Screening for anti-neoplastic enzymes producing halophilic bacterial extract and their antioxidant activity due to carotenoid synthesis

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Abstract

Out of 1127 halophilic bacteria, salt-loving microorganisms from different geographical locations in India isolated, twenty-eight strains were studied for antioxidant qualities for its usage in cancer medicines. The methanolic extracts were extracted and analyzed for anti-cancer effect through anti-neoplastic enzymes (L-asparaginase and L-glutaminase) screening, while antioxidant capabilities were investigated for fifteen isolates using both radical and non-radical assays.. The extracts also have enhanced radical scavenging ability against the stable 2,2'azinobis (3-ethylbenzothiazoline-6-sulfonic acid) disodium salt radical cation (86.34±0.007 mM TE/g extract), nitric oxide (64.79±0.004 µg/mL), phosphomolybdenum (1.69±0.024 mg AAE/g extract), ferric reducing power (28.19±0.012 mM Fe (II) E/mg) and showed better Ic_{50} values on 1,1-diphenyl- 2-picrylhydrazyl (5.48 mg/ml) assay. All of the studies has been done three times, and the results are significantly better than the standards utilized. Finally, four potent halophilic isolates from various antioxidant activities were chosen. These isolates could synthesize carotenoids that possessed anti-oxidant activities when grown in the halophilic medium was identified by UV/Visible spectra analysis. Secondary metabolite investigations using GC-MS study and LC-MS/MS analysis confirmed that isolated strains had the potential to produce compounds that might be used primarily as therapeutics. The enormous anti-oxidant potential of the strains *Staphylococcus arlettae*, *Bacillus subtilis*, *B. tequilensis*, and *B. haynesii* extracts could be used as a natural free radical scavenger to prevent oxidative-stress-related illnesses like cancer, neurodegeneration, as well as in the food safety markets in general.

1. Introduction

Halophilic microorganisms exposed to high amounts of reactive oxygen species in the oceans have been shown to manufacture antioxidants as a significant defense against free radical-mediated toxicity, protecting their tissues from oxidative stress-related damage. Moreover, In human tissue cells, a variety of endogenous and exogenous factors produce free radicals (such as hydroxyl-OH or peroxyl-ROO radicals), which cause significant oxidative damage and may contribute to aging, carcinoma, and other related illnesses (Reaven and Witzum, 1996). Similarly, antioxidants work via giving, reducing power, metal chelating ability, inhibiting -carotene bleaching, hydrogen to radicals, quenching singlet oxygen, and free radical scavenging activity, among other methods (Chisté et al.,2011). Furthermore, the increased desire for natural molecules to replace the widely used synthetic ones drives the hunt for new natural sources. Therefore, the generation of antioxidants from microbial origin represents a huge possibility in this context. *In vitro* assays can only evaluate antioxidant activity for their specific reaction system, hence its applicability to *in vivo* therapeutic effects is unknown. As a result, it is recommended that antioxidant activity be measured using more than one type of antioxidant test, with at least one assay having biological relevance.

Carotenoids produced by certain microbes have structural differences from those present in foods, such as a higher number of carbon atoms and hydroxyl groups, all of which contribute to their high antioxidant potential (Klassen and Foght 2011). Most carotenoids, for example, 40-carbon (C_{40}) terpenoids, have

isoprenoids (unsaturated hydrocarbon) as their major structural element. A common differentiation is made between "carotenes," that are purely aliphatic (beta-carotene, lycopene), and "xanthophylls," which have polar end groups showing an oxidative step in their synthesis (e.g., lutein, zeaxanthin) (Khachik et al., 1995). Colorants, feed additives, antioxidants, precursors of vitamin A, anti-tumor promoters of *in vitro* antibody formation, and heart disease preventative agents are just a few of the uses for carotenoids. As a result, they're widely used as pigments and functional additives in the dietary, pharmaceutical, medical, and cosmetic industries (Li et al., 2012; Cabral et al., 2011). Discovering alternative sources for anticancer enzyme production and widening the perspective for enzyme-producing strains can help in the quest for novel anticancer enzyme qualities that minimise immune reactivity to these enzymes during therapy.

This study found halophilic/halotolerant bacteria from various hypersaline environments throughout the world. They were then tested to see if they might produce extracellular anticancer enzymes like L-asparaginase and L-glutaminase. As previously said, this category of bacteria's enzymes may offer novel properties in terms of employing them to treat cancerous cells with enzymes due to their distinct traits. Enzyme activity was assessed, and the effect of antioxidants against free radicals and oxides was explored in the case of potent strains. The high antioxidant activity was due to the carotenoid synthesis, later identified by UV/Visible spectra, LC-MS/MS, and GC-MS analysis. There is limited data regarding the carotenoid profile of halophilic and halotolerant microorganisms in the literature, and none about the antioxidant capacity of carotenoids from *S. arlettae* and *B. tequilensis*. The current work underlines the biotechnological importance of bacteria found in saltpans, as well as the fact that these microbes are likely a source for the identification of antioxidants, anticancer, neuroprotective compounds, etc. Additional research will be done to thoroughly describe the bioactive fraction’s composition and activity *in vivo* testing. Many halophilic bacterial species have yet to be discovered, many potential bioactivities have yet to be investigated, and it is still a work in progress to isolate and identify the molecules that are responsible for these functions.

2. Materials And Methods

2.1 Chemicals, solvents and reagents

Methanol, calcium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, l-asparagine, l-glutamine, sodium chloride, glucose, magnesium sulfate, phenol, bromothymol blue, hydeochloric acid, acetate buffer, ferric chloride, DPPH(2,2-diphenyl-2-picrylhydrazyl) solution, ABTS (2,2'azinobis (3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, ethanol, trolox, (TPTZ) 2,4,6-Tris (2-pyridyl)-s-triazine, rutin, ferrous sulfate, sodium nitroprusside, Griess reagent, phosphate buffer, sulphuric aide, sodium phosphate, ammonium molybdate, dimethyl sulphoxide, ascorbic acid, ammonium acetate, acetonitrile, dichloromethane, triethylamine

2.2 Bacterial cultures

The halophilic bacteria (1127) under study were isolated from different Indian solar salterns (Goa, Kerala, Thoothukudi, Marakkanam, and Kanyakumari). Based on the relative physiological parameters like

temperature, pH, NaCl concentration, incubation time (data not shown), twenty-eight isolates were chosen for investigation. The halophilic isolates are; AD03-*Staphylococcus sciuri* subsp. *carnaticus* GTCC 1227 (NCBI GenBank ID: MW012639), AD08-*Stenotrophomonas maltophilia* ATCC 13637 (NCBI GenBank ID: MW012640), AD09-*Staphylococcus xylosu*s JCM 2418 (NCBI GenBank ID: MW012641), AD11-*Staphylococcus arlettae* ATCC 43957 (NCBI GenBank ID: MW012642), AD14-*Bacillus subtilis* subsp., *subtilis* BGSC3A28 (NCBI GenBank ID: MW012643), AD15-*Bacillus piscis* 16MFT21 (NCBI GenBank ID: MW012644), AD23-*Bacillus tequilensis* 10b (NCBI GenBank ID: MW012645), AD28-*Bacillus haynesii* NRRL B-41327 (NCBI GenBank ID: MW012646), AD29-*Pseudomonas zhaodongensis* SCSIO_43767 (NCBI GenBank ID: MW012647), AD35-*Staphylococcus* sp. CTSP32 (NCBI GenBank ID: MW012648), AD36-*Staphylococcus sciuri* DSM 20345 (NCBI GenBank ID: MW012649), AD37-*Bacillus paramycoides* MCCC 1A04098 (NCBI GenBank ID: MW012650), AD39-*Staphylococcus sciuri* DSM20345 (NCBI GenBank ID: MW012651), AD40-*Bacillus subtilis* subsp. *inaquosorum* BGSC3A28 (NCBI GenBank ID: MW012652), AD43-*Staphylococcus sciuri* DSM 20345 (NCBI GenBank ID: MW012653), AD44-*Bacillus tequilensis* 10b (NCBI GenBank ID: MW012654), AD45-*Bacillus subtilis* subsp. *spizizenii* NBRC 101239 (NCBI GenBank ID: MW012655), AD49-*Staphylococcus xylosus* JCM 2418 (NCBI GenBank ID: MW012656), AD50-*Staphylococcus xylosus* KL 162 (NCBI GenBank ID: MW012657), AD51-*Staphylococcus saprophyticus* ATCC 15305 (NCBI GenBank ID: MW012658), AD61-*Staphylococcus edaphiicus* CCM 8730 (NCBI GenBank ID: MW012659), AD162-*Staphylococcus edaphicus* CCM 8730 (NCBI GenBank ID: MW012660), AD263a-*Bacillus subtilis* subsp. *inaquosorum* BGSC3A2B (NCBI GenBank ID: MW012661), AD263b-*Staphylococcus cohnii* GH 137 (NCBI GenBank ID: MW012662), AD464-*Enterobacter hormaechei* subsp. *xiangfangensis* 10–17 (NCBI GenBank ID: MW012663),AD665- *Pseudomonas mendocina* NC15 10541 (NCBI GenBank ID: MW012664), AD770-*Ochrobactrum intermedium* NBRC 15820 (NCBI GenBank ID: 020173), AD1077-*Oceanobacillus aidingensis* AD7-25 (NCBI GenBank ID: 020174). All the growth media, reagents, and solvents of analytical grade were used in the study.

2.3 Culture conditions and maintenance

The bacterial isolates were grown in 250 mL Starch Glycerol Nitrate (SGN) broth (medium composition previously reported). The pH of the medium was measured, and it was added with 10% and 15% (w/v) NaCl before being incubated at 30°C for 5–7 days with constant shaking at 120 rpm. A UV-Visible double beam spectrophotometer (Shimadzu, Kyoto, Japan, UV-2450) with an optical density of 640 nm was used to monitor bacterial growth on a regular basis.

2.4 Preparation of extract

The bacterial extract was prepared using the modified procedure of Prathiba and Jayaraman, 2018. Pure bacterial culture (40 mL) was collected when the isolated organisms reached the stationary phase of growth and centrifuged at 6450 g for 15 min. After centrifugation, the supernatant was disposed, and the bioactive metabolite-containing pellets were rinsed with distilled water and centrifuged to separate the supernatant. Methanol (40 mL) was used to collect the probable metabolite-containing protein and lipophilic substances discharged from the cells, which were centrifuged at 6450 g for 15 minutes. This procedure is repeated until the cell pellet obtained is colorless. After that, some of the methanolic extracts

were kept in liquid form, while others were dried in rotary vapour and solid powder was obtained for further research.

2.5 Anti-neoplastic enzyme production

For bacterial growth and enzyme production, various screening procedures were optimized. Rapid plate assay method was performed for screening isolates for L-asparaginase and L-glutaminase enzyme production (Gulati et al, 1997). The isolates obtained were plated out on Modified M-9 medium containing: $CaCl_2.2H_2O$, 0.15 g; $Na_2HPO_4.2H_2O$, 6.0 g; KH_2PO_4, 3.0 g; L-asparagine or L-glutamine, 5.0 g; NaCl, 30.0 g; Glucose, 2.0 g; $MgSO_4.7H_2O$, 0.5 g, and agar, 15.0 g in 1 L of distilled water. The medium was prepared, pH was checked and adjusted to 7.0. Stock solution of 2.5 mL of 3% (w/v) pH indicator, phenol, or bromothymol blue in ethanol was added to the media and incubated at 37°C for 48 hours. After 24 and 48 hours of incubation, a change in the colour of the media around the streaking colonies emerged, which was used as a sign of L-asparaginase and L-glutaminase enzyme production. A negative control medium comprising no amino acids was used for each enzyme.

2.6 In vitro antioxidant assays

2.6.1 DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-2-picrylhydrazyl) technique was used to assess the antioxidant potential of the methanol extract (1958). As a result, 200 µl of methanolic extract and 1 mL of DPPH were mixed together (0.1 mM in methanol). The reaction mixture was then centrifuged before being incubated for 30 minutes at 25 degrees Celsius. Using a Systronics AU2701 double beam spectrophotometer, the absorbance was measured at 517 nm. The standard was ascorbic acid (from 10 to 50 g/mL). The % scavenging activity was estimated using the formula below,

Radical scavenging activity % = Ac − As /Ac x 100

where Ac and As are the absorbances of the control (200 µl methanol) and sample, respectively.. The IC_{50} values were obtained by plotting linear regression, with the ‘x coordinate’ representing the concentration of halophilic methanol extracts studied and the ‘y coordinate’ calculating the average percentage of reducing capacity from triplicates.

2.6.2 Reducing Power Assay - FRAP

The reducing power of the halophilic extract was estimated using the Pulido et al. technique (2000). The ferric reducing ability of plasma (FRAP) assay was performed to assess the cell extract’s ferric reducing activity. The FRAP reagent was made up of 2.5 mL of 20 mM 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, 25 mL of 0.3 M acetate buffer, and 2.5 mL of 20 mM FeCl3. 6H2O (pH – 3.6). The procedure was as follows: a blank reading at 593 nm was taken using a Systronics AU2701 double-beam spectrophotometer after a 5-minute incubation at 37°C with freshly prepared FRAP reagent. Then, in 900 µl of FRAP reagent, 10–100 g/mL of cell extract, 1–10 g/mL of standard, and distilled water of 90 µl were

added. After the reaction was started, absorbance values were taken as soon as the FRAP reagent was added. The absorbance was compared to the absorbance changes of a Fe (II) standard solution that was examined at the same time. Three trials were used to compute the results, which were expressed as micromoles ferric reducing activity of the extracts per gram of dried weight. The positive control used was a ferrous sulfate. Methanolic solutions with known Fe (II) concentrations ranging from 100 µM to 2000 µM were used to produce the calibration curve ($FeSO_4.7H_2O$). The quantity of antioxidant that can reduce ferric-TPTZ to the same extent as 1 mM ferrous sulfate was defined as the parameter equivalent concentration. An equivalent concentration is the amount of antioxidants that generates an increase in absorbance in the FRAP experiment equal to the predicted absorbance value of a 1 mM concentration of Fe (II) solution.

2.6.3 ABTS Activity

Re et al. (1999) used the 2,2'azinobis (3-ethylbenzothiazoline-6-sulfonic acid) disodium salt radical cation (ABTS•+) decolorization test to assess the overall antioxidant activity of the samples. 7 mM aqueous ABTS solution was initially prepared by reacting it with 2.4 mM potassium persulfate at ambient temperature for 12–16 hours in a dark setting. After incubation at 30°C, the mixture was diluted with ethanol (1:89 v/v) to get an absorbance of 0.700 ± 0.002 at wavelength 734 nm, which was employed in the experiment. The free radical - quenching reaction was started by mixing 5 µl of methanolic extracts with 1 mL of diluted ABTS solution and incubating for 30 minutes in the dark at room temperature. Triplicate measurements were taken at each dilution of the standards and sample extracts, and the absorbance was noted at 734 nm against a blank using a Systronics AU2701 double-beam spectrophotometer. The positive control used as trolox. All of the reagents were present in the control, with the exception of the extract. An equal volume of pure water was added to keep the overall volume steady. A Trolox calibration curve was created using the decrease in absorbance of the ABTS solution in the Trolox presence (final concentration 0–15 µM). By comparing the ratios of the gradients of the concentration plots of the extracts and Trolox throughout a linear concentration range, the ABTS radical scavenging activity of the halophilic extracts and standard was measured. The antioxidant capacity was measured in mM Trolox equivalent (TE) per gram of sample methanolic extracts.

2.6.4 Nitric oxide production from sodium nitroprusside: assay of nitrite production

At physiological pH, sodium nitroprusside produces NO (nitric oxide), which interacts with oxygen to yield nitrite ions, which can be quantified using the Griess reagent (Green et al., 1982; Marcocci et al., 1994a) (Marcoci et al., 1994b). Because nitric oxide scavengers compete with oxygen, nitric oxide generation is reduced (Marcoci et al., 1994b). In phosphate-buffered saline, different quantities of cell extract were mixed with sodium nitroprusside (5mM) and incubated at 25°C for 2h and 30 minutes. A control mixture with no sample and no blank (saline buffer) was used in the same way. At regular gaps, a sample of the incubation solution (0.5 mL) was taken and diluted with 0.5 mL Griess reagent (1 percent sulphanilamide, 2 percent H3PO4, and 0.1 percent N-(1-naphthyl) ethylenediamine dihydrochloride). The absorbance of the chromophore generated after nitrite diazotization with sulphanilamide and subsequent coupling with

naphthyl ethylenediamine was measured at 546 nm and compared to the absorbance of a reference solution of potassium nitrite treated with Griess reagent in the similar way. As a positive control, rutin was used.

2.6.5 Phosphomolybdenum assay

The antioxidant activity of the methanolic extract was determined using Prieto et al. (1999)'s green phosphomolybdenum complex synthesis technique. Three replicates of 100 µl of sample was combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and various amounts of standard in a test tube (ascorbic acid in 1 mM dimethyl sulphoxide). The negative control was made up of 3 mL of reagent solution and an adequate amount of sample, which was incubated under similar conditions as the other samples. The test tubes were wrapped in foil and incubated in a water bath at 95°C for 1h and 30 minutes. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm. The findings were measured in milligrams of ascorbic acid equivalents per gramme of extract. The reference standard used was ascorbic acid.

2.7 Characterization of halophilic extract

The isolates *Staphylococcus arlettae* ATCC 43957, *Bacillus subtilis* subsp., *subtilis* BGSC3A28, *Bacillus tequilensis* 10b, and *Bacillus haynesii* NRRL B-41327 methanolic cell extracts were individually analyzed for UV-Visible Spectroscopy, LC-MS, and GC-MS as these halophilic isolates almost showed valuable antioxidant activity in the above performed *in vitro* radical scavenging assay.

2.7.1 UV-Vis Spectroscopy

A double beam spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) was used to document UV spectrum absorbance of methanolic cell extracts (5mg/mL) of the above four isolates between 200 to 800 nm. The control used was methanol. The optical density (OD) of the samples in nm ($\lambda_{max)}$ was used to estimate the number of antioxidants present in the extract fs.

2.7.2 Liquid Chromatography-Mass Spectroscopy (LC-MS/MS)

To decrease isomerization and oxidation of carotenoids caused by UV irradiation, sample extracts were handled on ice, wrapped in aluminium foil. For chromatographic separation, a microsyringe (20 µl, Triple Quadrupole, LCMS 8040; Shimadzu; Kyoto; Japan) was used to inject 10 l of the filtered material into the manual injector at a flow rate of 2 ml/min. Using isocratic elution at a flow rate of 0.8 ml/min. The mobile phase consisted of 70:20:10 v/v/v acetonitrile-dichloromethane-methanol, 20 mM ammonium acetate, and 0.1 percent triethylamine. The column was kept at a constant temperature of 250°C, and UV detection was done at 450 nanometres. The extracts were put into a reverse phase column with a 20-liter injection volume Mass spectra in the mass range of 100–1300 m/z were acquired in both positive and negative ion modes. The mass spectrometer's settings were set as follows: The nebulizer pressure was 30 pounds per square inch, the drying gas flow was 20 litres per minute, and the temperature of the drying

gas was 250 degrees Fahrenheit. 2500 V was applied across the capillary. The voltage of the detector was set to 1.3 kV. Ions were detected in both protonated and deprotonated forms in scan mode. The flow rate was maintained at 2 ml/min. Carotenoids were identified using the retention period, UV spectrum, and characteristics of the mass spectrum and its MS/MS fragments. For the data analysis, the class's VP integration software was utilized. An UV-visible detector was employed to track all of the carotenoids at 450 nm.

2.7.3 Gas Chromatography-Mass Spectroscopy Analysis

Thermo GC - Trace Ultra Version: 5.0 was used to analyse the GC-MS methanolic extracts. Helium was employed as the carrier gas, with a flow rate of 1.0 ml min1. The temperature of the injection port was fixed at 250°C. The temperature in the column oven was kept at 70°C for 2 minutes before being programmed at 10°C min1 to 260°C for 0 minutes and then at 5°C min1 to 280°C for 9 minutes. The chemicals detected in the active extract were compared using the National Institute of Standards and Technology's NIST 20.library database.

3. Results And Discussion

3.1 Culture Analysis

The collected water samples resulted in a total of 28 distinct pure isolates at different geographical locations in India (Fig. 1). The methods for isolation and identification were reported previously (Rathakrishnan and Gopalan, 2022). Phylogenetic analysis of each isolate was done using PCR amplified 16S rRNA gene sequence data against a database of known species using the National Centre for Biotechnological Information (NCBI) server (https://www.ncbi.nlm.nih.gov/gene/) and found that bacteria of the genus *Staphylococcus* were the most common, accounting for 46.42% of the isolates. The genus *Bacillus* accounted for 32% followed by the genus *Pseudomonas* 7%. There were other isolates from the genera *Stenotrophomonas*, *Enterobacter*, *Ochrobactrum*, and *Oceanobacillus* accounting for 3.5% of the total isolates. All isolates have a pairwise nucleotide sequence similarity of 96 percent to 99 percent. The bacterial isolate codes, lengths of sequences, percentage similarity to closest matched strains, and accession numbers compared were listed (supplementary file). To assess their phylogenetic locations, the 16S rRNA gene sequences of each strain were evaluated, and a phylogenetic tree with gene accession numbers was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 11. The readings were classified using the RDP classifier tool based on specified phylogenetic bacterial species. Firmicutes and proteobacteria were identified as prominent groupings in the phylogenetic tree (supplementary file). The genus Firmicutes was the most common among the isolated strains. The phylogenetic tree also shows that only five isolates were distinct from the rest of the isolates and their near relatives.

3.2 Growth optimization and pigment production of halophilic isolates

Starch Glycerol Nitrate (SGN) medium (100 ml) was prepared in a 250 mL Erlenmeyer flask. Different optimizing growth parameters, such as pH (3–11), temperature (20, 25, 30, and 35°C), and salinity, were tested separately (5, 10, 15, 20, 25, and 30 ppt) (data not shown). All of the isolates studied were salt tolerant and could grow optimally in culture conditions containing 15% sodium chloride (NaCl) (w/v). The pigmentation in culture was due to the presence of yeast extract in the medium that influenced different colorations of the culture broth. Although the isolates used in this study were from diverse habitats, their responses to salt concentration and temperature in terms of growth and pigment production were similar. At 30°C, cell growth and pigment synthesis were at their peak, and a pH of 9 was considered ideal. *Bacillus haynesii*, *B. tequilensis*, *Pseudomonas zhaodongensis*, *Bacillus paramycoides*, and *Staphylococcus saprophyticus* showed intense and huge pigmentation yield in 10% (w/v) NaCl concentration. Notably, *B. tequilensis* was able to adapt quickly, multiplying in only 18 hours. The strains showed a considerable increase in pigment biomass when the concentration of NaCl was increased from 5–10% (w/v), demonstrating the importance of continuous pigment synthesis in defending the cell from osmotic stress conditions. In comparison to 0% NaCl (w/v), bacterial strains *Staphylococcus cohnii*, *Enterobacter hormaechei*, and *Oceanobacillus aidingensis* took longer to attain OD_{640} at higher salt concentrations.

3.3 Bioactive properties of extracts

Later, in the stationary phase end, a total of 28 halophilic extracts were collected from the isolates. The extracts were immiscible with ethyl acetate, hexane, isopropyl alcohol, chloroform, cyclohexane, ethanol, petroleum ether, acetone, tween-80, toluene, and butyl alcohol but miscible with DMSO and methanol. All of the isolates' methanolic extracts were kept at 4°C until needed, while the dry powder form was kept at -80°C. Biological studies used to assess the anticancer potential of these cell-free supernatants obtained from the growth medium were tested as detailed below.

3.4 Screening of extracellular anti-neoplastic enzyme-producing halophilic isolates

The ability to manufacture L-asparaginase and L-glutaminase enzymes is predominantly tested in halophilic isolates. The medium's colour changed from yellow to pink, indicating the formation of L-asparaginase and L-glutaminase. When L-asparaginase and L-glutaminase activities were tested in the cell culture, it was discovered that both enzymes were released into the culture media. There were seven isolates from Thoothukudi, five from Marakkanam, seven from Goa, four from Ernakulam, and five from Kanyakumari among the selected ones. Most of the positive strains were gram-positive cocci (46.4%). L-glutaminase showed the highest activity and was the most prevalent anti-tumor enzyme found in the tested strains, accounting for 57% of the bacterial strains (Table 1). The majority of anticancer enzyme-producing isolates stemmed from the genera *Halomonas*, *Marinobacter*, and *Bacillus*, which are all common in saline habitats (Ventosa.,1988) In some of the strains, the combination antitumor activity (L-asparaginase and L-glutaminase) was also identified. Both the enzyme production can be observed in the middle stationary phase. These enzymes have the potential therapeutic outcomes in anticancer behavior, therefore, allowing us to select fifteen halophilic strains for antioxidant studies. We infer from the

previous study report that temperature, pH, and NaCl concentration had a significant impact on the production of both enzymes (Pejman et al.,2016). Furthermore, sucrose and glucose were the best carbon sources for *Vibrio* sps and *Rhodococcus* sps to synthesise L-asparaginase and L-glutaminase, respectively (Zolfaghar et al., 2019). According to Distasio et al.,*Bacillus subtilis* RSP-GLU produced the most L-glutaminase at 37.1°C and pH 7 (Sathish and Prakasham, 2010), while *Vibrio succinogenes* has the most L-asparaginase activity at pH 7.3. (1976).

Table 1
Distribution of anti-neoplastic enzymes based on geographical location and cellular morphology

Sample source/cell shape	L-asparaginase	L-glutaminase
Goa	4	6
Ernakulam	2	3
Marakkanam	3	5
Thoothukudi	4	5
Kanyakumari	5	5
Gram-positive rod	4	4
Gram-positive cocci	5	8
Gram-negative rod	3	4

3.5 Radical Scavenging activity

3.5.1 2,2-Diphenylpicrylhydrazyl radical scavenging assay

The use of DPPH (1,1-diphenyl-2-picrylhydrazyl), an electron transfer method to test radical scavenging capacity, revealed that all chosen halophilic bacterial extracts can suppress free radicals. The antiradical activity of the selected halophilic extracts was tested using DPPH, a stable free radical with an absorption band at 515 nm that can be greatly quenched in the presence of protonated radical scavengers (Blois, 1958). The reduction of free radicals is indicated by the darkening of the purple colour of DPPH to yellow. The DPPH experiment yielded a positive result, indicating that these compounds can serve as antioxidants by transferring electrons to reactive species like DPPH or ions with a high oxidation number. The scavenging capacity rose as the amount of extract used in the experiment increased. The as the concentration of sample at which the inhibition rate hits 50% is the Ic_{50} value. Antioxidant value increases as the IC50 value decreases. The IC50 of typical antioxidants (Table 2) was found to be significantly lower than that of cell extracts, indicating that the isolate AD23 (Ic_{50} value 5.48 mg/ml) has high free radical scavenging action than ascorbic acid and the lowest activity was observed in AD03 (Ic_{50} value 75.87 mg/ml).

Table 2
Results of various *in vitro* antioxidant analyses (mean ± SD of triple assessment, n = 3)

Isolate	DPPH (IC_{50}* µg/mL)	FRAP (µM Fe(II)E*/mg extract)	ABTS(mM TE*/mg extract)	Nitric oxide radical scavenging activity (%) (µg/mL extract)	Phosphomolybdenum reduction (mg AAE*/g extract)
Standard	6.35^{c}	27.66 ± 0.083^{e}	43.78 ± 0.114^{o}	51.61 ± 0.011^{d}	1.857 ± 0.473^{a}
AD03	75.87^{p}	28.19 ± 0.012^{c}	79.41 ± 0.014^{f}	24.71 ± 0.004^{i}	$0.512 \pm .039^{o}$
AD09	6.00^{b}	28.15 ± 0.002^{b}	84.04 ± 0.017^{d}	20.22 ± 0.006^{k}	1.318 ± 0.017^{g}
AD11	8.46^{i}	46.49 ± 0.567^{m}	43.17 ± 0.042^{p}	11.83 ± 0.027^{o}	1.676 ± 0.584^{d}
AD14	10.05^{l}	32.08 ± 0.045^{g}	86.34 ± 0.007^{a}	35.58 ± 0.025^{g}	1.69 ± 0.024^{c}
AD15	9.01^{j}	66.14 ± 0.020^{p}	65.42 ± 0.013^{k}	40.82 ± 0.006^{f}	1.548 ± 0.079^{e}
AD23	5.48^{a}	48.72 ± 0.035^{o}	72.06 ± 0.003^{i}	19.47 ± 0.011^{l}	1.038 ± 0.019^{l}
AD28	6.50^{d}	35.72 ± 0.015^{h}	70.29 ± 0.006^{j}	12.73 ± 0.004^{n}	1.482 ± 0.033^{f}
AD29	13.87^{p}	47.10 ± 0.015^{n}	86.19 ± 0.012^{b}	7.11 ± 0.008^{p}	1.049 ± 0.029^{j}
AD35	31.05^{m}	38.67 ± 0.037^{j}	73.97 ± 0.032^{h}	64.79 ± 0.004^{a}	1.097 ± 0.075^{i}
AD37	9.72^{k}	46.36 ± 0.012^{l}	53.15 ± 0.023^{n}	43.07 ± 0.003^{e}	1.053 ± 0.115^{k}
AD51	8.24^{h}	38.45 ± 0.030^{i}	79.13 ± 0.021^{g}	52.43 ± 0.002^{c}	1.314 ± 0.180^{h}
AD162	7.30^{f}	25.40 ± 0.016^{a}	62.79 ± 0.081^{l}	22.47 ± 0.004^{j}	0.242 ± 0.108^{p}
AD263	17.48^{n}	40.20 ± 0.042^{k}	80.22 ± 0.046^{e}	28.08 ± 0.001^{h}	1.777 ± 0.0630^{b}

± standard deviation, % - % of Inhibition, *IC_{50} - sample concentration when the inhibition rate hits 50%,* Fe (II)E (ferrous ion equivalents), *TE- Trolox Equivalents, *AAE- Ascorbic Acid Equivalents. Statistically significant at $p < 0.05$ where $^{a>b>c>d>e>f>g>h>i>j>k>l>m>n>o>p}$ in each column.

Isolate	DPPH (IC_{50}* µg/mL)	FRAP (µM Fe(II)E*/mg extract)	ABTS(mM TE*/mg extract)	Nitric oxide radical scavenging activity (%) (µg/mL extract)	Phosphomolybdenum reduction (mg AAE*/g extract)
AD464	6.80^{e}	28.89 ± 0.030^{d}	60.69 ± 0.00^{m}	15.35 ± 0.002^{m}	0.805 ± 0.046^{n}
AD665	7.46^{g}	29.81 ± 0.080^{f}	86.19 ± 0.011^{c}	56.55 ± 0.007^{b}	0.878 ± 0.116^{m}

± standard deviation, % - % of Inhibition, *IC_{50} - sample concentration when the inhibition rate hits 50%,* Fe (II)E (ferrous ion equivalents), *TE- Trolox Equivalents, *AAE- Ascorbic Acid Equivalents. Statistically significant at $p < 0.05$ where $^{a>b>c>d>e>f>g>h>i>j>k>l>m>n>o>p}$ in each column.

3.5.2 Ferric reducing antioxidant power (FRAP) assay

This is a colorimetric assay that evaluates plasma's ability to convert the deep blue ferric tripyridyltriazine (TPTZ) complex to its ferrous form (TPTZ-Fe (III) into TPTZ-Fe (II)), resulting in a change in absorbance. The lower value indicates that the antioxidant activity is high. When compared to ordinary ferrous sulfate, AD162 showed to have stronger ferric reducing antioxidant activity among the halophilic isolates. The ferric reducing power of the methanol extracts was 28.190.012, 28.150.002, and 25.400.016, mM Fe (II) E/mg for isolates AD03, AD09, and AD162, respectively, while isolate AD23 exhibited the least gain in ferric reducing capacity (Table 2). Standard synthetic antioxidants have much lower radical scavenging and reduction power than these species (Ferrous sulfate). Although FRAP is a simple, reliable, and cost-effective method, it is unable to detect species that function via radical quenching (H transfer), such as glutathione and proteins, which include SH group-containing antioxidants like thiols (Prior, 2000 and Huang Do, 2005).

3.5.3 ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ABTS, a hydrogen atom transfer technique, was used to assess the antioxidant activity of the sample methanolic extracts at varying salt concentrations. Because it is soluble in both aqueous and organic solvents and is unaffected by ionic strength, the ABTS radical can be utilised to measure the antioxidant capacity of hydrophilic and lipophilic compounds in test samples (Roginsky and Lissi.,2005). The antioxidant changes the colorless ABTS free radical into a stable blue-colored ABTS free radical. Spectrophotometry can be used to measure the color change at 734 nm. When ABTS interacts with ferryl myoglobin, a fairly stable blue-green hue, measured at 600 nm, is formed. Color generation is inhibited to a proportional degree by antioxidants in the fluid sample (Badrinath et al.,2010). As seen, all halophilic extract's scavenging effect increased when compared to the control except AD11(43.17 ± 0.042 mM TE/mg extract). The highest activity was observed in AD14, AD29 and AD665 were 86.34 ± 0.007, 86.19 ± 0.011, 86.19 ± 0.011 mM TE/mg extract, respectively while the standard represented 43.78 ± 0.114 mM TE/mg extract (Table 2). The higher concentrations of phenolics and tannins, that appeared to behave as

excellent radical quenchers, could explain the total antioxidant activity reported. As a result, the halophilic extract's total antioxidant capacity can be attributed to its free radical scavenging activity, which is comparable to natural and common antioxidant vitamin C. All of the analyses were carried out in three different ways.

3.5.4 Nitric oxide

Nitric oxide is a bio-supervisory molecule in the human system (immune, neurological, and cardiovascular) (Pick, 2012). Nitric oxide, or reactive nitrogen species, such as N_2O_4, NO_2, NO_3^-, N_3O_4, and NO_2^+, are extremely reactive when they combine with oxygen or superoxide. Many cellular components have their structure and functional behavior altered by these substances. The nitric oxide radical scavenging activity was calculated as a (%) value and the values are represented in standard deviation. The methanolic extract of AD35 showed maximum scavenging of 64.79 ± 0.004 µg/mL whereas the standard rutin at the same concentration exhibited 51.61 ± 0.011 µg/mL (Table 2). The scavenging activity also may aid to stop the cascade of reactions that are harmful to human health and are triggered by excess NO production. Cancer, inflammation, and other pathological disorders have all been linked to nitric acid (Moncada., 1991). Halophilic extracts can be utilized to scavenge reactive nitrogen species in the human body because they demonstrate good performance.

3.5.5 Phosphomolybdenum assay

The phosphomolybdenum approach works by converting Mo (VI) to Mo (V) and generating a green phosphate/Mo (V) complex with an absorption maxima at 695 nm, where an elevated absorbance indicates a higher overall scavenging capacity (Prieto et al., 1999). This approach was utilized to assess the antioxidant capabilities of methanolic extracts of halophilic isolates spectrophotometrically, with rutin as a positive reference molecule. When compared to all other solvent extracts, rutin had significantly the highest activity (1.857 ± 0.473 mg AAE/g extract), although methanolic extracts had higher activities (1.676 ± 0.584 and 1.69 ± 0.024 mg AAE/g extract by AD14 and AD11 halophilic isolates, respectively) (Table 2). The lowest activity is exhibited in AD162 (0.242 ± 0.108 mg AAE/g extract). The increased activity of the crude extracts could be attributable to the inclusion of additional chemicals, and the extraction solvents have also been reported to have remarkable outcomes in terms of Mo reduction (VI).

3.6 UV-Visible Spectroscopy

The methanol extract's UV–vis spectra are similar to conventional carotenoids spectra. The broad-band centered at 400–500 nm is the most prominent feature of these spectra (Fig. 2). These are carotenoids in the bacterioruberin class, such as zeaxanthin, beta-carotene, lutein, canthaxanthin, and lycopene, that have antioxidant properties and provide organisms yellow, orange, pink, and red hues. The antioxidant activity of carotenoids is assumed to have a role in many disease prevention, and certain of them, such as lycopene and beta-carotene, are widely known for their antioxidant properties (Squillaci et al.,2017). Although the carotenoids from *Haloterrigena turkmenica* have substantial antioxidant properties, their output is insufficient for widespread application. In the perspective of this, the carotenoid pigments of psychrotolerant *Sphingobacterium antarcticus* and mesophilic *Sphingobacterium multivorum* were

characterized, and different characteristics such as their *in vivo* location, production, and *in vitro* interactions with membranes were investigated by Jagannatham et al., (2000). Carotenoids also have biological roles and activities in humans and animals, such as light absorbers, anticancer, scavengers of active oxygen, oxygen transporters, and promoters of *in vitro* antibody formation (Kaulmann and Bohn, 2014; Gupta and Prakash, 2014; Ascenso et al., 2014).

3.7 Liquid Chromatography-Mass Spectroscopy (LC-MS/MS) analysis

Among other probable sources, antioxidant metabolites extracted from halophilic bacteria have received the greatest interest in recent years. LC-MS/MS was used to separate and mass characterize the halophilic extract in methanol. The existence of unlike components in the pigment, which may be carotenoids, was identified via LC-MS/MS analysis. In positive ionisation mode, ions correspond to protonated molecules [M + H]+, while in negative ionisation mode, ions correspond to deprotonated molecules [M-H]-. The list of carotenoids has been identified using reference standards. LCMS/MS analysis identified twenty components, where all exhibit biological activity. The chromatogram of LC-MS/MS analysis of the above four isolates is depicted (Fig. 3) that illustrates retention time, mass to charge ratio along percentage intensity. The molecular formula, structure, and biological activity of identified carotenoids are tabulated (supplementary file). β-Carotene, a carotenoid synthesis intermediate, was discovered to be generated from lycopene (Chi et al., 2015; Rodriguez-Saiz et al., 2010;). Furthermore, the production of 3-hydroxy cyclic carotenoids and epoxy carotenoids is known to be caused by hydroxylation of hydrocarbon carotenoids (Ambati et al., 2010). In the pharmaceutical sector, LC-MS/MS is a particularly useful approach for quickly screening medication contaminants and degradation products.

3.8 Gas Chromatography-Mass Spectroscopy Analysis

Several key organic volatile substances and their derivatives were discovered as a result of this investigation. The goal of this study was to use GC-MS to identify an antioxidant molecule from an organic solvent extract of four halophilic isolates. Because the isolates coded AD11, AD14, AD23 and AD28 varied from other isolates in terms of tested biochemical activity, it was chosen for completion of this study. Based on the match factor (comparison of unknown's spectra against library's known spectra), the main constituent of halophilic crude extract, containing unique chemical compounds in the first five numbers having 60–70% match is presented. Table 3 shows the compound name, retention time (RT), molecular formula, and structure reported in the crude extract. Some of our chemical components have been determined to have medicinal importance in pharmaceutical domains when their mass spectrum were compared to those in the NIST 20 library database (supplementary file). The GC-MS analysis characterized many organic compounds that had pharmaceutical applications like anti-oxidant, anticancer, neuroprotective, etc. The peak at the retention time of 9.54 and 9.48 was confirmed as thiazolo and thiophene with the molecular formula of $C_{14}H_8N_4S_2$ and $C_{38}H_{12}S$ reported to have antioxidant properties. These secondary metabolites provide a new research route for identifying chemical elements with anticancer activity in the future. Microalgae produce active extracts with

antioxidant and antibacterial properties, according to Krishnakumar et al (2013). In terms of retention time and peak area percentage, a few other minor chemical compounds were also detected. Based on the findings, methanol was chosen as the best organic solvent for extracting bioactive substances from halophilic bacteria for biomedical and pharmaceutical applications.

4. Conclusion

The hunt for unique antioxidants in halophiles appears to be especially interesting, according to the findings of this study, as they have unique adaptations such as greater stability in extreme conditions and microbial products that are very stable and useful in a range of disciplines. Following our first findings, we're working to identify the anti-oxidants and prove their functionality. To our knowledge, such a huge number of halophilic bacteria have been examined after screening for various bioactivities for the first time in India. Both the extracellular extracts from the culture medium and the cellular extracts had significant antioxidant activity, scavenging DPPH and ABTS radicals, chelating iron, scavenging NO radicals, and reducing molybdenum. Halobacteria from salterns will aid in the development of more profitable eco-friendly pharmacological goods for the pharmaceutical industry as well as in food industry. Because a variety of antioxidant assays were used, the results represent a comprehensive assessment of halobacteria's antioxidant ability. These findings suggest that halobacterial extracts could be a viable source of antioxidant chemicals that could be used as natural food preservatives, colorants, and supplements, and also producers of leads for cosmetic and pharmaceutical formulations to avoid oxidative damage.

Declarations

Conflict of interest

The authors state that there were no commercial or financial relationships that may be considered as a potential conflict of interest during the research.

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Tables

Table 3 is available in the Supplemental Files section.

Figures

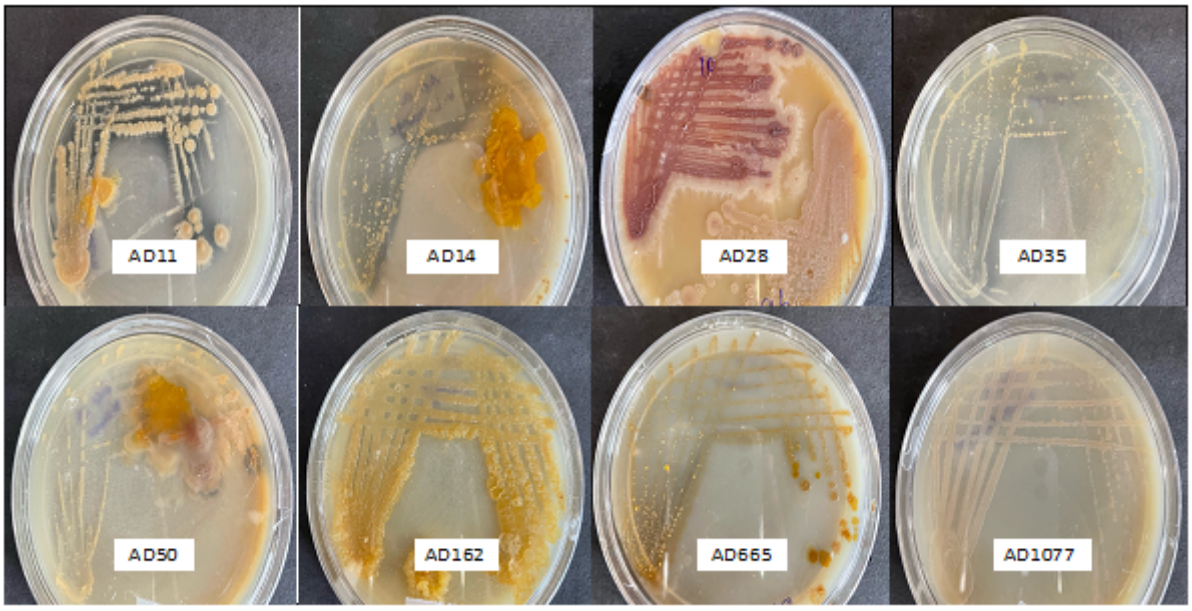

Figure 1

Single colony (streaked) cultures of the pigmented halophilic/halotolerant isolates were obtained from different solar salterns of Thoothukudi, Marakkanam, and, Kanyakumari, Tamil Nadu; Ribander, Goa; Ernakulum, Kerala, India. The isolates were grown in Starch Nitrate Medium agar and few isolates used in the study were able to produce halocin forming zone of clearance in the medium is found.

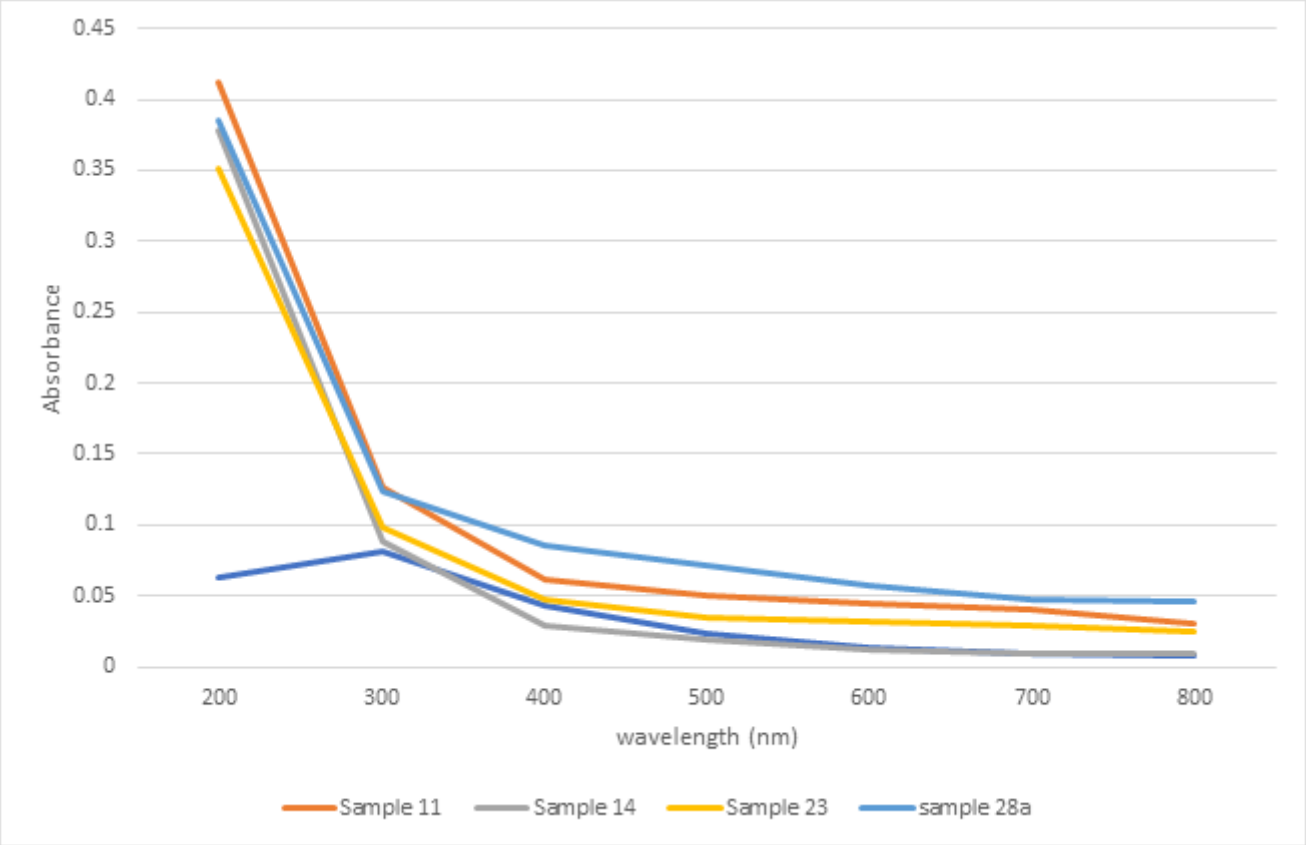

Figure 2

Spectrophotometric analysis of methanolic extracts from various halophilic isolates showing the presence of bacterioruberin class of carotenoids.

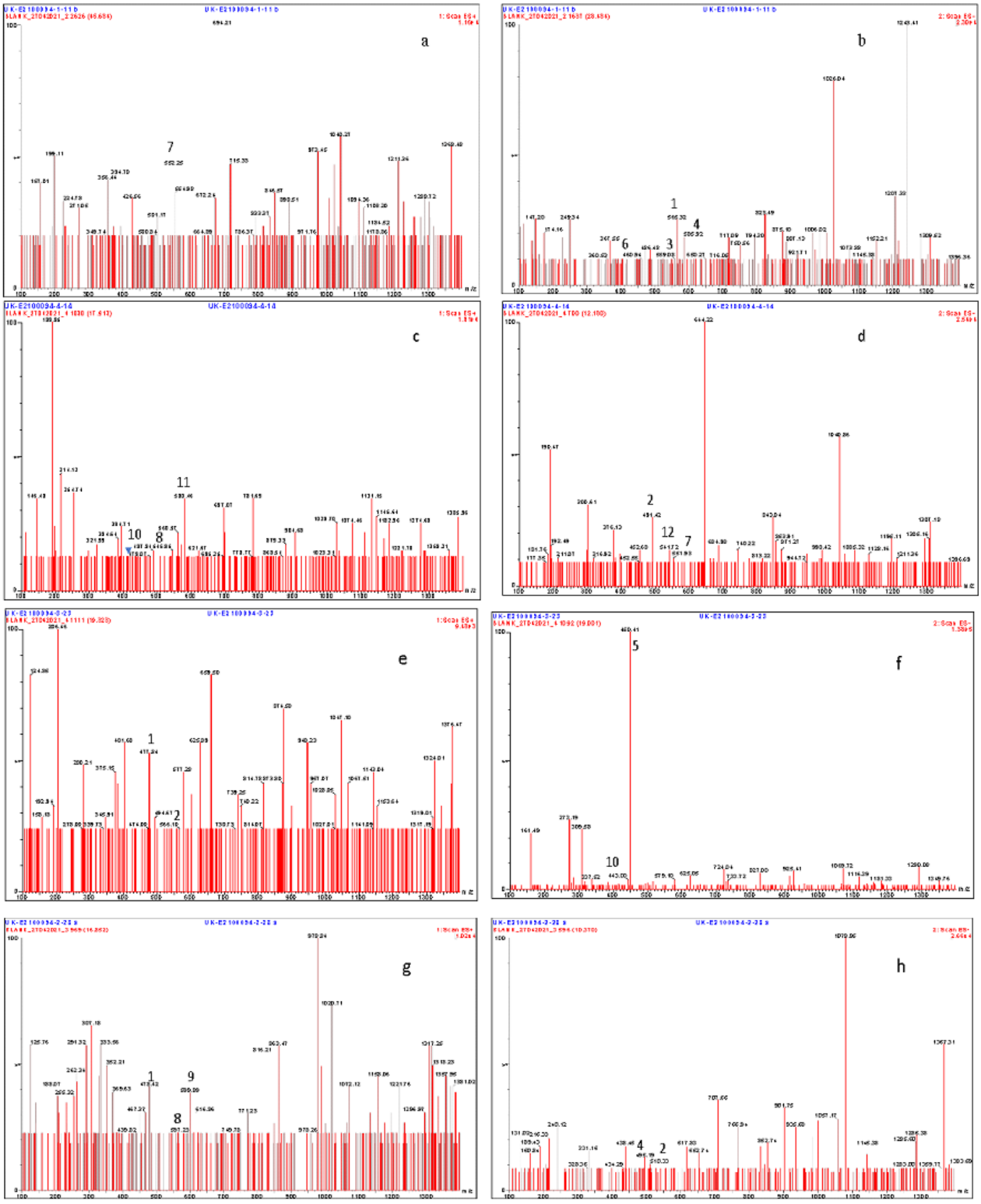

Figure 3

LC-MS/MS profiles of selected halophilic strains. The left side shows a positive ion chromatogram and to the right is a negative ion chromatogram. (a) & (b), (c) & (d), (e) & (f), (g) & (h) belong to the mass spectra of AD11, AD14, AD23 and AD28 respectively. The numerical values represent the following antioxidant compounds: 1- Zeaxanthin, 2-Neoxanthin, 3- Canthaxanthin, 4- Lutein, 5- Beta-Carotene, 6-Epoxide, 7-Cryptoxanthin, 8-Violaxanthin, 9- Astaxanthin, 10- Alpha-Carotene, 11- Antheraxanthin, and 12- E-Carotene

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table3ListofcompoundsidentifiedfromthesamplesusingGC.docx
- Supplementaryfile.docx