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Hepatoprotective effects of *Tinospora cordifolia* extract against bleomycin-induced toxicity in mice

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Tinospora cordifolia (Willd.) Hook.f.&Thomson extract has previously been reported to alleviate appearance of liver alterations. The current study examined the antioxidant activity, therapeutic potential and action of the *T. cordifolia* extract to modulate and protect the liver alterations in bleomycin (BLM)-induced toxicity in ICR/w mice models. The hypothesis was that *T. cordifolia* extract would protect the liver alterations by inhibiting lipid peroxidation, lowering biochemical parameters, decreasing ROS production and reducing oxidative stress levels. Hepatocellular toxicity was induced by intraperitoneal injection of mice once daily with BLM (0.069 U/mL; 0.29 U/kg bw.) for a period of 4 weeks. The *T. cordifolia* was administered once a day for 4 weeks, 2 h prior at dose (80 mg/mL; 0.295 mg/kg/day). BLM intoxication produced oxidative stress in which the antioxidant system functioned incorrectly and ROS production significantly increased. The *T. cordifolia* extract provided significant hepatic protection against BLM toxicity by improving SOD, CAT ($p < 0.04$), MDA and total cholesterol (TC) levels and decreasing ROS in the group receiving BLM ($p < 0.05$), leading to reduced membrane lipid peroxidation. In conclusion, the *T. cordifolia* extract facilitated recovery from BLM-induced hepatic injury by suppressing oxidative stress damages. Therefore, the *T. cordifolia* stimulates antioxidant-scavenging activity and lipid peroxidation reduction in liver. Our results make it appropriate to propose the use of the *T. cordifolia* extract as a possible addition to the treatment of chronic liver alterations associated with BLM-induced toxicity.

Keywords: *T. cordifolia;* oxidative-scavenging imbalance; hepatotoxicity, mice.

INTRODUCTION

Tinospora cordifolia (Willd.) Hook.f. & Thomson. (*T. cordifolia, Guduchi*) belongs to the family Menispermaceae and is used as a protective antioxidant. Ayurveda, India's traditional health system, recommends that the whole plant be used for therapeutic purposes. The extract from *T. cordifolia* has various active components in the structure such as alkaloids, steroids, diterpenoid lactones, aliphatics, glycosides, etc [1]. Moreover, *T. cordifolia* inhibits lipid peroxidation [2], stimulates bile secretion, activates immune effector cells, e.g., differentiation of T cells and B cells [3] and has diuretic properties. Some experimental studies indicate that *T. cordifolia* extract significantly reduces chemotherapy–induced toxicity, cell membrane oxidation, and has a protective role against neurodegenerative changes in the rat hippocampus [4, 5]. In addition, Sharma and Padney [6] have determined that *T. cordifolia* extract has strong antioxidant, anti-inflammatory, anti-arthritic, anti-allergic, anti-diabetic, antimalarial, immunomodulatory, antineoplastic and hepatoprotective properties. There is also evidence that *T. cordifolia* extract reduces damage to cellular oxidative stress and has free radical scavenging activity against reactive oxygen and nitrogen species (ROS/RNS) [7]. In the study of Sangeetha *et al.* [7] the antioxidant activity of *T. cordifolia* was attributed to the presence of tannins and phenolic compounds. The presence of alkaloids such as choline, tinosporin, isocolumin, etc. in aqueous or alcoholic extract of *T. cordifolia* shows detoxification effects and protection against toxin-induced disorders in the mice kidneys [8]. Treatment with *T. cordifolia* extract effectively increases intestinal absorption, hepatoprotection and the regulated alcohol-induced multivitamin deficiency [9]. Phytochemical analysis has shown that *T. cordifolia* extract protects carbon tetrachloride hepatotoxicity in animals [10, 11], and alleviates cisplatin –induced nephrotoxicity *in vivo* [11, 12]. Although *T. cordifolia* extract has different medicinal properties, its protective role has not yet been evaluated against bleomycin-induced toxicity, if any.

Bleomycin (BLM), is an antitumor antibiotic that has been identified as a medicine that induces

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reactive oxygen species (ROS/ $\bullet O^{-2}$, H_2O_2, •OH, NO•); cell membrane instability, lipid and protein peroxidation, inflammatory responses in the lung (fibrosis) [13]; and as a result toxic products are generated [13, 14].

The present study attempts to elucidate the hepatoprotective efficacy of *T. cordifolia* extract in regulation against experimentally BLM-induced toxicity on free radical production and changes in oxidative stress in the liver cells of male IRC/ w mice.

MATERIALS AND METHODS

Chemicals and preparation of T. cordifolia extract

Bleomycin sulfate (EP 9041-93-4), Carboxy-Ptio.K, and other chemicals were purchased from Sigma Aldrich Co., USA, and were of analytical grade. *T. cordifolia* fine powder (ABC Limited, India; identified by a plant taxonomist) was kinetically extracted (for 48 h in 100% ethanol, v:v). The total filtrate was dried using a rotary evaporator (Buchi B-480, India) at 400c and was lyophilized (Iishin Lab Co. Ltd, USA) to crude extract. The *T. cordifolia* extract was stored in air-tight glass bottle at 8°C, and it was used as a practical approach to protect against BLM- intoxication.

Maintenance of animals

Male ICR/w mice weighing approximately 45-50 ± 3.0 g were obtained from the 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 BFSA and Trakia University, Stara Zagora, Bulgaria (131/ 6000-0333/ 09.12.2016). The mice were housed in polypropylene cages at a temperature of 18–20°C and under a light/dark period of12/12 h daily. They were fed on a standard commercial feed (Indusrial, Bulgaria), after 10 days of acclimatization and free access to tap water. The essential cleanliness conditions were also maintained. The lyophilized *T. cordifolia* was dissolved in distilled H_2O and preserved at 4^0C until use.

Experimental protocol

Mice were divided into 4 groups (n=6) for a period of 29 days and drugs administration were through intraperitoneal (i.p.) injection as follows:

I) Group I (CG) (no treatment);

II) Group II (BLM) (BLM 0.069 U / ml; 0.34 U / kg body weight in saline (250 µl) was given i.p. and completed on day 16. After day 17 the animals were given BLM at a schedule up to the 28th day [15];

III) Group III (*T. cordifolia extract* 80 mg/ml; 0.295 mg/kg body weight in distilled H_2O (250 µl) was given once daily i.p. continued on schedule for up to 28 day);

IV) Group IV (*T. cordifolia* +BLM) *T. cordifolia* extract (80 mg/ml) + BLM (0.34 U/kg) (antioxidant was injected once daily 2 h before the antibiotic and continued on schedule for up to 28 days).

Additionally, the physiological status and behavior of animals were monitored daily. After the last drug administration, the mice were given rest and on the next day, they were sacrificed under anesthesia (Nembutal 50 mg/kg i.p.). The liver samples were removed, washed in phosphate buffer saline (pH=7.4, 4°C) homogenized and analyzed for biochemical parameters, and ROS production. The fresh blood (1.1-1.5 cm^3) was collected directly from the heart in cold plasma-containers (5 cm^3 Monovette, Germany). After centrifugation of blood samples at 4000 rpm, 4°C for 10 min, 200 µl of plasma from each group were investigated directly for TC estimation.

Biochemical analyses of hepatocellular antioxidant status

The liver tissue lipid peroxidation (malondialdehyde concentration (MDA)) was estimated by the method of Plaser *et al.*, 1966 [16], and the activities of superoxide dismutase (SOD) and catalase (CAT) were analysed using the method described by Sun *et al*., 1988 [17] and by Aebi, 1984 [18], respectively. The TC in blood was estimated using a commercially available diagnostic kit (AM-2035-KA, 2017). The biochemical analyses were performed on a UV–VIS spectrophotometer-400 (TERMO Sci., RS232C, Stratagene, USA).

Electron paramagnetic resonance (EPR) in vivo evaluation of ROS production

ROS production in the liver samples was investigated by *in vivo* EPR (X-Band, Emx^{micro} spectrometer, Bruker) method according to Shi *et al*. (2005) [19]. Briefly, to 100 µl plasma and 100 mg of spleen were added 900 µl of 50 mM N-t-butyl-alpha-phenylnitron (PBN) dissolved in dimethyl sulfoxide (DMSO) and centrifuged at 4000 rpm/ 10 min at 4ºC, with settings: 3505 g centerfield, 6.42 mw microwave power, 5 g modulated amplitude, 1-5 scans. All experiments were made in triplicate.

Statistical analysis

The processing of the spectra was performed using Bruker Win-EPR and Sim-fonia software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student- t- test to determine significant difference among data groups. The data were expressed as means ± standard error (SE). A value of p< 0.05 was considered significant.

RESULTS AND DISCUSSION

Changes in biochemical enzymes after exposure to BLM, both alone and in combination with *T. cordifolia* extract, showed a significant change in oxidative / pro-oxidative activity. BLM exposure (Fig. 1) produced statistically significant decrease in SOD (6.88±0.46 IU/gHb, *p*<0.03) and CAT (5.433±0.91n IU/gHb, p<0.05) activities, compared to CG (16.28±1.35 IU/gHb). In addition, *T. cordifolia* extract showed a statistically significant increase in the levels of both antioxidant enzymes (SOD: 18.49±3.16 IU/gHb; CAT: 14.83±1.21 IU/gHb,), compared to untreated CG (p<0.05) and to BLM treated (p<0.05) group. Moreover, administration of the *T. cordifolia* extract 2 h before BLM treatment showed a protective effect on the hepatic cells in SOD (p<0.05) and CAT (p<0.003) activities.

The BLM –induced toxicity increased oxidative stress disorders and inflammatory responses, due to the destructive free-oxygen production and leading to highly lipid peroxidation. Experimentally, BLM has been used to induce chronic toxicity in mice models at a dose of 0.069 U/mL; 0.29 U/kg bw dissolved in saline and to produce oxidative hepatocellular changes [21].

A number of studies have reported the isolation and protection of active biomolecules from plant antioxidants against chemotherapy-induced damage and toxicity [6, 20, 22] as effective inhibitors of ROS. There is evidence that plant extracts containing alkaloids and glycosidic compounds are potent inhibitors of various oxidative processes, exhibit significant antioxidant activity, prevent lipid peroxidation and restore SOD and CAT activity [23].

Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion ($\bullet O_2^-$) to H_2O_2 and O_2, while catalase (CAT) reduces the H_2O_2 levels into H_2O molecules [24]. In our experiment, it was shown that SOD activity and CAT activity were statistically significantly decreased in the BLM group compared to controls (p <0.05 *vs*. CG).

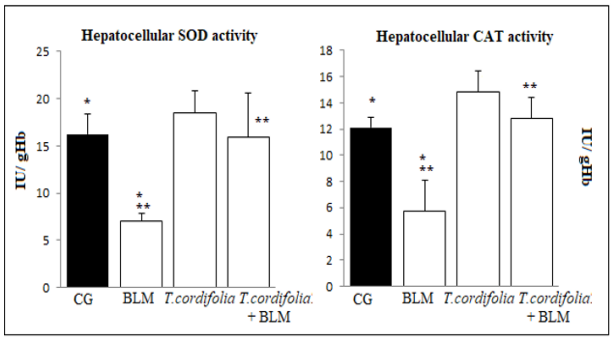

Figure 1. Levels of SOD and CAT activity in liver homogenates. *T. cordifolia extract* and its constituents, alkaloids and glycosides, regulate antioxidant biochemical enzymes in chronic BLM-model. Liver samples were collected from all sacrificed animals. The experiments were repeated three times. *p < 0.05 *vs*. the CG group; **p < 0.05 *vs*. the BLM group (n = 6).

The decrease in endogenous antioxidant enzymes is likely to be associated with increased oxidative damage that contributes to the inflammatory response of BLM administration. In addition, *T. cordifolia* extract contains alkaloids and glycosides and has the potential to reduce oxidative stress damage by inactivating H_2O_2 and by inhibiting inflammatory responses.

These results simultaneously support the claim that treatment with 80 mg / mL *T. cordifolia* provides protection against the effects of BLM-induced stress; and indicate the protective role of *T. cordifolia* in liver tissues [6, 25].

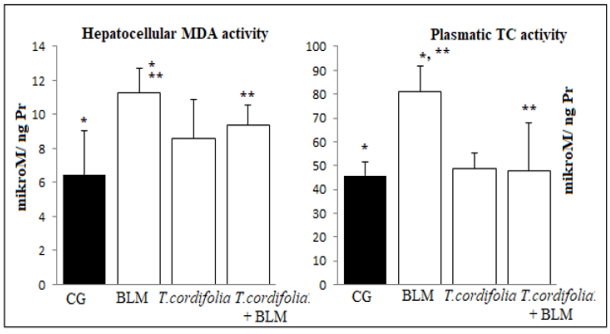

Figure 2. Levels of MDA in liver homogenates and levels of TC in plasma. *T. cordifolia extract* and its constituents normalize hepatocellular/ plasmatic levels of lipid accumulation in chronic BLM-model. Liver samples were collected from all sacrificed animals. *C longa* extract BIPF-model. The experiments were repeated three times. *p < 0.003 and p < 0.05 *vs*. the CG group, respectively; **p < 0.05 *vs*. the BLM group (n = 6).

Oxidative stress is associated with an imbalance between the production and purification of ROS products. ROS overproduction and BLM-induced oxidative damages contribute to hepatocyte injuries and these processes increase cell lipid damage and induce hepatic cell malformation [26, 27]. To investigate the effects of *T. cordifolia* extract on

hepatic lipid accumulation, we measured MDA levels in liver homogenates and total plasma cholesterol (TC) in all tested groups (Fig. 2).

However, MDA (11.6±4.16 μM/ng Pr *vs*. 6.16±1.03 μM/ng Pr *vs*; p< 0.003, t-test) and TC (80.56±11.12 μM/ng Pr *vs*. 45.7±7.03 μM/ng Pr; p< 0.05, t-test) levels all significantly increased in the BLM model, compared with the CG. The combination of *T. cordifolia* and BLM (80.56±11.12 μM/ng Pr *vs*. 45.7±7.03 μM/ng Pr; p< 0.05, t-test) correspondingly reduced the increased plasma lipid concentrations, in MDA (9.253±0.91 μM/ng Pr *vs*. 11.6±4.16 μM/ng Pr; p< 0.05, t-test) and TC (47.56±11.12 μM/ng Pr *vs*. 80.56±11.12 μM/ng Pr; p< 0.05, t-test), compared to BLM treatment. Consistent with these findings, we found comparable values in the plasmatic lipid peroxidation between the *T. cordifolia* extract and controls. In accordance with our results, other investigations report inhibition in the lipid peroxidation process, prevention of tissue damages thereby maintaining the membrane integrity and free-radicals reduction in chemo-induced toxicity, after *T. cordifolia* extract application [28-30].

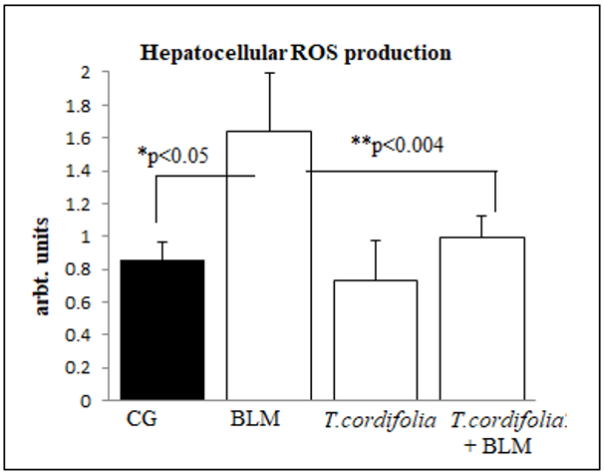

Figure 3. *In vivo* ROS radical production. Liver samples were collected from all sacrificed animals. Results were calculated by double integration of the corresponding EPR spectrum immediately registered in liver homogenates (expressed in arbitrary units/ *arbt. units*). The experiments were repeated three times. *p < 0.05 *vs.* the CG group; **p < 0.004 *vs.* the BLM group (n = 6).

Banerjee *et al*. [31] commented that intracellular, endogenous ROS expression in peripheral blood of patients suffering from persisting polyarthralgia post CHIK infection was significantly scavenged by *ex vivo* treatment with *T. cordifolia* leaf extract. To confirm the efficacy of *T. cordifolia* extract containing alkaloids and glycosides, reduction of the BLM-induced toxicity in liver homogenates was evaluated. Figure 3 shows the EPR spectra of ROS products in liver homogenate measured in arbitrary units.

The results demonstrate the highly toxic effects of BLM administration, and showed a statistically significant increase of ROS production in hepatic cells (1.72 ± 0.901 *vs* 0.858 ± 0.21 a.u., p<0.05, *t*-test), relative to the CG. However, the ROS products levels were close to that in CG in the group treated with *T. cordifolia* (0.739 ± 0.14 *vs*. 0.858 ± 0.21 a.u., t-test), or with a combination of *T. cordifolia* + BLM (0.997 ± 0.33 *vs*. 0.858 ± 0.21 a.u., t-test). The EPR method indicated an increased ROS concentration in hepatocytes. The statistically significant decrease in ROS production in hepatic cells was observed in *T. cordifolia* + BLM combination (0.923 ± 0.5 a.u. *vs* 1.72 ± 0.901, p<0.004, *t*-test), in comparison to the BLM administration. However, *T. cordifolia* extract administration completely ameliorated the ROS production and hepatic pro-oxidative effect in BLM-intoxicated mice (p < 0.05). Different investigations have suggested that the plant extract has a protective effect against damages in hepatic function due to direct antioxidant [32] and free radical scavenging mechanisms and regulation of ROS production [31-33]. Moreover, Baskaran *et al*. [25] reported that *T. cordifolia* extract regulates free radicals levels and lipid peroxidation by countering Cd-induced oxidative stress and by controlling enhanced ROS production effected over tissue glycoproteins in liver cells and hepatotoxicity.

CONCLUSION

Finally, our results indicated that *T. cordifolia* extract treatment stimulated endogenous antioxidant activity, reduced lipid peroxidation and scavenged ROS products. These results make it appropriate to propose the use of the *T. cordifolia* extract as a possible addition to the treatment of chronic hepatotoxicity associate with chemo-induced oxidative damages.

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

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