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Amelioration of bleomycin-induced pulmonary toxicity in murine models by a semiquinone glucoside derivative isolated from *Bacillus sp.* INM-1

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The mechanism of action of bleomycin (BLM) is related to the ability to cause DNA fragmentation and myelo-depressive and immunosuppressive activity. In the present study, pulmonary protective effects of a semiquinone glucoside derivative (SQGD), a bacterial metabolite isolated from *Bacillus sp. INM-1*, were evaluated in a model of BLM-induced oxidative toxicity in IRC/white male mice. Mice were divided into four groups, i.e., (1) untreated controls; (2) SQGD-treated (40 mg/kg b. wt. i.p., every two days) mice; (3) BLM (0.34 U/kg b.wt., i.p., every two days); and (4) SQGD (40 mg/kg b.wt., i.p.) administered 2 h prior to BLM-administration (0.34 U/kg b.wt., i.p.). Till the end of the 28 experimental days of BLM administration, no mortality rate (-1) was observed in mice. Reactive oxygen species (ROS) and lung tissue homogenates of the treated animals were subjected to ascorbate radical estimation and ROS production and the level of three oxidative stress markers, i.e., the GSH ratio, and antioxidative capability of SOD and CAT were estimated. Treatment with SQGD protected BLM-induced pulmonary injury by suppressing oxidative stress with significant ($p< 0.05$) reduction of ascorbate radicals and ROS products in the lungs, and enhancement of the GSH ratio and SOD and CAT. Reduction in oxidative disorders was observed in healthy mice which were treated with SQGD only, compared to controls. Thus, it can be concluded that SQGD treatment alone and in combination SQGD+BLM neutralized BLM-induced pulmonary toxicity associated with oxidative damage caused by the anticancer drug not only by reducing lipid peroxidation but also by improving antioxidant status of lungs. Therefore, SQGD has a potential therapeutic effect as a strong radical-scavenger in the prevention and alleviation of pulmonary fibroses.

Key words: SQGD, BLM, lipid-peroxidation, pulmonary protection

INTRODUCTION

Bleomycin (a cytostatic-glycopeptidyl antibiotic) is a small peptide isolated from *Streptomyces verticillatus* in 1966 by Umezawa *et al.* and its mechanism of action is breaking the DNA-double binding region by the free-radical overproduction, which is oxygen and iron subordinate [1]. It has been shown to be an effective antitumor agent used for treatment of a variety of malignancies, predominantly germ cell tumors, testicular carcinoma and Hodgkin lymphoma [2, 3]. The drug caused partial marrow suppression, but pulmonary toxicity (PT) was a major adverse effect, because BLM damaged lung cells by inducing lipid peroxidation [4]. The serious limitation of BLM therapy is deposition in the pulmonary cells, activation of the anti-inflammatory cytokines IL-18 and IL-1 beta that result in BLM-induced lung injuries and idiopathic pulmonary fibrosis (called also fibrosing alveolitis) (IPF) [2, 3, 5]. Dempsey *et al.* specified IPF as a progressive disease characterized by lung alveoli (fibrotic) changes in the pulmonary structure that lead to decreased gas exchange and pulmonary cramps [6]. Also, Giri *et al.* [7] found that intratracheal BLM infusion in rodents gave a reliable and versatile therapeutic potential for the study of BIPF in animals and humans. Recently it has been reported that BLM induction in mice results in an explosive pulmonary inflammatory response in the alveolar wall, characterized by leukocyte infiltration, fibroblast proliferation and increased content of collagen and other matrix components [8, 9, 10]. In addition, the BLM-generated ROS in lung tissues were another factor partially responsible for induced cytotoxicity and carcinogenesis [11].

Given the mechanism of action of BLM and anti-inflammatory treatments, the use of antioxidant therapy may be a potential therapeutic strategy for clinical applications. Semiquinone glucoside derivative (SQGD) (deposited in NCBI gene bank with accession number EU 240544.1), isolated

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from a radio-resistant bacterium *Bacillus sp. INM-1* [12, 13], was used as anti-inflammatory and BLM injury protective agent in the present study. SQGD has already been characterized and evaluated for its antioxidant activity, immune-stimulatory properties and protective activities against gamma radiation-induced damages [12-17] and modulate immune-stimulatory cytokines [16, 17]. The present research was focused on the amelioration of the SQGD antioxidant potential to overcome BLM-induced pulmonary toxicity in terms of antioxidant enzymes (Glutathione (GSH); superoxide dismutase (SOD); catalase (CAT)) and intracellular ascorbate ($Asc^{\cdot}$) radicals and ROS production in lungs in male RCS/b mice.

EXPERIMENTAL

SQGD characterization

SQGD characterization (strong, single symmetrical signal, g= 2.0056 ± 0.0002 registered in powder) was carried out using electron paramagnetic resonance (EPR) spectroscopy [18] and other extraction procedures described previously [12, 13]. The type strain *Bacillus sp. INM-1, MTCC No. 1026, IBG-21* was deposited at the Institute of Microbial Technology, Chandigarh and INMAS, Delhi, India as reference.

Materials

The BLM solution (($C_{55}H_{84}N_{17}O_{21}S_3$ sulfate) EP 9041-93-4; dose 0.069U/ml); EDTA-containers (5 cm^3 Monovette, Germany); phosphate-buffered saline (PBS, pH 7.4); reduced GSH-ELISA commercial kit (Catalog No- CS0260, 2–8^{0}C); 5,5'-dithiobis (2-nitrobenzoic acid); nembutal; spin-trap dimethyl sulfoxide (DMSO) D4540-500 ml and N-*tert*-butyl-alpha-phenylnitrone (PBN) were purchased from Bristol-Myers Squibb Co., and Sigma Aldrich Company.

Instruments

The spectrophotometric measurements were performed on the UV–VIS spectrophotometer-400 (320 nm-750 nm), TERMO Sci., RS232C, Stratagene, USA. For the ELISA kit was used Urit-660 A spectrophotometer, Germany. The direct and spin-trapping EPR analyzes were carried out in triplicate on an EMX^{micro} spectrometer (Bruker, IFC-11007) Germany.

Animals used

Yung 24 male RCS/b mice (n=6; average weight 30 g; 8 weeks of age, specific pathogen-free, second line) were obtained from the Vivarium, Medical Faculty, Stara Zagora, Bulgaria and placed in polypropylene cages. Animals were fed with laboratory chow diet (standard conditions 20-22^{0}C and 12 h light/12 h dark; humidity of 40–60%). Food and tap water were provided *ad libitum*. The regulations and rules of the Research Ethics Commission of the Medical Faculty, Trakia University and the European directive 210/63/EU from 22.09.2010 were strictly followed during experiments.

Animal model of BLM-induced lung toxicity, SQGD administration and treatment protocol

Bleomycin-induced lung toxicity and co-treatment with SQGD: the male mice were once i.p. injected early in the morning with a BLM solution in cold saline of 250 μl (0.24 U/kg b.w.) continued on schedule from the 1st to the 28th day of the start of the experiment, into the lower part of the stomach by needle number 1, while breathing evenly. Pre-treatment with SQGD was given with i.p. injections at a concentration of 40 mg/ml (0.131 mg/kg bw) for 28 days, daily, in the early morning 2 h prior to BLM-injection, from the 1st to the 28th day.

Each of the mice was allowed to recover for 2 days under normal laboratory conditions, the body weight being measured every day during the first week and then weekly until they were killed on day 29 with lethal i.p. injection of nembutal (50 mg/kg). The toxicological symptoms, physiological status, and behavior (after 24 h) of the IRC/b mice were monitored daily. The freshly isolated lung tissue (un-extravasation with cold 0.9% saline) collected on ice were homogenized and after addition of solvents the samples were centrifuged at 4000 rpm for 10 min at 4°C and 300 μl of supernatant was prepared for biochemical analysis. The fresh blood (2 cm^3) was collected directly from the heart in cold EDTA-containers and centrifuged at 4000 rpm for 10 min at 4°C, and 200 μl of plasma from each group was placed at - 4^{0}C until used.

Experimental groups: The study was performed with 4 groups, with 6 animals per group: (1) control mice treated with 300 μL of cold PBS, pH=7.4; (2) animals that received SQGD before eating; (3) animals that received BLM before eating; (4) animals that received SQGD i.p. 2 h prior to BLM.

Superoxide dismutase assay

The activity of plasmatic and lung cellular SOD was assayed using Sun *et al*., 1988 method [19]. The absorbance was recorded at 420 nm for 3 min with 30 s intervals and calculated with the help of kinetics of the reaction mixture.

Catalase assay

CAT activity in the plasma and lung homogenate was assessed using Aebi method [20]. The absorbance was recorded at 240 nm for 1 min at 15 s intervals in both blank and test samples.

Glutathione assay

GSH levels in plasma and lung cells were estimated using the method of Akerboom and Sies, 1981 [21]. Reduced GSH was assessed by a continuous reduction of DTNB expressed as nanomoles of GSH per milliliter of protein. Blank without blood/ tissue homogenate was prepared similarly and the absorbance was recorded at 412 nm.

In vivo ascorbate levels

The method used according to Buettner & Jurkiewicz [22] was to evaluate the ascorbate levels ($Asc^{\bullet}$) and its protection against BLM-induced toxicity by SQGD pre-treatment. In brief, 200 mg of lung samples and 100 µl of plasma were homogenized in cold DMSO (10% w/v) and centrifuged at 4000×g for 10 min at 4°C. Supernatants were transferred into Eppendorf tubes, analyzed and EPR spectrum was registered in the lungs and blood plasma (*arbitrary units*). The spin-adduct formed between DMSO and generated $Asc^{\bullet}$ radicals was recorded in real time.

In vivo evaluation of ROS production

Lung tissue homogenates (100 mg) and 100 µl of plasma were homogenized with 900 µl of 50 mM spin-trap PBN dissolved in DMSO using ultrasound sonicator at one cycle for 1 min. After 5 min of incubation in cold, the suspension was centrifuged at 4000 rpm for 10 min at 4ºC and the EPR spectrum was registered (*arbitrary units*). The formation of ROS production in the supernatants was estimated as described earlier [23] with some modifications [18, 24].

Statistical analysis

The data obtained were processed by the statistical program Statistica Version 6.1 (StaSoft, Inc., USA) and presented as mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA and Student t-test to determine significant differences among data groups. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

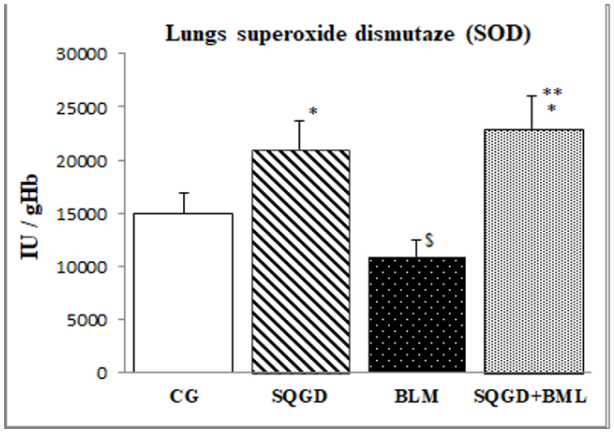
In this study, we demonstrated that i.p. SQGD inhibits effectively significant pulmonary changes following BLM-administration in mice. We also showed that SQGD regulated the antioxidant defense system and significantly decreased oxidative stress changes in lungs in combination SQGD + BLM- injured animals compared to BLM-administered alone. Furthermore, similarly un-limited intraperitoneal bioavailability of natural antioxidants parallels with previous findings in animals [25]. In his investigation Chandler, 1990 [26] reported that the ROS generated in the lung cells after BLM administration were probably responsible for its cytotoxic changes after cancer treatment. Therefore, the protective effects of bacterial metabolite on mortality and chronic inflammatory response were not surprising given the antioxidant ability to protect different tissues and organs of ROS adverse effects [12-18, 27]. In our study in the group treated with saline, and in the SQGD alone and SQGD + BML treated groups no deaths were recorded. 28 days of BLM-induction statistically insignificant, only 16% of the mice died, and death occurred 19 days after the start of the experiment possibly due to respiratory failure.

SQGD alone provided protection against 5-fluorouracil-induced immunosuppression and recovered the spleen, bone marrow and haematopoiesis [27]. In addition, SQGD has been shown to stimulate antioxidant production against radiation-induced ROS by enhancing antioxidant enzymes, and also provides increased antioxidant status at renal [17] and small intestine systems [15]. Significant increases in endogenous SOD activity were observed in lungs of mice treated with SQGD alone and in pretreatment with the antioxidant in the SQGD+BML group, compared to untreated controls ($p< 0.004$) and BML-administration ($p< 0.05$) at 28 days (Fig. 1a).

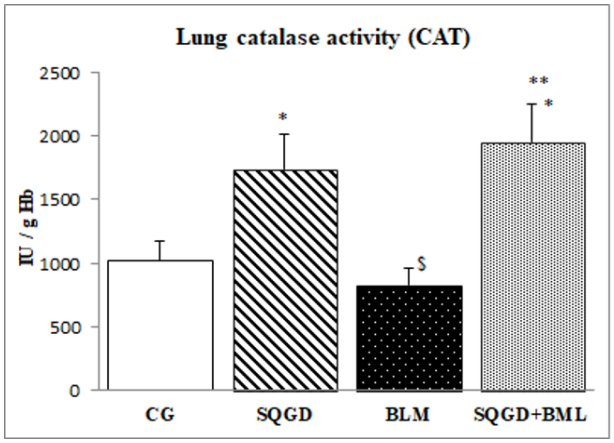
Maximal increase (23000.18 ± 408.52 UI/g of Hb) in SOD enzyme was observed at 28 days in lungs in the SQGD+BML group. The results demonstrated that SQGD significantly regulates SOD induction and alone does not induce oxidative stress changes by scavenging superoxide radicals. Moreover, SQGD accelerates SOD activity even after BLM pulmonary induction by its stable antioxidant properties [12, 17].

Hydrogen peroxide (H_2O_2) induces toxicity and increases oxidative disturbances in accumulation in the pulmonary cellular system. For the H_2O_2 decomposition to normal oxygen and water, the involvement of another endogenous enzyme - CAT is necessary. Results of the study demonstrated a slight increase in CAT activity (Fig. 1b) in lungs in mice treated with SQGD at 28 days of treatment (23.076±1.22 l mol/min/mg *vs.* 23.076±1.22), compared to controls. Though BLM-administered

animals showed significant decrease in CAT enzyme ($p<0.05$) as compared to controls (Fig. 1b), a significant increase in enzyme activity was evident in BLM mice pretreated by SQGD, as compared to BLM and control groups.



a)



b)

Figure 1. SQGD influence on SOD (a) and CAT activities against BLM-induced toxicity in lungs tissues. **1a)** *p< 0.003 SQGD treated *vs* CG; $p< 0.05 BLM-treatment vs control; **p< 0.05 SQGD+BLM group *vs* CG and BLM- group; **1b)** *p< 0.004 SQGD treated *vs* CG; $p< 0.05 BLM- treatment *vs* control; **p< 0.05 SQGD+BLM group *vs* CG and BLM- group.

We assume that the bacterial metabolite SQGD has the potential to increase the CAT activity levels in alveolar cells and to modulate the oxidative stress induced by BLM-induced toxicity. Earlier reports demonstrated that the SQGD intensity effect on pulmonary metabolic detoxifying enzymes (SOD and CAT activities) was due to the acceleration of the antioxidant protective mechanisms in a biological system [12, 17]. Concomitantly, the present study suggests that SQGD may inhibit BLM-induced pulmonary toxicity by immunosuppressive effects or by other unknown mechanism characteristic of the extremophilic action [17, 28, 29].

Meister, 1994 [30] draw attention to a tripeptide thiol GSH as an intracellular antioxidant and drug-toxicity protector against the oxidative changes caused by ROS. Significant increase in GSH levels in lungs ($p<0.05$) was observed at 28 days in the SQGD treated group and in SQGD+BLM, compared to controls (Fig. 2).

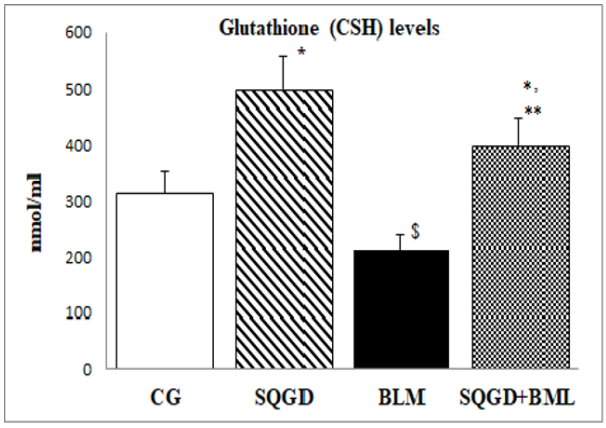


Figure 2. SQGD influence on CSH level against BLM-induced toxicity in lungs tissues. *p< 0.05 SQGD treated vs CG; $p< 0.05 BLM-treatment *vs* control; **p<0.05 SQGD+BLM group *vs* CG and BLM- group.

In contrary, a significant decrease ($p< 0.05$) in the GSH levels was observed in the BLM-administrated mice, compared to controls and SQGD groups. In accordance with our results, Mishra *et al.*, 2013 [17] commented that SQGD, as an excellent antioxidant increased organ GSH levels by free radical scavenging even at 10 Gy radiation exposure.

In addition to antioxidant status, the GSH function in the reduction of dehydroascorbate to ascorbate in tissues (lung, liver, kidney, brain, eye) was examined by different researchers in *in vitro/ ex vivo* animal systems [30, 31]. Buettner and Moseley, 1993, using EPR spin trapping investigated the ascorbate radicals presence, and found that BLM becomes a redox-inactive form incapable to break the DNA strand [32]. The EPR results of the ascorbate ($Asc^{\cdot}$) radicals assessment are shown in Fig. 3.

Twenty-eight days after BLM challenge, the lungs (0.218±0.06 a.u. *vs* 0.323±0.008 a.u.; $p<0.005$) and plasma samples (0.1988±0.0016 a.u. *vs* 0.299±0.01 a.u.; $p<0.004$) of the IRC/b mice showed statistically significant lower levels in $Asc^{\cdot}$, than the same in controls. In contrary, an increase in $Asc^{\cdot}$ expression in both lungs and plasma in the SQGD ($p<0.004$) and SQGD + BLM groups ($p<0.005$), was registered compared to untreated

controls and BLM-group. This study demonstrated that SQGD protects the reaction cycle of dehydroascorbate to ascorbate in alveolar tissues and blood against the BLM cytotoxicity in the lung by suppressing additional oxidative stress. Present findings were in complete agreement with the protective effect of the antioxidant fraction of SQGD alone and in combination in organs/ cells and in blood samples against lomustine-induced cytotoxicity [33] or in the regulation of stress-mediated signaling by transcriptional factor NF-kb and provided a gain support to the present research [14, 15].

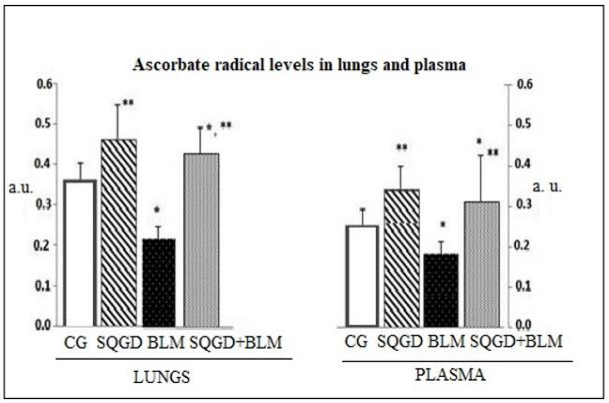


Figure 3. SQGD effect in BLM-treatment on ascorbate levels in the lungs and plasma. Mice pretreated with SQGD 2 h i.p. before BLM-treatment. All values shown are mean ± SE. Values significantly different: *p< 0.05 lungs and *p< 0.004 plasma BLM treated *vs* CG; $^{\$}$p< 0.05 SQGD *vs* BLM; **p< 0.05 SQGD+BLM group *vs* BLM-group.

Increased ROS generation, as well as changes in antioxidant/ prooxidant cells capabilities lead to tissue oxidative damage [34]. BLM administration stimulates the lipid macromolecules damages, resulting in excessive release of larger amounts of superoxide anion and nitric oxide from alveolar macrophages and oxidative burden imposed on pulmonary tissues [35]. The ameliorative SQGD activity on BLM-exposure in animals is shown in Fig. 4. In these analyses, the EPR method indicates a significant two-fold elevation in pulmonary ROS production/ lipid peroxidation in the BLM group, compared with controls and SQGD alone, in both lungs ($p < 0.05$) and blood ($p < 0.005$) samples. Moreover, better effects were observed in the SQGD + BLM groups, in both ($p < 0.004$). Our results are in accordance with Kara *et al*., 2007 other authors that have reported higher lipid peroxidation after BLM-exposure [36].

Earlier reports suggested that antioxidants possessed protective effect against pulmonary functional injuries, owing to direct antioxidant and free-radical scavenging mechanisms and ROS regulation [33-35].

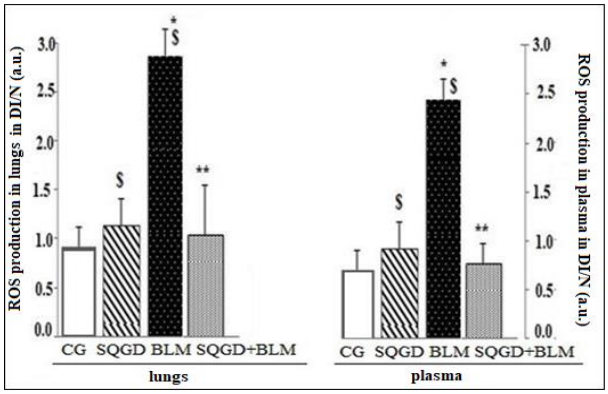


Figure 4. SQGD effect in BLM-treatment on ROS production in the lungs and plasma. Mice pretreated with SQGD 2 h i.p. before BLM-treatment. All values shown are mean ± SE. Values significantly different: *p< 0.05 lungs and *p< 0.004 plasma BLM treated *vs* CG; $^{\$}$p< 0.05 SQGD *vs* BLM; **p< 0.05 SQGD+BLM group *vs* BLM- group.

CONCLUSION

In conclusion, the study demonstrated that bacterial antioxidant SQGD significantly ameliorates the development of BLM-induced pulmonary toxicity and oxidative stress. The results suggest that SQGD pretreatment markedly suppresses the toxic exhibitions, by scavenging ascorbate radicals and ROS production and normalizing the enzyme protective system. However, further studies are needed to clarify the SQGD effect and its combinations in IPF therapy, applied to experimental animal models.

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REFERENCES

1. H. Umezawa, K. Maeda, Y. Okami, *Cancer*, **19** (1966).
2. K. Jules-Elysee, D. A. White, *Clin. Chest. Med*., **11**, 1 (1990).
3. J. M. O'Sullivan, R. A. Huddart, A. R. Norman, J. Nicholls, D. P. Dearnaley, A. Horwich, *Ann Oncol.*, **14**, 91 (2003).
4. J. Hay, S. Shahzeidi, G. Laurent, *Arch Toxicol*., **65**, 2 (1991).
5. T. Hoshino, M. Okamoto, Y. Sakazaki, S. Kato, H. A. Young, H. Aizawa, *Am. J. Respir. Cell. Mol. Biol.*, **41**, 6 (2009).
6. O. J. Dempsey, K. M. Kerr, L. Gomersall, H. Remmen, G. P. Currie, *QJM.*, **99** (2006).
7. S. N. Giri, R. Blaisdell, R. B. Rucker, Q. Wang, D. M. Hyde, *Environm Health Persp.*, **102**, 10 (1994).

8. F. Vatansever, W. C. M. A. de Melo, P. Avci, D. Vecchio, M. Sadasivam, A. Gupta, M. R. Hamblin, *FEMS Microbiol. Rev*., **37**, 6 (2013).
9. D. J. Schrier, R. G. Kunkel, S. H. Phan, *Am. Rev. Respir. Dis.*, **27 (**1983).
10. E. Cavarra, P. A. Martorana, B. Bartalesi, S. Fineschi, F. Gambelli, M. Lucattelli, L. Ortiz, G. Lungarella, *European Resp. J*., **17** (2001).
11. L. Nici, A. Santos-Moore, C. Kuhn, P. Calabresi, *Amer. Cancer Soc.*, **83**, 9 (2000).
12. R. Kumar, D. D. Bansal, D. D. Patel, S. Mishra, Y. Karamalakova, A. Zheleva, V.Gadjeva, R. K.Sharma, *Mol. Cell. Biochem*. **349**, 1 (2011).
13. R. Kumar, R. K. Sharma, D. D. Bansal, S. Mishra, L. Miteva, Z. Dobreva, V. Gadjeva, S. Stanilova. *Cell Immunol*., **267**, 1 (2011).
14. D. D. Patel, D. D. Bansal, S. Mishra, R. Arora, R. K. Sharma, S. K. Jain, R. Kumar, *Environ. Toxicol.* doi:10.1002/tox.21781 (2013).
15. D. D. Patel, D. D. Bansal, S. Mishra, R. Arora, A. Sharma, S. K. Jain, R. Kumar, *Mol. Cell Biochem*., **370**, 1-2 (2012).
16. S. Mishra, D. D. Bansal, P. K. Malhotra, D. Sudheer, K. Reddy, S. J. Vishawdeep, D. D. Patel, A. K. Gupta, P. K. Singh, S. Javed, R. Kumar. *Environ. Toxicol*., **29**, 12 (2014).
17. S. Mishra, D. S. Reddy, V. S. Jamwal, D. D. Bansal, D. D. Patel, P. Malhotra, A. K. Gupta, P. K. Singh, S. Jawed, R. Kumar, *Mol. Cell. Biochem.*, **379**, 1 (2013).
18. A. Zheleva, Y. Karamalakova, G. Nikolova, R. Kumar, R. Sharma, V. Gadjeva, *Biotechnol. & Biotechnol. Equipm*., **26**, 1 (2012).
19. Y. Sun, L.W. Oberley, Y. Li., *Clin Chem*., **34**, 3 (1988).
20. H. Aebi, *Meth. Enzymol*., **105** (1984).
21. T. P. Akerboom, H. Sies, *Methods Enzymol.,* **77** (1981).
22. G. R. Buettner, B. A. Jurkiewicz, *Free Radic. Bio Med*., **14**, 49 (1993).
23. H. H. Shi, Y. X. Sui, X. R. Wang, Luo Yand, L. L. Jia, *Comp. Biochem. Physiol. C Toxicol. Pharmacol*., **140**, 115 (2005).
24. V. G. Gadjeva, G. D. Nikolova, B. G. Grigorov, A. M. Zheleva, A. N. Tolekova, M. I. Vasileva, *Advances in Biol. Chem*., **4** (2014).
25. M. R. Smith, S. R. Gangireddy, V. R. Narala, C. M. Hogaboam, T. J. Standiford, P. J. Christensen, A. K. Kondapi, R. C. Reddy, *Am. J. Physiol/ Lung Cell Mol. Physiol.*, **298**, 5 (2010).
26. D. B. Chandler, *Clin. Chest Med.*, **11** (1990).
27. S. Mishra, P. Malhotra, A. K. Gupta, P. K. Singh, S. Javed, R. Kumar, *J. Immunotoxicol*., **12**, 1 (2015).
28. R. Kumar, D. D. Patel, D. D. Bansal, S. Mishra, A. Mohammed, R. Arora, A. Sharma, R. K. Sharma, R. P. Tripathi, in: Sustainable biotechnology, O. V. Singh, S. P. Harvey (eds.), Springer, Netherlands, 2010, p. 279.
29. S. H. Phan, J. C. Fantone, *Laboratory Investigation; J. Technical Methods and Pathol,* **50**, 5 (1984).
30. A. Meister, *Cancer Res.*, **54** (1994).
31. W. W. Wells, D. P. Xu, Y. Yang, P. A. Rocque, *J. Biol. Chem.*, **265** (1990).
32. G. R. Buettner, P. L. Moseley, *Free Rad. Res. Communic*., **19**, 1 (1993).
33. Y. Karamalakova, G. Nikolova, R. K. Sharma, R. Kumar, R. Arora, A. Zheleva, V. Gadjeva, *Defence Life Sci. J.*, **2**, 3 (2017).
34. K. Razavi-Azarkhiavi, M. Ali-Omrani, R. Solgi, P. Bagheri, M. Haji-Noormohammadi, N.Amani, M.-R. Sepand, *Pharm. Biol.*, **52**, 10 (2014).
35. A. Serrano-Mollar, D. Closa, N. Prats, S. Blesa, M. Martinez-Losa, J. Cortijo, J. M. Estrela, E. J. Morcillo, O. Bulbena, *British J. Pharmacol.*, **138** (2003).
36. H. Kara, F. Karatas, T. Tug, H. Canatan, A. Karaoglu, *Exp. Toxicol. Pathol*., **62**, 3 (2010).