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In Vivo Antioxidant Activities of Thermomyces sp Pigment in Albino Mice

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Abstract

In the present study, *In vivo* antioxidant activity of fungal pigment extract of *Thermomyces* sp was evaluated in animal model using albino mice. The pigment extract was orally administrated at doses of 25, 50, 100 and 200 mg/kg for 30 days. The Enzymatic antioxidant such as Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) non enzymatic antioxidant Glutathione (GSH) was analyzed in kidney and liver tissues. The fungal pigment treated groups recorded higher antioxidant activity compare to control animals.

Highlights

Fungal pigments were shown to display a high antioxidant activity under *in vitro* conditions. The fungal pigment isolated from *Thermomyces* sp fungi used as powerful antioxidant and natural colourant for food and fabrics. The induction of antioxidant enzymes and scavenging of free radicals may account for the mechanism of action of the extract as an antioxidant.

Keywords: *Thermomyces* sp, CAT, SOD, GSH, GPx

Reactive oxygen and nitrogen species play key roles in normal physiological process, including cellular life/death process, protection from pathogens, various cellular signaling pathways, and regulation of vascular tone (Valko *et al.,* 2007) .Oxidative stress is caused by an insufficient capacity of biological systems to neutralize excessive free radical production, which can contribute to human diseases and aging, including cardiovascular disease, neurodegerative disease and age-related cognitive decline , obesity and insulin resistance, as well as immune system dysfunction (Swerdlow, 2007; Larbi *et al.,* 2007). Cellular oxidative damage is a well-established general mechanism for cell and tissue injury and primarily caused by reactive oxygen species (ROS). These ROS can bind with most normal cellular components; they react with unsaturated bonds of membrane lipids, denature proteins, and attack nucleic acids (Martinez , 2007). A disturbance of the balance between formation of active oxygen metabolites and the rate at which they are scavenged by enzymic and nonenzymic antioxidants is referred to as oxidative stress (Jayaprakasha and Bhimanagouda,2007)[r]. It has been suggested that oxidative stress plays an important role in some physiological conditions and in many diseases, including diabetes mellitus (DM), myocardial infarction and carcinogenesis. Cells and biological fluids have an array of protective antioxidant mechanisms such as glucose-6-phosphate dehydrogenase,

superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and reduced glutathione, for both preventing the production of free radicals and repairing oxidative damage (Lu *et al.,* 2007)

While oxidative damage no doubt plays a significant pathological role in human disease, it is suggested that the intake of food-derived antioxidants may reduce oxidative damage and have a corresponding beneficial effect on human health (Mahfuz *et al.,* 2007) .In oriental countries, filamentous fungi such as *Aspergillus* and *Rhizopus*, are usually inoculated into the solid culture of steamed soybean, rice or barley for koji preparation. The prepared koji was then used to prepare traditional fermented food products (Ribeiro *et al.,* 2008). So for, limited information exists concerning the antioxidant effect of fungi, Hence, the present study aimed to assess the antioxidant potential of pigment by estimating the activity of enzymatic and non-enzymatic antioxidants on oral administration.

Materials and Methods

Fungal extract preparation

A loop full of *Thermomyces* sp from the PDA slants was inoculated into 10 ml of broth. After 2 or 3 days of growth the inoculum was transferred to 3 lit and 5 lit flasks containing the potato dextrose medium. The extracellular pigments that are excreted in the broth after 5 days of growth was harvested by filtration using Whatman No 1 filter paper. The culture extract was concentrated using vacuum rotary evaporator. The concentrated solutions were then lyophilized to get the dryness and stored at -20ºC until they were utilized for assays.

Experimental animals and housing conditions

Young male and female mice (approximately 25 g) were employed for the *in vivo* antioxidant studies. The animals were obtained from the KMCH College and Pharmacy and maintained in Institute Animal House Facility. The Institute is recognized for animal studies by the ethical committee. The animals were acclimatized for approximately one week and assigned to five groups, all consisting of 7 animals of each group. The animals were housed in polycarbonate cages (seven mice /cage) on soft chip bedding, which was changed twice per week. For drinking water, tap water was provided. They were housed in a room maintained at 25 ± 2 ºC with a relative humidity of 60–70% and exposed to a light and dark cycle of 12 h duration.

Animal experiments were carried out based on the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment

Mice were fed with commercial diet (Ms / Amrut – laboratory animal feed, Pranav agro industrial Ltd) obtained from Banagalore. The pigment was orally administered at levels based on body weight. The animals were observed daily for signs of adverse effects and were weighed at the start and on weekly intervals for 5 weeks.

Tissue sample preparations

At the end of the 4 weeks period, animals were sacrificed after an overnight fasting, by exsanguinations under ether anaesthesia. The liver and kidney tissues of each animal were removed, cleaned, excised, rinsed in ice-cold saline, and a 10% w/v homogenate was prepared using 0.1 M tris buffer, pH 7.0, centrifuged at 10,000 X g for 20 min at 4 °C. The supernatant obtained was used for the estimation of catalase. Further, the homogenate was centrifuged at 1000 X g for 20 min at 4°C and the supernatant was used for estimation of SOD, GSH and glutathione.

Estimation of catalase

The catalase activity was assayed by the method of Sinha, 1972 . The reaction mixture (1.5 ml) contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of supernatant of centrifuged tissue homogenate and 0.4 ml of 0.2 M hydrogen peroxide. The hydrogen peroxide solution was left out in control tubes. After incubation for 1 min at 37ºC, the reaction was stopped by addition of 2 ml of potassium dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1: 3 ratio). Samples were kept in boiling water bath for 10 min, finally cooled and the absorbance was read at 570 nm against control. The standard was taken and treated in the same manner. Catalase activity was expressed as units/mg protein.

Estimation of superoxide dismutase

Superoxide dismutase in tissues of kidney and liver in mice was assyed by the method of Kakkar *et al.,* 1984. The assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.025 mol/L), 0.1ml phenazine methosulphate (186 mM), 0.3 ml NBT (300 mM), 0.2 ml NADH (780 mM) and approximately diluted enzyme preparation and water in a total volume of 3 ml. After

incubation at 30ºC for 90 sec, the reaction was terminated by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The colour intensity of the chromogen in the butanol layer was measured at 560 nm against n-butanol and concentration of SOD was expressed as units/mg protein.

Determination of Reduced Glutathione (GSH)

To measure the reduced glutathione (GSH) 0.2 ml of tissue homogenate was mixed with 1.8 ml of EDTA solution. To this 3.0 ml precipitating reagent (1.67 g of met phosphoric acid, 0.2 g of EDTA disodium salt, 30 g sodium chloride in 1 L of distilled water) was added, mixed thoroughly and kept for 5 min before centrifugation. To 2.0 ml of the supernatant, 4.0 ml of 0.3-M disodium hydrogen phosphate solution and 1.0 ml of DTNB (5, 5-dithio bis 2-nitro benzoic acid) reagent were added and was read at 412 nm. The activities were expressed as units/mg protein(Lakshmi and Rajagopal, 1998)

Estimation of glutathione peroxidase(GPx)

Glutathione peroxidase activity was measured by the method of Rotruck *et al.,* (1973). Briefly, the reaction mixture contained 0.2 ml of 0.4 M phosphate buffer (pH 7.0), 0.2 ml of 10 mM sodium azide, 0.1 ml tissue homogenate, 0.2 ml reduced glutathione, and 0.2 ml of 0.2 mM hydrogen peroxide. The contents were incubated for 10 min at 37^0 C, 0.4 ml 10% TCA was added to stop the reaction and centrifuged at 3200 rpm for 20 min. The supernatant (0.2 ml) was assayed for glutathione contents using 0.5 ml of DTNB reagent (19.8 mg 5,5'-dithiobisnitrobenzoic acid in 1% sodium citrate) and 4 ml of disodium hydrogen phosphate solution. A blank was prepared with disodium hydrogen phosphate solution and 1 ml DTNB reagent. Suitable aliquots of the standard were taken and treated in the same manner. Samples were measured in triplicate. The activities were expressed as units/mg protein.

Results and Discussion

Enzymes are one of the potential active ingredients in pharmaceutical and cosmetic products. SOD has a high capacity of removing free radicals that are one of the main causes for skin aging. It is an essential enzyme, which protects cells from oxidative damage by catalyzing the reduction of the O_2 to H_2O_2 and molecular oxygen (O_2) (Meng *et al.,* 2010; Masoodi *et al.,* 2009). The superoxide dismutase activity of pigment treated group was assessed in kidney and liver of mice in both sexes. The mice liver recorded high antioxidant activity based on the dose in male and female. The SOD activity in kidney of control mice showed less activity of 2.57 U/mg of protein but it increased to 9.21 U/mg of protein in 200 (mg/kg) of male mice (Table 1).

Catalase activity in kidney and liver were increased in mice fed with yellow pigment in both sexes of animals. Catalase is an enzymatic antioxidant widely distributed in all animal tissues including RBC and liver. Catalase decomposes

Table 1: Catalase and superoxidase dismutase activity of mice fed with *Thermomyces* sp pigment

S.No	Experimental group (mg kg^{-1} body weight)	*In vivo* antioxidant assay			
		Catalase (CAT) U/mg of protein		Superoxidase dismutase (SOD) U/mg of protein	
		Kidney	Liver	Kidney	Liver
Male					
1.	Control	26.97 ± 0.16	38.03 ± 0.08	2.57 ± 0.15	5.92 ± 0.19
2.	25	35.21 ± 0.43	43.69 ± 0.39	4.86 ± 0.08	9.24 ± 0.21
3.	50	36.91 ± 0.41	46.19 ± 0.28	5.03 ± 0.08	12.91 ± 0.19
4.	100	40.43 ± 0.16	49.15 ± 0.20	7.13 ± 0.46	13.90 ± 0.13
5.	200	43.77 ± 0.23	52.09 ± 0.52	9.21 ± 0.06	16.28 ± 0.35
Female					
1.	Control	26.14 ± 0.14	36.66 ± 0.29	2.22 ± 0.12	5.32 ± 0.42
2.	25	32.52 ± 0.25	44.03 ± 0.54	3.57 ± 0.26	8.83 ± 0.25
3.	50	36.52 ± 0.26	45.88 ± 0.52	5.25 ± 0.15	12.35 ± 0.29
4.	100	38.94 ±0.08	48.44 ± 0.18	6.95 ± 0.15	14.18 ± 0.53
5.	200	42.38 ± 0.49	52.26 ± 0.53	8.93 ± 0.55	17.82 ± 0.45

hydrogen peroxide and helps to protect the tissues from highly reactive hydroxyl radicals (Qingming *et al*., 2010; Xiao, 2009). Reduction in the activity of catalase may result in many deleterious effects due to the accumulation of hydrogen peroxide. Administration of yellow pigment increased in the enzymatic antioxidant activities.A dose dependent increase in the level of catalase was observed in both the kidney and liver (25 - 200 mg kg^{-1} body weight) supplemented groups when compared to the control. The increase in kidney catalase levels varied from 35.21 to 43.77 U/mg of protein in 50 - 200 mg kg^{-1} of male mice (Krishnaraju *et al.,* 2009). The liver catalase level also increased in dose dependent manner in the range of 26.14 to 42.38 U/mg of protein in female mice (Table 1).

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C (Karuna *et al*.,2009; Verma *et al.,* 2010). Administration of thiol compounds such as glutathione, cysteine and methionine have been shown to protect against oxidative stress in humans and animals. The level of total glutathione level was observed in the *Thermomyces* sp., (25, 50, 100 and 200 mg kg-1 body weight) supplemented groups when compared to the control group. The increase in glutathione level observed in liver (65.5 U/mg of protein) and kidney (47.9 U/mg of protein) treated with 200 mg kg^{-1} of *Thermomyces* sp. was remarkably higher compared to the control group 41.70 and 28.29 U/mg of protein respectively(Filomena Conforti *et al.,* 2008). Minimum difference was observed in the antioxidant level of male and female (Table 2).

Glutathione peroxidase catalyses the reductions of hydrogen peroxide in the presence of glutathione to form water and oxidized glutathione. GSH-PX is also known to detoxify lipid peroxides and thereby inhibit lipid peroxidation (Jimoh *et al.,* 2009; Madhavan *et al.,* 2010). The glutathione peroxidase activity in treated group of female mice ranged from 2.17 to 4.81 U/mg of protein in kidney and liver it ranged from 1.88 to 5.19 U/mg of protein. The male also exhibited the same pattern Table 2)

In vitro antioxidant activity of fungal pigments showed radicals scavenging abilities on DPPH, NO, and superoxide radicals. The scavenging effects were powerful, close to the positive controls used in respective assays (Poorniammal, 2010). In the present study, *in vivo* free radical scavenging activities indicated that fungal pigment have significant radicals scavenging abilities on catalase, GSH, GPx and superoxide radicals. Therefore, fungal pigments should be explored as novel potential antioxidants. On the other hand, the fungal pigment showed non toxic properties when applied to food and beverages (Poorniamma *et al.,* 2011). Further studies are needed to better characterize the important active constituents responsible for the free radical scavenging activity.

Table 2: Glutathione peroxidase and reduced glutathione activity of mice fed with *Thermomyces* sp pigment

S.No	Experimental group mg kg^{-1} body weight	*In vivo* antioxidant assay			
		GSH U/mg of protein		GPx U/mg of protein	
		Kidney	Liver	Kidney	Liver
Male					
1	Control	28.29 ± 0.18	41.70 ± 0.21	1.55 ± 0.05	1.18 ± 0.07
2	25	33.19 ± 0.32	45.93 ± 1.70	2.33 ± 0.13	1.41 ± 0.05
3	50	38.30 ± 0.25	53.80 ± 0.41	2.98 ± 0.06	1.72 ± 0.04
4	100	45.15 ± 0.94	59.89 ± 0.40	3.21 ± 0.11	2.30 ± 0.08
5	200	47.99 ± 0.56	65.55 ± 1.17	5.62 ± 0.26	4.87 ± 0.47
Female					
1	Control	27.18 ± 0.19	41.63 ± 0.46	1.38 ± 0.03	1.08 ± 0.02
2	25	32.61 ± 0.27	47.14 ± 0.43	2.17 ± 0.07	1.88 ± 0.02
3	50	37.52 ± 0.22	52.74 ± 0.10	2.99 ± 0.11	2.43 ± 0.06
4	100	42.72 ± 0.10	56.96 ± 1.42	4.10 ± 0.13	2.96 ± 0.14
5	200	46.63 ± 0.26	63.38 ± 0.62	4.81 ± 0.29	5.19 ± 0.32

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