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Alternatives to classic solvents for the isolation of bioactive compounds from *Chrysochromulina rotalis*

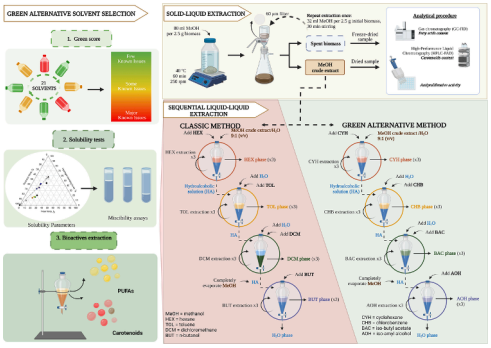
M.A. González-Cardoso [a], M.C. Cerón-García [a,b,*], E. Navarro-López [a,b], A. Molina-Miras [a,b], A. Sánchez-Mirón [a,b], A. Contreras-Gómez [a,b], F. García-Camacho [a,b]

[a] Department of Chemical Engineering, University of Almería, Almería 04120, Spain
[b] Research Center in Agrifood Biotechnology (CIAMBITAL) University of Almería, Spain

HIGHLIGHTS

- Downstream for first time to isolate bioactives from *Chrysocromulina rotalis*.
- Replacing classic hazardous solvents with other safer alternatives.
- Antiproliferative potential containing fucoxanthin and fatty acids among others.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper demonstrates a sequential partitioning method for isolating bioactive compounds from *Chrysochromulina rotalis* using a polarity gradient, replacing classic and hazardous solvents with greener alternatives. Seventeen solvents were evaluated based on their Hansen solubility parameters and for having a similar polarity to the solvents they would replace, four of which were selected as substitutes in the classic fractionation process. Considering the fatty acid and carotenoid recovery yields obtained for each of the solvents, it has been proposed to replace hexane (HEX), toluene (TOL), dichloromethane (DCM) and *n*-butanol (BUT) with cyclohexane, chlorobenzene, isobutyl acetate and isoamyl alcohol, respectively. In addition, cytotoxic activity was observed when the TOL and DCM solvent extracts were tested against tumour cell lines, demonstrating the antiproliferative potential of compounds containing, for example, fucoxanthin, fatty acids, peptides, isoflavonoids or terpenes, among others.

* Corresponding author at: Department of Chemical Engineering, University of Almería. Carretera Sacramento s/n. E04120, Almería, Spain.
E-mail address: mcceron@ual.es (M.C. Cerón-García).

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1. Introduction

The microalgae sector has great potential to further the blue bioeconomy and achieve the objectives of the European Green Deal. With well-designed bioprocesses, the microalgae are "regenerative" by nature in their use of photosynthesis to transform solar energy and nutrients into plant biomass. Microalgae are a source of various products such as food, food additives, nutraceuticals, pharmaceuticals, cosmetics, and materials. Marine microalgae are a potential resource for anticancer drugs and have an enormous exploitation potential (Martínez et al., 2022).

Although many innovative methodologies have been developed to efficiently extract metabolites from microalgae biomass (Saini & Keum, 2018; Soštarič et al., 2012), some strains do not need pretreatment to extract the metabolites; this is why organic solvents have traditionally been used in microalgae biotechnology for such a purpose (Cerón-García et al., 2018b; Hladnik et al., 2022; Ramluckan et al., 2014 and Ventura et al., 2017). Because solid–liquid extraction (SLE) is the most efficient and sustainable approach to extract lipids from microalgae biomass, and to apply less toxic and more environmentally friendly solvents such as ethanol, ethyl acetate and water (among others), it has already been successfully applied for their recovery. However, selection is one of the most critical factors for efficient extraction since it is the polarity of the compound to be extracted from the biomass that must be considered. However, to isolate different target compounds, it is necessary to use more than one extraction or fractionation process with solvents of different polarity and solubility parameters, which allows selective separation of each compound. In this regard, a solvent-partitioning process was for the first time successfully applied in a systematic way to crude extracts from marine dinoflagellate biomass (López-Rodríguez et al., 2021). The process provided five fractions with increasing polarity HEX, carbon tetrachloride (CCl_4), DCM, BUT and water, where the target metabolite was obtained in a clear-cut way in the BUT fraction. The remaining fractions contained compounds of well-known families distributed in them according to their polarity (carotenoids, fatty acids etc.).

However, recent strict environmental legislation regarding air emissions and waste management is driving the implementation of green chemistry strategies in all industrial sectors, including biotechnology (Chen et al., 2020). One of the twelve principles of green chemistry refers to the "use of safer solvents" (Kerton and Marriott, 2013). In this regard, solvent selection guides from prestigious pharmaceutical companies and scientific societies have been published in recent years to promote more sustainable solvents (Alder et al., 2016; Joshi and Adhikari, 2019; Diorazio et al., 2016; Prat et al., 2013; ACS GCI-PR, 2011; Prat et al., 2014; Prat et al., 2016). These guidelines were developed according to similar criteria based on the risk to safety, health, and the environment. Although there may be discrepancies, all of them consider benzene, chloroform, carbon tetrachloride, dichloromethane, diethyl ether, and hexane among the least recommended solvents.

Within microalgae, species found in the haptophyte phylum possess biotechnological potential for providing active pharmaceutical ingredients. For example, *Isochrysis* sp. produces large amounts of fucoxanthin (Crupi et al., 2013), a promising carotenoid for cancer therapy as it has been shown to inhibit the growth of cell lines in several types of cancers, such as colorectal cancer (Méresse et al., 2020;). *I. galbana* produces a β-glucan that could be a potential antitumor agent against myeloid leukaemia (Sadovskaya et al., 2014). Most haptophytes produce β-carotene, diadinoxanthin and fucoxanthin as the main carotenoids, and to a lesser extent or only in some species, zeaxanthin, diatoxanthin and fucoxanthin esters (Takaichi, 2011).

Regarding fatty acids, in two classes of haptophytes, the highest production of the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) was found with respect to the total fatty acids (Jónasdóttir, 2019), and many species of this group also accumulate relatively high amounts of docosahexaenoic acid (DHA) (Lang, et al., 2011), with *E. huxleyi* being the most representative (Jónasdóttir, 2019). These omega-3 fatty acids help prevent cardiovascular disorders (Allayee et al., 2009; Schuchardt, et al., 2010).

Chrysochromulina is of particular interest for the metabolites it can produce; specifically, *C. polylepis* and *C. tobin* produce compounds of great pharmacological interest: polyketides, and non-ribosomal peptides (Hovde et al., 2015). Many species within this genus are rich in carotenoids, as well as unsaturated fatty acids (Seoane et al. 2009; Bigelow et al., 2013), such as fucoxanthin and its derivatives and polyunsaturated fatty acids with anticancer bioactivities (Jóźwiak et al., 2020).

This work proposes an alternative isolation method to obtain bioactive compounds from *Chrysochromulina rotalis* by partitioning in sequential gradient, carried out by substituting the hazardous classic solvents with alternative solvents while maintaining recovery yields similar to those obtained with the classic solvents. The antiproliferative activity is measured in the extracts isolated by sequential-gradient partitioning of *C. rotalis* crude methanolic extract to determine if they are effective for biorefining.

2. Materials and methods

2.1. Microalgae biomass

The marine microalga *Chrysochromulina rotalis* BMCC18 was used (LT560338 – GenBank accession number). It was provided by the Basque Microalgae Culture Collection (Spain). The biomass used for this work was obtained from a culture grown in a pilot-scale 80 L tubular photobioreactor with LED lighting (data not shown) provided by the microalgae cultures collection of the Department of Plant Biology and Ecology at the University of the Basque Country (UPV). The wet biomass was dried in a vacuum freeze dryer (Cryodos 50, Telstar) and approximately 15 g of dry biomass was used for this work. The lyophilised biomass was stored at –22 °C until being processed by different procedures.

2.2. Selection of alternative solvents

The main criterion for selecting the solvents used in this study was their "green score" as alternatives to four conventional reference solvents used in microalgae metabolite fractionation and isolation (Fig. 1), namely, HEX, TOL, DCM and BUT (López-Rodríguez et al., 2021). To this end, publicly available solvent selection guides developed by different companies and institutions (Alder et al., 2016; Joshi and Adhikari, 2019; Diorazio et al., 2016; Prat et al., 2013; ACS GCI-PR, 2011) were used. So as not to lose solvent capacity, those with similar solubility parameters were selected according to Hansen (2014) (HSP) (Table 1). These parameters are represented in a ternary diagram (see Fig. 2). The total solubility parameter (δ_T) for pure solvents is given by the equation:

$$\sqrt{\delta_d^2 + \delta_p^2 + \delta_h^2} \quad (1)$$

where δ_d represents the dispersion force energy, δ_p the energy of the intermolecular dipolar force, and δ_h the energy of the hydrogen bonds between molecules.

The temperature adjustment for the solubility parameter was carried out as proposed by Barton (1983):

$$\left(\frac{\delta_1}{\delta_2}\right)^2 = \frac{T_2}{T_1} \quad (2)$$

where δ_1 and δ_2 represent the solubility parameters adjusted to each temperature, T_1 is the reference temperature (25 °C) and T_2 the extraction temperature (40 °C).

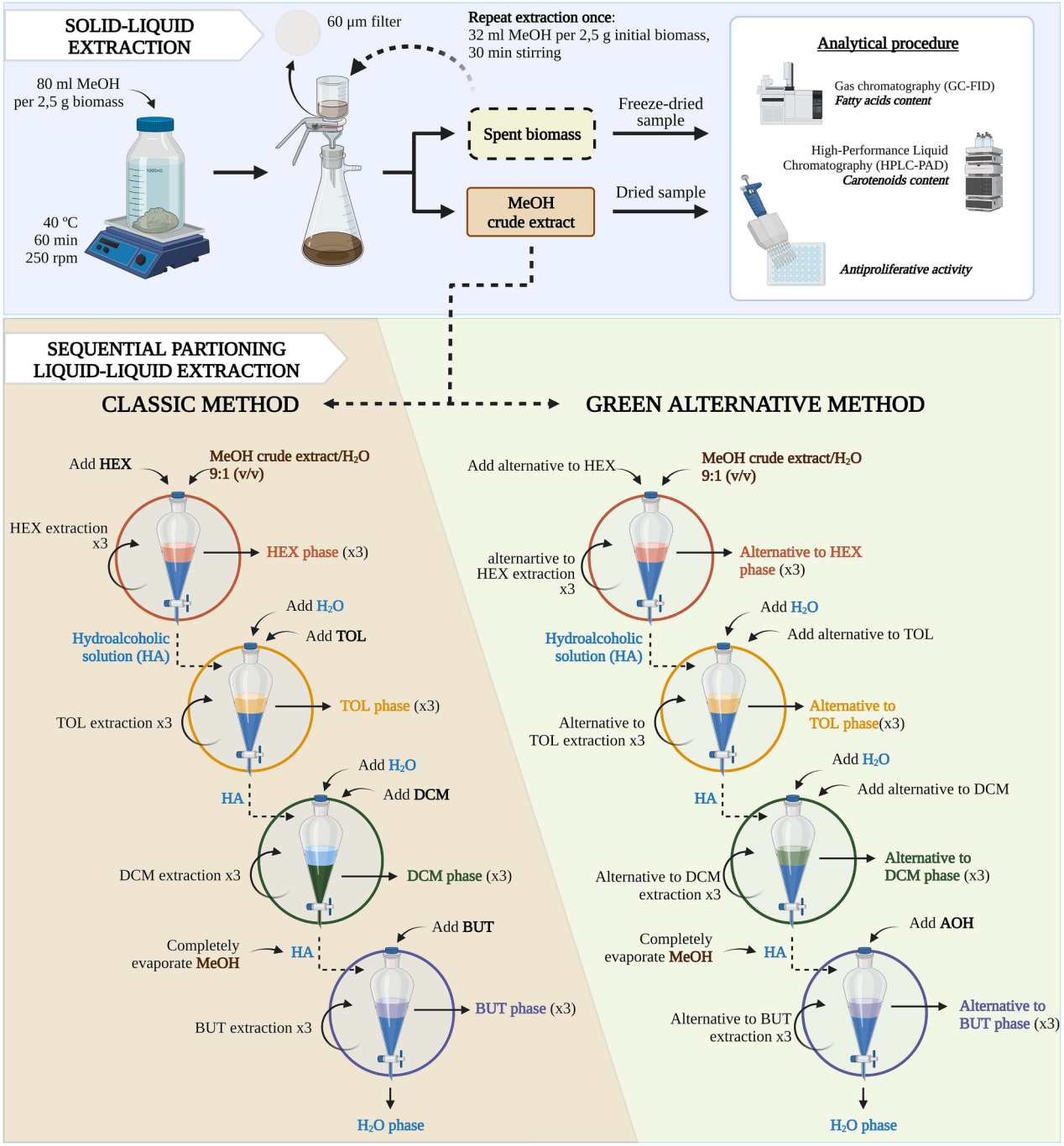

Fig. 1. Strategy followed for the isolation and recovery of compounds with high added value from microalgal biomass (MeOH: Methanol; HEX: Hexane; TOL: Toluene; DCM: Dichloromethane; BUT: *n*-Butanol) comparing classic solvents with alternatives one.

2.3. Solubility tests

For each green solvent, solubility tests were carried out in methanol–water mixtures at different proportions to allow total immiscibility between both phases, taking as a reference the hydroalcoholic phase ratios used in fractionation with HEX, TOL, DCM and *n*-butanol (9:1, 7:3, 6:4 and 0:10, respectively). The tests were performed in graduated test tubes, adding to each of them 1 mL of the different methanol–water mixtures, 1 mL of the solvent to be tested, and a drop of Trypan blue (Thermo Fisher) to colour the aqueous phase and provide better phase visualization. The tubes were shaken in a vortex and left to rest for 5 min until the phases were completely separated. The immiscibility in methanol–water was then evaluated by observing whether or not the volume of each phase was maintained or if one of the two phases had more volume than the other.

2.4. Extracting the metabolites of interest with alternative solvents

The criteria followed to select a single solvent from among all the alternative solvents was to select those that recovered a higher content of carotenoids and fatty acids by liquid–liquid extraction (LLE) compared to the four control tests performed in the same way with HEX, TOL, DCM and BUT. The protocol was carried out entirely in darkness to avoid the degradation of the carotenoids. 10 mg of dry biomass were weighed in a Pyrex tube and mixed with 2 mL of methanol (Panreac) in a water bath at 40 °C for 2 min, stirring in a vortex every 20 s. The tubes

Table 1
Hansen's solubility parameters (δ_d, δ_p, δ_h) and the total solubility parameter (δ_t) of the different solvents tested along with the temperature-adjusted parameter (δ_T).

Solvent	CAS No.	Nomenclature	δ_d[1]	δ_p[1]	δ_h[1]	δ_t	δ_T
Hexane	110–54-3	HEX	14.9	0.0	0.0	14.9	14.5
Cyclohexane	110–82-7	D1	16.8	0.0	0.2	16.8	16.4
Heptane	142–82-5	D2	15.3	0.0	0.0	15.3	14.9
Isooctane	540–84-1	D3	14.1	0.0	0.0	14.1	13.8
Pentane	109–66-0	D4	14.5	0.0	0.0	14.5	14.1
Toluene	108–88-3	TOL	18.0	1.4	2.0	18.2	17.5
Chlorobenzene	108–90-7	D5	19.0	4.3	2.0	19.6	19.1
Methylcyclohexane	108–87-2	D6	16.0	0.0	1.0	16.0	15.6
Dichloromethane	75–09-2	DCM	18.2	6.3	6.1	20.2	19.7
t-Amyl methyl ether	994–05-8	D7	15.2	4.5	4.4	16.5	16.1
n-Butyl acetate	123–86-4	D8	15.8	3.7	6.3	17.4	17.0
Cyclopentyl methyl ether	5614–37-9	D9	16.7	4.3	4.3	17.8	17.3
Ethyl acetate	141–78-6	D10	15.8	5.3	7.2	18.2	17.7
Isobutyl acetate	110–19-0	D11	15.1	3.7	6.3	16.8	16.4
Methyl isobutyl ketone (MIBK)	108–10-1	D12	15.3	6.1	4.1	17.0	16.6
n-Butanol	71–36-3	BUT	16.0	5.7	15.8	23.2	22.6
t-Amyl alcohol	75–85-4	D13	15.3	6.1	13.3	21.2	21.2
Benzyl alcohol	100–51-6	D14	18.4	6.3	13.7	23.8	23.8
Dimethyl carbonate	616–38-6	D15	15.5	8.6	9.7	20.2	20.2
Isoamyl alcohol	123–51-3	D16	15.8	5.2	13.3	21.3	21.3
1-pentanol	71–41-0	D17	15.9	5.9	13.9	21.9	21.9

[1] Hansen (2014).

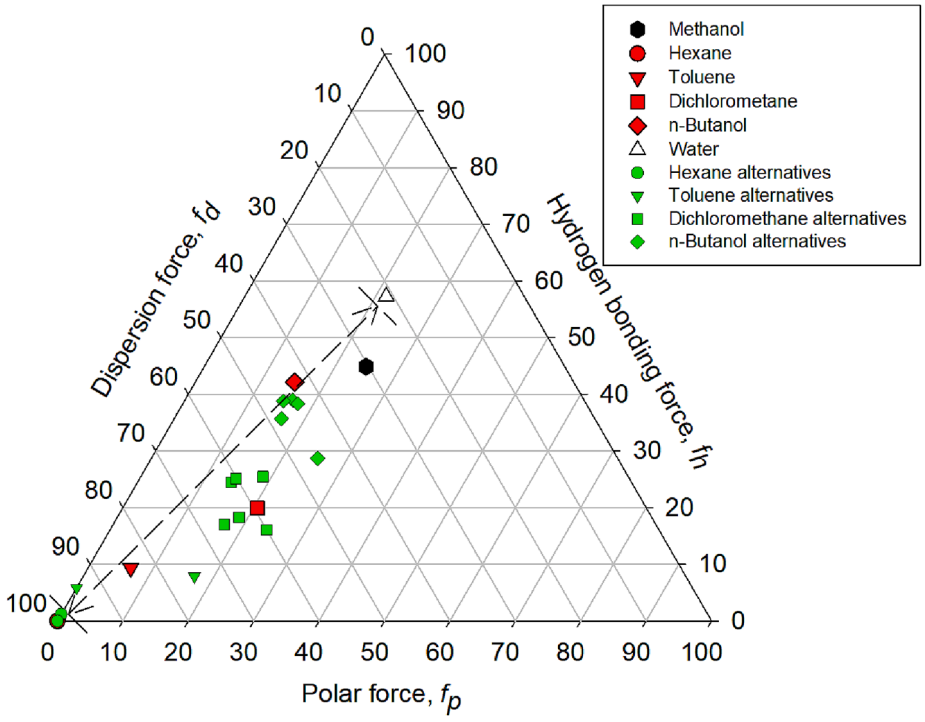

Fig. 2. Ternary diagram depicting classic solvents (red symbols) and various alternative solvents (green symbols) found in the literature.

were centrifuged in a Mixtasel-BLT tube centrifuge (J.P. Selecta) for 3 min at 4000 rpm. From each tube, 1.5 mL of the supernatant was transferred to another tube, and HPLC grade water (Honeywell) was added to adjust each to the methanol–water proportion determined above. To this methanol–water mixture, the same volume of the corresponding alternative solvent was added and vortexed for 1 min at room temperature. It was left to rest for a few minutes until both phases were stabilized, either at room temperature or at 5 °C to facilitate separation. Two 0.5 mL aliquots were taken from the organic phase to further analyse the fatty acids and carotenoids. The aliquots were completely dried in a thermoblock at 42 °C while applying nitrogen to avoid sample degradation, and afterwards they were stored at −20 °C until analysis. All the extraction experiments and analysis were performed in duplicate. Data points are averages, and vertical bars are standard deviations (SD) for duplicate samples.

2.5. Extraction and isolation of the metabolites of interest by fractionation

The methodology followed in this work is a modified version of that previously described by López-Rodríguez et al. (2021) for the microalgae *Amphidinium carterae*. The most important modification was to replace the carbon tetrachloride with TOL as a solvent with a similar polarity, and to adjust the MeOH/water phase with which it is in contact to carry out the LLE from 80:20 to 70:30 (v/v), in accordance with the TOL solubility in that phase. In summary, two sequential-gradient

partitioning experiments were performed: one as a control using HEX, TOL, DCM, and BUT, and the other with the alternative solvents, each substituting the solvents in the control test, which were chosen following the extraction results. First, the SLE of the dry biomass was carried out in a glass bottle, using methanol (Panreac) in a proportion of 80 mL per 2.5 g of biomass. Nitrogen was introduced into the mixture to prevent degradation of the biomass components. The SLE was then performed between the biomass and methanol at 40 °C for 1 h, at 250 rpm. Subsequently, the depleted biomass was separated twice by vacuum filtration with filter paper in a Büchner funnel, and then filtrated through nylon mesh filters with a pore size of 60 μm (Millipore). All the depleted biomass was recovered in a single container and SLE was repeated with 32 mL of methanol for 30 min. No biomass pretreatment was carried out since it has been shown that cell breakage using KOH degrades fucoxanthin and its derivatives (Cerón-García et al., 2018a). This mixture was filtered again in the same way into separate aliquots for analysis in triplicate. The methanolic extract was subjected to sequential-gradient partitioning (Fig. 1).

2.6. Determination of carotenoids

The carotenoids were analysed by reverse phase HPLC using a Shimadzu SPDM10AV high-performance liquid chromatograph (Shimadzu, Japan), equipped with a diode array detector (DAD), applying the method adapted by Cerón-García et al., 2018a with the extraction conditions used in it for *Isochrysis galbana* due to the similarity between the two strains. The modification consisted of eliminating the potash treatment. The different extracts were eluted in a gradient at a rate of 1 mL/min using a ramp with different polarities. β-carotene (Sigma Chemical Co., St. Louis, USA) and fucoxanthin, dinoxanthin, diadinoxanthin, diatoxanthin, diadinochrome, echinenone and non-polar chlorophyll type c2 from *Chrysochromulina* sp. (DHI; Hørsholm, Denmark) were used as standards with calibration curves to quantify the concentrations. The carotenoid analysis was performed with LabSolutions software.

2.7. Determination of fatty acids

To determine the saponifiable lipid (SL) content, the *C. rotalis* biomass was transesterified directly to convert into methyl esters (FAME), which were then analysed by gas chromatography following the method described by Rodríguez-Ruiz et al. (1998), using an Agilent Technologies 6890 gas chromatograph (Avondale, PA, USA), equipped with a Flame Ionization Detector (FID).

2.8. Antiproliferative activity assay against human tumour cells

Assays were performed to evaluate the antiproliferative activity of samples obtained from *C. rotalis* against a panel of four different human tumour cell lines (i.e., ATCC®NSCLC A549 lung carcinoma, HT-29 ATCC®HTB-38 colon adenocarcinoma, MDA-MB-231 ATCC®HTB-26 breast adenocarcinoma, and PSN-1 ATCC® CRL-3211 pancreatic adenocarcinoma). All the cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the bioassays were performed according to Abreu et al. (2019). The results were expressed as the growth inhibition percentage for all four cell lines − 100 % means that all the cells were lysed (a strong cytotoxic effect) compared to the same number of cells at the beginning of the assay (the control); 0 means that there are the same number of cells as at the beginning of the assay (no growth) and −100 % means that there are as many cells as the control (samples do not inhibit growth). The results presented standard deviations of<10 % in all cases. None of the dry extracts from the solvents were toxic to the cancer cell lines.

2.9. Statistical analyses

The significant difference analysis was performed using a one-way analysis of variance (ANOVA) test. Fisher's least significant difference (LSD) method was used to discriminate between the means at the 95.0 % confidence level to determine the effect on the carotenoid and fatty acid contents in each classic and alternative solvent. Statgraphics Centurion XVII (version 17.2.04) statistical software (2014, Statpoint Technologies, Inc., Warrenton, VA) was used to analyse the statistical significance of the data.

3. Results and discussion

3.1. Identification of alternative solvents for assay

The solvents initially proposed as alternatives are shown in the ternary diagram of Fig. 2 in which solvents are located according to their Hansen solubility parameters, the aim being to obtain simple and quick identification of possible substitutes to the classic solvents. Of all the solvents proposed, seventeen were finally selected for testing: four alternatives to HEX, two for TOL, six for DCM and five for BUT (Table 1). Those that did not show similar Hansen solubility parameters (i.e. did not meet the criteria) were discarded.

For the less polar zone, many of the proposed solvents were located just above HEX in Fig. 2, thus predicting very similar performance in terms of metabolite extraction yield from the biomass. In the case of TOL, very few valid solvents were close in the diagram, meaning that one would not expect a very similar response to that of TOL from the two solvents finally tested. Quite a few options were found to replace both DCM and BUT, and some even had to be ruled out due to the volume of tests that had already been proposed with all the selected solvents.

3.2. Solubility tests

Table 2 shows the results obtained from the solubility tests performed with the different alternative solvents selected. In almost all the tests, immiscibility of the solvent was obtained against the reference methanol–water ratio. Only with D10, D11, and D12 were additional tests needed against the methanol–water mixtures at slightly higher or

Table 2
Results observed after the solubility tests with the alternative solvents.

Conventional solvents	Alternative solvents selected	MEOH/H_2O ratio	Immiscibility
Hexane	Cyclohexane	9:1	++
	Heptane	9:1	++
	Isooctane	9:1	++
	Pentane	9:1	++
Toluene	Chlorobenzene	7:3	++
	Methylcyclohexane	7:3	++
DCM	*Tert*-amyl methyl ether	6:4	++
	n-Butyl acetate	6:4	++
	Cyclopentyl methyl ether	6:4	++
	Ethyl acetate	6:4	–
		5:5	–
		4.5:5.5	–
		4:6	++
	Isobutyl acetate	6:4	+
		5.5:4.5	++
		5:5	+
	Methyl isobutyl ketone	6:4	–
		5:5	+
		4.5:5.5	++
***n*-Butanol**	*Tert*-amyl alcohol	0:10	++
	Benzyl alcohol	0:10	++
	Dimethyl carbonate	0:10	++
	Isoamyl alcohol	0:10	++
	1-pentanol	0:10	++

(++, totally immiscible; +, partially miscible; -, miscible).

lower ratios. This is because these DCM substitutes are closer to the polarity of methanol, and therefore dissolve in it more readily than less polar solvents such as HEX or TOL.

For the LLE experiments, only mixtures of MeOH/H_2O with total immiscibility were selected for each solvent.

3.3. Content and profile of fatty acids and carotenoids in the biomass studied

The total fatty acid content of the *C. rotalis* microalgal biomass was 17.84 ± 1.0 wt% of dry biomass. Table 3 shows the fatty acid and carotenoid profile of the biomass, including its classification into saturated, monounsaturated and polyunsaturated fatty acids and polar, medium and non-polar carotenoids, respectively. The lipid composition of microalgae depends on several variables, such as the culture conditions, harvest time, and the method and solvents chosen to extract the lipids from the microalgae biomass (Ríos et al., 2013). This total fatty acid content matches the value for *C.* sp. *P5.5* (17.1 %) reported by Bigelow et al. (2013). Other strains show the fatty acid content decreases by half, as with *C. Kappa* (9.57 %) or *C. parva Lackey* (7.88 %) (Bigelow et al., 2013). Most of the fatty acid content with respect to the total fatty acids of this microalgae are saturated or monounsaturated fatty acids (69.3 %), while the PUFAs, mainly 18:4n3 (11.1 %), 20:5n3 (1.2 %) and 22:6n3 (5.7 %), represent 29.4 % of the total fatty acids. The distribution of fatty acids varies greatly depending on the strain (Bigelow et al., 2013) since this author reports data on PUFAs from 1.72 to 11.72 % of the total fatty acids. PUFAs are structural fatty acids and behave as primary metabolites; however, high irradiances can cause a decrease in PUFAs (Aguilera-Sáez et al., 2019). At low irradiances, the formation of polar lipids (PL) is induced (Thompson, 1996). Conversely, Aguilera-Sáez et al. (2019) report that at high irradiances, a conversion of PL to neutral lipids (NL: saturated and unsaturated) can occur. Hence the differences in the content and distribution of our results compared to those reported by Bigelow et al. (2013), since these authors grew the cultures at low incident irradiance (100 μEs $^{-1}m^{-2}$) compared to those in our work (600 μE·s $^{-1}$·m^{-2}).

C. rotalis mainly contains carotenoids similar to those encountered in the genus *Chrysochromulina* such as the main ones - fucoxanthin, diadinoxanthin, diatoxanthin, non-polar chlorophyll (similar to the c2 of *C. rotalis)* and β-carotene, along with the minor ones, 19′-butanoyloxyfucoxanthin, and 4-keto-19′-hexanoyloxyfucoxanthin, as described by Seoane et al. (2009). In our results, *C. rotalis* in the selected culture system accumulated at most 3.4 % d.w. of total carotenoids (Table 3). Of these, fucoxanthin together with its derivatives (4-keto-hex-fucoxanthin and Hex-fucoxanthin) represent about 50 % of the total carotenoid content; it is also noteworthy how diatoxanthin represents almost 30 % of the total while equinenone, found as a new carotenoid in this strain, accounted for 9 %.

Table 3
Fatty acids and carotenoids profile (percentage with respect to the total fatty acid and carotenoid content in the biomass, respectively) of the main fatty acids and carotenoids identified from the *Chrysochromulina rotalis* biomass and their classification into saturated (SAFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acid classes or their classification into polar, medium polar and non-polar carotenoids.

Fatty acid	SLs in biomass	Carotenoid (CAs)	CAs in biomass
14:00	21.8 ± 0.3	But-fucoxanthin	0.5 ± 0.0
16:00	15.0 ± 0.2	Fucoxanthin	26.9 ± 0.1
16:1n7	6.4 ± 0.1	4-keto-hex-fuco	17.5 ± 0.0
16:2n4	0.8 ± 0.0	Hex-fucoxanthin	2.9 ± 0.0
16:3n4	0.5 ± 0.0	Diadinoxanthin	2.9 ± 0.0
18:00	0.4 ± 0.0	Diadinochrome	0.9 ± 0.0
18:1n9	24.4 ± 0.4	Diatoxanthin	29.4 ± 0.1
18:1n7	1.2 ± 0.0	np.chlc2-Cp	3.1 ± 0.0
18:2n6	6.3 ± 0.1	Equinenone	9.0 ± 0.0
18:3n3	3.8 ± 0.0	β-carotene	6.8 ± 0.0
18:4n3	11.1 ± 0.1		
20:1n9	1.3 ± 0.0		
20:5n3	1.2 ± 0.0		
22:6n3	5.7 ± 0.0		
Σ SAFAs	37.2 ± 0.3	Σ Polar CAs (xanthophylls)	47.9 ± 0.1
Σ MUFAs	32.1 ± 0.2	Σ Medium polar CAs	36.3 ± 0.0
Σ PUFAs	29.4 ± 0.2	Σ Non-polar CAs	15.8 ± 0.0

3.4. Comparison of the LLE performance of high value-added products between the classic and alternative solvents

It was possible to choose a substitute solvent for each of the classic solvents described in Section 2.4 based on the fatty acid and carotenoid contents extracted. The methanolic extract contained 11.02 ± 0.10 % d.w. of fatty acids, and 2.45 ± 0.10 % d.w. of carotenoids, which means that 62 % and 72 %, respectively, of the total biomass was extracted, similar to values reported by López-Rodríguez et al. (2021). This result is in line with another previous work, which reported that the best results for extracting fatty acids and carotenoids are achieved with solvents having temperature-adjusted solubility parameters between 16.5 and 19.5 [$MPa^{1/2}$] (López-Rodríguez et al., 2020) and not above, as is the case, for example, with methanol at 28 [$MPa^{1/2}$]. Regarding the distribution of fatty acids in terms of saturated, monounsaturated and PUFAs, this is maintained from the biomass to the methanol extract; however, for carotenoids, the percentage decreases in more polar carotenoids.

Fig. 3 shows a comparison of the total contents of fatty acids (A) and carotenoids (B) extracted with the alternative and classic solvents tested. Only the D1 alternative solvent tested had an individual fatty acid extraction yield very similar to that of HEX (the control) without statistically significant differences (p-value < 0.05) (Fig. 3A), with even a certain amount of DHA being extracted - this is not achieved with HEX since PUFAs, such as EPA and DHA, are usually part of the PLs (Jiménez-Callejón et al., 2022), and D1 has a higher polarity than HEX (16.8 and 14.9, respectively).

The content of fatty acids extracted with D4 was 30 % higher, far above all the others (D2 and D4) showing statistically significant differences, $p > 0.005$.

Analysing Fig. 3A, which shows the yields obtained using the TOL substitutes, one can appreciate that both proposed alternative solvents achieved a higher fatty acid extraction yield than that obtained with TOL (the control). Similar recovery yields were obtained with D5 and D6, and so either would be a good candidate for fatty acid extraction. Different lowercase letters in Fig. 3A for TOL indicate p-value > 0.05.

Among the alternative solvents for substituting DCM, none presented an improvement in fatty acid recovery performance, all of them statistically with a p-value > 0.05, the highest amounts of fatty acids being recovered with DCM (the control). There are only minor exceptions, such as for the 14:0, 16:0 and 18:0 contents, for which a slight improvement in performance can be seen with D10; this is not ultimately reflected in the overall recovery performance. Therefore, the tested solvents are not suitable alternatives to DCM based on the extracted fatty acid content.

Finally, in Fig. 3A for the *n*-butanol substitute solvents, one can observe similar total fatty acid contents in all the extracts coming from the five tested solvents, with the results obtained for D15 standing out above all the others. In addition to achieving a higher fatty acid recovery yield than the rest of the alternative solvents, D15 also presents a slight improvement in the total fatty acid content extracted with respect to that with BUT (the control), the only one without statistically significant differences (p-value < 0.05).

The results show that quite similar carotenoid yields were obtained when using HEX and the greener substitutes, except for D4 (Fig. 3B). The total carotenoid contents extracted with D1, D2 and D3 were similar with no statistically significant differences (p-value < 0.05); however, D4 was slightly higher while there was a small decrease when using D3 but with no statistically significant differences (p-value < 0.05). As for

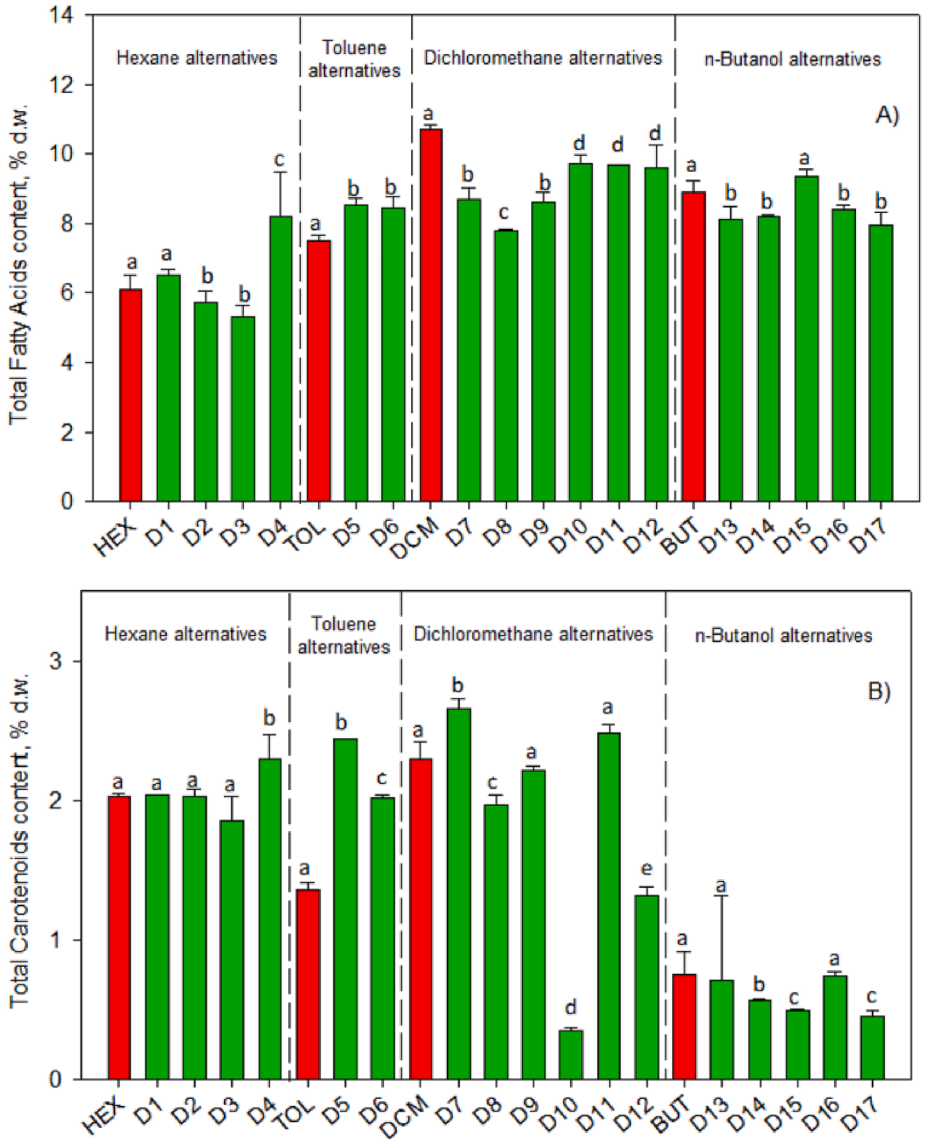

Fig. 3. Total contents of fatty acids (A) and carotenoids (B) extracted with the alternative solvents tested (marked in green) and with the classic reference solvents (marked in red). Nomenclature for the classic and alternative solvents (D) are the same as presented in Table 1. Results are shown as mean ± SE (n = 3). Different lowercase letters indicate significant differences ($p < 0.05$) within each treatment (comparing each classic solvent with its alternative).

the alternative solvents proposed for TOL, a clear improvement can be observed in the carotenoid extraction obtained using D5 or D6 compared to the other solvents, both with a p-value > 0.05. This resulted in the total extracted content for TOL being well below that obtained with the other solvents.

As for the DCM substitute solvents, D7 and D11 stand out in the individual contents of some carotenoids: diatoxanthin and np.chlc2-Cp for the former, and 4-keto-Hex-fucoxanthin for the latter. For both solvents, this translates into a higher total content of extracted carotenoids than when using DCM. Although there were good fatty acid extraction yields obtained when using D10, this was not repeated with the carotenoids. The BUT results reveal that no green solvent tested improved the extraction of carotenoids. Only with D13 is a total content obtained which is close to that obtained with BUT, followed by D16 with p-values < 0.05. The analysis of the individual carotenoid extraction profiles highlights the large amount of diatoxanthin recovered with BUT compared to the other solvents. Likewise, the np.chlc2-Cp content in D13, which indicates that these compounds are more polar; however, it remains well below the other solvents because BUT exceeds the solubility parameter limit at which more carotenoids are extracted (López-Rodríguez et al., 2021).

3.5. Solvent partition process comparing the classic and alternative solvents

Fig. 4A shows the different fatty acid extraction yields when using HEX or TOL as classic solvents and their possible substitutes, selected for having lower toxicity (D1 and D5, respectively). Using HEX as the solvent, a fatty acid extraction yield of almost 73.3 % was obtained after the first extraction, which increased to 79.3 % after the three extractions (4.3 % and 1.7 % in the second and third extractions, respectively). It is not economically viable to increase the number of stages from one to three. This extraction yield was considerably higher than that obtained by other authors for different microalgae species, such as *Nannochloropsis gaditana* when using HEX as the solvent (Navarro-López et al., 2016). In this case, the authors reported an extraction yield of 44 %, and this was improved upon using mixtures with a much higher polarity, such as HEX:isopropanol (3:2 v/v). This difference is due to the PL and NL contents of both species. In the case of *Nannochloropsis*, the authors reported the content of PL (mainly glycolipids) as being 57.4 % whereas

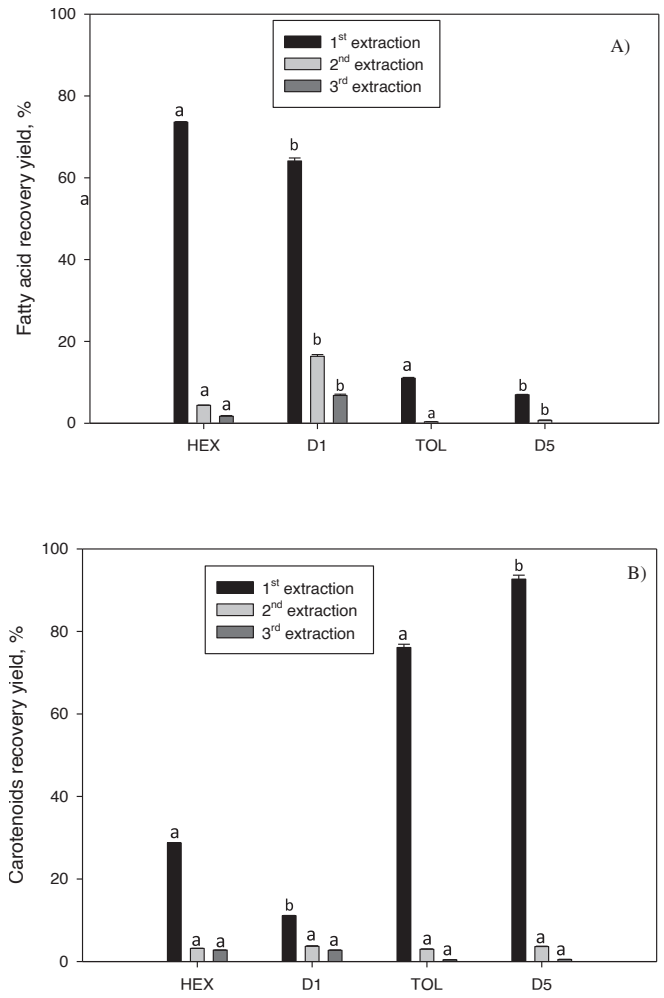

Fig. 4. Comparison of the recovery of fatty acids (A) and carotenoids (B) obtained by fractionation with classic solvents (HEX and TOL) and with alternative solvents (D1 (cyclohexane) and D5 (chlorobenzene). Results are shown as mean ± SE (n = 3). Different lowercase letters indicate significant differences ($p < 0.05$) within each treatment (comparing each classic solvent with its alternative in each extraction).

in the case of *Chrysochromulina,* with its high percentage of saturated fatty acids (14:0 and 16:0 mainly) and cultured under higher available irradiance, the NL content is expected to be higher than the polar lipid content, hence the high extraction yields when using a non-polar solvent such as HEX (Navarro-López et al., 2016). Regarding the use of D1 as an alternative solvent to HEX, the latter being traditionally used in extractions, one can see in Fig. 4A how the first extraction yield is slightly lower than that of HEX with statistically significant differences, but the overall result is a slightly higher fatty acid extraction yield after 3 extractions.

Both TOL and D5 have much higher polarities than that of HEX (18.2 and 19.6 $MPA^{1/2}$ versus 14.9 $MPA^{1/2}$), so the extraction yields should be much lower, given that the major fraction of lipids in *Chrysochromulina* must be NL. Therefore, contrary to what happens when extracting carotenoids (which are more polar) (Fig. 4B), extraction with polar solvents reduces the yield to values well below that observed when using non-polar solvents such as HEX.

The fatty acid recovery yields corresponding to the subsequent stages, both for the classic fractionation (fractions of DCM and BUT) and the green fractionation (fractions of D11 and D16) have been omitted from the graph, since they accounted for<2 % of the total recovery in both cases.

In the subsequent stages of both fractionations, no carotenoids were recovered. The recovery yields of TOL and D5 were corrected for with a factor calculated from the extraction yield obtained in small-scale extractions with both solvents.

3.6. Evaluation of the extraction yields with the alternative solvents

Once all the results for the fatty acid and carotenoid contents obtained by each of the solvents were analysed, we chose D1, D5, D11 and D16 as the green solvents to replace HEX, TOL, DCM, and BUT, respectively.

Fig. 5 shows the correlation between the temperature-adjusted solubility parameter (δ_T) and the total content of carotenoids and fatty acids obtained from the extraction. Optimal carotenoid extraction was achieved using solvents with δ_T values between 13 and 16 [$MPa^{1/2}$]. The maximum value of carotenoids, 1.4 % d.w., was obtained using D5, with $\delta_T = 15.5$ [$MPa^{1/2}$]. However, the maximum fatty acid extraction was retarded to 15 [$MPa^{1/2}$]. Optimal fatty acid extraction was achieved using solvent δ_T values between 14 and 16 [$MPa^{1/2}$]. Similar values were described by López-Rodríguez et al. (2020) with *A. carterae*.

3.7. Evaluation of the antiproliferative activity of the extracts obtained

Despite the fact that producing low-value bulk commodities from microalgae is not yet economically feasible, valorising different biomass fractions for the production of valuable specialty or niche products appears to be a more promising option, and the only exception to this trend is the production of high-value products such as carotenoids and phycobiliproteins (Sarkar et al., 2020; Lam et al., 2018). Therefore, to bring pragmatism to the microalgae-based multi-product biorefinery, it is indispensable to co-produce primary and secondary metabolites from the microalga selected (Park et al., 2022). Nonetheless, the added value of microalgal metabolites strongly depends on their potential range of commercial application. Thus, the pharmaceutical, cosmetics and food supplements for health, and disease prevention sectors are those that currently provide the highest revaluation of microalgal biomass. For this reason, it would be recommendable that those microalgae proposed as feedstock for biorefining are potentially applied in one or more of the aforementioned sectors. Indeed, microalgae assessment is commonly carried out based on the varied bioactivities of interest that their extracts may present, antitumor activity being one of them (Lauritano et al., 2016).

In line with the above, the antiproliferative activity of the cellular extracts obtained in this work was used as a proxy to compare the extraction performance of bioactives that have antitumor potential.

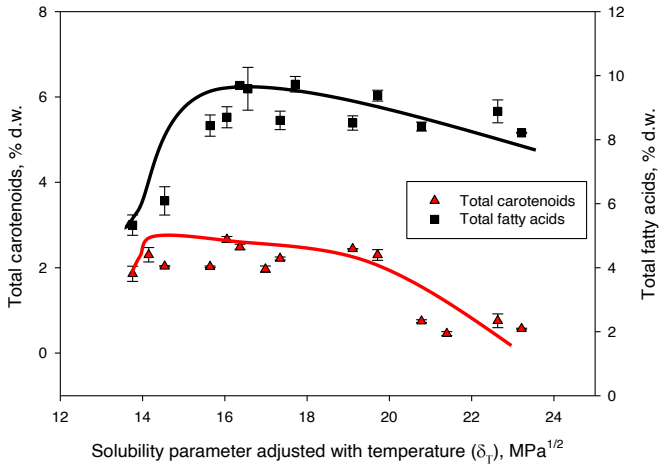

Fig. 5. Correlation between the extraction of different families of compounds (carotenoids and fatty acids) with the temperature-adjusted solubility parameter.

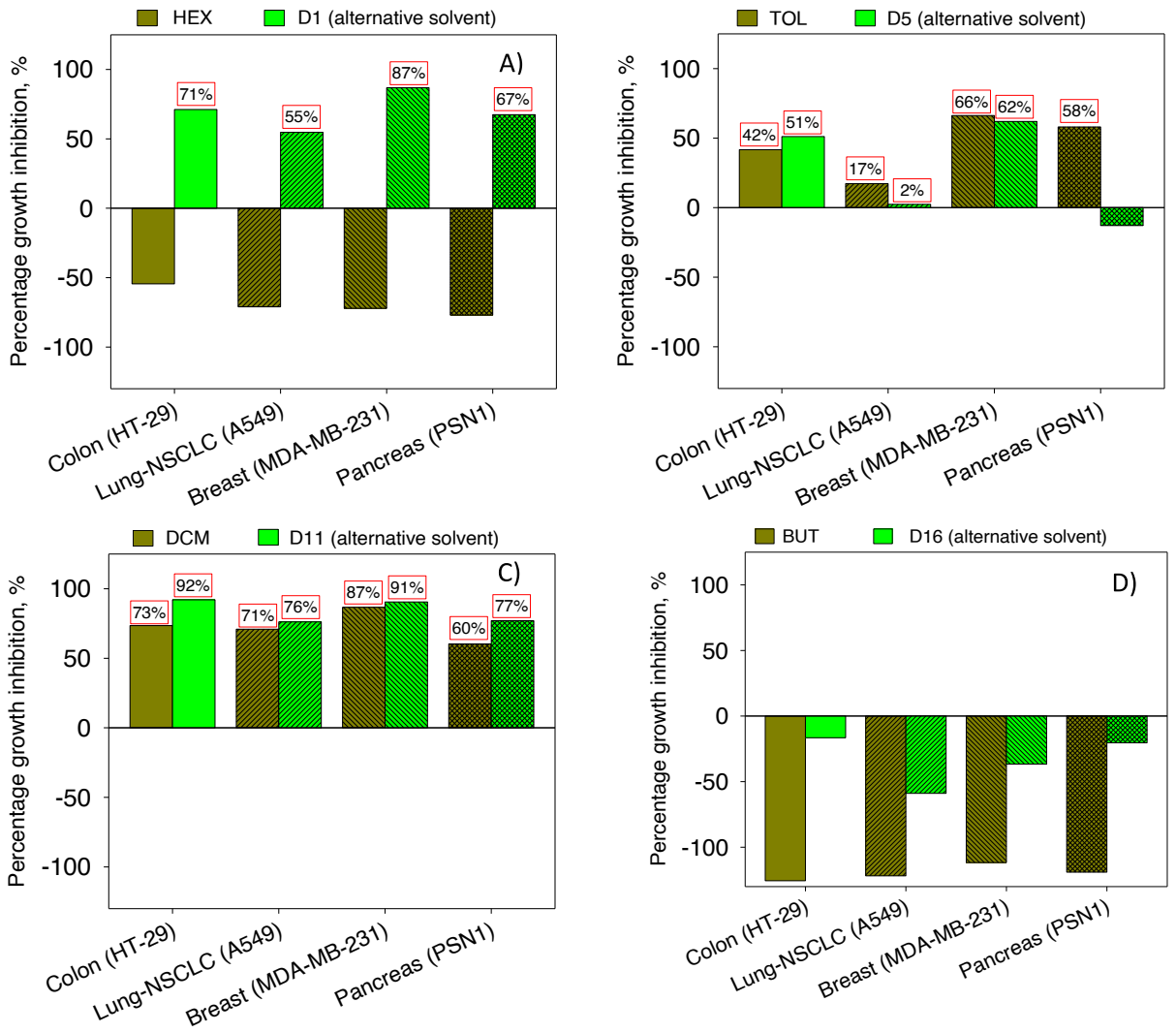

Fig. 6. Results from the antiproliferative assays. Survival percentages of the different human tumour cell lines in extracts prepared from classic solvents and green solvents: (A) HEX (hexane) vs D1 (cyclohexane); (B) TOL (toluene) vs D5 (chlorobenzene); (C) DCM (dichloromethane) vs D11 (isobutyl acetate); and (D) BUT (*n*-butanol) vs D16 (isoamyl alcohol). Data points are averages, and vertical bars are standard deviations (SD) for duplicate samples. Points without SD bars indicate that the SD was smaller than the symbol.

Fig. 6 shows the results of the antiproliferative tests on the fractionated extracts obtained from the sequential-gradient partitioning of the *C. rotalis* crude extract by LLE, both for the classic solvents and for the previously chosen alternative solvents. For comparative purposes, the classic solvent has been represented against the selected alternative solvent (Fig. 6A-D).

The results can be divided into two groups: (i) Fig. 6A-B for the most apolar solvents, where virtually all of the fatty acids and carotenoids are extracted (as shown in Fig. 3); and (ii) Fig. 6C-D for the more polar solvents, in which only some trace amounts of fatty acids and carotenoids were detected. Regarding Fig. 6A-B, antiproliferative activity can be appreciated in all the solvents except HEX. In principle, this result would be expected taking into account the antiproliferative activity of the fatty acids (Table 3) and carotenoids present in the biomass, as has been widely reported in the literature. For example, palmitoleic acid (16:1n7), hexadecadienoic acid (16:2n4), oleic acid (18:1n9), linoleic acid (18:2n6), α-linoleic acid (18:3n3), stearidonic acid (18:4n3), 20:5n3 (EPA) and 22:6n3 (DHA) fatty acids exhibit strong antitumor activity against a variety of cancer cell lines (Jóźwiak et al., 2020; Zhu et al., 2021; Evans et al., 2009). Similarly, the antitumor activity of the main carotenoids present in *C rotalis* (i.e., fucoxanthin) is well documented. Furthermore, microalgae have been recently considered as a lucrative source of fucoxanthin (Ashokkumar et al., 2023). Both the number of fatty acids and carotenoids with antiproliferative activity, and the proportion of each one in the lipid and carotenoid profile of microalgal biomasses, can make a difference in the screening programs for microalgal extracts with antitumor activity. In fact, the percentage of microalgae species whose apolar extracts exhibit antitumor activity is low (Lauritano et al., 2016). The antitumor potency of the extracts in Fig. 6A-B could be modulated both by the recovered amount of each fatty acid and carotenoid, and by the synergistic effect between them. This last aspect has not yet been studied in the literature nor is it the objective of this study. However, this may be a plausible reason for the lack of antitumor activity from the hexane extract (Fig. 6A).

In relation to Fig. 6C-D, the moderately polar solvents DCM and D11 (the alternative homologue) showed strong antiproliferative activity, while the more polar ones (BUT and D16) did not.

The above discussion does not preclude the possibility that other undetermined metabolites might contribute to the antitumor activity of the extracts in Fig. 6A-D. A few microalgae have been reported to produce other added high-value metabolites with anticancer activity (Ferdous and Yusof, 2021a,b; Skjånes et al., 2021). Thus, the DCM and D11 fractions from *C. rotalis* may have compounds of medium polarity such as peptides or isoflavonoids, while in the TOL and D5, only low polarity

metabolites such as terpenes may be found. Nevertheless, the appearance of new unknown compounds responsible for the activity cannot be ruled out. Therefore, future work will be aimed at dereplicating the extracts to identify known and unknown compounds, the latter requiring the arduous work of isolation and identification by NMR. The antiproliferative activity measured in several of the extracts obtained by sequential-gradient partitioning of *C. rotalis* crude methanolic extract is one more incentive for selecting this microalga as a feedstock in biorefinery studies.

4. Conclusions

A sustainable and more environmentally friendly method is proposed to isolate bioactive and high value-added compounds with solvents considered for their green score and reduced operating costs derived from using them. The final solvents selected were D1 for HEX, D5 in place of TOL, D11 for DCM and D16 in place of BUT. Antiproliferative activity can be seen in all the solvents except HEX. The antiproliferative activity of fucoxanthin (mainly) and the fatty acids obtained in the extracts isolated by sequential-gradient partitioning of *C. rotalis* crude methanolic extract makes it an effective selection as a feedstock in biorefinery approaches.

CRediT authorship contribution statement

M.A. González-Cardoso: Methodology, Data curation, Formal analysis, Writing – original draft. **M.C. Cerón-García:** Conceptualization, Supervision, Writing – review & editing, Project administration. **E. Navarro-López:** Methodology, Software, Formal analysis. **A. Molina-Miras:** Methodology, Software, Formal analysis. **A. Sánchez-Mirón:** Software. **A. Contreras-Gómez:** Software. **F. García-Camacho:** Conceptualization, Writing – review & editing, Project administration.

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.

Data availability

The authors do not have permission to share data.

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