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Algal extracts as potential antiproliferative agents. Preliminary evaluation in canine tumour cell lines

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ABSTRACT

Algal extracts as potential antiproliferative agents. Preliminary evaluation in canine tumour cell lines

Different bioactive compounds with proven cytotoxic, antiproliferative, antioxidant, anti-inflammatory and, immunomodulatory effects have been isolated from different marine sources, especially from macro and microalgae. Consequently, algae preparations are commonly and traditionally used as alternative medicines.

Canine mastocytoma and lymphoma are among the most frequently occurring neoplasms in dogs. Cancer chemotherapy is one of the most commonly adopted therapeutic approach in pets oncology. However, the adverse side effects of various chemotherapeutic drugs as well as, drug resistance developed by the tumours over time, are of primary concern.

In the present study, we investigate the antiproliferative and cytotoxic potential of the dichloromethane-methanolic (1:1 v/v) crude extract of four marine macroalgae, *Plocamium cartilagenium* (red alga), *Gracilaria cervicornis* (red alga), *Laminaria ochroleuca* (brown alga) and, *Halopithys incurvus* (red alga), from the Moroccan coast, using *in vitro* models of canine cancer, i.e. mastocytoma and lymphoma cell lines (C2 and CLBL-1 cells respectively).

The cytotoxic activity of the extracts was assessed using three different assays: Alamar Blue, Sulforhodamine B, and Neutral Red Uptake, which assess cell viability, proliferation, and permeability. The IC_{50} and R^2 values (goodness of fit) were determined. In this preliminary comparative screening, the maximum percentage of cytotoxicity was induced by *G. cervicornis* and *P. cartilagenium*. The cytotoxic activity (IC_{50}) of these extracts in C2 cells was 26.14 $\mu g.mL^{-1}$ and 50.94 $\mu g.mL^{-1}$ respectively; in CLBL-1 cells 11.65 $\mu g.mL^{-1}$ and 10.69 $\mu g.mL^{-1}$.

From the IC_{50} values here obtained we can conclude that *G. cervicornis* displayed greater effectiveness against both the canine neoplasms, with CLBL-1 having a higher susceptibility to both extracts. Further studies are needed for bioactive compound isolation and a more exhaustive elucidation of their (main) respective cytotoxic mechanisms of action.

Keywords : Canine cell lines, Algae extract, Cytotoxicity, Alamar Blue, Sulforhodamine B, Neutral Red Uptake.

List of Acronyms

AB - Alamar Blue

CLBL-1 - Human Diffuse Large B-cell Lymphoma

DNA - De-oxyribose Necleic Acid

EDTA - Ethylene Di-amine Tetraacetate

EGF - Epidermal Growth factor

FBS - Foetal Bovine Serum

G.C. - *Gracilaria cervicornis*

GC-MS - Gas Chromatography- Mass Spectrometry

GSH - Glutathione

H.I. - *Halopithys incurvus*

IARC - International Agency for Research on Cancer

IC_{50} - Maximal inhibitory concentration 50%

L.O. - *Laminaria ochroleuca*

LC-MS - Liquid Chromatography- Mass Spectrometry

MCT - Mast Cell Tumours

NEAA - Non Essential Amino Acid

NRU - Neutral Red Uptake

P.C. - *Plocamium cartilagenium*

P/S - Penicillin/ Streptomycin

PBS - Phosphate Buffer Saline

R^2 - Goodness of fit

ROS - Reactive Oxygen Species

RTK - Receptor Tyrosine Kinases

SRB - Sulforhodamine B

TCA - Trichloro Acetic Acid

TKIs - Tyrosine Kinase Inhibitors

WHO - World Health Organisation

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1. INTRODUCTION

1.1 Cancer: a worldly threat

The 21st century's advancement in science and technology, made a tremendous progress in the medical field, especially in the case of "deadly" diseases, whose ultimate result is the death of the person. Cancer is such a disease, with utmost priority. Globally, it is one of the leading causes of death, which can happen due to one of the following three reasons or altogether: (a) an incorrect lifestyle (unhealthy diet); (b) environmental pollution (c) genetic predisposition (*Sakthivel, R., & Devi, K. P., 2019*). Under normal situations a balance is usually maintained between cellular proliferation and programmed cell death; however a mutation in DNA can cause alterations in this balance and makes the cells divide in an uncontrolled way, thereby resulting in the formation of a malignant mass (tumour) that can spread to different locations (metastasized) (*Suarez-Jimenez, G. M. et al., 2012*). Efforts have been made consistently, to fight against this disease. Chemotherapy is considered, together with radiotherapy and immunotherapy, as one of the options for cancer treatment, even though most of anticancer drugs are cytotoxic to normal cells, too. Actually in the past few years the advancements in cellular and molecular biology led to the introduction of potent anticancer agents that are capable of targeting cancerous tissues with minimum side effects (*Kaur, K. et al., 2014*).

More than one hundred physical, chemical, and biological carcinogens have been mentioned by the World Health Organization (WHO) and the International Agency of Research on Cancer (IARC), in 2014 Oncological research led to the discovery of new carcinogens and their mechanisms of action. As affirmed by Serota and Yuppa, in their review of- "The Emperor of all maladies, The Biography of Cancer ", by Siddhart Mukherjee, "We must focus on prolonging life, rather than eliminating death". Since the history of cancer is evolving, brand new discoveries happen daily in laboratories all around the world, and one thing we have to keep in mind is that cancerous cells are a *"more perfect version of normal cells they grow faster and adapt better to their respective environment"*(*Serota, R., 2011 , Yuppa. D.P., 2012*).

1.2 Natural products as anticancer drugs

Considering their remarkable diversity, natural products are always considered a rich reservoir of bioactive compounds having therapeutic properties. It is quite astonishing to know that, about half of the drugs used for the anticancer treatment have been natural products or either semi-than fully synthetic compounds directly derived from them. A notable number of products are commonly extracted from plants, animals, microorganisms, and even from the marine population. It is estimated there are more products with different chemical diversities that can be useful for the treatment of about 87% of human ailments *(Newman, D. J., & Cragg, G. M., 2016, Ramasamy, K., & Agarwal, R., 2008*). Some anticancer drugs originate from natural sources: - irinotecan, vincristine, etoposide, and paclitaxel are derived from plants, actinomycin D, mitomycin C from bacteria while, bleomycin, is of marine origin. Some of these compounds are still used for cancer therapy and they will continue to play an important role in the unforeseen future (*Huang, M. et al., 2021*). From ancient times, natural products isolated from medicinal plants have been used, and the first use was dated back to Mesopotamia in 2600 BC (*Borchardt, J. K., 2002, Cragg, G. M., & Newman, D. J., 2013*).

Vinca alkaloids from the plant *Catharanthus roseus* are the second most used anticancer drugs. They were first used in combination with other anticancer drugs, for the treatment of lymphoma, leukemia, advanced testicular cancer, lungs, and breast cancer. These alkaloids are generally classified as cell cycle-specific cytotoxic drugs. In addition, they also have the ability to prevent *in vitro* malignant angiogenesis. They disrupt the microtubules' function by developing synergy with tubulin and thereby leading to cell death. At present, the new synthetic vinca alkaloid vinflunine, has gained approval in Europe for cancer treatment (*Dey, P. et al., 2020*).

However, the process of botanical drug discovery and development suffers of a number of challenges such as., the availability, the isolation and identification of the bioactive compound and its exact mechanism of action, the difficulty in specimen collection, and the incompatibility toward the high throughput screening (*Dutta, S. et al., 2019*) Additionally, the identified active principle is not effective principle is not enough and does not match the expectations. Finally, their approval rate by the US Food and Drug Administration is quite low (*Scannell, J. W. et al., 2012*).

As a whole, these reasons at least in part justify why the pharmaceutical companies shifted their focus toward the manufacturing of synthetic drugs or semi-synthetic derivatives. The chemical structure of some of the successful anticancer agents derived from plants is shown in **Figure 1**.

1.3 Marine natural products as anticancer drugs

About 70% of the earth's surface is covered by oceans, which are the richest sources of various bioactive compounds. It has also been reported that the marine ecosystem is more biologically diverse than the tropical rainforest (*Tadesse, S., 2018*). As to Darwin's theory of "Survival of the fittest", and each organism's adaptation to its immediate environment, a large number of species have evolved by chemical means to protect themselves from predators. Thus, this adaptation resulted in the synthesis of secondary metabolites, which include terpenoids, polyketides, alkaloids, sugars and, peptides (*Haefner, B., 2003*).

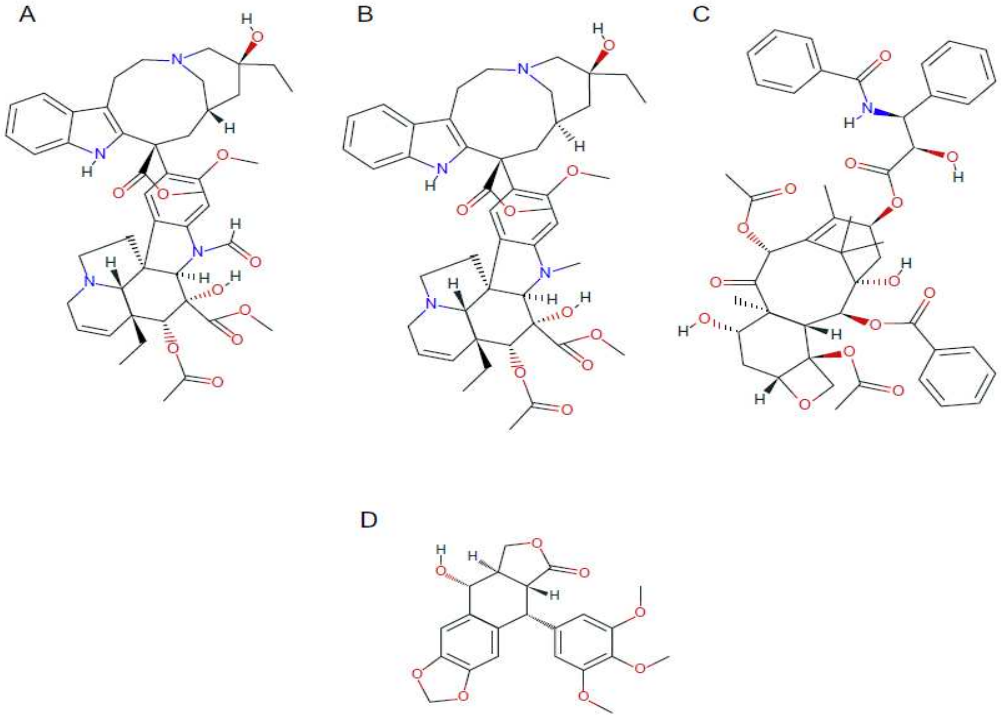

Figure 1. Chemical structure of some successful anticancer agents obtained from plants. (A) Vincristine (B) Vinblastine (C) Paclitaxel (D) Podophyllotoxin.

As compared with terrestrial natural products, the identification, study, and usage of marine natural products are in their growing phase (*Simmons, T. L. et al., 2005*). Studies showed that these compounds can affect carcinogenesis either *in vitro* than *in vivo.* They also exhibited greater affinity and specificity to interact with specific intracellular signaling pathways, like endoplasmic reticulum stress induction, autophagy, mitochondrial dysfunction, apoptosis, and inflammation (*Alves, C., & Diederich, M., 2021*). The xanthophyll carotenoid, astaxanthin, that can be found in many marine species like microalgae, fungi, bacteria, sea urchins, sea snails, etc., decrease the level of certain markers of stemness, i.e., the homebox protein NANOG (NANOG) and the octamer-binding transcription factor 4(Oct-4), in breast carcinoma cells. It also shows antiproliferative effects in the aggressive prostate cancer DU145 cells, triggering cell death by apoptosis and reducing invasion and migration abilities (*Galasso, C. et al., 2017, Ahn, Y. T. et al., 2020, Sun, S. Q. et al., 2020*). The flaccidoxide-13-acetate diterpenoid, another compound of interest, isolated from the marine soft coral *Sinularia gibberosa*, was proved to decrease cell viability, migration, and invasion in hepatocellular carcinoma (*Wu, Y. J. et al., 2020*). Jiang and co-workers studied the cytotoxic properties of three compounds isolated from *Penicillium chrysogenum* LD 201810, a marine alga-associated fungus: a pentaketide derivative (penilactonol A), and two hydroxy-phenylacetic acid derivatives [(2′R)-stachyline B and (2′R)-westerdijkin A]. This latter one showed significant cytotoxic activity against the hepatocellular carcinoma cell line HepG2 (*Jiang, L.L.. et al., 2020*).

Marine organisms are also source of tyrosine kinase receptor inhibitors. A brominated tyrosine derivative from the sponge *Verongia aerophobia* (Aeroplysinin-1), showed an inhibitory effect on the purified epidermal growth factor (EGF) kinase activity, blocking EGF-stimulated proliferation either in cancer cell lines by inducing apoptosis at high nanomolar concentrations than *in vivo* by suppressing angiogenesis (*Kreuter, M. H. et al., 1990, Hinterding, K. et al., 1998, Rodríguez-Nieto, S. et al., 2002*).

Overall, more than thousands of bioactive compounds with diverse biological activity have been reported in the past 20 years; this clearly proves the marine environment as an excellent and potentially rich source of bioactive compounds. However, one of the biggest challenges in bioprospecting and pharmacological application of marine natural products is their low extraction yield, which could compromise the supply chain in the pre-clinical and clinical trials, fundamental steps in the drug development process. Many strategies have been adopted for overcoming such a problem, e.g., aquaculture/cultivation, genetic engineering,

semi-synthesis, synthesis, and chemical modifications (*Lindequist,U., 2016*).Some anticancer compounds isolated from some marine organisms and their mechanisms of action are shown in **Table 1**.

1.4 Marine Seaweeds

Over the past few decades, promising progress have been made in the isolation and screening of biological activity of seaweeds, and some of them have been proved to possess a potential medicinal value (*Koyanagi, S. et al., 2003*). Moreover, they have also been proved to be an excellent source of bioactive compounds having anticancer, anti-inflammatory, antiviral, cytotoxic, and anti-mitotic activities (*Bhosale, S. H. et al., 2002, Naqvi, Solimabi, S. A. et al., 1981*). Among the various bioactive molecules with potential therapeutic effects we include polyphenols (*Li, Y. X. et al., 2011*), terpenoids (*Culioli, G. et al., 2004*), polysaccharides (*Wijesekara, I. et al., 2011*), and meroterpenoids (*Valls, R. et al., 1993*).

Table 1. List of some important anticancer bioactive compounds isolated from marine natural products.

Bioactive compound	**Source**	**Mechanism of action**	**References**
Cytarabine	sponge	inhibition of DNA synthesis	[1,2]
Dolastatin 10	sea hare	pro-apoptotic effects and inhibition of microtubule assembly	[1,2]
Ecteinascidin 743	tunicate	alkylation of DNA	[1,2]
Aplidine	tunicate	inhibition of cell cycle progression	[1,2]
Halichondrin B	sponge	interaction with microtubule function	[1,2]
Spirulan	alga	inhibition of heparanase	[3]

Tolyprophin	alga	Inhibition of sterol *O*-acyl transferase	[3]
Stypodiol	alga	promotion of tubulin polymerization	[3]

**For references:-*

1: Fenical, W., 1982.

2: Schwartsmann, G. et al., 2001

3: Mayer, A. M., 1999

Seaweeds (also known as marine microalga) are primitive plant species that lack true stems, roots, and leaves, unlike higher plants; instead, the body of seaweed (thallus), comprises holdfast, stipe, and blade. They are responsible for the production of about half of the world's oxygen and are also the primary producers of the ocean food chain. The marine algae include the invisible unicellular microalgae, which form the phytoplankton together with cyanobacteria, and multicellular macroalgae, generally called as seaweed, which is larger, more visible, and complex. Seaweeds can be found along the coastal areas, on the floor of continental shelves, and sometimes washed up in the intertidal zones. Traditionally, marine algae can be classified into *Rhodophyta* (red algae), *Phaeophyta* (brown algae), *Chlorophyta* (green algae), *Cyanophyta* (blue-green algae), *Prophyta* (dinoflagellates), *Chrysophyta* (diatoms and golden brown algae), and, finally, *Chlorophyta* (microscopic green algae). A broad classification of algae is shown in **Figure.2**.
(https://en.wikipedia.org/wiki/Marine_primary_production).

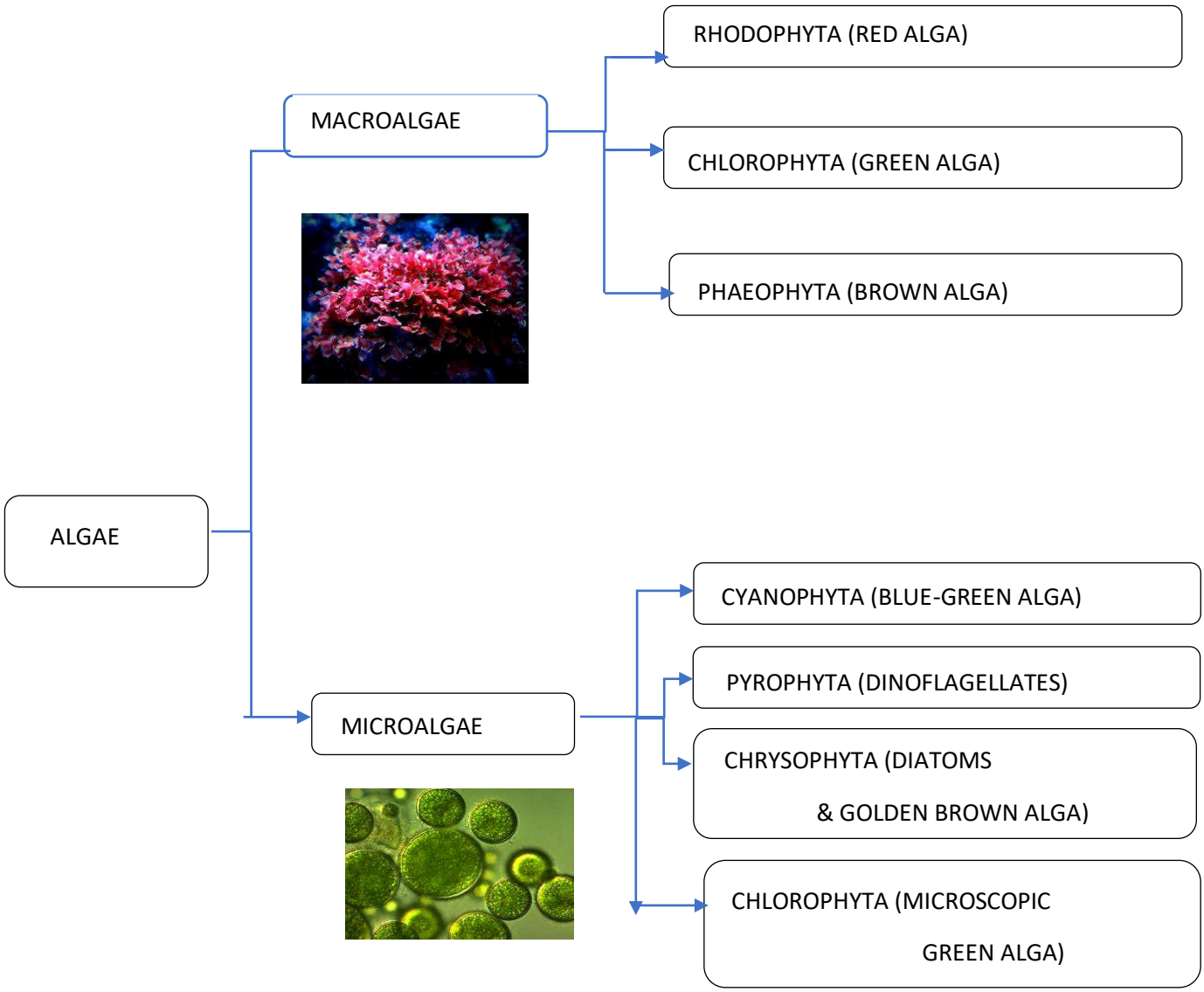

Figure 2. Classification of marine seaweed.

It has been estimated that about 150,000 seaweeds are present in the intertidal zones and the tropical water of the ocean. Some seaweeds can be consumed directly and some can be utilized for the production of substances used as food ingredients, fertilizers, and animal feed supplements, too. Among the seaweed most common extracts we include; alginates, agar-agar, and carrageenan. Furthermore, seaweed extracts may contain significant levels of plant growth hormones such as auxins, gibberellins, and cytokines. *Ascophyllum nodosum* is a seaweed harvested from mineral-rich water, and it is considered as the best seaweed extract (*Bocanegra, A. et al., 2009, Pal, A. et al., 2014, De Almeida, C.L.F. et al., 2011*).

Studies showed that the green seaweeds contain sulfuric acid polysaccharides and sulfated galactans, whereas the red algae contain floridean starch, carrageenan, mucopolysaccharide, and water-soluble sulfated galactans. Fucoidan or sulfated fucose, laminarian or β-1,3-glucan, and alginic acid are the main components of brown seaweed. The seaweed polysaccharide is effective as an antitumour, antiviral, and anticoagulant agent, preventing large intestine cancer, diabetes, and obesity; moreover, it decreases, low-density lipid cholesterol in rats (*De Almeida, C.L.F.et al., 2011*).

1.5 Anticancer and antioxidant potential of seaweeds

The general composition of marine algae includes carotenoids, pigments, terpenes, polyphenols, phycobiliproteins, phlorotannins, and polysaccharides. The type and the amount of these constituents vary with the species and also with algae ability to interact with the environmental factors such as, water pressure, salinity, and radiation (*Gutiérrez-Rodríguez, A. G. et al., 2018*). Along with the aforementioned compounds, algae, also are a rich source of minerals like iron, zinc, magnesium, and iodine, as well as vitamins, lipids, and fiber. Some studies showed that among all the compounds mentioned above, terpenes, polysaccharides, and polyphenols, are biologically relevant and potentitially responsible for anticancer activity (*Senthilkumar, K. et al., 2013, Atashrazm, F. et al., 2015, Peng, J. et al., 2011*). Polysaccharides isolated from the red alga, *Gracilariopsis lemaneiformis*, comprise 3,6-anhydro-L-galactose and D-galactose, and a linear structure of repeated disaccharide agarobiose units. These compounds were, cytotoxic on A549 (human lung cancer), B16 (mouse melanoma), and, MKN-28 (gastric cancer) cell lines (*Kang, Y. et al., 2017*). The sulfated polysaccharides extracted from the brown alga *Sargassum vulgare* showed antiangiogenic activity and inhibited cell proliferation in HeLa and B16 cell lines (*Dore, C.M.P.G. et al., 2013*). Polysaccharides isolated from *Ulva lactuca*, a widely distributed green alga, exhibited *in vitro* and *in vivo* activity against breast cancer in Wistar rats along with a chemopreventive effect (*Abd-Ellatef, G. E. F. et al., 2017*). Fucoidans are sulfated polysacahrides found in number of brown algae. One of these polysaccharides, isolated from *Fucus evanescens*, exhibits anti-tumour and anti-metastatic effects in C57B1/6 mice transplanted with lung adenocarcinoma (*Alekseyenko, T.V. et al., 2007*). Another one, extracted from the brown seaweed *Cladosiphon okamuranus* expesses strong and dose-dependent pro-apoptotic activity on MCF-7 cells by activating caspase-3 and inducing DNA

fragmentation (*Teruya, T. et al., 2007*). Production of ROS is an important matter of concern. ROS over production results in cellular damage, genomic stability and, carcinogenecity.

Production of Reactive Oxygen Species (ROS) is an important matter of concern. Indeed, the increasing amounts of ROS, along with the incompetency of the cellular antioxidant response, are the main reason for the onset of cellular oxidative stress. Basically, ROS can interact with a number of cellular targets including lipids, proteins and, DNA, ultimately resulting in cellular damage, genomic instability, and progression of tumourigenic cells (*Kashyap, D. et al., 2019*).

Cells possess various anti-ROS mechanisms of defense, essentially referable to the prevention of ROS, excessive production or the activation of antioxidants (e.g., enzymes) to convert them into harmless species. The sulfated polysaccharide, fucoidan, mostly localized in the cell wall of marine brown algae, show a high ROS scavenging activity *in vitro* without causing any cytotoxic effect at the concentration of $2mg.mL^{-1}$. Furthermore, it seems to increase the production of glutathione (GSH) and restored the catalase activity in HepG2 cell line (*Chale-Dzul, J. et al., 2017*). The carotenoid fucoxanthin and the polyphenolic compound phloroglucinol, both from the brown seaweeds, show anticancer and antioxidant effects on two human colorectal cancer cell lines (HCT116 and HT-29), without causing any effect on normal cells (*Lopes-Costa, E. et al., 2017*). The ethanol extract of the red algae *Callophyllis japonica*, subdues the H_2O_2 induced apoptosis and increases the cellular antioxidant enzyme activity. The aqueous extract of *Gracilaria tenuistipitata* (a red algae), increases the recovery of H1299 (an epithelial-*like* lung carcinoma cell line) cells from H_2O_2-induced DNA damage; moreover, it inhibits cell proliferation (*Lee, J. C. et al., 2013, Ji, Y. et al., 2012*). Data from animal model studies reveal that the hot water extraction of the green alga, *Ulva reticulata*, reduced hepatic stress in albino rats through free radical scavenging effects (*Balaji Raghavendra Rao, H. et al ., 2004*).

1.6 Properties of seaweeds of our interest:

1.6.1 *Gracilaria cervicornis*

The genus *Gracilaria* belongs to red algae (phylum-*Rhodophyta*). It is known for its economic importance as an agarophyte and also because of its use as a food product for humans and various species of shellfish. It is generally found in warm waters, and cannot

survive at temperatures below 10 ºC and it is present in all the oceans except the Arctic. It is the largest genera of red algae in terms of the number of species (https://en.wikipedia.org/wiki/Gracilaria),(*McLachlan, J., & Bird, C. J., 1984*). Previous studies have been proved that these algae are particularly enriched with fatty acids; in particular, several *Gracilaria* species, including *G. cervicornis*, mostly contain hexadeconic acid (50.49%), tetradeconic acid (5.32%) and, octadeconis acid (4.66%) (*Torres, PB., 2017, de Andrade Tomaz, A. C. et al., 2012*). Nevertheless, the alga composition can be influenced by the extraction protocol and the solvents used for the extraction (*Martins, R. M. et al., 2018*). The dichloromethane/methanol extract (3:1) of *G.cervicornis* showed fungistatic activity against candida species when used in combination with commercial antifungals like fluconazole (*Sampaio, T.M.M. et al., 2022*). Furthermore, many *Gracilaria* species show anti-proliferative, anti-inflammatory, and antioxidant properties. The foremost *Gracilaria* species and their biological effects are reported in **Table 2.**

Table 2. Main *Gracilaria* species and their biological effects.

Species	**Biological activity**	**Cell line or rodent species**	**References**
G.tenuistipitata	Anti-proliferative	Ca9-22 human oral squamous carcinoma	[1]
G.corticata	Anti-proliferative	MCF-7 human breast cancer	[2]
G.edulis	Anti-carcinogenic	A549 human lung adenocarcinoma	[3]
G.cornea	Anti-inflammatory (inhibits paw edema induced by carrageenan and dextran)	Rat	[4]

G. changii	Anti-inflammatory (inhibits TNF-α level of response and TNF-α and IL-6 gene expression)	Rat	[5]
G. fisheri	Anti-tumour (delayed cell migration; inhibits EGFR and MAPK phosphorylation	HuCCA-1 Human cholangiocarcinoma cells.	[6]

**For reference:-*

1: (Yeh, C.C. et al., 2012)

2: (Namvar, F. et al., 2014)

3: (Sakthivel, R. et al ., 2016)

4: (Coura, C.O. et al., 2015)

5: (Shu, M. H. et al ., 2013)

6: (Sae-Lao, T. et al., 2017)

1.6.2 *Plocamium cartilagineum*

Plocamium cartilagineum, from the family, *Plocamiaceae*, is an erect red alga that grows about 10 cm in height. Distributed worldwide, it has been extensively studied. *Plocamium* species are generally famous for the presence of halogenated monoterpenes, more chlorinated in comparison to compounds found in other taxa of red macroalgae. The most abudant monoterpenes identified in *Plocamium cartilagenium* are Meretensene and violacene. These compounds show insecticidal activity against tomato moths and cereals green bugs (*Argandoña, V. et al., 2000, Shilling, A. J. et al., 2021*). A number of polyhalogenated terpenes from *Plocamium cartilagineum*, are cytotoxic against CT26 (murine colon adenocarcinoma), SW480 (human colon adenocarcinoma), HeLa (human

cervical adenocarcinoma), and SkMel28 (human malignant melanoma) cell lines (*de Inés, C. et al., 2004*). Other species of the genus *Plocamium*, showed beyond doubt anti-proliferative effects in WHCO1 (human esophageal cancer) cell line (*Antunes, E.M. et al., 2011*).

1.6.3 *Laminaria ochroleuca*

Large brown alga in the order *Laminariales*, also called 'golden kelps', due to their blade coloration. It is frequently found in the northern hemisphere, from Morocco to the south of England (https://en.wikipedia.org/wiki/Laminaria_ochroleuca). Different species of *Laminaria* are used in food and cosmetics industries due to their flavor and other biological properties (*Kim, S. K. (Ed.)., 011*). The primary constituents of these brown algae are polysaccharides, and the sulfated polysaccharide from *sssas* exhibits high anti-cancer activity in a human colon cancer cell line (HTC-116), with a half-maximal inhibitory concentration (IC_{50}) of 0.114 $mgmL^{-1}$), as well as in human malignant melanoma, breast adenocarcinoma, and leukemia. Some authors have reported that sulfated polysaccharides from brown algae have diverse applications as an antioxidant, anticoagulant, antiviral, anti-hyperlipidemic, and anti-tumour agents (*Abdala Díaz, R. T. et al., 2019*). Another component of *Laminaria* species, laminarin (a storage glucan), showed anti-proliferative and pro-apoptic effects in the human colon adenocarcinoma cell line (HT-29) (*Park, H. K. et al., 2012*). Some important Laminaria species with noticeable effects in **Table 3.**

1.6.4 *Halopitys incurvus*

Halopitys incurvus or *Halopithys incurva* is a red seaweed belonging to the family *Rhodomelaceae.* The generic name of this particular genus means "sea pine", and the term "incurvus" explains the shape of the branches ("bending inwards" or "bowed"). This particular algae is quite rare, but it can be found, all throughout the year. It is particularly present in the Mediterranean sea, and generally on rocky substrates (*Batters, E.A.L., 1902.*, *Vasarri, M. et al., 2020*). Among seaweeds, the red algae contain the highest amount of halogenated compounds. In particular, *H. incurvus* is rich in total phenolic content with proven anti-oxidant properties (*Chibi, F. et al., 2018*). Previous studies showed that *H. incurvus* extracts possess antibacterial, antiplasmodial, antiviral and, well-fortified anti-inflammatory properties (*Oumaskour, K. H. A. D. I. J. A. et al., 2013, Spavieri, J,. et al.,*

2013, Allmendinger, A. et al., 2010). In HT-29 human adenocarcinoma cell line, it exhibits polysaccharide-mediated immunomodulatory effects (*Díaz, R. T. A., Chabrillón, M. et al., 2011*), along with antitumour and antioxidant potential (*Zbakh, H. et al., 2014*). This alga is a candidate of interest; nevertheless, little is known about its biological activity.

Table 3. Main *Laminaria* species and their biological effects.

Species	**Biological activity**	**Cell line or rodent species**	**References**
L. digitata	Anticancer (proapoptotic)	HT-29 Human colon cancer	[1]
L. sacchaina	Anti-inflammatory (inhibts leukecyte recruitment and neutrophils adhesion to platelets *in vivo*	Rat	[2]
L. cichorioides	Anti-tumour(inhibits cell growth	DLD-1 and HT-29 human colon cancer	[3]
L. japonica	Anti-tumour	A375 and BGC823 Human melanoma and gastric carcinoma	[4]

**For reference:-*

1: (Hussain, E. et al., 2016)

2: (Croci, D.O. et al., 2011)

3: (Imbs, T. I. et al., 2009)

4: (Peng, Z. et al., 2012

e. *Ellisonadia elongata*

Ellisonadia elongata is a, red limestone alga with calcareous consistency. Also known as “common coral”, it belongs to the family of *Corallinaceae,* red algae. It is branched, erect, grows up to 10-12 cm in height, and mainly survives on sea rocky bottoms (https://it.wikipedia.org/wiki/Ellisolandia_elongata). Very little information about its biological activity exists. However, *E. elongata* methanolic extracts show the highest antibacterial activity against *Staphylococcus aureus*. Moreover, it exhibits a remarkable antioxidant scavenging effect (*Sahnouni, F. et al., 2021*). Finally, *E. elongata* is considered as a good bioindicator of organic pollutants exposure in marine ecosystems (*Sabri, H. et al., 2020*).

1.7 Canine Mastocytoma

Based on the published literature, the mast cell tumours (MCT) is the most common skin cancer of dogs, and can be cutaneous or subcutaneous. This tumour may affect dogs of any age, but usually middle or old-aged ones. These tumours arise when the mast cells proliferate in an uncontrolled manner and clump together in clusters generally in the form of skin bumps. The tumours appear small and not significant, many are benign but can be really serious and life-threatening (*London, C. A., & Seguin, B., 2003*). Mast cells arise as precursors of bone marrow and then migrate to the peripheral tissues, and under the influence of local cytokines may differentiate in mature mast cells. These ones contain cytoplasmic granules in which bioactive molecules such as histamine and heparin are present and responsible for hypersensitivity, allergy, and inflammatory reactions. Only upon the activation of mast cells by any physical, chemical stimuli, or through the activation of immune response mechanisms, the above-mentioned cellular components are released (*London, C. A., & Seguin, B., 2003, Blackwood, L. et al., 2012*).

1.7.1 Treatment:

The treatment approaches to canine MCTs depend on tumour stage, histological grade, and clinical feature. Likewise to many other cancers, surgery, chemotherapy, radiation therapy and adjuvant chemotherapy, or a combination of these ones, can be used. However, the number of masses and their location, the tumour grade, the presence or absence of metastasis, and, finally, the unpredictable biological behavior of MCT should also be considered when deciding the treatment of choice (*Blackwood, L. et al., 2012*). For dogs with localized, non-metastatic MCT (grade I and II) surgery is the recommended treatment; on the other hand, grade III tumours are invariably malignant and even after surgical removal, there is a high chance of relapse spread (metastasis) to lymph nodes and other organs including bone marrow. The use of radiotherapy is restricted to cases where surgical removal is not possible or to reduce the incidence of recurrence and it is most often used as a post-operative ancillary therapy after incomplete excision (*London, C. A., & Seguin, B., 2003, Blackwood, L. et al., 2012*). Chemotherapy is used to delay or prevent metastatic disease in addition to surgical removal of the tumour, or to delay the progression of existing metastatic disease.

Several anticancer drugs and protocols with a single agent or a combination of two or even more cytostatic drugs are available. Since MCT releases, histamine, the use of antihistamines may be of help to reduce symptoms. To counteract the acid-increasing

substances released by MCT acid blockers (anti-H2 drugs) are used. Corticosteroids such as prednisone decrease in inflammation, and mitigate the tumour undesirable effects. The combined use of prednisone and vinca alkaloids such as vinblastine is one of the most commonly used therapeutic approach, but the alkylating agent lomustine is also used, either alone, in combination or in alternating use. When choosing the proper anticancer drug, care should be given to avoid potential side effects. Vinblastine, prednisone, and lomustine may cause myelosuppression and gastrointestinal toxicity. Hence, a hematological examination must be done prior each use. Furthermore, lomustine may also provoke hepatotoxicity; hence, a routine monitoring of liver enzymes is required (*Blackwood, L. et al., 2012*).

1.7.2 Receptor tyrosine kinase (RTK), tyrosine kinase inhibitor (TKIs) and canine MCT

Receptor tyrosine kinases are cell surface receptors, having a higher affinity to growth factors, cytokines, and hormones. They are responsible for the activation of various downstream signaling pathways fundamental in normal cellular processes. Dysregulation of RTK gene, such as, overexpression, mutation, chromosomal translocation, or autocrine signaling may led to the development and progression of different types of cancers. Recent studies showed that *KIT* mutations may be associated with the development of canine MCT (about 25-30% of moderate to high-grade tumours) (*Blackwood, L. al., 2012, Garrett, L. D., 2014, Webster, J. D. et al., 2006*). In these cases, the binding either of a growth factor (the stem cell factor) or of a ligand to the receptor is no longer needed for activating protein kinases, thereby resulting in tumour growth and angiogenesis (*Webster, J. D.. et al., 2006*).

One widely accepted and successful treatment approach for MCT is to target the RTK by using “TKI”, which induce a competitive mechanism of inhibition toward ATP, necessary for the activation of RTKs; as a consequence, they inhibit the RTK autophosphorylation and the downstream signal transduction. In canines, two TKIs have been approved by the European Medical Agencies (EMA): toceranib phosphate and masitinib. Toceranib is approved for use in recurrent non-resectable grade II/III MCT; however, masitinib is approved for use in non-resectable grade II/III MCT with an established *KIT* mutation, to be confirmed before the onset of the treatment (*Yancey, M. F. et al., 2010, Ogilvie, G. K. et al., 2011*). Likewise, to other cytostatic drugs, the use of TKIs cannot be considered as an alternative to surgical resection; moreover, their use may provoke side effects, too.

1.8 Canine Lymphoma

Lymphomas are one of the most frequent tumours of canines. Although canine lymphoma is often viewed as a single disease, it actually encompasses many clinically and morphologically distinct subtypes (*Zandvliet, M., 2016).* According to the cell of origin, they are classified into B-cell and T- cell lymphomas. B-cell lymphoma is the most common canine lymphoma, and it is similar to human diffuse large b-cell lymphoma (DLBCL). In short, the proliferation of malignant lymphocytes led to the development of intermediate to high-grade lymphoma. It can affect any dogs of middle-sized to larger breeds. Although the exact cause still remains obscure, some environmental factors and a genetic susceptibility are believed to play an important role in lymphoma carcinogenicity (*Teske, E., 1994*). Interestingly, canine lymphoma shows molecular, signaling, pathologic features, and incidence similarities with human non-Hodgkin lymphoma (NHL); hence it is considered as a useful comparative, large animal model of carcinogenesis, especially in disease progression and possible treatments (*Marconato, L. et al., 2013, Seelig, D. M. et al., 2016*). Lymphoma can affect different organs; however, in more than half of cases, lymphoma affects the peripheral lymph nodes. On the other hand, very rarely it develop in the chest cavity, skin, intestines, or other organs. The RTK *KIT,* which plays an important role in cellular proliferation, survival, and differentiation, shows a typically low pattern of expression in canine lymphoma; by contrast, a higher expression has been observed in shown some high-grade T-cell lymphomas (*Teske, E., 1994*).

1.9 Canine MCT and lymphoma cell lines (C2 and CLBL-1, respectively)

Nowadays, comparative oncological studies are considered relevant not only for veterinary and medical oncologists, but also in preclinical sciences. Indeed, following the unveiling of morphologic, histologic, and biological similarities between human cancers and spontaneously occurring pets malignancies, companion animals are increasingly proposed as suitable animal models in drug development and preclinical studies, (*Zorzan, E. et al., 2018*).

The canine cell line C2 was established from a naturally occurring MCT in a mixed-breed dog, that was transplanted and propagated in BALB/c nude mice (*Lazarus, S. C. et al., 1986*). These cells grow in suspension, and are widely used to study in vitro the canine MCT

biology. It possesses all the general features of an aggressive MCT, including KIT-mutations and has a doubling time of 21 hrs. (*Sekis, I.. et al., 2009*).

With regards to canine lymphoma, the CLBL-1 cell line was obtained from a fine needle aspirate of a dog with confirmed stage IV DLBCL cells grew without the addition of growth factors and the cell line was established after two months of continuous culturing (*Rütgen, B. C. et al., 2010*). It represents the first canine cell line developed from a fine needle aspirate of a malignant peripheral lymph node. These cells grow in suspension in small clusters of 10-20 cells. Under standardized cell culture conditions, CLBL-1 shows a doubling time of about 31hrs. This cell line is an interesting candidate for specific research on canine DLBCL but also for NHL (*Rütgen, B. C. et al., 2010*).

2. SCOPE OF THE THESIS

Humans and canines share not the only the same environment but also some pathologies, and particularly a deadly non-contagious one, i.e. cancer. A number of studies have also characterized the genetic risk of carcinogenesis among canines, and how this latter sometimes is very similar to that of some human cancer. Osteosarcoma, bladder cancer, and human non-Hodgkin's lymphoma are some of tumours sharing similarities between the two species.

Concerning the various treatment strategies, the adverse side effects caused by anticancer drugs, the drug resistance developed by tumours over time-along with the usage of expensive medicines, are the primary concerns, that initiated the need for identifying an alternative sources, more specific and less toxic than anticancer agents. Several research efforts are underway to discover anticancer drugs obtained from plant natural substances or other alternative sources; in some instances, - a number of biologically active substances with known chemical interaction have successfully identified. In such a context, the research on the marine environment, which represents about a lion's share of the earth's terrain, has proven to be a rich source of diverse compounds of interest. Specifically, there have been a plethora of attempts to associate the antiproliferative, antioxidant, anti-inflammatory, as well as the immunomodulatory effects of seaweed extracts to various cancer cell lines of both humans and animals. The present study falls under this umbrella, as it aims at assessing the ability of candidate algal extracts to inhibit the proliferation of two canine cancer cell lines established from two very common canine tumours, i.e. mast cell tumour and lymphoma. These cell lines, but especially the lymphoma one, can be (are) used as a suitable comparative in vitro model for human tumours cush as mastocytosis and non-hodgkin's lymphoma.

Specifically, in the present work, we aimed to make a very preliminary evaluation of the effect of four algal extracts, i.e. *G. cervicornis*, *P. cartilagenium*, *L. ochroleuca*, and, *H. incurvus* on C2 and CLBL-1 cell lines. The study was carried out in two steps:

a) Preliminary identification, for each algal extract, of the most useful set of concentrations to be used in cytotoxicity testing.
b) Analysis of cytotoxicity of each crude algal extract, using Alamar Blue, Sulforhodamine B and, Neutral Red Uptake assays, following the treatment of cells for different times with a range of concentration; therefore determination of the corresponding IC_{50} values.

3. MATERIAL AND METHODS

3.1 Cell Culture: Growth and Maintenance

Canine Mastocytoma (C2) and Canine Large B-cell Lymphoma (CLBL-1), two suspension cell lines were used throughout the experiments. Both of them were grown in T-25 and T-75 cm^2 flasks (SARSTEDT AG&Co., KG Numbrecht, Germany) and incubated at a temperature of 37 ºC and a 5 % CO_2 atmosphere. Splitting/ Sub-culturing of C2 cell lines were done every 2 days, to obtain a concentration of 250,000 cells/mL in the new flask and for CLBL-1 sub-culturing is done every 3 days, for 500,000 cells/mL.

3.1.1. Preparation of Culture Medium for C2 and CLBL-1 cell lines

Reagents used:

Roswell Park Memorial Institute 1640(RPMI 1640)(Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA); Decomplemented Foetal Bovine Serum (FBS)(Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA); 2 mM Alanine-Glutamine (Sigma, Merk kGaA, Darmstadt, Germania); Penicillin / Streptomycin (P/S) (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA); 1mM Sodium pyruvate (Sigma-Aldrich, Merk kGaA, Darmstadt, Germany); NEAA(Non-Essential Amino Acids).

Protocol Followed:

For C2 and CLBL-1 cell lines RPMI 1640 medium is used. The medium and the other reagents mentioned above are previously warmed in the water bath, maintained at a temperature of 37 ºC. C2 cells were maintained in RPMI 1640 supplemented with FBS (previously decomplemented by heating in the water bath for 30 minutes at 56 ºC), Alanine-Glutamine, P/S, and 1 mM Sodium Pyruvate(**Table 4**). Whereas, for CLBL-1 cells, RPMI 1640 was supplemented with FBS (previously decomplemented by heating in the water bath for 30 minutes at 56 ºC), Alanine-Glutamine, P/S, and NEAA. (**Table 5**).

Reagent	Final Concentration
FBS	10%
Alanine-Glutamine	2mM(1%)
P/S	1%
Sodium Pyruvate	1%

Table 4 – RPMI 1640 - Medium Composition used for C2 (Complete Culture Medium)

Reagent	Final Concentration
FBS	10%
Alanine-Glutamine	2mM(1%)
P/S	1%
NEAA	1%

Table 5- RPMI 1640-Medium Composition used for CLBL-1

3.1.2 Cell Thawing for C2 and CLBL-1

Protocol Followed:

- The cryovial with the frozen cells is taken from the liquid nitrogen container and thawed by gently swirling it in the water bath preheated at 37 ºC (< 2 minutes).
- Transfer the contents of the cryovial to a falcon containing 9 mL of the preheated (37 ºC) complete culture medium.
- Centrifuge at 1200 rpm for 5 minutes and remove the supernatant using a vacuum pump.
- Resuspend the pellet in 5 mL of the complete medium, and transfer the cell suspension to a T-75 polystyrene flask with a 15 mL medium.
- The cells were incubated at 37 ºC & a 5% CO_2 atmosphere.

3.1.3. Cell Freezing (C2 and CLBL-1)

3.1.3.1. Preparation of the Freezing medium:

Reagents Used :

Complete culture medium, Foetal Bovine Serum (FBS)(Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA); Di-Methyl Sulfoxide (DMSO)(Sigma-Aldrich, Merk kGaA, Darmstadt, Germany).

Protocol Followed :

Add and properly mix the reagents mentioned, according to the proportion listed below (**Table 6**) to obtain the desired final volume.

Reagent	Final Concentration
Culture medium	70%
FBS	20%
DMSO	10%

Table 6 – Freezing Medium Composition

3.1.3.2. Cell Freezing

Protocol Followed:

- Warm the previously prepared freezing medium, in a water bath at 37 ºC.
- Observe the cells under the microscope, to evaluate their growth stage and possible bacterial, yeast, or fungal contamination.
- Transfer the cells to a falcon and centrifuged at 1200 rpm for 5 minutes.
- Remove the supernatant and resuspend it in 6 mL of the culture medium. Cell counting, to determine the desired volume to be added to the freezing medium.
- Centrifuge again, at 1200 rpm for 5 minutes and remove the supernatant, and resuspend the pellet in the freezing medium.
- Transfer the contents to a cryogenic vial and the cell line, passage number, date, and operator's name were mentioned on the vial.

- For a slow freezing process, store the vial in a cryogenic storage box containing isopropanol, and keep it at -80 °C, for 24 hours(hrs); finally, transfer the vial into a liquid nitrogen tank for a long time of storage.

3.1.4. Cell Counting

Cell counting is an integral part of cell culture maintenance, where we can establish or evaluate cell growth rates, as well as make new cell cultures with known cell numbers. It helps not only to get the total viable cell number but also an indication of the health of the cell culture. Trypan blue test is the most common method for cell counting, which relies on the principle of the integrity of cell membrane as an indicator of cell viability. Trypan blue dye is taken up and retained by only the dead cells, which do not have an intact cell membrane. Burker chamber is used for the calculation of cell numbers following the formula :

$$\text{Number of cells /mL} = N * 5 * 10{,}000 \quad [\textbf{formula 1}]$$

Where N : the mean number of cells counted in five squares

5 : the dilution factor

10,000 : the conversion factor of the Burker chamber.

The volume of cell suspension needed to get the desired number of cells/mL, for both splitting and plating, can be calculated by the formula :

$$C_1 * V_1 = C_2 * V_2 \quad [\textbf{formula 2}]$$

Where C_1 : the initial concentration of cells/mL

V_1 : initial volume (the volume of cell suspension with the desired cell numbers)

C_2 : the final concentration of cells/mL

V_2 : final volume of cell suspension

Reagents Used:

Trypan Blue solution 0.4% (Life Technologies Corporation, NY,USA); Phosphate Buffer Saline (PBS)1X + EDTA.

Protocol Followed:

For counting in Burker chamber:

Prepare a mixture using Trypan blue, PBS 1X + EDTA, and cell suspension according to the volumes reported in **Table 7**. Transfer 10 µl from the above-mentioned solution to the Burker chamber. Count the cells from 5 squares by observing them under the microscope. The total number of cells/mL is calculated using formulas 1 and 2.

Reagents	Volume used
Trypan Blue dye	500 µL
PBS 1x+EDTA	300 µL
Cell suspension	200µL

Table 7 – Composition of Trypan Blue dye exclusion test mixture

3.1.5 Cell Splitting

Reagents used:

RPMI 1640 (Complete culture medium), Trypan Blue solution 0.4 % (Life Technologies Corporation, NY, USA) ; Phosphate Buffer Saline (PBS)1X + EDTA.

Protocol Followed:

- The culture medium should be previously warmed in the water bath at 37º C.
- The cells were observed under the microscope and the flask with sufficient cell growth will be selected for sub-culturing.
- Transfer the cell suspension from T-25 or T-75 cm^2 flasks, prepared two days (48 hours) before for C2 and three days before for CLBL-1, into a falcon.

- Centrifuge the falcon at 1200 rpm for 5 minutes and remove the supernatant using a vacuum pump and resuspend the pellet in 6 mL or 7 mL of complete medium, based on the size of the pellet formed.
- Cell counting using counting chambers, such as Burker Chamber. Calculate the required volume of the cell suspension and the remaining volume of the culture medium to be added according to formulas 1 and 2 to a T-25 or T-75 cm^2 flask.

3.2. Cytotoxicity Assays

3.2.1. Alamar blue Assay

Alamar blue(AB) is one of the widely used cytotoxicity assays, as an indicator of cellular viability, where the blue-colored, non-fluorescent compound Resazurin, is reduced by the living cell's mitochondrial, cytosolic, or microsomal enzymes into a pink-colored, highly fluorescent Resorufin. The quantity of resorufin, which in turn is proportional to the fluorescence produced, can be correlated with higher cellular viability. (*O'brien, J. et al ., 2000*)

3.2.1.1. Preparation of Alamar Blue reagent

Preparation of Alamar blue reagent should be done in the dark, as it is light-sensitive.

Reagents used:

Resazurin (Sigma-Aldrich, Merk kGaA, Darmstadt, Germany); Phosphate Buffer Saline (PBS)(pH 7.3)

Protocol Followed:

a. Transfer 0.15 mg of high purity resazurin in PBS (pH 7.3), following the proportion : 0.15 mg/mL.
b. Filter -sterile the resazurin solution, through a 0.2 µm pore-sized filter, into a sterile, light protected container.
c. Store the resazurin solution away from light at 4 ºC for frequent use or at -20 ºC for long-term storage.

3.2.1.2. Alamar blue assay for cells

Reagents used :

Alamar blue (prepared following the protocol reported in section 3.2.1.1)

Protocol Followed:

a. For the assay to be performed, transfer 100 µL of cell suspension into the wells. (30,000 cells /well) (4.2 and 4.3)
b. Add the test compound (algal extract) and vehicle control (DMSO 1%), and the treatment to appropriate wells to make a final volume of 150 µL in each well.
c. Incubate the cells for 48 hrs, 37 ºC, and 5% CO_2.
d. After 48 hrs of incubation, remove the plates from the incubator and add 30 µL of the Alamar Blue reagent solution.
e. Shake the plate for 10 seconds and incubate for another 3 hrs, under the standard cell conditions.
f. After 3 hrs of incubation, the fluorescence, be measured at 544 nm (excitation wavelength) and 590 nm (emission wavelength), using VICTOR^{TM} X4 Multilabel Plate Reader.

3.2.2. Sulforhodamine B–Assay

A fast and sensitive colorimetric assay for the determination of drug-mediated cytotoxicity in both suspension and attached cells. Sulforhodamine-B (SRB), is a bright pink aminoxanthine dye that binds to protein's basic amino acid residues, of trichloro acetic acid (TCA) – fixed cells, which is a mild acidic condition. The dye is then released under basic conditions (pH 10.5), and its colorimetric determination provides an approximate cell number based on the cellular protein content which can directly be related to viable cell numbers determined from a standard curve (*Skehan, P. et al., 1990*).

Reagents used :

Acetic acid (1%) (M = 17.5) (Sigma–Aldrich, Co., 3050 Spruoe Street ,St. Louis, MO 63103 USA); Sulforhodamine-B(0.4%)(Sigma-Aldrich,Merk kGaA, Darmstadt, Germany); Trichloro acetic acid (TCA) (163.39 g/mol) (AppliChem, GmbH, Ottoweg 10b, Darmstadt); Tris buffer (pH 10.5), Ultrapure water.

3.2.2.1. Preparation of Reagents

- Dissolve high purity acetic acid in Ultrapure water, at the rate of 1 %.
- Dissolve the required amount of SRB (0.4%) in 1 % acetic acid. As it is light sensitive, preparation of SRB and the SRB addition should be carried out away from light.
- Prepare the required amount of TCA (50%) and Tris buffer(50%, 10mM, pH 10.5) in Ultra pure water.

3.2.2.2. SRB assay for the cells

Protocol Followed:

a. For the assay to be performed, transfer 100 µL of cell suspension into the wells. (30,000 cells /well) (4.2 and 4.3).
b. Add 50 µL test compound (algal extract dissolved in DMSO), and 50 µL of vehicle control (RPMI 1640+DMSO) in appropriate wells to make a final volume of 150 µL in each well.
c. Incubate the cells for 48 hrs, 37^{o} C, and 5% CO2.
d. After 48 hrs of incubation, remove the plates from the incubator and centrifuge at 2000 rpm for 5 minutes, to make the cells sediment at the bottom of the wells.
e. Add 37.5 µL of 50 % cold TCA on top of the medium in each well (fixing the cells at the bottom under a slightly acidic condition)
f. Incubating at 4^{o} C for 1 hr.
g. After 1 hr of incubation, remove the supernatant
h. Washing with 100 µL ultrapure water, 3 times.
i. Dry the plate in the heater at 37°C for 5-10 minutes.
j. Add 100 µL of SRB (0.4%), to each well to be assessed.
k. Incubate at room temperature for 30 minutes.
l. After incubation, remove the dye carefully and wash it with 100 µL of 1 % acetic acid, 4 times.
m. Dry the plate in the heater at 37 ^{o}C, for 5-10 minutes without the cover.
n. Add 200 µL of 10 mM of Tris buffer pH 10.5(releasing the dye under mild basic conditions).
o. Incubate at room temperature for 5 minutes on a shaker.

p. The absorbance is to be measured at 570nm and 630 nm (which is the reference wavelength) in the MULTISKAN^{TM} GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.2.3. Neutral Red Uptake Assay

Neutral red assay helps to determine the cellular proliferation., an in vitro quantification of xenobiotic–induced cytotoxicity. The assay is based on the living cell's ability to uptake the weak, cationic dye neutral red, only in the lysosomes of living cells. And after the extraction of the dye from the cells, using ethanol-acetic acid, the absorbance can be measured, and the intensity of red color released from the cells can be directly linked to the concentration of viable cells with intact membranes (*Babich, H., & Borenfreund, E., 1990*).

Reagents used:

Neutral red dye(Sigma-Aldrich, Merk kGaA, Darmstadt, Germany); Neutral red destain solution; RPMI 1640(without FBS) (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA); Ethanol 96%; Glacial acetic acid 1% (M = 17.5) (Sigma – Aldrich, Co., 3050 Spruoe Street, St. Louis, MO 63103 USA); PBS (pH. 7.3)

3.2.3.1. Preparation of reagents:

a. Neutral red dye (0.05 mg/mL)

Add the required amount of neutral red powder in RPMI(without FBS) to make a final concentration of 0.05 mg/mL. Incubate overnight at 37 ºC and centrifuged at 4000 rpm for 5 minutes before using, to avoid the precipitated crystals. It should be prepared one day before use and stored away from light.

b. Neutral red destain solution

50% Ethanol (96%), 49% deionized water, and 1% glacial acetic acid are used for the preparation.

3.2.3.2. Neutral red assay for cells

Protocol Followed:

a. For the assay to be performed, transfer 100 µL of cell suspension into the wells. (90,000 cells /well) (4.2 & 4.3).

b. Add 50 µL test compound (algal extract dissolved in DMSO), and 50 µL of vehicle control (RPMI 1640+DMSO) to appropriate wells to make a final volume of 150 µL in each well.
c. Incubate the cells for 48 hrs, 37 ºC, and 5% CO_2 atmosphere.
d. After 48 hrs of incubation, remove the plates from the incubator, and transfer the content of each well into a V-shaped 96-well plate.
e. Centrifuge at 2000 rpm for 5 minutes.
f. After centrifugation, carefully remove the supernatant.

Take the neutral red dye solution from the incubator and centrifuge at 4000 rpm for 5 minutes to avoid any precipitated crystals. The addition of neutral red and further steps should be done away from a light source.

g. Add 150 µL of neutral red dye solution to each well and resuspend.
h. Incubate for 3 hrs at 37 ºC, 5% CO_2 atmosphere.
i. After the required incubation, centrifuge the plate again at 2000 rpm for 5 minutes.
j. Remove the supernatant and add 150 µL of PBS and resuspend
k. Centrifuge at 2000 rpm for 5 minutes.
l. Remove the supernatant and add 150 µL of neutral red-destain solution and resuspend.
m. Wait for 15 minutes and transfer the content of each well to 96 flat bottom well plates
q. Read the absorbance in a spectrophotometer at 540 nm and 630 nm (which is the reference wavelength) in the MULTISKAN^{TM} GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.3. Validation Phase for C2

The validation or documentation phase is an integral part of the experiment. It has to be done as a preliminary evaluation. The three main objectives of this phase include:

a. Calculating the number of cells required for plating
b. Determining the possible time of incubation of each 96-well plate (24 hrs / 48 hrs/ 72 hrs) after the treatment addition.
c. Evaluating the best incubation time for Alamar blue, NR and SRB assays.

Reagents used:

Alamar blue reagent (3.2.1.1); SRB Reagents(3.2.2); NRU Reagents(3.2.3)

Protocol Followed :

a. Set up three 96–well plates, with a range of cellular concentrations, need to be assessed. Six technical replicates of each concentration are maintained. The initial volume (i.e., the higher concentration of cells = 80,000/150 µL) of cell suspension with the desired number of cells is calculated using Formula 2 and then serial dilutions were prepared. The scheme for the addition of cell suspension in each well is represented in **Table 8.**

C2	1	2	3	4	5	6	7	8	9
(AB)	Ab.B.	625	1,250	2,500	5,000	10,000	20,000	40,000	80,000

Table 8. Schematic representation of the addition of C2 cell suspension in each well for AB assay

(Ab.B. : Absolute Blank (only the medium))

b. The final volume in each well is 150 µL (cell suspension with the desired cell numbers) and incubate at 37 °C, in a 5% CO_2 atmosphere, where the first plate is incubated for 24hrs, and the second and third plate is for 48 and 72hrs respectively.

c. Remove the plates after the specific incubation period and add 30 µL of Alamar blue reagent to each well to be assessed.

d. Shake the plates for 10 seconds at room temperature, away from light.

e. Incubate at standard cell culture conditions for 2, 3, and 4 hours for each plate respectively.

f. For validating the cell numbers for SRB and NRU assays, the protocol for the addition of cell suspension is the same as that mentioned above for Alamar blue (from a-e). The scheme for the addition of cell suspension in each well is as in **Table 9.**

C2 (SRB and NRU)	1	2	3	4	5	6	7	8	9
	Ab.B.	625	1,250	2,500	5,000	10,000	20,000	40,000	80,000

Table 9. Schematic representation of the addition of C2 cell suspension in the wells for SRB and NRU assays.

(Ab.B. : Absolute Blank (only the medium))

g. After the required period of incubation of plates for 24, 48, and 72hrs, the assays are performed as per the methods mentioned in 3.2.2 and 3.2.3 for SRB and NRU.

h. For AB, after the required incubation, shake the plate again for 10 seconds and the fluorescence is measured at 544 nm (excitation wavelength) and 590 (emission wavelength), in the spectrophotometer (VICTOR^{TM}X4 Multilabel Plate Reader) (Perkin Elmer, Waltham, MA, USA).

3.4. Validation Phase for CLBL-1

The validation or documentation phase is an integral part of the experiment. It has to be done as a preliminary evaluation. The three main objectives of this phase include:

a. Calculating the number of cells required for plating
b. Determining the possible time of incubation of each 96-well plate (24 hrs / 48 hrs/ 72 hrs) after the treatment addition.
c. Evaluating the best incubation time for Alamar blue, SRB and NRU assays respectively.

Reagents used:

Alamar blue reagent ((3.2.1.1); SRB Reagents(3.2.2); NRU Reagents(3.2.3)

Protocol Followed:

a. Set up three 96–well plates, with a range of cellular concentrations, need to be assessed. Six technical replicates of each concentration are maintained. The initial volume (i.e., the higher concentration of cells = 120,000 cells/ 150 µL(AB and SRB), but for NRU, the higher concentration of cells = 240,000 cells/150µL) of cell suspension with the desired number of cells is calculated using Formula 2 and then serial dilutions were prepared. The scheme for the addition of cell suspension in each well is as follows in **Table 10.**

CLBL-1(AB)	1	2	3	4	5	6	7	8
	Ab.B.	1875	3750	7,500	15,000	30,000	60,000	120,000

Table 10. Schematic representation of the addition of CLBL-1 cell suspension in the wells for AB assay

(Ab.B. : Absolute Blank (only the medium))

b. The final volume in each well is 150 µL (cell suspension with the desired cell numbers) and incubate at 37 °C, in a 5% CO_2 atmosphere, where the first plate is incubated for 24 hours, and the second and third plate is for 48 and 72 hours respectively.

c. Remove the plates after the specific incubation period, and add 30 µL of Alamar blue reagent.

d. Shake the plates for 10 seconds at room temperature, away from light.

e. Incubate at standard cell culture conditions for 2, 3, and 4hrs for each plate respectively.

f. For validating the cell numbers for SRB and NRU assays, the protocol followed is the same as that mentioned above for Alamar blue (from a-e). The scheme for the addition of cell suspension in each well is showed. **(Table 11 and 12).**

CLBL-1(SRB)	1	2	3	4	5	6	7	8
	Ab. B.	1875	3750	7,500	15,000	30,000	60,000	120,000

Table 11. Schematic representation of the addition of CLBL-1 cell lines for SRB assay.

CLBL-1(NRU)	1	2	3	4	5	6	7	8	9
	Ab.B.	1875	3750	7,500	15,000	30,000	60,000	120,000	240,000

Table 12. Schematic representation of the addition of CLBL-1 cell lines for NRU Assay.

(Ab.B. : Absolute Blank (only the medium))

g. After the required period of incubation of plates for 24, 48, and 72hrs, the assays are performed as per the methods mentioned in 3.2.2 and 3.2.3 for SRB and NRU.

h. For AB, after the required incubation, shake the plate again for 10 seconds and the fluorescence is measured at 544 nm (excitation wavelength) and 590 (emission wavelength), in the spectrophotometer (VICTOR^{TM}X4 Multilabel Plate Reader) (Perkin Elmer, Waltham, MA, USA).

4. CYTOTOXICITY ASSAY STUDIES

The algal extracts of five different algae (Moroccan origin), *Gracilaria cervicornis* (GC), *Plocamium cartilagineum* (PC), *Laminaria ochroleuca* (LO), *Halopithys incurvus* (HI), and *Ellisonadia elongata* (EE) have been extracted and the antiproliferative activity was evaluated in both C2 and CLBL–1 cell lines. These cell lines were incubated with the seaweed extracts, and the half maximal inhibitory concentration (IC_{50}) was determined using three cytotoxic endpoints; Alamar Blue (AB), for understanding the cellular metabolic activity; Sulforhodamine-B (SRB), for analyzing cellular permeability; and Neutral Red Uptake Assay (NRU), evaluates lysosomal uptake.

Cells were seeded in 96 flat bottom well plates and treated with different concentrations of algal extracts. For each alga, the concentrations used were also different. During the initial stages of the experiment, as part of the preliminary evaluation, different concentrations of extracts have been used and they were incubated for a period of 24 hrs,48 hrs, and 72 hrs at 37 ^{o}C and 5% CO_2. In the later stages, it has been decided to continue with 48 hrs, as the incubation temperature for all extracts.

The whole experiment can be divided into two parts namely the “Screening assays” and the “Main assays”. In the first part, the extracts from all five different algae were used, in different concentrations for each alga, and their antiproliferative activity was analyzed by using only the Alamar Blue cytotoxicity assay. After the analysis, the most effective ones were selected for further investigation and the cellular toxicity and viability were determined using all three assays mentioned above.

4.1 Preparation of Algal Extract

Algal extracts were prepared from dried algal powder (from Morocco). The extraction protocol can be divided into three main steps., Maceration, Filtration, and Evaporation.

Reagents used:

CH_2Cl_2 : Dichloromethane; MeOH : Methanol

Protocol Followed:

From a known mass of Algae Powder:

Step 1: Maceration

- In an Erlenmeyer flask of 500 mL, put 50 g of powder (X4 Erlenmeyer, 200 g in total),
- Add CH_2Cl_2 /MeOH (1:1, V/V) to the algae powder in a 50:50 ratio,
- Macerate for 72 hrs on a shaker in a closed Erlenmeyer flask (1 st Maceration),

Step 2: Filtration

A: Filtration of the first maceration

- Filter the mixture on Whatman filter paper (φ 185 mm),
- After having filtered the whole mixture, recover the extract (filtrate) and keep it at room temperature protected from light, then proceed to a second maceration of the same powder under the same conditions (Solvent, Time ...),

B: Filtration of the second maceration

- Filter the mixture on Whatman paper (φ 185 mm),
- Recover the extract (filtrate) obtained from the first and second maceration then proceed to evaporation.

Step 3: Evaporation

- Evaporate the mixture recovered in tubes on an evaporation plate (Turbo Vap LV),
- Weigh the empty tubes then gather the extracts obtained in a single tube,
- Calculate the total mass of extract obtained.

4.2. Seeding of cells in 96-well plates

The two cell lines, C2 and CLBL–1 were seeded on 96-well plates with a concentration of 30,000 cells /well for AB and SRB assays and 90,000 cells /well for NRU assay respectively. The total cellular concentrations for each assay were obtained from the validation assay (3.3 and 3.4).

Reagents used:

Complete culture medium; Trypan Blue solution 0.4% (Life Technologies Corporation, NY,USA); Phosphate Buffer Saline (PBS) 1X EDTA.

Protocol Followed:

- Transfer the cell contents from previously prepared 25 or 75 cm^2 flasks, into a sterile falcon, and centrifuge at 1200 rpm for 5 minutes.
- Discard the supernatant and care should be taken not to disturb the cell pellet.
- Resuspend the pellet in 6 mL or 7 mL of complete medium and homogenize the suspension.
- Calculate the required number of cells using the Burker chamber and transfer the required amount of cell suspension into a new medium. Then the cells are ready for plating.
- Add 100 µL of cell suspension into the 96-well plates. Shake them gently and they are ready for the treatment addition.

4.3. Cell incubation with the treatment (algal extracts)

Reagents used:

Di-Methyl Sulfoxide (DMSO) 100%(Sigma-Aldrich, Merk kGaA, Darmstadt, Germany), RPMI 1640 (complete culture medium).

Protocol Followed:

Weigh the required amount of raw algal extract in a sterile, clean Eppendorf and dissolve it using DMSO (100%) as a solvent. Prepare the stock solution using a complete culture medium, and make serial dilutions. 50 µL of the algal extract dilutions were added into each well, according to various concentrations to be assessed. The plates were then, incubated for a period of 48 hrs, at 37 ºC, in a humidified atmosphere with a 5% CO_2.

4.4. Screening Examinations - Alamar Blue Cytotoxicity Assay – Using C2 Cell Lines

For the screening examinations of each alga, two independent cell culture experiments each one run in sextuplicate has been used within a selected concentration range. The concentration ranges were decided based on the preliminary evaluations, in which the concentration of extracts with very low, medium, and, very high antiproliferative activity were selected. This particular range has been used for further assays.

Screening assays were confirmed using the Alamar Blue cell viability assay. The readings were measured in terms of fluorescence, in VIKTOR™ X4 Multilabel Plate Reader.

4.4.1. Screening assay - *Gracilaria cervicornis* (G.C.)

Cells of passage 14 (from two different cryovials) have been used for the assays and a final concentration of algal extract from 4.16 µg/mL to 166.67 µg/mL has been evaluated. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay must be maintained throughout.

Reagents Used:

Di-Methyl Sulfoxide (DMSO)100%; Complete culture medium; Trypan Blue solution 0.4 % (Life Technologies Corporation, NY,USA) ; Phosphate Buffer Saline (PBS) 1X + EDTA.

Protocol Followed:

4.4.1.1. Dilution of Algal extract:

a) Stock solution 100X: Prepare a stock solution by dissolving the algal extract in 100% DMSO, so that they get a final concentration of 200 mg/mL.
   The proportion followed for making the stock solution: 100 mg of algal extracts in 500 µL of DMSO (100 %).

b) Stock solution 1X: Make a dilution of 1:100, to dilute DMSO from 100 % to 1%, by transferring 100 µL of the 100X solution to 9.9 mL of RPMI 1640, thereby making an initial concentration of 2 mg/mL.

c) Prepare serial dilutions to reach a final concentration of 4.16µg, 8.33µg, 16.67 µg, 25µg, 41.66µg, 83.33µg, 166.67µg in the wells.

d) Addition of 100 µl of the cell suspension, using a multi-channel pipette, to each well, except the first column where, only the culture medium is added, which is considered as "absolute blank". Cell dilution and cell counting were done as per the procedure mentioned above (3.1.4).

4.4.1.2. Plating of cells and treatment addition in 96-well plates.

50 µL of the treatment from each concentration was used for plating. A total volume of 150 µL is added to each well of the 96-well plates. The final concentration of extracts will be three times lower than the actual concentration that we prepare. This is because, 50 µL, is added to 100µL cell suspension to make a final volume of 150 µL. (In other words, 50 µL in a total volume of 150 µL in the well). Hence a ratio of 1:3 is followed in the preparation of the treatment.

For example., 500µg/mL/ 3 = 166.6 µg/mL in the well. (Conc. of algal extract)

The first column represents the absolute blank which contains 150µL of the medium. The second column, where 100µL of cell suspension and 50µL of complete culture medium without DMSO, is the control, for assessing whether the DMSO has any toxic effect on the cell viability. Finally, the third column, which contains 100µL cell suspension and 50µL of solvent (RPMI 1640+ DMSO 0.33%), is the control for the treatment to be examined.

4.4.2. Screening assay – *Plocamium cartilagineum* (P.C.)

Cells in passages 14 and 15 have been used for the assays and a concentration range from 4.16 µg/mL to 166.67 µg/mL has been used. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay was maintained throughout.

For *Plocamium*, the protocol followed, as reported in 4.4.1.

4.4.3. Screening assay – *Halopithys incurvus* (H.I.)

Cells, in passages 14 and 16 were used for plating. All the procedures for plating cells and the treatment addition were done under sterilized conditions. A concentration range from 20.83 µg/mL to 1.33 mg/mL was used.

Reagents Used:

Di-Methyl Sulfoxide (DMSO)100% ; Complete culture medium (RPMI 1640) ; Trypan Blue solution 0.4 % (Life Technologies Corporation, NY,USA) ; Phosphate Buffer Saline (PBS) 1X + EDTA.

Protocol Followed:

4.4.3.1. Dilution of Algal extract:

a. Stock solution 100X: Prepare a stock solution by dissolving the algal extracts in 100% DMSO, to obtain a final concentration of 400 mg/mL.
   The proportion followed for making the stock solution: 200 mg of algal extracts in 500 µL of DMSO (100 %).

b. Stock solution 1X: Make a dilution of 1:100, to dilute DMSO from 100 % to 1%, by transferring 100 µL of the 100X solution to 9.9 mL of RPMI 1640, thereby making an initial concentration of 4 mg/mL.

c. Prepare serial dilutions to reach a final concentration of 20.83µg, 83.33µg, 166.66µg, 333.33µg, 666.67µg, 1µg, and 1.33µg in the wells.

d. Addition of 100 µL of the cell suspension, using a multi-channel pipette, to each well, except the first column where, only the culture medium is added, which is considered as "absolute blank". Cell dilution and cell counting were done as per the procedure mentioned above (3.1.4).

4.4.3.2. Plating of cell suspension and treatment in 96-well plates.

50µL of the treatment from concentration 20.83µg to 1.33µg was used for plating as per the procedures mentioned in 4.4.1.2.

4.4.4. Screening assay – *Laminaria ochroleuca* (L.O.)

C2 cell lines from Passage numbers 25 and 28 were used for the analysis. A concentration range from 200 µg/mL to 700 µg/mL. Sterile conditions are to be maintained through the experiments.

Reagents Used:

Di-Methyl Sulfoxide (DMSO) 100%; Complete culture medium; Trypan Blue solution 0.4 % (Life Technologies Corporation, NY,USA) ; Phosphate Buffer Saline (PBS) 1X + EDTA.

Protocol Followed:

4.4.4.1. Dilution of Algal extract:

a. Stock solution 100X: Prepare a stock solution by dissolving the algal extracts in 100% DMSO, to achieve a resulting concentration of 210 mg/mL.
   The proportion followed for making the stock solution: 105 mg of algal extracts in 500 µL of DMSO (100 %).
b. Stock solution 1X: Make a dilution of 1:100, to dilute DMSO from 100 % to 1%, by transferring 100 µL of the 1000X solution to 9.9 mL of RPMI 1640, thereby making an initial concentration of 2.1 mg/mL.
c. Prepare serial dilutions to reach a final concentration of 200µg followed by 300µg, 400µg, 500µg, 600 µg, and 700µg in the wells.
d. Addition of 100 µL of the cell suspension, using a multi-channel pipette, to each well, except the first column where, only the culture medium is added, which is considered as "absolute blank". Cell dilution and cell counting were done as per the procedure mentioned above (3.1.4).

4.4.4.2. Plating of cell suspension and treatment in 96-well plates.

50 µL of the treatments from 200µg to 700 µg were used for plating as per the procedures mentioned in 4.4.1.2.

4.4.5. Screening assay – *Ellisonadia elongata*

As compared with the other algae, *Ellisonadia elongata*, has a structure (thallus) based on calcium carbonate and is considered a coral reef alga. Because of its structural properties, it makes them difficult to be solubilized in the solvents like DMSO and also in Ethanol, which caused its analysis, unsuccessful. So we have decided to carry out the analysis with the other four algae.

The schematic representation of the various treatment concentrations of all the four algae in the well is represented below in **Table 13.**

	1	2	3	4	5	6	7	8	9	10	11	12
G.C.	Ab.B.	Cells	Cells+ Solv.	4.16µg	8.33 µg	16.67µg	25 µg	41.66 µg	83.33 µg	166.67µg		
P.C.	Ab.B.	Cells	Cells+ Solv.	4.16µg	8.33 µg	16.67µg	25 µg	41.66 µg	83.33 µg	166.67µg		
H.I.	Ab.B.	Cells	Cells+ Solv.	20.83 µg	83.33 µg	166.66 µg	333.33 µg	666.67µg	1mg	1.3mg		
L.O.	Ab.B.	Cells	Cells+ Solv.	200µg	300 µg	400µg	500 µg	600 µg	700 µg			

Table 13. Schematic representation of the addition of cell suspension in each well for different concentrations of algal extracts

The plating is as follows: 100µL cell suspension and 50µL of the treatment prepared.

Column 1: Absolute Blank (Ab.B.) – Only the medium

Column 2 – Column 10: 100 µl of cell suspension.

Column 2 : Cells + culture medium (50µL)

Column 3 : Cells + Solvent (RPMI 1640+ DMSO 0.33%)

After the plating with treatment and cell suspension, the plates were incubated for a period of 48 hrs, 37 ºC, and 5% CO_2 atmosphere.

Alamar Blue Analysis

After 48 hours of incubation, the plates were prepared for Alamar blue analysis. As described in 3.2.1.2.

4.5. Screening Examinations - Alamar Blue Cytotoxicity Assay – Using Clbl-1 Cell Lines

G. cervicornis and *P. cartilagenium* were used for the screening examinations. One biological replicate for each extract and sextuplicates has been used within a selected concentration range. Screening assays were confirmed using the Alamar Blue cell viability assay. The readings were measured in terms of fluorescence, in VIKTOR™ X4 Multilabel Plate Reader.

4.5.1. Screening assay - *Gracilaria cervicornis* (G.C.) (1% DMSO)

Cells of passage number 24 has been used for the assays and a final concentration of algal extract from 2.08 µg/mL to 83.33 µg/mL has been evaluated. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay must be maintained throughout.

Reagents Used:

Di-Methyl Sulfoxide (DMSO)100%; Complete culture medium; Trypan Blue solution 0.4 % (Life Technologies Corporation, NY,USA) ; Phosphate Buffer Saline (PBS) 1X + EDTA.

Protocol Followed:

4.5.1.1. Dilution of Algal extract:

a) Stock solution 100X: Prepare a stock solution by dissolving the algal extract in 100% DMSO, so that they get a final concentration of 200 mg/mL.
   The proportion followed for making the stock solution: 100 mg of algal extracts in 500 µL of DMSO (100 %).
b) Stock solution 1X: Make a dilution of 1:100, to dilute DMSO from 100 % to 1%, by transferring 100 µL of the 100X solution to 9.9 mL of RPMI 1640, thereby making an initial concentration of 2 mg/mL.
c) Prepare serial dilutions to reach a final concentration of 2.08 µg, 4.16 µg, 8.33µg, 16.67 µg, 25µg, 41.66µg, 83.33µg in the wells.
d) Addition of 100 µl of the cell suspension, using a multi-channel pipette, to each well, except the first column where, only the culture medium is added, which is considered as “absolute blank”. Cell dilution and cell counting were done as per the procedure mentioned above (3.1.4)

4.5.1.2. Plating of cells and treatment addition in 96-well plates.

50µL of the treatment from concentration 2.08 µg to 83.33 µg was used for plating as per the procedures mentioned in 4.4.1.2.

4.5.2. Screening assay – *Plocamium cartilagineum* (P.C.)

Cells in passage number 24 has been used for the assays and a concentration range from 2.08 µg/mL to 83.33 µg/mL has been used. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay was maintained throughout.

For *P. cartilagenium*, the protocol followed, as reported in 4.5.1.

4.5.3. Screening assay - *Gracilaria cervicornis* (G.C.) (0.5% DMSO)

For CLBL-1 cell lines, it was observed that, a concentration of 1% of DMSO, induces toxicity for the cell lines. In order to avoid the bias in cytotoxicity, an additional assay to be performed, with a decreasing concentration of 0.5% DMSO was used.

Reagents Used :

Di-Methyl Sulfoxide (DMSO)100%; Complete culture medium; Trypan Blue solution 0.4 % (Life Technologies Corporation, NY,USA) ; Phosphate Buffer Saline (PBS) 1X + EDTA.

Protocol Followed :

4.5.3.1. Dilution of Algal extract :

a) Stock solution 100X: Prepare a stock solution by dissolving the algal extract in 100% DMSO, so that they get a final concentration of 200 mg/mL.
   The proportion followed for making the stock solution: 100 mg of algal extracts in 500 µL of DMSO (100 %).
b) Stock solution 1X: Make a dilution of 1:100, to dilute DMSO from 100 % to 1%, by transferring 100 µL of the 100X solution to 9.9 mL of RPMI 1640, thereby making an initial concentration of 2 mg/mL.
c) Prepare further dilutions, so as to decrease the DMSO concentration from 1% to 0.5%.

d) Prepare serial dilutions to reach a final concentration of 2.08 µg, 4.16 µg, 8.33µg, 16.67 µg, 25µg, 41.66µg, 83.33µg in the wells.

e) Addition of 100 µl of the cell suspension, using a multi-channel pipette, to each well, except the first column where, only the culture medium is added, which is considered as "absolute blank". Cell dilution and cell counting were done as per the procedure mentioned above (3.1.4)

4.5.3.2. Plating of cells and treatment addition in 96-well plates.

50µL of the treatment from concentration 2.08 µg to 83.33 µg was used for plating as per the procedures mentioned in 4.4.1.2.

The schematic representation of the various treatment concentrations of all the four algae in the well is represented in **Table 14.**

	1	2	3	4	5	6	7	8	9	10	11	12
G.C.	Ab.B.	Cells	Cells+ Solv.	2.08 µg	4.16 µg	8.33µg	16.67 µg	25 µg	41.66 µg	83.33 µg		
P.C.	Ab.B.	Cells	Cells+ Solv.	2.08 µg	4.16 µg	8.33µg	16.67 µg	25 µg	41.66 µg	83.33 µg		
G.C. (a)	Ab.B.	Cells	Cells+ Solv.	2.08 µg	4.16 µg	8.33µg	16.67 µg	25 µg	41.66 µg	83.33 µg		

Table 14. Schematic representation of the addition of CLBL-1 cell suspension in each well for different concentrations of algae extracts

The plating is as follows: 100µL cell suspension and 50µL of the treatment prepared.

Column 1: Absolute Blank (Ab.B.) – Only the medium

Column 2 – Column 10: 100 µl of cell suspension.

Column 2 : Cells + culture medium (50µL)

Column 3 : Cells + Solvent (RPMI 1640+ DMSO 0.33%)

*For G.C (a) : DMSO concentration reduced to 0.5%

After the plating with treatment and cell suspension, the plates were incubated for a period of 48 hrs, 37 ºC, and 5% CO_2 atmosphere.

Alamar Blue Analysis

After 48 hours of incubation, the plates were prepared for Alamar blue analysis. As described in 3.2.1.2.

4.6. Main Assays – Alamar Blue, Sulforhodamine -B & Neutral Red Analysis – Using C2 Cell Lines

Main assays were done as a confirmatory analysis, and also as a continuation of the screening examinations. The results obtained from the screening analysis for *Gracilaria cervicornis*, *Plocamium cartilagineum*, *Halopithys incurvus*, and *Laminaria ochroleuca*, from Alamar blue cytotoxicity assay, were evaluated and the most effective two algae, *Gracilaria cervicornis*, and *Plocamium cartilagineum* were selected for the main assays.

In this section of the research, along with Alamar Blue, the other tests including Sulforhodamine-B and Neutral Red were also used to evaluate the effect of G.C. and P.C. on C2. For all the assays three independent biological experiments each one run in sextuplicate were considered.

4.6.1. Main assays - *Gracillaria cervicornis*

Cells have been used from passage 16 to 22 in these assays evaluating GC cytotoxicity with a concentration range from 4.16 µg/mL to 166.67 µg/mL. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay was to be maintained throughout.

Reagents Used:

Di-Methyl Sulfoxide (DMSO) 100 %; Complete culture medium (RPMI 1640) ; Trypan Blue solution 0.4 % (Sigma – Aldrich Co., St. Louis, Missouri, USA) ; Phosphate Buffer Saline (PBS) 1x + EDTA.

Protocol Followed:

Is the same as the screening assay for G.C. (4.4.1)

4.6.2. Main Assay – Plocamium cartilagineum

Cells from passages 28 to 31 have been used to test this extract with a concentration range from 4.16 µg/mL to 166.67 µg/mL. The experiments were conducted under the Laminar Air Flow Chamber and sterility of the assay was to be maintained throughout.

Reagents Used:

Di-Methyl Sulfoxide (DMSO) 100 % ; Complete culture medium (RPMI 1640) ; Trypan Blue solution 0.4 % (Sigma – Aldrich Co., St.Louis, Missouri, USA) ; Phosphate Buffer Saline (PBS) 1x + EDTA.

Protocol Followed:

As the same as that of the screening assay for Plocamium cartilagineum. (4.4.2).

For both G.C. and P.C. after plating with the treatment and the cell suspension the plates were incubated for a period of 48 hrs, 37 ºC, 5% CO_2 atmosphere. After incubation, all three assays, AB, SRB, and NRU were performed.

4.7. Data Analysis

After measuring the absorbance and fluorescence the data were extracted in the form of an Excel file. The computation of data in Microsoft Excel is as follows:

4.7.1. Data analysis for the assays performed for C2 and CLBL-1 cell lines

a. Export the data from the plate reader and open the file in excel

b. Find the mean value of the absolute blank and subtract the other values from the mean value.

c. A new set of values was obtained and further calculations were done using these values.

d. Calculating the outliers by analyzing Quartile 1(Q1) and Quartile 3(Q3).

e. Calculate the cut-off values, the cut-off inferior and superior using the formula:

Cutoff inferior = Q1-1.5*(Q3-Q1)

Cut off Superior = Q1+1.5*(Q3-Q1)

f. Eliminate the cut-off values from ‘c’, and calculate new mean values.

g. Calculate the percentage of viability and cytotoxicity using the new mean values.

Viability% = (Mean Value/Control)*100

(Control is the cells + DMSO(0.33%))

Cytotoxicity% = 100- Viability.

h. The cytotoxicity and viability percentages of DMSO have also been calculated using the same formula indicated above. Here, the control is cells + Medium.

i. Create a final graph using the percentage of cytotoxicity and concentration used.

The final dose-response curve was created using GraphPad Prism Software(Version.5, San Diego, CA, USA). By using the cytotoxicity rate of the treated cells as compared with the vehicle control (DMSO), a non-linear regression [log(inhibitor) vs. normalized response, variable slope] was obtained. The half maximal inhibitory concentration (IC_{50} and the goodness of fit (R^2) values were provided by the software.

5. RESULTS AND DISCUSSION

The present study was designed to evaluate the cellular effects of four different algae collected from the Moroccan coast, on two canine cancer *in-vitro* models of MCT and lymphoma, C2 and CLBL-1 cell lines, respectively. The 1:1 (v/v) dichloromethane-methanolic extract of algae was used throughout the experiment. Cells were incubated in 96-well plates with algal extracts at the required time-temperature combinations. For each extract, cell and vehicle control (DMSO) were included. The antiproliferative activity, the cellular viability and cytotoxicity were evaluated by using three assays, AB, SRB, and NRU. Noteworthy, in the preliminary screening the cell viability was determined only by using AB.

For each extract, the corresponding IC_{50} value and the R^2 (goodness of fit) values were calculated using GraphPad prism 5 software.

5.1. Screening assay (AB)

The dichloromethane-methanolic extracts (four) belonging to *Rhodophyta* (*G. cervicornis*, *P. cartilagenium*, *H. incurvus*) and *Phaeophyta* (*L. ochroleuca*) families of algae were tested on C2 cells and using the AB tests analysis. *G. cervicornis* and *P. cartilagenium* exhibited a low IC_{50} value, 29.7 $\mu g \cdot mL^{-1}$ and 26.57 $\mu g \cdot mL^{-1}$ respectively. Both dose-response curves showed a R^2 value of 0.99. (**Figure 3a-b**). Dose-response curves obtained with increasing concentrations of *L. ochroleuca* and *H. incurvus* showed higher IC_{50} values (363.5 $\mu g \cdot mL^{-1}$ and 484.8 $\mu g \cdot mL^{-1}$) (**Figure 4a-b**); hence, these two algal extracts showed a comparatively minor antiproliferative effects on canine C2 cell line.

On CLBL-1 cell lines, a typical dose-response curve was produced by both *G. cervicornis* and *P. cartilagenium* with an IC_{50} value of 11.65 $\mu g \cdot mL^{-1}$ and 10.69 $\mu g \cdot mL^{-1}$, respectively. **(Figure 5a-b).** We have obtained the results for CLBL-1, only from one biological replicate and more assays have to be performed for the confirmation of the result. However, it was also observed that, the vehicle control, DMSO, is having a cytotoxic effect on the cells. On further analysis with a decreasing concentration of DMSO, from 1% to 0.5%, a decreasing effect of cytotoxicity on CLBL-1 cell lines was obtained **(Figure 5c)**. Hence, more assays could be performed by decreasing the concentration of the vehicle control (DMSO), to assure that the solvent does not interfere with the toxic effect produced by the extract, in general and the bias can be avoided.

Consequently, by comparing the effect of extracts on both cell lines, C2 and CLBL-1, *G. cervicornis* and *P. cartilagenium* were considered as the most suitable and qualified candidates for confirmatory analysis.

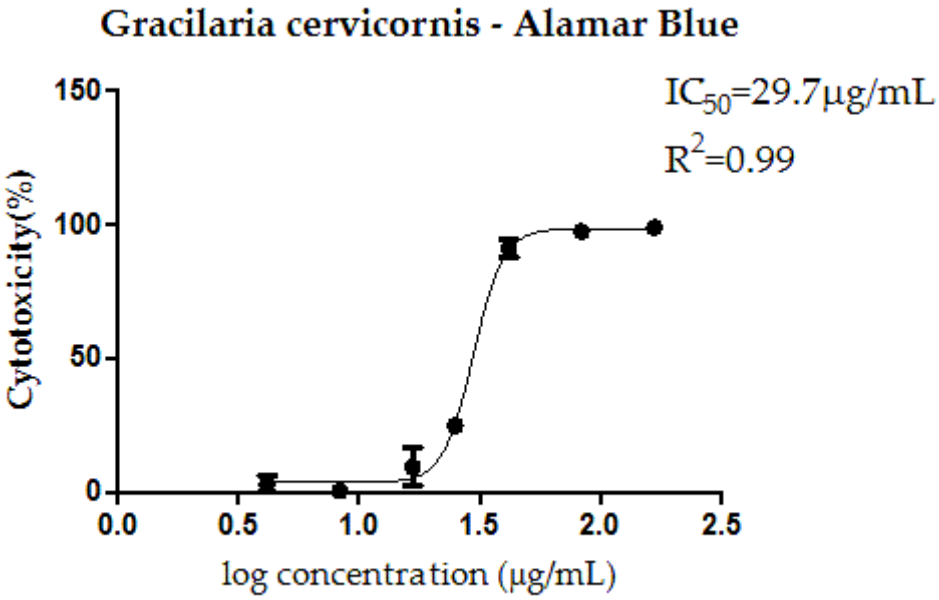

Figure 3a. Dose response curve for *G. cervicornis* on C2, after AB assay

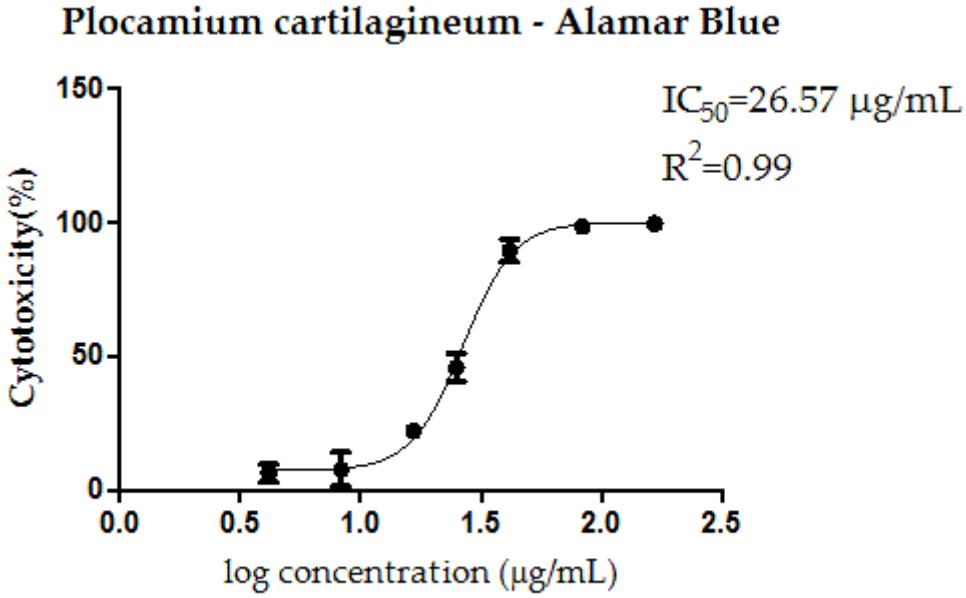

Figure 3b. Dose response curve for *P. cartilagenium* on C2, after AB assay

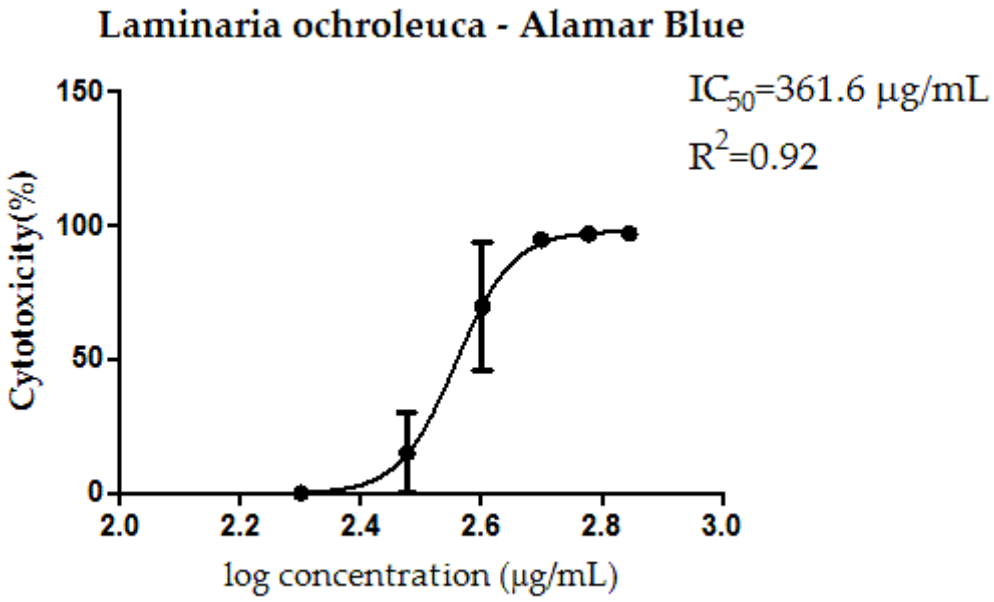

Figure 4a. Dose response curve for *L. ochroleuca* on C2, after AB analysis

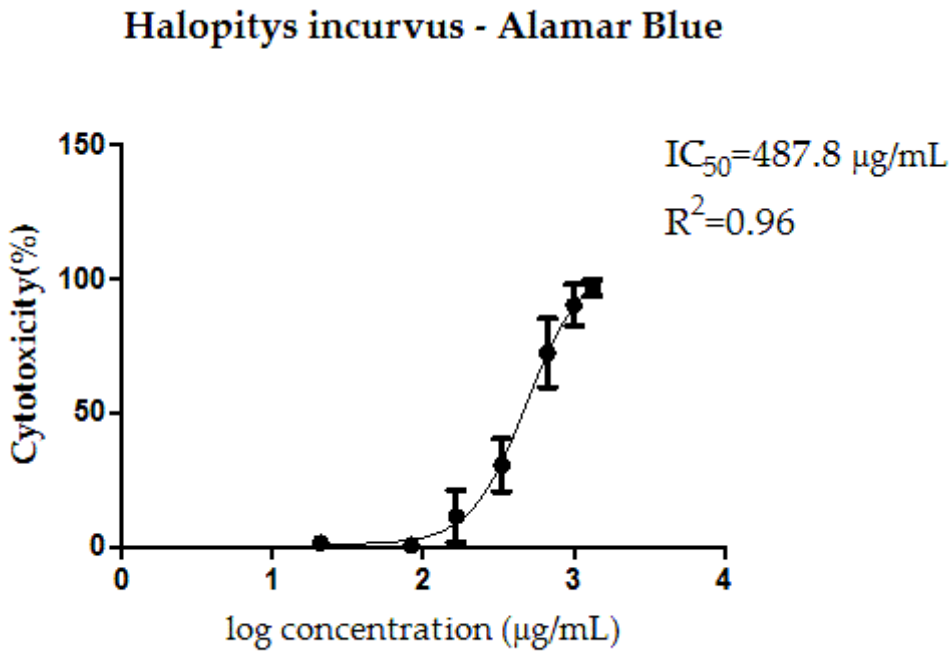

Figure 4b. Dose response curve for *H. incurvus* on C2, after AB analysis

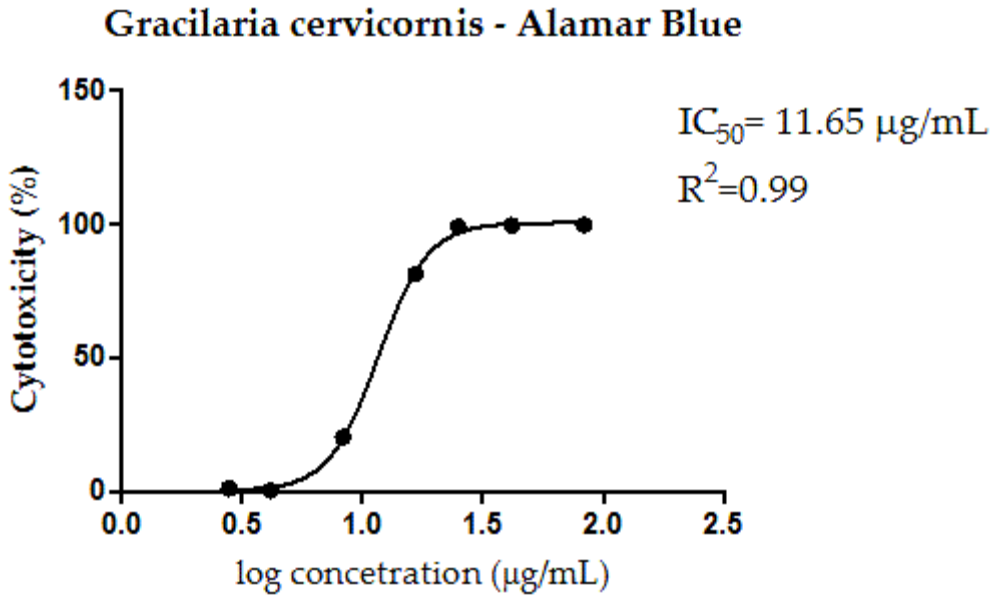

Figure 5a. Dose response curve for *G. cervicornis* on CLBL-1, after AB analysis

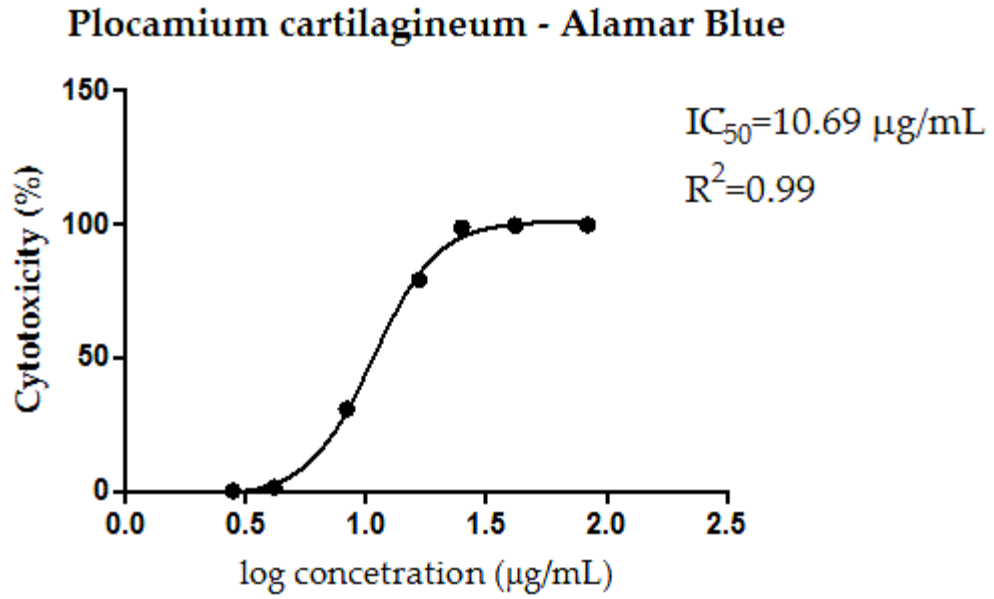

Figure 5b. Dose response curve for *P.cartilagenium* on CLBL-1, after AB analysis

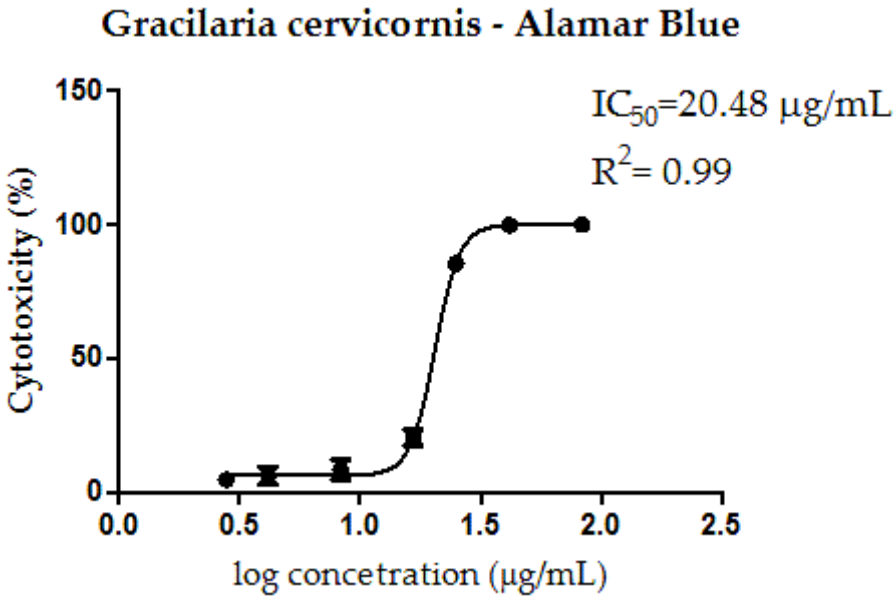

Figure 5c. Dose response curve for *G. cervicornis* on CLBL-1, after AB analysis, with a decreasing concentration of DMSO

5.2. Confirmatory analysis (AB, SRB, and NRU)

The biological activity of aforementioned extracts was further evaluated using three cytotoxic tests on C2. Overall, both *G. cervicornis*, *P. cartilagenium* confirmed their cytotoxic activity on C2, and we have obtained a typical sigmoidal dose-response curve in all three assays.

The alga, *G. cervicornis*, generated a typical sigmoidal dose response curve from all the three tests. In terms of both IC_{50} and R^2 values, it can be ascertained that, AB is the most sensitive assay, with a 76% of variability between the values produced with NRU assay. The IC_{50} and R^2 values for the three tests are presented in **Table 15.**

Cytotoxicity Assay	IC_{50} value	R^2 value
AB (**Figure 6a**.)	26.14 µg·mL^{-1}	0.96
SRB (**Figure 6b.**)	20.05 µg·mL^{-1}	0.93
NRU (**Figure 6c.**)	34.55 µg·mL^{-1}	0.87

Table 15. IC_{50} and R^2 values for C2, produced by *G. cervicornis*

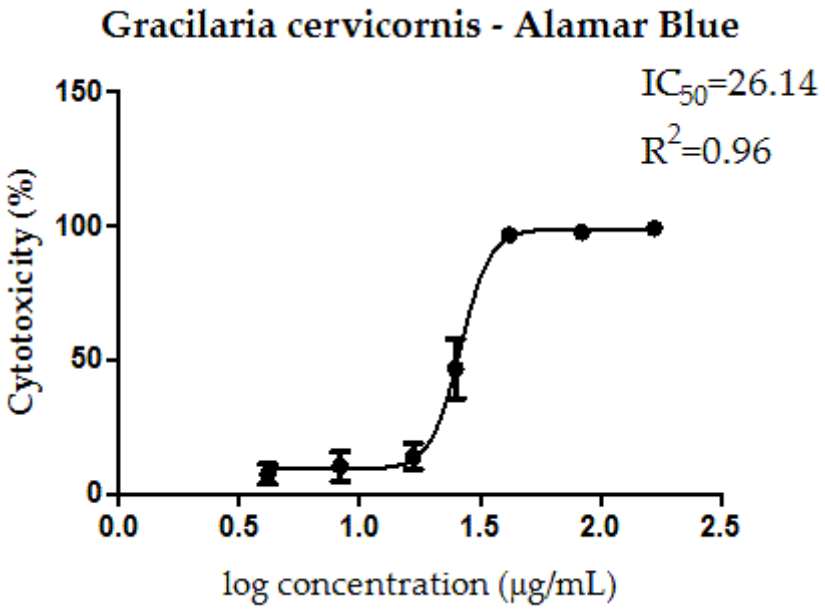

Figure 6a. Dose response curve for *G. cervicornis*, on C2, after AB analysis

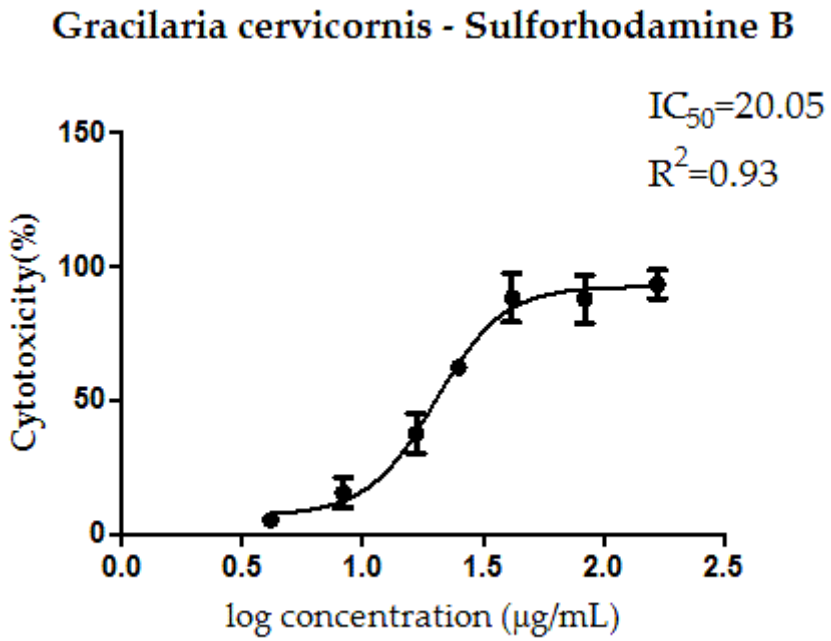

Figure 6b. Dose response curve for *G. cervicornis*, on C2, after SRB analysis

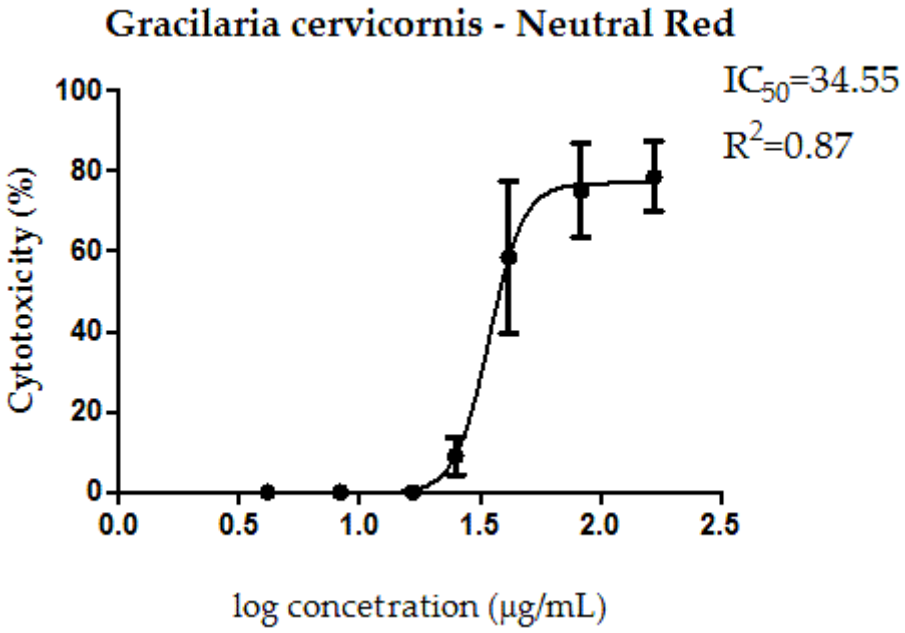

Figure 6c. Dose response curve for *G. cervicornis*, on C2, after NRU analysis

For *P. cartilagenium*, all the three tests produced the sigmoidal curve, with varying IC_{50} and R^2 values. The values are represented in **Table 16.**

Cytotoxicity Assay	IC_{50} value	R^2 value
AB (Figure 7a.)	50.90 µg·mL^{-1}	0.99
SRB (Figure 7b.)	43.60 µg·mL^{-1}	0.94
NRU (Figure 7c.)	77.75 µg·mL^{-1}	0.89

Table 16. IC_{50} and R^2 values for C2, produced by *P. cartilagenium*

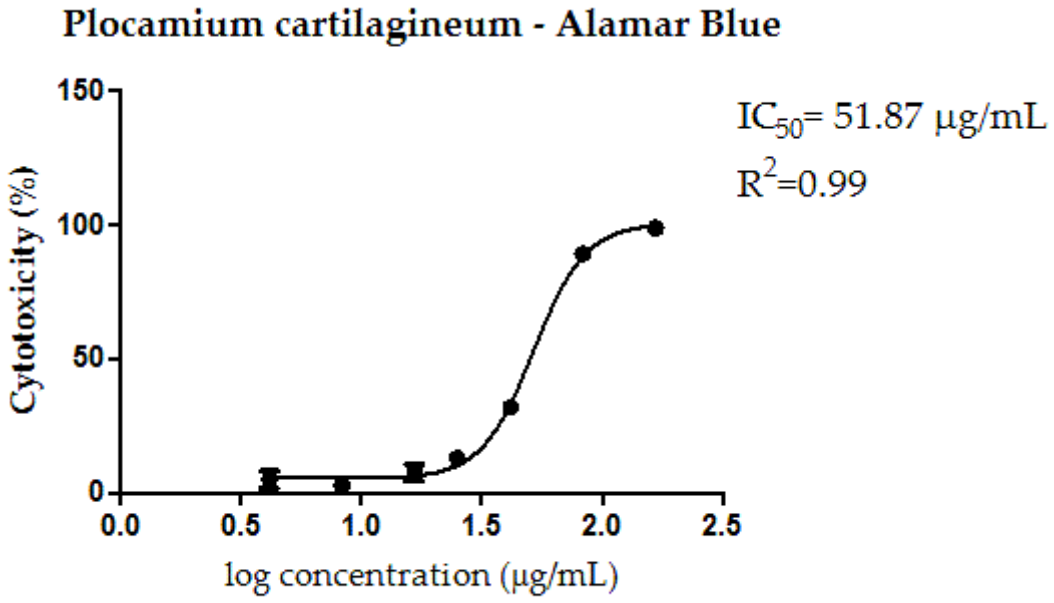

Figure 7a. Dose response curve for *P. cartilagenium*, on C2, after AB analysis

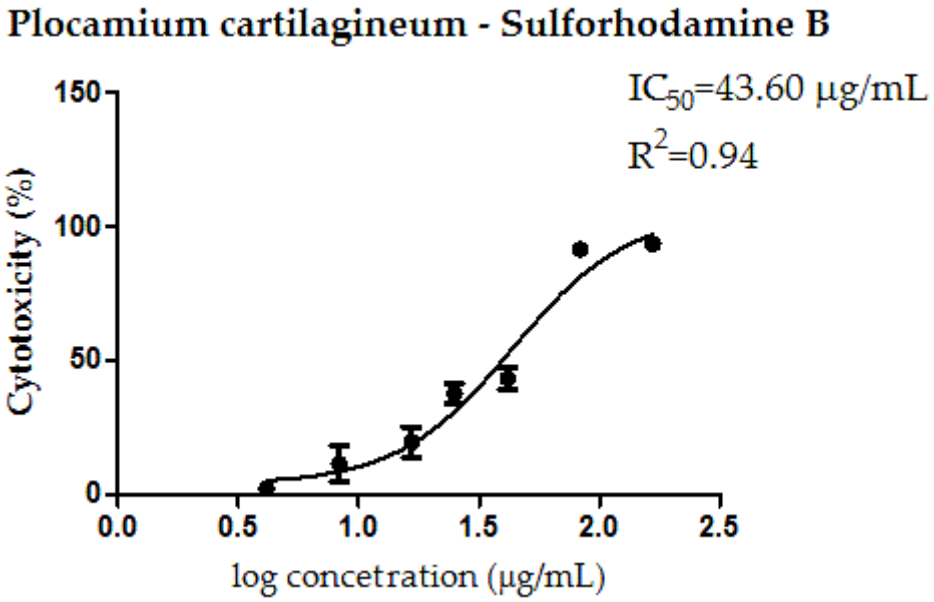

Figure 7b. Dose response curve for *P. cartilagenium*, on C2, after SRB analysis

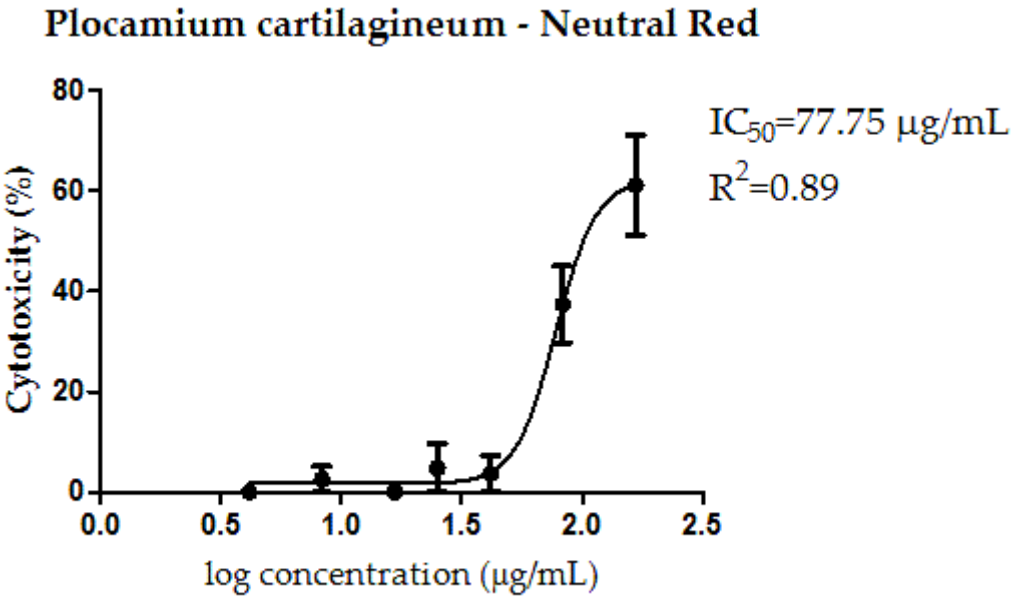

Figure 7c. Dose response curve for *P. cartilagenium*, on C2 after NRU analysis

From the above assays, it can be confirmed that, *G. cervicornis* exerts the maximum effectiveness in terms of antiproliferation. The sensitivity of the cells in producing a particular response, is also to be considered. CLBL-1 is the most susceptible among the two, that could be identified by the IC_{50} values produced by the same extracts used for C2.

Natural products have been in the mainstay of cancer chemotherapeutic procedures. Plants have played a major role in the development of highly sophiscated traditional medicinal systems. In the late 1960s, Vinblastine and Vincristine from the plants *Podophyllum peltatum* (mayapple) and *Catharanthus roseus* (Vinca rosea) respectively, have contributed to the long -term remission of many cancers (*Dhyani, P. et al., 2022*). Their discovery contributed to the development of a significant drug, Paclitaxel (Taxol), which extracted from the bark of Pacific yew, Taxus brevifolia and it still remains to be one of the best plant based cancer treatment available (*Gallego-Jara, J. et al., 2020*). Plant based drug discovery also resulted in the development of many anticancer drugs, presently in clinical use. It also provides a platform to better understand the complex synergestic interaction between the various constituents of anticancer herbs (*Khazir, J. et al., 2014*). The production of natural products from marine organisms was essentially non-existent, until the mid-1960s'. Since then, a large number of products with different therapeutic properties have been listed from seaweeds, marine microbes, sponges, soft corals and, marine invertebrates. Among these, marine algae, with diverse constituents having proven therapeutic value, have created a great interest in the medical filed (*McCauley, E. P. et al., 2020*). Microalgae and macroalgae with excellent anti-oxidant and anti-proliferative properties have been studied on different *in vitro* cell culture models of both animals and humans (*McCauley, E. P. et al., 2020, Kang, K. H., & Kim, S. K., 2013*).

Nevertheless, to our knowledge this is one of the few (if any) studies assessing the potential cytotoxic effects of crude algal extracts on canine tumour cell lines obtained from two of the most common canine neoplasia, MCT and lymphoma (*Ivashkiv, B. B. et al., 2020, Moore, A. S., 2016*). We made a preliminary evaluation of Rhodophyta and Phaeophyta that differs in biomolecule constituents having anticancer and antiproliferative properties. Overall, the extracts of *G. cervicornis and P. cartilagenium* inhibited both the C2 and CLBL-1 cell growth, albeit with varying IC_{50} values.

With regard to the highest activity shown by *G. cervicornis*, we retain important to know that various biomolecules have already been identified. Lectins with acaricidal activity (*Singh, R. S., & Walia, A. K., 2018*) toxic polycavernosides (*Yotsu-Yamashita, M. et al., 2004*), cyclopropane containing cerebrosides with a weak cytotoxicity against melanoma cell lines (*Sun, Y. et al., 2006*), chlorinated fatty acid metabolites (*Shoeb, M., & Jaspars, M., 2003*) and, phycobiliproteins with antioxidant activity (*Tello-Ireland, C. et al., 2011*), have been so far isolated from *Gracilaria spp*. Even though not many cytotoxic in vitro studies

have been reported in particular, for *G. cervicornis*, other species proved to have cytotoxic activities against Ca9-22 (oral cancer) cells in a dose dependent manner (*Yeh, C.C. et al., 2012*), human lung adenocarcinoma cells (A549) with an IC_{50} value of 24.5 ± 19.1 $\mu g.mL^{-1}$(*Sakthivel, R. et al., 2016*). Whereas, *G. cervicornis* has made considerable attention in providing fungistatic activity against the opportunistic microorganism *Candida spp.* (*Sampaio, T.M.M. et al., 2022*). The aqueous extract of some *Gracilaria* species also confirmed the anti-inflammatory activity (*Chalini, K. et al., 2017*). It is also reported that red macroalgae can also be used as a source of potential anti-allergic therapeutics (*Vo, T. S. et al., 2012*) which makes this study more interesting, as MCT produces a large number of allergic mediators, which can even lead to life-threatening anaphylaxis.

Furthermore, former studies have characterized the part played by *KIT* proto-oncogene in tumourigenesis (*da Costa, R. M. G., 2015*), and its role as a potential molecular target for the treatment of canine MCT (*Sheikh, E. et al., 2022*). Interestingly, this co-relates with the study by Shuai-Yu Wang and colleagues in 2015, where a bromophenol derivative isolated from the marine red alga *Rhodomela confervoides*, produced inhibitory response against different RTKs. (*Wang, S. al., 2015*). This confides the fact, that the inhibitory effect shown by the algae require additional ligand-receptor studies, as they both belong to the red algae family, with respect to MCT.

Like *G. cervicornis*, *P. cartilagenium* also belongs to the family of red alga. The dichloromethane extract (1 $mg \cdot mL^{-1}$, 24 hrs) of *P. cartilagenium* exhibited a strong anti-proliferative activity against human hepatocellular carcinoma cells (HepG2), with an 85% decrease in cell viability, but with a higher IC_{50} value of 852.7 $\mu g.mL^{-1}$ (*Alves, C. et al., 2016*). Interestingly, this extract seems to be most effective against C2 and CLBL-1 cell lines than HepG2, as it produces a lower IC_{50} value of 50.90 $\mu g.mL^{-1}$ and 10.69 $\mu g.mL^{-1}$ for C2 and CLBL-1 respectively. Studies showed the capability of the aqueous extract of *P. cartilgenium*, in inhibiting the replication of Herpes Simplex Virus type-1 (HSV-1), with an IC_{50} value of 6.4 $\mu g.mL^{-1}$, which explains its potential antiviral properties (*Rhimou, B. et al., 2010*). The dichloromethane extract of *P. cartilagenium* induced a higher antiproliferative activity on human colorectal cancer (Caco-2) cells with 95% reduction of the cells (*Alves, C. et al., 2018*).

It is difficult to arrive at a definitive conclusion regarding the reason behind *P. cartilagenium* anti-proliferative and cytotoxic effects; indeed, many constituents with

possibly potential cytotoxic effects have been isolated, e.g., monoterpenes, polyterpenes and, sulfated polysaccharides (*Sabry, O. M. et al., 2017, Bagchi, B. et al., 2012*). Nevertheless, the biological effect here obtained might be related to the presence of halogenated monoterpenes, that have been proved to be cytotoxic against human colon adenocarcinoma cellss (SW480 cell line) (*de Inés, C. et al., 2004*).

Additionally, the other two algae *L. ochroleuca* and *H. incurvus* have also been shown anticancer activities on a number of cell lines. Boujaber et al, (*Boujaber, N. et al., 2019*) studied the anti-tumour activity of dichloromethane-methanolic extract of *L. ochroleuca* collected from the Moroccan coast, and evidenced 100% activity against human buccal epidermal carcinoma cells (KB). On the other hand, the acetone-methanolic extract of *L. ochroleuca*

collected from the same coast, showed maximum cytotoxicity and anti-proliferative effect against HT-29 human colon cancer cells, with an IC_{50} value of 88.26 $\mu g \cdot mL^{-1}$ (*Zbakh, H. et al., 2014*). Although they have been proved to be cytotoxic, in our experimental conditions they did not produce a very high effect on C2 cells.

Overall, some differences in the biological activity can be related to various environmental conditions to which the algae are subjected that, in turn, can lead to the production of different amounts of compounds potentially giving cytotoxicity. As an example, some authors have linked the production of compounds with toxic and anti-proliferative properties to temporal and spatial variations, depending on the community or season, focusing on the importance of various biotic and abiotic factors (*Martí, R. et al., 2004, Taskin, E. et al., 2010*)

As a whole (and clearly), these results are very preliminary. Looking forward, we need to: (a) better characterize from the analytical point of view (e.g., more purified extracts) at least the two extract proved as cytotoxic; (b) to get preliminary information about the mechanism of action of these extracts (e.g., by RNA-seq investigations); (c) to make some more specific confirmatory studies, for as examples, investigate pro-apoptotic mechanisms by using annexin-V flow cytometry, check for ROS formation by fluorescent dyes (d) to study in particular, from the toxico-kinetics point of view. Some of these developments are currently considered in our lab.

6. CONCLUSION

The present study was one in a series that probed the anticancer activity of algal extracts on *in vitro* models (established cell lines). Though, a number of papers about the cytotoxic activity of red and brown algae have already been published, little is known about the effects produced by the algae extracts we selected for this particular study, and especially on the canine cancer cell lines, i.e. C2 and CLBL-1.

In the first part of the thesis, the best experimental conditions were determined using a wide range of concentrations and different time of exposure. Then, cell cytotoxicity was assessed by a more specific range of concentrations, a fixed time of exposure and three cytotoxicity assays. Overall, the crude algal extracts showed a marked cytotoxic effect on these target cell lines. Encouragingly, two out of the four algae tested, namely *G. cervicornis*, and *P. cartilagenium*, showed a comparatively higher cytotoxicity. Both, cell lines were susceptible, but more valuable results were obtained with the CLBL-1 cell line.

Clearly, further investigations aiming at clarifying and supporting *G. cervicornis*, and *P. cartilagenium* cytotoxic effects are required. As an example, extracts purification and isolation of the bioactive molecule responsible for such a cytotoxicity, using GC-MS and LC-MS. Furthermore, studies aiming at identifying the effects of these extracts on cell transcriptome. In this way, the molecular mechanisms, behind the algal action can be carved out.

From the future perspective, with the discovery of new natural sources, it could be possible, the development of an alternative cancer strategy and can serve as a comparative model for human oncological investigations.

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