**Universidade de Aveiro**
**Ano 2011/2012**

Departamento de Química

**Maria João
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**Cianobactérias marinhas: avaliação do
potencial anticancerígeno**

**Marine cyanobacteria: evaluation of the
anticancer potential**

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**Marine cyanobacteria: evaluation of the anticancer potential**

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação da Professora Doutora Maria do Rosário Fidalgo Martins, e co-orientada pelo Professor Doutor Manuel António da Silva Santos.

**o júri**

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**resumo**      Durante as últimas décadas, as cianobactérias têm ganho importância
devido à sua capacidade de sintetizar metabolitos secundários com
actividade biológica, úteis no tratamento de inúmeras doenças, tal
como o cancro. A caracterização dos metabolitos secundários com
aplicações farmacológicas tem sido maioritariamente feita com
cianobactérias filamentosas marinhas, do género *Lyngbya*, *Microcoleus*
e *Symploca,* recolhidas em áreas tropicais. Contudo, a investigação em
cianobactérias marinhas que ocorrem em baixas densidades e em
regiões temperadas, como a costa Portuguesa, tem sido largamente
negligenciada. Neste sentido, este trabalho teve como objectivo avaliar
o potencial anticancerígeno de cianobactérias isoladas da costa
Portuguesa e mantidas no laboratório de Ecotoxicologia, Genómica e
Evolução do Centro Interdisciplinar de Investigação Marinha e
Ambiental, Porto. Cinco estirpes de cianobactérias pertencentes aos
géneros *Cyanobium*, *Leptolyngbya*, *Romeria* e *Synechocystis*,
LEGE06098 (*Cyanobium* sp.), LEGE06113 (*Cyanobium* sp.),
LEGE06102 (*Leptolyngbya cf. halophila*), LEGE06013 (*Romeria* sp.) e
LEGE06155 (*Synechocystis salina*), foram cultivadas em condições
laboratoriais. A partir da biomassa liofilizada foi obtido um extracto
bruto usando metanol e diclorometano, o qual foi posteriormente
fraccionado usando hexano, acetato de etilo e metanol, originando as
fracções A, B e C, respectivamente, de forma a separar compostos
com polaridades crescentes. Com o extracto bruto e as fracções foram
realizados ensaios de citotoxicidade em quatro linhagens humanas
tumorais: osteosarcoma (MG63), adenocardinoma de cólon (RKO e
HT29) e neuroblastoma (SH-SY5Y). A citotoxicidade foi avaliada
através da redução do bromide 3-(4,5-dimetil-tiazol-2-il)-2,5-difenil-
tetrazolio (MTT) e do ensaio da lactato desidrogenase (LDH). A
ocorrência de Apoptose/Necrose foi investigada pelo método de
exclusão dos corantes, usando Iodeto de Propídeo e Hoechst 33342.
De entre o extracto bruto e as fracções preparadas de cada estirpe de
cianobactérias, a fracção com a maior percentagem de acetato de etilo,
fracção B, revelou uma maior percentagem de inibição das células
tumorais, sendo, portanto, promissora relativamente ao isolamento de
compostos bioactivos. De entre as estirpes de cianobactérias incluídas

no estudo, a estirpe *Synechocystis salina*, LEGE06155, revelou ser a mais interessante para o isolamento de potenciais compostos bioactivos.

**keywords**

**abstract**

In the last few decades, cyanobacteria have gained significant importance due to their ability to synthesize secondary metabolites with biological activity, useful in the treatment of a role of diseases, such as cancer. Characterization of bioactive metabolites with pharmacological applications has mainly been performed with marine filamentous cyanobacteria of the genera *Lyngbya*, *Microcoleus* and *Symploca* collected in tropical areas. However, the research on marine cyanobacteria that occur in low densities in temperate regions such as the Portuguese coast has been largely overlooked. In this work we aimed to assess the anticancer potential of marine cyanobacteria isolated from the Portuguese coast. Five cyanobacteria strains from the genera *Cyanobium*, *Leptolyngbya*, *Romeria* and *Synechocystis*, LEGE06098 (*Cyanobium* sp.) and LEGE06113 (*Cyanobium* sp.), LEGE06102 (*Leptolyngbya cf. halophila*), LEGE06013 (*Romeria* sp.), and LEGE06155 (*Synechocystis salina*), respectively, were cultivated under laboratory conditions. From freeze dried biomass a crude extract was obtained using methanol and dichloromethane and fractionated using hexane, ethyl acetate and methanol, yielding fractions A, B and C with increasing polarity. The crude extract and the fractions were tested for cytotoxicity in four human tumor cell lines: osteosarcoma (MG63), colon adenocarcinoma (RKO and HT29) and neuroblastoma (SH-SY5Y). Cytotoxicity was evaluated by the reduction of the bromide 3-(4,5-dimetil-tiazol-2-il)-2,5-difenil-tetrazolio (MTT) and the Lactate Dehydrogenase (LDH) assay. Apoptosis/Necrosis was investigated by the dye exclusion method, using both Propidum Iodide and Hoechst 33342. Among the crude extract and prepared fractions, the fraction containing the higher percentage of ethyl acetate, fraction B, from each cyanobacteria strain revealed the highest percentage of inhibition of tumor cells growth, and is, therefore, promising in terms of isolation of bioactive compounds. Among the cyanobacteria strains, the *Synechocystis salina* strain, LEGE06155, revealed to be an interesting strain for the isolation of bioactive compounds.

# Table of contents

Natural products have been played an enormous role in the development of modern medicines. Over the years, with numerous investigations on bioactive natural products, it has been possible to gather many of the most important chemotherapeutic agents for the treatment of human diseases (BOOPATHY and KATHIRESAN, 2010, NEWMAN and CRAGG, 2007).  In fact, along with their derivatives, natural products represent more than 50% of the drugs in clinical use (BOOPATHY and KATHIRESAN, 2010). Beside its importance as pharmacological agents, natural products have been also providing lead structures for the development of novel synthetically derived drugs (PROKSCH [et al.], 2002).

Higher plants and terrestrial microorganisms have been considered the richest sources of natural drugs (PROKSCH [et al.], 2002), yielding many useful organic compounds, such as aspirin or penicillin (BURJA [et al.], 2001).  Marine products, whether due to evolution or environmental features specific to the oceans, retain structural features which are quite different from the terrestrial ones (VILLA and GERWICK, 2010).

By the early 1960's, the oceans began to be seen as a totally new and untouched source of potentially useful compounds (BURJA [et al.], 2001). The narrow ocean fringe and the deep sea vent communities harbor most of the world's species, making them the most species rich and biologically productive regions of the world. This intense concentration of biodiversity shapes these regions into highly competitive and complex habitats (SIMMONS [et al.], 2005). Such intense competition has provided those species with chemical tools, by which they can defend against predation or the overgrowth of competing species (WILLIAMS [et al.], 1989). The chemical adaptation of organisms is usually associated with secondary metabolites, such as alkaloids and terpenoids (SIMMONS [et al.], 2005). These marine natural products must have a strong toxic profile to show any effect, considering that they are rapidly diluted in the water (HAEFNER, 2003).

Over the last few decades, through the combined efforts of pharmaceutical companies and academic institutions it could be possible to isolate and identify more than 10 000 new marine-derived natural products (BOOPATHY and KATHIRESAN, 2010).

Among marine organisms, invertebrates including sponges, soft corals, sea fans, tunicates, corals and molluscs, fungi and sediment-derived bacteria, have served a tremendous source of structurally unprecedented bioactive secondary metabolites with anti-cancer, anti-inflammatory and anti-infective properties (Table 1) (SIMMONS [et al.], 2008, CRAGG [et al.], 2009, BOOPATHY and KATHIRESAN, 2010). Considering prokaryotes between 1997 and 2008, the great majority of the marine bacterial isolated compounds were obtained from the Actinobacteria (256 compounds), Cyanobacteria (220 compounds), Proteobacteria (78 compounds), Firmicutes (35 compounds) and Bacteroidetes (34 compounds) (Table 2; WILLIAMS, 2009). It is now also known that bacteria and cyanobacteria present in some marine invertebrates, either assimilated by grazing or growing in association in a symbiotic or commensal relationship, are frequently the true source of the secondary metabolites isolated from those invertebrates (SIMMONS [et al.], 2008).

**Table 1.** Some natural bioactive products isolated from marine invertebrates and their pharmacological application adapted from FUSETANI, 2000.

| Organisms | Compound | Disease area | Reference |
|---|---|---|---|
| **Porifera** | | | |
| *Agelas mauritianus* | KRN7000 | Cancer | (KOBAYASHI [et al.], 1995) |
| **Coelenterata** | | | |
| *Pseudopterogorgia elisabethae* | Methopterosin | Inflammation | (CORREA [et al.], 2009) |
| **Mollusca** | | | |
| *Dolabella auricularia* | Dolastatin 10 | Cancer | (NEWMAN [et al.], 1994) |
| | LU-103793 | Cancer | (DE ARRUDA [et al.], 1995) |
| *Conus magnus* | Ziconitide | Pain | (SNUTCH and DAVID, 2006) |
| **Bryozoa** | | | |
| *Bugula neritina* | Bryostatin 1 | Cancer | (KENNEDY [et al.], 1992) |
| **Urochordata** | | | |
| *Trididemnum solidum* | Didemnin B | Cancer | (RINEHART [et al.], 1981) |
| | Dehydrodidemnin B | Cancer | (URDIALES [et al.], 1996) |
| *Aplidium albicans* | Ecteinascidin 743 | Cancer | (RINEHART [et al.], 1990) |
| *Ecteinascidia turbinata* | | | |
| **Chordata** | | | |
| *Squalus acanthias* | Squalamine | Cancer | (SILLS [et al.], 1998) |

**Table 2.** Some marine bacterial compounds with pharmacological application adapted from WILLIAMS, 2009.

| Compound | Biological target | Potential therapeutic uses | Reference |
|---|---|---|---|
| **Actinomycetes** | | | |
| Marynomicin | Cytotoxic | Oncology | (KWON [et al.], 2006) |
| Abyssomicin | ℗-Aminobenzoic acid | Infectious disease | (BISTER [et al.], 2004) |
| Proximicin | Antiproliferative | Oncology | (YE [et al.], 1990) |
| SS-228 Y | Antibacterial | Infectious disease | (OKAZAKI [et al.], 1975) |
| Thiocoraline | DNA polymerase | Oncology, infectious disease | (TRIMURTULU and FAULKNER, 1994) |
| Salinosporamide A | Proteosome inhibitor | Oncology, malaria, Alzheimer's disease | (FELING [et al.], 2003) |
| **Proteobacteria** | | | |
| Bryostatin | Protein kinase C | Oncology | (SUDEK [et al.], 2007) |

Cyanobacteria have been considered as one of the most promising groups of organisms from which novel bioactive products with pharmaceutical applications can be isolated (BOOPATHY and KATHIRESAN, 2010).

Cyanobacteria (blue-green algae) are ancient gram-negative photosynthetic prokaryotes, placed within the *Eubacteria* group. This group constitutes the first level organisms in food chains in water ecosystems, as a consequence of their photosynthetic capacity (SAINIS [et al.], 2010). Moreover, they also have the ability of anaerobic metabolism and the capacity to use elemental sulfur for anaerobic dark respiration (COHEN and GUREVITZ, 2006). They also have an important role in the marine nitrogen cycle, by fixing atmospheric nitrogen (N), and in $CO_2$ dynamics (WHITTON and POTTS, 2002). Within the cyanobacteria group 150 genera and about 2000 species are described (VAN APELDOORN [et al.], 2007).

In terms of morphology, cyanobacteria are either single celled, colonial or filamentous, and in most species, each cell is surrounded by a cell wall made of peptidoglycan and lipopolysaccharide layers, which, in turn, is surrounded by a gelatinous or mucilaginous sheath (DUY [et al.], 2000). These organisms commonly reproduce by fission, budding, trichome breakage, hormogonia formation or akinetic germination. Other strategies involve conjugation, transformation, and transduction (RAI, 1990). Regarding the physiology, cyanobacteria exhibit versatile physiology and a wide ecological tolerance which contributes to their competitive success over a broad spectrum of environments (COHEN and GUREVITZ, 2006). In fact, these organisms can be found in a wide range of habitats, such as ice fields, hot springs and deserts being especially common in freshwater, brackish and marine environments (MARTINS [et al.], 2005). Considering the aquatic environments, it is impossible to fully separate freshwater and marine cyanobacteria, considering that some species are capable to grow in both environments, and produce similar or different natural products (BURJA [et al.], 2001).

In aquatic habitats, cyanobacteria are able to reproduce quite rapidly, when exposed to specific conditions, which can result in a very high cell density (blooms), that can cause severe changes in the water quality (VASCONCELOS, 2006). These bloom

conditions are intrinsically connected with nutrients, light and temperature, and have direct consequences in water transparency, oxygen levels and release of toxins (VASCONCELOS, 2006).

The first studies concerning the production of bioactive compounds by cyanobacteria were based on the production of toxins and in the occurrence of intoxication episodes. The cyanobacteria responsible for toxin poisoning episodes include around 40 genera, being the main ones *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis*, *Lyngbya*, *Nostoc* and *Oscillatoria* (*Planktothrix*) (CARMICHAEL, 2001). Toxin production by cyanobacteria seems to be significantly variable, either within and between species and strains, and may be related to various factors such as growing phase, light intensity, temperature, pH, oxygen saturation, nutrient concentration, and trace metals, such iron and zinc (LUKAC and AEGERTER, 1993).

Cyanotoxins are biotoxins, accountable for acute and (sub)chronic poisonings of wild and domestic animals and even humans (VAN APELDOORN [et al.], 2007). An extensive research concerning toxins has been performed with freshwater cyanobacteria strains as a consequence of toxic blooms and intoxication episodes with mammals, aves and reptiles. Toxic blooms and toxicological studies are, however, rare for marine cyanobacteria since these phenomena are scarcer and more localized (NAGARAJAN [et al.], 2011). The mechanisms by which cyanotoxins exert their toxic effect are distinct since they are structurally diverse chemicals (ZEGURA [et al.], 2011). In terms of chemical structure, cyanotoxins are grouped in cyclic peptides, alkaloids and lipopolyssacharides (LPS) (VAN APELDOORN [et al.], 2007). In terms of toxicity they are classified in five functional groups, hepatotoxins, neurotoxins, cytotoxins, dermatoxins and irritant toxins (WIEGAND and PFLUGMACHER, 2005), as described in Table 3.

**Table 3.** Main toxins produced by Cyanobacteria.

| Chemical structure | Toxin | Source organism(s) | Mechanism of action | Reference |
|---|---|---|---|---|
| Alkaloids | Aplysiatoxins | *Lyngbya majuscula* | **Dermatoxin** Inflammatory activity | (MYNDERSE [et al.], 1977) |
| | Lyngbyatoxins | *Lyngbya majuscula* | **Dermatoxin** Inflammatory activity | (CARDELLINA [et al.], 1979) |
| | Cylindrospermopsin | *Cylindrospermopsis raciborskii* | **Hepatotoxin** Blocks protein synthesis | (TERAO [et al.], 1994) |
| | Anatoxin-a | *Anabaena flos-aquae* | **Neurotoxin** Binding irreversibly to acetylcholine receptor s | (SPIVAK [et al.], 1980) |
| | Anatoxin-a(S) | *Anabaena flos-aquae* | **Neurotoxin** Inhibition of Ach-esterase activity | (MAHMOOD and CARMICHAEL, 1986) |
| | Saxitoxin | *Anabaena flos-aquae* | **Neurotoxin** Binding and blocking the sodium channels in neural cells | (NARAHASHI, 1972) |
| Cyclic Peptides | Microcystins | *Microcystis aeruginosa* *Anabaena* sp. *Planktothrix agardhii* *P. rubescens* *Oscillatoria agardhii* | **Hepatotoxin** Inhibition of protein serine/threonine phosphatases 1 and 2A (PP1 and PP2) | (HONKANEN [et al.], 1990) |
| | Nodularins | *Nodularia spumigena* | **Hepatotoxin** Inhibition of protein serine/threonine phosphatases 1 and 2A (PP1 and PP2) | (YOSHIZAWA [et al.], 1990) |
| Lipopolysaccharide endotoxins (LPS) | | *Microcystis aeruginosa* *Microcystis* sp. *Phormidium* spp. | **Dermatotoxin** Inflammatory activity | (RAZIUDDIN [et al.], 1983) (MIKHEYSKAYA [et al.], 1977) |

In the last decades, with the improvement of molecular biology tools, complete sets or clusters of biosynthetic pathway genes have been cloned and examined at the molecular genetic level, thus clarifying the pathways by which complex secondary metabolites are assembled, although only a few have been elucidated so far (HERRERO and FLORES, 2008).

To date, most of bioactive metabolites isolated from cyanobacteria have either been polyketides, non-ribossomal peptides, or a mixture of both (BARRIOS-LLERENA [et al.], 2007). The mechanism for non-ribosomal biosynthesis of peptides is performed by a family of enzymes known as non-ribosomal peptide synthetases (NRPS), which assemble to large multi-enzyme complexes (DITTMANN [et al.], 2001). These enzymes include a modular structure, with each module being responsible for the activation, thiolation, modification and condensation of one specific amino acid substrate (KLEINKAUF and VON DOHREN, 1996). Similarly to NRPS, modular polyketide synthases (PKS) are multifunctional megasynthases arranged into repeated functional units and each unit catalyzes all distinct steps of enzyme reactions for the polyketide chain elongation (DITTMANN [et al.], 2001). As an example, in Figure 1, it's clarified the biosynthesis of the toxin barbamide, which uses a mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) system (RAMASWAMY [et al.], 2006).
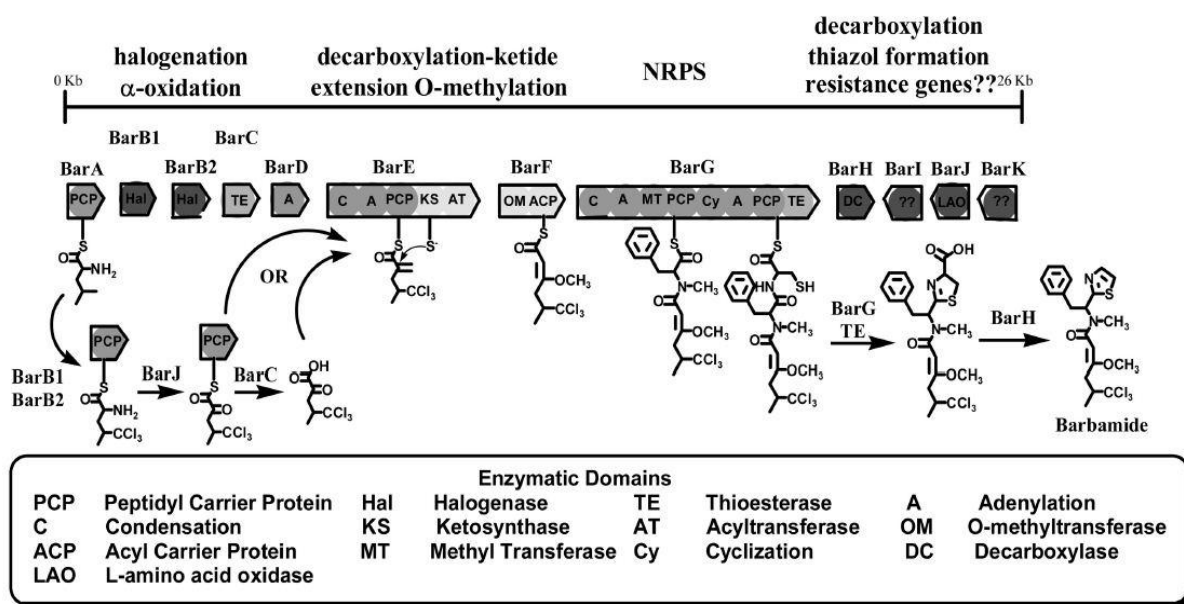


**Figure 1.** Generic architecture of the biosynthetic gene cluster and proposed biosynthesis of barbamide (RAMASWAMY [et al.], 2006).

## 2.1.Biomedical potential of cyanobacteria natural products

Despite the negative impact on ecosystems, an increasing number of studies show that cyanobacteria toxins and other natural products have important characteristics which can outcome in pharmaceutical leads against a wide role of diseases. In fact, cyanobacteria started to be considered medicinally useful as early as 1500 BC, when *Nostoc* species were used to treat gout, fistula and even several forms of cancer (PIETRA, 1990). The exploration of marine cyanobacteria as a source of pharmacological active compounds has been mainly confined to filamentous species that naturally occur in large densities in tropical areas (TAN, 2007). It is also at tropical areas, such as Papua New Guinea, Guam and Palau that the major marine cyanobacteria collections are localized (Figure 2).
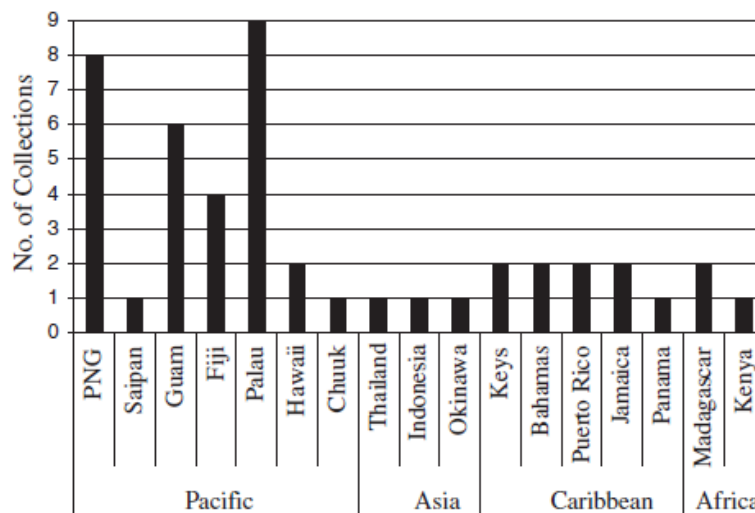


**Figure 2.** Geographical distribution of marine cyanobacteria collections from which secondary metabolites have been reported (PNG - Papua New Guinea) (TAN, 2007).

In a review by GERWICK [et al.], 2008, involving nearly 800 compounds isolated from marine cyanobacteria, it was found that the great majority was produced by filamentous species, being approximately half of such compounds provided by the order Oscillatoriales, as clarified in Figure 3. Among the Oscillatoriales, the genus *Lyngbya* seems to be the major contributor, being the specie *Lyngbya majuscula* the most important one (Figure 4). In a recent work by ENGENE [et al.], 2012 some strains previously included in the genus *Lyngbya,* are now placed in a new genus, *Moorea* gen.

nov.. Both genus share morphological characteristics and the misidentification was a consequence of using traditional morphology-based taxonomic systems. As such, most strains identified as *Lyngbya majuscula* are now included in the genus *Moorea*.
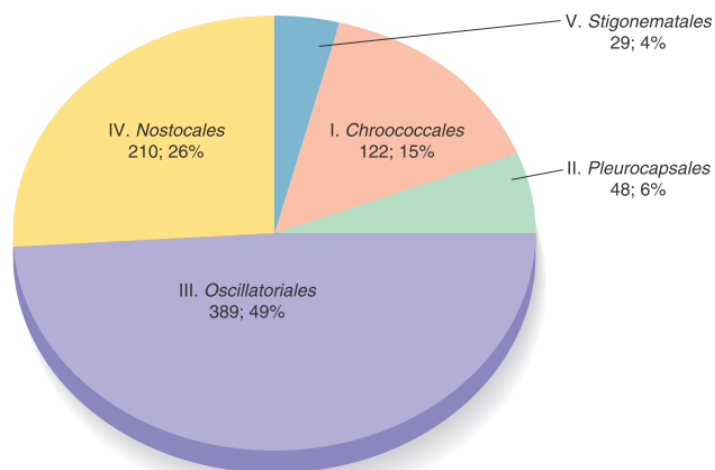


**Figure 3.** Marine cyanobacteria orders from which nearly 800 secondary metabolites have been reported (GERWICK [et al.], 2008).
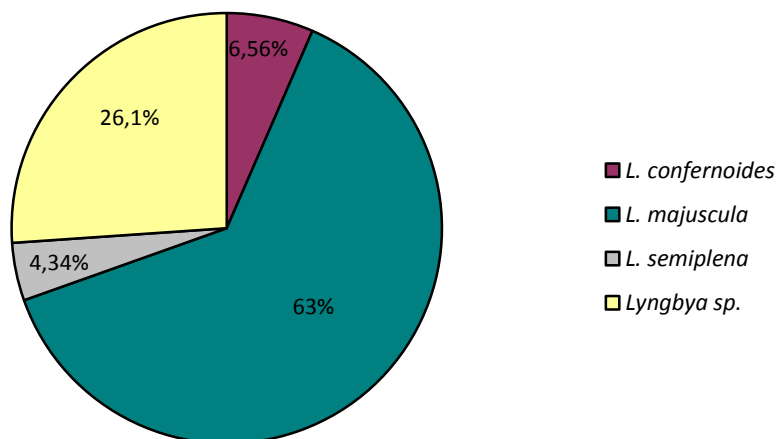


**Figure 4.** Secondary metabolites from the genus *Lyngbya*, published from January 2001 to December 2006 (adapted from TAN, 2007).

Over the past few decades, the biomedical investigation on marine natural products has converged mainly on a few areas: central nervous system (CNS), membrane-active toxins and ion channels effectors, anticancer and antiviral agents, tumor

promoters, anti-inflammatory agents, inhibitors of proteases, and metabolites that control micro-filament-mediated processes (ATTAWAY and ZABORSKY, 1993, NAGARAJAN [et al.], 2011).

Considering that cancer is a major cause of death within the human population, increasing with changing life style, diet and even global warming, we have been witnessed an enormous increase in research concerning anticancer drug discovery (FOLMER [et al.], 2010). The ability to inhibit the growth of cancer cells has been one of the most explored bioactivity, especially in what concerns to cyanobacteria compounds (NAGARAJAN [et al.], 2011). In the past decades there was a significant breakthrough in the number of anticancer lead compounds from distinct marine life entering human clinical trials (SIMMONS [et al.], 2005). In Table 4 there are presented some compounds isolated from marine cyanobacteria that revealed anticancer activity.

Over the last years, a significant number of cyanobacteria natural products have entered several clinical trials. Unfortunately, many of those compounds were dropped from those trials, due to the development of undesired secondary effects on cancer patients, like the case with dolastatin 10 that revealed neurological complications (PITOT [et al.], 1999, SIMMONS [et al.], 2005). Nonetheless, the structure of cyanobacteria natural products can serve as structural templates for the synthesis of highly potent synthetic analogues with reduced cytotoxicity (TAN, 2010).

Many of the molecules presented in Table 4 target specific macromolecules or enzymes associated with cell proliferation. By disrupting the microtubules/actin filaments in eukaryotic cells and inhibiting crucial proteases implicated in tumor progression, invasion and metastasis, the compounds may control the proliferation of cancer cells, thus being invaluable as potential anticancer drugs (NAGARAJAN [et al.], 2011).

Marine cyanobacteria ability to kill cancer cells can rely on two mechanisms: whether by inducing apoptotic death, or by affecting the cell signaling through activation of the members of protein kinase c family of signaling enzymes (BOOPATHY and KATHIRESAN, 2010).

11

**Table 4.** Marine cyanobacteria compounds with anticancer activity.

| Source | Compound | Biological activity | Anticancer activity | Reference |
|---|---|---|---|---|
| *Leptolyngbya* sp. | Coibamide A | ND | Cytotoxicity against NCIH460 lung and mouse neuro-2a cells | (MEDINA [et al.], 2008) |
| *Lyngbya bouillonii* | Apratoxin A | Target the Heat Shock Protein (HSP) and the secretory pathway | Cytotoxicity against a panel of cancer cell lines | (SHEN [et al.], 2009) (LIU [et al.], 2009) (LUESCH [et al.], 2001) |
| *Lyngbya majuscula* | Curacin A | Potent inhibitor of cell growth and mitosis, binding at the colchicine site of tubulin | Potent anti-proliferative and cytotoxic activity against colon, renal and breast–cancer derived cell lines | (GERWICK [et al.], 1994) (VERDIER-PINARD [et al.], 1998) |
| *Lyngbya majuscula* | Hectochlorin | Actin-disrupting agent | Cytotoxicity against colon, melanoma, ovarian and renal cell lines | (MARQUEZ [et al.], 2002) |
| *Lyngbya majuscula* | Jamaicamides | Blocks sodium channels | Cytotoxicity against H-460 human lung and neuro-2a mouse neuroblastoma cell lines | (EDWARDS [et al.], 2004) |
| *Lyngbya majuscula* | Lagunamides | May trigger caspase-mediated apoptosis | Cytotoxic against a panel of cancer cell lines | (TRIPATHI [et al.], 2012) |
| *Lyngbya majuscula* | Somocystinamide | Activation of the death-inducing signaling complex in cell membranes, thus activating caspase 8 and 3 | Stimulates apoptosis in a number of tumor cell lines | (WRASIDLO [et al.], 2008) |
| *Lyngbya* sp. | Bisebromoamide | Inhibition of phosphorylation of ERK, an extracellular signal-related protein kinase | Cytotoxic against a panel of cancer cell lines | (TERUYA [et al.], 2009) |
| *Nostoc linckcia* | Cryptophycin 1 | Disruption of microtubule structure | Cytotoxicity against human tumor cell lines and human solid tumors | (SMITH [et al.], 1994) |
| *Symploca* sp. | Dolastatin 10 | Bind to tubulin, causing cell cycle arrest in metaphase | Antiproliferative agent against murine PS leukemia cells | (PETTIT [et al.], 1987) |
| *Symploca* sp. | Largazole | Class I histone deacetylases (HDACs) inhibitor | Antiproliferative activity | (YING [et al.], 2008) (TAORI [et al.], 2008) |

Apoptosis is an essential death mechanism used by both cells and tissues that is controlled by sequential actions of a specific set of proteins which are conserved throughout multicellular organisms and translate death-inducing signals into cell-disassembling biochemical processes (AL-RUBEAI and FUSSENEGGER, 2004). It is also a silent process considering that dismantles the target cell but does not indiscriminately propagate to the surrounding cells (SALIDO and ROSADO, 2009).

Apoptosis can be initiated by a variety of environmental stimuli, both physiological and pathological, leading to structural changes (KERR [et al.], 1972), such as rounding up and hypercondensation of the cell, reduction of cellular and nuclear volume, modification of cytoplasmatic organelles and violent plasma membrane blebbing (KROEMER [et al.], 2005). Along with morphological changes, apoptotic cells also undergo considerable biochemical modifications just as protein cleavage, protein cross-linking, degradation of the chromosomal DNA into small fragments, and phagocytic recognition (HENGARTNER, 2000). Another biochemical hallmark is the expression of cell surface markers that allow the phagocytic recognition, as a result of the movement of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to the outer layers of the plasma membrane (BRATTON [et al.], 1997). Other proteins are also exposed on the cell surface during apoptosis, which include Annexin I (ARUR [et al.], 2003) and calreticulin (GARDAI [et al.], 2005). The cell fragmentation into membrane-enclosed vesicles, known as apoptotic bodies, and their subsequent engulfment by macrophages or neighboring cells prevents an inflammatory response, opposed to what can be observed in necrotic events (ELMORE, 2007).

Caspases are intracellular cysteine proteases, which play a crucial role in apoptosis (HENGARTNER, 2000). Two classes of caspases were described in apoptosis: initiator (caspases 8 and 9) and effector caspases (caspases 3, 6 and 7) (AL-RUBEAI and FUSSENEGGER, 2004). These proteins are produced as inactive zymogens, which require proteolytic processing in order to become active proteases (SHI, 2001).

The activation of apoptosis is performed by two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway, both converging in the same terminal or executioner pathway (ELMORE, 2007).

The extrinsic signaling pathway involves transmembrane receptor-mediated interactions in order to trigger apoptosis (ELMORE, 2007). Binding of death receptors from the tumor necrosis factor receptor superfamily (TNF) (such as fibroblast-associated (Fas) receptor) to their respective ligands initiates ligation of the receptors and transmission of the apoptotic signals through death domains (DDs), death effector domains (DED), and caspase recruitment domains (CARD) (BUDIHARDJO [et al.], 1999). The interaction between DEDs of the adaptor molecules and of procaspases 8 and 10 enables formation of the intracytoplasmic death-inducing signaling complex (DISC). The binding of DISC to procaspase 8 initiates the caspase cascade. Such events can be regulated by Flip (FLICE inhibitory protein), and the inhibitors of apoptosis (IAPs), affecting both initiator and effector caspases. The actions of IAPs are antagonized by the binding of mitochondria-derived activator of caspase (DIABLO), therefore neutralizing their inhibitory actions on the caspases. (HUSSEIN [et al.], 2003)

The intrinsic signaling pathway is a mitochondrial initiated event and includes non-receptor mediated stimuli which produce intracellular signals acting directly on targets within the cell (ELMORE, 2007). Induction of the pro-apoptotic Bcl-2 homology domains 3 (BH3)-only proteins, including Bcl-2-interacting domain (Bid), Bcl-$X_L$/Bcl-2-associated death promoter (Bad), PMA-induced protein (Noxa) and p53-upregulated modulator of apoptosis (PUMA), can send the signals to the mitochondria. Then, assembly of pro-apoptotic Bcl-2-associated X protein (Bax) and Bcl-2 antagonist killer 1 (Bak) occurs into the pores in the outer mitochondrial membrane. This process involves changes in mitochondrial permeability and the release of various factors involved in apoptosis, such cytochrome *c*, apoptosis-inducing factor (AIF), and DIABLO. (HUSSEIN [et al.], 2003) An apoptosome is formed as a consequence of the interaction between cytochrome *c* and Apaf1 (apoptotic protease activating factor 1), in a dATP/ATP-dependent fashion, which

will recruit and activate caspase 9 (JIANG and WANG, 2004). The IAP family of proteins interacts with mature caspases, inhibiting their enzymatic activity, with DIABLO being responsible for relieving IAPs inhibitory effect, allowing the occurrence of apoptosis (SHI, 2001).

Both pathways culminate in the activation of executioner caspases (caspase 3, 6 and 7), at the execution phase, the final pathway of apoptosis (ELMORE, 2007). These caspases activate cytoplasmatic endonucleases and proteases, responsible for degrading the nuclear material, and both nuclear and cytoskeletal proteins (SLEE [et al.], 2001). The action of caspases on their substrates ultimately leads to the morphological and biochemical changes associated with apoptosis, as clarified in Figure 5.
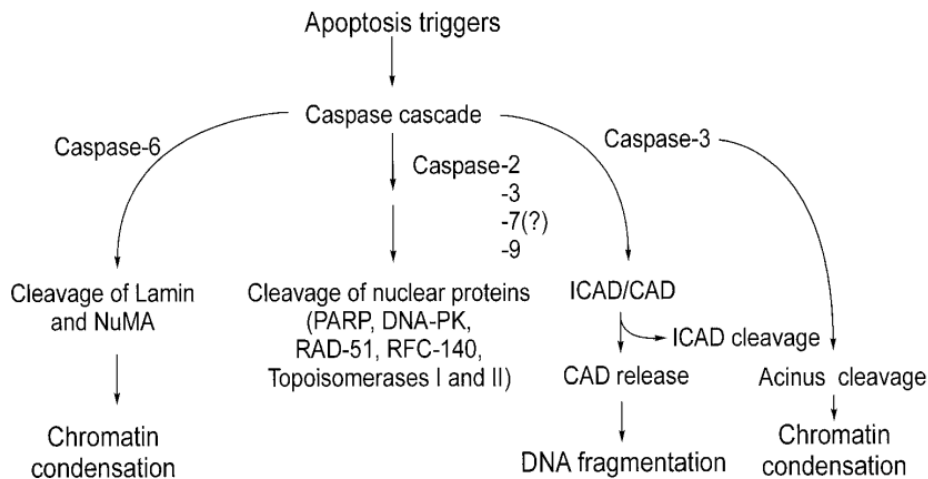


**Figure 5.** Role of executioner caspases in apoptosis. (ROBERTSON [et al]., 2000)

The last step in apoptosis is the uptake of apoptotic cells. Since there is no release of the cellular constituents, there is essentially no inflammatory response during these events (ELMORE, 2007).

Taking into consideration the scarcity of inflammatory response during apoptosis, this mechanism is thus considered a preferred form of cancer cell death in cancer treatments and, therefore, both modulation of apoptotic pathways and selective induction of apoptosis by chemical agents result in an attractive approach for cancer therapy (ZHENG [et al.], 2011).

### 3.1.1. The role of cyanobacteria compounds in apoptosis

Some cyanobacteria compounds are already described to induce apoptosis by several mechanisms. MCDERMOTT [et al.], 1998, observed biochemical changes and DNA fragmentation, in a variety of cell types (hepatocytes, fibroblasts, endothelial cells, promyelocytes, lymphocytes and epithelial cells) when exposed to the toxin microcystin-LR, although the mechanism by which it occurred wasn't elucidated. CHEN [et al.], 2005 studied the effect of the same toxin in mouse hepatocytes and were able to clarify the mechanism behind apoptosis, which begins through the BID-BAX-BCL-2 pathway, and is followed *via* a reactive oxygen species pathway, but only when exposed to higher doses. Although the great majority of the studies were performed with microcystins, other compounds have already been associated with apoptosis, like somocystinamide A, which is also a potent inductor of apoptosis, through a mechanism involving the activation of caspase 8 (WRASIDLO [et al.], 2008). This particular aspect of somocystinamide is quite interesting, since numerous malignant tumors maintain expression of caspase 8, thus being an attractive target for tumor suppression (BARNHART [et al.], 2004).

More recently, Lagunamides, a potent cytotoxic cyclic depsipeptide isolated from *L. majuscula*, was found to successfully kill a variety of cancer cell lines through apoptosis, presumably *via* the intrinsic apoptotic pathway, thus constituting a potential drug in cancer treatments (TRIPATHI [et al.], 2012).

Despite the fact that many other cyanobacteria compounds are reported to be associated with apoptosis, not every mechanism by which they induce this phenomenon is fully explained.

Necrosis is a cell death mechanism that occurs as a consequence of severe trauma, such as hypoxia, ischemia or nutrient deprivation. However, it can also be triggered when ligands bind to their respective receptors (TNFR1, Fas and Trail receptor and pathogen recognition receptors (PRR)) leading to signaling events. (VANLANGENAKKER [et al.], 2008) Necrosis may occur during mammalian development and in adult tissue homeostasis (GALLUZZI [et al.], 2007). Moreover, necrosis is a mechanism that disturbs many adjacent cells (SEARLE [et al.], 1982).

Unlike apoptosis, its morphological characteristics involve rapid cytoplasmatic swelling, disintegration of the cell organelles and permeabilization and collapse of the plasma membrane. Other cellular changes in necrosis consist of early signs of mitochondrial malfunction, such as loss of calcium homeostasis, organelle perinuclear clustering and activation of some proteases and lysosomal rupture. (GALLUZZI [et al.], 2007) Episodes of DNA degradation may also occur (VAUX, 1993). The plasma membrane rupture leads to the release of intracellular factors, such as cytokines, which will trigger a local inflammation (VANLANGENAKKER [et al.], 2008). Besides the morphological characteristics, there is not a clear biochemical marker that can be linked to necrosis (GALLUZZI [et al.], 2007).

A macropinocytotic mechanism is responsible for the engulfment of parts of the necrotic cell by phagocytes (KRYSKO [et al.], 2008), although this uptake is delayed and less efficient than in apoptosis (VANLANGENAKKER [et al.], 2008).

The main purpose of this work was to evaluate the anticancer potential of marine cyanobacteria strains isolated from the Portuguese coast and belonging to the genera *Cyanobium*, *Leptolyngbya*, *Romeria* and *Synechocystis*, since these genera have rarely been studied on what concerns production of interesting pharmacological compounds. Five cyanobacteria strains of the cyanobacteria culture collection of the Laboratory of Ecotoxicology Genomics and Evolution (LEGE) of the Interdisciplinary Center of Marine and Environmental Research (CIIMAR-Porto) were selected for cytotoxicity screening in four human tumor cell lines, osteosarcoma (MG63), colon adenocarcinoma (RKO and HT29) and neuroblastoma (SH-SY5Y). From the cyanobacteria biomass, a crude extract and organic fractions were prepared in order to separate compounds with different polarities. Considering cytotoxicity, it was also an objective to assess the potential of induction of apoptosis. In order to perform the objectives, the work comprised four fundamental tasks:

1. Culture of cyanobacteria strains for biomass production.
2. Preparation of cyanobacteria extracts in order to separate fractions with different compounds.
3. Evaluation of the cytotoxic profile of the extracts in tumor cell lines.
4. Evaluation of the cytotoxic mechanisms by the potential induction of apoptosis.

## 5.1.Cyanobacteria strains

The cyanobacteria strains included in this study belong to the genera *Cyanobium*, *Leptolyngbya*, *Romeria* and *Synechocystis*. Strains were isolated from samples collected along the Portuguese coast, were identified by morphological characteristics and molecular tools and are maintained at the Laboratory of Ecotoxicology Genomics and Evolution (LEGE) at the Interdisciplinary Center of Marine and Environmental Research (CIIMAR-Porto) (Table 5).

**Table 5.** Cyanobacteria strains.

| Strain code | Genera | Origin |
|---|---|---|
| LEGE06013 | *Romeria* | Foz do Arelho |
| LEGE06098 | *Cyanobium* | Martinhal |
| LEGE06102 | *Leptolyngbya* | S. Bartolomeu do Mar |
| LEGE06113 | *Cyanobium* | Aguda |
| LEGE06155 | *Synechocystis* | S. Bartolomeu do Mar |

### 5.1.1.Cyanobacteria culture conditions

Cyanobacteria were cultivated in 4 liters of Z8 medium (KOTAI, 1972), enriched with 20g/L NaCl (Annex I). The cultures were maintained with permanent aeration, at 25$^{\circ}$C and with a 14:10h light:dark photoperiod, according to MARTINS [et al.], 2005. Cells were harvested by centrifugation at 4600 rpm, after a growing period of about one month. The concentrated biomass was washed twice with distilled water to remove the remaining NaCl and then frozen at -20$^{\circ}$C, lyophilized and stored at -20$^{\circ}$C.

Approximately 1g of dry weight from each cyanobacteria strain was repeatedly extracted for 6 times, during 10 minutes, with a methanol:dichloromethane (1:2) solution. After the extraction, the solvents were evaporated in a rotary evaporator, and the extract was ressuspended in isooctane:ethanol (1:1). After nitrogen drying, the extract was dissolved again in isooctane:ethanol (1:1) in order to obtain an extract concentration of 50mg/mL. This extract constituted the crude extract. A portion of the crude extract (150mg) was fractionated by silica gel vacuum liquid chromatography in disposable chromatography columns using a stepwise gradient of increasing polarity, starting with 100% hexane and finishing with 100% methanol, rendering 3 fractions, fraction A (hexane fraction), fraction B (ethyl acetate fraction) and fraction C (methanol fraction), as clarified in Table 6. The solvents gradient allows the separation of compounds with increasing polarities.

**Table 6.** Solvent gradient used in the compounds extraction.

| Fraction A | Fraction B | Fraction C |
|---|---|---|
| 100% hexane | 60% ethyl acetate in hexane | 75% methanol in ethyl acetate |
| 20% ethyl acetate in hexane | 80% ethyl acetate in hexane | 100% methanol |
| 40% ethyl acetate in hexane | 100% ethyl acetate | 100% methanol |
| | 75% ethyl acetate in methanol | |

The solvents present in the fractions were evaporated using the rotary evaporator and the sediments were dissolved in isooctane:ethanol (1:1), dried in nitrogen, and stored at -20$^{o}$C. All fractions and the crude extract were dissolved in isooctane:ethanol (1:1) in order to obtain a concentration of 10mg/mL. A portion of this material was further dried in nitrogen and dissolved in dimethyl sulfoxide (DMSO) to yield a stock concentration of 10mg/mL. Dilutions were performed in order to obtain also stock concentrations of 1mg/mL and 0,1mg/mL. Stocks were stored at -4$^{o}$C.

**5.3. Cytotoxicity of the crude extract and fractions in human tumor cell lines**

**5.3.1. Cell lines**

In order to assess the anticancer potential of the crude extract and fractions of the marine cyanobacteria strains, cell viability/proliferation assays were conducted with human cancer cell lines. The osteosarcoma cell line (MG63), the colon adenocarcinoma cell lines (RKO and HT29) and the neuroblastoma cell line (SH-SY5Y) were included in this study (Table 7). HT29 and SH-SY5Y cell lines were purchased from Sigma-Aldrich. MG63 and RKO cell lines were obtained from Prof. Maria Helena Fernandes at Dental Faculty of Dental Medicine, University of Porto.

**5.3.2. Cell culture**

Cells lines included in this study are presented in Table 7, along with the information of the culture medium. All cell lines were grown in an incubator at 37°C, in a 5% $CO_2$ atmosphere.

**Table 7.** Cell lines used in the work. FBS - Foetal Bovine Serum.

| Cell type | Cell line | Description | Culture medium |
|---|---|---|---|
| Tumoral cell lines | MG63 | Human osteosarcoma | **DMEM Glutamax** 10% FBS 100IU/mL penicillin 10mg/mL streptomycin |
| | RKO | Colon adenocarcinoma | **DMEM Glutamax** 10% FBS 100IU/mL penicillin 10mg/mL streptomycin |
| | HT29 | Colon adenocarcinoma | **McCoy´s** 2mM Glutamine 10% FBS 100IU/mL penicillin 10mg/mL streptomycin |
| | SH-SY5Y | Human neuroblastoma | **Ham´s F12** 2mM Glutamine 1% non-essential amino acids 15% FBS 100IU/mL penicillin 10mg/mL streptomycin |

### 5.3.3. Cytotoxic assays

The cellular viability/proliferation was evaluated by the reduction of the bromide 3-(4,5-dimetil-tiazol-2-il)-2,5-difenil-tetrazolio (MTT), which is a colorimetric assay based on the ability of reductase enzymes to reduce MTT within the mitochondria, rendering purple formazan products. Since this conversion is only performed by active enzymes, it allows evaluating the cell viability and proliferation.

To perform the MTT assay, cells were seeded in 96 multiwell plates, at a $10^4$cell/cm$^2$ density, in 100μL of growth medium. After 24 hours of adherence to the wells, the cells were exposed to the crude extract and fractions A, B and C from each cyanobacteria strain, at a final concentration of 100μg/mL, 10μg/mL and 1μg/mL, for a period of 24, 48 and 72 hours, and at 37$^o$C and 5% $CO_2$. After each incubation time, cells were incubated with 10μL of a 0,5mg/mL MTT solution, for 4 hours in the incubator. By the end of this period, the medium was removed and the produced formazan crystals were dissolved in 100μL DMSO. The plates were then read for absorvance at 550nm in a "GEN5™ - Multi – detection Microplate Reader" (Biotek). All tests were run in triplicate and were averaged. Cytotoxicity is expressed as a percentage of cell viability considering 100% viability in the control (cells treated with 1% DMSO in culture medium).

Extract/fractions that induced cytotoxic activity were further tested by the Lactate Dehydrogenase (LDH) assay. LDH assay relies on the reduction of NAD by LDH in active living cells and their consequent release from damaged or destroyed cells. The resulting reduced NAD is then used in the stoichiometric conversion of a tetrazolium dye, which is measured spectrophotometrically.

To perform LDH assay, cells were seeded in 96 multiwell plates at a $2x10^4$cell/cm$^2$ density. After 24 hours of adherence to the well, the cells were exposed to the selected extracts, at a final concentration of 100μg/mL, 10μg/mL and 1μg/mL, for a period of 24, 48 and 72 hours, and at 37$^o$C and 5% $CO_2$. Control cells were exposed to 1% DMSO in the culture medium.

After each incubation period, the LDH activity was measured by the In vitro toxicological assay kit TOX7 (Sigma Aldrich), according to the manufacturer's instructions. With this kit the released and total LDH is measured. Briefly, the assay was performed in

sextiplicate. Lysis solution was added onto three wells so as to provide a control of total LDH release. The additional wells give a measure of LDH released to the medium. After an incubation period of 45 minutes with the lysis solution in 5% $CO_2$ and 37⁰C, the samples were centrifuged at 250g for 4 minutes. 25µL aliquots from each individual well were transferred to a new 96 multiwell plate. After the addition of the reconstituted substrate mixture, the plates were incubated at room temperature for 30 minutes, in the darkness. The reaction was stopped with 1N HCl, and the plates were then read at 490 nm, and at 690nm for background absorbances values in a "GEN5™ - Multi – detection Microplate Reader" (Biotek).

After subtracting the blanks from the absorbance values, the percentage LDH released was given by the following formula:

$$\% \, LDH \; released = \frac{LDH \; release}{Total \; LDH \; release} \; x \; 100$$

### 5.3.4.Apoptosis/Necrosis assay

Apoptosis was investigated by the dye exclusion method, using both Propidium Iodide and Hoechst 33342 staining in fixed cells. Cells were plated onto round microscope cover slips inside 24 multiwell plates in a $2,59x10^4$ cell/$cm^2$ density, in 500µL of culture medium. After 24 hours of adherence to the wells, the cells were exposed to the selected extracts, at a final concentration of 100µg/mL, 10µg/mL and 1µg/mL, for a period of 24, 48 and 72 hours, and at 37⁰C and 5% $CO_2$. Control cells were exposed to 1% DMSO in the culture medium. After each incubation period, cells were washed twice in phosphate-buffered saline (PBS), followed by incubation with 5µL/mL Propidium Iodide and Hoechst 33342, for 10 minutes at 37⁰C, with minimal light exposure. After 2 washes with PBS, cells were fixed with 36% Formaldehyde for 10 minutes at room temperature. After the fixation, the cover slips were placed in microscope slides using Mowiol$^®$ 4-88 mounting solution and visualized by fluorescence microscopy. Since Propidium Iodide cannot move through intact cell membranes, it will only stain cells dead by necrosis. Hoechst 33342 will stain the nuclei and, in apoptotic cells, this organelle will appear fractionated.

## 5.4. Statistical analysis

Results are expressed as mean ± standard deviation (SD) from three replicates. In order to verify if the data followed a normal distribution, it was used the R program (R Core Team, 2012). To conclude whether any significant relationship exists among the obtained results, it was used the Mann Whitney test, executed with SPSS Statistics 20 (SPSS, IBM Corp, 2012). Values of $p \leq 0.05$ were considered as significant.

For the last decades, the medicinal potential of marine cyanobacteria has engendered an enormous interest by researchers. The putative potential of these organisms as a source of anticancer agents has been one of the most emerging and exciting areas. However, while most of the studies regarding the production of bioactive compounds are focused in filamentous forms that grow in large densities at tropical areas, such as *Lyngbya*, genera that grow in low densities in temperate regions have been largely overlooked. In these cyanobacteria are included the genera *Cyanobium*, *Leptolyngbya*, *Romeria* and *Synechocystis*. With this work we aimed to provide an insight into the potential of five cyanobacteria strains isolated from the Portuguese coast and belonging to the genera *Cyanobium*, *Leptolyngbya*, *Romeria* and *Synechocystis* to provide compounds with anticancer properties. This study constitutes a first screening on the cytotoxic effects of the five marine cyanobacteria strains against cancer cell lines.

In this study the cancer cell lines MG63, RKO, HT29 and SH-SY5Y were exposed to the crude extract and the organic fractions from the cyanobacteria strains at concentrations of 100, 10 and 1μg/mL for a period of 24, 48 and 72 hours.

In order to assess the cytotoxic activity of the extracts, two of the most frequently used methods for quantitating cell viability were performed, the MTT and the LDH assay (FOTAKIS and TIMBRELL, 2006). Primarily, cell viability was measured by the MTT reduction assay, which relies on the spectrophotometric detection of dark formazan products produced by metabolically active cells after incubation with MTT. The resulting optical density (OD) is proportional to the number of healthy viable cells. Based on the ODs obtained from each experience, the percentage of viable cells was determined. Secondly, the LDH release assay was performed with the cyanobacteria crude extracts and fractions that showed a more pronounced toxicity by the MTT assay. LDH release is commonly used as a marker for necrotic cells since when cells are lethally injured, the enzyme is released to the medium.

In Figure 6 there are presented the results obtained in the MTT cytotoxicity assay for the MG63 cell line exposed to the crude extract and fraction A, B and C of the five cyanobacteria strains. Concerning these results, fraction B from strain LEGE06155 was found to be highly cytotoxic, with an almost complete reduction in cell viability at a 100µg/mL concentration, and a significant time-dependent reduction in cell viability at 10µg/mL. This reduction in cell viability can be explained by the presence of toxic compounds that caused permeabilization of the cell membrane leading to a rapid mechanism of cell death. A time and dose-dependent reduction in the cell viability was also observed for the crude extract. Comparing the results from the crude extract and fraction B it is evident that the crude extract induced cell mortality in a lower extent than fraction B. These results can be due to the presence of cytotoxic compounds present in fraction B with such toxic effect being attenuated by the presence of other compounds, such as compounds potentially present in fractions A and C, since with these fractions an increase in the cell viability was registered.

For strains LEGE06098 and LEGE06113, a time and dose-dependent decrease was observed in the cell viability with the crude extract. Since cell death was also registered for fraction A, B and C, we can suggest a synergistic effect between compounds. In a work conducted by PICHARDO [et al.], 2006 , where microcystins and other cyanobacteria extracts were exposed to fish cells, the authors also observed a more pronounced damaged with the extracts than with the pure toxin, and they also suggested a synergistic action of the cyanobacteria compounds.

In strains LEGE06013 and LEGE06113 a higher reduction in the cell viability was also observed for the fraction B, which emphasises the presence of cytotoxic compounds. Although in LEGE06113 a time dependent inhibition was also observed, with LEGE06013 an increase in the cell viability occurred after 72 hours, which point towards the existence of stimulatory compounds that allow the cells to grow.
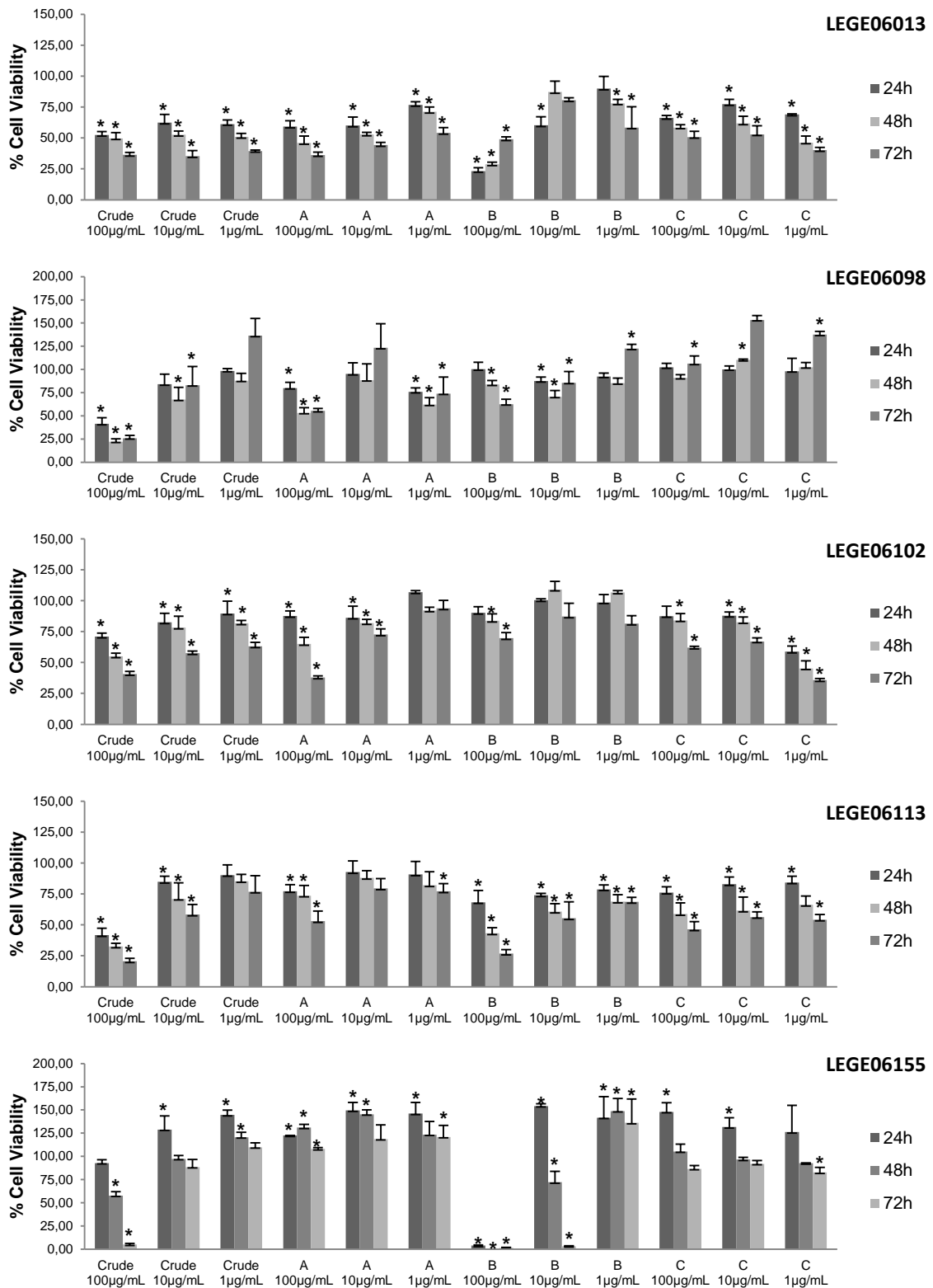
**Figure 6.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on MG63 cells. Data represents results from the MTT assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)

In figure 7 there are represented the MTT results for the RKO cell line exposed to the crude extract and fractions A, B and C of the cyanobacteria strains. For this cell line, a strong toxic effect was observed with the fraction B of the cyanobacteria strain LEGE06155 at a concentration of 100 µg/mL. Also a significant reduction in the cell viability was registered with the same fraction and concentration in the other cyanobacteria strains after 24 hours with a recovery of cells after 48 hours and, more pronounced, after 72 hours. Following the same cytotoxicity pattern as in MG63, the crude extract also induced toxicity to a lesser extent.

Considering the different tested strains against RKO, LEGE06155 was found to be the most toxic.


In Figure 8, there are presented the results for HT29 cell line exposed to the crude extract and fractions A, B and C of the cyanobacteria strains.

Fraction B continues to held a major percentage of cell inhibition with a parallel inhibition of cell viability in the crude extract.

In LEGE06013, fraction B decreased cell viability after 24 hours. However, after 48 hours the cytotoxicity was absent, followed by a second decrease in the viability after a 72 hours treatment.

Considering LEGE06098, an interesting event occurred, where fraction A in its lowest concentration revealed the strongest cytotoxicity. It is known that some compounds, particularly environmental pollutants, trigger cell death at low doses in *in vitro* models (ORRENIUS [et al.], 2011). Knowing this, it may be possible to explain this particular case based on those assumptions, considering that the highest concentration also succeed in decreasing cell viability, although other studies would have to be conducted in order to confirm this hypothesis.

In LEGE06102, fraction B induced inhibition, in a time dependent manner. Even though fraction B also showed cytotoxicity in LEGE06113 and LEGE06155, this effect was more pronounced after 24 hours for LEGE06113 with a recovery in the cell viability, and only after 48 hours in LEGE06155 wich suggests a better endurance by HT29 to this particular strain. In strains LEGE06102, LEGE06113 and LEGE06155 the inhibitory effect

on cellular growth with fraction B was dose-dependent and the same toxicity pattern was observed with the crude extract. Once again we suspected that the toxic effect is due to compounds present in fraction B.

The results obtained by the MTT assay and considering the SH-SY5Y cell line are presented in Figure 9.

The cytotoxicity induced by the cyanobacteria crude extract and fractions to this cell line follows the same pattern as in MG63 and RKO, with fraction B being responsible for a higher decrease in cell viability, once again, particularly in LEGE06155. Considering strains LEGE06013 and LEGE06102, fraction B revealed also cytotoxic effects, but such was only observed after 48 hours with a recovery in cell viability after 72 hours, which suggests again the presence of growing stimulatory compounds. In LEGE06113, the highest concentration of fraction A also induced a decrease in cell viability and also after a 48 hours exposure.
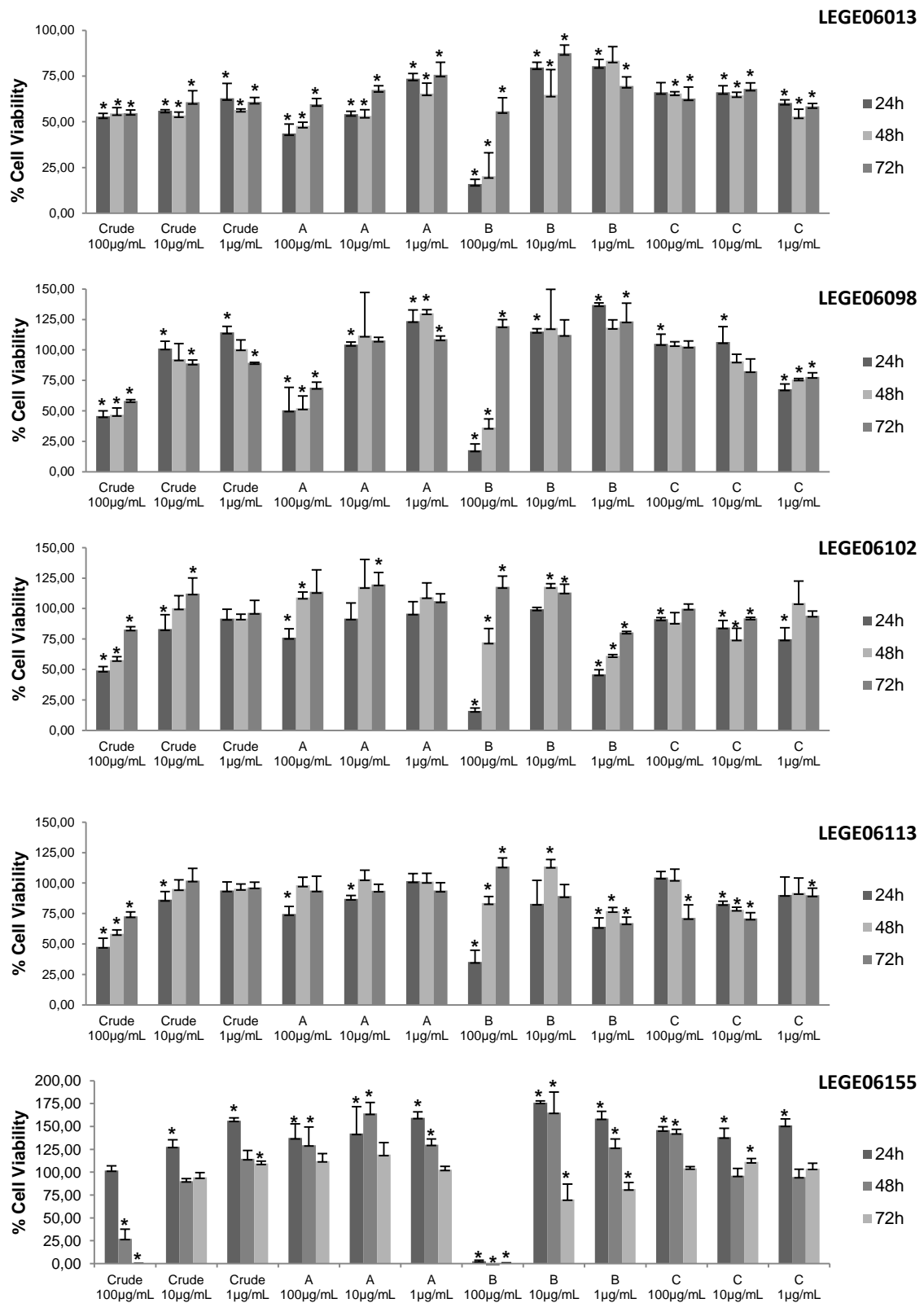
**Figure 7.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on RKO cells. Data represents results from the MTT assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)

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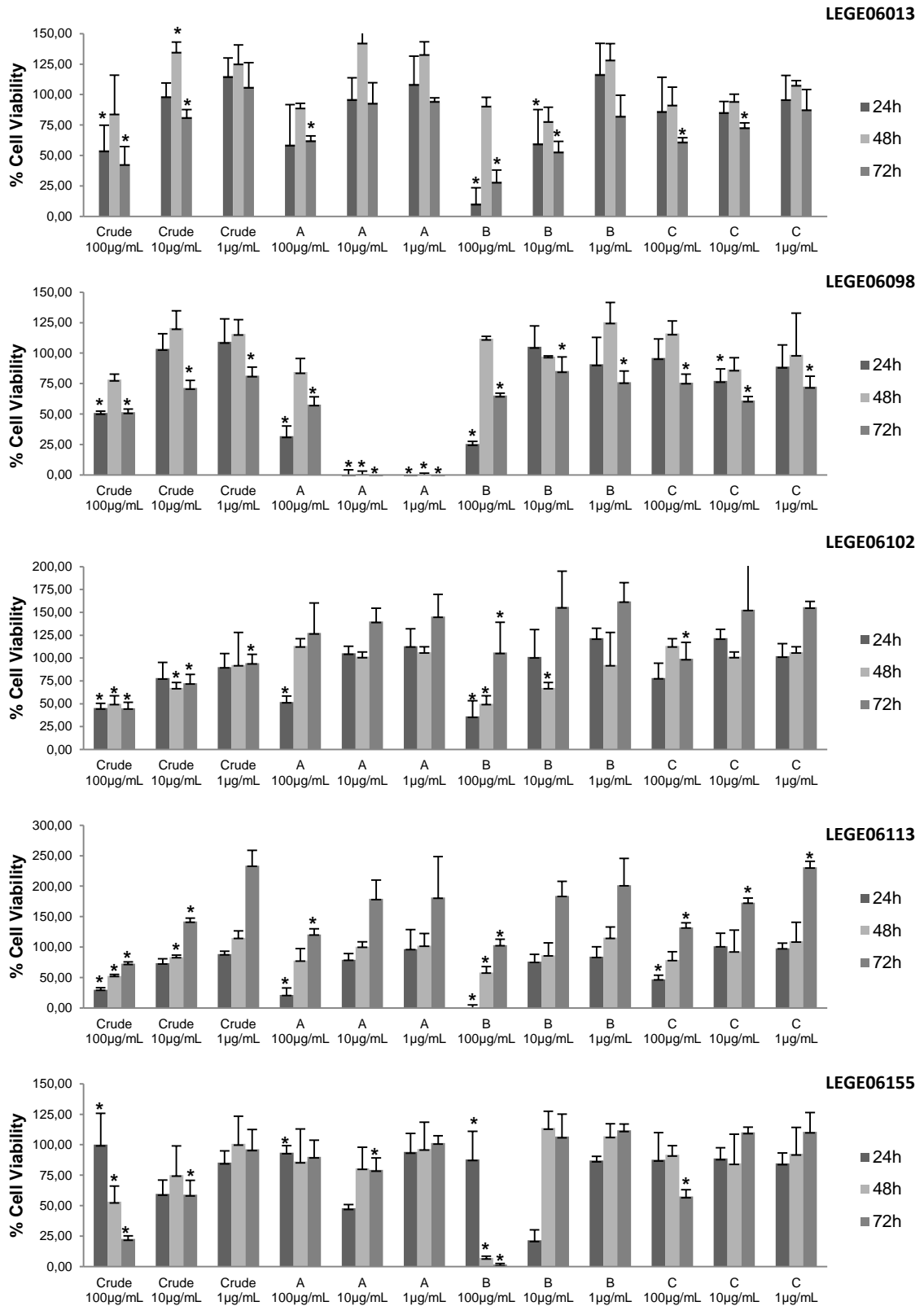
**Figure 8.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on HT29 cells. Data represents results from the MTT assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)
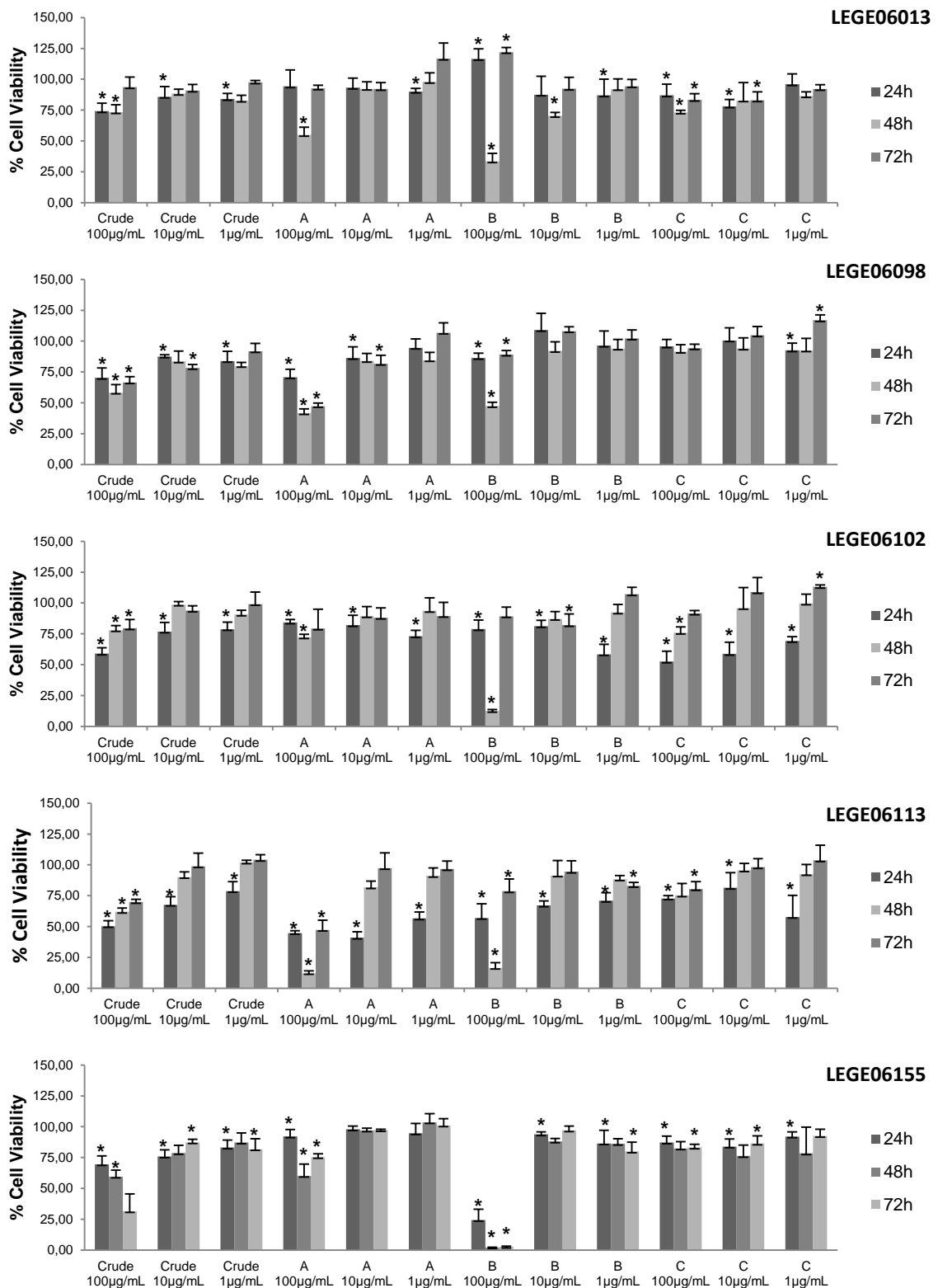
**Figure 9.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on SH-SY5Y cells. Data represents results from the MTT assay as mean ± Standard Deviation (SD) of triplicate determinations. ($p \leq 0.05$)

In order to perform a detailed evaluation concerning the differences between the control and the rest of data groups, pairwise comparisons were made for each exposure time, inside each concentration, treatment, cell line and cyanobacteria strain against the controls. Since the sample size was insufficient to fulfill the assumptions for parametric methods, these comparisons were done using a non parametric method, the Mann-Whitney test (Annex II).

The global effect of the different experimental factors against the "Viability" variable was assessed using a generalized linear model. Previously, a general linear model was applied, although it couldn't be possible to normalize the model error (residues) after data transformation with the Box-Cox function. Therefore, a generalized linear model was adjusted, which integrates a non normal distribution to the model error (residues). After testing the error adjustment to several distributions (Gamma, Weibull, Poisson) the Poisson distribution was the only optimum fit ($\chi2$ = 1.01; $df$ = 2; $p$ = 0.6). First, a model integrating all possible interactions between the factors was fit. Then, a new model was fit excluding all interactions that didn't result as significant. The analysis of deviance results on the definitive model is presented in Table 8.

**Table 8.** Results for the analysis of deviance of the generalized linear model made for Viability ($n$=2338). $\chi^2$ – chi-squared; df – degrees of freedom

| Factor/co-variable/interaction | $\chi^2$ | df | p |
|---|---|---|---|
| Treatment (fixed factor) | 38.5 | 12 | <0.001 |
| Cell line (fixed factor) | 229.8 | 3 | <0.001 |
| Cyanobacteria strain (fixed factor) | 31.1 | 4 | <0.001 |
| Time (co-variable) | 74.1 | 1 | <0.001 |
| Cell line*Cyanobacteria strain | 28.3 | 12 | 0.005 |

Results from Table 8 indicate that the treatment, the cell line, the cyanobacteria strain and the time, have significant effects in the viability ($p$ values≤0.05). The only significant interaction found in the model was for the cell line and the cyanobacteria strain ($p$ value≤0.05).

The differences between each level factor were analyzed using Tukey's pos-hoc method with the cell line and the cyanobacteria strain (Annex III, Table 17 and 18), and using Dunnett test with each treatment (Table 9).

**Table 9.** Results from Dunnett's post-hoc analysis for the treatment factor.

| Comparison | Difference estimation | z | p |
|---|---|---|---|
| A 100µg/mL - Control | -0.36 | -2.5 | 0.12 |
| A 10µg/mL - Control | -0.17 | -1.2 | 0.88 |
| A 1µg/mL - Control | -0.12 | -0.9 | 0.98 |
| B 100µg/mL - Control | -0.69 | -4.2 | <0.001 |
| B 10µg/mL - Control | -0.14 | -1.1 | 0.95 |
| B 1µg/mL - Control | -0.12 | -0.8 | 0.99 |
| C 100µg/mL - Control | -0.71 | -4.3 | 0.59 |
| C 10µg/mL - Control | -0.27 | -1.9 | 0.72 |
| C 1µg/mL - Control | -0.18 | -1.3 | 0.51 |
| Crude 100µg/mL - Control | -0.23 | -1.6 | <0.001 |
| Crude 10µg/mL - Control | -0.21 | -1.5 | 0.39 |
| Crude 1µg/mL - Control | -0.25 | -1.7 | 0.86 |

Considering all the comparisons between the treatments, only the highest concentration of the fraction B and the crude extract showed a significant effect in decreasing cell viability ($p$ values≤0.05), even though there were found differences in the viability for every treatment towards the control values. In fact, fraction B revealed to be the most cytotoxic fraction. This fraction containing the higher percentage of ethyl acetate is, between the tested fractions, the one with an intermediate polarity and the one that is supposed to comprise compounds such as peptides. Most of the cyanobacteria bioactive compounds described are of peptide nature, which led us to assume the presence of potential interesting compounds in this fraction.

Regarding all results from the MTT assay, all four tumor cell lines appear to reveal similar sensitivity to the different cyanobacteria strains, and LEGE06115 was the most effective in decreasing cell viability, with growth inhibitions around 100%.

In what concerns the different exposure periods to the extracts, MG63 appears to be more sensitive with the increase in the time exposure, with RKO showing an opposing response, where the inhibitory effect was more pronounced after 24 hours of exposure to the extracts, followed by an increasing recovery in the cell viability. As for the SH-SY5Y cell line, the extracts only revealed considerable cytotoxic activity after 48 hours, although this effect was lost after 72 hours. Among all the cell lines, HT29 was the only that revealed different susceptibility concerning the different tested cyanobacteria strain, not following a pattern in terms of time exposure.

It is worth pointing out that most of the tested fractions in the lowest concentrations promoted an increase in the cell viability, compared to control values. As such, it would be interesting to evaluate whether it could be due to an hormetic response. Hormesis is characterized by a biphasic dose-response, assuming a U-shaped or inverted U-shape dose response (DAVIS and SVENDSGAARD, 1990). Hormetic responses rely on low-dose stimulation and high-dose inhibition, and such events have already been described in several biological models (CALABRESE and BALDWIN, 2003). Such low-dose stimulation is a consequence of a homeostasis shift which leads to a moderate overcompensation response, with maximum stimulations between 30% and 60% (CALABRESE, 2005), similar to what was observed in this work. Moreover, there are already described hormetic responses in a variety of cancer cell lines, including HT29 (VAN DER WOUDE [et al.], 2003) and MG63 (DZIAK [et al.], 2003).

In order to attest the results from the MTT assay, the LDH assay was perfomed with the extracts that revealed considerable decreases in the cell viability. The integrity of the cell membrane is a parameter for cell death. Since LDH is rapidly released into the culture medium when the cell membrane is damaged, by measuring the activity of the enzyme released to the medium we can infer about cell viability. As such, an increase in the number of dead cells should be translated in a increase of the release of LDH to the medium.

In Figures 10, 11, 12 and 13 the results obtained with the LDH assay in terms of percentage of LDH released to the culture medium in the four cancer cell lines are presented. LDH values from the crude extract and the fractions from each cyanobacteria strains were compared with the corresponding control values. It is an overall finding that the results seem to be controversial since in some cases a higher percentage of LDH released should be expected, according to the decrease in cell viability obtained with the MTT assay. Also some data seemed to reveal some inchoerence such as the lack of LDH released after a 48 hours incubation and not after 24 and 72 hours (Figure 10 and 12).
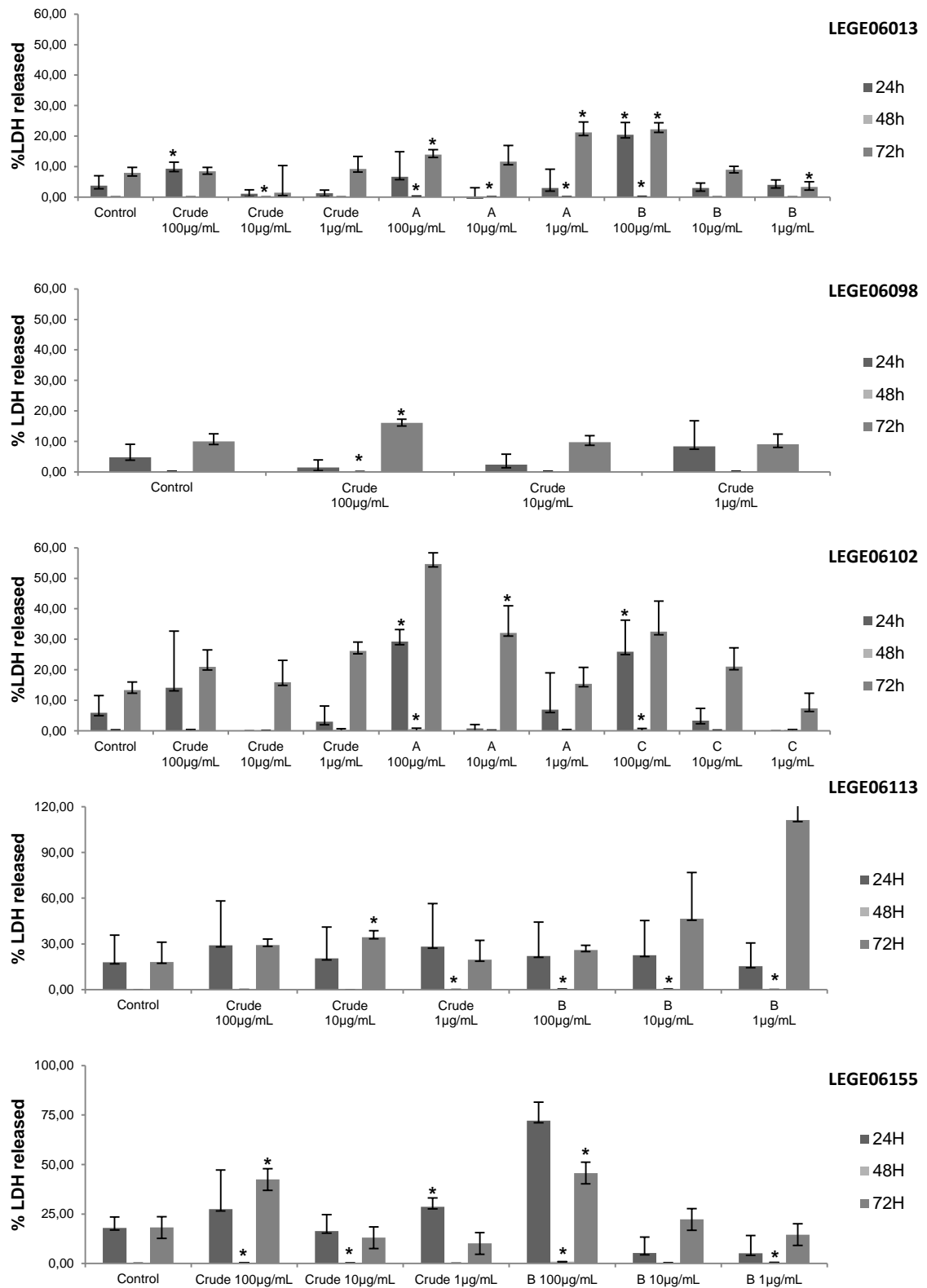
**Figure 10.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on MG63 cells. Data represents results from the LDH assay as mean ± Standard Deviation (SD) of triplicate determinations. ($p \leq 0.05$)

Considering the results for the MG63 cell line presented in Figure 10, fraction A and B from LEGE06013 showed lower cytotoxicity levels, compared with the MTT assay. However, if we take into consideration the control values, those fractions at 100μg/mL succeed in increasing the percentage of LDH released, meaning that a decrease in the cell viability occurred. In LEGE06098, the crude extract was tested, and also an increase in the released enzyme, comparing to control values, occurred. MG63 was also exposed to the crude extract and fraction A and C from LEGE06102, and results reveal a decrease in cell viability since an increase of the LDH released to the medium was registered compared to the control.

For LEGE06113, it was tested the crude extract and the fraction B. A slight increase in LDH released was observed with the crude extract and a higher percentage was achieved with fraction B. Although higher percentages of LDH released should be expected according to the MTT results, the proportion in terms of cytotoxicity seems to be similar. For LEGE06155, the crude extract and fraction B were assayed for LDH release. After 24 hours, both the crude extract and the fraction B at 100μg/mL succeed in increasing LDH released. Although the obtained results do not appear consistent, since no effect was obtained after 48 hours, a higher percentage of released enzyme was observed following the same pattern as with the MTT assay.

Figure 11 includes results from LDH assay for RKO cell line. The obtained results suggest that none of the extracts from LEGE06013, LEGE06098, LEGE06102 and LEGE06113 achieved similar toxicity values as in the MTT assay. However, for fraction B from LEGE06013 and LEGE06102, it was observed an increase in the cell death when compared to the control.

The crude extract and fraction B from LEGE06098 were the only tested extracts that didn't show an increase in the released enzyme. For LEGE06113, a slight increase in the percentage of LDH released was obtained for the crude extract and fraction B, with fraction B showing a higher effect.

The crude extract and fraction B from LEGE06155 revealed a very strong cytotoxic effect in RKO, in a dose dependent manner. However, the crude extract only revealed a higher effect after 72 hours, while fraction B had an earlier action.

LDH results for HT29 are presented in Figure 12. The crude extract and fraction B from LEGE06013 were exposed to HT29 and both succeed in increasing LDH release in a dose and time-dependent manner. In LEGE06098, there was tested the crude extract and fraction A and B, and for all of them was detected an increase in the enzyme, with fraction B showing a higher release.

Both the crude extract and fraction B tested for LEGE06102 increased LDH released, only after 24 hours. For the crude extract, a more pronounced toxicity was found at the lowest concentration. In LEGE06113, the crude extract, fraction A and B led to a decrease in cell viability, although the fractions had a stronger effect towards HT29 than the crude extract.

For LEGE06155, the crude extract and fraction B were tested and both achieved an increase in the released enzyme. It was also found an inconsistency for LEGE06113 and LEGE06155 in terms of time exposure, since very low levels of LDH were detected after 48 hours, contrary to what can be observed after 24 and 72 hours.


Results for SH-SY5Y from LDH assay are presented in Figure 13. For LEGE06013 and LEGE06102, it was tested the fraction B, which showed a more pronounced toxicity profile at 100µg/mL. In LEGE06098, the crude extract and fraction A were tested and the crude extract only showed cytotoxicity at 1µg/mL and after 72 hours. With fraction A, the highest values in LDH released were achieved also in the lowest concentration and after 72 hours. This same fraction also achieved to decrease cell viability in MTT, although in a dose dependent manner.

In LEGE06113, it was only observed an increase in the percentage of LDH released in fraction A. Fraction B was also tested for this strain, however it did not succeed in increasing the percentage of the released enzyme compared to control values. LEGE06155 was tested for the crude extract and fraction B, and both revealed a cytotoxicity effect, particularly at a 100µg/mL concentration.
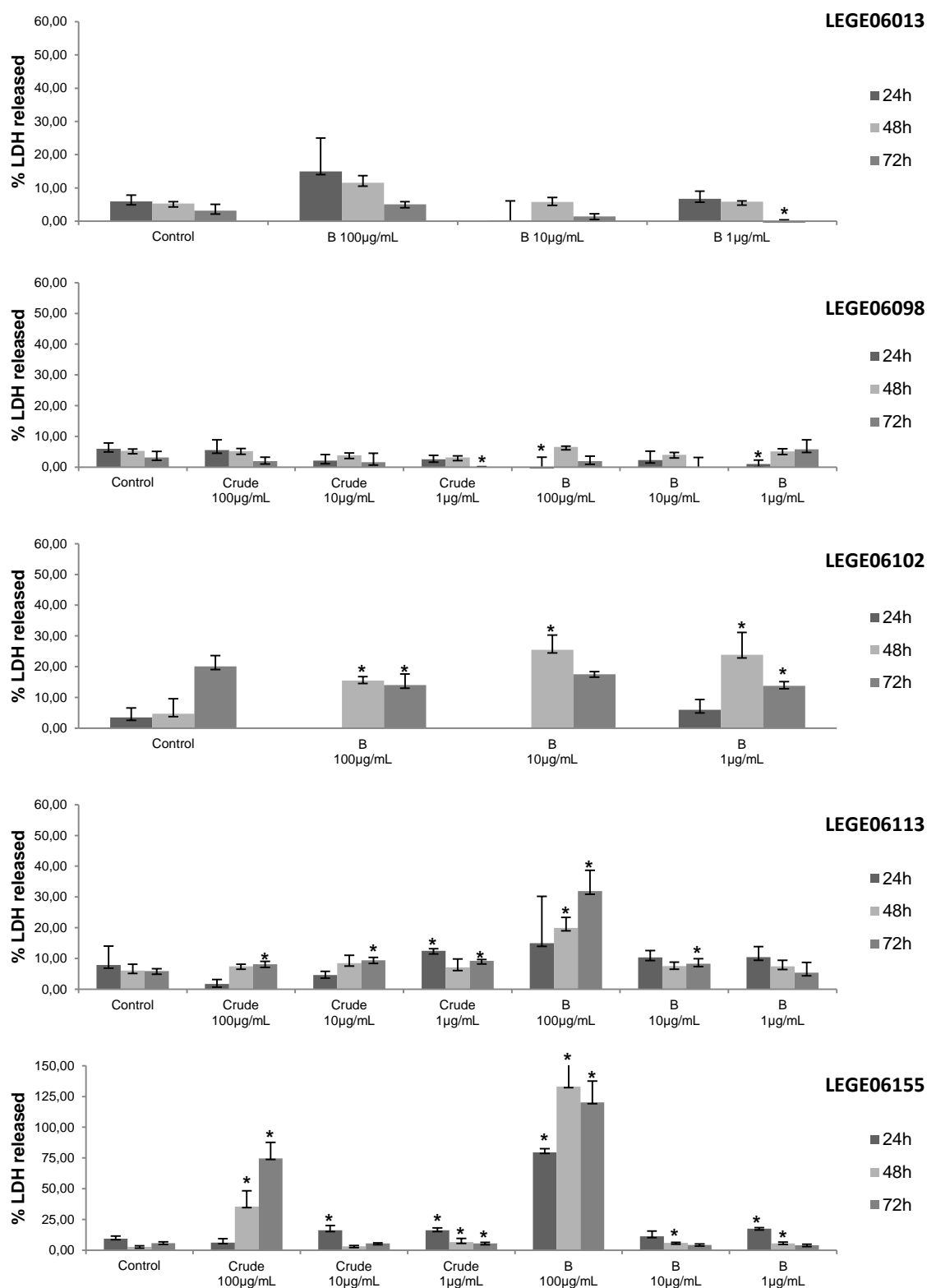
**Figure 11.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on RKO cells. Data represents results from the LDH assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)
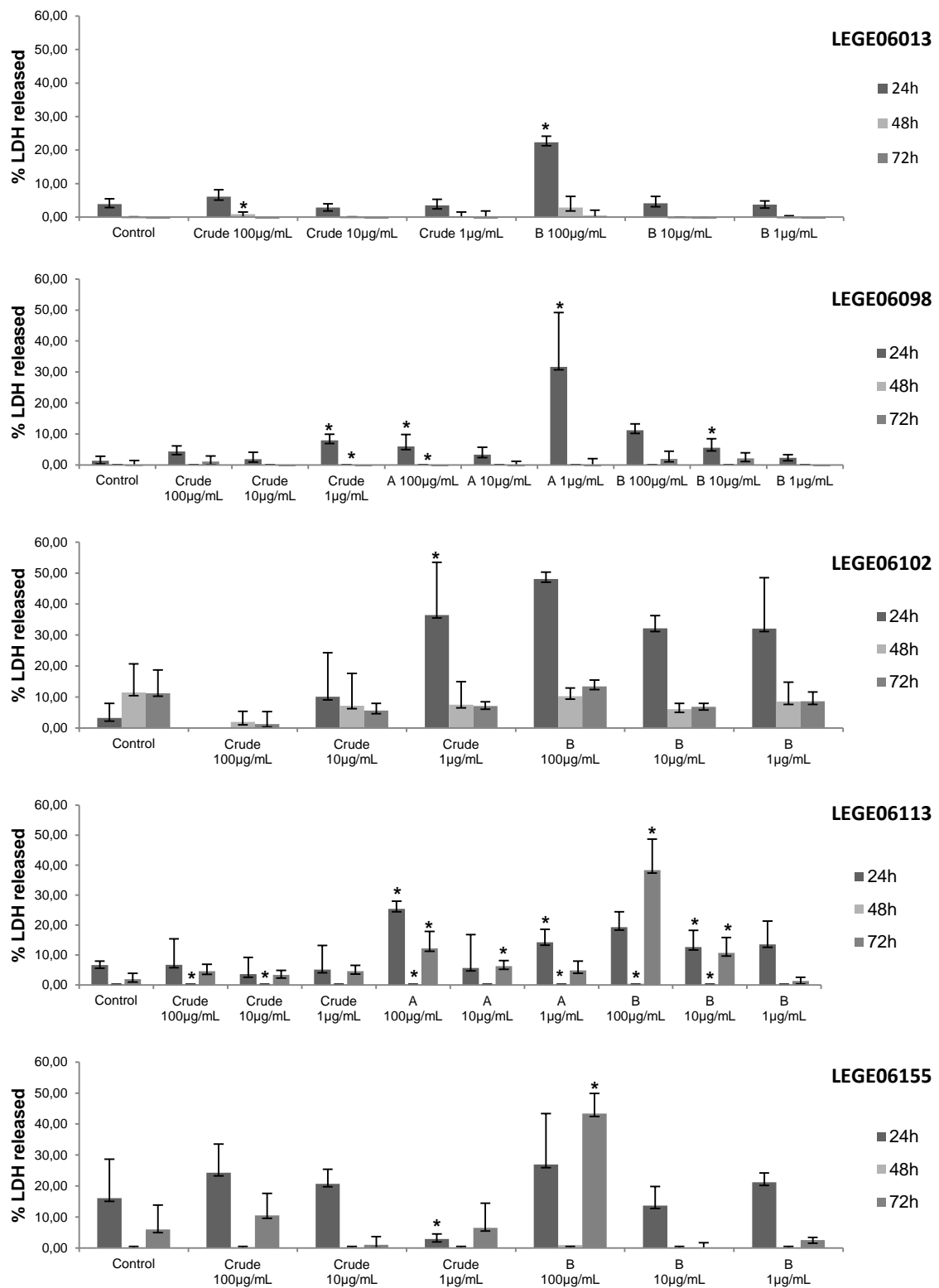
**Figure 12.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on HT29 cells. Data represents results from the LDH assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)
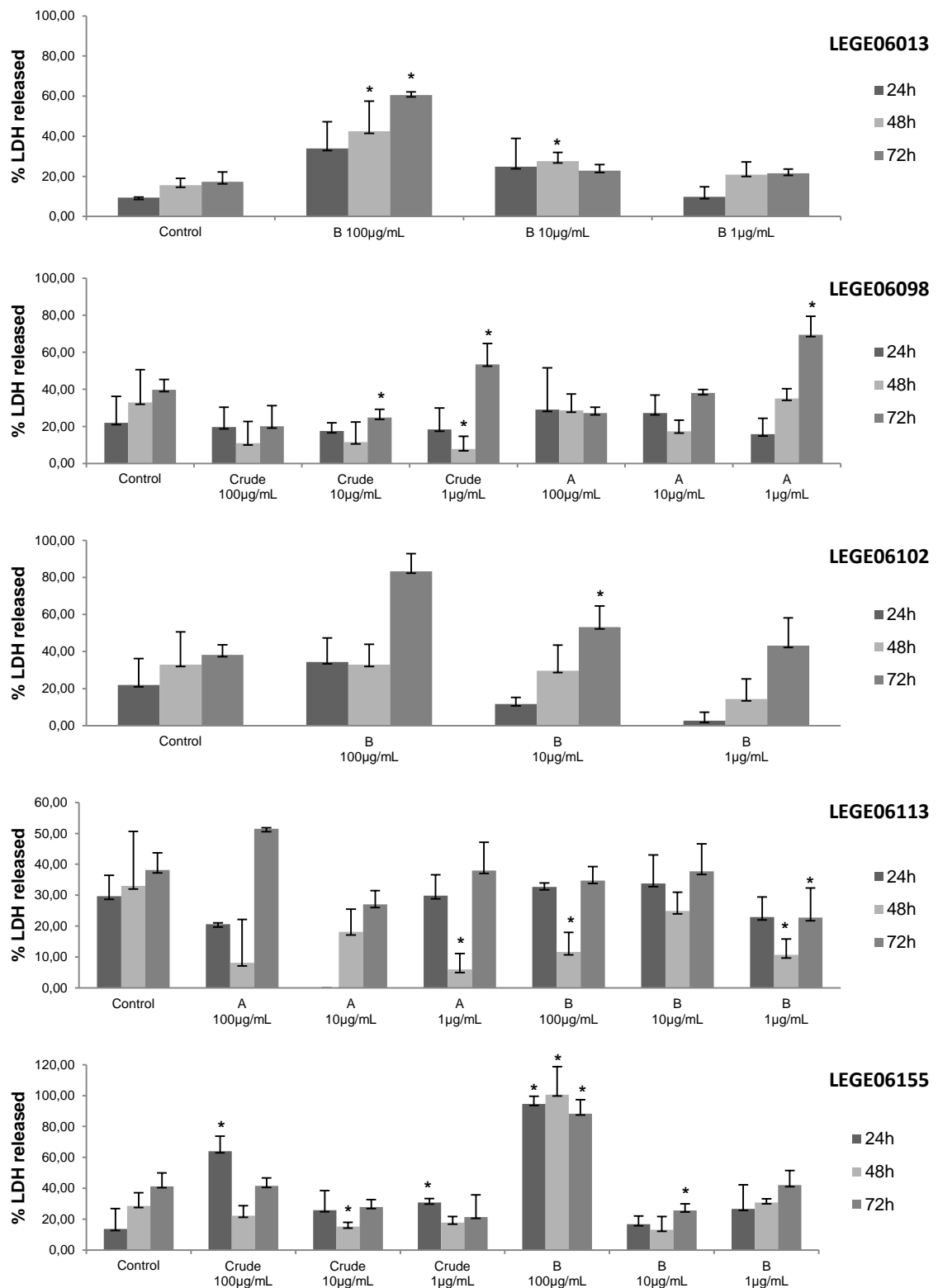
**Figure 13.** Cytotoxicity of cyanobacteria crude extract and fractions A, B, C from the five cyanobacteria strains (LEGE06013, LEGE06098, LEGE06102, LEGE06113, LEGE06155) on SH-SY5Y cells. Data represents results from the LDH assay as mean ± Standard Deviation (SD) of triplicate determinations. (p≤0.05)

An evaluation in the differences between the control and the rest of data groups was also performed for LDH results, and pairwise comparisons were made for each exposure time, inside each concentration, treatment, cell line and cyanobacteria strain against the controls, using the Mann-Whitney test (Annex IV).

The global effect of the different experimental factors against the "Viability" variable was assessed using a generalized linear model. A general linear model was previously applied, although it couldn't be possible to normalize the model error (residues) after data transformation with the Box-Cox function. A generalized linear model was adjusted, integrating a non normal distribution to the model error (residues). After testing the error adjustment to several distributions (Gamma, Weibull, Poisson) the Negative Binomial distribution was the only optimum fit ($\chi 2$ = 76,5; $df$ = 85; $p$ = 0.73). A model integrating all possible interactions between the factors was fit. Then, a new model was fit excluding all interactions that didn't result as significant. The analysis of deviance results on the definitive model is presented in Table 10.

**Table 10.** Results for the analysis of deviance of the generalized linear model made for Viability (n=1252). χ2 – chi-squared; df – degrees of freedom

| Factor/co-variable/interaction | $\chi^2$ | $df$ | $p$ |
|---|---|---|---|
| Treatment (fixed factor) | 357.3 | 12 | <0.001 |
| Cell line (fixed factor) | 347.9 | 3 | <0.001 |
| Cyanobacteria strain (fixed factor) | 263.3 | 4 | <0.001 |
| Time (co-variable) | 49.6 | 1 | <0.001 |
| Cell line*Cyanobacteria strain | 86.5 | 24 | <0.001 |
| Treatment*Cell line | 234.0 | 30 | <0.001 |
| Treatment*Cyanobacteria strain | 178.8 | 12 | <0.001 |
| Treatment*Cell line*Cyanobacteria strain | 133.3 | 54 | <0.001 |

Considering the statistical analysis performed with the LDH results, we can not make any conclusions concerning the individual effect of each factor (treatment, cell lines and cyanobacteria strains), since the analysis of deviance found significant interactions between all the factors ($p$ values≤0.05).

Regarding the MTT and LDH assays, we present a summary in Table 11 of the overall results considering the percentage of decrease in cell viability for the MTT assay and the percentage of LDH released for the LDH assay. Although, in general, the assays are in accordance, the MTT assay seemed to be more sensitive than the LDH assay. The difference in the sensitivity with both assays follows, however, the same pattern obtained by other authors. In a work by FOTAKIS [et al.], 2006, four different cytotoxic assays were compared against a human hepatoma cell line, and they also observed different results for the MTT and LDH assays, being also the MTT considered more sensitive. The authors suggested that such differences could be associated with the different mechanisms behind the two assays. The LDH assay relies on the release of the LDH enzyme to the cell culture supernatant as a consequence of the plasma membrane injury, while the MTT assay is essentially based on the enzymatic conversion of MTT within the mitochondria. Also, DIAS [et al.], 2009 exposed microcistin-LR and other purified extracts from *Microcystis aeruginosa* to kidney epithelial cells and differences between the MTT and the LDH assay were also found. Based on their results, the authors considered the MTT assay more sensitive, proposing that the mitochondria is primarily affected by this toxin.

| Cyanobacteria Strains | | Cancer cell lines | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | MG63 | | RKO | | HT29 | | SH-SY5Y | |
| | | MTT | LDH | MTT | LDH | MTT | LDH | MTT | LDH |
| *Cyanobium* sp. | LEGE06098 | +++ | - | +++ | - | +++ | + | +++ | +++ |
| *Cyanobium* sp. | LEGE06113 | +++ | +++ | +++ | + | +++ | ++ | ++ | ++ |
| *Leptolyngbya cf. halophila* | LEGE06102 | +++ | ++ | +++ | + | +++ | ++ | +++ | +++ |
| *Synechocystis salina* | LEGE06155 | +++ | +++ | +++ | +++ | +++ | ++ | +++ | +++ |
| *Romeria* sp. | LEGE06013 | +++ | + | +++ | - | +++ | + | +++ | +++ |

**Table 11.** Resume of the cytotoxic effects induced by the cyanobacteria extracts in the cancer cell lines. For MTT – no activity, + 20 to 40%, ++ 40 to 60%, +++ more than 60% of decrease in cell viability. For LDH – no activity, + 20 to 40%, ++ 40 to 60%, +++ more than 60% of LDH released.

Since MTT and LDH assay don't provide direct information about the mechanism behind cell death, it was perform an Apoptosis/Necrosis assay, using Hoechst 33342 and Propidium Iodide as fluorochromes. Propidium Iodide only enters in membrane damaged cells, while Hoechst 33342 always enters the cells and stains the nucleus, revealing a fragmented organelle in apoptotic cells. By using simultaneously both fluorochromes it will allow distinguishing between necrotic and apoptotic cells. Due to constraints regarding the high number of cells and extract amount required for this assay, it was only performed for the crude extract from LEGE06098 and fraction B from LEGE06013 towards the MG63 cell line, fraction B from LEGE06102 against the RKO cell line and fraction B from LEGE06155 for the HT29 cell line. In Figure 7, results from this assay after exposure of the crude extract at 100μg/mL from LEGE06098 to the MG63 cell line, suggest that cells died from necrotic events since it was observed a propidium iodide staining in the cells exposed to the extract. The nucleus didn't appear in a fragmented manner, which doesn't suggest the occurrence of apoptosis. Results from the other cell lines are not shown, but identical effects were found in all of them.

Considering again the MTT and LDH assays and the particular case of strain LEGE06098, the results obtained suggested that the decrease in the cell viability observed by the MTT assay were not due to a loss of integrity of the cell membrane, since low percentages of LDH released were found in the culture medium. However, results from the Propidium Iodide and Hoechst 33342 staining showed that the cell membrane was, indeed, compromised. These results led us to the hypothesis that some compounds might interfere with the activity of the LDH with a consequent inactivation of the enzyme as observed by WEYERMANN [et al.], 2005 when they found that the compounds that were being tested in fibroblasts were acting as LDH inhibitors.
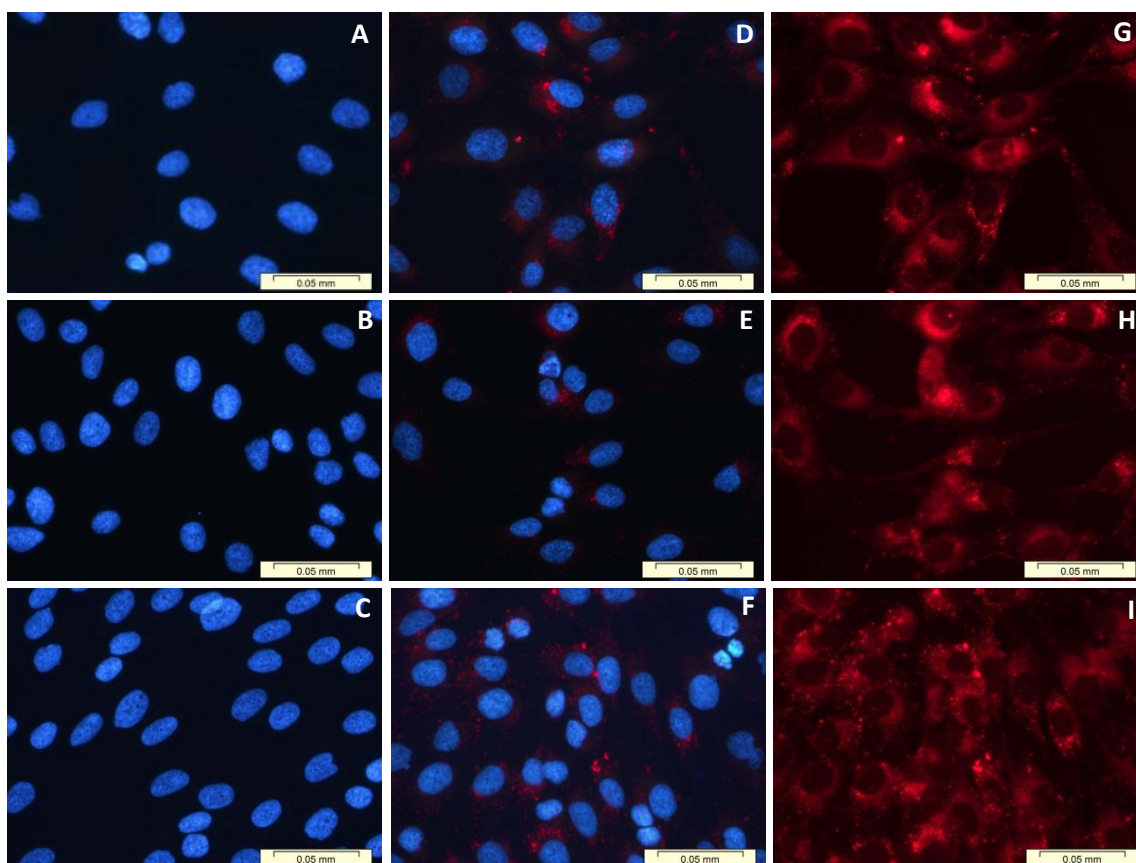
**Figure 14.** Cytotoxicity induced by the crude extract at 100µg/mL from LEGE06098 in the MG63 cell line, revealed by the propidium iodide/Hoechst 33342 staining. **A**, **B**, **C** - control cells after 24, 48 and 72 hours, respectively. There was no staining with Propidium Iodide. **D**, **G** - cells after a 24 hours treatment. **E**, **H** – cells after a 48 hours treatment. **F**, **I** – cells after a 72 hours treatment.

Results from MTT and LDH assays and propidium iodide/hoechst 33342 staining led us to suppose that a mixture of cytotoxic compounds present in the tested cyanobacteria strains may induce a severe shock in the cells that leads to a very fast cell death by membrane disruption. Since the crude extract and fractions constitute a mixture of compounds, we cannot dismiss the fact that isolated compounds can trigger apoptosis, which will be the preferred type of cell death in cancer cells.

## 7. Conclusions and future perspectives

Marine cyanobacteria are an important source of medicinal bioactive metabolites and many studies have led to the discovery of cyanobacteria compounds with remarkable anticancer properties. Most studies concerning marine cyanobacteria secondary metabolites have been conducted with cyanobacteria from tropical areas that occur in large densities, being, however, important to explore also other latitudes such as the temperate ones. With this work we found that marine cyanobacteria genera largely overlooked for the production of bioactive compounds can also be considered potential prolific as a source of therapeutical agents.

In this work, marine cyanobacteria strains from the LEGE collection LEGE06013 (*Romeria* sp.), LEGE06098 (*Cyanobium* sp.), LEGE06102 (*Leptolyngbya cf. halophila*), LEGE06113 (*Cyanobium* sp.) and LEGE06155 (*Synechocystis salina*) sampled from the Portuguese coast, revealed cytotoxic activity against human tumor cell lines osteosarcoma (MG63), colon adenocarcinoma (RKO and HT29) and neuroblastoma (SH-SY5Y). All tested cyanobacteria strains decreased cell viability in every tumor cell line, however, the strain LEGE06155 seems to be the most promising one for further toxicological studies and isolation of bioactive compounds. The observed inhibitory effect on cellular growth followed a pattern in most of the cell lines, in which the fraction containing the higher percentage of ethyl acetate (fraction B) was the most powerful in reducing cell viability in a dose dependent manner. This fraction is, therefore, a promising fraction for the isolation of bioactive compounds. Taking into consideration results from the MTT and LDH assays and the propidium iodide/Hoechst 33342 staining, we suggest that the cytotoxic compounds acted in a very strong manner, leading to a necrotic cell death. Since a most toxic fraction and cyanobacteria strain are now identified, and considering that the fractions represent a cocktail of compounds, it is now interesting to test sub-fractions, in order to localize more specifically the compounds.

Even though cyanobacteria strains revealed anticancer properties, it will be important to expose the cytotoxic fractions against normal cell lines, in order to evaluate their suitability as anticancer drugs.

As a final remark we consider that the cyanobacteria strains included in this study are promising organisms in terms of production of compounds which anticancer potential.

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**Annex I |** Z8 medium composition

| Solution Concentration (mL/L) | | Composition | | | |
|---|---|---|---|---|---|
| | | Reagent | g/L | | |
| **A** | 10 | NaNO$_3$ | 46,7 | | |
| | | Ca(NO$_3$)$_2$.4H$_2$O | 5,9 | | |
| | | MgSO$_4$.7H$_2$O | 2,5 | | |
| **B** | 10 | K$_2$HPO$_4$ | 3,1 | | |
| | | Na$_2$CO$_3$ | 2,1 | | |
| | | Reagent | mL/L | 100 mL | |
| **Fe-EDTA** | 10 | FeCl$_3$ | 10 | FeCl$_3$.6H$_2$O | 2,8 g |
| | | | | HCl (0.1 N) | 100 mL |
| | | EDTA-Na | 9,5 | EDTA | 3,9 g |
| | | | | NaOH (0.1 N) | 100 mL |
| | | Reagent | mL/L | g/L | |
| **Micronutrients** | 1 | Na$_2$WO$_4$.2H$_2$O | 10 | 0,33 | |
| | | (NH$_4$)$_6$Mo$_7$O$_{24}$.2H$_2$O | | 0,88 | |
| | | KBr | | 1,2 | |
| | | KI | | 0,83 | |
| | | ZnSO$_4$.7H$_2$O | | 2,87 | |
| | | Cd(NO$_3$).4H$_2$O | | 1,55 | |
| | | Co(NO$_3$)$_2$.6H$_2$O | | 1,46 | |
| | | CuSO$_4$.5H$_2$O | | 1,25 | |
| | | NiSO$_4$(NH$_4$)$_2$SO$_4$.6H$_2$O | | 1,98 | |
| | | Cr(NO$_3$)$_3$.9H$_2$O | | 0,41 | |
| | | V$_2$O$_5$ | | 0,089 | |
| | | Al$_2$(SO$_4$)$_3$K$_2$SO$_4$.24H$_2$O | | 4,74 | |
| | | H$_3$BO$_3$ | 100 | 3,1 | |
| | | MnSO$_4$.4H$_2$O | | 2,23 | |

**Table 13.** Results from the Mann-Whitney test for the MG63 cell line, regarding the MTT assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,500 | 0,400 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 4,000 | 0,500 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,500 | 0,150 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 3,000 | 0,350 |

| Organism | Fraction | Time | | | |
|---|---|---|---|---|---|
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | | 100 | 2,000 | 0,200 |
| | | 48 | 10 | 0,000 | 0,050 |
| | | | 1 | 4,000 | 0,500 |
| | | | 100 | 0,000 | 0,050 |
| | | 72 | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | 24 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,300 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction C | 24 | 100 | 1,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06113) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |

| | | | | | |
|---|---|---|---|---|---|
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,300 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |

**Table 14.** Results from the Mann-Whitney test for the RKO cell line, regarding the MTT assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (μg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 4,000 | 0,500 |
| | | | 10 | 0,500 | 0,100 |

| Species | Extract | Time (h) | Conc. | | |
|---|---|---|---|---|---|
| | | | 1 | 0,000 | 0,050 |
| Leptolyngbya cf. halophila (LEGE06102) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 3,000 | 0,350 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| Cyanobium sp. (LEGE06113) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| Synechocystis salina (LEGE06155) | Crude | 24 | 100 | 3,500 | 0,400 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |

| | Fraction | Time | | | |
|---|---|---|---|---|---|
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | 72 | 10 | 2,000 | 0,200 |
| | | 72 | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | 24 | 10 | 0,000 | 0,050 |
| | | 24 | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | 48 | 10 | 0,000 | 0,050 |
| | | 48 | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | 72 | 10 | 1,000 | 0,100 |
| | | 72 | 1 | 2,500 | 0,250 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | 24 | 10 | 0,000 | 0,050 |
| | | 24 | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | 48 | 10 | 0,000 | 0,050 |
| | | 48 | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | 72 | 10 | 0,000 | 0,050 |
| | | 72 | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | 24 | 10 | 0,000 | 0,050 |
| | | 24 | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | 48 | 10 | 3,000 | 0,350 |
| | | 48 | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | 72 | 10 | 0,000 | 0,050 |
| | | 72 | 1 | 3,000 | 0,350 |

**Table 15.** Results from the Mann-Whitney test for the HT29 cell line, regarding the MTT assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,300 |
| | Fraction A | 24 | 100 | 1,000 | 0,100 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,500 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,500 | 0,250 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction C | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,500 | 0,600 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,500 | 0,400 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 3,000 | 0,300 |
| | | | 10 | 3,500 | 0,450 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 4,000 | 0,500 |

| | | | | | |
|---|---|---|---|---|---|
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 3,000 | 0,300 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,300 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,300 |
| | Fraction C | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,300 |
| | | | 1 | 4,000 | 0,500 |
| *Cyanobium* sp. (LEGE06113) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,500 | 0,150 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 4,500 | 0,600 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |

| Species | Fraction | Time | Conc. | Value 1 | Value 2 |
|---|---|---|---|---|---|
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,500 | 0,250 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction C | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 2,000 | 0,200 |

**Table 16.** Results from the Mann-Whitney test for the SH-SY5Y cell line, regarding the MTT assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 3,000 | 0,350 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction A | 24 | 100 | 3,000 | 0,300 |
| | | | 10 | 0,500 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,500 | 0,400 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,500 | 0,400 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,500 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,500 | 0,250 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction C | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 2,000 | 0,200 |

| | | | | | |
|---|---|---|---|---|---|
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,500 | 0,150 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,500 | 0,400 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06113) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |

| | | | | | |
|---|---|---|---|---|---|
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 4,000 | 0,500 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,500 | 0,250 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,500 | 0,450 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,300 |
| | | | 1 | 2,500 | 0,250 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |

**Annex III**

**Table 17.** Results from Tukey's post-hoc analysis for the cell line factor, concerning the data obtained from the MTT assay.

| Comparison | Difference estimation | z | p |
|---|---|---|---|
| MG63 – HT29 | 1.39 | 7.3 | <0.001 |
| RKO – HT29 | 1.02 | 5.1 | <0.001 |
| SH-SY5Y – HT29 | -0.08 | -0.3 | 0.99 |
| RKO – MG63 | -0.37 | -2.8 | 0.03 |
| SH-SY5Y – MG63 | -1.47 | -7.5 | <0.001 |
| SH-SY5Y - RKO | -1.09 | -5.4 | <0.001 |

**Table 18.** Results from Tukey's post-hoc analysis for the cyanobacteria strain factor, concerning the data obtained from the MTT assay.

| Comparison | Difference estimation | z | p |
|---|---|---|---|
| LEGE06098 – LEGE06013 | -0.20 | -0.8 | 0.93 |
| LEGE06102 – LEGE06013 | -0.04 | -0.2 | 1 |
| LEGE06113 – LEGE06013 | -0.05 | -0.2 | 1 |
| LEGE06155 – LEGE06013 | -0.06 | -0.2 | 0.99 |
| LEGE06102 – LEGE06098 | 0.16 | 0.6 | 0.97 |
| LEGE06113 – LEGE06098 | 0.16 | 0.6 | 0.97 |
| LEGE06155 – LEGE06098 | 0.15 | 0.6 | 0.98 |
| LEGE06113 – LEGE06102 | -0.01 | -0.03 | 1 |
| LEGE06155 – LEGE06102 | -0.01 | -0.05 | 1 |
| LEGE06155 – LEGE06113 | -0.01 | -0.03 | 1 |

**Table 19.** Results from the Mann-Whitney test for the MG63 cell line, regarding the LDH assay data

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,500 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,500 | 0,250 |
| | | 72 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction A | 24 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 1,000 | 0,150 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 3,500 | 0,400 |
| | | | 1 | 2,500 | 0,250 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Crude | 24 | 100 | 3,500 | 0,400 |
| | | | 10 | 1,500 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,100 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction C | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,500 | 0,400 |
| | | | 1 | 1,500 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,500 | 0,400 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| *Cyanobium* sp. (LEGE06113) | Crude | 24 | 100 | 1,000 | 0,200 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 1,000 | 0,200 |

| | | | 100 | 3,000 | 0,500 |
| | | 48 | 10 | 4,500 | 1,000 |
| | | | 1 | 0,000 | 0,050 |
| | | | 100 | 1,000 | 0,200 |
| | | 72 | 10 | 0,000 | 0,050 |
| | | | 1 | 4,000 | 0,500 |
| | | | 100 | 3,000 | 0,350 |
| | | 24 | 10 | 3,500 | 0,400 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | | 100 | 0,000 | 0,050 |
| | | 48 | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | | 100 | 2,000 | 0,200 |
| | | 72 | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,100 |
| | | | 100 | 4,000 | 0,500 |
| | | 24 | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | | 100 | 0,000 | 0,050 |
| | Crude | 48 | 10 | 0,000 | 0,050 |
| | | | 1 | 4,500 | 1,000 |
| | | | 100 | 0,000 | 0,050 |
| | | 72 | 10 | 3,000 | 0,350 |
| *Synechocystis salina* (LEGE06155) | | | 1 | 3,000 | 0,350 |
| | | | 100 | 0,000 | 0,100 |
| | | 24 | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction B | | 100 | 0,000 | 0,050 |
| | | 48 | 10 | 3,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | | 100 | 0,000 | 0,050 |
| | | 72 | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |

**Table 20.** Results from the Mann-Whitney test for the RKO cell line, regarding the LDH assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Fraction B | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,100 |
| | | | 10 | 3,000 | 0,600 |
| | | | 1 | 1,000 | 0,200 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | | 48 | 100 | 3,000 | 0,600 |
| | | | 10 | 0,000 | 0,100 |
| | | | 1 | 0,000 | 0,100 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,400 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,100 |
| | | | 1 | 2,500 | 0,500 |
| | | 72 | 100 | 3,000 | 0,350 |
| | | | 10 | 2,000 | 0,150 |
| | | | 1 | 1,000 | 0,200 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Fraction B | 24 | 100 | 1,500 | 0,200 |
| | | | 10 | 1,500 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| *Cyanobium* sp. (LEGE06113) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 4,000 | 0,500 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |

**Table 21.** Results from the Mann-Whitney test for the HT29 cell line, regarding the LDH assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| Romeria sp. (LEGE06013) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,500 | 1,000 |
| | | | 1 | 1,500 | 2,000 |
| | | 72 | 100 | 4,500 | 1,000 |
| | | | 10 | 4,500 | 1,000 |
| | | | 1 | 3,000 | 0,500 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 1,500 | 2,000 |
| | | | 10 | 3,000 | 0,500 |
| | | | 1 | 3,000 | 0,500 |
| | | 72 | 100 | 4,500 | 1,000 |
| | | | 10 | 4,500 | 1,000 |
| | | | 1 | 4,500 | 1,000 |
| Cyanobium sp. (LEGE06098) | Crude | 24 | 100 | 1,000 | 0,100 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 3,000 | 0,500 |
| | | | 10 | 1,000 | 0,150 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 3,500 | 0,400 |
| | | | 10 | 1,500 | 0,200 |
| | | | 1 | 1,500 | 0,200 |
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,300 |
| | | | 1 | 0,000 | 0,100 |
| | | 72 | 100 | 1,500 | 0,200 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction B | 24 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 48 | 100 | 1,500 | 0,150 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 2,000 | 0,150 |
| | | 72 | 100 | 2,500 | 0,250 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 1,500 | 0,200 |
| Leptolyngbya cf. halophila (LEGE06102) | Crude | 24 | 100 | 1,000 | 0,300 |
| | | | 10 | 2,500 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 1,000 | 0,100 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | Fraction B | 24 | 100 | 0,000 | 0,100 |
| | | | 10 | 2,500 | 0,250 |
| | | | 1 | 0,000 | 0,100 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 4,000 | 0,500 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| Cyanobium sp. (LEGE06113) | Crude | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,300 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |

| | Fraction | Time (h) | Conc. | | |
|---|---|---|---|---|---|
| | Fraction A | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,500 | 0,250 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction B | 24 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,400 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,150 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 3,000 | 0,350 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | | 72 | 100 | 2,000 | 0,200 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction B | 24 | 100 | 2,000 | 0,200 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 3,000 | 0,350 |
| | | | 10 | 1,500 | 0,150 |
| | | | 1 | 1,500 | 0,150 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,150 |
| | | | 1 | 4,000 | 0,500 |

**Table 22.** Results from the Mann-Whitney test for the SH-SY5Y cell line, regarding the LDH assay data.

| Cyanobacteria strain | Treatment | Exposure Time (h) | Concentration (µg/mL) | Mann-Whitney U | Sig. |
|---|---|---|---|---|---|
| *Romeria* sp. (LEGE06013) | Fraction B | 24 | 100 | 0,000 | 0,167 |
| | | | 10 | 0,000 | 0,167 |
| | | | 1 | 2,000 | 0,667 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 2,000 | 0,200 |
| | | 72 | 100 | 0,000 | 0,050 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 2,000 | 0,200 |
| *Cyanobium* sp. (LEGE06098) | Crude | 24 | 100 | 4,000 | 0,500 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 4,000 | 0,500 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 0,000 | 0,050 |
| | Fraction A | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,500 | 0,400 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 2,000 | 0,400 |
| | | | 1 | 0,000 | 0,050 |
| *Leptolyngbya cf. halophila* (LEGE06102) | Fraction B | 24 | 100 | 3,000 | 0,350 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 1,000 | 0,100 |
| | | 48 | 100 | 4,000 | 0,500 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 4,500 | 0,600 |
| *Cyanobium* sp. (LEGE06113) | Fraction A | 24 | 100 | 0,000 | 0,100 |
| | | | 10 | 0,000 | 0,167 |
| | | | 1 | 1,000 | 0,333 |
| | | 48 | 100 | 1,000 | 0,100 |
| | | | 10 | 2,000 | 0,200 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 0,000 | 0,100 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 4,000 | 0,500 |
| | Fraction B | 24 | 100 | 3,000 | 0,600 |
| | | | 10 | 2,000 | 0,400 |
| | | | 1 | 1,000 | 0,200 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 72 | 100 | 3,000 | 0,350 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 0,000 | 0,050 |
| *Synechocystis salina* (LEGE06155) | Crude | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 3,000 | 0,350 |
| | | | 1 | 0,000 | 0,050 |
| | | 48 | 100 | 2,000 | 0,200 |
| | | | 10 | 0,000 | 0,050 |
| | | | 1 | 1,000 | 0,100 |
| | | 72 | 100 | 4,000 | 0,500 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 1,000 | 0,100 |
| | Fraction B | 24 | 100 | 0,000 | 0,050 |
| | | | 10 | 4,000 | 0,500 |
| | | | 1 | 3,000 | 0,350 |
| | | 48 | 100 | 0,000 | 0,050 |
| | | | 10 | 1,000 | 0,100 |
| | | | 1 | 3,000 | 0,350 |
| | | 72 | 100 | 0,000 | 0,050 |

| | | | 10 | 0,000 | 0,050 |
|---|---|---|---|---|---|
| | | | 1 | 3,000 | 0,350 |