain interest from the translational research perspective. It is worth noting that rats and mice are the most commonly used preclinical species for testing new potential protective agents against drug-induced toxicity and for elucidating the mechanism through which these agents exert their protective action [45,46]. In choosing between the two rodent species, experimenters working in drug discovery and development should consider, among others [47], that the translational validity of a rat and a mouse model may not be identical [48]. For instance, in the case of hepatotoxicity induced by acetaminophen, it has been clearly demonstrated that the mouse model is superior to the rat model, because it more closely resembles the mechanisms and severity of acetaminophen-induced hepatotoxicity occurring in humans [45,46]. Regarding DIC, there is evidence of species-specificity for its pattern of biotransformation and excretion [49–51]. However, with respect to the

mechanisms of hepato-renal toxicity of this drug, currently available information does not allow to establish whether differences of translational importance actually exist between the mouse and rat models. Future studies replicating these findings across rodent species under comparable experimental conditions will be valuable in addressing this question. Such research will, in turn, help reduce the risk of failure when advancing new hepato-renal protective agents against DIC toxicity to clinical trials [48].

4.3. Protective effects of pre-treatment with LO and CIT on DIC-induced hepato-renal injury

Apart from the new insights discussed above, the main innovation of the present study lies in demonstrating that pre-treatments with LO and CIT effectively protect mice from kidney and liver damage induced by DIC toxicity. Notably, similar studies using rats have not yet been conducted, highlighting the unique contribution of this research.

It is worth noting that the two herbal products, LO and CIT, did not demonstrate equal effectiveness in exerting their protective potential.

With particular regard to LO, it demonstrated the ability to counteract the detrimental effects of DIC on nearly all of the biochemical and histological parameters examined; only the DIC-induced reduction in albumin levels showed limited sensitivity to LO's protective activity. For most of the hepato-renal endpoints responsive to LO (with the exception of increased serum globulin levels and decreased liver SOD levels), the protective activity of LO was dose-dependent. The lower dose (10 mg/kg) proved less effective, or in some cases ineffective when compared to the higher dose (100 mg/kg), depending on the specific parameter. Notably, at the high dose (100 mg/kg), LO exerted a protective effect comparable to that of SILY for most of the variables investigated. The only exceptions were serum albumin levels, where LO100 performed worse than SILY, and serum urea and ALP levels, where LO100 performed even better than SILY. Finally, it seems high likely that the well-documented antioxidant properties of LO [28] have played an important role in its hepato-renal protective potential in the acute DIC-induced toxicity model used in the present study. This is supported by the observation that the mitigating (or even preventive) effects of LO100 on the alterations induced by DIC in serum biochemical markers of kidney and liver health, as well as in the histological appearance of both tissues, occurred alongside lower tissue levels of MDA and higher tissue levels of SOD and CAT compared to the DIC-intoxicated mice.

Similar to LO, CIT was effective in counteracting the negative effect of DIC on nearly all serum biochemical markers of kidney and liver health (with the exception of albumin levels), and in mitigating the DIC-induced alterations in the histological appearance of both tissues examined. In addition, for some of these CIT-responsive variables, the protective activity of CIT was found to be dose-related; the lower dose (10 mg/kg) was either less effective (as seen in serum ALP levels) or ineffective (in serum levels of ALT and total proteins, as well as in histopathological scores) in comparison with the higher dose (100 mg/kg). However, for the other CIT-responsive variables (serum urea, creatinine, and globulin levels), no dose-effect relationship could be observed for CIT, as both tested doses of this phytocompound demonstrated equal efficacy. The mitigation effect of CIT100 was comparable to that of LO100 for all serum biochemical parameters related to liver health (ALT, ALP, total protein, and globulin levels), as well as for the kidney histopathological scores. However, the protective influence of CIT100 was less effective than that of LO100 for the serum biochemical parameters related to kidney function (urea and creatinine levels) and liver histopathological scores. Interestingly, in parallel with these findings, CIT100, unlike LO100, showed minimal mitigating effects on the alterations induced by DIC toxicity in the oxidative status parameters of the kidney (as evidenced by increased MDA levels and decreased SOD and CAT levels). Moreover, regarding the DIC-induced alterations in hepatic oxidative status, CIT100 was as effective as LO100 in mitigating or preventing changes in SOD and MDA levels, but - unlike LO100 - it was almost unable to exert any mitigation on the DIC-induced decrease of liver CAT levels. Overall, these results suggest that under the conditions of the present study, pre-treatment with CIT100 provided less effective control of the DIC-induced hepato-renal oxidative damage compared to pre-treatment with LO100. This led to a less efficient preservation of kidney function and, correspondingly, a reduced maintenance of liver structural integrity.

A plausible explanation for the different protective potential of the two herbal products tested in our study is that LO is a mixture of several bioactive compounds. Although CIT is its main phytoconstituent, as our analysis confirmed consistently with previous reports [25,27,28], other minor components (including, but not limited to geranyl acetate, citronellal, citronellol) may probably exert synergistic interactions [28]. Consistent with our findings, CIT was reported to be ineffective compared to LO in promoting the healing of ethanol-induced gastric ulcers in mice [26].

Despite the differences in their protective efficacy, it is noteworthy that both LO and CIT demonstrated safety at the highest and most effective dose tested in the present study. This was evidenced by the finding that none of the investigated variables showed remarkable alterations in the mice receiving either LO100 or CIT100 without any DIC treatment. This finding may be of particular importance in the perspective of the safe integration of these herbal medicinal products into conventional medical practices [2].

Besides being of interest to human medicine, our findings concerning the efficacy of LO and CIT at safely preventing the hepato-renal toxicity of DIC may also be of interest to veterinary medicine. Indeed, DIC is registered for veterinary use in certain countries [52] and, in this view, veterinary patients are at risk of developing DIC-induced kidney and/or liver injury just like human patients [49]. In addition, accidental acute poisoning by DIC may occur especially in pets, as a consequence of ingestion of their owners' DIC-containing medications [53]. Last but not least, it is known that certain wild necrophagous bird species, such as vultures, can become intoxicated when feeding on the carcasses of DIC-treated animals, which likely contain residues of the drug [16,52,54].

In the light of these considerations, it should be acknowledged as a limitation of the present study that the protective potential of the tested herbal products against DIC-induced hepato-renal toxicity has been evaluated from a prophylactic point of view in a model of acute toxicity. Further studies testing the ability of LO and CIT to reverse an already established drug-induced damage (thereby acting as therapeutic agents) [13] and/or to prevent toxicity associated with long-term use of this anti-inflammatory drug are needed.

As a further potential limitation of this study, the parameters that were examined to assess the protective activity of the herbal products of interest against DIC-induced hepato-renal toxicity were relatively few, though all relevant and meaningful. For instance, inflammatory markers or other markers of liver and kidney function were not included. Therefore, some observations may have been missed, leading to a still partial characterization of the actual hepato-renal protective potential of SILY, LO and CIT in the context of this mouse model of acute DIC-induced toxicity. More extensive evaluations need to be carried out in future studies, along with deeper exploration of the mechanisms that - at a molecular level - account for this protective efficacy.

5. Conclusions

In summary, the findings of our study provide evidence that pre-treatment with LO, CIT, or SILY is able to mitigate DIC-induced kidney and liver injuries in mice, at least in part by reducing oxidative stress and related alterations in the tissues. The protective efficacy of LO, especially at the higher dose (100 mg/kg), was comparable to that of the reference product SILY but more pronounced than CIT, particularly concerning the ability to preserve liver structural integrity and kidney function.

On the whole, the present study adds to the existing literature that provides scientific support for the traditional use of lemongrass in the management of kidney and liver disorders, and identifies the administration of LO (or CIT) as a further promising approach to offer protection against hepato-renal toxicity of DIC (in addition to the variety of synthetic drugs and herbal products with antioxidant properties already examined). Given the rapidly growing lemongrass cultivation industry, with an estimated market value of 7 billion dollars, projected to double in the next five years [28], our findings contribute to making LO a promising green candidate with nephroprotective and hepatoprotective properties for further research and development.

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CRediT authorship contribution statement

Masoumeh Houshyar: Writing – original draft, Visualization, Resources, Formal analysis. **Mohaddeseh Abouhosseini Tabari:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Claudia Zizzadoro:** Writing – review & editing, Visualization, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Roberta Cardone:** Writing – review & editing, Visualization. **Giuseppe Crescenzo:** Writing – review & editing, Methodology, Funding acquisition. **Navideh Mirzakhani:** Visualization, Validation, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Atefeh Araghi:** Validation, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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