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AMELIORATIVE EFFECT OF *PUNICA GRANATUM* L. AGAINST BLEOMYCIN INDUCED PULMONARY FIBROSIS IN RATS

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ABSTRACT: Aim: Bleomycin (BLM), a potent anticancer agent widely used in the treatment of cancer is an antibiotic isolated from *Streptomyces verticillus*. BLM has been reported to cause pulmonary fibrosis that limits the chemotherapeutic efficiency. The present study aims to evaluate the efficacy of the aqueous extract of pomegranate (*Punica granatum*) against BLM induced pulmotoxicity in rats. **Methods**: The experimental rats were divided to 5 groups. Aqueous extract of *Punica granatum* peel at 250 and 500 mg/kg, was administered to rats of group III and IV respectively. The rats were induced with BLM. Group I served as normal control and group II as BLM control. Vitamin C at 250 mg/kg b.wt was administered to group V rats. **Results**: Treatment with aqueous extract of *Punica granatum* peel (250 and 500 mg/kg, orally) proved to be protective against BLM induced pulmonary fibrosis by normalizing the levels of glycoproteins (hexose, hexosamine and sialic acid) and by improving the activity of antioxidant enzymes- superoxide dismutase (SOD) and catalase (CAT). The extract also enhanced pulmonary glutathione (GSH) content and suppressed the levels of lipid peroxides in a dose dependent manner with 500 mg dose revealing more defending effect in line with the standard antioxidant, Vitamin C. **Conclusion**: The observed results indicate that the pomegranate extract at both the doses were effective in curbing the toxic insult of BLM.

INTRODUCTION: Gastric and lung cancer are common diseases that pose a serious global threat to health. Chemotherapy agents such as bleomycin, mitomycin and methotrexate may cause pulmonary toxicity, while radiotherapy may lead to radiation pneumonitis [1, 2]. Bleomycin (BLM) is a glycopeptide that binds iron and oxygen *in vivo* to produce an active drug, effective in cancer treatment. BLM treatment is often limited by severe side effects.

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Accumulation of the drug in the skin and lung have been detected after intravenous infusion and become major sites of BLM toxicity. Resistance to BLM in other tissues can be associated with the presence of a bleomycin hydrolase enzyme that is found in lower concentration in skin and lung that may explain the unique sensitivity of these tissues to BLM toxicity.

It has been reported that the major constraint in BLM therapy is the potential for developing of pulmonary toxicity [3]. Lung injury seriously hampers full implementation of treatment, limiting the potential benefits of therapy. The early phase of lung injury is characterized by inflammation (alveolitis), while the late phase is characterized by the organization and deposition of collagen with remodeling (pulmonary fibrosis) [1, 2].

BLM-induced lung fibrosis in animals is a popular model for the study of human lung fibrosis [4]. BLM is believed to induce both single-and double-strand DNA cleavage in neoplastic cells [5]. The chemotherapeutic mechanism results from the chelation of iron ions with oxygen, which leads to production of DNA-cleaving superoxide, and also hydroxide free radicals [6-8]. The increased production of reactive oxygen species (ROS) may be critical to lead to BLM induced pulmonary toxicity, and may eventually lead to lung fibrosis[9-11]. In recent literature, the presence of several ROS has been found in clinical cases of idiopathic pulmonary fibrosis (IPF) [12], and decreased production of ROS has been shown to protect mice against bleomycin-induced pulmonary fibrosis [13].

Studies have reported that, BLM induces diffuse alveolar damage, inflammation and pulmonary fibrosis in animal models [14 -16]. In addition, a reduction in antioxidants has been reported in IPF lungs, and the resulting oxidant-antioxidant imbalance has been suggested in the progression of IPF [9, 14, 17].

Recent studies have reported that phytochemicals derived from plants are effective in protecting against BLM induced pulmonary fibrosis and injury [15,16,18,19].

Punica granatum Linn, commonly referred to as pomegranate, is a mediterranean small tree. In the Indian subcontinent's ancient ayurveda system of medicine, the pomegranate has extensively been used as a source of traditional remedies for thousands of years. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments [20].

Numerous studies have been conducted on pomegranate because of its antioxidant effects. The high antioxidant activity of the extracts from the various parts of pomegranate fruit including peel, juice and seeds have been reported. This antioxidant activity has been said to be the result of a high level of phytochemicals [21, 22, 23].

The present study was undertaken to evaluate the potential of the peel of the fruit *Punica granatum* Linn against BLM-induced lung injury.

MATERIALS AND METHODS:

Preparation of extract:

The *Punica granatum* fruit was purchased from local market, Coimbatore, Tamilnadu, India. The fruits were washed thoroughly with saline and distilled water. The peel of the fruit were collected, shade dried and ground to yield fine powder. 10 g of the powder was extracted with 100 ml of water at 100^0C for 4 hours, centrifuged at 5000 rpm for 15 minutes and filtered through Whatman No.1 filter paper. The residue was extracted twice with 100 ml portions of water, as described above. The extracts were combined and vacuum evaporated. The extract obtained after vacuum evaporation was freeze dried and stored at 4^0C until further use.

Chemicals:

Bleomycin was procured from Cipla Ltd, Mumbai and Vitamin C was obtained from Himedia, Bangalore, India. All other chemicals used in this study were obtained commercially and were of analytical grade.

Experimental Design:

The animals were divided into 5 groups (Group I – V) of six animals each. Pulmonary toxicity was induced subcutaneously (s.c) dose of BLM [24]. **Group I (**Control) animals were on normal pelleted diet, **group II** rats were induced with BLM at 15 mg/kg body weight (b.wt), subcutaneously.), three times a week for a total period of 4 weeks. Aqueous extract of *Punica granatum* (PGAE) at 250 mg and 500 mg /kg b.wt was administered orally to the animals (**group III and IV** respectively) for 7 days prior and during BLM induction as in group II. Standard antioxidant, vitamin C at 250 mg/kg b.wt / day was given orally, to the **group V** animals for 7 days prior and during BLM induction as in group II.

Biochemical Analysis:

The experimental animals were subjected to fasting for a period of 12 hours after the last dose of BLM. At the end of 12 hours fasting the animals were sacrificed, whole blood was collected and lung were excised and washed in saline.10% homogenate of the lung tissues were prepared with 0.1 M Tri-HCl buffer, pH 7.4 and centrifuged at 3000 rpm for 15 min at 4ºC for cytosolic separation. Plasma was prepared from whole blood.

The levels of plasma glycoproteins- Hexose and Sialic acid were determined by the method of Niebes [25] and Hexosamine as described by Wagner [26].

The activity of the pulmonary antioxidant enzymes - superoxide dismutase (SOD) was assessed according to the method of Das et al [27] and Catalase (CAT) by the method of Sinha [28], Glutathione (GSH) content of pulmonary tissues were assessed using Ellman's reagent according to the method described by Ellman [29]. Protein levels were determined as described by Lowry [30]. The lipid peroxidation status was determined by measuring the MDA content according to the method of Niehus and Samuelsson [31].

Histopathological Examination:

The lung tissue of each animal were dissected out and then fixed in buffered formalin for 12 hours and processed for histopathological examination. Four µm-thick paraffin sections were stained with hematoxylin and eosin for light microscope examination using conventional protocol.

Statistical analysis:

The data are expressed as mean ± S.D. Statistical comparison was done at significance level, $p<0.05$ using SPSS package version 10.0. One way ANOVA followed by post hoc analysis of LSD was performed.

RESULTS:

The levels of plasma glycoproteins- hexose, hexosamine and sialic acid in the experimental animals were determined (**Table 1**). The results revealed a marked ($p<0.05$) 2-fold rise in the plasma glycoprotein levels in plasma of the BLM intoxicated rats (group II). The treatment with (PGAE) to the animals of group III and IV at 250 mg/kg and 500 mg/kg b.wt, respectively, resulted in a significant ($p<0.05$) drop in the glycoprotein content in the plasma in a dose dependent manner. Significant decline in the levels of the glycoproteins was also observed in rats treated with vitamin C (group V) as compared against BLM-control animals (group II).

One of the major mechanisms of BLM toxicity has been reported to be due to ROS. The effect of PGAE on the activity of the antioxidant enzymes (SOD and CAT), pulmonary GSH content and lipid peroxidation levels were assessed and presented in **Table 2**.

A significant diminution in the activity of SOD, CAT and GSH were observed in the BLM control animals as compared to the normal animals. BLM induction resulted in a multiple fold increase in the pulmonary lipid peroxidation status. Prior treatment and co-admin

istration of PGAE (250 mg and 500 mg/kg b.wt) resulted in marked improvement in the activity of SOD and CAT with significant ($p<0.05$) raise in the levels of GSH in a dose dependent manner. The extract was able to efficiently lessen the elevated MDA content. Vitamin C normalized ($p<0.05$) the activity of the enzymes- SOD and CAT, increased the pulmonary GSH content and depressed the lipid peroxide levels in the lung tissue of the group V animals.

TABLE 1: EFFECT OF AQUEOUS EXTRACT OF *PUNICA GRANATUM* ON THE LEVELS OF PLASMA HEXOSE, HEXOSAMINE AND SIALIC ACID

Groups	Hexose (mg/dl)	Hexosamine (mg/dl)	Sialic acid (mg/dl)
Control	93.81 ± 5.09[b]	24.77 ± 1.51[b]	29.90 ± 1.08[b]
Bleomycin (15 mg/kg b.wt)	187.88 ± 7.61[a]	55.39 ± 2.38[a]	64.82 ± 3.49[a]
PGAE (250 mg/kg b.wt) + Bleomycin	132.49 ± 7.34[b]	40.91 ± 2.44[b]	47.45 ± 2.32[b]
PGAE (500 mg/kg b.wt) + Bleomycin	100.07 ± 6.23[b]	27.01 ± 0.79[b]	30.13 ± 1.42[b]
Vitamin C (250 mg/kg b.wt) + Bleomycin	98.17 ± 5.81[b]	26.33 ± 1.09[b]	33.92 ± 1.11[b]

Values are expressed as mean ± SD for six animals.

Group comparison and statistical significance at $p<0.05$:

[a]: Group I vs. II, III, IV, V

[b]: Group II vs. I, III, IV, V.

TABLE 2: EFFECTS OF AQUEOUS EXTRACT OF *PUNICA GRANATUM* ON THE ANTIOXIDANT STATUS AND MDA CONTENT

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GSH (µg/mg protein)	MDA (nmoles /min/mg protein)
Control	7.38 ± 0.41[b]	16.04 ± 0.55[b]	4.89 ± 0.20[b]	1.73± 0.08[b]
Bleomycin (15 mg /kg b.wt)	2.83 ± 0.10[a]	6.10 ± 0. 31[a]	2.38 ± 0.12[a]	2.24 ± 0.11[a]
PGAE (250mg/kg b.wt) +Bleomycin	4.77 ± 0.19[ab]	8.34 ± 0.37[b]	4.43 ±0.21[ab]	1.92 ± 0.05[b]
PGAE (500mg/kg b.wt) +Bleomycin	7.20 ± 0.35[b]	15.96 ± 0.74[b]	5.01 ± 0.23[ab]	1.70 ± 0.07[b]
Vitamin C (250mg/kgb.wt)+Bleomycin	6.7 ± 0.51[b]	15.58 ± 0.79[b]	4.94 ± 0.28[ab]	1.72 ± 0.05[b]

V alues are expressed as mean ± SD for six animals.
Group comparison and statistical significance at $p<0.05$: [a]: Group I vs. II, III, IV, V [b:] Group II vs. I, III, IV, V

The results of the histopathological sectioning of the pulmonary tissue are presented in **Figure 1 (a-e)**. The lung tissue sectioning of group I animals presents normal lobular architecture with normal appearance and no obvious abnormality (**Figure 1a).** The pulmonary tissue of the bleomycin control animals reveals severe hemorrhage and necrosis. Tissue also presents intense scarring and infiltration of inflammatory cells (**Figure 1b).**

The lung tissue sectioning of the animals administered with 250 mg/kg b.wt PGAE presents mild necrosis and milder infiltration of inflammatory cells (**Figure 1c**). **Figure 1d**- represents the lung section of the group IV animals treated with 500 mg/kg b.wt PGAE. The sectioning indicates the absence of necrosis and considerable reversal to normal architecture and very mild inflammation. Treatment with standard, vitamin C has effectively protected the cells form BLM toxicity as presented in **Figure 1e**. The section presents negligible inflammation.

Histopathological analysis:

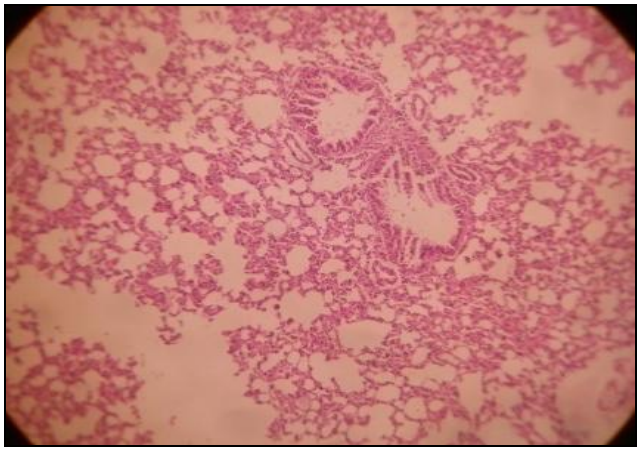

FIGURE 1a: GROUP I (CONTROL)

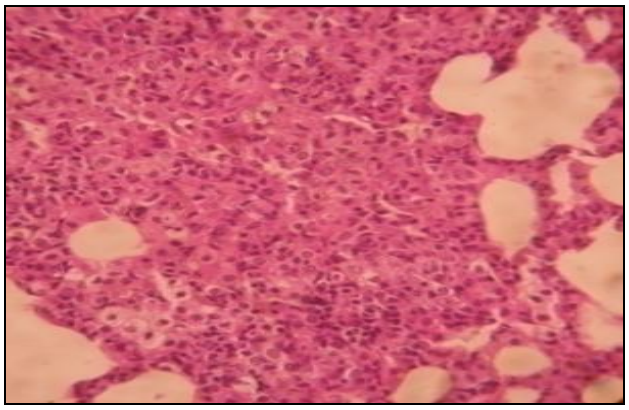

FIGURE 1b: GROUP II (BLEOMYCIN CONTROL)

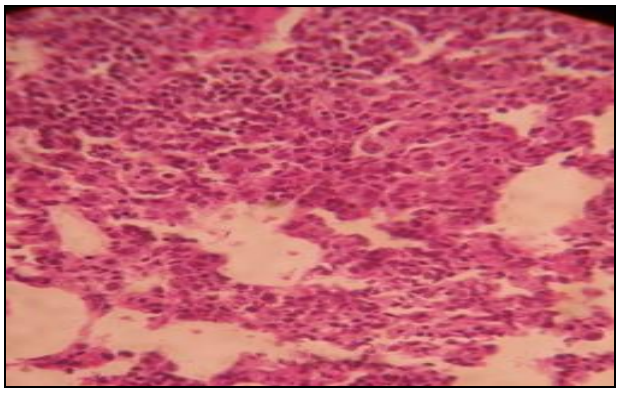

FIGURE 1c: GROUP III
250 mg/kg b.wt PGAE+ BLEOMYCIN)

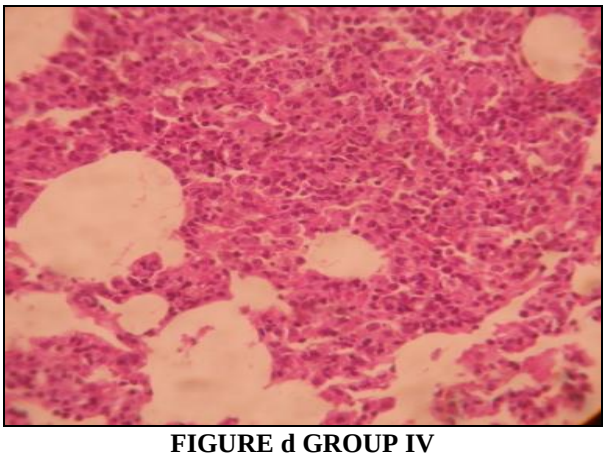

FIGURE d GROUP IV
(500 mg/ kg b.wt PGAE+BLEOMYCIN)

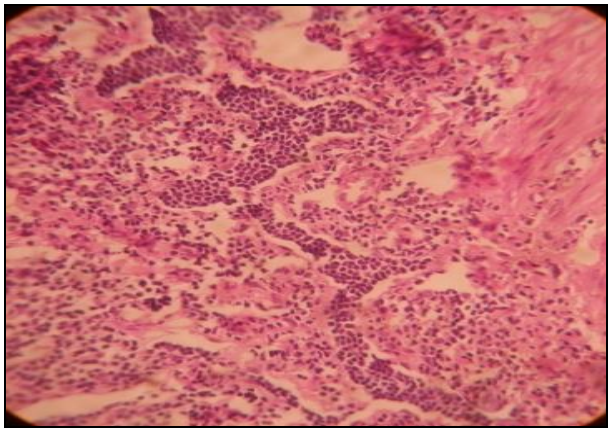

FIGURE 1e: GROUP V
(VITAMIN C 250 mg/kg b.wt +BLEOMYCIN)

DISCUSSION: Bleomycin is a commonly used chemotherapeutic agent that is reported to induce dose-dependent pulmonary fibrosis upon long-term administration [32]. Pulmonary fibrosis is characterized by failure of alveolar re-epithelialization, persistence of fibroblasts/myo-fibroblasts, and deposition of extra cellular matrix (ECM) and distortion of lung architecture. Glycoproteins comprise the connective tissue component of the ECM and play a vital role in the pathogenesis of pulmonary fibrosis. Glycoproteins are predominantly protein in nature with one or more heterosaccharide chains that contains hexose, hexosamine, sialic acid and fucose.

Lung inflammation is considered to be a major contributing factor in the induction of pulmonary fibrosis [14, 15, 33]. ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical are reported as mediators of lung inflammatory processes [33].

BLM induced pulmonary injury and fibrosis has been reported and documented using several animal models [33-35]. The experimental models have been widely used for studying the underlying mechanisms involved in the sequence of pulmonary fibrosis and the action of various drugs on this progression [36-38]. The interaction of BLM with DNA is postulated to initiate the inflammatory and fibro proliferative changes through a concerted action of various cytokines leading to collagen accumulation in the lung [36, 39-42].

The elevation in the levels of glycoprotein components as observed in the study on BLM induction may be due to the secretion of cell membrane glycoconjugates into the circulation [14, 36, 40] and may also be due to increased deposition of macromolecular components, which may be a physiological adjustment to the pathological process. Elevated levels of glycoprotein components have been already reported during pulmonary fibrosis [14,16,36,43]

Natural antioxidants, such as polyphenols from green tea extracts are known for their capability of reducing the expression of ECM genes [44].

Notably, studies have reported that phytochemicals as - neferine, naringin, luteolin and paeonol have inhibitory effects on pulmonary fibrosis, due to their actions as anti-inflammatory agents, anti-oxidants and inhibitors of cytokines and NF-κB [45-48].

In the present study the administration of PGAE (250 and 500 mg/kg b.wt) evidenced reduction in the levels of glycoproteins - hexose, hexosamine and sialic acid in the plasma that is suggestive of the protective effect of the extract. PGAE may have contributed to the decline in plasma glycoproteins either by suppressing the ECM genes or countered against the ROS produced by BLM and inflammation as well.

Previous studies have demonstrated that BLM generates ROS and initiates inflammation and fibro proliferative changes via a concerted action of various cytokines leading to collagen accumulation in the lung [49]. BLM has also been reported to result in depletion of endogenous antioxidant defenses thereby increasing the risk of oxidant mediated tissue injury [33, 50].

Several studies suggest that a number of antioxidant and detoxification enzymes as MnSOD, catalase, glutamate cysteine ligase, thioredoxin, glutaredoxin, and heme-oxygenase 1 are low/ absent in the fibrotic lesions of pulmonary fibrosis [33, 51].

As in line with the previous reports in the study similar reduction in the activity of antioxidant enzymes were observed ($p<0.05$) in animals that were induced with BLM as compared to group I animals.

The decreased levels of antioxidant viz., SOD and CAT activities may be due, in part, to an overwhelming oxidative modification of the enzymatic proteins by excessive ROS generation. More so, reduction in the activities of these enzymes may stem from decrease in their rate of synthesis. Inhibition of this protective mechanism results in enhanced sensitivity to free radical induced cellular damage.

PGAE supplementation to the animals (group III and IV) markedly improved the enzyme activities ($p<0.05$) dose dependently with the 500 mg dose of PGAE presenting the enzyme activity close to control (group I) similar to supplementation of Vitamin C.

The outcome of PGAE administration increases the activities of SOD and CAT in BLM induced rats and thus curbs the accumulation of excessive free radicals and protects the lung from BLM induced toxication. Similar results were reported by Teixeira et al [34]. Silymarin was able to effectively improve the antioxidant status in bleomycin-induced mice [51].

Imbalance between oxidant and antioxidant defense mechanisms has been reported to contribute to the incidence of pulmonary fibrosis [11-13]. Glutathione (GSH), an intracellular thiol acts as a non-enzymatic antioxidant and provides a protection to the lung from oxidative damage imposed by endogenous or exogenous lung toxicants [51, 52]. However, its depletion in the lung by a fibrogenic agent, such as BLM as shown in the present study may be associated with the risk of lung damage [35, 51.]

Co-administration of PGAE with BLM reversed GSH depletion and subsequent lung damage. The ability of PGAE to prevent depletion of GSH stores in the pulmonary tissue suggests that the antifibrotic activity of the extract and this could have been in part by acting as ROS scavenger or by enhancing GSH synthesis.

The results of our study were in accordance with the previous studies with antioxidants. Alpha-lipoic acid was found to improve the levels of antioxidants in BLM induced rats [52.] Boswellic acid [53] supplementation on BLM induction was found to improve the levels of GSH in the lung tissue. Saba et al [54] reported that ellagic acid was able to offer protection in BLM induced rats, it effectively improved GSH levels.

Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. LPO is considered as the prime rationale that instigates lung injury [35]. The most commonly used lipid peroxidation markers are TBARS as MDA.

The elevated MDA content in the lung tissue proves the increased oxidative stress due to BLM induction. The decrease in MDA levels by co administration of PGAE at 250 mg/kg b.wt and 500 mg/kg b.wt suggest the capacity of the extract in combating the effect of BLM induced toxicity.

Teixeira et al [34] reported that N-acetylcysteine was able to reduce the levels of MDA that was elevated upon BLM induction. Boswellic acid was reported by Ali and Mansour [53] to improve the antioxidant status and reduce the levels of MDA. Zhou et al[55] reported that the essential oil from *Citrus reticulate* reduced lipid peroxidation and improved antioxidant status.

BLM induction distorted the architecture of the lung tissue which included moderate to severe hemorrhages, areas of increased thickening of alveolar septa, infiltration of inflammatory cells and fibroplasias. It has been reported previously that BLM induction results in the similar structural changes [43, 56]. PGAE at both the doses efficiently reversed the abnormal histology of the lung to near normal architecture revealing its protective effects against BLM induced histological alterations. PGAE was able to reduce the infiltration of inflammatory cells and reduce the deposition of glycoproteins comparable to vitamin C.

Similar results were reported on treatment with quercetin in BLM induction. Quercetin showed to have ameliorative effect on the inflammatory lesions and reduce the thickening observed in the alveolar septa as well that were developed by BLM treatment [57].

Thus by improving the activities of the antioxidant enzymes and GSH levels, and decreasing lipid peroxidation, PGAE was found to ameliorate the effects of BLM and offered protection. The protective effects of the extract from the peel of *Punica granatum* could be attributed to the phytochemicals harbored.

CONCLUSION: The results observed thus indicate the aqueous extract from the peel of *Punica granatum* at both doses (250 mg/kg b.wt and 500 mg/kg b.wt) effectively ameliorated the toxic effect of BLM in a dose dependent manner. Thus it could be suggested the extract could be explored further in chemotherapy and in combating side effects of chemotherapeutic drugs.

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