Dual mode of action of *Talaromyces purpureogenus* CFRM02 pigment to ameliorate alcohol induced liver toxicity in rats

Saritha Gopal Pandit

Department of Microbiology & Fermentation Technology, CSIR- Central Food Technological Research Institute, Mysuru 570 020, Karnataka, India

Krishna Prashanth Ramesh Mekala

Department of Microbiology & Fermentation Technology, CSIR- Central Food Technological Research Institute, Mysuru 570 020, Karnataka, India

Mohankumari H. Puttananjaiah

Grain Science & Technology, CSIR- Central Food Technological Research Institute, Mysuru 570 020, Karnataka, India

Muthukumar Serva Peddha

Department of Biochemistry, CSIR- Central Food Technological Research Institute, Mysuru 570 020, Karnataka, India

Mohan A Dhale (✉ dhalemohan@yahoo.com)

Department of Microbiology & Fermentation Technology, CSIR- Central Food Technological Research Institute, Mysuru 570 020, Karnataka, India

Short Report

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Abstract

Talaromyces purpureogenus CFRM02 pigment exhibited antioxidant activity by scavenging free radicals. The alcohol feeding lead to free radical generation causing pathophysiological processes of alcoholic liver disease (ALD) and alcoholic hepatitis. The *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 number of neutrophils help in the 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 *T. purpureogenus* CFRM02 pigment represents a novel protective and therapeutic strategy against ALD.

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 (Lieber 2004). The excess accumulation of the metabolic end-products of the alcohol metabolism can cause oxidative stress, lipid peroxidation, inflammation and promote fat accumulation (Ceni et al. 2014). Progression of ALD is a multifactorial and multistep process, involves alcohol metabolism and secondary mechanisms such as oxidative stress, endotoxin, cytokines and immune regulators (Seth et al. 2011). However, the pathogenic and systemic inflammation mechanism underlying the development of ALD is unclear/unknown (Ceni et al. 2014; Gustot and Jalan 2019).

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 (Ceni et al. 2014; Dey and Cederbaum 2006; Tan et al. 2020). *Monascus purpureus* NTU 568 fermented rice alleviates ALD injury by inhibiting oxidative stress and inflammation (Cheng and Pan 2011). Compounds isolated from the culture extracts of *Talaromyces* and *Penicillium* sp. have shown their potential as antioxidative, anticancer and antimicrobial agents (Liu et al. 2016; Nicoletti and Trincone 2016). The *T. purpureogenus* CFRM02 red pigment have demonstrated the various mechanism of antioxidant activity and modulated the harmful effects of ROS by scavenging the free radicals generated *in vitro* (Pandit et al. 2019; Pandit et al. 2020). 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 (Sarin et al. 2019). Recent studies have demonstrated the nontoxic effects of *T. purpureogenus* CFRM02 pigment (Pandit et al. 2020). While the ameliorative effect of *T. purpureogenus* CFRM02 pigment against pathogenesis of ALD remains unclear. Based on previous results, we hypothesized hepatoprotective effects through 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 Method

Chemicals

The bengal gram husk (BegH) was procured from the local market. Analytical grade solvents hexane and methanol were purchased from 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) from Thermo Scientific. TRIS (hydroxy methyl) amino methane (Trizma base), were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Sample preparation and animal experiment

The red pigment was extracted from *T. purpureogenus* CFRM02 fermented BegH (Pandit et al. 2018). The experimental protocol was approved by Institutional Animal Ethical Committee of CSIR-Central Food Technological Research Institute, Mysuru-570020 (IAEC No CFT/IAEC/83/17) 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. Thirty male Wistar rats (*Rattus norvegicus* $\approx$ 150 g, 4-5 week old) were housed 3 per cage and maintained at a controlled ambient temperature (23 ± 2 °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 (160mg/kg/day); Group D (Rx1): Normal Diet + ethanol (4 g/kg/day) + red pigment (300mg/kg/day); Group E (Rx2): Normal Diet + ethanol (4 g/kg/day) + red pigment (600mg/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 μ m sections were obtained using CM 3050S 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 (Bankoglu et al. 2016).

Histopathology

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

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 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 ($13.37 \pm 0.21 \times 10^3/\mu\text{L}$) compared to the rats fed with alcohol only. Whereas the increase in the red blood cell (RBC) count ($9.37 \pm 0.12 \times 10^6/\mu\text{L}$) 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 $43.56 \pm 0.28\%$) 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 ($42.6 \pm 0.48\%$ and 17.36 ± 0.13 pg) respectively. The platelet counts were significant higher in the rats fed with pigment ($870.6 \pm 18.4 \times 10^3/\mu\text{L}$) and metadoxine ($739.2 \pm 25.3 \times 10^3/\mu\text{L}$) compared to the control ($563.6 \pm 52.9 \times 10^3/\mu\text{L}$) and alcohol ($613.6 \pm 41.3 \times 10^3/\mu\text{L}$) 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 ($30.0 \pm 1.52\%$). 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.

Table 1

Haematological parameters of rats fed with alcohol and *T. purpureogenus* CFRM02 red pigment extract.

	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 ^a	14.36±0.33 ^b	14.14±1.58 ^b	13.45±0.164 ^b	13.37±0.21 ^b
MCV(fL)	53.44±1.95 ^{ab}	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 ^e	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 ^a	1.07±0.13 ^a	1.20±0.40 ^a
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 Concentration; MCHC – Mean Cell Corpuscular Haemoglobin Concentration, Hematocrit (Hct), PLT – platelet. DC – differential count, dL, deciliter; fL, femtoliter; µl, microliter; pg, pictogram; 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 $p \leq 0.05$.					

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, 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 300mg did not show any infiltration of neutrophils. While the infiltration of neutrophils in the hepatocytes were observed in rats fed with 600mg pigment (Fig. 2). The haematological analysis also have evidenced the increase in neutrophil concentration (Table 1).

Discussion

Alcohol is emerging as the common cause of chronic liver disease worldwide and responsible for 47.9% of all chronic liver deaths (Rosato et al. 2016). Research into ALD therapy, has been ongoing for more than five decades. However, the cellular and molecular mechanisms underlying the progression of ALD are not yet fully understood. There are no efficacious therapeutic strategies for ALD treatment (Singal et al. 2016; Singal and Shah 2019). 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 (Vuittonet et al. 2014). 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 (Singal et al. 2016).

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* (Pandit et al. 2019; Pandit et al. 2018). To investigate the hepatoprotective effects of *T. purpureogenus* CFRM02 pigment, ALD was induced in the male Wistar rat by administering an alcohol for 6 weeks, resulting in oxidative stress. A significant increase in the liver-to-body weight ratio in the ALD group compared with control group indicated alcoholic hepatitis and steatosis in the ALD group (data not shown). Alcohol related oxidative stress plays multiple roles in liver disease pathogenesis (Tan et al. 2020). This leads to the accumulation or generation of free radicals or reactive oxygen species (ROS) as observed in the figure 1. Metadoxine, pentoxifylline and several other drugs are prescribed for the treatment of ALD as antioxidant. Similarly, the antioxidant mechanism was observed in the treatment of ALD by the drugs N-acetyl 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 (Tang et al. 2021). 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 regeneration in severe AH (Liu et al. 2021). 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 (Shi X et al. 2019). Similarly, in this study, neutrophils infiltration in the hepatocytes has been observed (Fig. 2, 600mg) 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 (Singal and Shah 2019), but not the antioxidant mechanism.

Earlier reports on *T. purpureogenus* CFRM02 pigment have shown antioxidant and non toxic characteristics (Pandit et al. 2019; Pandit et al. 2020). The results of this studies have demonstrated the amelioration of ALD by scavenging free radicals (as antioxidant) and increased neutrophil infiltration (liver regenerative effect) by feeding the pigment to rats. The increased neutrophils concentration could help in the liver regeneration caused by alcoholic hepatositis (AH).

Conclusions

The *T. purpureogenus* CFRM02 pigment extract have shown the hepatoprotective effects against the ALD in oxidative stress. The red pigment extract of scavenged the free radicals generated by the alcohol induced stress. 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 dual mode of action of *T. purpureogenus* CFRM02 red pigment to ameliorate alcoholic hepatitis suggested the applications in food and nutraceuticals.

Declarations

CRedit authorship contribution statement

Saritha Pandit: Methodology, Software, Investigation, Data curation, Writing – original draft,

Krishana Prashant: Investigation, Visualization Mohankumari: Conceptualization, Methodology, Resources, Writing- review & editing. Muthukumar: Methodology, Resources, Data curation, review & editing. Mohan Dhale: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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References

Bankoglu EE, Tschopp O, Schmitt J, et al. (2016) Role of PTEN in Oxidative Stress and DNA Damage in the Liver of Whole-Body Pten Haplodeficient Mice. *PLoS One* 11:e0166956

Ceni E, Mello T, Galli A (2014) Pathogenesis of alcoholic liver disease: Role of oxidative metabolism. *World Journal of Gastroenterology* 20:17756–17772

Cheng C, Pan T-M (2011) Protective effect of Monascus-fermented red mold rice against alcoholic liver disease by attenuating oxidative stress and inflammatory response. *Journal of Agricultural and Food Chemistry* 59:9950–9957

Dey A, Cederbaum AI (2006) Alcohol and Oxidative Liver Injury. *Hepatology* 43:S63-S74

Gustot T, Jalan R (2019) Acute-on-chronic liver failure in patients with alcohol-related liver disease. *Journal of Hepatology* 70:319–327

Lieber CS (2004) Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. *Alcohol* 34:9–19

Liu H, Li X-M, Liu Y, Zhang P, Wang J-N, Wang B-G (2016) Chermesins A-D: meroterpenoids with a drimane-type spirosesquiterpene skeleton from the marine algal-derived endophytic fungus *Penicillium chermesinum* EN-480. *Journal of Natural Products* 79:806–811

Liu K, Wang F-U, Xu R (2021) Neutrophils in liver diseases: pathogenesis and therapeutic targets. *Cellular & Molecular Immunology* 18:38–44

Nicoletti R, Trincone A (2016) Bioactive Compounds Produced by Strains of *Penicillium* and *Talaromyces* of Marine Origin. *Marine Drugs* 14:37

Pandit SG, Mekala K-PR, Puttananjaiah MH, Dhale MA (2019) *Cicer arietinum* (Bengal gram) husk as alternative for *Talaromyces purpureogenus* CFRM02 pigment production: Bioactivities and identification. *LWT*:108499

- Pandit SG, Puttananjaiah MH, Harohally NV, Dhale MA (2018) Functional attributes of a new molecule-2-hydroxymethyl-benzoic acid 2'-hydroxy-tetradecyl ester isolated from *Talaromyces purpureogenus* CFRM02. *Food Chemistry* 255:89–96
- Pandit SG, Puttananjaiah MH, Peddha MS, Dhale MA (2020) Safety efficacy and chemical profiling of water-soluble *Talaromyces purpureogenus* CFRM02 pigment. *Food Chemistry*:125869
- Rosato V, Abenavoli L, Federico A, Masarone M, Persico M (2016) Pharmacotherapy of alcoholic liver disease in clinical practice. *The International Journal of Clinical Practice* 70:119–131
- Sarin SK, Pande A, Schnabl B (2019) Microbiome as a therapeutic target in alcohol-related liver disease. *Journal of Hepatology* 70:260–272
- Seth D, Haber PS, Syn W-K, Diehl AM, Day CP (2011) Pathogenesis of alcohol-induced liver disease: Classical concepts and recent advances. *Journal of Gastroenterology and Hepatology* 26:1089–1105
- Shi X, DeLucia AL, Bao J, Zhang P (2019) Alcohol abuse and disorder of granulopoiesis. *Pharmacology & Therapeutics* 198:206–219
- Singal AK, Kodali S, Vucovich LA, Darley-Usmar V, Schiano TD (2016) Diagnosis and treatment of alcoholic hepatitis: a systematic review. *Alcoholism: Clinical and Experimental Research* 40:1390–1402
- Singal AK, Shah VH (2019) Current trials and novel therapeutic targets for alcoholic hepatitis. *Journal of Hepatology* 70:305–313
- Tan HK, Yates H, Lilly K, Dhanda AD (2020) Oxidative stress in alcohol-related liver disease. *World Journal of Hepatology* 27:332–349
- Tang J, Yan Z, Feng Q, Yu L, Wang H (2021) The Roles of Neutrophils in the Pathogenesis of Liver Diseases. *Frontiers in Immunology* 12:625472
- Vuittonet CL, Halse M, Leggio L, et al. (2014) Pharmacotherapy for alcoholic patients with alcoholic liver disease. *American Journal of Health-System Pharmacy* 71:1265–1276

Figures

Figure 1

The levels of ROS generated in the hepatocytes were detected by staining the liver sections with fluorescent dye DHE. Control group; EtOH, alcohol-induced liver disease group; Metadoxine (160mg/kg/day) fed group; 300mg and 600mg, *T. purpureogenus* CFRM02 red pigment extract fed group.

Figure 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). Hematoxylin and eosin staining shows neutrophil infiltration (arrows) in the liver of red pigment extract fed (600mg/kg bw) rats (bottom lane).