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SHORT COMMUNICATION

Characterisation and evaluation of anticancer, antimicrobial and antioxidant activity of the pigment from *Pyxidicoccus* sp. S252

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Myxobacteria, a group of δ-proteobacteria, are known to be prolific producers of bioactive secondary metabolites. *Pyxidicoccus* sp. S252, a myxobacterial strain, produces an orange pigment. The pigment was extracted from the cells, and its bioactive potential was evaluated. The pigment extract inhibited Gram-positive bacteria and fungi (*Alternaria* sp. and *Fusarium* sp.) and exhibited antioxidant properties. The methanol extract of the pigment inhibited human osteosarcoma cells in vitro, with an IC50 of 87 µg/mL against MG-63 cells. The active fraction of the pigment implicated in antimicrobial activity was identified as a flexirubin-type pigment.

Keywords: Bioactive, Cytotoxic, Myxobacteria, Pigment, *Pyxidicoccus* sp. S252

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Introduction

Pigments or synthetic colourants are essential components of food, fabric and pharmaceutical industries. In 1856, the first synthetic dye (mauve) was made. Synthetic colourants gained much attraction in everyday life because of their cost-effective nature and more stability[1]. The serious side effects of synthetic pigments on the environment and human health have deterred their use[2]. Due to the carcinogenic nature of some synthetic colourants, a ban has been imposed on them, namely Blue FCF, Blue No.1, and No.2[3]. This paved the way for the discovery of non-toxic biodegradable pigments from natural sources. The therapeutic uses of natural pigments have prompted researchers to seek novel pigments. Plants, animals, and microorganisms are the main sources of natural pigments. Fast growth, uncomplicated processing, and independence from weather make bacteria and fungi more advantageous than other natural pigment sources[4]. Because plant pigment production is seasonal, some pigments are commercially produced by microbes. For example, carotenoids are produced on low carbon sources by bacteria (*Streptomyces chrestomyceticus*, *Flavobacterium* sp) and fungi (*Blakeslea trispora*, *Phycomyces blakesleeanus*, *Phaffia* sp., and *Rhodotorula* sp.)[5]. Carotenoids possess biological properties like antioxidant, provitamin A, and inhibition of cancer cells. Further, lycopene (a carotenoid) is the strongest antioxidant secreted by the fungus *Blakeslea trispora* and *Phycomyces blakesleeanus*[6]. More than 50 *Monascus* pigments have been identified, with several significant activities within biological systems[7]. Thus, biodegradability, low toxicity, and better therapeutic efficacy make bio-pigments safe alternatives to synthetic pigments. Among different sources of bio-pigments, bacteria are the favoured due to their ease of cultivation and genetic manipulation[8].

Bacterial pigments are commercially produced for the food, fabric, and cosmetic industries. Besides imparting colour to any substance, these pigments have antimicrobial, antioxidant, antiviral, and anticancer activities[8]. The bacterial pigment anthocyanin induces apoptosis in cancer cells and has anti-inflammatory effects. Violacein, a cytotoxic pigment isolated from *Chromobacterium violaceum* shows antileishmanial and anticancer activities[9]. Another well-known pigment prodigiosin, obtained from *Serratia marcescens*, acts as a cytotoxic agent against various cell lines[10].

Myxobacteria are Gram-negative, rod-shaped, soil-dwelling bacteria with a unique life cycle that includes a multicellular stage, the fruiting bodies[11]. For more than thirty-five years, they have been known as prolific producers of novel secondary metabolites with several bioactive properties[12]. Carotenoids and DKxanthenes (a family of yellow pigments) implicated in developmental regulation and protection against oxidative damage have been reported in *Myxococcus* spp.[13,14].

In this study, a pigment was isolated from *Pyxidicoccus* sp. S252, a myxobacterium isolated from soil in India[15]. The pigment was partially purified by thin layer chromatography (TLC) and characterised by LC/MS and FTIR. The bioactivity of

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the pigment was assessed against bacteria, fungi, and human cancer cells.

Materials and Methods

Growth conditions and pigment extraction

Pyxidicoccus sp. S252 (accession number MCC 3273) was procured from the National Centre for Microbial Resource (NCMR). It was cultured in CY broth (0.3% casitone, 0.1% yeast extract, 0.1% $CaCl_2 \cdot 2H_2O$; pH 7.4) at 30°C.

To extract the pigment, a 1% overnight-grown seed culture was inoculated into CY medium and incubated at 30°C/160 rpm for 5 days. The culture was centrifuged at 5,000 rpm for 15 min, and the cell pellet was resuspended in methanol and sonicated. The suspension was subjected to centrifugation and the supernatant was retrieved, dried, weighed, and resuspended in methanol to prepare the methanol extract (ME) of the pigment.

Thin layer chromatography

A TLC plate (silica sheet) was used to separate the components in the pigment extract. A thin strip (9.8 cm×2.5 cm) was cut, and a line was marked about 1.5 cm from the bottom edge. After applying a spot of ME on the marked line, the TLC plate was placed inside the solvent chamber containing the solvent mixture. The solvent system used for the separation of ME was chloroform:hexane:acetone (8:2:0.5). As the solvent reached the top edge of the sheet, the solvent front was marked with a pencil[16]. An iodine chamber was used for developing the TLC, and Rf values were calculated as:

$$\text{Rf} = \frac{distance\ \ travelled\ \ by\ the\ solute}{distance\ \ travelled\ \ by\ the\ solvent}$$

Cytotoxicity assay

Human cancer cell lines were procured from the National Centre for Cell Science (NCCS), Pune and maintained in their respective culture media as follows: Dulbecco's Modified Eagle's Medium (DMEM) for osteosarcoma (MG-63), McCoy's 5a Medium for colorectal carcinoma (HCT-116) and Roswell Park Memorial Institute Medium (RPMI) for prostate adenocarcinoma (PC-3) supplemented with 10% fetal bovine serum (FBS) and was maintained at 37°C in CO_2 incubator (5% CO_2; 90% RH).

A Sulforhodamine B (SRB) assay was performed to evaluate ME cytotoxicity across different cell lines. 7,500 cells were seeded per well in the microtiter plates and incubated for 24 h (MG-63 and HCT-116) and 48 h (PC-3). ME was added in the wells in quadruplicate and incubated for 48 h at 37°C. The cells were fixed using ice-cold 10% (w/v) trichloroacetic acid at 4°C for 1 h, washed, dried, and stained with 100 µL of 0.04% (w/v) SRB at room temperature for 1 h[17]. The cells were washed with 1% acetic acid to remove the unbound SRB, and the plate was air-dried. Thereafter, 100 µL of 10 mM Tris solution was added to each well, and absorbance was measured at 510 nm using a microplate reader[18]. Cell growth inhibition was evaluated by:

$$\%\ \text{cell growth} = \frac{\text{Absorbance sample}}{\text{Absorbance untreated}} \times 100;$$

$$\%\ \text{growth inhibition} = 100 - \%\ \text{cell growth}^{19}.$$

Characterisation of the pigment

A reagent spray test was conducted to examine the nature of the fractionated spots on the TLC plate. Freshly prepared sulfuric acid-acetic anhydride reagent, or Liebermann-Burchard (LB) reagent, was used as follows: 50 mL of pure ethanol was mixed with 5 mL each of acetic anhydride and concentrated sulfuric acid while cooling on ice. After 5–10 minutes of warming the plate at 100°C, the sprayed plate was examined for the confirmatory spots of terpenes[20].

Spot 1 was eluted from the silica plate by scraping and eluting in methanol. The spot was applied to the HPLC column (Waters 2795) using a 100 µL syringe and eluted with methanol containing 0.1% formic acid (v/v). The flow rate was kept at 0.1 mL/min. Mass spectrometric analysis was then carried out with a quadrupole time-of-flight (Q-Tof) mass spectrometer (Sophisticated Analytical Instrumentation Facility, Chandigarh) equipped with electrospray ionisation (ESI) in positive mode, with a scan range of *m/z* 100-1500.

The infrared spectrum of the fractionated spot 1 was acquired using a Spectrum Two™ FTIR spectrometer (PerkinElmer Inc., USA) and examined as a KBr pellet. The frequency of the spectral area examined ranged from 450-4000 cm^{-1}.

Antifungal activity assay

The potential of ME and the compounds extracted from the spots separated on TLC as antifungal agents was tested against *Fusarium oxysporum* (MTCC 7677) and *Alternaria alternata* (lab isolate). The vegetative cells of each fungal strain were spread on potato dextrose agar (PDA) plates, and wells (6 mm) were punched with the sterile tips[21]. A 100 µL of ME (1 mg/mL) or the eluted fractions (100 µg/mL) were added to the wells, and the plates were incubated at

room temperature. Methanol and cycloheximide (1 mg/mL) were used as negative and positive controls, respectively. The clear zones surrounding the wells were measured to assess the antifungal activity.

Antibacterial activity assay

The evaluation of antibacterial activity was carried out against both Gram-negative (*E. coli* DH5α MTCC 1652) and lab-isolated Gram-positive bacteria (*Bacillus* sp. and *Lysinibacillus* sp.) using the well-diffusion assay[22]. The freshly grown test cultures were spread on Czapek Yeast Extract agar medium, and wells (6 mm) were punched. 100 µL (1 mg/mL) of ME or 100 µL of the eluted fractions (100 µg/mL) were added to the wells, incubated at 37°C for 16 h, and the zones of inhibition were measured. Kanamycin (1 mg/mL) and methanol were used as positive and negative controls, respectively.

Antioxidant activity

Ferrous reducing antioxidant capacity assay

The reducing potential of the pigment was estimated by following the standard method[23]. To 0.25 mL of the ME (0.5 mg/mL - 1.5 mg/mL), 0.625 mL of potassium buffer (0.2 M) and 0.625 mL of 1% potassium ferricyanide solution were added. After incubation for 20 min at 50°C, 0.625 mL of 10% trichloroacetic acid (TCA) was added and the mix was centrifuged. To the supernatant, an equal volume of distilled water and 0.36 mL of 0.1% ferric chloride solution were added followed by measurement of absorbance at 700 nm.

DPPH radical scavenging assay

The scavenging activity of the pigment was measured by the method of Blois[24]. The violet/purple colour of DPPH in methanol fades to yellow colour by the pigment's hydrogen atom donation. To 1.6 mL of ME (500-1500 µg/mL), 2.4 mL of 0.1 mM DPPH solution prepared in methanol was added. The mixture was vortexed and incubated in dark at RT for 30 min. DPPH scavenging was measured by taking absorbance at 517 nm. The percentage DPPH radical scavenging activity was calculated as:

$$\%\ \text{DPPH radical scavenging activity} = \frac{A0 - A1}{A0} \times 100$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of ME.

Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical analysis was carried out using an unpaired t-test in GraphPad Prism software (version 8.0), with a p-value ≤ 0.05 (statistically significant).

Results and Discussion

Extraction of pigment from *Pyxidicoccus* sp. S252

An orange-colored methanol extract (ME) was obtained from S252 (Fig. 1a). ME was spotted and resolved on the silica sheet and showed five spots after development of the plate with iodine (Fig. 1b), with Rf values as listed in Table 1.

In vitro cytotoxicity of the pigment extract

ME was screened for anticancer activity and showed a distinct cytotoxic effect on the MG-63, HCT-116, and PC-3 cell lines in a dose-dependent manner. A significant antiproliferative activity was observed against all tested cell lines, with the most potent activity (93.5% cell death at 250 µg/mL) against MG-63 cells, with an inhibitory concentration (IC50) of 87.1±5.2 (Fig. 2).

Most probable mechanism of action for the antiproliferative effect of natural products is the

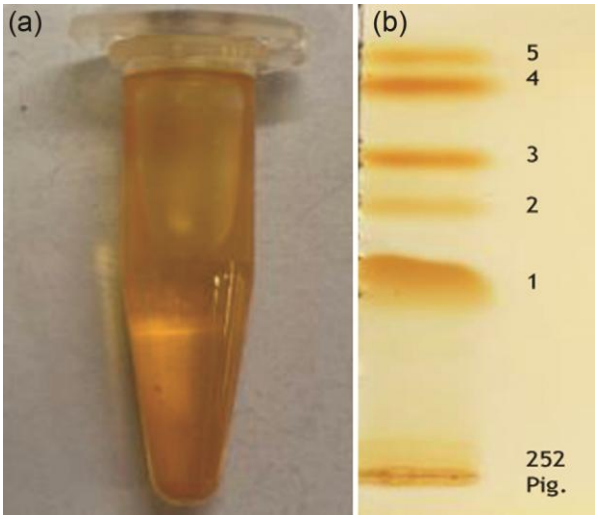

Fig. 1 — (a) ME of S252, and (b) Thin layer chromatography of ME showing five distinct spots.

Table 1 — Rf values of different spots of ME separated by TLC

Spots	Rf value
1	0.421
2	0.526
3	0.61
4	0.736
5	0.789

induction of apoptosis[25]. Lin *et al.* conducted a study on pigments derived from 12 bacterial strains and showed that the main cause of cytotoxicity in HeLa cells is apoptosis[26]. A similar mechanism has been confirmed in pigments such as anthocyanins, prodigiosins, and violaceins. To the best of our knowledge, the pigment molecule from any of the myxobacterial strains has not been reported to have antiproliferative activity against human cancer cells. Pigments, myxochromides B3 and A3, isolated from *M. virescens* and *M. flavescens*, were evaluated against a panel of 28 cancer lines and failed to show cytotoxic effects against any of them[27].

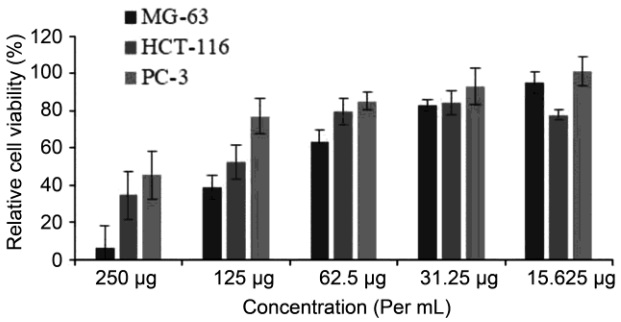

Fig. 2 — Antiproliferative activity of the methanolic extract of the pigment in osteosarcoma (MG-63), colon cancer (HCT-116), and prostate cancer (PC-3) cell lines.

Antifungal activity

The antifungal activity of ME was examined against *A. alternata* and *Fusarium oxysporum* by measuring the zone of inhibition around the wells after 3 days of incubation (Fig. 3; Table 2). ME showed comparable zones of inhibition to those of the tested fungal strains. As ME showed promising activity against both phytopathogenic fungi, the compounds in the fractionated spots were eluted from the TLC plates, and their growth inhibition was assessed against the test fungi. Only spots 1 and 5 showed inhibition zones against both fungi (Fig. 3; Table 2). *A. alternata* and *F. oxysporum* are phytopathogenic fungi that lead to pre- and post-harvest yield loss, implicating economic loss[28]. The use of chemical fungicides is hazardous to human and animal health, making it imperative to seek natural antifungals that are environmentally sustainable for controlling phytopathogens. The antifungal mechanism of action of known microbial pigments, such as violaceins, is not well understood. It is known that these pigments generate reactive oxygen species (ROS), which further disrupt the fungal cell wall and reduce hyphal growth. DKxanthenes (DKX), a class of yellow pigments, isolated from *Myxococcus stipitatus* DSM 14675, inhibited the growth of *Candida albicans*, *Aspergillus niger* and *Rhizopus stolonifer*[29].

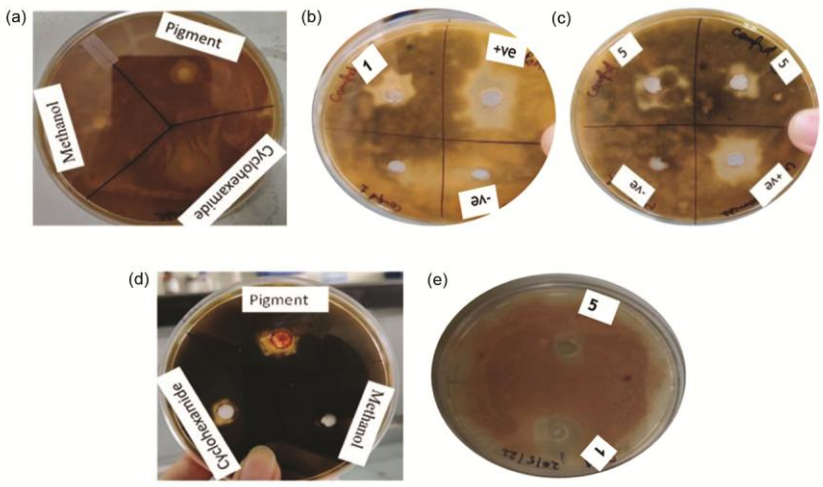

Fig. 3 — Antifungal activity of ME and purified fractions against (a-c) *Alternaria alternata*, and (d-e) *Fusarium oxysporum*, respectively.

Table 2 — Zones of inhibition of the tested samples against *Alternaria alternata* and *Fusarium oxysporum*

Sample	Concentration (mg/mL)	*Alternaria alternata* (mm)	*Fusarium oxysporum* (mm)
ME	1	9.2±0.54	10±0.31
Spot 1	0.1	12±0.12	12±0.32
Spot 5	0.1	9.2±0.25	9±0.08
Cycloheximide (control)	1	12.2±0.12	11.6±0.92

Antibacterial activity

The pigment extracted from the S252 strain showed no activity against the Gram-negative bacterium (*Escherichia coli*). In contrast, growth inhibition was observed in *Bacillus* sp. and *Lysinibacillus* (Fig. 4). The zone of inhibition in *Bacillus* sp. and *Lysinibacillus* was 9±0.32 and 11±0.25 mm, respectively. Spots 1, 2, and 4 also showed inhibitory activity, with clear zones of 8±0.15 mm.

Disruption of the bacterial cell membrane in Gram-positive bacteria is the primary mechanism of pigments' antibacterial activity. This disruption suppresses quorum sensing (QS) systems as in *Chromobacterium violaceum*, preventing biofilm formation, reducing membrane potential, increasing cell contents leakage as in *Lachnum* YM30 melanin pigment against *Vibrio parahaemolyticus* and *Staphylococcus aureus*[30], and boosting non-protein nitrogen absorption. Previous literature also showed less effect of pigments on Gram-negative bacteria than Gram-positive microbes due to the presence of liposaccharide layer in the earlier[31]. The pigment antibiotics, myxochromide B_3 and A_3, extracted from *M. virescens* and *M. flavescens*, did not show antibacterial activity against *Bacillus megaterium* and *Escherichia coli*.

Antioxidant activity

Ferrous reducing antioxidant capacity assay

The Fe^{3+} to Fe^{2+} reduction test was used in this investigation to determine the pigment extract's reducing capacity. The extract showed an increase in absorbance with increasing concentration, suggesting an increase in the pigment's reducing power.

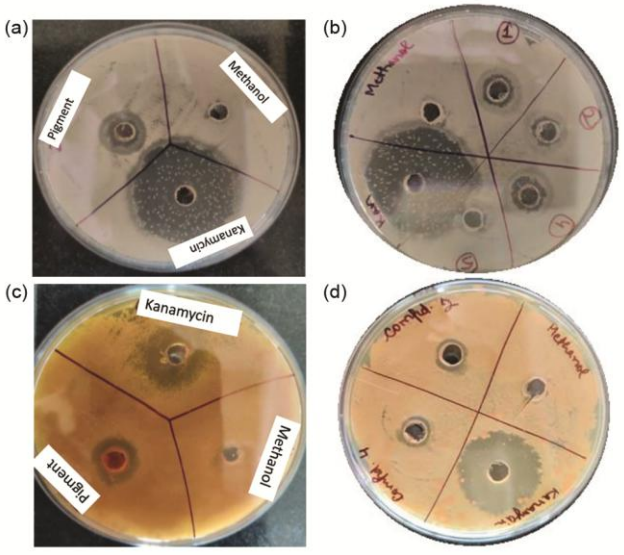

Fig. 4 — Antibacterial activity of ME and purified fractions against (a-b) *Bacillus subtilis*, and (c-d) *Lysinibacillus*, respectively.

DPPH radical scavenging assay

The antioxidant activity increased significantly with the concentration of the pigment extract, showing 20.07, 48.8, and 73.51 at 250, 500, and 1000 µg, respectively. Ascorbic acid, as a positive control, showed 98% activity at a concentration of 1000 µg. Further studies on the purified pigment will assess its potential as an antioxidant for biotechnological applications. DKxanthene-534, isolated from *Myxococcus Xanthus* DK1050, showed three-fold lower antioxidant activity in the DPPH assay than ascorbic acid. The possible mechanism of its activity involves free oxygen scavenging and preventing the entry of ROS into the hydrophobic lipid bilayer (the site of oxygen degradation)[32].

Characterisation of the active fraction of the pigment

Spot 1 in the TLC fractionation showed both antifungal and antibacterial activity; it was subjected to chemical analysis. The spray test conducted with Liebermann-Burchard (LB) reagent on the TLC plate indicated the presence of terpenes (carotenoid) in spot 1. The spot was eluted from the TLC plate and resuspended in methanol; it was characterised using Fourier transform infrared spectroscopy (FTIR) and LC/MS-MS. The spectrum of spot 1 revealed a major peak at a retention time of 0.77 min from the HPLC column (Fig. 5a). Further, mass analysis depicted two peaks at *m/z* 481 and 525 (Fig. 5b). The difference in mass of parent and product ion may represent a formic acid adduct [HCOOH], and peaks are analogous to spectral data of carotenoids[33].

Analysis by FTIR revealed the broad band at 3496 cm-1 as a distinctive O-H stretching band of a hydroxyl group (Fig. 6). The CH and CH2 stretching of aliphatic groups is typically associated with the shoulder at 2853 cm^{-1}. Conjugated C=C bonds are thought to be indicated by the combination of bands at 2116 cm^{-1} and 1642 cm^{-1}. Carbonyl groups exhibit sharp peaks between 1750 and 1610 cm^{-1}. Keto groups can also be attributed to the peak at 1650 cm^{-1}, as they typically exhibit bands in FTIR at 1650–1670 cm^{-1} or lower values. The weak band represents the C-O bond at 1017 cm^{-1}. Additionally, the asymmetric C-H stretching in alkyl hydrocarbons, which correlates to the flexirubin class of pigment, is responsible for the absorption bands from 893 to 526 cm^{-1}[(Ref. 34)]. The pigment isolated from S252

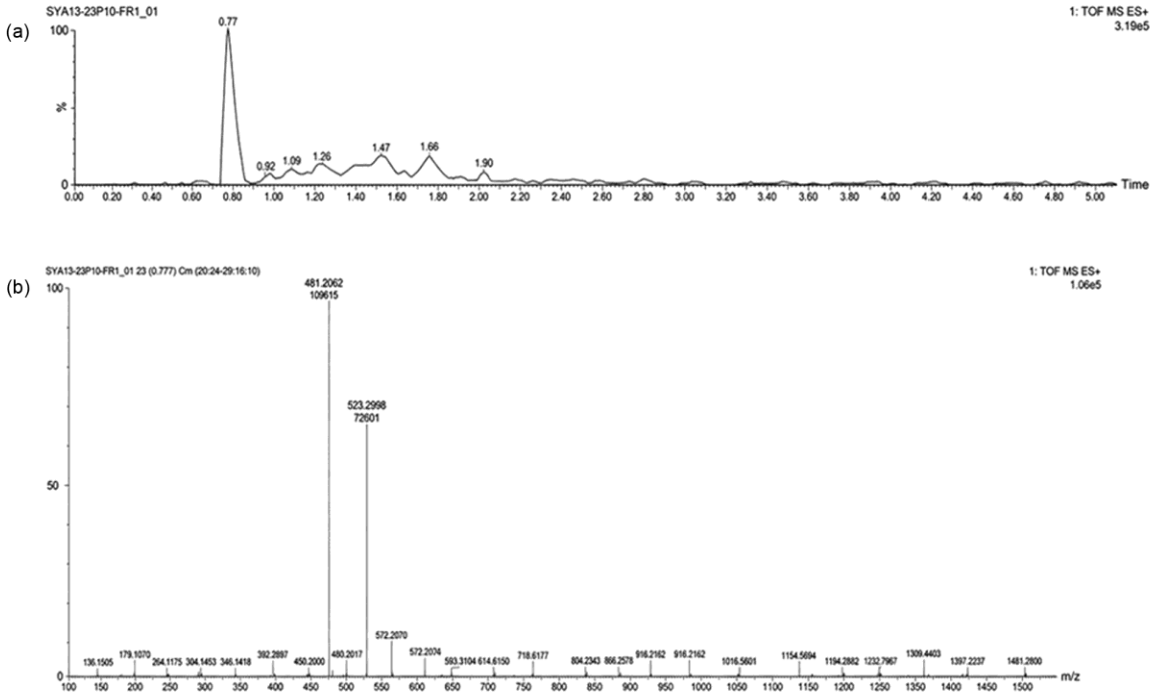

Fig. 5 — (a) HPLC profile, and (b) Mass spectrum (MS) of the fractionated spot 1 from the pigment extract of S252.

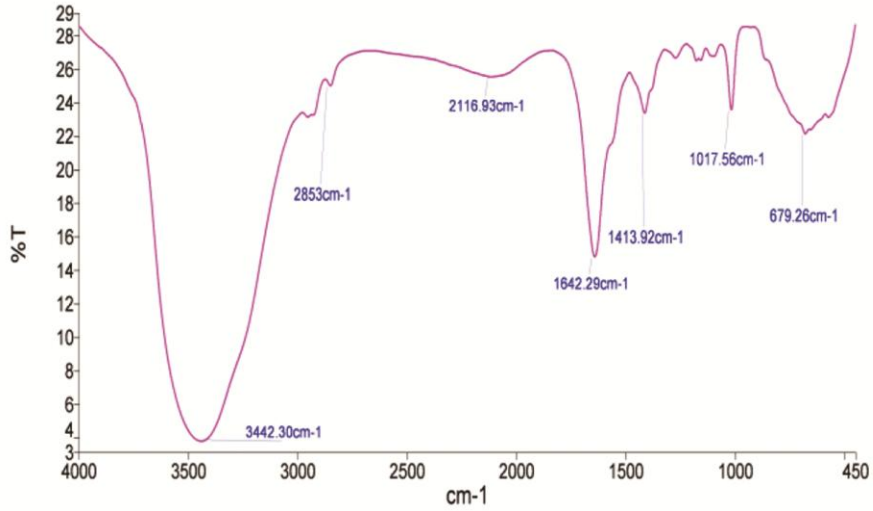

Fig. 6 — FTIR spectrum of the fractionated spot 1 from the pigment extract of S252.

might be a member of the flexirubin group, albeit more analysis is required to determine the precise chemical structure.

Conclusion

According to the current investigation, the orange pigment produced by S252 exhibits inhibitory activity against bacteria (*Bacillus subtilis* and *Lysinibacillus*) and fungi (*A. alternata* and *F. oxysporum*), making this pigment a potential antimicrobial agent for applications such as food additives. The pigment extract also strongly inhibited the proliferation of MG-63 cells *in vitro*, compared with HCT-116 and PC-3 cells. The pigment extract also showed strong antioxidant activity, suggesting the possible applications in the cosmetics industry. A component of the pigment extract with antimicrobial activity was identified as a flexirubin-like compound. Further

investigation into this pigment can provide insight into its use as an eco-friendly alternative to chemically synthesised pigments.

Conflict of interest

All authors declare that they do not have any conflict of interest.

AI use disclosure

Authors declare that no AI tool has been used for any purpose in this research paper.

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