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Updated pigment composition of *Tisochrysis lutea* and purification of fucoxanthin using centrifugal partition chromatography coupled to flash chromatography for the chemosensitization of melanoma cells

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Abstract :

Tisochrysis lutea (T. lutea, ex T. Isochrysis galbana or T-Iso) is a marine haptophyte that was first isolated from Tahiti seawater. Because of its high content in lipids, this tropical species is commonly used in aquaculture to feed fishes, crustaceans and molluscs larvae. It is also a rich source of fucoxanthin with a high potential for nutraceutical, cosmetic and pharmaceutical applications. The purpose of the present study was to detail the pigment composition of T. lutea and to develop an efficient process to recover highly purified fucoxanthin. Using ultra performant liquid chromatography coupled to diode arrays and high-resolution mass spectrometry detectors (UPLC-DAD-MS/MS), we demonstrated for the first time the presence of echinenone, 3-hydroxy-echinenone and chlorophyll c2-monogalactosyldiacylglycerol [18:4/14:0] in unstressed cultures of T. lutea. The chemotaxonomic relevance of this updated pigment composition was discussed in relation to the Haptophyta phylum. A two-step purification of fucoxanthin was then optimized using centrifugal partition chromatography coupled to flash chromatography. This process allowed the efficient isolation of fucoxanthin (purity > 99%), that was further assessed as a low-toxicity antineoplastic and chemosensitizing natural product in human chemoresistant melanoma cells. This carotenoid exerted an antiproliferative activity in A2058 melanoma cells and reversed in vitro their chemoresistance to dacarbazine, a DNA-alkylating agent clinically used for the treatment of metastatic melanoma.

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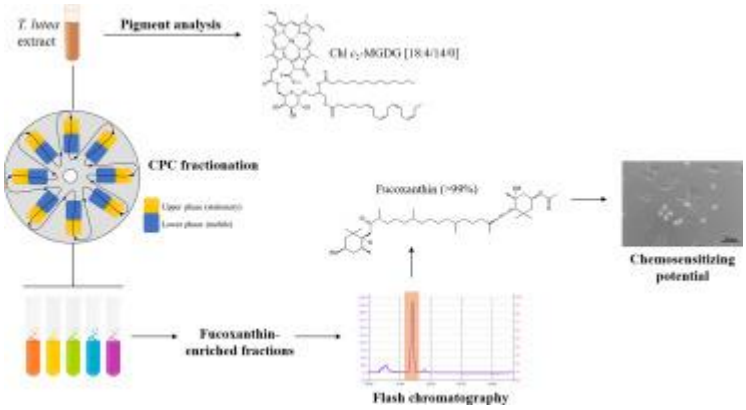
Graphical abstract



Highlights

► Echinenone, 3-hydroxy-echinenone and chlorophyll c2-MonoGalactosylDiacylGlycerol [18:4/14:0] in *Tisochrysis lutea* ► Chemotaxonomic relevance of these pigments for species identification in the Haptophyta phylum ► Development of a two-step purification of fucoxanthin (purity > 99%) using CPC coupled to flash chromatography. ► Reversion of dacarbazine chemoresistance in human melanoma cells *in vitro* by fucoxanthin purified from *T. lutea*.

Keywords : Tisochrysis lutea, Carotenoids, Pigments, Centrifugal partition chromatography, Melanoma, Multidrug resistance

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1. Introduction

Tisochrysis lutea (*T. lutea*), formerly named *Isochrysis* affinis *galbana* or T-*Iso* is a marine brown microalga (Haptophyta) originally isolated from tropical seawater (Tahiti, French Polynesia) [1]. This species is widely used in aquaculture because it contains a high amount of polyunsaturated fatty acids (PUFAs), notably docosahexaenoic acid (DHA). *T. lutea* also presents a promising nutraceutical and pharmaceutical potential [2–4] because of its high content in carotenoids and vitamins [5,6]. Preliminary analyses of its pigment composition by our research group have shown high amounts of fucoxanthin and unidentified carotenoids (named Car43 and Car48) of chemotaxonomic interest [7].

Several biological and pharmacological activities have been demonstrated for fucoxanthin, including antioxidant, anti-obesity, anti-diabetic, anti-inflammatory and anticancer effects [8–11]. This marine carotenoid is especially able to induce *in vitro* apoptosis in a wide variety of cancer cells and to prevent *in vivo* tumor initiation, growth, metastasis and angiogenesis [10,12]. It also enhanced the cytotoxicity of anticancer drugs in leukemia, colon, liver, breast and cervical cancer cells, suggesting a potential use as natural chemosensitizer in adjuvant therapy [13–16]. It has high commercial value related to cosmetic, pharmaceutical and nutraceutical applications; for example, food supplements containing fucoxanthin are sold in nutrition shops for anti-obesity indication [17]. Moreover, previous *in vivo* studies have shown that this compound has no significant oral toxicity [18,19].

However, the high scale availability of fucoxanthin for the pharmaceutical market is hampered by the complexity of its extraction and purification from seaweeds and microalgae. Conventional procedures are based on simple maceration using dimethyl sulfoxide (DMSO) or acetone. Extracts are then subjected to classical purification methods including silica gel column chromatography, preparative thin layer chromatography and/or high-performance liquid chromatography (HPLC) [2,20–22]. Although efficient to obtain highly purified carotenoids, these techniques are usually time-consuming, require large volumes of toxic organic solvents, may be limited by pigment retention or chemical transformations occurring on the stationary phase and can only be performed with small amounts of sample, providing low recovery yields.

Centrifugal partition chromatography (CPC) may represent an alternative to overcome these problems. This technique, based on the partition of compounds between two immiscible liquid phases, does not generate polluted silica, requires low volumes of solvents, allows fast and complete sample recovery without degradation of targeted metabolites, and scale-up to batch production is feasible. Though widely used for the purification of bioactive products from

plant extracts, CPC has rarely been applied to the separation of metabolites from algae and microalgae. Isolation of fucoxanthin from the seaweed *Eisenia bicyclis* has been reported, nevertheless up to date, there is no report of such process from microalgae extract [23].

In this context, this paper aims to detail the exhaustive pigment composition of *T. lutea* using ultra performant liquid chromatography coupled to diode arrays and high-resolution mass spectrometry detectors (UPLC-DAD-MS/MS), to optimize a purification process including CPC for high yield recovery of fucoxanthin from *T. lutea* ethanolic extract, to evaluate the antiproliferative activity of this carotenoid toward chemoresistant human melanoma cells and the ability to revert their chemoresistance to dacarbazine, a DNA-alkylating agent clinically used to treat metastatic melanoma.

2. Materials and methods

2.1. Microalgae culture, harvest and freeze-drying

Tisochrysis lutea CCAP 927/14 was grown in a commercial 16 L photobioreactor LUCY© (Synoxis algae, Le Cellier, France) containing 0.2 µm filtered and autoclaved seawater enriched with Walne's medium 4 $mL.L^{-1}$. The culture was realized in batch condition with an increasing irradiance from 74 to 300 µmol photons $m^{-2}.s^{-1}$ from day 0 to day 3 in a climate room at 18 °C. A pH 9 regulation was maintained with a regulated CO_2 injection. After 9 days, cells reached a concentration of 4.07×10^7 $cell.mL^{-1}$. At this early stationary phase, 16 L of culture were harvested by centrifugation. The microalgae paste was frozen at -80°C and freeze-dried before extraction.

2.2. Sonication-assisted extraction of T. lutea pigments

T. lutea lyophilized biomass was subjected to sonication-assisted extraction using absolute ethanol as solvent (2 g of biomass/500 mL of solvent). Extraction was performed using an ultrasonic processor (50W, 30 kHz, UP50H model, Hielscher, Germany), with 100% amplitude and continuous pulse cycle, for 30 min and under constant stirring. In order to limit thermal degradation of pigments by the heat generated at the sonicator tip, the extraction procedure was entirely performed on ice. The pigment extract was filtered through a PVDF 0.22 µm membrane and solvent was evaporated in amber vials (45 °C, vacuum). Five hundred and three mg of *T. lutea* dried ethanol extract (Tl-EtOH) were recovered, corresponding to a 25.15% pigment extraction yield (w/w) as compared to the starting biomass. This extraction procedure was repeated to obtain a sufficient amount of extract to perform all analyses, CPC

separation and pharmacological tests. This pigment extraction was carried out iteratively, to obtain grams of Tl-EtOH, that were stored at -20 °C until chemical characterization and pharmacological assays.

2.3. Scanning electron microscopy (SEM)

SEM analysis was performed in order to evaluate cell morphology after freeze-drying and sonication-assisted extraction. Cells were placed on a conductive double layer carbon support and examined in environmental mode without metal coating, using a Philips-FEI Quanta 200 ESEM/FEG (USA) microscope, equipped with a FEG canon delivering 1 to 30 kV beam current [24].

2.4. Pigment analysis

Pigment composition was determined using an Acquity UPLC H-Class (Waters, Milford, USA) coupled to a Waters 2996 photodiode array detector or a Xevo G2 S Q-TOF mass spectrometer, equipped with an electrospray ionization (ESI) source (Waters, Manchester, England). The chromatographic system consisted of a quaternary pump (Quaternary Solvent Manager, Waters) and an automatic injector (Sample Manager-FTN, Waters) equipped with a 10 µL sample loop. Tl-EtOH was dissolved in methanol to obtain a 1 $mg.mL^{-1}$ solution and 5 or 10 µL (for MS or UV analysis, respectively) were injected in a C18 column (Acquity UPLC BEH C18, Waters) (2.1 x 50 mm, 1.7 µm), using a flow rate of 300 $\mu L.min^{-1}$. The elution gradient was composed of solvents A (0.001% formic acid in water) and B (0.001% formic acid in methanol) as follows: 0 – 1 min, 80% B; 1 – 2 min, 80-81% B; 2 – 5 min, 81% B; 5 – 7 min, 81-81.5% B; 7 – 10 min, 81.5% B; 10 – 11 min, 81.5-83% B; 11 – 14 min, 83% B; 14 – 16 min, 83-85% B; 16 – 20 min, 85% B; 20 – 23 min, 85-90% B; 23 – 27 min, 90% B; 27 – 29 min, 90-95% B; 29 – 35 min, 95% B; 35 – 37 min, 95-98% B; 37 – 43 min, 98% B; 43 – 44 min, 100% B; 44 – 48 min, 100% B; 48 – 48.5 min, 100-20% B; 48.5 – 53 min, 20% B [25,26]. Column and injector were kept at 25 and 7 °C, respectively, during all analyses. UV spectra acquisition was performed in a 300-800 nm interval, with a 5 Hz acquisition frequency and 1.2 nm resolution. All MS analyses were performed in the positive ionization mode, with MS^E function in centroid mode. Final ESI conditions were: source temperature 120 °C, desolvation temperature 500 °C, cone gas flow-rate 50 $L.h^{-1}$, desolvation gas flow-rate 300 $L.h^{-1}$, capillary voltage 3.0 kV, sampling cone voltage 35 V, and source compensation 80 V. The instrument was set to acquire over the *m/z* 250 – 2000 range with a scan time equal to 0.5 s. Ramp collision energy was from 10 to 30 V and mass spectrometry calibration was performed before analysis

using 0.5 mM sodium formate solution. Leucine Enkephalin (M = 555.62 Da, 1 ng.µL^{-1}) was used as lock-mass. Mass error between experimental and theoretical parent or fragment ions was calculated as [(experimental *m/z* – theoretical *m/z*) / theoretical *m/z*] x 10^6. Quantitative analysis of fucoxanthin was performed under the same conditions, using an analytical standard (>99% purity, Sigma Aldrich, France). A calibration curve (0–1000 µg.mL^{-1}, R^2 = 0.9994, UV detection at 450 nm) allowed the determination of the fucoxanthin content in extracts and fractions. All quantitative analyses were performed in triplicate.

2.5. Selection of CPC biphasic solvents system

The selection of the solvents system was achieved in two steps. At first, a range of Arizona-derived systems comprising various proportions of cyclohexane, ethyl acetate, methanol, water and of several ternary mixtures of cyclohexane, methanol and water were evaluated by shake-flask method. Briefly, an aliquot of Tl-EtOH was solubilized in the solvent systems previously equilibrated into small vials, vigorously shaken, then kept still until complete separation of the two phases (if no emulsion had been formed). The upper and lower phases were monitored by TLC with an additional spot of fucoxanthin standard. As ternary mixtures seemed more selective, they were chosen for the second step, consisting of determination of the partition coefficient *K*. For each system, *K* values for fucoxanthin were calculated using the area under curve (AUC) obtained for both phases by UPLC-DAD performed using the above-mentioned conditions (2.4) (Table 1). Finally, the system cyclohexane/methanol/water (6/6/1, v/v/v) was selected. The upper phase was defined as the stationary phase and the lower phase as the mobile one (descending mode).

Table 1. Determination of *K* values for fucoxanthin (Fuco) in different solvents systems, as $[Fuco]_{upper}$ / $[Fuco]_{lower}$.

Solvent system	**Solvent proportion CHex/MeOH/H_2O (v/v/v)**	**Volume ratio of upper and lower phases**	***K*-value**
1	5:5:1	0.67	0.93
2	6:6:1	0.67	1.20
3	6:5:1	0.5	1.16
4	7:7:1	0.33	1.77
5	8:8:1	0.33	1.30

CHex (cyclohexane), MeOH (methanol).

2.6. CPC experiment

CPC separation was performed on a SCPC-250+1000-B (Gilson/Armen Instrument, Saint-Avé, France) by using the 250 mL rotor containing 1953 twin-cells, equipped with a gradient pump and a 10 mL injection loop 6-ways valve. The rotor was initially filled with distilled water; all the following steps were performed in descending mode. The upper stationary phase was injected with a flow rate of 30 mL.min^{-1} and a rotation speed of 500 rpm. Then, the system was equilibrated with the lower mobile phase at 15 mL.min^{-1} and 1600 rpm. The retention of the stationary phase was calculated as 68% (170 mL retained in the rotor). Tl-EtOH (750 mg) was solubilized in equal volumes of stationary and mobile phases (5 mL each) and manually injected through the 10 mL loop. The flow rate was set up at 10 mL.min^{-1} and rotation speed kept at 1600 rpm. After 80 min of elution, the extrusion was achieved by pumping the stationary phase at 20 mL.min^{-1} and 1600 rpm for 30 min. Fractions were collected every 10 and 20 mL during elution and extrusion respectively, and pooled when their TLC profile were similar. These chromatographic conditions were adapted from previous investigations [27,28].

2.7. Flash chromatography purification

Fucoxanthin-enriched CPC fractions were transferred to flash liquid chromatography as previously reported [25]. Fractions were separately solubilized in methanol and added to 3 g of Celite® 545 (Sigma-Aldrich®, France). After manual homogenization and complete solvent evaporation, the mixture was put in a pre-column, which was connected at the top of a PF-C18 column (20 g, 15 µm). The purification was performed using an Interchim Puriflash® PF430 system (France), equipped with an automatic collector (10 mL per tube) with a mobile phase composed of a ternary solvent gradient: A (MeOH/H_2O, 80:20, v/v), B (acetonitrile/H_2O, 90:10, v/v) and C (isopropanol). Elution was monitored at 450 nm, using a fixed flow rate (5 mL.min^{-1}) and the following gradient program: 0 – 5 min, 100% A; 5 – 9 min, 100% B; 9 – 45 min, 30% B and 70% C; 45 – 50 min, 100% C; 50 – 55 min, 100% C; 55 – 60 min, 100% B; 60 – 65 min, 100% A; 65 – 70 min, 100% A. Peaks corresponding to fucoxanthin were collected and fucoxanthin content was determined by UPLC-DAD as above mentioned.

2.8. Antimelanoma activity of fucoxanthin purified from T. lutea

2.8.1. Antiproliferative activity

The antiproliferative activity of purified fucoxanthin was evaluated on A2058 cells (ATCC® CRL-11147™), expressing the BRAF V600E oncogenic mutation. These are highly invasive and metastatic human melanoma cells, deriving from a lymph node metastasis, tumorigenic at 100% frequency in nude mice and chemoresistant to some anticancer drugs [29]. Cells were grown in 75 cm^2 flasks containing DMEM culture medium supplemented with 10% foetal calf serum (FCS) and 1% antibiotics (penicillin-streptomycin) (Dominique Dutscher, France). Cells were kept at 37 °C in a 5% CO_2 humidified atmosphere during all experiments. Fucoxanthin was solubilized in DMSO and then diluted in the cell culture medium. Initially, melanoma cells (2000/well) were added into 96-well microplates and treated with increasing concentrations of fucoxanthin (1 – 100 µM) for 72 h. The final DMSO concentration was equal to or lower than 1% and tested as negative control. After treatment, cell viability was determined by the MTT assay as previously described [24,29] and IC_{50} value was calculated from at least three independent measurements.

2.8.2. Chemosensitizing effect

In order to evaluate the ability of fucoxanthin to sensitize A2058 cells to chemotherapy, several concentrations (1 – 100 µM) of vemurafenib (BRAF inhibitor, Selleckchem®, France) and dacarbazine (DNA alkylating agent, Sigma-Aldrich®, France) were tested alone or combined with fucoxanthin at its ½IC_{50} (7.5 µM). After 72h of treatment, cell viability was measured by the MTT assay and results were expressed as IC_{50}. The combination index (CI) were calculated according to the Chou-Talalay method [30], using the free software CompuSyn® (version 1.0). Briefly, CI < 1.0 indicates synergism, CI > 1.0 indicates antagonism and CI ≅ 1.0 indicates an additive effect.

2.9. Statistical analysis

Data were expressed as mean ± standard error of the mean and analysed by unpaired Student's *t* test (n=3 or more) using the software GraphPad Prism® 6.0. Values of $p<0.05$ were considered statistically significant. IC_{50} values were calculated by nonlinear regression analysis.

3. Results and Discussion

Fucoxanthin is a major metabolite found in most algal classes of the red and brown lineages, contributing to more than 10% of all carotenoids production estimated in nature [31].

Industrial scale extraction is mainly achieved from brown seaweeds such as *Eisenia bicyclis*, *Laminaria japonica*, *Undaria pinnatifida* and *Sargassum* sp. [31,32]. Nevertheless, microalgae, particularly diatoms [33] and haptophytes [34] are progressively being considered as alternative to seaweed because of their higher fucoxanthin content [35], growth performance, metabolic plasticity and possible optimization by mutation/selection/transformation/improvement of culture conditions to obtain better yields all along the year. Among these species, *T. lutea* has been highlighted as a promising source of fucoxanthin for commercial purposes [36].

3.1. Sonication-assisted extraction of T. lutea pigments

Various processes have been developed to extract pigments from microalgae, depending on the cell wall solidity (silicified frustule, calcified coccoliths, cellulosic theca) and potential presence of extracellular structures (EPS, mucus) [24,37,38]. A suitable pigment extraction protocol should combine simplicity, low cost, high speed, eco-efficiency, reproducibility, possible selectivity based on the biochemical characteristics of the targeted compound, protection from thermal and photic damage, high yield and scalability to the industrial level. Although *T. lutea* does not contain a hard cell wall, it is covered with a dense layer of thin calcified scales similar to those found in *Isochrysis galbana* [1]. To ensure maximum pigment extraction efficiency, freeze-dried *T. lutea* biomass was subjected to sonication-assisted extraction. SEM observation of *T. lutea* before extraction revealed in fact that most cells were already open following the freeze-dried process of concentrated biomass coming from the photobioreactor (Fig. 1A and B). Probably the freezing step was sufficient to break most of the cells, whose membranes merged during the water sublimation step. Some salt crystals coming from the algal cell culture medium could also be observed in the freeze-dried microalgae powder. Occasionally, intact cells exhibiting a spherical to ovoid shape, coherent with the expected cell morphology, and a length varying from 4 to 7 µm could be observed, suggesting that the sonication step could improve the extraction yield (Fig. 1A and B). After sonication in ethanol, no clear morphological change was observed and fusion or tearing of cell membranes could also be noticed (Fig. 1C and D). In conclusion, the combination of freeze-drying and sonication in ethanol allowed to break most *T. lutea* cells and favored the exposure of the intracellular content to the extraction solvent to achieve an efficient pigment recovery.

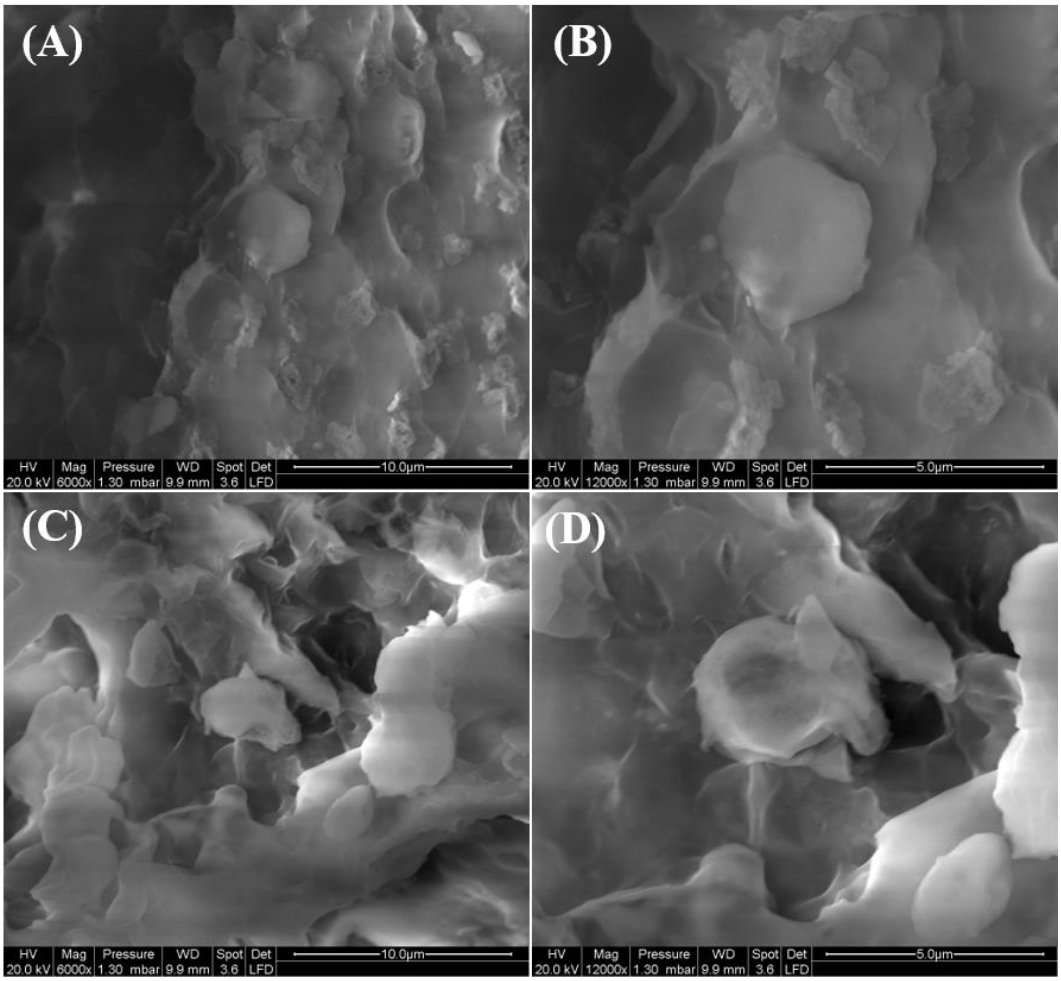

Fig. 1. Scanning electron microscopy analysis of freeze-dried *T. lutea* cells before (A and B) and after sonication-assisted extraction (C and D). Magnification ×6000 (A and C) and ×12000 (B and D).

3.2. Pigments composition of T. lutea

UPLC-DAD analysis of Tl-EtOH provided a chromatogram containing eighteen peaks (300–800 nm, full scan) (Fig. 2). The first pigment eluting as a major peak at 5.51 min was identified as fucoxanthin ($C_{42}H_{58}O_6$), with a maximal absorption wavelength at 453 nm in UPLC solvents and a peak at *m/z* 681.4131 in its mass spectrum, identical to the sodium adduct $[M+Na]^+$ expected for this compound (Fig. 3). Its high resolution MS/MS spectrum also showed fragment patterns at *m/z* 641.4222 and 581.4007, corresponding to $[M+H-H_2O]^+$ and $[M+H-H_2O-AcOH]^+$, respectively (Table 2) [36].

In accordance with previous reports, fucoxanthin isomers, diadinoxanthin, diatoxanthin, β,β-carotene, chlorophyll *a*, pheophytin *a* and chlorophylls c_1 and c_2 were also identified in Tl-EtOH [7,39]. Diadinochrome, 3-hydroxy-pheophytin *a* and epimers were also detected but considered as pigment derivatives produced by thermal damage or chemical reaction with

ethanol during the sonication step. These derivatives are indeed absent or in minute amount within phytoplankton living cells [24,25].

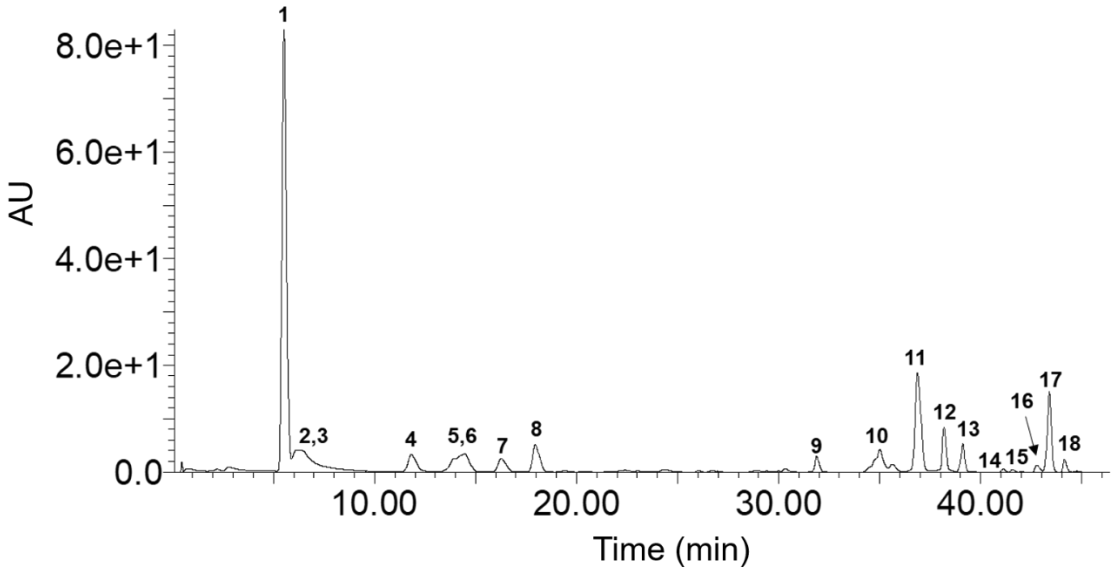

Fig. 2. UPLC-DAD chromatogram (full scan, 300-800 nm) of Tl-EtOH. Peak characterization is shown in Table 2.

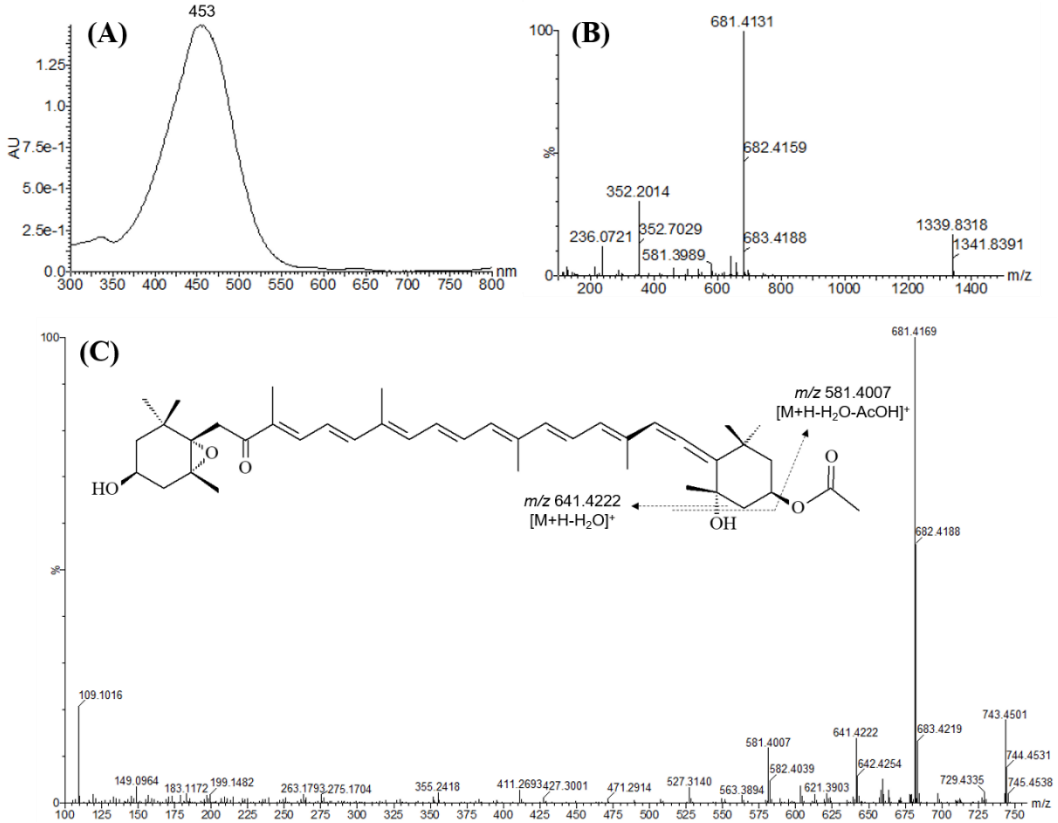

Fig. 3. UV, MS (ESI^+) and MS/MS (ESI^+) spectra (A, B and C, respectively) of fucoxanthin identified in Tl-EtOH (peak 1, Fig. 2).

Table 2. Pigments identified in *T. lutea* extract (Tl-EtOH) by UPLC-DAD-MS/MS analysis. Peak numbering according to Fig. 2.

Peak	Pigment	RT (min)	Molecular formula	λ_{max} (nm)	Band III/II ratio (%)	Experimental *m/z* (Δ, ppm)			MS^2 fragments *m/z*
						$M^{\cdot+}$	$[M+H]^+$	$[M+Na]^+$	
1	Fucoxanthin	5.51	$C_{42}H_{58}O_6$	453	-	-	-	681.4131 (0.0)	641.4222, 581.4007
2	Chlorophyll c_2	6.24	$C_{35}H_{28}O_5N_4Mg$	452, 586, 635	-	-	609.1971 (2.8)	631.1799 (2.5)	549.1777
3	Chlorophyll c_1	6.49	$C_{35}H_{30}O_5N_4Mg$	453, 584, 639	-	-	611.2125 (3.3)	633.1941 (3.6)	551.1903
4	Diadinoxanthin	11.56	$C_{40}H_{54}O_3$	425, 445, 476	61.11	582.4072 (0.2)	-	-	567.3815, 490.3434
5	Diadinochrome	13.52	$C_{40}H_{54}O_3$	408, 429, 456	95.65	582.4074 (0.2)	-	605.3972 (0.2)	502.3440, 221.1538, 181.1233
6	Fucoxanthin isomer	14.4	$C_{42}H_{58}O_6$	453	-	658.4225 (1.2)	-	681.4126 (1.0)	527.3135, 467.2901
7	Diadinochrome isomer	15.99	$C_{40}H_{54}O_3$	408, 429, 456	73.68	582.4073 (0.0)	-	605.3971 (0.0)	502.3439, 221.1540, 181.1221
8	Diatoxanthin	17.85	$C_{40}H_{54}O_2$	427, 451, 480	33.33	566.4128 (0.7)	-	689.4018 (0.0)	474.3496, 119.0862
9	3-hydroxy-echinenone	31.82	$C_{40}H_{54}O_2$	456	-	566.4124 (0.0)	-	589.4020 (0.3)	474.3490, 209.1338, 119.0863
10	Echinenone	34.74	$C_{40}H_{54}O$	465	-	550.4179 (0.7)	-	573.4070 (0.4)	458.3551, 203.1428, 119.0852
11	Chlorophyll *a*	36.49	$C_{55}H_{72}O_5N_4Mg$	431, 665	-	892.5333 (2.2)	-	915.5225 (2.8)	614.2379 555.2235, 481.1874
12	Chlorophyll *a* epimer	37.95	$C_{55}H_{72}O_5N_4Mg$	430, 665	-	892.5334 (2.1)	-	-	614.2377, 555.2289, 481.1897

13	Chl c_2-MGDG [18:4/14/0]	39.12	$C_{76}H_{96}O_{14}N_4Mg$	458, 590, 635	-	-	1313.6840 (0.9)	-	549.4880
14	Hydroxy-pheophytin *a*	41.48	$C_{55}H_{74}O_6N_4$	405, 508, 534, 663	-	-	887.5660 (3.0)	-	609.2703
15	Hydroxy-pheophytin *a* epimer	41.95	$C_{55}H_{74}O_6N_4$	407, 504, 533, 666	-	-	887.5662 (2.8)	-	609.2719
16	β,β-Carotene	42.60	$C_{40}H_{56}$	422, 448, 472	46.67	536.4390 (1.5)	-	-	444.3734
17	Pheophytin *a*	43.16	$C_{55}H_{74}O_5N_4$	408, 504, 535, 665	-	-	871.5724 (1.5)	-	593.2762, 533.2554,
18	Pheophytin *a* epimer	43.98	$C_{55}H_{74}O_5N_4$	408, 507, 538, 666	-	-	871.5721 (1.8)	-	593.2762, 533.2554,

RT: retention time. Chl c_2-MGDG: chlorophyll *c*2-monogalactosyldiacylglyceride ester [18:4/14:0].

Echinenone and 3-hydroxy-echinenone were also identified in Tl-EtOH. The retention time of 3-hydroxy-echinenone (peak 9) was significantly lower than that of echinenone (peak 10) in accordance with a gain of polarity due to hydroxylation (Fig. 2). After comparing the chromatographic and spectral data, we found that these two pigments corresponded to the unidentified carotenoids Car43 and Car48 respectively, previously described by our research group in the strain T-*Iso* CCAP 927/14 [7].

Interestingly, another non-described pigment for *T. lutea* was detected in the Tl-EtOH chromatogram (Fig. 2). Peak 13, eluting at 39.12 min, showed a characteristic chlorophyll *c* UV spectrum, with three maximum absorption bands at 458, 590 and 635 nm. Its retention time was shifted compared to chlorophylls c_2 and c_1 (6.24 and 6.49 min, respectively), corresponding to a chromatographic behavior typical of a non-polar chlorophyll *c* derivative [40,41]. Its high-resolution mass spectrum revealed a peak at *m/z* 1313.6840, corresponding to the protonated adduct $[M+H]^+$ for the molecular formula $C_{76}H_{96}O_{14}N_4Mg$ (Table 2). The fragmentation pattern at *m/z* 549.4880 indicated a consistent similarity for a chlorophyll c_2 derivative. Taken together and in comparison with literature data [42], these results allowed the identification of peak 13 as Chl c_2-MGDG (chlorophyll c_2-monogalactosyldiacylglyceride ester [18:4/14:0]) (Fig. 4).

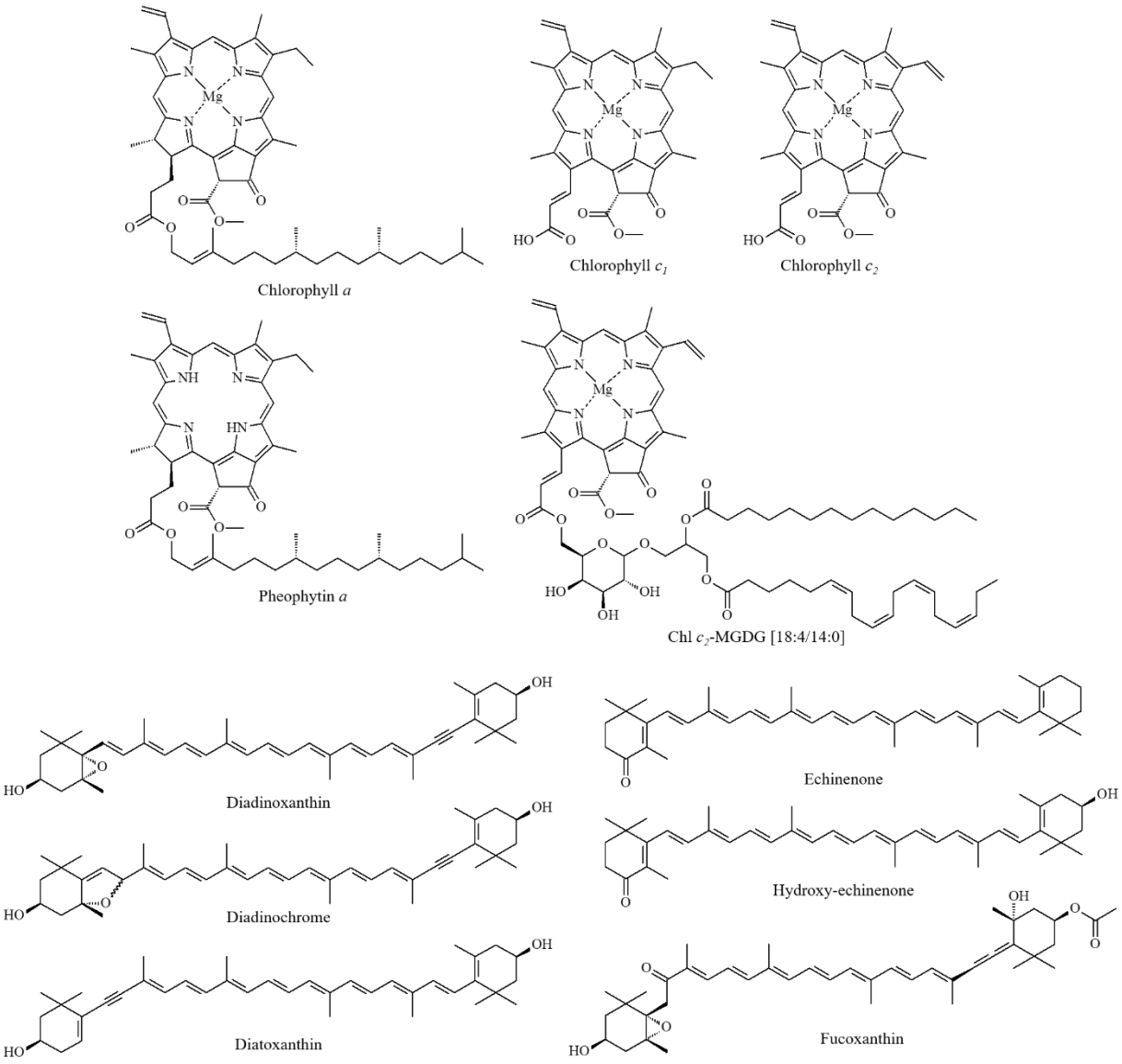

Fig. 4. Chemical structures of the main pigments found in Tl-EtOH.

3.3. Physiological and chemotaxonomic relevance of T. lutea pigments composition

Previously named *Isochrysis* affinis *galbana* (T-*Iso*), *T. lutea* (T-*Iso* CCAP 927/14) contains chlorophylls *a*, c_1 and c_2, fucoxanthin and β,β-carotene as light-harvesting pigments for photosynthesis. Diatoxanthin and diadinoxanthin are photoprotective compounds involved in heat dissipation and protection of photosystem from harmful effects of lights [42]. Based on their pigment profile, *T. lutea* and *I. galbana* have been grouped in a taxonomic cluster named HAPTO-3 [7,42], characterized by the presence of common carotenoids (diatoxanthin, diadinoxanthin and fucoxanthin) [7,39]; however two previously unidentified carotenoids were only detected in *T. lutea* (T-*Iso* CCAP 927/14) [7]. In this work, we identified these metabolites, previously named Car43 and Car48, as 3-hydroxy-echinenone and echinenone, respectively, in

line with data reporting their occurrence in T-*Iso* cultivated under nitrogen, sulfur and magnesium-limited conditions [43]. As echinenone and 3-hydroxy-echinenone are absent in *I. galbana*, they can be considered as robust chemotaxonomic markers for *T. lutea*, allowing species differentiation through pigment analysis without the need for genetic analysis required by their morphological similarities [1].

In this study, we also describe for the first time the identification of a non-polar chlorophyll c_2 derivative (Chl c_2-MGDG [18:4/14:0]) in *T. lutea*. Haptophytes may contain several kinds of chlorophyll *c*, notably chlorophylls c_1, c_2 and c_3. Chlorophyll c_2 is the most common type encountered in haptophytes species and some non-polar derivatives have been reported [34,41,44]. Phytylated chlorophyll *c*-like derivatives have been described in *Emiliania huxleyi*, *Prymnesium parvum* and *Isochrysis galbana* [45,46]. Chlorophyll c_2-monogalactosyldiacylglyceride are also common in haptophytes. A high-molecular weight chlorophyll c_2-galactolipid was isolated from *Chrysochromulina polylepis* (now renamed *Prymnesium polylepis* CCMP 286) containing two myristic acid residues (Chl c_2-MGDG [14:0/14:0]) [41]. Chl c_2-MGDG [18:4/14:0] containing one octadecatetraenoic acid (18:4) and one myristic acid (14:0) unit was reported as a novel marker in *E. huxleyi* [40], but it was also detected in other haptophyte species such as *P. parvum*, *Ochrosphaera neopolitana* and *Phaeocystis antartica* [34]. In the same study, Chl c_2-MGDG [18:4/14:0] was also identified as a marker of *I. galbana* strains (haptophyte pigment type 3). Our data now prove that this non-polar chlorophyll c_2 derivative is also present in *T. lutea*, confirming its wide distribution in the haptophyte group.

The occurrence of non-polar chlorophyll *c* derivatives has been used in oceanographic investigations as a chemical indicator for the presence of haptophytes in natural waters [47]. Particularly, the identification of metabolites containing a galactolipid linked to a pigment can be considered as a highly discriminant chemotaxonomic signature for the rapid and accurate identification of species or taxonomic groups within a mix of natural phytoplankton populations [40]. In addition to their chemotaxonomic interest, these molecules also ensure a fundamental biochemical role in microalgae. Chl c_2-MGDG [18:4/14:0] could indeed have a light-harvesting function, participate to the assembly of light-harvesting complexes [48] and act as a transporter of chlorophyll c_2 from the MGDG-rich lipid bilayer located in the inner chloroplast envelope membrane to its final location in the light-harvesting pigment complexes of the thylakoids [34,49]. A complete taxonomic analysis for pigments found in Tl-EtOH is given in Table 3.

Table 3. Overview and taxonomic importance of pigments identified in *T. lutea* extract (Tl-EtOH) for the haptophytes.

Peak	Pigment	Taxonomic coherence	Reference
1	Fucoxanthin	Extensively reported in haptophytes, notably in HAPTO-3	[7,34,36,41,42]
2	Chlorophyll c_2	Extensively reported in haptophytes	[7,34,42,46]
3	Chlorophyll c_1	Extensively reported in haptophytes	[7,34,42,46]
4	Diadinoxanthin	Extensively reported in haptophytes, notably in HAPTO-3	[7,34,41,42]
5	Diadinochrome	Produced by diadinoxanthin rearrangement in weakly acid solutions	[7,34,41,42]
6	Fucoxanthin isomer	Isomerization of fucoxanthin	[7,42]
7	Diadinochrome isomer	Isomerization of diadinochrome	[24,42]
8	Diatoxanthin	Extensively reported in haptophytes, notably in HAPTO-3	[7,34,41,42]
9	3-hydroxy-echinenone	Reported in T-*Iso* under nutrient-depleted conditions; not reported in *I. galbana*	[43]
10	Echinenone	Reported in T-*Iso* under nutrient-depleted conditions; not reported in *I. galbana*	[43]
11	Chlorophyll *a*	Ubiquitary in photosynthetic algae	[7,24–26,42]
12	Chlorophyll *a* epimer	Epimerization of Chl *a*	[24,25,42]
13	Chl c_2-MGDG [18:4/14/0]	Reported in haptophytes such as *I. galbana*, *E. huxleyi*, *P. parvum*, *O. neopolitana* and *P. antartica*	[34,40,42]
14	Hydroxy-pheophytin *a*	Hydroxylation of pheophytin *a* during extraction	[24,25,42]
15	Hydroxy-pheophytin *a* epimer	Epimerization of hydroxy-pheophytin *a*	[24,25,42]
16	β,β-Carotene	Ubiquitary in photosynthetic algae	[24–26,42]
17	Pheophytin *a*	Pheophytination of Chl *a* during extraction	[25,42]
18	Pheophytin *a* epimer	Epimerization of pheophytin *a*	[24,25,42]

HAPTO-3: taxonomic group of haptophytes according to [42] and [7]. Chl c_2-MGDG: chlorophyll *c*2-monogalactosyldiacylglyceride ester [18:4/14:0].

3.4. Fucoxanthin purification procedure

3.4.1. CPC fractionation of *T. lutea* ethanolic extract

Besides the optimization of microalgae culture conditions and extraction, the development of efficient purification methods is needed in order to reduce the isolation costs and the deleterious environmental impact. CPC, when rationally used, appears as an efficient eco-friendly alternative for metabolites isolation or concentration. In our case, we decided to use this technique during a first step of fractionation aiming to quickly access to enriched fractions.

The determination of an appropriate solvents system was at first carried out by shake-flask method. Considering the low polarity of the targeted compound, the tested biphasic mixtures were of two types, at first derived from Arizona (cyclohexane, ethyl acetate, methanol, water), then composed of cyclohexane, methanol with a small proportion of water. After TLC examination, these ternary mixtures seemed to be more convenient and were chosen for *K* values calculation. They all were quite close, between 0.93 with 1.77 (Table 1). Other criteria were then examined, duration of emulsion after addition of extract and flask-shaking as well as relative volumes of upper and lower phases. System 1 was ruled out because the emulsion lasted longer than for other systems (45 s). For systems 2-5, these durations were similar (approximately 20 s). System 2 was finally selected because the relative volume of upper/lower phases (ratio 2/3) were the most satisfactory for further use and save (filling rotor with upper phase, equilibration and elution with lower phase, extrusion with upper phase) without having to prepare another volume of solvents mixture.

Fucoxanthin is a relatively non-polar metabolite. In order to obtain good retention and quality of separation, descending mode with the more polar lower phase as the mobile one was chosen to run the experiment.

After collection in descending mode, 16 pooled fractions were obtained (F1 to F16) on the basis of TLC profile, where an additional spot corresponding to a standard of fucoxanthin was added (Fig. 5). F1 to F5 looked like an orange amorphous paste, indicating a high content of carotenoids. After UPLC-DAD analysis, F2 (66.7 mg), F3 (50.7 mg) and F4 (61.6 mg) were found to contain 24.8, 65.7 and 36.1% (w/w) of fucoxanthin, respectively (Table 4, Fig. 6). The fucoxanthin content of previous and following fractions decreased abruptly (1.8% and 1.9% for F1 and F5, respectively), assessing the efficiency of the selected solvents system. The major impurities were identified as chlorophylls c_1 and c_2. Some fractions also contained minor carotenoids, such as diadinoxanthin, diatoxanthin and diadinochrome. However, non-polar pigments (e.g. chlorophyll *a*, pheophytin *a*, echinenone, 3-hydroxy-echinenone, Chl c_2-MGDG

[18:4/14/0], β,β-carotene and derivatives) were not detected in fractions F1 to F5, suggesting that a single CPC run was able to separate polar from non-polar pigments, resulting in fucoxanthin-enriched fractions.

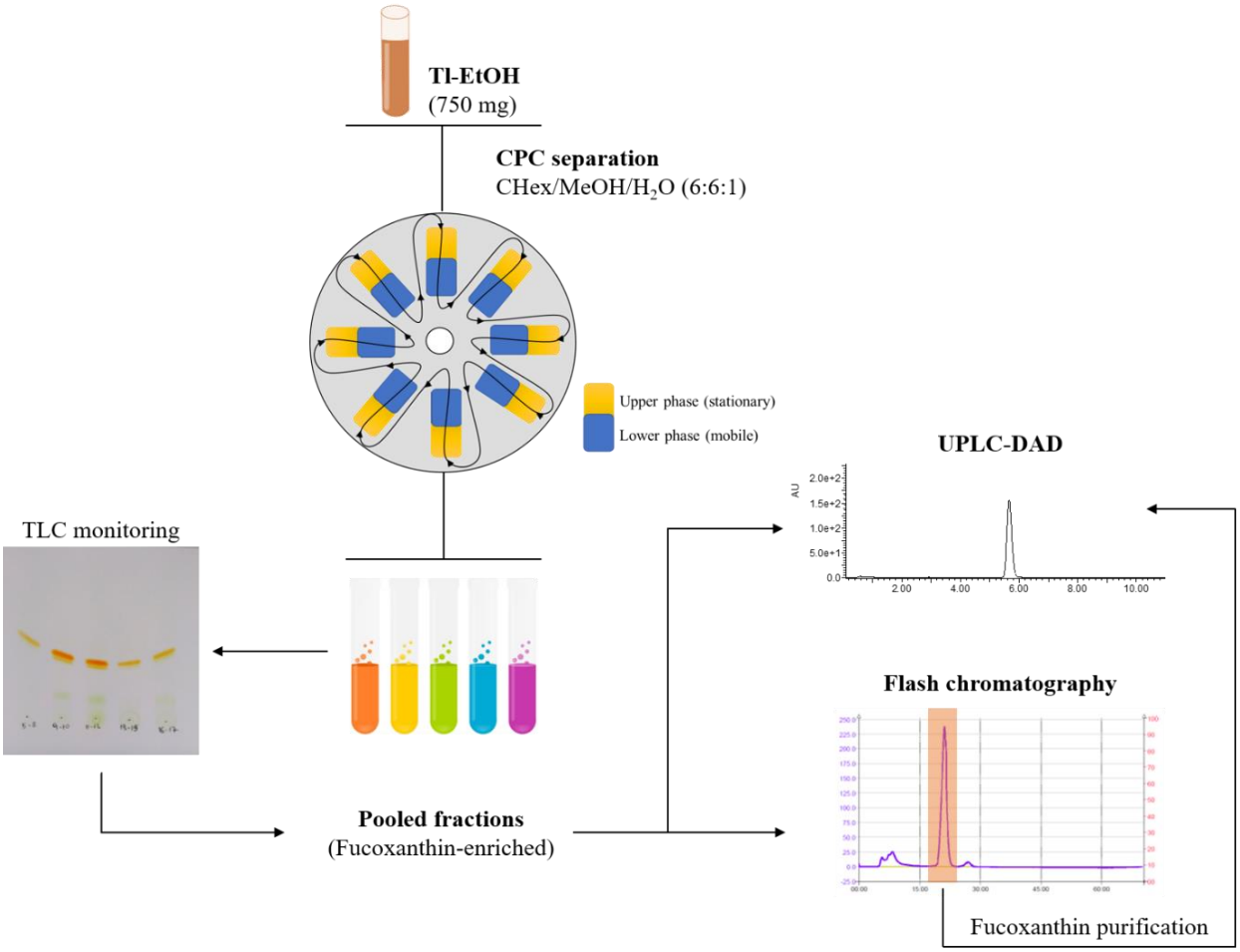

Fig. 5. Flowchart of the CPC-concentration and flash chromatography purification of fucoxanthin from *T. lutea* ethanol extract (Tl-EtOH). CPC fractions were monitored by TLC and UPLC-DAD, final yield and purity were determined by UPLC-DAD.

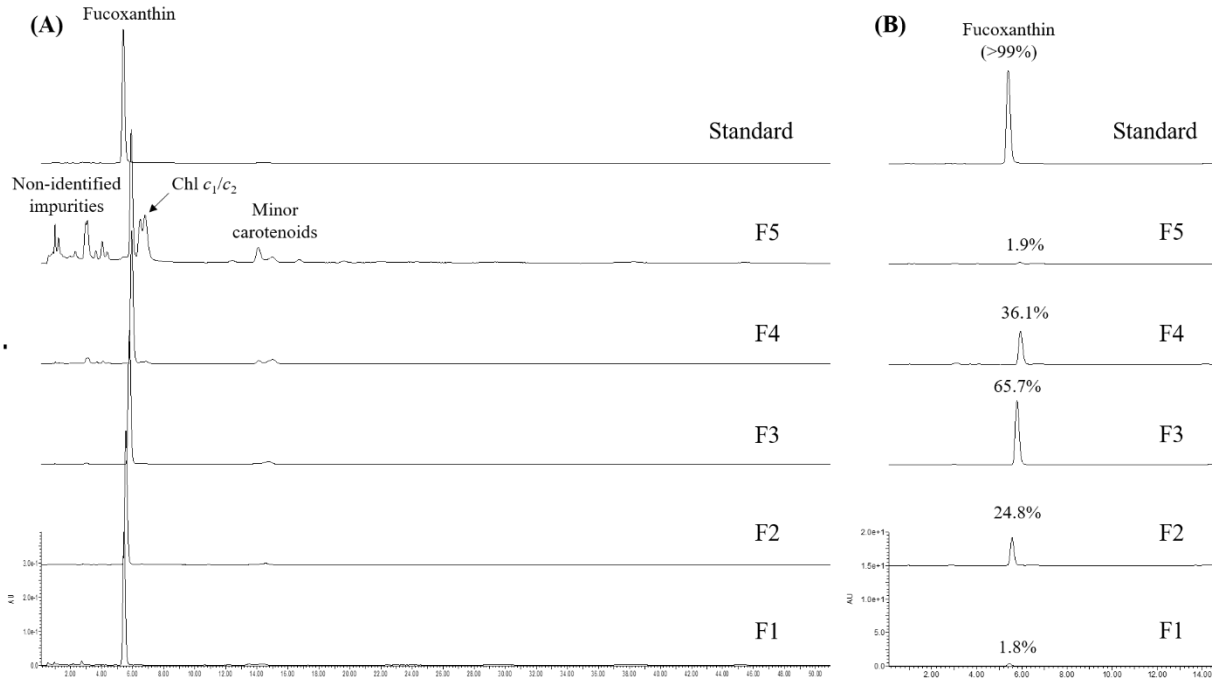

Fig. 6. (A) UPLC-DAD chromatograms at 450 nm of fucoxanthin-enriched fractions (F1 to F5) obtained after CPC separation. (B) Fucoxanthin content (%, w/w) was calculated after normalization of data *vs* a calibration curve obtained with a commercial standard (purity>99%, Sigma-Aldrich®). All measurements were performed in triplicate.

3.4.2. Flash chromatography isolation of fucoxanthin

Due to their close polarity, the solvents system of the CPC procedure was not able to completely separate fucoxanthin from chlorophylls c_1 and c_2. Thus, in order to increase the purity of fucoxanthin-enriched fractions, F1 to F5 were subjected to a final refining step by flash chromatography. The separation conditions were adapted from an optimized method previously reported for microalgae pigments purification [25]. After recovery as the major compound from fractions F1 to F4 (but undetectable from fraction F5 where chlorophylls were dominant) (Fig. 7), fucoxanthin was further quantified by UPLC-DAD, confirming its high purity (>99%) when isolated from F2, F3 and F4 (Fig. 8). Retention time and UV absorption spectrum were in accordance with those observed for the analytical standard.

Recovered fucoxanthin amounts (Table 4) were proportionally higher than those reported in previous studies involving the use of CPC or high-speed countercurrent chromatography (HSCCC) using macroalgae such as *L. japonica, U. pinnatifida, S. fusiforme* or *E. bicyclis* as starting material [23,50]. Purification of fucoxanthin from *L. japonica, U. pinnatifida, S. fusiforme* using HSCCC has provided yields varying from 1.13 to 74 mg/100 g of extract (purity index from 86.8 to 94.8%) [50]. In another study, CPC was used for fucoxanthin purification from *E. bicyclis*, providing a yield of 33.3 mg/100 g of extract (purity

index = 98%) [23]. In the present work, fucoxanthin recovery was significantly higher compared to these previous investigations (1,186.7 mg/100 g of extract, purity index > 99%). However, it is important to note that the employed extraction methods (microwave-assisted extraction or maceration) and solvents (ethanol or acetone) were not the same, which may also interfere with the purification yields of the compound.

Considering that the separation conditions can be further optimized, these first results validate the rational use of CPC as an efficient eco-friendly method for the high yield extraction and pre-purification of fucoxanthin from microalgae.

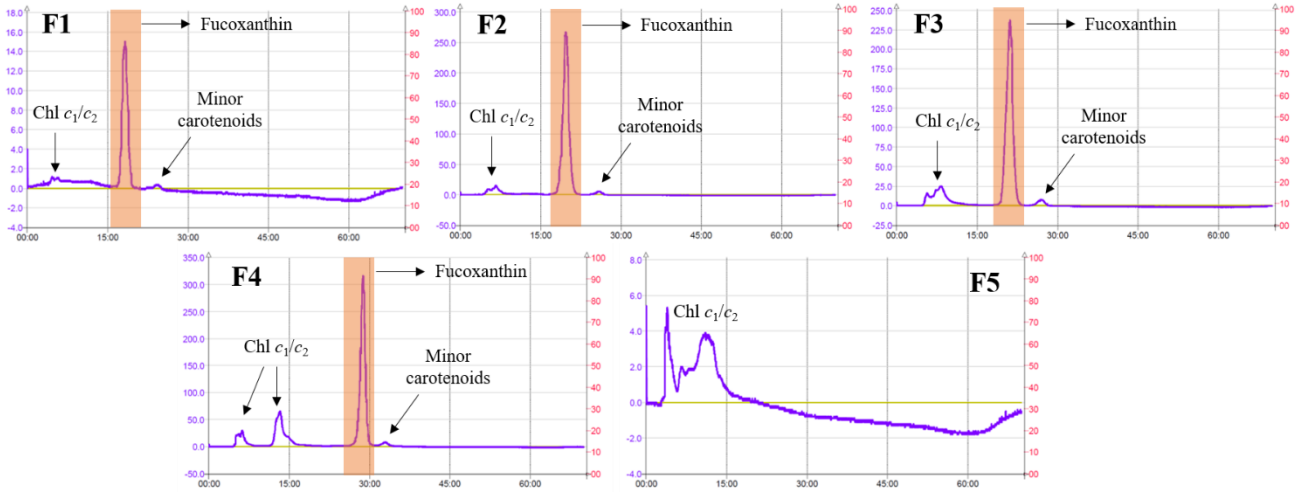

Fig. 7. Flash chromatography of F1 to F5. Purification was monitored at 450 nm. The peak corresponding to fucoxanthin (highlighted in orange) was collected for further analysis by UPLC-DAD.

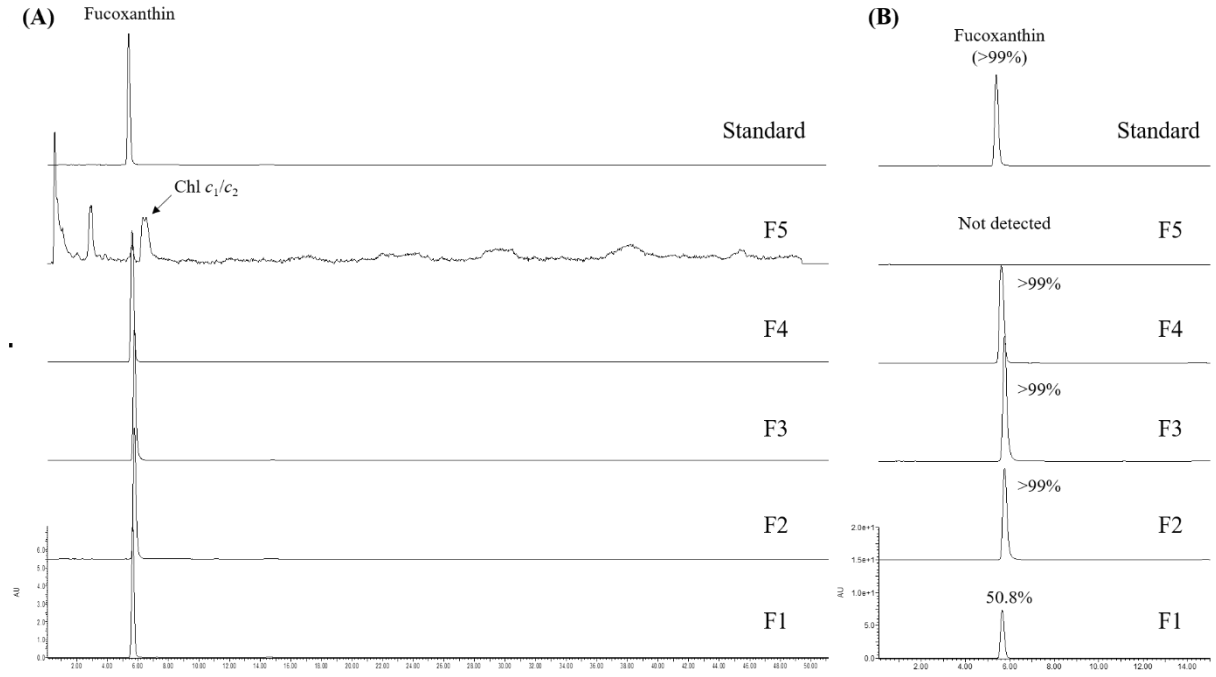

Fig. 8. (A) UPLC-DAD chromatograms at 450 nm of fucoxanthin-enriched fractions (F1 to F5) obtained after final flash chromatography of CPC pooled fractions. (B) Fucoxanthin purity (%, w/w) was calculated after normalization of data *vs* a calibration curve obtained with a commercial standard (purity>99%, Sigma-Aldrich®). All measurements were performed in triplicate.

Table 4. Fucoxanthin content (mass and massic percentage) in CPC pooled fractions and after final purification by flash chromatography. Fucoxanthin dosage was performed in triplicate by UPLC-DAD analysis at 450 nm, using a commercial standard (purity >99%, Sigma-Aldrich®) (R^2=0.9994).

CPC separation (injection of 750 mg Tl-EtOH)			**Flash chromatography purification (fucoxanthin peak collection)**		
CPC fraction	Dried mass (mg)	Fucoxanthin content (%, w/w)	Injected dried mass (mg)	Recovered fucoxanthin (mg)	Fucoxanthin purity (%, w/w)
F1	56.4	1.8	22.6	0.5	50.8
F2	66.7	24.8	48.7	2.9	>99
F3	50.7	65.7	35.7	2.7	>99
F4	61.6	36.1	51.2	3.3	>99
F5	28.5	1.9	22.9	-	-

3.5. Chemosensitizing activity of T. lutea purified fucoxanthin in melanoma cells

A large number of *in vitro* and *in vivo* studies have established the high cytotoxic, cytostatic, antimetastatic and antiangiogenic activities of fucoxanthin in various tumor models, including melanoma [10,51]. This compound was also combined to anticancer drugs to improve their cytotoxicity [15,16]. In coherence with this strategy, we recently highlighted that some carotenoids and apocarotenoids from algae and plants not only induce cytotoxicity in melanoma cells, but also sensitize them to the cytotoxic effect of chemotherapy, improving the effectiveness of anticancer drugs or reversing their chemoresistance [25,29,52]. As fucoxanthin chemosensitizing activity in melanoma cells was never assessed before, we evaluated its ability, after purification from *T. lutea*, to sensitize A2058 human melanoma cells to the cytotoxic effect of dacarbazine and vemurafenib, two drugs used for the clinical treatment of metastatic melanoma.

Fucoxanthin induced a concentration-dependent growth inhibition, with an IC_{50} calculated to 14.67 µM (Fig. 9A). Photomicrographs of control and treated cells demonstrated

a moderate dose-dependent reduction in cell density, confirming an antiproliferative activity (Fig. 9D).

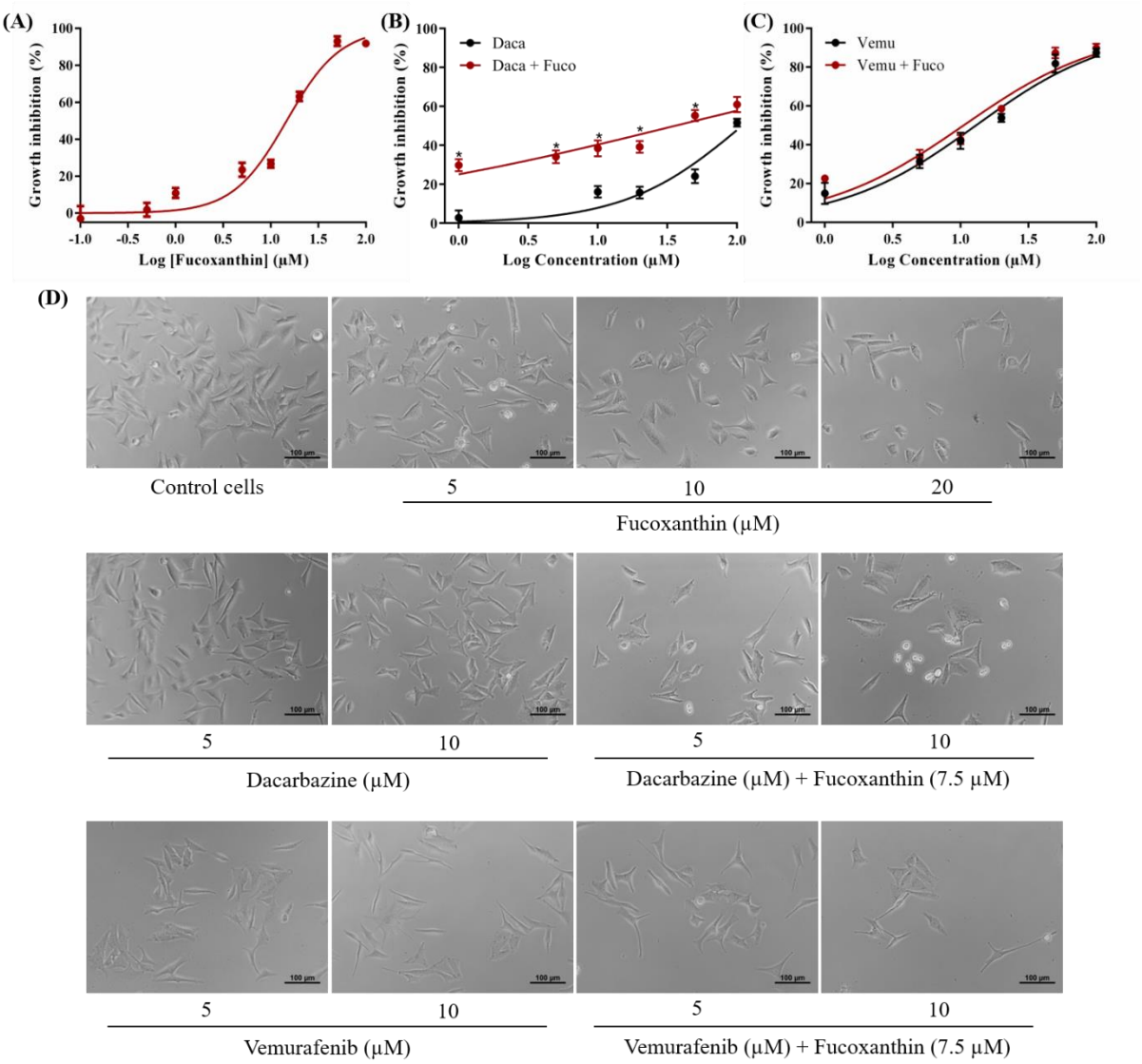

Fig. 9. Antiproliferative activity of fucoxanthin (A), dacarbazine (Daca), vemurafenib (Vemu) and the respective combined treatments (B and C) in the MTT assay. A2058 cells were grown for 72h with increasing concentrations of the antimelanoma drugs (1–100 µM) in the absence or presence of fucoxanthin (½IC_{50}, 7.5 µM). Photomicrographs (D) show reduction of cell density promoted by combined therapies compared to monotherapies and control untreated cells. Data are presented as mean ± SEM, *p<0.05 according to unpaired Student's t test, from at least three measurements.

To assess a chemosensitizing potential, cells were exposed to fucoxanthin combined to vemurafenib or dacarbazine. Vemurafenib (Vemu) is a BRAF inhibitor recently introduced for the targeted therapy of advanced melanoma, while dacarbazine (Daca) is an alkylating agent

classically used in combination with other anticancer drugs for the treatment of metastatic melanoma. In a first step, cells were treated with increasing concentrations of vemurafenib or dacarbazine (1-100 µM) for 72h, then cell viability was determined by the MTT assay. A2058 cells were sensitive to vemurafenib (IC_{50} = 12.97 µM), but resistant to dacarbazine (IC_{50} > 100 µM) (Fig. 9 and Table 5). Then, combined treatments were applied. When associated to dacarbazine, fucoxanthin at its half IC_{50} (7.5 µM) restored cell sensitivity to this alkylating agent, reducing its IC_{50} value to 35.74 µM. Photomicrographs also showed an important decrease in cell density compared to dacarbazine treatment alone and relevant morphological changes induced by combined therapy, such as cell shrinkage and rounding. In contrast, no significant enhanced antiproliferative effect was observed with the combination Fuco + Vemu (Table 5).

Table 5. Antiproliferative activity of fucoxanthin (Fuco), vemurafenib (Vemu), dacarbazine (Daca) and combined treatments (Vemu + Fuco and Daca + Fuco). Data are expressed as IC_{50} values and 95% confidence interval, from at least three measurements (n=3). For combined treatments, increasing concentrations of vemurafenib or dacarbazine (1–100 µM) were associated to ½IC_{50} of fucoxanthin (7.5 µM).

Monotherapy	**IC_{50} (µM)**	**Combined treatment**	**IC_{50} (µM)**
Fuco	14.67 (12.78 – 16.84)	-	-
Vemu	12.97 (10.66 – 15.78)	Vemu + Fuco	10.51 (8.24 – 12.52)
Daca	> 100	Daca + Fuco	35.74 (22.47 – 56.83)

Combination indexes (CI) were calculated using the Chou-Talalay method in order to characterize the chemosensitizing effect of fucoxanthin [30] as additive (CI = 1), synergistic (CI < 1) or antagonistic (CI > 1) on the antiproliferative activity of vemurafenib or dacarbazine. Fuco + Daca treatment resulted in an additive effect at 10 and 20 µM dacarbazine, and synergistic behavior at 50 and 100 µM dacarbazine. In contrast, Fuco + Vemu association promoted antagonistic effects at almost all doses, except at 50 and 100 µM vemurafenib where the effect was considered as synergistic (Fig. 10). These data suggest that fucoxanthin reversion of dacarbazine chemoresistance in A2058 melanoma cells may be linked to the facilitation of dacarbazine access to the intracellular environment that could stimulate dacarbazine-induced DNA-alkylation. For example, an integration of fucoxanthin in the cytoplasmic membrane [53] may facilitate the penetration of the alkylating agent. These data are in agreement with the previously reported chemosensitizing activity of fucoxanthin in other tumor cell lines [15,16].

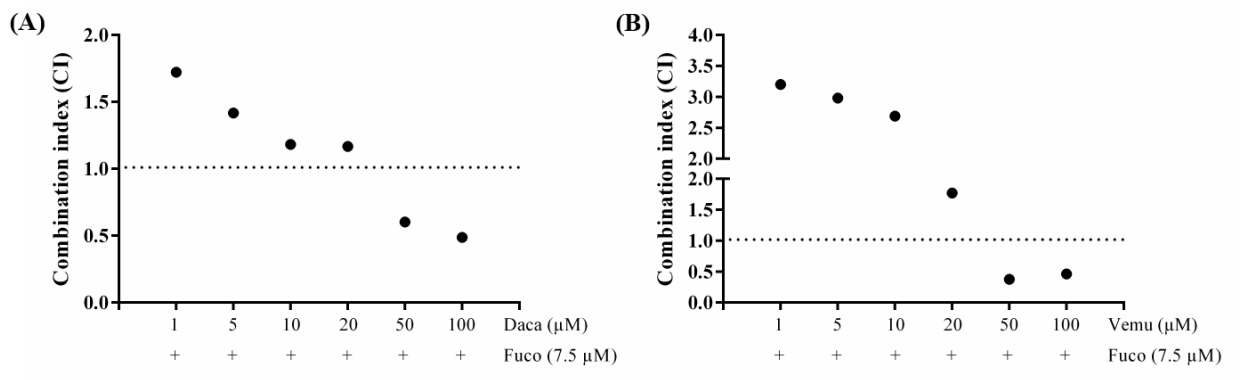

Fig. 10. Combination index (CI) calculated for the association of fucoxanthin (Fuco) with dacarbazine (Daca, A) and vemurafenib (Vemu, B) in the MTT assay. CI = 1 indicates additive effect, CI < 1 indicates synergistic effect, while CI > 1 indicates antagonistic effect according to Chou-Talalay method [30].

4. Conclusion

In summary, this study has described the complete pigments profile of *T. lutea* extract, including fucoxanthin as its major metabolite and two previously mentioned but unidentified carotenoids of taxonomic relevance, echinenone and 3-hydroxy-echinenone. A nonpolar chlorophyll c_2 derivative (Chl c_2-MGDG [18:4/14:0]) was also characterized for the first time in *T. lutea*. This outcome provides valuable chemotaxonomic information and increases the diversity of chlorophyll patterns for the Haptophyta phylum. Moreover, an off-line coupling CPC-flash chromatography was successfully applied to purify fucoxanthin, which exhibited promising chemosensitizing potential in treatment of melanoma cells. Although further process optimization remains desirable, this is the first report of isolation of this compound from microalgae using a step of CPC enrichment, which may be useful to its industrial scale production.

Acknowledgements and conflicts of interest

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(PAHRB, La Rochelle University) for the scientific and technical contribution to this paper. The authors declare no conflict of interest.

Statement of Informed Consent, Human/Animal Rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Credit Author Contributions Statement

RGOJ (PhD student), LP, RG, AJ (Postdoc) and JR (Postdoc) were in charge of bibliographic search, CPC and flash chromatography experiments and cell culture experiments. EN was responsible for microalgae growth in photobioreactor and biomass collection. RG, PEB and AB performed the High-Resolution Mass Spectrometry analysis. RGOJ and LP wrote the manuscript. LP obtained the research funding, supervised the work and takes full responsibility for the scientific integrity of the work. All authors have read the final manuscript and approved the submission.

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