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Therapeutic use of *Curcuma longa L*. extract against Bleomycin- induced chronic oxidative stress

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The elaborate of Bleomycin- induced pulmonary fibrosis (BIPF) has been associated with inflammation and necrosis of the alveolocytes, production of free- radicals and induction of chronic oxidative stress. The aim of the study was to establish the radical- scavenging capacity and antioxidant activity of *Curcuma longa* extract, in Bleomycin models in mice and to determine the therapeutic potential and action against similar oxidative stress induced conditions. BIPF was induced with a single (injection in every two days) of Bleomycyn in dose 0.34 U/kg body weight. Till the end of the experimental period (twenty-eight days) after Bleomycyn administration, the mortality rate was not observed in ICR mice.

Lipid peroxidation and total cholesterol levels were significantly reduced in *C. longa* extract+ Bleomycyn group, compared to Bleomycyn treated group ($p< 0.05$). SOD and CAT plasma antioxidant enzymes expression were increased in *C. longa* + Bleomycyn group, compared to Bleomycyn -treated group ($p< 0.05$). In the other hand, nitric oxide scavenging expression increased in Bleomycyn ($p<0.05$) compared to the other groups ($p< 0.05$).

In conclusion, the treatment with *C. longa* extract stimulates endogenous antioxidant activity, reduction of lipid peroxidation and scavenging of nitric oxide (NO•). These results make it appropriate to propose the use of this plant extract as a possible addition to the treatment of acute and chronic pulmonary diseases associate with oxidative stress.

Keywords: pulmonary fibrosis; oxidative- scavenging imbalance; SOD, CAT, NO•.

INTRODUCTION

Bleomycin is an antitumor antibiotic isolated from *Streptomyces verticillatus*, has been used for treatment of squamous cell carcinomas, testicular carcinomas, lymphomas, and malignant pleural effusions by inducing tumor cell death and inhibiting tumor angiogenesis [1]. It is a small peptide, and contains at opposite ends of molecule, a DNA-binding region and an iron-binding region. The iron is an essential cofactor for free radical generation and the bleomycin cytotoxic activity. Moreover, bleomycin forms a complex with Fe^{2+}, which is oxidized to Fe^{3+}, and resulting in the oxygen reduction to reactive oxigen spicies (ROS) and reactive nitrogen spicies (RNS) [2]. The ROS/RNS induction results in instability of cellular membrane, lipid and protein peroxidation, inflammatory reactions in the lungs and pulmonary fibrosis [3]. Bleomycyn often used as a representative model for pulmonary fibroses (BIPF) and possessed acute and chronic oxidative stress in terms of ROS. In the body, antibiotic is rapidly metabolized by complex of extracellular matrix deposition, oxidative changes, and pulmonary remodeling [4], resulting in the generation of the toxic products, which subsequently cause lung-toxicity.

Curcuma longa L. (*C. longa*) contains 0.1% turmerin, 5-kDa heat-resistant, non-cyclic peptide. It is a member of the ginger family (Zingiberaceae) with different therapeutic actions, including anti-inflammatory, anti-infectious, anti-fibrotic, antioxidant and anticancer, in animal models [5, 6]. There is also evidence that *C. longa* treatment can protect against liver injury, cardiovascular, neoplastic, metabolic and pulmonary diseases, caused by antibiotics, etc. [7]. Turmerin possessed protective effect against ROS mediation, membrane lipid peroxidation, DNA damages and mutagenicity. The inhibitory/therapeutic potential was shown against type 2 diabetes and in oxidative organ damages against venom phospholipase A2 in animals [8, 9].

The aim of the present study was to establish the radical- scavenging capacity, the levels of nitric oxide and antioxidant activity of *Curcuma longa* extract, in Bleomycin models in mice and to determine the therapeutic potential and action against chronic lung damages.

EXPERIMENTAL

Chemicals

Bleomycin Sulfate ($C_{55}H_{84}N_{17}O_{21}S_3$, EP 9041-93-4), Carboxy-Ptio.K probe and other chemicals purchased from Sigma Aldrich Co., USA was of

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analytical grade and all solvents were distilled prior to use. The *C. longa* fine powder was obtained from authentic source, ABC Limited, India. The powder was kinetically extracted (48 hrs/100% ethanol). The total filtrate was dried using rotary evaporator (Buchi B-480, India) at 400c and was lyophilized (Iishin Lab Co. Ltd, USA) to crude extract. The extract was stored in air tight glass bottle and kept at 28°C.

Animals and diet

Thirty male pathogen-free ICR mice weighing 47-50 ± 2.0g were obtained from Medical Faculty, Trakia University, (Suppliers of Laboratory Animals), Stara Zagora, Bulgaria. The animal procedures were in accordance with *Directive 2010/63/EU* on the protection of animals used for experimental and other scientific work, and approved by the *Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/ 6000-0333/ 09.12.2016).* Animals were housed in polypropylene cages at a temperature of 18–23 ±2°C and under a light period of 12–12 hrs daily. They were fed on a standard commercial feed (Indusrial, Bulgaria), after 10 days acclimatization on humidity 55% and free access to tap water. The lyophilized *C. longa* was dissolved in d. $H_2O$and preserved at 4°C until use.

Bleomycin administration and treatment protocol

Mice were divided into four groups (n=6 animals in each group). For period of 28 days and administration of the drugs were administrated through intraperitoneal injection (i.p.). The first group was control group -the mice were on standard diet. In the second group mice were treated with Bleomycin sulfate. Bleomicyn were given i.p. (0.069 U/ml) 0.34 U/kg body weight dissolved in 250 µl saline every other day for a period of 28 days. To study the Bleomicine toxicity we used the mouse models of bleomycin- induced pulmonary fibrosis of Walters *et al.* [10]. The third group consists from mice treated with C. longa extract that was administrated i.p. (40 mg/ml) 0.200 mg/kg body weight once a day for a period of 28 days. In the fourth group (C. longa + Bleomicyn), mice were pretreated for 2 hours i.p. with C. longa in doses of (40 mg/ml) 0.200 mg/kg body, once a day. The Bleomicyn was administrated every other day in dose (0.069 U/ml) 0.34 U/kg body weight dissolved in 250 µl saline. The duration of the experiment was 28 days.

To ensure that pulmonary fibrosis is developed, there was an additional fifth group (n= 6). The mice from this group received i.p. BLM in doses (0.069 U/ml) 0.34 U/kg body weight dissolved in 250 µl saline every other day for a period of 16 days. The mice were sacrificed, dissected and by histopathological examination was registered the Bleomicyn induced pulmonary fibrosis (the results are not presented).

Additionally, the physiological status and behavior of animals were monitored daily.

Blood samples

On 29 days after beginning of the experiment, the animals were sacrificed under anesthesia (Nembutal 50 mg/kg i.p.) and the fresh blood (1.3-2cm^3) was collected directly from the heart in cold EDTA-containers (5 cm^3 Monovette, Germany). After centrifugation of blood samples at 4000rpm at 4°C for 10 min, 200µl of plasma from each group were stored at 4ºC were studied.

Instruments

The biochemical analyses were performed at UV–VIS spectrophotometer-400 (TERMO Sci., RS232C, Stratagene, USA). The Electron Paramagnetic measurements (EPR) were performed an X-Band, Emx^{micro} Spectrometer (Bruker, Germany) with settings: 3505 g centerfield, 6.42 mw microwave power, 5 g modulated amplitude, 1-5 scans. All experiments were made triplicate.

Estimation of plasma lipid peroxidation

The lipid peroxidation products in plasma of all groups experimented animals were assessed through thiobarbituric acid reactive substances (TBARS) assay by using the Plaser *et al.* [11] method which measures reactive malondialdehyde products (MDA) at 532 nm, and the results were expressed in µmol/ml.

Estimation of plasma antioxidant enzymes

The activities of superoxide dismutase (SOD) and catalase (CAT) were analyzed using method described by Sun *et al.* [12] and by Aebi [13], respectively, measured as IU/gHb.

Estimation of plasma EPR ex vivo evaluation of nitric (NO•) radicals

Plasma NO• radicals were studied by methods of Yoshioka *et al.* [14] and Yokoyama *et al.* [15], adapted for EPR estimation of the spin-adduct formed between Carboxy- Ptio. K and generated radicals.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, Inc. and the results were expressed as means ± S.E. All data were expressed as mean ± SE and obtained by one-way ANOVA. $p>0.05$ was considered statistically significant. To define which

groups are different from each other we have used LSD post hoc test.

RESULTS AND DISCUSSION

Chronic oxidative stress and inflammation arise from the induction of bleomycin, due to the destructive production of free radicals and causing severe lipid peroxidation [7, 9]. Researches have been reported on isolation and protection of active biomolecules from plant antioxidants against drug damage and toxicity, as effective inhibitors of ROS and RNS [16, 17]. Many studies mentions plant antioxidants and small organic compounds as protectors and therapeutic agents, but the role of plant peptides, contained even in small amounts, it is not fully understood and explored. Vittal et al. [18] have reported a peptide-mediated inhibition of MK2 affects inflammatory and fibrotic responses, against BIPF. But none of the studies are focused on inhibition of free radicals generated by BLM and preventing the chronic damage by natural compositions, containing peptides.

In this study, we showed that the application of *C. longa* extract contains 0.1% peptide turmerin, pointedly inhibited chronic blood inflammation in BLM model and reduced oxidative stress levels, which was induced by free radical formation.

C. longa L. extract regulate body weight gain in the chronic toxicity model

The 28 days after the start of the experiment, the average weight of the BIPF chronic model showed a significant decrease ($p < 0.05$) compared to controls. In contrast, the use of plant extract in the C. longa treated group and the C. longa + Bleomicyn treated group significantly increased the weight. No significant differences were observed regarding daily food consumption (*results are not present*).

C. longa L. extract regulate plasma lipid peroxidation in the chronic toxicity model

To investigate the effects of *C. longa* L. extract on lipid accumulation, we measured the levels of MDA (Fig. 1), and total cholesterol (TC) (Fig. 2) in plasma from the four tested groups. However, plasmatic MDA and TC levels all significantly increased ($p< 0.05$) in the BLM model, compared with the untreated controls, and combination with *C. longa* extract correspondingly reduced the increased plasma lipid profiles. Consistent with these findings, we found no difference in the lipid peroxidation between the controls and *C. longa* extract-treated group.

According to our results, Chethankumar [9] found that pretreatment with peptide-containing *C. longa* extract showed 95% decrement in plasma lipid peroxidation. Hassan et al. [19] commend that *C. longa* ethanol extract inhibited lipid peroxidation process, in maximal concentration.

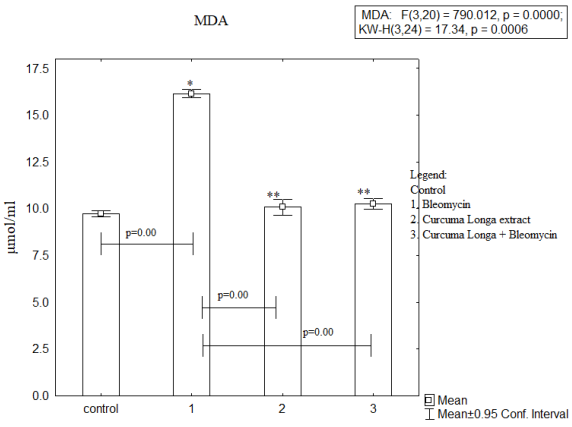

Fig. 1. Levels of MDA in plasma. *C longa* extract normalize plasma levels of MDA , lipid accumulation in chronic BIPF-model. Blood samples were collected from all sacrificed animals. The experiments were repeated three times. * $p < 0.05$ vs. the CG group; #$p < 0.05$ vs. the BLM group ($n = 6$).

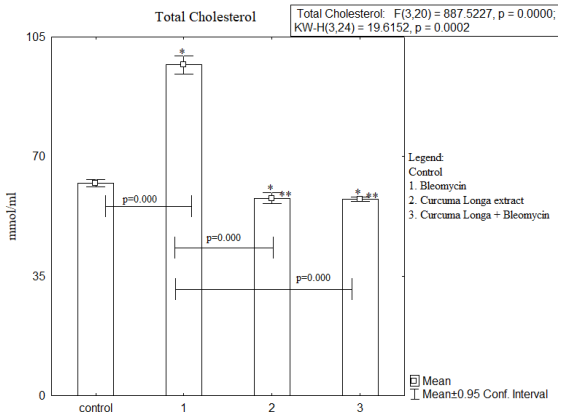

Figure 2. The TC in plasma. *C longa* extract normalize plasma levels of TC lipid accumulation in chronic BIPF-model. Blood samples were collected from all sacrificed animals. The experiments were repeated three times. * $p < 0.05$ vs. the CG group; #$p < 0.05$ vs. the BLM group ($n = 6$).

C. longa extract increased plasma antioxidant system function in the chronic toxicity model

Compared to the controls, plasmatic SOD and CAT activity were significantly decreased in the BLM group ($p<0.00$). The activity of SOD (Fig.3) and CAT levels (Fig. 4) increased in *C. longa* treated groups, compared to untreated controls. In addition, the Bleomicyn induced decrease in SOD and CAT activity recovered after treatment with *C. longa* extract.

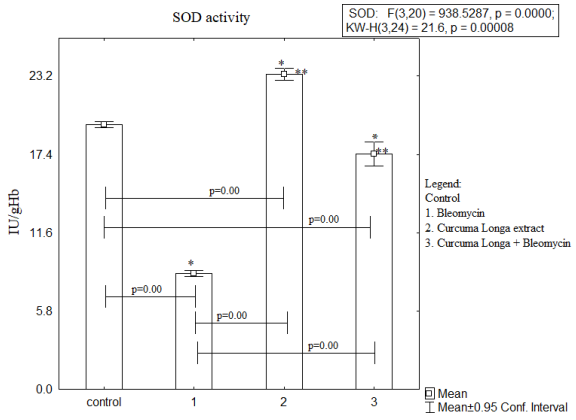

Figure 3. SOD levels in plasma. *C longa* extract and its constituent, turmerin regulate antioxidant system function and in chronic BIPF-model. Blood samples were collected from all sacrificed animals. The experiments were repeated three times. *p < 0.05 vs. the CG group; #p < 0.05 vs. the BLM group (n = 6).

As major antioxidant enzymes in the body, SOD converts and catalyzes the dismutation of superoxide anion radicals into H_2O_2, whereas CAT convert and reduced levels of H_2O_2 into water molecules [20]. In our experiment, SOD and CAT activity was shown to be statistically reduced in the Bleomicyn group, compared to controls ($p< 0.05$ vs the control). The loss of SOD and CAT activity probably reflects the increased oxidative stress, contributing to the chronic inflammatory state of the Bleomicyn administration. Interestingly, previous studies suggest that reduced SOD activity resulted in H_2O_2 lower levels, which thus directly affected CAT activity [20].

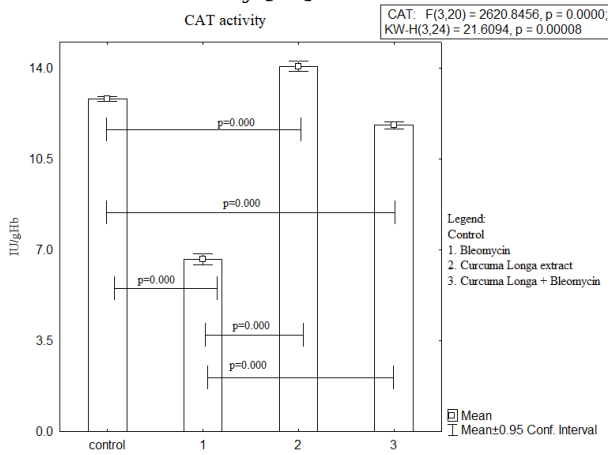

Figure 4. CAT levels in plasma. *C longa* extract and its constituent, turmerin regulate antioxidant system function and in chronic BIPF-model. Blood samples were collected from all sacrificed animals. The experiments were repeated three times. *p < 0.05 vs. the CG group; #p < 0.05 vs. the BLM group (n = 6).

In addition, *C. longa* extracts have been frequently studied with regard to protective functions. In the present study, the SOD and CAT activity was increased and normalized in the groups treated with *C. longa* extract alone and in *C. longa* + Bleomycin. These results indicate that *C. longa* extract, containing peptide turmerin with an antioxidant property are able to reduce H_2O_2 damages, inhibit inflammatory reactions and the induced chronic oxidative stress, caused by Bleomicyn [20-22].

C. longa extract regulate the oxidative redox imbalance and ex vivo nitric (NO•) radicals

Noguchi *et al.* [23] comment that increased expression of endogenous nitric oxide radicals (NO•) and all others NOS isoforms in plasma led to a deterioration of pulmonary fibrosis in a BLM-treated mice and in patients with pulmonary fibrosis. *Ex vivo* nitric oxide (NO•) is gaseous free radical, formed from its precursor, L-arginine [23] and was identified as a endogenous marker maintaining respiratory homeostasis, and play an important role in the development of pulmonary fibrosis [24]. To confirm the efficacy of *C. longa* extract, containing turmerin in neutralizing oxidative toxicity generated by Bleomicyn, NO• radical levels in blood plasma (Fig.5) were evaluated.

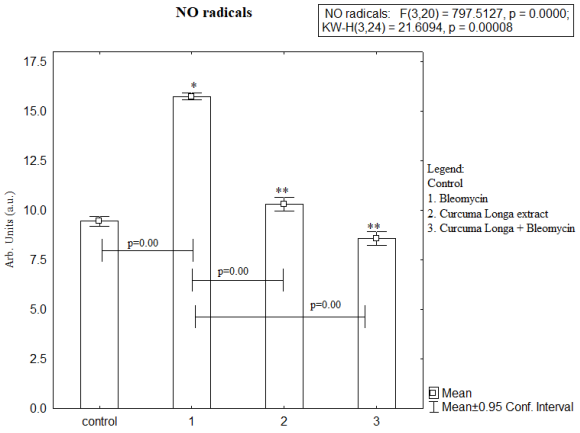

Figure 5. *Ex vivo* nitric oxide (NO•) free radical formation. Blood samples were collected from all sacrificed animals. Results were calculated by double integration of the corresponding EPR spectrum immediately registered in plasma (expressed in arbitrary units/ arbt. units). The experiments were repeated three times. *p < 0.05 vs. the CG group; #p < 0.05 vs. the BLM group (n = 6).

The results clearly demonstrate the toxic effects of BLM, and showed an almost two-fold increase of NO• radicals (15.78 ± 0.93 vs 9.558 ± 0.96 a.u., *p<0.004), in plasma relative to the controls. However, *C. longa* extract administration ameliorated the oxidative effect of BLM in

Bleomicyn intoxicated male mice (8.291 ± 0.7 vs 15.78 ± 0.93 a.u., *p<0.05).

Throughout this study, *C. longa* extract with active component peptide showed strong antioxidant activity and scavenging effect against the accumulation of ROS/ RNS, resulting in the protective effect against Bleomicyn induced chronic stress. In other hand, the protective activity of NO and all others NOS isoforms against BIPF have been demonstrated in different studies [23, 25, 26], and could be possible that NO/NOS may protect against the development of pulmonary fibrosis.

CONCLUSION

Finally, our results indicated that *C. longa* treatment stimulates endogenous antioxidant activity, reduced lipid peroxidation and scavenging nitric oxide (NO•). These results make it appropriate to propose the use of this plant extract as a possible addition to the treatment of acute and chronic pulmonary diseases associate with oxidative stress.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

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