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ORIGINAL ARTICLE

A novel red pigment from marine *Arthrobacter* sp. G20 with specific anticancer activity

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Abstract

Aims: Bacterial pigments are promising compounds in the prevention and treatment of various cancers. In the current study, the antioxidant, cytotoxic and antimicrobial effects of a red pigment obtained from a marine bacterial strain were investigated.

Methods and Results: Optimization of the pigment production by the marine strain was conducted using the one-factor-at-a-time approach. Chemical identification of the pigment was achieved by UV-visible, FTIR and HPLC analyses. The biological activities of the pigment were evaluated by DPPH, MTT and microbroth dilution assays. The strain was identified as *Arthrobacter*, and its pigment was related to carotenoids. The EC_{50} antioxidant activity of the pigment was evaluated as 4·5 mg ml^{-1}. It showed moderate anticancer effects on an oesophageal cancer cell line, KYSE30, while no inhibition was observed on normal HDF (human dermal fibroblasts) cells. The pigment had no antibacterial effects on the four tested strains.

Conclusion: The antitumour activity of a carotenoid-related pigment from *Arthrobacter* sp. was reported for the first time.

Significance and Impact of the Study: Marine environments are interesting sources for the identification of novel bioproducts. The identification of carotenoid pigments from marine bacteria with remarkable antioxidant and anticancer activities would result in better insights into the potential pharmaceutical applications of carotenoids and marine environments.

Introduction

Cancer is a group of debilitating diseases in which cells begin to divide and spread into the surrounding tissues is an uncontrolled manner. The development of cancer in the body is a dynamic and long-term process affected by factors like chemical pollutants, diets, hormonal and many other (unknown) factors (Pan and Ho 2008). The increasing industrial applications of carcinogenic compounds, along with changes in the lifestyle, and extended life expectancy have resulted in the recognition of cancer as a big threat to public health, and it accounts as one of the leading causes of death all over the world. Chemotherapy is one of the useful methods for the clinical treatment of cancer patients. Some synthetic drugs are used in chemotherapy, but they usually have severe side effects as they affect both cancerous and normal cells. Therefore, it is necessary to develop anticancerous compounds with selective activity against tumour cells (Chang *et al.* 2011).

Pigments with various chemical formulas are colourful materials as a result of selective visible wavelength absorption. The main sources of biological pigments are plants and flowers. They have been shown to possess various biological effects like antibacterial, anticancerous and antioxidant activity. Flavonoids and quinones obtained from citrus and vegetables are some examples of plant pigments with antitumour activity (Dai and Mumper 2010). However, there is a growing interest in bacterial pigments in comparison to plant sources because of some

properties like rapid production regardless of seasonal-geographical conditions and specific pharmaceutical characteristics (Venil *et al.* 2013). Bacterial pigments have great applications in food, textile, cosmetic and pharmaceutical industries (Rao *et al.* 2017). In addition to being environmental friendly, these pigments can apply as natural colour to foods and have antioxidant and cancer prevention health benefit (Tuli *et al.* 2015). Prodiginin, a red pigment produced by *Serratia* spp., and violacein, a purplish black pigment produced by *Chromobacterium* spp., are two well-known bacterial pigments with antibacterial (Lapenda *et al.* 2015), antiviral (Cazoto *et al.* 2011) and anticancer (Montaner *et al.* 2000) properties.

Marine environments cover two-thirds of the planet's surface; they are interesting sources for the identification of novel bioactive compounds. Mudit and El Sayed recently reviewed marine- derived compounds with potential anticancer activities (Mudit and El Sayed 2016). Several of these entities have bacterial sources (Chang *et al.* 2011). Aqueous environments are also interesting habitats for isolating cold adaptive micro-organisms in which pigmentation is a common trait for various protection purposes (De Maayer *et al.* 2014). The aim of the current study was to explore the biological activities of a red pigment extracted from a psychrophilic bacterial strain isolated from the Caspian Sea.

Materials and methods

Isolation and identification of pigmented bacterium

Samples were obtained from 15 m depth of the Caspian Sea (36·46°N 51·02°E). They were aseptically pooled and diluted up to 10^{-5}, and inoculated on the artificial sea water (ASW) agar medium containing (g l^{-1}): NaCl 10, $MgSO_4$ 0·52, $FeSO_4$ 0·01, $MgCl_2{\cdot}6H_2O$ 0·19, $(NH_4)_2SO_4$, 0·98, $CaCl_2{\cdot}2H_2O$ 0·19, KCl, 0·15, peptone 10, yeast extract 5, glucose 2 and agar 15 (Stafsnes *et al.* 2010). The pH was adjusted to 8, and the plates were kept at 20°C for at least 1 month. A red-pigmented bacterium designated as strain G20 was obtained in the isolation procedure. The extraction of genomic DNA, PCR amplification of 16S rRNA and phylogenetic analysis were carried out as described later (Makhdoumi *et al.* 2015).

Preparation of the pigment extract

The bacterial cells were grown in the ASW broth medium in a 1000-ml Erlenmeyer flask and kept in a shaker incubator (150 rpm and 20°C) until the culture medium turned red. The bacterial cells were harvested following centrifugation at 4000 rpm for 20 min. The cell pellets were extracted with 95% (v/v) methanol until the pellets became colourless. The methanolic extract was collected and evaporated under reduced pressure below 40°C in a rotary evaporator (Heidolph, Germany), and then it was freeze-dried (Freeze dryer, Christ Alpha 1·2 LD Plus, Germany). Thin layer chromatography (TLC) was developed using silica plates (Merck, Darmstadt, Germany). The concentrated pigment was dissolved in a methanol solution, spotted on a silica gel sheet and developed with a mobile phase of ethanol: Diethyl ether (1:4 v/v) at room temperature in the dark. For preliminary chemical characterization, the UV-visible spectrum of the pigment was recorded between 200 and 800 nm, using methanol as a blank. High-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) consisting of a 1525 binary pump and a 2489 UV/vis detector set at 440 nm, was used. Separation was conducted on an InertSustainSwift C18 column (150 mm length, 4·6-mm internal diameter and 5-μm particle size) and the data were analysed with the Breeze 2 software (Waters). Linear gradient elution chromatography was done using solvent systems A (methanol) and B (water) at ambient temperature. The gradient program used was as follows: initial, A–B (20:80, v/v), and 0–60 min, linear change to A–B (80:20, v/v) at 1% methanol/min gradient speed. The flow rate was kept constant at 1 ml min^{-1}, and 20 μl of the methanol-water (50%, v/v) extract of pure pigment was applied. Crocin—a well-known plant carotenoid—was used as the reference pigment. Fourier transform infrared spectroscopy (FTIR) was measured in cuvettes with windows of calcium florid. The transformed spectres were background-corrected for the solvent (Aust *et al.* 2003).

Enhancing pigment production

To increase the pigment production, amounts of different factors including the carbon source (1% w/v of glucose, fructose, maltose and starch), temperature (10–40°C), pH (6–9) and incubation time (0–120 h) were varied one at a time in an ASW basal broth medium (20°C, pH 8). Each subsequent factor was examined after taking into account the previously optimized factor(s). In each experiment, the absorbance of methanolic extract was measured at 490 nm, and the bacterial growth was determined by the measurement of the absorbance at 600 nm (Shimadzu, Japan).

Antioxidant activity

The antioxidant capacity of the pigment was studied following a method described by Blois (1958). Briefly, two-fold serial dilutions of the pigment prepared in dimethyl sulfoxide were added to a 0·1 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma, USA) methanolic solution, and then left in darkness at 30°C for 30 min. The absorbance

of the resulting solutions was measured colorimetrically at 517 nm. A sample containing only the solvent and the DPPH without the pigment was used as a control. The scavenging effect on the DPPH was determined as follows:

$$\text{DPPH scavenging effect } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the pigment extract.

Cytotoxic activity

Cell lines and cell culture

KYSE-30 (a human oesophageal squamous carcinoma cell line) cells were obtained from the Pasteur Institute (Tehran, Iran) and cultured in the Roswell Park Memorial Institute medium (RPMI 1640, Gibco, Scotland) with 10% fetal bovine serum (FBS, Gibco, Scotland). Human dermal fibroblasts cells as a gift from the Royan Institute (Tehran, Iran) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Scotland) and enriched with fetal bovine serum (FBS, Gibco, Scotland). Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2 in the air.

Cell viability assay

Cell viability was determined by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann 1983). For this, 4000 KYSE30 cells and 8000 HDF cells were incubated in 96-well plates, in the absence (control) or presence (test) of various concentrations of the pigment in a final volume of 200 μl for 24, 48 and 72 h. A total of 20 μl of MTT (diluted in PBS) was added to each well, and after 4 h of incubation, the precipitated purple MTT formazan crystals were dissolved in 150 μl DMSO, and the absorbance was recorded at 540 nm (Stat Fax, America). The cell viability was measured by the following formula (Maheswarappa *et al.* 2013):

$$\text{Viablity} = [(A_T)/A_C] \times 100$$

where A_C was the absorbance of the control reaction and A_T was the absorbance in the presence of the pigment extract. Treated and control cells were observed under a light inverted microscope (Olympus, Japan) for monitoring the morphological changes after 24, 48 and 72 h.

Antibacterial activity

The effects of the pigment on the growth of the selected microbial strains were evaluated in a liquid Mueller Hinton medium using the microbroth serial dilution approach (Kim *et al.* 2007). *Staphylococcus aureus* (PTCC1431), *Bacillus subtilis* (PTCC1023), *Pseudomonas aeruginosa* (PTCC1074) and *Escherichia coli* (PTCC 1330) were selected as reference strains. Microbial growth was determined by measuring the turbidity at 600 nm.

Data analysis

All quantitative experiments were repeated at least three times. The data analysis was conducted using GraphPad Prism version 6·0 and the IC_{50} and EC_{50} values were obtained from nonlinear regression (Mosaddik *et al.* 2004; Deng *et al.* 2013).

Results

Isolation and identification of pigmented bacterium

Strain G20 was obtained from 15 m depth of Caspian Sea coastal water. The novel strain was Gram-negative, nonmotile and rod shaped. The cells of strain G20 formed convex and round colonies with an entire edge on the solid ASW medium after 2 days (Fig. 1a). The preliminary chemical characterization of the pigment extract is presented in Fig. 1b–d. As seen in Fig. 1b, the UV–Vis spectrum of pigment showed the maximum absorption at 491 nm, which is in the range of reports for various carotenoids (Sutthiwong *et al.* 2014). The FTIR spectrum for pigment (Fig. 1c) showed characteristic absorption frequencies of the carbonyl group at 1737 cm^{-1}, alkene group at 1635 cm^{-1}, aromatic ether group at 1264 cm^{-1}, CH_2 (bending) at 1466 cm^{-1}, mono-substituted benzene group at 721 cm^{-1} and the hydroxyl group at 3000 cm^{-1}. Further signals were identified as CH_3/CH_2 stretch vibrations at 2962 cm^{-1} and 2851 cm^{-1}. The IR spectrum of the pigment extract is similar to the spectrum of purified lycopene (Aghel *et al.* 2007). Comparing the HPLC chromatogram of the novel pigment solution with the chromatogram of the crocin solution (Fig. 1d) showed the major peak at the retention time of 30·536 min, followed three minutes later by a smaller but marked peak, which is comparable with crocin, a plant carotenoid that is found in the flowers of *Crocus* and *Gardenia*. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain G20 is a member of the genus *Arthrobacter* (Fig. 2). The closest relatives of strain G20 were *Arthrobacter echini* AM23^T, *Arthrobacter agilis* KCTC 3200^T and *Arthrobacter pityocampae* Tp2^T with a gene sequence similarities of 99·4%.

Enhancing pigment production by one-factor-at-a-time approach

The effects of various levels of selected factors including a carbon source, temperature, pH and incubation time

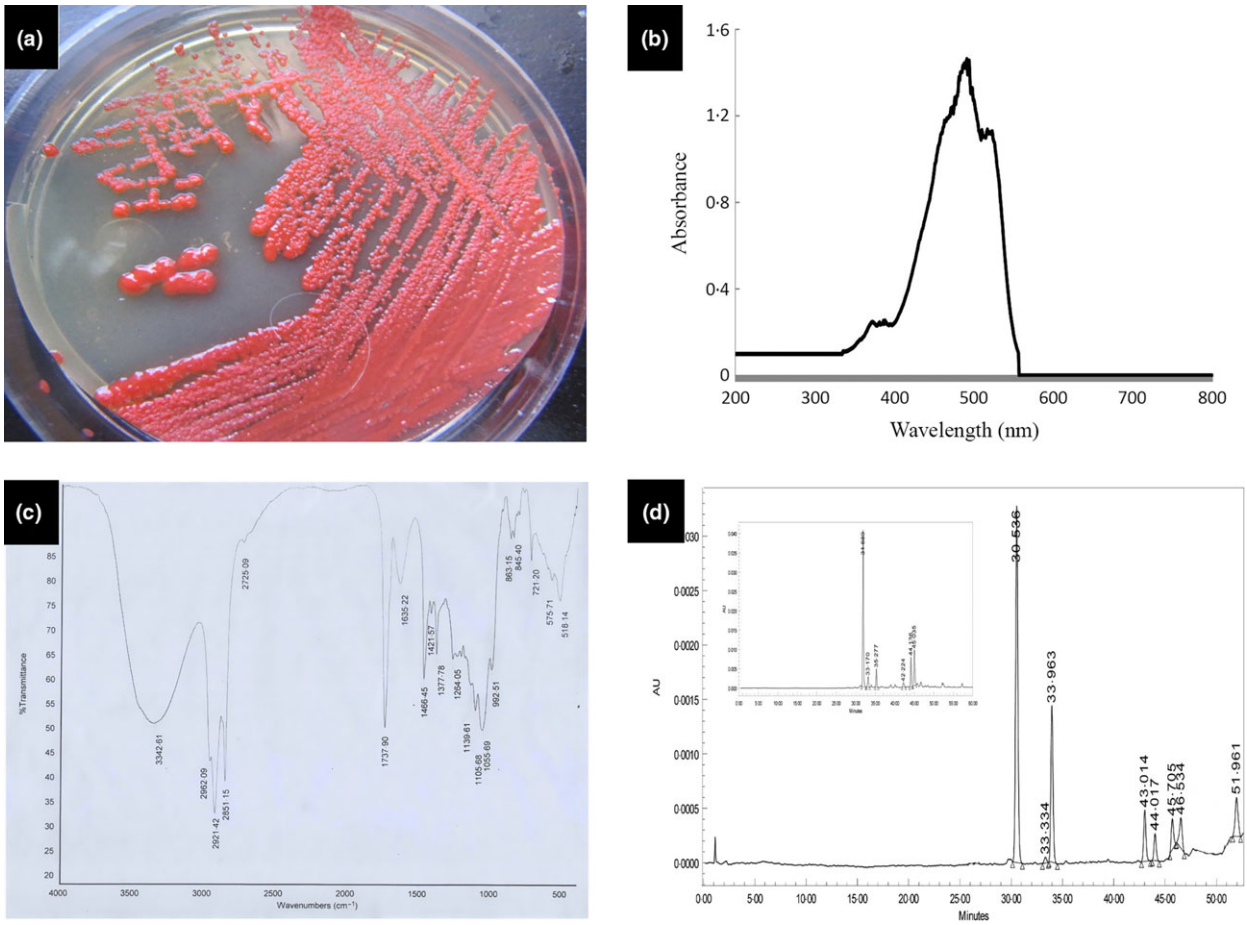

Figure 1 Some characteristics of the novel pigment. (a) Pure culture of pigment producing *Arthrobacter* sp. G20 grown on ASW medium. (b) UV/vis spectrum of pigment. (c) FTIR spectrum of methanolic extract. (d) HPLC analysis of novel pigment in comparison to crocin (top-left corner). [Colour figure can be viewed at wileyonlinelibrary.com]

on the bacterial growth and pigmentation are presented in Fig. 3a–d. The bacterial growth was not affected ($P<0{\cdot}05$) by a range of carbon source (mono-, di- and polysaccharides). However, most of the pigment production was observed in the media supplemented by maltose. As observed in Fig. 2d, the pigment production does not coincide with the bacterial growth, and the highest concentration of the pigment was obtained at the stationary phase. The alkaline condition strongly reduced the bacterial growth and pigmentation, and the highest amount of pigment production was observed at a pH around 7. The strain was psychrophile, and it could not grow over 30°C. The maximum amounts of bacterial growth and pigmentation were achieved at 20°C. Optimization procedure (carbon source, maltose; temperature, 20°C; pH, 7: and incubation time, 72 h) resulted in 2·7 and 2·1 times increased bacterial growth and pigmentation (Fig. 3e) and yielded 0·84 g l^{-1} of pigment production.

Antioxidant activity

The novel carotenoid pigment has the ability to act as an antioxidant, and its EC_{50} scavenging activity of DPPH was equal to 4·5 mg ml^{-1}. This amount is comparable to well-known antioxidants like β-carotene and α-tocopherol, for which the EC_{50} scavenging capacities are reported as 3·5 and 1·5 mg ml^{-} respectively (Correa-Llantén *et al.* 2012).

Cytotoxic activity

As revealed by the MTT assay, the pigment extract had no detectable effects on the viability of the normal HDF cells (Fig. 4a,e) while it showed a moderate antitumour effect on the oesophageal cancer cells (Fig. 4b,e). The IC_{50} values of the pigment on the KYSE30 cells were calculated as (μg ml^{-1}) 1321, 668 and 366 after 24, 48 and 72 h respectively. Microscopic observation revealed that after

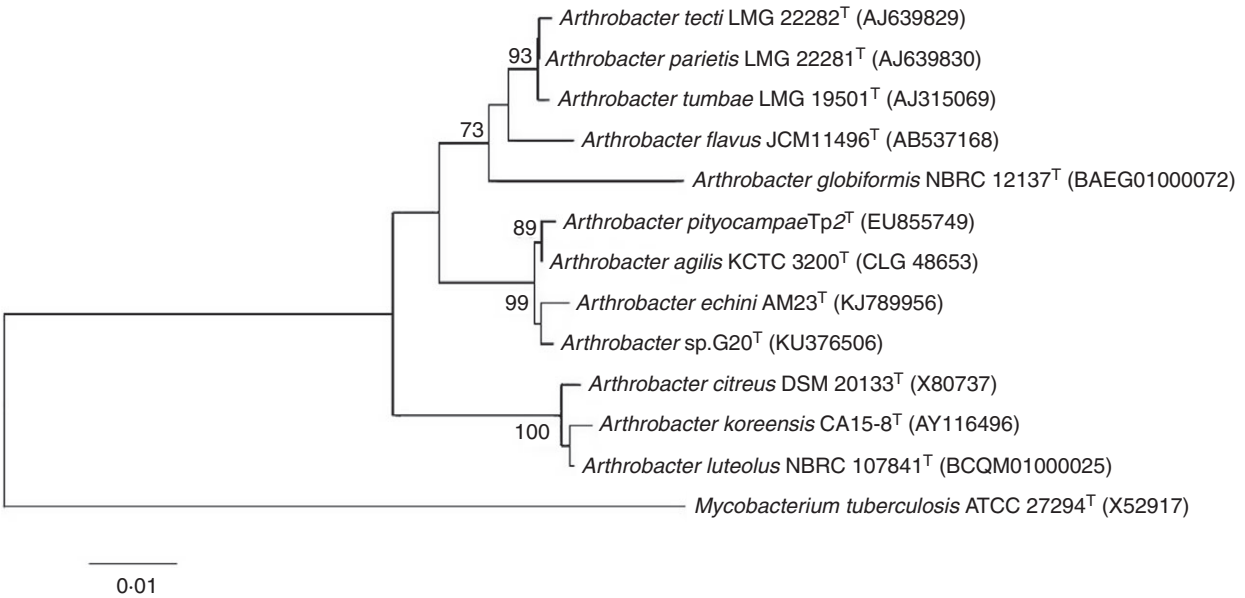

Figure 2 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing of the novel strain and other identified *Arthrobacter*.

treatment with the pigment, cells turned to round shapes in comparison with control cells (Fig. 4c,d).

Antibacterial effects

The novel carotenoid-related pigment obtained in the current study did not display significant antibacterial activity. Only a weak activity against *E. coli* was observed at a high pigment concentration (MIC_{50} equal to 25 mg ml^{-1}) (Data not shown).

Discussion

In this study, the biological activities of a novel pigment obtained from the marine *Arthrobacter* sp. were investigated. Based on the chemical characterization and compared to other known pigments, it was suggested as a member of carotenoids. The genus *Arthobacter*, which was first introduced by Conn and Dimmik (Conn and Dimmick 1947) is phenotypically heterogeneous, and its 89 species (Parte 2013) have been isolated from various sources such as soil, fresh water, fermented food, clinical specimens, paintings, air, ocean sediment, etc. Except for some species like *A. scleromae*, *A. histidinolovorans* and *A. albus*, the bacteria of this genus produce pigments with a great variety of hues and structures (Huang *et al.* 2005). Riboflavin (yellow), indigoidine (blue), indochrome (blue) and porphyrins (red) are examples of various structures observed in this genus (Sutthiwong *et al.* 2014). *A. roseus* and *A. agilis* are two *Arthrobacter* species isolated from cold environments, which contain red carotenoid pigments (Reddy *et al.* 2000, 2002). It was stated that pigmentation is responsible for the cold adaptation of these psychrotrophic bacteria through cell membrane stabilization and radiation protection (Chattopadhyay and Jagannadham 2001). Although carotenoid production is demonstrated in this genus, there are no reports so far about the biological activities of carotenoids obtained from *Arthrobacter* spp.

As regards the enhancement of the pigment production by optimization of the culture condition, maltose was the most suitable carbon source in comparison to glucose and starch. As reported previously psychrophilic *Arthrobacter*, unlike mesophilic ones, could not hydrolase starch (Reddy *et al.* 2000), which explains why starch is not a suitable carbon source for pigment production by this psychrophilic strain. Our results indicated that more complex carbon sources (like maltose) are more desirable than simple monosaccharide (glucose and fructose) for the production of this secondary metabolite.

As Fong *et al.* (2001) stated, carotenoid production is an adaptation strategy of psychrophilic bacteria to decreasing growth temperatures. We also observed the best bacterial growth at 20°C and the best pigment production yield (ratio of pigment production to biomass) at 15°C.

Free radicals—which contain a single unpaired electron in their molecular structures—are generated in the human body for various endo/exogenous reasons. These molecules adversely alter cellular lipids, proteins and DNA, and trigger a number of human diseases like

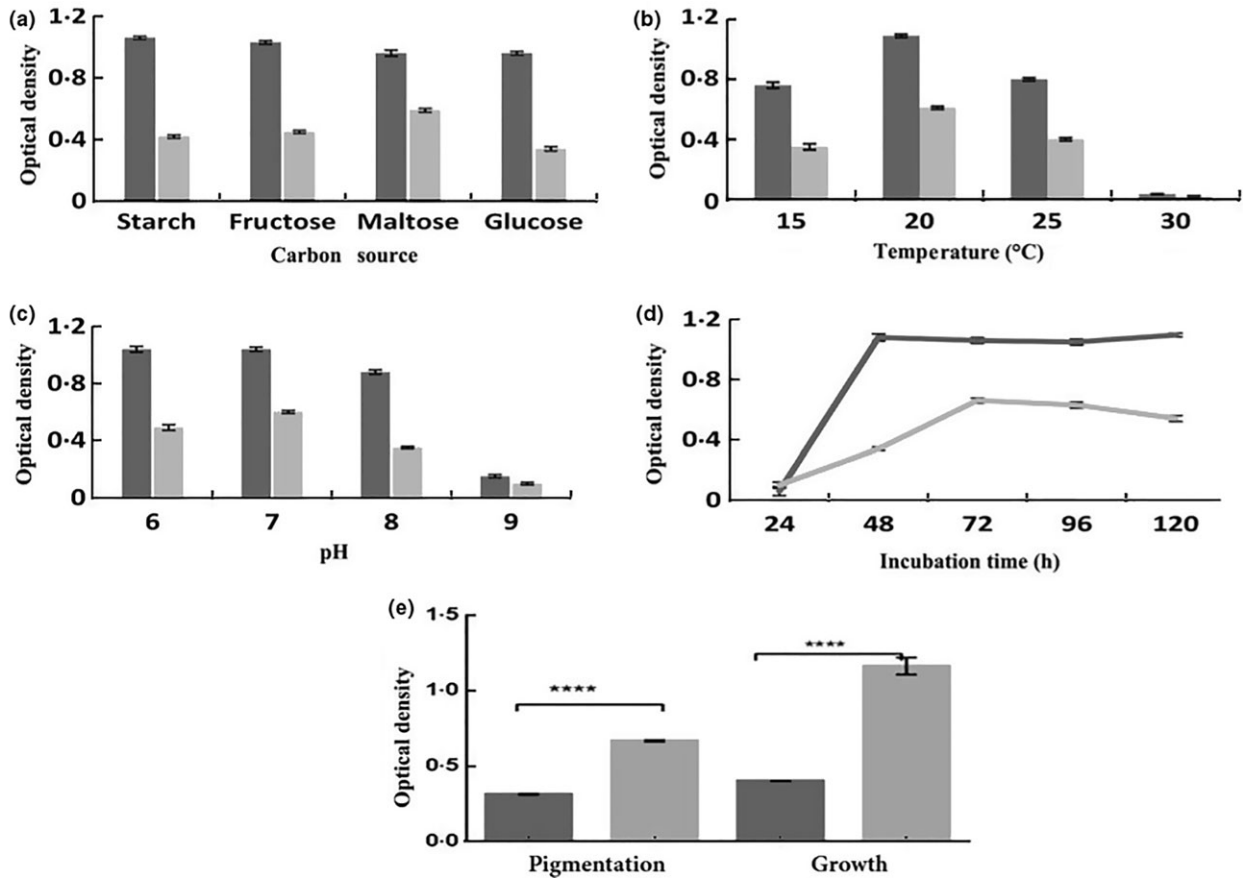

Figure 3 Effects of different factors on the growth (dark grey) and pigment production (light grey) by *Arthrobacter* sp. G20. (a) Carbon source (20°C, pH = 8); (b) temperature (pH = 8); (c) initial pH and (d) incubation time. (e) Comparison of pigment production and bacterial growth before (dark grey) and after (light grey) optimization. ****$P < 0{\cdot}0001$ (un-pair ANOVA with Tukey *post hoc* test).

cancers. Antioxidants like carotenoids are compounds that donate an electron to rampaging free radicals and neutralize them. Therefore, they are well-known as preventing agents for cancers and other diseases in the pharmaceutical and food industries. The antioxidant capacity of our novel pigment is comparable to well-known antioxidants like β-carotene and α-tocopherol. These data suggest that *Arthrobacter* spp. are interesting sources for the bacterial-based antioxidant production.

There are remarkable reports about the role of antioxidants in lowering the risk of a variety of cancers. However, some scientists have argued about their role in cancer treatment. This is because of the fact that tumour development is accompanied by decreasing the intracellular reactive oxygen species (ROS). In cancerous cells, ROS acts as an apoptosis-promoting agent and the excess of antioxidants can block these cancer-preventive mechanisms and develop cancer (Salganik 2001). Recently Schoenfeld *et al.* (2017), demonstrated that the alterations of redox-active iron metabolisms in cancer cells in comparison to normal cells are responsible for the specific activity of ascorbic acid (antioxidant agent) against nonsmall-cell lung cancer (NSCLC) and glioblastoma (GBM) cells. It is understood from this example that an excess of antioxidants can also kill cancer cells but by using a different mechanism. There are some other reports that indicated that carotenoids harbour both anticancer and antioxidant activity, and could promote the apoptosis cascade and kill the tumour cells (Chen *et al.* 2015). For instance, Lycopene suppresses the development of gastric cancer (Velmurugan *et al.* 2005) and induces apoptosis in smoke-induced lung cancer (Seren *et al.* 2008). In the current study, we observed that the treated cancerous cells were rounded, which is a morphological characteristic of apoptotic cells. However, the exact mechanism of its action remains to be clarified.

The tested pigment did not show remarkable antibacterial activity. There are no uniform reports about the antibacterial activity of carotenoids. The differences in their activities may be attributed to the various pigment formulas, as well as different bacterial cell structures. Some pigments could inhibit the growth of bacteria like

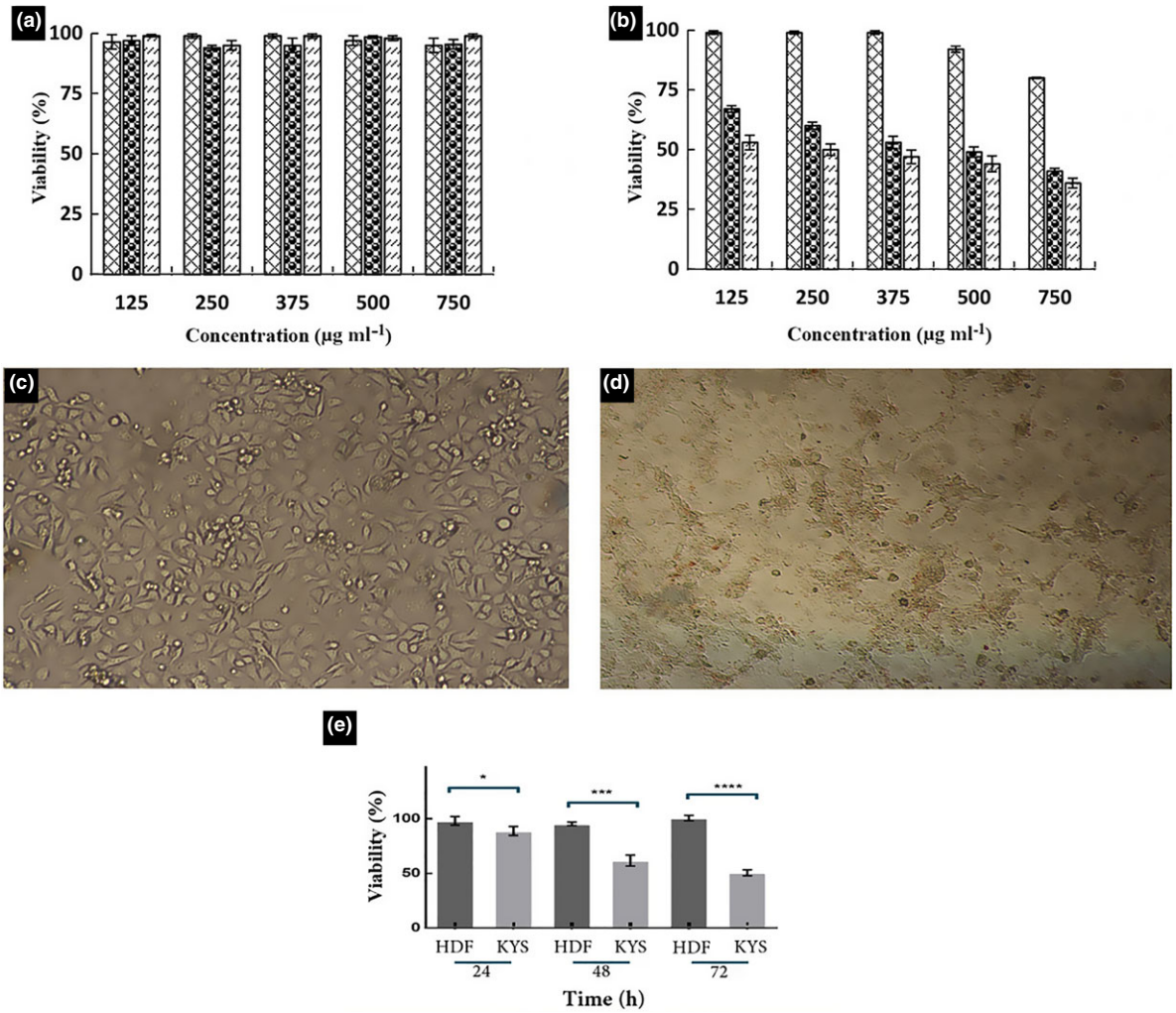

Figure 4 Effect of red pigment on cell viability and morphology. (a) Effect on normal HDF cell viability after 24 (diamond), 48 (sphere) and 72 (diagonal) h. (b) Effect on cancerous KYSE30 cell viability after 24 (diamond), 48 (sphere) and 72 (diagonal) h. (c) Light microscopic images of nontreated KYSE30 cells. (d) Light microscopic images of treated KYSE30 cells with pigment (750 μg/ml) after 72 h. (e) Comparison the viability of HDF (dark grey) and KYS30 (light grey) cells after treatment by pigment extract. $^{*}P < 0{\cdot}05$, $^{***}P < 0{\cdot}01$, $^{****}P < 0{\cdot}0001$ (unpaired *t*-test). [Colour figure can be viewed at wileyonlinelibrary.com]

Bacillus cereus (Al-Abd *et al.* 2015), but others did not affect *B. subtils* (Keceli *et al.* 2013) and *Salmonella typhimurium* (Al-Abd *et al.* 2015). While the pigment did not have a direct antibacterial effect, the possibility that a carotenoid such as this could enhance immune responses of a host organism is entirely consistent with the work of Pechinskii and Kuregyan (2014).

In conclusion, a novel marine and cold adaptive *Arthrobacter* sp. was isolated from the Caspian Sea. This strain produced a nonwater-soluble red pigment. The chemical characterization of the pigment suggested that it belongs to carotenoids. The pigment showed remarkable antioxidant activity. The specific antiproliferating effect against a KYSE30 cancerous cell line in comparison with normal HDF cells was observed. This is the first report on the pharmaceutical potential of carotenoid pigments from *Arthrobacter* spp. However, further studies are required to determine the structure and the exact mechanisms involved in the function of this pigment.

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Conflict of Interest

There are no conflicts of interest.

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