Saudi Journal of Biological Sciences 16 (2023) 103825

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Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Some novel bioactivities of *Virgibacillus halodenitrificans* carotenoids, isolated from Wadi El-Natrun lakes

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ARTICLE INFO

Article history:
Received 11 August 2023
Revised 16 September 2023
Accepted 29 September 2023
Available online 5 October 2023

Keywords:
Halophilic
Carotenoids
Antioxidant
Antidiabetes
Anticancer
Antiviral

ABSTRACT

Carotenoids come in second among the most frequent natural pigments and are utilized in medications, nutraceuticals, cosmetics, food pigments, and feed supplements. Based on recent complementary work, Virgibacillus was announced for the first time as a member of Wadi El-Natrun salt and soda lakes microbiota, identified as Virgibacillus halodenitrificans, and named V. halodenitrificans DASH; hence, this work aimed to investigate several *in vitro* medicinal bioactivities of V. halodenitrificans DASH carotenoids. The carotenoid methanolic extract showed antioxidant activity based on diphenylpicrylhydrazyl (DPPH) scavenging capacity with a half-maximal concentration (IC_{50}) of 1.6 mg/mL as well as nitric oxide (NO) scavenging action expressed by an IC_{50} of 46.4 μg/mL. The extract showed considerable inhibitory activity for alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) enzymes (IC_{50} of 100 and 173.4 μg/mL, respectively). Moreover, the extract displayed selective anticancer activity against Caco-2 (IC_{50} = 138.96 μg/mL) and HepG-2 cell lines (IC_{50} = 31.25 μg/mL), representing colorectal adenocarcinoma and hepatoblastoma. Likewise, the extract showed 98.9 % clearance for human hepatitis C virus (HCV) using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), HCV-NS5B polymerase activity inhibition (IC_{50} = 27.4 μg/mL), and selective inhibitory activity against human coronavirus (HCoV 229E) using the plaque reduction assay (IC_{50} = 53.5 μg/mL). As far as we can tell, the anticancer, antiviral, and antidiabetic attributes of Virgibacillus carotenoids are, de novo, reported in this work which accordingly invokes further exploration of the other medicinal, biotechnological, and industrial applications of Virgibacillus and haloalkaliphilic bacteria carotenoids.

1. Introduction

(Fayez et al., 2022) Carotenoid pigments are counted as the second most naturally produced pigments (Nisar et al., 2015), produced by eukaryotic organisms adopting photosynthesis, like algae and plants, some fungi, and some prokaryotes including cyanobacteria, archaea, and bacteria (Foong et al., 2021). The stressful environment, survival, signaling, as well as competition are the triggers of microbial carotenoid synthesis (Lyakhovchenko et al., 2021; Hou et al., 2016). Generally, carotenoid pigments' chemical structure comprises a 40-carbon skeleton of eight isoprene entities containing a frequent polyene chain having nine conjugated double bonds with a group at each chain end. Carotenoid pigments color can be yellow, orange, red, or even colorless (Maoka, 2020; Yatsunami et al., 2014). Nonetheless, Rodrigo-Baños et al. (2015) stated that despite their capacity for producing both 40-carbon and 50-carbon carotenoids, the potential of haloarchaea as carotenoid producers has not attracted much interest.

Microbial carotenoids have gained a rising interest, compared with chemically synthesized carotenoids, due to their natural character and safe application, medical properties, and independence on geographical and seasonal variations; thus, yield prediction and control is feasible (Nakashima et al., 2005). Also, microbes

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2023.103825
1319-562X/

can synthesize carotenoids from waste; therefore, enabling reducing environmental pollution (Kamla et al., 2012). In the same context, carotenoid production, particularly by halophilic bacteria and archaea, is considered advantageous for the simpler extraction because lysis can be achieved easily by lowering the NaCl concentration (Naziri et al., 2014). Carotenoid consumption and human health proved them as powerful antioxidants; hence, antitumor agents; moreover, some carotenoids act as pro-vitamin A, while others demonstrate regulatory activities in several tissues as they may protect against cancer, heart disorders, and degenerative diseases including macular degeneration (Gammone et al., 2015). Also, some carotenoids are involved in the functioning of the eyes, cardiovascular system, and immune system, as well as general fitness and interspecies communication (Harrison & Quadro, 2018; Saint et al., 2018). For carotenoids driven from halobacteria and haloarchaea, the biotechnological applications included their use as food and cosmetics colorants, feed additives, vitamin A source, (*in vitro*) boosters of antibody production, antioxidants, antitumor drugs, and cardiopathy-preventing agents (Vílchez et al., 2011; Naziri et al., 2014). β-Carotene, lutein, astaxanthin, canthaxanthin, fucoxanthin, β-cryptoxanthin, lycopene, and violaxanthin are among the most crucial carotenoids for their biomedical and biotechnological applications. Europe is considered the major market for carotenoids with 45 % of worldwide sales (Rodrigo-Baños et al., 2015).

Despite the growing interest in carotenoids, only a few studies during the 20th century last half focused on halobacteria carotenoids in comparison with other organisms' carotenoids (Naziri et al., 2014). Besides, the bioactivities of *Virgibacillus* carotenoids are not sufficiently investigated, and there is a rising necessity for novel sources of these influential bioactive compounds. Kusmita et al. (2021a) reported antibacterial action for the carotenoid pigment extract of *Virgibacillus salarius* strain 19.PP.Sc1.6 (isolated from a marine soft coral known as *Sinularia* sp.) towards methicillin-resistant *Staphylococcus aureus* (MRSA) along with *Escherichia coli* with multidrug-resistance at a dilution of 4, 6, and 8 % of the methanolic extract. Moreover, Kusmita et al. (2021b) investigated the possibility of using carotenoids from *V. salaries* 19.PP.Sc1.6 for the formulation of skin care anti-aging cream, where three formulations were examined for stability, antioxidant, and sunscreen potentials. The cream formulation of *V. salaries* 19.PP.Sc1.6 carotenoid pigment extract exhibited improved stability contrasted with the oil-containing cream formulation incorporating an anionic emulsifier. Similarly, Fayez et al. (2022) reported an antibacterial trait for the carotenoid pigment extract of *Virgibacillus halodenitrificans* DASH (the current study strain), at 20 μg/mL towards *S. aureus, Pseudomonas aeruginosa,* and *E. coli*; besides, antifungal action towards *Candida albicans*, and a dose-dependent anti-biofilm trait towards *P. aeruginosa* and *S. aureus* at concentrations as low as 20 and 10 μg/mL, respectively.

Based on a complementary recent work (Fayez et al., 2022), the study strain *V. halodenitrificans* DASH was recognized for the first time as a part of the microbiota of the soda and salt lakes of Wadi El-Natrun. Analytical characterization of its carotenoids was conducted where thin layer chromatography (TLC), liquid chromatography-mass spectroscopy (LC-MS), ultraviolet–visible (UV–vis) absorbance, Fourier-transform infrared (FTIR) spectroscopy, and Raman spectroscopy have revealed the investigated carotenoids to consist of a mixture of lutein, β-carotene, as well as β-Apo-8′-carotenal. In this regard, the current work aimed to investigate some *in vitro* bioactivities, of a medicinal nature, of *V. halodenitrificans* DASH methanolic carotenoid extract, a step expected to pave the way for additional research on the prospective medicinal, industrial, and biotechnological applications of the carotenoids of *Virgibacillus*, in particular, and generally carotenoids from bacteria associated with the insufficiently explored Wadi El-Natrun soda and salt lakes located at the central north of Egypt, fed from underground seepage of water from The Nile River, characterized by a high evaporation rate, and interestingly has mineral salt content, pH values (8.5–9.5) and a salinity (283–540 g/L) conditions (El-Ghani et al., 2015) which make such harsh niche a highly potential source of microbial isolates with impactful bioactivities; therefore, medicinal and industrial applications.

2. Materials and methods

2.1. The study strain history and growth conditions

The haloalkaliphilic bacterial study strain *V. halodenitrificans* was earlier isolated (Fayez et al., 2022) from the hypersaline Egyptian ecosystem of Wadi-El-Natrun soda and salt lakes on IRAM agar plates (IRAM, 1988) at 30 °C after 7 days. The medium composition (g/L) is agar-agar (15 g), peptone (5 g), NaCl (116.8 g), yeast extract (4 g), $MgSO_4{\cdot}7H_2O$ (20 g), $CaCl_2{\cdot}6H_2O$ (0.2 g), and KCl (5 g). The pH value was adjusted to 9.0 ± 0.2. For growth and maintenance, the strain was grown at 30 °C for 7 days in 100 mL of liquid IRAM medium dispensed in 250 mL Erlenmeyer flask under shaken conditions (150 rpm) using a rotary evaporator until an optical density (OD) value of one was reached. OD was measured at 600 nm utilizing a spectrophotometer (JENWAY6305, UK). The accession number MN795630 was specified for the 16S rRNA sequence at GenBank.

2.2. Carotenoid pigment extraction

The cell pellet of 7-day old 100 mL culture was obtained by a 15 min centrifugation (24000 × g). Successive extraction utilizing a cold acetone-methanol mixture (7:3 v/v) was conducted. The extract was dried overnight at 45 °C. The resultant residues were weighed and re-suspended in methanol (Hegazy et al., 2020). TLC, LC-MS, UV–vis absorbance, FTIR spectroscopy, and Raman spectroscopy have shown the inspected carotenoids as a mixture of lutein, β-carotene, as well as β-Apo-8′-carotenal.

2.3. Carotenoid extract antioxidant activity

2.3.1. DPPH scavenging (1, 1-diphenyl-2-picrilhydrazyl)

The antioxidant impact of the carotenoid methanolic extract was estimated by assessing the DPPH free radical scavenging ability outlined by Rajendiran et al. (2017). Hundred microliter aliquots of the pigment extract, representing 6.25, 12.5, 25, 50, 100, and 200 mg/mL concentrations, were separately added to an equal volume of DPPH solution (0.1 mM in methanol). Following 30 min under dark conditions, the UV–vis absorbance (517 nm) was recorded. The %scavenging ability was carried out in triplicates and conveyed as the concentration triggering half-maximal inhibition (IC_{50}). The DPPH control was constituted by mixing equal volumes (100 μL) of the DPPH solution and methanol. The %inhibition triggered by each extract dilution was determined through the formula described by Stankovic (2011) as follows:

$$\%\boldsymbol{Scavenging} = (\mathbf{1} - (\boldsymbol{A_{Test}}/(\boldsymbol{A_{Control}})) \times \mathbf{100}$$

$\mathbf{A}_{\mathrm{Test}}$ is the mean of the UV–vis absorbances of test sample replicates, and $A_{control}$ is the mean of the UV–vis absorbances of the DPPH control sample replicates. The positive control was represented by ascorbic acid at the two-fold serial dilution range of 200–6.25 μg/mL.

2.3.2. Nitric oxide (NO) scavenging

In this trial, antioxidant activity assessment was related to the fact that sodium nitroprusside spontaneously generates NO, in aqueous solutions adjusted to physiological pH, which converts into nitrite ions, estimated using Griess reagent, through its interaction with oxygen (Garret, 1964). The %scavenging by the extract was evaluated as described by Serafim et al. (2012) according to which 50 µL of each of the two-fold serial dilutions of the extract (200–6.25 µg/mL) was dispensed into a 96-well plate, along with an equal volume of sodium nitroprusside solution (10 mM). Distilled water was adopted as a negative control. Incubation proceeded under light at room temperature. After 90 min, immediate determination of the UV–vis absorbance (at 490 nm), using a 96-well microplate spectrophotometer, followed Griess reagent (100 µL) addition. Trials were triplicated, and the activity was expressed in terms of the IC_{50}. The %inhibition triggered by each extract dilution was determined through the formula:

$$\%\boldsymbol{NOscavenging} = ((\boldsymbol{A_{Control}} - \boldsymbol{A_{Test}})/\boldsymbol{A_{Control}}) \times \mathbf{100}$$

$A_{Control}$ represents the mean of the UV–vis absorbances of the negative control sample replicates, while A_{test} is the mean of the UV–vis absorbances of test sample replicates. The positive control was represented by ascorbic acid at the two-fold serial dilution range of 200–6.25 µg/mL.

2.4. Carotenoid extract antidiabetic activity

2.4.1. Alpha-amylase (α-amylase) inhibition

The assay was conducted as per Etsassala et al. (2020). Two-fold serial dilutions were made in the range of 4–0.016 mg/mL of the methanolic extract. A mixture of 250 µL each dilution and an equal volume of α-amylase solution (0.5 mg/mL of 0.02 M sodium phosphate buffer) was prepared. Incubation proceeded at 25 °C. After 10 min, the addition of 250 µL of starch solution (1 % in 0.02 M sodium phosphate buffer) was followed by an additional 10 min incubation. The reaction stop was attained through 500 µL addition of dinitrosalicyclic acid. A 5 min boiling water incubation was followed by room temperature cooling. For preparing negative control tubes, the pigment extract was replaced with dimethyl sulfoxide (DMSO). In each tube, distilled water (5 mL) was added before UV–vis absorbance (540 nm) was determined. Trials were triplicated, and activity was expressed in terms of the IC_{50}. The estimation of the % inhibition triggered by the extract relied on the formula:

$$\%\boldsymbol{Inhibitionof\,\alpha - Amylaseactivity} = ((\boldsymbol{A_{Control}} - \boldsymbol{A_{Test}})/\boldsymbol{A_{Control}}) \times \mathbf{100}$$

$\mathbf{A_{Control}}$ represents the mean of the UV–vis absorbances of the negative control replicates, while A_{Test} is the mean of the UV–vis absorbances of the test replicates.

2.4.2. Alpha-glucosidase (α- glucosidase) inhibitory assay

The test was conducted utilizing p-nitrophenyl-α-d-glucopyranoside (p-PNP) as a substrate as described by Zhang et al. (2014). Briefly, a mixture encompassing 10 µL of pancreatin solution (10 mg/mL), 50 µL of 50 mM phosphate buffer (at physiological pH), and 20 µL of the methanolic extract (4, 2, 1, 0.5, 0.25, 0.063, 0.031, and 0.016 µg/mL) was subjected to a 5 min incubation at 37 °C, subsequently, 20 µL addition of 4-nitrophenyl-β-D-glucopyranosiduronic acid (1 mM), a substrate, was achieved. At 37 °C, a 30 min incubation proceeded. Reaction stop was achieved through 50 µL addition of 0.1 M sodium carbonate, a step based on which a yellow color develops due to p-nitrophenol formation, followed by UV–vis absorbance determination at 405 nm utilizing a 96-well microplate spectrophotometer. For the control wells, the extract was replaced with DMSO. Trials were triplicated, and activity was expressed in terms of the IC_{50}. The α-glucosidase inhibitory effect was estimated using the same equation described in Section 2.5.1.

2.5. Carotenoid extract anticancer activity

2.5.1. Thawing, culture, and maintenance of the frozen human cell lines

Mainly, the guide of animal cell culture, proposed by The American Type Culture Collection (ATCC) (Website 1), was adopted for this purpose, and cell lines were purchased from Vacsera Co., Egypt. Human colorectal adenocarcinoma Caco-2 cell line (RRID: CVCL_0025, ATCC® HTB-37™), human hepatoblastoma cell line HepG-2 (RRID: CVCL_0027, ATCC® Cat. No. HB-8065™), and human cervical adenocarcinoma cell line WISH (RRID: CVCL_1909, HeLa derivative, ATCC CCL-25) were maintained using DMEM (Dulbecco's Modification of Eagle's Medium), with glucose and L-glutamine concentrations of 4.5 g/L and 4 mM (Lonza, Britain), respectively. Fetal bovine serum (FBS) supplement (Sigma, USA) was added (10 % v/v) to DMEM to constitute a complete culture medium. Human peripheral blood mononuclear cells (PBMNCs) were grown as suspended cells in Roswell Park Memorial Institute (RPMI)-1640 medium (Lonza, Britain), with stable L-glutamine, and supplemented with FBS (10 % v/v), as a complete culture medium.

A frozen liquid nitrogen-stored vial of each of the three cell lines was thawed by water bath (Bicasa, Italy) immersion for maximally 2 min at 37 °C; then, the cell suspension of each cell line was transferred slowly to a 7 mL of complete culture medium in a tissue culture-grade polystyrene flask (25 cm^2). Cells were allowed to attach and grow as a monolayer in a CO_2 incubator (Nuaire, Germany) at 95 ± 5 % humidity conditions, 5 ± 1 % CO_2, and 37 ± 1 °C for 24 h following which, the culture medium was replaced with a new medium aliquot. Cells were fed every 2–3 days until 70 % confluence was reached. Cell maintenance, passaging, and treatment were conducted in a class II biosafety cabinet (Bicasa, Italy).

Cells were checked daily for their confluence, morphological, and adhesive properties using an inverted phase contrast microscope (Zeiss, Germany). At 70 % confluence, adherent cells were detached through trypsinization, as described by (Rafehi et al., 2011). The resultant suspension of single cells was subjected to a 10 min centrifugation (4 °C) at 600 × g using a cooling centrifuge (Heraeus, Germany). The culture supernatant was discarded. The cellular pellet was converted into a single-cell suspension for the subsequent cell count procedures (using Neubauer improved hemocytometer) and subculture in a complete culture medium to inoculate new flasks or for dispersion into the 96-well plates. Thawed cells were passaged twice-thrice in 25 cm^2 tissue culture flasks before being plated for any assay.

2.5.2. Isolation of PBMNCs

PBMNCs were adopted as normal cells and isolated by density gradient centrifugation (Bøyum, 1968) where 5 mL of fresh human peripheral blood was heparinized with 50 µL of heparin (2500 I.U/mL). Four milliliters of the separation medium Histopaque®-1077 was pipetted in a sterile Falcon tube (15 mL); then, the anticoagulated blood was gently and slowly layered onto the Histopaque®-1077 layer using a sterile 5 mL pipette. Centrifugation was conducted in a swinging-bucket rotor at 20 °C and 400 × g for 20 min after which the buffy coat layer was withdrawn using a sterile Pasteur pipette; then, washed with phosphate buffer saline in a sterile Falcon tube. A 10 min centrifugation proceeded at 400 × g and 20 °C. The supernatant was removed carefully. The washing step was repeated at 200 × g to get rid of platelets. The resultant cell pellet was converted into a single-cell suspension

using 1 mL of complete RPMI-1640 medium, before applying cell count using Neubauer improved hemocytometer.

2.5.3. Determination of the cytotoxic and or antiproliferative effect of the carotenoid extract

The cell suspension dispensing into 96-well plates ($1.5x10^4$ cell/well) was followed by incubation under the same culture conditions (Section 2.5.1) for 24 h after which the adherent cells were treated with two-fold serial dilutions of the extract (1000–31.25 μg/mL of DMEM complete culture medium). After 48 h, the addition of 100 μL of a membrane (0.22 μm)-filtered MTT solution (0.5 mg/mL DMEM) (Sigma, USA) followed the removal of the exhausted medium. Incubation proceeded for 3 h after which supernatants discarding, DMSO (100 μL) addition, and UV–vis absorbance determination at 490 nm (instead of 540 nm as described by (Vijayarathna & Sasidharan, 2012) using a 96-well microtiter plate spectrophotometer (BIO-RAD, Japan), were accomplished.

MTT assay was also conducted to evaluate the extract's impact on the cellular viability of PBMNCs (as reference normal cells) isolated as described earlier. For this purpose, Molaee et al. (2017) method was slightly modified**.** PBMNCs count was adjusted to $2x10^5$ cells /mL of RPMI-1640 complete culture medium, before being dispensed as 100 μL ($2x10^5$ cells) aliquots into 96-well plate wells. Extract serial dilutions (2000–62.5 μg/mL of RPMI-1640 complete culture medium) were added to the wells, and some wells were left untreated as control wells. After 48 h of 5 % CO_2 incubation, each well content was transferred to an Eppendorf tube before centrifuging at room temperature at 400 × g for 10 min. Supernatants were carefully discarded; then, the MTT solution (100 μL) was dispensed into the Eppendorf tubes incubated for 4 h at 37 °C. Afterward, supernatants were discarded, and DMSO was dispended into the tubes as 100 μL aliquots to solubilize the developed formazan crystals; then, each tube content was transferred into a 96-well plate, and the UV–vis absorbance of wells was determined utilizing the 96-well microtiter plate spectrophotometer at 490 nm. Trials were duplicated, and the anticancer activity was conveyed as the IC_{50}. To evaluate the selectivity; hence, the safety of the extract, the selectivity index value (SI) was addressed by dividing the extract's concentration triggering half-maximal cytotoxicity (CC_{50}) against the PBMNCs, by the extract IC_{50} value reported against each cell line separately as described by Nogueira & Estólio do Rosário (2010).

2.6. Antiviral potential of the carotenoid extract on human hepatitis C virus (HCV) and human coronavirus HCoV 229E

2.6.1. The extract's in vitro anti-HCV activity using reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

First, it was planned to apply the MTT test to determine the effective concentration (EC_{100}) value which is the highest concentration tolerated by PBMNCs without affecting the cellular viability for both the extract and the standard anti-HCV drug ribavirin. Cells were separately treated for 72 h with dilutions of the extract (500, 200, and 100 mg/mL) or the standard anti-HCV drug (600, 300, 150, and 75 mg/mL) dissolved in DMEM complete culture medium. For the negative control wells, the cells were treated with only DMEM complete culture medium.

After determining the sublethal concentrations of the extract and ribavirin, a quantitative RT-qPCR assessment of the extract's impact on the number of HCV copies in HCV-infected PBMNCs was conducted. For this purpose, PBMNCs were suspended in RPMI-1640 complete culture medium before being seeded as 200 μL aliquots into the wells of a 24-well tissue culture plate ($2x10^5$ cells/well). Excluding negative control wells, all wells were co-incubated with HCV-infected serum (genotype 4a, 3 × 10^3 copies/mL) in RPMI-1640 complete culture medium in a CO_2 incubator for 2 h after which the medium was renewed with RPMI-1640 complete culture medium for positive control wells (virus-infected untreated cells). For the treated wells, the medium was renewed with RPMI-1640 complete culture medium and the carotenoid extract or standard drug each at the EC_{100}. After 72 h, COBAS® Ampliprep/COBAS® TaqMan® (CAP-CTM) analyzer (Roche Diagnostics, USA) Was used to quantitatively check the cells for intracellular HCV levels following the manufacturer's instructions (Mosmann, 1983). Trials were triplicated.

2.6.2. In vitro inhibitory action of the extract on HCV-NS5B polymerase-dependent HCV replication

The influence of the carotenoid extract on HCV RNA-dependent polymerase NS5B (HCV-NS5B) activity was investigated as explained by Hegazy et al. (2020). The test involves the incubation of an HCV-NS5B (genotype 1b-Con1)-Δ21-His 6-polymerase enzyme with a template of heteropolymeric RNA (i.e., with a 3′-end dideoxycytidine) and radiolabeled nucleotides. An HCV-infected plasma sample was considered the positive control, while an HCV-negative plasma sample represented the negative control. Radiolabeled nucleotides incorporation was quantitatively recorded in both control cells as well as cells treated with a series of concentrations of the carotenoid extract (80, 40, 20, 10, and 5 μg/mL) or the standard anti-HCV drug ribavirin (200, 100, 50, 25, and 12.5 μg/mL) utilizing TopCounti NXT scintillation counter (Perkin Elmer, Wellesley, MA). Trials were triplicated, and the enzyme inhibitory activity was conveyed as the IC_{50}.

2.6.3. In vitro anti-HCoV 229E activity of the pigment extract using plaque reduction assay

This assay assesses the antiviral activity in cell culture systems by checking the viral yield reduction implied by viral plaque formation reduction. HCoV 229E and African green monkey kidney cells Vero E6 (RRID:CVCL_0059, ATCC® CRL-1586™), regarded as the gold standard cell line for coronaviruses' propagation and titration, were provided by Nawah Scientific, Egypt.

First, it was intended to specify the range of safe concentrations of the pigment extract using the MTT test described earlier in section 2.6.1; therefore, serial dilutions of the extract (1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.9, and 1.95 μg/mL) were tested for their effect on Vero E6 cells viability after 72 h, a step based on which the extract CC_{50} was determined, for the future step of assessing the extract selectivity by calculating the SI as well as defining the highest extract concentration tolerated by Vero E6 cells to be assessed for its effect on reducing the virus-induced plaques. Vero E6 cells were cultured in DMEM complete culture medium (1.2×10^4 cells/well) in a 96-well plate for 24 h before cell treatment with the pigment extract‘s dilutions.

After determining the highest concentration of the extract tolerated by Vero E6 cells, the anti-HCoV 229E activity was evaluated by assessing the level of inhibition of the virus-induced viral plaques (areas of killed or morphologically altered Vero E6 cells) as a virus-induced cytopathic effect. For this purpose, the plaque reduction test was run as specified by Hayden et al. (1980). Cells were grown in the 6-well plates for 24 h as a confluent monolayer at 95 ± 5 % humidity atmosphere with 5 ± 1 % CO_2 at 37 ± 1 °C. The hundred-fold diluted virus stock was incubated with three dilutions of the sample (25, 12.5, and 6.25 μg/mL) for 30 min. Following exhausted culture medium removal and inoculation with the virus/sample mixtures (100 μL/well), incubation was extended for1 h (to allow virus particle adsorption); then, 1.5 mL of the complete culture medium with 2 % agarose was layered on the cells. After medium solidification, plates were incubated for 72 h under the same culture conditions defined earlier. Formalin (10 % v/v in distilled water) fixation was adopted for 2 h after which crystal

violet (0.1 % wt/vol in distilled water) staining was applied. Plaques were checked and counted microscopically before the % plaque reduction, relative to the control (virus-infected, untreated) wells, was reported as follows:

$$\%Plaquereduction = (untreated\,viruscount - treated\,viruscount)/untreated\,viruscount \times 100$$

Control cells (untreated and uninfected) and virus controls (untreated and virus-infected) were included in the experiment. Trials were triplicated, and the anti-HCoV 229E activity was expressed in terms of IC_{50}.

2.7. IC_{50} calculation

The carotenoid pigment extract antioxidant, antidiabetic, and anticancer effects, in addition to the inhibitory effect on HCV-NS5B polymerase and HCoV 229E plaque formation, was expressed as IC_{50} values calculated through regression analysis based on dose–response curves using MS-Excel (2010).

2.8. Declaration

All blood samples, plasma, and sera were provided by Mabaret El-Asafra laboratories.

3. Results

3.1. Antioxidant activity

For the DPPH scavenging effect, the extract showed an IC_{50} of 1.6 mg/mL (Fig. 1a), compared with ascorbic acid positive control which showed an IC_{50} of 7.2 μg/mL. For NO scavenging activity, the maximal attained activity was 82.1 % at 200 μg/mL, where the extract realized an IC_{50} of 46.4 μg/mL (Fig.1b), while ascorbic acid realized an IC_{50} of 16.0 μg/mL.

3.2. Antidiabetic activity

The carotenoid pigment extract of *V. halodenitrificans* DASH was screened for its inhibitory impact on α-amylase as well as α-glucosidase. At 800 μg/mL, the extract showed % 86.8 ± 1.6 α-amylase inhibition (Fig. 2a) with an IC_{50} of 95.9 μg/mL, while the %α-glucosidase inhibition by the extract was 95 ± 1, corresponding to an IC_{50} of 173.4 μg/mL (Fig. 2b). To our knowledge, no antidiabetic activity has been reported earlier for *Virgibacillus* carotenoids.

3.3. In vitro anticancer activity

The cytotoxic and or antiproliferative effect of the *V. halodenitrificans* DASH carotenoid extract against PBMNCs as well as Caco-2, HepG-2, and WISH cells was assessed after 48 h using the MTT test. Dose-response curves describing the action of several dilutions of the extract on the cellular viability of each cell line are illustrated in Fig. 3a. The IC_{50} values of the carotenoid extract were estimated as 138.96, 31.25, 653.11, and 1000 μg/mL against Caco-2, HepG-2, WISH, and PBMNCs, respectively (Fig. 3b), with SI values of 7.2, 32.0, and 1.53 as reported for Caco-2, HepG-2, and WISH cell lines, respectively.

3.4. Antiviral activity

3.4.1. Anti-HCV activity using RT-qPCR

The antiviral effect of the investigated carotenoid pigment extract was evaluated in comparison with that of the standard anti-HCV drug ribavirin. First, the highest concentration (EC_{100}) of the extract and ribavirin, tolerated by PBMNCs as HCV host cells, was defined after 72 h through the MTT test as 200, and 300 mg/mL, respectively; then, the carotenoid extract and ribavirin were separately incubated, at the earlier mentioned concentrations, with the HCV-infected PBMNCs. Quantification of the viral load after 72 h in treated HCV-infected PBMNCs as well as untreated HCV-infected PBMNCs revealed that the carotenoid extract eliminated HCV by approximately 98.8 ± 0.2 %, and the antiviral drug (ribavirin) has achieved a seemingly entire HCV elimination (Fig. 4a).

3.4.2. Inhibitory potential against HCV-NS5B polymerase-dependent HCV replication

Interestingly, the extract realized a 50 % reduction in HCV-NS5B polymerase activity at a concentration (IC_{50}) of 27.4 μg/mL even lower than that reported for the standard drug ribavirin that realized a 50 % reduction in HCV-NS5B polymerase activity at 68.9 μg/mL, a result implying that the investigated carotenoid extract, compared with the standard drug ribavirin, showed an apparent, higher inhibition to the activity of HCV-NS5B polymerase (Fig. 4b).

3.4.3. In vitro anti-HCoV 229E activity using the plaque reduction assay

Based on the cellular viability assessed by the MTT test in the current work, 25 μg/mL was selected as the highest concentration of the pigment extract to be tested for anti-HCoV 229E activity, which was associated with cellular viability of almost 86 % as estimated from the dose–response curve (Fig. 5a). Accordingly, the virus-infected cells were separately treated with three extract dilutions (25, 12.5, and 6.25 μg/mL). A maximal inhibitory effect of 25 % reduction in the number of HCoV 229E-induced plaques, as a virus-induced cytopathic effect, was reported for the pigment extract at 25 μg/mL. The IC_{50} derived utilizing the dose–response curve (Fig. 5b) was 53.5 μg/mL.

3.4.4. Selectivity of the carotenoid pigment extract as an antiviral agent

The possibility of applying the carotenoid extract of *V. halodenitrificans* DASH as a safe anti-HCoV 229E agent was addressed by calculating the SI of the extract, which was reported as 25.5 based on an estimated IC_{50} of 53.5 μg/mL against HCoV 229E as well as a CC_{50} value of 1364.4 μg/mL reported against PBMNCs (Fig. 4). Similarly and in agreement with the previously reported SI value, the SI was interestingly calculated as 49.8 using the IC_{50} value (27.4 μg/mL) associated the extract inhibitory action on HCV-NS5B polymerase activity.

4. Discussion

Considering the safe, natural characteristics of microbial carotenoids and their fast, economical, and environmentally friendly production, together with the insufficient research on the health benefits and pharmaceutical applications of carotenoids produced by haloalkaliphilic bacteria in general (Maeda et al., 2015; Lau & Kwan, 2022) and particularly *Virgibacillus* reported by Fayez et al. (2022) as a good candidate for large scale**,** inexpensive, and fast production of carotenoids, investigative efforts should be intensified to uncover the expanding issues about their medicinal and industrial significance.

Oxidative stress has clearly been reported for the induction and even progression of various disorders, especially cancer, diabetes mellitus, and cardiovascular diseases (Lenaz, 2012). The antioxidant activity reported in the present work for the carotenoid extract of *V. halodenitificans* DASH seems consistent with the fact that carotenoids are broadly known for their distinct antioxidant

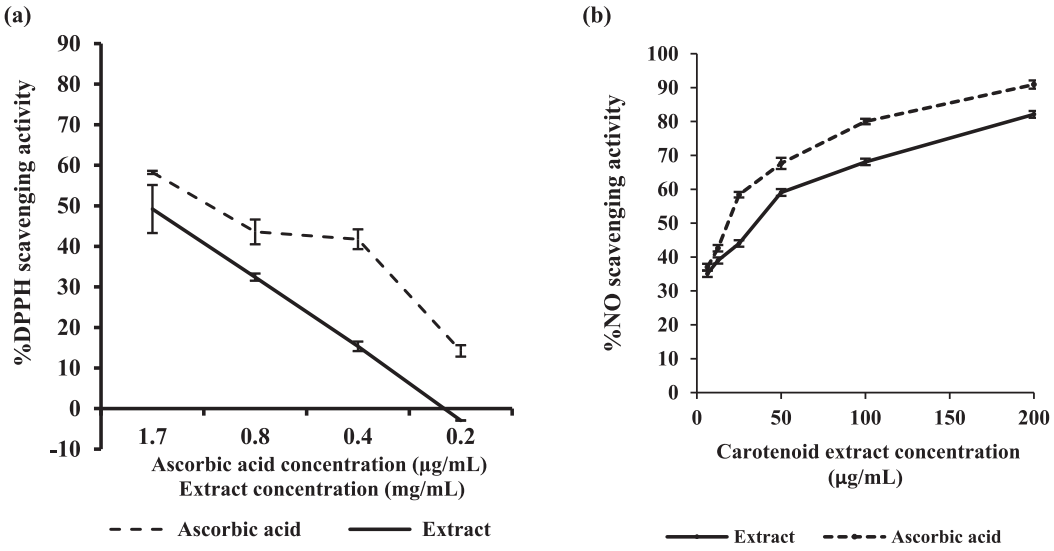

Fig. 1. Scavenging activity of *V. halodenitrificans* DASH carotenoid extract on DPPH radical (a) and NO radical (b). Error bars represent the mean ± the standard deviation (SD) of three replicates.

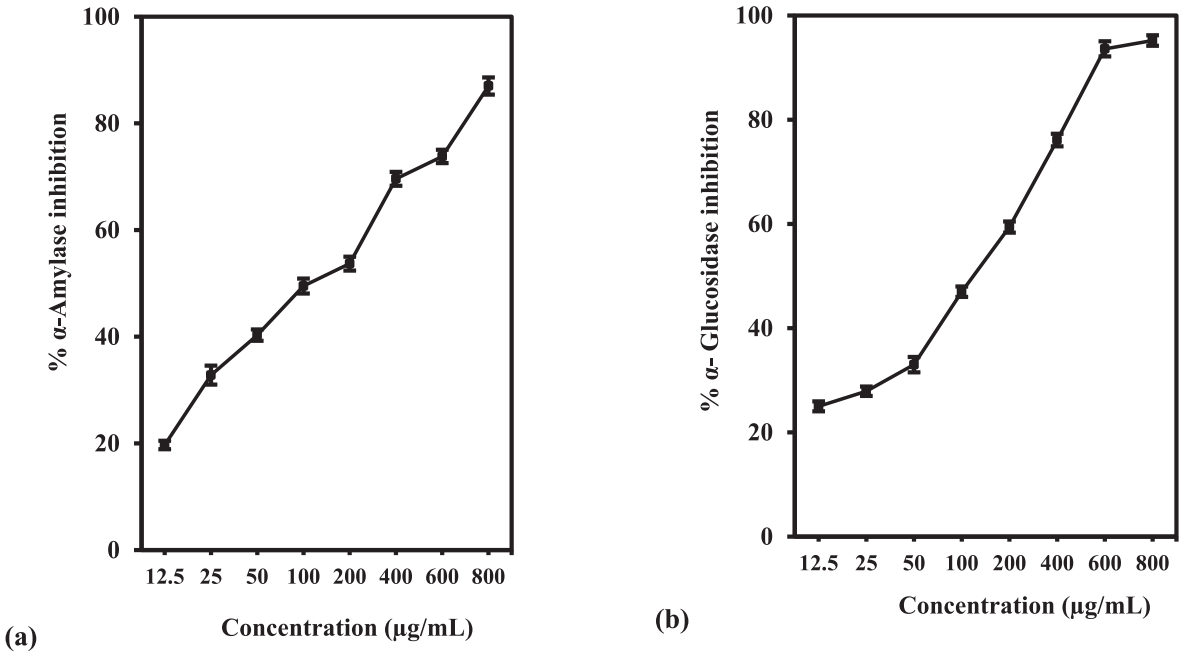

Fig. 2. *In vitro* α-amylase (a) and α-glucosidase (b) inhibitory potential of *V. halodenitrificans* DASH carotenoid extract. Error bars represent the mean ± SD of three replicates.

criteria ascribed to the plentiful hydroxyl groups as well as conjugated double bonds (Mandelli et al., 2012; Yang et al., 2015) in addition to the ability to remove unpaired electrons (Mortensen et al., 2001); therefore, carotenoids are able to quench free radicals like reactive oxygen (RO) and reactive nitrogen species generated by nearly all organisms, like as superoxide, peroxyl, hydroxyl, and nitric oxide radicals (Bayir, 2005). In the current study, the carotenoid pigment produced by *V. halodenitrificans* DASH exhibited antioxidant activity against DPPH (IC_{50} = 1.6 mg/mL) and NO (IC_{50} = 46.4 µg/mL). Similarly, several reports recorded the antioxidant traits of halophilic archaea and bacteria carotenoids (Squillaci et al., 2017; Hou & Cui, 2018; Flores et al., 2020; Hegazy et al., 2020; Lizama et al., 2021). For *Virgibacillus*, Arunachalam & Appadorai (2013) reported *Virgibacillus* sp. (an isolate from a marine sponge designated as Callyspongia *diffusa*) for producing non-carotenoid secondary metabolite(s), with antioxidant potential (DPPH scavenging) represented by an IC_{50} of 857.5 µg/mL, and NO scavenging with an IC_{50} value of 1353.3 µg/mL.

α-Amylase, along with α-glucosidase, performs a pivotal role in carbohydrate digestion (Dona et al. 2010; Ch'ng et al. 2019). Using α-amylase and α-glucosidase inhibitors slows down carbohydrate digestion's last steps; hence, prevents glucose entry into the blood circulation; accordingly, is considered an efficient approach for reducing postprandial hyperglycemia (Moloto et al., 2020), also known as type 2 diabetes mellitus (T2DM). Bacterial glucosidase inhibitors' importance for diabetes treatment has increased due to their effectiveness and the adverse effects triggered by synthetic α-amylase together with α-glucosidase inhibitors, for instance, diarrhea, bloating, and abdominal ache (Chiasson et al., 2002).

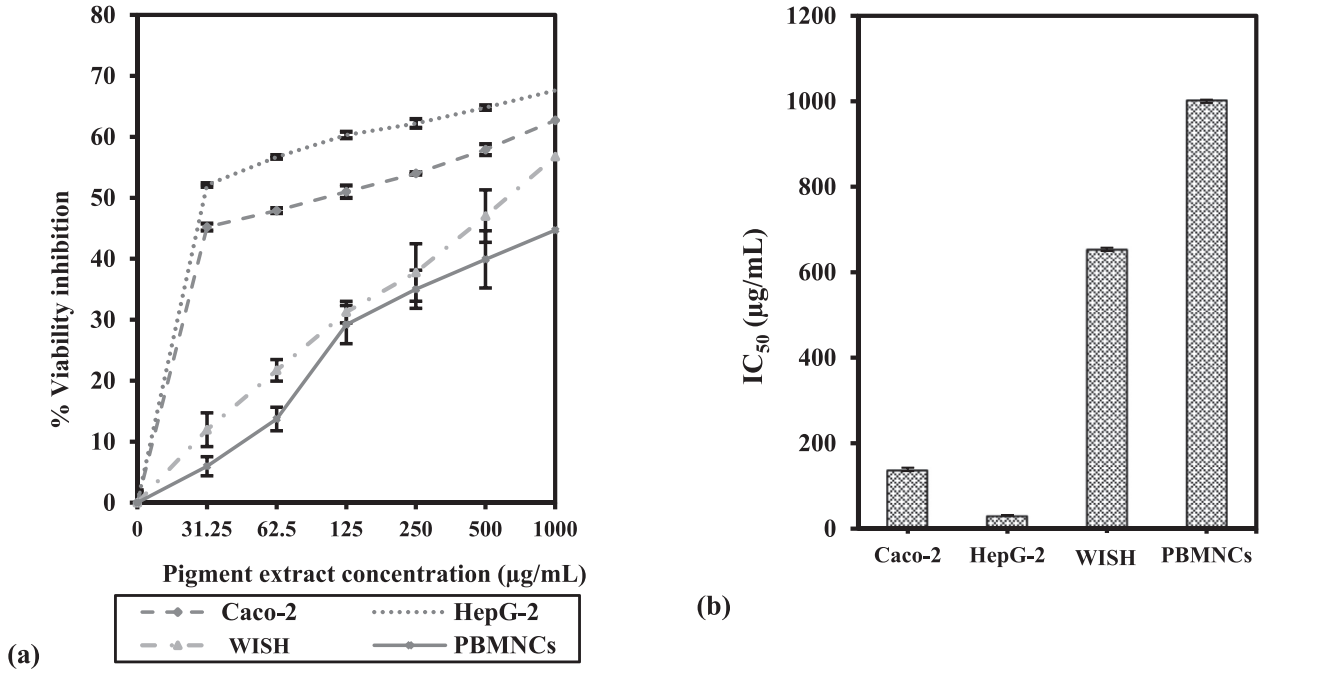

Fig. 3. Cytotoxic and or antiproliferative activity of *V. halodenitrificans* DASH carotenoid extract using MTT test. (a) Dose-Response curves of the extract against Caco-2, HepG-2, WISH, and PBMNCs, (b) The extract IC_{50} values against the investigated cell lines. Error bars represent the mean ± averaged deviation of two replicates.

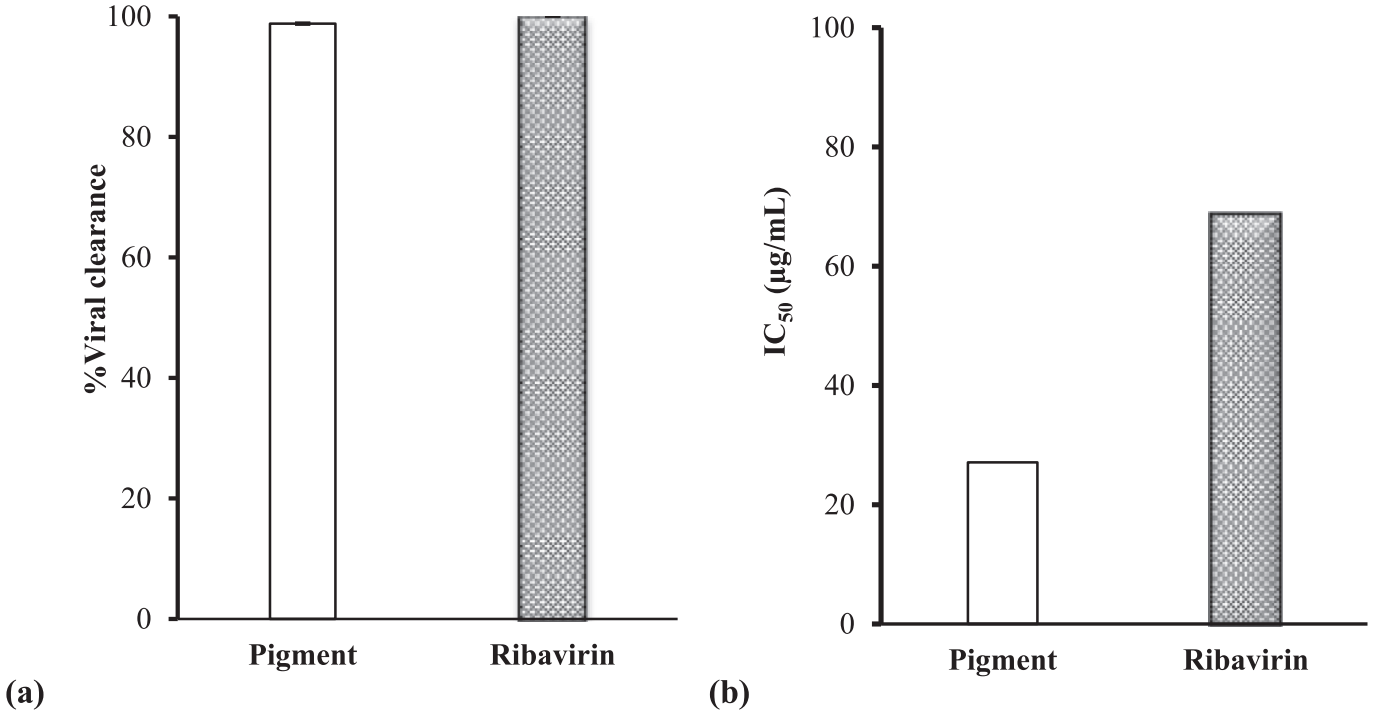

Fig. 4. Anti-HCV activity of carotenoid extract of *V. halodenitrificans* DASH. (a) The %viral clearance using RT-qPCR in HCV-infected PBMNCs after treatment with the carotenoid extract or ribavirin, (b) IC_{50} values reported for the pigment extract and ribavirin for HCV-NS5B polymerase inhibition. Error bars represent the mean ± SD of three replicates.

The anti-inflammatory coupled with antioxidant features are responsible for such antidiabetic trait because, in T2DM, a rise in RO species occurs as a result of hyperglycemia and a decline in antioxidant response, while carotenoids play a regulatory role in the redox balance; thus, preventing new RO species formation and inhibiting cell membrane-associated lipid peroxidation (Abbasian et al., 2018). In addition, carotenoids were proven to affect obesity directly associated with T2DM (Lin et al., 2016). Through our work, the potential antidiabetic activity of *Virgibacillus* carotenoids has been reported, to our knowledge, for the first time, while several studies highlighted the antidiabetic activity of carotenoids, in general, including Kawee-Ai et al. (2019) who reported lutein (IC_{50} = 70 µmol/L), zeaxanthin (IC_{50} = 53.5 µmol/L), as well as fucoxanthin (IC_{50} = 4.75 mmol/L) for reducing α-

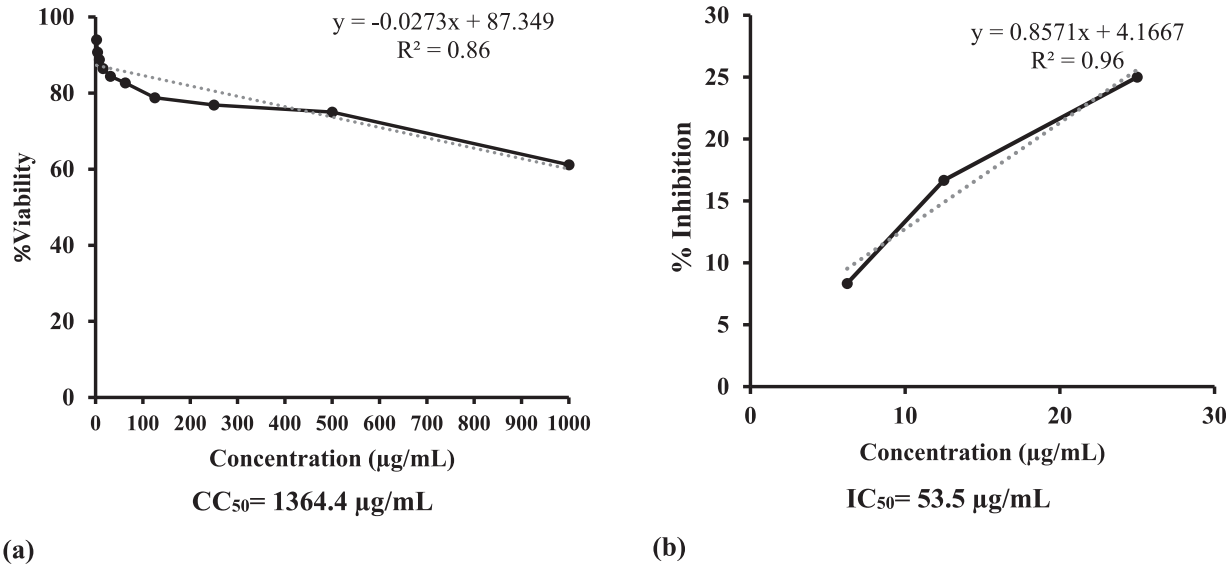

Fig. 5. (a) The dose–response curve of *V. halodenitrificans* DASH carotenoid extract against PBMNCs using MTT test, (b) The dose–response curve of the extract against HCoV 229E using plaque reduction assay.

glucosidase activity. Moreover, certain studies have documented that ingesting some carotenoids, like α-carotene, β-cryptoxanthin, and zeaxanthin, reduces T2DM risk (Sugiura et al., 2015). For microbial carotenoids, Metwally et al. (2022) reported that *Kocuria* sp. RAM1 carotenoids displayed concentration-dependent suppression to α-glucosidase activity up to 90 % at 1000 µg/mL whereas, in the current work, at 800 µg/mL, α-amylase and α-glucosidase suppression of almost 87 % and 95 %, respectively were recorded for *V. halodenitrificans* DASH carotenoid extract. In the same context, Giani et al. (2022) have recently reported antioxidant, hypoglycemic, and antilipidemic traits for the carotenoid extracts of the haloarchaeon *Haloferax mediterranei* where the higher carbon availability in the culture media was associated with higher proportions of all-*trans*-bacterioruberin, 5-*cis*-bacterioruberin, and a double isomeric bacterioruberin as well as a decrease in 9-*cis*-bacterioruberin and 13-*cis*-bacterioruberin; subsequently, an increase in the investigated extract biological activities.

The current work demonstrated considerably selective anticancer activity for the carotenoid extract of *V. halodenitrificans* DASH towards human colorectal carcinoma and hepatoblastoma cell lines Caco-2 and HepG-2, as proven by the sufficiently high SI values (7.2 and 32.0, respectively) measured against human reference normal cells (PBMNCs) using the MTT test after 48 h. The sufficiently high SI values reflect how safe the application of carotenoids as therapeutic anticancer agents is. However, for the cervical adenocarcinoma cell line WISH, the anticancer activity seemed less selective as implied by the SI value (1.53) less than two which is not acceptable according to Nogueira & Estólio do Rosário (2010) who suggested the acceptable SI value for a safe application of a natural compound to be > two; however, Famuyide et al. (2019) considered a potential bioactive agent as non-toxic with SI value > 1. Epidemiological jointly with experimental studies have demonstrated that carotenoids have anticancer characteristics against varied cancer cell lines, for instance, breast, colon, cervix, prostate, lymphoblastic leukemia, and liver (Ajila & Brar, 2012; Gloria et al., 2014). The main cancer chemoprevention mechanisms triggered by carotenoids include alterations in pathways controlling cell death or cell growth, including immune modification, hormone control, growth factor-mediated signaling, mechanisms regulating cell differentiation, cell cycle, and apoptotic events (Tanaka et al., 2012). At the prophylactic level, a lycopene and β-cryptoxanthin-rich diet was shown to be protective against prostate carcinoma, including the aggressive type, amongst European-American as well as African American males considered in PCaP (the North Carolina-Louisiana research project of prostate cancer) (Antwi et al., 2016). For halophilic bacteria carotenoids, recently, combined therapy of doxorubicin and halophilic β-carotene and halophilic lutein was reported for its pro-apoptotic effect (oxidative stress-preceded) on two breast carcinoma cell lines (MCF-7 and MDA-MB-231). Similarly, a combination of halophilic astaxanthin with carbendazim acted synergistically against MCF-7 cells (Atalay et al., 2018; Giani et al., 2021). Also, Hegazy et al. (2020) reported significant anticancer activity for *Natrialba* sp. M6 (a haloalkaliphilic archaeon) carotenoids towards four human carcinoma cell line models (Caco-2, HepG-2, MCF-7, and WISH), where the carotenoid extract exhibited higher selectivity (SI > 2) contrasted with the standard anticancer drug 5-fluorouracil (SI = 1.7), with IC_{50} values range of 21.18–38.24 µg/mL in contrast to the current work IC_{50} estimations of the carotenoid extract, reported as 139 µg/mL against Caco-2, 31 µg/mL against HepG-2, and 653 µg/mL against WISH. To our knowledge, no anticancer activity has previously been reported for *Virgibacillus* carotenoids.

HCV, as an RNA virus, is the most common type of hepatitis viruses leading to liver fibrosis and cirrhosis as well as liver cancer. The global approximation of HCV infection is around 150 million cases. An ideal anti-HCV therapeutic agent mainly targets blocking HCV replication with a minimal level of side effects; accordingly, the persistent demand for innovative, safe bioactive compounds should be tackled, despite the effectiveness of the already adopted synthetic anti-HCV drugs (Habashy & Abu-Serie, 2019). β-Carotene role in reducing HCV-induced hepatosteatosis by inhibiting viral RNA replication, as well as the role of provitamin A activation in decreasing RO species and aborting hepatocellular carcinoma progression caused by human HCV and hepatitis B virus (HBV), has been reported (Yadav et al., 2002). With this respect and considering halophile-driven carotenoids, it was exposed recently that haloarchaea, including *Natrialba* sp., seems prospective candidates for carotenoid production, where the *in vitro* trials showed that the carotenoid extract, which predominantly consists of C_{50} bacterioruberin, has entirely cleared HCV, and 89.4 % of HBV has been

eliminated, whereas the antiviral drug sofosbuvir eliminated 96 % of HCV, and lamivudine eliminated 77 % of HBV from infected PBMNCs, by inhibiting HBV and HCV replication ascribed to polymerase suppression (Hegazy et al., 2020), a result close to the 98.8 % HCV clearance from PBMNCs interestingly recorded in the present study for *V. halodenitrificans* DASH carotenoid extract, using RT-qPCR, and comparable with the seemingly complete HCV clearance reported for the standard drug ribavirin. Moreover, this result was confirmed by the HCV-NS5B polymerase inhibition, which eventually suppresses HCV replication, corresponding to an IC_{50} of 27.4 μg/mL and 68.9 μg/mL of the carotenoid extract and ribavirin, respectively.

HCoV 229E has been previously identified as the second most frequent reason behind the common cold following rhinoviruses in adults (Mäkelä et al., 1998) and belongs to coronaviruses (CoVs) considered as a genus of the *Coronaviridae* family and classified as positive-stranded RNA viruses. In this work, the half maximal reduction in HCoV 229E-induced plaque formation by *V. halodenitrificans* DASH carotenoid extract was estimated as 53.5 μg/mL, a result coinciding with that attained by Xiao et al. (2020) who demonstrated that carotenoids could be prospective agents for remedying coronavirus disease 2019 (COVID-19) through blocking Akt1 pathways which plays a vital function in clearing acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-triggered infections. Also, Yim et al. (2021) recorded a significant antiviral activity (IC_{50} = 87 μM) for siphonaxanthin produced by the green alga *Codium fragile* against SARS-CoV-2 pseudovirus by interfering with virus entry. As for the antidiabetic and anticancer activities, no antiviral activity has been formerly reported for *Virgibacillus* carotenoids. Luckily, the opportunity to apply the carotenoid makeup of *V. halodenitrificans* DASH as a safe anti-HCoV 229E agent was addressed by calculating the SI of the methanolic extract, against PBMNCs, as 25.5, a result interestingly suggesting high selectivity of the investigated carotenoid extract based on the earlier mentioned Nogueira & Estólio do Rosário (2010) report which recommended an SI value > 2 for a bioactive compound to be safe and even considering the more strict Weerapreeyakul et al. (2012) report which suggested an SI value $\geq$ 3, as well as the most strict Peña-Morán et al. (2016) report which endorsed an acceptable SI value of $\geq$ 10 for considering a bioactive compound selective.

5. Conclusion

This work is considered the first report on the anticancer, along with antiviral features, of the carotenoid asset of *Virgibacillus*, as well as their inhibitory impact exceeding α-amylase to include α-glucosidase; moreover, the current report is one of the few reports generally tackling the biological activities associated with *Virgibacillus*. Additionally, the carotenoid pigment extract of the haloalkaliphilic *V. halodenitrificans* DASH, recognized based on recent complementary work for the first time as a member of Wadi El-Natrun salt lakes microbiota, showed antioxidant activity that jointly with α-glucosidase and α-amylase suppressing action implies its potential as a natural prophylactic and therapeutic agent for oxidative stress and T2DM. Moreover, the extract showed a selective anti-HCoV 229E activity, along with anti-HCV activity, and displayed a selective anticancer activity against human colorectal carcinoma and hepatoblastoma cell lines Caco-2 and HepG-2, a result implying the potential application of carotenoid pigments of *V. halodenitrificans* DASH as an alternative prophylactic and therapeutic anticancer and antiviral agents. Accordingly, and considering the insufficient research on the medicinal applications of carotenoids produced generally by haloalkaliphilic bacteria and archaea and particularly by *Virgibacillus*, the current work evokes investigating the fractionated carotenoids of haloalkaliphilic bacteria associated with the local, harsh, and less explored habitats including *Virgibacillus*, as therapeutic and prophylactic agents. Also, it calls for uncovering other possible medicinal and industrial applications of halophilic carotenoids together with designing and testing halophilic carotenoid fractions-based formulations for pharmaceutical and clinical investigations.

CRediT authorship contribution statement

Doaa Fayez: Writing – original draft, Conceptualization, Methodology, Formal analysis, Investigation. **Asmaa Youssif:** Methodology, Formal analysis, Investigation. **Soraya Sabry:** Conceptualization, Methodology. **Hanan Ghozlan:** Conceptualization, Methodology. **Fatma El-Sayed:** Formal analysis, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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