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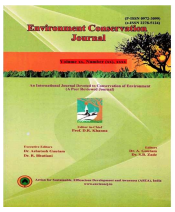

Evaluating anti-microbial and anti-oxidative potential of red biopigment from *Monascus purpureus*

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ABSTRACT

In the present study, *Monascus* red biopigment produced by solid-state fermentation was evaluated for its anti-microbial and antioxidative potential. The antibacterial activity through Scanning Electron Microscopy against *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus* was found to show morphological damage in some cells, as evidenced by the outflow of cell contents, deep craters, burst cells, and cell death at concentration of 200 µg/ml of biopigment. Moreover, antibacterial activity through agar well diffusion method against *Bacillus cereus, Staphylococcus aureus, Klebsiella pneumonia* and *Pseudomonas aeruginosa* was in range of 2-6 mm by varying concentration of biopigment from 1 to 20 mg/ml.Next, the antifungal activity of the extracted biopigment was in the range of 2-9 mm for *Aspergillus flavus, Fusarium oxysporum* and *Alternaria alternata.* In addition, antioxidant efficacy of red biopigment through DPPH, ABTS and FRAP assay results was found to show 59.69 %, 91.1 %, and 15.22 % free radical scavenging activity. The results of this study revealed that red biopigment has potential to modulate the antimicrobial and antioxidative activity.

Introduction

The food colorants, based on "natural" biopigments, are highly preferable over present day used "synthetic" chemical colors, which are generally "toxic" in nature and may cause various allergic and intolerance reactions (Weiss, 2012; Tkaczyk *et al.,* 2020). On contrary, "natural" biopigments serve additional health benefits with a number of anti-cancers, anti-microbial and therapeutic properties (Park and Kim 2011; Lin *et al.,* 2019). The biopigments are derived from plants and microorganisms such as *Monascus purpureus* (*M. purpureus*) and *Rhodotorula* etc. (Marova *et al.,* 2012; Vendruscolo *et al.,* 2016), among which, *M. purpureus*, a filamentous fungus, is highly versatile and produces several bioactive secondary metabolites of polyketide origin, such as mixture of three (orange, yellow and red) biopigments, monacolins K (Lovastatin) etc. (Chen *et al.,* 2015; Chen *et al.,* 2017). The biopigment is commonly produced by fermentation method with both solid-state and submerged cultivation, among which, solid state fermentation (SSF) offers high yield of biopigment due to the use of solid substrate, generally agricultural products, for release of biopigment (Thomas *et al.,* 2013). On contrary, the biopigments in submerged cultivation are mainly retained intracellularly, which impedes further development and evolution of biopigment (Velmurugan *et al.,* 2011). Among two, the red biopigment is of high demand in food industry due

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to its extensive use in meat products to replace synthetic nitrites colorants (Yu *et al.,* 2015).

Monascus spp naturally produces other secondary metabolites, chemical constituents (monascin, monascopyridines, monapurpyridines A, monacolin) and phytochemical such as flavonoids, polyphenols, terpenoids in significant quantities (Wild *et al.,* 2002; Hsu *et al.,* 2012; Cheng *et al.,* 2013; Ji *et al.,* 2018). Additionally, polyphenolic components of higher fungi like *M. purpureus,* etc. protected alongside oxidative loss through preventing or reducing free radicals as well as reactive oxygen species *in vitro*. Molecules like flavonoids, phenolics, phenylpropanoids in addition strongly polymerized molecules (i.e. tannins) and acetate accumulate naturally end products through shikimate pathways, signifying the extensively antioxidant activity in a generally distributed sub-group and can be applied in the food, health, and cosmetics industries (Smith *et al.,* 2015; Tan *et al.,* 2018). These pigments and chemical components are secondary metabolites with a common skeleton of azaphilone might be having antimicrobial and antioxidative activity (Chen *et al.,* 2017; Patakova *et al.,* 2017; Wu *et al*., 2019).

Keeping in view of the importance of *Monascus* pigments and their associated biochemical activities, the current study was aimed to reveal the antimicrobial (antibacterial and antifungal) potential including anti-oxidative activity of biopigment. In this manuscript the effect of red *Monascus* biopigment was evaluated on bacterial pathogens through scanning electron microscopy and agar well assay including fungal pathogens through agar well asasay. Next, the antioxidative effect of *Monascus* red biopigment was evaluated through DPPH, ABTS and FRAP assay. The current study specifies the antimicrobial and antioxidative effect of *Monascus* red biopigment.

Material and Methods

The red biopigment was produced at a pilot-scale using tray-type fermenter from *M. purpureus* (MTCC-369) using broken rice as substrate through solid-state fermentation. Subsequently, red biopigment was extracted from *Monascus* fermented biomass through static extraction method (Roy, 1967) with 60% ethanol at 60°C for 80 min at 10,000 rpm. The extracted red biopigment was collected and stored in refrigerated conditions (-20°C) till further analysis.

Antibacterial activities: Antibacterial activity of red biopigment against various bacterial pathogens such as *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 96, *Streptococcus mutans* MTCC 890*, Listeria monocytogenes* MTCC 1143*, Klebsiella pneumoniae* MTCC 109*, Proteus mirabilis* MTCC 425*, Pseudomonas aeruginosa* MTCC 741*, Salmonella typhi* MTCC 733*, Shigella flexneri* MTCC 1457 and *Escherichia coli* MTCC 723 was determined using agar well diffusion assay and scanning electron microscopy. The bacterial cultures were revived on Brain Heart Infusion broth incubated at 37°C for 24 hrs. The stock cultures were preserved at 4°C in a refrigerator and sub-cultured every three weeks.

Scanning Electron Microscopy (SEM): The overnight grown bacterial cultures of *B. cereus, E. coli, and S. aureus* was used to perform the SEM for antibacterial evaluation as described previously (Chen *et al.,* 2018). The reaction mixture of red biopigment was prepared by mixing 1 mg/ml with DMSO. For the antibacterial assay, 2 ml microcentrifuge tubes were taken and the reaction mixture was prepared by adding 200 µg of 1 mg/ml red biopigment mixed each tube along with 100 µl of the bacterial culture. After mixing the reaction mixture make the final volume was 1.5 ml with BHI broth. BHI broth as such served as a negative control whereas BHI broth with culture instead worked as a positive control. The mixture was vortexed and incubated at 37ºC for 96 hrs. After incubation bacterial cells were harvested at 8000 rpm for 10 min. Cell pellets were washed 3 times with phosphate buffer saline (10 mM PBS), after washing cells were re-suspended in normal saline and made smear on a glass coverslip. Briefly, cells were fixed with 3 % glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.2) at 4°C for 24 hrs. After fixation, cells were washed for 3x10 min in 0.1 M phosphate buffer and Post-fixation, with 2 % osmium tetroxide (in 0.1 M phosphate buffer pH = 7.2) for 4 hrs at room temp in a light-tight container. The samples were then washed in 0.1 M phosphate buffer (3 × 10 min.) and dehydration was done with a graded ethanol solutions in water – 30 %, 50 %, 70 %, 80 %, 90 %, 96 %, 100 % for 5-15 min each; 2 x 100 % ethanol for 15-30 min each. The treated samples were dried in a desiccator and

metal coated by an ion spray instrument (MSP-2S, IXRF, USA) followed by analysis using S-3400 scanning electron microscopy carried out at Electron Microscopy Centre of Dairy Microbiology Division, ICAR-NDRI Karnal, Haryana.

Agar well diffusion assay: The overnight grown bacterial cultures of *B. cereus*, *S. aureus*, *S. mutans, L. monocytogenes, K. pneumoniae, P. mirabilis, P. aeruginosa, S.Typhi, S. flexneri* and *E. coli* was used to evaluate the antibacterial activity of biopigment (Vendruscolo *et al.* 2014). The reaction mixture of red biopigment was prepared by mixing 1 mg/ml, 5 mg/ml, and 20 mg/ml with Dimethylsulfoxide (DMSO) and DMSO as such without biopigment served as control. For the antibacterial assay, Brain Heart Infusion agar plates were prepared and 100 µl of 24 hrs grown test bacterial culture was seeded on the surface of Petri plates. Wells of approximately 10 mm was bored using a cork borer and sealed with soft agar. Then 100 µl samples of various concentration i.e. 1 mg/ml, 5 mg/ml, and 20 mg/ml of biopigment were introduced in each well. Petri plates were incubated at 37^{o}C for 48 hrs. Growth of bacterial culture was observed at 6, 12, 24, 48 hrs and diameters of zones of inhibition were recorded after 48 hrs. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Antifungal Activity: The Antifungal activity of red biopigment was estimated using the agar well assay method described by Ferdes *et al.* (2009) with slight modification. In brief, five fungal species *Aspergillus flavus* NCIM535*, Penicillium chryosogenum* ATCC66564*, Mucor azygosporus* ATCC15087*, Fusarium oxysporum* NCIM1008*,* and *Alternaria alternata* ATCC6663 were procured from the Institute of Microbial Technology, Chandigarh, India. The culture was revived on Potato Dextrose Agar incubated at 30°C for 7 days. The stock culture was preserved at 4°C in a refrigerator and sub-cultured every three weeks. The reaction mixture of red biopigment was prepared by mixing 1 mg/ml, 5 mg/ml, and 20 mg/ml with DMSO and DMSO alone served as control. For the antifungal assay, PDA plates were prepared and wells of approximately 10 mm were bored using a cork borer and sealed wells with soft agar. The fungal culture bit size of 8 to 10 mm was placed onto the center of the Petri plate and 100 µl of different dilutions was introduced in each well around the fungal bit. The Petri plates were then incubated at 30^{o}C for 7 days. The growth of fungal species was observed each day and diameters of zones of inhibition were recorded after 7 days. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the well.

Anti-oxidative activity: Radical scavenging activity was determined using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) assay, 2, 2‟-Azino-Bis-(3–Ethylbenzthiazoline–6-Sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP) assay.

DPPH assay: The antioxidant activities of the *Monascus* red biopigments were determined by the activities of 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) free radical scavenging activity assay (Tan *et al.,* 2018) with minor modifications. In brief, the reaction mixture was prepared by using various concentration 2, 10, 20, 40 60, 80, 160, 320, 640 µl/ml of 5 mg/ml red biopigment combined with 2 ml of the DPPH ethanol solution (0.05 mM in ethanol). Ethanol served as blank whereas DMSO instead of sample functioned as control. The mixture was vortexed and incubated for 60 min at room temp in the dark. Following incubation, reaction mixtures were centrifuged (8000 rpm for 10 min) and each reaction mixture (200 µl) was transmitted to a 96 well plate, and absorbance was measured at 517 nm on ELISA plate reader (Thermo Fisher, Scientific). Positive controls included BHT, Ascorbic acid, and Quercetin. The concentration of sample that resulted in free radical scavenging activity was recorded.

$$\textbf{DPPH scavenging ability}\ (\%) = \left(1 - \frac{(S - B)}{C}\right) \times 100$$

Where; A_{sample} = Absorbance of the test sample, A_{blank} = Absorbance of blank, $A_{control}$ - Absorbance of control

ABTS Assay: The freshly prepared ABTS solution was prepared by oxidation of 7.4 mmol/L ABTS with 4.90 mmol/l potassium persulfate according to Tan *et al.* (2018) with minor modification. In brief, the reaction mixture was prepared 5 mg/ml of red biopigemt at various concentration of 2, 10, 20, 40

60, 80, 160, 320, 640 µl/ml. 50µl of different dilution of red biopigment was mixed with 200 µl ABTS solution. Ethanol served as blank whereas DMSO instead of sample served as control. The mixture was vortexed and incubated for 5 min at room temp in the dark. Absorbance was measured at 734 nm on an ELISA plate reader. All the values were normalized with control. The positive controls included BHT, Ascorbic acid, and Quercetin. The scavenging ability of the ABTS was estimated by following Equation:

$$\textbf{ABTS free radical scavenging activity}\ (\%) = \left(1 - \frac{(S - B)}{C}\right) \times 100$$

FRAP assay: The freshly prepared FRAP (Xiao 2015) reagent consisted of 300 mM acetate buffer (pH 3.4), 100mM 2,4,6-tripydyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in ratio of 10:1:1. In brief, the reaction mixture consist of 5 mg/ml of red biopigemt at various concentration of 2, 10, 20, 40 60, 80, 160, 320, 640 µl/ml. 50µl of each dilution was mixed with 200 µl of FRAP reagent and incubated at 37ºC temp for 30 min in the dark. An ELISA plate reader was used to measure the absorbance at 517 nm. Different concentrations of ferrous sulphate (0-1mM) were used to create the standard curve (Figure 1) by following the same procedure; just replacing the sample with the known concentration of ferrous sulphate solution (Ferrous sulphate 1mM= 1.51 mg/10ml) (Shokryazdan *et al.,* 2018). The FRAP antioxidant activity was expressed as mM ferrous sulphate equivalent using a standard curve prepared by ferrous sulphate. The values were normalized with control. BHT, Ascorbic acid, and quercetin were used as positive control.

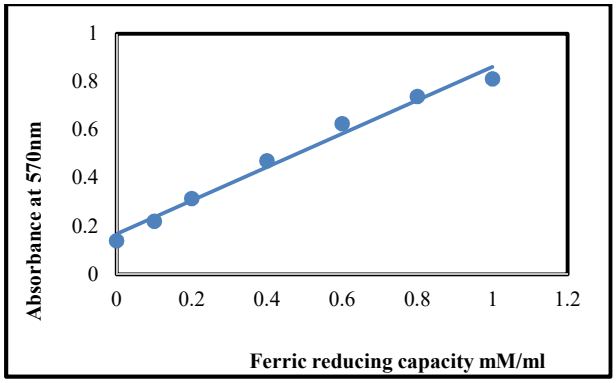


Figure 1: Standard curve of ferrous sulphate.

Statistical analysis: The collected research data in triplicate was analyzed for the mean values and standard error through Microsoft Excel. To determine the significance of the collected data one-way analysis of variance (ANOVA) tests were used in SPSS (16.0v). Differences in means of less than 0.05 % were measured statistically significant.

Results and Discussion

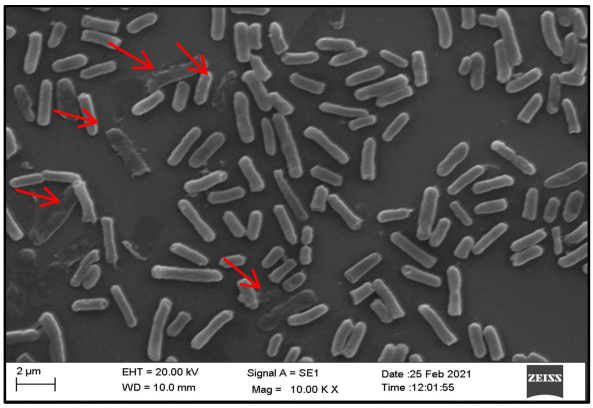
Antibacterial activity

The antibacterial activity of extracted red biopigment was determined against strains of Gram-positive and Gram-negative food pathogenic bacteria by the scanning electron microscopy (SEM) and agar well diffusion method.

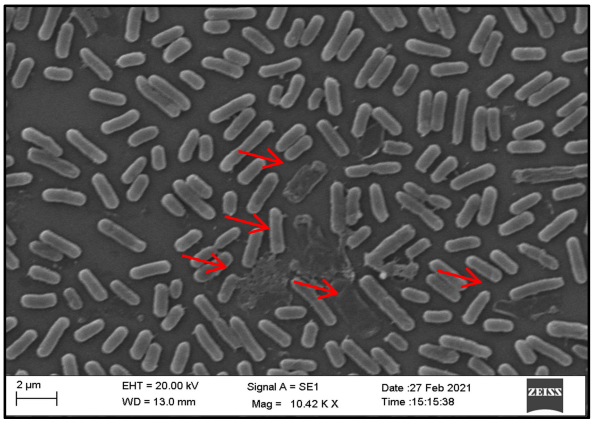
Scanning Electron Microscopy (SEM)

The antibacterial activity was observed in 200 µg/ml of extracted biopigment against *Bacillus cereus*, *Escherichia coli*, and *Staphylococcus aureus* after 96 hrs of incubation at 37ºC temperature. Morphological changes were analyzed and are shown in (Figure 2). Bacterial cells in the control group were healthy and smooth, with no signs of damage. In contrast, *B. cereus*, *E. coli*, and *S. aureus* cells treated with *Monascus* biopigment were found to show morphological damage in some cells, as evidenced by the outflow of cell contents, deep craters, burst cells, and cell death (Figure 2 a,b and c). Furthermore, the total numbers of cells into given suspension were reduced with treatment of biopigment in comparison to the control cells. The extracted biopigment was observed to be slightly effective based on SEM results. Zhao *et al.* (2016) also demonstrated that a 2.5 mg/ml concentration of orange pigment derived from *M. purpureus* damage *E. coli* bacterial cells, resulting in cell death. Likewise,100 µl of red biopigment from *M. purpureus* MTCC 1090 was also found to be effective against *S. aureus*, *E. coli*, *Klebsiella pneumonia*, and Providencia by Bi and Gajalakshmi (2018).Furthermore, Feng *et al.* (2019) used scanning electron microscopy and transmission electron microscopy to analyze that 10 mg/ml *Monascus* pigment has antibacterial activity against *S. aureus*. The antibacterial properties of *Monascus* pigment can be due to disruption to bacterial cell membranes, which allows some cellular components such as proteins and DNA to escape, resulting in bacterial cell death (Feng *et al.,*2019).As a result, *Monascus* biopigment could

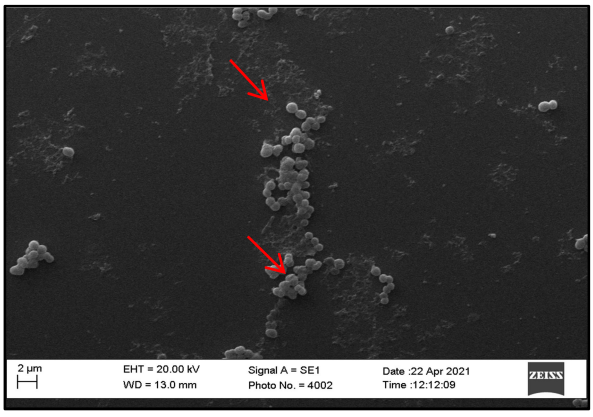
be used as a natural food preservative in the food industry.



(a): *Bacillus cereus*



(b): *Escherichia coli*

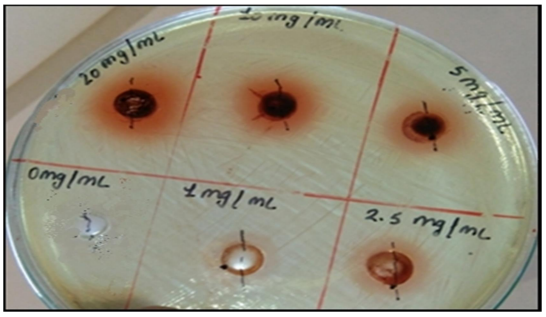


(c): *Staphylococcus aureus*

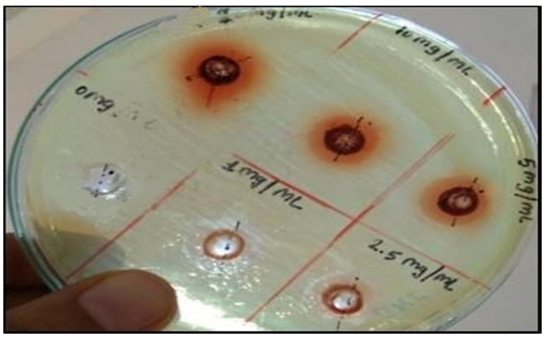
Figure 2: Morphological changes of a) *B. cereus,* b) *E. coli* andc) *S. aureus* after treatment with red biopigment for 96 hrs with 200 µg/ml of biopigment. Red arrows indicate formation deep craters and cell death.

Agar well diffusion assay: The antibacterial activity of the extracted biopigment was tested against *B. cereus*, *S. aureus, K. pneumonia* and *Pseudomonas aeruginosa* by varying the concentration from 1, 5 and 20 mg/ml (Figure 3). The zone of inhibition produced by biopigment at varying concentration was in the range of 2-6 mm for *B. cereus,* 2-5 mm for *S. aureus,* 2-4 mm for *K. pneumonia* and again 2-6 mm against *P. aeruginosa* (Figure 3 a-d). Whereas, biopigment exhibited no activity against *Sreptococcus mutans, Listeria monocytogenes, Proteus mirabilis*, *Salmonella typhi, Shigella flexneri, and E. coli* (Table 1). Based on the agar well assay, extracted biopigment was found to be slight effective against *B. cereus*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa.* The results were corroborated with Mukherjee and Kumar (2011), who demonstrated that extracted pigment from *M. purpureus* NFCCI 1756 strain exhibited the antibacterial activity against *Bacillus megaterium*, *Bacillus mycoides* and *B. subtilis* ranging from 1.26 to 1.36 cmand no activity against *Salmonella typhimurium*, *S. typhi*, and *E. coli.* Similarly, Vendruscolo *et al.* (2014) reported that extracted biopigment from *M. purpureus* CCT 3802 was effective against *S. aureus* and *E. coli* while showed no antagonostic activity against *Salmonella enteritidis*. Ferdes *et al.* (2009) demonstrated that pigment extracted from *M. purpureus* M5 strain exhibited antibacterial activity against *Bacillus subtilis*, *P. aeruginosa*, *E. coli* species ranging from 8 to 12 mm. Antibacterial activity of *Monascus* pigments demonstrated the preservative properties of natural food colorants.

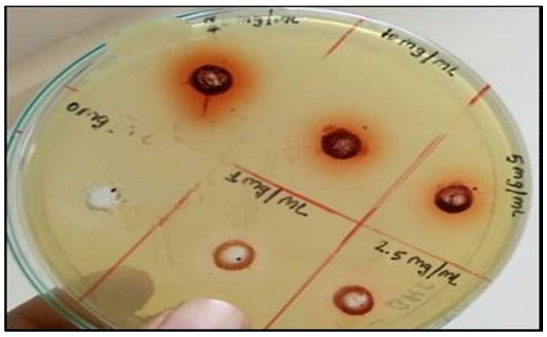
Antifungal activities: The antifungal activity of the extracted biopigment was tested against *Aspergillus flavus, Penicillium chryosogenum, Mucor azygosporus, Fusarium oxysporum,* and *Alternaria alternata* by varying the concentration from 1, 5 and 20 mg/ml (Figure 4) (Table 2). The zone of inhibition produced by biopigment at varying concentration was in the range of 2-8 mm for *A. flavus,* 3-9 mm for *F. oxysporum* and 2-6 mm for *A. alternata* (Figure 4 a, d and e). Biopigment did not show any antifungal activity against *P. chryosogenum* (Figure 4 b) and *M. azygosporus* (Figure 4 c). In addition, DMSO which was used as a solvent, thereby served as



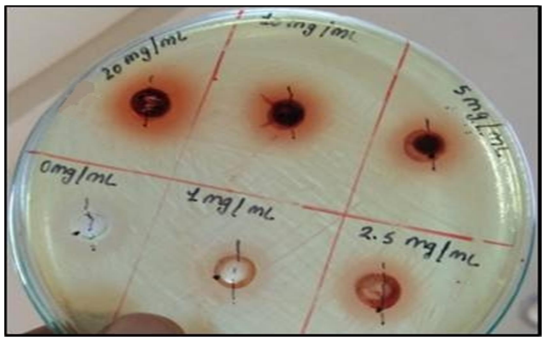
(a): *Bacillus cereus*



(b): *Staphylococcus aureus*



(c): *Klebsiella pneumonia*



(d): *Pseudomonas aeruginosa*

Figure 3: Zone of inhibition produced by different concentration of biopigment against bacterial pathogens (a) *B. cereus* MTCC 1272 (b) *S. aureus* (c) *K. pneumoniae* (d) *P. aeruginosa.*

control, exhibited no activity against the fungal pathogens. Based on the agar well assay, extracted biopigment was the most effective against *A. flavus, F. oxysporum, A. alternata*. The antifungal activity of extracted pigment from *M. purpureus* against some fungal strains might be due to monascidin A, the main compound responsible for the inhibitory activity (Ferdes *et al.,* 2009). The results were corroborated with Ferdes *et al.* (2009), who demonstrated that pigment extracted from *M. purpureus* M5 strain exhibited the antifungal activity against *Aspergillus*, *Mucor*, *Penicillium*, and *Fusarium* species. In addition, another study conducted by Cheng *et al.* (2011) showed that chemical constituents extracted from *M. purpureus* BCRC 38038 were also found to be effective against *Candida albicans* and *Saccharomyces cerevisiae* by a TLC bioautographic approach. Antifungal activity of *Monascus* pigments demonstrated the preservative properties of this natural food biopigment.

Screening for antioxidative potential of *Monascus* biopigment: The antioxidant efficacy of red biopigment were tested in this study using 2, 2 Diphenyl-1- Picrylhydrazyl (DPPH) free radical scavenging assay, 2, 2 -Azino-Bis-(3–Ethylbenzthiazoline–6-Sulfonic acid) assay (ABTS) and ferric reducing antioxidant power (FRAP) assay.

Diphenyl-1-Picrylhydrazyl (DPPH) assay: Basically, DPPH molecule is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule that gives rise to the deep violet color. When a solution of DPPH is mixed with tested antioxidant substance, that can donate a hydrogen atom, the intensity of violet color gets reduced. Therefore, in order to evaluate the antioxidant efficacy through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored (Alam *et al.,* 2013). The results showed that there was a significant difference between concentrations of biopigment on the antioxidant ability at 5 % (0.05 %) level of significance. The results revealed that 640 µl of 5 mg/ml of extracted biopigment and positive control BHT, ascorbic acid and quercetin had higher free radical scavenging activities as compared to other concentration (Figure 5a-b). In particular, 1 to 640 µl/ml of 5 mg/ml red biopigment significantly increased antioxidant ability ranging from 1.16 to 59.69 % (51.45 fold).

Table 1: Antibacterial activity of extracted red biopigment of *M. purpureus*

Test Bacteria	Red biopigment concentration (mg/ml)				
	1	2.5	5	10	20
	Zone of inhibition (mm)				
Bacillus cereus MTCC 1272	2	2	2	4	6
Staphylococcus aureus MTCC 96	2	2	2	3	5
Streptococcus mutans MTCC 890	-	-	-	-	-
Listeria monocytogenes MTCC 1143	-	-	-	-	-
Klebsiella pneumoniae MTCC 109	2	2	2	3	4
Proteus micrabilis MTCC 425	-	-	-	-	-
Pseudomonas aeruginosa MTCC 741	2	3	3	5	6
Salmonella typhi MTCC 733	-	-	-	-	-
Shigella flexneri MTCC 1457	-	-	-	-	-
Escherichia coli MTCC 723	-	-	-	-	-

- Nil

Table 2: Antifungal activity of extracted red biopigment of *M. purpureus*

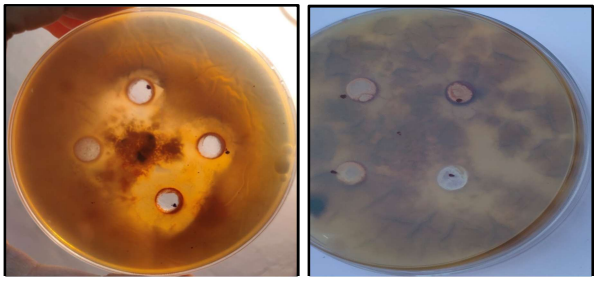
Test Fungi	Red biopigment concentration (mg/ml)		
	1	5	20
	Zone of inhibition (mm)		
Aspergillus flavus NCIM535	2	4	8
Penicillium chryosogenum ATCC66564	-	-	-
Mucor azygosporus ATCC15087	-	-	-
Fusarium oxysporum NCIM1008	3	4	9
Alternaria alternata ATCC6663	2	4	6

- Nil

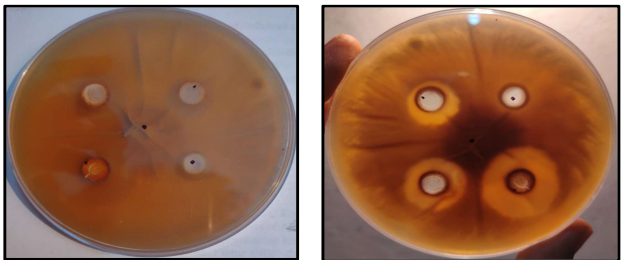
(Figure 5a). Similarly, 1 to 640 µl/ml of 5 mg/ml BHT, ascorbic acid and quercetin showed significantly increased antioxidant ability ranging from 3.77 to 45.33 % (12.02 fold), 2.40 to 38.52 % (16.05 fold) and 3.40 to 40.52 % (11.91 fold) (Fig 5 b). The antioxidant activity of red biopigment might be due to the reduction of hydroperoxide, inactivation of free radicals or complex formating with metal ions or combination there of (Lee *et al.* 2008). Moreover, this good antioxidant activity of red biopigment might be attributed to the presence of phytochemical nutrients (Wang and Wixon, 1999). The results imply that these active extracts may contain constituents with strong proton-donating abilities (Olivero *et al.,* 2010).

The results corroborated to Tan *et al.* (2018), who reported that 1 mg/ml red yeast *Monascus* pigment extracted from *Monascus ruber* CGMCC 10910 has the highest antioxidant activity of 32.84 %. In addition, red yeast rice extracted from *M. purpureus* CICC 40942 were found to show 9.739 ± 0.652 mg AEE/g of sample scavenging activity (Huang *et al.,* 2017). According to a comparable investigation, several *M. purpureus* extracts have DPPH radical scavenging activity ranging from 69.18 to 77.33 % at 100 mg/ml concentration of sample (Lin *et al.,* 2019). The good antioxidant activity of red biopigment might be attributed to the presence of many peptides and some metabolites produced by *Monascus* species during the fermentation and pigment derived from polyketides (Cheng *et al.,* 2016).

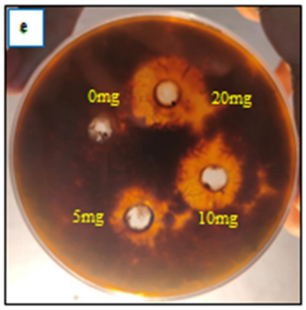
2,2'-Azino-Bis-(3–Ethylbenzthiazoline–6-Sulfonic acid) assay (ABTS): 2, 2′-azinobis (ethyl benzothiazoline 6-sulfonate) is oxidized by oxidants to its radical cation, ABTS•−, which is intensely colored, and antioxidant capacity is measured as the ability of test compounds to



(a) ***Aspergillus flavus*** ***(b) Penicillium chryosogenum***



(c)*Mucor azygosporus* **(d)** ***Fusarium oxysporum***



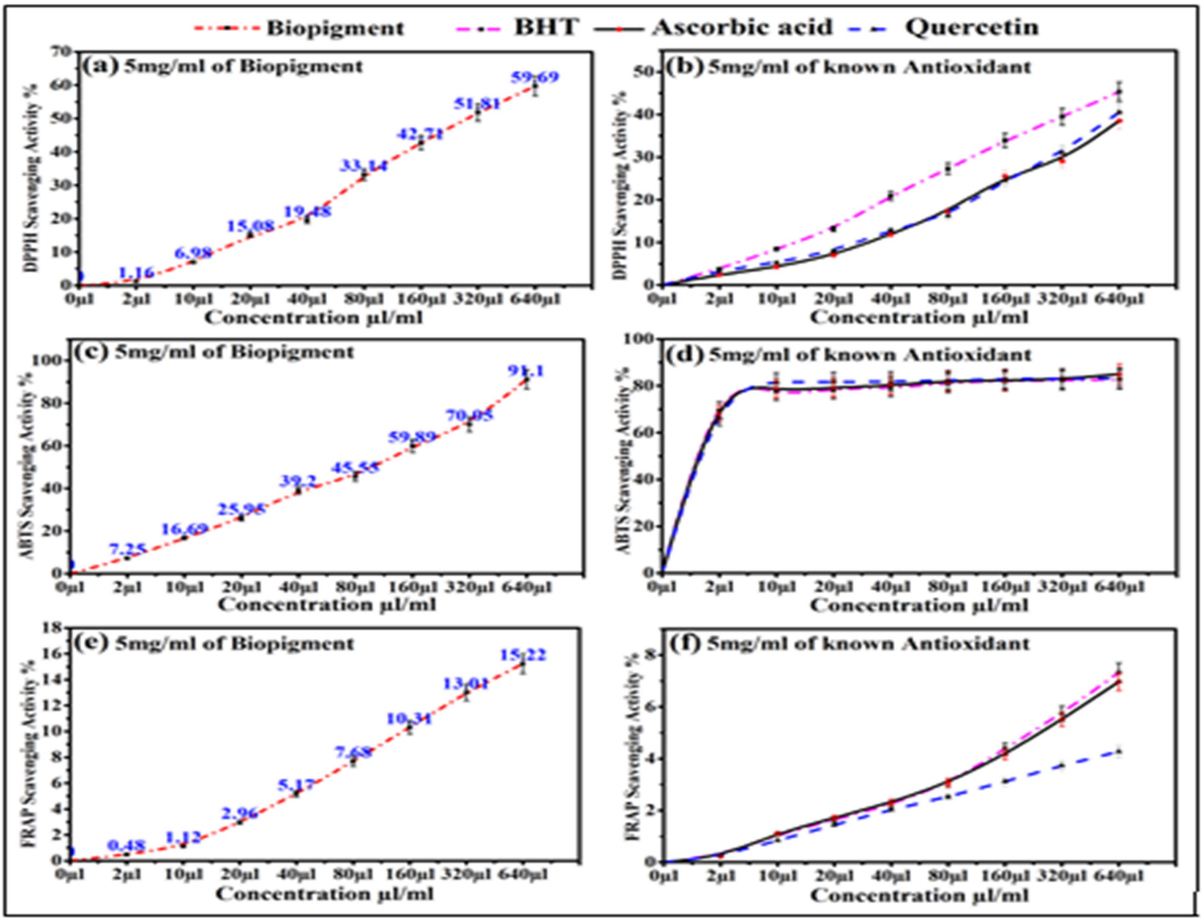
(e) Alternaria alternata

Figure 4: The inhibition zone (mm) produced by different concentration of *Monascus* biopigment against (a) *A. flavus,* (b) *P. chryosogenum,* (c) *M. azygosporus,* (d) *F. oxysporum,* and (e) *A. alternate.*

decolorize the ABTS radical directly. The results showed that 640 µl of 5mg/ml of extracted biopigment and positive control BHT, ascorbic acid and quercetin had higher free radical scavenging activities as compared to other concentration (Figure 5 c-d). There was a significant difference between concentration of biopigment and the antioxidant ability at 5 % (0.05 %) level of significance. In particular, 1 to 640 µl/ml of 5 mg/ml red biopigment significantly increased antioxidant ability ranging from 7.25 to 91.1 % (12.56 fold) (Figure 5 c). While, 1 to 640 µl/ml of 5 mg/ml BHT, ascorbic acid and quercetin showed significantly increased antioxidant ability ranging from 69.66 to 82.77 % (1.18 fold), 68.29 to 84.99 % (1.24 fold) and 66.22 to 83.19 % (1.25 fold) (Figure 5 d). The antioxidant action of red biopigment could be attributed to hydroperoxide reduction, free radical inactivation, metal ion complexing, or a combination of these factors Lee *et al.* (2008). Furthermore, the presence of phytochemical elements may be responsible for red biopigment's high antioxidant activity Wang and Wixon, (1999).

The results corroborated to earlier studies by Tan *et al.* (2018) showing that 1 mg/ml red yeast *Monascus* pigment extracted from *Monascus ruber* CGMCC 10910 has the highest antioxidant activity of 84.92 %. The antioxidant activity of *Monascus* fermented rice bran produced by *Monascus pilosus* KCCM60084 increased from 25 to 75 % at concentration of 0.25, 0.5, and 1 mg/ml of test samples (Cheng *et al.,* 2016). According to a comparable study, red yeast rice extracted from *M. purpureus* CICC 40942 were also found to show 9.739 ± 0.652 mg AEE/g of sample scavenging activity (Huang *et al.,* 2017). Due to several metabolites formed during the fermentation, the results suggest that the *Monascus* red pigment may contain components with considerable antioxidant potential (Olivero *et al.,* 2010; Cheng *et al.,* 2016).

Ferric Reducing Antioxidant Power (FRAP) assay: FRAP assay is widely-used to directly test the total antioxidant potential of several foods and plant extracts based on the reduction of complexes of 2,4,6-32 tripyridyl-s-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3.6H_2O$), which are almost colorless. The solution eventually turns slightly brownish forming blue ferrous complexes after complete reduction. The results showed that 640 µl of 5 mg/ml of extracted biopigment and positive control BHT, ascorbic acid and quercetin had higher free radical scavenging activities as compared to other concentration. There was a significant difference between concentrations of biopigment on the antioxidant ability at a 5 % (0.05 %) level of significance. In particular, 1 to 640 µl/ml of 5 mg/ml red biopigment significantly increased antioxidant ability ranging from 0.48 to 15.22 % (31.74 fold) (Figure 5 e). Whereas, 1 to 640 µl/ml of 5 mg/ml BHT, ascorbic acid and quercetin showed significantly increased antioxidant ability ranging from 0.26 to 7.32 % (28.15 fold), 0.22 to 6.96 % (31.63 fold) and 0.24 to 4.27 % (17.79 fold) (Figure 5 f). The increased antioxidant activity could be



Error bars are the mean ± standard deviation from independent variations

Figure 5: Free radical DPPH, ABTS and FRAP scavenging activity of red biopigment with respect to positive controls.

attributable to the increased polyphenol and flavonoid contents. Furthermore, fermentation can generate a large number of small peptides and other secondary metabolites with high antioxidant potential (Wang and Wixon, 1999; Cheng *et al.,* 2016).

The results corroborated to Chang *et al.* (2016) who reported that 1 mg/ml extract from *Monascus* fermented rice bran by *Monascus ruber* CGMCC 10910 demonstrated 15 to 38 % reducing activity at concentration of 0.25, 0.5, and 1 mg/ ml of test samples. In addition, according to a comparable study, extract of red yeast rice produced from *M. purpureus* CICC 40942 were found to show 0.023 ± 0.002a mmol Fe^{2+}/g of sample reducing activity (Huang *et al.,* 2017). The results imply that extracted biopigment may contain constituents with strong proton-donating abilities (Olivero *et al.,* 2010).

Conclusion

The efficacy of *Monascus* biopigment as an agent that selectively inhibits bacterial and fungal pathogens. The antibacterial activity through Scanning Electron Microscopy and agar well assay against *B. cereus*, *E. coli*, and *S. aureus, K. pneumonia* and *P. aeruginosa* was observed show morphological damage in some cells and zone of inhibition produced. Whereas, biopigment exhibited no antibacterial activity against *S. mutans, L. monocytogenes, P. mirabilis*, *S. Typhi* and *S. flexneri.* Next, the antifungal activity of the extracted biopigment was observed zone of inhibition produced for *A. flavus, F. oxysporum* and

A. alternata. Biopigment did not show any antifungal activity against *P. chryosogenum* and *M. azygosporus*. *Monascus* biopigment demonstrated the potential antimicrobial effect through inhibition of bacterial cells and fungal pathogens. In addition, antioxidative assay DPPH, ABTS and FRAP based results showed that there was a significant difference between various concentrations of biopigment on the antioxidant ability. Considering these observations, it appears that red biopigment may be a useful supplement for antimicrobial and therapeutic related diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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