An Acad Bras Cienc (2022) 94(Suppl. 4): e20211585 DOI 10.1590/0001-3765202220211585
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Crosstalk between biological and chemical diversity with cytotoxic and cytostatic effects of *Aphanothece halophytica in vitro*

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Abstract: Different solvent extracts from *Aphanothece halophytica (A. halophytica)* were evaluated for their cytotoxic effects against four human cancer cell lines. The samples demonstrated different percentages of cyanobacteria species populations. The samples containing 100% *A. halophytica* and 90% *A. halophytica* showed a significant cytotoxic effect in human breast cancer cells MDA231. The cytostatic effect was demonstrated in MDA231 and human glioblastoma T98G cells regardless of the treatment, resulting in a significant cell cycle arrest in the S phase. The chemical profiles of the extracts were proven to be diverse in qualitative and quantitative compositions. This variability was dependent on the *A. halophytica´s* abundance in each extract. The 100% *A. halophytica* extract induced cytotoxic and cytostatic effects in breast cancer cells, and those could be associated with the predominance of fatty acids, hydrocarbons and phthalates, indicating that *A. halophytica* is an interesting source of novel compound with anticancer effect.

Key words: *Aphanothece halophytica*, biodiversity, secondary metabolites, cytotoxic, cytostatic.

INTRODUCTION

Over the past decades, the use of natural products for chemoprevention and therapy has gained great importance (Cuzick 2017, Goyal et al. 2017). Studies have demonstrated that pharmacological active marine-derived compounds have potent biological activity with little or no side effects (Sawadogo et al. 2015, Castro-Carvalho et al. 2017). Also, the significance of cyanobacteria in drug discovery has increased over the past two decades (Bérdy 2013).

Cyanobacteria are prokaryotic organisms found in a wide range of ecosystems of terrestrial and aquatic environments, including extreme systems (Madigan et al. 2010). This high ability to inhabit different ecosystems has given these organisms a strong evolutionary advantage such as the ability to produce secondary metabolites, many of them with unique structures. These compounds have different ecological functions acting as chemical defenses as well as signals or cues in organism interactions (Pereira & da Gama 2008, Hay 2009). Their production is influenced by different factors such as biotic and abiotic factors (Pereira et al. 2004, Sudatti et al. 2011, Cahill et al. 2019/3). The high chemical diversity makes these organisms one of the most interesting phylums to produce biologically active compounds. They are targets of research in the biomedical area, and their potential as antibacterial, antifungal, antiprotozoal, anti-inflammatory and mainly anticancer has also

been shown (Rastogi & Sinha 2009, Singh et al. 2011, Dixit & Suseela 2013, Singh et al. 2016).

According to the World Health Organization, cancer is the second leading cause of death. In 2018, 18.1 million new cases were estimated. For 2040, 29.5 million new cases are estimated (WHO 2018, IARC 2020). In Brazil, cancer is the second leading cause of death, and for the 2020-2022 biennium, 680,000 new cases are estimated (INCA 2020). There are several treatment strategies, but the drugs present cytotoxicity, as well as many adverse effects. Also, some cancers are resistant, and the cure rates are still unsatisfactory, which highlights the importance of the development of drugs (DeVita & Chu 2008).

Aphanothece halophytica (A. halophytica) is a halotolerant cyanobacteria found in a wide range of salinity from 0.25 to 3.0 M NaCl and in extreme alkaline conditions up to an external pH of 11.0 due to the production of osmoprotective molecules such as trehalose, glycine betaine, glycerol and regulation of Na^+/H^+ channels (Hibino et al. 1999, Waditee et al. 2003, Laloknam et al. 2006). These cyanobacterial species have the ability to survive in conditions of intense light, limited O_2 and CO_2 diffusion, low nutritional concentration, long periods of desiccation, temperature, salinity and high exopolysaccharide production (Badger et al. 2006). Polysaccharides, fatty acids, sterols, alkaloids, and phthalates have been described for the Aphanothece species, as well as the biological activity of some of them (Zheng et al. 2006, Vishwakarma 2013, Du et al. 2019). For example, *A. halophytica* extracts showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Vishwakarma 2013). Also, the exopolysaccharides of the *A. halophytica* showed anti-viral and anti-cancer effects inducing apoptosis in HeLa cells and a protective and therapeutic effect in rats with H1N1-induced pneumonia (Zheng et al. 2006, Ou et al. 2014). There are rare studies about the biotechnological potential of the chemical compounds of the halophilic cyanobacteria *Aphanothece halophytica* as strategies for cancer treatment.

To evaluate the antiproliferative potential of *A. halophytica* metabolites, extracts from the same population of this organism collected at different months of the year were evaluated against different human cancer cell lines *in vitro*.

MATERIALS AND METHODS

Cyanobacterial samples

The samples of cyanobacteria were collected manually at a depth of 5 cm with a sieve in the crystallizer reservoir of an active salt processing company, located in the city of Cabo Frio-RJ (22°52'31.2"S, 42º04'20.2"W). Three samplings were performed in 2019's fall, a period of higher biomass occurrence, on March, April and May. The biological material was analyzed by brightfield microscopy and identified according to Komarek and Anagnostidis (Anagnostidis & Komárek 1988). Two different species were identified: *A. halophytica* and *A clathrata*, and their abundance in each sample varied depending on the month: March (90% *A. halophytica* and 10% *A. clathrata*), April (100% *A. halophytica*) and May (85% *A. halophytica* and 15% *A. clathrata*).

Extract preparation

Samples were lyophilized and extracted with a mixture of ethyl acetate: methanol (1:1) for 30 minutes in an ultrasound bath, followed by static extraction for 2 hours at room temperature. All materials were filtered, the solids were retained in a paper filter and the filtrate was separated. The solids were extracted again using the proportion of the same solvent and

the same procedures were repeated twice. The filtrates were combined, and the solvents were evaporated using a rotary evaporator under reduced pressure, keeping the bath temperature at 45 °C (Seddeck et al. 2019). The extracts were then kept at -20°C. For cytotoxic and cytostatic assays, samples were defrosted and sonicated for 5 min at room temperature, then filtered (0.22µm) for sterilization.

Cell culture

Human cancer cell lines, T98G (glioblastoma), MDA231 (breast), A549 (lung), K562 (leukemia) and a healthy human fibroblast (BJ-5ta) were cultured with Dulbecco's Modified Eagle Medium, Fetal Bovine serum 10% and penicillin-streptomycin (100 units/mL) as American Type Culture Collection instructions. Media were monitored daily and replaced with fresh media at least two times per week. After washing cells in the flask with PBS (pH 7.0), cells were harvested with 0.05% trypsin for 4 min at 37°C. Trypsin was then neutralized with a complete growth medium and the cell suspension was centrifuged at 400×g for 5 min. After the removal of the supernatant, the cell pellet was resuspended in a complete growth medium and the cell density was calculated using the Neubauer chamber.

Cytotoxicity assay

The T98G (glioblastoma), A549 (lung cancer), MDA231 (breast cancer), K562 (leukemia) and BJ-5ta (fibroblast) (*American Type Culture Collection*) cells (15,625 cells/cm^2) were cultured with increasing concentrations of all three cyanobacterial samples (62.5, 125, 250, 500 and 1,000µg/mL). A stock solution of all extracts previously dissolved in dimethyl sulfoxide (DMSO) was used to obtain a final DMSO concentration of 0.5% in each sample. After 72h, the spectrophotometric mitochondrial activity technique was performed by converting the MTT salt (5mg/mL) to formazan crystal (Mosmann 1983). After the dissolution of these crystals by DMSO, the optical density was recorded at 570nm (Beckman Coulter). The activity was converted to a percentage, considering the optical density of the control cells (with 0.5 % DMSO) as 100%.

Iodide propidium staining

The T98G, A549, MDA231 and K562 cells (15,625 cells/cm^2) were cultured with the highest concentration (1,000µg/mL) of cyanobacterial samples for 72 hours and then fixed with a citrate buffer (10mM). Afterwards, the propidium iodide (1µg/mL) and RNAse (20µg/mL) were added for 1 hour to DNA intercalating. The fluorescence (FL-3) was analyzed by flow cytometry (Cyan ADP, Becton & Dickinson) (Honorato et al. 2020). At least 10,000 events were acquired and the percentage of cells in each phase of the cell cycle (G0/G1, S and G2/M) was observed and fragmented DNA (subG0).

GC-MS chemical profiles

All cyanobacterial lipophilic extracts were analyzed with Gas Chromatography coupled to Mass Spectrometry (GC-MS), and their chemical profiles were obtained. Before use, the extracts were solubilized in dichloromethane (HPLC grade, Tedia), filtered in a 0.45-µm PTFE syringe filter (pore width of 0.45µm; diameter of 15mm; Millipore, USA) to remove any insoluble constituents. The solvent was evaporated, and the samples were subsequently lyophilized overnight to eliminate humidity. The remaining material was resuspended in ethyl acetate (HPLC grade, Tedia) to a final concentration of 1 mg/mL, and then injected. All samples were analyzed in duplicate. GC profiles of *A. halophytica* samples were obtained on a GC-2010 Shimadzu coupled to a QP-2010 ULTRA mass spectrometer with an AOC 20i autosampler using an RTX-1 capillary

column (30m × 0.25mm; film thickness 0.25µm; Restek) and equipped with a flame ionization detector (FID). The method used had an injection flow of 1.20 mL/min in split mode, with a ratio of 1:5. Helium gas was used as the carrier gas. The injector temperature was 280°C. The column was programmed to remain at 40°C for 1 minute and heated at 150°C for 3 minutes, followed by a temperature ramp-up to 300°C at a rate of 8°C/min. The detection was performed in the full-scan mode, using a mass range of 60–450 *m/z*. The comparison of the chemical profiles of all samples was performed based on mass spectra data and retention time. The compounds were identified by comparing the mass spectra of each substance with those available in the NIST05 Mass Spectral and Wiley Registry of Mass Spectral Data (when showing similarity index higher than 85%), and with MS spectra and MS fragmentation pattern published in the literature, generating Table II.

Statistical analysis

Statistical analysis was performed using the *GraphPad Prism 6.0* software (Windows). Data were evaluated for normality tests such as Kolmogorov-Smirnov and Shapiro-Wilk. Hence, the two-way ANOVA with Dunnett's post-test parametric test was used. All the results were representative of three independent experiments represented by the mean and standard deviation (SD), which is considered as significant when $P < 0.05$.

RESULTS

Algal taxonomy

The cyanobacteria population in the controlled water reservoir was variable at the time and dominated by the species *A. halophytica*. A small amount of *A. clathrata* was observed in two samples. The brightfield microscopical analysis of the biological sample collected in March 2019 was composed of 12.70 x 10^6 cells/mL of *A. halophytica* and 1.27 x 10^6 cells/mL of *A. clathrata* (90% *A. halophytica*). The sample from April 2019 was composed exclusively (13.87 x 10^6 cells/mL) of *A. halophytica* (100% *A. halophytica*), the sample from May 2019 presented 28.75 x 10^6 cells/mL of *A. halophytica* and 4.31 x 10^6 cells/mL of *A. clathrata* (85% *A. halophytica*).

Cytotoxic potential of *A. halophytica* extracts

The MTT colorimetric assay demonstrated that the treatments in each human cell line resulted in different responses (Figure 1). 100% *A. halophytica* and 90% *A. halophytica* samples showed a significant mitochondrial activity reduction specifically in MDA231 cells with IC_{50}= 597.9 µg/mL and IC_{50}= 690.8µg/mL, respectively (Figure 1b) (Table I). The 85% *A. halophytica* sample showed no effect in mitochondrial activity in any cancer cell type. The three samples showed no effect in the human fibroblast cells (healthy cells) (data not shown).

The DNA fragmentation analysis showed a significant percentage of cell death in the MDA231 and K562 cells (Figure 2). MDA231 cells treated with the 85% *A. halophytica* sample demonstrated a significant percentage of DNA fragmentation when cultured with the highest concentration (1.000 µg/mL) (14.86 ± 2.65%) (Figure 2b). K562 cells treated with the 100% *A. halophytica* sample presented 27.75± 11.22% cells with fragmented DNA (Figure 2d). The 90% *A. halophytica* sample did not show any significant DNA fragmentation in any cancer cells. The T98G and A549 cells demonstrated DNA fragmentation lower than 10% of the population (Figure 2a, c).

Cyanobacterial extracts induced cell cycle arrest regardless of biodiversity

In the cell cycle evaluation, cyanobacterial extracts showed significant changes in the

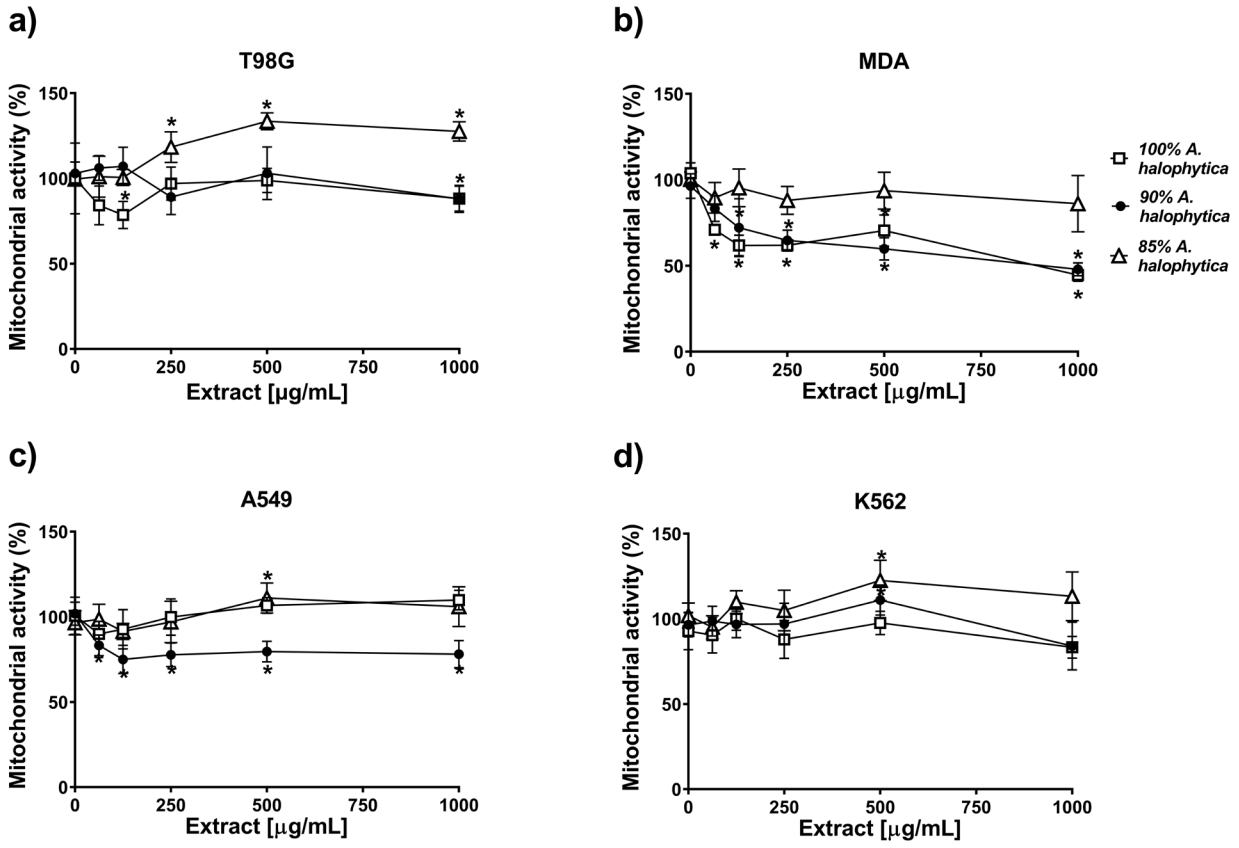

Figure 1. *In vitro* evaluation of the cytotoxic activity of *Aphanothece halophytica* extracts. Viability of human T98G(a), MDA231(b), A549(c), K562(d) cells cultured with crude cyanobacterial extracts after 72 hours by the MTT method. Representative result of 3 independent experiments performed in eight replicates, being the mean ± standard deviation. Two-way ANOVA with Dunnett's post-test relative to control; * P <0.05.

Table I. Inhibitory Concentration from the cyanobacterial extracts.

	100% *A. halophytica*	90% *A. halophytica*	85% *A. halophytica*
Cells Line	IC_{50} (µg/mL)		
T98G	-	-	-
MDA231	597.9	690.8	-
A549	-	-	-
K562	-	-	-

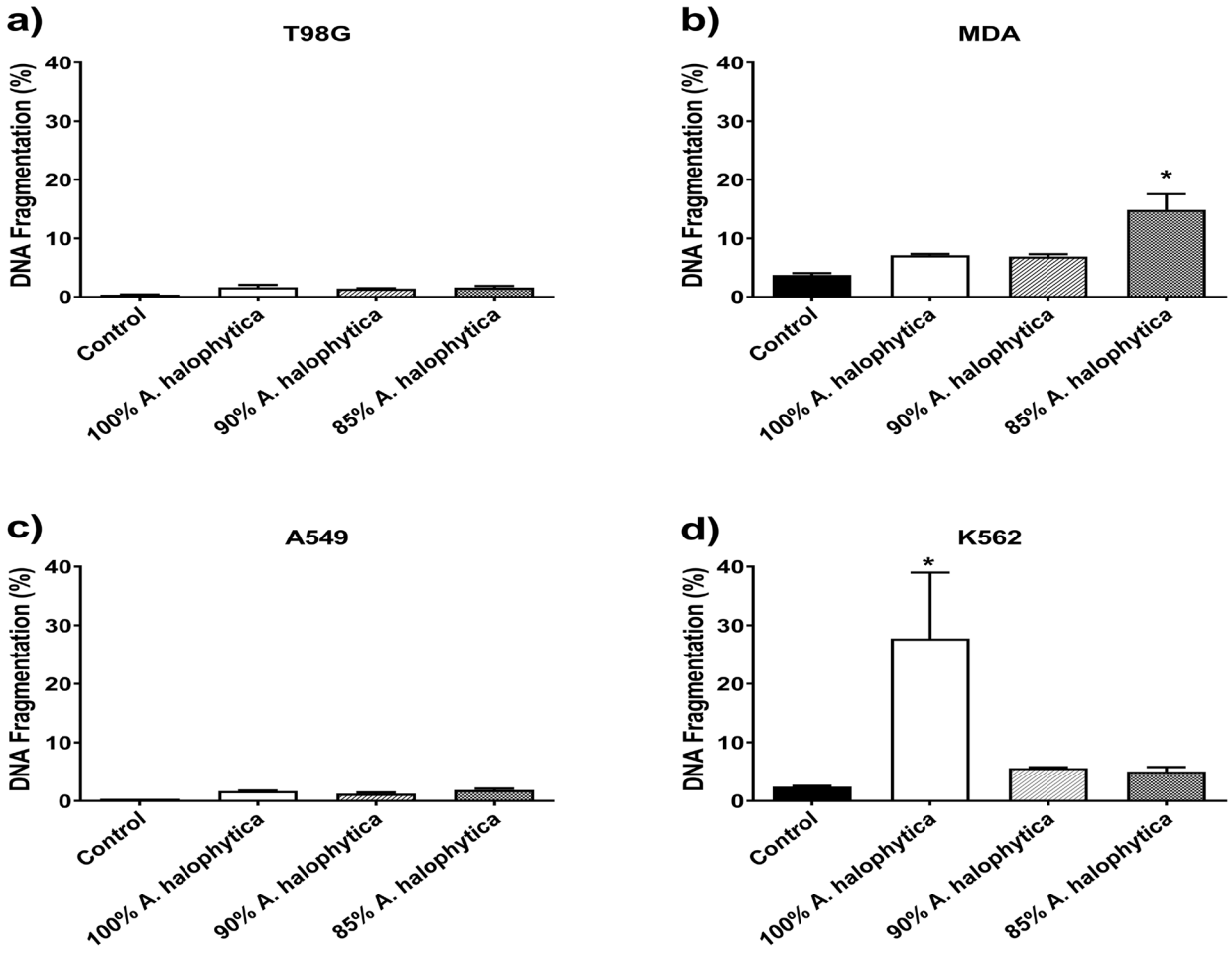

Figure 2. DNA fragmentation induced by *Aphanothece halophytica* extract. T98G (a), MDA231(b), A549(c) and K562(d) cells cultured with extracts of cyanobacteria after 72 hours with their highest concentration [1000µg/mL]. Representative result of flow cytometry experiment considering a minimum of 10,000 events, being the mean ± standard deviation. Two-way ANOVA with Dunnett's post-test relative to control; * P <0.05.

G0/G1 and S phases distribution of the T98 cells and all cell cycle phases distribution in MDA231 cells (Figure 3a, b). After incubation with 1,000µg/mL, the proportion of G0/G1 phase in T98G cells significantly decreased to 58.67 ± 0.87%, 59.83 ± 1.17% and 53.31 ± 3.50% with the *100% A. halophytica, 90% A. halophytica* and *85% A. halophytica* extracts, respectively, when compared to the control group with 71.15 ± 0.05% cells. That corroborated the increase in proportions of cells in S phase as 27.33 ± 0.23%, 28.71 ± 1.11% and 30.67 ± 2.09% in the 100% *A. halophytica,* 90% *A. halophytica* and 85% *A. halophytica* extracts respectively, when compared to the control group with 17.57 ± 0.16% cells (Figure 3a). Moreover, the percentages of MDA231 cells in the G0/G1 phase were significantly lower than the control, being 53.63 ± 1.25%, 57.80 ± 0.75% and 54.03 ± 0.29% in the *100% A. halophytica, 90% A. halophytica* and *85% A. halophytica* extracts respectively, and 66.21 ±1.82% cells in the control. The MDA231 percentage of cells in the S phase was significantly higher, being 23.11 ± 0.34%, 23.85 ± 1.34% and 23.15 ± 2.32% in the 100% *A. halophytica,* 90% *A. halophytica* and 85% *A. halophytica* extracts respectively,

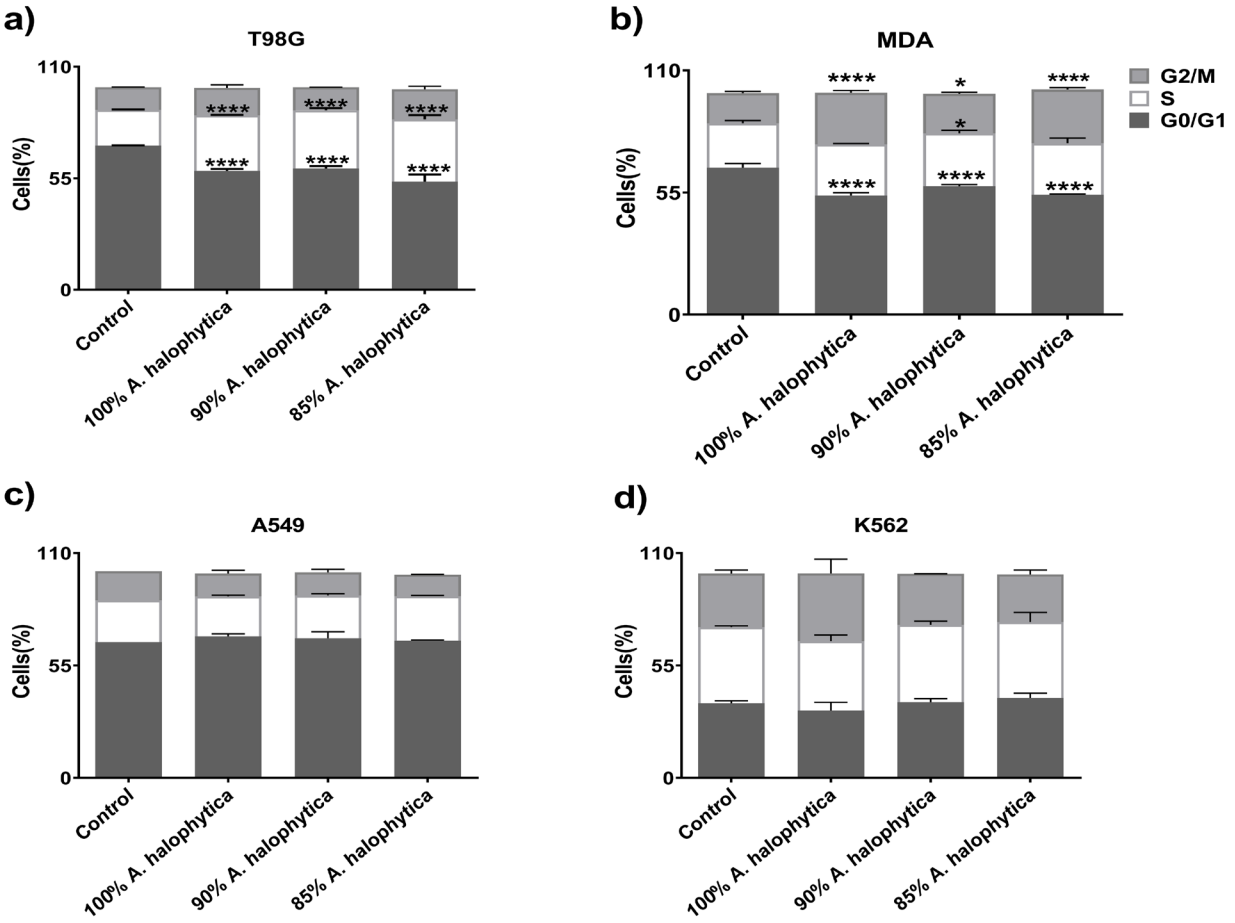

Figure 3. ***Aphanothece halophytica*** **extracts induced cell cycle arrest. T98G (a), MDA231(b), A549(c) and K562(d) cells cultured with cyanobacterial extracts after 72 hours with their highest concentration [1000µg/mL]. Representative result of flow cytometry experiment considering a minimum of 10,000 events, being the mean ± standard deviation. Two-way ANOVA with Dunnett's post-test relative to control; * P <0.05, **** P <0.0001.**

compared to the control group 19.98 ± 1.25% cells. Also, the three cyanobacterial extracts showed a significant change in the proportion of MDA231 cells in the G2/M phase, demonstrating 23.22 ± 0.96%, 17.83 ± 0.65% and 24.23 ± 0.79% in the treatment of 100% *A. halophytica,* 90% *A. halophytica,* and 85% *A. halophytica* samples, respectively, compared to 13.59 ± 0.76% in the control (Figure 3b). Meanwhile, the A549 and K562 cells did not show any significant changes in the cell cycle phase distribution (Figure 3c, d).

GC-MS chemical profiles

The chemical composition of each cyanobacterial extract was variable depending on the period of collection and on the abundance of *A. halophytica* and *A. clathrata* in the extracts. The GC-MS chromatograms showed differences in the qualitative and quantitative analysis of compounds in all three samples (Figure 4). In total, 29 major substances were annotated in the soluble dichloromethane fraction of all cyanobacterial extracts. Among them, nine compounds, with the same retention time and mass spectra, were observed (Table II) in all three samples: 3,7,11,15-Tetramethyl-2-hexadecen-1-ol

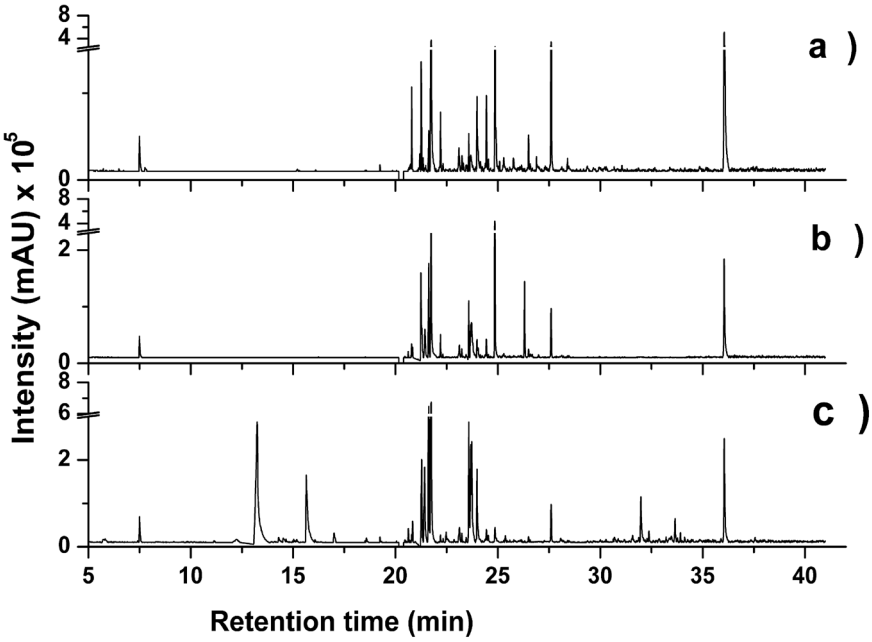

Figure 4. *Chemical profiles of extracts of cyanobacteria obtained through GC-MS. (a) 100% of A. halophytica, (b) 90% of A. halophytica and (c) 85% of A. Halophytica.*

(4), Octathiocane (13), Hexadecanoic acid (14), 9,12-Octadecadienoic acid, methyl ester (17), Stearic acid (20), (*Z*)-Tricos-9-ene (21), Diisooctyl phthalate (26) and two unknown compounds with t_R 7.48 min (1) and t_R 36.04 min (29). On the other hand, five substances (8, 11, 16, 23 and 25) were only observed for the 100% *A. halophytica* sample. Whereas only two substances 22 and 24 were observed in the 90% *A. halophytica*, being the tributyl acetylcitrate (22), the major substance in the sample. Finally, five substances (2, 3, 5, 27 and 28) were only observed in the 85% *A. halophytica* sample. The classes of metabolite compounds identified in this work belong to fatty acids and their derivatives (40%), hydrocarbons (13%), oxygenated compounds (10%), phthalates (3%), alkaloids (3%), chlorinated derivatives (3%) and sulfur compound derivatives (3%). In all samples, fatty acids and their derivatives, oxygenated compounds such as alcohols and aldehydes, as well as hydrocarbons, were the most abundant classes of compounds. Hexadecanoic acid (14) and its derivative (9) were annotated as the major compounds in all samples. Octathiocane (13), a sulfur compound, was observed in a high amount in all extracts, and Chlorobenzoic acid was the major compound in the 85% *A. halophytica* sample (Table II).

DISCUSSION AND CONCLUSION

In this study, the anticancer potential of three cyanobacterial extracts against different human cancer cell lines was demonstrated and it was correlated with the chemical composition of each sample. These extracts demonstrated a different composition of cyanobacterial species being *A. halophytica* and *A. clathrata* in different proportions. All three extracts were tested *in vitro* for cytotoxicity against different human cancer cell lines. The variability of species composition indicated that the homogenate of 100% of *A. halophytica* had the most significant effects in a wider range of cancer cell types than other sample mixtures, an effect that could be due to its chemical composition and abundance.

The extracts were tested *in vitro* for the cytotoxicity on four human cancer cell lines, and the extracts with a percentage of *A. halophytica* equal or higher than 90% had a cytotoxic effect

Table II. CG-MS chemical characteristics of secondary metabolites in the cyanobacterial extracts.

No.	t_R (min)	Annotated compounds	Main ions fragments (*m/z*)	Relative abundance in *A. halophytica* samples		
				100%	90%	85%
1	7.48	NI		**1.86**	**2.02**	**1.42**
2	13.18	4-Chlorobenzoic acid	$M^{+\cdot}$ (^{37}Cl/ ^{35}Cl) 158/ 156; 141/139; 113/111	-	-	17.22
3	15.68	NI		-	-	5.42
4	**20.32**	**3,7,11,15-Tetramethyl-2-hexadecen-1-ol**	**123; 95; 81; 68**	**0.62**	**3.03**	**2.77**
5	20.62	Citronellyl isobutyrate	123; 109; 95; 81	-	-	0.45
6	20.78	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	261; 205; 189; 57	2.65	0.80	-
7	20.83	3,7-Dimethyloct-6-enyl propanoate	123; 95; 81	-	0.71	0.68
8	21.18	NI		0.64	-	-
9	21.25	Hexadecanoic acid, methyl ester	$M^{+\cdot}$ 270; 239; 227; 74	4.25	7.18	-
10	21.29	9,12-Octadecadien-1-ol (Z,Z)	252; 95; 81; 67	-	3.20	4.77
11	21.33	NI		0.62	-	-
12	21.43	(*Z*)-Octadec-9-enoic acid	236; 97; 83; 56	-	4.54	5.69
13	**21.61**	**Octathiocane**	**$M^{+\cdot}$256; 224; 192; 64**	**1.96**	**8.73**	**12.16**
14	**21.73**	**Hexadecanoic acid**	**$M^{+\cdot}$ 256; 129; 73**	**19.58**	**13.98**	**16.70**
15	22.19	(*E*)-Icos-3-ene	125; 111; 83; 55	2.38	1.56	-
16	23.09	(*E*)-Icos-9-ene	97; 83; 55	1.31	-	-
17	**23.57**	**9,12-Octadecadienoic acid, methyl ester**	**280; 95; 81; 67**	**1.48**	**5.08**	**5.82**
18	23.66	(*Z*)-Octadec-6-enoic acid	264; 97; 83; 55	-	2.94	4.97
19	23.72	Oleic acid	264; 97; 83	-	2.28	6.39
20	**23.97**	**Stearic acid**	**$M^{+\cdot}$284; 241; 185**	**3.82**	**1.22**	**4.14**
21	**24.43**	**(*Z*)-Tricos-9-ene**	**139; 125; 111; 97**	**3.01**	**1.25**	**0.37**
22	24.81	Tributyl acetylcitrate	329; 185; 129	-	19.88	-
23	24.85	Isoamyl laurate		11.66	-	-
24	26.29	bis(2-ethylhexyl) hexanedioate	259; 241; 129	-	6.39	-
25	26.49	1-Nonadecene	125; 111; 97; 83: 57	1.43	-	-
26	**27.59**	**Diisooctyl phthalate**	**279; 167; 149**	**13.09**	**4.02**	**1.52**
27	31.99	3-(4-hydroxyphenyl)-2-phenyl-6,7-dihydro-5*H*-indazol-4-one	$M^{+\cdot}$ 304; 276; 247	-	-	2.59
28	33.65	NI		-	-	0.98
29	**36.04**	**NI**		28.21	11.19	5.94

[NI]-Not Identified.

on the MDA231 cancer cells. The cyanobacterial extracts had an selective cytotoxicity, since when the IHF cells were exposed to the same treatment conditions as the tumor cell lines, they did not had a reduction in cell viability, which indicates that the cyanobacterial extracts weren't toxic to the healthy cell.

The 100% *A. halophytica* and the 85% *A. halophytica* caused a significant DNA fragmentation in the K562 and MDA231 cancer

cells lines respectively; by necrotic events, as it was observed by the presence of the PI staining the cells. These results suggest that the decrease in the cell viability observed in the MTT assay caused by the sample 100% *A. halophytica* and the 90% *A. halophytica* in the MDA231 cancer cells wasn't because of loss of the cell membrane integrity but by another mechanism, maybe inactivating some enzyme, as was observed in a research made by Weyermann et al. 2005.

The cell cycle arrest mechanism was already described for several cyanobacterial compounds, and those were all associated with cell accumulation in the G1 phase in the human cervical cancer cell (HeLa cell line), increase in the number of cells in the G1 phase with little change in G2/M in breast cancer cells (MDA-MB-435 cell line) and accumulation in S and G2/M phase in leukemia cells (CEM cell line) (Ma et al. 2006, Medina et al. 2008, Khan et al. 2009). In our study we observed that the treatment with the cyanobacterial extracts led to an alteration in the percentage distribution of the cell cycle phases in the T98G and MDA231 cancer cell lines. The extracts led to a reduction in the number of cells in the G0/G1 phase and an increase in the number of cells in the S phase in these two cell lines, but the 90% *A. halophytica* extract also led to a significantly increase in the number of cells in the G2/M phase in the MDA231 cancer cell line.

Approximately 30 % of cyanobacterial extracts have been reported to cause damage to mammal cells in vitro (Surakka et al. 2005). These damages can be caused due to the presence of specific secondary metabolites or synergic effects of compounds that affect the cell metabolism.

The chemical composition of the dichloromethane fractions of cyanobacteria extracts was investigated by GC-MS analysis, and compounds belonging to different classes of natural products were annotated. The samples were characterized by fatty acids and their derivatives, hydrocarbons, alkaloids, oxygenated compounds, as alcohols and aldehydes, in addition to chlorinated and sulfur compounds.

Fatty acids and their derivatives are one of the most studied compounds in cyanobacteria. Here, they were the most representative group of compounds in all extracts, and some of them were identified in *A. halophytica* as Hexadecanoic acid, methyl ester (9), Hexadecenoic acid (14) and Stearic acid (20) (Jones & Yopp 1979, Catarina Guedes et al. 2011), and its abundance seems to be influenced by abiotic factors as salinity (Oren et al. 1985). Some compounds of this chemical class had demonstrated anticancer activities as antiproliferative and pro-apoptotic effects in A549, HeLa, PC3, MCF-7 and MDA-MB-231 cancer cells (Bonesi et al. 2018), anticancer potential in both prostate (Gu et al. 2013) and colorectal cells (Song et al. 2014).

Hydrocarbons had also already been identified in cyanobacteria; their compounds, such as heptadecane, have been observed as major components in different species (Tsuchiya et al. 1981, Ozdemir et al. 2004, Khairy & El-Kassas 2010). This class of compounds was the second most common in the cyanobacteria extracts, with the sample containing only *A. halophytica* with the highest number of hydrocarbons (4 compounds).

Phthalates esters, another group of metabolites identified in the extracts, displayed an inhibitory effect on the ATPase domain of human topoisomerase IIa on hepatocellular carcinoma HepG2 cells (Selvakumar et al. 2019). In recent years, these compounds have been identified as natural compounds in different marine and aquatic organisms, as cyanobacteria (Namikoshi et al. 2006, Babu & Wu 2010). The amount of each compound was dependent on the abundance of cyanobacteria in the

extracts. Diisooctyl phthalate (26) was detected in all extracts, but in higher concentration only in the 100% *A. halophytica* sample and *A. clathrata* absent. Whereas Tributyl acetylcitrate (22), a compound widely used as a phthalate substitute plasticizer, was observed only in the sample containing 90% of *A. halophytica*, being the major compound in the extract.

Some oxygenated compounds have been described in the literature with anticancer effect in breast cancer cells (MCF-7 cell line) acting in the free radical scavenging (Jaikumar et al. 2016). These compounds inhibited the proliferation of myeloma cells (RPMI-8266 cells) inducing apoptosis (Park et al. 2014) and had cytotoxic effect on lung adenocarcinoma cells (A549 cell line) through necroptosis via caspase-3 (Sansone et al. 2014).

Alkaloids chlorinated and sulfur compounds were also identified in the extracts, and they were dependent on the abundance of species. The alkaloids have a great anticancer potential in the prostate (PC-3 cell line), colon (HT-29 cell line) and breast cancer cells (MCF-7), inducing G2/M cell cycle arrest, apoptosis, and autophagy inhibition (Kim et al. 2012, Dyshlovoy et al. 2018). The sulfur compound identified in all extracts was octathiocane (13) and the concentration of this compound proportionally increased with the abundance of *A. clathrata*. This chemical class is found in some freshwater cyanobacteria species such as *Leptolyngbya* sp. (Hamilton et al. 2018). *Synechocystis* sp. has a sulfur mobilization which is one of the key steps in ubiquitous Fe-S clusters and the modification and synthesis of some biomolecules. The sulfur mobilization stands out as a mechanism that allows cyanobacteria to adapt to different environmental conditions (Campanini et al. 2006). Chlorinated compounds are described in cyanobacteria, as the hectochlorin in *Lyngbya majuscula* (Marquez et al. 2002) and the carbamide cyclophane from the cyanobacteria *Nostoc* sp that had anticancer activity in breast adenocarcinoma cells (MCF-7 cell line) (Bui et al. 2007).

The temporal variability of the abundance of cyanobacteria species in the water hypersaline reservoir, containing *A. halophytica* and *A. clathrata* in different proportions, was also observed in the chemical composition of all samples. Some compounds were identified only in one of the samples and others in all of them, but the amount varied between the samples. The 100% *A. halophytica* sample had more significant results in cancer cells than the other two samples that had a mixture of *A. halophytica* and *A.clathrata,* suggesting that the diversity could lead to a loss in the anticancer effect. The biological and chemical diversity observed could stem from biotic, abiotic and also by the interaction between these two factors (Sudatti et al. 2011). Environmental factors such as salinity, pH and temperature play a significant role in the distribution of species and can modulate the levels of metabolites (Çelekli et al. 2014). Another observation was that a possible competition between the two species of *Aphanothece* inhibits or reduced the production of the compounds 6, 8, 11, 15, 16, 23, 25, 26 and 29; some studies indicate that the competitiveness and species complementarity could be crucial factors for biomass and lipid productivity (Omirou et al. 2018).

In conclusion, the results showed a seasonal variation among the species of cyanobacteria in the crystallizer reservoir, and this biological variation led to a chemical variation among the metabolites produced by the cyanobacteria species. The cyanobacterium extract from *A. halophytica* induced a selectively antiproliferative effect. The chemical profiles and the biological assays inferred that the *A. halophytica* extract is a promising source

to further investigation to identify the exact compound with antiproliferative effect.

Acknowledgments

The authors report no conflict of interest. We appreciate the financial support provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Brazilian Ministry of Health.

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LIST OF ABBREVIATION

µg	microgram
A clathrata	*Aphanothece clathrata*
A. halophytic	*Aphanothece halophytica*
cm^2	square centimetre
CO_2	Carbon Dioxide
DMSO	Dymethyl sulfoxide
DNA	Deoxyribonucleic acid
GC-MS	Gas Chromatography Coupled to Mass Spectrometry
H^+	Hydrogen
IC50	Inhibitory Concentration 50%
mg	milligram
min	Minutes
mL	Millilitres
MS	Mass Spectral
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na^+	Sodium
NaCl	Sodium Chloride
O_2	Oxygen
PBS	Phosphate Buffer Solution
pH	Potential of Hydrogen
RNAse	Ribonuclease
SD	Standard deviation
t_R	Retention time

How to cite
SILVA IVG, SILVA KL, MAIA RC, DUARTE HM, COUTINHO R, NEVES NHCB, SOARES AR & LOPES GPF. 2022. Crosstalk between biological and chemical diversity with cytotoxic and cytostatic effects of *Aphanothece halophytica in vitro*. An Acad Bras Cienc 94: e20211585. DOI 10.1590/0001-3765202220211585.

Manuscript received on December 15, 2021; accepted for publication on April 04, 2022

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