


Bleomycin-Induced Damage in Rat Lung: Protective Effect of Grape Seed and Skin Extract

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Abstract

Introduction: Bleomycin is an effective chemotherapeutic agent with main side effects including lung fibrosis which limited its clinical use. The aim of this study is to evaluate the protective effect of grape seed and skin extract (GSSE) against bleomycin-induced oxidative damage and inflammation in rat lung, by assessing respiratory index (RI), oxidative and nitrosative stress (SOD and XO activity, NO), fibrotic mediators (hydroxyproline and collagen), apoptosis (cytochrome C and LDH), inflammation (IL-6, TNF- α and TGF- β 1), and histological disturbances.

Methods: Rats were pre-treated during three weeks with vehicle [ethanol 10% control] or GSSE (4 g/kg) and then administered with a single dose of bleo (15 mg/kg bw) at the 7th day.

Results: Bleo disturbed lung function through the accumulation of hydroxyproline and collagen, decreased SOD activity but increased XO activity as well as GSH and NO levels. Bleo also increased the pro-inflammatory cytokines IL-6, TNF- α , and TGF- β 1, and pro-apoptotic cytochrome C factor and induced severe histological alterations of lung parenchyma. Interestingly GSSE pre-treatment efficiently counteracted most of the bleo-induced lung tissue damages.

Conclusion: Data suggest that GSSE exerts anti-oxidant, anti-inflammatory, and anti-fibrosis properties that could find potential application in the protection against bleo-induced lung fibrosis.

Keywords

bleo, pneumotoxicity, GSSE, oxidative stress, inflammation

Introduction

Bleomycin (bleo) is an anthracycline antibiotic largely used as a chemotherapeutic agent in the treatment of a variety of human cancers such as lymphomas, head and neck cancer, and testicular and ovarian cancer.¹ The therapeutic options for bleo-induced lung toxicity are very limited and lung inflammation is a major underlying component of a wide variety of pulmonary fibro-proliferative disorders.

Pulmonary fibrosis constitutes a severe form of lung toxicity, characterized by altered cellular composition of the alveolar region with excessive deposition of collagen.² Although the mechanism of bleo-induced lung fibrosis is still unresolved,³ it is generally admitted that upon binding to DNA and in the presence of catalytic iron, bleo is able to

generate ROS and RNS that induce DNA damage, initiating the inflammatory and fibro proliferative change via a concerted action of various cytokines leading to collagen accumulation and finally cell death by both apoptotic and necrotic pathways. There are currently few treatments available for pulmonary fibrosis. Therefore, identification of new therapeutic approaches is of critical importance.

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GSSE is a complex polyphenolics mixture containing bioactive components such as proanthocyanidins, flavonoids, and stilbenes⁴ exhibiting potent pharmacological properties against various biotic or abiotic stressors, inducing multi-organ dysfunction such as cardiotoxicity, nephrotoxicity, hepatotoxicity, and neurotoxicity.⁵ GSSE is also protective against toxicity linked to the use of antineoplastic drugs as doxorubicin⁶ or cisplatin⁷ and exerted beneficial effects on bleo-induced lung fibrosis.⁸ Proanthocyanidins from grape seeds are endowed with anti-inflammatory properties on experimentally induced inflammation in rodent, and their mechanism of action is likely linked to free radical scavenging activity, metal chelation, antioxidative, and mitigation of pro-inflammatory processes.

The aim of our study is to evaluate the preventive role of GSSE (Li et al, 2001) against bleo-induced lung toxicity and fibrosis, thanks to its antioxidant and anti-inflammatory properties.

Material and Methods

Chemicals

Trichloroacetic acid (TCA) CAS 76-03-9, hydrogen peroxide (H₂O₂) CAS 7722-84-1, 2-methoxyphenol (guaiacol) CAS 90-05-1, bovine catalase CAS 9001-05-2 and 4-(1-Hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine) CAS 120003-76-1, and 2,4-dinitrophenyl hydrazine (DNPH) CAS 119-26-6 were purchased from Sigma-Aldrich Co (Germany).

Composition of GSSE

GSSE was processed from a grape cultivar (Carignan) of *Vitis vinifera* from northern Tunisia. Seeds and skin were dried and grounded separately with an electric mincer (FP3121 Moulinex) until a fine powder was obtained. Powder mixture containing grape seed (50%) and skin (50%) was dissolved in 10% ethanol at dark, vigorously vortexed for 15 min, and centrifuged at 10,000 g for 15 min at 4°C for debris elimination and supernatant containing soluble polyphenols was ready to use. Total phenolic content was determined by the Folin-Ciocalteu colorimetric method,⁹ flavonoids and condensed tannins according to Dewanto et al¹⁰ and Sun et al,¹¹ respectively. GSSE composition was established by HPLC-MS/MS analysis. Briefly, liquid chromatography was performed using a Perkin Elmer system series 200 equipped with a binary micro-pump. The analyses were carried out on a C18 column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm, particle size 5 μm). The mobile phase A was .1% formic acid in water and the mobile phase B was .1% formic acid in acetonitrile. Elution was performed at a flow rate of 1 mL min⁻¹ and an injection volume of 20 μL with LOD .1 mg.L⁻¹. Tandem mass spectrometry (MS/MS) was carried out using a 3200 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex Forster city USA) equipped with an electrospray ionization (ESI) interface. Data were acquired and processed with Analyst 1.5.1 software. The detector was set in the negative ion mode.

The ion trap mass spectrometer was operating in the m/z 50–1700 mass range.

Animals and Experimental Design

Twenty-four male Wistar rats (195–200 g) from Pasteur Institute (Tunis) were used in agreement with the ethics committee of Tunis University and with NIH guidelines. They were provided with food and water ad libitum and maintained in animal house at fixed temperature of 22 ± 2°C with a 12 h light–dark cycle.

Rats were randomly divided into four groups of six animals each that were daily treated by intraperitoneal (ip) route: Group 1: control: rats receiving 10% ethanol for 21 days; Group 2: GSSE: rats receiving 4 g/kg GSSE for 21 days; Group 3: bleo: rats receiving a single dose of bleo (15 mg/kg) at day 7; and Group 4: bleo + GSSE: rats treated both with GSSE and a single dose of bleo at day 7. At the end of the treatment, rats were anesthetized with urethane [40 mg/mL] and sacrificed and their blood was collected into heparinized tubes and processed for plasma biomarkers determination. Lungs were isolated, homogenized in PBS buffer pH 7.4 with an ultrathurax T25 homogenizator, and centrifuged (15 min at 10,000 g 4°C) and the resulting supernatant was used for lung lipidemia and oxidative stress analyses.

Function Measurements

A volumetric respirometer at constant pressure (Jeulin Everex, France) was used to determine respiratory index measured at 0, 7, and 13 days post-bleo treatment.¹²

Oxidative Stress Parameters

The superoxide dismutase (SOD) activity (E.C.1.15.1.1) was determined according to Misra and Fridovich (1972).¹³ Reaction mixture consists in 50 mM phosphate buffer Na₂CO₃/NaHCO₃ pH 10, 10 μL lung supernatant, and 10 μL bovine catalase (0.44 U/ml). The reaction was initiated by the addition of epinephrine (20 μL) and the rate of reaction was measured by following changes in absorbance at 480 nm at 30 s interval for 5 min, and activity was expressed as U/min/mg protein.

Xanthine oxidase (XO) activity (EC1.1.3.22) was determined by monitoring uric acid formation from xanthine according to De Souza et al.¹⁴ A .12 mL aliquot of xanthine solution (250 mM) was added to a test tube containing 10 μL lung homogenate and .54 mL potassium oxonate (1 mM) in 50 mM sodium phosphate buffer pH 7.4 that was previously warmed at 35°C for 15 min. The reaction was stopped after 30 min by addition of .1 mL .6 M HCl, and after centrifugation at 3000 g for 5 min, absorbance was measured at 295 nm, and XO activity was expressed as μmol uric acid formed/minute (U)/mg protein.

Lung GSH content was determined according to Ellman¹⁵ using GSH reductase. Briefly, the reaction of GSH with

Elman's reagent 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) gives rise to a product that can be quantified spectrophotometrically at 405 nm, and results are expressed as nmol GSH/g tissue.

Measurement of NO

NO level was measured by the method of Green et al (1982),¹⁶ using Griess reagent. .5 mL of lung homogenate was precipitated with 200 μ L 30% sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000xg with an equal volume of Griess reagent containing 1% sulfanilamide in 5% phosphoric acid/0.1% naphthalene ethylene-diamine dihydrochloride that was incubated for 10 min in the dark. Absorbance was read at 543 nm using sodium nitrite as standard and NO level was expressed as mol nitrite/mg tissue.

Lung Hydroxyproline and Collagen Content

The hydroxyproline content was measured according to the method described in the hydroxyproline assay kit (obtained from Sigma-Aldrich Co (Germany)). Briefly, 100 μ L lung homogenate were added to 100 μ L concentrated hydrochloric acid (12 M) into a microplate well and hydrolyzed at 120°C for 3 h. 50 μ L of supernatant from each sample and standard well was evaporated to dryness under vacuum to which was added 100 μ L of chloramine T/oxidation buffer mixture, incubated at room temperature for 5 min. After addition of 100 μ L DMAB reagent to each sample and standard well and incubation for 90 min at 60°C, absorbance was measured at 560 nm using a microplate reader. Lung collagen content was determined based on hydroxyproline assay and expressed using the following equation:

Lung collagen content = hydroxyproline \times 13.5¹⁷ and results are expressed as μ g collagen per mg wet lung tissue.

Pro-Inflammatory Cytokines Measurement

IL-6 pro-inflammatory cytokine in lung tissue supernatants was determined using quantikine IL-6 R immunoassay (R&D Systems, Minneapolis, MN). TNF- α and TGF- β 1 were determined using TNF- α ELISA kit (catalog number DY2279, R&D Systems, USA) and TGF- β 1 ELISA kit (catalog number MB100B, R&D Systems, Minneapolis, MN), respectively, according to manufacturer's protocols.

Cytochrome C and LDH Activity

The determination of cytochrome C, an important mediator of the apoptotic pathway, was carried out according to Margoliash and Schejter.¹⁸ LDH activity (E.C.1.1.1.1.), which is a relevant biomarker of bleo-induced lung fibrosis, was determined according to Bergmeyer¹⁹ using a commercial kit from Biomaghreb Tunisia.

Protein Determination

Total proteins were measured according to Lowry et al (1951) with BSA as standard.²⁰

Histopathology

Lung samples were collected and fixed in 4% (v/v) formalin, dehydrated with alcohol baths, and embedded into paraffin. Four micrometer sections were colored with hematoxylin-eosin (HE) and examined under light microscope.

Statistical Analysis

STATISTICA program (version 8.0) was used to develop the statistical analysis. All obtained results were described as mean \pm standard deviation. The observed variation was examined by one-way ANOVA ($P < .05$). First of all, we verified the prerequisites for the analysis of variance (normality of distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). Once relevant modifications were found, Tukey's test was applied to fix which values diverged significantly. (*) indicated significance vs control and (x) vs bleo.

Results

GSSE Composition

Polyphenolics levels found in seed and skin flour from Carignan cultivar are shown in Table 1. Total phenolics and flavonoids are slightly higher in seeds than skins, whereas condensed tannins are much represented in skins (3-fold). Among the 15 phenolic compounds identified within seeds and skins, only 2,5-dihydroxybenzoic, caffeic, and ferulic acids were much abundant in skins than seeds (Table 1).

Determination of Respiratory Index (RI)

Following i.p. treatment, bleo induced the significant decrease of RI at days 7 and 13 and GSSE failed to produce any significant protection of the altered lung function (Table 2).

Lung Oxidative and Nitrosative Stress

Bleo significantly decreased SOD enzyme activity by -27.66% (Figure 1A) and increased XO activity by $+79.64\%$ (Figure 1B). Bleo also increased GSH by $+58.76\%$ (Figure 1C) and NO level by $+277.6\%$ (Figure 1D) and GSSE restored efficiently these oxidative and nitrosative stress biomarkers close to control.

Lung Fibrotic Mediators

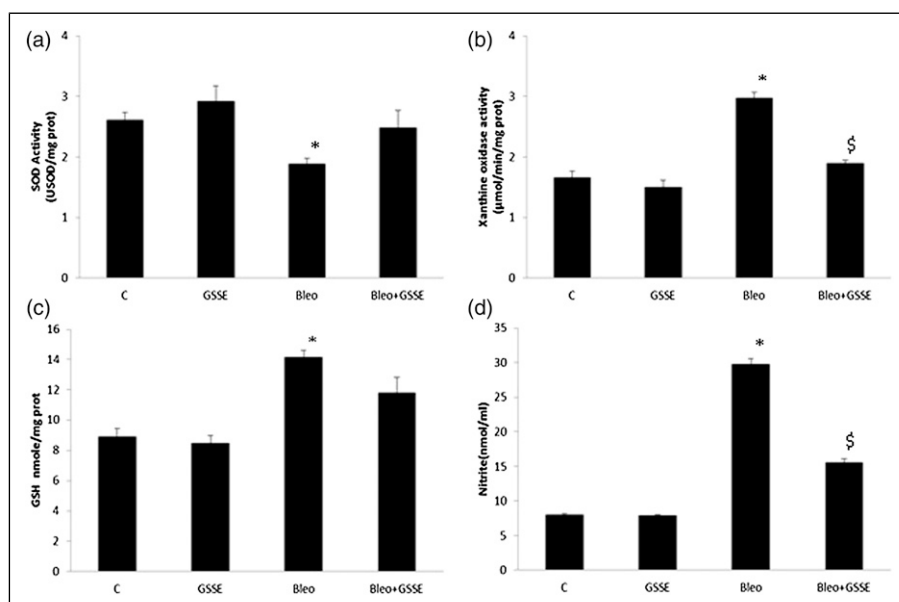
We further sought to determine the effect of bleo on lung hydroxyproline (Figure 2A) and collagen (Figure 2B). Bleo highly increased hydroxyproline by $+361.01\%$ as well as

Table 1. LC-MS/MS data of phenolic compounds found in GSSE.

Compounds	m/z Negative Mode [M-H] ⁻	MS ² Fragment	Relative Abundance (%)	
			Seed	Skin
Catechin	289	245/108.8/122.8	2.27 ± .07	.36 ± .04
Epicatechin	289	245/108.8/122.8	2.85 ± .18	.37 ± .01
Procyanidin dimer	577	289.3/407.4	.47 ± .03	ND
Procyanidin trimer	865	577	ND	ND
Quercetin	301	150.8/120.9	.64 ± .02	.47 ± .02
Resveratrol	227	184.6/143	.14 ± .01	ND
Rutin	609	300.1	1.51 ± .01	.50 ± .02
Vanillin	151	135.7/108.1	10.7 ± 0.2	7.75 ± .39
Gallic acid	169	124.7/78.9	50.3 ± 0.5	32.8 ± 1.2
p-Coumaric acid	163	119/93	ND	.38 ± .03
Rosmarinic acid	359	160.8/197.1	ND	.75 ± .02
2,5-dihydroxybenzoic acid	152	108.7/90.7	30.6 ± 0.5	52.0 ± 0.2
Caffeic acid	179	135	ND	2.8 ± 0.1
Chlorogenic acid	353	191	ND	.34 ± .02
Ferulic acid	193	134/89	.55 ± .02	1.46 ± .11

Table 2. Effect of bleo and GSSE on respiratory index.

Days	C	GSSE	Bleo	Bleo + GSSE
0	8.55 ± 0.33	8.53 ± 0.26	8.57 ± 0.23	8.55 ± 0.19
6	8.53 ± 0.28	8.72 ± 0.22	8.55 ± 0.33	8.67 ± 0.30
14 (7th day after bleo treatment)	8.60 ± 0.19	8.96 ± 0.32	6.38 ± 0.24	6.84 ± 0.19
20	8.71 ± 0.29	9.12 ± 0.35	4.43 ± 0.11	4.46 ± 0.021

**Figure 1.** Effect of GSSE on bleo-induced lung SOD [A] and XO [B] activities, GSH level [C], and NO metabolite [D]. Rats were daily treated with bleo with or without GSSE [4 g/kg bw]. Results are expressed as mean ± SEM. [n = 6] P < .05 was considered significant: * for bleo vs control, and § for bleo +GSSE vs bleo.

collagen by 360.63% which were significantly but only partially corrected by GSSE by -28%.

Lung Inflammation

We also assessed the bleo-induced lung inflammation and found that bleo increased the pro-inflammatory IL-6 cytokine reaching +322% (Figure 3A), TNF- α by +280% (Figure 3B), and TGF- β 1 by +313% (Figure 3C). GSSE efficiently corrected IL-6 till lower level than control, whereas GSSE corrected TNF- α and TGF- β 1 close to control level.

Lung Pro-Apoptotic Mediators

LDH level was significantly higher in bleo-exposed lung when compared to control (Figure 4A) which was abrogated till near control upon GSSE. Furthermore, bleo slightly increased cytochrome C activity (Figure 4B), and unexpectedly, GSSE which acted in concert with bleo, further increased the cytochrome C pro-apoptotic mediator activity.

Histopathology

We also investigated the effect of bleo on lung architecture and found that bleo induced the thickening of alveolar septa as well as an interstitial infiltration by inflammatory cells, and GSSE reduced the severity of bleo-induced lung damages (Figure 5).

Discussion

Our study mainly showed that bleo altered overall lung architecture and respiratory index and provoked an oxidative/nitrosative stress and chronic inflammation, and an increase in hydroxyproline and collagen deposition finally leading to fibrosis.^{21,22}

Oxidative stress is a prominent mechanism associated with the pathogenesis of lung fibrosis,²³ and in our case, bleo induced an oxidative stress status within the lung as assessed by depressed SOD activity and GSH level and increased XO activity.

Superoxide dismutase (SOD) catalyzes the conversion of superoxide free radical anions (O_2^-) to H_2O_2 ²⁴ and

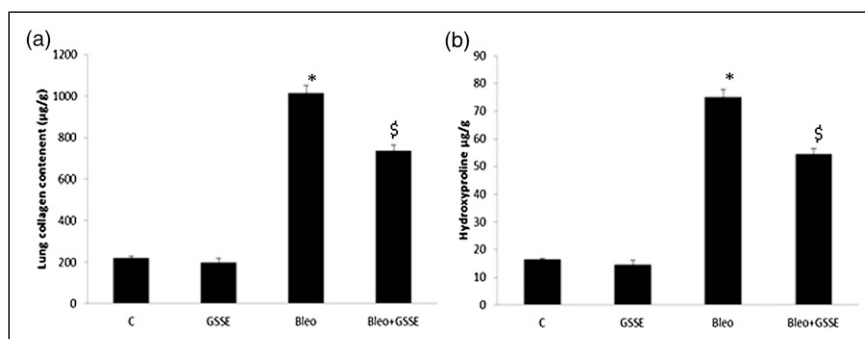


Figure 2. Effect of GSSE on bleo-induced lung fibrotic mediators as hydroxyproline [A] and collagen [B].

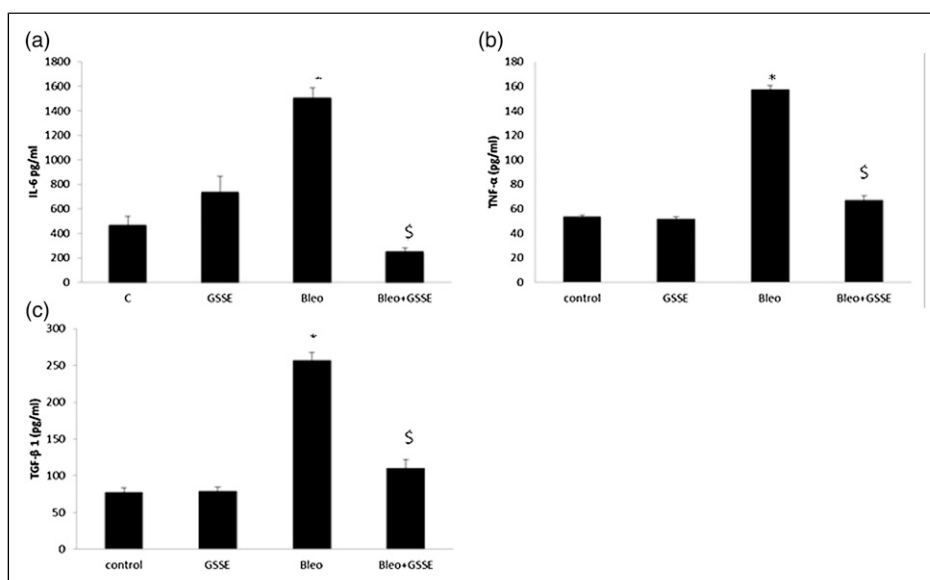


Figure 3. Effect of GSSE on bleo-induced lung inflammation: IL-6 [A], TNF- α [B], and TGF- β 1 [C].

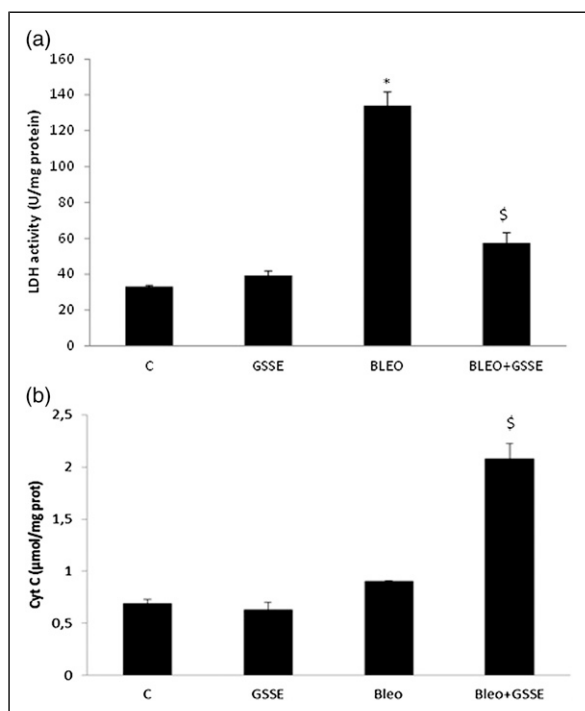


Figure 4. Effect of GSSE on bleo-induced lung pro-apoptotic mediators as LDH [A] and cytochrome C activity [B].

glutathione is an intracellular antioxidant that protected the lung from endogenous or exogenous lung toxicants.²⁵ Moreover, XO is a cytosolic metalloflavoprotein implicated in the pathogenesis of a wide spectrum of diseases, which is an important source of oxygen-free radicals and cell damage.

Allopurinol inhibition of XO to patients with chronic obstructive pulmonary disease was able to alleviate oxidative stress through lowering glutathione and²⁶ lipid oxidation.²⁷ Another study carried out on reactive nitrogen species (RNS) confirmed that anticancer drugs increased NO production and lung inflammation.²⁸ Furthermore, NO reacts with O_2^- to form peroxynitrite leading to acute lung injury.^{29,30}

Bleo highly induced cytokines such as TNF- α , TGF- β 1, and IL-6 which promote the progression to lung fibrosis as previously reported.³¹ Interestingly, IL-6 eliciting both pro-inflammatory and profibrotic effect was shown to be elevated in mice suffering from pulmonary fibrosis.^{32,33}

Besides inflammation, bleo induced cytotoxicity and apoptosis as indicated by increased LDH activity and release of cytochrome C to the cytosol.³⁴ Moreover, LDH is enhanced in rat lung upon intratracheal administration of bleo.³⁵

Lung neutrophils may also release pro-inflammatory and pro-apoptotic cytokines to injure adjacent cells, and our current study demonstrates high level of cytochrome C, a relevant marker for apoptosis that reflected the deleterious effect of bleo into the lung. Unexpectedly, our study showed that pretreatment with GSSE promoted cytochrome C activity, which is in disagreement with Song et al (2014) who

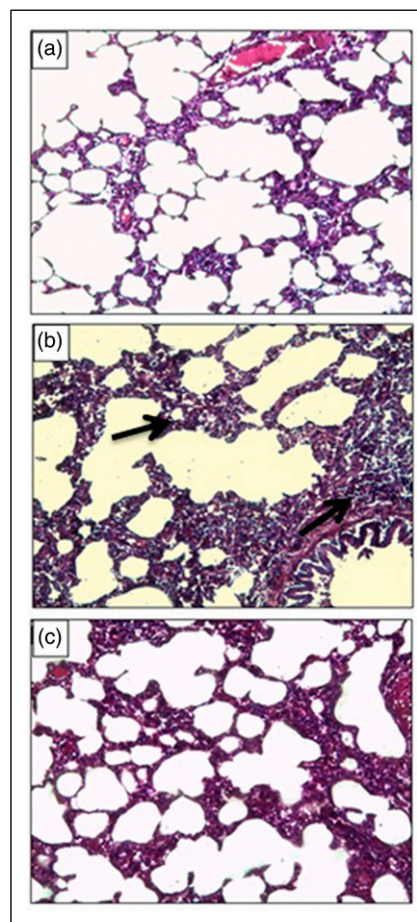


Figure 5. Rat lung histology: control [A], bleo [B], and bleo + GSSE [C].

demonstrated that astaxanthin, a powerful antioxidant, inhibits apoptosis of alveolar epithelial type II cells by suppressing the release of cytochrome C into the cytosol.³⁶ However, our data are in full agreement with the recently described synergistic effect of quercetin and apigenin with cisplatin leading to increased cytochrome C and apoptotic leukemia cell death.³⁷

In this context, recent studies have shown that the inhibition of Rho/Rho-kinase signaling decreases fibroblast expression of anti-apoptotic BCL-2 proteins and promotes IPF lung fibroblast apoptosis while decreasing lung fibrosis in vivo.³⁸ In our present case, bleo-treated animals were characterized by thickening of lung alveolar septa, inflammatory cell infiltration, and loss of alveolar-capillary membrane integrity, leading to breathing disturbance.

Undoubtedly, the most relevant result drawn from the current study is the protection afforded by high dosage GSSE against bleo-induced lung fibrosis. GSSE protection was demonstrated to occur through anti-inflammatory and anti-oxidative pathways that occurred in various experimental settings including prevention against lithium neurotoxicity³⁹ or arsenic cardiotoxicity.⁴⁰

Pretreatment with GSSE significantly reduced the level of hydroxyproline and collagen deposition as well as the severity of the histological signs, which are consistent with previous studies in the field.^{41,42} Other studies have shown that bleo increased lung hydroxyproline content which was reduced using various antioxidants as ellagic acid,²³ pyrrolidine dithiocarbamate,²⁴ and GSE.⁴³ Bleo-induced oxidative stress into the lung is associated with a higher risk of lung damage, and GSSE prevented lung depletion of endogenous antioxidant by acting either as ROS scavenger or enhancer of endogenous antioxidant.

Our results are in accordance with a previous study suggesting that ellagic acid pretreatment affected NO production into the lung treated with bleo or cisplatin.²³ Administration of curcumin to nicotine-treated rats decreased NO level into the lung,⁴⁴ as did GSSE to lithium-treated rats.³³ GSSE preventing bleo-induced lung IL-6, TNF- α , and TGF- β 1 highlighted its anti-inflammatory ability. As bleo-induced ROS and pro-inflammatory cytokines, GSSE might also decrease ROS production in lung macrophages. Moreover, as GSSE significantly decreased lung LDH, this result fully corroborated previous data obtained with gossypol, an effective polyphenol at both preventing and treating bleo-induced pulmonary fibrosis, emphasizing LDH as a potential therapeutic target for pulmonary fibrosis.⁴⁵

In conclusion, due to its multitargeted effect and safety, high dosage GSSE should be tested in large scale trials either as nutritional supplement into various food recipes in a preventive and holistic approach of lifestyle-related diseases or alternatively packaged into nanoformulation to be used as a more suitable galenic form into chemotherapeutic protocol or tested as an adjuvant in clinical trials.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. Einhorn LH. Curing metastatic testicular cancer. *Proc Natl Acad Sci USA*. 2002;99(7):4592-4595.
2. Kinnula VL, Raivio KO, Linnainmaa K, Ekman A, & Klockars M. Neutrophil and asbestos fiber-induced cytotoxicity in cultured human mesothelial and bronchial epithelial cells. *Free Radic Biol Med*. 1995;18(3):391-399.
3. Erdem A, Congur G. Impedimetric detection of in situ interaction between anti-cancer drug bleomycin and DNA. *Int J Biol Macromol*. 2013;61:295-301.
4. Khanal RC, Howard LR, Prior RL. Procyanidin content of grape seed and pomace, and total anthocyanin content of grape pomace as affected by extrusion processing. *J Food Sci*. 2009;74(6):H174-H182.
5. NassiriAsl M, Hosseinzadeh H. Review of the pharmacological effects of *Vitis vinifera*[Grape] and its bioactive compounds. *Phytother Res: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*. 2009;23(9):1197-1204.
6. Postescu ID, Chereches G, Tatomir C, Daicovicu D, Filip GA. Modulation of doxorubicin-induced oxidative stress by a grape [*Vitis vinifera* L.] seed extract in normal and tumor cells. *J Med Food*. 2012;15(7):639-645.
7. Yousef MI, Saad AA, El-Shennawy LK. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. *Food Chem Toxicol*. 2009;47(6):1176-1183.
8. Agackiran Y, Gul H, Gunay E, Akyurek N, Memis L, Gunay S, Ide T. The efficiency of proanthocyanidin in an experimental pulmonary fibrosis model: comparison with taurine. *Inflammation*. 2012;35(4):1402-1410.
9. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*. 1965;16(3):144-158.
10. Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J Agric Food Chem*. 2002;50(10):3010-3014.
11. Sun B, Ricardo-da-Silva JM, Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. *J Agric Food Chem*. 1998;46(10):4267-4274.
12. Scholander PF, Iversen O. New design of volumetric respirometer. *Scandinavian journal of clinical and laboratory investigation*. 1958;10(4):429-431.
13. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 1972;247(10):3170-3175.
14. De Souza MR, Paula CA, Resende MLP, Grabe-Guimarães A, Filho JDS, Saúde-Guimarães DA. Pharmacological basis for use of *Lychnophora trichocarpa* in gouty arthritis: anti-hyperuricemic and anti-inflammatory effects of its extract, fraction and constituents. *J Ethnopharmacol*. 2012;142:845-850.
15. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82:70-77.
16. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and (15N) nitrate in biological fluids. *Anal Biochem*. 1982;126(1):131-138.
17. Kliment CR, Englert JM, Crum LP, Oury TD. A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal. *Int J Clin Exp Pathol*. 2011;4(4):349.
18. Margoliash E, Schejter A. Cytochrome C. *Adv Protein Chem*. 1966;21:113-286.

19. Bergmeyer HU. New values for the molar extinction coefficients of NADH and NADPH for the use in routine laboratories [author's transl]. *Zeitschrift für klinische Chemie und klinische Biochemie*. 1975;13(11):507-508.
20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265-275.
21. Guo C, Atochina-Vasserman E, Abramova H, et al. Role of NOS2 in pulmonary injury and repair in response to bleomycin. *Free Radic Biol Med*. 2016;91:293-301.
22. Khan S, Parvez S, Chaudhari B, Ahmad F, Anjum S, Raisuddin S. Ellagic acid attenuates bleomycin and cyclophosphamide-induced pulmonary toxicity in Wistar rats. *Food Chem Toxicol*. 2013;58:210-219.
23. Zaaan MA, Zaki HF, El-Brairy AI, Kenawy SA. Pyrrolidine dithiocarbamate attenuates bleomycin-induced pulmonary fibrosis in rats: modulation of oxidative stress, fibrosis, and inflammatory parameters. *Exp Lung Res*. 2016;42(8-10):408-416.
24. Verma G, Marella A, Shaquiquzzaman M, Alam MM. Immunoinflammatory responses in gastrointestinal tract injury and recovery. *Acta Biochim Pol*. 2013;60(2):143-149.
25. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. *Biomed Pharmacother*. 2003;57(3-4):145-155.
26. Heunks LM, Viña J, Van Herwaarden CL, Folgering HT, Gimeno A, Dekhuijzen PR. Xanthine oxidase is involved in exercise-induced oxidative stress in chronic obstructive pulmonary disease. *Am J Physiol Regul Integr Comp Physiol*. 1999; 277(6):R1697-R1704.
27. Vega VL, Mardones L, Maldonado M, et al. Xanthine oxidase released from reperfused hind limbs mediate kupffer cell activation, neutrophil sequestration, and hepatic oxidative stress in rats subjected to tourniquet shock. *Shock [Augusta, Ga]*. 2000; 14(5):565-571.
28. Sayed-Ahmed MM, Mansour HH, Gharib OA, Hafez HF. Acetyl-L-carnitine modulates bleomycin-induced oxidative stress and energy depletion in lung tissues. *J Egypt Natl Cancer Inst*. 2004;16(4):237-243.
29. Hua-Huy T, Le-Dong NN, Duong-Quy S, et al. Increased exhaled nitric oxide precedes lung fibrosis in two murine models of systemic sclerosis. *J Breath Res*. 2015;9(3):036007.
30. Gurujeyalakshmi G, Wang Y, Giri SN. Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. *Nitric Oxide*. 2000;4(4):399-411.
31. Kandhare AD, Bodhankar SL, Mohan V, Thakurdesai PA. Effect of glycosides based standardized fenugreek seed extract in bleomycin-induced pulmonary fibrosis in rats: decisive role of Bax, Nrf2, NF- κ B, Muc5ac, TNF- α and IL-1 β . *Chem Biol Interact*. 2015;237:151-165.
32. Mezni A, Aoua H, Khazri O, Limam F, Aouani E. Lithium induced oxidative damage and inflammation in the rat's heart: Protective effect of grape seed and skin extract. *Biomed Pharmacother*. 2017;95:1103-1111.
33. Saito M, Yoshida K, Hibi M, Taga T, Kishimoto T. Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. *J Immunol*. 1992; 148(12):4066-4071.
34. Mungunsukh O, Griffin AJ, Lee YH, Day RM. Bleomycin induces the extrinsic apoptotic pathway in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2010;298(5): L696-L703.
35. Jin C, Zhou D, Lv F. Beneficial effects of early [but not late] intervention of heme oxygenase-1 on bleomycin-induced pulmonary fibrosis in mice. *Respir Physiol Neurobiol*. 2011;175(2): 239-246.
36. Song X, Wang B, Lin S, et al. Astaxanthin inhibits apoptosis in alveolar epithelial cells type II in vivo and in vitro through the ROS dependent mitochondrial signalling pathway. *J Cell Mol Med*. 2014;18(11):2198-2212.
37. Mahbub AA, Le Maitre CL, Haywood-Small S, NeilCross ANA, Jordan-Mahy N. Polyphenols enhance the activity of alkylating agents in leukaemia cell lines. *Oncotarget*. 2019; 10(44):4570-4586.
38. Fan Y, Cheng J, Zeng H, Shao L. Senescent cell depletion through targeting BCL-family proteins and mitochondria. *Front Physiol*. 2020;1549.
39. Mezni A, Khazri A, Khazri O, Limam F, Cosette P, Aouani E. Neuroprotective activity of grape seed and skin extract against Lithium exposure using proteomic research. *Mol Neurobiol*. 2017;54(4):2720-2730.
40. Sfaxi I, Charradi K, Limam F, El May MV, Aouani E. Grape seed and skin extract protects against arsenic trioxide induced oxidative stress in rat heart. *Canadian journal of physiology and pharmacology*. 2016;94(2):168-176.
41. Liu Y, Lu F, Kang L, Wang Z, Wang Y. Pirfenidone attenuates bleomycin-induced pulmonary fibrosis in mice by regulating Nrf2/Bach1 equilibrium. *BMC Pulm Med*. 2017;17(1):1-11.
42. Hemmati A, &Nazari Z, &Hasanvand N, Aghel N, Mohammadian B. protective effect of grape seed extract against the fibrogenic effect of bleomycin in rat lung. *Iranian Journal of Pharmaceutical Sciences*. 2006;2(3):143-150. <https://www.sid.ir/en/journal/ViewPaper.aspx?id=85210>
43. Liu Q, Jiang JX, Liu YN, Ge LT, Guan Y, Zhao W, Xie QM. Grape seed extract ameliorates bleomycin-induced mouse pulmonary fibrosis. *Toxicology letters*. 2017;273:1-9.
44. Kalpana C, Sudheer AR, Rajasekharan KN, Menon VP. Comparative effects of curcumin and its synthetic analogue on tissue lipid peroxidation and antioxidant status during nicotine-induced toxicity. *Singapore medical journal*. 2007;48(2):124.
45. Bale S, Venkatesh P, Sunkoju M, Godugu C. An adaptogen with afeirin A ameliorates in vitro and in vivo pulmonary fibrosis by modulating the interplay of fibrotic, matricellular proteins, and cytokines. *Front Pharmacol*. 2018;9:248.
46. Li WG, Zhang XY, Wu YJ, Tian X. Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds. *Acta Pharmacol Sin*. 2001;22:1117-1120.