UNIVERSITÀ DI SIENA 1240

Department of Biotechnology, Chemistry and Farmacy

REGIONAL PHD SCHOOL IN BIOCHEMISTRY AND MOLECULAR BIOLOGY – BIBIM 2.0
XXXIII CYCLE

PhD coordinator
Prof. Lorenza Trabalzini

# Characterization of an extract of the jellyfish *Cassiopea andromeda*, Forsskål, 1775 (Scyphozoa, Cnidaria) and study of its antioxidant activity, cytotoxicity, and ability to modulate Gap Junction Intercellular Communications in cancer cells

AD: Life Sciences
S.S.D: BIO/10 Biochemistry

Tutor: University of Siena (UniSi)
Prof. Annalisa Santucci
Tutor: National Research Council
Institute of Sciences of Food Production (CNR-ISPA) Unit of Lecce
Dr. Antonella Leone

PhD Student
Dr. Gianluca De Rinaldis

ACADEMIC YEAR
2019/2020

# Funding

This PhD thesis was developed at the Institute of Sciences of Food Production of the National Research Council, Unit of Lecce (CNR- ISPA) under the supervision of Dr. Antonella Leone and under the Operational Agreement (Prot. no. 306 of 08/02/2018). between CNR-ISPA and Prof. Annalisa Santucci of University of Siena

All the costs of research activities and one year of research grant were funded by the EU project "GoJelly – *A gelatinous solution to plastic pollution*" funded by European Union's H2020-BG-2017-1 call topic, under Grant Agreement n°774499.

https://gojelly.eu/

The abroad research activity was covered by Erasmus Plus Programme, University of Siena and RECETOX centre.

# *Table of Contents*

## *Funding*

*Table of Contents* i
**Abstract** **iii**

**1 Introduction** **1**
1.1 Marine jellyfish biomasses 1
1.1.1 Invasive Alien Species 2
1.1.2 Cassiopea andromeda species 3
1.2 Jellyfish exploitation: from direct food use to bioprospecting 9
1.2.1 Bioprospecting of marine natural products 9
1.2.2 Jellyfish compounds and related bioactivities 10
1.2.3 Anticancer activity of jellyfish extracts 13
1.2.4 GJIC and cancer, and jellyfish extracts as anti-proliferative agents 15

**2 Materials and Methods** **20**
2.1 Reagents, materials, and devices 20
2.2 Jellyfish samples, extraction, and fractioning 22
2.2.1 Soluble compounds extraction and sequential hydrolysis of insoluble compounds 23
2.2.2 Aqueous soluble extraction (PBS extraction) 23
2.2.3 Hydroalcoholic extraction 24
2.2.4 Hydrolysis of insoluble compounds 25
2.2.5 Hydroalcoholic extract fractionation 25
2.3 Biochemical characterization 28
2.3.1 Protein content 28
2.3.2 Collagen content 28
2.3.3 Total phenolic content 29
2.3.4 In vitro antioxidant capacity 30
2.4 Human breast cancer cell cultures 30
2.4.1 Cells treatment with fractions of the jellyfish hydroalcoholic extract 32
2.4.2 Cells viability assessment by MTS assay 33
2.4.3 Apoptosis evaluation in cell cultures 33
2.5 Murine cancer cells cultures 33
2.5.1 Cell viability assessment by 3-dye cytotoxicity assay 35
2.5.2 Assessment of GJIC by scrape loading-dye transfer (SL/DT) assay 37
IMAGEJ macro algorithm 43
2.6 Statistical analysis 47

**3 Results and Discussion** **48**
**3.1 Biochemical characterization of Cassiopea andromeda jellyfish biomass** **48**
3.1.1 Jellyfish biometric data 48
3.1.2 Jellyfish biomass composition 49
3.1.3 Jellyfish soluble compounds 50

*Whole jellyfish* 50
*Umbrella and Oral Arms* 51
*3.1.4 Umbrella and Oral Arms biochemical characterization* 53
*3.1.5 Partial characterization of extracted soluble compounds* 54
*Protein content of the aqueous and hydroalcoholic extracts* 54
*Phenol content of the aqueous and hydroalcoholic extracts* 57
*Antioxidant activity of the aqueous and hydroalcoholic extracts* 59
*3.1.6 Enzymatic sequential digestion of insoluble proteinaceous compounds* 61
*Pepsin-hydrolyzed proteins* 61
*Total phenol content of pepsin-hydrolyzed fractions* 62
*Antioxidant activity of pepsin-hydrolyzed fractions* 63
*Collagenase-hydrolyzed proteins* 65
*Total phenol content of collagenase-hydrolyzed fractions* 67
*Antioxidant activity of collagenase-hydrolyzed fractions* 68
***3.2 Bioactivity of the hydroalcoholic extracts fractions of Umbrella and Oral Arms*** **70**
*3.2.1 Hydroalcoholic (80% ethanol) extraction* 70
*Fractionation of the hydroalcoholic extracts* 71
*3.2.2 Hydroalcoholic extract biochemical characterization* 72
*3.2.3 Bioactivity of the hydroalcoholic extracts fractions of Umbrella and Oral Arms* 76
*3.2.3.1 Effect on viability of MCF-7 and MB-MDA-231 cell cultures* 76
*Preliminary evaluation of apoptotic effect* 80

***(mobility abroad section)***

***3.3 Evaluation of the effect of jellyfish extracts fractions on GJIC in murine cell models, WB-ras and WB-neo*** **82**
*3.3.1 Evaluation of cell viability by jellyfish extracts fractions on WB cell models* 83
*3.3.2 Effect of jellyfish extracts fractions on GJIC functionality in WB cell models* 92

***4 Conclusions*** **97**

***5 Acknowledgements*** **99**

***6 References*** **100**

# Abstract

The blooms of Mediterranean jellyfish are becoming increasingly frequent causing damage to ecosystems and to human productive activities, on the other hand they can represent abundant bio-resources easily accessible to the inhabitants of coastal communities.

The studies presented here aim to the biochemical characterization of the biomass of the invasive zooxanthellatae jellyfish *Cassiopea andromeda,* Forsskål, 1775 (Cnidaria, Rhizostomeae) collected in the port area of Palermo (Sicily, Italy), and on the possible biological activities of some partially purified extracts.

The first part of the thesis includes the composition analysis and a preliminary biochemical characterization of the jellyfish *Cassiopea andromeda.* Aqueous (PBS) and hydroalcoholic (80% ethanol) soluble components were obtained from the whole jellyfish and from both oral arms and umbrella. The insoluble components were hydrolysed by sequential enzymatic digestion with pepsin and collagenase. Both aqueous and hydroalcoholic extracts containing soluble compounds, as well as the hydrolysed peptides were biochemically characterized for their proteins and phenols contents, and for their antioxidant activity. A notable yield as well as a considerable antioxidant activity was detected in almost all assessed samples mainly in the extracts containing soluble compounds. The PBS extracts from oral arms samples have a higher yield in organic matter and are richer in proteins and phenols as compared to the hydroalcoholic extracts, whereas the hydroalcoholic extracts, showed a stronger antioxidant activity than aqueous soluble compounds.

The second part of the thesis deepens the study of the hydroalcoholic extracts, which show the greatest antioxidant activity and have been further fractionated, biochemically characterized and their biological activity has been evaluated on different human and murine cancer cell systems. When the hydroalcoholic extract was fractionated, the resulting hydrophilic fractions obtained from both umbrellas and oral arms samples, resulted to be rich in proteins and phenols, with a strong antioxidant activity in oral arms fractions. The potential cytotoxic activity of four jellyfish fractions of the hydroalcoholic extract were assayed on human breast cancer cells (MCF-7 and MB-MDA-231 cells lines) and on two rat

liver epithelial cells, WB-*ras* and WB-*neo* cells lines. In order to elucidate the action mechanism of the cytotoxicity against cancer cells, the ability of the four jellyfish extract fractions to modulate the function of the Gap Junction mediated Intercellular Communication (GJIC) was also assessed on both rat liver epithelial cell lines, WB-*ras* and WB-*neo* cell lines. When administrated on human cancer cells, the lipophilic and hydrophilic fractions resulted to strongly reduce the cell viability of both MCF-7 and MB-MDA-231 cells lines, with a stronger effect of the hydrophilic fractions than the lipophilic ones. As expected, the cytotoxic effect was more evident in MCF-7 cells than in the resistant MB-MDA-231 cells, however a notable cytotoxic effect was also exerted on the resistant MB-MDA-231 cell line.

The cytotoxic effect on cancer cells was also confirmed by assessment of the two fractions on rat liver cell lines, the *ras*-transformed epithelial cells (WB-*ras*) and the normal epithelial cells (WB-*neo*) where the lipophilic fractions resulted to be more cytotoxic than the hydrophilic ones, thus proving the presence of possible lipophilic bioactive compounds in jellyfish tissues or in their endosymbiotic zooxanthellae. The same fractions have also demonstrated the ability to improve GJIC in the non-communicating tumorigenic WB-*ras* cells and had no effect on GJIC in the well-communicating WB-*neo* cells.

In conclusion, all the results suggest that the invasive zooxanthellatae *Cassiopea andromeda* jellyfish, like other Mediterranean blooming jellyfish, represent a valuable biomass and a sustainable source of compounds with biological activity for current and future applications. However, other studies and research will lead to a better understanding of the biology and the potential use of the upside-down *Cassiopea andromeda* jellyfish.

# 1. *Introduction*

## *1.1. Marine jellyfish biomasses*

In the last decades, an increase of jellyfish "blooms" - the sudden and abnormal increase of jellyfish population – has been recorded in most coastal areas of the world including the Mediterranean Sea (Roux et al., 2013; Brotz et al., 2012; Richardson et al., 2009; Purcell and Benovic, 1999). Although a direct causality has not yet been demonstrated, climate change, anthropogenic factors such as overfishing, eutrophication, modification of the marine ecosystems, jointly with the ability of jellyfish to survive under a wide range of environmental conditions, seems related to the increase of jellyfish populations (Boero et al., 2008). However, several natural conditions like winds, currents, tidal changes, and water parameters can also trigger jellyfish blooms. Several jellyfishes, indeed, are positively affected by the increase in temperature that help them to expand their habitat (Boero et al., 2016). Jellyfish belongs to the phylum of Cnidaria and their blooms are also facilitated by their particular life cycle. In general, Cnidaria have two morphotypes, the benthic polyp stage that has the ability to asexually produce massive jellyfish populations – the adult pelagic morphotype, sexually actives - in a very short time and to survive unfavourable periods at polyp stage. These outbreaks periodically become an issue to marine and maritime human activities with ecological, economic, and social impacts, including loss of biodiversity and biomass (Vilà and Hulme, 2018; Bellard et al., 2016; Gallardo et al., 2016; Walsh et al., 2016).

Although jellyfish have a role in the marine food web as carbon source, however, they negatively impact on marine ecosystems being predators of zooplankton. Jellyfish populations act indirectly, depleting the resources usually eaten by fish larvae and other organisms and, directly by eating fish eggs, larvae and small fishes, also due to the large dimensions that some jellyfish species can reach (Condon et al., 2011; Arai, 2005; Purcell and Arai, 2001). The major negative impacts of jellyfish blooms on human activities relate to damage to aquaculture, human well-being, tourism, and damage to industrial facilities and represent a multi-billion-euro problem (Bosch-Belmar et al., 2020; Bosch-Belmar et al., 2017; Ferguson et al., 2010; Baxter et al., 2011).

Considering the Mediterranean Sea, many jellyfish species are able to form blooms, such as *Pelagia noctiluca*, started to be a tourism problem along the beach in the early 1980s (CIESM, 2001). The jellyfish *Cotylorhiza tuberculata* is also a Mediterranean species that creates blooms following the rule of "*the warmer the better*" sometime with public distress along the impacted coasts as was showed in the coastal lagoon of Mar Menor, Spain (Ruiz et al., 2012).

Another Mediterranean species with large population fluctuations is the *Rhizostoma pulmo,* which occurs with large blooms along Adriatic and Ionian Seas and in other coastal areas of the Mediterranean Sea (Basso et al., 2019). *R. pulmo* specimens can reach dimension up to 60 cm in the umbrella diameter and several kg of weight (Lilley et al., 2009). This species causes blooms with more than ten specimens per square meter, about 50 thousand specimens per square kilometre and with an estimated biomass of about 300 tons per square kilometre (Leone et al., 2015).

Beside the native Mediterranean jellyfish, invasive species are becoming frequent in the Mediterranean basin.

### *1.1.1. Invasive Alien Species*

An aspect connected with the global changes is indeed the transportation of organisms across the oceans and the alteration of their marine natural distribution (Occhipinti-Ambrogi and Galil, 2010). Invasive Alien Species (IAS) are defined as "animals and plants that are introduced accidentally or deliberately into a natural environment where they are not normally found, with serious negative consequences for their new environment". Marine ecosystem changes as well as anthropogenic changes to the environment lead to the introduction of IAS in the Mediterranean Sea, an area where environmental phenomena happen more quickly and with more strength than into the Oceans (Boero, 2015). It was observed that there are no other seas or areas where marine species invasion is not counterbalanced like in the Mediterranean Sea, where marine IAS are completely and rapidly replacing their Mediterranean counterparts (Galil et al., 2014). Marine invasive alien species can naturally enter the Mediterranean basin, facilitated by climate change or through human dispersion as ballast water or fouling or even by voluntary introduction thus

impacting on the biodiversity (Killi et al., 2020). The IAS represent a real concern for the European Union (EU) which has, as a priority target, the reduction of the biodiversity decline (EU Regulation 1143/2014 on Invasive Alien Species, https://eur-lex.europa.eu/). The core of this regulation is a list of all alien species, marine and terrestrial, that represent a concern for Europe (https://ec.europa.eu/). The list was established in 2016 and regularly updated (last update in 2019).

Member states or the European commission, indeed, can propose the addition of new species to the list based on the evaluation by a scientific panel or an IAS committee. So, if the species falls within the criticality criteria, there will be a new update of the list of invasive alien species (https://ec.europa.eu/environment/nature/invasivealien/). However, many IAS are still outside the list since they are still not considered a concern by the EU government, nonetheless they may become an issue in the future. This is the case of several marine invasive alien species, which are more cryptic, less known, or still not considered in the literature or not completely recognized as critical by the EU governments.

A number of alien jellyfish species are already detected. The Atlantic *Physalia physalis* appeared in the Mediterranean Sea in summer 2010 (Prieto et al., 2015) as well as the *Chrysaora quinquecirrha*, able to eat more than one hundred eggs and larvae per day (Purcell and Arai, 2001; Purcell et al., 1994). Some of these species are able to consistently reach high densities, such as *Rhopilema nomadica* which represent up to 60% of trawl's by-catch in the Eastern Mediterranean coasts (Turan et al., 2011). However, among the Mediterranean invasive jellyfish, the most abundant Lessepsian jellyfish are *Cotylorhiza erythraea*, *Marivagia stellata*, *Phyllorhiza punctata*, *Rhopilema nomadica* and the *Cassiopea andromeda,* some of which have not yet been included in the list.

### *1.1.2. Cassiopea andromeda species*

The benthic scyphozoan *Cassiopea andromeda,* Forsskål, 1775 (Cnidaria, Rhizostomeae) (Fig. 1) also known as the upside-down jellyfish (Galil et al., 2017; Galil et al., 1990) is a Mediterranean Lessepsian invasive alien species that is still not proposed for the IAS list, because it is not still recognized as an issue by both

the EU governments and scientists, probably due to its low dispersion rate and localization in the Mediterranean area and along the Italian coasts (Giakoumi et al., 2019).

***Figure 1.*** The up-side down *Cassiopea andromeda* jellyfish © Raimond Spekking / CC BY-SA 4.0 (via Wikimedia Commons)

*Cassiopea andromeda*, as jellyfish, belongs to the phylum Cnidaria, that with more than 10k species is one of the most ancient animal lineages and includes benthic and pelagic individuals that live mostly in the marine environment (Hooper et al., 2011; Zhang et al., 2011; Marques and Collins, 2004; Ruppert et al., 2003). Despite their size, forms, toxicity and habitat variety, Cnidarians have common features: a gastrovascular cavity and a body composed of an ectoderm and endoderm layer separated by a mesoglea, a gelatinous matrix that usually represent the main tissue of these organisms, and the cnidocytes which represent the key feature of the phylum, also giving its name. Cnidocytes ("stinging cells") are specialized cells containing organelle-like structures called cnidocysts or nematocysts (stingers). These cells are present around the mouth and along the

tentacles, serving to immobilize prey with toxins contained within specialized organelles.

The phylum is subdivided into 4 classes, Hydrozoa, Anthozoa, Cubozoa and Scyphozoa with the last one mostly represented by true-jellyfish, individuals with a bell-shape "medusoid" called medusae (El-Bawab, 2020). Among the four Scyphozoa orders, the Rhizostomeae order have peculiar jellyfish lacking tentacles and having eight oral arms instead of four. This order is also the main one of interest for commercial fishing for human consumption, especially in China.

The genus *Cassiopea* is a planktonically dispersed and stationary jellyfish that is usually associated with shallow water and around mangroves in tropical and sub-tropical waters with its bell resting on the sea floor. It currently includes nine molecular recognized species even though other species are still considered by morphological analysis only (Arai et al., 2017; Morandini et al., 2016; Holland et al., 2004). Among the jellyfish, *Cassiopea andromeda* shows a unique posture, upside-down as it is also called, with the exumbrella "adherent" to the seafloor (Fig. 2-3).

***Figure 2.*** Usual posture on the seafloor of the up-side down *Cassiopea andromeda* jellyfish © Shannon Tompkins / CC BY-NC-ND 2.0 (via Flickr)

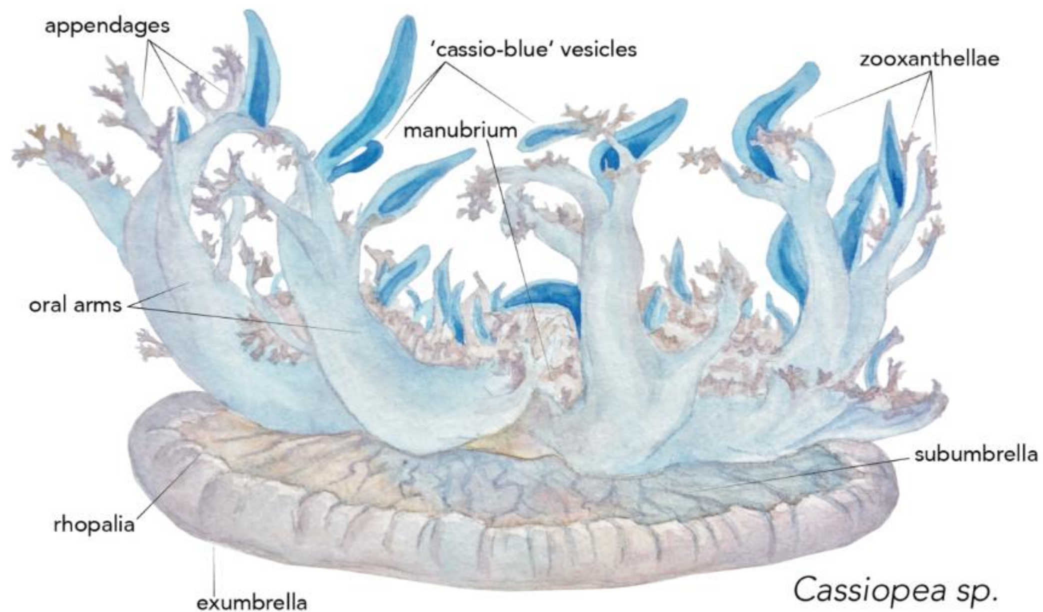


***Figure 3.*** Drawing of the morphological features of schyphozoan upside-down jellyfish, *Cassiopea andromeda.* © Orphistotheutis on Tumblr.

The first records of *Cassiopea andromeda* species from the central Mediterranean Sea were in Malta harbour (Schembri et al., 2010) and in Tunisia (Amor et al., 2015). In the last years, *Cassiopea andromeda* reached also eutrophic waters of the Spanish coasts (Rubio, 2017) and finally, it was detected in the harbour of Palermo, Sicily, Italy (Cillari et al., 2018). Currently, small blooms are occurring almost every year inside this dock (personal communication). This observation strengthens the hypothesis that boat shipping from the red sea and between Mediterranean ports is the true vector responsible for the *Cassiopea andromeda* invasion, as first suggested by Schembri et al. 2010. The *Cassiopea sp.* found into the Palermo Harbour were first recognized by morphological features but also by molecular analysis as *Cassiopea andromeda* species (Maggio et al., 2019). (Fig. 4)

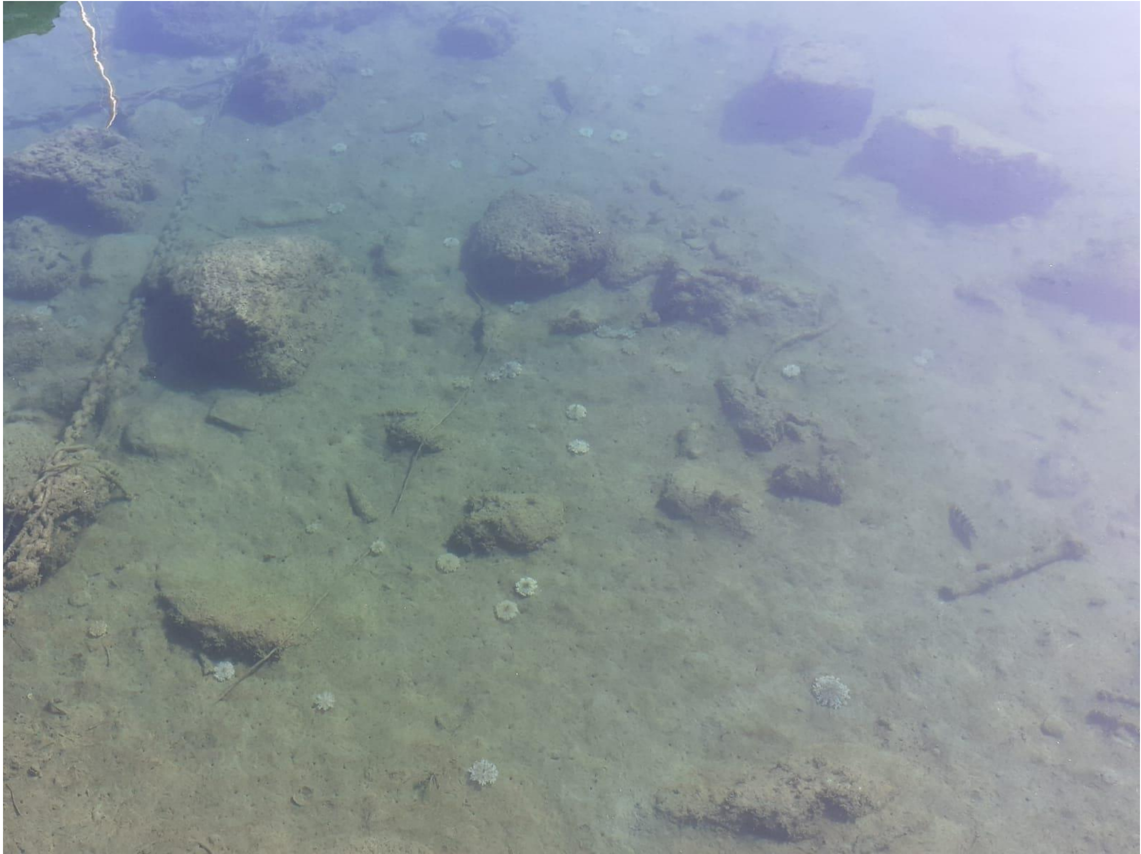

***Figure 4.*** *Cassiopea andromeda* lying on the sea floor of the Palermo Harbour providing sunlight to its photosynthetic symbiotic algae. © Tony Scontrino.

Regarding morphological features, *Cassiopea andromeda* has an umbrella that is usually yellow brown with several white spots and lines. Oral arms are of the same colours but usually darker than umbrella, with extended tentacles. Every appendage, other than to host the zooxanthellae, displays several vesicles that exhibit different vivid colours, from brown to green, or violet, and the so called "cassio-blue pigment" (Bulina et al., 2004; Blanquet and Phelan, 1987).

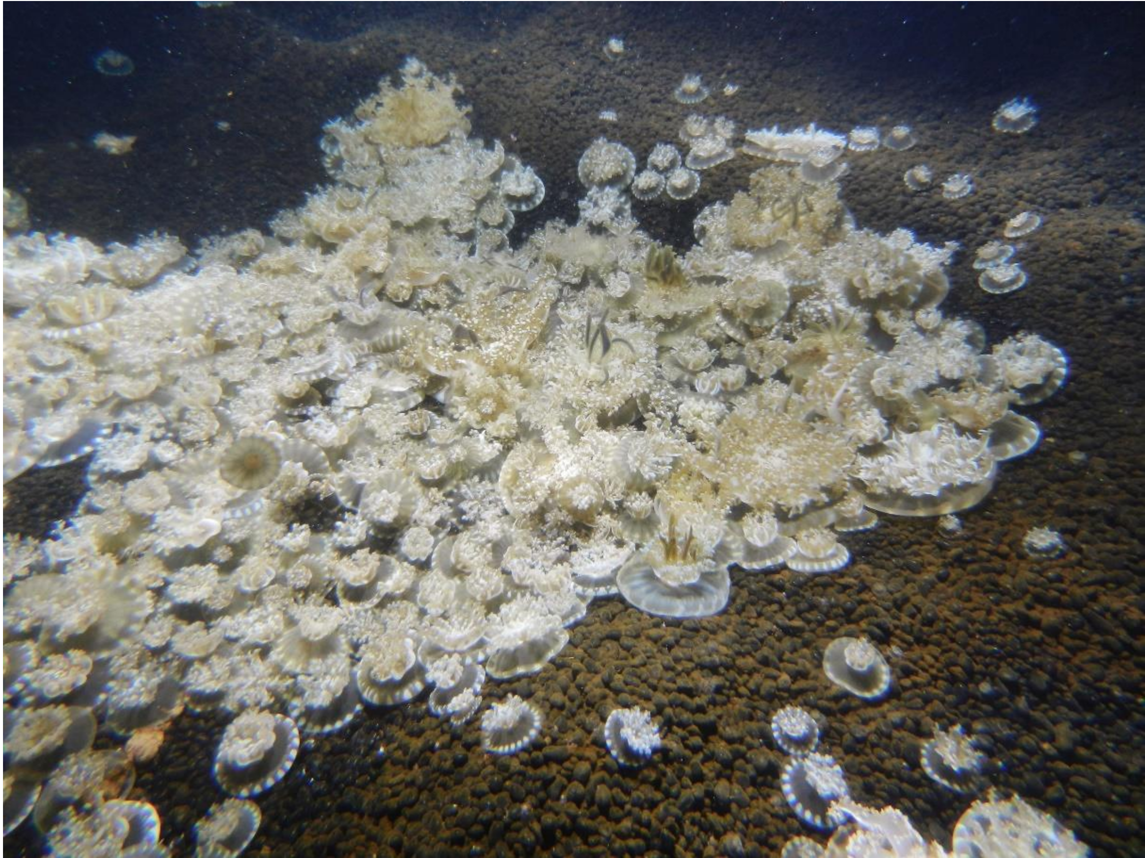

***Figure 5.*** An assemblage of the up-side-down *Cassiopea andromeda* jellyfish – shot taken at the Inner Harbour of Baltimore, Maryland by Travis S. © CC BY-NC 2.0 (via Flickr)

The peculiar body posture of *Cassiopea* (Fig. 5) has a trophic reason. Like corals and other marine species, also some jellyfish species like *C. andromeda* host endosymbiotic microalgae present all over the body tissues (Lampert et al., 2012), and are called zooxanthellae. These unicellular microalgae are photosynthetic dinoflagellates and most of them belong to the genus *Symbiodinium* (Lee et al., 2015). As photosynthesizing organisms, *Symbiodinium* grow by fixing carbon using light energy, being autotrophs is closely connected to the success of this process. *Symbiodinium microadriaticum*, was identified by molecular tools as the endosymbiotic zooxanthellae associated with several Mediterranean and invasive jellyfish, like *Cotylorhiza tuberculata* and *Cassiopea andromeda* jellyfish (Banha et al., 2020; LaJeunesse et al., 2009).

Although *Cassiopea andromeda* specimens exhibit a colourful appearance of their appendages, these colours are not related to the presence of difference clades of the symbiotic microalgae, because *C. andromeda* hosts a single *S. microadriaticum*

clade although displaying different colours between brown, green, violet, and blue (Lampert et al., 2012).

### 1.2. *Jellyfish exploitation: from direct food use to bioprospecting*

These large Mediterranean jellyfish biomasses, invasive or endemic, are not yet exploited, unlike some of Asiatic species that have been exploited for millennia for human food uses (Brotz and Pauly, 2017). The market value of this kind of commodity is considerable. Following the Food and Agriculture Organization of the United Nations (FAO STAT) statistics, jellyfish have a worldwide growing business market of about 100 million USD with ton's value around 2500 USD, but these values are clearly underestimate if compared with the Chinese data, that declares a number of tons more than fifteen times higher (Chenjin, 2017). In Europe, there is no tradition of the food use of jellyfish, no food market related to jellyfish as food, or any other type of use of jellyfish by humans.
Recently, the Mediterranean species *Rhizostoma pulmo* was suggested as raw material for human foods (Bleve et al., 2019), however eating jellyfish is not yet permitted for humans, and jellyfish are still labelled as "novel food" under the current European Regulation (EU Regulation 2015/2283 of 25/11/2015 http://eur-lex.europa.eu/eli/reg/2015/2283/oj).
Other studies on Mediterranean jellyfish species forming blooms (Leone et al., 2019; 2015; 2013), consider that the jellyfish outbreaks could be turned into a source of value-added healthy food or as source of compounds for nutraceutical, cosmeceutical, or pharmaceutical uses.

#### 1.2.1. *Bioprospecting of marine natural products*

Natural products discovered thousands of years ago are still used for human benefit. Bioprospecting (or biodiversity prospecting) is the exploration of natural sources for small molecules, macromolecules and biochemical and genetic information that could be developed into commercially valuable products for overall benefits of the society. Up to now, terrestrial organisms have played an important role as source of bioactive compounds. However, taking into account

that the marine biodiversity is far to be completely explored, and that the discovered biodiversity is much greater than that on land, there is a big expectation on new marine natural products in the years to come (Leal et al., 2012). Marine bioprospecting of new natural products has yielded several thousand novel molecules in the last decades and natural products represent a fundamental step in the new drug discovery process, with over the 30% of the natural or derived chemicals approved by the Food and Drug Administration, arising from natural products (Paterson and Nelson, 2017).

Since 1950's, the process of evaluating and isolating natural compounds from marine organisms began to be successful. Despite more than 40 thousand marine compounds discovered and the fact that the seas with their organisms are now clearly the greatest resource for the discovery of new bioactive compounds (Carroll et al., 2019; Jiménez, 2018; Deshmukh et al., 2017; Leal et al., 2016; Malve et al., 2016; Newman and Cragg, 2016b), a still limited number of new marine compounds have seen important (industrial or pharmaceutical) applications. They display interesting bioactivities in different application fields as cosmeceutics or nutraceuticals (Fusetani, 2010). Specifically, until 2014, only 12 marine compounds or their derivatives were used in clinical trials and only eight other compounds of marine origin were approved as drugs by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (Martins et al., 2014).

It is expected that the unique features of marine biodiversity, and the new tools now available for marine bioprospecting research will lead to the discovery of numerous new marine natural products with high bioactivity (Carroll et al., 2019; Khalifa et al., 2019).

### *1.2.2. Jellyfish compounds and related bioactivities*

Marine invertebrates like sponges are one of the most studied group of marine organisms, excluding marine bacteria. However, together with bacteria, they represent just a small amount of marine life that has been studied (Gerwick and Moore, 2012). Cnidarians, like jellyfish, sea anemones and corals have also been studied, with the Anthozoan orders that seem to have the highest number of organisms able to produce interesting compounds. Then, marine jellyfish with

their huge amount of biomass, currently represent a societal issue due to their seasonal outbreak (Brotz et al., 2012), anyway they could also represent a valuable source of bioactive compounds (D'Amico et al., 2017; Leone et al., 2015; Leone et al., 2013).

The peptide "aurelin" from *Aurelia aurita,* for example showed to be a very promising antimicrobial agent against bacteria (Ovchinnikova et al., 2006), as well as a novel polypeptide from *Cyanea capillata* jellyfish tentacles exerts growth-promoting healing properties in human cell cultures (Wang et al., 2019), and a metalloproteinase from *Rhizostoma pulmo* exerts good anticoagulant activity (Rastogi et al., 2017). Overall, jellyfish have been relatively under-exploited with a still limited number of jellyfish derived natural products (Carroll et al., 2019; Blunt et al., 2011-2018). In general, studies were focused on the proteinaceous compounds of jellyfish, anyway other non-proteinaceous component could be potentially interesting bioactive compounds, as the new polysaccharide (JSP-11) from *Rhopilema esculetum,* that displayed the ability to stimulate the human immune response via several regulating signalling pathways (Li et al., 2017).

Scyphomedusae, indeed, are made up of water for more than 95% (Lucas et al., 2011) while salt, proteins (mainly collagen) and other organic non-proteinaceous compounds (as lipids) are minor components. The percentage of these compounds can be very different between jellyfish species, with species of the order Rhizostomeae having more proteins than the other Scyphomedusae (Merquiol et al., 2019).

Probably, the most famous technological development from jellyfish components is the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria*, first isolated in 1962 (Stepanenko et al., 2009; Shimomura, 2005). After the discovery of this bioluminescent protein, improved synthesized versions of that GFP have been developed and are extensively used as a powerful tool (biomarker) for fluorescence imaging (Shaner et al., 2005).

Other jellyfish proteins that have been studied are the large toxic proteins (phospholipase and metalloproteases are the most common components) which constitute many jellyfish venoms (Merquiol et al., 2019). In most jellyfish species these proteins have not yet been defined in detail. However, these venoms have a wide range of effects on humans likely due to the diverse composition and

accordingly, they display a wide range of potent effects, like anti-tumoral or antioxidant activities (Ayed et al., 2012; Li et al., 2012).

Currently, marine collagen is considered a good alternative to the classical terrestrial source of collagen, mainly from bones and skin of porcine and bovine animals (Lin, 2011), due to the lack of risk of transfer of bovine spongiform encephalopathy (BSE). Collagen is a structural macro-protein (made by three polypeptide chains arranged in three helixes) and the main protein in the extracellular matrixes. The complexity of the structure allows amino acids to form about 20 different type of collagen (Ferreira et al., 2012) and every collagen form plays a different role in different tissues. Collagen accounts for up to 30% of proteins in mammals (Tong et al., 2000) and for up to 60% in jellyfish (Khong et al., 2016; Addad et al., 2011).

Among marine organisms, the yield of collagen obtained from jellyfish is usually greater when obtained by pepsin hydrolysis that seems the most effective extraction process (Leone et al., 2015; Barzideh et al., 2014a). Jellyfish collagen seems highly biocompatible for human body (Jankangram et al., 2016) and it seems to have similar biological effects on human cells, like the mammalian type I collagen (Addad et al., 2011). Moreover, collagen and collagen hydrolysate (collagen peptides) have been shown to exert several effects like immunomodulatory, antioxidant and wound healing ability (De Domenico et al., 2019; Felician et al., 2019; Sugahara et al., 2006). For example, collagenous peptides from the umbrella of rhizostomous jellyfish (*Rhopilema asamushi*) were able to restore collagen and elastin fibres after UV damages (Fan et al., 2013). A *Chrysaora sp*. collagenous peptide displayed a strong "angiotensin I" converting enzyme (ACE) inhibitory activity (Barzideh et al., 2014b) and others obtained from *Nemopilema nomurai* jellyfish boost the immune system by activating the production of inflammatory cytokines (Putra et al., 2015). In addition, the collagen peptides obtained from *Rhopilema esculentum* showed several interesting bioactivities (Felician et al., 2019; Cheng et al., 2017).

Not only proteins or derived peptides from jellyfish biomasses have peculiar bioactive properties. Zooxanthellatae species, such as *Cotylorhiza tuberculata,* are indeed characterized of a higher amount of lipids and phenols due to the presence of the endosymbiotic microalgae *Symbiodinium microadriaticum* (Leone et al., 2013). Jellyfish are very low in lipids, which are usually used as storage

units, primarily in the gonads and digestive tracts , and do not undergo transformation during the storage (Iverson, 2009). As for fatty acids (sub-units of complex lipids), poly-unsaturated fatty acids (PUFAs) are usually more abundant than mono-unsaturated fatty acids (MUFAs) in jellyfish. Literature data reported that the zooxanthaellatae *Cotylorhiza tuberculata* jellyfish has higher amount of lipids than the *Rhizostoma pulmo* and *Aurelia coerulea* jellyfish (Leone et al., 2015). Furthermore, phenol compounds, which are usually referred to as secondary metabolites produced by terrestrial plants, are widespread in nature including the marine environment, with marine micro and macroalgae that usually have the greatest amount of polyphenols (Capillo et al., 2018; Gall et al., 2015; Goiris et al., 2012).

The well-known antioxidant activity of phenol compounds depends in large part on the amounts of phenol rings (aromatic ring) and the hydroxyl groups (functional groups) they carry on (Heim et al., 2002). Their activity is related to the ability to scavenge the reactive oxygen species (ROS) that are naturally produced into an organism. Some phenols and polyphenols are now well recognized as protector against cancer and other diseases (Hussain et al., 2016; Pandey and Rizvi, 2009; Arts and Hollman, 2005; Graf et al., 2005). Moreover, polyphenols are able to act on cell signalling (Corona G et al., 2007; Khan et al., 2006), growth inhibition (Corona et al., 2009; Wang W et al., 2000), apoptosis induction (Fini et al., 2008; Mantena et al., 2006; Fabiani et al., 2002) and as modulator of enzymatic activity (Adams and Chen, 2009). However, there is only few literature data on jellyfish phenols (Leone et al., 2019, 2015; Costa et al., 2019), such as the jellyfish *Cotylorhiza tuberculata* that has endosymbiotic microalgae, which are known to contain phenol compounds (Leone et al., 2013).

### *1.2.3. Anticancer activity of jellyfish extracts*

Cancer is among the leading causes of death worldwide. In 2018, there were 18.1 million new cases and 9.5 million cancer-related deaths worldwide (Global Cancer Observatory, https://gco.iarc.fr/ ). Cancer risk factors, such as alcohol use, dietary factors, and obesity, can activate pathways leading to cellular stress, resulting in an abnormal production of reactive species (Kurutas, 2016). This cellular response induces an oxi/nitrosative stresses that alter the natural balance

between reactive species and antioxidants enzymes and scavengers (polyphenols, vitamins, and others) mostly obtained from extracellular sources (Devi et al., 2014; Ziech et al., 2010). This imbalance can alter or completely change the cellular dynamics, starting or promoting carcinogenesis (Isnaini et al., 2018; Hecht et al., 2016).

A greater emphasis has been also placed to the pathogenesis of cancer and its prevention and treatment by natural antioxidants (Kruk et al., 2015). Chemotherapeutic drugs for the treatment of cancer discovered since the mid-nineteenth century are effective and can help to recover, but usually with non-avoidable adverse side effects (Carr et al., 2008). The reduction of these side effects and the discoveries of new drugs spur researchers to find novel chemotherapeutic agents from natural sources.

Among the biological activities, anticancer and/or anti-proliferative activity were attributed to several jellyfish extracts or compounds. Partially purified venom demonstrated anti-tumoral properties against human cancer cells (Ayed et al., 2012), although there are still large gaps about the biological activities of jellyfish venoms (Badré, 2014). Several jellyfish proteins and peptides have been also demonstrated to show antioxidant capacity and other important biological effects, including anti-tumoral ones (De Domenico et al., 2019; Zhang Q. et al., 2018; Leone et al., 2015; Barzideh et al., 2014b; Leone et al., 2013; Zhuang et al., 2012, 2009; Addad et al., 2011; Ding et al., 2011; Morais et al., 2009; Yu et al., 2006). *Cotylorhiza tuberculata* (Macri, 1778), one of the most common zooxanthellatae jellyfish in the Mediterranean Sea, contain phenol and other antioxidant compounds. The extracts of this jellyfish exert antioxidant ability and a cell-specific cytotoxicity against MCF-7 breast cancer cells but not against HEKa (non-malignant) epidermal keratinocytes (Leone et al., 2013). Phytochemicals with antioxidant activity (Zhou et al., 2016; Leone et al., 2012) might prevent and treat cancer and other human diseases (Rosillo et al., 2016) or might be useful for the protection against cancer therapy-induced side effects (Zhang Q.Y. et al., 2018; Mohan et al., 2013).

Also, *Cassiopea andromeda* venom exerts haemolytic and acetylcholinesterase inhibitory activities (Mohebbi et al., 2018; Nabipour et al., 2017) and induce ROS-mediated apoptosis in fresh breast tumour tissue (Mirshamsi et al., 2017). Furthermore, a lipophilic extract from *C. andromeda* showed potent inhibitory

activity against HIV-1 protease (Ellithey et al., 2014), and *C. andromeda* crude methanolic extract had antibacterial properties against 5 strains of bacteria isolated from marine environments (Bhosale et al., 2002). No other cancer research data are available for the so-called upside-down C. *andromeda* jellyfish.
Discovering anti-cancer molecules from marine organism represent the trend of the marine pharmacology, a new discipline that explores the marine environment searching lead compounds for potential pharmaceuticals. In this framework the marine jellyfish could also become an option for anticancer drug discovery. It is now recognized a link between cancer and imbalance of the oxidative status of the cells.

#### *1.2.4. GJIC and cancer, and jellyfish extracts as anti-proliferative agents*

Cell to cell communication across gap junctions is essential in maintaining the homeostatic balance of multicellular organisms, and aberrant Intercellular Gap Junctional Communication (GJIC) has been implicated in tumour promotion and many chronic diseases. Since the oxidative stress has also been implicated in similar conditions such as cancer, a link between oxidative stress and GJIC functionality has long been reported (Upham et al, 1997). Gap Junction Intercellular Communication (GJIC) are intercellular channels that allow the exchange of compounds usually less than 1 kDa in mass (signalling molecules, ions, nucleotides, and other small or tiny compounds e.g., glucose) (Alexander and Goldberg, 2003). Gap junction channel functions in multicellular organisms includes homeostasis regulation, cell adhesion, cell migration, as well as endocrine, paracrine and autocrine signalling transduction, and the extrinsic apoptotic pathway. Gap junctions, also modulate the gene expression of adjacent cells via exchange signalling molecules, being the base of the extracellular communication network between cells and other tissues (Zong et al., 2016; Trosko, 2011; Goodenough and Paul, 2009; Mese et al., 2007; Evans and Martin, 2002).
Abnormal GJIC seems to be implicated in the development of cancer (Mesnil et al., 2005; Trosko and Ruch, 2002, 1998) anyway they can play very different roles in the early and late stages of carcinogenesis (Fig. 6 and 7) (Zefferino et al., 2019). As GJIC acts as tumor suppressor in the initial stages of tumorigenesis, their

activation (increase of cell-cell communication) in the promoter phase could have an anti-tumor-promotion activity.

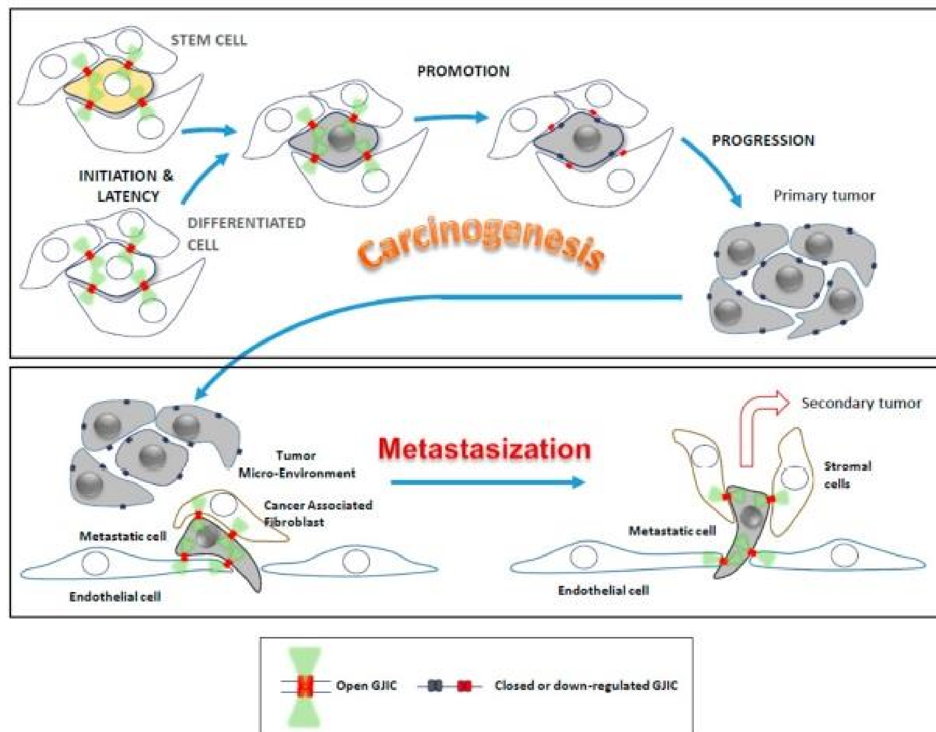


*Figure 6.* Different roles of GJIC in early and late stages of carcinogenesis involving either stem cells or differentiated cells. Intact (not inhibited) GJIC acts as tumor suppressor in the initial stages of tumorigenesis, it is inhibited in the promoter phase, and it is reactivated (re-opened) during the metastasizing stage. In migrating tumor cells, re-expression of Connexins promote tumor metastasis, enabling cell-to-cell communication between tumor cells and cancer-associated fibroblasts, as well as endothelial and stromal cells. The same might happen in the secondary tumor. © Zefferino et al., 2019; [https://doi.org/10.3390/cells8080896](https://doi.org/10.3390/cells8080896) (CC BY 4.0)

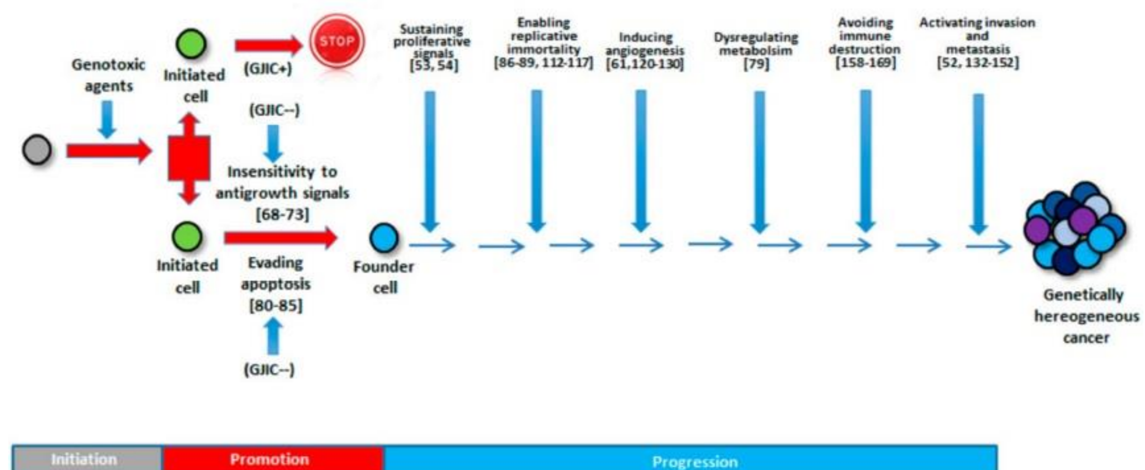


*Figure 7.* Involvement of GJIC in the different steps of carcinogenesis. Carcinogenesis is characterized by "initiation," "promotion," and "progression" phases. After a stem/progenitor cell is initiated by genotoxic agents, this is followed by promotion of cell growth. If functional

GJIC is expressed, the initiated cell will be stopped at first step (Initiation). On the other hand, if these initiated cells are exposed, chronically, to agents that down regulate GJIC, they will lose sensitivity to antigrowth signals and evade apoptosis, giving rise to the so-called "founder" cell. During the progression phase, these cells would proliferate, accumulate, and accrue sufficient genetic/epigenetic changes that will allow them to acquire all the hallmarks of cancer. At the end, a tumor will be a mass of genetically heterogeneous cells. References showing the role of GJIC during all the described phases are indicated in square brackets (see Zefferino et al. 2019). GJIC+: Gap Junction Intercellular Communication functioning; GJIC–: Gap Junction Intercellular Communication inhibited. © Zefferino et al., 2019; https://doi.org/10.3390/cells8080896 (CC BY 4.0)

Cancer cells indeed often display a loss of GJIC due to the dysregulation of connexin expression and unlike normal cells, are not inhibited by the contact with the adjacent cells and are not able to terminally differentiate or to enter apoptosis, neither to stop proliferating; therefore, GJIC are regarded as a target for cancer preventive agents (Trosko, 2019; Trosko, 2007; Trosko, 2003; Trosko and Chang, 2001). Thus, a well-regulated GJIC process seems to be a strategic chemoprevention therapy (Leone et al., 2012; Trosko and Chang, 2000). Since to reverse the downregulation of GJIC a fundamental key of an anticancer drugs (Lee et al., 2010a, 2010b; Trosko and Ruch, 2002; Kang et al., 2002; Trosko and Chang, 2001; Sai et al., 2000, 2001), the GJIC modulation has been proposed as an evaluation tool of the antitumor mechanism of natural bioactive compounds (Leone et al., 2012).

Gap junctions have been shown to be involved in the apoptotic process (Wilson et al., 2000), and a good strategy would be to prevent the down regulation of GJIC, by tumour promoter agents (Romo et al., 2019; Sovadinova et al., 2016) or to force the up-regulation of GJIC in non-communicating cancer cells and, several natural compounds are able to modulate GJIC, allowing cell-cell communication and inducing apoptosis through the passage of chemical signals (Leone et al., 2019, 2013, 2012; Li et al., 2012; Zhao et al., 2011; Fornelli et al., 2007; Lee et al., 2006, 2004; Ale-Agha et al., 2002; Chaumontet et al., 1997). The apoptosis induced by a transient cell-cell communication in cancer is able to reduce the number of cancer cells. This finding inspired the growing interest in GJs as novel therapeutic targets (Gakhar et al., 2010; Salameh et al., 2005) and many natural compounds with anti-tumor activity could act in this way (Fig. 8).

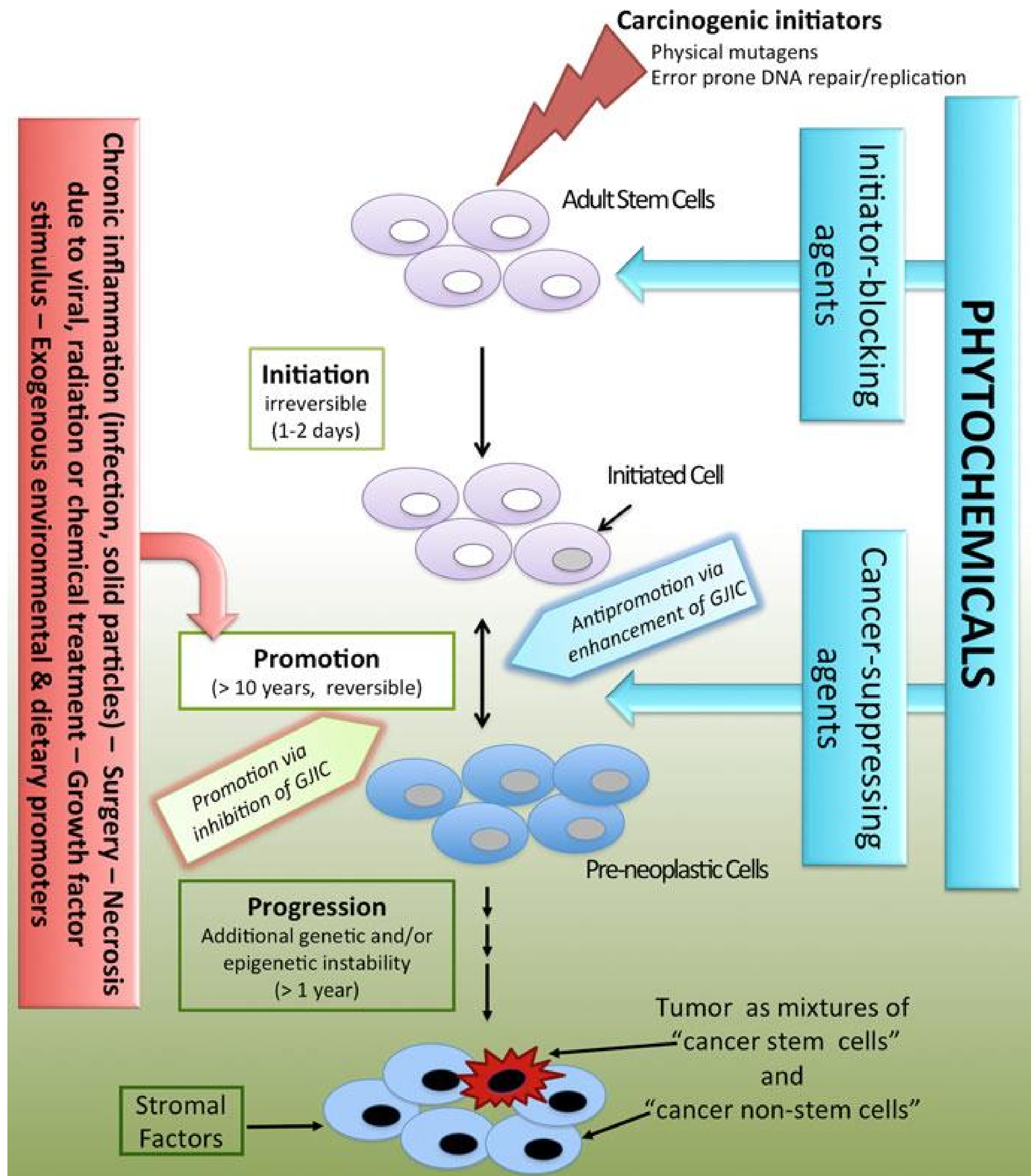


*Figure 8.* Scheme of postulated actions of phytochemicals in cancer chemoprevention. Courtesy from Leone et al., 2012, Phytochem Rev DOI 10.1007/s11101-012-9235-7

The action mechanisms of these natural chemopreventive agents are yet not known in detail, but it is hypothesized that there is an induction of signal transduction due to oxidative stress that alter gene expression at all levels (Leone et al., 2012; Upham et al., 2009).

Several marine compounds from microalgae and cyanobacteria are able to regulate GJIC (Nováková et al., 2011; Stahl and Sies, 2005). It was demonstrated

that extracts of the jellyfish *Cotylorhiza tuberculata* are able to exert their anti-proliferative activity through the positive modulation of GJIC in cancer cell model (MCF-7) (Leone et al., 2013).

In the present thesis two main works are presented.

**1)** The biochemical characterization of an Italian population of the jellyfish *Cassiopea andromeda*, which was studied for its biochemical composition and antioxidant activity in whole jellyfish and their different body parts (umbrella and oral arms), also aqueous and hydroalcoholic extracts were tested for proteins and phenols content and antioxidant activity.

**2)** An in-depth study on the composition and bioactivity of partially purified fractions of an hydroalcoholic extract of umbrellas and oral arms of *C. andromeda*; the biological activity, namely the antioxidant and anti-proliferative activity, was evaluated on human (MCF-7 and MB-MDA-231) and murine (WB-*ras*) cancer cell lines and on murine (WB-*neo*) non-tumorigenic cell line; it was also assessed their ability to modulate the functionality of the gap junction intercellular communications (GJIC), as possible action mechanism.

# 2. *Materials and Methods*

## *2.1. Reagents, materials, and devices*

*Reagents and materials for Jellyfish extraction*

Ethanol and Acetonitrile were purchased from Sigma-Aldrich (Merck Life Science srl, Milan, Italy). Dulbecco's phosphate buffered saline (DPBS) without calcium and magnesium (GIBCO) was purchased from Thermo Fisher Scientific, Waltham, MA, USA.

*Reagents and materials for cells culture*

Antibiotic antimycotic solution (100×) containing 10000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml were purchased from Sigma-Aldrich (Merck Life Science srl, Milan, Italy). While the recovery cell culture freezing medium (GIBCO), trypsin-EDTA 0.05% solution (GIBCO), RPMI 1640, no glutamine, no phenol red (GIBCO), Dulbecco's Modified Eagle Medium (DMEM) with 1 g/l D-glucose, with pyruvate, no L-glutamine, no phenol red (GIBCO), dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium, no phenol red (GIBCO), fetal bovine serum, FBS (GIBCO), L-Glutamine 200 mM (GIBCO), fetal bovine serum (FBS), south America origin (Biosera), L-Glutamine 100x 200 mM (Biosera), trypan blue stain (0.4%), cell counting chamber slides (Invitrogen, Carlsbad, CA, USA) were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Cell culture flasks 25 $cm^2$ (Cellstar) were purchased from Greiner bio one, SD100 cellometer – cell counting chambers were purchased from Nexcelom Bioscience Ltd.

*Reagents and materials for biochemical characterization*

For biochemical assays like protein and phenols quantification and antioxidant capacity determination, bovine serum albumin (BSA), 2,20-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3,4,5-Trihydroxybenzoic acid (gallic acid), potassium persulfate, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) and Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Merck Life Science srl, Milan, Italy). While the Bio-Rad protein assay dye reagent concentrates was purchased from Bio-Rad laboratories (Munich, Germany) and the 96 Well clear

polystyrene round-bottom microplates were purchased from Corning®, NY, USA.

*Reagents and materials for cells culture assays*

Annexin V Alexa Fluor™ 488 conjugate, 1X Annexin V Binding buffer and Propidium Iodide - 1.0 mg/ml Solution in Water were purchased from Thermo Fisher Scientific, Waltham, MA, USA. While 96 Well TC-treated flat-bottom microplates and 75 $cm^2$ rectangular canted neck cell culture flask with vented cap were purchased from Corning®, NY, USA. MTS CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI, USA).

Hoechst, Trihydrochloride, Trihydrate - 10 mg/ml Solution in Water (Invitrogen™) and 5-Carboxyfluorescein diacetate (5-CFDA-AM) (Eugene, molecular probes by life technologies) were purchased from Thermo fisher scientific.

Propidium Iodide 10 mg, Lucifer Yellow CH dilithium salt 100 mg, Neutral Red (NR) solution, 100 ml, sterile filtered, 7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt (Resazurin - RES) 1 g, phorbol 12-myristate 13-acetate (TPA), Sodium butyrate (NaBut) and tissue culture test plate 96F, growth enhanced treated, sterile, were purchased from Sigma-Aldrich.

Micro knife blades, pro stencil knife blade #17 were purchased from Proedge Blades, Paterson, NJ.

Formaldehyde 36-38% 2500 ml was purchased from Lachner sro, Czech Republic.

Iwaki microplate 48 wells with lid, flat bottom, tissue culture treated, polystyrene, science products dept. was purchased from Asahi glass co ltd, Japan.

Cell culture microplate 96 wells, polystyrene, flat bottom (chimney well), µclear, black, tissue culture treated, lid with condensation rings, sterile and cell culture flasks 25 $cm^2$, Cellstar were purchased from Greiner bio one.

*Main devices*

Infinite M200, quad4 monochromator™ detection system was purchased from TECAN Life Sciences Group (Männedorf, Switzerland). Countess™ Automated Cell Counter was purchased from Invitrogen, Carlsbad, CA, USA. Buchi R-205

with Vacuum pump V-710 and Vacuum controller V-850 was purchased from Buchi, Cornaredo MI, Italy. Freezone 4.5L Dry System, Labconco Co. was purchased from Thermo Scientific, Kansas City, MO, USA. Zeiss LSM 5 Pascal laser scanning confocal microscope (ZEN software) was purchased from Carl Zeiss, Munchen, Germany. Forma™ Direct Heat $CO_2$ Incubators was purchased from Thermo Scientific, Kansas City, MO, USA. Cellometer Auto T4 Bright Field Automated Cell Counter (Nexcelom Bioscience) with cellometer autocounter software ver. 3.3.8.3 was purchased from Nexcelom Bioscience Ltd. Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek) with GEN5 software was purchased from BioTek (Germany). Axio Observer Z1 widefield fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with Hamamatsu digital camera C11440-22CU orca flash 4.0, X-CITE series 120PC Q Lumen dynamics, tissue diagnostics automated platform and tissueFAXS cell analysis system 4.2 software were purchased from Carl Zeiss AG (Germany). Milli-Q water purification system was purchased from Millipore, Bedford, MA.

## 2.2. *Jellyfish samples, extraction, and fractioning*

*Sampling*

*Cassiopea andromeda* (Forsskål, 1775) jellyfish employed in this work were collected inside the harbour "la Cala" of Palermo, Sicily, Italy. Jellyfish were found in November 2017 at a depth between 0.5 and 2 m. Samples container was then transferred to laboratory and after biometric measurement (weight and bell diameter) several samples were separated in oral arms and umbrella of each specimen. The jellyfish samples were immediately frozen in liquid nitrogen and stored at −80 °C until lyophilization. Lyophilized jellyfish samples (Fig. 9) were stored in small tubes at -20 °C until use.

***Figure 9***. Lyophilized *Cassiopea andromeda* jellyfish samples. Each couple of tubes represent a different specimen divided into Umbrella and Oral Arms.

### 2.2.1. *Soluble compounds extraction and sequential hydrolysis of insoluble compounds*

*Cassiopea andromeda* lyophilized tissues samples (whole organisms or oral arms and umbrella) were finely powdered with mortar and pestle and liquid nitrogen and the resulted dry powder was subjected to PBS extraction and further sequential hydrolysis (Fig. 10).

### 2.2.2. *Aqueous soluble extraction (PBS extraction)*

Aqueous soluble compounds were extracted with 16 volumes (w/v) of phosphate buffer saline (PBS) pH 7.4 and stirred for 2 h, at 4°C, then centrifuged at 9000 × g for 30 min at 4 °C. After supernatant separation, soluble compounds were then analysed for protein and phenols content and antioxidant activity, with biochemical assays as further described.

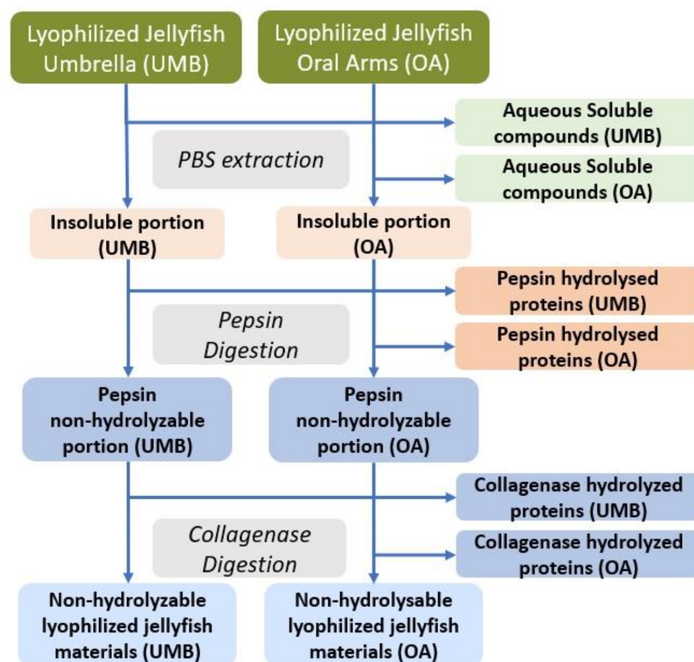


***Figure 10***. PBS extraction and sequential hydrolysis methodology (Leone et al., 2013)

### 2.2.3. *Hydroalcoholic extraction*

*Cassiopea andromeda* lyophilized tissues samples (oral arms and umbrella) powdered in liquid nitrogen were also subjected to hydroalcoholic extraction by stirring in 16 volumes (w/v) of 80% ethanol, by a rotary tube mixer with speed at 25 rpm, for 16 h at 4 °C. Samples were then centrifuged at 9000 × g for 30 min, at 4 °C, the supernatants were separated from the insoluble material (Fig. 11), an aliquot of each supernatant was used for biochemical assays.

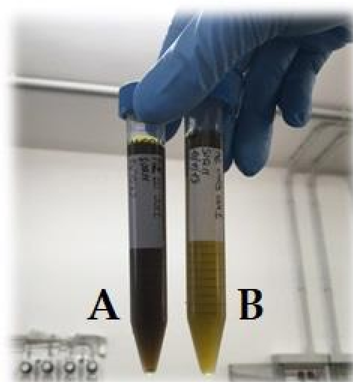


***Figure 11.*** Tubes with the extracts of *Cassiopea andromeda* jellyfish after the hydroalcoholic extraction from the insoluble material. The darkest extract (A) was from the Oral Arms and the lightest one (B) was from the Umbrella.

Hydroalcoholic extracts from whole jellyfish or umbrellas and oral arms were concentrated by vacuum rotary evaporator (Buchi R-205 with Vacuum pump V-710 and Vacuum controller V-850) at low temperature, treated under a stream of nitrogen gas in order to completely evaporate the ethanol, and then lyophilized at -40 °C in a chamber pressure of 0.110 mbar (Freezone 4.5L Dry System). All the operations were performed in light-protected conditions and at 4 °C in order to limit loss of activity. Some samples were subjected to both aqueous and hydroalcoholic extraction, in different sequence in order to verify their efficiency.

### 2.2.4. *Hydrolysis of insoluble compounds*

The insoluble compounds present in the pellets after aqueous or hydroalcoholic extraction were then subjected to sequential enzymatic hydrolyses with 1 mg/ml of pepsin (enzyme/pellet ratio of 1:50, w/w) in an acidic environment (0.5 M acetic acid) and stirred for 48 h at 4 °C. After the enzymatic digestion, the sample was centrifuged at 9000 × g for 30 min and the pepsin-hydrolysed compounds in the supernatants were stored for further biochemical quantification and evaluation analysis. The residual pellet was washed two times with bi-distilled water and then, the insoluble pepsin non-hydrolysable portion was subjected to a second enzymatic digestion with 6 mg/ml of collagenase (enzyme/substrate ratio of 1:50, w/w) with TES buffer 50 mM, pH 7.4 and 0.36 mM of $CaCl_2$, and stirred for 5 h at 37 °C. After the end of the hydrolysis, the sample was centrifuged at 9000 × g for 30 min, and the collagenase-hydrolysed peptides present in the supernatants were stored for further biochemical quantification and evaluation analysis. The residual pellet after collagenase digestion was considered as not-hydrolysable jellyfish material (Fig. 10).

### 2.2.5. *Hydroalcoholic extract fractionation*

The hydroalcoholic extracts obtained as above described were subject to the fraction's separation protocol, a described in Leone et al. (2013) by a cold-induced acetonitrile/water (ACN/$H_2O$) phase separation (Fig. 12).

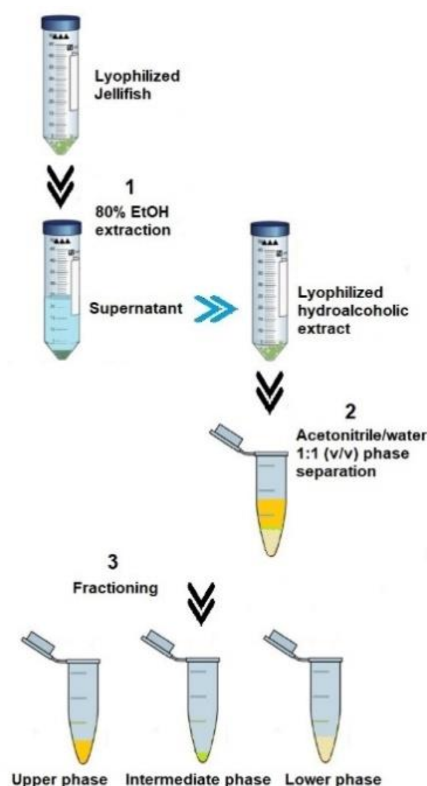


*Figure 12.* Steps of extraction and phase separation from the lyophilized jellyfish samples (Leone et al., 2013)

Briefly, each dried extract was weighed and 1 ml of iced acetonitrile/$H_2O$ 1:1 (v/v) was added to 50 mg of dried extract. After stirring by using Vortex mixer, the suspension was left on ice for 30 minutes to facilitate a phase separation. The ACN/$H_2O$ phase separation drives the separation of complex mixture of compounds into 3 distinct phases (Fig. 13), an upper phase that is rich in ACN, an intermediate phase, and a lower phase rich in water, but still containing enough acetonitrile to avoid freezing at -20 °C for at least one week (Shao et al., 2017).

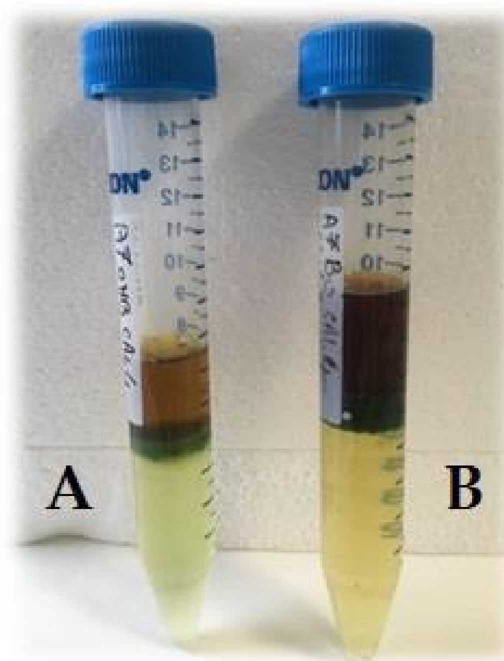


*Figure 13.* Three distinct phases, after 30 min on ice and ACN/$H_2O$ separation; the tube A shows the lightest colours of the Umbrella phases, while the tube B shows the darkest colours of the Oral Arms phases.

The three phases Upper Phase (UP), Intermediate Phase (IP) and Lower Phase (LP) were then better separated by centrifugation at 9000 x g (15 min at 4 °C) (Fig. 14), and the Upper and Lower phases were then separately analysed. Protein concentration, antioxidant activity and phenol content were evaluated.

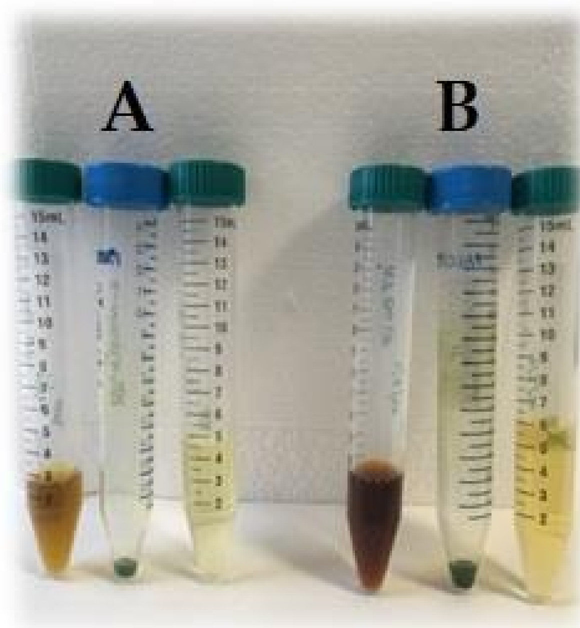


*Figure 14.* Fractions obtained by acetonitrile precipitation from hydroalcoholic extracts. From the left: Upper, Intermediate, and Lower Phases of the umbrella (A) and oral arms (B) of the same specimen.

## 2.3. *Biochemical characterization*

### 2.3.1. *Protein content*

The well-known Bradford colorimetric assay (Bradford, 1976) was adapted to 96-well round bottom microplate (Corning) and used to determine protein concentration. Analysis was carried out by Infinite M200 (TECAN Life Sciences Group), quad4 monochromator™ detection system. A calibration curve using bovine serum albumin (BSA) was used for the protein concentration estimation. The absorbance value read at 595 nm were plotted on a linear standard curve (from 1 to 16 μg/ml of BSA) to evaluate the amount of the proteins in each sample; this step was done in triplicate and the protein concentration in the samples (μg/μl) was then used to define the tested concentrations for cytotoxicity assays. The BioRad bradford reagent uses a brilliant dye (Coomassie blue G250) that binds proteins, increasing its absorbance wavelength value. The colour of the reaction shifts from red to a brilliant blue in a linear way with the amount of proteins. This new deprotonated complex is stable for an hour at least, but with an absorbance wavelength value that shift from 470 to 595 nm (the plateau of the absorbance is reached in ten minutes). Of course, not all the aminoacidic residues (mainly arginine, lysine, and histidine, but also tyrosine, tryptophan, and phenylalanine) are able to interact with the dye reagent, each of them with a different strength (Compton and Jones, 1985); This means that, just the absorbance of the amount of them that interact with the Coomassie Brilliant Blue G250 dye reagent is correlated with the absorbance of the plotted BSA curve.
The amount of μl of sample used for each well was different (usually between 1 to 10 μl), depending on the expected concentration of protein samples and value obtained. Appropriate blanks (PBS, 80% ethanol, Acetonitrile or Acetonitrile/Water, 1:1, v/v) were prepared with the same volume of the relative samples and MilliQ water needed. For each well a fixed final volume (300 μl) and Dye reagent (60 μl) were used.

### 2.3.2. *Collagen content*

As Bradford assay, Lowry's method is one of the most used procedure for protein quantification, but both standard methods lead to unreliable amount evaluations

when applied to collagen samples due to the presence of the triple helix and the specific amino acid content in collagen. A modified version by Kiew and Don, 2013 of the Lowry's method firstly developed by Hartree, 1972 has been used to evaluate and compare with the Bradford assay the amount of collagen in collagenase hydrolysed samples. The assay was carried out by preparing two different reagents: reagent A, 40 mg of potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6$-4 $H_2O$) and 1 g of sodium carbonate ($Na_2CO_3$) in 10 ml of 0.5 M sodium hydroxide (NaOH) and B, 20 mg of potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6$-4$H_2O$) and 30 mg of copper (II) sulphate pentahydrate ($CuSO_4$-5$H_2O$) in 1 ml of 0.1 M NaOH. Forty-five µl of reagent A and 5 µl of reagent B were added and mixed with 50 µl of samples and then incubated at 50 °C for 20 min without light. Then, 150 µl of 2N 1:15 Folin Ciocalteu's reagent was added to the samples, mixed and incubated at 40 °C for 20 min and, the absorbance was read at 630 nm. A solution (200 µl) of 1 mg/ml of collagen was used as control. All samples and controls were evaluated in triplicate.

### *2.3.3. Total phenolic content*

The content of total phenols was determined by a modified Folin-Ciocalteu colorimetric method (Magalhaes et al., 2010).

This methodology is based on an oxidation/reduction (redox) reaction (Prior et al., 2005). The transfer of an electron in an alkaline medium from a phenolic compound to molybdenum to form a blue complex is the principle of this colorimetric method. The test solutions containing 50 µl of sample were mixed with 50 µl (1:4) of Folin Ciocalteu's phenol reagent and with 100 µl of 0.35 M Sodium hydroxide (NaOH). After 5 min, at room temperature in the dark, the absorbance was spectrophotometrically measured at 720 nm (the higher wavelength minimizes possible interference from the sample matrix, which is often coloured). The calibration curve was plotted versus concentrations of gallic acid ranging from 0 to 40 µg/ml, used as standard. The results were expressed as µg of gallic acid equivalents (GAE) per gram of dry extract.

### 2.3.4. *In vitro antioxidant capacity*

The total antioxidant capacity was assayed by TEAC (Trolox Equivalent Antioxidant Capacity) method (Re et al., 1999) based on the scavenging of the blue/green ABTS radical 2,20-azinobis-(3-ethyl-benzotiazolie-6-sulfonic acid), that is converted into a colourless product by natural antioxidants present into the samples.

The assay was adapted to 96-well microplate (Corning) for Infinite M200 (TECAN Life Sciences Group). This methodology needs the pre-formation of the radical cation (ABTS+ solution) prior to the addition of the antioxidant. Appropriate blanks with the relative solvent were run in each assay and a Trolox calibration curve was prepared under the same conditions of the samples. Briefly, 10 μl of each sample was added to 200 μl of ABTS+ solution, were stirred and the absorbance at 734 nm was read after 6 min. The antioxidant activity was expressed as nmol of Trolox equivalents (TE) per g of dry weight, mg of contained proteins and μg of gallic acid equivalents.

### 2.4. *Human breast cancer cell cultures*

Breast cancer cell line (MCF-7), containing the estrogenic receptor, was obtained from the European Collection of Cell Cultures (ECACC, London, UK), while triple-negative breast cancer cell line (MB-MDA-231) was a kind gift of Dr. Daniele Vergara of University of Salento (Lecce, Italy).

Cell lines (Fig. 15) were routinely grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin in 75 $cm^2$ plastic flasks (Corning) at 37 °C in a 5% $CO_2$ humidified atmosphere.

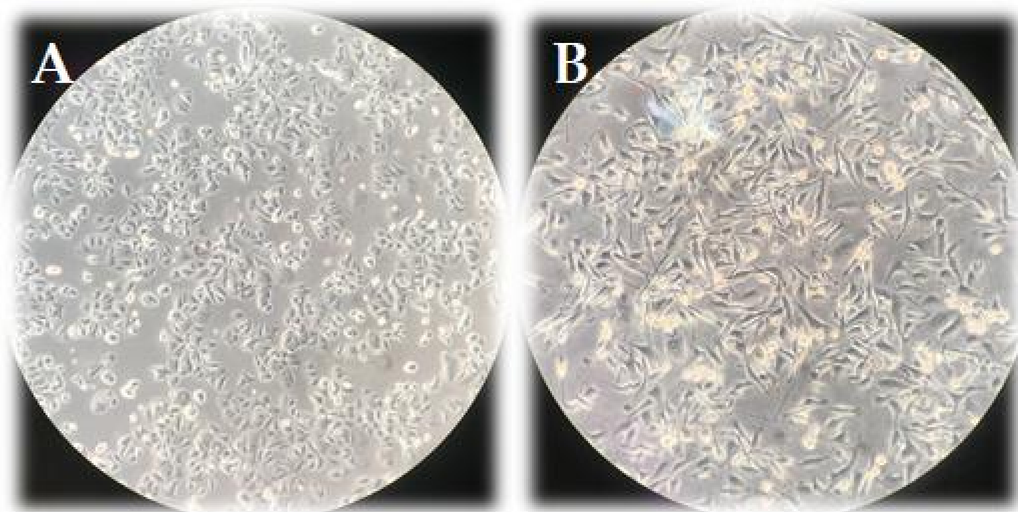


***Figure 15.*** Morphological aspect of MCF-7 (A) and MB-MDA-231 cells (B) observed by phase contrast microscopy at 20X. MCF-7 with luminal epithelial phenotype whereas MB-MDA-231 with an elongated fibroblast-like phenotype.

Thawing and freezing procedures were carried out at the start of the research and when needed during the years, to avoid high number of passages. Cells were grown until 70% – 80% confluence, then passaged (about twice a week) by trypsinization: after washing with sterile Dulbecco Phosphate Buffer Saline (DPBS), cells monolayer was disrupted by incubation at 37 °C with 2 ml of pre-warmed 0.05% Trypsin-EDTA solution. Cell viability was assayed by Trypan blue dye exclusion assay associated to automated cell counting (Countess® Automated Cell Counter, Invitrogen Carlsbad, CA, USA), as suitable method to assess and constantly monitor the real number of live and dead cells (Fig. 16) between passages.

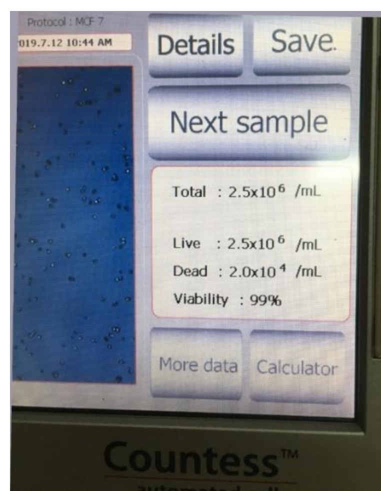


***Figure 16.*** Automated cell counter with MCF-7 protocol. Cells were incubated with Trypan blue stain (1:1) and so counted into the instrument.

After trypsinization, cells were pelleted by centrifugation at 200 x g for 5 minutes. The supernatant was discarded, and the cell pellet resuspended with 4 ml per flask of fresh medium.

### *2.4.1. Cells treatment with fractions of the jellyfish hydroalcoholic extract*

MCF-7 and MB-MDA-231 cells were seeded in flat bottom 96-well plates (Corning) at 25 × $10^4$ cells/well and 40 × $10^4$ cells/well respectively in 200 µl of RPMI medium supplemented with FBS, L-glutamine and antibiotics, and allowed to attach for 24 h at 37 °C in a 5% $CO_2$ humidified atmosphere. Cell culture were treated with 100 µl/well of supplemented medium containing the jellyfish sample at the following protein concentration 0.0001, 0.0005, 0.005, and 0.01 µg/µl and 0.0005, 0.005, 0.015, and 0.05 µg/µl for UP and LP, respectively. For negative controls, the tested compounds were replaced with medium or with the relative solvent at the same concentrations present in the fractions. Both controls were included in each plate.
Since some of the tested compounds (jellyfish extract fractions) were coloured, in order to verify and eliminate the possible interference with the colorimetric assay, the tested compounds in the medium without cells were included in each experiment. The absorbance at 490 nm only of the used jellyfish fractions was evaluated and subtracted to the absorbance in the treated cells during cytotoxicity calculation. The cells were incubated for 24 h at 37 °C and 5% $CO_2$ and assayed for vitality by MTS assay according to the scheme presented in Figure 17.

***Figure 17.*** Experimental scheme of the cell treatments with UPs and LPs. Controls without treatments and controls with treatments but without cells were run for each experiment (n=4). Cells viability was analysed using MTS assay.

### 2.4.2. *Cells viability assessment by MTS assay*

Cell viability in treated and not treated cell culture were assayed by MTS assay. Twenty microliters of CellTiter 96® Aqueous One Solution Reagent were added to each well. When the absorbance of the controls reaches similar values between cells near 1 (about an 1 h for MCF-7 and 2 h 30′ for MB-MDA-231, due to a different ability to metabolize MTS) the cell viability was determined as assessment of metabolic activity (indirect measure of viable cell number) by measuring the absorbance at 490 nm using a TECAN M200. The assay was performed in quadruplicate for four independent experiments and the results were expressed as a percentage of the relative control.

### 2.4.3. *Apoptosis evaluation in cell cultures*

In order to evaluate possible pro-apoptotic effect of jellyfish extract fractions, Annexin V Alexa Fluor™ 488 conjugate and Propidium Iodide (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit. Invitrogen®) were used on cells seeded in 96-Well TC-Treated Microplates (Corning®) and treated with different concentrations of jellyfish fractions. Labelling of cells was performed according to the manufacturer's instructions. Fluorescent cells were immediately detected by laser scanning confocal microscope (LSM Pascal, Carl Zeiss, Munich, Germany). Digital images were recorded for each treatment and controls and pictures showing cells stained with propidium iodide (PI) and Annexin V Alexa Fluor™ 488 conjugate or with both the dyes (co-localization) were considered for the assessment.

## 2.5. *Murine cancer cells cultures*

The following activities were carried out at the Research Centre for Toxic Compounds in the Environment (RECETOX) of the Masaryk University (Brno, Czech Republic) with SECANTOX group, Cell and Tissue Toxicology Lab, that is part of the Environmental Toxicology Division of RECETOX. The experimental work was carried out using lyophilized extracts previously obtained at ISPA-

CNR (Italy), as described, and previously tested on human breast cancer cell cultures.

Rat liver epithelial cells (WB-*neo* and WB-*ras*) were selected from the liquid nitrogen storing facility of RECETOX for the evaluation of the UPs and LPs effects on Gap Junction Intercellular Communication (GJIC) (Fig. 18).

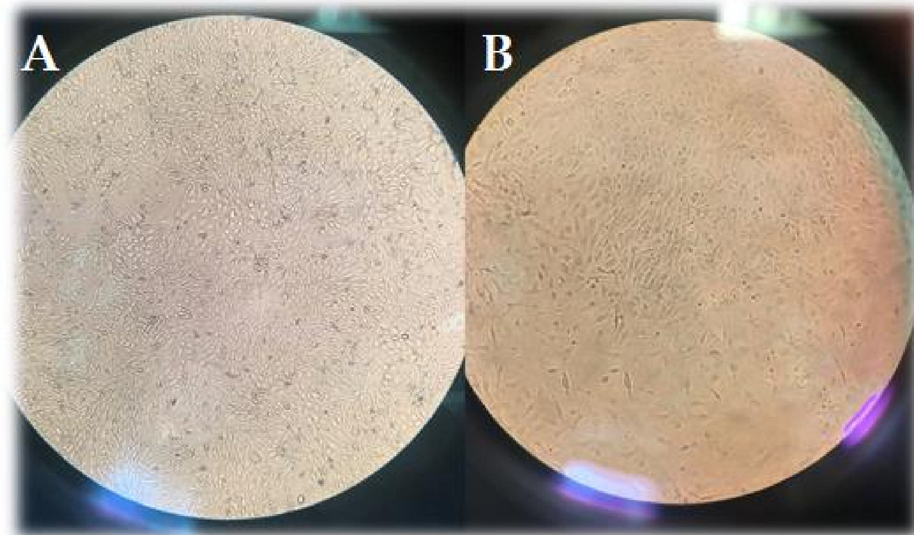


***Figure 18.*** Morphological aspect of WB-*ras* (A) and WB-*neo* cells (B) observed by phase contrast microscopy at 20x. WB-*neo* maintain the morphological aspect of WB-F344 cells, while WB-*ras* cells have an altered shape.

WB-*neo* and WB-*ras* cells were derived from WB-F344 rat liver epithelial cells via transfection of H-*ras* oncogene (WB-*ras*) or blank plasmid (WB-*neo*). WB-*neo* cells have a high expression of Cx43 (Connexin-43) and so a strong communication via GJs in vitro, while WB-*ras* have completely lost this capability (Ruch et al., 1993).

These two cell lines have been widely used as cell system to analyse the modulation of the functionality of the gap junction intercellular communications (DeoCampo et al., 2000; Hayashi et al., 1998; De Feijter et al., 1996, 1990; You et al., 1995). The two cell lines were routinely grown in DMEM medium supplemented with 7.5% FBS (Biosera) and 2 mM L-glutamine (Biosera) without antibiotics/antimycotics in 25 $cm^2$ plastic flasks (Cellstar) at 37 °C in a 5% $CO_2$ humidified atmosphere. Cells were passaged at 90% confluence about twice a week by trypsinization: cells monolayer washed with sterile PBS was treated by incubation for less than 3 minutes at 37 °C with 0.5 ml of pre-warmed 0.05% Trypsin-EDTA solution. After trypsinization and the addiction of 2 ml of supplemented medium, three drops of detached cells were directly dispensed

into new 25 $cm^2$ plastic flask and resuspended with 6 ml of fresh medium. While, when cells were needed for experiments, after trypsinization and the addiction of 2 ml of supplemented medium, the number of cells was evaluated by pipetting 20 μl of the resulting suspension (without Trypan Blue dye) into a cell counting chamber SD100 slide (Nexcelom Bioscience) and evaluated with a bright field automated T4 cell counter (Nexcelom Bioscience) with the related "Cellometer Autocounter" software (three drops of the same batch were however added into a new 25 $cm^2$ plastic flask and resuspended with 6 ml of fresh medium). Then, after done the appropriate dilution for the experiments, cells were seeded into 48 or 96 wells plates.

### *2.5.1. Cell viability assessment by 3-dye cytotoxicity assay*

The 3-dye cytotoxicity assay was used for cell viability determination (Boaru et al., 2006) in WB-*ras* and WB-*neo* cell cultures. This assay uses three dyes able to evaluate the break of several central cellular processes.
In detail:

- Resazurin (RES) (Sigma-Aldrich) cell permeable and poorly fluorescent dye is converted into the highly fluorescent product resorufin (excitation/emission wavelengths 530/590 nm) by the cellular dehydrogenase activity of the metabolically active cells, thus highlighting the residual metabolic reductive potential of the cells.
- The cell permeable probe 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) (Thermo fisher scientific) is cleaved by esterases and it is retained inside cells with intact membranes as a fluorescent product (excitation/emission wavelengths 485/520 nm); it is useful to evaluate the esterase activity itself and the membrane integrity and is an indirect measure of cell membrane integrity.
- Neutral Red (NR) (Sigma-Aldrich) is accumulated in intact lysosomal system; being the lysosomal system important for the maintenance of intracellular pH gradients, NR labels viable cells (Repetto et al. 2008; Guidelines for the Testing of Chemicals test n° 432 - OECD 2019). NR and the relative background were measured at wavelengths of 540 nm and 690 nm, respectively.

The dye resazurin (RES) is used in the Alamar blue® Assay (O'Brien et al., 2000; Borra et al., 2009), the reagent was made by dissolving 100 μg/ml (436 μM) of resazurin sodium salt powder in PBS solution. The solution was sterilized by filtration with 0.2 μm filter and stored at -20 °C. The 5-carboxyfluorescein diacetate acetoxymethyl ester (5-CFDA-AM) dye was prepared at the concentration of 2-10 μM and stored at -20 °C. Conversely, the Neutral Red 0.33% (3.3 g/l in DPBS) was a ready-to-use solution from Sigma (see above for details) and was stored at -4 °C.

Both WB-*ras* and WB-*neo* cell lines were seeded with 100 μl/well of supplemented medium at 2.5 x $10^4$ cells/$cm^2$ into black 96 wells plates (Fig. 19). After 24 h of growing, cells were treated with 100 μl/well of supplemented medium containing the jellyfish fractions at the following six final protein concentrations 0.0002 (C6), 0.0004 (C5), 0.001 (C4), 0.0025 (C3), 0.006 (C2), and 0.015 (C1) μg/μl and 0.0005 (C6), 0.0001 (C5), 0.003 (C4), 0.008 (C3), 0.02 (C2), and 0.05 (C1) μg/μl for UP and LP, respectively. For negative controls (M), the tested compounds were replaced with medium.

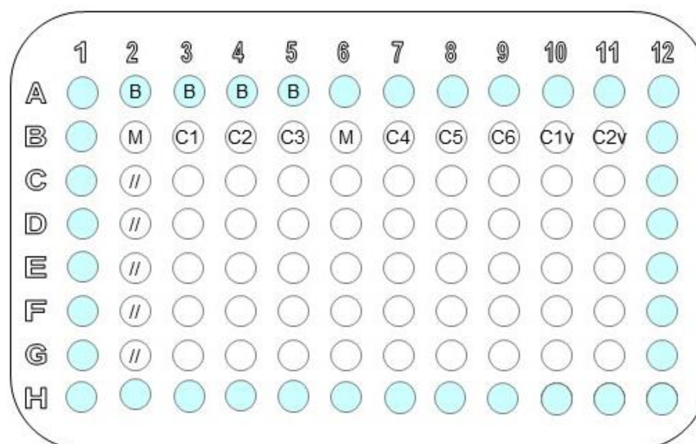


***Figure 19.*** Schematic view of the plate design. WB-*ras* cells were seeded from B2 to D11 and WB-*neo* from E2 to G11. All the external wells were with medium only to reduce evaporation. Column 2 and column 6 were filled with cells treated with supplemented medium only (negative controls - M), while, in the columns 3, 4, 5, and 7, 8, 9 cells were treated with different final concentrations of the tested jellyfish fractions (from C1 to C6, see above for details). In the columns 10 and 11 cells were treated with the same amount of the relative solvents of the first and second highest tested final concentrations. Wells from A2 to A5 were used for blanks during the assay.

To rule out any doubts about the cytotoxicity of the solvents (Acetonitrile and Acetonitrile/water, 1:1 v/v), two cytotoxicity evaluations (3-dyes assay) were done with the following final concentration between 0.29 to 5% v/v (0.29%, 0.44%,0.66%, 0.99%, 1.48%, 2.22%, 3.33%, 5% v/v), with both cell lines. Anyway,

we used as solvent control the solvent concentration present at the two highest concentrations of UP and LP fractions. Both negative and solvent controls were included in every experimental plate (Fig. 19). Then, after 48 h of treatment, cells were washed twice with PBS since the residual FBS from the medium seems interfering with the assay. Then cells were incubated for 30 minutes at 37 ºC with 100 µl of phenol red-free DMEM medium (without supplements) with 2.5% (v/v) RES and 4 µM 5-CFDA-AM without light. After that, cells were analyzed for fluorescence at 485/520 nm and 530/590 nm using SynergyMX reader (BioTek) with GEN5 software.

Cells were then washed with PBS and incubated at 37 ºC for 90 minutes with 100 µl of phenol red-free DMEM medium (without supplements) with 5% (v/v) of the ready-to-use 0,33% Neutral Red (NR) solution. Cells were then washed three times with PBS to remove the not incorporated dye, and then lysed with a solution of 1% Acetic acid in ethanol/water 50% (v/v) solution and stirred by an orbital shaker for 15 min (150 rpm) without light. At the end, the absorbance at 540 nm and 690 nm were evaluated using SynergyMX reader (BioTek) with GEN5 software. Blanks (B) were deducted, and the results were normalized with the not-treated controls (M) and expressed as percentage of viable cells on the total cells. The assay was performed in triplicate for three independent experiments (Fig. 20).

***Figure 20.*** Experimental scheme of the cell's treatments with UPs and LPs. Controls without treatments and blanks were run for each experiment (n=3). Cells viability was evaluated with 3-dyes cytotoxicity assay.

### *2.5.2. Assessment of GJIC by scrape loading-dye transfer (SL/DT) assay*

The Gap Junction Intercellular Communication (GJIC) functionality in the monolayer of cells was assessed by scrape loading-dye transfer assay. The protocol here used was developed by Dydowiczová et al. (2020), which modified and improved the original protocol developed by El-Fouly et al. (1987). This

methodology has been widely used to evaluate the functionality of the intercellular communication gap-mediated of many cells (Leone et al., 2013; Vinken et al., 2009; Abbaci et al., 2008; Trosko and Ruch et al., 2002). It is based on the evaluation of the number of communicating cells as compared to the control. Cell-cell communication is assessed by loading with the fluorescent dye Lucifer yellow non- permeant the membrane but able to pass through Gap Junction channels, the number of labelled cells and the diffusion area is representative of GJIC functionality (Fig. 21).

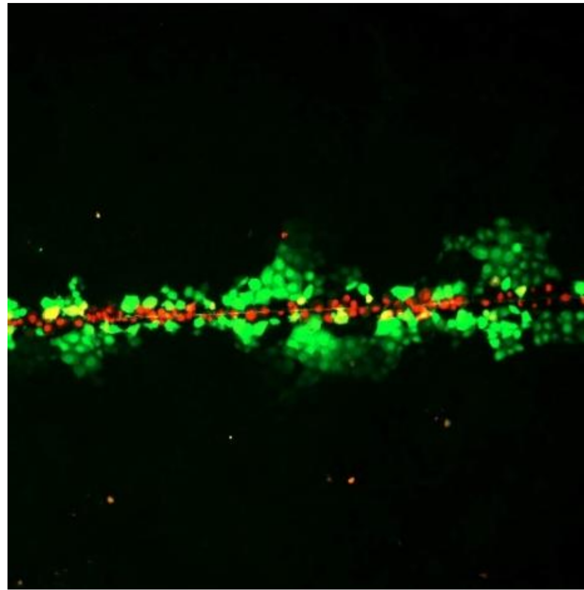

***Figure 21***. Overlapping between Lucifer Yellow (green area/cells) and the Propidium Iodide dyes (red area/cells) in WB-*ras* cells.

Both WB-cell lines were seeded with 250 µl/well of supplemented medium at 3 x $10^4$ cells/cm$^2$ into 48 wells IWAKI plates and treated with 150 µl/well of supplemented medium containing the jellyfish fractions at the following five protein final concentration in the well 0.0002 (C6), 0.0004 (C5), 0.001 (C4), 0.0025 (C3), 0.006 (C2) µg/µl and 0.0005 (C6), 0.0001 (C5), 0.003 (C4), 0.008 (C3), 0.02 (C2) µg/µl for UP and LP, respectively (Fig. 22).

*Figure 22*. Experimental scheme for the evaluation of the effects of UPs and LPs on GJIC. Negative control and positive controls (Sodium butyrate (Nabut) 1.6 mM and phorbol 12-myristate 13-acetate (TPA) 25nM) were run for each experiment (n=3) for both cell lines. TPA was added 30 min before the assay to temporary stop the communication between cells. Gap Junction Intercellular Communication was evaluated with SL/DT assay.

For positive controls, tested compounds were replaced with the same volume of supplemented medium only. The communications between WB-*ras* cells were activated with 150 µl of medium supplemented with Sodium butyrate (Nabut) in ethanol (1.6 mM) as a positive control. Phorbol 12-myristate 13-acetate (TPA) in ethanol (25 nM) was used as a positive control to temporary stop the communications between WB-*neo* cells (it must be added no more than 30min before the evaluation of the communication). All controls were included on each plate (Fig. 23).

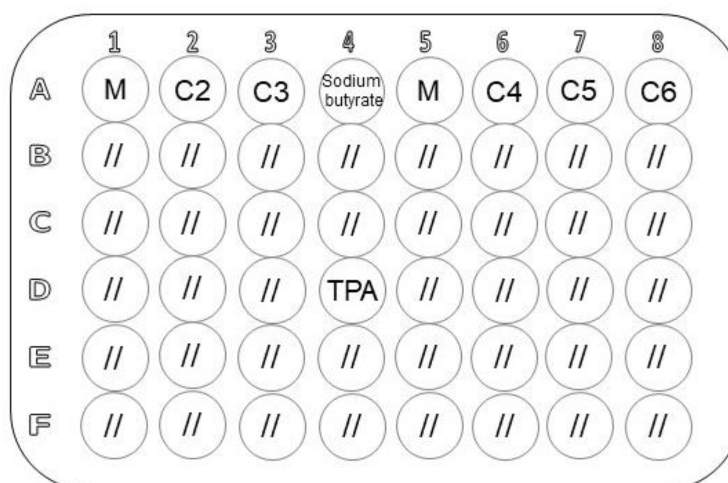


*Figure 23.* Schematic view of the plate design. WB-*ras* cells were seeded from A1 to C8 and WB-*neo* from D1 to F8. Column 1 and column 5 were filled with cells treated with supplemented medium only (negative control – M), while in the columns 2,3 and 6,7,8 cells were treated with different final concentrations of the tested jellyfish fractions (from C2 to C6, see above for details). Wells A, B, C, of the column 4 were filled with cells treated with Sodium butyrate (NaBut) as a positive control (1.6 mM), while wells D, E, F were filled with cells treated with phorbol 12-myristate 13-acetate as a positive control (TPA - 25 nM). Space between wells was filled with medium only to reduce evaporation.

After 24 h of cells growing and 48 h of treatment, cells were washed twice with phosphate-buffered saline (PBS) solution supplemented with calcium (0.68 mM)

and magnesium (0.49 mM) to avoid cells detachment and then, 150 µl Ca-Mg/PBS solution with Lucifer Yellow (1 mg/ml), Propidium Iodide (1 mg/ml) and Hoechst (10 mg/ml) was added to each well.

Scrape-loading dye transfer assay was carried out by two parallels cuts (Fig. 24) with a microsurgical steel scalpel blade to allow the membrane non-permeant Lucifer yellow dye to enter the cells.

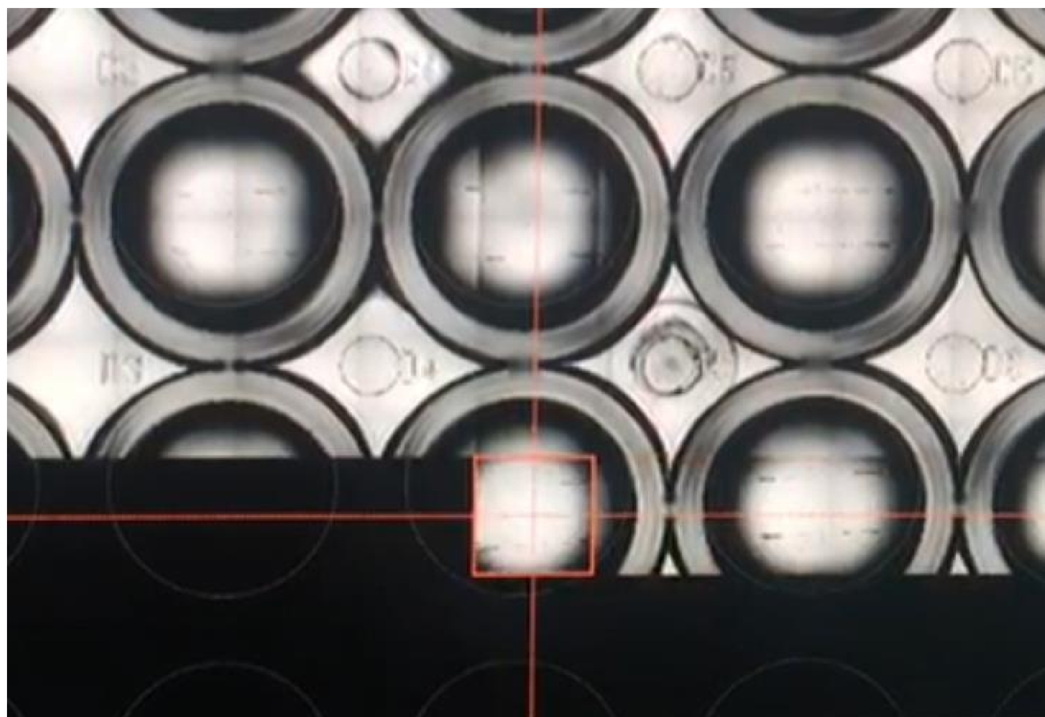

***Figure 24.*** Image from Tissuefaxs cell analysis software during the automated acquisition (preview) of the 48 wells plate at 2.5X. Parallel scrapes (manual made with a microsurgical steel scalpel blade) were visible in each well.

After ten minutes of dye exposure, the dye entered the damaged cells and in communicating cells by passing through their functional Gap Junction channels, then cells were washed twice and fixed with 4% formaldehyde solution. Propidium iodide was used in order to label died cells. Cells were analysed by a Zeiss AXIO OBSERVER Z1 wide-field epifluorescence microscope (Fig. 25) equipped with Hamamatsu digital camera and a tissue diagnostics automated platform with the associated Tissuefaxs cell analysis software.

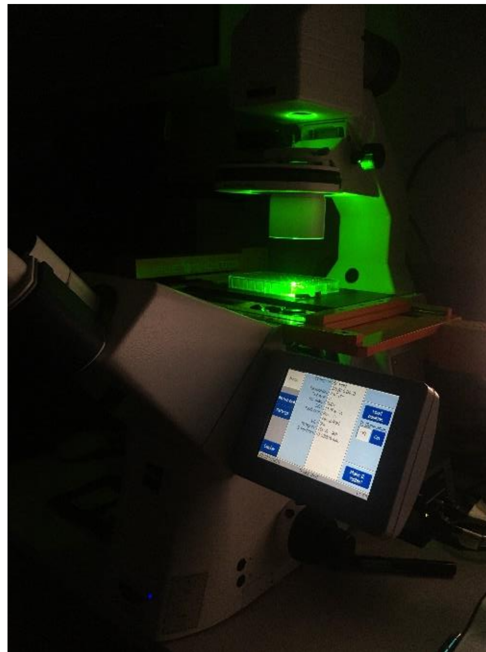

***Figure 25.*** Zeiss AXIO OBSERVER Z1 during the automated acquisition of a 48 wells plate.

Three replicates were carried out for each treatment and the relative controls; six replicates were made for negative (no treatment) and positive controls. Three independent experiments with each cell line were performed.
Analysis of communicating cells were performed on both scrapes of each well. Each scrape was divided into three different sections. Four images were acquired at 20x for each section (Fig. 26) and were related to the used stains (Hoechst, Lucifer Yellow, Propidium iodide) and the bright field. The Hoechst (blue, fluorescent dyes used to stain DNA) and Bright field images were taken as control. 24 images were acquired each well (3 different areas along each cut, two parallel cuts each well), and 1152 images were acquired each 48 wells plate.

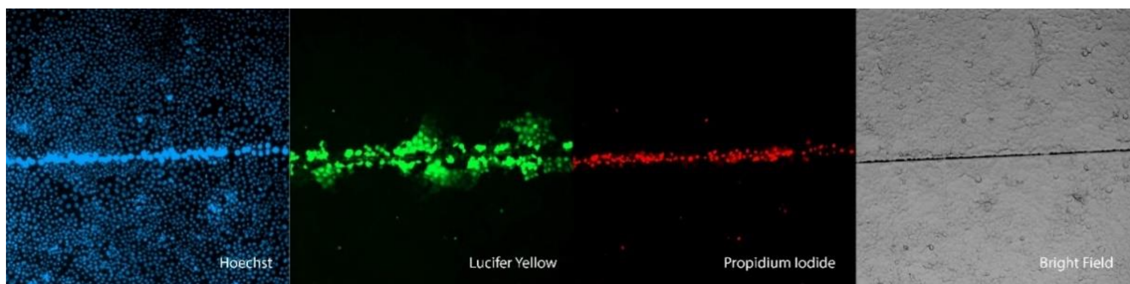


***Figure 26.*** Example of a set of images of a scrape. In order from left to right: Hoechst, Lucifer Yellow, Propidium Iodide and Bright field images. WB-*ras* cells.

The Lucifer Yellow area of each image was quantified with IMAGEJ (Fig. 27), then was subtracted to the relative Propidium Iodide area (Fig. 28), mediated, and finally normalized with the relative mediated values of the negative control

(no treatment) and expressed as a percentage. The same ImageJ macro algorithm was used.

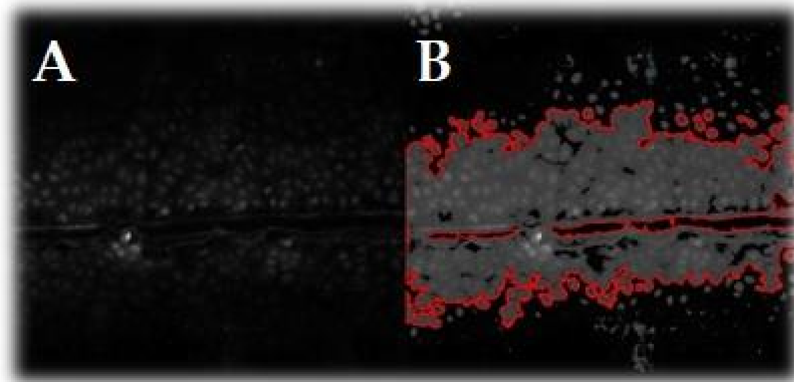


***Figure 27.*** Example of quantification of lucifer yellow area made with ImageJ on the same scrape section. Native lucifer yellow image (A), elaborated with ImageJ (B). WB-*neo* cells.

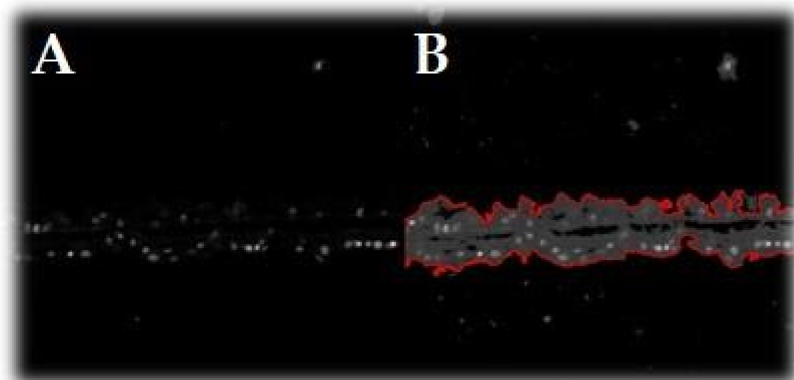


***Figure 28.*** Example of quantification of Propidium iodide area made with ImageJ on the same scrape section. Native propidium iodide image (A) and elaborated with ImageJ (B). WB-*neo* cells.

*IMAGEJ macro algorithm*

The protocol previously described, and the following relative ImageJ macro algorithm were developed by Dydowiczová et al. (2020).

```
// Initial variables
var scale = 0.72;
var minSize = 1000;
var maxNoImgs = 500;
var visualizedParticles = 0;
Dialog.create("THE GJIC ANALYZER");
Dialog.addString("Folder name", "C:\\Users\\Dell\\Desktop\\B2B
titrace\\40k\\LY\\", 100);
Dialog.addString("Results table name", "Fun with GJIC", 100);
Dialog.addNumber("Maximum number of images", maxNoImgs);
Dialog.addNumber("Minimal size", minSize);
Dialog.addNumber("Scale (um/px)", scale);
Dialog.show();
gjicdir = Dialog.getString;
targetTable = Dialog.getString;
maxNoImgs = Dialog.getNumber;
minSize = Dialog.getNumber;
scale = Dialog.getNumber;
//gjicdir = getDirectory("Choose gjic Source Directory ");
//targetdir = getDirectory("Choose Destination Directory ");
File.mkdir(gjicdir + "\\control");
setBatchMode(false);
gjicList = getFileList(gjicdir);
gjicAreas = newArray();
outFilePath = gjicdir + "results.xls";
headerString = "name of image\t" + "no_particles\t" + "sum_area\t";// +
"%area\t";
File.saveString(headerString, outFilePath);
File.append(" ", outFilePath);
analyseImages();
saveResults();
endScript();
function analyseImages () {
	for (i=0; i<gjicList.length; i++) {
		if (i < maxNoImgs) {
			if (indexOf(gjicList[i], '/') == -1) {
```

```
                    run("Clear Results");
                    showProgress(i+1, gjicList.length);
                    gjicImage = gjicdir + gjicList[i];
                open(gjicImage);
                original=getTitle();
                open(gjicImage);
                run("Set Scale...", "distance=1 known=scale pixel=1
unit=Ä• Å¼Ë• m global");
                    run("8-bit");
                    run("Invert");
                    run("Enhance Contrast...", "saturated=5");
                    //setThreshold(0, 237);
                    run("Auto Threshold", "method=Mean");
                     //run("Fill Holes");
                    run("ROI Manager...");
                    run("Analyze Particles...", "size="+ minSize + "-5000" +
"show=[Overlay Outlines] display summarize add");
                    run("Add Image...","image=["+original+"] x=0 y=0
opacity=80");
                    print("Close this window to continue!");

                    checkPause();
                    function checkPause(){
                        if (!isOpen("Log")){
                            //if (roiManager("count") > 0) {
                            indexes = newArray();
                            for (j = 0; j< roiManager("count"); j++){
            indexes = Array.concat(indexes, j);
};
                            noResults = nResults();
                            sum_area = 0;
//sumP_area = 0;
            for (r = 0; r < noResults; r++) {
                    sum_area = sum_area + getResult("Area",r);
                //umP_area = sumP_area + getResult("%Area",r);
                            }
File.append(original + "\t" + noResults + "\t" + sum_area, outFilePath);
                            roiManager("Select", indexes);
                            roiManager("Set Color", "red");
                            roiManager("Set Line Width", 5);
                            //saveAs("Measurements", gjicdir +
removeExtension(gjicList[i]) + "_gjic.csv");
```

```
						//run("Add Image...","image=["+original+"] x=0 y=0 opacity=80");
						saveAs("Jpeg", gjicdir + "control\\" + removeExtension(gjicList[i]) + "_gjic.jpg");

						truncateROIManager();

						//}
						run("Close All");
						// trash garbage
						call("java.lang.System.gc");
					}else{
						wait(100);
						visualiseParticles();
						checkPause();
					}
				}
			}
		}
	}
}
function truncateROIManager(){
	particlesToDelete = roiManager("count");
	if (particlesToDelete > 0 ) {
		indexes = newArray();
		for (i = 0; i< particlesToDelete; i++){
			indexes = Array.concat(indexes, i);
		};
		roiManager("Select", indexes);
		roiManager("Delete");
	}
};
function saveResults () {
	selectWindow("Summary");
	//saveAs("Text", gjicdir + "results.csv");
	/*
	headerString = "name of image\t" + "area\t";
	resultsPath = gjicdir + "results.csv";
	File.saveString( headerString, resultsPath);
	Array.print(gjicAreas);
	for (i=0; i<gjicList.length; i++) {
		imageResultRow = gjicList[i] + "\t" + gjicAreas[i];
```

```
			print(imageResultRow)
			File.append(imageResultRow, resultsPath);
		}
		*/
}
function removeExtension (nameWith) {
		parts=split(nameWith, ".");
		return parts[0]
}

function endScript () {
		selectWindow("ROI Manager");
		run("Close");
		selectWindow("Results");
		run("Close");
		print("Well done!!! Your analysis is completed.")
};
function visualiseParticles () {
		//setBatchMode(true);
		particlesCount = roiManager("count");
		if (visualizedParticles != particlesCount ){
			print("noOfParticles" + particlesCount);
			print("visualizedParticles" + visualizedParticles);
			indexes = newArray();
			for (i = 0; i< particlesCount; i++){
				indexes = Array.concat(indexes, i);
			};
			Array.print(indexes);
			roiManager("Select", indexes);
			roiManager("Set Color", "red");
			roiManager("Set Line Width", 5);
			visualizedParticles = particlesCount;
			roiManager("Update");
			print ("number of particles changed to",  visualizedParticles);

			//updateResults();
			run("Clear Results");
			roiManager("Measure");
		}
		//setBatchMode(false); }
```

### 2.6. *Statistical analysis*

Comparisons between treated cells and the related controls were analysed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test using Prism 6.0 (GraphPad) and statistical functions of Excel (Microsoft). Statistical significance correlations were evaluated at several p (see Results and Discussion). IC50 values [log (inhibitor) vs normalized response] were calculated using Prism 6.0 (GraphPad).

# 3. *Results and Discussion*

## 3.1 *Biochemical characterization of Cassiopea andromeda jellyfish biomass*

### 3.1.1 *Jellyfish biometric data*

*Cassiopea andromeda* specimens were sampled in two different locations of Palermo (Sicily, Italy) harbour. All samples showed similar features, biometric data revealed that jellyfish specimens were almost homogeneous in size (Table 1) with the umbrella diameter ranged from 13.5 to 17.5 cm, with a mean of 15.0 ± *1.3* cm, and the fresh weight (FW) of the whole specimen (umbrella and oral arms) ranged from 153.2 to 301.9 g, with a mean of 233.5 ± *52.6* g.

***Table 1.*** Biometric data of 9 *Cassiopea andromeda* specimens, with mean and standard deviation (SD).

| **Item** | **Specimen** | **Diameter (cm)** | **Fresh Weight (g)** | **Dry Weight (g)** | **Yield (% of FW)** |
|---|---|---|---|---|---|
| A1 | CAN4 | 15.0 | 241.0 | 18.3 | 7.59 |
| A2 | CAL5 | 14.0 | 234.5 | 16.4 | 6.99 |
| A3 | CAN1 | 17.5 | 299.9 | 22.7 | 7.57 |
| A4 | CAL1 | 16.0 | 301.9 | 20.7 | 6.86 |
| A5 | CAL2 | 14.0 | 185.4 | 14.4 | 7.77 |
| A6 | CAL3 | 14.0 | 206.5 | 15.4 | 7.46 |
| A7 | CAL4 | 16.0 | 280.8 | 22.7 | 8.08 |
| A8 | CAN2 | 13.5 | 153.2 | 11.7 | 7.64 |
| A9 | CAN3 | 15.0 | 198.5 | 15.3 | 7.71 |
| **Mean** | | **15.0** | **233.5** | **17.5** | **7.52** |
| SD | | ± 1.3 | ± 52.6 | ± 3.9 | ± 0.4 |

The resulting total dry weight (DW) of the jellyfish ranged from 11.7 g to 22.7 g, with a mean of 17.5 ± *3.9* g. As a consequence, the dry weight referred to the corresponding fresh weight ranged from 6.86% to 8.08%, with the mean of yield of 7.52% of the fresh weight. This value is remarkable when compared with the yield range found in other species of jellyfish of the Rhizostomeae order, for which previously reported percentages varied between 2 and about 7% (De Domenico et al., 2019; Khong et al., 2016; Leone et al., 2015; Lucas, 2009; De Souza et al., 2007).

Based on our data, the biomass yield of the whole zooxanthellatae *Cassiopea andromeda* jellyfish in terms of dry weight compared to the fresh weight (Table 1), seems to be in the higher range of those found in various jellyfish species, indeed, the biomass yield ranged between 1.1 to 10.5%, 2.2 to 3%, and 4.1 to 6.8% for *Periphylla periphylla*, *Aurelia sp*. and *Rhizostoma pulmo*, respectively, only *Cotylorhiza tuberculata,* a zooxanthellatae jellyfish as *C. andromeda*, has a larger range of percentage maybe due to the different content of microalgae (Khong et al., 2016; Leone et al., 2015; Lucas, 2009).
In addition, Leone et al. (2015) found that dry weight percentage for some species could increase with the increasing dimension of the specimens. The yield data here presented are related to specimens with a diameter in the range of 13 cm to 17 cm, as a consequence a higher yield could be expected for this jellyfish species considering that the size of *Cassiopea andromeda* can grow up to 20 - 25 cm of diameter, depending on the environment and the amount of available nutrients (Thè et al., 2020). Interestingly, *C. andromeda* populations that invaded the shrimp's farms in the Brazilian coasts, seem more stable than the natural population living in the mangrove, maybe due to the environmental intra-annual stability in the farm that promotes jellyfish growth, in addition shrimp farm-associated jellyfish are three times larger than those in the mangroves, regardless of season (Thé et al., 2020).

### *3.1.2 Jellyfish biomass composition*

In order to characterize the biomass of *Cassiopea andromeda* jellyfish present in Sicily (Italy), protocols for extraction and the analysis of soluble and insoluble components were applied. Furthermore, due to the particular trophic behaviour and position of this species, each different specimen of *C. andromeda* has been separated into umbrella and oral arms, to verify their possible different composition. Each umbrella and oral arms samples of different specimen of *C. andromeda* was separately freeze-dried and then analysed to characterize the composition of each part of jellyfish tissues. Soluble fractions were analysed for proteins amount, phenol content and antioxidant activity. The insoluble compounds, mainly composed by proteins, especially collagen, were subjected

to enzymatic digestion and then analysed for proteins amount, phenol content and antioxidant activity.

### *3.1.3 Jellyfish soluble compounds*

To establish the presence of extractable compounds and their chemical nature, two different protocols for the extraction of soluble components were applied as shown in Figure 29. Two kind of extraction solutions, phosphate-buffered saline (PBS) as a standard aqueous system and a solution of 80% ethanol were used to solubilize hydro-soluble compounds and compounds soluble in hydroalcoholic solutions, respectively (Leone at al., 2015; Leone at al., 2013).

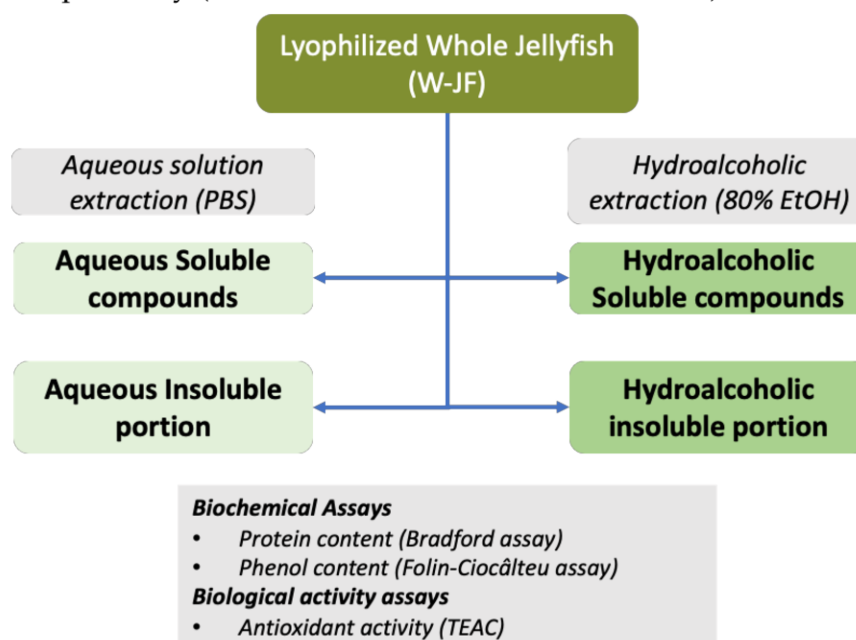


***Figure 29.*** Scheme of the experimental approach for the extraction of soluble compounds from *Cassiopea andromeda* jellyfish samples.

*Whole jellyfish*

A first analysis to assess the yield of extraction from jellyfish tissues, were conducted on the biomass of the whole jellyfish (WJ). In order to verify the extraction efficiency of the two solvent systems, the extraction yield was estimate in terms of amount (g) of dried extract compared to the dry weight of the lyophilized whole jellyfish (% DW), for each extraction solution type (Table 2).

*Table 2.* Yield of extraction with aqueous solution (PBS) and hydroalcoholic solvent (80% ethanol); the lyophilized extracts were compared to the lyophilized whole jellyfish samples of *Cassiopea andromeda*. (*) The yield compared to fresh weight (% FW) was theoretically calculated. Data are expressed as mean ± Standard Deviation, *SD* (n=6).

| **Extraction Solvents** | **Whole jellyfish (WJ)** | | | |
|---|---|---|---|---|
| | **WJ DW (g)** | **Extract DW (g)** | **Yield (%DW)** | **Yield * (% FW)** |
| | Mean ± *SD* | | | |
| **PBS** | 1.004 ± *0.015* | 0.725 ± *0.011* | **72.2** ± *1.4* | **5.4*** |
| **80% Ethanol** | 1.517 ± *0.051* | 0.650 ± *0.009* | **49.36** ± *0.9* | **3.7*** |

Data shows that a considerable amount of dry extract was recovered after extraction by both the solvent systems. Extraction by the saline solution PBS yields the 72.2% ± 1.4 (w/w on DW), while 80% Ethanol solution was able to extract only 42.8 % ± 0.9. The mean of the DW/FW ratio of 7.52%, showed in Table 1, was considered to theoretically calculate the yield in term of fresh weight (FW). The yield was 5.4% and 3.7% as compared to the fresh weight (FW) for PBS and 80% ethanol extract, respectively. The data demonstrated that aqueous solution (PBS) is able to extract a higher amount of compounds than hydroalcoholic solution (80% ethanol) from dried biomass of *Cassiopea andromeda*.

Leone et al. (2013) showed that the hydroalcoholic extract from *Cotylorhiza tuberculata*, a zooxanthaellatae jellyfish, obtained by 80% ethanol extraction in the same condition of this study provided a similar yield of 43.6 ± *4.1*% (w/w of DW) compared to the dry biomass and a higher yield of 11.7 ± *1.7*% (w/w of FW) compared to the fresh biomass (Leone et al., 2013), indicating similarity in term of yield independently from the species and the size of the jellyfish. To our best knowledge, no data are available on PBS or other saline solution extractions on *C. andromeda* jellyfish.

*Umbrella and Oral Arms*

Umbrellas and oral arms of different specimens of *Cassiopea andromeda* jellyfish were separately analyzed in order to estimate the yield of organic matter extracted from the two jellyfish body parts.

Table 3 shows the average yields of the extractions from umbrella using PBS and 80% ethanol solutions. The dry weight of PBS aqueous extract (g) and the 80% ethanol hydroalcoholic extract (g) are compared to the dry weight of the lyophilized jellyfish umbrella. Again, the extraction yield obtained by PBS was higher than those obtained by 80% ethanol giving a percentage of 64.73 ± *0.02*% and 41.60 ± *0.03*% (% DW ± *SD)*, respectively.

***Table 3.*** Yield of extractions with aqueous solution (PBS) and hydroalcoholic solvent (80% ethanol) compared to the lyophilized umbrella (UMB) samples of *Cassiopea andromeda*. Data are expressed as mean ± Standard Deviation, *SD*, (n=6).

| **Extraction Solutions** | **Jellyfish Umbrella (UMB)** | | |
|---|---|---|---|
| | **DW of UMB (g)** | **Extract DW (g)** | **Yield (%DW ± *SD*)** |
| | | Mean ±*SD* | |
| **PBS** | 0.952 ± *0.155* | 0.616 ± *0.1* | **64.73** ± *0.02* |
| **80% Ethanol** | 0.515 ± *0.172* | 0.214 ± *0.071* | **41.60** ± *0.03* |

Therefore, the same analysis was performed on oral arms tissues of *C. Andromeda*. As shown in Table 4, a surprisingly high extraction yield was obtained with PBS saline solution, which was 95.83 ± *0.005*% of DW of the lyophilized oral arms. This value was one and half times higher than the yield obtained in umbrella samples (64.73% of DW) showed in Table 3**.** Conversely, the extraction yield obtained by hydroalcoholic solution in oral arms was 43.50 ± *0.005*% of the DW, that was comparable to the yield value obtained with umbrella samples (41.60 %, Table 3).

***Table 4.*** Yield of extractions with aqueous solution (PBS) and hydroalcoholic solvent (80% ethanol) compared to the lyophilized oral arms (OA) samples of *Cassiopea andromeda*. Data are expressed as mean ± Standard Deviation, *SD* (n=6).

| **Extraction Solvents** | **Jellyfish Oral Arms (OA)** | | |
|---|---|---|---|
| | **DW of OA (g)** | **Extract (g)** | **Yield (%DW± *SD*)** |
| | | Mean ±*SD* | |
| **PBS** | 0.978 ± *0.15* | 0.937 ± *0.011* | **95.83** ± *0.01* |
| **80% Ethanol** | 1.003 ± *0.08* | 0.436 ± *0.040* | **43.50** ± *0.01* |

Noteworthy is the difference between oral arms and umbrella samples in terms of yield. Given that the yields of both aqueous and hydroalcoholic extracts of the whole jellyfish were quite high, ranging from approximately 50 to 70% of dry weight (Table 2), it appears that the presence of extractable compounds are distributed asymmetrically in the jellyfish body parts. The extracts of oral arms samples of C. *andromeda,* indeed, showed significantly higher yields, as both aqueous and hydroalcoholic extracts, compared to the jellyfish umbrellas' samples.

Generally, jellyfish body parts have a different composition due to their diverse role. In pelagic Cnidarian species, umbrellas have well developed muscle cell organization useful for active contraction and movement, indeed, the pulsations of the bell are relied on as their mode of locomotion (Park et al., 2015; Costello et al., 2008; McHenry and Jed, 2003; Colin and Costello 2002), while the tentacles are typically used for capturing prey (Purcell, 2012; Pitt et al., 2008).

A different case is represented by the upside-down jellyfish *Cassiopea*, which have a less active locomotion being more similar to sessile organisms. The contractile nature of the umbrella and oral arms in *Cassiopea andromeda* specie was studied by mathematical models and fluid dynamics (Hamlet, 2012, 2011). The oral arms seem to have active contractile capacity and a particular structure/body that make them different from the pelagic jellyfish. The absence of a primary mouth, with the presence of secondary mouths and mucus secretions between the appendages of the oral arms could be factors promoting this unusual lifestyle. Therefore, a higher yield in organic matter at the level of oral arms would therefore seem to be more plausible for this jellyfish species. Furthermore, initial evidence suggests that heterotrophic feeding behavior is more important than the photoautotrophic compounds (mainly lipids) provided by symbiotic zooxanthellae (Banha et al., 2020; Thè et al., 2020; Latyshev et al., 1991).

### *3.1.4 Umbrella and Oral Arms biochemical characterization*

Umbrellas and oral arms of different specimens of jellyfish *Cassiopea andromeda* were separately analyzed in order to verify if the two body parts present different chemical composition and content of bioactive compounds.

To partially characterize the class of soluble compounds present in umbrella and oral arms, the protein and phenol content were evaluated in the aqueous (PBS) and hydroalcoholic (80% ethanol) extracts of the two jellyfish body parts.

### 3.1.5 *Partial characterization of extracted soluble compounds*

To evaluate the efficiency of extraction, a strategy of single and multiple extractions was used as showed in the scheme in Figure 30, to evaluate the extraction efficiency of different molecules, such as proteins and phenols, from jellyfish tissues.

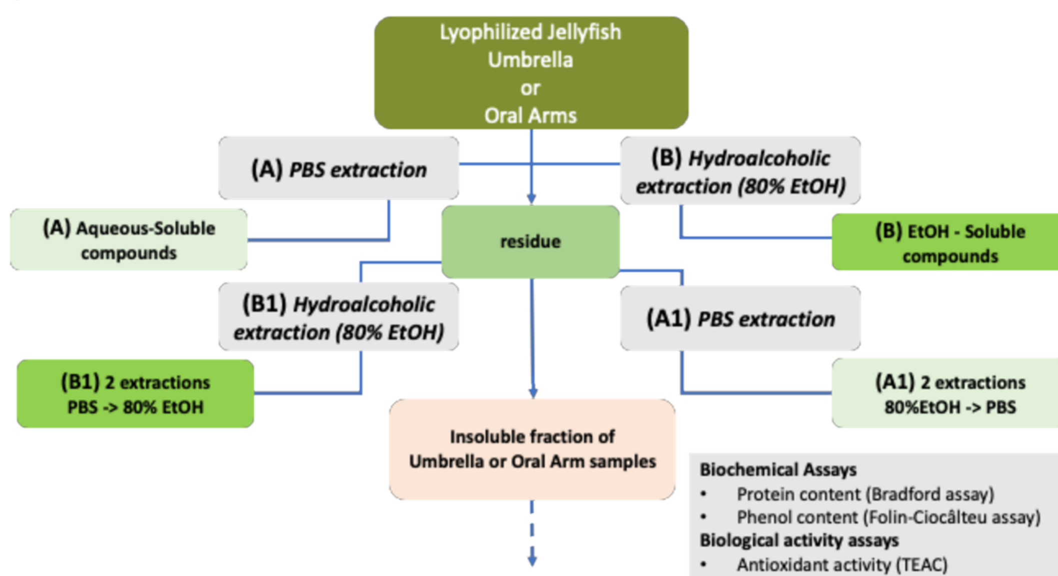


***Figure 30.*** Scheme of the experimental approach used to evaluate the extraction efficiency in terms of proteins and phenols content.

*Protein content of the aqueous and hydroalcoholic extracts*

The total amount of soluble proteins in aqueous solution (PBS, Treatment A in Figure 30) and in hydroalcoholic solution (80% ethanol, Treatment B) was evaluated by the Bradford assay. As shown in Figure 31, PBS extraction is more efficient than ethanol (80%) ones, because a significantly higher amount of protein were extracted by PBS solution than 80% ethanol in both umbrellas and oral arms samples. Indeed, an amount of 44.72 ± *3.1* and 73.21 ± *5.9* mg of proteins per g of DW (mg/g DW) in umbrella and oral arms samples, respectively (Fig. 31, A), were measured in the PBS extracts, while the amount of proteins extracted by

80% ethanol were of 3.55 ± *0.68* mg/g DW in umbrella and 7.03 ± *3.37* mg/g DW in oral arms samples. (Fig. 31, B). Furthermore, a significant difference between protein content in umbrella and oral arms were evident for the PBS extracts, while for 80% ethanol extracts no differences were found (Fig. 31).

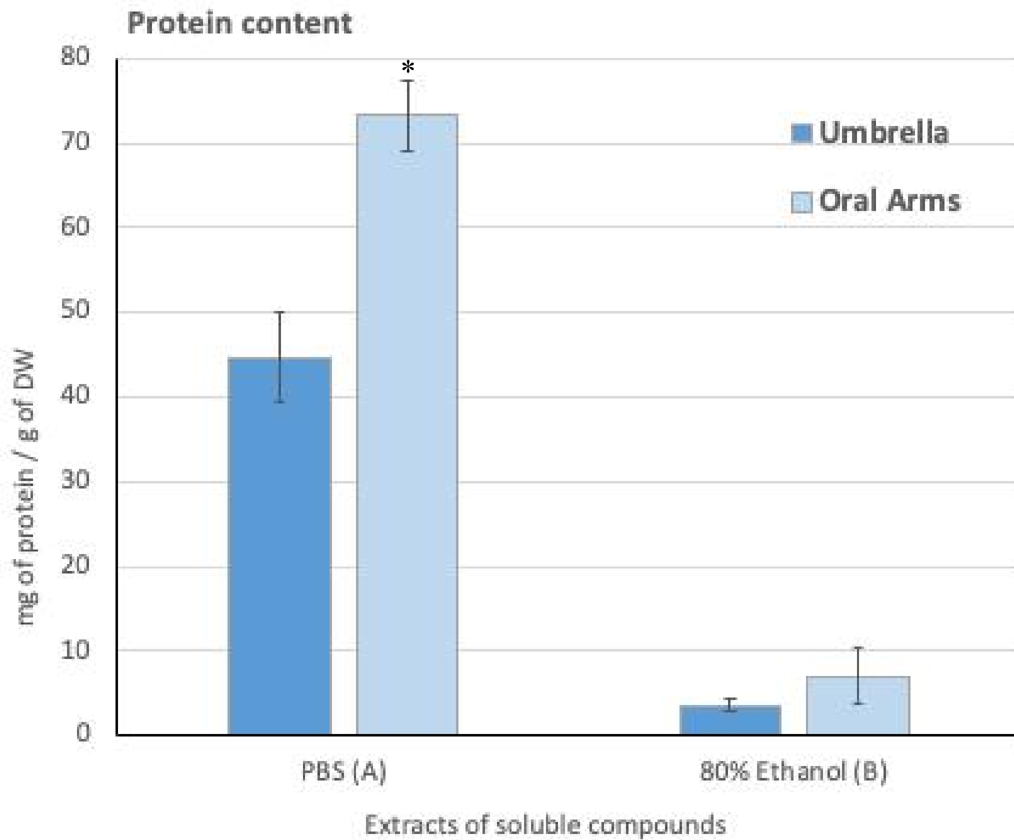


***Figure 31.*** Protein content in PBS (A) and 80% ethanol (B) extracts from umbrella and oral arms samples of *Cassiopea andromeda*. Data are expressed as mg of proteins per g of dry weight of the lyophilized samples. Data are mean (n=6), Bars represent ± standard deviation *(SD).*

In order to optimize the extraction process and verify the effect of multiple extractions with the two solutions, the insoluble residue (pellet) of jellyfish samples (from both umbrella and oral arms) obtained by 80% ethanol extraction were re-extracted with PBS (Treatment A1). In parallel, the sample residues previously extracted with PBS, were subjected to a second extraction with 80% ethanol (Treatment B1). Figure 32 shows the protein content of the extracts resulting from multiple extractions, A1 and B1 as shown in the scheme in Figure 30. The jellyfish samples extracted with PBS after the hydroalcoholic extraction (A1) contained 4.33 ± *0.67* mg of proteins / g DW and 4.42 ± *0.39* mg of proteins / g DW, in umbrella and oral arms, respectively (Fig. 32, A1). The extraction with

80% ethanol preceded by the extraction with PBS (B1), provided a very low amount of protein, which contents were 0.62 ± *0.02* mg and 1.95 ± *0.68* mg/g of DW in the umbrella and oral arms, respectively (Fig. 32-B1). No difference between protein content of umbrella and oral arms was found for each extraction type (Fig. 32).

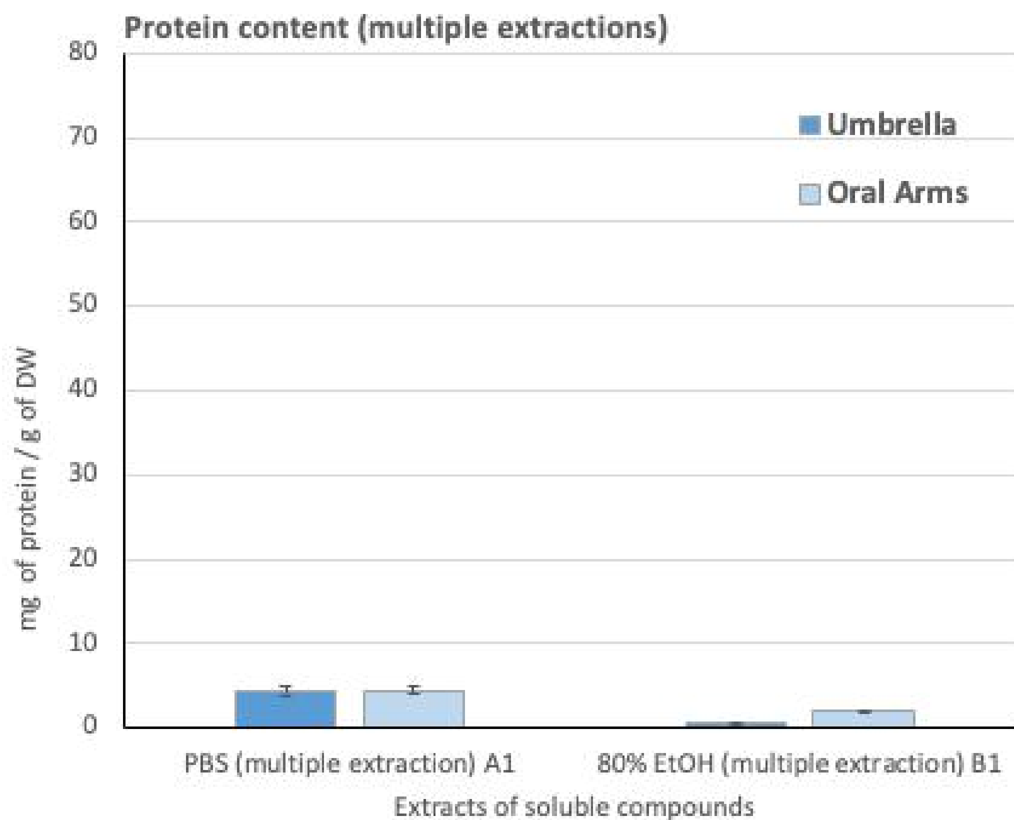


***Figure 32.*** Protein content in PBS extracts performed on samples after 80% ethanol extraction (multiple extraction, A1) or in 80% ethanol extracts after PBS extraction (multiple extraction, B1) from umbrellas and oral arms' samples of *Cassiopea andromeda*. Data are expressed as mg of proteins per g of dry weight of the lyophilized samples. Data are mean (n=6); Bars represent ± standard deviation *(SD).*

It is notable that the pre-extraction with 80% ethanol prevents the protein extraction with PBS, maybe due to the protein denaturation action of the 80% ethanol solution and the protein precipitation in insoluble forms (Dos Santos et al., 2017). Therefore, the most efficient extraction solution is PBS, while the extraction with 80% ethanol both as direct and second extraction, provided a lower total extraction yield, but likely more specific and selective (Lim et al., 2020).

*Phenol content of the aqueous and hydroalcoholic extracts*

The amount of total phenolic compound content was evaluated as micrograms of gallic acid equivalent (GAE) per g of jellyfish dry weight (μg GAE/g DW) and was measured in all the four extracts. Namely, extracts obtained directly from dried jellyfish biomass in PBS (A) and 80% ethanol (B) (Fig. 30) and extracts in PBS performed after 80% ethanol extraction (A1) as well as 80% ethanol extraction after PBS extraction (B1) (Fig. 30) in umbrella and oral arms of *Cassiopea andromeda* samples.

As shown in Figure 33, the first extraction in aqueous solution (A) was able to extract about 1785 ± *380* μg of GAE/g of DW from umbrellas and about 3851 ± *450* μg of GAE/g of DW from oral arms, while the 80% ethanol solution (B) was able to extract 1358 ± *340* GAE/g of DW from umbrella and 2483 ± *201* GAE/g of DW from oral arms This confirmed that a difference between umbrella and oral arms in extractable compounds is evident also for the class of phenols. In addition, the statistical analysis indicates no significant difference between extractable phenols by PBS and ethanol solutions from umbrella, while a significantly higher amount of PBS extractable phenols was detected in oral arms as compared to 80% ethanol extraction solution.

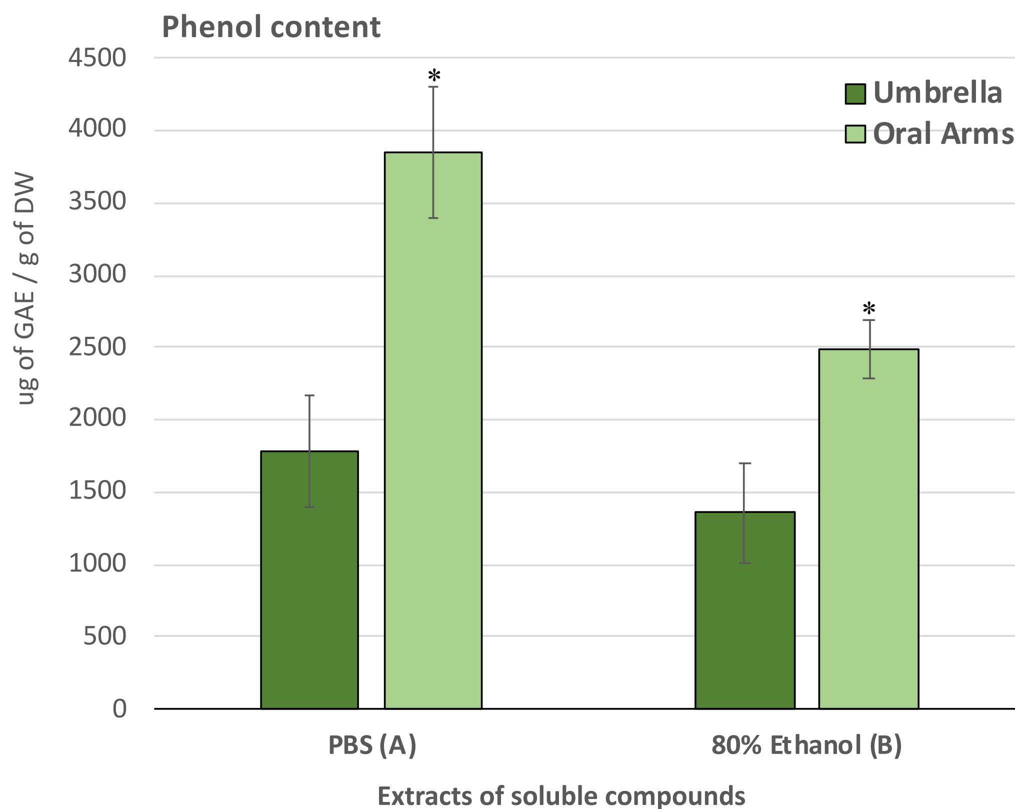


***Figure 33.*** Content of phenol compounds in PBS (A) and in 80% ethanol (B) extracts from umbrella and oral arms samples of *Cassiopea andromeda*. Data are expressed as µg of gallic acid equivalent (GAE) per g of dry weight of extract. Data are mean (n=6), Bars represent ± standard deviation *(SD).*

The total content of phenols in the extracts obtained from a second extraction after a previous complementary extraction (A1 and B1) are shown in Figure 34. From one third to one sixth of the quantity presents in the first extraction solution was still extractable in the subsequent extraction. No difference was detected for extractable phenols in oral arms with both PBS after ethanol extraction (A1) and with 80% ethanol extraction after PBS extraction (B1), while significant less phenol compounds were extracted in 80% ethanol after PBS extraction (B1) in umbrella samples.

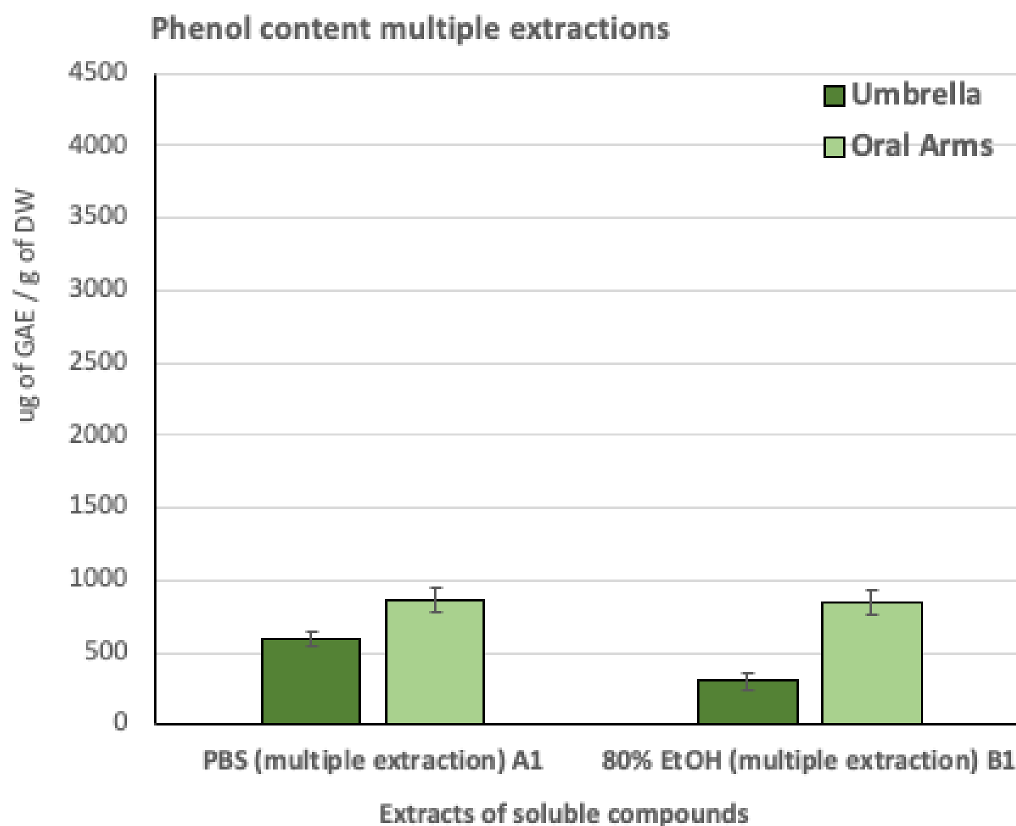


***Figure 34.*** Total phenol content in PBS extracts after twice 80% ethanol extraction (A1) (multiple extraction PBS -> 80% ethanol) and in 80% ethanol extracts after PBS extraction (B1) (multiple extraction 80%ethanol -> PBS) from umbrellas and oral arms' samples of *Cassiopea andromeda*. Data are expressed as µg of gallic acid equivalent (GAE) per g of dry weight of extract. Data are mean (n=6); Bars represent ± standard deviation (SD).

*Antioxidant activity of the aqueous and hydroalcoholic extracts*

A significant antioxidant activity, measured as radical scavenging activity, was detected in all four jellyfish extracts, (Fig. 35 A, B and Fig. 36, A1 B1). In order to avoid an incorrect measurement due to the different extraction capacity of the solvents used and the different compounds contents of the samples (umbrellas and oral arms), the antioxidant activity was expressed as Trolox equivalent per mg of proteins (TE/mg proteins) instead than per mg of DW. This normalized data is able to express the qualitative differences among samples related to their antioxidant capability.

No differences in the antioxidant activity between umbrella and oral arms samples were found (Fig. 35, A and B) in both types of extraction. It was noticeable that the antioxidant activity of the 80% ethanol extracts (Fig. 35, B) were significantly higher than PBS extracts (Fig. 35, A), regardless of the origin from umbrellas' or oral arms' samples. This indicated nonsignificant qualitative

differences in the content of antioxidant compounds between body parts (oral arms and umbrella); however, it indicated that the hydroalcoholic extraction is selective for compounds with high antioxidant activity.

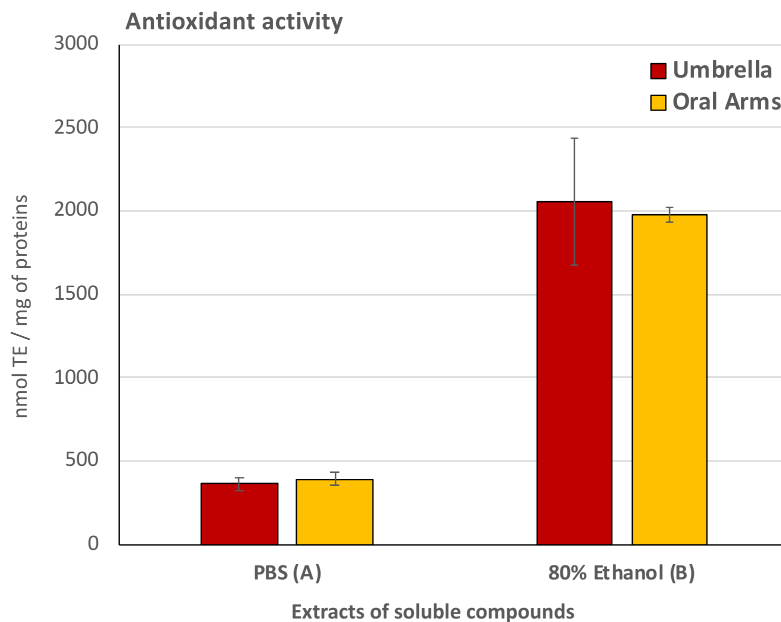


***Figure 35.*** Total antioxidant activity in jellyfish extracted with phosphate-buffered saline (PBS, A) or 80% ethanol (B) from freeze-dried umbrella or oral arms of *Cassiopea andromeda* jellyfish. Antioxidant activity is expressed as nmol of Trolox eq. (TE) per mg of proteins. Data are mean (n=6); Bars represent ± standard deviation (SD).

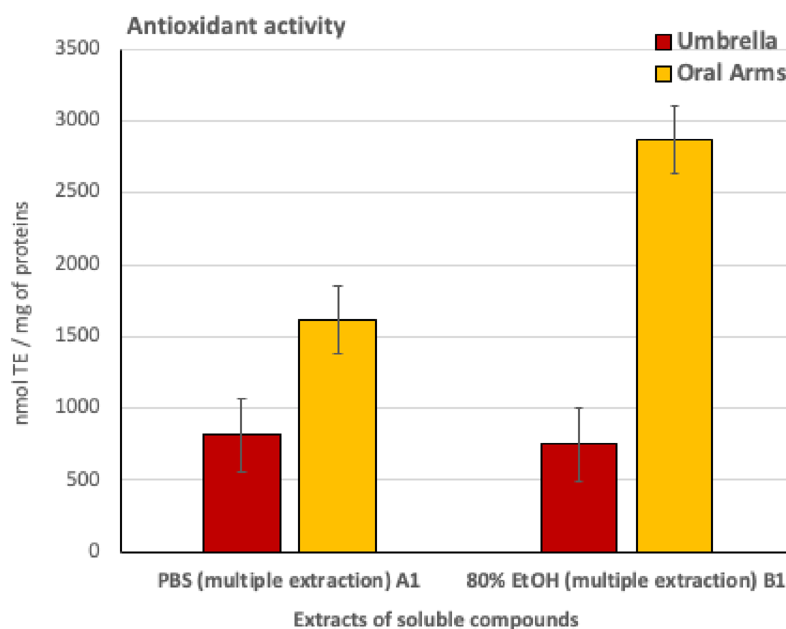


***Figure 36.*** Total antioxidant activity in umbrella or oral arms *Cassiopea andromeda* jellyfish extracts with phosphate-buffered saline (PBS) after 80% ethanol extraction (A1) or with 80% ethanol after PBS extraction (B1). Antioxidant activity is expressed as nmol of Trolox eq. (TE) per mg of proteins. Data are mean (n=6); Bars represent ± standard deviation (SD).

### 3.1.6 *Enzymatic sequential digestion of insoluble proteinaceous compounds*

The insoluble material resulting from the aqueous (PBS, Treatment A) and hydroalcoholic (80% Ethanol, Treatment B) extractions, were subjected to sequential enzymatic hydrolysis by pepsin digestion followed by collagenase hydrolysis (De Domenico et al., 2019; Leone et al., 2015).
Pepsin is the main human digestive enzymes present in the gastric juice and it breaks down a wide range of proteinaceous compounds (Blanco et al., 2017). Its optimal temperature is 37°C but to preserve samples it has been used at 4°C for a longer time. In addition, it cleaves the non-triple collagen domains without damaging the triple helix (Jongjareonrak et al., 2005).
The undigested insoluble proteins by pepsin, mainly consisting of the triple helix domains of collagen were then hydrolyzed by bacterial collagenase as in Leone et al. (2015).
The sequential process has been preferred instead of a combined enzymatic hydrolysis to have a better control of the digestion parameters and of the resulting peptide composition (Grienke et al., 2014).

*Pepsin-hydrolyzed proteins*
The digestion with pepsin was performed as first step on insoluble proteins obtained as pellet after the aqueous (PBS, treatment A) or hydroalcoholic extractions (80% ethanol, treatment B) carried out on jellyfish umbrella and oral arms. As for the extractable soluble proteins, the amount of peptides obtained by pepsin-digestion of insoluble proteins in oral arms was higher as compared to the umbrellas fraction, in both extraction protocols, as shown in Figure 37. Indeed, the amount of pepsin-hydrolysates from the umbrella was 8.55 ± *0.8* mg/g DW after PBS extraction (treatment A) and 9.61 ± *0.95* mg/g of DW after the hydroalcoholic extraction (treatment B) . The amount of pepsin-hydrolysates from oral arms were 11.99 ± *1.0* and 13.39 ± *1.2* mg/g of DW after treatment A (PBS) and treatment B (80% ethanol), respectively.
No significant differences in the amounts of proteins digested with pepsin were detected between samples pretreated with aqueous or hydroalcoholic solutions, in both umbrella and oral arms. Therefore, our data demonstrated that the

amounts of insoluble protein digestible by pepsin were independent of the type of extraction previously carried out.

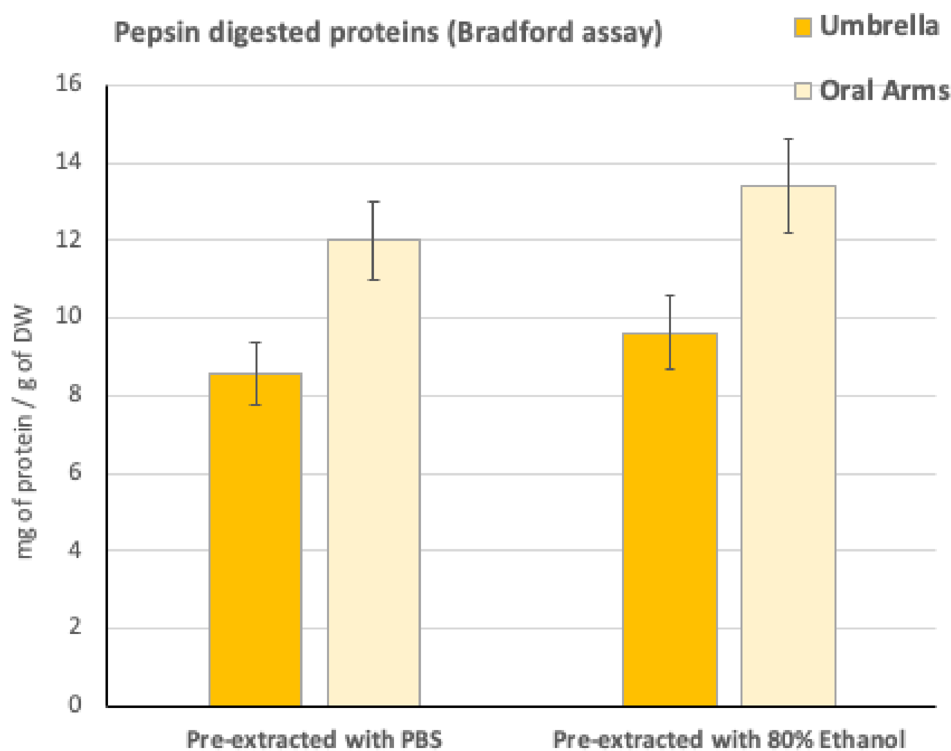


***Figure 37.*** Amount of proteins hydrolysed with pepsin in samples of umbrella and oral arms of *Cassiopea andromeda*, pre-extracted with phosphate-buffered saline (PBS) or with 80% ethanol. Protein content is expressed as mg per gram of dry weight. Data are mean (n=6) Bars represent ± standard deviation *(SD).*

*Total phenol content of pepsin-hydrolyzed fractions*

A notable amount of total phenols was found in the pepsin hydrolized fractions from jellyfish umbrellas and oral arms (Fig. 38). An amount of 2285 µg and 2738 µg of gallic acid equivalent per g of dry weight (GAE/g DW) were found in pepsin hydrolyzed fractions of umbrella and oral arms, respectively, after PBS extraction (Treatment A). While 3256 µg and 4838 µg GAE/g of DW were found in hydrolized fractions from umbrella and oral arms, respectively, in sample subjected to the extraction with 80% ethanol (treatment B).

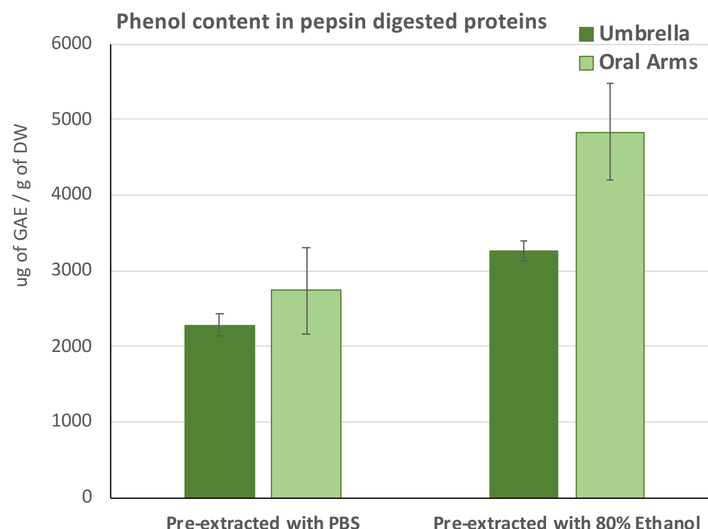


***Figure 38.*** Content of phenol compounds in umbrella and oral arms samples of *Cassiopea andromeda* hydrolysed by pepsin after extraction of soluble compounds with PBS or 80% ethanol. Data are mean (n=6); Bars represent ± standard deviation (SD).

No differences were found between the samples, either between the body parts of the jellyfish or between the type of extraction previously performed, confirming that the pre-extraction treatments did not affect the phenol content of the *C. andromeda* samples digested with pepsin.

The presence of phenol compounds is usually an index of several protective biological properties including strong antioxidant and chemo-preventive activities (Lee et al., 2016; Wang et al., 2013; Manach et al., 2004; Nijveldt et al., 2001). They are usually found in great amounts into plants, vegetables and other terrestrial and marine organisms like macro and microalgae and, in our case their strong presence is surely due to the well-known symbiosis with the microalgae of the genus *Symbiodinium microadriaticum*.

*Antioxidant activity of pepsin-hydrolyzed fractions*

A surprising high antioxidant activity was also detected in pepsin hydrolyzed fractions of umbrellas and oral arms of *C. andromeda.* In Figure 39 values of 2780 nmol TE / and 2327 nmol TE / mg of proteins were found in umbrellas and oral arms, respectively, of jellyfish samples previously extracted with PBS. On the contrary, the antioxidant activity values found in samples of umbrellas and oral

arms previously extracted with the 80% ethanol were 3644 nmol TE / mg and 3424 nmol TE / mg of proteins; these values were significantly higher than samples pre-extracted with PBS.

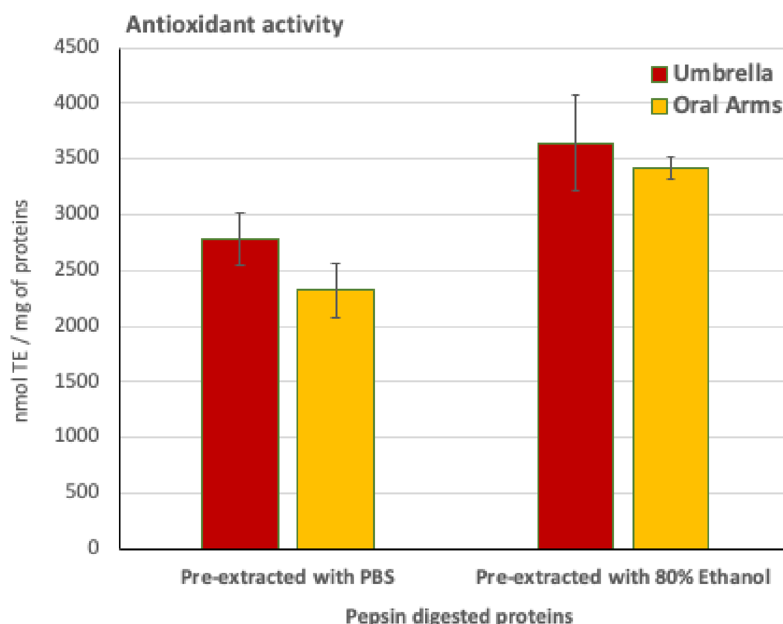


***Figure 39.*** Total antioxidant activity in umbrella or oral arms samples of *Cassiopea andromeda* jellyfish hydrolysed by pepsin after PBS or 80% ethanol extraction. Antioxidant activity is expressed as nmol of Trolox eq. (TE) per mg of proteins. Data are mean (n=6); Bars represent ± standard deviation (SD).

The amount of jellyfish nutritional components is highly influenced by environmental factors, but usually proteins are the major jellyfish components, apart from the zooxanthellatae jellyfish species where lipids are usually higher (Khong et al., 2016; Leone et al., 2015; Hsieh et al., 2001). Proteins hydrolysates are known to exert a wide spectrum of biological functions including anti-proliferative, anti-cancer, anti-hypertensive, hypocholesterolemic, anti-inflammatory and antioxidant (Zhang et al., 2018; Liu et al., 2016; Fan et al., 2013; Zhuang et al., 2012-2009; Hsu et al., 2011), and all these biological activities are mainly attributed to peptides (Etemadian et al., 2020). Due to their well accepted antioxidant effects that prevent or reduce oxidative stress associated to body diseases (Pisoschi et al., 2021; Lorenzo et al., 2018; De Domenico and Giudetti, 2017; Adebiyi et al., 2009; Gulcin et al., 2004), natural peptides are gaining attention in the last decades. Natural peptides can be valuable replacement for the problematic synthetic antioxidants widely used nowadays, because, although

the latter are usually more effective, they are toxic to the body for long-term use (Gajanan et al., 2016; Shahidi and Zhong, 2015;). Rather, natural peptides penetrate the cells more easily due to the amino acids sequence, composition, and the lower weight of them, creating the condition for a lower osmotic pressure difference (Gorguc et al., 2020; Karoud et al., 2019; Zamora-Sillero et al., 2018).

*Collagenase-hydrolyzed proteins*

The biggest part of jellyfish protein hydrolysates by the endopeptidase pepsin is mainly composed of insoluble collagen deprived of the triple helix (Jongjareonrak et al., 2005), indeed, collagen usually account for up to 60% of dry weight (Khong et al., 2016; Addad et al., 2011).

Several studies focused on jellyfish hydrolysed collagen which have shown to exert a wide range of biological effects like immune-stimulation and antioxidant activity (De Domenico et al., 2019; Barzideh et al., 2014b; Sugahara et al., 2006).

In order to evaluate the amount of collagen in umbrella and oral arms of *Cassiopea andromeda*, the residual biomass after pepsin digestion was subjected to enzymatic hydrolysis with collagenase, a bacterial endopeptidase able to hydrolyse the collagen jellyfish triple helix producing smaller peptides (De Domenico et al., 2019; Leone et al., 2015).

Figure 40 and Figure 41 show the amounts of collagenase digested proteins evaluated by Bradford assay (Bradford et al., 1976) and also by a modified Lowry's method (Komsa-Penkova et al., 1996).

From the Bradford dosage (Fig. 40) the amount of proteins digested by collagenase, likely fibrillar collagen, was about 12.8 ± *0.26* and 10.1 ± *0.32* mg / g of DW lyophilized umbrella and oral arms samples, previously extracted with PBS (Fig. 40-A).

A similar amount of collagen, namely 9.6 ± *0.14* and 11.9 ± *0.45* mg / g of DW was measured in samples of umbrella and oral arms, respectively, previously extracted by hydroalcoholic solution (Fig. 40-B). No significant differences between umbrella and oral arms as well as between pre-extraction type were found.

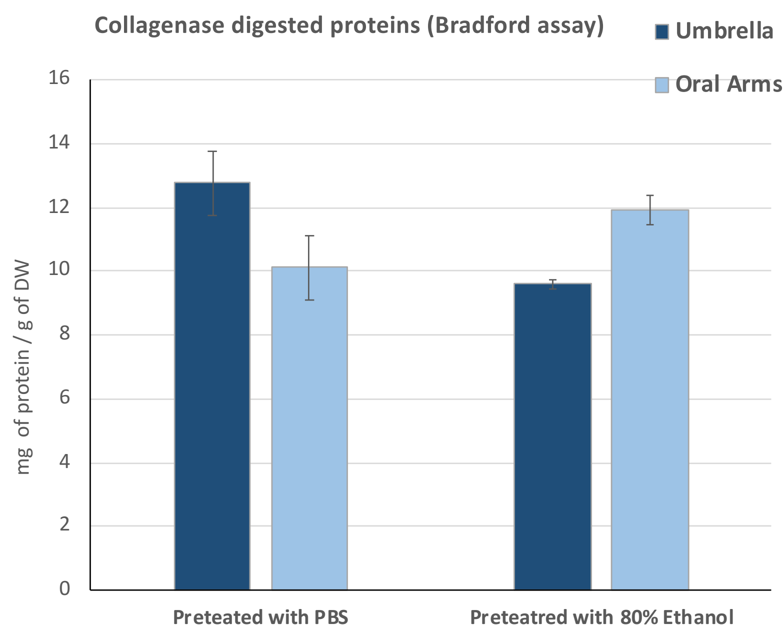


***Figure 40.*** Amount of proteins hydrolysed with collagenase estimated by Bradford assay, in umbrella and oral arms samples of *Cassiopea andromeda*, pre-treated with phosphate-buffered saline (PBS) or with 80% ethanol. Protein content is expressed as mg per gram of dry weight. Data are mean (n=6); Bars represent ± standard deviation (SD).

The evaluation of protein amount by Bradford assay were performed on collagenase digested samples to ensure conformity among protein content data in the other fractions. However, taking into account that the standard Bradford assay and also an its improved version (Lopez et al., 1993) were unable to accurately estimate protein contents in collagen rich samples, due to the action mechanism of the dye. Then, a simple and sensitive Lowry's method, first developed by Komsa-Penkova et al. (1996) and then slightly modified by Kiew and Don (2013), was used to determine the amount of collagen in our jellyfish collagenase hydrolysed samples.

Data of protein content in collagenase hydrolysed samples evaluated by Lowry assay are shown in Figure 41. As expected, values measured by the modified Lowry method were found three times higher than the values resulting from Bradford assay (Fig. 40). Fibrillar collagen concentration was of about 32.6 ± *1.5* and 34.6 ± *1.0* mg / g of DW of lyophilized umbrella and oral arms, respectively, in samples pre-treated with PBS. In umbrella and oral arms samples pre-treated with 80% ethanol, the amounts were about 22.0 ± *1.08* and 29.8 ± *0.47* mg / g of DW, respectively.

Independently from the absolute values, no difference between umbrella and oral arms as well as between pre-extraction type were found also by measuring the protein content with Lowry's assay in collagenase-hydrolysed samples.

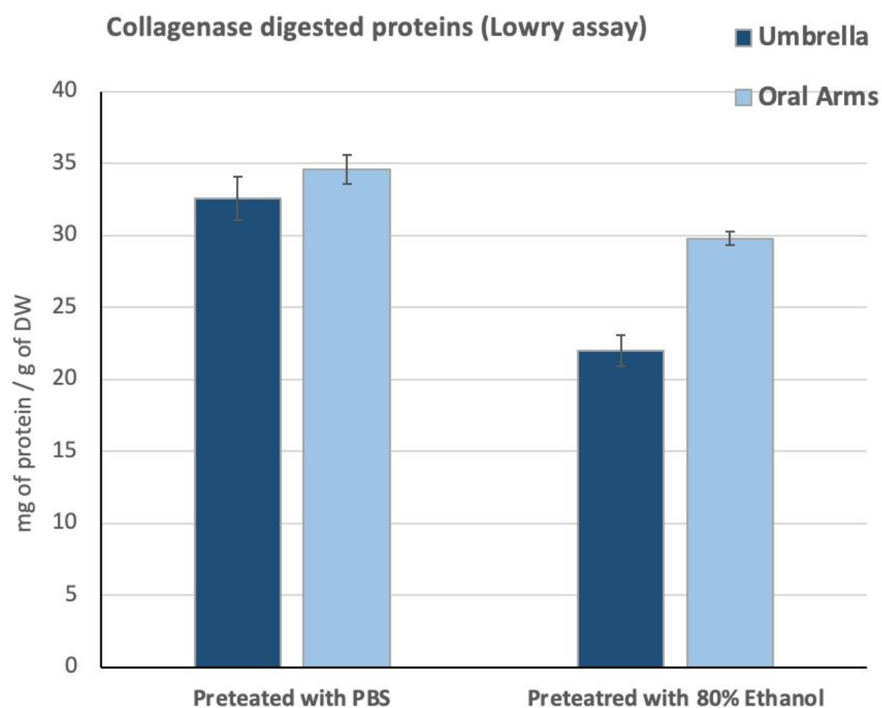


***Figure 41.*** Amount of proteins hydrolysed with collagenase estimated by Lowry assay, in samples of umbrella and oral arms of *Cassiopea andromeda*, pre-treated with phosphate-buffered saline (PBS) or with 80% ethanol. Protein content is expressed as mg per gram of dry weight. Data are mean (n=6); Bars represent ± standard deviation (SD).

*Total phenol content of collagenase-hydrolyzed fractions*

The fraction of collagen hydrolyzed by collagenase contained less amount of phenols as compared to the fraction containing pepsin-hydrolyzed proteins (Fig. 38 and 42). Collagenase-hydrolyzed fractions from umbrella and oral arms display no difference between the jellyfish body parts either between the used extraction solvents in the previous extraction by PBS or 80% ethanol. Data of phenols content in collagenase hydrolysed samples evaluated by Folin-Ciocalteu assay are shown in Figure 42. The amount of gallic acid equivalent (GAE) per g of dry weight were found to be 1103 and 1132 in the umbrella and in the oral arms, respectively, in samples previously subjected to PBS extraction (Fig. 42, A). Similar values, of 950 and 1197 µg GAE / g DW were then found in the samples of umbrella and oral arms subjected to extraction with 80% Ethanol (Fig. 42, B). No significant differences between the parts of the jellyfish body or between the

type of pre-extraction were found, indicating that the phenol content is a minoritarian and stable part of this fraction.

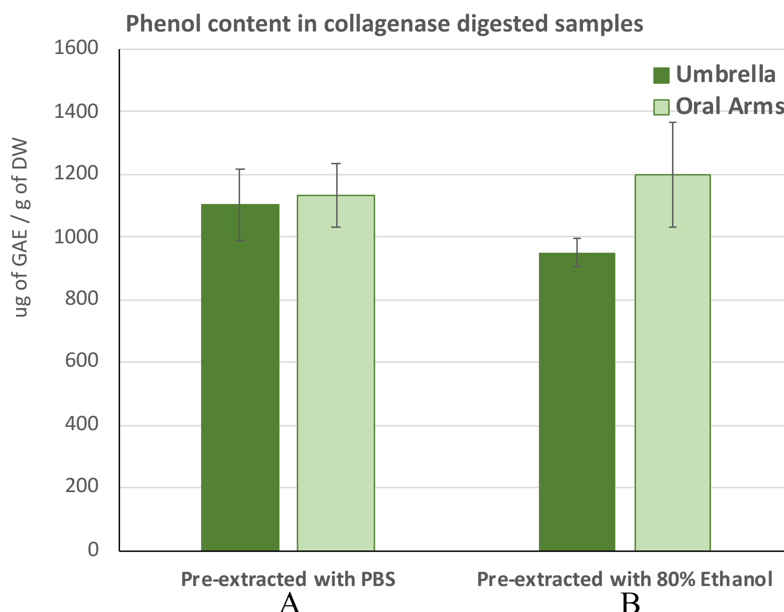


***Figure 42***. Amount of phenols in fractions hydrolyzed with collagenase derived from umbrella and oral arms of *Cassiopea andromeda*, pre-treated with phosphate-buffered saline (PBS) or with 80% ethanol and estimated by Folin-Ciocalteu assay. Phenols content is expressed as µg GAE / g per gram of dry weight. Data are mean (n=6); Bars represent ± standard deviation (SD).

*Antioxidant activity of collagenase-hydrolyzed fractions*

Hydrolyzed collagen samples from both oral arms and umbrella, as well as derived from both types of pre-extraction, also showed antioxidant activity (Fig. 43) even though it was lower than the antioxidant activity found in pepsin hydrolyzed samples (Fig. 39). As expected, the detected antioxidant activity was similar in samples of umbrellas and oral arms and no differences were detected between samples that were subjected to PBS extraction or 80% ethanol.

Indeed, the amount of antioxidant activity expressed as nmol of Trolox equivalents per mg of proteins were 683 nmol TE and 911 nmol TE / mg proteins in *C. andromeda* umbrella and oral arms samples, respectively, in samples pretreated with PBS. The values of antioxidant activity were 837 nmol TE and 842 nmol TE / mg of proteins in umbrella and oral arms, respectively, in jellyfish samples extracted with 80% Ethanol (Fig. 43).

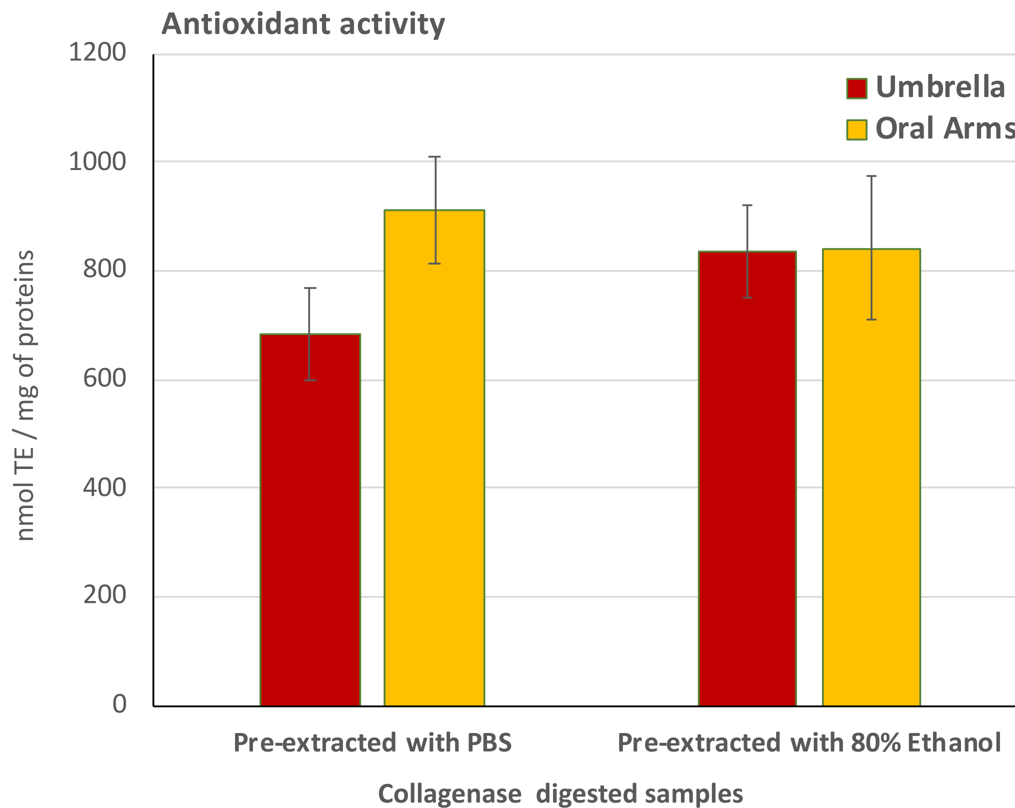


***Figure 43.*** Total antioxidant activity in umbrella and oral arms samples of *Cassiopea andromeda* jellyfish hydrolysed by collagenase after PBS (A) or 80% ethanol (B) extraction. Antioxidant activity is expressed as nmol of Trolox eq. (TE) per mg of proteins. Data are mean (n=6); Bars represent ± standard deviation (SD).

## 3.2 *Bioactivity of the hydroalcoholic extracts fractions of Umbrella and Oral Arms*

In order to deepen the aspects of the biological properties of *Cassiopea andromeda* and, possibly isolate active fractions with pro-apoptotic and anti-proliferative activity on cancer cells, an in-depth study on fractions coming from the hydroalcoholic extract of oral arms and umbrella of the jellyfish *C. andromeda* were carried out.

### 3.2.1 *Hydroalcoholic (80% ethanol) extraction*

The previous biochemical characterization of the biomass of jellyfish here described evidenced that a very high antioxidant activity was recorded in the hydroalcoholic extract of *Cassiopea andromeda*, mainly in the oral arms. In order to characterize the biological activity of the different jellyfish tissues, the extractions of compounds soluble in hydroalcoholic solution were performed from freeze-dried samples of umbrella (UMB) and oral arms (OA) of six out nine specimens of *C. andromeda*. Hydroalcoholic-soluble compounds were extracted with an 80% ethanol solution and the amount of total extract obtained from umbrella and oral arms, expressed as grams of dry weight (DW) and as percentage of the lyophilized samples are showed in Table 5.

***Table 5.*** Dry weight and yield of the hydroalcoholic extracts from umbrellas and oral arms of different specimens of *C. andromeda*. DW, Dry weight; SD, Standard deviation

| | | **UMBRELLA (UMB)** | | | **ORAL ARMS (OA)** | | |
|---|---|---|---|---|---|---|---|
| **Item** | **Specimen** | **DW (g)** | **Extract (g)** | **Yield (%DW)** | **DW (g)** | **Extract (g)** | **Yield (%DW)** |
| A1 | CAN4 | 0.931 | 0.382 | **41.1** | 1.142 | 0.56 | **49** |
| A3 | CAL1 | 0.615 | 0.204 | **33.2** | 0.761 | 0.356 | **46.8** |
| A4 | CAL3 | 0.636 | 0.264 | **41.6** | 1.059 | 0.464 | **43.8** |
| A5 | CAN2 | 0.393 | 0.164 | **41.7** | 0.946 | 0.408 | **43.2** |
| A7 | CAL4 | 0.822 | 0.36 | **43.8** | 1.052 | 0.459 | **43.6** |
| A8 | CAN3 | 0.852 | 0.356 | **41.8** | 0.95 | 0.423 | **44.5** |
| **Mean ±*SD*** | | **0.708 ±*0.2*** | **0.243 ±*0.1*** | **40.5 ±*3.7*** | **0.985 ±*3.7*** | **0.445 ±*0.1*** | **45.1 ±*2.3*** |

A quite constant amount of hydroalcoholic extract was obtained from different samples. The amount of dried extract from UMB samples was on average 0.243 ± *0.08* g, which was about 40.5% of the DW of starting biomass, while the amount of extract from OA was two times higher than the UMB extract being on average 0.445 ± *0.07* g, which was about the 45.1% of the dried initial biomass (Table 5).

*Fractionation of the hydroalcoholic extracts*

The jellyfish hydroalcoholic extracts (80% ethanol) contain a mixture of heterogeneous compounds, so, in order to obtain partially purified fractions a first separation was performed by protein precipitation. A 50% acetonitrile (ACN) solution was used to efficiently precipitate proteins larger than ~40 kDa (Leone et al., 2013; Romitelli et al., 2007; Ghasemi et al., 2007; Alpert and Shukla, 2003).

This phase separation methodology was used for the first time to partially characterize the hydroalcoholic extract of the whole jellyfish *Cotylorhiza tuberculata* by Leone et al. (2013).

This method is useful for separating different classes of components eventually present in a hydroalcoholic-soluble extract of complex samples, such as biomass of zooxanthellatae jellyfish. Indeed, the use of a mild organic solvent (FDA, 2012) as the solution acetonitrile : water (ACN:$H_2O$) and the avoidance of freeze-thaw cycles, damaging protein structures, resulted in an advantageous method for composite organisms (Leone et al., 2013).

After resuspension in acetonitrile/water solution, a three phases separation was obtained (Fig. 44-A) in a similar way to that observed by Leone et al. (2013) in *Cotylorhiza tuberculata* samples.

A lipophilic upper phase (UP) and a hydrophilic lower phase (LP) separated by a semisolid, green coloured, intermediate phase (IP) were obtained. After an accurate separation (Fig. 44-B), the characterization of the three phases of the jellyfish extract was attempted.

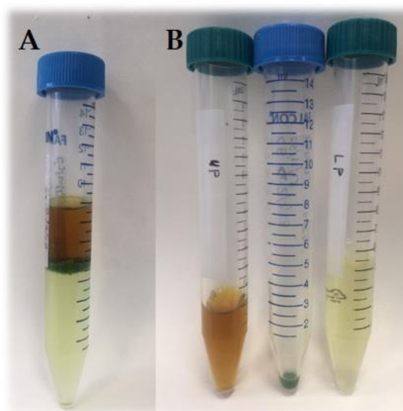


***Figure 44.*** (A) Three phases separation of the total hydroalcoholic extract of *C. andromeda* jellyfish after phase separation with acetonitrile/water solution; In (B) the obtained separated fractions .

### *3.2.2 Hydroalcoholic extract biochemical characterization*

Intermediate phase (IP) fractions were characterized of a bright green colour, that was hypothesised is due to the protein-pigment complexes of the symbiotic zooxanthellae, as highlighted for IPs and LPs in *Cotylorhiza tuberculata* jellyfish (Leone et al., 2013). However, comparing the colours of the IPs and LPs between *C. andromeda* and *C. tuberculata* it was clear that the Lower Phases of *C. andromeda* are not characterized by green colour.
The unique consistency of the intermediate phase (IP) was not useful for accurate analysis due to the insolubility in most of the solvents suitable for the study of biological activity (Fig. 45), so both Umbrella and Oral Arms IP fractions were not characterised in this work. The lipophilic upper phase (UP) and the aqueous lower phase (LP) were characterized for protein and phenol contents and for their antioxidant activity.

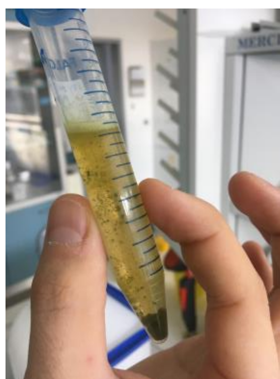

***Figure 45.*** Intermediate phase (IP) of *C. andromeda* showing an almost completely lack of solubility in Ethanol.

*Protein content*

Table 6 shows the amount of proteins detected in the UP and LP phases from both umbrella (UMB) and oral arms (OA), expressed as mg of proteins per gram of dried extract. The data confirm that the fractions obtained from the extract coming from the oral arms' samples give a higher protein content as compared to the fractions coming from the umbrellas' extract. This clearly indicated that composition of the hydroalcoholic extract of the oral arms significantly differs from that of umbrellas.

The content of proteins in the UP (lipophilic fraction) of *C. andromeda* umbrella was estimated about 0.88 ± *0.28* mg of proteins per gram of dried weight, while in the LP (hydrophilic fraction) were 2.30 ± *1.38* mg/g of DW. The protein content of UP and LP from oral arms extract were 2.09 ± *0.72* mg and 3.53 ± *1.46* mg of proteins per gram of dried weight, respectively (Table 6).

***Table 6.*** Amount of proteins, and Mean ± Standard Deviation (SD) in the upper phase (UP) and in the lower phase (LP) from both umbrella (UMB) and oral arms (OA) expressed as mg per g of Dry Weight (DW)

| Item | Specimen | UMBRELLA (UMB) | | ORAL ARMS (OA) | |
|---|---|---|---|---|---|
| | | Upper phase (UMB-UP) | Lower phase (UMB-LP) | Upper phase (OA-UP) | Lower phase (OA-LP) |
| | | mg / g of DW | | | |
| A1 | CAN4 | 0.797 | 2.011 | 1.726 | 2.704 |
| A3 | CAL1 | 0.41 | 0.508 | 1.375 | 2.791 |
| A4 | CAL3 | 0.873 | 1.837 | 3.06 | 2.808 |
| A5 | CAN2 | 0.986 | 1.576 | 1.488 | 2.268 |
| A7 | CAL4 | 0.978 | 3.656 | 2.896 | 4.605 |
| A8 | CAN3 | 1.249 | 4.225 | 1.997 | 6.014 |
| **Mean ±SD** | | **0.882 ±0.277** | **2.302 ±1.384** | **2.090 ±0.722** | **3.532 ±1.461** |

The evaluation of the amount of proteins in the different parts of jellyfish showed that oral arms contained more than double the proteins of the Umbrella if we consider the mg of proteins per g of Dry Weight, and more than three times if we consider the total extracted amount of proteins. Indeed, about 5.27 ± *2.23* mg of proteins per gram of dry extract (29.488 mg of total extracted proteins) and 2.29

± *1.07* mg of proteins per gram of dried extract (8.196 mg of total extracted proteins), was detected in the OA and UMB, respectively.
The distribution between the two phases (Table 6) also confirms the fact that the upper phases are mainly made up of non-polar or less-polar acetonitrile soluble compounds, being this protocol useful to separate proteins between phases in hydroalcoholic extracts of jellyfish tissue samples (Leone et al., 2013).

*Phenolic compounds*

The phenolic compounds, that are widespread between plant and plant-derived foods and that are one of the most effective antioxidants known in nature (Li et al., 2014; Weichselbaum and Buttriss, 2010; Tsao, 2010; D'Archivio et al., 2007), were found mostly in the oral arms (about 2400.1 ± *162* μg GAE/g of DW against the 1220.8 ± *261* μg GAE/g of DW in UMB). Furthermore, phenolic compounds were found mostly in the LP, as already found by Leone et al. (2013) in *C. tuberculata*. Indeed, the UMB-UP of *C. andromeda* contained about 226.9 ± *110.5* μg GAE/g DW, while the UMB-LP 693.7 ± *122.2* μg GAE/g of DW. In oral arms, furthermore, the OA-UP showed 374.9 ± *103.1* μg GAE/g DW and well 1399.1 ± *299* μg of gallic acid equivalents per g of dried weight in the LP (Table 7). This confirms that compounds present in the oral arms and soluble in the hydroalcoholic extract are different from that present in the umbrella.

***Table 7.*** Amount of phenolic compounds and Mean ± Standard Deviation by a modified Folin-Ciocalteu in the upper phase (UP) and in the lower phase (LP) from both umbrella (UMB) and oral arms (OA) expressed as μg of gallic acid equivalents (GAE) per g of dry weight (DW).

| **Item** | **Specimen** | **UMBRELLA (UMB)** | | **ORAL ARMS (OA)** | |
|---|---|---|---|---|---|
| | | **Upper phase (UMB-UP)** | **Lower phase (UMB-LP)** | **Upper phase (OA-UP)** | **Lower phase (OA-LP)** |
| | | μg GAE / g DW | | | |
| A4 | CAL3 | 255.4 | 827.5 | 425.1 | 1826.9 |
| A5 | CAN2 | 370.9 | 763.1 | 488.7 | 1365 |
| A7 | CAL4 | 129.9 | 619.7 | 331.1 | 1259 |
| A8 | CAN3 | 151.4 | 564.7 | 254.5 | 1145.5 |
| **Mean ±SD** | | **226.9 ±110** | **693.7 ±122** | **374.9 ±103** | **1399.1 ±299** |

*Antioxidant activity*

The evaluation of the antioxidant activity (expressed as Trolox Equivalents per g of dried extract) in the fractionated hydroalcoholic extracts showed the same trend, with the highest values in LPs, and in general in OA extracts (Table 8). In detail, the UP and LP of the oral arms have the higher antioxidant activity values (mean values of 1650 and 15592 nmol of Trolox equivalents (TE) per g of dried extracts, respectively) compared with the respective UP and LP of the umbrella (mean values of 911 and 8786 nmol of Trolox equivalents (TE) per g of dried extracts, respectively).

***Table 8.*** Values are Means ± Standard Deviation (n=4) of antioxidant activity by TEAC in the upper phase (UP) and in the lower phase (LP) from both umbrella (UMB) and oral arms (OA) expressed as nmol of Trolox Equivalents and normalized per g of dry weight, per mg of proteins and per µg of gallic acid equivalents.

| | **UMBRELLA (UMB)** | | **ORAL ARMS (OA)** | |
|---|---|---|---|---|
| | **Upper phase (UMB-UP)** | **Lower phase (UMB-LP)** | **Upper phase (OA-UP)** | **Lower phase (OA-LP)** |
| **Mean ±SD (nmol TE/g of DW)** | 911 ±275 | 8786 ±338 | 1650 ±156 | 15592 ±2288 |
| **Mean ±SD (nmol TE/mg of proteins)** | 887 ±274 | 3700 ±1626 | 727 ±331 | 4230 ±1551 |
| **Mean ±SD (nmol TE/ µg of GAE)** | 4214 ±1579 | 12365 ±2448 | 4365 ±331 | 10935 ±2632 |

Moreover, Table 8 shows that the antioxidant activity (AA) of the compounds in the fractions when were normalized to proteins or phenols contents (i.e., expressed as nmol TE/ mg of proteins or as nmol/TE per µg of GAE) were always higher in the lower phases (LP) than in the upper phase (UP). Since the LP showed the highest antioxidant capacity when normalized to phenols, we can reasonably suppose that the nature of this AA is related to compounds with active phenolic groups and to the high reactivity of these species (Roleira et al., 2015). Furthermore, since phenols are mainly produced by plants and microalgae (Jerez-Martel et al., 2017; Hattenschwiler and Vitousek, 2000) and the microalgae are equally present all over the body of *C. andromeda* jellyfish (Lampert et al., 2012), it can be supposed that the higher amount of phenols and the related antioxidant activity detected in the oral arms compared to the umbrella, is due to

the photosynthetic function of endosymbiotic zooxanthellae and to the habit of this jellyfish to stay upside-down on the sea floor (Jantzen et al., 2010). The better exposition to the light for the photosynthetic algae located into the oral arms.

### *3.2.3 Bioactivity of the hydroalcoholic extracts fractions of Umbrella and Oral Arms*

In order to evaluate the putative biological activity in cell systems of the fractions of extracts from *C. andromeda* jellyfish, a set of assays on human and murine cell culture systems were selected. The analyses performed on human cell cultures were carried out at the Institute of Sciences of Food Production of National Research Council (CNR-ISPA) of Lecce, Italy, while the assays performed on murine cell cultures were carried out during a period of abroad activity at the Research Centre for Toxic Compounds in the Environment (RECETOX) of the Masaryk University, Brno, Czech Republic.

#### *3.2.3.1 Effect on viability of MCF-7 and MB-MDA-231 cell cultures*

Considering the high antioxidant activity found in the extracts of jellyfish *C. andromeda* and the fact that various cell biological activities are related to the antioxidant activity, jellyfish extracts were analysed in their ability to reduce the cell viability on two well-established human breast cancer cells lines, MCF-7 and MB-MDA-231, with different features.

MCF-7 is a commonly used breast cancer cell line, that has been propagated for many years by multiple groups for studies on the first partially reversible breast cancer cell features (Comsa et al., 2015). It proves to be a suitable model cell line for breast cancer investigations worldwide, including those regarding anticancer drugs (Shirazi, 2011). MB-MDA-231 is a highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks oestrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification (Chavez et al., 2010; Liu et al., 2003). MB-MDA-231 is an aggressive form of breast cancer with limited treatment options, similarly to other invasive cancer cell lines, the invasiveness of the MB-MDA-231 cells is mediated by proteolytic degradation of the

extracellular matrix. The fractions of the hydroalcoholic extracts corresponding to the Upper (UP) and Lower Phases (LP) of umbrella (UMB) and oral arms (OA) of *C. andromeda* were assayed for their cytotoxic effects on cell cultures by a 24h-cell-treatment (Fig. 46-47) by MTS assay (see material and methods).

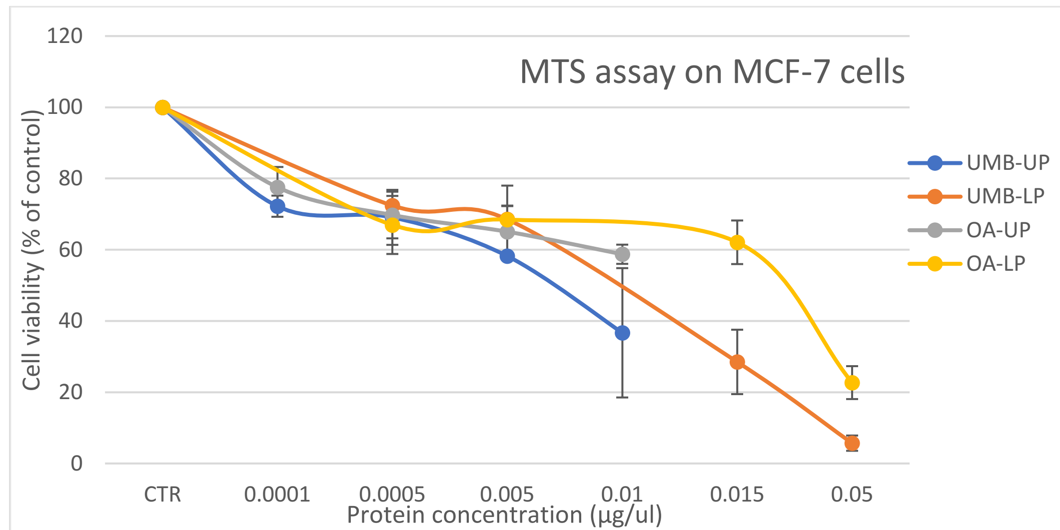


***Figure 46.*** Cell viability in MCF-7 cells treated with different concentrations of the fractions UP and LP of extracts from umbrella (UMB-UP, UMB-LP) and oral arms (OA-UP, OA-LP) of the jellyfish *C. andromeda*. Values are Mean ± Standard Deviation (n=3) of two independent experiments. ANOVA test followed by Dunnett's post-test was used to compare each treatment with the corresponding control (CTR). All differences were highly significant $p < 0.001$

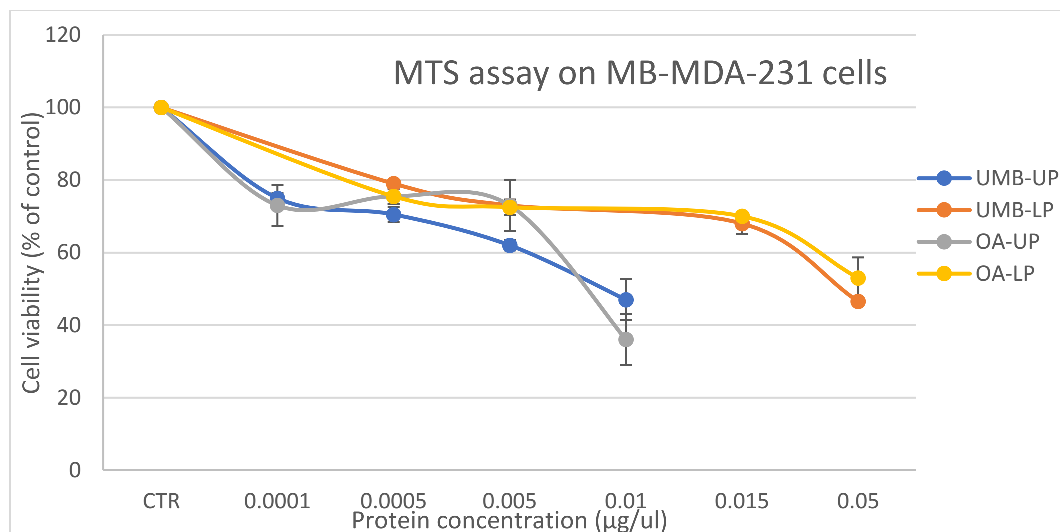


***Figure 47.*** Cell viability in MB-MDA-231 cells treated with different concentrations of the fractions UP and LP of extracts from umbrella (UMB-UP, UMB-LP) and oral arms (OA-UP, OA-LP) of the jellyfish *C. andromeda*. Values are Mean ± Standard Deviation (n=3) of two independent experiments. ANOVA test followed by Dunnett's post-test was used to compare each treatment with the corresponding control (CTR). All differences were highly significant $p < 0.001$

A dose-response assay was set up and a different range of amounts of the UPs and LPs, due to their different chemical composition and protein content, was assayed. In particular the UPs were tested in the range between 0.0001 and 0.01 µg/µl of contained proteins and the LPs were tested between 0.0005 and 0.05 µg/µl, all tested concentrations of both fractions (UP and LP) were compared to the corresponding controls (CTR).
Figure 46 and Figure 47 show that all used concentrations of both UP and LP fractions, significantly reduce the cell viability in both tumor cell cultures and a dose dependent cytotoxicity was exerted by all jellyfish extracts fractions ($p < 0.001$).
In particular, Lower Phase fractions from both umbrella (UMB-LP) and oral arms (OA-LP) at the lowest concentration (0.0005 and 0.005 µg/µl) showed similar effect on both MCF-7 and MB-MDA-231 cell cultures, reducing the cell viability to about 70% in MCF-7 (Fig. 46) and 77% in MB-MDA-231 (Fig. 47). When administrated on cells at 0.015 µg/µl just the UMB-LP induces a considerable decrease in vitality (28.5%) on MCF-7 cells, while the OA-LP weakly reduces vitality to about 62% of the control (Fig. 46), as well as on MB-MDA-231 both UMB-LP and OA-LP phases administered at this concentration do not induce a strong reduction of vitality, with a reduction of cell viability of 68% and 70% of the control, respectively. Noteworthy, both UMB-LP and OA-LP phases, were highly cytotoxic at the highest tested concentration (0.05 µg/µl) against MCF-7 showing a cell viability reduced to 6% and 23%, respectively, as compared to the controls (Fig. 46). Remarkably, at the same concentration, both UMB-LP and OA-LP fractions were less cytotoxic against MB-MDA-231, indeed, the cell viability was 46.5% and 53% of the control, respectively (Fig. 47).
Similarly, a dose-response effect for the upper phase fractions from both the umbrella and oral arms (UMB-UP and OA-UP) was also detected in both tumor cell types. Although, the upper phases (UMB-UP and OA-UP) showed effects with a more similar trend between MCF-7 and MB-MDA-231 cells than the hydrophilic fractions, LPs (Fig. 46-47).
When were administered at the lower tested concentrations (0.0001 and 0.0005 µg/µl) both UMB-UP and OA-UP induced similar effects in both MCF-7 and MB-MDA-231 cell cultures, with a registered cell viability of about 70.5 and 74% of the controls in MCF-7 cells (Fig. 46) and 72.5 and 74% in MB-MDA-231,

respectively (Fig. 47). However, at the higher tested concentration (0.01 μg/μl), there was a different effect of OA-UP, which reduced MCF-7 cell viability to 59% of the control while was 36% in MB-MDA-231 cells. No significant differences were detected between the effect of UMB-UP on the two cell lines, MCF-7 and MB-MDA-231 (37% and 47%, respectively) (Fig. 46 - 47).
In order to compare the effects of the four fractions of jellyfish extracts on the two cancer cell types, the $IC_{50}$ values for each treatment and cell type was evaluated (Table 9).

***Table 9***. IC50 values (μg/μl) calculated from the MTS assay data.

| Cell Line | Umbrella (UMB) | | Oral Arms (OA) | |
|---|---|---|---|---|
| | **Upper phase (UMB-UP)** | **Lower phase (UMB-LP)** | **Upper phase (OA-UP)** | **Lower phase (OA-LP)** |
| | IC50 (μg/μl) | | | |
| **MCF-7** | 0.0064 | 0.0093 | 0.0123 | 0.0165 |
| **MB-MDA-231** | 0.0075 | 0.0282 | 0.0077 | 0.0337 |

Significantly, data of IC50 (Table 9) show that the cell type most sensitive to almost all tested jellyfish extract fractions was MCF-7 cell line while MB-MDA-231 where more resistant except for the treatment with OA-UP.
In particular, MB-MDA-231 were up to three times more resistant than MCF-7 to both hydrophilic fractions, UMB-LP and OA-LP, of the jellyfish extract. The IC50 for MCF-7 was 0.0093 μg/μl and 0.0165 μg/μl for UMB-LP and OA-LP, respectively, while for MB-MDA-231 cells the IC50 was 0.0282 μg/μl and 0.0337 μg/μl for UMB-LP and OA-LP, respectively.
Noteworthy, the lipophilic UP fractions were really effective on both cell types, indeed concentrations of 0.0064 μg/μl and 0.0075 μg/μl were able to be cytotoxic to the 50% of cell population of MCF-7 and MB-MDA-231, respectively. The data regarding the fraction OA-UP indicated an opposite trend but they need to be confirmed with additional experiments.
The two cancer system models here used, where MB-MDA-231 are representative of highly metastatic cancer cells rather resistant to chemotherapy treatments, while MCF-7 cells are sensitive to certain compounds and used as model for testing the treatments, give interesting results. These data indicated that all

jellyfish extract fractions contain compounds putatively efficient against both breast cancer cells. Treatments with the hydroalcoholic extract fractions of *C. andromeda* here studied cause significant cell death in both cell lines, however the partially purified lipophilic fractions (UPs) are cytotoxic at lower doses also on MB-MDA-231 cells, this is remarkable given the resistance feature of this cancer cell system model. In addition to the specific composition, perhaps this action could be due, at least in part, to the lipophilic nature of the UP fractions, since drugs and several chemotherapeutic agents must be lipophilic to have a good absorption and to penetrate cellular membranes (Rezano et al., 2020; Liu et al., 2009) or because the antioxidant action is exerted at level of membrane functions.

*Preliminary evaluation of apoptotic effect*

In order to verify if the cytotoxicity of the jellyfish extract is due to a nonspecific toxic effect or is the effect of apoptosis induction, a preliminary qualitative experiment with Annexin V and Propidium Iodide labelling was performed on MCF-7 cells treated with two concentrations (0.0005 μg/μl and 0.01 μg/μl) of the jellyfish fraction OA-UP (Fig. 48).

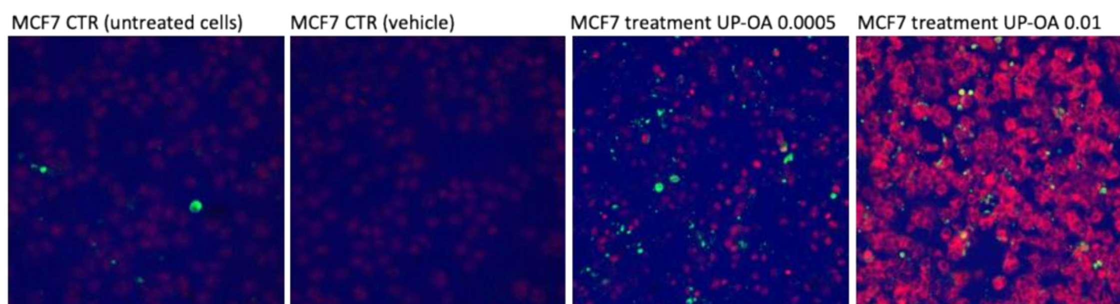


***Figure 48.*** Confocal images of apoptosis detected by labelling with Alexa Fluor® 488 conjugate Annexin V (green) and propidium iodide (red), in MCF-7 cells treated 0.0005 μg/μl and 0.01 μg/μl of the jellyfish extract fraction UP-OA for 24h and compared with untreated cells (medium) and cells treated with 0.025% acetonitrile as vehicle control (vehicle). All the pictures are the matching images from red and green channel fluorescence detectors. Images shown are representative of two independent experiments, each done in quadruplicate.

The preliminary results showed that the cytotoxicity of the OA-UP jellyfish extract fraction at 0.0005 μg/μl was mainly due to an apoptotic effect as demonstrated from the of green spot (early apoptosis) and green (Annexin V) and red (Propidium Iodide) labelled cells in MCF-7 cultures showed in the images in Figure 48. At higher concentration (0.01 μg/μl) a higher number of cells

were labelled with Propidium Iodide (red) showing not only cells in late apoptosis (green and red labelled cells) but also a number of necrotic or died cells (cells coloured in red by the Propidium Iodide). This demonstrated that at lower concentrations the cytotoxic mechanism of UP-OA is due to the pro-apoptotic effect while at higher concentrations other mechanisms may also intervene.

### *3.3 Evaluation of the effect of jellyfish extracts fractions on GJIC in murine cell models, WB-ras and WB-neo*

*The following part presents results obtained by research activity carried out at Research Centre for Toxic Compounds in the Environment (RECETOX) of the Masaryk University, Brno, Czech Republic with SECANTOX group, Cell and Tissue Toxicology Lab that is part of the Environmental Toxicology Division of RECETOX, managed by Dr. Pavel Babica and Dr. Iva Sovadinová (group leaders). The mobility abroad was carried out thanks to the RECETOX centre, the Erasmus Plus Programme and the University of Siena that covered the material and human costs.*

Considering the effect on cell viability observed in MCF-7 and MB-MDA-231 cell lines (previously described), in order to investigate the mechanism of action of the cytotoxic effect of active jellyfish fractions, the study was extended to the evaluation of the effect of *Cassiopea andromeda* extracts on the Intercellular Communication mediated by Gap Junction (GJIC).
Based on the observed effect on the cell viability on breast cancer cell models, MCF-7 and MB-MDA-231, the effect of the two fractions, UPs and LPs, of the extract of *C. andromeda* was studied on the GJIC functionality by using the Scrape Loading / Dye Transfer assay on two cell lines derived from WB-F344 rat liver epithelial cell lines, WB-*neo* and WB-*ras*.
Most of the in vivo tumor promotion assays were done in rat liver, specifically in the Fischer 344 rats, the WB-344 cell line was designed to match in vitro experiments in liver cell line from the same rat strain. Also, the WB-344 cell line is an immortalized diploid cell line that is non tumorigenic (Tsao et al., 1994), has been well characterized around the world for its expressed gap junction genes and its ability to perform GJIC via all available techniques, has been tested with all kinds of tumor-promoting chemicals for their ability to block GJIC (Trosko et al., 1993)
The WB cell lines were obtained by transformation with either a retrovirus-containing the neutral gene *neo* (WB-*neo*) or an activated *H-ras* oncogene (WB-*ras*). The WB-*neo* cells strongly express Connexin 43, one of the structural proteins forming gap junction channels, and strongly communicate via GJs in vitro. While

WB-*ras*, being oncogene transformed cells, completely lost their capability to communicate via GJIC (Ruch et al., 1993). Moreover, WB cells were able to create a good monolayer of cells, essential factor for the success of the scrape loading dye transfer assay.
Since this is the first time that jellyfish extract of *C. andromeda* are used on this WB-*neo* and WB-*ras* cell systems, accurate preliminary cytotoxic tests were performed, since cell viability is an essential requirement for the correct evaluation of cell-cell communication and GJIC function. Therefore, as first step we have defined the toxic concentration limits of the hydroalcoholic fractionated extracts of *Cassiopea andromeda* and the corresponding solvents (Vehicles) in which they are solubilized, in order to set the non-cytotoxic concentration range of the fractions that will evaluate for their ability to modulate GJIC.

### 3.3.1 *Evaluation of cell viability by jellyfish extracts fractions on WB cell models*

A series of experiments were carried out in order to establish the higher non-toxic final concentration in this specific experimental model.
The effect of 8 different final concentrations (between 0,29% to 5%) of the two used solvents ACN (Acetonitrile) and ACN/$H_2O$ 1:1 v/v in the extraction of the phases has been evaluated as first step to define the toxic concentration limits for both cell lines, WB-*ras* (tumorigenic *ras*-oncogene transformed) and WB-*neo* (non-tumorigenic). To do that, the 3-dyes cytotoxicity assay was selected instead of MTS assay, due to the ability of this test to evaluate different effects, which cannot be observed with the MTS assay used on MCF-7 and MB-MDA-231. This assay makes use of three dyes able to evaluate the disturbance or disruption of three important cellular processes, namely, the Resazurin dye (RES) highlights the residual metabolic reductive potential of the cells, the 5-carboxyfluorescein diacetate acetoxymethyl ester dye (5-CFDA-AM) highlights the membrane integrity and the functionality of esterases, finally the neutral red dye (NR) highlights viable cells, highlighting the functioning of the cellular lysosomal system.
After 24h of cells growth and 48h of cells treatment, acetonitrile showed a mild cytotoxic effect at final concentration of about 1.5 and 2.5% for WB-*neo* and WB-

*ras*, respectively, whereas it showed a high cytotoxicity at a concentration of 5% for both cell lines.

After the definition of the toxic concentration limits of solvent for both cell lines, the Upper (UMB-UP and OA-UP) and Lower Phases (UMB-LP and OA-LP) were assayed for their cytotoxic effects (Fig. 49-56). The UPs were tested with the same conditions (24h of cells growth and 48h of cells treatment) and at the final concentrations of 0.0002, 0.0004, 0.001, 0.0025, 0.006 and 0.015 µg/µl of contained proteins and the LPs at 0.0005, 0.0001, 0.003, 0.008, 0.02 and 0.05 µg/µl given that these final concentrations are in the range of the final concentration used in MCF-7 and MB-MDA-231 cells and have already been used in a previous work of evaluation of GJIC of the same type of extracts obtained from *C. tuberculata* in MCF-7 and human non cancer keratinocytes (Leone et al., 2013).

All tested concentrations of all phases and the solvent controls were normalized and compared to the respective non treated controls (NCs). Each experiment was conducted with two solvent controls at the two respective highest concentrations (0.006 and 0.015 µg/µl and 0.02 and 0.05 µg/µl for UPs and LPs, respectively) and the results (vehicle only) were showed as dots in the graphs.

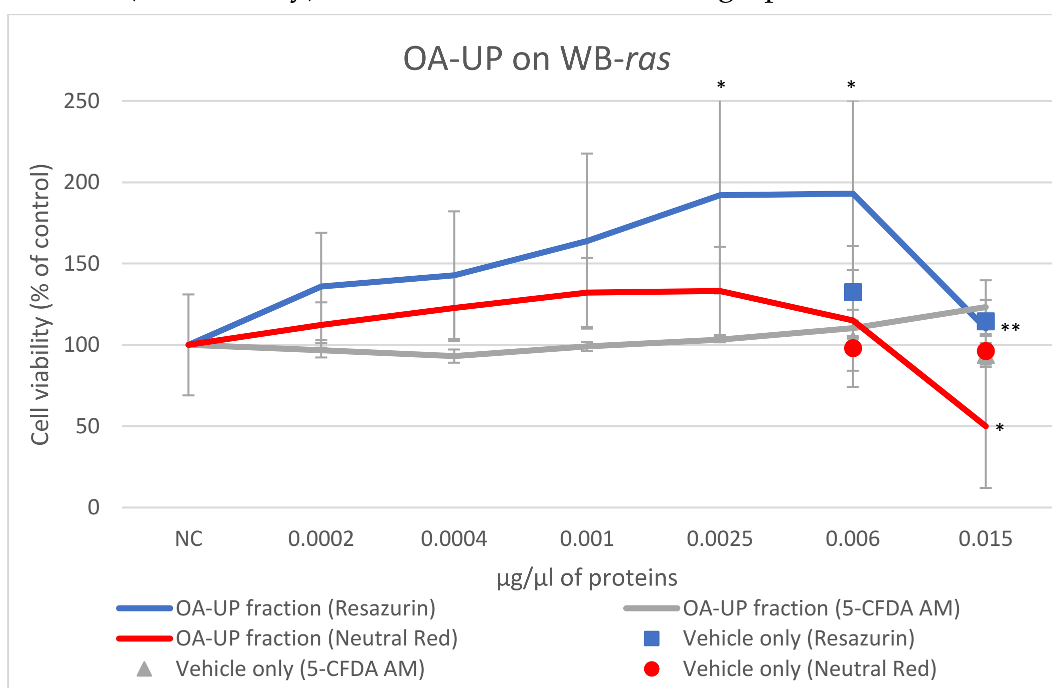


***Figure 49.*** The effect of OA-UP fraction on cell viability of WB-*ras* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05, **p<0.01.

As first, the effect of the upper phase of the hydroalcoholic extract of oral arms (OA-UP) was evaluated on WB-*ras* (tumorigenic cell line) (Fig. 49). At the highest tested concentration (0.015 µg/µl), OA-UP showed a high cytotoxic effect (Neutral Red dye). When administrated at concentration of 0.001, 0.0025 and 0.006 µg/µl, a notable increase of the metabolic reductive potential (Resazurin dye) and a mild increase in the activity of lysosomal system (Neutral Red dye) were estimated. The enzymatic activity and membrane integrity were stable for all the tested concentrations as demonstrated by 5-CFDA AM dye (Fig. 49).
The upper phase of the Umbrella (UMB-UP) showed the same trend of the OA-UP on WB-*ras* cells (Fig. 50) with the Neutral Red dye that highlight the defection of the lysosomal system, so the reduction of the cell viability at the highest tested concentration (0.015 µg/µl). However, the Resazurin dye and the Neutral Red showed that the UMB-UP increases the metabolic reductive potential and the activity of the lysosomal system, respectively, above all, at the used concentration of (0.001, 0.0025 and 0.006 µg/µl). The enzymatic activity and membrane integrity (5-CFDA AM dye) were stable for all the tested concentrations. (Fig. 50).

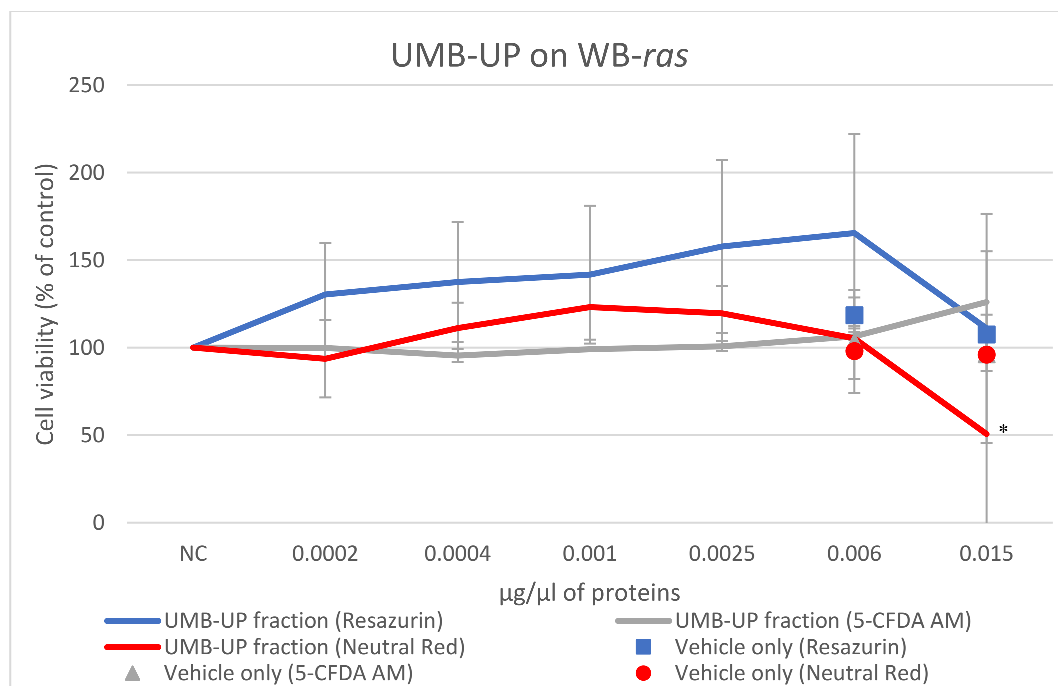


***Figure 50.*** The effect of UMB-UP fraction on cell viability of WB-*ras* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05.

Conversely, no sign of reduced cell viability was observed after administration of the lower phase of the oral arms (OA-LP) on WB-*ras* cells (Fig. 51), not even at the high concentration tested (0.05 µg/µl) (Fig. 51). However, the OA-LP showed to increase the metabolic reductive potential (Resazurin) of the cells at the concentration of 0.02 and 0.008 µg/µl, but also at 0.0032 µg/µl and at the higher (0.05 µg/µl). The membrane integrity and the functionality of esterases (5-CFDA AM) were not affected at all the tested concentrations except for the highest (0.05 µg/µl) that showed an increased esterases activity.

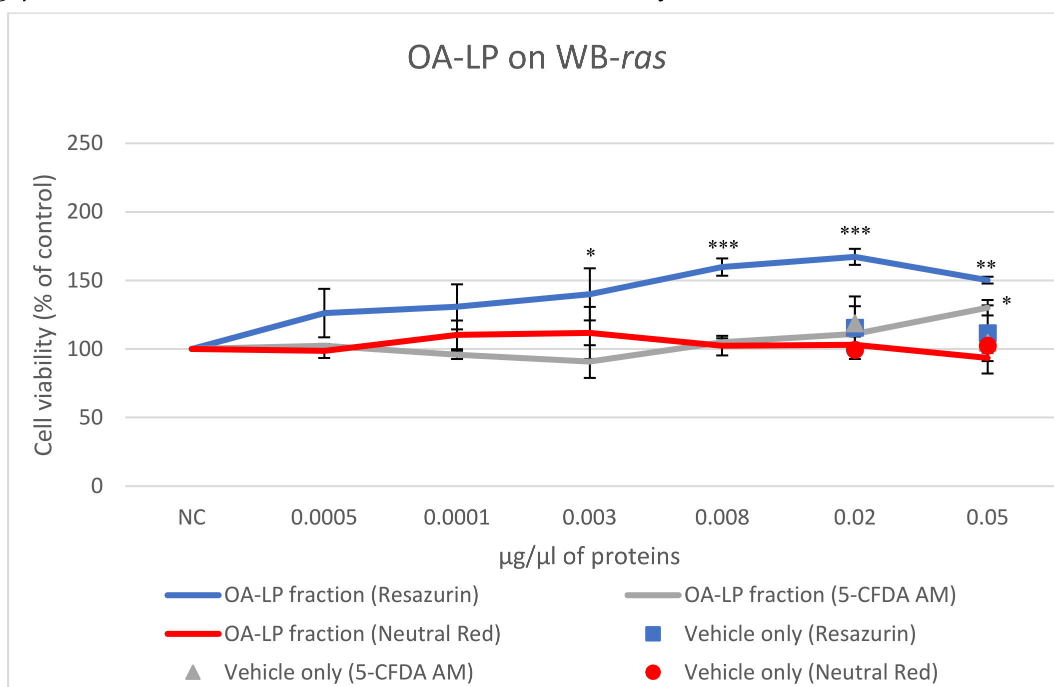


***Figure 51.*** The effect of OA-LP fraction on cell viability of WB-*ras* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ±standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05, **p<0.01, ***p<0.001.

Again, the lower phase of the umbrella (UMB-LP) did not affect WB-*ras* cells vitality, not even at the high concentration tested 0.05 µg/µl (Fig. 52). The UMB-LP increases the metabolic reductive potential (Resazurin) at the two higher concentration 0.05 and at 0.02, and in less amount at 0.008 µg/µl. The 5-CFDA AM dye showed again no sign of variation in membrane integrity and/or esterases functionality except at the highest concentration of 0.05 µg/µl, where there was an increase of the esterases functionality.

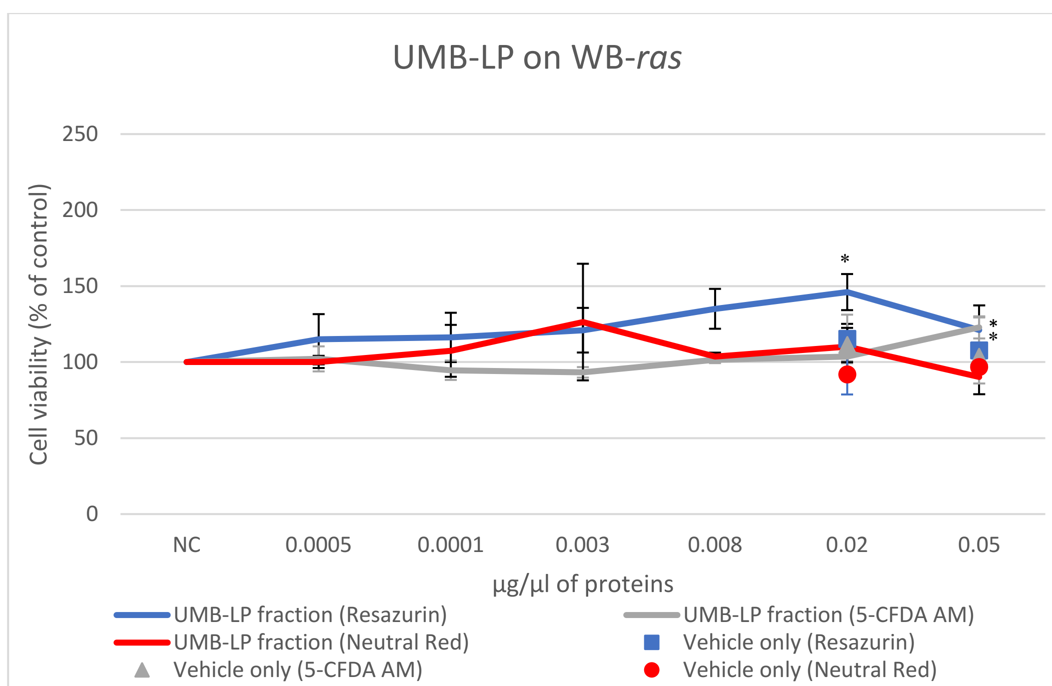


***Figure 52.*** The effect of UMB-LP fraction on cell viability of WB-*ras* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ±standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05.

Then, after the evaluation of the cytotoxicity effects on WB-*ras* cells, the cytotoxic effects of all hydroalcoholic fractionated phases were evaluated on WB-*neo* cells. Isogenic to WB-*ras*, as we already reported, WB-*neo* are non-tumorigenic cells transfected via a blank plasmid that express higher levels of connexins and have a strong cell-cell communication, which lacks into their neoplastic WB-*ras* counterparts. However, these cells, like usually happen with normal cells, are more sensitive to compounds, as already seen with the results of our solvent cytotoxicity assay.

The effects of the upper phase of the oral arms (OA-UP) were evaluated on WB-*neo* (Fig. 53). The Neutral Red dye highlight the cytotoxicity of the OA-UP at the highest tested concentration (0.015 µg/µl), with the defection of the lysosomal system, while it showed to increase the metabolic reductive potential (Resazurin dye) above all at the concentration of 0.006, 0.0025 and 0.001 µg/µl) before to drastically decrease at the higher concentration of 0.015 µg/µl. The enzymatic activity and membrane integrity were stable for all the tested concentrations. (Fig. 53, 5-CFDA AM dye).

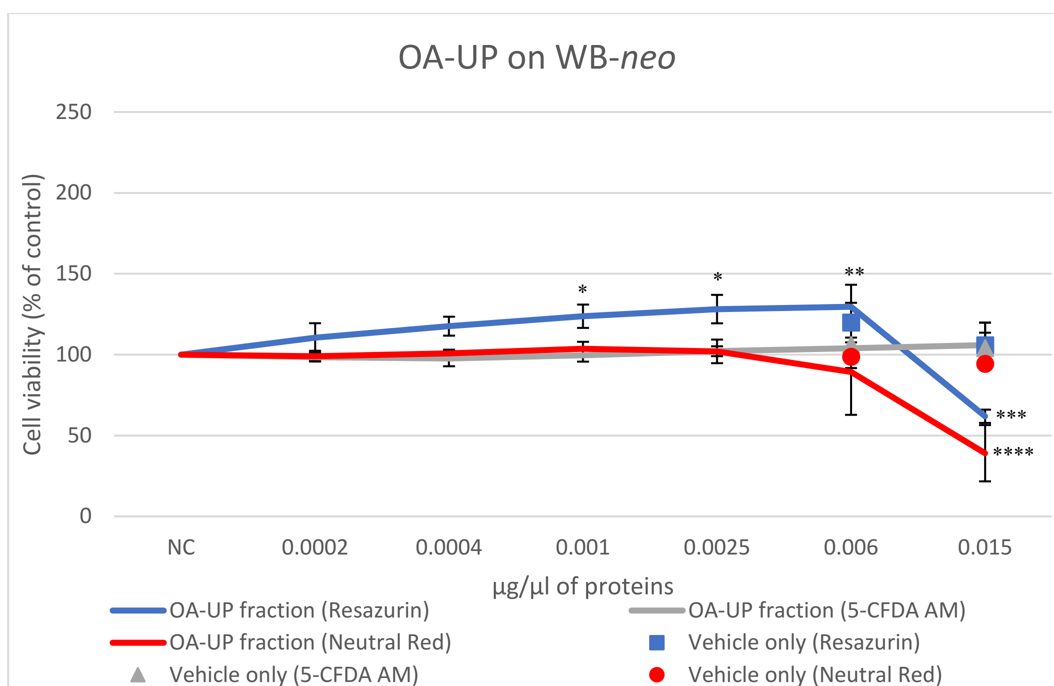


***Figure 53.*** The effect of OA-UP fraction on cell viability of WB-*neo* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The upper phase of the Umbrella (UMB-UP) showed the same trend of the OA-UP on WB-*neo* cells (Fig. 53) with the reduction of the cell viability, at the highest concentration of 0.015 µg/µl (Fig. 54, Neutral Red). However, the UMB-UP showed to increase the metabolic reductive potential (Resazurin dye) at the concentration of 0.006 and 0.0025 µg/µl, like the OA-UP but not in a statistically significant way. The esterase functionality and membrane integrity related values were stable for all the tested concentrations. (Fig. 54, 5-CFDA AM).

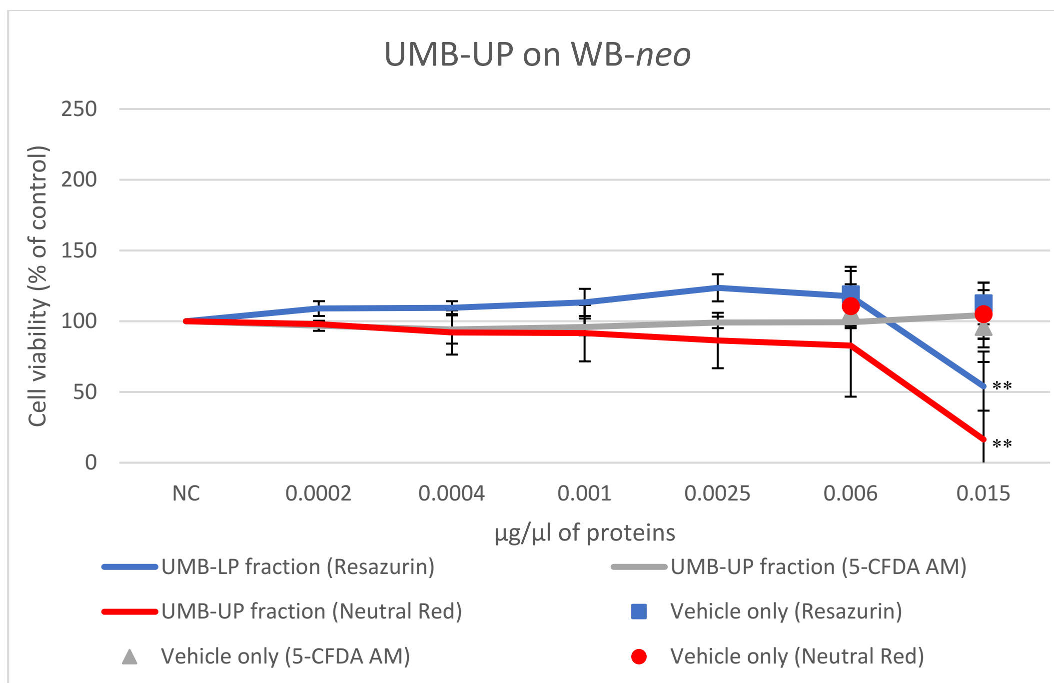


***Figure 54.*** The effect of UMB-UP fraction on cell viability of WB-*neo* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), **p<0.01.

The lower phase of the oral arms (OA-LP) showed no sign of defection of the lysosomal system on WB-*neo* cells at the highest concentration of 0.05 µg/µl (Fig. 55 – Neutral Red).

However, an increase of the metabolic reductive potential (Resazurin dye) and the esterase functionality (5-CFDA AM) was detected at the higher concentration (0.05 µg/µl) and at the concentration of 0.02 µg/µl (Fig. 55).

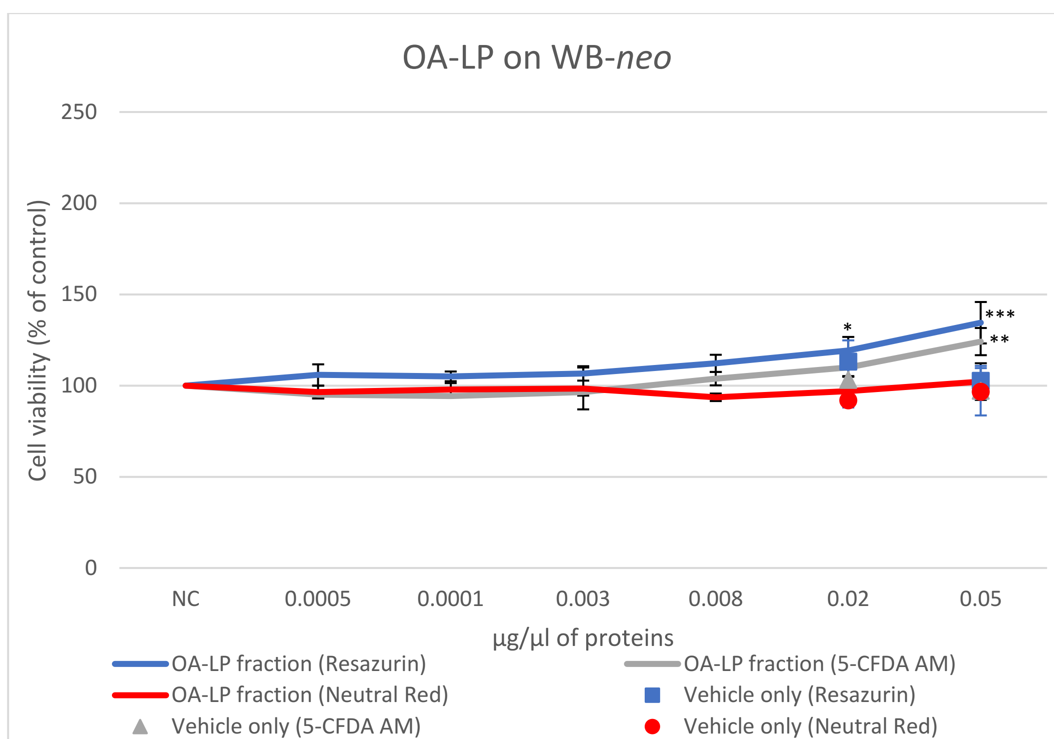


***Figure 55.*** The effect of OA-LP fraction on cell viability of WB-*neo* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05, **p<0.01, ***p<0.001

The lower phase of the umbrella (UMB-LP) showed the same trend displayed by the lower phase of the oral arms (OA-LP) (Fig. 55), with no sign of reduction of cell viability at the highest concentration of 0.05 µg/µl (Fig. 56 – Neutral Red) and with an increase of the metabolic reductive potential (Resazurin dye) and the esterase functionality (5-CFDA AM) detected at the higher concentration (0.05 µg/µl) and at 0.02 µg/µl (Fig. 58).

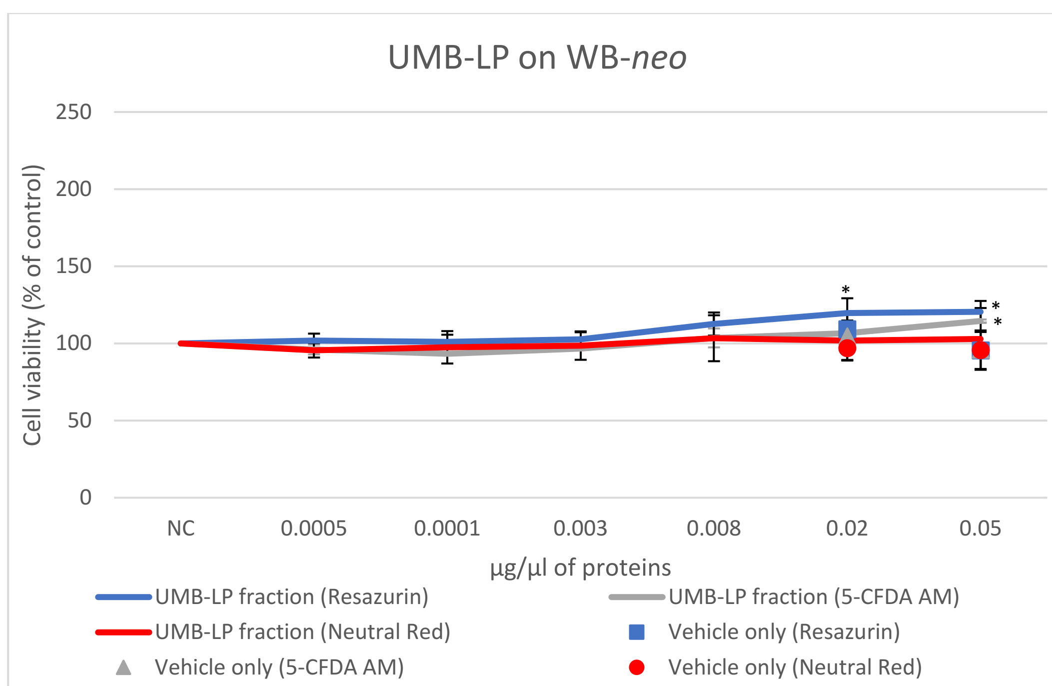


***Figure 56.*** The effect of UMB-LP fraction on cell viability of WB-*neo* cells was measured by the 3-dyes cytotoxicity assay. The effect of the vehicle alone at the two highest concentrations was also shows as dots. Values are mean of three independent experiments performed in three technical replicates ± standard deviation, with one specimen (A8). ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05.

With both cell lines (WB-*ras* and WB-*neo*), the UPs (UMB-UP and OA-UP) showed to be more cytotoxic, with the defection of the lysosomal system and the alteration of the cellular pH at the higher tested concentration of 0.015 μg/μl, if compared to the effects showed by LPs (UMB-LP and OA-LP) at the higher tested concentration of 0.05 μg/μl, that seems not alter the cellular vitality in both cell lines. Moreover, both the UPs showed an increase in reduction of resazurin that relates to an increased mitochondrial activity, where resazurin is mainly reduced. All phases (UMB-UP, UMB-LP, OA-UP, OA-LP) cannot be tested at higher concentrations, since the solvents, and in particular the ACN:H2O (1:1, v:v) starts to be highly cytotoxic at a final concentration higher than 2.5 and 1.5% for WB-*ras* and WB-*neo* cells, respectively, as we already defined with the solvent cytotoxicity assay.

It is important to highlight, that due to the different concentration of proteins in the different fractions, the volume of compound used were different. The final solvent concentrations at the higher tested concentrations were about 3.6% for UMB-UP, 3% for UMB-LP, 2.5% for OA-UP and 2.3% for OA-LP. This means, that

all phases at the higher tested concentrations were probably already toxic due to a too higher amount of solvent for WB-*neo*, while just the UMB phases were probably already toxic due to solvent amount for WB-*ras*.
However, at concentrations of 0.02 µg/µl for LPs and 0.006 µg/µl for UPs, the solvents' percentages were below the previously defined toxicity limits for both, WB-*ras* and WB-*neo* cells.
Considering that cytotoxic solvent concentrations have to be eliminated and that the tested jellyfish fractions showed non-cytotoxic effects in a suitable range of concentrations were selected for the scrape loading dye transfer assay (SL/DT assay) to test the GJIC mediated cell-cell communication. In particular, were evaluated the following five concentrations: 0.0002, 0.0004, 0.001, 0.0025 and 0.006 µg/µl for the Upper Phases, UMB-UP and OA-UP, and 0.0005, 0.0001, 0.003, 0.008 and 0.02 µg/µl for the Lower Phases, UMB-LP and OA-LP.

### 3.3.2 *Effect of jellyfish extracts fractions on GJIC functionality in WB cell models*

Cell-cell communication have essential role in homeostasis and cell growth, intercellular communication mediated by gap junctions, able to allow the passage of ions and small regulatory molecules, have a pivotal role in carcinogenesis and have been regarded as a target for tumor-promoter as well as anti-tumor promoter agents. (Zefferino et al., 2019; Trosko et al., 1994). Here we evaluate the effect of compounds present in *C. andromeda* jellyfish extract on GJIC on WB-*neo* and WB-*ras* cell lines, in order to assess whether their mechanism of action underlying the cytotoxic activity on cancer cells is mediated by modulation of GJIC.
Gap junctional intercellular communication (GJIC) has been evaluated *in vitro* (24h of cells growth and 48h of cells treatment) with the concentrations of UPs and LPs of *Cassiopea andromeda* previously established, on WB-*ras* (tumorigenic *ras*-oncogene transformed) and WB-*neo* (non-tumorigenic) cell models derived from WB-F344 rat liver epithelial cells (De Feijter et al., 1990). Data are referred as percentage of the controls treated with medium only (NC, negative control). Figure 57 shows that concentrations from 0.001 to 0.02 µg/µl of the two LP fractions are able induce an increasing of GJIC functionality on the non-

communicating WB-*ras* cells as compared to the control. A dose-response effect was also evident with a stronger effect at concentrations of 0.008 µg/µl, where the GJIC functionality in presence of UMB-LP and OA-LP reached values of 184% and 140% of the control value, respectively. This evidence the ability of the jellyfish compounds to restore GJIC functionality in this model of non-communicating cancer cells.

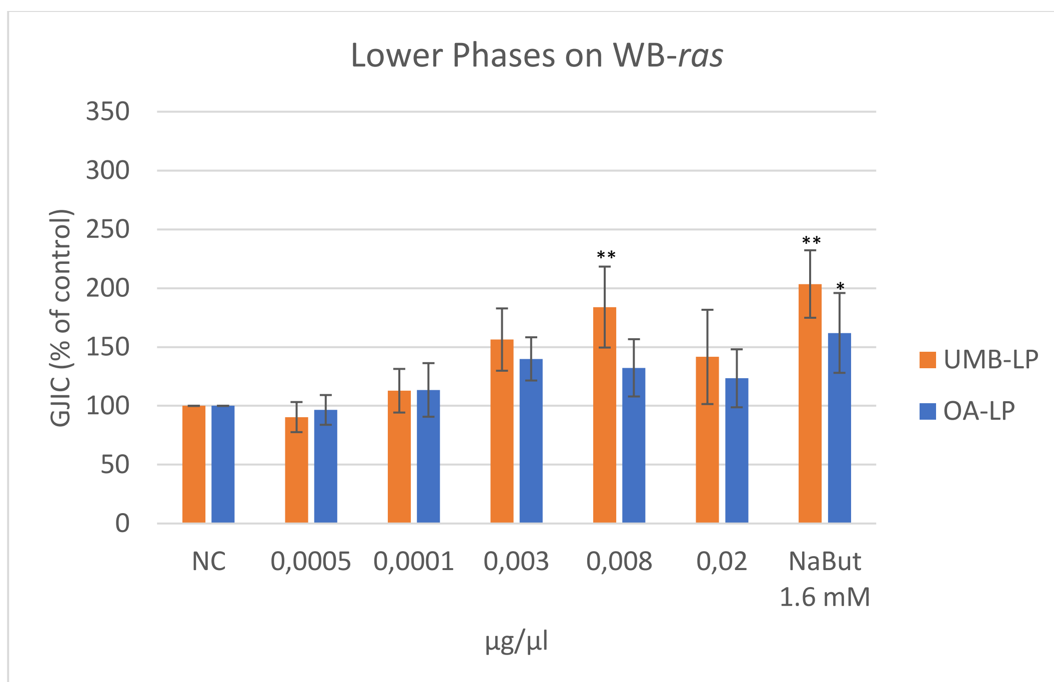


***Figure 57.*** Evaluation of the Gap Junction Intercellular Communication functionality by SL/DT assay on WB-*ras* cells with both the fractions of jellyfish UMB-LP and OA-LP . Values are mean of three independent experiments performed in three technical replicates ± standard deviation with one specimen (A8). Nabut (Sodium butyrate) 1.6 mM was used as positive control. ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *p<0.05, **p<0.01.

Also, the jellyfish fractions corresponding to Upper Phases (UMB-UP and OA-UP) induced a remarkable increasing of the functionality of GJIC, already at concentration of 0.001 µg/µl, with values of 164% and 171% of the control value for UMB-UP and OA-UP, respectively and at concentration of 0.0025 µg/µl, with a value of about 169% for both UMB-UP and OA-UP (Fig. 58).

The higher tested concentration (0.006 µg/µl) of both the UP fractions, UMB-UP and OA-UP, however, exerted a toxic effect in both cell lines with a massive detachment of the cells during the assay, that did not allow to perform the GJIC evaluation.

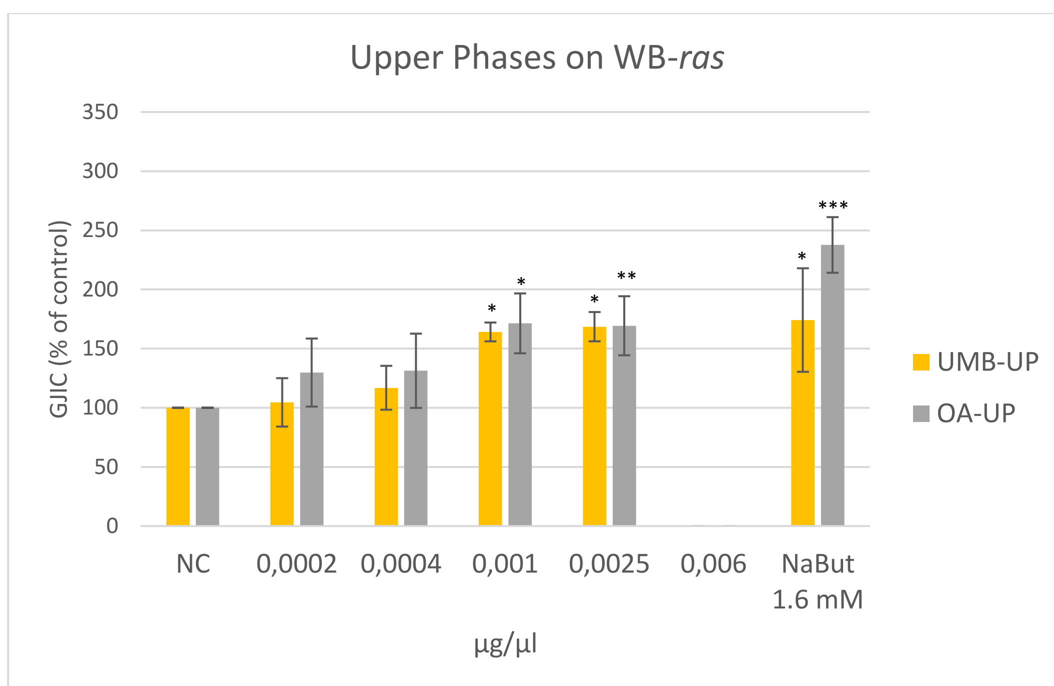


***Figure 58.*** Evaluation of the Gap Junction Intercellular Communication functionality by SL/DT assay on WB-*ras* cells assayed with both the upper phases (UMB-UP and OA-UP. Values are mean of three independent experiments performed in three technical replicates ± standard deviation with one specimen (A8). Nabut (Sodium butyrate) 1.6 mM was used as positive control. ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), *$p<0.05$, **$p<0.01$, ***$p<0.001$.

All the tested fractions did not exert effects on GJIC in WB-*neo* cells (Fig. 59 and 60), except for a mild and overall non-significant decrease of GJIC in WB-*neo* cells treated with LPs and UPs at 0.02 µg/µl and 0.0025 µg/µl concentrations, respectively. This effect is likely due to the sensitivity of WB-*neo* cells to acetonitrile (the solvent vehicle of the jellyfish fractions) and could only be a toxic effect related to the solvent. Since the WB-*neo* are well communicating cells with a well-established GJ-mediated cell-cell communication, it is notable that concentration active on non-communicating WB-*ras* cells are neutral on WB-*neo*.

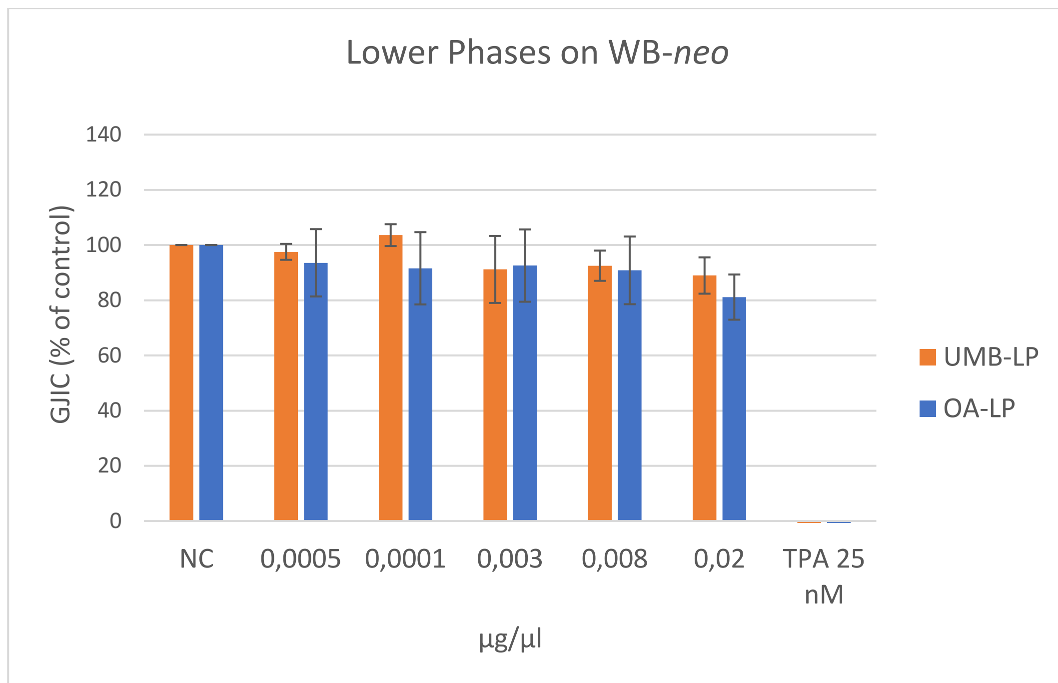


***Figure 59.*** Evaluation of the Gap Junction Intercellular Communication obtained by Scrape Loading / Dye Transfer assay with both the lower phases (UMB-LP and OA-LP on WB-*neo*. Values are mean of three independent experiments performed in eighteen technical replicates ± standard deviation with one specimen (A8). TPA (phorbol 12-myristate 13-acetate) 25 nM was used just 30′ before the assay to temporary stop communication between cells. ANOVA statistic test followed by Dunnett's post-test was used to compare each treatment with the control (NC), ($p<0.05$).

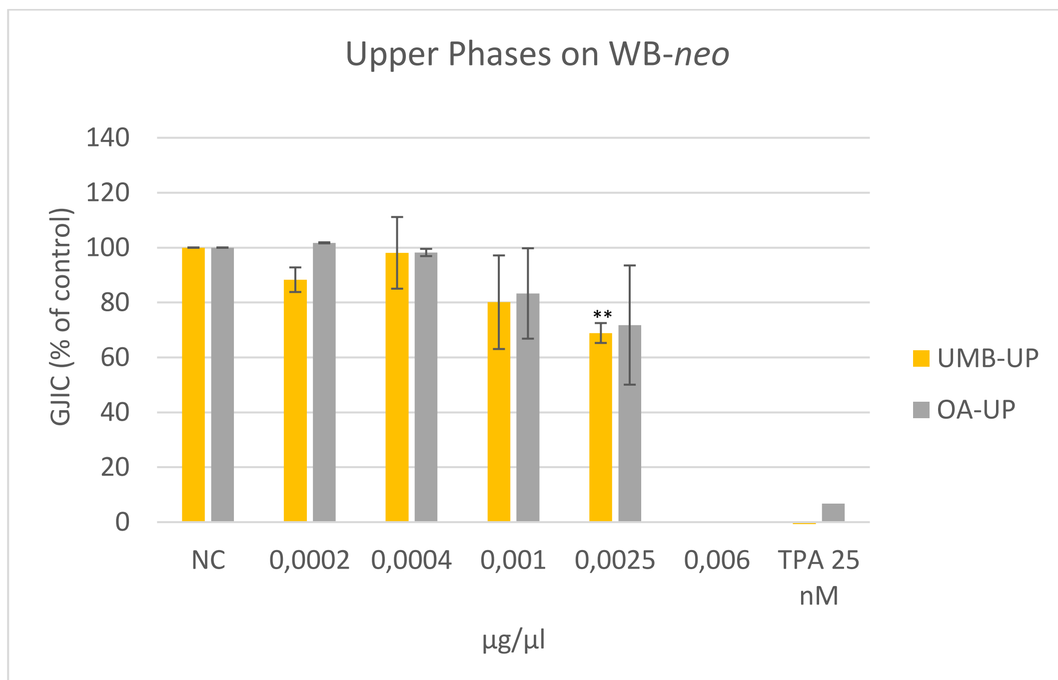


***Figure 60.*** Evaluation of the Gap Junction Intercellular Communication obtained by Scrape Loading / Dye Transfer assay with both the lower phases (UMB-UP and OA-UP on WB-*neo*. Values are mean of three independent experiments performed in eighteen technical replicates ± standard deviation with one specimen (A8). TPA (phorbol 12-myristate 13-acetate) 25 nM was used just 30′ before the assay to temporary stop communication between cells. ANOVA statistic

test followed by Dunnett's post-test was used to compare each treatment with the control (NC), $^{**}p<0.01$.

The four *Cassiopea andromeda* fractions of the jellyfish extract were clearly able to induce a significant increase of GJIC functionality in WB-*ras* cell and a cytotoxic effect on tumorigenic cell line likely mediated by the modulation of GJIC can be hypothesized.

Based on the presented results it is not possible to establish if the GJIC restoration is related to the enhancement of the permeability of the existing gap junctions or to the increase of the expression of connexins. However, taking into account the concentrations of the hydroalcoholic fractionated *C. andromeda* extracts that displayed the restoration of the gap junction intercellular communication on tumorigenic cells, and the fact that, these results were also in line with the results previously obtained with *Cotylorhiza tuberculata* on MCF-7 and HEKa cells (Leone, et al., 2013), it can be speculated that the fractions of the hydroalcoholic extract contains compounds able to exert a GJ restoration effect on tumorigenic cells.

# 4 Conclusions

The findings showed in this thesis suggest that jellyfish including invasive alien species, contain a number of compounds useful as leading compounds in bioprospecting. The jellyfish *Cassiopea andromeda* could be regarded as a novel source of natural compounds for current and future applications in natural product exploitation,

The increasing presence in the Mediterranean Sea of jellyfish that form blooms every year, represent a valuable biomass that need to be studied. Moreover, these biomasses, with their rich load of important antioxidant compounds (De Domenico et al., 2019) and compounds having related bioactivities as anti-cancer (Leone et al., 2013) and anti-inflammatory activities, are receiving a growing interest as dietary components. In this perspective, *Cassiopea andromeda* jellyfish, could represent an available and sustainable biomass, also given their characteristic to host autotrophic zooxanthellae, and of their possible breeding, for which further research studies in this direction are necessary.

Other aspects of *Cassiopea andromeda* jellyfish and its future uses for biomedical and pharmaceutical applications must be enhanced. In fact, both lipophilic (UPs) and hydrophilic (LPs) fractions of the jellyfish extract here isolated and evaluated for their effect on cell viability and pro-apoptotic effects on breast cancer cells (MCF-7 and MB-MDA-231 cell lines) need to be biochemically characterized and further studied. Here it was demonstrated that the action mechanism of the cytotoxic effect against cancer cells can pass though the pro-apoptotic effect and the modulation of GJICs as showed on WB-*ras* and WB-*neo* murine cancer system. All jellyfish fractions induced an improvement of the intercellular communication mediated by gap junction on tumorigenic non-communicating WB-*ras* cells, while were ineffective and not toxic on the well-communicating WB-*neo* cells.

These new evidence of the biological properties of the *Cassiopea andromeda* jellyfish, as the ability to modulate the gap-junctional permeability of the tumorigenic WB-*ras* cells rather than against non-malignant WB-*neo* cells, must be analysed in depth. Indeed, the GJIC represents a target for potential chemotherapeutic compounds, since alterations of this function are strictly

related to the carcinogenesis process, particularly at the tumor-promotion phases. Finally, it is necessary to analyse the cellular and molecular effects of individual compounds rather than complex extracts in order to clarify the action mechanism(s).

# 5 *Acknowledgements*

First and foremost, I would like to thank my tutors Prof. Annalisa Santucci (UniSi) and Dr. Antonella Leone (ISPA-CNR) without whom my PhD would not have been a reality.

I would like to thank my supervisor Dr. Antonella Leone for giving me the opportunity to work within the EU GoJelly project and for giving me her foresight, for guiding the PhD project, patience supervision, and corrections for the PhD Thesis. Her perfect and professional attitude to the work, sets a good example for me and inspires me to be better in the future work.

I would like to thank Prof. Pavel Babica and the specialist Dr. Eliška Sychrová for their extraordinary assistance and advices during my abroad period at RECETOX, Brno, Czech Republic.

I would like to express my sincere gratitude to Dr. Stefania De Domenico for support in experimental and writing, Dr. Mar Bosch-Belmar, Dr. Giacomo Milisenda and Prof. Stefano Piraino for *Cassiopea andromeda* fresh and lyophilized samples.

I would like to thank my family for their selfless support during my whole education.

Especially, I would like to thank my girlfriend to feel loved, supported and for pushing me to go further.

# 6 References


1. Abbaci, M.; Barberi-Heyob, M.; Blondel W.; Guillemin, F.; Didelon, J. Advantages and limitations of commonly used methods to assay the molecular permeability of gap junctional intercellular communication. *Biotechniques* **2008,** 45, 33–58. https://doi.org/10.2144/000112810;
2. Adams, L.S.; Chen, S. Phytochemicals for breast cancer prevention by targeting aromatase. *Frontiers in Bioscience* **2009**, 14, 3846–3863. https://doi.org/10.2741/3493;
3. Addad, S.; Esposito, J.Y.; Faye, C.; Ricard-Blum, S.; Lethias, C. Isolation, characterization, and biological evaluation of jellyfish collagen for use in biomedical applications. *Marin Drugs* **2011**, 9(6), 967–983. https://doi.org/10.3390/md9060967;
4. Adebiyi, A.P.; Adebiyi, A.O.; Yamashita, J.; Ogawa, T.; Muramoto, K. Purification and characterization of antioxidative peptides derived from rice bran protein hydrolysates. *European Food Research and Technology* **2009**, 228, 553–563. https://doi.org/10.1007/s00217-008-0962-3;
5. Ahmet, G.; Gençdağ, E.; Yılmaz, F.M. Bioactive peptides derived from plant origin by-products: Biological activities and techno-functional utilizations in food developments – A review. *Food Research International* **2020**, 136, 109504, https://doi.org/10.1016/j.foodres.2020.109504;
6. Ale-Agha, N.; Stahl,W.; Sies, H. (-)-Epicatechin e_ects in rat liver epithelial cells: Stimulation of gap junctional communication and counteraction of its loss due to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. *Biochemical Pharmacology* **2002**, 63, 2145–2149. https://doi.org/10.1016/S0006-2952(02)01021-3;
7. Alexander, D.B.; Goldberg, G.S. Transfer of biologically important molecules between cells through gap junction channels. *Current Medicinal Chemistry* **2003**, 10(19), 2045–2058. https://doi.org/10.2174/0929867033456927;
8. Alpert, A.K.; Shukla, A.J. Precipitation of Large, High-Abundance Proteins from Serum with Organic Solvents. *Association of Biomolecular*

*Resource Facilities* ***2003***, http://polylc.com/Downloads/ABRF_2003_SEC_poster.pdf;

9. Amor, K.O.B.; Rifi, M.; Ghanem, R.; Draeif, I.; Zaouali, J.; Souissi, J.B. Update of alien fauna and new records from Tunisian marine waters. *Mediterranean Marine Science* **2015**, 17(1), 124–143. https://doi.org/10.12681/mms.1371;
10. Arai, M.N. Predation on pelagic coelenterates: a review *Journal of the Marine Biological Association of the United Kingdom* **2005**, 85, 523-536. http://www.seaturtle.org/PDF/olAssUK.pdf;
11. Arai, Y.; Gotoh, R.O.; Yokoyama, J.; Sato, C.; Okuizumi, K.; Hanzawa, N. Phylogenetic relationships and morphological variations of upside-down jellyfishes, *Cassiopea spp.* inhabiting Palau Islands. *Biogeography* **2017**, 19, 133–141. https://www.jstage.jst.go.jp/article/biogeo/19/0/19_133/_pdf;
12. Arts, I.C.; Hollman, P.C. Polyphenols and disease risk in epidemiologic studies. *The American Journal of Clinical Nutrition* **2005**, 81(1), 317-325. https://doi.org/10.1093/ajcn/81.1.317s;
13. Ayed, Y.M.; Bousabbeh, H.B.; Mabrouk, M.; Morjen, N.; Marrakchi, H. Bacha Impairment of the cell-to-matrix adhesion and cytotoxicity induced by the Mediterranean jellyfish *Pelagia noctiluca* venom and its fractions in cultured glioblastoma cells. *Lipids in health and disease* **2012**, 11, 84. https://doi.org/10.1186/1511X-11-84;
14. Badré, S. Bioactive toxins from stinging jellyfish. *Toxicon* **2014**, 91, 114-125. https://doi.org/10.1016/j.toxicon.2014.09.010;
15. Banha, T.N.S.; Mies, M.; Güth, A.Z.; Pomory, C.M.; Sumida, P.Y.G. Juvenile *Cassiopea andromeda* medusae are resistant to multiple thermal stress events. *Marine Biology* **2020**, 167-173. https://doi.org/10.1007/s00227-020-03792-w;
16. Barzideh, Z.; Latiff, A.A.; Gan, C.; Alias, A.K. ACE inhibitory and antioxidant activities of collagen hydrolysates from the ribbon Jellyfish (*Chrysaora sp*.). *Food Technology and Biotechnology* **2014b**, 52(4), 495–504. https://doi.org/10.17113/b.52.04.14.3641;
17. Barzideh, Z.; Latiff, A.A.; Gan, C.Y.; Benjakul, S.; Karim, A.A. Isolation and characterisation of collagen from the ribbon jellyfish (*Chrysaora sp*.).

Int. *Journal of Food Science and Technology* **2014a**, 49, 1490–1499. https://doi.org/10.1111/ijfs.12464;

18. Basso, L.; Rizzo, L.; Marzano, M.; Intranuovo, M.; Fosso, B.; Pesole, G.; Piraino, S.; Stabili, L. Jellyfish summer outbreaks as bacterial vectors and potential hazards for marine animals and human's health? The case of *Rhizostoma pulmo* (Scyphozoa, Cnidaria). *Science of The Total Environment* **2019,** 692, 305-318. https://doi.org/10.1016/j.scitotenv.2019.07.155;
19. Baxter, E.J.; Sturt, M.M.; Ruane, N.M.; Doyle, T.K.; McAllen, R.; Harman, L.; Rodger H.D. Gill damage to Atlantic salmon (*Salmo salar*) caused by the common jellyfish (*Aurelia aurita*) under experimental challenge. *Plos One* **2011**, 6, 18529. https://doi.org/10.1371/journal.pone.0018529;
20. Bellard, C.; Cassey, P.; Blackburn, P.M. Alien species as a driver of recent extinctions. *Biology Letters* **2016**, 12, 1-4. https://doi.org/10.1098/rsbl.2015.0623;
21. Bhosale, S.H.; Nagle, V.L.; Jagtap, T.G. Antifouling potential of some marine organisms from India against species of Bacillus and Pseudomonas. *Marine Biotechnology* (NY) **2002**, 4(2), 111-118. http://doi.org/10.1007/s10126-001-0087-1;
22. Blanco, M.; Vázquez, J.A.; Pérez-Martín, R.I.; Sotelo, C.G. Hydrolysates of fish skin collagen: An opportunity for valorising fish industry by-products. *Marine Drugs* **2017**, 15, 131. https://doi.org/10.3390/md15050131;
23. Blanquet, R. S.; Phelan, M.A. An unusual blue mesogleal protein from the mangrove jellyfish *Cassiopea xamachana. Marine Biology* **1987**, 94, 423–430. https://doi.org/10.1007/BF00428249;
24. Bleve, G.; Ramires, F.A.; Gallo, A.; Leone, A. Identification of safety and quality parameters for preparation of jellyfish based novel food products. *Foods* **2019**, 8, 263, 1-13. https://doi.org/10.3390/foods8070263;
25. Blunt, J.W.; Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2018**, *35*, 8–53. https://doi.org/10.1039/c7np00052a;
26. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2012**, *29*, 144–222. https://doi.org/10.1039/c2np00090c;

27. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2013**, *30*, 237–323. https://doi.org/10.1039/c2np20112g;
28. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2014**, *31*, 160–258. https://doi.org/10.1039/c3np70117d;
29. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2015**, *32*, 116–211. https://doi.org/10.1039/c4np00144c;
30. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2016**, *33*, 382–431. https://doi.org/10.1039/c5np00156k;
31. Blunt, J.W.; Copp, B.R.; Keyzers, R.A.; Munro, M.H.G.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2017**, *34*, 235–294. https://doi.org/10.1039/c6np00124f;
32. Blunt, J.W.; Copp, B.R.; Munro, M.H.G.; Northcote, P.T.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2011**, *28*, 196–268. https://doi.org/10.1039/C005001F;
33. Boaru, D.A.; Dragoş, N.; Schirmer, K. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures and cell lines: a comparative study *Toxicology* **2006**, 218, 134-148. https://doi.org/10.1016/j.tox.2005.10.005;
34. Boero, F. The future of the Mediterranean Sea ecosystem: towards a different tomorrow. *Rendiconti Lincei* **2015**, 26, 3-12. https://doi.org/10.1007/s12210-014-0340-y;
35. Boero, F.; Bouillon, J.; Gravili, C.; Miglietta, M.P.; Parsons, T.; Piraino, S. Gelatinous plankton: irregularities rule the world (sometimes). *Marine Ecology Progress Series* **2008**, 356, 299-310. http://www.int-res.com/abstracts/meps/v356/p299-310/;
36. Boero, F.; Brotz, L.; Gibbons, M.J.; Piraino, S.; Zampardi, S. 3.10 Impacts and effects of ocean warming on jellyfish. In: Laffoley, D.; Baxter, J.M. (Ed.). Explaining ocean warming: Causes, scale, effects, and consequences. Full report. Gland, Switzerland: *International Union for*

*Conservation of Nature* **2016,** 213-237. https://www.academia.edu//48664613/jellyfishIUCN.pdf;

37. Borra, R.C.; Lotufo, M.A.; Gagioti, S.M.; Barros, F.D.M.; Andrade, P.M.; Repetto, G.; Peso, A.; Zurita, J.L. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols* **2008**, 3, 1125–1131. http://dx.doi.org/10.1038/nprot.2008.75;
38. Borra, R.C.; Lotufo, M.A.; Gagioti, S.M.; Barros, F.D.M.; Andrade, P.M. A simple method to measure cell viability in proliferation and cytotoxicity assays. *Brazilian Oral Research* **2009**, 23(3), 255-262. https://doi.org/10.1590/s1806-83242009000300006;
39. Bosch-Belmar, M.; Azzurro, E.; Pulis, K.; Milisenda, G.; Fuentes, V.; Yahia, O.K.D.; Micallef, A.; Deidun, A.; Piraino, S. Jellyfish blooms perception in Mediterranean finfish aquaculture. *Marine Policy* **2017**, 76, 1–7. https://doi.org/10.1016/j.marpol.2016.11.005;
40. Bosch-Belmar, M.; Milisenda, G.; Basso, L.; Doyle, T.K.; Leone, A.; Piraino, S. Jellyfish Impacts on Marine Aquaculture and Fisheries. *Reviews in Fisheries Science & Aquaculture*, **2020**. http://doi.org/10.1080/23308249.2020.1806201;
41. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **1976,** 72, 248–254. https://dx.doi.org/10.1016/0003-2697(76)90527-3;
42. Brotz, L.; Cheung, W.W.L.; Kleisner, K.; Pakhomov, E.; Pauly, D. Increasing jellyfish populations: trends in Large Marine Ecosystems. *Hydrobiologia* **2012**, 690, 3–20. https://doi.org/10.1007/s10750-012-1039-7;
43. Brotz, L.; Pauly, D. Studying jellyfish fisheries: toward accurate national catch reports and appropriate methods Mariottini, G.L. (Ed.). Jellyfish: ecology, distribution patterns and Human Interactions, *US Nova Publishers, New York* **2017**, 313-329 https://www.researchgate.net/publication/3124;
44. Bulina, M.E.; Konstantin, A.; Lukyanov, I.V.; Yampolsky, D.M.; Chudakov, D.B.; Staroverov, A.S.; Shcheglov, N.G. Lukyanov, S.; Lukyanov, G. New Class of Blue Animal Pigments Based on Frizzled and

Kringle Protein Domains. *The Journal of Biological Chemistry* **2004**, 279, 43367-43370. https://doi.org/10.1074/jbc.C400337200;

45. Capillo, G.; Savoca, S.; Costa, R.; Sanfilippo, M.; Rizzo, C.; Lo Giudice, A.; Albergamo, A.; Rando, R.; Bartolomeo, G.; Spanò, N.; Faggio, C. New insights into the culture method and antibacterial potential of *Gracilaria Gracilis*. *Marine Drugs* **2018**, 16, 492. https://dx.doi.org/10.3390%2Fmd16120492;
46. Carr, C.; Ng, J.; Wigmore, T. The side effects of chemotherapeutic agents *Current Anaesthesia & Critical Care* **2008**, 2, 70-79. https://doi.org/10.1016/j.cacc.2008.01.004;
47. Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine natural products. *Natural Products Reports* **2019**, 36, 122–173. https://doi.org/10.1039/C8NP00092A;
48. Chaumontet, C.; Droumaguet, C.; Bex, V.; Heberden, C.; Gaillard-Sanchez, I.; Martel, P. Flavonoids (apigenin, tangeretin) counteract tumor promoter-induced inhibition of intercellular communication of rat liver epithelial cells. **Cancer Letters 1997**, 114, 207–210. https://doi.org/10.1016/s0304-3835(97)04664-8;
49. Chavez, K.J.; Garimella, S.V.; Lipkowitz, S. Triple Negative Breast Cancer Cell Lines: One Tool in the Search for Better Treatment of Triple Negative Breast Cancer. *Breast Disease* **2010**, 32(1- 2):35–48. https://doi.org/10.3233/bd-2010-0307;
50. Cheng, X.; Shao, Z.; Li, C.; Yu, L.; Raja, M.A.; Liu, C. Isolation, Characterization, and evaluation of collagen from jellyfish *Rhopilema esculentum* Kishinouye for use in haemostatic applications. *Plos One* **2017**, 12(1), e0169731. https://doi.org/10.1371/journal.pone.0169731;
51. Chenjin. China Statistical yearbook of Marine fisheries (in Chinese). *Beijing* **2017.** http://www.stats.gov.cn/tjsj/ndsj/2017/indexeh.htm;
52. CIESM. Gelatinous Plankton Outbreaks: Theory and Practice. *CIESM Workshop Series* **2001**, 14, 112. http://www.ciesm.org/online/monographs/Naples.html;
53. Cillari, T.; Andaloro, F.; Castriota, L. First documented record of *Cassiopea* cf *andromeda* (Cnidaria: Scyphozoa) in Italian waters. *Cahiers de*

*Biologie Marine* **2018**, 59, 193–195. https://dx.doi.org/10.21411/CBM.A.1037AA;
54. Clark, A. Natural Products, in Foye's Principles of Medicinal Chemistry, *T.L.L. Williams, D.A. (Ed.). Lippincott Williams & Wilkins* **2002**, 24-36. https://www.amazon.it/Foyes-Principles-M31;
55. Colin, S.P.; Costello, J.H. Morphology, swimming performance and propulsive mode of six co-occurring hydromedusae. *Journal of Experimental Biology* **2002,** 427-437. https://jeb.biologists.org/content/jexbio/205/3/427.full.pdf;
56. Compton, S.J.; Jones, C.G. Mechanism of Dye Response, and interference in the Bradford Protein Assay. *Analytical Biochemistry* **1985**, 151, 369–374. https://doi.org/10.1016/0003-2697(85)90190-3;
57. Comsa, S.; Cimpean, M.; Raica, M. The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research. *International Journal of Cancer Research and Treatment* **2015**, 35(6), 3147-3154. http://ar.iiarjournals.org/content/35/6/3147.long;
58. Condon, R.H.; Steinberg, D.K.; del Giorgio, P.A.; Bouvier, T.C.; Bronk, D.A.; Graham, W.A.; Ducklow, H.W. Jellyfish blooms result in a major microbial respiratory sink of carbon in marine systems Ducklow. *Proceedings of the National Academy of Sciences* **2011**, 201015782. http://doi.org/10.1073/pnas.1015782108;
59. Corona G.; Deiana, M.; Incani, A.; Vauzour, D.; Dessi, M.A.; Spencer, J.P.E. Hydroxytyrosol inhibits the proliferation of human colon adenocarcinoma cells through inhibition of ERK1/2 and cyclin D1. *Molecular Nutrition and Food Research* **2009**, 53, 897–903. https://doi.org/10.1002/mnfr.200800269;
60. Corona, G.; Deiana, M.; Incani, A.; Vauzour, D.; Dessi, M.A.; Spencer, J.P.E. Inhibition of p38/CREB phosphorylation and COX-2 expression by olive oil polyphenols underlies their anti-proliferative effects. *Biochemical and Biophysical Research Communications* **2007**, 362, 606–611. https://doi.org/10.1016/j.bbrc.2007.08.049;
61. Costa, R.; Capillo, G.; Albergamo, A.; Li Volsi, R.; Bartolomeo, G.; Bua, G.; Ferracane, A.; Savoca, S.; Gervasi, T.; Rando, R.; Dugo, G.; Spanò, N.C. A Multi-screening Evaluation of the Nutritional and Nutraceutical

Potential of the Mediterranean Jellyfish *Pelagia noctiluca*. *Marine Drugs* **2019**, 17(3), 172. https://doi.org/10.3390/md17030172;

62. Costello, J.H.; Colin, S.P.; Dabiri J.O.; Medusan morphospace: phylogenetic constraints, biomechanical solutions, and ecological consequences. *Invertebrate Biology* **2008**, 127, 265-290. https://doi.org/10.1111/j.1744-7410.2008.00126.x;
63. D'Amico, P.; Leone, A.; Giusti, A.; Armani, A. Jellyfish and humans: not just negative interactions. In: Mariottini, G.L. (Ed.). Jellyfish: ecology, distribution patterns and human interactions. *US Nova Publishers, New York* **2017**, 331–352. http://hdl.handle.net/11568/795143;
64. D'Archivio, M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R. Polyphenols, dietary sources and bioavailability. *Annali dell'Istituto Superiore di Sanità* **2007**, 43, 348–361. http://old.iss.it/publ/anna/2007/4/434348.pdf;
65. De Domenico, S.; De Rinaldis, G.; Paulmery, M.; Piraino, S.; Leone, A. Barrel Jellyfish (*Rhizostoma pulmo*) as Source of Antioxidant Peptides *Marine Drugs* **2019**, 17, 134. https://doi.org/10.3390/md17020134;
66. De Domenico, S.; Giudetti, A. Nutraceutical intervention in ageing brain. *Società Italiana di Gerontologia e Geriatria* **2017**, 65, 79–92. http://www.jgerontology-geriatrics.com/article/download/146/176;
67. De Feijter, A.W.; Matesic, D.F.; Ruch, R.J.; Guan, X.; Chang, C.C.; Trosko, J.E. Localization and function of the connexin 43 gap-junction protein in normal and various oncogene-expressing rat liver epithelial cells. *Molecular Carcinogenesis* **1996**, 16, 203-212. https://doi.org/10.1002/(sici)1098-27mc4%3E3.0.co;2-g;
68. De Feijter, A.W.; Ray, J.S.; Weghorst, C.M.; Klaunig, J.E.; Goodman, J.I.; Chang, C.C.; Ruch, R.J.; Trosko, J.E. Infection of rat liver epithelial cells with v-Ha-*ras*: correlation between oncogene expression, gap junctional communication, and tumorigenicity. *Molecular Carcinogenesis* **1990**, 3(2), 54-67. https://doi.org/10.1002/mc.2940030203;
69. De Souza, L.M.; Iacomini, M.; Gorin, P. A. J.; Sari, R. S.; Haddad, M. A.; Sassaki, G. L. Glyco and sphingophosphonolipids from the medusa *Phyllorhiza punctata*: NMR and ESI-MS/MS fingerprints. *Chemistry and*

*Physics of Lipids* **2007**, 145, 85–96. https://doi.org/10.1016/j.chemphyslip.2006.11.001;

70. DeoCampo, N.D.; Wilson, M.R.; Trosko, J.E. Cooperation of bcl-2 and myc in the neoplastic transformation of normal rat liver epithelial cells is related to the downregulation of gap junction-mediated intercellular communication. *Carcinogenesis* **2000**, 21, 1501-1506. https://doi.org/10.1093/carcin/21.5.501;
71. Deshmukh, S.K.; Prakash, V.; Ranjan, N. Marine fungi: a source of potential anticancer compounds. *Frontiers in Microbiology* **2017**, 8, 2536. https://doi.org/10.3389/fmicb.2017.02536;
72. Devi, G.R.; Allensworth, J.L.; Evans, M.K.; Sauer, S.J. The role of oxidative stress in breast cancer. In: *Cancer - Academic Press* **2014**, 3–14. https://doi.org/10.1016/B978-0-12-405205-5.00001-5;
73. Ding, J.F.; Li, Y.Y.; Xu, J.J.; Su, X.R.; Gao, X.: Yue, F.P. Study on effect of jellyfish collagen hydrolysate on anti-fatigue and anti-oxidation. *Food Hydrocolloids* **2011**, 25(5), 1350–1353. https://doi.org/10.1016/j.foodhyd.2010.12.013;
74. Dos Santos, R.; Carvalho, A.L.; Roque, A.C.A. Renaissance of protein crystallization and precipitation in biopharmaceuticals purification. *Biotechnology Advances* **2017**, 35, 41–50. https://doi.org/10.1016/j.biotechadv.2016.11.005;
75. Dydowiczová, A., Brózman, O., Babica, P. et al. Improved multiparametric scrape loading-dye transfer assay for a simultaneous high-throughput analysis of gap junctional intercellular communication, cell density and viability. Sci Rep 10, 730 (2020). https://doi.org/10.1038/s41598-020-57536-3
76. El-Bawab, F. Chapter 5 - Phylum Cnidaria, Invertebrate Embryology and Reproduction, *Academic Press* **2020**, 172-306. https://doi.org/10.1016/B978-0-12-814114-4.00005-9;
77. El-fouly, M.H.; Trosko, J.E.; Chang, C.C. Scrape-loading and dye transfer—a rapid and simple technique to study gap junctional intercellular communication. *Experimental Cell Research* **1987**, 168, 422–430. https://doi.org/10.1016/0014-4827(87)90014-0;
78. Ellithey, M.S.; Lall, N.; Hussein, A.A.; Meyer, D. Cytotoxic and HIV-1 enzyme inhibitory activities of Red Sea marine organisms. *BMC*

*Complementary Medicine and Therapies* **2014**, 14, 77. http://www.doi.org/10.1186/1472-6882-14-77;.

79. Etemadian, Y.; Ghaemi, V.; Shaviklo, A.R.; Pourashouri, P.; Mahoonak, A.R.S.; Rafipour, F. Development of animal/plant-based protein hydrolysate and its application in food, feed and nutraceutical industries: State of the art. *Journal of Cleaner Production* **2021**, 278. https://doi.org/10.1016/j.jclepro.2020.123219;

80. Evans, W.H.; Martin, P.E. Gap junctions: Structure and function (Review). *Molecular Membrane Biology* **2002**, 19, 121–136. https://doi.org/10.1080/09687680210139839;

81. Fabiani, R.; De Bartolomeo, A.; Rosignoli, P.; Servili, M.; Montedoro, G.F.; Morozzi, G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *European Journal of Cancer Prevention* **2002**, 11, 351–358. https://doi.org/10.1097/00008469-200208000-00006;

82. Fan, J.; Zhuang, Y.; Li, B. Effects of collagen and collagen hydrolysate from jellyfish umbrella on histological and immunity changes of mice photoaging. *Nutrients* **2013**, 5, 223–233. https://dx.doi.org/10.3390%2Fnu5010223;

83. FAO STAT (Food and Agriculture Organization of the United Nations Statistics Division). *FAO yearbook: fishery and aquaculture statistics* **2015**. https://doi.org/10.5860/CHOICE.50-5350;

84. Felician, F.F.; Yu, R.H.; Li, M.Z.; Li, C.J.; Chen H.Q.; Jiang, Y.; Tang, T.; Qiab, W.Y.; Xu, H.M. The wound healing potential of collagen peptides derived from the jellyfish *Rhopilema esculentum*. *Chinese Journal of Traumatology* **2019**, 12-20. https://doi.org/10.1016/j.cjtee.2018.10.004;

85. Ferguson, H.W.; Delannoy, C.M.J.; Hay, S.; Nicolson, J.; Sutherland, D.; Crumlish, M. Jellyfish as vectors of bacterial disease for farmed salmon (*Salmo salar*). *Journal of Veterinary Diagnostic Investigation* **2010**, 22, 376-382. https://doi.org/10.1177/104063871002200305;

86. Ferreira, A.M.; Gentile, P.; Chiono, V.; Ciardelli, G. Collagen for bone tissue regeneration. *Acta Biomaterialia* **2012**, 8, 3191–3200. https://doi.org/10.1016/j.actbio.2012.06.014;

87. Fini, L.; Hotchkiss, E.; Fogliano, V.; Graziani, G.; Romano, M.; B. De Vol, E.; Qin, H.; Selgrad, M.; Boland, C.R.; Ricciardiello, L. Chemopreventive properties of pinoresinol-rich olive oil involve a selective activation of the ATM-p53 cascade in colon cancer cell lines. *Carcinogenesis* **2008**, 29, 139–146. https://doi.org/10.1093/carcin/bgm255;
88. Fornelli, F.; Leone, A.; Verdesca, I.; Minervini, F.; Zacheo, G. The influence of lycopene on the proliferation of human breast cell line (MCF-7). *Toxicology In Vitro* **2007**, 21, 217–223. https://doi.org/10.1016/j.tiv.2006.09.024;
89. Fusetani, N. Biotechnological potential of marine natural products. *Pure and Applied Chemistry* **2010**, 82, 17–26. https://doi.org/10-1351/PAC-COM-09-01-11;
90. Gajanan, P.G.; Elavarasan, K.; Shamasundar, B.A. Bioactive and functional properties of protein hydrolysates from fish frame processing waste using plant proteases. *Environmental Science and Pollution Research* **2016**, 23, 24901-24911. https://doi.org/10.1007/s11356-016-7618-9;
91. Gakhar, G.; Hua, D.H.; Nguyen, T.A. Combinational treatment of gap junctional activator and tamoxifen in breast cancer cells. *Anticancer Drugs* **2010**, 21, 77–88. https://doi.org/10.1097/cad.0b013e328333d557;
92. Galil, B.S.; Gershwin, L.A.; Zorea, M.; Rahav, A.; Rothman, S.B.; Fine, M.; Lubinevsky, H.; Douek, J.; Paz, G.; Rinkevich, B. *Cotylorhiza erythraea* Stiasny, 1920 (Scyphozoa: Rhizostomeae: Cepheidae), yet another erythraean jellyfish from the Mediterranean coast of Israel. *Marine Biodiversity* **2017**, 47, 229-235. https://doi.org/10.1007/s12526-016-0449-6;
93. Galil, B.S.; Marchini, A.; Occhipinti-Ambrogi, A.; Minchin, D.; Narščius, A.; Ojaveer, H.; Olenin, S. International arrivals: widespread bioinvasions in European Seas. *Ethology Ecology & Evolution* **2014**, 26, 152-171. https://dx.doi.org/10.1080%2F03949370.2014.897651;
94. Galil, B.S.; Spanier, E.; Ferguson, W.W. The Scyphomedusae of the Mediterranean coast of Israel, including two Lessepsian migrants new to the Mediterranean. *Zoologische Mededelingen* **1990**, 64, 95-105. https://repository.naturalis.nl/document/149481;
95. Gall, E.A.; Lelchat, F.; Hupel, M.; Jegou, C.; Stiger-Pouvreau, V. Extraction and purification of phlorotannins from brown algae. *Methods*

*in Molecular Biology* **2015**, 1308, 131–143. https://link.springer.com/protocol/10.1007%2F978-1-4939-2684-8_7;

96. Gallardo, B.; Clavero, M.; Sánchez, M.I.; Vilà M. Global ecological impacts of invasive species in aquatic ecosystems. *Global Change Biology* **2016**, 22, 151-163. https://doi.org/10.1111/gcb.13004;
97. Gerwick, W.H.; Moore, B.S. Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology. *Chemistry & Biology* **2012**, 19, 85-98. https://doi.org/10.1016/j.chembiol.2011.12.014;
98. Ghasemi, A.; Hedayati, M.; Biabani, H. Protein precipitation methods evaluated for determination of serum nitric oxide end products by the Griess assay. *Journal of Research in Medical Sciences* **2007**, *1*, 43–46. https://www.researchgate.ey=AS%3A42%401478166098860;
99. Giakoumi, S.; Katsanevakis, S.; Albano, P.G.; Azzurro, E.; Cardoso, A.C.; Cebrian, E.; Deidun, A.; Edelist, D.; Francour, P.; Jimenez, C.; Mačić, V.; Occhipinti-Ambrogi, A.; Rilov, G.; Sghaier, Y.R.; Management priorities for marine invasive species. *Science of The Total Environment* **2019**, 688, 976-982. https://doi.org/10.1016/j.scitotenv.2019.06.282;
100. Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *Journal of Applied Phycology* **2012**, 24, 1477–1486. https://doi.org/10.1007/s10811-012-9804-6;
101. Goodenough, D.A.; Paul, D.L. Gap junctions. *Cold Spring Harbor Perspectives in Biology* **2009**, 1, 2576. https://doi.org/10.1101/cshperspect.a002576;
102. Gorguc, A.; Gencdag, E.; Yilmaz, F.M. Bioactive peptides derived from plant origin by-products: Biological activities and techno-functional utilizations in food developments–A review. *Food Research International* **2020**, 109504. https://doi.org/10.1016/j.foodres.2020.109504;
103. Graf, B.A.; Milbury, P.E.; Blumberg, J.B. Flavonols, flavones, flavanones, and human health: epidemiological evidence. *Journal of Medicinal Food* **2005**, 8(3), 281-90. https://doi.org/10.1089/jmf.2005.8.281;

104. Grienke, U.; Silke, J.; Tasdemir, D. Bioactive compounds from marine mussels and their effects on human health. *Food Chemistry* **2014**, 142, 48–60. https://doi.org/10.1016/j.foodchem.2013.07.027;
105. Gulcin, I.; Şat, İ.G.; Beydemir, Ş.; Elmastaş, M.; Küfrevioğlu, Ö.İ. Comparison of antioxidant activity of clove (*Eugenia caryophylata*) buds and lavender (*Lavandula stoechas*). *Food Chemistry* **2004**, 87, 393–400. http://10.1016/j.foodchem.2003.12.008;
106. Hamlet, C.; Miller, L.A. Feeding Currents of the Upside-Down Jellyfish in the Presence of Background Flow. *Bulletin of Mathematical Biology* **2012**, 74, 2547–2569. https://doi.org/10.1007/s11538-012-9765-6;
107. Hamlet, C.; Santhanakrishnan, A.; Miller, L.A. A numerical study of the effects of bell pulsation dynamics and oral arms on the exchange currents generated by the upside-down jellyfish *Cassiopea xamachana*. *Journal of Experimental Biology* **2011**, 214, 11, 1911-1921. https://doi.org/10.1242/jeb.052506;
108. Hartree, E.F. Determination of protein—Modification of Lowry method that gives a linear photometric response. *Analytical Biochemistry* **1972**, 48, 2, 422–427. https://doi.org/10.1016/0003-2697(72)90094-2;
109. Hattenschwiler, S.; Vitousek, P.M. The role of polyphenols in terrestrial ecosystem nutrient cycling. *Trends in Ecology & Evolution* **2000**, 15(6), 238–243. https://doi.org/10.1016/S0169-5347(00)01861-9;
110. Hayashi, T.; Nomata, K.;Chang,C.C.; Ruch, R.J.;Trosko J.E. Cooperative effects of v-myc and c-Ha-*ras* oncogenes on gap junctional intercellular communication and tumorigenicity in rat liver epithelial cells. *Cancer Letters* **1998**, 128, 145-154. https://doi.org/10.1016/S0304-3835(98)00060-3;
111. Hecht, F.; Pessoa, C.F.; Gentile, L.B.; Rosenthal, D.; Carvalho, D.P. The role of oxidative stress on breast cancer development and therapy. *Tumor Biology* **2016**, 37, 4281–4291. https://doi.org/10.1007/s13277-016-4873-9;
112. Heim, K.E.; Tagliaferro, A.R.; Bobilya, D.J. Flavonoid antioxidants: chemistry, metabolism, and structure-activity relationships. *Journal of Nutritional Biochemistry* **2002**, 13, 10, 572–584. https://doi.org/10.1016/s0955-2863(02)00208-5;

113. Hesketh, G.G.; Van Eyk, J.E.; Tomaselli, G.F. Mechanisms of gap junction traffic in health and disease. *Cardiovascular pharmacology* **2009**, 54, 263–272. https://dx.doi.org/10.1097%2FFJC.0b013e3181ba0811;
114. Holland, B.S.; Dawson, M.N.; Crow, G.L.; Hofmann, D.K. Global phylogeography of *Cassiopea* (Scyphozoa: Rhizostomeae): molecular evidence for cryptic species and multiple invasions of the Hawaiian Islands. *Marine Biology* **2004**, 145, 1119–1128 https://doi.org/10.1007/s00227-004-1409-4;
115. Hooper, J.N.A.; Van Soest, R.W.M.; Pisera, A. Phylum Porifera Grant, 1826. In: Zhang Z-Q, editor. Animal biodiversity: an outline of higher-level classification and survey of taxonomic richness, *Zootaxa* **2011**, 3148, 13–8. https://www.mapress.com/zootaxa/2011/f/zt03148p018.pdf;
116. Hsieh, Y.P.; Leong, F.M.; Rudloe, J. Jellyfish as food. *Hydrobiologia* **2001**, 451(1–3), 11-17. https://doi.org/10.1023/A:1011875720415;
117. Hsu, K.; Li-Chen, Ch.; Eunice, C.H.; Jao, L.C. Antiproliferative activity of peptides prepared from enzymatic hydrolysates of tuna dark muscle on human breast cancer cell line MCF-7. *Food Chemistry* **2011**, 126, 617-622. https://doi.org/10.1016/j.foodchem.2010.11.066;
118. Hussain, T.; Tan B.; Yin, Y.; Blachier, F.; Tossou, M.C.B.; Rahu, N. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us?. *Oxidative Medicine and Cellular Longevity* **2016**, 9. https://doi.org/10.1155/2016/7432797;
119. Isnaini, I.; Permatasari, N.; Mintaroem, K.; Prihardina, B.; Widodo M.A. Oxidants-Antioxidants Profile in the Breast Cancer Cell Line MCF-7. *Asian Pacific Journal of Cancer Prevention* **2018**, 19(11), 3175-3178. https://doi.org/10.31557/APJCP.2018.19.11.3175;
120. Iverson, S.J.; Tracing aquatic food webs using fatty acids: From qualitative indicators to quantitative determination. In Lipids in Aquatic Ecosystems; Arts, M.T.; Kainz, M.; *Eds. Springer Science and Business Media LLC: Berlin, Germany,* **2009**, 281–308. https://link.springer.com/chapter/10.1007/978-0-387-89366-2_12;
121. Jankangram, W.; Chooluck, S.; Pomthong, B. Comparison of the properties of collagen extracted from dried jellyfish and dried squid.

*African Journal of Biotechnology* **2016**, 15, 642–648. https://doi.org/10.5897/AJB2016.15210;

122. Jantzen, C.; Wild, C.; Rasheed, M.; El-Zibdah, M.; Richter, C. Enhanced pore-water nutrient fluxes by the upside-down jellyfish *Cassiopea sp*. in a Red Sea coral reef. *Marine Ecology Progress Series* **2010**, 411:117-125. https://doi.org/10.3354/meps08623;
123. Jerez-Martel, I.; García-Poza, S.; Rodríguez-Martel, G.; Rico, M.; Afonso-Olivares, C.; Gómez-Pinchetti, J.L. Phenolic Profile and Antioxidant Activity of Crude Extracts from Microalgae and Cyanobacteria Strains. *Journal of Food Quality* **2017**. https://doi.org/10.1155/2017/2924508;
124. Jiménez, C. Marine natural products in medicinal chemistry. *ACS Medicinal Chemistry Letters* **2018**, 9, 959–961. https://doi.org/10.1021/acsmedchemlett.8b00368;
125. Jongjareonrak, A.; Benjakul, S.; Visessanguan, W.; Tanaka, M. Isolation, and characterization of collagen from bigeye snapper (*Priacanthus macracanthus*) skin. *Journal of the Science of Food and Agriculture* **2005**, 85, 1203–1210 https://doi.org/10.1002/jsfa.2072;
126. Kang, K.S.; Lee, Y.S.; Kim, H.S. Di-(2-ethylhexyl) phthalate-induced cell proliferation is involved in the inhibition of gap junctional intercellular communication and blockage of apoptosis in mouse Sertoli cells. *Journal of Toxicology and Environmental Health, Part A* **2002**, 65, 447–459. https://doi.org/10.1080/15287390252808109;
127. Karoud, W.; Sila, A.; Krichen, F. Martinez-Alvarez, O.; Bougatef, A. Characterization, Surface Properties and Biological Activities of Protein Hydrolysates Obtained from Hake (*Merluccius merluccius*) Heads. *Waste and Biomass Valorization* **2019**, 10, 287–297. https://doi.org/10.1007/s12649-017-0069-9;
128. Khalifa, S.A.M.; Elias, N.; Farag, M.A.; Chen, L.; Saeed, A.; Hegazy M.E.F.; Moustafa. S.; El-Wahed A.; Al-Mousawi, S.M.; Musharraf, S.G.; Chang, F.R.; Iwasaki, A.; Suenaga, K.; Alajlani, M.; Göransson, U.; El-Seedi, H.R. Marine natural products: a source of novel anticancer drugs. *Marine Drugs* **2019**, 17(9), 491–522. https://doi.org/10.3390/md17090491;
129. Khan, N.; Afaq, F.; Saleem, M.; Ahmad, N.; Mukhtar, H. Targeting multiple signalling pathways by green tea polyphenol (−)-

epigallocatechin-3-gallate. *Cancer Research* **2006**, 66, 2500–2505. https://doi.org/10.1158/0008-5472;

130. Khong, N.M.; Yusoff, F.M.; Jamilah, B.; Basri, M.; Maznah, I.; Chan, K.W.; Nishikawa, J. Nutritional composition, and total collagen content of three commercially important edible jellyfish. *Food Chemistry* **2016**, 196, 953–960. https://doi.org/10.1016/j.foodchem.2015.09.094;
131. Kiew, P.L.; Don, M.M. Modified Lowry's Method for Acid and Pepsin Soluble Collagen Measurement from Clarias Species Muscles. **2013**. http://dx.doi.org/10.4172/scientificreports678;
132. Killi, N.; Tarkan, A.S.; Kozic, S.; Copp, G.H.; Davison, P.I.; Vilizzi L. Risk screening of the potential invasiveness of non-native jellyfishes in the Mediterranean Sea. *Marine Pollution Bulletin* ***2020***, 150, 110728. https://doi.org/10.1016/j.marpolbul.2019.110728;
133. Komsa-Penkova, R.; Spirova, R.; Bechev, B. Modification of Lowry's method for collagen concentration measurement. *Journal of Biochemical and Biophysical Methods* **1996**, 32, 1, 33-43. https://doi.org/10.1016/0165-022X(95)00046-T;
134. Kruk, J.; Kubasik-Kladna, K.; Aboul-Enein, H.Y. The Role Oxidative Stress in the Pathogenesis of Eye Diseases: Current Status and a Dual Role of Physical Activity. *Mini-Reviews in Medicinal Chemistry* **2015**, 16(3), 241-57. http://doi.org/10.2174/1389557516666151120114605;
135. Kurutas, E.B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition Journal* **2016**, 15, 71. https://dx.doi.org/10.1186%2Fs12937-016-0186-5;
136. LaJeunesse, T.C.; Loh, W.; Trench, R.K. Do introduced endosymbiotic dinoflagellates 'take' to new hosts? *Biological Invasions* **2009**, 11, 995–1003. https://doi.org/10.1007/s10530-008-9311-5;
137. Lampert, K.P.; Burger, P.; Striewski, S.; Tollrian, R. Lack of association between colour morphs of the Jellyfish *Cassiopea andromeda* and zooxanthella clade. *Marine Ecology* **2012**, 33, 364–369. https://doi.org/10.1111/j.1439-0485.2011.00488.x;

138. Latyshev, N.A.; Naumenko, N.V.; Svetashev, V.I.; Latypov, Y.Y. Fatty acids of reef-building corals. *Marine Ecology Progress Series* **1991**, 76, 295–301. https://www.int-res.com/articles/meps/76/m076p295.pdf;

139. Leal, M.C.; Hilário, A.; Munro, M.H.G.; Blunt, J.W.; Calado, R. Natural products discovery needs improved taxonomic and geographic information. *Natural Product Reports* **2016**, 33, 747–750. https://doi.org/10.1039/C5NP00130G;

140. Leal, M.C.; Puga, J.; Serôdio, J.; Gomes, N.M.; Calado, R. Trends in the Discovery of New Marine Natural Products from Invertebrates over the Last Two Decades – Where and What Are We Bioprospecting? *Plos one* 2012, 7, 1, e30580. https://doi.org/10.1371/journal.pone.0030580;

141. Lee DE; Kang, N.J.; Lee, K.M.; Lee, B.K.; Kim, J.H.; Lee, K.W.; Lee, H.J. Cocoa polyphenols attenuate hydrogen peroxide induced inhibition of gap-junction intercellular communication by blocking phosphorylation of connexin 43 via the MEK/ERK signaling pathway. *The Journal of Nutritional Biochemistry* **2010a**, 21, 680–686 https://doi.org/10.1016/j.jnutbio.2009.03.014;

142. Lee, D.E.; Shin, B.J.; Hur, H.J.; Kim, J.H.; Kim, J.; Kang, N.J.; Kim, D.O.; Lee, C.Y.; Lee, K.W.; Lee, H.J. Quercetin, the active phenolic component in kiwifruit, prevents hydrogen peroxide-induced inhibition of gap-junction intercellular communication. *British Journal of Nutrition* **2010b**, 104, 164–170. https://doi.org/10.1017/S0007114510000346;

143. Lee, J.H.; Oh, M.; Seok, J.H.; Kim, S.; Lee, D.B.; Bae, G.; Bae, H.I.; Bae, S. Y.; Hong Y.M.; Kwon, S.O.; Lee, D.H.; Song C.S.; Mun J.Y.; Chung, M.S.; Kim, K.H. Antiviral Effects of Black Raspberry (*Rubus coreanus*) Seed and Its Gallic Acid against Influenza Virus Infection. *Viruses* **2016**, 8, 157. https://doi.org/10.3390/v8060157;

144. Lee, K.W.; Lee, H.J. Biphasic effects of dietary antioxidants on oxidative stress-mediated carcinogenesis. *Mechanisms of Ageing and Development* **2006**, 127, 424–431. https://doi.org/10.1016/j.mad.2006.01.021;

145. Lee, S.Y.; Jeong, H.J.; Kang, N.S.; Jang, T.Y.; Jang, S.H.; Lajeunesse, T.C. *Symbiodinium tridacnidorum* sp. nov., a dinoflagellate common to Indo-Pacific giant clams, and a revised morphological description of *Symbiodinium microadriaticum* Freudenthal, emended Trench & Blank.

*European Journal of Phycology* **2015**, 50, 2, 155-172. https://doi.org/10.1080/09670262.2015.1018336;

146. Leone, A.; Lecci, R.; Durante, M.; Meli, F.; Piraino, S. The bright side of gelatinous blooms: nutraceutical value and antioxidant properties of three mediterranean jellyfish (Scyphozoa). *Marine Drugs* **2015**, 13, 8, 4654–4681. https://doi.org/10.3390/md13084654;
147. Leone, A.; Lecci, R.M.; Durante, M.; Piraino, S. Extract from the zooxanthellatae jellyfish *Cotylorhiza tuberculata* modulates gap junction intercellular communication in human cell cultures. *Marine Drugs* **2013**, 11, 5, 1728–1762. https://doi.org/10.3390/md11051728;
148. Leone, A.; Lecci, R.M.; Milisenda, G.; Piraino, S. Mediterranean jellyfish as novel food: effects of thermal processing on antioxidant, phenolic, and protein contents. *European Food Research and Technology* **2019**, 245, 1611–1627. https://doi.org/10.1007/s73248-6;
149. Leone, A.; Longo, C.; Trosko, J.E. The chemopreventive role of dietary phytochemicals through gap junctional intercellular communication. *Phytochemistry reviews* **2012**, 11, 2–3, 285–307. https://doi.org/10.1007/s11101-012-9235-7;
150. Li, A.; Li, S.; Zhang, Y.; Xu, X.; Chen, Y.; Li, H. Resources and Biological Activities of Natural Polyphenols. *Nutrients* **2014**, 6, 6020–6047. https://doi.org/10.3390/nu6126020;
151. Li, Q.M.; Wang, J.F.; Zha, X.Q.; Pan, L.H.; Zhang, H.L.; Luo, J.P. Structural characterization, and immunomodulatory activity of a new polysaccharide from jellyfish. *Carbohydrate Polymers* **2017**, 159, 188-194. https://doi.org/10.1016/j.carbpol.2016.12.031;
152. Li, R.; Yu, H.; Xing, R.; Liu, S.; Qing, Y.; Li, K.; Li, B.; Meng, X.; Cui, J.; Li, P. Isolation, identification and characterization of a novel antioxidant protein from the nematocyst of the jellyfish *Stomolophus Meleagris. International Journal of Biological Macromolecules* **2012**, 51, 274-278. https://doi.org/10.1016/j.ijbiomac.2012.05.015;
153. Li, X.; Wang, Q.; Yang, J.; Pan, Y.; Chen, Q.; Yan, X.; Wang, D.; Zhou, X.; Wu, Y. Up-regulation of the gap junction intercellular communication by tea polyphenol in the human metastatic lung carcinoma cell line. *Journal of Cancer Therapy* **2012**, 3, 64–70. http://dx.doi.org/10.4236/jct.2012.31009;

154. Lilley, M.K.S.; Houghton, J.D.R.; Hays, G.C. Distribution, extent of inter-annual variability and diet of the bloom-forming jellyfish *Rhizostoma* in European waters. *Journal of the Marine Biological Association of the United Kingdom* **2009**, 89, 39-48. https://doi.org/10.1017/S0025315408002439;

155. Lim, D.G.; Lee, J.C.; Kim, D.J.; Kim, S.J.; Yu, H.W.; Jeong, S.H. Effects of precipitation process on the biophysical properties of highly concentrated proteins. *Journal of Pharmaceutical Investigation* **2020**, 50, 493–503. https://doi.org/10.1007/s40005-020-00471-2;

156. Lin, Z. In vitro Evaluation of Natural Marine Sponge Collagen as a Scaffold for Bone Tissue Engineering. *International Journal of Biological Sciences* **2011**, 7, 968–977 https://dx.doi.org/10.7150%2Fijbs.7.968;

157. Liu, H.; Zang, C.; Fenner, M.H.; Possinger, K.; Elstner, E. PPAR gamma ligands and ATRA inhibit the invasion of human breast cancer cells in vitro. *Breast Cancer Research and Treatment* **2003,** 79(1):63-74. https://doi.org/10.1023/a:1023366117157;

158. Liu, X.M.; Zhang, Y.; Shi, R.; Qiao, W.; Tang, Z. Sun Production of the angiotensin I converting enzyme inhibitory peptides and isolation of four novel peptides from jellyfish (*Rhopilema esculentum*) protein hydrolysate. *Journal of the Science of Food and Agriculture* **2016**, 96, 3240-3248. https://doi.org/10.1002/jsfa.7507;

159. Liu, Z.; Wang, S.; Hu, M. Chapter 11 - Oral Absorption Basics: Pathways, Physico-chemical and Biological Factors Affecting Absorption, Editor(s): Yihong Qiu, Yisheng Chen, Geoff G.Z. Zhang, Lirong Liu, William R. Porter, Developing Solid Oral Dosage Forms. *Academic Press* **2009**, 263-288, https://doi.org/10.1016/B978-0-444-53242-8.00011-4;

160. Lopez, J.M.; Imperial, S.; Valderrama, R.; Navarro, S. An improved Bradford protein for collagen proteins. *Clinica Chimica Acta* **1993**, 220, 91-100. https://doi.org/10.1016/0009-8981(93)90009-S;

161. Lorenzo, J.M.; Munekata, P.E.S.; Gomez, B.; Barba, F.J.; Mora, L.; Pérez-Santaescolástica, C.; Toldrá, F. Bioactive peptides as natural antioxidants in food products—A review. *Trends in Food Science & Technology* **2018**, 79, 136–147. https://doi.org/10.1016/j.tifs.2018.07.003;

162. Lucas, C.H. Biochemical composition of the mesopelagic coronate jellyfish *Periphylla periphylla* from the Gulf of Mexico. *Journal of the Marine*

*Biological Association of the United Kingdom* **2009**, *89*, 77–81. https://doi.org/10.1017/S0025315408002804;

163. Lucas, C.H.; Pitt, K.A.; Purcell, J.E.; Lebrato, M.; Condon, R.H. What's in a jellyfish? Proximate and elemental composition and biometric relationships for use in biogeochemical studies. *Ecology* **2011**, *92*, 1704–1704. https://doi.org/10.1890/11-0302.1;
164. Magalhaes, L.M.; Fernando, S.; Marcela S.; Salette, R.; Lima José L.F.C. Rapid microplate high-throughput methodology for assessment of Folin-Ciocalteu reducing capacity. *Talanta* **2010**, 83, 441–447. https://doi.org/10.1016/j.talanta.2010.09.042;
165. Maggio, T.; Allegra, A.; Bosch-Belmar, M.; Cillari, T.; Cuttitta, A.; Falautano, M.; Milisenda, G.; Nicosia, A.; Perzia, P.; Sinopoli M.; Castriota, L. Molecular identity of the non-indigenous *Cassiopea* sp. from Palermo Harbour (central Mediterranean Sea). *Journal of the Marine Biological Association of the United Kingdom* **2019**, 99, 1765-1773. https://doi.org/10.1017/S0025315419000924;
166. Malve, H. Exploring the ocean for new drug developments: marine pharmacology. *Journal of Pharmacy and Bioallied Science* **2016**, 8, 2, 83–91. https://dx.doi.org/10.4103%2F0975-7406.171700;
167. Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* **2004**, 79, 727–747. https://doi.org/10.1093/ajcn/79.5.727;
168. Mantena, S.K.; Baliga, M.S.; Katiyar, S.K. Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis* **2006**, 27, 1682–1691 https://doi.org/10.1093/carcin/bgl030;
169. Marques, A.C.; Collins, A.G. Cladistic analysis of Medusozoan and cnidarian evolution. *Invertebrate Biology* **2004**, 123(1), 23–42. https://doi.org/10.1111/j.1744-7410.2004.tb00139.x;
170. Martins, A.; Vieira, H.; Gaspar, H.; Santos, S. Marketed marine natural products in the pharmaceutical and cosmeceutical industries: tips for success. *Marine Drugs* **2014**, 12(2), 1066–1101. https://doi.org/10.3390/md12021066;

171. McHenry, M.J.; Jed, J. The ontogenetic scaling of hydrodynamics and swimming performance in jellyfish (*Aurelia aurita*). *Journal of Experimental Biology* **2003**, 206, 4125-4137. https://doi.org/10.1242/jeb.00649;
172. Merquiol, L.; Romano, G.; Ianora, A.; D'Ambra, I. Biotechnological Applications of Scyphomedusae. *Marine Drugs* **2019**, 17, 604. https://doi.org/10.3390/md17110604;
173. Mese, G.; Richard, G.; White, T.W. Gap junctions: basic structure and function. *Journal of Investigative Dermatology* **2007**, 127, 2516–2524. https://doi.org/10.1038/sj.jid.5700770;
174. Mesnil, M.; Crespin, S.; Avanzo, J.L.; Zaidandagli, M.L. Defective gap junctional intercellular communication in the carcinogenic process. Biochim. Biophys. Acta Biomembr. 2005, 1719, 125–145. http://dx.doi.org/10.1016/j.bbamem.2005.11.004;
175. Mirshamsi, M.R.; Omranipour, R.; Vazirizadeh, A.; Fakhri, A.; Zangeneh, F.; Mohebbi, Seyedian, R.; Pourahmad, J. Persian Gulf Jellyfish (*Cassiopea andromeda*) Venom Fractions Induce Selective Injury and Cytochrome C Release in Mitochondria Obtained from Breast Adenocarcinoma Patients. *Asian Pacific Journal of Cancer Prevention* **2017**, 18(1), 277–286. https://dx.doi.org/10.22034%2FAPJCP.2017.18.1.277;
176. Mohan, A.; Narayanan, S.; Sethuraman, S.; Krishnan, U.M. Combinations of plant polyphenols & anti-cancer molecules: A novel treatment strategy for cancer chemotherapy. *Anti-Cancer Agents in Medicinal Chemistry* **2013**, 13, 281–295. https://doi.org/10.2174/1871520611313020015;
177. Mohebbi, G.H.; Nabipour, I.; Vazirizadeh, A.; Vatanpour, H.; Farrokhnia, M.; Maryamabadid, A.; Bargahia, A. Acetylcholinesterase inhibitory activity of a neurosteroidal alkaloid from the upside-down jellyfish *Cassiopea andromeda* venom. *Revista Brasileira de Farmacognosia* 2018, 28, 568-574. https://doi.org/10.1016/j.bjp.2018.06.002;
178. Morais, Z.B.; Pintao, A.M.; Costa, I.M.; Calejo, M.T.; Bandarra, N.M.; Abreu, P. Composition and in vitro antioxidant effects of jellyfish *Catostylus tagi* from Sado estuary (SW Portugal). *Journal of Aquatic Food Product Technology* **2009**, 18, 1–2, 90–107. https://doi.org/10.1080/10498850802581799;

179. Morandini, A.C.; Stampar, S.N.; Maronna, M.M.; Da Silveira, F.L. All non-indigenous species were introduced recently? The case study of *Cassiopea* (Cnidaria: Scyphozoa) in Brazilian waters. *Journal of the Marine Biological Association of the United Kingdom* **2016**, 97, 321–328. https://doi.org/10.1017/S0025315416000400;
180. Nabipour, I.G.; Mohebbi, H.; Vatanpour, H.; Vazirizadeh, H. Hematological parameters on the effect of the jellyfish venom *Cassiopea andromeda* in animal models. *Data Brief* **2017**, 11, 517-521. https://doi.org/10.1016/j.dib.2017.02.054;
181. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs from 1981 to 2014. *Journal of Natural Products* **2016b**, 79, 629–661. https://doi.org/10.1021/acs.jnatprod.5b01055;
182. Nijveldt, R.J.; Van Nood, E.; Van Hoorn, D.E.; Boelens, P.G.; Van Norren, K.; Van Leeuwen, P.A. Flavonoids: a review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition* **2001**, 74:418–425. https://doi.org/10.1093/ajcn/74.4.418;
183. Nováková, K.; Babica, P.; Adamovský, O.; Bláha, L.; Nováková, K. Modulation of gap-junctional intercellular communication by a series of cyanobacterial samples from nature and laboratory cultures. *Toxicon* **2011**, 58, 76–84. https://doi.org/10.1016/j.toxicon.2011.05.006;
184. O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry* **2000**, 267(17), 5421-5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x;
185. Occhipinti-Ambrogi, A.; Galil, B.S. Marine alien species as an aspect of global change. *Advances in Oceanography and Limnology* **2010**, 1:1, 199-218. https://doi.org/10.1080/19475721003743876;
186. Organisation for Economic Co-Operation and Development (OECD). Test No. 432: In Vitro 3T3 NRU Phototoxicity Test; OECD Guidelines for the Testing of Chemicals, Section 4; Organisation for Economic Co-Operation and Development (OECD): Paris, France, **2019**; ISBN 9789264071162. https://www.oecd.org/env/ehs/testing/019.pdf;
187. Ovchinnikova, T.V.; Balandin, S.V.; Aleshina, G.M.; Tagaev, A.A.; Leonova, Y.F.; Krasnodembsky, E.D.; Men'shenin, A.V.; Kokryakov,

V.N. Aurelin, a novel antimicrobial peptide from jellyfish *Aurelia aurita* with structural features of defensins and channel-blocking toxins. *Biochemical and Biophysical Research Communications* **2006**, 348, 514–523. https://doi.org/10.1016/j.bbrc.2006.07.078;

188. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity* **2009**, 2(5), 270–278. https://doi.org/10.4161/oxim.2.5.9498;
189. Park, S.G.; Kim, B.; Lee, J.; Huang, W.X.; Sung, H.J. Dynamics of prolate jellyfish with a jet-based locomotion. *Journal of Fluids and Structures* **2015**, 57, 331-343. https://doi.org/10.1016/j.jfluidstructs.2015.07.002;
190. Paterson, R.; Nelson, L. (Eds.), Bioprospecting. Success, Potential and Constraints. *Springer* **2017**. https://www.springer.com/gp/book/9783319479330;
191. Pisoschi, A.M.; Pop, A.; Iordache, F.; Stanca, L.; Predoi, G.; Serban, A.I. Oxidative stress mitigation by antioxidants - An overview on their chemistry and influences on health status. *European Journal of Medicinal Chemistry* **2021**, 209, 112891. https://doi.org/10.1016/j.ejmech.2020.112891;
192. Pitt, K.A.; Clement, A.L.; Connolly, R.M.; Thibault-Botha, D. Predation by jellyfish on large and emergent zooplankton: Implications for benthic–pelagic coupling. *Estuarine, Coastal and Shelf Science* **2008**, 76, 4, 827-833, https://doi.org/10.1016/j.ecss.2007.08.011;
193. Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry* **2005**, 53(10), 4290-302. https://doi.org/10.1021/jf0502698;
194. Purcell, J.E. Jellyfish and Ctenophore Blooms Coincide with Human Proliferations and Environmental Perturbations. *Annual Review of Marine Science* **2012**, 4, 1, 209-235. https://doi.org/10.11;
195. Purcell, J.E.; Arai, M.N. Interactions of pelagic cnidarians and ctenophores with fish: a review. *Hydrobiologia* **2001**, 451, 27-44. https://doi.org/10.1023/A:1011883905394;
196. Purcell, J.E.; Benovic, A. Potential links of jellyfish to eutrophication and fisheries. In: Ecosystems at the Land-Sea Margin: Drainage Basin to Coastal Sea (Malone, T.; Malej A. et al., Eds). *American Geophysical Union*

*Washington, DC, USA,* **1999**, 55, 241–263. https://doi.org/10.1029/CE055p0241;

197. Purcell, J.E.; Nemazie, D.A.; Dorsey, S.E.; Houde, E.D.; Gamble Predation mortality of bay anchovy (*Anchoa mitchilli*) eggs and larvae due to scyphomedusae and ctenophores in Chesapeake Bay. *Marine Ecology Progress Series* **1994**, 114, 47-58. https://doi.org/10.3354/meps114047;
198. Putra, A.B.N.; Nishi, K.; Shiraishi, R.; Doi, M.; Sugahara T. Jellyfish collagen stimulates maturation of mouse bone marrow-derived dendritic cells. *Journal of Functional Foods* 2015, 14, 308-317. https://doi.org/10.1016/j.jff.2015.02.008;
199. Rastogi, A.; Sarkar, A.; Chakrabarty, D. Partial purification and identification of a metalloproteinase with anticoagulant activity from Rhizostoma pulmo (Barrel Jellyfish). *Toxicon* **2017**, 132, 29-39. https://doi.org/10.1016/j.toxicon.2017.04.006;
200. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine* **1999**, 26, 1231–1237. https://doi.org/10-1016/S0891-5849(98)00315-3;
201. Rezano, A.; Kuwahara, K.; Yamamoto-Ibusuki, M. Breast cancers with high DSS1 expression that potentially maintains BRCA2 stability have poor prognosis in the relapse-free survival. *BMC Cancer* **2013,** 13, 562. https://doi.org/10.1186/1471-2407-13-562;
202. Rezano, A.; Ridhayanti, F.; Rangkuti, A.; Gunawan, T.; Winarno, G.; Wijaya, I. Cytotoxicity of Simvastatin in Human Breast Cancer MCF-7 and MB-MDA-231 Cell Lines. *Asian Pacific Journal of Cancer Prevention* 2020, 21(S2), 33-42. https://doi.org/10.22034/APJCP.2020.21.S2.33;
203. Ribeiro-Rodrigues, T.M.; Martins-Marques, T.; Morel, S.; R. Kwak, B.; Girão H. Role of connexin 43 in different forms of intercellular communication – gap junctions, extracellular vesicles, and tunnelling nanotubes. *Journal of Cell Science* **2017**, 130, 3619-3630. https://doi.org/10.1242/jcs.200667;
204. Richardson, A.J.; Bakun, A.; Hays, G.C.; Gibbons, M.J. The jellyfish joyride: causes, consequences, and management responses to a more

gelatinous future. *Trends in Ecology & Evolution* **2009**, 24, 6, 312-322. https://doi.org/10.1016/j.tree.2009.01.010;

205. Roleira, F.M.; Tavares-da-Silva, E.J.; Varela, C.L. Costa, S.C.; Silva, T.; Garrido, J.; Borges F. Plant derived and dietary phenolic antioxidants: anticancer properties. *Food Chemistry* **2015**, 183, 235-258. https://doi.org/10.1016/j.foodchem.2015.03.039;

206. Romitelli, F.; Santini, S.A.; Chierici, E.; Pitocco, D.; Tavazzia, B.; Amorini, A.M.; Lazzarino, G.; Di Stasio, E. Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC-MS: the importance of a correct removal of proteins in the Griess assay. *The Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences* **2007**, 851, 257-267. https://doi.org/10.1016/j.jchromb.2007.02.003;

207. Rosillo, M.A.; Alarcón-de-la-Lastra, C.; Sánchez-Hidalgo, M. An update on dietary phenolic compounds in the prevention and management of rheumatoid arthritis. *Food & Function* **2016**, 7(7), 2943–2969. https://doi.org/10.1039/C6FO00485G;

208. Roux, J.P.; Van Der Lingen, C.D.; Gibbons, M.J.; Moroff, N.E.; Shannon, L.J.; Smith, A.D.; Cury, P.M. Jellification of Marine Ecosystems as a Likely Consequence of Overfishing Small Pelagic Fishes: Lessons from the Benguela. *Bulletin of Marine Science* **2013**, 89, 249–284. https://doi.org/10.5343/bms.2011.1145;

209. Rubio, M. Una medusa tropical, nueva amenaza para la biodiversidad del mar Menor. **2017.** https://elclickverde.com/reportajes/unael-mar-menor;

210. Ruch, R.J.; Madhukar, B.V.; Trosko, J.E.; Klaunig, J.E. Reversal of *ras*-Induced Inhibition of Gap-Junctional Intercellular Communication, Transformation, and Tumorigenesis by Lovastatin. *Molecular carcinogenesis* **1993**, 7:50-59 https://doi.org/10.1002/mc.2940070109;

211. Ruiz, J.; Prieto, L.; Astorga, D. A model for temperature control of jellyfish (*Cotylorhiza tuberculata*) outbreaks: A causal analysis in a

Mediterranean coastal lagoon, *Ecological Modelling* **2012**, 233, 59-69, 0304-3800. https://doi.org/10.1016/j.ecolmodel.2012.03.019;

212. Ruppert, E.E.; Fox, R.S.; Barnes, R.D. Invertebrate zoology: a functional evolutionary approach. *Belmont: Thomson Learning,* **2003**. https://www.amazon.it/Invertebrate-Zoology-F030259827;
213. Sai, K.; Kang, K.-S.; Hirose, A.; Hasegawa, R.; Trosko, J.E.; Inoue, T. Inhibition of apoptosis by pentachlorophenol in v-myc-transfected rat liver epithelial cells: Relation to down-regulation of gap junctional intercellular communication. *Cancer Letter* **2001**, 173, 163–174. https://doi.org/10.1016/s0304-3835(01)00616-4;
214. Sai, K.; Kanno, J.; Hasegawa, R.; Trosko, J.E.; Inoue, T. Prevention of the downregulation of gap junctional intercellular communication by green tea in the liver of mice fed pentachlorophenol. *Carcinogenesis* **2000**, 21, 1671–1676. https://doi.org/10.1093/carcin/21.9.1671;
215. Salameh, A.; Dhein, S. Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? *Biochimica et Biophysica Acta* **2005**, 1719, 36–58. https://doi.org/10.1016/j.bbamem.2005.09.007;
216. Schembri, P.J.; Deidun, A.; Vella, P.J. First record of *Cassiopea andromeda* (Scyphozoa: Rhizostomeae: Cassiopeidae) from the central Mediterranean Sea. *Marine Biodiversity Records* **2010**, 3, e6. https://doi.org/10.1017/S1755267209990625**;**
217. Shahidi, F.; Zhong, Y. Measurement of antioxidant activity. *Journal of Functional Foods* **2015**, 18, 757-781. https://doi.org/10.1016/j.jff.2015.01.047;
218. Shaner, N.C.; Steinbach, P.A.; Tsien, R.Y. A guide to choosing fluorescent proteins. Nature Methods 2005, 2, 905-909. https://doi.org/10.1038/nmeth819;
219. Shao, G.; Agar, J.; Giese, R.W. Cold-induced aqueous acetonitrile phase separation: A salt-free way to begin quick, easy, cheap, effective, rugged, safe *Journal of Chromatography A* **2017**, Volume 1506, 128-133. https://doi.org/10.1016/j.chroma.2017.05.045;

220. Shimomura, O. The discovery of aequorin and green fluorescent protein. *Journal of Microscopy* **2005**, 217, 1-15. http://mcb5068.wustl.edu/mcb/prace/shimomura2005.pdf;

221. Shirazi, F.H. :Remarks in Successful Cellular Investigations for Fighting Breast Cancer Using Novel Synthetic Compounds. In: Breast Cancer – Focusing *Tumor Microenvironment. Stem Cells and Metastasis* (Gunduz M, Gunduz E (eds.). Rijeka, InTech, pp. 85-102 **2011**. https://www.researchgate.net/profile/.../publication/.pdf

222. Stahl, W.; Sies, H.; Bioactivity and protective effects of natural carotenoids. *Biochimica et Biophysica Acta* **2005**, 1740, 101–107. https://doi.org/10.1016/j.bbadis.2004.12.006;

223. Stepanenko, O.V.; Verkhusha, V.V.; Kuznetsova, I.M.; Uversky, V.N.; Turoverov, K.K. Fluorescent proteins as biomarkers and biosensors: throwing colour lights on molecular and cellular processes. *Current Protein & Peptide Science* ***2009***, 9, 338-369. https://www.ingentaconnect.com/content/b0;

224. Sugahara, T.; Ueno, M.; Goto, Y.; Shiraishi, R.; Doi, M.; Akiyama K.; Yamauchi, S. Immunostimulation Effect of Jellyfish Collagen. *Bioscience, Biotechnology, and Biochemistry* **2006**, 70:9, 2131-2137. https://doi.org/10.1271/bbb.60076;

225. Thé J, Barroso, H.dS.; Mammone, M.; Viana, M.; Servulo, C.; Melo, B.; Mies, M.; Banha, T.N.S; Morandini, A.C.; Rossi, S.; Soares, de Olivera M. Aquaculture facilities promote populational stability throughout seasons and increase medusae size for the invasive jellyfish *Cassiopea andromeda. Marine Environmental Research* **2020**, 162, 105161. https://doi.org/10.1016/j.marenvres.2020.105161;

226. Tong, L.M.; Sasaki S.; McClements, D.J.; Decker, E.A. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *Journal of Agricultural and Food Chemistry* **2000**, 48(5), 1473-1478. https://doi.org/10.1021/jf991342v;

227. Trosko, E.J.; Ruch, R.J. Cell-cell communication in carcinogenesis. Front. Biosci. 1998, 3, d208–d236. http://dx.doi.org/10.2741/A275;

228. Trosko, J.E. Stem cells and cell-cell communication in the understanding of the role of diet and nutrients in human diseases. *Journal of Food Hygiene*

*and Safety* **2007**, 22, 1–14. https://www.koreascience.or.kr/article/4539114431.page;

229. Trosko, J.E. The gap junction as a "Biological Rosetta Stone": implications of evolution, stem cells to homeostatic regulation of health and disease in the Barker hypothesis. *Journal of Cell Communication and Signaling* **2011**, 5(1), 53–66. https://dx.doi.org/10.1007%2Fs12079-010-0108-9;
230. Trosko, J.E.; Chang, C.C. Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and chemotherapy of cancer. *Mutation Research* **2001**, 480–481, 219–229. https://doi.org/10.1016/S0027-5107(01)00181-6;
231. Trosko, J.E.; Chang, C.C. Modulation of cell-cell communication in the cause and chemoprevention/chemotherapy of cancer. *BioFactors* **2000**, 12, 259–263. https://doi.org/10.1002/biof.5520120139;
232. Trosko, J.E.; Chang, C.C.; Madhukar, B.V. The role of modulated gap junctional intercellular communication in epigenetic toxicology. *Risk Analysis* **1994**, 14, 3, 303-312. https://doi.org/10.1111/j.1539-6924.1994.tb00245.x;
233. Trosko, J.E.; Madhukar, B.V.; Chang, C.C. Endogenous and exogenous modulation of gap junctional intercellular communication: toxicological and pharmacological implications. *Life Sciences* **1993**, 53:1-19. https://doi.org/10.1016/0024-3205(93)90606-4;
234. Trosko, J.E.; Ruch, R.J. Gap junctions as targets for cancer chemoprevention and chemotherapy. *Current Drug Targets* **2002**, 3, 465–482. https://doi.org/10.2174/1389450023347371;
235. Tsao, M.S.; Smith, J.D.; Nelson, K.G.; Grisham, J.W.A. Diploid epithelial cell line from normal adult rat liver with phenotypic properties of oval cells. *Experimental Cell Research* **1984**, 154:38-52. https://doi.org/10.1016/0014-4827(84)90666-9;
236. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, 2, 1231–1246. https://doi.org/10.3390/nu2121231;
237. Turan. C.; Gürlek, M.; Özbalcılar, B.; Yağlıoğlu, D.; Ergüden, D.; Öztürk, B.; Güngör, M.; Jellyfish bycatch data by purse seine, trawl and net fisheries during March–April 2011 in the Mediterranean coasts of Turkey, (pp. 1–7) In First National Workshop on Jellyfish and Other

Gelatinous Species in Turkish Marine Waters, Bodrum, 20–21 May 2011. Turkish Marine Research Foundation **2011** (In: Turan, C., Öztürk, B. eds.) (Istanbul, Turkey) https://www.researchgate.net/pub_of_Turkey;

238. Upham, B.L.; Kang, K.S.; Cho, H.Y.; Trosko, J.E. Hydrogen peroxide inhibits gap junctional intercellular communication in glutathione sufficient but not glutathione deficient cells. *Carcinogenesis* **1997,** 18, 37-42. https://doi.org/710.1093/carcin/18.1.37;
239. Upham, B.L.; Trosko, J.E. Oxidative-dependent integration of signal transduction with intercellular gap junctional communication in the control of gene expression. *Antioxidants & Redox Signaling* **2009**, 11, 297–307. https://doi.org/10.1089/ars.2008.2146;
240. Vilà, M.; Hulme, P.E. Impact of Biological Invasions on Ecosystem Services. *Springer, Berlin, Germany* 2018, https://www.springer.com/gp/book/9783319451190;
241. Vinken, M.; Doktorova, T.; Decrock, E.; Leybaert, L.; Vanhaecke, T.; Rogiers, V. Gap junctional intercellular communication as a target for liver toxicity and carcinogenicity. *Critical Reviews in Biochemistry and Molecular Biology* **2009**, 44, 201–222. https://doi.org/10.1080/10409230903061215
242. Walsh, J.R.; Carpenter, S.R.; Vander Zanden M.J. Invasive species triggers a massive loss of ecosystem services through a trophic cascade. *Proceedings of the National Academy of Sciences* **2016**, 113, 4081- 4085. https://doi.org/10.1073/pnas.1600366113;
243. Wang, C.; Wang, B.; Wang, B.; Wang, Q.; Liu, G.; Fan, C.; Zhang, L. A novel granulin homologue isolated from the jellyfish *Cyanea capillata* promotes proliferation and migration of human umbilical vein endothelial cells through the ERK1/2-signaling pathway. *International Journal of Biological Macromolecules* ***2019***, 135, 212-225, https://doi.org/10.1016/j.ijbiomac.2019.05.101;
244. Wang, W.; Heideman, L.; Chung, C.S.; Pelling, J.C.; Koehler, K.J.; Birt, D.F. Cell-cycle arrest at G2/M and growth inhibition by apigenin in human colon carcinoma cell lines. *Molecular Carcinogenesis* **2000**, 28, 102–110. https://doi.org/10.1002/1098-2744(200006)28:2%3C102::AID-MC6%3E3.0.CO;2-2;

245. Wang, Z.J.; Ohnaka, K.; Morita, M.; Toyomura, K.; Kono, S.; Ueki, T.; Tanaka, M. Kakeji, Y.; Maehara, Y.; Okamura, T.; Ikejiri, K.; Futami, K.; Maekawa, T.; Yasunami, Y.; Takenaka, K.; Ichimiya, H.; Terasaka, R. Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study. *World Journal of Gastroenterology* **2013**, 19, 2683–2690. https://dx.doi.org/10.3748%2Fwjg.v19.i17.2683;

246. Weichselbaum, E., Buttriss, J.L. Polyphenols in the diet. *Nutrition Bulletin* **2010**, 35, 157–164. https://doi.org/10.1111/j.1467-3010.2010.01821.x;

247. Willecke, K.; Eiberger, J.; Degen, J.; Eckardt, D.; Romualdi, A.; Guldenagel, M.; Deutsch, U.; Söhl, G. Structural and functional diversity of connexin genes in the mouse and human genome. *Biol Chem.* **2002**, 383(5), 725–737. https://doi.org/10.1515/BC.2002.076;

248. You, Y.S.; Layhe, B.; Matesic, D.F.; Chang, C.C.; De Feijter, A.W.; Lockwood. L.; Welsch, C.W.; Klaunig, J.E.; Trosko, J.E. Inhibition of gap junctional intercellular communication and malignant transformation of rat liver epithelial cells by neu oncogene. *Carcinogenesis* **1995**, 16, 311-317. https://doi.org/10.1093/carcin/16.2.311;

249. Yu, H.H.; Liu, X.G.; Xing, R.E.; Liu, S.; Guo, Z.Y.; Wang, P.B.; Li, C.P.; Li, P.C. In vitro determination of antioxidant activity of proteins from jellyfish *Rhopilema esculentum*. *Food Chemistry* **2006**, 95(1), 123–130. https://doi.org/10.1016/j.foodchem.2004.12.025;

250. Zamora-Sillero, J.; Gharsallaoui, A.; Prentice, C. Peptides from Fish By-product Protein Hydrolysates and Its Functional Properties: an Overview. *Marine Biotechnology* **2018**, 20, 118–130. https://doi.org/10.1007/s10126-018-9799-3;

251. Zefferino, R. Piccoli, C.; Di Gioia, S.; Capitanio, N.; Conese, M. Gap Junction Intercellular Communication in the Carcinogenesis Hallmarks: Is This a Phenomenon or Epiphenomenon? *Cells* **2019**, 8(8), 896; https://doi.org/10.3390/cells8080896;

252. Zhang, Q.; Song, C.; Zhao, J.; Shi, X.; Sun, M.; Liu, J.; Fu, Y.; Jin, W.; Zhu, B. Separation and characterization of antioxidative and angiotensin converting enzyme inhibitory peptide from jellyfish gonad hydrolysate. *Molecules* **2018**, 23(1), 94. https://doi.org/10.3390/molecules23010094;

253. Zhang, Q.Y.; Wang, F.X.; Jia, K.K.; Kong, L.D. Natural product interventions for chemotherapy and radiotherapy-induced side effects. *Frontiers in Pharmacology* **2018**, 9, 1253, https://doi.org/10.3389/fphar.2018.01253;
254. Zhang, Z.; Animal biodiversity: An introduction to higher-level classification and taxonomic richness. *Zootaxa* **2011**, 3148, 7-12. https://doi.org/10.11646/zootaxa.3148.1.3;
255. Zhao, Y.; Yu, L.; Xu, S.; Qiu, F.; Fan, Y.; Fu, G. Down-regulation of connexin43 gap junction by serum deprivation in human endothelial cells was improved by (-)-Epigallocatechin gallate via ERK MAP kinase pathway. *Biochemical and Biophysical Research Communications* **2011**, 404, 217–222.
256. Zhou, Y.; Zheng, J.; Li, Y.; Xu, D.P.; Li, S.; Chen, Y.M.; Li, H.B. Natural Polyphenols for Prevention and Treatment of Cancer. *Nutrients* **2016**, 22, 515. https://doi.org/10.3390/nu8080515;
257. Zhuang, Y.; Hou, H.; Zhao, X.; Zhang, Z.; Li, B. Effects of collagen and collagen hydrolysate from jellyfish (*Rhopilema esculentum*) on mice skin photoaging induced by UV Irradiation. *Journal of Food Science* **2009**, 74(6), 187–188. https://doi.org/10.1111/j.1750-3841.2009.01236.x;
258. Zhuang. Y.; Sun, L.; Zhang, Y.; Liu, G. Antihypertensive effect of long-term oral administration of jellyfish (*Rhopilema esculentum*) collagen peptides on renovascular hypertension. *Marine Drugs* **2012**, 10(2), 417–426. https://doi.org/10.3390/md10020417;
259. Ziech, D.; Franco, R.; Georgakilas, A.G.; Georgakila, S.; Malamou-Mitsi, V.; Schoneveld, O.; Pappa, A.; Panayiotidis, M.I. The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. *Chemico-Biological Interactions* **2010**, 188 (2), 334-339. https://doi.org/10.1016/j.cbi.2010.07.010;
260. Zong, L.; Zhu, Y.; Liang, R.; Zhao, H.B. Gap junction mediated miRNA intercellular transfer and gene regulation: a novel mechanism for intercellular genetic communication. *Scientific Reports* **2016**, 6, 19884. https://doi.org/10.1038/srep19884;