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Therapeutic efficacy of galangin and piperine individually and in combination with the bleomycin on body weights and hematology alterations in c57bl/6 mice

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Abstract
This study was conducted to know the therapeutic efficacy of Galangin and Piperine individually and in combination with the Bleomycin-induced oxidative stress. Fifty C57BL/6 male mice of 6-7 weeks of age weighing ~28-30 g were divided into seven groups of six animals each. Group 1 served as normal control; Group 2 served as disease Control -Bleomycin sulfate [BLM] @ 1.5 IU/ Kg bwt, oropharyngeal route, Single dose on 0th day; Group 3 treated with Galangin @ 5 mg/kg bwt/orally/daily per se; Group 4 is Piperine @ 50 mg/kg bwt/orally/daily per se; Group 5 treated Bleomycin sulfate + Galangin @ 5 mg/kg bwt/orally/daily; Group 6 Bleomycin sulfate + Piperine @ 50 mg/kg bwt/orally/daily and Group 7 Bleomycin sulfate + Galangin @ 2.5 mg/kg bwt/orally/daily + Piperine @ 25 mg/kg bwt/orally/daily. Blood samples were collected at the end of the experiment to evaluate hematological parameters body weights were measured on the 7th, 14th, and 21st day. The study results of treatment groups (5, 6 and 7) have revealed a significant (p>0.05) increase in body weights compared to group 2 at different time intervals. Similarly, TLC was significantly higher, and Hemoglobin and Total erythrocyte count were lower in group 2 compared to treatment groups 5, 6 and 7. In conclusion, among treatment groups, the combination groups (7) had shown a better ameliorative effect than the individually treated group.

Keywords: Bleomycin (BLM), galangin (GA), piperine (PIP), DC (disease control), and oxidative stress

1. Introduction
Pulmonary fibrosis is a pathological condition which accompanies a wide range of inflammatory conditions of the airways. The severe and lethal pulmonary fibrosis is prevalent in patients with diseases such as Idiopathic Pulmonary Fibrosis (IPF), acute respiratory distress syndrome, and unusual interstitial possible mechanisms involving reactive oxygen species (ROS) damage to the elements of extracellular matrix or through activation of metalo proteinases [1]. After tissue injury and the onset of scarring or fibrosis, initiating repair processes involves multiple aspects of the host inflammatory response, including oxidative stress [2]. Many models have been established to study pulmonary pathophysiology. The standard agent for induction of experimental pulmonary fibrosis in animals is Bleomycin [3, 4]. Bleomycin hydrolase, a Bleomycin-inactivating enzyme, critically influences the effects of this drug on different tissues. The lungs maintain low levels of the enzyme and therefore are more susceptible to Bleomycin-induced tissue injury [5].
Galangin, one of the most important and naturally active flavonoids, exists in high concentrations in honey, *Alpinia galanga* and *Alpinia officinarum*, herbs that have been used as a spice for a variety of ailments [6]. It has been demonstrated that galangin exhibits antioxidant, anti-inflammatory and anti-fibrotic activities in various disorders [7].
Alkaloids have long been used as a reservoir for drug discovery infrastructure. Among the spices, black pepper is a well-known distinctive spice worldwide. The pungent taste of pepper and its many pharmacological properties are attributed to Piperine (PIP), one of its major alkaloids [8]. PIP has been demonstrated to impart protection against oxidative stress-mediated cellular damage by quenching ROS, free radicals, and reactive metabolic intermediates [9, 10].
In view of the above beneficial effects of GA and PIP, the present study was designed and carried out to evaluate the therapeutic efficacy of GA and PIP individually and in combination in BLM-induced pulmonary toxicity causing oxidative stress in C57BL/6 mice for a period of 21 days

2. Materials and Methods

2.1 Chemicals

Bleomycin sulfate was obtained from Cipla Pvt. Ltd. India Galangin and Piperine Tokyo Chemical Industry Co. Ltd

2.2 Experimental animals

The mice strain used for the study was C57BL/6, a classic murine model for Bleomyc in induced pulmonary toxicity, of 6-7 weeks of age weighing ~28-30 g were procured from Vyas Labs, Hyderabad (CPCSEA:2085/PO/Rc.Bi.Bt/S/19/CPCSEA). This experimental study was approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science, Hyderabad (IAEC, Approval No. CPCSEA 40/24/C.V.Sc, Hyd, IAEC.MICE/ dated 012.06.2021). These animals were kept in polypropylene cages and maintained with a 12 hrs dark/light cycle under hygienic conditions having ambient temperature (22–24 °C) at Animal house in the Department of Veterinary Pharmacology and Toxicology. All animals were placed on commercial standard pellet feed (M/s. VRK Nutritional solutions, Hyderabad) and provided water ad libitum throughout the experiment.

2.3 Experimental protocol

Fifty healthy adult male C57BL/6 mice, aged 6-7 weeks, were procured and acclimatized for 15 days before beginning the study. The mice were randomly divided into seven groups consisting of 6 mice in each group. Millipore (reverse osmosis) water was employed for oral gavage. All the groups were maintained as per the following treatment schedule for three weeks as follows (Table1)

Table1: Experimental design

Group	Treatment
1.	Control (0.9% saline) oropharyngeal route, Single dose on 0th day [NC]
2.	Bleomycin sulphate @ 1.5 IU/Kg bwt, oropharyngeal route, Single dose on 0th day [DC]
3.	Galangin @ 5 mg/kg bwt/orally/daily [GA]
4.	Piperine@ 50mg/kg bwt/orally/daily [PIP]
5.	Bleomycin sulphate + Galangin @ 5 mg/kg bwt/orally/daily [BG]
6.	Bleomycin sulphate + Piperine@ 50 mg/kg bwt/orally/daily [BP]
7.	Bleomycin sulphate + Galangin @ 2.5 mg/kg bwt/orally/daily + Piperine @ 25 mg/kg bwt/orally/daily [BGP]

2.4 Body weights and blood collection

Body weights were measured on the 7th, 14th, and 21st day of the experimental period. Blood collection was carried out at the end of the experimental period of 3 weeks. Feed withdrawal before 12hrs of the blood collection. Blood from animals was collected (approximately 20µL) with EDTA-coated vacationers and the estimation for TLC, TEC, Hemoglobin, and Haematocrit through an Automated blood analyzer (ABX M ESV 60 -Horiba medicals, Japan).

3. Results and Discussion

3.1 Average body weights (g)

The average body weights were recorded on the 7th, 14th and 21st day of the experimental period. The average body weights of DC was significantly (p<0.05) lower (ranging from 28.76±1.71 to 19.85±0.81) than that of NC mice (ranging from 31.18±0.29 to 32.87±0.78) from 7th, 14th and 21st day of the experimental period. The treatment group BGP (30.33±0.34 to 31.59±0.83) showed a significant (p<0.05) increase in average body weights when compared to treatment group BG and BP (27.65±0.17 to 29.65±0.64 and 26.31±0.85 to 29.23±0.56 respectively). Similar trends of results were obaserved in between groups on the 7th, 14th and 21st day. However, the body weights of per se groups (3 and 4) were comparable to group 1(Table 2**).**

3.2 Total Erythrocyte Count (TEC)

The TEC (10^6/mL) of DC (7.52±0.31) was significantly (p<0.05) lower than the NC (9.64±0.23), although treatment groups, BG (8.37±0.25), BP (8.48±0.78) and BGP (8.93±0.49) showed a significant (p<0.05) greater values respectively when compared to DC. There was also a no significant difference between NC and per se groups GPS (9.12±0.48) and PPS (9.02±0.49) respectively (Table 3)

3.3 Total Leukocyte Count (TLC)

The TLC (10^3/cu.mm) of DC (8.79±0.11) was significantly (p<0.05) higher than the NC (3.63±0.08), although treatment groups, BG (6.79±0.21) and BP (6.36±0.15) and BGP (5.72±0.19) respectively, showed a significant (P<0.05) lower values when compared to DC. There was also a no significant difference in between NC and per se groups GPS (4.04±0.10) and PPS (4.28±0.18) respectively. (Table 3)

3.4 Hemoglobin (Hb)

The Haemoglobin (g/dL) of DC (10.14±0.04) was significantly (P<0.05) lower than the NC (14.48±0.05), although treatment groups, BG (12.61±0.02), BP (12.47±0.08) and BGP (13.39±0.09) showed a significant (P<0.05) greater values respectively when compared to DC. There was also a no substantial difference between NC and per se groups GPS (14.28±0.09) and PPS (14.45±0.07). (Table 3)

3.5 Haematocrit (%)

The Haematocrit of DC (38.47±0.25) was significantly (P<0.05) lower than the NC (45.17±0.24), although treatment group BGP (42.98±0.48), BG (45.67±0.66) and BP (41.28±0.54) showed a significant (P<0.05) greater values respectively, when compared to DC. There was also a no considerable difference between NC and per se groups GPS (44.28±0.21) and PPS (45.67±0.24) respectively (Table 3)

Table 2: Mean values of the body weights (g) in different groups of mice

Group	Treatment	7th day	14th day	21st day
1.	NC	31.18 ± 0.29^{a}	32.51 ± 0.84^{a}	32.87 ± 0.78^{a}
2.	BLM @1.5 U/kg Bwt (DC)	28.76 ± 1.71^{gA}	24.48 ± 0.88^{fB}	19.85 ± 0.81^{fC}
3.	GA @ 5 mg/kg Bwt/p.o/daily (GPS)	29.47 ± 1.13^{f}	28.05 ± 0.80^{d}	28.32 ± 0.25^{d}
4.	PIP @ 50 mg/kg Bwt/p.o/daily (PPS)	29.08 ± 0.64^{e}	28.75 ± 0.75^{e}	27.24 ± 0.92^{e}
5.	BLM+ GA @ 5 mg/kg Bwt/p.o/daily (BG)	27.65 ± 0.17^{c}	28.25 ± 0.82^{c}	29.65 ± 0.64^{c}
6.	BLM + PIP @ 50 mg/kg Bwt/p.o/daily (BP)	26.31 ± 0.85^{dA}	28.12 ± 0.75^{c}	29.23 ± 0.56^{cB}
7.	BLM+ GA @ 2.5 mg/kg Bwt/p.o/daily + PIP @ 25 mg/kg Bwt/p.o/daily (BGP)	28.33 ± 0.34^{bA}	29.54 ± 0.64^{b}	31.59 ± 0.83^{bB}

Values are Mean ± SE (n=6); Two way ANOVA with Duncan's post hoc test (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups; small alphabets represents vertical comparison and Capital letters represents horizontal comparision.

Group	Treatment	TEC (10^6/mL)	TLC (10^3/cu.mm)	Hb(g/dL)	Haematocrit (%)
1.	NC	9.64 ± 0.23^{a}	3.63 ± 0.08^{a}	14.48 ± 0.05^{a}	45.17 ± 0.24^{a}
2.	BLM @1.5U/kg Bwt (DC)	7.52 ± 0.31^{b}	8.79 ± 0.11^{b}	10.14 ± 0.04^{b}	38.47 ± 0.25^{b}
3.	GA @ 5mg/kg Bwt/p.o/daily (GPS)	9.12 ± 0.48^{a}	4.04 ± 0.10^{a}	14.28 ± 0.09^{a}	44.28 ± 0.21^{a}
4.	PIP @ 50mg/kg Bwt/p.o/daily (PPS)	9.02 ± 0.49^{a}	4.28 ± 0.18^{a}	14.45 ± 0.07^{a}	45.67 ± 0.24^{a}
5.	BLM+ GA @ 5mg/kg Bwt/p.o/daily (BG)	8.37 ± 0.25^{a}	6.79 ± 0.21^{c}	12.61 ± 0.02^{c}	42.36 ± 0.66^{c}
6.	BLM + PIP @ 50mg/kg Bwt/p.o/daily (BP)	8.48 ± 0.78^{a}	6.36 ± 0.15^{c}	12.47 ± 0.08^{c}	41.28 ± 0.54^{c}
7.	BLM+ GA @ 2.5mg/kg Bwt/p.o/daily + PIP @ 25mg/kg Bwt/p.o/daily (BGP)	8.93 ± 0.49^{a}	5.72 ± 0.19^{cd}	13.39 ± 0.09^{c}	42.98 ± 0.48^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS). Means with different alphabets as superscripts differ significantly ($P<0.05$) among the groups.

BLM is an antibiotic that acts through the induction of the DNA strand breaks and is categorized as one of the cytotoxic and/or anti-tumor antibiotics. Oxidative stress is a critical biomarker in various disorders, representing an imbalance between the antioxidant enzymes [11, 12]. Many authors studied lung-related toxicity using the LPS model [13], but Bleomycin-induced toxicity showed severe inflammation and fibrosis [14]. In this study, examine the effect of Bleomycin on haemotological parameters and its amelioration with the combination of PIP and GA. The results of the current study showed that there was a significant reduction of blood indices in the group receiving BLM mice. On other hand the treatment groups revealed a substantial reversal in their values. As per the literature it is suggested that BLM could able to bind with Iron (Fe^{+2}), resulting in causing anemia by decreasing in the concentration of Fe^{+2} in the erythrocytes. Further it was suggested that oxidative stress could also contribute to release of free radicles like H_2O_2 and other peroxides damage to the erythrocyte's membrane. Therefore, the present study results are in the previous results [15]. The abnormal alternations in the blood indices indirectly effecting the feed and water intake and weight loss may be due to the rise in pro-inflammatory cytokines and increased oxidative stress leading to rapid catabolism and lethargy. Animals in normal control (NC/group 1) showed a normal range of body weight throughout the study, while the per se groups (3, 4) showed a significant increase in body weight compared to NC and it may be due to the antioxidant and anti-inflammatory properties of PIP and GA. Treatment groups (BG/ 5, BP/ 6 & BGP/ 7 showed a significant difference in increased body weight compared to DC. Moreover, the combination group BGP showed a significant difference compared to 5 & 6 which is also evident from the study. The effect of BLM on hematology revealed a significantly lower value of TEC and Hb, and a sharp increase in the TLC in the DC compared to the NC and per se groups. TLC indicates a range of conditions, including infections, inflammation, injury and immune system disorders a significant change in hematological parameters may be due to dysfunction of the hemopoietic system.

Further, the anemia resulted in the reduction of iron concentration, thereby compromising hemoglobin synthesis. The chemotactic effect of various chemokines and cytokines secreted by activated macrophages at the site of injury is the probable possibility of oxidative damage induced cellular lysis resulting in the reduced TEC, Hb and an increase in TLC. These findings are in agreement with Khan *et al.* (2020) [17].

The treatment group BGP showed significant improvement in hematological parameters compared to BG and BP. The haemoprotective effect of GA and PIP may be possibly due to enhanced free radical scavenging activity as well as antioxidant properties [18] and antiinflammatory efficacy [19]. As cited earlier, no literature was found till now on GA and PIP individually and in combination with BLM induced toxicity.

4. Conclusions

The results of this study revealed an improvement in body weight, TEC and Hb and decrease in TLC in BG and BP treated groups individually and the combination group BGP showed better amelioration in male C57BL/6 mice due to the antioxidant potentical of the compounds.

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7. Conflicts of Interest

The authors declare no conflict of interest

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