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Research Article

EVALUATION OF ANTI-INFLAMMATORY PROPERTY OF MELANIN FROM MARINE *BACILLUS* SPP. BTCZ31

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ABSTRACT

Objectives: To evaluate the anti-inflammatory property of melanin from marine *Bacillus* spp. BTCZ31.

Methods: Radical scavenging property of melanin was determined by 2,2-Diphenyl-1-picrylhydrazyl and metal chelation assays, which was further confirmed by electron paramagnetic resonance (EPR) spectroscopy. Anti-inflammatory property of melanin was explored *in vitro* in RAW264.7 cell line using cyclooxygenase (COX), Lipoxygenase (LOX), Myleoperoxidase (MPO), cellular nitrite inhibitory assays. Cytotoxicity of melanin was determined using 3-(4,5 dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay.

Results: BTCZ31 melanin showed radical scavenging activity of 67.55% and ferrous ion chelating activity of 97.88%. EPR spectrum showed sharp peaks indicating the presence of unpaired electrons. Melanin inhibited the activity of COX and LOX enzyme with IC_{50} values of 104.34 µg/mL and 10.5 µg/mL, respectively. It also reduced the activity of MPO and cellular nitrite levels. Cytotoxic concentration of melanin was found to be 105.4 µg/mL (IC_{50}).

Conclusion: *Bacillus* spp. BTCZ10 melanin can be a potential anti-inflammatory agent. Further *in vivo* evaluations are needed for confirming the activity, leading to therapeutic applications.

Keywords: Anti-inflammatory, Antioxidant, *Bacillus* spp., Bacteria, Marine, Melanin.

INTRODUCTION

Free radicals are highly unstable, reactive species that contain one or more unpaired electrons. Reactive oxygen species (ROS) includes oxygen radicals like superoxide (O^{2-}•), hydroxyl (OH•), peroxyl (RO^{2}•), hydroperoxyl (HO^{2}•) and certain non-radical oxidizing agents like hydrogen per oxide (H_2O_2), ozone and hypochlorous acid (HOCl) [1]. ROS are understood to be involved in the etiology of many diseases such as ageing, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and also includes inflammation as indicated by the signs of oxidative stress [2]. Antioxidants acts as physical barriers, which prevent ROS generation or ROS access to important biological sites (UV filters, cell membranes); chemical traps/sinks that "absorb" energy and electrons, quenching ROS (carotenoids, anthocyanidins); catalytic systems that neutralize or divert ROS (antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase); binding/inactivation of metal ions to prevent generation of ROS (ferritin, ceruloplasmin, catechins); and chain breaking antioxidants, which scavenge and destroy ROS (ascorbic acid, tocopherols, uric acid, glutathione, and flavonoids) [3].

Inflammation is body's natural response induced by tissue injury or infection and functions to combat external invaders. During inflammatory response, the endothelial permeability increases, blood leukocytes influx into the interstitium, oxidative burst, and release of cytokines (interleukins and tumor necrosis factor-α (TNF-α) occur. At the same time, there is also an induction of the activity of several enzymes like oxygenases, nitric oxide (NO) synthases, peroxidases as well as the arachidonic acid metabolism. These responses are also accompanied by an increase in expression of adhesion molecules like intercellular adhesion molecule and vascular cell adhesion molecules. An antioxidant molecule can minimize the ROS production and thereby help in reducing the inflammatory response [4]. Inflammation is also accompanied by increase in pain in arthritis, sprains, and other conditions and is commonly treated by non-steroidal anti-inflammatory drugs such as ibuprofen and naproxen, which can cause severe side-effects including heart attacks and stroke [5].

Melanins are brown to black colored complex pigments produced mainly via amino acid tyrosine and are widely distributed in living forms, where they have several biological functions such as photo protection, thermoregulation, free radical sinks, cation chelators, and antibiotics. In plants melanin is incorporated in their cell walls as strengtheners [6], whereas in humans it not only determines the skin color, but also plays an important role in protecting against UV radiation [7]. In the microbes, it protects against environmental stresses. Melanins are well known for its antioxidant property, which can help reduce ROS generation [8]. Minimizing ROS minimizes inflammation as both these properties are interrelated to each other. There are no reports of bacterial melanins with anti-inflammatory activity. In this study, we are exploring the antioxidant and anti-inflammatory property of bacterial melanin produced by a marine bacteria *Bacillus* spp. BTCZ31.

METHODS

Cell lines and bacterial isolates

L929 and RAW 264.7 cell lines were maintained in Dulbecco's modified eagles media (Himedia, India) supplemented with 10% fetal bovine serum (Invitrogen, USA) and grown to confluence at 37°C at 5% CO_2 in a CO_2 incubator (Eppendorf, Germany).

Melanin producing *Bacillus* spp. BTCZ31 was obtained from marine sediments collected from 96.47 m depth (9.59°N, 75.39°E) during the Sagar Sampada cruise no 305 in the Arabian Sea on the West coast of India. The bacteria and its pigment were characterized (unpublished data).

Extraction and purification of melanin

Tyrosine basal broth [9] was used for melanin production. Melanin production was monitored spectrophotometrically at 400 nm [10] using synthetic melanin (Sigma, USA) as standard. The cell free supernatant

from the production broth was acidified to pH 2 with 1 N HCl, allowed to stand for a week at room temperature (RT) (28±0.5°C), followed by boiling for 1 h and centrifuged (Thermo Scientific, USA) at 7000 rpm for 10 minutes. Resultant black pellet was washed thrice with 15 mL of 0.1 N HCl, followed by water. To this pellet, 10 mL of ethanol was added and the mixture was incubated in a boiling water bath for 10 minutes, kept at RT for 1 day, washed twice in ethanol, air dried [11] and used for further analysis.

Antioxidant activities

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Free radical scavenging activity of melanin was determined using DPPH radical scavenging assay [12], wherein 1.0 mL of 0.135 mM DPPH in methanol was mixed with 10 µL of melanin at concentration ranging from 20 to 100 µg/mL, mixed well and left in the dark at RT for 30 minutes. Absorbance was measured spectrophotometrically (Shimadzu, Japan) at 517 nm. Ascorbic acid (standard antioxidant) served as positive control.

The ability to scavenge DPPH radical was calculated as follows:

Free radical scavenging activity (%) = $A_{control} - A_{sample}/A_{control} \times 100$

Where $A_{control}$ is the absorbance of the DPPH + methanol, and A_{sample} is the absorbance of the free radical solution with melanin/standard antioxidant.

Metal chelation activity

Chelating potential of ferrous (Fe^{2+}) ions by melanin was estimated as per Dinis *et al.* [13]. Melanin (25-100 µg/mL) or standard ethylene diamine tetra acetic acid (EDTA) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), mixture was shaken vigorously and left standing at RT for 10 minutes. Absorbance of the solution was read at 562 nm (Shimadzu).

The percentage inhibition of ferrozine – Fe^{2+} complex formation was calculated as:

$A_c - A_s/A_c \times 100$

Where A_c was the absorbance of the control, and A_s was the absorbance of the melanin/standard.

Electron paramagnetic resonance (EPR) spectroscopy

EPR spectrum of melanin was obtained with JEOL Model JES FA200 (X-Band) EPR spectrophotometer. The EPR spectral conditions included: frequency 9.12 GHz; modulation frequency, 100.00 kHz; power, 0.99800 mW; field center, 326.296 mT; and sweep time 2.0 minutes. Proportionality factor (g factor) was calculated by the following equation [14]:

$h\upsilon = g\beta H$

Where h is Planck's constant, υ is microwave frequency, H is magnetic field and β is a constant, Bohr magneton.

Anti-inflammatory activities

RAW 264.7 cells were then grown to 60% confluence followed by activation with 1 µL lipopolysaccharide (LPS) (1µg/mL). LPS stimulated RAW cells were exposed with different concentration (6.25, 12.5, 25, 50, 100 µg/mL) of melanin solution. Diclofenac sodium, a standard anti-inflammatory drug in varying concentration corresponding to the sample was also added and incubated for 24 hours. After incubation the anti-inflammatory assays were performed using the cell lysate.

Cyclooxygenase (COX) activity

The COX activity was assayed by the method of Walker and Gierse [15]. The cell lysate was incubated in Tris-HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 minutes incubation at 37°C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, COX activity was determined by reading absorbance at 632 nm (Shimadzu).

Percentage inhibition of the enzyme was calculated as,
% inhibition = Absorbance of control−Absorbance of test/Absorbance of control × 100

Lipoxygenase (LOX) activity

The determination of LOX activity was as per Axelrod *et al.* [16]. Briefly, the reaction mixture (2 mL final volume) contained Tris-HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The LOX activity was monitored as an increase of absorbance at 234 nm (Shimadzu), which reflects the formation of 5-hydroxyeicosatetraenoic acid.

Percentage inhibition of the enzyme was calculated using the formula:

% inhibition = Absorbance of control – Absorbance of test/Absorbance of control × 100

Myeloperoxidase (MPO) activity

Cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethyl ammonium bromide (HTAB). Homogenized mixture was frozen in liquid nitrogen and thawed. After freeze thawing 3 times, the samples were centrifuged at 2000 g (Sigma, Germany) for 30 minutes at 4°C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H_2O_2 [17]. The change in absorbance at 460 nm (Shimadzu) was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25°C.

Cellular nitrite levels

Cellular nitrite level was estimated by the method of Lepoivre *et al.* [18]. To 0.5 mL of cell lysate, 0.1 mL of sulfosalicylic acid was added, vortexed for 30 minutes and then centrifuged at 5000 rpm (Sigma,) for 15 minutes. The protein-free supernatant was used for nitrite estimation. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris-HCl buffer and mixed well. To this, 530 µL of Griess reagent was added, incubated in the dark for 10-15 minutes, and the absorbance was read at 540 nm (Shimadzu) against a Griess reagent blank. Sodium nitrite solution was used as standard. The amount of nitrite present in the samples was estimated from the standard curve.

Cytotoxicity studies

Different concentration (6.25, 12.5, 25, 50 and 100 µg/mL) of BTCZ31 melanin were added to L929 cells and incubated for 24 hours. The percentage difference in viability was determined by standard 3-(4,5 dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [19] after 24 hours of incubation. The cells were washed with phosphate buffer saline (PBS), added 30 µL of MTT solution, and incubated at 37°C for 3 hrs. MTT was removed by washing with PBS and 200 µL of dimethyl sulfoxide (DMSO) was added to the culture. Incubation was at RT for 30 minutes until the cell were lysed and color was obtained was read at 540 nm using DMSO as blank in a microplate reader (ErbaLisaScan II, Germany).

Statistical analysis

The data are expressed as Mean ± standard deviation (n=3). Statistical significance was determined by one-way ANOVA using GraphPad Prism Software. At 95% confidence interval, $p<0.05$ were considered to be significant.

RESULTS

Purified *Bacillus* spp. BTCZ31 melanin was previously characterized using chemical and spectroscopic characterizations (unpublished data)

to confirm its identity and used for further studies. The DPPH radical scavenging activity of pure melanin from *Bacillus* spp. BTCZ31 was comparable with the activity of standard antioxidant ascorbic acid. The results revealed that 100 µg/mL bacterial melanin exhibited 67.55% radical scavenging activity, which was comparable to that of standard antioxidant ascorbic acid showing an activity of 77.32% (Fig. 1a). Sharp peaks in the EPR spectra (Fig. 1c) of melanin indicated the presence of unpaired electrons, which can trap free radicals. Proportionality factor (g factor) of BTCZ31 melanin was 1.98, which was as good as the g value of organic radicals. Antioxidant efficiency of BTCZ31 melanin was also reflected in its metal scavenging property. Melanin even at lower concentration of 25 µg/mL had shown 92.39% Fe^{2+} scavenging activity (Fig. 1b), which increased as the concentration increased. 100 µg/mL of melanin showed a scavenging activity of 97.84% which was equivalent to that of the standard EDTA (99.64%).

Purified melanin from *Bacillus* spp. BTCZ31 inhibited COX and LOX enzymes effectively at increasing concentrations. Melanin inhibited COX enzyme with an IC_{50} value of 104.34 µg/mL, while LOX enzyme was inhibited with an IC_{50} value of 10.5 µg/mL. 100 µg/mL of BTCZ31 melanin inhibited COX and LOX enzymes at 47.92 and 69.48% ($p \leq 0.0001$), respectively. MPO activity was reduced significantly ($p \leq 0.0001$) as the concentration of melanin increased from 6.25-100 µg/mL. Cellular nitrite levels, an indicator of NO produced during inflammation had reduced (647.54 µg/mL Lysate) as melanin concentration increased (Fig. 2). MTT assay revealed that BTCZ31 melanin inhibited growth of L929 cells, with IC_{50} being 105.4 µg/mL (Fig. 3).

DISCUSSION

In this study, the evaluation of melanin as a potent anti-inflammatory agent was carried out. Antioxidant ability of melanin is the major factor determining its ability to act as a good anti-inflammatory agent. Many previous reports [11,20] discussed the immense antioxidant potential of bacterial melanin. Manivasagan *et al.* [20] reported 90% radical scavenging activity for melanin (3.5 mg/mL) from *Actinoalloteichus* spp. *Klebsiella* spp. Melanin [11] was reported to have 70% (50 µg/mL) free radical scavenging activity. Here *Bacillus* spp. BTCZ31 melanin (100 µg/mL) showed 67.55% scavenging activity which was comparable with the earlier reports. Like ferritin, ceruloplasmin, catechins etc., melanin also binds to metal ions and minimizes the generation of ROS [3]. Therefore, antioxidant potential of melanin can also be related to its metal chelating ability. Moreover BTCZ31 melanin had shown nearly cent percent Fe^{2+}chelating activity, which further reflects its ability to scavenge free radicals. EPR peaks of melanin indicated the presence of unpaired electrons, which can trap free radicals. Proportionality factor (g) of BTCZ31 melanin (1.98) was similar to that of melanin from *Aspergillus bridgeri* (2.005) [21] and free electron in vacuum (2.002) [14]. This also confirmed the radical scavenging ability of BTCZ31 melanin.

COX and LOX enzymes have significant roles to play in the regulation of inflammatory responses [22]. Anti-inflammatory drugs and agents decrease this response by suppressing the production pathway of the inflammatory mediators, which in turn block the initiation and progression of inflammation-associated diseases [23]. In the present study, the *in vitro* experiments showed the dose-dependent inhibition of COX, LOX, MPO and NO activity in RAW 264.7 cell lines by BTCZ31 melanin. COX is a rate limiting enzyme in inflammation as it involved in the conversion of arachidonic acid to prostaglandins, which are associated with many inflammatory diseases. The inhibition of COX by BTCZ31 melanin was significant. LOX enzyme which produce leukotrienes is important in the pathophysiology of inflammatory diseases [24]. At lower concentration, BTCZ31 melanin inhibited LOX activity. Myeloperoxidase catalyzes the formation of HOCl by the oxidation of H_2O_2 during inflammatory response [25]. The addition of BTCZ31 melanin decreased the MPO activity considerably. The decrease in cellular nitrite level [26] after BTCZ31 melanin treatment indicates its capability for use as a potential ant-inflammatory agent. There are only few reports on anti-inflammatory properties of melanin. Avramidis *et al.* [27], reported that grape melanin interfered with the prostaglandin as well as the leukotriene and/or complement system mediated inflammation. This supports our findings. *Bacillus spp.* BTCZ31 melanin was also less toxic to cells, which suggests its feasibility in therapeutic

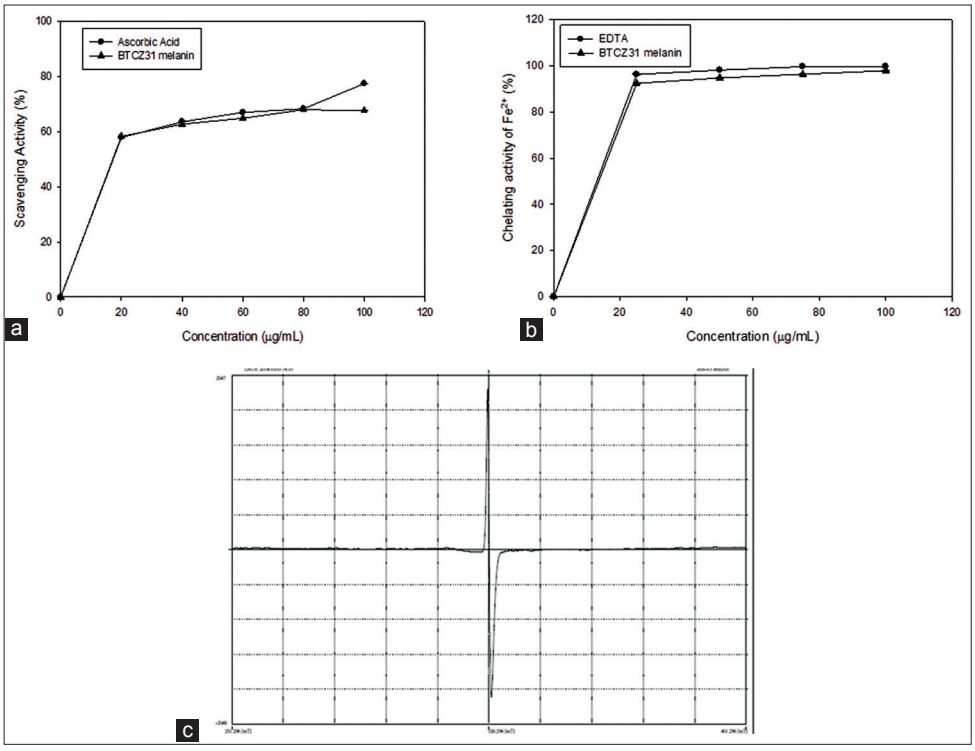

Fig. 1: (a) Radical scavenging activity of *Bacillus* spp. BTCZ31 melanin compared to standard antioxidant ascorbic acid (b) Fe^{2+} chelating potential melanin compared to standard ethylene diamine tetra acetic acid (c) Electron paramagnetic resonance spectrum of melanin

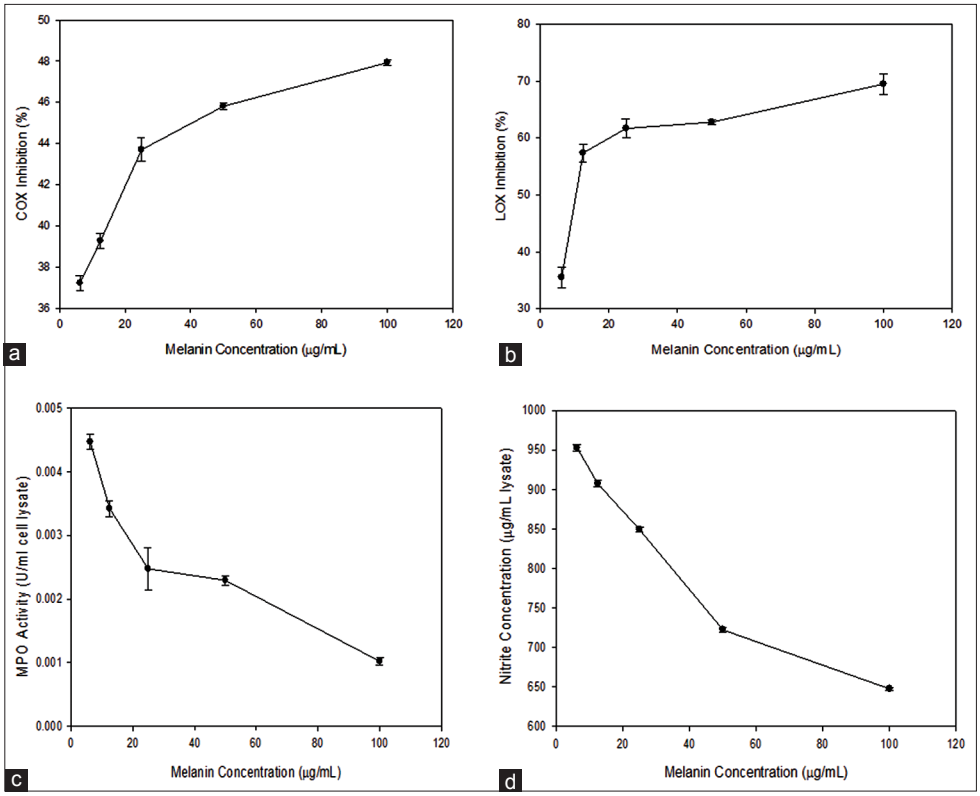

Fig. 2: Effect of BTCZ31 melanin on the activities of (a) Cyclooxygenase (b) Lipoxygenase (c) Myeloperoxidase and (d) Cellular nitrite levels

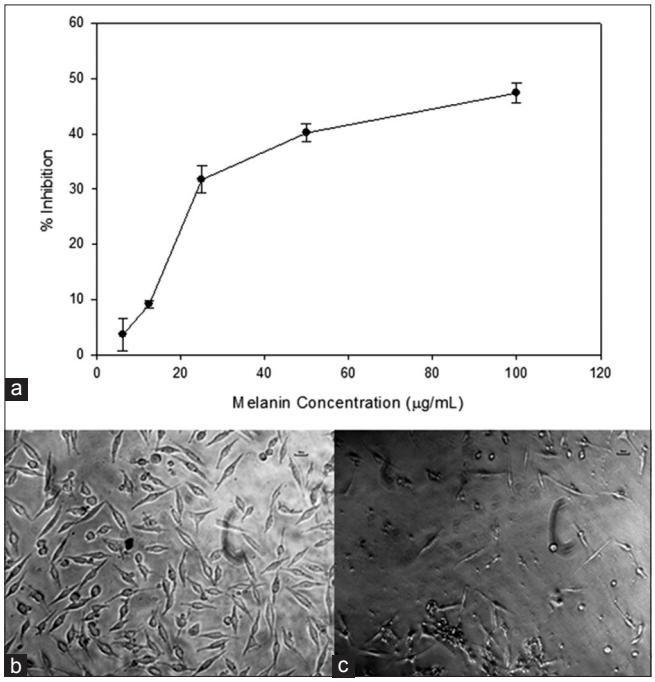

Fig. 3: Cytotoxic effect of *Bacillus* spp. BTCZ31 melanin (a) Phase contrast micrographs (×20 magnification) showing the cytotoxic effect (b) Control (c) Treated (100 µg/mL)

applications.

In conclusion, bacterial melanin from *Bacillus* spp. BTCZ31 showed good anti-inflammatory potential *in vitro*. However, further *in vivo* studies are needed to explore the molecular mechanisms underlying the inhibition of key enzymes, which participate in inflammatory responses.

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