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ORIGINAL RESEARCH ARTICLE

Bioactive metabolites produced by *Spirulina subsalsa* from the Baltic Sea

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Summary Cyanobacteria are known producers of compounds with possible medical applications. So far, the biotechnological potential of *Spirulina subsalsa* has been explored in few studies. They were mainly focused on the use of this cyanobacterium as a bioremediation agent. In our study, seven fractions from Baltic-derived *S. subsalsa* CCNP1310 were obtained and their cytotoxic effect on the T47D breast cancer cell line as well as inhibitory effects against elastase, trypsin, thrombin, chymotrypsin, and carboxypeptidase A were examined. Four fractions revealed a significant decrease in relative viability of cancer cells. Two inhibited the activity of chymotrypsin and one carboxypeptidase A, but at a moderate level. No effect was observed against other tested proteases. Active fractions were screened with liquid chromatography tandem mass spectrometry (LC—MS/MS) optimized for the detection of peptides, for preliminary characterization of bioactive compounds. We identified three groups of compounds which share the same fragment ions and are possibly linked with effects observed in conducted tests. Our research indicates for the first time that compounds produced by Baltic strain of *S. subsalsa* not only have high activity against T47D cancer cells but also seem to work selectively as they do not have strong inhibitory effect against the tested enzymes. That indicates the existing potential of the *cyanobacterium* to be used as a source of important cytotoxic agents.

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1. Introduction

Cyanobacteria, as primary producers, are important components of the phytoplankton community. In eutrophic ecosystems, and under favorable conditions, they can develop into blooms. The ecosystem consequences of the blooms are complex and still not fully recognized (Havens, 2008; Peng et al., 2017; Šulčius et al., 2017). At least part of the effects of cyanobacteria on co-occurring organisms can be attributed to the activity of secondary metabolites produced and released to the environment (Karjalainen et al., 2007). Recently, these metabolites have attracted a lot of scientific attention, as beside environmental significance they can find application in different branches of our life, including agriculture and food industry. Cyanobacterial products are also seriously considered as potential drugs and cosmetics (Rastogi and Sinha, 2009; Singh et al., 2011). For this reason, the species from *Arthrospira* genus, mainly *Arthrospira platensis*, have been widely explored (Ciferri and Tiboni, 1985; Gademann, 2011; Hosseini et al., 2013). Unfortunately, in the majority of the reports, the cyanobacterium was wrongly classified as belonging to *Spirulina* genus. The situation is further complicated by the fact that products from *Arthrospira* are sold on the market under the name of *Spirulina*. To solve the problem, Tomaselli et al. (1996) suggested that in scientific published materials only the proper generic name of the cyanobacterium could be used. In all other cases, such as commercial products, the common name "Spirulina" could be accepted, if the correct scientific denomination of the organism is provided.

Spirulina genus was primarily described by Turpin (1827) as *Spirulina oscillarioides*. The author observed filamentous structures, usually in clusters or in fine mats which were macroscopically visible. The tightly and regularly screw-like coiled filaments were composed of cylindrical cells and intensely motile (rotating) trichomes. Later, Stizenberger (1852) introduced morphologically similar genus named *Arthrospira*. In subsequent years, the classification of these two genera remained a matter of dispute. Main morphological criteria dividing these two organisms are: trichome diameter, type of helicity, visibility of cross walls, cell wall pore pattern, mode of trichome fragmentation and presence of cylindrical bodies (Vonshak and Tomaselli, 2000). In recent years, the taxonomic classification of cyanobacteria has undergone extensive revision with the advent of phylogenetic analyses based on molecular sequence data. These analyses confirmed and justified the separation of these two genera. Currently, *Spirulina* is classified to Spirulinales order, while *Arthrospira* belongs to Oscillatoriales order (Komarek et al., 2014).

Spirulina subsalsa Oersted ex Gomont, used in our work, was first described by Gomont in 1892. The cyanobacterium occurs in salt and fresh waters all over the world (*S. subsalsa* Oersted ex Gomont:: Algaebase). In the Baltic Sea, it was reported for the first time by Witkowski in 1993. The trichomes of Baltic-derived *S. subsalsa* are 3.0–5.0 μm wide and form tightly coiled spirals. They glide with typical oscillatory, screw-like movements over the substrate. The cyanobacterium forms mat structures, usually of blue-green color but in the Baltic pinkish-red filaments were also observed (Włodarska-Kowalczuk et al., 2014). *S. subsalsa* was found to be one of the components of cyanobacterial blooms that have harmful effects on lesser flamingos in lake Bogoria, Kenya (Ballot et al., 2004; Krienitz et al., 2003) and on blue shrimps, after they were stocked into pre-cleaned algal raceways (Lightner, 1978). However, there is no evidence that it produces any of the known cyanotoxins. Krienitz et al. (2003) and Ballot et al. (2004) found that *S. subsalsa* is one of the most abundant components of cyanobacterial communities in Kenyan lakes yet neither produces microcystins nor anatoxin-a, which were identified in flamingo stomach contents and their fecal pellets. Lightner (1978) did not identify the agent responsible for blue shrimps hemorrhagic enteritis, however, results of his work suggested that under certain conditions *S. subsalsa* produces a weak toxin that is mildly toxic to shrimps, but only when relatively large quantity of fresh cyanobacterial biomass was consumed.

So far, the potential biotechnological application of *S. subsalsa* has been explored in few studies. They were mainly focused on the use of this cyanobacterium as bioremediation agent for the accumulation of pollutants and industrial leftovers (Chakraborty et al., 2011; Huang and Zhihui, 2002; Jiang et al., 2015; Zhang et al., 2014; Zhihui and Guolan, 2000). *S. subsalsa* was also used as a biosensor for toxicity assessment of estuarine waters (Campanella et al., 2001). This species is also a source of polyhydroxyalkanoates (PHA), the environmentally friendly biopolymers which can find application among others in the production of implants and artificial tissues (Shrivastav et al., 2010).

In previous screening studies, *S. subsalsa* CCNP1310 was found to produce biologically active metabolites such as commercially important enzymes and unknown enzyme inhibitors, as well as agents promoting the growth of several bacterial strains (Mazur-Marzec et al., 2015). The results of the tests indicated that this organism is a promising candidate for further studies into the potential application of its metabolites. However, in the tests, crude extracts, containing a complex mixture of metabolites were used. The rich matrix of the samples could have attenuating or enhancing effects on the results of the performed assays. Therefore, the aim of our current work was to extend the existing knowledge of the activity of metabolites produced by the Baltic cyanobacterium *S. subsalsa* CCNP1310 by fractionation of crude extract (1) and application of series of biochemical assays (2). Attempts to characterize the active agent(s) were also made.

2. Material and methods

The list of reagents used in the study for extraction, separation and biochemical tests is presented in supplementary materials.

2.1. Culture, extraction, and fractionation of *S. subsalsa*

S. subsalsa, from Culture Collection of Northern Poland, strain CCNP1310 (accession number in GenBank KJ161437) was isolated from Puck Bay in 2009. Monospecies culture of the cyanobacterium was grown for biomass in Z8 medium (Kotai, 1972; Mazur-Marzec et al., 2015) supplemented with NaCl and $MgSO_4$ to obtain salinity at 7 [PSU], at room tem-

perature (25 ± 1°C) and continuous light of 10 [μM photons m^{-2} s^{-1}] provided by standard cool white fluorescent lamps (36 [W]). Biomass was harvested between 3 and 4 weeks of the culture, in the mid exponential growth phase. Freeze-dried cyanobacterial biomass (2 g) was extracted with 75% methanol (20 ml) by vortexing for 10 min, followed by 10 min bath sonication. Then, the extract was centrifuge (10,000 × *g*) for 10 min. The obtained supernatant was dissolved in water so that the methanol content did not exceed 15%. The sample was loaded onto the 10-[g] SPE cartridge (Sep-Pak; C18 cartridge, Waters, Milford, USA). The cartridge was first washed with MiliQ water and then the sorbed substances were eluted with aqueous solutions of methanol, gradually increasing the strength of the eluent from 40% to 100%, at 10% step. The collected fractions were evaporated to dry residue.

2.2. Screening for enzyme inhibitors

The enzyme inhibition assays were performed according to protocols described by Pluotno and Carmeli (2005), Ocampo Bennet (2007), and Kwan et al. (2009). The detailed conditions of the five enzymatic assays are presented in Table 1. Serial dilutions (from 10 times to 100,000 times) of the solid-phase extraction (SPE) fractions were prepared in MiliQ water. Standard inhibitors were dissolved either in water or in 1% DMSO (as indicated by the producer); the enzyme and the substrate were dissolved in a buffer solution or in water (Table 1). The mixtures containing the sample or standard inhibitor (positive control), enzyme and buffer were preincubated in microplates for 5—20 min. Then, the substrate solution was added and the mixture was incubated for the next 10—20 min, depending on the enzyme (Table 1). The absorbance was measured with a microplate reader (Versa Max Tunable Microplate Reader, Sunnyvale, USA). Data were obtained from three independent experiments; in each test, the samples were assayed in triplicate.

2.3. Cytotoxic activity

Human breast adenocarcinoma cell line was obtained from CLS Cell Lines Service GmbH (Eppelheim, Germany). Monolayer cultures of T47D cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics mixture (penicillin and streptomycin). Cells were incubated at 37°C in CO_2 (5%) incubator (New Brunswick Galaxy 170s, Eppendorf, Germany). Cell viability was determined by MTT method as described by Felczykowska et al. (2015). For this purpose, T47D cells were seeded at a density of 4×10^3 (for 24 h of incubation) and 2×10^3 (for 48 h of incubation) per well of 96-well plate and allowed to attach overnight. Next, the medium was replaced with a fresh portion of medium containing *S. subsalsa* SPE fractions at concentrations 25, 50, 100 and 200 μg ml^{-1}. Then, 100 μl of MTT solution (4 mg ml^{-1}) was added to each well. After 2 h of incubation, the medium was removed and formazan was dissolved in 100 μl of added DMSO. The absorbance of the reaction mixtures was measured at 570 nm (with reference wavelength 660 nm) with a microplate reader. Data from three independent experiments were collected. In MTT assay, cell viability drop below 50% was considered as significant.

2.4. Mass spectrometry analysis

The SPE fractions were transferred to chromatographic vials and analyzed by liquid chromatography tandem mass spectrometry (LC—MS/MS) as described in Mazur-Marzec et al. (2015). The instrument was equipped with Agilent 1200 (Agilent Technologies, Waldboronn, Germany) coupled online to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP5500, Applied Biosystems, Sciex, Concord, ON, Canada). A turbo ion source (550°C, 5.5 kV) operating in positive mode was used. To determine the content of the SPE fractions, the information-dependent acquisition method (IDA) was used. Enhanced product ion spectra (EPI) were acquired at the interval 50—1000 Da with 50 V collision energy and 20 V collision energy spread. Data were gathered and processed with Analyst QS (Version 1.5.1, Applied Biosystems/MDS Analytical Technologies, Concord, ON, Canada, 2008).

3. Results

As a result of solid phase extraction, eight fractions from *S. subsalsa* extract were collected. In the text, they were denoted by numbers corresponding to the methanol concentration [%] in the eluent.

3.1. Inhibition of enzyme activity

Inhibitory activity of SPE fractions against proteases was assessed using elastase, trypsin, thrombin chymotrypsin and carboxypeptidase A. Results of the assays are shown in Table 2. No inhibitory effect was observed for elastase, trypsin or thrombin. Fractions 70 and 80 revealed moderate effect for chymotrypsin. Fraction 90 inhibited carboxypeptidase A, but only at the highest concentration.

3.2. Cytotoxicity against human breast cancer cells

The activity of eight SPE fractions from *S. subsalsa* extract toward T47D human breast cancer cell line was evaluated using MTT viability test. As shown in Fig. 1, fractions 40, 60, 80 and 90, revealed the highest potency in decreasing T47D cancer cell viability. The strongest effect was observed for fraction 40 and 60 with IC_{50} values 25 μg ml^{-1}, yet concentration-dependency in case of those fractions were low. Cytotoxic and concentration-dependent effects were observed for fractions 80 and 90 with IC_{50} values 100 μg ml^{-1}. Of all tested samples, the highest decrease in T47D cancer cell viability was recorded for fraction 90 applied at concentration 200 μg ml^{-1} (mean relative viability 17.5%).

3.3. LC—MS/MS analysis

The content of the active fractions was analyzed with LC—MS/MS system optimized for the detection of peptides. Using IDA mode, the *m/z* values, retention time and a peak area of the detected ions were determined (Table 3). For the most intensive peaks, which could be responsible for the observed

Table 1 Enzyme inhibition assay.

	Chymotrypsin	Trypsin	Elastase	Thrombin	Carboxypeptidase-A
Enzyme	Chymotripsin 0.1 mg ml^{-1} Solvent: buffer solution Volume: 10 μl	Trypsin 1 mg ml^{-1} Solvent: buffer solution Volume: 10 μl	Elastase 75 μg ml^{-1} Solvent: buffer solution Volume: 10 μl	Thrombin 0.5 mg ml^{-1} Solvent: water Volume: 10 μl	Carboxypeptidase-A 1.6 μM Solvent: buffer solution Volume: 10 μl
Inhibitor (or sample)	Aprotinine 50–500 μg ml^{-1} Solvent: water Volume: 10 μl	Aprotinine 10–60 μg ml^{-1} Solvent: water Volume: 10 μl	Elastatinal 5–125 μg ml^{-1} Solvent: 1% DMSO Volume: 10 μl	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) 60–2400 μg ml^{-1} Solvent: water Volume: 10 μl	CPI 5–100 μg ml^{-1} Solvent: water Volume: 10 μl
Buffer	50 mM Tris–HCl 100 mM NaCl 1 mM $CaCl_2$, pH 7.5 Volume: 100 μl	50 mM Tris–HCl, 100 mM NaCl, 1 mM $CaCl_2$, pH 7.5 Volume: 100 μl	0.2 M Tris–HCl, pH 8.0 Volume: 150 μl	0.2 M Tris–HCl, pH 8.0 Volume: 170 μl	50 [mM] Tris–HCl, pH 7.5 Volume: 160 μl
Preincubation	5 min at 25°C	5 min at 25°C	20 min at 30°C	10 min at 25°C	10 min at 25°C
Substrate	Suc-Gly-Gly-pnitroanilide 2 mM Solvent: buffer solution Volume: 100 μl	Nα-benzoyl-L-arginine-4-nitroanilide hydrochloride BAPNA; 2 mM Solvent: buffer solution Volume: 100 μl	N-succinyl-Ala-Ala-Ala-p-nitroanilide 2 mM Solvent: buffer solution Volume: 30 μl	N-p-tosyl-Gly-Pro-Lys-p-nitroanilide acetate salt 0.5 mg ml^{-1} Solvent: buffer solution Volume: 20 μl	N-4-Metoxyphenylazoformyl-Phe-OH potassium salt; 0.2 mM Solvent: 1% DMSO Volume: 20 μl
Incubation	20 min at 25°C	20 min at 25°C	10 min at 30°C	10 min at 25°C	10 min at 25°C
Wavelength	405 nm	405 nm	405 nm	405 nm	350 nm
Reference	(Ocampo Bennet, 2007)	(Pluotno and Carmeli, 2005)	(Kwan et al., 2009)	(Ocampo Bennet, 2007)	(Ocampo Bennet, 2007)

Table 2 Inhibition of serine proteases by SPE fractions from *Spirulina subsalsa* CCNP1310; Inhibitory effects against the enzymes were studied by microplate test method as described in Section 2. Results are mean of tests done in triplicate; "+" inhibition observed up to 100 x dilution; "–" no effect observed.

Fraction	0	40	50	60	70	80	90	100
Elastase	–	–	–	–	–	–	–	–
Trypsin	–	–	–	–	–	–	–	–
Thrombin	–	–	–	–	–	–	–	–
Chymotrypsin	–	–	–	–	+	+	–	–
Carboxypeptidase A	–	–	–	–	–	–	+	–

activity, the product ion spectra were collected (Supplementary materials Fig. 1S). We identified three groups of ion peaks which share the same fragment ions and are possibly linked with effects observed in conducted tests. Presence of ions characterized by m/z at 677 (in fractions 40 and 50) and 832, (in fraction, 90) (1) and doubly charged ions with m/z 602 (fractions 60 and 70) and 594 (fraction 60) (2), corresponds to cytotoxicity revealed in MTT test. Ion peak at m/z = 649 was detected in fractions 70 and 80 which showed inhibitory activity against chymotrypsin (3).

4. Discussion

In the search for agents effective in the treatment of incurable or/and persistent diseases, the marine environment with its unexplored biodiversity appears to be of high importance. Seas and oceans are inhabited by 92.3 million species and marine organisms make up 95% of the biomass on Earth. However, only 10% of publications on biodiversity refers to marine organisms and only slightly over 0.1% of published genomes describe marine species (Titilade and Olalekan, 2015). Also, the knowledge about secondary metabolites produced by marine organisms is limited. Among marine microorganisms, cyanobacteria are one of the most effective producers of compounds which show strong and diverse biological activity. It was estimated that approximately 24% of marine natural products commercially available for biomedical research are of cyanobacterial origin (Gerwick and Moore, 2012). Due to diverse mechanisms of action and potent activity, the compounds are highly attractive as potential drug candidates (Felczykowska et al., 2015; Gerwick and Moore, 2012; Herfindal et al., 2005; Humisto et al., 2016; Mazur-Marzec et al., 2015; Oftedal et al., 2010). Bioactive cyanobacterial products were mainly identified in organisms from tropical waters. However, recent studies revealed that also cyanobacteria from temperate regions, such as the Baltic Sea, can be explored as sources of metabolites with the potential biotechnological application.

In order to select natural products with desired activity, screening studies with application of different bioassays are conducted. In the tests, the effect of metabolites on the specific proteolytic activity of the enzyme can be studied. Proteases are fundamental for the functioning of live structures and occur in all organisms (Patel, 2017). Compounds, which deregulate activity of such proteases as chymotrypsin, trypsin, elastase, and thrombin, can find application in the treatment of several metabolic disorders, such as urticaria, contact dermatitis, asthma, inflammatory bowel disease, blood clogging, neurological disorders or cancer (Patel, 2017; Sapio and Fricker, 2014).

Several strains of Baltic cyanobacteria were proven to produce metabolites with inhibitory effect against serine proteases (Table 4). Neither of the previously tested crude extracts from 27 Baltic cyanobacteria (Mazur-Marzec et al., 2015), nor SPE fractions from *S. subsalsa* CCNP1310 examined in this study, were active against elastase. That can indicate the rare occurrence of elastase inhibitors among metabolites produced by these microorganisms. Separated fractions from

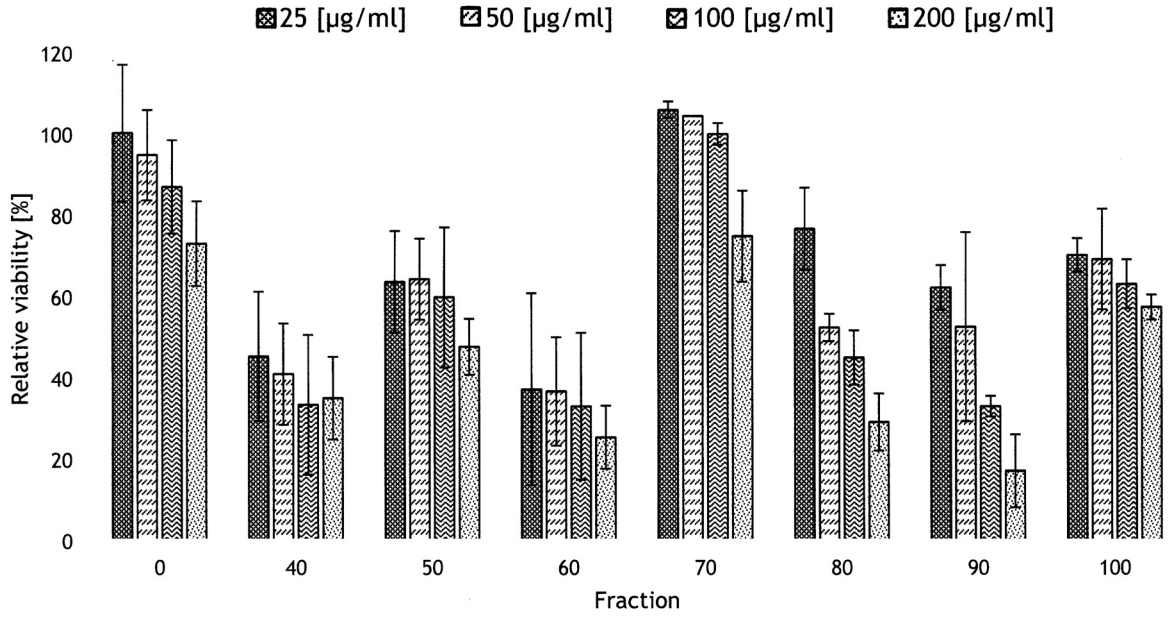

Figure 1 The effects of SPE fractions from *Spirulina subsalsa* CCNP1310 extract on the viability of T47D human breast cancer cells assayed by MTT method as described in Section 2. Each bar represents a mean (±SD) of three experiments performed in triplicate.

Table 3 Characteristics of ions detected by LC–MS/MS in Spirulina CCNP1310 fractions.

Fraction	Retention time [min]	Peak area of extracted ion	*m/z*	Fragment ions
40	9.23	2.81×10^{10}	677	70, 86, 103, 120
	10.45	1.11×10^{10}	633	271, 289, 299, 317
	10.50	2.29×10^{9}	573	
	10.93	3.18×10^{9}	557	195, 213, 281, 299, 367, 385, 453, 471
50	4.63	2.89×10^{9}	621	70, 170, 243, 534, 541, 626, 643
	5.59	1.47×10^{9}	518	127, 151, 210, 271, 415, 474
	9.24	1.19×10^{10}	677	70, 86, 103, 120
	9.47	5.28×10^{9}	500	127, 145, 379, 397, 456
	11.68	6.92×10^{9}	659	120, 379, 395, 511, 572, 599
60	9.68	2.59×10^{10}	1203/602[a]	70, 112, 138, 180, 209, 350, 378
	10.35	3.75×10^{9}	1187/594[a]	70, 138, 180, 209, 350, 595, 633, 762, 886
70	9.68	6.41×10^{9}	1203/602[a]	70, 112, 138, 180, 209, 350, 378
	9.79	6.51×10^{9}	1050/524[a]	86, 136, 183, 262, 308, 326, 432, 478, 562, 747, 860, 975
	10.45	2.31×10^{9}	547	105, 173, 323, 349, 385, 529
	11.10	4.25×10^{9}	647	159, 323, 385, 347, 485
	11.78	1.01×10^{10}	649	109, 171, 259, 421, 487
80	1.96	1.15×10^{9}	641	109, 139, 183, 297, 413, 527, 605
	11.09	3.78×10^{9}	647	86, 104, 125, 166, 184, 502
	11.55	5.79×10^{9}	649	109, 171, 259, 421, 487
	11.79	1.71×10^{10}	650	347, 487
90	9.71	1.25×10^{10}	832	70, 86, 103, 120
100	11.59	1.34×10^{9}	883	347, 405, 467, 482, 500, 629, 657, 721
	11.65	3.28×10^{9}	500	144, 236, 438, 482, 500

[a] Doubly charged ions.

CCNP1310 had also no effect against trypsin and thrombin, although such activity was previously revealed in the crude extract from this cyanobacterium. The possible explanations for this discrepancy are that during SPE procedure some compounds were lost and/or some were separated into several different fractions. As a consequence, their concentration in the tested samples could have been too low to exert any effect on the enzyme. We revealed the moderate activity of fractions 70 and 80 from *S. subsalsa* CCNP1310 against chymotrypsin for the first time. As in both fractions, ion peak at $m/z = 649$ was detected, the link between its presence and the inhibitory activity against chymotrypsin can be assumed. Carboxypeptidase A was found to be inhibited by components of fraction 90 and this result confirms previous effects observed in tests performed on a crude extract from the cyanobacterium (Mazur-Marzec et al., 2015).

To discover anticancer natural products, cell based methods are the most common. The T47D cell line applied in our tests derives from a 54-year-old woman and is one of the most widely used cell line in breast cancer studies. It was shown to be an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer (Yu et al., 2017). Cytotoxic activity against cancer cells have been revealed in many strains of cyanobacteria, but only in several cases, the active agents were identified (Rastogi and Sinha, 2009; Singh et al., 2011). The range of cellular targets of cytotoxic cyanobacterial metabolites is wide and include cytoskeletal structures, as well as enzymes (Table 5).

In addition, there are numerous natural anticancer agents whose mechanism of action has not been elucidated yet, e.g.: dragonamides, minutissamides, and almiramides (Humisto et al., 2016).

As for Baltic cyanobacteria, Herfindal et al. (2005) and Oftedal et al. (2010) found that extracts from benthic strains of *Anabaena* sp. induced apoptosis of acute myeloid leukemia cells. Further research of Humisto et al. (2016) confirmed that Baltic-derived *Anabaena* strains produce the most promising leukemia drug candidate. In studies carried out by Felczykowska et al. (2015), ethanol extracts from Baltic strains of *Pseudanabaena* sp., *Microcystis aeruginosa*, and *Pseudanabaena cf. galeata* had cytotoxic activity toward breast cancer cells and uterine cervix origin, but not against normal dermal fibroblasts. Although in several studies a potential of Baltic cyanobacteria to synthesized novel bioactive compounds was demonstrated, the metabolites responsible for cytotoxic effects were not identified. In our work, we proved that some SPE fractions from of *S. subsalsa* CCNP1310 cells contain metabolites cytotoxic against human breast cancer cells, line T47D. As active fractions were separated by non-active one, we presume that *S. subsalsa* CCNP1310 produces more than one cytotoxic agent.

Mass spectrometry analysis conducted in our study revealed that in the TIC of fraction 90, only one major ion peak was present, which strongly links it with the observed cytotoxic activity against the T47D cells. The ion detected in IDA mode is characterized by m/z 832, and fragment ions at m/z 70, 86,

Table 4 Marine cyanobacterial metabolites of known inhibitory properties against selected proteases.

Compound	Species	Class of compound	Place of isolation	Target (protease)	Reference
Bouillomides A–B	*Lyngbya bouillonii*	Depsipeptides	Guam	Elastase, chymotrypsin	Rubio et al. (2010)
Kempopeptin A	*Lyngbya* sp.	Cyclic depsipeptide	Grassy Kay	α-Chymotrypsin, elastase	Taori et al. (2008)
Kempopeptin B	*Lyngbya* sp.	Cyclic depsipeptide	Grassy Kay	Trypsin	Taori et al. (2008)
Largamides A–C	*Lyngbya confervoides*	Cyclic depsipeptides	No data	Elastase	Plaza and Bewley (2006)
Largamides D–G	*Oscillatoria* sp.	Cyclic depsipeptides	No data	α-Chymotrypsin	Plaza and Bewley (2006)
Lyngbyastatin 4	*Lyngbya confervoides*	Cyclic depsipeptide	Florida	α-Chymotrypsin, elastase	Matthew et al. (2007)
Lyngbyastatin 5–7	*Lyngbya* spp.	Cyclic depsipeptides	South Florida	Elastase	Taori et al. (2007)
Lyngbyastatin 8–10	*Lyngbya semiplena*	Cyclic depsipeptides	Guam	Elastase	Kwan et al. (2009)
Molassamide	*Dichothrix utahensis*	Depsipeptide	Molasses	α-Chymotrypsin, elastase	Gunasekera et al. (2008)
Pompanopeptin A	*Lyngbya confervoides*	Cyclic peptide	Florida	Trypsin	Matthew et al. (2008)
Symplocamide	*Symploca* sp.	Cyclic peptide	Papua New Guinea	Chymotrypsin, trypsin	Linington et al. (2008)
Tiglicamides A–C	*Lyngbya confervoides*	Cyclic depsipeptides	Florida	Elastase	Matthew et al. (2009)
Somamide B	*Lyngbya majuscula* *Schizothrix assemblage*	Cyclic depsipeptide	South Florida	Elastase, chymotrypsin	Taori et al. (2007)
Aeruginosins	*Microcystis viridis*, *Oscillatoria agardhii*, *Microcystis aeruginosa*	Lipopeptide	No data	Trypsin, thrombine	Ersmark et al. (2008)
Pitipeptolides A- B	*Lyngbja majuscula*	Cyclic depsipeptides	No data	Elastase	Costa et al. (2012)
Anabenopeptin	*Oscilatoria agardhii*	Cyclic peptides	No data	Trypsin	Burja et al. (2001)
Aeruginosins	*Nodularia spumigena*	Linear peptides	Baltic Sea	Trypsin, thrombine	Fewer et al. (2013)
Spumigins	*Nodularia spumigena*	Linear peptides	Baltic Sea	Trypsin	Fewer et al. (2009)
Anabenopeptines	*Nodularia spumigena*	Cyclic peptides	Baltic Sea	Trypsin, carboxypeptidase A, elastase	Spoof et al. (2015)
Pseudoaeruginosins	*Nodularia spumigena*	Peptide	Baltic sea	Trypsin	Fewer et al. (2013)

Table 5 Marine cyanobacterial metabolites of known structure and anticancer properties.

Compound	Species	Place of isolation	Molecular targets	Cancer cell line	Reference
Apratoxin A	*Lyngbya majuscula*	Finger's Reef, Apra Harbor, Guam	JAK/STAT pathway; inhibits Hsp90 function	KB, LoVo	Luesch et al. (2001b)
Aurilide	*L. majuscula*	Papua New Guinea	Prohibitin 1	NCI-H460, Neuro-2a	Han et al. (2006)
Bisebromoamide	*Lyngbya* sp.	Okinawa Prefecture	Actin filaments	HeLa	Teruya et al. (2009)
Carmaphycins A and B	*Symploca* sp.	Curaçao	Proteasome	H-460, HTC-116	Pereira et al. (2012)
Curacin A	*L. majuscula*	Curaçao	Microtubules	MCF-7, MDA-MB231, PC-3, OV-2008	Wipf et al. (2004)
Dolastatins 10 and 15	*Symploca* sp.	Ulong Channel, Palau	Microtubules	KB, LoVo	Luesch et al. (2001a)
Hoiamide D	*L. majuscule* and *Phormidium gracile*	Papua New Guinea	Inhibits MDM2/p53 interaction	NCI-H460, Neuro-2a	Pereira et al. (2009)
Isomalyngamides A and A1	*L. majuscula*	Taiwan	Inactivation of cellular kinases	MCF-7, MDA-MB-231	Chang et al. (2011)
Lagunamide A	*L. majuscula*	Pulau Hantu, Singapore	Prohibitine 1	P388, A549, PC3, HCT8, SK-OV3, HCT8, MCF7	Tripathi et al. (2012)
Largazole	*Symploca* sp.	Key Largo, Florida	Class 1 histone deacetylase	MDA-MB-231, U2OS, HT29, IMR-32	Taori et al. (2007)
Somocystinamide A	*L. majuscula*	Somo Somo, Fiji	Caspase-8-dependent cell death pathway	Neuro-2a	Nogle and Gerwick (2002)
Symplocamide A	*Symploca* sp.	Sunday Island, Papua New Guinea	Chymotrypsin	H-460, Neuro-2a	Linington et al. (2008)
Tubercidin	*Plectonema radiosum*	Fiji	Microtubules	KB, HL-60	Stewart et al. (1988)

120. These low mass ions are probably immonium ions of Pro (or Arg), Leu/Ile and Phe, respectively, and indicate the peptidic structure of the compound. Trace amounts of the ion in fraction 80 was detected in enhanced ion product mode. As ion at *m/z* 677, present in cytotoxic fractions 40 and 50, also gave these product ions, it can be concluded that it represents structural analogue of the same class of peptide as a compound in fraction 90. The TIC of fraction 60, which was also active in MTT assay against the breast cancer cells, was characterized by the presence of major ion peak at *m/z* 602 and less intense ion peak at *m/z* 594. Both ions have several fragment ions in common, therefore they can also be classified to the same group of compounds. Ion peak at *m/z* 602 can be observed also in TIC of fraction 70. This fraction, however, was not active in MTT assay, which can be explained by the four-times lower content of the compound.

In our study, the activity of *S. subsalsa* metabolites against T47D cells was revealed. Although an extract from a small amount (2 g) of lyophilized material was used, significant effects were observed even at 25 $\mu g\ ml^{-1}$ concentration of the tested material. In similar studies conducted on crude extracts from 1 g of Baltic-derived cyanobacteria, activity against cancer cells was also observed, but never at concentrations lower than 50 $\mu g\ ml^{-1}$ (Felczykowska et al., 2015). This could indicate that *S. subsalsa* metabolites are highly active and exert an effect on T47D cells even at small amounts. Low amounts of bioactive compounds produced by organisms might make the process of their identification difficult. In such cases, the mass cultivation of cyanobacteria is indispensible to isolate sufficient quantities of the active molecule and to proceed to further steps of drug discovery process.

Cytotoxic activity of metabolites of *S. subsalsa* has never been reported. Our research indicates that compounds produced by this cyanobacterium not only have strong activity against T47D cancer cells but also seem to work selectively, as they do not have strong inhibitory effect against the tested enzymes, especially in the case of fractions 40 and 60. Although in conducted research, the compounds responsible for the observed activity were not unequivocally identified, some characteristic features of their structure were described. This improves the quality of the work, compared to studies where only activity of crude cyanobacterial extract was documented. The tests showed for the first time the existing potential of the Baltic *S. subsalsa* to be used as a source of important cytotoxic agents. In view of these promising results, further studies are worth to be continued.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.oceano.2017.11.003.

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