Dual Mode of Action of *Talaromyces purpureogenus* CFRM02 Pigment to Ameliorate Alcohol Induced Liver Toxicity in Rats

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Abstract

*Talaromyces purpureogenus* CFRM02 pigment exhibited antioxidant activity by scavenging free radicals. The alcohol feeding leads to free radical generation causing pathophysiological processes of alcoholic liver disease (ALD) and alcoholic hepatitis. *T. purpureogenus* CFRM02 pigment administered to rats ameliorated the ALD by scavenging ROS. The haematological analysis revealed the increased neutrophil circulation. The neutrophil infiltration was observed in the hepatocytes of the rats fed with pigment (600 mg/kg body weight). The increase in the number of neutrophils helps in liver regeneration caused by alcoholic hepatitis. The dual mechanism of action of pigment, antioxidant and liver regeneration through neutrophil production is attributed to alleviate the ALD. These results suggested that *T. purpureogenus* CFRM02 pigment represents the protective and therapeutic strategy against ALD.

Keywords *Talaromyces purpureogenus* · Pigment · Alcohol-toxicity · Hepatoprotective · Free radical · Neutrophils

Key Points *T. purpureogenus* CFRM02 pigment scavenged ROS and increased neutrophils. Antioxidant and liver regeneration by neutrophil mediated mechanism of action. Pigment signifies a protective and therapeutic effect against ALD.

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Introduction

The World Health Organization Global Status Report on Alcohol and Health (2018) states that more than 3 million people died as a result of harmful practice of alcohol in 2016. Alcoholic liver disease (ALD) is caused by prolonged high alcohol intake and contributing significantly to the prevalence, illness and mortality. ALD is a major etiologic factor in causing fatty liver, alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. The excess accumulation of the metabolic end-products of the alcohol metabolism can cause oxidative stress, lipid peroxidation, inflammation and promote fat accumulation [2]. Progression of ALD is a multifactorial and multistep process that involves alcohol metabolism and secondary mechanisms such as oxidative stress, endotoxin, cytokines and immune regulators [3]. However, the pathogenic and systemic inflammation mechanism underlying the development of ALD is unclear/unknown [2, 4]. There are emerging evidences that alcohol induced oxidative stress plays a very important role in the pathogenesis through free radicals and inflammatory response that directly causes liver damage [2, 5, 6]. Monascus purpureus NTU 568 fermented rice alleviates ALD injury by inhibiting oxidative stress and inflammation [7]. Compounds isolated from the culture extracts of Talaromyces and Penicillium sp. have shown their potential as antioxidative, anticancer and antimicrobial agents [8, 9]. The T. purpureogenus CFRM02 red pigment has demonstrated the various mechanism of antioxidant activity and modulated the harmful effects of ROS by scavenging the free radicals generated in vitro [10, 11]. The fungal pigments continue to provide new chemical entities showing novel biological activities. These microbial metabolites can find applications against infectious diseases and metabolic disorders, including T lymphocytes and neutrophils [12]. Recent studies have demonstrated the nontoxic effects of T. purpureogenus CFRM02 pigment [11]. While the ameliorative effect of T. purpureogenus CFRM02 pigment against pathogenesis of ALD remains unclear. Based on previous results, we hypothesized hepatoprotective effects through an antioxidant mechanism. Interestingly we also observed the neutrophil infiltration in hepatocytes. Apparently, the neutrophil infiltration regenerated the liver against alcohol induced toxicity in rats.

Materials and Methods

Chemicals

The bengal gram husk (BegH) was procured from the local market. Analytical grade solvents hexane and methanol were purchased form Merck, Mumbai, India. The culture medium for cultivation of T. purpureogenus CFRM02 such as potato dextrose agar (PDA) was obtained from Hi-Media Laboratories, Mumbai, India. Dihydroethidium (Hydroethidine) and TRIS (hydroxy methyl) amino methane (Trizma base) were obtained from Thermo Fisher Scientific and Sigma Chemical Co., St. Louis, MO, USA respectively.

Sample Preparation and Animal Experiment

The red pigment was extracted form T. purpureogenus CFRM02 fermented BegH [13]. The experimental protocol was approved by Institutional Animal Ethical Committee of CSIR-Central Food Technological Research Institute, Mysuru-570,020 (IAEC No CFT/IAEC/83/17) as per the guidelines of Committee for Control and Supervision of Animals
Thirty male Wistar rats (Rattus norvegicus \( \approx 150 \text{ g, } 4–5 \text{ week old} \) were housed 3 per cage and maintained at a controlled ambient temperature (23 \( \pm 2 \text{ °C} \)). The rats were randomly divided into five groups with 6 rat in each. Group A (Control): Normal Diet without alcohol; Group B (ALD): Normal Diet + ethanol group (4 g/kg/day); Group C: Normal Diet + ethanol (4 g/kg/day) + Metadoxine (160 mg/kg/day); Group D (Rx1): Normal Diet + ethanol (4 g/kg/day) + red pigment (300 mg/kg/day); Group E (Rx2): Normal Diet + ethanol (4 g/kg/day) + red pigment (600 mg/kg/day). The control group was given orally an equivalent volume of distilled water. At the end of experimental period (after 6 weeks), the rats were euthanized and blood was collected for further analysis.

### Haematological Analysis

Packed Cells Volume (PCV), Hemoglobin (Hb), Platelet (PLT), Total White Blood Cell Count (TWBC) were determined directly using a fully automated haematology analyser (Pentra-XL 80, Horiba ABX, USA) for blood cell profiling.

### Fluorescence Detection of Free Radicals

The frozen liver tissue blocks were fixed and 5–8 \( \mu \text{m} \) sections were obtained using CM 3050 S cryo-microtome. From each group, several successive sections were taken for microscopic observation. For in situ detection of ROS in the liver, dihydroethidium was used. To compare the oxidative stress in liver tissues, ROS levels were imaged using dihydroethidium (Ex = 588 nm and Em = 615 nm). Nonfluorescent dihydroethidium is oxidized by ROS to yield red fluorescent product. The images were then captured using a fluorescence microscope (Nikon, Tokyo, Japan), 10x objective, equipped with DFC 300 FX digital camera [14].

### Histopathology

The histopathological changes of the liver were observed by light microscopy. Liver tissues were fixed in 10% formalin, and sections at 5\( \mu \) were stained with hematoxylin and eosin.

### Statistical Analysis

All experiments were performed in triplicate, the results expressed as the mean \( \pm \) SEM and statistical differences between samples was determined by Duncan’s multiple range test. The \( p \) values < 0.05 were regarded as statistically significant. The statistical analyses were performed using Graph Pad Prism 7 (Graph Pad Software, Inc., La Jolla, CA).

### Results

#### Haematological Parameters

The haematological parameters of ALD induced rats and treated with red pigment are presented in Table 1. Rats fed with alcohol showed increase in haemoglobin (Hb)
concentration (15.34 ± 0.15 g/dL), and decrease in WBC concentration (13.37 ± 0.21 10^3/µL) compared to the rats fed with alcohol only. Whereas the increase in the red blood cell (RBC) count (9.37 ± 0.12 10^6/µL) was observed in the alcohol fed group compared to the control, standard and pigment fed groups. The MCV in rats fed with pigment (57.14 ± 1.18) and metadoxine (53.36 ± 1.24) were higher as compared to alcohol (51.91 ± 1.30 fL) fed groups. Similarly the HCT (44.34 ± 0.24 and 43.56 ± 0.28%) and MCH (18.12 ± 0.16 and 18.14 ± 0.05 pg) concentrations were significantly (p < 0.05) higher in the rats fed with pigments and metadoxine compared to the rats fed with alcohol (42.6 ± 0.48% and 17.36 ± 0.13 pg) respectively. The platelet counts were significant higher in the rats fed with pigment (870.6 ± 18.4 10^3/µL) and metadoxine (739.2 ± 25.3 10^3/µL) compared to the control (563.6 ± 52.9 10^3/µL) and alcohol (613.6 ± 41.3 10^3/µL) fed groups. Interestingly, no significant difference was observed among control, alcohol and metadoxine fed rats, but the neutrophils counts were significantly higher in the rats fed with the pigment (30.0 ± 1.52%). The results indicated that treatment of *T. purpureogenus* CFRM02 red pigment increased the Hb, MCV, platelets and neutrophils number compared to the alcohol fed group.

### ROS in Liver Tissue

Investigating the change of ROS contents was important to know the degree of damage in the liver tissue, and ROS are critical mediators of liver damage. In the current investigation, it was observed that ALD caused oxidative stress in the liver as revealed by increasing in ROS levels production (Fig. 1). The microscopic studies revealed that,

| Groups | Control | Alcohol | Standard | RX1 | RX2 |
|--------|---------|---------|----------|-----|-----|
| Hb (g/dL) | 13.56 ± 0.15^a | 14.26 ± 0.15^b | 15.52 ± 0.26^c | 14.32 ± 0.22^b | 15.34 ± 0.15^c |
| RBC (10^6 / µL) | 7.29 ± 0.12^a | 09.37 ± 0.12^d | 08.37 ± 0.16^c | 8.57 ± 0.12^c | 07.64 ± 0.25^b |
| WBC (10^3 / µL) | 10.39 ± 0.17^b | 14.36 ± 0.33^b | 14.14 ± 1.58^b | 13.45 ± 0.16^b | 13.37 ± 0.21^b |
| MCV(µL) | 53.44 ± 1.95^b | 51.91 ± 1.30^a | 53.36 ± 1.42^a | 56.05 ± 1.35^bc | 57.14 ± 1.88^c |
| MCH (pg) | 18.48 ± 0.27^c | 17.36 ± 0.13^a | 18.12 ± 0.16^b | 18.28 ± 0.08^bc | 18.14 ± 0.05^b |
| MCHC (%) | 35.26 ± 0.05^c | 32.68 ± 0.22^a | 33.32 ± 0.22^b | 35.4 ± 0.25^c | 35.32 ± 0.19^c |
| HCT (%) | 38.56 ± 0.34^a | 42.6 ± 0.48^b | 43.56 ± 0.28^c | 46.3 ± 0.16^a | 44.34 ± 0.24^d |
| PLT (10^3 / µL) | 563.6 ± 52.9^a | 613.6 ± 41.3^b | 739.2 ± 25.3^c | 950.6 ± 32.2^e | 870.6 ± 18.4^d |
| Neutrophils (%) | 21.27 ± 1.23^a | 21.27 ± 1.00^a | 20.00 ± 1.52^a | 20.00 ± 1.25^a | 30.00 ± 1.52^b |
| Monocyte (%) | 1.17 ± 0.26^a | 1.30 ± 0.16^a | 1.20 ± 0.40^b | 1.07 ± 0.13^a | 1.20 ± 0.40^b |
| Eosinophils (%) | 2.00 ± 0.11^c | 1.00 ± 0.10^a | 1.54 ± 0.12^b | 2.17 ± 0.23^c | 1.54 ± 0.12^b |
| Basophils (%) | 0.00 ± 0.00^a | 0.00 ± 0.00^a | 0.00 ± 0.00^a | 0.00 ± 0.00^a | 0.00 ± 0.00^a |

Hb – haemoglobin, RBC – red blood cells, WBC – white blood cells, MCV – Mean Corpuscular Volume, MCH – Mean Cell Corpuscular Haemoglobin; MCHC – Mean Cell Corpuscular Haemoglobin Concentration, Hematocrit (Hct), PLT – platelet. DC – differential count, dL, deciliter; fL, femtoliter; µl, microliter; pg, picrogram; Data are expressed as mean ± S.D (n = 6). One way ANOVA Statistical analysis performed using SPSS 25.0 software. All the groups were compared using Duncan’s test for differentiation at p ≤ 0.05
there was strong observable red (DHE) fluorescence (Fig. 1) emission from the liver cryo sections in the alcohol consumed group, indicating the generation of free radicals. The generated free radicals causes cell damage through lipid peroxidation and protein oxidation mechanism that disposes tissue damage in the liver. Whereas the generation of free radicals was significantly inhibited in the rats fed orally with alcohol and red pigment group. These results indicated the red pigment scavenged the free radicals generated due to the alcoholic oxidative stress.

**Histopathology of Rat Hepatocytes**

The rats fed with the alcohol has shown the discolouration of liver (Fig. 2). The alcoholic effects on liver were normalised by feeding the pigment to the rats as compared to the control and metadoxine. The histological section of control rat liver has shown regular morphology, neat arrangement, distinct nuclei and well-defined cell boundaries (Fig. 2). The hepatocytes of the rats fed with ethanol were noticeably injured, with cellular degeneration, fatty vacuolation, and other negative changes (Fig. 2 EtOH). Whereas in hepatocytes fed with low and high dose of pigment have shown the decreased necrotic cells and fatty vacuoles. The integrity of cellular boundaries increased and hepatocytes resemble to have normal structure as compared to the control and metadoxine treated rats. The rats fed with metadoxine and 300 mg did not show any infiltration of neutrophils. While the infiltration of neutrophils in the hepatocytes were observed in rats fed with 600 mg pigment (Fig. 2). The haematological analysis also have evidenced the increase in neutrophil concentration (Table 1).
Discussion

There are no efficacious therapeutic strategies for ALD treatment [15, 16]. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) has supported many consortia to conduct clinical trials to examine novel targets and to develop viable treatments for the management of ALD through drugs and nutrition [17]. Accordingly, drugs were classified based on mechanism of action like, (a) acting on the gut-liver axis, (b) anti-inflammatory agents, (c) antioxidants, and (d) regenerative benefits [15].

The T. purpureogenus CFRM02 red pigment have demonstrated the various mechanism of antioxidant activity and scavenged the free radicals generated in vitro and in vivo [10, 13]. Alcohol related oxidative stress plays multiple roles in liver disease pathogenesis [6]. This leads to the accumulation or generation of free radicals or reactive oxygen species (ROS) as observed in the Fig. 1. The level of pro-inflammatory and anti-inflammatory genes (supplementary Table 1) expression were affected due to ROS generation in alcohol fed rats and normalised by feeding the T. purpureogenus CFRM02 pigment (data not shown). For the treatment of ALD, metadoxine, pentoxifylline and several other drugs are prescribed as antioxidant. Similarly, the antioxidant mechanism was observed in the treatment of ALD by the drugs N-aceytyl cysteine but no long term effect was observed. The fluorescence microscopic studies of liver sections revealed a significant reduction in ROS in rats treated with metadoxine and fed with pigment compared to the ALD group (Fig. 1). These results confirmed T. purpureogenus CFRM02 pigment the ameliorate of ALD through antioxidant mechanism.

Interestingly, neutrophil concentration was increased by non-infectious cytokine cascade in rats fed with pigment (Table 1). Whereas, rats treated with metadoxine drug did not show any neutrophil elevation (Table 1). Neutrophils are the largest population of circulating leukocytes and play critical roles in immunity, inflammation, free radicals and cytokines production [18]. Even though many reports are on the negative roles of neutrophils in alcoholic hepatitis, but some studies have drawn different conclusions. The neutrophils are able to clear necrotic hepatocytes and essential source of hepatocyte growth factor (HGF) in patients with severe alcoholic hepatitis, thus participating in hepatocyte

![Fig. 2 Effects of T. purpureogenus CFRM02 red pigment extract and metadoxine in alcohol-induced liver disease in rats fed with ethanol. The liver colour has changed from dark red to pale red (above lane). Hema-toxylin and eosin staining shows neutrophil infiltration (arrows) in the liver of red pigment extract fed (600 mg/kg bw) rats (bottom lane)](image-url)
regeneration in severe AH [19]. It has been reported that, treatment of granulocyte colony stimulating factor (G-CSF) increase neutrophil counts and improve their function, there by substantially increase the survival of patients with severe alcoholic hepatitis or liver failure [20]. Similarly, in this study, neutrophils infiltration in the hepatocytes has been observed (Fig. 2, 600 mg) in the rats fed with red pigment extract of T. purpureogenus CFRM02 (Table 1). This kind of mechanism of ALD amelioration was observed in the pharmaceutical agent pilgrastism [16], but not the antioxidant mechanism. The results of this studies have demonstrated the amelioration of ALD by scavenging free radicals (as antioxidant) generated due the alcoholic stress. The increased neutrophils concentration could help in the liver regeneration caused by alcoholic hepatositosis (AH). Our earlier reports on T. purpureogenus CFRM02 pigment have shown antioxidant and non toxic characteristics [10, 11].

In conclusion, the alcoholic hepatitis in rats was controlled by feeding the red pigment by dual mode of action, (i) antioxidant and (ii) through neutrophil infiltration. The hepatoprotective effects of T. purpureogenus CFRM02 red pigment to ameliorate ALD suggested the applications in food and nutraceuticals.

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**Author Contributions** SGP: Methodology, Software, Investigation, Data curation, Writing – original draft, KPMR: Investigation, Visualization MHP: Conceptualization, Methodology, Resources, Writing- review & editing. MSP: Methodology, Resources, Data curation, review & editing. MD: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, funding acquisition.

**Declarations**

**Ethical Approval** The experimental protocol was approved by Institutional Animal Ethical Committee of CSIR-Central Food Technological Research Institute, Mysuru-570020 as per the guidelines of Committee for Control and Supervision of Animals for Experiments (CPCSEA), Ministry of Environment, Forests, and Climate Change, Government of India.

**Consent to Participate** I confirm that the final manuscript has been seen and approved by all the authors. The undersigned author transfers all copyright ownership of the manuscript to Applied Biochemistry and Biotechnology in the event the work is published.

**Consent for Publication** We hope that you will find our manuscript acceptable for publication in the above journal.

**Conflict of Interest** The authors declare no competing interests.

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