Effect-directed analysis reveals inhibition of zebrafish uptake transporter Oatp1d1 by caulerpenyne, a major secondary metabolite from the invasive marine alga *Caulerpa taxifolia*

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Abstract

Caulerpa taxifolia is a marine alga of tropical and subtropical distribution and a well-known invasive species in several temperate regions. Its invasiveness mainly stems from the production of secondary metabolites, some of which are toxic or repellent substances. In this study we investigated the possible inhibitory effects of *C. taxifolia* secondary metabolites on the activity of two zebrafish (*Danio rerio*) uptake transporters that transport organic anions (Oatp1d1) and cations (Oct1). Both transporters were transiently transfected and overexpressed in human embryonic kidney HEK293T cells. Transport activity assays using lucifer yellow (LY) and 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) as model substrates were applied for the determination of Oatp1d1 and Oct1 interactors. A two-step Effect-Directed Analysis (EDA) procedure was applied for the separation and identification of compounds. We identified caulerpenyne (CYN) as the major metabolite in *C. taxifolia* and reveal its potent inhibitory effect towards zebrafish Oatp1d1 as well as weak effect on zebrafish Oct1 transport. The observed effect was confirmed by testing CYN purified from *C. taxifolia*, resulting in an IC_{50} of 17.97 µM, and a weak CYN interaction was also determined for the zebrafish Oct1 transporter. Finally, using Michaelis-Menten kinetics experiments, we identified CYN as a non-competitive inhibitor of the zebrafish Oatp1d1. In conclusion, this study describes a novel mechanism of biological activity in *C. taxifolia*, shows that CYN was a potent non-competitive inhibitor of zebrafish Oatp1d1, and demonstrates that EDA can be reliably used for characterization of environmentally relevant complex biological samples.

Keywords: *Caulerpa taxifolia*, caulerpenyne, Oatp1d1, Oct1, zebrafish, effect-directed analysis (EDA)

1. Introduction

Some marine green algae belonging to the genus *Caulerpa* are well-known invasive species. Among them, *Caulerpa taxifolia* (M. Vahl) C. Agardh has been described as one of the most invasive in several temperate regions due to its tendency to spread rapidly and negatively impact different ecosystems (Boudouresque et al., 1992, 1995; Meinesz, 1999). Since it was first discovered outside its native habitat in 1984 near the Oceanographic Museum in Monaco, *C. taxifolia* has been found throughout the Mediterranean, California and southeastern Australia (Meinesz et al., 2001; Schaffelke et al., 2002; Creese et al., 2004). After 2009, a significant decline in the abundance of *C. taxifolia* meadows was recorded at most of the invaded areas, probably due to large annual changes in the temperature of the Mediterranean Sea, as *C. taxifolia* exhibits greater sensitivity to colder temperatures (Tejada and Sureda, 2013; Montefalcone et al., 2015). Nevertheless, this marine species still remains in the focus of research studies due to its unique characteristics and invasive potential.

The complex composition of secondary metabolites is thought to be one of the greatest advantages of the *Caulerpa* species, largely contributing to their invasive behavior. Most have been shown to be toxic or repulsive, and it is mainly believed that they serve the *Caulerpa* species as a chemical defense mechanism against herbivores and in interspecies competition (Klein and Verlaque, 2008). The diverse chemical structure of these metabolites includes mainly bisindole alkaloids, sesquiterpenoids and diterpenoids, with aldehyde and/or enol acetate functional groups (Guerrerio et al., 1992, 1993; Smyrniotopoulos et al., 2003). Various biological effects attributed to the functional groups of these compounds have been described, including antimicrobial, insecticidal, antifouling, ichthyotoxic, feeding deterrent, anti-inflammatory, cytotoxic, and growth regulatory properties (Paul and Fenical, 1982; Paul et al., 1987; Fischel et al., 1995; Smyrniotopoulos et al., 2003; de Souza et al., 2009; Alarif et al., 2010; Nagaraj and Osborne, 2014). Caulerpenyne (CYN), a unique acetylenic sesquiterpen, has been identified as a major secondary metabolite in *C. taxifolia* (Amico et al., 1978). Various minor metabolites have also been identified. They are produced during CYN transformation processes such as hydrolysis, degradation, deacetylation or oxidation (Guerriero et al., 1992; Lemée et al., 1993; Guerriero and D'Ambrosio, 1999). Toxic effects of secondary metabolites from the genus *Caulerpa* have been studied in the context of cellular detoxification pathways, especially their interaction with biotransformation processes (Schröder et al., 1998; Uchimura et al., 1999a, 1999b; Sureda et al., 2006, 2009; Feline et al., 2012; Tejada et al., 2013). Nevertheless, a mechanistic understanding of the toxic effects of secondary metabolites from the genus *Caulerpa* on the cellular and/or molecular level is still

lacking. It is therefore important to identify the biologically active substances responsible for the observed toxic effects and understand the mechanisms of their toxic action.

Effect-Directed Analysis (EDA) is a powerful multidisciplinary diagnostic tool developed in the field of environmental science. EDA combines the use of advanced chemical and biological methods to identify, characterize and prioritize the toxicants present in complex environmental matrices. Sometimes also called "effect-based" or "effect-oriented", it integrates stepwise fractionation procedures together with biotesting and chemical analyses to reduce sample complexity, detect toxic effects and ultimately identify specific chemicals of concern (Brack, 2003; Brack et al., 2003, 2005). So far the EDA approach has been exclusively utilized for the identification and evaluation of environmental contaminants present in non-biological complex environmental samples (sediment, surface water, wastewater, soil). This study is the first to use the EDA approach for the characterization of a complex biological sample.

One of the key factors of the cellular detoxification system are biological membranes, which play a vital role in the uptake and elimination of various endo- and xenobiotics (Simkiss, 1995). Uptake transporters are responsible for the uptake of these compounds into cells via membranes, contributing to the phase 0 of the cellular detoxification mechanism (Klassen and Lu, 2008; König et al., 2013). Organic Anion Transporting Polypeptides (OATPs), Organic Cation Transporters (OCTs), and Organic Anion Transporters (OATs) are major groups of polyspecific uptake transporters in the cell (Koepsell and Endou, 2004; König, 2011; Roth et al., 2012; Koepsell, 2013). However, despite their toxicological relevance in mammals, knowledge on the presence of polyspecific uptake transporters in non-mammalian organisms is scarce, and their potential ecotoxicological relevance is still poorly understood. We recently characterized the first uptake transporter of organic anions in zebrafish, named Oatp1d1, and demonstrated its interaction with various environmentally relevant compounds (Popovic et al., 2014). Continuing along this line of research, an uptake transporter of organic cations, Oct1, was also identified in zebrafish, and our first data indicated that Oct1 may function as an integral part of cellular defense against potentially hazardous organic endo- and xenobiotic cationic compounds through biliary and renal excretion (Mihaljevic et al., 2016).

Consequently, as the influence of secondary metabolites from the genus *Caulerpa* on the uptake phase of cellular detoxification has not been investigated, the primary goals of this study were (1) to determine the potential inhibitory effects of *C. taxifolia* secondary metabolites on the activity of two zebrafish uptake transporters: the anionic transporter Oatp1,

and the cationic transporter Oct1, and (2) to perform a preliminary identification of the biologically active compounds responsible for the observed interaction by applying the EDA approach. A modified EDA protocol using non-selective and non-target preparation of the samples along with specific biological assays was applied, allowing the identification of both the mechanism of toxic action and the chemical identity of toxic substances present in a highly complex biological material.

2. Material and Methods

2.1. Chemicals

Lucifer yellow (LY), 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+), Trypsin-EDTA solution and Hepes were purchased from Sigma-Aldrich, St. Louis, MO, USA. Dulbecco's Modified Eagle's Medium (DMEM) (Powder, High Glucose, Pyruvate), Fetal Bovine Serum (FBS) and Phosphate Buffer Saline (PBS) were purchased from Gibco Invitrogen, Life technologies, CA, USA. Silica gel, solvents and salts were purchased from Kemika, Zagreb, Croatia. Purified CYN standard was acquired from Dr. Philipe Amade (Equipe Molécules Bioactives, University of Nice-Sophia Antipolis, France).

2.2. Collection of *C. taxifolia* and sample extraction

The complete workflow of our EDA study is presented in Figure 1. In the following sections, we describe the critical steps used subsequently in this investigation. *C. taxifolia* sampling was done by scuba diving in September 2013 at Stari Grad Bay, Adriatic Sea, Croatia (43.1833°N, 16.5838°E) at depths ranging from 8-12 meters (a detailed image of the sampling site is provided in the Supplementary material, Fig. S1). After the collection, fronds and stolons of *C. taxifolia* were cleaned from sediment impurities and stored at -20°C. One day after, the algal material was transported by car to Zagreb (Ruđer Bošković Institute) in a cooling box. Upon arrival to the laboratory, the algal samples were immediately stored at -20°C until further extraction. For obtaining *C. taxifolia* extracts, 5 g of algal fronds were added into two 50-mL Falcon tubes (a total of 10 g) and dissolved in 30 mL of dichloromethane (DCM):2-propanol (PrOH) mixture (1:1). Samples were further homogenized using a Polytron homogenizer (PT 20 OD, Kinematic GmbH 600 Lucerne/Switzerland) for 1 min at a 0.5 power setting, and then manually mixed for 1 min, put on ice and additionally vortexed for 5 min. After this step, the samples were centrifuged (Universal 32 R, Hettich Zentrifugen, Germany) twice for 5 min at 2500 x *g* and the water/lipophilic layer was removed by pipetting. Supernatant was evaporated under a nitrogen

stream using a TurboVap system (Caliper Life Sciences, Hopkinton, MS, USA) at 45°C in order to obtain a dry residue. Dry residues of algal extracts (100 mg) were weighed and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 30 mg/mL. The concentrations of *C. taxifolia* extracts used for the final transport activity measurements ranged from 1.5 to 30 µg/mL and from 15 to 300 µg/mL for the Oatp1d1 and Oct1 transporters, respectively. These concentrations were selected following an initial experiment (data not shown) in which inhibitory effects on Oatp1 and Oct1 transport were evaluated within a wide concentration range (0.1-350 µg/mL).

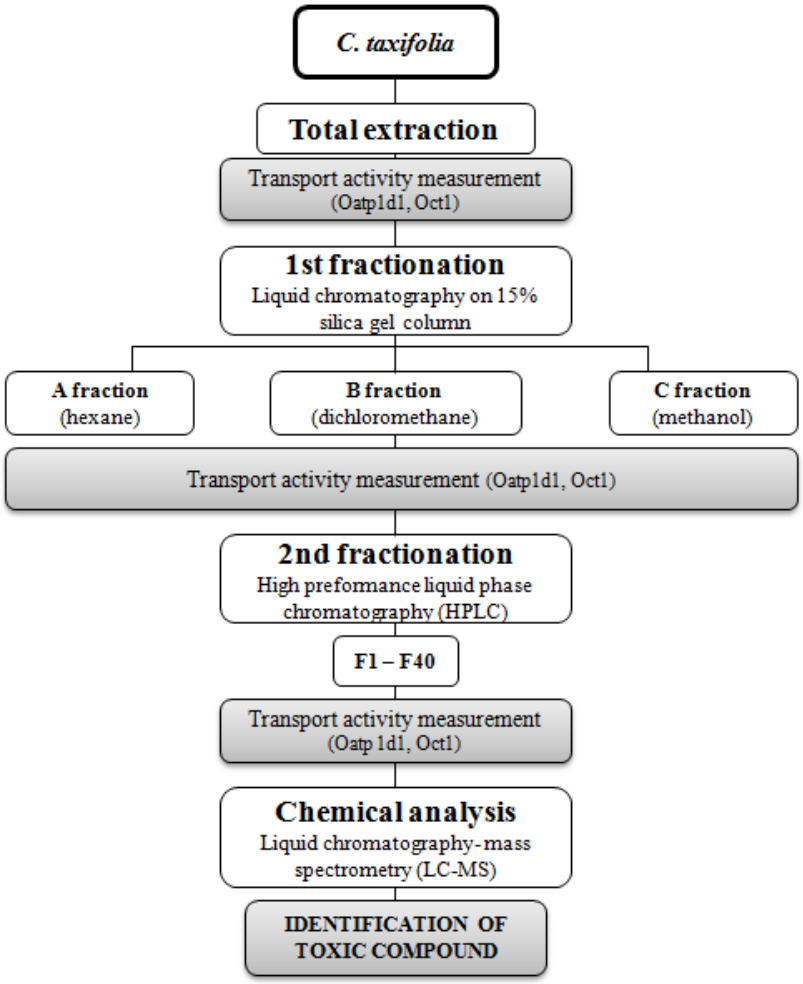


Fig. 1. Schematic presentation of the EDA workflow used for determination of interaction of *C. taxifolia* with the zebrafish uptake transporters (Oatp1d1, Oct1).

2.3. Two-step fractionation of the algal material

The initial separation of chemical compounds from the *C. taxifolia* extract was performed using column liquid chromatography (LC) with 15% silica gel in order to obtain three major

fractions (designated A, B and C) in the first fractionation step. This so-called ABC fractionation included the use of three different solvents – hexane (HEX), DCM and methanol (MeOH) – which enabled the separation of compounds according to their polarity, thus providing the A (non-polar), B (medium polar) and C (polar) fraction.

Upon the initial fractionation step, preparative high-performance liquid chromatography (HPLC) was used in the second fractionation step for further separation of algal B and C fractions into 40 sub-fractions. Details on the applied chemical fractionation of the algal material can be found in the Supplementary material.

2.4. Chemical analyses

The ABC fractions and selected HPLC sub-fractions that showed the most potent inhibition effect on the activity of the uptake transporters were further analyzed by highly sensitive analytical liquid chromatography coupled to high-resolution mass spectrometry (LC-MS). A Waters Acquity ultra-performance liquid chromatography (UPLC, Waters Corp., Milford, MA, USA) system coupled to hybrid quadrupole-time-of-flight/mass spectrometry (QTOFMS Premier, Waters Corp., Milford, MA, USA) equipped with an electrospray ionisation source was used to perform the separation and detection of present constituents (Terzic and Ahel, 2011). Nine B (B22-B30) and 13 C (C16-C28) sub-fractions of *C. taxifolia* obtained by HPLC were analyzed by LC-MS. The methodological details regarding chemical identifications are provided in the Supplementary material.

2.5. Caulerpenyne determinations

Stock solutions of purified CYN (10 mg) were prepared in MeOH for analytical LC-MS analysis (1 mg/mL) and bioassays (10 mg/mL) and stored at -80°C. For LC-MS analyses, the CYN standard was diluted 1:100 in MeOH, resulting in a 10 ng/µL CYN solution and 1% of DMSO was added to 1 mL of MeOH. A concentration response series was used to test the effect of the purified CYN on zebrafish Oatp1d1 and Oct1 transporter activity within the range of 0.03-270 µM.

2.6. Transport activity measurement

The inhibition of transport activity was determined in the transiently transfected human embryonic kidney HEK239T cell line overexpressing either the Oatp1d1 or the Oct1 uptake transporter cloned from zebrafish liver (Popovic et al., 2013; Mihaljević, 2015). The inhibition effect of *C. taxifolia* preliminary extracts and fractions was determined according to

the transport activity assay protocols described in detail by Popovic et al. (2013), using LY as a zebrafish Oatp1d1 and ASP+ as a zebrafish Oct1 model substrate. HEK293T cells were maintained in DMEM growth medium (pH 7.4) with high glucose and 10% FBS at 37°C and 5% CO_2 humidity. Forty-eight hours before transfection, HEK293T cells were seeded to 48-well plates with density between 1.9 and 2.0 x 10^5 cells/mL in a final volume of 250 µL/well. Oatp1d1 and Oct1 transporter gene sequences sub-cloned into a eukaryotic pcDNA3.1(+)/His plasmid were transiently transfected to HEK293T cells using a polyethyleneimine (PEI) reagent in a 1:1 ratio (Tom et al., 2008). After rapid vortexing (3 x 3 sec) and incubation (15 min) at room temperature, 25 µL/well of PEI and plasmid solution were added to the cells containing 225 µL/well of DMEM (without FBS). Incubation with transporter gene sequences lasted for 4 h after which the medium was replaced with 250 µL/well of DMEM with 10% FBS. Twenty four hours after transfection, HEK293T cells with Oatp1d1 and Oct1 overexpression were exposed to 200 µL/well of incubation medium for 15 min at 37°C. After preincubation, 100 µL/well of medium was removed and replaced with 50 µL/well of sample serial dilution in duplicate. Then, 50 µL/well of the model substrate, diluted in incubation medium, was added to the wells. In previous studies, the transport affinities (*Km*) and transport rates (*Vmax*) of zebrafish Oatp1d1-mediated uptake of LY and Oct1-mediated uptake of ASP+ were determined by Michaelis-Menten kinetics, resulting in *Km* values of 41.7 and 25.97 µM, respectively (Popovic et al., 2013; Mihaljević, 2015). For transport activity measurements, the final concentrations of Oatp1d1-specific substrate LY and of Oct1-specific substrate ASP+ used were below the obtained *Km* values and set to 10 and 15 µM, respectively. The incubation time for LY and ASP+ was 5 min for each substrate at 37°C. After incubation, the cells were washed two times with ice-cold incubation medium (250 µL/well) and incubated with 250 µL/well of 0.1% sodium dodecyl sulfate (SDS) for 30 min at 37°C for cell lysis. Fluorescence of transport specific substrates was measured in 96-well black microplates using a microplate reader (Infinite M200, Tecan, Salzburg, Austria) at specific wavelengths of 425/540 nm for LY and 485/590 nm for ASP+, respectively. The eukaryotic vector pcDNA3.1(+)/His without cloned genes (mock cells) was also transfected into the HEK293T cells in order to determine non-transporter-specific uptake. Oatp1d1- and Oct1-transfected cells exposed only to incubation medium were used as additional negative control. The maximal amount of DMSO used as solvent in all of the tested samples never exceeded 1%.

2.7. Determining the type of interaction of caulerpenyne with the Oatp1d1 transporter

The interaction type of CYN with zebrafish Oatp1d1 was determined by Michaelis-Menten kinetics. The activity of the Oatp1d1 transporter in HEK293T transfected cells was measured at 3 different CYN concentrations (IC_{30}, IC_{50}, IC_{70}) and 7 concentrations of the LY model substrate (5-500 µM). A shift in the *Km* and *Vmax* values of Oatp1d1 transport was determined after 15 minutes of incubation of LY with CYN. After incubation, the cells were washed with 250 µL of cold incubation medium and incubated with 250 µL of 0.1% SDS for 30 min at 37°C for cell lysis. As a control, the activity of the Oatp1d1 transporter in HEK293T cells was measured in the presence of only model substrate LY. The uptake into vector-transfected HEK293T cells (mock cells) was subtracted to obtain transporter-specific uptake. The fluorescence of the LY model substrate was measured in 96-well black microplates at 425/540 nm using a microplate reader (Infinite M200, Tecan, Salzburg, Austria). The *Km* increase and no changes in *Vmax* indicated CYN as a substrate (competitive inhibition); no changes in *Km* and *Vmax* decrease indicated non-competitive inhibition; *Km* and *Vmax* decrease indicated un-competitive inhibition. The measurements were done in duplicates and conducted in 3 independent experiments. The results of the representative experiment are shown in the Results section. To obtain a linear calibration curve, fluorescent dye LY was dissolved in 0.1% SDS, in the cell matrix dissolved in 0.1% SDS, and in the incubation medium to obtain the linear calibration curves. Bradford assay was used to measure total protein concentration (Bradford, 1976). Based on total protein measurements and the obtained linear calibration curves, the uptake of LY was calculated and expressed as nmol of substrate per µg of protein per minute.

2.8. Statistical analysis

Results were expressed as inhibition percentages of LY/ASP+ uptake from obtained duplicates with mean ± standard deviation (SD) or standard error mean (SEM) values. All of the studies were performed in 2-4 independent experiments. Concentration-dependent LY and ASP+ uptake was calculated using the equation (1):

$$(1) \qquad I_i = ((F_i - F_m) / (F_c - F_m)) \times 100$$

where I_i is the percentage of inhibition for the test concentration i, F_i is the mean fluorescent value for the test concentration i, F_m is the mean fluorescent value for the mock and F_c is the mean fluorescent value for the control.

Results were recorded and calculated in Microsoft Office Excel 2007 and statistical analysis was made in GraphPad Prism 5 for Windows. Serial dilutions were log transformed and the results were analyzed by non-linear regression method used for obtaining dose-response curves, with 95% confidence intervals (CI). Results were expressed as IC_{50} values that designate the concentrations that cause 50% of the maximal observed inhibition. IC_{50} values were calculated from sigmoidal curves using the equation (2):

$$(2) \qquad y = b + (a - b) / (1 + 10^{((LogIC50 - x) * h)}) \tag{2}$$

where y is the response, b is the minimum (bottom) of response, a represents the maximum (top) response, hillslope (h) is the slope of the curve, $LogIC_{50}$ is the halfway response from bottom to top and x is the logarithm of inhibitor concentration.

The kinetic parameters, *Km* and *Vmax* values were calculated using the Michaelis-Menten equation,

$$(3) \qquad V = (V_{max} \text{ x } [S]) / ([S] + K_m) \tag{3}$$

where V is the velocity (nanomoles of substrate per microgram of proteins per minute), *Vmax* is the maximal velocity, [*S*] is the substrate concentration (micromoles) and *Km* is the Michaelis-Menten constant.

3. Results

3.1. Inhibition of zebrafish Oatp1d1 and Oct1 transporters by *C. taxifolia* extract

The uptake activity of zebrafish Oatp1d1 and Oct1 transporters was inhibited by the *C. taxifolia* extract. Dose-response curves were obtained for both transporters, clearly showing that the related transporter-specific activities were significantly inhibited by the algal extract resulting in a 94% inhibition of LY uptake by Oatp1d1 and a 100% inhibition of ASP+ uptake mediated by Oct1 at the highest applied concentration of the extract (Fig. 2). The determined and calculated IC_{50} values for the *C. taxifolia* extract, i.e. extract concentrations that caused a 50% inhibition of model substrate transport, were 3.5 µg/mL for Oatp1d1 and 44 µg/mL for Oct1 (Fig. 2). Therefore, significantly higher inhibitory potential of was determined for *C. taxifolia* extract towards the zebrafish Oatp1d1 than towards Oct1.

In the following step of our study, the obtained major ABC fractions were tested on the inhibition of Oatp1d1 and Oct1 transport activity. A wide concentration series was tested for

all major fractions, and our data showed that all of the three major fractions of *C. taxifolia* extract showed significant inhibition of zebrafish Oatp1d1 uptake activity (Fig. 3). ABC fractions obtained from *C. taxifolia* reduced anion transporter activity to below 10% uptake, as determined at the highest concentrations. The lowest uptake, i.e. the highest inhibitory effect, was determined for *C. taxifolia* C fraction which yielded an almost complete inhibition of the Oatp1d1-mediated uptake of model substrate LY. An Oatp1d1 transporter inhibitory response was observed in the concentration range between 2 and 40 µg of extract per mL of exposure medium. The obtained and calculated IC_{50} values for all of the three major fractions of *C. taxifolia* were: 4.84, 9.18 and 6.3 µg/mL for the A, B and C fractions, respectively (Fig. 3). In comparison, the inhibition of Oct1 uptake activity was much weaker. Fraction A showed the weakest effect on the Oct1 transporter, causing approximately a 50% inhibition of ASP+ uptake at maximal concentration (Fig. 3). On the other hand, the B and C fractions demonstrated a stronger effect on the Oct1 transporter in the concentration range between 0.4-100 µg/mL by reducing the uptake of ASP+ to approximately 20-30% at the maximal applied concentrations. The obtained and calculated IC_{50} values for the B and C fractions of *C. taxifolia* showed that fraction C had the lowest IC_{50} value of 27.89 µg/mL, while the B fraction IC_{50} value was 52.63 µg/mL (Fig. 3). The IC_{50} value for fraction A could not be calculated due to its far too weak inhibitory effect.

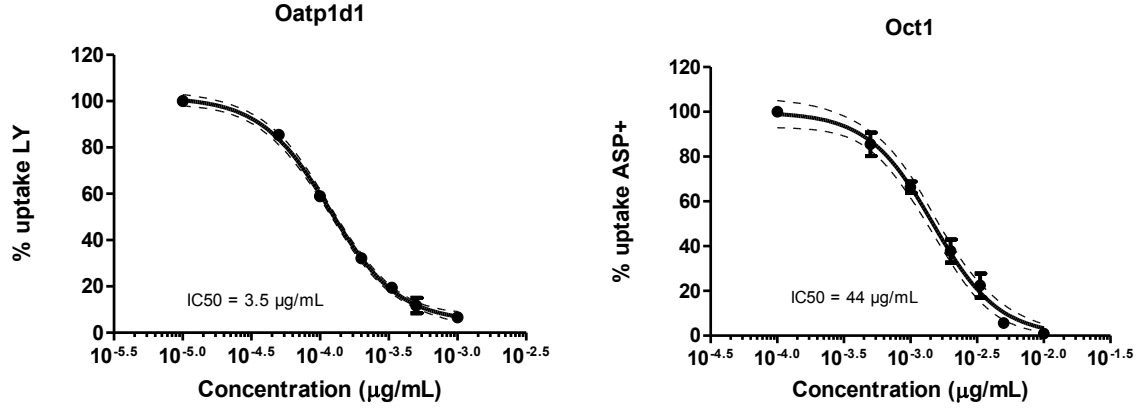
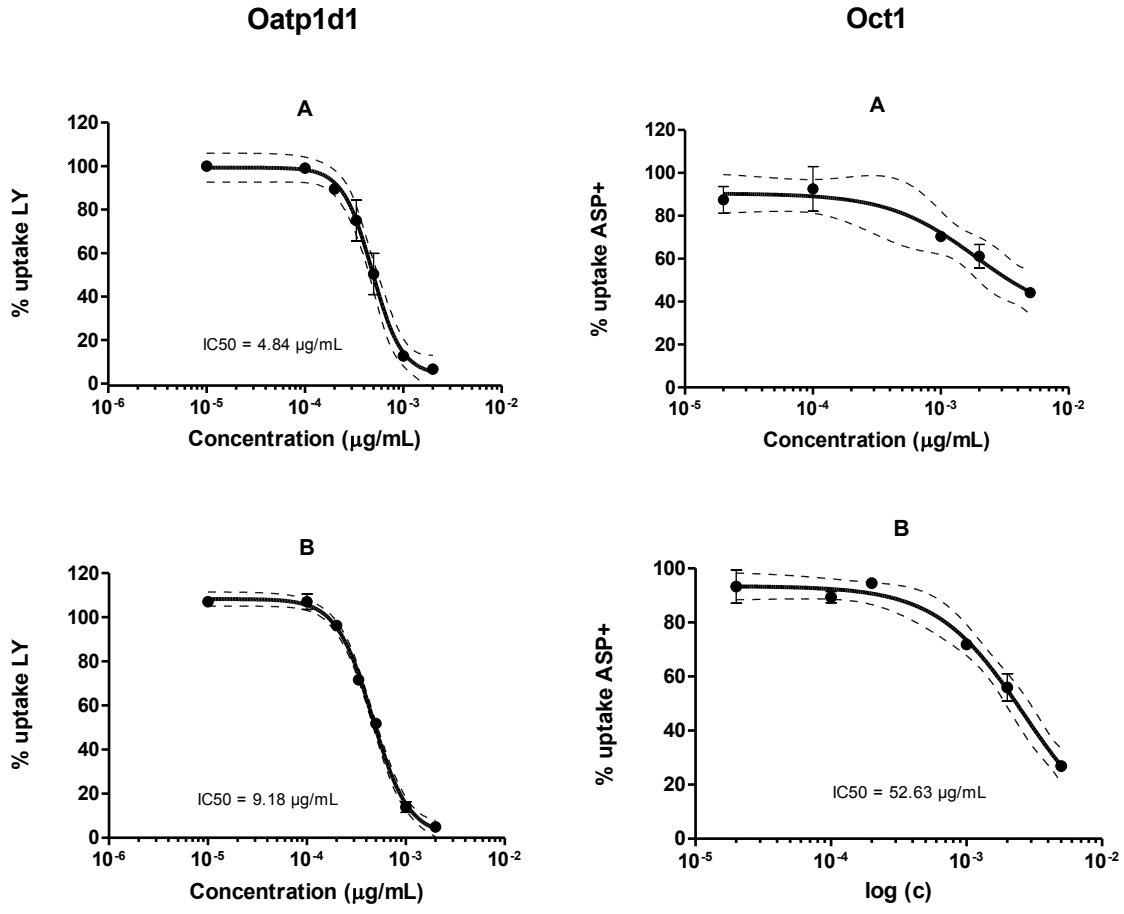


Fig. 2. Concentration-dependent inhibition of the zebrafish Oatp1d1 and Oct1 transport activities by *C. taxifolia* extract. Results are shown as percentages of Lucifer yellow (LY) (Oatp1d1) or 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) (Oct1) uptake in human embryonic kidney HEK293T cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. *C. taxifolia* extract was tested in 2 independent experiments.

3.2. Inhibition of zebrafish Oatp1d1 and Oct1 transporters by algal HPLC sub-fractions

After the initial testing of the *C. taxifolia* extract and the major ABC fractions, the major fractions that caused a positive inhibitory response were subjected to a final HPLC fractionation step which resulted in 40 sub-fractions. Since major fractions B and C demonstrated inhibitory effects towards both transporters they were further processed to the HPLC fractionation step. The obtained HPLC sub-fractions were selected and screened for Oatp1d1 and Oct1 inhibitory potential. The selection was carried out according to (1) the availability of the sub-fractions' material; (2) the amount of substances in sub-fractions as initially determined chromatographically (additional data available in Supplementary material); and (3) making sure that the whole sub-fractionation series is as evenly covered by the tested sub-fractions as possible, without large gaps in the series. The tested sub-fractions included 24 B fractions (B2, B3, B6-B9, B12, B15-B30, B38), and 22 C fractions (C2, C3, C5, C6, C16-C31, C34, C38).



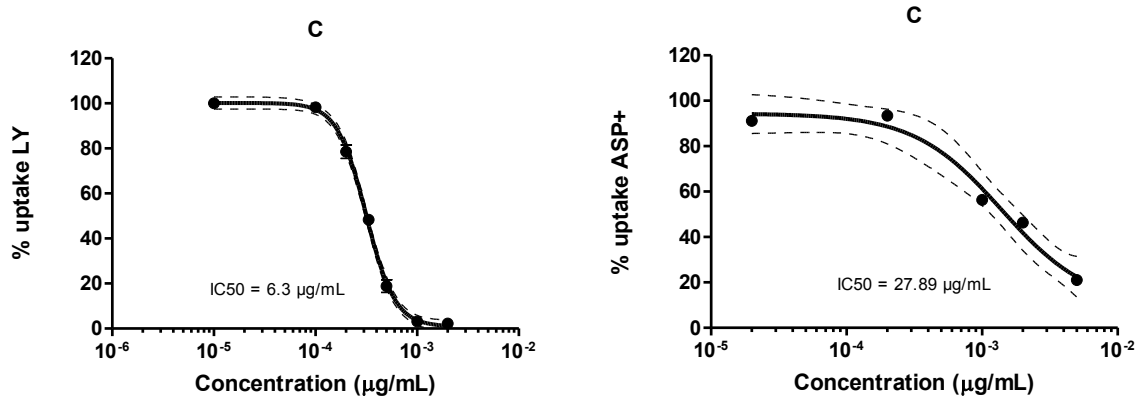


Fig. 3. Inhibition of zebrafish Oatp1d1 and Oct1 transporters by *C. taxifolia* ABC fractions. Results are shown as percentages of Lucifer yellow (LY) (Oatp1d1) or 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) (Oct1) substrate uptake in human embryonic kidney HEK293T cells. Mean ± SD values with 95% CI are shown, as calculated from duplicates. ABC fractions were tested in 2-3 independent experiments.

A total of 15 out of the 24 tested B sub-fractions demonstrated an inhibitory effect on Oatp1d1 transporter activity (Supplemental Fig. S2; Fig. 4). At maximal extract concentration (i.e., minimal dilution limited by the amount of the DMSO solvent not higher than 1%), sub-fractions B16-B30 reduced zebrafish Oatp1d1 uptake activity to below 10% (Supplemental Fig. S2). More potent inhibition was observed in the C compared to B sub-fractions. Inhibition of Oatp1d1 activity was determined in 15 out of the 22 tested *C. taxifolia* C sub-fractions (Supplemental Fig. S3 and S4; Fig. 4). The highest determined inhibitory effects resulted in a ≤5% uptake of LY as determined for the C24 and C29 fractions (Supplemental Fig. S3). Concentrations of the C24 and C29 sub-fractions demonstrating the highest inhibitory effect ranged 0.025-5 µg/mL. Dose-response curves were also obtained for the C16-C23, C25-C28, and C30 sub-fractions (Supplemental Fig. S4). From the obtained data, the IC_{50} values were calculated, clearly showing a more potent Oatp1d1 inhibitory potential of the C sub-fractions. As presented on Figure 4, the strongest Oatp1d1 inhibitory potential was observed for *C. taxifolia* C sub-fractions, with the lowest IC_{50} values determined for the C24 (1.29 µg/mL) and C29 (1.08 µg/mL) sub-fractions. Among the B sub-fractions, the most potent inhibitory effect on the Oatp1d1 transporter was detected in the B28 and B29 sub-fractions, with respective IC_{50} values of 3.91 and 3.83 µg/mL (Fig. 4).

Seven B and eight C *C. taxifolia* sub-fractions caused over 50% of the inhibition of zebrafish Oct1 transport. The response was observed mostly in sub-fractions 16 to 34, and the

corresponding fractions resulted in the reduction of Oct1 uptake to 30-40% of the ASP+ uptake determined in non-treated cells (Supplemental Fig. S5). Sub-fraction B27 showed the highest Oct1 inhibitory potency resulting in the lowest observed uptake (17%), while B8 was almost equally potent with 19% of the ASP+ uptake remaining (Supplemental Fig. S5). Since most B and C sub-fractions of *C. taxifolia* had no effect on Oct1 transporter activity, the related IC_{50} values could not be calculated reliably. Consequently, IC_{50} values were calculated for four B sub-fractions of *C. taxifolia*: B7, B8, B27 and B30 (7.14, 11.33, 10.16 and 14.12 µg/mL, respectively).

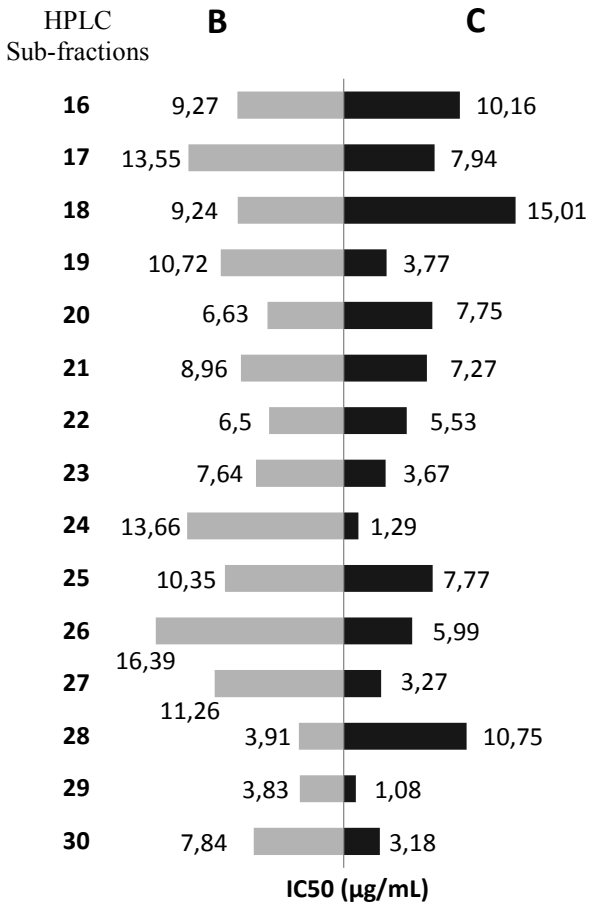


Fig. 4. Zebrafish Oatp1d1 inhibitory potential of *C. taxifolia* B and C high-performance liquid chromatography (HPLC) sub-fractions calculated and expressed as IC50 values (µg/mL).

3.3. Initial HPLC determinations

The chromatographic pattern of B sub-fractions that exhibited an inhibitory effect on zebrafish Oatp1d1 transporter activity matches those that showed an effect on the Oct1 transporter. The inhibition effects were most pronounced from the B22 to B30 sub-fractions,

in accordance with the initial HPLC chromatogram that revealed significant peaks in all of these sub-fractions (Supplemental Fig. S6). The highest peak was observed during minute 25 of elution, and the strongest Oatp1d1 inhibition effects were observed in the B28 and B29 sub-fractions, while B27 showed the strongest inhibition effect towards the Oct1 transporter (Supplemental Fig. S6). Several higher peaks were also detected during the first 2-3 min of elution, but the related sub-fractions did not show any effect on the tested transporters. The HPLC chromatogram of the C sub-fractions revealed high amounts of different chemical compounds over a 14-35 min time interval (Supplemental Fig. S7). Again, the effects on the Oatp1d1 and Oct1 transporters were detected within the same time range and were observed from sub-fractions C16 to C34. The strongest effects were observed in C24 and C29 for Oatp1d1 inhibition and C22 for Oct1 inhibition (Supplemental Fig. S7). Therefore, the observed biological effects were consistent with the preliminary chemical analysis of *C. taxifolia* B and C sub-fractions.

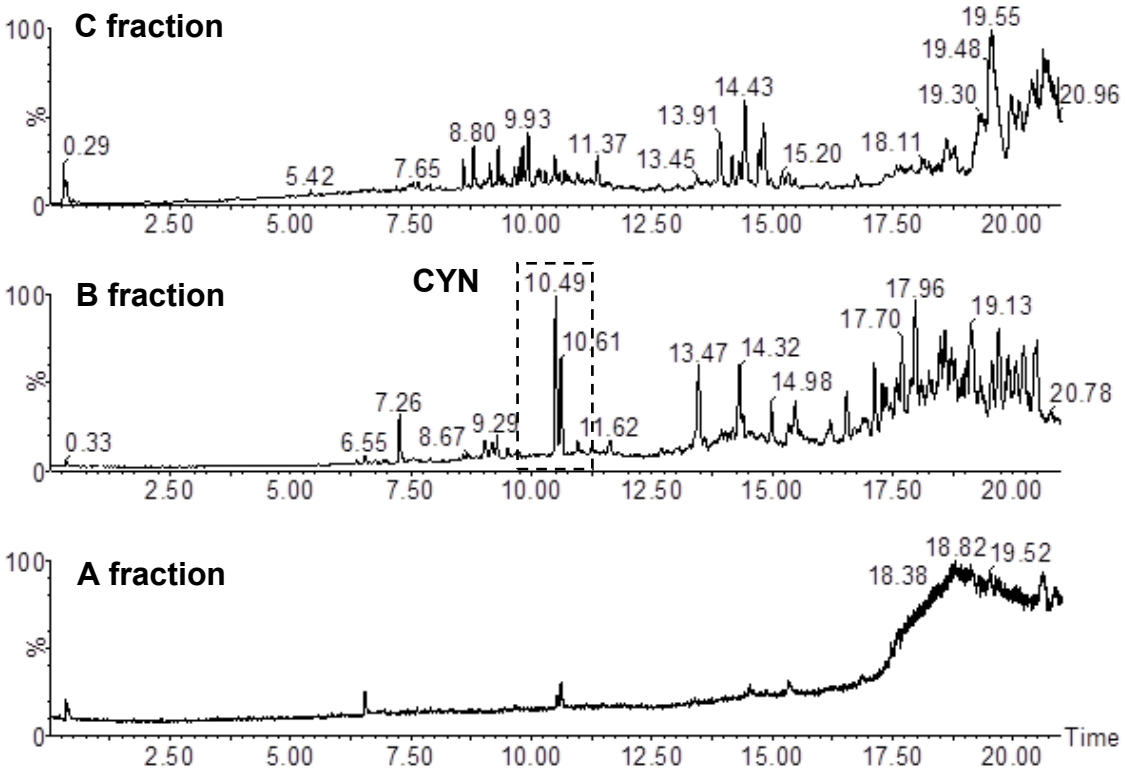


Fig. 5. LC/MS chromatograms of the major hexane (A fraction), dichloromethane (B fraction) and methanol (C fraction) fractions of the total extract of *Caulerpa taxifolia*. The dominant peak for caulerpenyne (CYN) at 10.49 min was recorded in B fraction.

3.4. LC-MS analyses

The total ion current (TIC) LC-MS chromatogram of purified CYN showed a dominant CYN peak at 10.47 min (Supplemental Fig. S8). Positive electrospray ionization (ESI) mass spectra yielded ionization by sodium adduct ion formation, producing an ion at m/z 397.162 exclusive to CYN (Supplemental Fig. S8). Results of LC-MS analyses conducted on ABC fractions of *C. taxifolia* indicated a major CYN metabolite at 10.49 min in the TIC LC-MS chromatogram of fraction B (Fig. 5). Concentration of CYN determined in the B fraction was 119 µg/µL. Although numerous peaks were also recorded by LC-MS analysis of the A and C fractions, no presence of CYN was found in any of these fractions. The formation of the CYN producing ion on the mass spectrum occurred at m/z 397.162 in positive ion mode, responding to the obtained mass spectrum values for purified CYN. LC-MS analyses of subsequent HPLC sub-fractions further confirmed that CYN was the dominant compound detected in the B sub-fractions, as well as that the dominant peak of CYN was detected at 10.47 min in the TIC LC-MS chromatogram in sub-fraction B26 (Fig. 6). Concentration of CYN in the B26 sub-fraction was 87 µg/µL, and the following sub-fraction B27 had 1.5 µg/µL. Other sub-fractions did not even contain the characteristic CYN signal. Therefore, 2 out of the 9 tested B sub-fractions of *C. taxifolia* contained CYN. As expected, CYN was not detected in the C sub-fractions. However, numerous chromatographic peaks other than CYN were also detected in the HPLC sub-fractions indicating the presence of various chemical compounds.

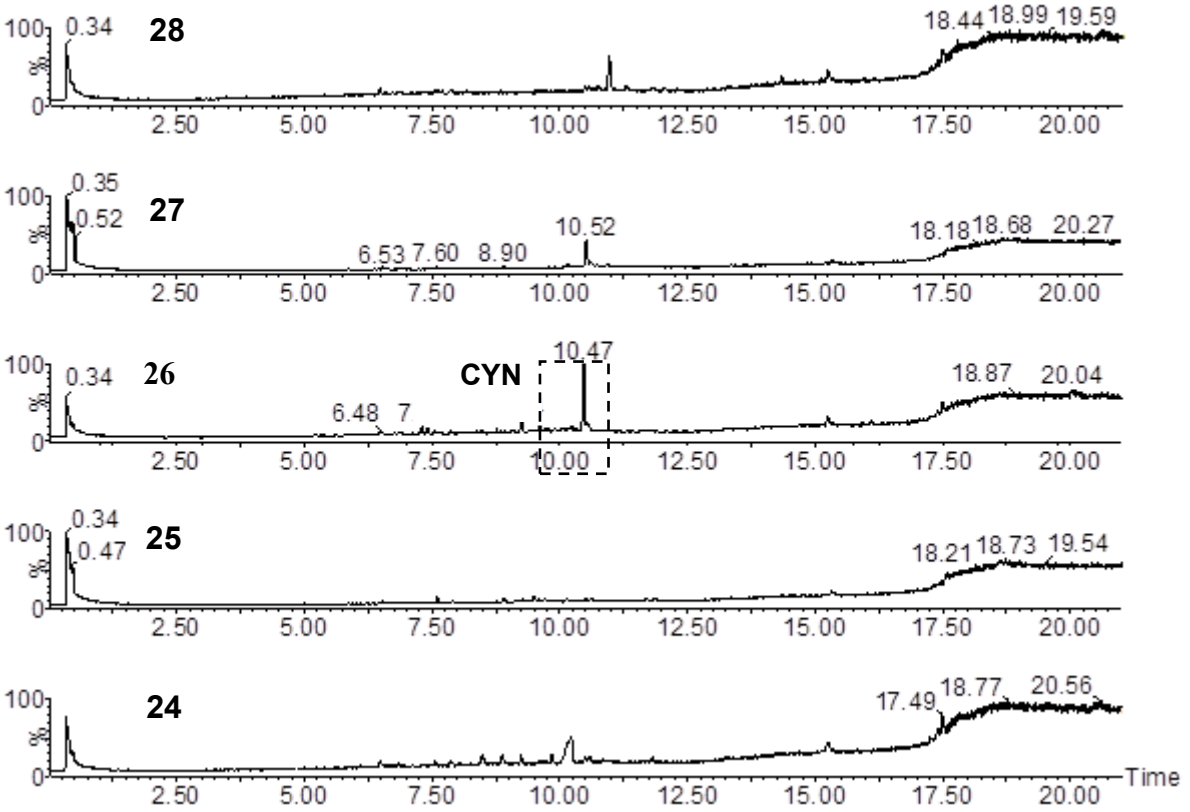


Fig. 6. LC/MS chromatograms of selected high-performance liquid chromatography (HPLC) B sub-fractions from *Caulerpa taxifolia* showing total ion current (TIC) chromatograms. Caulerpenyne (CYN) is predominantly eluted in sub-fractions B26 and B27.

3.5. Interaction of caulerpenyne with uptake transporter activity

The sub-fractions that were shown to contain CYN (Fig. 6) – B26 and B27 – reduced zebrafish Oatp1d1 uptake activity to below 10% (Fig. 7). Furthermore, purified CYN showed a high inhibitory effect on the transport activity of zebrafish uptake transporter Oatp1d1 within the concentration range from 1.33 to 267 μM (Fig. 8). At the highest CYN concentration, the inhibition of Oatp1d1 uptake was 85% compared to non-treated cells, resulting in a calculated IC_{50} value of 17.97 μM. On the contrary, only a weak interaction of CYN with the Oct1 uptake transporter was observed, where at the highest CYN concentration (267 μM), the uptake of ASP+ substrate decreased moderately (26%) in comparison to non-treated cells (Fig. 8).

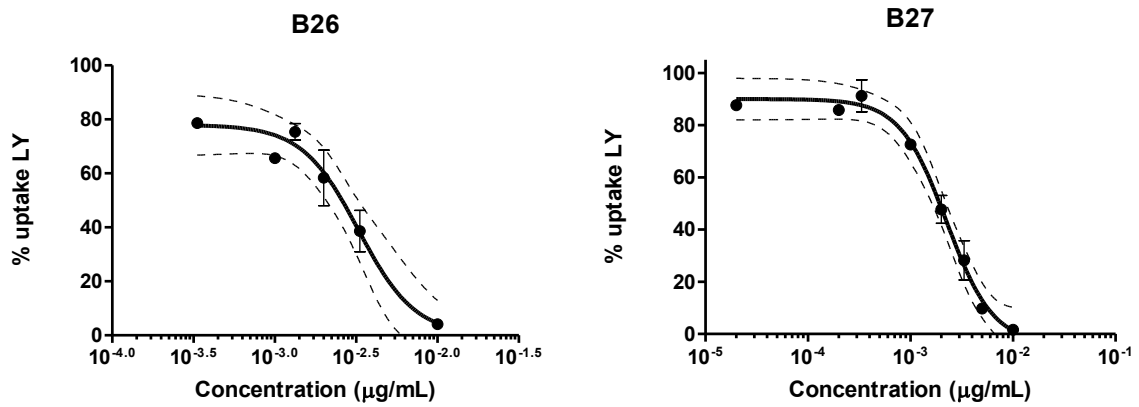


Fig. 7. Dose-response curves of *C. taxifolia* B26 and B27 high-performance liquid chromatography (HPLC) sub-fractions. Caulerpenyne (CYN) was detected in those fractions by liquid chromatography – mass spectrometry (LC-MS) analyses showing inhibitory effect on zebrafish Oatp1d1 transport. Results are expressed as percentage of Lucifer yellow (LY) model substrate uptake. Sub-fractions were tested in 3 independent experiments in monoplicates. Mean ± SEM values with 95% CI are shown as calculated from independent monoplicates.

3.6. Type of interaction of caulerpenyne with the Oatp1d1 transporter

The obtained kinetic parameters *Km* and *Vmax* of LY uptake showed a non-competitive type of inhibition of zebrafish Oatp1d1 transport by CYN. Compared to control (*Vmax* 20.09 nmol/µg protein/min), a significant decrease of *Vmax* value was determined in the presence of different concentrations of CYN (Table 1, Fig. 9). At higher concentrations, the calculated *Vmax* IC_{30}, IC_{50} and IC_{70} values were 16.19, 14.83 and 5.73 nmol/µg protein/min, respectively (Table 1). The 95% CI for the obtained *Km* values of LY uptake in the presence of CYN overlapped with the control *Km* values, showing that apparent LY affinity was maintained and was equivalent to the actual LY affinity (Table 1). Therefore, the observed decrease in the *Vmax* and no change in the *Km* kinetic constant classify CYN as a non-competitive inhibitor of the Oatp1d1 transporter. Representative dose-response curves of non-competitive inhibition are shown on Fig. 9.

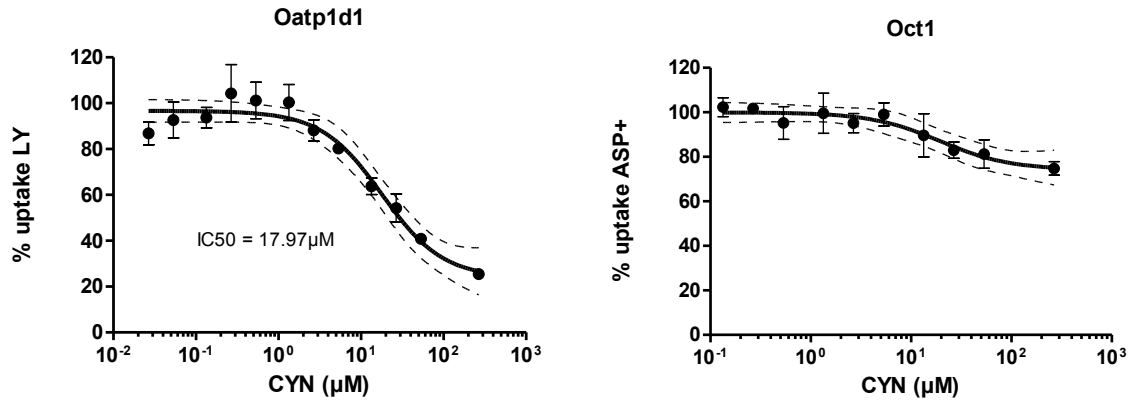


Fig. 8. Concentration-dependent inhibition of zebrafish Oatp1d1 and Oct1 transport activities by purified caulerpenyne (CYN). Results are shown as percentages of Lucifer yellow (LY) and 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) uptake in human embryonic kidney HEK293T cells. Mean ± SD values with 95% CI are shown as calculated from duplicates. CYN was tested in 2 independent experiments.

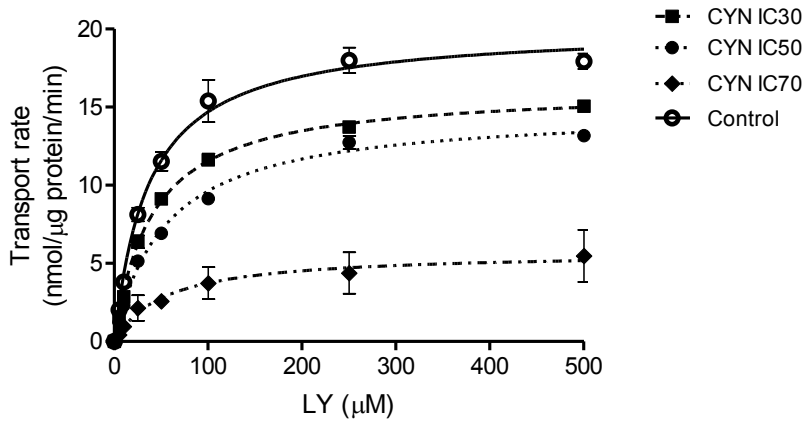


Fig. 9. Determination of the type of interaction of caulerpenyne (CYN) with zebrafish Oatp1d1 transporter. Michaelis-Menten kinetics of Lucifer yellow (LY) mediated transport by HEK293T/Oatp1d1 overexpressing cells was determined either in the absence (Control), and the presence of 3 different concentrations of non-competitive inhibitor CYN (IC30, IC50, and IC70). The shift in kinetic parameters (*Km* and *Vmax*) of LY was determined using LY concentration range from 5 to 500 µM (7 concentrations). Dose-response curves were calculated using nonlinear regression. The mean velocity ± SD values are shown expressed as transport rate (nmol/µg protein/min), as calculated from duplicates. Michaelis-Menten analyses were done in 3 independent experiments.

Table 1. Basic kinetic parameters obtained in determining the type of interaction of caulerpenyne (CYN) with zebrafish Oatp1d1 transporter using Michaelis-Menten kinetics experiments and Lucifer yellow (LY) as the model substrate. *Km* values with 95% CI are expressed in μM and *Vmax* values with 95% CI are expressed as transport rate (nmol/μg protein/min).

Interactor	*Km* (LY)	95% CI	*Vmax* (LY)	95% CI
Control (without CYN)	36.73	30.15 - 43.31	20.09	19.08 - 21.09
CYN IC30	40.25	36.73 - 43.77	16.19	15.79 - 16.60
CYN IC50	54.16	46.31 - 62.01	14.83	14.18 - 15.49
CYN IC70	53.54	16.90 - 90.18	5.73	4.531 - 6.928

4. Discussion

Studies regarding the biochemical and nutritional composition of macroalgae have demonstrated that *C. taxifolia* possesses significant amounts of primary metabolites such as proteins and lipids, but that it also contains carbohydrates, fiber and ash (Setthamongkol et al., 2015). Except for the production of primary metabolites, other studies have implied that secondary metabolites synthesized by the *Caulerpa* species probably play a major role as evolutionarily-developed chemical defense mechanisms against various herbivores, epiphytes and other competitors (Paul and Fenical, 1986; Pohnert and Jung, 2003; Erickson et al., 2006). Our study showed that *C. taxifolia* secondary metabolites interact with phase 0 cellular detoxification mechanisms through the inhibition of related transport proteins. It is the first report related to this mechanism of potentially toxic action of the *Caulerpa* species, as no studies on the effects of *Caulerpa* metabolites on the activity of (eco)toxicologically relevant uptake transmembrane transporters have yet been published to the best of our knowledge.

Our previous work showed that both of the transmembrane transporters addressed in this study may have an important role in the uptake of various environmentally and physiologically relevant chemicals in zebrafish (Popovic et al., 2014; Mihaljević, 2015). More specifically, interaction with steroid hormones such as dehydroepiandrosterone sulfate (DHEAS), estrone-3-sulfate (E3S), 17α-ethynilestradiol (EE2), and E17ß-glucuronide was determined for the Oatp1d1 transporter, while Oct1 was shown to transport androstenedione, progesterone, and testosterone (Popovic et al., 2013; Mihaljević, 2015). Furthermore, xenobiotics such as 1-methyl-4-phenylpyridinium (MPP+), tetrapentylammonium (TPA), tetrabutylammonium (TBA), prazosin, mitoxantrone and cimetidine have been recognized as

potent interactors of the Oct1 transporter, whereas perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), nonylphenol, gemfibrozil, diclofenac and caffeine as potent interactors of the Oatp1d1 transporter (Popovic et al., 2014; Mihaljević, 2015).

Non-fractionated, total *C. taxifolia* extract showed the highest impact on the zebrafish Oatp1d1 uptake transporter, with IC_{50} values of 3.5 μg/mL (Fig. 2). Relevant interaction with the zebrafish cationic transporter Oct1 was also demonstrated, although the determined inhibitory response was significantly lower (IC_{50} 44 μg/mL; Fig. 2). Therefore, in the initial phase of our EDA study, we were able to characterize the substance(s) of concern as polar anionic and cationic compound(s), and the observed interaction with both anion and cation transporters indicated the presence of multiple bioactive compounds in *C. taxifolia.* Subsequent testing of the major ABC fractions further confirmed a complex composition of *C. taxifolia* secondary metabolites. Significant Oatp1d1 inhibitory potential was determined in all three major fractions, with comparable inhibitory potencies (Fig. 3). These data indicated that different types of chemical compounds with distinct polarity features able to cause the same or similar inhibitory effect on uptake transporters were present in *C. taxifolia*. The inhibitory effect of *C. taxifolia* ABC fractions on the Oct1 transporter was observed in the B and C fractions while the A fraction showed very weak interaction with the transporter (Fig. 3). The inhibitory responses of the B and C fractions were significantly weaker compared to Oatp1d1 inhibitory effect as was the case in the initial, total extract (Fig. 3). These data indicate that more polar compounds cause an inhibitory effect on the Oct1 transporter. Nevertheless, we observed a decrease in the Oct1 inhibitory effect of *C. taxifolia* B and C fractions in comparison with the effect of the total extract that caused an almost 100% inhibition of Oct1 transporter activity. That may be attributed to the possible loss of sample material, especially cationic compounds, when extracts were processed through a silica gel column (Figs. 2 and 3).

Oatp1d1 and Oct1 transporters belong to different superfamilies of transporters (SLC21 and SLC22), transport two different types of polar compounds (anions and cations), depend on different transport gradients (pH and Na^+), and differ in the complexity of the binding between the active site of the transporter and the interactor (Popovic et al., 2013; Mihaljević, 2015). In the *C. taxifolia* samples, polar compounds were responsible for the inhibitory effect observed for both uptake transporters. Nevertheless, although cationic compounds were present in the *C. taxifolia* extract and subsequent fractions, they seemed to be weak interactors with the Oct1 transporter while the anionic compounds present in the *C. taxifolia* samples were very strong interactors with Oatp1d1. In general, Octs exhibit variety and complexity in

559 interacting with their interactors, demonstrating different types of interaction with their
560 substrates and inhibitors that can deviate from classical types of inhibition. This could
561 contribute to the observed lower inhibitory effect of *C. taxifolia* samples on the Oct1
562 transporter.

563 The strong inhibitory effects determined in the major fractions led to the selection of the
564 most potent B and C fractions for the second fractionation step. Data on testing related HPLC
565 sub-fractions produced during the second fractionation step of B and C major fractions
566 confirmed a strong inhibitory interaction with the Oatp1d1 transporter (Fig. 4). As expected,
567 the zebrafish Oct1 transporter was less responsive to *C. taxifolia* sub-fractions, as was the
568 case with the initial extract and its major fractions (Supplemental Fig. S5). The most potent
569 Oatp1d1 inhibitory activity was determined in sub-fractions C24 and C29, while among
570 several of the most responsive B sub-fractions, B28 and B29 showed the most potent (Fig. 4).
571 The initial chromatogram for the *C. taxifolia* C sub-fractions revealed a series of overlapping
572 peaks displaying the very complex nature of the chemical compounds present in these
573 fractions (Supplemental Fig. S7). The C fraction possessed a more complex composition of
574 metabolites in comparison to the B sub-fractions, and based on these initial insights it appears
575 that the *C. taxifolia* substance(s) responsible for the majority of the adverse effects on the
576 Oatp1d1 uptake transporter were polar and dominantly anionic substances. In general, the
577 presence of chemical compounds in the most responsive sub-fractions of *C. taxifolia* (C24,
578 C29, B28 and B29) corresponded well with the chemical composition of compounds detected
579 on the related initial chromatograms (Supplemental Figs. S6 and S7).

580 The following step in our EDA study encompassed the first attempts at the identification
581 of chemical compound(s) in the active HPLC sub-fractions of *C. taxifolia*. Using UPLC
582 coupled with QTOFMS, we were able to obtain LC-MS chromatograms and mass spectrums
583 characteristic for CYN- producing ions at m/z 397.162 (Supplemental Fig. S8). This result
584 was in accordance with previous data on CYN detection at m/z 392 and 397 by ionspray
585 ionization mass spectrometry method, as reported by Raffaelli et al. (1997). Likewise, LC-MS
586 analyses of the ABC fractions of *C. taxifolia* total extract revealed CYN as a dominant
587 metabolite eluted only in the B fraction (Fig. 5). Except for being a dominant intracellular
588 secondary metabolite, a recent study showed CYN to be also present in elevated amounts at
589 the surface of the algae indicating its potential role as a surface defense compound or a natural
590 antifouling metabolite (Cirri et al., 2016). LC-MS chromatograms of fraction A did not show
591 any dominant peaks or peaks characteristic for CYN (Fig. 5). Consequently, this fraction was
592 considered of minor toxicological importance for our EDA workflow. Apart from CYN,

numerous other chemical compounds were also detected by LC-MS analysis (Fig. 5), indicating that other biologically active metabolites such as epoxides and alcoholic compounds may also be present. Further analytical analyses and confirmation studies with corresponding standards are clearly needed to test these observations.

Detailed LC-MS analyses of HPLC B and C sub-fractions of *C. taxifolia* extract revealed CYN as a dominant compound in sub-fractions B26 and B27 (Fig. 6; Supplemental Fig. S9). Accordingly, among the sub-fractions tested by our bioassays, a strong Oatp1d1 inhibitory effect was detected in sub-fractions B26 and B27 (Fig. 7) with IC_{50} values of 16.39 and 11.26 μg/mL; respectively (Fig. 4). Purified CYN proved that CYN was indeed a potent Oatp1d1 inhibitor, with a determined low IC_{50} value of 17.97 μM (Fig. 8). Finally, Michaelis-Menten LY uptake kinetics analysis revealed that CYN is a non-competitive inhibitor of the zebrafish Oatp1d1 transporter, accomplishing this by significantly increasing the Oatp1d1 transport rate and keeping *Km* unchanged (Fig. 9). As expected, the weak inhibitory effect of purified CYN on the zebrafish Oct1 transporter correlates well with the previously observed low Oct1 inhibitory potency of HPLC sub-fractions. Other highly potent sub-fractions to zebrafish Oatp1d1 transport activity were B28 and B29, where no presence of CYN was detected (Figs. 4 and 6). This suggests that CYN-like chemical entities could also be responsible for the inhibitory effects observed in other B sub-fractions, implying the presence of yet unidentified but potent Oatp1d1 interactors.

Considering the interaction potency of CYN, data from our previous work on the interaction between the zebrafish Oatp1d1 transporter and environmentally relevant contaminants such as PFOS, PFOA, nonylphenol, gemfibrozil, diclofenac, and caffeine, showed that the highest affinity (*Ki* values) for the Oatp1d1 transporter were in the range of 0.2 to 13 μM (Popovic et al., 2014). These compounds have been classified as very strong (*Ki* < 1) and strong (*Ki* = 1-20 μM) interactors (Popovic et al., 2014). In this study, the LY concentration (10 μM) used as a model substrate for Oatp1d1 transport activity measurements was 4 times lower than the *Km* value (41.7 μM) determined by Michaelis-Menten kinetics of zebrafish Oatp1d1 mediated uptake of LY by Popovic et al. (2013), which means that the CYN IC_{50} value was similar to the *Ki* value. Therefore, based on the obtained data, CYN can be regarded as a strong inhibitor of the zebrafish Oatp1d1 transporter, with an IC_{50} value of 17.97 μM.

Finally, apart from *Caulerpa* species, there are other aquatic organisms that also possess the ability to produce a wide range of secondary metabolites with various biological effects. For example, the interaction of cyanotoxins, secondary metabolites produced by specific

strains of cyanobacteria, with the fish Oatp1d1 transporter was reported in several studies (Meier-Abt et al., 2007; Steiner et al., 2014; Faltermann et al., 2016). These studies demonstrated that Oatp1d1 from the little skate, rainbow trout and zebrafish does transport microcystin-LR. Furthermore, it has also been shown that Oatp1d1 is an uptake transporter of nodularin (Faltermann et al., 2016). Therefore, although cyanobacterial microcystins and CYN from *C. taxifolia* are structurally different chemical compounds, certain results indicate that Oatp1d1 is included in the transport of these important environmental toxins.

5. Conclusions

This study attempted to determine a novel mechanism behind the inhibitory action of *C. taxifolia* on phase 0 cellular detoxification which may help to gain a better understanding of the invasive potential of *Caulerpa* species. The first reliable indications of chemical characteristics potentially responsible for the observed inhibitory effects towards ecotoxicologically relevant uptake transport proteins in zebrafish were successfully obtained. CYN, a dominant secondary metabolite in *C. taxifolia* is a potent non-competitive inhibitor of zebrafish Oatp1d1. Additional, CYN-like chemical substances are the most plausible new candidates for inhibitory effects observed for both types of uptake transporters, although further EDA studies directed at the identification and confirmation of specific (eco)toxic chemical entities in investigated complex biological samples are clearly needed. Finally, the concept used in this study demonstrated that a modified EDA procedure can be used as a reliable multidisciplinary diagnostic tool allowing for a detailed characterization of environmentally relevant complex biological samples and potential identification of new biologically active substances.

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Abbreviations: ASP+ - 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide; CYN – caulerpenyne; EDA – effect-directed analysis; ESI – electrospray ionization; HEK293T - human embryonic kidney cells; HPLC – high-performance liquid chromatography; LC-MS – liquid chromatography-mass spectrometry; LY – lucifer yellow; OATs – organic anion transporters; OATPs – organic anion transporting polypeptides; OCTs – organic cation

661 transporters; PEI – polyethyleneimine; QTOFMS – quadrupole-time-of-flight/mass
662 spectrometry; TIC – total ion current; UPLC – ultra-performance liquid chromatography
663
664 **7. References**

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