PROTECTIVE ACTIVITY OF LEMNA MINOR L. IN CHRONIC BLEOMYCIN-INDUCED LUNG INFLAMMATION

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Abstract: This research investigates the probable effects of induced chronic (28 days) lung inflammations by Bleomycin (BLM) and its oxidative-toxicity protection by the aquatic extract of Lemna minor L. (LME). Balb/c male mice were in every two days exposed to: (1) a controlled normal diet, (2) an LME treatment (120 mg/kg bwt, i.p.), (3) a BLM treatment (0.34 U/kg bwt, i.p.), and (4) an LME (120 mg/kg bwt, i.p.) administered two hours prior to the BLM. At the 30 experimental days of chronic BLM administration, the mice were sacrificed and fresh lung tissue was collected for biochemical determination and EPR analysis. The BLM treatment significantly increased the biochemical indices two-fold (SOD, CAT, MDA, TC) than controls. Furthermore, lung/alveolar cell experiments were performed to investigate the LME modulative and oxidative-protection effect. The results revealed that LME alone and in combination (LME + BLM) inhibited BLM expression by significantly reducing EPR-ascorbate ($p < 0.05$), ROS production ($p < 0.05$), and by enhancing enzymatic antioxidants. As a conclusion, our results indicated that chronic BLM toxicity and lung inflammation could be neutralized by long-term LME treatment. Therefore, LME + BLM prevented the detrimental impacts of BLM and have proved to have a potential therapeutic effect on the oxidative stress biomarkers, antioxidant enzymes and alleviation of lung inflammations.

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Introduction

Clinically, the classical anticancer agent Bleomycin (BLM) presents good results. However, it subsequently induces interstitial pulmonary toxicity and lung inflammation. According to Hara et al. (2020), the cytostatic-glycopeptide antibiotic BLM produced by *Streptomyces verticillus* was used in malignancies, testicular carcinoma, Hodgkin lymphoma, etc. Jureczko and Przysaś (2019) note that in vivo, BLM activated a complex with $\text{Fe}^{2+}/\text{Fe}^{3+}$ containing enzyme formations that generate reactive oxygen species ($\text{ROS}/\cdot\text{O}_2^-$, H_2O_2 , $\cdot\text{OH}$, $\text{NO}\cdot$), damaged cellular components, and lead to DNA destruction. Karamalakova et al. (2019) commented on the acceleration of instability in the cell membrane, lipid and protein peroxidation, and progression of lung inflammations, all after BLM administration. In investigations of Karamalakova et al. (2019), Rago et al. (2019), Hara et al. (2020) and Karamalakova et al. (2020), BLM has been used in experimental murine models as an aggressive and progressive lung inflammation activator and pulmonary fibrotic administrator. BLM-lung toxicity symptoms such as cough, thoracic pain, cyanosis, pleuritic pain and pleural rubbing are typical indicators according to Hosseini et al. (2018). Moreover, Hosseini et al. (2018), Karamalakova et al. (2019) and Karamalakova et al. (2020) specify that free radicals, oxidative stress and inflammatory cytokines (tumor necrosis factor (TNF), interleukin IL-1, 18, 22, 17a) are fibroblast effectors and inducers of BLM-lung inflammation. Due to poor anti-inflammatory therapies, there is greater interest in the co-relationship between BLM-induced lung inflammation and plant antioxidant fibrotic remodeling.

An aquatic, fast-growing, freshwater plant Lemna minor L. (LME, duckweed, Araceae family) is associated with high toxicological and pharmaceutical capacity by Gomes et al. (2017). The author illustrates the antioxidant abilities of L. minor that allow it to absorb ciprofloxacin-induces toxic effects and reduce hydrogen peroxide (H_2O_2) accumulation alongside the increased oxidative stress.

The LME antioxidant effect originating from the southeastern Bulgarian region is based on 32 biologically active constituents like phytosterols (52.8 mg/kg), hydrocarbons (23.1 mg/kg), aldehydes

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and ketones (20.2 mg/kg), protein (21.80 %), lipids (11.1 mg/kg), etc., as reported by Vladimirova & Georgiyants (2014) and Velichkova (2019). Traditionally, *L. minor* is used as an antiscorbutic, diuretic depurative, and is also effective during colds, according to Al-Snai (2019). In addition, Al-Snai (2019) retrospectively defined *L. minor*'s internal uses in the treatment of human diseases, such as for respiratory tract and tissue inflammations and autoimmune conditions; it works mainly as an anti-inflammatory antioxidant.

The present study was to investigate the potential and mitigative roles of *L. minor* water extract as an antioxidant in order to overcome BLM-induced lung inflammation by way of intracellular free-radical production and antioxidant enzymes in the experimental mice model.

Materials and methods

- **Chemicals:** The BLM solution (EP 9041-93-4/dose, 0.34 U/kg), Dimethyl sulfoxide (DMSO), N-tert-butyl- α -phenylnitrone (PBN), phosphate-buffered saline (PBS, pH 7.4), 5,5'-dithiobism (2-nitrobenzoic acid), Nembutal and commercial ELISA kits (Catalog No-CS0260, 2–80C) were purchased from Sigma Chemical Co, St. Louis, USA.
- **Plant Extract:** Air-dried LME was ground into a fine powder, dissolved in 2 L distilled water, and macerated with constant stirring for 48 hours. The lyophilized aqueous LME was preserved at 4°C.

Experimental Animals and Protocol: Twenty-four Balb/c male mice (weight 33–35 g; 8 weeks of age) were obtained from the Vivarium Medical Faculty, Stara Zagora, Bulgaria and fed with laboratory chow diet (20–22°C and 12h light/12h dark; 47% humidity). Food and tap water were provided *ad libitum*. The experimental purposes were strictly followed by REC/MF, Trakia University and the European directive 210/63/EU from September, 2010. In the study, 4 groups were used (n = 6 animals per group);

- (1) Control group treated with 300 μ L cold PBS, pH 7.4, every day,
- (2) LME i.p. treatment daily, early in the morning,
- (3) i.p. BLM/saline solution (250 μ L) administrated in every two days,
- (4) the animals received LME i.p. 2 hrs prior to the BLM. After the two-day recovery of each mouse, they were sacrificed by anesthesia (Nembutal 50 mg/kg i.p.). The physiological status, body weight and behavior were monitored daily for the first week, and then, weekly. The lung tissues were removed in ice, washed in cold PBS/4°C, and homogenized at 4000 rpm/4°C/10 min. 500 μ L of supernatant was analyzed for biochemical assays and ROS products.

- **Biochemical Assay:** The lung cellular activity of superoxide dismutase (SOD), catalase (CAT), malondialdehyde concentration (MDA) and total cholesterol (TC) were estimated using the methods mentioned by Sun et al. (1988), Aebi (1984), and Plaser et al. (1966) respectively. The total cholesterol (TC) was estimated using a commercially available diagnostic kit (AM-2035-KA, 2017/TERMO Sci.).
- **Ex vivo EPR Evaluation:** Methods according to Buettner & Jurkiewicz (1993) and Shi et al. (2005), were employed to evaluate the ascorbate (Asc^{•-}) and ROS production and the LME protective effect against BLM-induced oxidative changes and lung inflammations. The registered EPR spectra in lung samples were real-time-formations between spin-adducts and they generated Asc^{•-} and ROS (*arbitrary units*).

Statistical Analysis: Bruker Win-EPR, Sim-fonia Softwar, Statistica 8.0 and StaSoft were used for EPR spectral processing and statistical analysis. One-way ANOVA and Student's T-Test were applied to determine the significant differences among the data groups. The results were expressed as means $\pm$ standard error (SE), and the $p < 0.05$ value was considered statistically significant.

Results

LME affects mortality and body weight at chronic chemo-toxicity

We did not register any deaths or behavioral changes in the mice, treated with either LME alone, or controls, or the LME + BLM combination. 30 days after the BLM induction, only 9% of the animals died and the deaths occurred 21 days after the commencement. This is statistically and presumably due to respiratory failure (data not shown). The average weight gain of the BLM-administrated group, 17 days after the beginning, notably decreased ($p < 0.05$) as compared to the controls, LME and the LME

+ BLM groups. The major differences were observed in regards to daily food consumption, especially in the LME and the LME + BLM groups.

LME stimulates pulmonary endogenous antioxidant system at chronic chemo-toxicity

To investigate the LME effects on BLM-induced toxicity, we measured endogenous antioxidant levels (Table 1) in lung tissues for all the tested groups. The max. statistically significant increase in the SOD activity was observed in the lung tissues of mice treated with only LME (14.01 ± 0.33 vs 13.981 ± 0.5 , $p < 0.005$, T-Test) and in the two-hour pre-treatment with the L. minor in the LME + BLM group (14.98 ± 0.8 vs 13.981 ± 0.5 , $p < 0.005$, T-Test), quite in contrast to the controls group. BLM-induced residual/chronic toxicity was demonstrated by a significant reduction in pulmonary SOD levels (4.336 ± 0.7 vs 13.981 ± 0.5 , $p < 0.005$, T-Test).

The CAT activity is present in Table 1. The results of the CAT activity demonstrate statistically significant regulation in the lung cells for both the LME alone (13.11 ± 3.18 vs 11.84 ± 2.12 , $p < 0.05$, T-Test) and the LME + BLM combination (11.074 ± 4.06 vs 11.84 ± 2.12 , $p < 0.005$, T-Test) groups, relative to the controls group and the BLM administration group (11.074 ± 4.06 vs 3.78 ± 0.13 , $p < 0.003$, T-Test) (Table 1). The BLM treatment resulted in a significant decrease in the CAT levels (3.78 ± 0.13 vs 11.84 ± 2.12 , $p < 0.05$) vs the controls on day 28 from the beginning of the experiment.

Table 1: The LME biochemical effect after BLM administration. All values shown are mean ± SE. Values significantly differ: p < 0.05 *vs controls and **vs BLM				
Groups	SOD	CAT	MDA; TC	
Control	13.981 ± 0.5	11.84 ± 2.12	2.545 ± 0.07; 50.5 ± 6.07	
BLM	4.336 ± 0.7 (p < 0.005) vs controls	3.78 ± 0.13 (p < 0.05) vs controls	4.248 ± 0.2; 82.9 ± 5.2 (p < 0.05) vs controls	
LME	14.01 ± 0.33 (p < 0.003) vs BLM	13.11 ± 3.18 (p < 0.003) vs BLM	2.406 ± 0.1; 52.01 ± 3.91 (p < 0.003) vs BLM	
LME + BLM	14.98 ± 0.8 (p < 0.005) vs controls	11.074 ± 4.06 (p < 0.05) vs controls (p < 0.003) vs BLM	3.311 ± 0.12; 53.3 ± 7.12 (p < 0.05) vs controls (p < 0.003) vs BLM	
Source: Author				

LME regulates pulmonary lipid accumulation at chronic chemo-toxicity

To investigate the LME effects on alveolar lipid accumulation, we studied lipid peroxidation (MDA) and total cholesterol (TC) in lung homogenates (Table 1). However, we registered a more significant increase in MDA (4.248 ± 0.2 vs 2.545 ± 0.07 , $p < 0.05$, T-Test) and TC (82.9 ± 5.2 vs 50.5 ± 6.07 , $p < 0.05$, T-Test) alveolar levels in the BLM treated animals as compared to the controls. The LME alone ($p < 0.05$, T-Test) reduced the increased pulmonary lipid concentrations in MDA (2.406 ± 0.1 vs 4.248 ± 0.2 , $p < 0.04$, T-Test) and TC (52.01 ± 3.91 vs 82.9 ± 5.2 , $p < 0.05$, T-Test) as compared to BLM administration.

For this study, the BLM-lipid peroxidation reduction in the lung tissues was evaluated to confirm the LME antioxidant efficacy (containing phytosteroids, phenols and flavonoids). Fig. 1 shows the $\text{Asc}^\cdot$ radicals and ROS production in alveolar homogenate measured by real-time EPR in arbitrary units. Twenty-eight days after highly-toxic BLM administration, the results revealed a significant two-fold decrease in $\text{Asc}^\cdot$ (0.63 ± 0.02 vs 1.42 ± 0.06 , $p < 0.05$ a.u., T-Test) levels and a two-fold increase in ROS production (2.09 ± 0.11 vs 0.77 ± 0.02 , $p < 0.04$ a.u., T-Test) in alveolar cells, relative to untreated controls. In contrast, the $\text{Asc}^\cdot$ expression (1.11 ± 0.4 vs 1.42 ± 0.06 , $p < 0.03$ a.u., T-Test) and ROS production (1.09 ± 0.08 vs 0.77 ± 0.02 , $p < 0.05$ a.u., T-Test) were non-significantly noted to be close to the controls in the LME treated group. The LME pre-treatment led to a marked suppression in the ROS peroxidation activation by BLM administration in the LME + BLM (1.13 ± 0.08 vs 2.09 ± 0.11 , $p < 0.05$ a.u., T-Test) combination.

Discussion

For the first time, we clearly demonstrated that the use of LME effectively inhibited serious cases of lung inflammations after chronic BLM administration in the experimental mice model. In addition to this, we observed the protective ability of LME, both as an endogenous antioxidant system regulator

and as an oxidative level reducer in the lung cells, especially in the form of the LME + BLM combination. According to Duecker et al. (2018), the endogenous and exogenous ROS progression is essential for mitochondrial inflammations observed in viral infections or in toxic substance induction. Increased oxidative disorders in the redox balance are mentioned in lung inflammations as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis by Duecker et al. (2018).

LME affects mortality and body weight and stimulates pulmonary endogenous antioxidant systems at chronic chemo-toxicity

Notable positive variances were observed regarding mortality, daily food consumption and mice behavior in the groups treated with LME and the LME + BLM combination. LME-treated BLM mice presented no signs of respiratory distress, enhanced survival, or reduction in body weight loss. These results suggest that LME possessed protective activity against BLM-induced chronic chemo-toxicity and lethality. Parallel to our results, Ekperusi et al. (2019) describe in detail the use of duckweed as an effective feed source for animal production that increases weight because of a high amino acid concentration.

Lu et al. (2018) and Ekperusi et al. (2019) determine that high sensitivity, fast growth, and antioxidant enzymatic activities of *L. minor* made it easier to inhibit acute or chronic induced toxicity in an experimental model. In addition, *L. minor* water extract was found to be an effective antioxidant with anti-radical possibilities in different in vitro assays as observed by Gülçin et al. (2010). In our study, a statistically significant ten-fold increase in SOD activity was observed in the two-hour pre-treatment with LME and in the LME + BLM ($p < 0.005$, T-Test) combination. Corresponding to our results, Gülçin et al. (2010) emphasize that LME water extract acts as an antioxidant and regulates the superoxide anion radical levels and oxidative changes in in vitro models.

Catalase (CAT) is an endogenous enzyme that converts hydrogen peroxide (H_2O_2) to normal oxygen and neutralizes toxicity-induced oxidative disturbances and inflammatory responses accumulated in the lung cells. We hypothesize that BLM-induced chronic toxicity and the consequent acute inflammatory processes in alveolar cells are progressively modulated by the LME aqueous extract. Moreover, LME has the potential to convert H_2O_2 to normal oxygen as follows the CAT regulation and the oxidative stress change amelioration. Hence, this research suggests that LME effectively inhibits BLM-induced lung inflammations with the infiltration of those inflammations by way of antioxidising and modularizing effects of monoclonal antibodies by unknown aquatic macrophyte mechanisms, as suggests Gülçin et al. (2010) and Ekperusi et al. (2019).

LME regulates pulmonary lipid accumulation and oxidative stress at chronic chemo-toxicity

The ROS imbalance is associated with oxidative stress presence. Toxic oxidative processes contribute to lung inflammations, increased alveolar lipid peroxidation damages and alveolar epithelium malformations, as records Hara et al. (2020). Our results demonstrate that LME-mediated reduction of free radicals and lipid peroxidation in lung tissues may be due to the antioxidant inhibition of inflammatory cells through the endothelial membrane directed at the site of inflammation. In correspondence to our results, Gülçin et al. (2010) in their studies in in vitro confirm 100% lipid peroxidation inhibition by $45 \mu g mL^{-1}$ *L. minor* aqueous extract besides a strong release of antioxidants — α -tocopherol (84.6%) and Trolox (95.6%), of the same concentration.

The active antioxidant components of *L. minor* probably work by employing a tolerance strategy that decreases the chronically-induced toxicity of BLM administration. The anti-inflammation and ROS suppressing ability of LME potentially restore the intracellular functions of the membrane bilayer (by both — thylakoids and mitochondria) of an aquatic plant, as first observed in the radiation protective study by Xie et al. (2019). This verifies LME as a possible protector of interstitial inflammatory cell infiltration and intra-alveolar collagen deposition after BLM administration.

Therefore, the *L. minor* protective action prevents tissue lipid peroxidation, while maintaining the pulmonary membrane integrity and endogenous regulation. Similar results were also observed in the Lu et al. (2018) experimental research.

Many investigators such as Buettner & Jurkiewicz (1993), Shi et al. (2005), Karamalakova et al. (2019) and Karamalakova et al. (2020) studied antioxidant status and oxidative stress damages in in vitro/in vivo animal systems by examining the dehydroascorbate — $Asc\cdot$ reduction and ROS concentration in organ homogenates, using EPR spin-trapping technics. BLM induction leads to an

Figure 1: The LME effect of BLM treatment on Ascorbate Radicals and ROS Production in the lungs. All values shown are mean $\pm$ SE. Values significantly differ: $p < 0.05$ *vs controls and **vs BLM.

Asc $\cdot$ and ROS redox imbalance and these are associated with the imminent increased cellular oxidative stress, as per Lu et al. (2018). Our results demonstrated LME enhancement in Asc $\cdot$ radical concentration and ROS production /lipid peroxidation associated with the cellular redox balance and the suppressing of additional oxidative pulmonary membrane injuries. The reduced SOD/CAT and ascorbate/ROS ratios can function as signals for the complete regulation of the protective antioxidant mechanisms after LME pre-treatment. The present findings show that LME-dependent reduction in oxidative disorders caused by chronically administered BLM was associated with the homeostatic balance restoration and the activated antioxidant enzymes that affect lung inflammation, in agreement with Lu et al. (2018), Karamalakova et al. (2019) and Karamalakova et al. (2020). Interestingly, L. minor maintains a highly efficient ROS scavenging antioxidant defense that limits direct intracellular ROS damages. Moreover, L. minor compounds have the potential to scavenge the oxyl and peroxy radicals associated with lipid peroxidation after gamma radiation probably in a bleomycin-induced lung injury model, as mentioned by Xie (2019).

Furthermore, the antioxidant-exogenous pre-treatment with L. minor could stimulate endogenous enzymatic synthesis and enhance radical-scavenging, H₂O₂, $\cdot$ O₂⁻, $\cdot$ OH formations, besides directly reducing lung inflammation. The protective capabilities of L. minor can be applied to environmental monitoring, livestock production, poultry as human food, and against metals and cytostatic drugs that induce oxidative stress; this was demonstrated in the studies of Lu et al. (2018), Ekperusi et al. (2019) and Jureczko & Przysaś (2019).

Conclusion

To sum up, it was observed that the L. minor treatment resulted in the alleviation of BLM-induced lung inflammation due to the: (1) regulation of antioxidant enzyme activities along with a reduction in BLM administration and alveolar cell inflammation; (2) increased ascorbate and decreased ROS production owing to the restabilization of the redox homeostasis; and (3) modulation of total cholesterol and lipid peroxidation. Moreover, our studies show another beneficial effect of L. Minor; it acts as an antioxidant-signaling molecule involved in retarding the toxic cellular malformations and lung inflammation.

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