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COLD-ADAPTED ASSOCIATED MARINE BACTERIA: A SOURCE OF NEW BIOMOLECULES WITH PHARMACEUTICAL APPLICATION

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1. INTRODUCTION

The term “bioactive molecule” refers to any "compound that has the ability to interact with one or more components of living tissues, presenting a wide range of probable effects, whose origin can be natural or synthetic (partial or total)". This was recently concluded by Guaadaoui and co-authors (Guaadaoui et al., 2014) who tried to define the concept of bioactive molecule, which remains not entirely clear at present.

The earliest evidence of the extraction and use of natural bioactive products has existed since ancient times. The text "Erbes Papyrus" (1500 BC) documents over 700 drugs of vegetable origin for therapeutic purposes. The "Chinese Materia Medica" (1100 BC) contains 52 prescriptions, and many others are reported in subsequent works such as "Shennong Herbal" (100 BC, 365 drugs) and "Tang Herbal" (659 a.D., 850 drugs). Friedrich Sertürner, an apprentice pharmacist, isolated about two centuries ago the first pharmacologically pure active compound, morphine, produced from poppy seeds (*Papaver somniferum*) (Borchardt et al., 2002; Hamilton et al., 2000). These discoveries probably represent the first steps taken towards the discipline that today we call natural product chemistry.

Today we are aware that the organisms present on Earth, in any habitat they have evolved, possess the ability to produce metabolites with unique properties, applicable in different scientific fields, as demonstrated by the continuous processing of yearbooks containing the list of innumerable molecules discovered and cataloged according to the organism of origin, with chemical-physical specifications and pharmacological properties, as shown by the periodical reviews published by Blunt et al. (2006) and Faulkner (2000).

1.1 PHARMACOLOGICAL POTENTIAL OF MARINE METABOLITES

The marine environment offers a series of compounds with unique molecular architectures that present a wide range of bioactivity, including antitumor, anti-inflammatory and antimicrobial activities.

Blunt et al. (2004) showed that, in the marine environment, sponges (37%), coelenterates (21%) and microorganisms (18%) represent the main sources for the development of compounds with applications in the medical-pharmaceutical field, followed by algae (9%), echinoderms and tunicates (6%), molluscs (2%) and bryozoans (1%).

The pharmaceutical industry has certainly benefited from research on marine metabolites, which, subjected to in vivo and in vitro assays, have allowed the development of drugs such as cytarabine, vidarabine and ziconotide (Gallimore, 2017).

The oceans cover the 70% of our planet surface, with an area of 360 million km^2 per 11 thousand meters of depth. Within this vast environment, for the most part still unexplored, the values of the chemical-physical properties mutate as the three spatial dimensions vary, such as latitude, longitude, and depth.

The result obtained is an extremely varied environment with unique ecosystems, within which life has developed, adapted and evolved also due to the tough competition between organisms, which aim to ensure an ecological niche that allows them to survive. What follows is those marine organisms, developing a natural and physiological set of secondary metabolites necessary to cope with this whole series of variables, have generated a pool of unique molecules of their kind, which show usefulness in biotechnological and pharmaceutical applications.

This richness, widely proven for terrestrial organisms with numerous scientific researches, has for marine organisms a particular singularity attributed to the adaptation strategies, to the different values of the chemical-physical parameters of the aqueous environment (high values of pressure, salinity, discrete variations of temperature, lack of nutrients and light, low oxygen levels or total substitution with other chemical elements on which to base one's metabolism) and more significant presence of halogens in the

marine aqueous medium, which makes these molecules different from the same isolated in a terrestrial environment (Paul et al., 2011).

Venter et al. (Venter et al., 2004) sequenced and studied the DNA extracted from 1,500 litres of surface seawater sampled off the coast of Bermuda, identifying over 1.2 million new genes; the discovery of such a large data set from a quantitatively limited sample has posed significant challenges in an emerging field such as that of marine molecular microbial ecology and evolutionary biology.

Carrying out a bibliographic study, it is possible to note that among all the new molecules of natural origin, isolated from marine organisms, these are widely represented by the category of terpenes, steroids, alkaloids, ethers and peptides.

Although in many cases the functions of these secondary metabolites in the same marine species are not known, other compounds play well-defined roles, such as trail markers, sexual attractants, antifouling substances or antifeedants. What is clear is that many of the most interesting marine secondary metabolites possess efficient activities largely unrelated to their in situ roles, such as anticancer, antiviral, immunosuppressive and antimicrobial agents, as well as neurotoxins, hepatotoxins and cardiac stimulants (Rinehart, 1992).

The microorganisms capable of producing these metabolites of interest are, at significant percentage, symbionts of Porifera. The largest number of isolated compounds is attributed to the Porifera phylum, around 4851 which today represent 30% of all natural marine products discovered in the last 50 years (Mehbub et al., 2014). Porifera are followed by Rhodophyta (700 compounds) and microorganisms, such as fungi and phytoplankton (350 compounds) (HU et al., 2011).

The number of compounds isolated from bacteria of marine origin is about 150. This number, however, low it is, does not take into account the microbial communities present inside and on the Porifera epithelium (Thakur et al., 2004) which, as described by Hentschel et al. (2006), can reach densities equal to 10^8 ~ 10^{10} bacteria *per* gram of dry sponge, and could therefore be partly responsible for the high number of compounds isolated from Porifera.

Furthermore, the chemical structure of these compounds has some similarities with that of other microbial products, which leads us to suppose that these compounds derive more from the symbiotic microorganisms than from the metabolism of the Porifera themselves. Recent studies (Wang, 2006) have in fact shown that microorganisms isolated from Porifera produce the same secondary metabolites observed in the sponges themselves.

The discovery of natural products of marine origin has increased over the last 20 years, with the number of new compounds discovered increasing every year.

A review shows that in 2010 over 15,000 new marine natural products were discovered with 8,368 new compounds registered between 2001 and 2010 (Faulkner, 2000; Blunt et al., 2006).

1.2 THE ACTIVITY OF BIOPROSPECTING IN THE MARINE ENVIRONMENT

In September 1991, an agreement between the National Institute for Biodiversity of Costa Rica (INBio) and the pharmaceutical company Merck & Co. established a supply to the latter of chemical extracts of plants, insects and microorganisms taken from the territory of the Costa Rica for Merck's drug screening program, against funds allocated to INBio for research, sampling and rights on any resulting commercial product; INBio also contributed by paying a share of the funds and rights obtained to the government's National Park Fund for the conservation of national parks in Costa Rica.

This agreement represented the first form of bioprospecting, or instead, the exploration of the biodiversity of genetic and biochemical resources present on the planet for commercial purposes (Reid et al., 1993).

The activity of bioprospecting in the marine environment was confined for a long time to the infralittoral plane (0-40m) of seas and oceans, as accomplished through the use of A.R.A. (Air Respirator) that, due to the scarce knowledge of the effects of scuba diving on human physiology and the technological development applied to diving equipment in those years, allowed a maximum operating depth of 40 meters.

Science has always been linked to the curiosity and the passion of knowing, typical of being human; technology, on the other hand, stems from the ability

to devise and build tools that expand the possibility of acting on the environment to better meet needs.

An example in the biological field is represented by the discovery of the DNA structure, which occurred in 1953 and the subsequent technological development of genetic engineering, whose potential is implemented over the years.

In 1872 the curiosity of oceanographers and naturalists, led by the officers of the British Navy, led them to carry out the first research expedition in the marine field, onboard the "HMS Challenger" corvette, on a 68,000-mile journey across the globe terrestrial.

The results obtained during the expedition constituted the pillars of modern oceanography, and the discovery of 4,700 new species (taken by dredges and nets) allowed the publication of the first marine biology text "Deep of the Sea" after 20 years.

This expedition gave way to the knowledge of the marine environment and the organisms present in it, as well as its enormous potential, giving rise to the desire to know more and more that ecosystem up to that point unknown (Cognetti et al., 2002).

Unfortunately, the technological gap of the time was the main responsible for the diversion of scientific attention towards a "more accessible nature", such as the terrestrial one, in order to satisfy the need for knowledge.

However, this "second choice" produced essential discoveries in the medical field, such as Penicillin, Cyclosporine, and Adriamycine.

In the second half of the nineteenth century, the development of underwater equipment and subsequently of remotely controlled vehicles (ROVs) for increasingly more in-depth investigations, supported by laboratory investigation tools, such as nuclear magnetic resonance (NMR) spectrometry, allowed the exploitation of that immense biological reserve hitherto confined to the depths of the sea, thus increasing the rate of discovery of new species and isolating an increasing number of natural marine products, with peculiar characteristics, year after year.

Consulting the marine scientific literature between 1978 and 1997 is possible to notice a 131% increase in scientific publications (2070 against 4791 publications), whose research focuses on natural marine products, with

a total of more than 7000 isolated metabolites, coming from bacteria (Kelecom, 2001).

Overall, around 15,000 pharmacologically active compounds have been isolated from marine species, many of which are structurally unique and absent in terrestrial organisms (Mehbub 2014; Murti et al., 2010).

1.3 ENVIRONMENTAL EXTREMES

Extreme marine environments cover over 50% of the Earth's surface and offer many opportunities to study biological responses and adaptations of organisms to stressful living conditions.

Environments are defined as extreme if one or more physic-chemical variables approach the limits of what is known to be tolerable by the most well-known life forms (for example extremely high or low temperatures or frequent variations of it, as well as pressure, concentrations of oxygen or salts, radiation levels, but also the presence of toxic compounds (Rothschild, 2001).

However, various organisms, defined precisely as extremophiles (MacElroy, 1974), grow optimally if these certain conditions exist (Table 1) since they have adapted over time.

Table 1. Classification of extremophiles (Rothschild & Mancinelli, 2001)

ENVIRONMENTAL PARAMETER	CLASSIFICATION	ENVIRONMENTAL REFEREE
TEMPERATURE	HYPERTHERMOPHILES	> 80° C
	THERMOPHILES	60°-80° C
	MESOPHILES	15°-60° C
	PSYCROTOLERANT	0°-15° C
	PSYCROPHILES	< 15° C
PRESSION	PIEZOPHILES	> 1 ATM
SALINITY	HALOPHYLES	2-5 M O 3-20% NACL
PH	ACIDOPHILES	< 2-3
	ALKALOPHILES	ε 9
DRYING	XEROPHILES	H_2O MINIMUM CONCENTR.
OXYGEN	ANAEROBIC	O_2INTOLERANT
	MICROAEROPHILES	O_2 MINIMUM CONCENTR.
	AEROBIC	O_2 TOLERANT

Terrestrial, marine, polar and deep-water ecosystems include both stable and unstable environments. In stable environments, such as polar ecosystems, well-adapted organisms live close to the limits of their physiological potential for long periods.

In less stable environments, such as hydrothermal sources, organisms intermittently test the limits of their physiological potential and develop different strategies to survive these stochastic variations (Carex, 2011).

The organisms present in these environments, which have developed strategies for adapting to these conditions, appear to be particularly sensitive to environmental changes, whether arising from the introduction of anthropogenic stressors or due to changes in their current environmental conditions (Catalan et al., 2006; Bellard et al., 2012); because of this, such organisms can also play a role of bioindicators of pollution and global change (Walther et al., 2002; Cavicchioli et al., 2011).

Polar environments are characterized by low and stable temperatures (Huntley et al., 1992) and influenced by critical seasonal variations in light intensity and related primary production (Clarke et al., 1991; Hagen et al., 2001). Arctic and Antarctic ecosystems, although accumulated by a range of temperatures close to zero, differ in many respects.

Marine polar ecosystems have long been considered desert environments with a low number of species capable of surviving the harsh conditions (Fogg, 1998).

However, in recent decades, scientific studies focused on Arctic and Antarctic ecosystems have increased, and today we know that polar ecosystems are home to a diverse community characterized by endemic species (Conover et al., 1991; Ingels et al., 2014).

1.4 "COLD-ADAPTED" MICROORGANISMS

In 1975 Morita classified microorganisms based on their cold tolerance in:

- *Psychrophiles*, lovers of the cold with an optimal growth temperature of 15° C or lower;
- *Psychrotrophs* or *psychrotolerants* with growth rates higher than temperatures above 20° C.

Millennia of exposure to environmental stresses such as low water temperatures with a relative lowering of metabolic activity, high photoperiod associated with high UVB radiation (280-325 nm), high salinity, osmotic stress, have generated a series of structural and physiological adaptations that have allowed evolution.

Examples are the Cold-Acclimation Proteins (CAPs), proteins synthesized following continuous exposure to low temperatures during the growth phases with the aim of maintaining metabolic functions and replacing denatured peptides due to cold (Griffiths, 2010); Antifreeze Proteins (AFPs), with the ability to modify the structure of ice crystals by inhibiting their recrystallization (at high temperatures below zero) and to promote thermal hysteresis (cooling of body fluids at temperatures below zero) (Berger et al., 1996; Yamashita et al., 2002) or the cryoresistant enzymes, capable of not denaturing at low temperatures (Gilbert et al., 2004).

Also, the exopolysaccharides (EPS) play a key role as they consist of monosaccharides rich in uronic acid which give the substance itself a gel consistency (highly aqueous, 99% water) which allows micro-organisms to adhere to the substrates, reducing of the freezing point guaranteeing the cell the availability of water in the liquid state and constitute a protective shell for extracellular enzymes (Privalov, 1990; Timasheff, 1992).

2. GENERAL CHARACTERISTICS OF THE ORGANISMS EXAMINED

2.1 PHYLUM MOLLUSCA

Mollusca (Cuvier, 1797) are organisms capable of colonizing different habitats, from tropical to polar regions.

The body is formed by a head-foot portion and a visceral mass; in the first, the sensory organs reside and the nutritional and locomotor functions take place, in the second the digestive, circulatory, respiratory and reproductive organs are contained.

The dorsal wall of the body has two lateral expansions able to form a protective mantle, between the mantle and the body wall there is a space called cavity of the mantle that houses the gills and, in some species, secretes a protective shell placed above the visceral mass. Some species of molluscs have a well-developed head with a specialized mouth and sense organs; inside the buccal cavity is present the radula, typical of molluscs, a ribbon-like membrane equipped with rows of thin teeth turned backwards and placed on protrusible cartilaginous support, known as an odontophore.

The foot varies according to the species, in some it is a ventral structure in the form of a sole in which the contraction waves produce a creeping movement; in others, as in bivalves, the foot is compressed laterally in the shape of an axe.

The shell has three layers, an external stratum corneum, known as the periostracum, formed by a very resistant protein called conchiolin; the intermediate prismatic layer and the internal mother-of-pearl layer are composed of calcium carbonate (respectively in prisms and laminates) pressed and immersed in a protein matrix.

The specimens with radula carry out the nutritional function through the scraping of the substrate conveying the particles obtained previously compacted with a mucous substance into the digestive tract.

In contrast, most bivalves are suspensory, which leads them to generate a continuous stream of water towards the gills with the aim of maintaining a

continuous exchange of oxygen and ensuring the transport of organic matter. The glandular cells of the gills secrete an abundant quantity of mucus, which imprisons the nutritive particles present; in the stomach mucous and food particles are kept in rotation by the crystalline stylus, which releases the digestive enzymes suitable for extracellular digestion. The ciliated walls of the stomach then divide the cell walls of the stomach by directing those that can be used towards the digestive gland for intracellular digestion. The shell has three layers, an external stratum corneum, known as the periostracum, formed by a very resistant protein called conchiolin; the intermediate prismatic layer and the internal mother-of-pearl layer are composed of calcium carbonate (respectively in prisms and laminates) pressed and immersed in a protein matrix.

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The phylum Mollusca includes a subphylum known as Conchifera, which groups these organisms according to the presence of protection (more or less represented) to the soft body. The subphylum is represented by 5 Classes:

- Monoplacophora, small molluscs with a low, rounded shell and a creeping foot (*Neopilinagalatheae*);
- Gastropoda, little movable or sedentary molluscs since most species have a univalve shell and poorly integrated locomotor organs (*Littorina punctata, Patella caerulea, Zonariapyrum*);
- Cephalopoda, molluscs with reduced shell (often internal) or absent, equipped with tentacles and arms, adapted to life in the pelagic and

benthic environment (*Nautilius pompilius, Spirula spirula, Argonauta argo*).

- Bivalvia, benthic molluscs equipped with a shell with 2 movable valves, joined by a hinge. The locomotor apparatus is frighteningly developed, the foot is not used for locomotion but to dig into the soft substrate (*Margaritifera margaritifera*, *Mytilus galloprovincialis*, *Ostrea edulis*)

- Scaphopoda, sedentary molluscs with an elongated body and tubular shell open at both ends (*Dentalium octangulatum*) (Hichman et al., 2007).

2.2 BACTERIAL COMMUNITIES ASSOCIATED WITH MOLLUSCA

Although little is known about the characteristics of the interactions between bacteria and mollusks, several studies have shown the presence of interactions between these organisms (Silverman et al. 1996; Cole et al., 1998; Cappello et al., 2007).

Molluscs, being filtering organisms, in addition to filtering nutritive particles, inevitably also accumulate microorganisms.

External envelope, internal tissues and organs or liquors of these animals enriched by nutrient compounds and adhesive substances can be considered as auspicious conditions for colonization, attachment and activity of associated microorganisms (Romanenko et al. 2008).

Microbial association of marine invertebrates have proven to be a rich source of biologically active substances with antimicrobial, cytotoxic, or antineoplastic activities that can be useful for biotechnological and pharmaceutical application (Austin, 1989; Fenical, 1993; Faulkner, 1999).

2.3 *MARGARITIFERA MARGARITIFERA*

The microbial community has been isolated from Bivalvia class belong to the *Margaritifera margaritifera* [Linneo, 1758] species. Also known as "pearl mussel of freshwater", it is a mollusc typical of the holarctic kingdom, which can reach dimensions between 10 and 13 cm in length.

Like all molluscs belonging to the Bivalvia class, *M. margaritifera* has two rigid valves joined by a movable hinge. The colour and thickness of the valves vary over time: the young specimens have thin shells and a yellowish-brown colour, unlike the adults, whose shell is thicker, black and shiny. At the base of the shell, whose interior is characterized by a pearly layer, there is an appendage, the foot, which allows it to dig, move and anchor in the ground. It has a siphon from which water draws, gills through which food particles filter, and a second siphon through which the previously filtered water is expelled (Degerman et al., 2006).

The mussels present a complex life cycle (Figure 1) with a high mortality level. It is estimated that only 1/100.000.000 larvae reach the adult stage; this loss is because the "encysting" phase on the gills of the host does not always occur because the host may not be suitable, leading the larva to disperse in the environment.

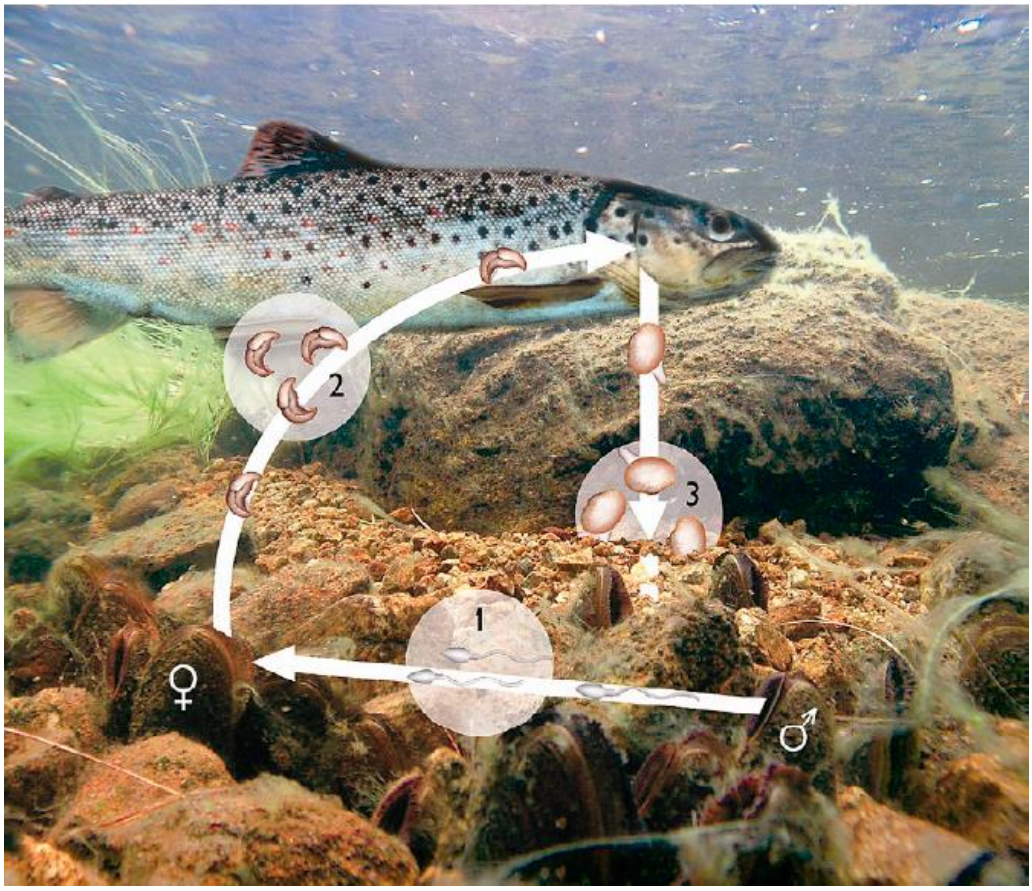


Figure 1. Life cycle of Margaritifera margaritifera

However, this loss is compensated by the longevity of the adult specimens (specimens were sampled at an age of 280 years) and by the high number of larvae produced by the individual (1-4 million larvae). Main actors of this life cycle are the Atlantic Salmon (*Salmo salar*) and the Sea Trout (*Salmo trutta*), which act as a host for the larvae, which adhere to the gills and exploiting this highly oxygenated anatomical portion, increase for the duration of about one year, following which they fall onto the sandy bottom (Figure 2).

Figure 2. Adult specimens of Margaritifera margaritifera on sandy substrate

This substrate will allow them to develop for another ten years until they reach sexual maturity, giving way to a reproductive period that will last over 75 years with an average of 200 million larvae produced (Oulasvirta et al., 2015; Skinner et al., 2003).

The conservation status is considered vulnerable (Figure 3) and, like most filtering organisms, is considered a good environmental bioindicator.

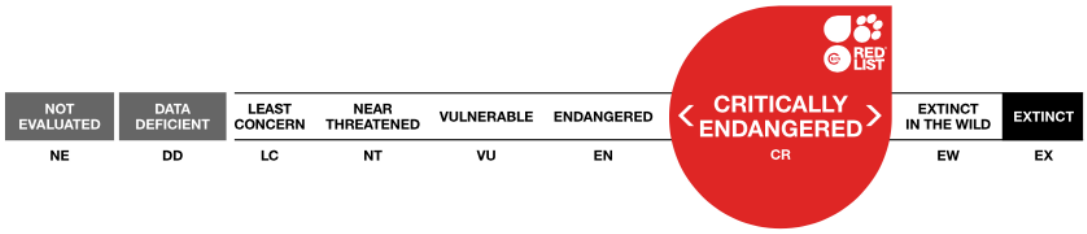


Figure 3. Conservation status of Margaritiferamargaritifera (www.iucnredlist.org)

2.4 PHYLUM PORIFERA

The Porifera, or sponges (Grant, 1836), from the latin "porters of pores", are multicellular structures, having bodies rich in pores and channels that operate the circulation of water.

Porifera involved the less evolved multicellular invertebrates, in fact in the sponges there is no cell organization in organs, and any form of symmetry is absent in the adult stage; it differs from all the other groups of invertebrates, which occupy a similar ecological niche, as they maintain a certain independence from protozoa due to their cellular constituents, which ensure that the cell mass pumps enough water to carry out all the commercial exchanges (Bergquist, 1978).

Porifera are able to colonize every type of environment, from the polar areas (Dayton et al., 1974), to the temperate and tropical zones (Reiswig, 1973; Wenner et al., 1983), from sciaphilous environments, such as caves, to tropical corals (Target and Schmahl, 1984).

Sponges are sessile benthic agents that live anchored to various substrates, can be erect or encrusting and assume various morphologies depending on the environmental characteristics, such as currents, substrates and wave motion.

They are suspending agents that efficiently filter the bacteria present in the surrounding environment, such as food source (Turon et al., 1997).

In the Porifera tissues, different microbial species have been found, so it has been hypothesized that the symbiotic bacteria were known, and therefore selected, during filtration (Wilkinson, 1978). Also, create a solitary life or form dense colonies that represent important habitats for other organs, both animal and plant.

The phylum Porifera consists of three classes called Hexactinellida or "glass sponges" because of their fragility, Calcarea, or calcareous sponges, and Demospongiae, a latter group belong to the majority of common species, with three different morphologies (Figure 4):

- Asconoid, with a single bag structure;
- Siconoid, with Czech background digits in the central cavity;

- Leukonoid, more evolved form, with internal chambers that amplifies the filtration process.

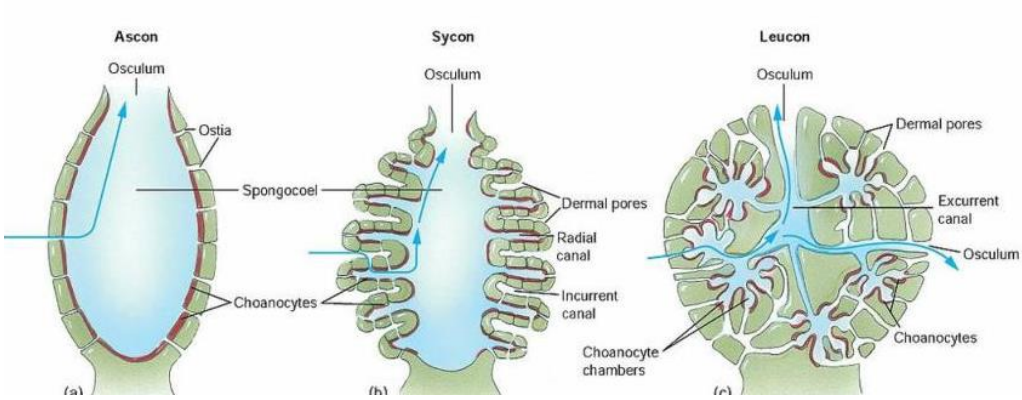


Figure 4. Three different body forms of Porifera

Different cellular layers, consisting of as many different cell types, form the body of a sponge. The external surface, or pinacoderm, is formed by epithelial cells known as pinacocytes, which act as a coating and which also extend to cover the part of the hosts, pores that open on the outer surface of the sponge and which constitute real channels of which the animal is permeated. The internal surface of the sponge is made up of particular flagellated cells, the choanocytes, which play a fundamental role both for sexual reproduction and for feeding, and group together to form chambers defined as choanoderm, within which nutrients accumulate.

Inside the choanoderm, the choanocytes allow to trap the particulate components, including the bacteria and algae (Bergquist, 1978), inside the mesohyl, a layer of connective tissue, thanks to the water filtration process that enters one of the you stayed in an elaborate aquifer system. Here, the food is digested by phagocytosis from another type of cell, the archaeocytes; these are totipotent cells capable of differentiating into any other cellular form of the sponge.

Once passed and filtered in the chamber, the water is expelled through the exhalant opening, the oculus (Figure 5). It has been estimated that thanks to this system, 24,000 liters of water per kilogram of sponge can be filtered every day (Vogel, 1977).

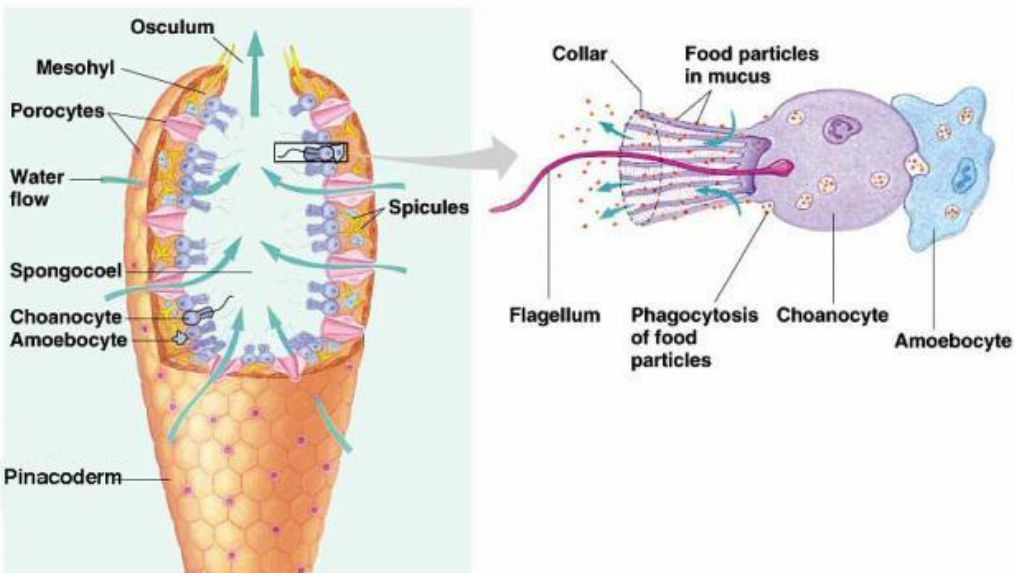


Figure 5 .Internal morphology of Porifera

The structural integrity of the Porifera is due to the presence of skeletal components, the spicules, consisting of silica or calcium carbonate, and the variety constitutes an important taxonomic parameter. Another contribution to the maintenance of the structure is provided by the spongin, a tissue of collagenous nature that allows the animal to reach considerable dimensions.

The different morphology of Porifera is associated with their ecological function; some species of Porifera associated with cyanobacteria have a flattened shape, which is assumed to ensure better reception of light for the photosynthetic symbiont (Sarà et al., 1998).

2.5 BACTERIAL COMMUNITIES ASSOCIATED WITH PORIFERA

Sponge-microorganism associations include a wide range of heterotrophic bacteria, cyanobacteria, facultative anaerobic bacteria, unicellular algae and Archea (Webster and Hill, 2001).

The benefits that these symbionts can provide to the sponge include: nutrition by direct incorporation of organic matter dissolved in seawater (Wilkinson and Garrone, 1980), nutrition by transfer of photosynthesis by symbiont cyanobacteria (Wilkinson, 1983), transport of metabolites through the mesoglea of sponges (Borowitzka et al., 1988), contribution to the structural rigidity of the sponge (Wilkinson et al., 1981), and assistance in chemical defense (Unson et al., 1994).

Furthermore, the optional anaerobic symbionts can metabolize a wide range of compounds and may be involved in the removal of waste products (Wilkinson, 1978a).

Sponges are widely recognized as a source of new bioactive secondary compounds and metabolites (Conte et al., 1994; Perry et al., 1994; Shigemori et al., 1994; Brantley et al., 1995; Hirota et al., 1996); the compounds isolated from sponges have recently been examined by Faulkner (2000).

Traditionally these compounds have been attributed to sponges, but there is a growing interest in the possibility that they may be, in some cases, synthesized by symbiotic bacteria within the sponge tissue (Stierle et al., 1988; Bulten-Poncè et al., 1997; Bewley and Faulkner, 1998; Schmidt et al., 2000). To date, there is evidence of this possibility based on studies between sponges and symbiont cells physically separated and analyzed. For example, polychlorinated compounds are unusually localized in the symbiont cyanobacteria of the sponge Dysidea herbacea, found in Australia (Unson and Faulkner, 1993), while in specimens of some sponges placed in Palau, symbiont cyanobacteria contain brominated biphenyl ether (Unson et al. , 1994).

The bacteria associated with the sponges, moreover, can be divided into three categories: exosymbionts, endosymbionts, present in the mesohyl, and intracellular symbionts, present in the cytoplasm and sometimes in the nucleus (Fuerst et al., 1999; Lee et al., 2001).

In addition, the bacteria associated with the sponges and those used as food particles can be distinguished by the presence of the latter only in the choanocyte chambers.

Two hypotheses have been proposed on the modality through which Porifera acquire the symbiotic bacteria: according to Friedrich et al. (1999) some bacterial strains are able to resist digestion and migration in the mesohyl, while according to Webster and Hill (2001) there is a vertical transmission of the symbionts through eggs or larvae.

2.6 *ANTARCTIC SPONGES*

The microbial community has been isolated from Porifera class belong to the *Calyx arcuaria* [Topsent, 1913]; *Haliclona virens* [Topsent, 1908]; *Haliclona (Rhizoniera) dancoi* [Topsent, 1913]; *Haliclona rudis* [Topsent, 1901] species.

The documentation concerning the morphology of these species is mostly non-existent and therefore it was not possible to investigate this characteristic as much as for *Margaritifera margaritifera*.

3. AIM OF THE WORK

The main objective of this project was the search for bioactive molecules, synthesized by microorganisms isolated from biological matrices (Porifera and bivalve molluscs), sampled in extreme environments, in order to exploit the potential of biosynthesised metabolites, changed during the process of adaptation to the particular environmental conditions, and test their effects in the biotechnological field.

The test will be performed in collaboration with other research institutes and organizations, including foreign, specialized in the pharmaceutical field, for the application of modern scientific protocols and/or new development, to identify biomolecules applicable in the medical-pharmaceutical field.

4. SAMPLING AREAS

4.1 THE ARCTIC

The Arctic is identified as the region situated beyond the 66°32'35"parallel of North latitude, corresponding to the Arctic Circle. However, this border has no geographical value; the border system that over the years has been used for to distinguish and highlight the Arctic zone from the subsequent subarctic zone is represented by the 10° C isotherm in July coinciding with the northern limit of tree growth.

This vast area of 21 million km^2 is occupied by 67% of the Arctic Ocean (14 million km^2) and the rest by land, such as Greenland, Iceland, Norway, Sweden, Finland, Russia, Alaska, Canada which mark the border with the sub-Arctic area (Figure 6).

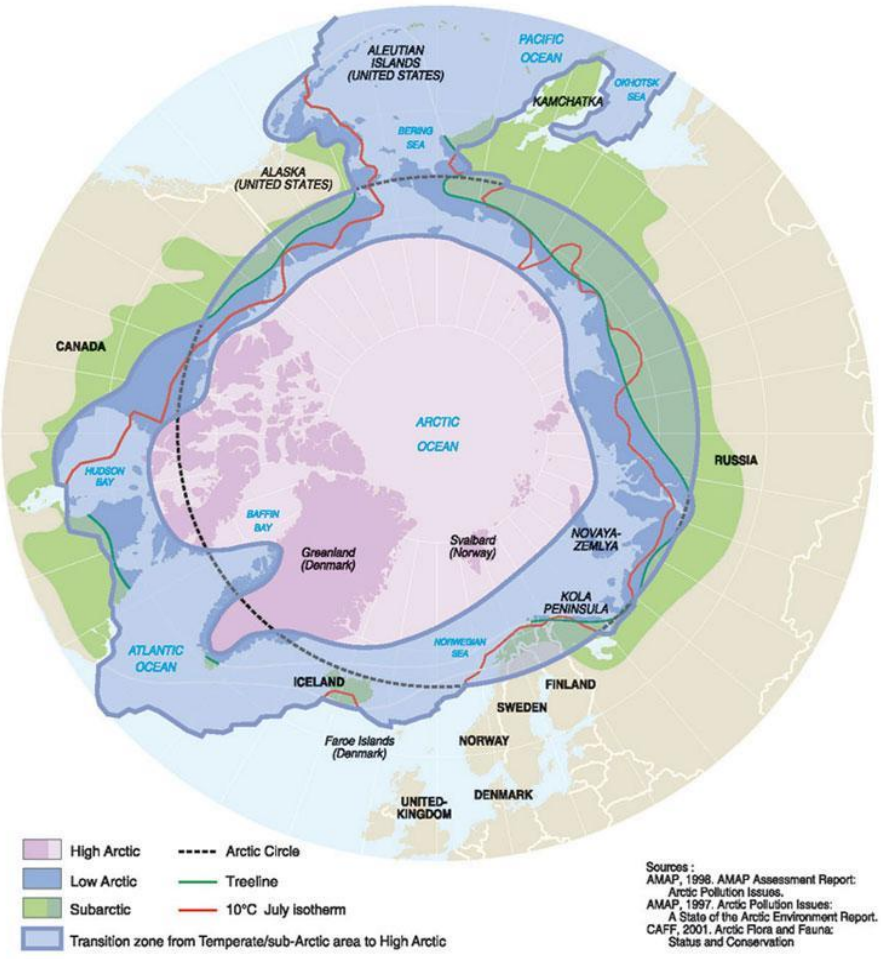


Figure 6. Arctic - North Pole

The extension of the multi-year Arctic ice presents small seasonal variations, between 7 and 17,106 Km^2 (summer-winter) with an average thickness of about 2-3 meters (Maykut, 1985). Solar radiation reaches the Arctic with a cyclical alternation between winter (October-February) and boreal summer (March-September) with average annual temperatures between -40° C and 0°C, respectively.

The utopia of the Arctic as a pristine region has now vanished for decades; the globalization, the increase of the naval routes passing through the Arctic Ocean, the exploitation of oil fields and the fallout of pollutants coming from the lower latitudes "Grasshopper Effect" (Figure 7) (Semeena et al., 2005), have determined a change of direction in scientific field; we have gone from supporting the image of an uncontaminated Arctic to starting international workgroups capable of developing multidisciplinary studies aimed at determining the degree of pollution present and undertaking environmental protection actions.

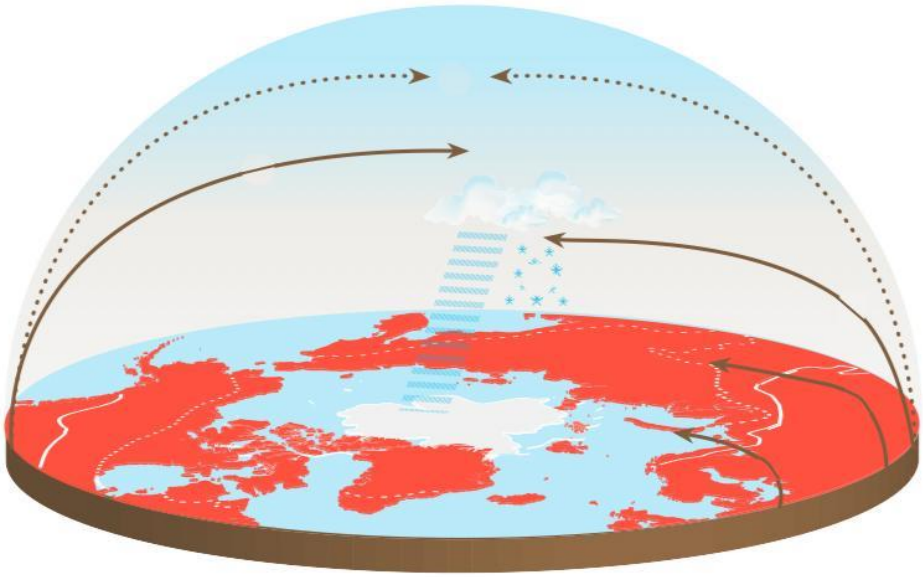


Figure 7. Grasshopper Effect

Work carried out in the past at the Laboratory of Ecology of the Marine Microbial Communities of the University of Messina, allowed to assess, through the microbial community associated with abiotic components and different biological matrices, the presence not only of pollutants (polychlorinated biphenyls, heavy metals) but also a high degree of adaptation

and tolerance to them, by the community itself (Caputo et al., 2019; Rappazzo et al., 2019).

Sampling in the Arctic area included three river courses distributed in Northern Norway, beyond the 70° north latitude (Figure 8), as described in the following text.

Figure 8. Sampling area in Arctic Norway

4.1.1 SKJELLBEKKEN

Skjellbekken, whose course extends for 18.9 km, branches off into small streams south-east of Mount Oksfjellet, crossing the Kiltjørnan valley; south of Lake Skjelvatnet, it further branches off to the mouth of Lake Hasetjørna, in the Pasvik Valley (Figure 9).

Figure 9. .Skjellbekken river (N 69°22.927 - E 29°27.765)

4.1.2 SPURVBEKKEN

The 6.6 km long Spurvbekken originates from the Spurvvatnet lake and flows east of Lake Vaggatem, in the Pasvik Valley (Figure 10).

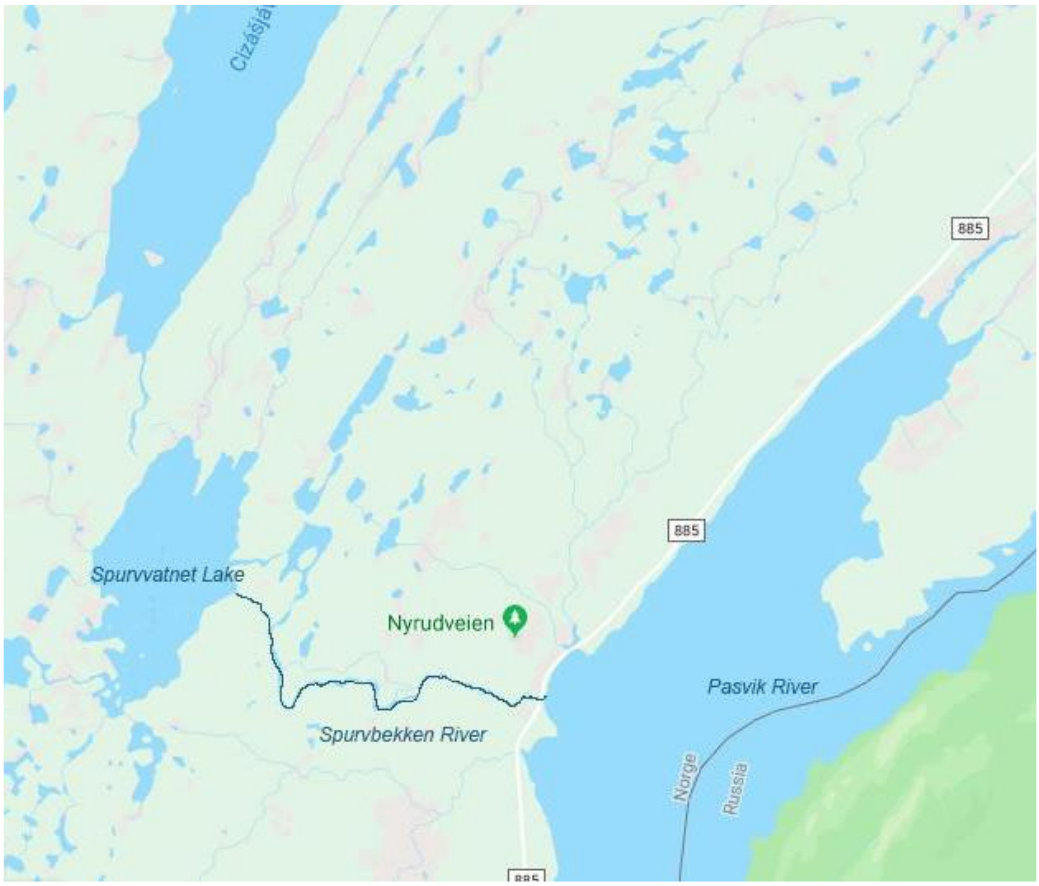


Figure 10. Spurvbekken river (N 69°18.112 - E 29°40.587)

4.1.3 KARPELVA

The Karpelva originates from Lake Evavatnet, flowing it feeds the Bissojohka and Beanajohka rivers, eventually flowing into the Jarfjord fjord, in the hamlet of Karpbukt (Figure 11).

The river is characterized by an alternation of rapids and sliding areas. Karpelva river basin has a mostly rocky substratum, consisting mainly of gneiss and granite.

Figure 11. Karpelva river (N 69°38.902 - E 30°25.549)

These three rivers are found in different climatic zones; this allows the comparison of the chemical-physical characteristics, as well as the comparison of the growth of the inhabiting organisms, in variable climatic conditions (Ylikorkko et al., 2015; Aspholm, 2013).

4.2 ANTARCTICA

Antarctica is the fifth continent by an extension (with a surface of 14 million Km^2), situated entirely beyond the 60° South latitude.

It has an almost circular shape, with an arm of land, known as the Antarctic Peninsula, which juts out towards South America, and two large diametrically opposite inlets, the Ross Sea and the Weddell Sea (Figure 12).

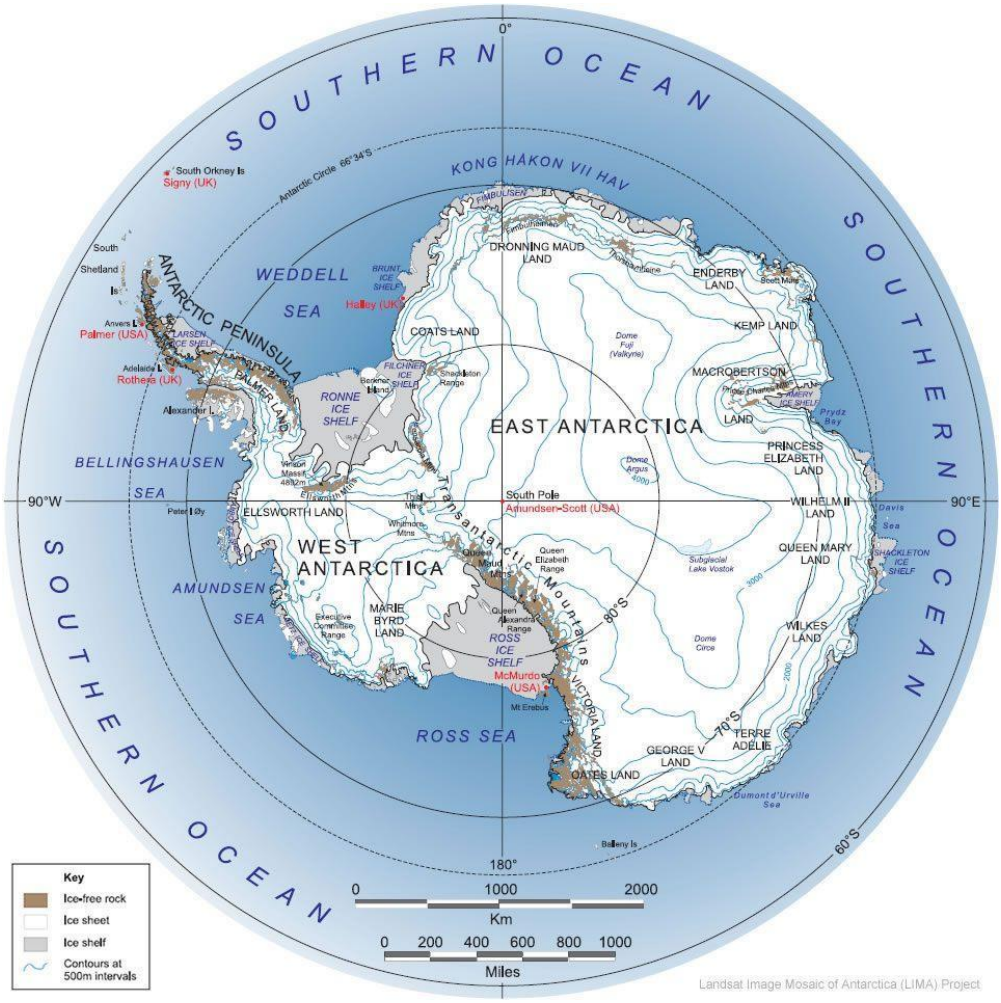


Figure 12. Antarctica - South Pole

The Antarctic continent is characterized by an alternating cyclic photoperiod; four months of light (October-February) coinciding with the southern summer, remaining in darkness during the rest of the year (austral winter), with average annual temperatures between -60°C and -28°C.

It's cold waters with temperatures between -1.86° C and +0.3° C (Dinnima et al., 2004) and rich in nourishment, as well as playing a vital role in the

worldwide ocean circulation, as it represents the driving force of the conveyor belt, constitute a unique ecosystem for the 8,200 catalogued species (RAMS - Register of Antarctic Marine Species), with a density that reaches $1.55 \cdot 10^5$ specimens per square meter.

Antarctica, with its chemical, physical and biological peculiarities, has attracted the scientific communities of every nation for decades now, coming to be considered an open-air laboratory.

Despite being an ecosystem with little human impact ($7 \cdot 10^{-5}$ inhabitants/km^2), recent studies have shown the presence of contaminants in the ice.

As described above, 8200 species have currently been studied and catalogued, a value that could double. This estimated but still unknown 50% is related to sampling difficulties.

However, this wealth does not cover all taxa; bryozoans, poriferas, and amphipods are the most representative at the expense of gastropods and bivalve molluscs. Some groups of fish and crustaceans are even absent (Table 1a, 1b) (Griffiths, 2010).

Table 1a. Numerical diversity of Antarctic species (RAMS)

TAXONOMIC GROUP	N SPECIES
CHROMISTA KINGDOM	256
PLANT KINGDOM	-
CHLOROPHYTA	24
RHODOPHYTA	70
PROTOZOA KINGDOM	-
DINOFLAGELLATE	75
FORAMINIFERA	179

Table 1b. Numerical diversity of Antarctic species (RAMS)

TAXONOMIC GROUP	N SPECIES
ANIMAL KINGDOM	-
PORIFERA	267
CNIDARIA	459
PLATYHELMINTHES	125
MOLLUSCA	740
ANELLIDA	536
CROSTACEA	2900
BRYOZOA	316
ECHINODERMATA	565
TUNICATA	114
OTHER INVERTEBRATES	586
FISHES	314
OTHER VERTEBRATES	284

The microbial community is also well represented in the Antarctic marine ecosystem, as described by Yakimov et al. (1999) and Michaud et al. (2004). As describe by Mock and Thomas (2005) it is responsible of degradation of DOM in the sea ice thought release more represented in terms of biomass and the main responsible in the primary production processes, degradation of the organic substance, transformation of the xenobiotics and responsible in the biogeochemical cycles.

Furthermore, the metabolic supply of Antarctic microorganisms presents adaptations to the environment, such as the ability to produce active enzymes at low temperatures and polyunsaturated fatty acids, such as to make them interesting for applications in the biotechnological field.

Specimens of 14 Antarctic sponge species were collected from 6 sites at Terra Nova Bay (Ross Sea Antarctica), namely Adelie Cove (AC; coordinates 74° 46’ 556’’S-164° 00’ 234’’E), Caletta (CAL; coordinates 74° 45’ 113’’S-164° 05’ 320’’E),Faraglioni (FAR; coordinates 74° 42’ 52’’S-164° 08’ 06.5’’E), Gondwana (GW; coordinates 74° 38 00.5’’S-164° 09’ 09.8’’E), Road Bay (RB; coordinates 74° 42.038’S-164° 08.167’E), and Tethys Bay (TB; coordinates 74° 41.698’S-164° 04’ 214’’E).

In this work, of the 14 specimens collected, only 4 specimens of Antarctic sponge species were analyzed examined (C. arcuaria; H. virens; H. dancoi; Haliclona rudis), collected in the sites shown in Figure 13; in detail, the 4 sponges above-mentioned was sampled in Gondwana and Caletta points.

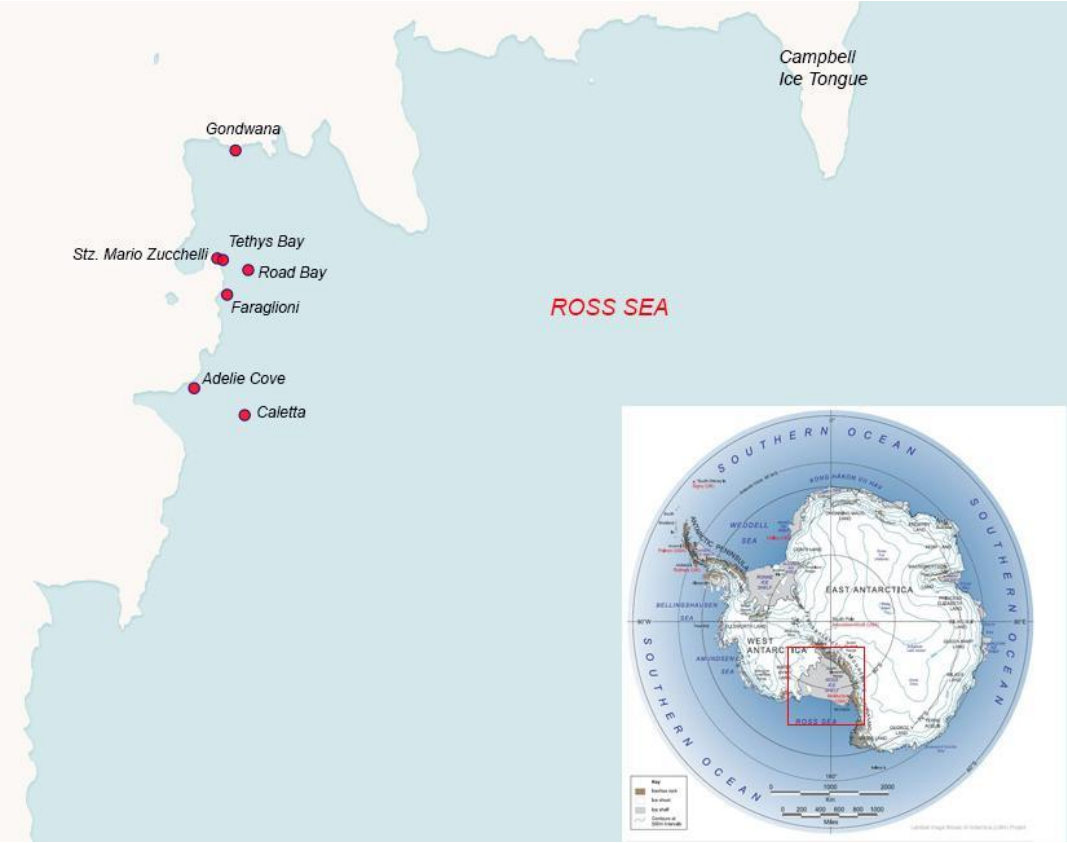


Figure 13. Antarctic sampling points

5. MATERIALS AND METHODS

5.1 ARCTIC ZONE – SAMPLING AND PRELIMINARY TREATMENT OF SAMPLES

M. margaritifera specimens (five per site) were collected by snorkelling at a depth varying between 50-140 centimetres; these were placed inside a container with distilled water to avoid osmotic shock and favour the release of the pseudofaeces (particles of undigested food associated with the mucus), from which the microbial community was then isolated (Figure 14).

Figure 14. Sample of pseudofaeces of Margaritifera margaritifera

At the end of this phase, the bivalves were transferred to a second container containing site water and kept at 4° C until transferred to the laboratory.

Once in the laboratory, weight and size measurements of each specimen were taken.

Subsequently, each mussel deprived of the valves, was homogenized using an Ultra Turrax® homogenizer, keeping it on ice to maintain the temperature low during the homogenization phases. Homogenates were serially diluted up to 10^{-4} in pre-sterilized (polycarbonate membranes; 0.22 μm porosity) site water (Figure 15) and aliquots (100μl) spread plated on Plate Count Agar (PCA), Reasoner's 2 Agar (R2A) and Tryptic Soya Agar (TSA). Plates were incubated in the dark at 4°C for 30 days under aerobic conditions. Bacterial colonies grown on the different media were randomly isolated and streaked at least three times before being considered pure. Cultures were routinely incubated at 4°C.

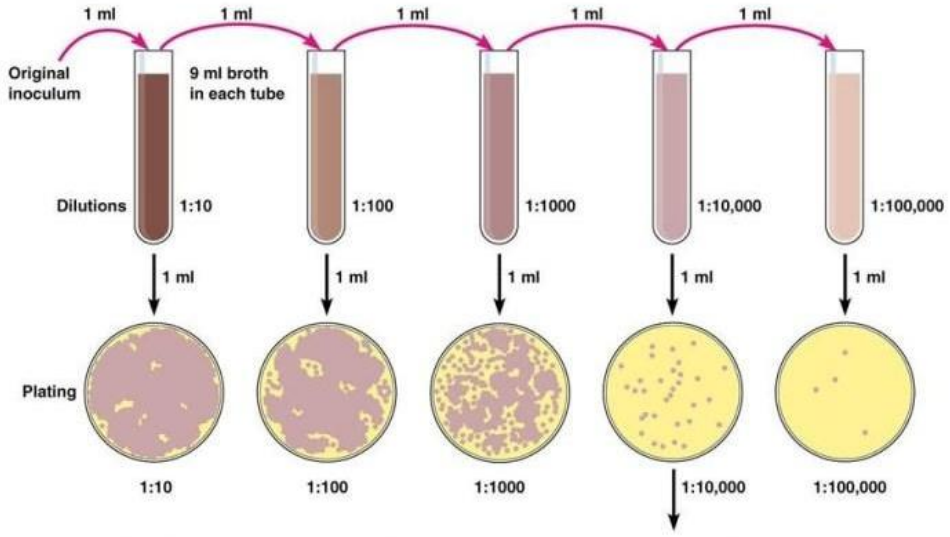


Figure 15. Serial diluitions

5.2 ANTARCTIC ZONE - SAMPLING AND PRELIMINARY TREATMENT OF SAMPLES

Sampling depths ranged between 25 and 200 m. Sponge specimen collection was authorized by the PRNA project. Sponge specimens were treated as previously described by *Mangano et al.* (2009). Briefly, organisms were immediately washed at least three times with filter-sterilized natural seawater to remove transient and loosely attached bacteria and/or debris.

Specimens were then placed into individual sterile plastic bags containing filter-sterilized natural seawater and transported directly to the laboratory at 4° C for microbiological processing (within 2 h after sampling). A fragment of each specimen was also preserved in 70% ethanol for taxonomic identification.

Bacterial isolation from sponge was carried out as previous described by *Mangano et al.* (2009). Briefly, a central core of the sponge body was cut using an EtOH sterilized corkborer or a sterile scalpel. The sponge fragment was then aseptically weighted and manually homogenized in 0.22 µm filtered seawater in a sterile mortar. Sponge extracts were serially diluted using filter-sterilized seawater. Aliquots (100 µl) of each dilution were spread in triplicate on Marine Agar 2216 (MA, Difco). Plates were incubated in the dark at 4°C for 1 month under aerobic conditions. Bacterial colonies grown on MA were randomly isolated and streaked at least three times before being considered pure. Cultures were routinely incubated at 4°C. All the bacterial strains isolated from the sponges were included in the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) ”Felice Ippolito”, and kept at the University of Messina (Italy).

5.3 ANTIBACTERIAL ACTIVITY

The antimicrobial activity was carried out at the Laboratory of Ecology of Marine Microbial Communities of the University of Messina and at the Institute for Biological Resources and Marine Biotechnologies (IRBIM) in Messina.

The antimicrobial activity was evaluated against 4 pathogens (target-strains) using the cross-streak method (Ivanova et al., 1998) only on the strains associated with the genus *Margaritifera margaritifera*; the antimicrobial activity of the Antarctic strains was the subject of Dr Lo Giudice in her post-doctoral thesis entitled "*Biodiversity and potential applications in biotechnology of Antarctic sponge-associated marine bacteria*".

The antibacterial activity was detected with the cross-strip method as described by Lo Giudice et al. (2007). In short, the Antarctic bacteria were striated on a third of an agar plate and incubated at 15 ° C, due to the psychrotrophic nature of the isolates, up to conspicuous growth (generally in 7-10 days, depending on the growth of the isolates). The indicator microorganisms were then striated perpendicularly to the initial strip and the plates were further incubated at 15 °C for 72/120h at 37 °C for one night and subsequently the presence of zones of inhibition was verified.

The antagonistic effect was indicated by the inability of the target strain to grow in the confluence area.

The inhibition, to be considered positive, must have a zone of inhibition of at least 5 mm between the producer strain and the target strain, and must be observed at least twice.

If the first two tests show ambiguous results, an additional test must be performed to reassess the inhibitory activity (Figure 16).

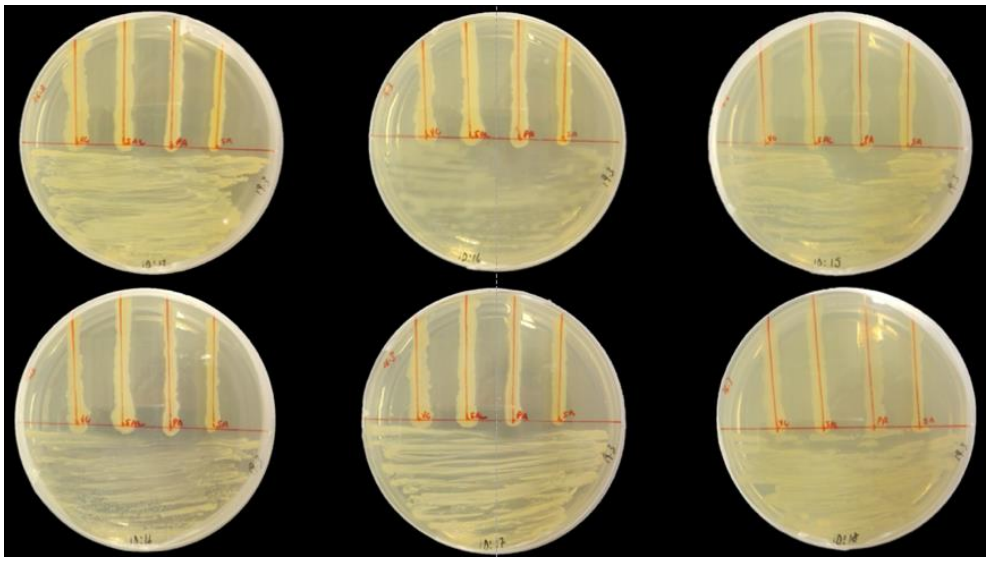


Figure 16. Cross-streak test

Four target-strains were tested:

- ***Staphylococcus aureus*** ATCC6538 (Rosenbach, 1884) is a Gram-positive aerobic microorganism with the ability to coagulate the blood, producing coagulases (enzymes capable of coagulating blood plasma). It is among the most widespread and dangerous human pathogens, both for its virulence and its ability to protect resistance to antibiotics. it is responsible for skin infections, pneumonia, endocarditis, osteomyelitis, septic arthritis, toxic shock syndrome, burned skin syndrome, food poisoning.
- The genus ***Salmonella enterica*** ATCC 14028 (Smith, 1885) is characterized by Gram-negative, asporigenic and facultative anaerobic bacilli, with the capacity to ferment glucose producing gas, degrading sulphurated proteins with the production of hydrogen sulfide, reducing nitrates.

  It is the most often isolated bacterial agent in case of foodborne and epidemic infections. It was reported for the first time in 1886, in a case of swine fever, it is present in nature with more than 2000 variants (the so-called serotypes).
- ***Vibrio cholerae*** ATCC 39315 (Pacini, 1854) is a non-invasive Gram-negative bacterium, anaerobic/anaerobic facultative inhabitant of two very different ecosystems: the aquatic environment and the human gut. The species include pathogenic and non-pathogenic strains that

receive and transfer clusters of genes coding toxins, colonization factors and antibiotic resistance, providing for the emergence of new pathogenic strains. It is an unusual enteropathogen, both for its tendency to cause an explosive epidemic and for its predilection in a pandemic spread.

- ***Pseudomonas aeruginosa*** *ATCC 27853* (Schröter, 1872) is a Gram-negative, optional anaerobic bacterium. The ubiquitous pathogen, resistant drug, which causes nosocomial infections especially in patients undergoing assisted ventilation, burns and chronic debilitation. Extra-hospital infections due to the bacterium have been found in patients with HIV and cystic fibrosis.

5.4 ANTIVIRAL ACTIVITY

The antiviral activity was carried out at Department of Molecular Medicine of the University of Padova.

A first phase of the antiviral activity test involved the evaluation of the degree of cytotoxicity of the sample to be tested against the virulent component.

This step is important to determine the maximum value of cytotoxicity of the sample since the aim is to test a sample that is cytotoxic towards the viral line and not the host cell, therefore, on the viral line, a smaller quantity than the concentration was sown. maximum of the sample, to prevent possible lysis of the host cell by the inserted sample, which would distort the test.

The cytotoxicity of the individual supernatants was evaluated in Vero cells (host cell represented by renal epithelial cells extracted from the grey-green Cercopithecus, *Chlorocebus aethiops*) using the previously described MTT Assay.

The cells were seeded in 96-well microtiter plates (12,000 cell/well) 24h before being treated with bacterial supernatants so that the next day there was a confluence of 70%; this allows the cells inside the cockpit to have enough space to replicate and to ensure that any death caused by competition does not distort the test.

The following day the cells were treated with three different amounts of each supernatant (1μL, 5μL, 10μL).

After 48h from the inoculum, 10μL of MTT was added to the final concentration of 5mg/mL and incubated for 4 hours at 37° C in a 5% CO_2 atmosphere. Subsequently 100μL/well of a 10% SDS solution and 0.001% of 10N HCl were added to dissolve the formazan crystals (indicators of the number of viable cells). The reaction was measured by spectrophotometric reading of the sample, at a wavelength of 620 nm. Every single experiment was performed in triplicate.

Once the maximum cytotoxicity was determined, a plaque test was performed which allowed us to demonstrate their ability to inhibit the viral replication of some viruses penetrating into the cells.

Three viruses were tested:

- ***Herpes simplex*** viruses are classified in type1 (HSV-1) and type2 (HSV-2) and belong to the Herpesviridae family. They are characterized by a short reproductive cycle, the destruction of the host cell and the ability to establish latency within the sensory ganglia (Whitley, 2004). They are responsible for diseases that cause orofacial lesions and brain infections responsible for herpetic encephalitis with an annual incidence of between 1/250.000 and 1/500.000 among children under 3 years and adults over 50 years.
- ***Human polyomavirus 3*** or known as ***KI polyomavirus*** (KIPyV) is one of the 5 viruses of the Polyomaviridae family, described in 2007 Allander et al. (2007). Pathogenicity and epidemiology are little known but some laboratory tests attribute the virus as responsible for acute respiratory diseases in children.
- ***Zika Virus*** belongs to the Flaviviridae family and was isolated in 1947 in monkeys in Uganda and 5 years later in the Ugandan and Tanzanian population. It appears as an asymptomatic infection with mild fever, rash, muscle pain and conjunctivitis that persist for about a week.

  This infection, however, if contracted during pregnancy, could be transmitted to the fetus, causing brain anomalies (microcephaly), miscarriage or triggering Guillain-Barrè Syndrome which causes progressive paralysis of the limbs.

  For the evaluation of possible antiviral activity, a biological test originally developed for the study of the bacteriophage from d'Herelle (Mosmann, 1983) in the early 1900s and adapted following the animal viruses by Dulbecco and Vogt (Alley et al., 1988).

  Specifically, the initial infection caused by a single viral particle on a cell can generate multiple cycles of infection in an area of infection called plaque.

5.4.1 PLATE TEST - HERPES SIMPLEX VIRUS

The plate was divided into 18 "windows" of 4 wells each (2 for negative control and 2 for the evaluation of the activity) and 12,000 cells / well (96-well microtiter plates) were ground into Dulbecco's Modified Eagle Medium (DMEM) with 10% of Bovine Fetal Serum (FBS) and 1% Penicillin / Streptomycin (P / S).

After 24 hours the cells were infected with the viral strains with different values of Multiplicity of Infection (M.O.I.):

- *Herpes simplex* Virus, M.O.I. equal to 0.01
- Polyomavirus, M.O.I. equal to $1.2 \cdot 10^3$
- Zika Virus, M.O.I. equal to 0.1

in Fetal Bovine Serum (FBS) at 2%, to provide the supplements needed for cell growth. A soil with a low concentration of serum is used to facilitate infection, as the serum tends to inhibit the entry of the virus into the cells.

Subsequently, the medium used for sowing the cells was removed and DMEM culture medium with 2% FBS with the virus inoculated and free of negative controls was added.

The medium inoculated with the virus was left to incubate at 37° C in a 5% CO_2 atmosphere for 90 minutes.

Subsequently, the soil was removed with the virus and replaced with DMEM medium at 10% FBS + 1% P / S.

Subsequently, 10 μL of supernatant per well was added and incubated at 37 ° C in a 5% CO2 atmosphere for 48h.

After 48 h of incubation the cells were fixed and stained simultaneously with a solution of Crystal Violet containing methanol (Figure 17).

Subsequently the soil was removed and 80μL of dye solution was added. The plate was rinsed with deionized water for 15 minutes under sterile conditions to remove excess dye.

Dry the plates under a chemical hood, they were observed to determine the presence and quantity of any plaques formed.

The presence of viral plaques indicates virus replication with subsequent lysis of host cells.

The M.O.I value measures the average amount of virus added per cell in an infection.

An M.O.I value of 1 indicates that 1 million virions have been added to a million cells.

However, this parameter does not indicate that each virion will infect a cell. To know the exact number of cells that will be infected, reference is made to a statistical calculation using the Poisson Distribution which allows calculating the number of times an event (infection) occurs in a time or space interval.

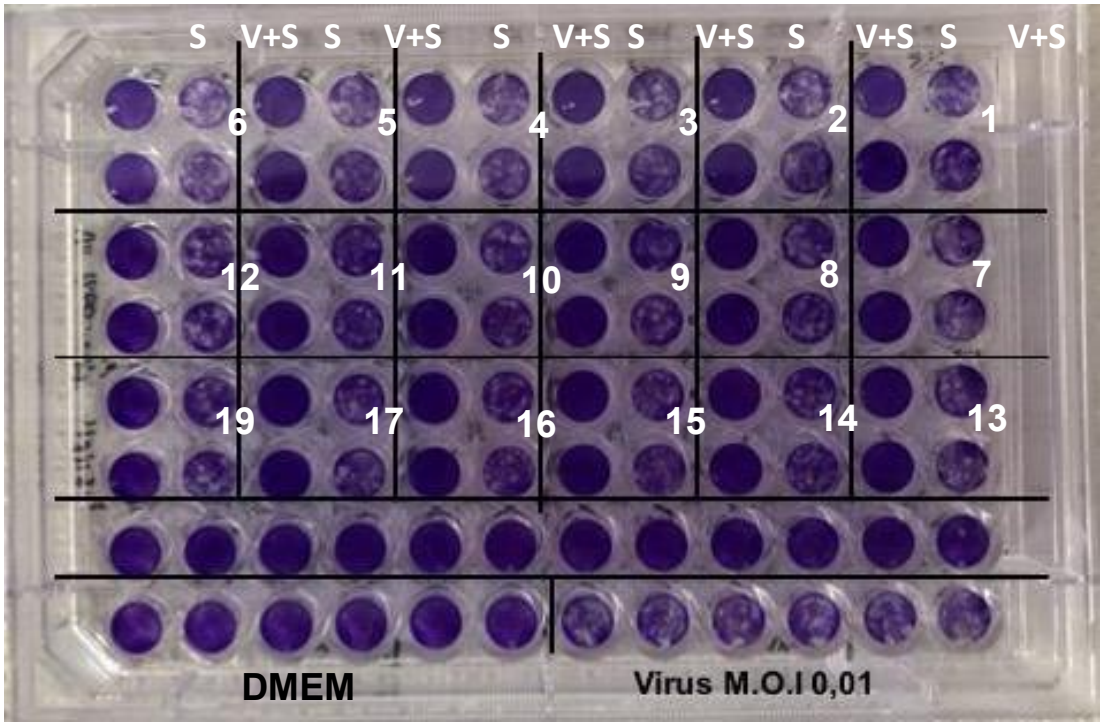


Figure 17. Plaques test on Herpes simplex virus

Using the same method, we proceeded to test the sample against Human Polyomavirus 3 (Figure 18) and Zika Virus (ZikV) (Figure 19). Unlike the assay for Herpes Simplex Virus with value M.O.I. 0.01 TCID50/mL, for THP and ZikV the values of M.O.I. was $1.2 \cdot 10^3$TCID50/mL and 0.1 TCID50/mL respectively.

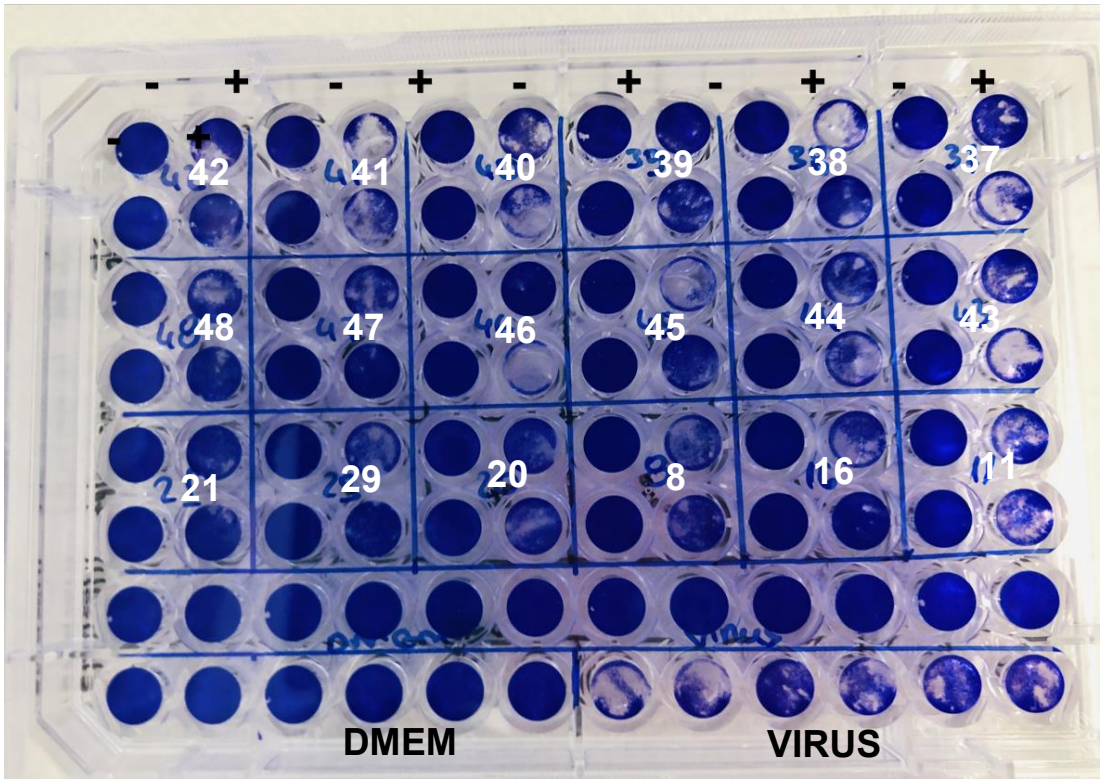


Figure 18. Plaques test on Human Polyomavirus 3

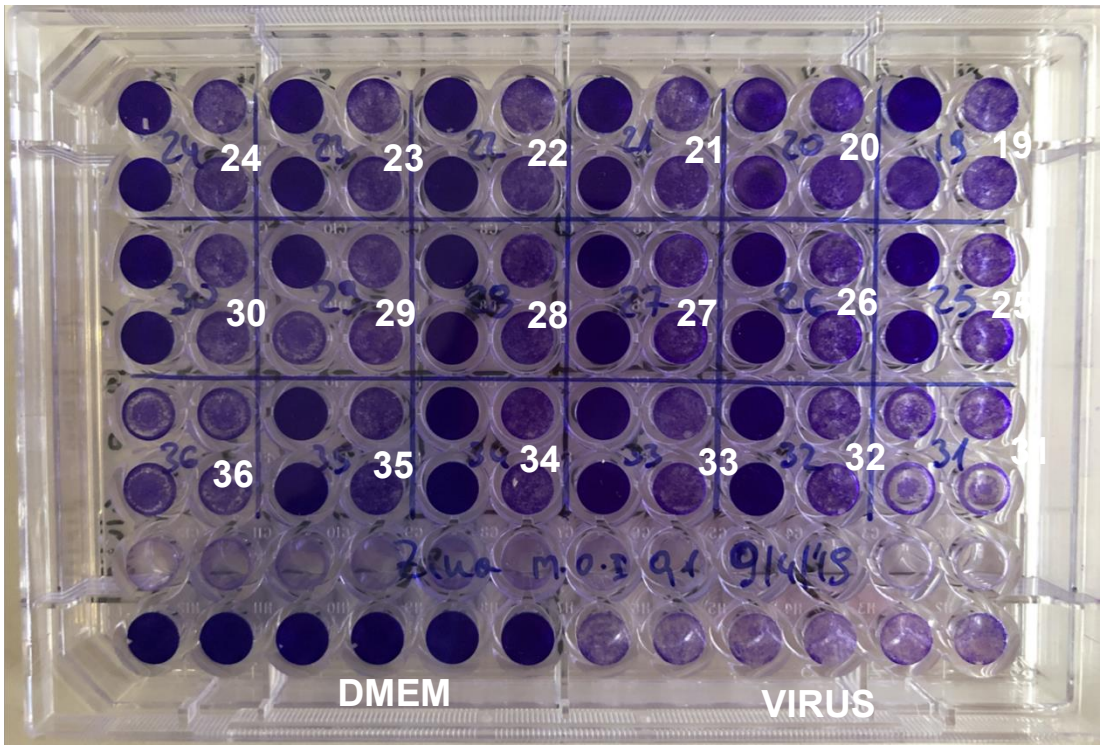


Figure 19. Plaques test on Zika virus

5.5 ANTIPROLIFERATIVE ACTIVITY

The antiproliferative activity carried out at Laboratory of Clinic Pharmacology of the University Hospital “G. Martino” in Messina and at BioLab of the Instituto Universitario de Bio-Orgánica "Antonio González" in Tenerife, was tested through two growth inhibition assays, mainly described in the bibliography: MTT Assay and SRB Assay against cancer cell lines:

- *A172: Human Brain Glioblastoma*
- A549: *Human Lung Epithelial Carcinoma*
- *C6: Rat Brain Glioblastoma*

A first analysis of the antiproliferative activity was applied directly on the supernatant of every single sample, to assess the presence/absence of activity.

Each was taken from the pure culture in a Petri dish and transferred, through a 20 μL loop, into a 1.5 mL eppendorf containing the same non-agarized culture medium (MB/TSB). Once bacterial growth was achieved in the new liquid medium, it was centrifuged at 13,000 rpm for 5 minutes, to separate the pellet from the supernatant.

Later, sterilized by 0.22 μm syringe filter was subjected to anti-proliferative investigation through the MTT assay.

5.5.1 MTT ASSAY

Is a colorimetric assay that measures the ability of mitochondrial succinate dehydrogenases to cleave the water-soluble tetrazole ring of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) molecule yellow, in formazan (E, Z)-5-(4,5-dimethylthiazol-2-yl)1,3 diphenylformazan salts insoluble in water and violet (Figure 20) (Hu et al., 2012; Block et al., 1946).

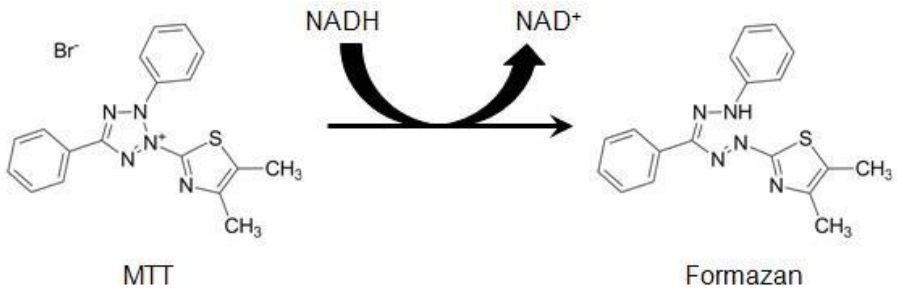


Figure 20. Mitochondrial reductase from MTT to Formazan

The cells with a metabolic activity will be able to split the tetrazole ring into formazan salts; the latter, of violet colour, due to their insolubility will not be able to pass the membrane accumulating in the cells and colouring it. In contrast, in a dead cell, having no metabolic activity, the tetrazole ring of the molecule will remain intact, thus maintaining the yellow colour.

Through the use of a spectrophotometer, the absorbance of the crystals of salts present in each cell was measured; the crystals were previously solubilized with DMSO and read at a wavelength of 570nm.

Absorbance was dependent on the concentration of the tested solution, resulting greater for live cells, which through the metabolic activity formed the insoluble formazan salts.

The protocol used involved the use of 96-well microtiter plates, in which the cancer cells were inoculated at different concentrations, following the scheme described in Figure 21.

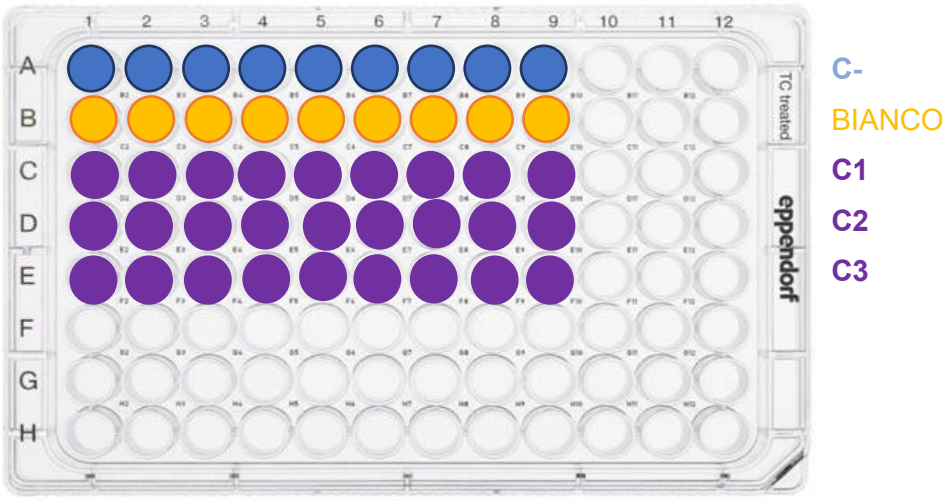


Figure 21. MTT 96 well plate scheme

To the cancer cells, a quantity of 3 mL of a solution of trypsin and EDTA was added and placed in incubation for 5 minutes to favour the detachment. Subsequently, they were centrifuged for 5 minutes at a different speed depending on the cell line (1.200 rpm for A172 and A549 and 1.300 rpm for C6) at a constant temperature of 25°C.

Once the centrifugation was over, the supernatant was removed and the obtained pellet was resuspended in the cellular medium, according to the number of pellets purchased.

Second, 100 μL of the offered mixture was taken and 200 μL of the physiological solution was added. Out of the total of 300μL an aliquot (100μL) was taken, to which 100μL of Trypan Blue were added.

The solution was placed in Burker's chamber to read the microscope and determine the average number of live cells present in 5 quadrants arranged in X (Figure 22), through the formula:

$$cvt/mL = \frac{live\ cell * 1000 * 6}{5}$$

live cell: n cell read in Burker's chamber;

1000: box quantiy

6: dilution factor*;

5: counted boxes

* I dilution 1:3 (cell:physiologic solut.);

* II diluizione 1:1 (cell + physiologic solution:trypanblue);

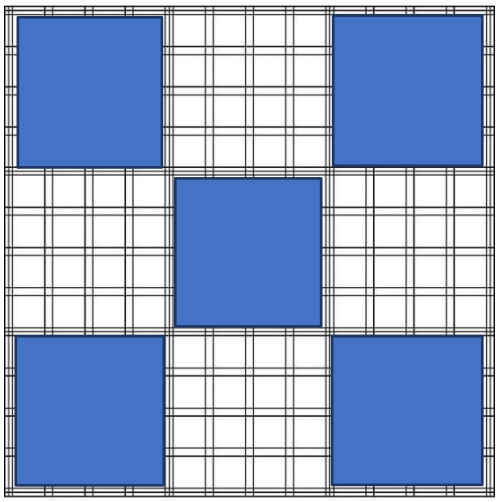


Figure 22. X reading in Burker's chamber

Subsequently, having obtained the number of cells present in the mix solution with Trypan Blue, the number of cells required (expressed in millions) was calculated, using the formula:

$$Tot\ n\ cell\ =\ n\ well\ *\ \left(n\frac{cell}{well}\right)$$

$$Tot\ n\ cell\ =\ 45\ *\ 40.000 = 1.800.000\ cell$$

Through a proportion between the number of cells present in the volume and the quantity required, the quantity of cell suspension to be taken was calculated so that the quantity of cells necessary for the correct performance is present in 150 μL (volume to be placed in each individual well) of the essay.

After the incubation period of 24 hours, the cells were observed to verify their adhesion to the bottom and aspirate the overlying medium.

Subsequently, different concentrations of the substance to be tested were dispensed into the wells in addition to the negative control (Ctr) and to the TSB (White) culture medium only:

- **Ctr**: 1,350 ml (9x150μL) culture medium of the cell line;
- **White**: 1,350 ml (9x150μL) of TSB culture medium;
- **C1**: 1,341 mL di colture medium + 9 µL of the compound to be tested
- **C5**: 1,305 mL di colture medium + 45 µL of the compound to be tested
- **C10**: 1,260 mL di colture medium + 90 µL of the compound to be tested

The plate was incubated for 24 hours at 37° C and 5% CO_2.

After 24 hours of incubation, the supernatant was aspirated from all the wells and MTT (200μL/well) was added at a concentration of 2 mg/ml.

The plate was re-incubated for 1 hour in the dark, as MTT is a photosensitive compound.

After incubation with MTT, the latter was aspirated and replaced with 100 μL of Dimethylsulfoxide (DMSO) per well.

Finally, the plate was placed inside the spectrophotometer by reading at a wavelength of 570 nm.

The MTT assay permitted to identify several strains that showed a mortality of the different cell lines, with average values close to 50% and in some cases even much below it.

5.5.2 MEDIUM-SCALE CULTURE AND SEQUENTIAL BIOASSAY GUIDED FRACTIONATION

The strains showing positive antiproliferative activity were selected to perform medium-scale cultures to identify the compounds (or secondary metabolites) responsible for the observed activity.

Specifically, six Pyrex® flasks with a volume of 2.8 litres, fitted with a screw cap with access for aseptic transfer, were filled with 1.8 litres of different Tryptic Soy Broth (TSB) and Marine Broth (MB), based on the origin of the sample. At the same time, a pre-culture of each single volume (equal to 10% of the total) was inoculated with the bacterial strain to be tested. Upon reaching the maximum value of optical density (indicating the maximum peak of the growth curve) monitored every 24 hours, the pre-cultures were transferred to the medium volume.

Through the aseptic transfer access, an aeration pump for the insufflation of air was connected utilizing a Teflon tube (previously sterilized) which allowed to guarantee the continuous agitation of the culture medium. Finally, to maintain the internal sterility of the culture at each aseptic access to each flask, a sterile RephiQuik® (RephiLe Bioscience Ltd.) filter was placed in 32 mm polypropylene with a 0.22 μm porosity (Figure 23). The average volumes were incubated at temperatures of 15° C.

Figure 23. Medium-size bacteria cultures

When the maximum optical density (OD) value of the cultures was reached (on average 10/15 days), the supernatants were separated from the pellets (Figure 24), utilizing a Sorvall™ RC 6 Plus centrifuge (Thermo Scientific), set at 5,000 rpm for 15 minutes at 4° C.

Figure 24. Supernatant and pellet of culture media

The supernatants (10 L) were separated from the pellets and combined in different flasks for further extraction.

The cells (pellet) were extracted using a mixture of AcOEt:Acetone (70:30) per litre. The solvent was placed in the pellet flask and stirred at 120 rpm. After 14 hours, the solvent mixture was filtered through a filter paper under vacuum and concentrated. Low-pressure evaporation was carried out using Rotavapor® R-200 (Buchi) at a temperature of 40°C, coupled to a Buchi Vacuum Pump V-700 to remove the excess of water in the samples, to obtain the biomass crude extract.

The culture media or supernatant was extracted through hydrophobic and polar interactions with an absorbent non-ionic resin (Hu et al., 2012). The resin used is the Amberlite® XAD7HP (Sigma-Aldrich) with a ratio of 20 g/L of culture medium.

Before use, the resin should be conditioned. The protocol of use foresees a phase of reactivation of the resin through alternate washings of MeOH and water according to the scheme shown in Table 2.

Table 2. Amberlite treatment

STEPS	DESCRIPTION	TIME
I	Wash in a beaker with MeOH and let stand	30'
II	Discard the MeOH and rinse it again with MeOH	10'
III	Discard the MeOH	-
IV	Wash with distilled water and let it rest	10'
V	Discard the water	-
VI	Repeat steps IV and V (x2)	-

After the conditioning step, the resin was added to the supernatant and stirred for 3 hours, to favour the adsorption of the secondary metabolites excreted to the culture medium. At the end of the incubation the resin was separated from the liquid medium by filtration under vacuum and transferred into a 5-litre flask containing a mixture of AcOEt:Acetone:MeOH (70:30:20). The adsorbed compounds were extracted from the resin after agitation for a period of 14 hours.

After that period, the resin is removed by filtration and the solvent is evaporated using Rotavapor® R-200 (Buchi) at 40°C, coupled to a BuchiVacuum Pump V-700 to remove the excess of water in the sample, to obtain the supernatant crude extract.

The samples obtained were stored in balloons, previously weighed, to determine their exact weight (Table 4). The samples were stored at 4ºC until used.

Table 4. ID-37 weight fractions

SAMPLE	FRACTION	WEIGHT (g)
ID-37	SUPERNATANT	18.58
	PELLET	1.718

Once the supernatant and the pellet extracts were obtained, the bioassay guided fractionation of both extracts proceeded according to the scheme shown in Figure 25.

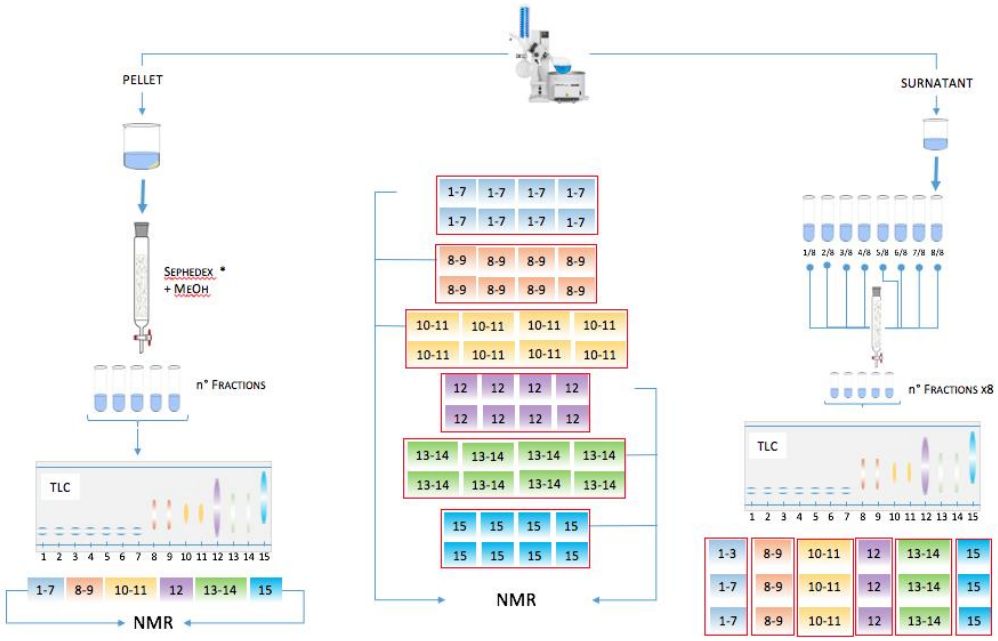


Figure 25. Sample fractionation workflow

Therefore, the next step involved the use of sequential chromatography which allowed to separate the extracts of the pellet and the supernatant obtained from the bacterial culture in the different fractions. After every chromatographic step, the analysis of the fraction content and bioactivity was performed. The activity results combined with the information provided ^{1}H NMR determine which fractions contain metabolites of interest.

5.5.3 CHROMATOGRAPHIC COLUMN (CC)

The chromatographic column system involves the use of a stationary phase and a mobile phase or eluent.

The stationary phase is packed inside a tubular glass column open at the top and closed in their lower part by a spout with a tap.

The separation of the mixture takes place by gravity.

To separate the sample under examination, a chromatographic column was used for molecular exclusion of 7 centimetres in diameter and 45 centimetres in length.

The stationary phase consisting of Sephadex® LH-20 (GE Healthcare), is a cross-linked dextran polymeric resin that makes the microspheres, with a diameter between 70-103 μm (average dry/wet value), porous and rich in channels; the hydroxypropylation process also confers the amphipathic characteristic (Figures 26, 27).

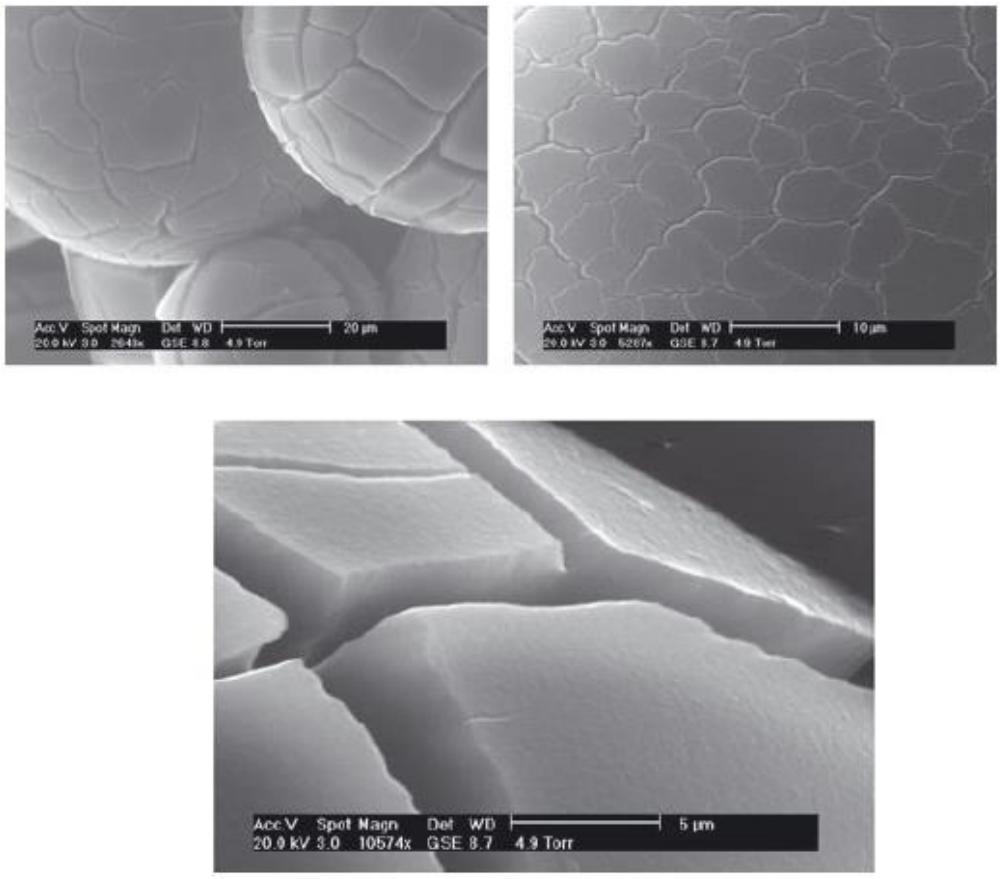


Figure 26. ESEM microscopy images of Sephadex LH-20 (Kale et al, 2006)

Figure 27. Chemical structure and dehydrated status of Sephadex LH-20

The eluent phase consists of a single solvent or solvent mixture, in which the sample should be completely solved (600 μL of MeOH or Chloroform). At this stage, the MeOH was shown to be the most suitable.

The column is packed with the stationary phase following several steps:

1. Suspension of Sephadex LH-20 in MeOH (4 ml/g) of the necessary quantity of resin in a beaker;
2. Transfer of the resin/solvent solution into the CC, with the aid of a glass rod to prevent the creation of air bubbles inside the CC which would lead to an incorrect packing of the resin;
3. Filling the space above the resin with MeOH;
4. Location at the entrance of the CC of a cap connected to a pump;
5. Increase in internal pressure, by a pump, to facilitate the flow of solvent and homogeneous packing of solid phase;
6. Opening the faucet, located at the bottom of the column due to the extraction of excess solvent;
7. Closure of the outflow tap and completed packing;
8. Adding the solvent to avoid dehydration of the resin (2-3 centimetres above the upper limit of the packed resin).

Once the CC was prepared, the dissolved sample in MeOH (5 ml) was inserted into it for separation.

As can be seen in the photo, with the flow of the solvent inside the CC, the sample is separated into its components based on the molecular size (Figure 28, 29).

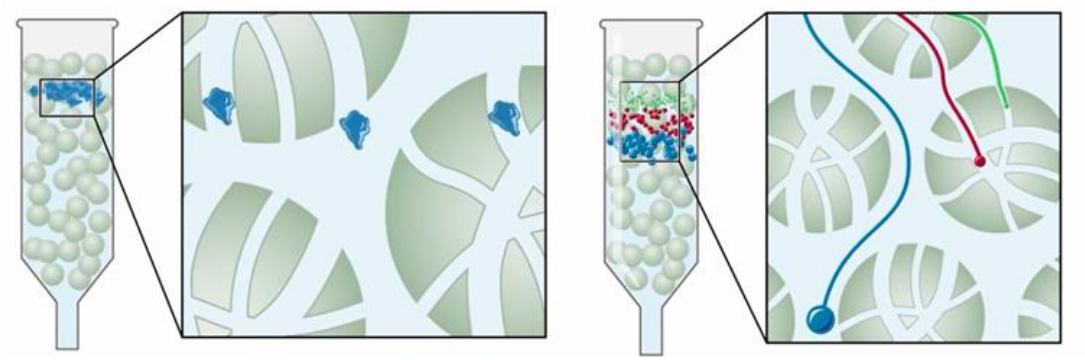


Figure 28. Sample downflow process throughout sephadex microspheres

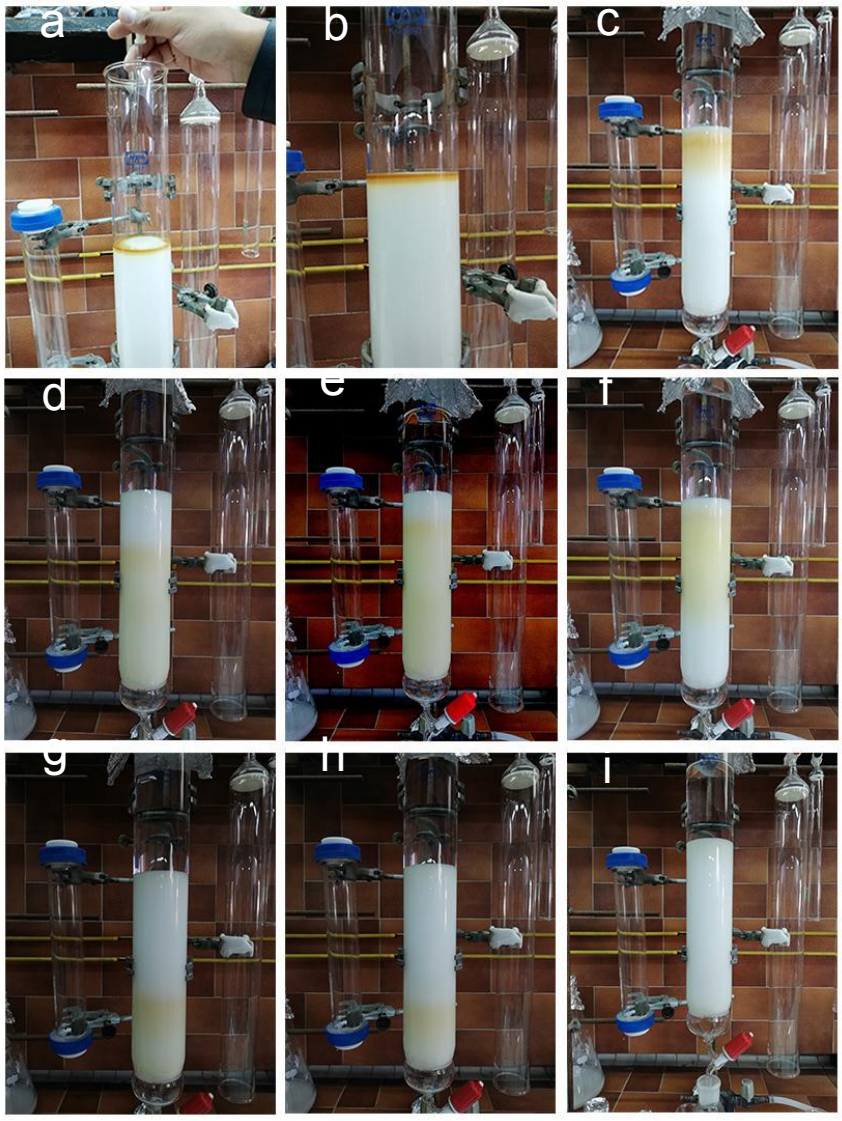


Figure 29. Sample fractionation process

The first fractions that were obtained should contain compounds with greater molecular weight, not retained inside the Sephadex than those contained in the latter fractions, trapped in the gel pores (Figure 30).

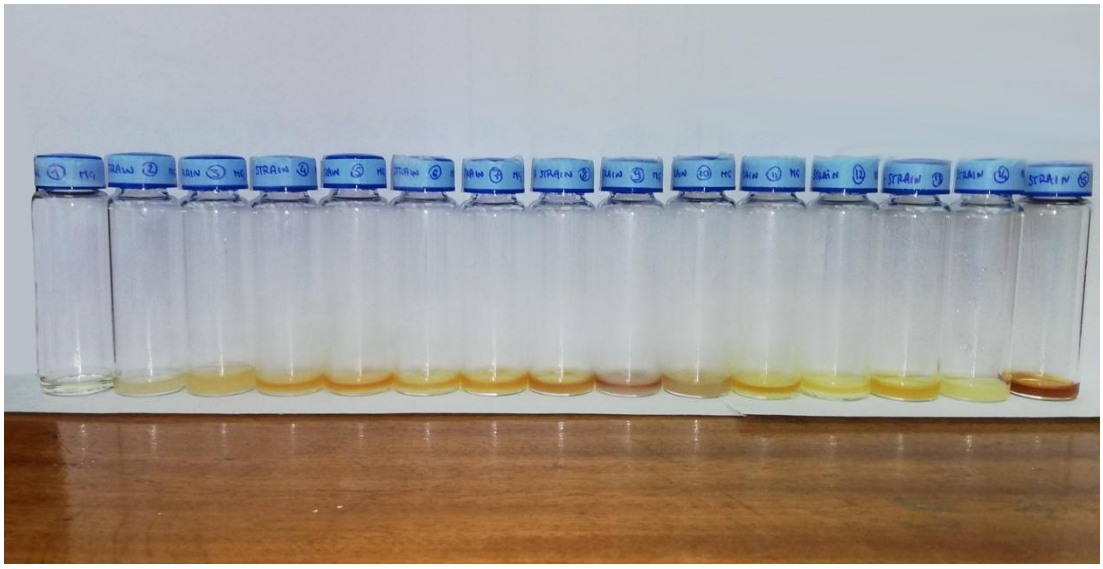


Figure 30. ID37 sample - Fractions pellet

Having obtained the different fractions of the sample, the next step was to compare them using the Thin Layer Chromatography (TLC) method before and after with the Nuclear Magnetic Resonance (NMR), to determine any chemically equal or similar fractions and combine them accordingly.

5.5.4 THIN LAYER CHROMATOGRAPHY (TLC)

TLC is a technique that allows the separation of a mixture into its compounds, widely used due to its ease of use and its speed of separation.

Like all chromatographic methods, it also consists of a stationary phase and an eluent phase. The stationary "plate" phase consists of an aluminium, plastic or glass sheet on which a layer of different absorbent material such as silica, cellulose, polyethyleneimine cellulose (PEI), aluminium oxide or magnesium silicate is present. The eluent phase consists of a solvent or solvent mixture.

The principle on which the TLC technique is based is that the mobile phase, flowing through the stationary phase, brings with it the components of the mixture separating them based on their ability to bind more with the solvent or with the stationary phase.

A more polar compound than the solvent will interact more with the stationary phase, stopping its stroke in the first few centimetres of it; otherwise, the less polar compounds will cover a larger portion of the solid phase with the solvent.

The tests performed involved the use of the normal phase TLC Silica Gel 60 F254 support from MerkKGeA, or reversed-phase TLC ALUGRAM® RP-18W / UV254 of Macherey-Nagel Gmbh& Co., a non-polar stationary phase used with a polar eluent phase, applying the following protocol in the Table 5 and schematized in Figure 31.

Table 5. TLC workflow.

STEP	DESCRIPTION
I	*Affix concentrated extract on the support (stationary phase), through a special capillary*
II	*Filling the ascending flow chamber with the solvent mixture (eluent phase)*
III	*Inserting the support in the chamber*
IV	*Collection of the support at the end of elution with excess solvent evaporation*
V	*Reading with a UV lamp at 2 wavelengths (254 nm and 365 nm)*

The result of the TLC can be visualized by UV detection (254 nm and 365 nm UV lamp) and developed after pulverization with a 10% solution of

phosphomolybdic acid in ethanol, followed by heating at 150ºC. The result obtained is shown in Figure 32.

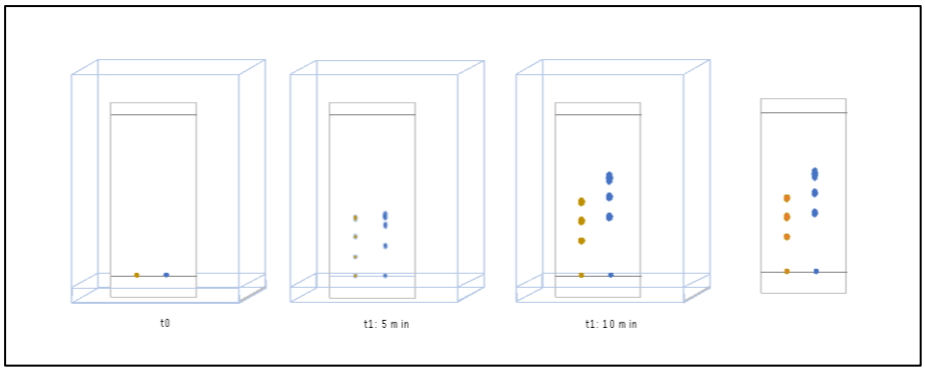


Figure 31. TLC steps

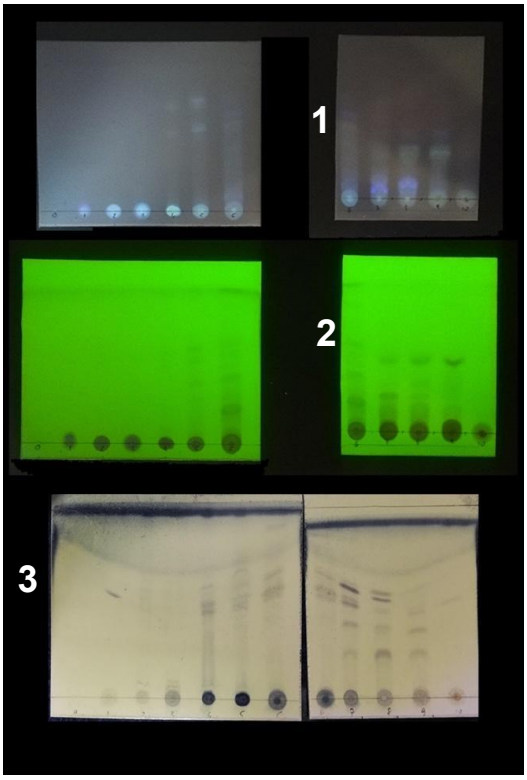


Figure 32. TLC detected at UV 254 nm (1), 365nm (2) and with 10% solution of phosphomolybdic acid: EtOH (3)

On some samples, further, checks were carried out using a phase-reversed TLC, through the Pre-coated TLC-Sheets support ALUGRAM® RP-18W / UV254 of Macherey-Nagel Gmbh&Co..

The latter differs because unlike "standard" TLC, a non-polar stationary phase and a polar eluent phase are used.

5.5.5 NUCLEAR MAGNETIC RESONANCE (NMR)

The NMR is an investigative technique discovered by Felix Bloch (Marion, 2013) and Edward Purcell (Marciano-Cabral et al., 2003) in 1946, which earned them the Nobel Prize for physics in 1952. To date, the use of nuclear magnetic resonance to determine the chemical composition of a product remains one of the tools most widely used.

The NMR spectrometer used in this research project was the Bruker®Avance 500 MHz and Bruker®Avance 600 MHz.

The scheme shown in Figure 33 allows identifying an internal central body, consisting of a superconducting magnet, a coil based on niobium-tin or niobium-titanium which allow obtaining an almost zero electrical resistance, when cooled to extremely low temperatures, in operation mode.

The superconductor is enclosed between two chambers respectively containing liquid helium (with the task of directly cooling the superconductor up to -269° C) and liquid nitrogen to avoid the thermal dispersion of the helium, with the surrounding environment. For further conservation of the internal temperature, there is a chamber (inside which the vacuum is created) interposed between the chambers containing the two gases and a final chamber, coated with Mylar®, polyethylene terephthalate with the function of thermal and electrical insulation.

Placed centrally on the top of the instrument, there is a cylinder that allows the insertion of glass tubes with a diameter of 5 millimetres, inside which the sample to be examined is inserted.

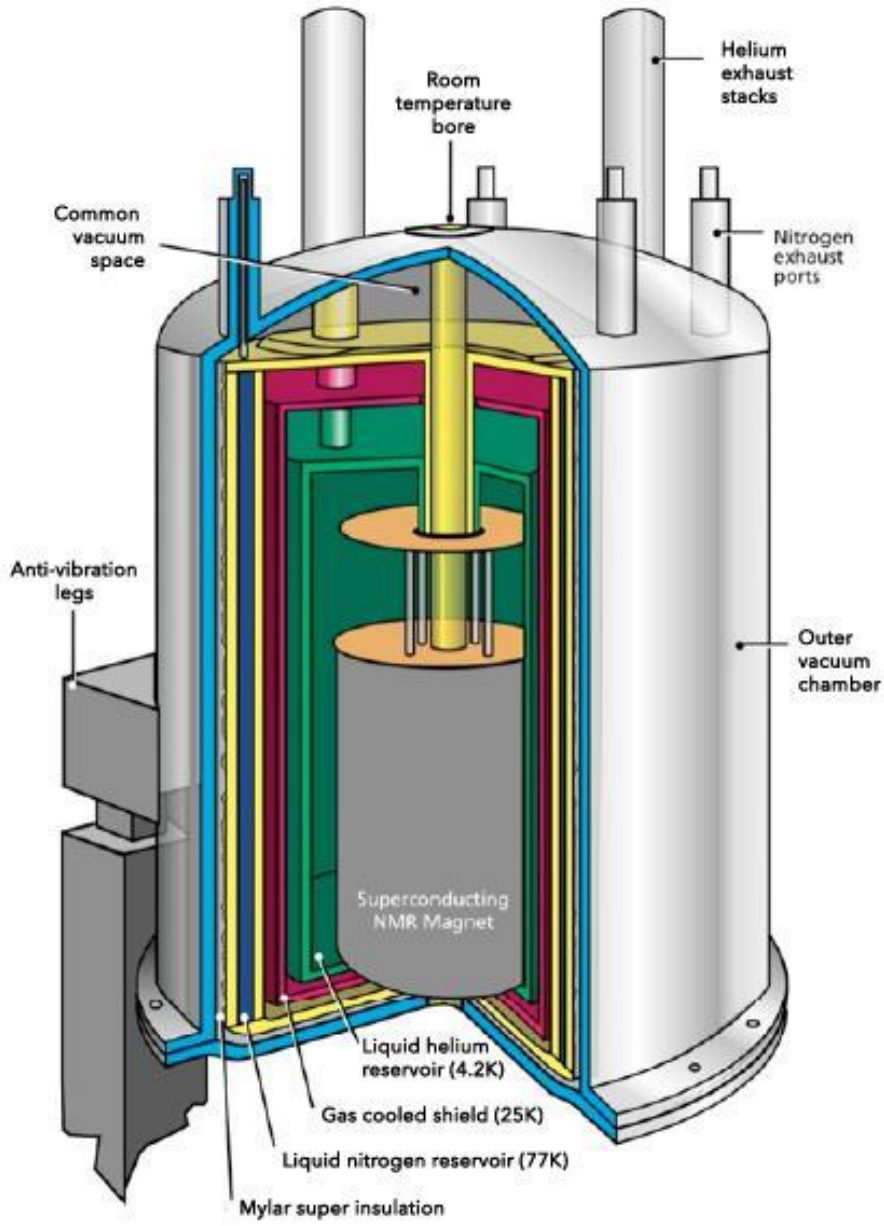


Figure 33. Sections constituting the NMR spectrometer

This investigation technique is based on the theory that some atomic nuclei and isotopes (^{1}H, ^{13}C, ^{15}N) in the presence of a static, uniform, magnetic field oriented towards the Z-axis (generated by the superconductor), absorb and re-emit an electromagnetic signal radiofrequency (RF) (Siddiqui et al., 2012).

The sample to be examined placed in the tube is introduced inside the magnetic field, where the nuclear spins of the molecule are oriented towards it, producing an excess of nuclei with aligned spins.

Subsequently, an RF pulse is applied to produce an excess of nuclei with spins opposite to the magnetic field (microseconds).

At the end of the pulse, the FID (Free Induction Decay) signal of the nuclei is recorded, while these fall within the original spin situation (about a second).

The FID (data related to time) is processed through the Fourier Transform, which returns an NMR spectrum (data related to the frequency), like the one shown in Figure 34.

Specifically, the aforementioned spectrum is a ^{1}H-NMR spectrum.

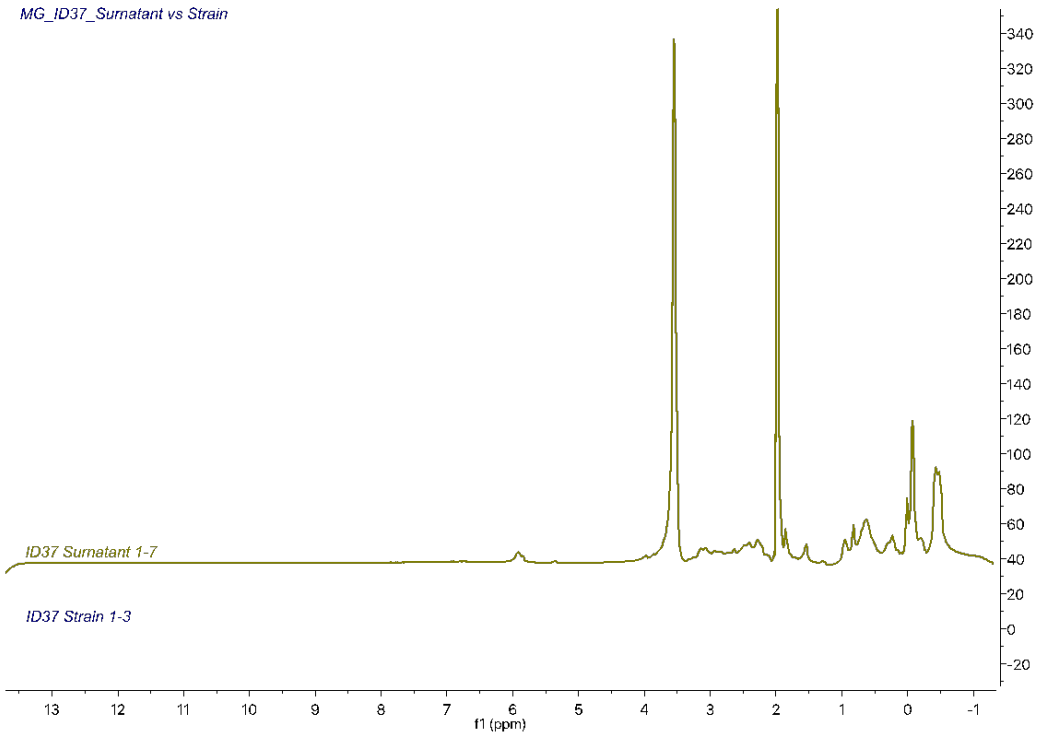


Figure 34. 1H-NMR spectrum of the sample ID37

However, the most important datum applied to the NMR technique is the chemical displacement dependent on the electronegativity of the hydrogen atoms bound to the different atoms.

Electronegativity, the cloud of charged particles around the bound hydrogen atom, when struck by a magnetic field (produced by the superconductor) generates a small opposing magnetic field, creating the chemical displacement of the H atoms making up the molecule.

Following the chemical shift, we will have that hydrocarbon hydrogens will be displayed in a value range between 0.9-1.7 ppm, carbonyl hydrogens in a range between 2-3 ppm, those related to halogens in an interval between 3-4 ppm and so on as shown in Figure 35.

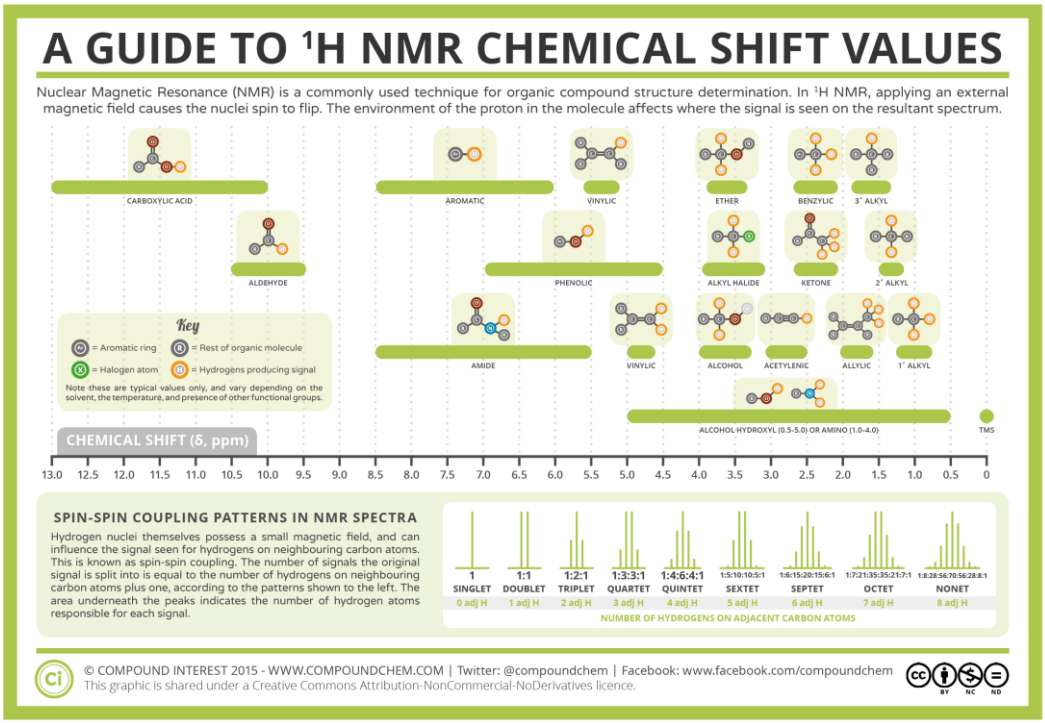


Figure 35. Chemical Shift 1H

A similar situation occurs for ^{13}C carbon (Figure 36).

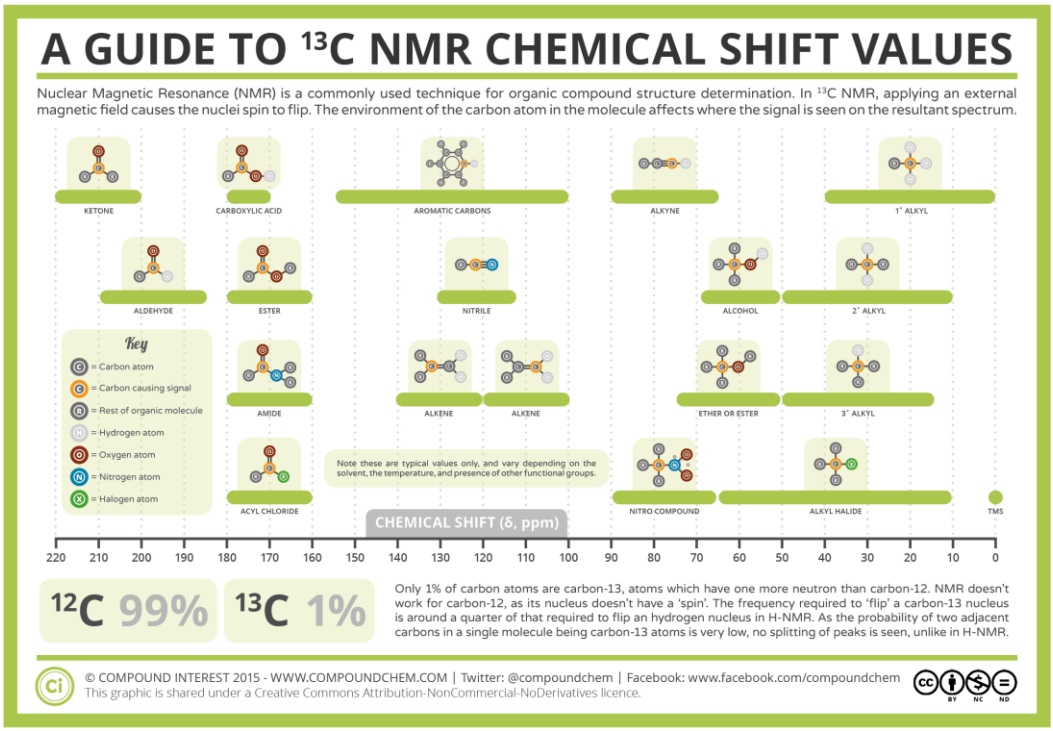


Figure 36. Chemical shift ^{13}C

Sometimes, however, the resonance spectra of protons (^{1}H) and carbons (^{13}C) may be insufficient because due to very similar resonance frequencies,

the signals obtained overlap, sometimes making it difficult to understand the molecules present in the sample.

For this reason, a series of two-dimensional (2D-NMR) homonuclear and heteronuclear correlation experiments are coupled to the one-dimensional experiment (1D-NMR) through chemical bonds. The most common 2D NMR experiments are:

- COrrelatedSpectroscopY (COSY);
- Heteronuclear Single-Quantum Correlation (HSQC);
- Heteronuclear Multiple Bond Correlation (HMBC);

and throughout the space:

- Rotating-frame OverhauserSpectroscopY (ROESY).

Unlike the 1D-NMR experiment, in the bidimensional apply to the sample, introduced in the magnetic field of the superconductor, a group of RF pulses with a delayed start, thus acquiring different values of FID.

This is, as for the 1D-NMR experiment, processed through the Fourier Transform, but in two dimensions, one representing the evolution time (time in which the impulse is applied) and the other the acquisition time (of the different FIDs).

The result obtained is a graphic representation in which we have:

- COSY, which relates the ^{1}H bonds present in the examined sample (Figure 37);
- HSCQ, which relates the chemical shift of the proton ^{1}H (dimension f2) with the chemical shift of the carbon ^{13}C (dimension f1) directly bound (Figure 38);
- HMBC, which correlates the chemical displacement of the proton ^{1}H (dimension f2) with the chemical displacement of the adjacent carbon ^{13}C (dimension f1), excluding the direct bonds (Figure 39);
- ROESY, which correlates the ^{1}H bonds of the sample in space, to return the spatial configuration of the molecule.

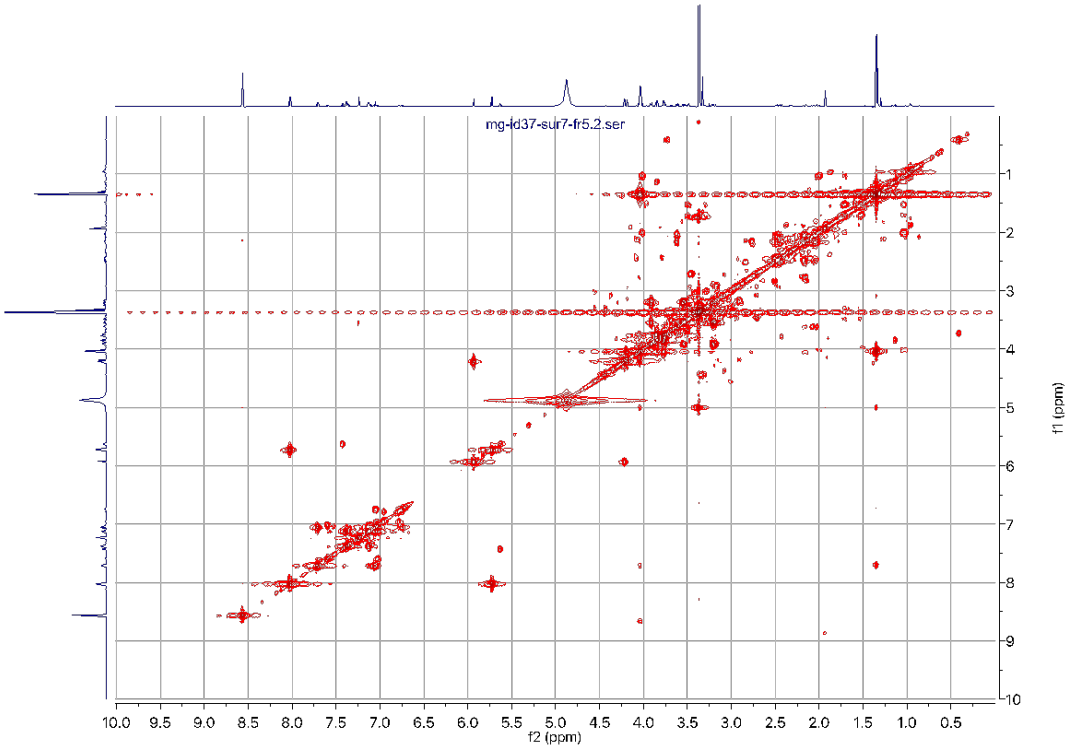


Figure 37. Correlated Spectroscopy (COSY)

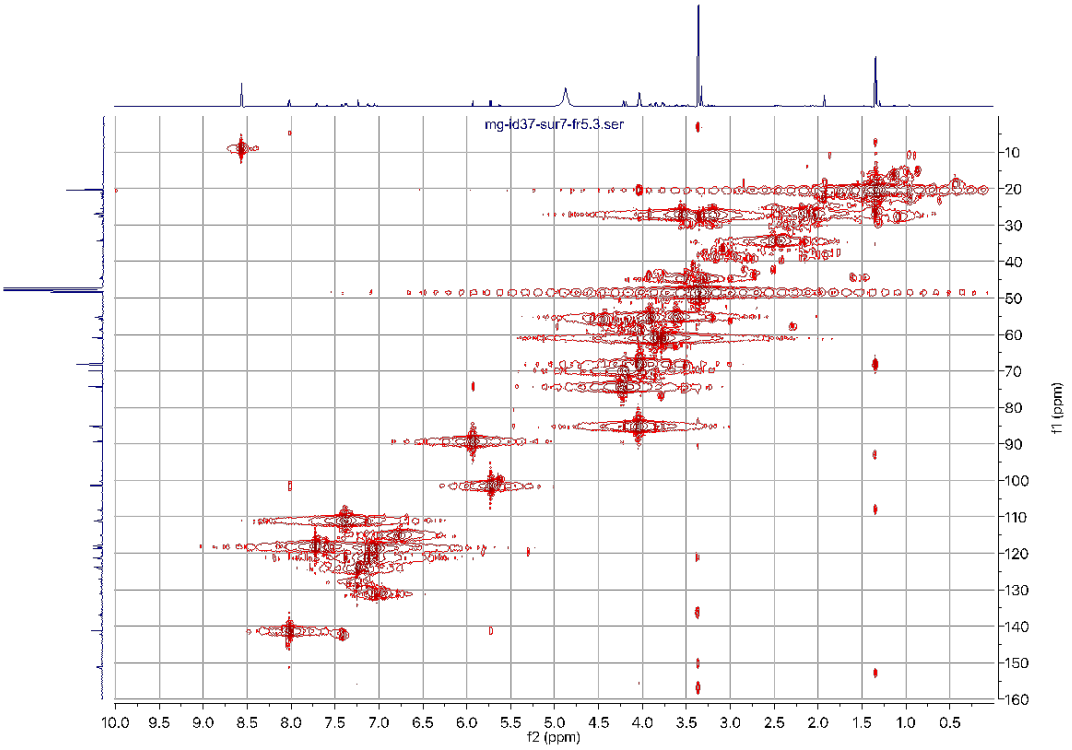


Figure 38. Heteronuclear Single-Quantum Correlation (HSQC)

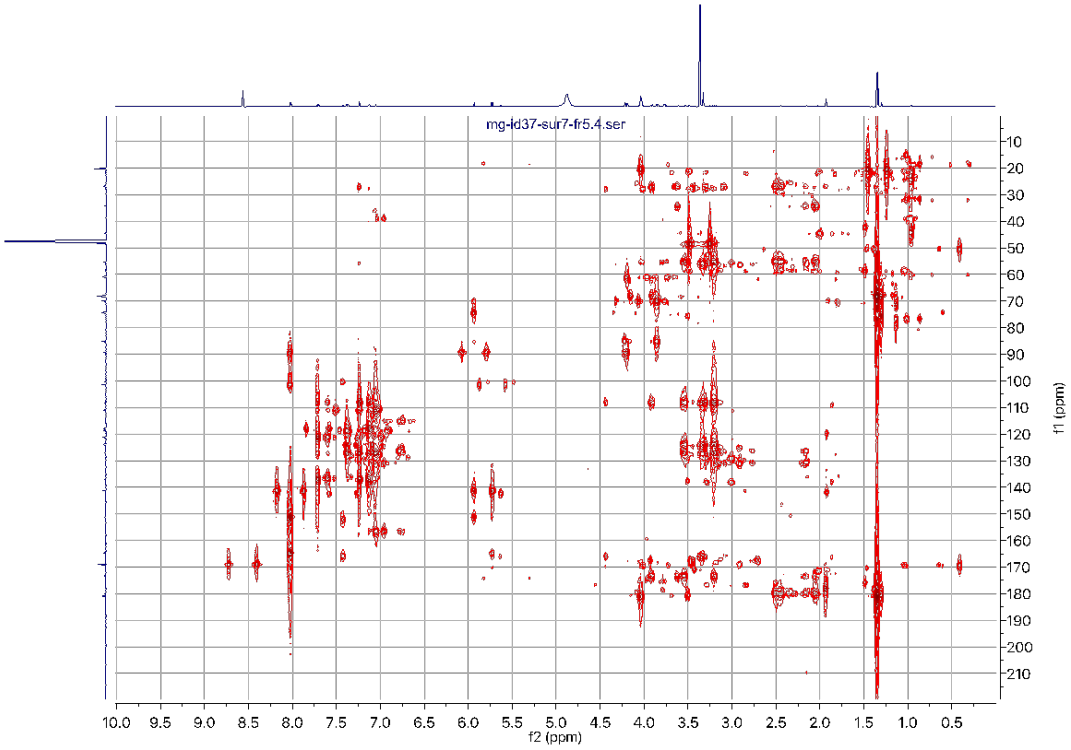


Figure 39. Heteronuclear Multiple Bond Correlation (HMBC)

5.5.6 SRB ASSAY

Sulforhodamine B is an aminoxanthene, a heterocyclic organic compound with fluorescent properties (bright pink) (Figure 40).

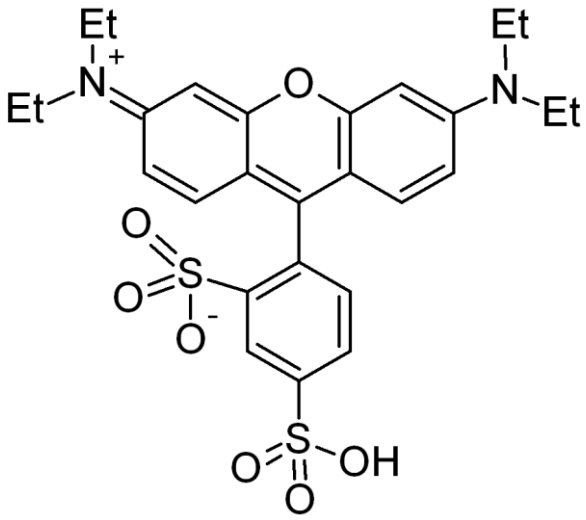


Figure 40. Sulforodamina B (SRB) molecule

It is able to bind the protein components of cells previously fixed to the culture plates with trichloroacetic acid (TCA); in fact, it has two sulfonic groups (-SO2-) which in an acid environment bind to the amino acid residues of proteins, thus allowing the density of the cell population to be assessed.

The protocol used included a first phase during which the cell lines on which to test the compound were transferred, in the liquid medium Sigma RPMI 1640 MEDIUM (resuspension) and performed cell counting using the VWR Moxi Z® instrument, It is able to bind the protein components of the cells previously fixed to the culture plates with trichloroacetic acid (TCA); in fact it has two sulfonic groups (-SO2-) that an acidic environment binds to the amino acid residues of proteins, thus allowing to assess the density of the population.

Following protocol, the liquid medium Sigma RPMI 1640 MEDIUM it was used to resuspending the cells; the VWR Moxi Z instrument was used to count the cells and determine their concentration to calculate the necessary quantity to insert in the well to have an equal cellular density in the same wells for all cell lines, such as:

- A549: *Human Lung Epithelial Carcinoma*
- HBL100: *Mammary Gland Epithelial Cancer*
- HeLa: *Uterine Cervix Epithelial Adenocarcinoma*
- SW1573: *Lung Alveolar Cell Carcinoma*
- T47D: *Mammary Gland Ductal Carcinoma*
- WiDr: *Human Colorectal Adenocarcinoma*

The cells were inoculated on 96-well microtiter plates in a volume of 200 µL per well at a density of 2500 cells/well (A549, HBL-100, HeLa and SW1573) and 5000 cells/well (T47D and WiDr), based on their growth times.

The cells were inoculated in the wells according to the scheme (Figure 41) constituting the "T0".

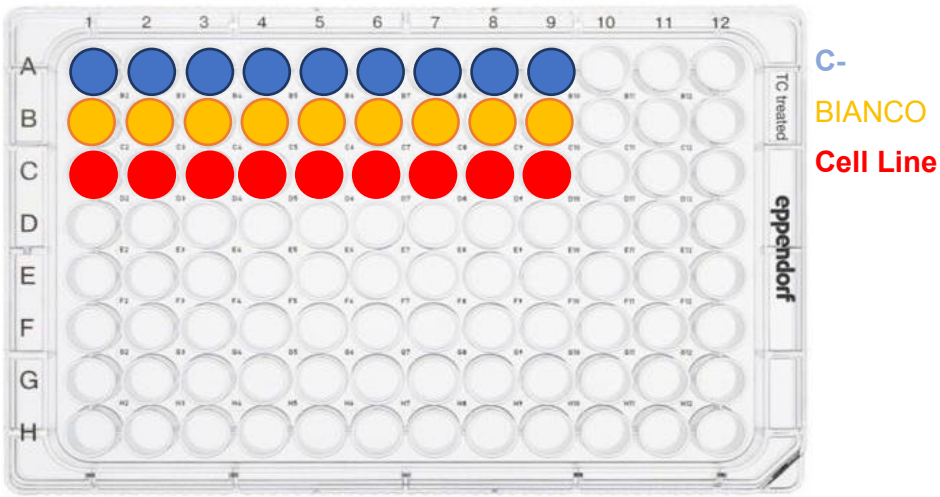


Figure 41. Sulforhodamine B 96 well plate scheme

The same operation was performed on a copy plate on which at the end of the 24-hour incubation period, the compound to be tested was inoculated at different concentrations (Figure 42).

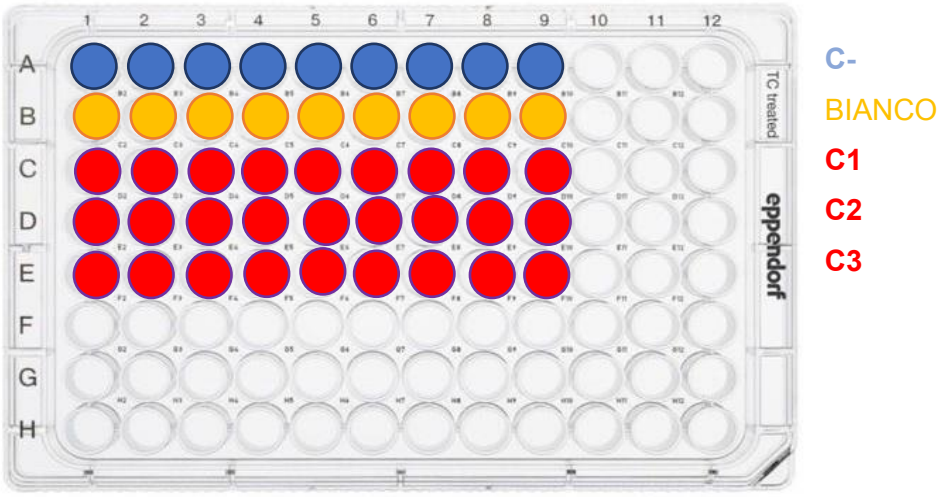


Figure 42. Sulforhodamine B 96 well plate scheme with different concentrations

The used sample concentrations were:

- 250 μg/mL
- 25 μg/mL
- 2.5 μg/mL

The plate was incubated for 48 hours at 37° C.

After the incubation time, the cellular metabolism was stopped inoculating 25μL of trichloroacetic acid (TCA, 50%) in each individual well and placing the plate in incubation for 60 minutes at 4° C.

The plate was rinsed with running water after the incubation time.

Then, SRB solution (0.4% w/v in 1% v/v acetic acid) was added to the plate, acting for 15 minutes at the end of which rinsing with 1% acetic acid was carried out to eliminate excess.

150 μL of Tris base (10 mM) was added in each individual well and subjected to a spectrophotometer to read the absorbance at a wavelength of 530 nm (λ absorbance of the protein) and at 620 nm (λ plate absorbance).

The value obtained by the difference between the absorbance at 620nm and at 530nm was subtracted with the absorbance value of the plate inoculated with untreated cell line, in order to evaluate the possible growth inhibition.

5.6 ANTIPARASITIC ACTIVITY

Simultaneously with the antiproliferative activity tests performed on the single fractions of the supernatants and pellets of the ID37 and CAL114 strains, a survey was carried out on the possible activity developed by the same strains against pests.

This work was carried out in collaboration with Instituto Universitario de Enfermedades Tropicales de Canarias of the Universidad de La Laguna.

The methods used in this work both for the study of trypanocidal and leishmanicidal activity and for the determination of cytotoxicity are based on the use of AlamarBlue® (El-Subbagh et al., 2009). It is an oxidation-reduction indicator that contains blue and non-fluorescent resazurin. This compound is reduced from viable cells to resorufin, pink and highly fluorescent, thanks to enzymes such as the reductases present in the mitochondria. The colour change is determined by a plate reader with which fluorescence and absorbance are measured at 570 and 630 nm. In this way, a quantitative measurement of cell viability is obtained.

To determine the activity of ID37 and CAL 144 fractions extract against these parasites, 96-well plates were used in which the final volume was 200µL (100µL of each extract at different concentrations + 100µL of parasites). In each plate, three wells were used as a control (pests without extract). The tests were performed in sterile conditions in a laminar flow chamber.

100µL of LIT medium and RPMI without phenol red (respectively for *T. cruzi* and *L. amazonensis*) are dispensed in all wells, including the three that were used as controls. In the first row of wells, 100µL more medium and extract were added, positioning first the necessary amount of medium and then the specific quantity of Maytenus extract to obtain the desired concentration. Subsequently, serial dilutions are performed by taking 100µL of the first row so that all wells remain with 100µL. Then, 100µL of parasites are added at a concentration of $2x10^5$ parasites/ml for T. cruzi or 10^6 parasites/ml for *L. amazonensis*. This completes the final volume of 200µL. Finally, 20mL of AlamarBlue® are added to each well and incubated for 72 hours in the oven at 26° C. After this time, the fluorescence is determined with the plate reader

Concerning The amoebicidal activity the destocking solutions were prepared by dissolving the extract in DMSO (Sigma Aldrich) and absolute ethanol at a concentration of 5 mg/ml.

The Acanthamoeba strain were incubated in PYG medium supplemented with gentamicin 10 µg/mL (BiochromAG, Cultek, Granollers, Barcelona, Spain) at 37° C with 5% CO_2.

The next step was to evaluate the activity with the same method AlamarBlue® described previously.

The parasites examined are four: *Acanthamoeba castellanii; Leishmania amazonensis; Leishmania donovani* and *Trypanosoma cruzi:*

- A genus ***Acanthamoeba*** is a group of nearly 25 named species that has a worldwide distribution. The genotype T4 nowadays is associated to *A. castellanii* species complex until future higher-resolution analyses are sufficient to more accurately differentiate between the morphologically described species within this genotype.

  Is an opportunistic pathogen which is the causal agent of sight-threatening ulceration of the cornea known as *Acanthamoeba keratitis* (AK) and, more rarely, an infection of the central nervous system called "*Granulomatous Amoebic Encephalitis*" (GAE). In the case of GAE, the infection reaches the CNS via the bloodstream from the upper respiratory tract, the conjunctiva, or a site of skin injury. The brain will present edema and multiple necrotic and hemorrhagic nodules in the cerebral hemispheres, brainstem, and cerebellum. The symptoms of AK are non-specific, and so it can be misdiagnosed as viral, bacterial, or fungal keratitis (Lorenzo-Morales et al., 2013; Martinez et al., 2014; Okwor et al., 2009).
- *Leishmania* spp. are obligate intracellular parasites in the family Trypanosomatidae. Leishmaniases are neglected tropical diseases transmitted by infected sandflies. Sand-flies belong to either *Lutzomyia* or *Phlebotomus* genera. There are roughly 30 documented species of *Leishmania*, with 20 of these able to cause disease in humans. Currently, approximately 12 million people are infected worldwide, which makes Leishmaniasis a global issue, affecting individuals in 98 countries. The disease is responsible for roughly 70,000 deaths per

year (Okwor et al., 2013), most of these attributed to the visceralizing form of the disease. *Leishmania amazonensis* is an intracellular protozoan parasite responsible for chronic cutaneous leishmaniasis (CL). CL is a neglected tropical disease responsible for infecting millions of people worldwide (Marin-Neto et al., 2007).

- ***Leishmania donovani*** is the causative agent of visceral leishmaniasis, traditionally known as kala-azar ("black fever", particularly in India). Clinical symptoms include pyrexia (recurring high fever which may be continuous or remittent), enlargement of spleen and liver, and heavy skin pigmentation which darkens the physical appearance (the reason for naming "black fever"). Where medical facilities are poor, mortality can be as high as 75–95% within 2 years of epidemics. The disease is often accompanied by complications with dysentery, tuberculosis, septicaemia and even HIV infection (Mcbride et al., 2005).
- ***Trypanosoma cruzi*** is a species of parasitic euglenoids that feed on blood and lymph causing disease or the probability of disease that varies with the organism. In humans it generates the Chagas disease that causes damage to heart tissue (Aizawa et al., 1987).

5.7 PHYLOGENETIC CHARACTERIZATION OF POSITIVE ISOLATES

The phylogenetic characterization of the ID37 isolate was performed by the Servicio de Genomica of the Universidad de La Laguna in Tenerife. The protocol was carried out through different phases detailed below.

5.7.1 PURIFICATION OF GENOMIC DNA

1. The cell pellets were resuspended in 200 µL of Lysis Buffer (40 mM Tris-HCl pH 7.8, 20 mM sodium acetate, 1 mM EDTA, 1% SDS, 0.04 µg/µL RNase-A (Promega), 0.04 µg/µL Lysozyme (Thermo Fisher Scientific), and incubated for 5 minutes at RT.
2. 66 µL of 5M NaCl was added and centrifuged (8000 x *g,* 10 min).
3. 200 µL of the supernatant was transferred to a new tube and 66 µL of Chloroform was added (Merk-Millipore). Both phases were mixed until a homogeneous solution was obtained.
4. It was centrifuged (8000 x *g,* 10 min), and 180 µL of the aqueous (upper) phase was transferred to a new tube.
5. 360 µL of absolute Ethanol (previously cooled to -20 ° C) was added and incubated 5 minutes in ice.
6. It was centrifuged (8000 x *g,* 10 min), the supernatant was removed and the pellet was washed twice with 500 µL of 70% Ethanol.
7. The open tubes were incubated in the laminar flow cabinet for 15 minutes to evaporate completely the remains of ethanol.
8. The precipitated DNA was resuspended in 50 µL of H_2O, keeping the O / N samples at 4° C.

The resulting genomic DNA samples were subjected to agarose gel electrophoresis for analysis.

For this, 10 µL of the DNA sample was mixed with 2 µL of 6X Loading Dye (Geneall) and the mixture was loaded into a well of an agarose gel (Agarose I™, VWR) 1%, prepared in TAE 1X buffer.

For DNA visualization, it was added 1X GelRed TM (Biotium) agarose gel.

Electrophoresis was developed for 20 minutes at 80V and 120 mA using 1X TAE as camera buffer.

The gel was then visualized using a UVP transilluminator TFM-20 and photographed (Figure 43).

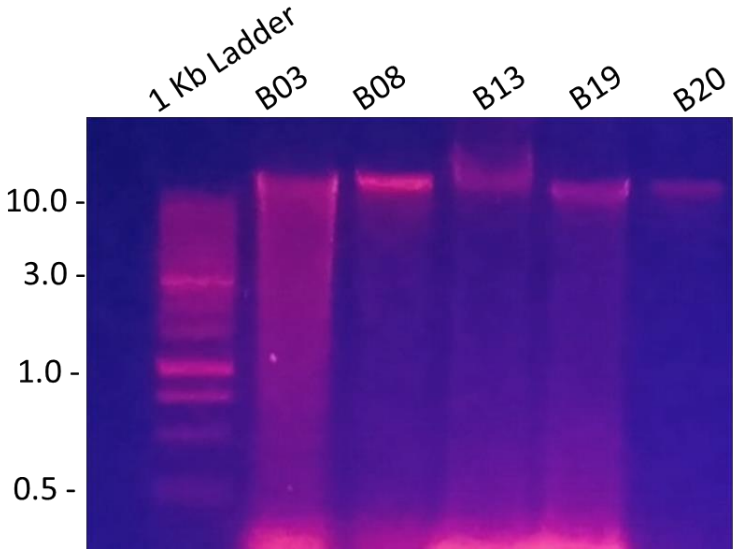


Figure 43. ***Agarose gel electrophoresis of purified genomic DNA.*** *To the left of the image the size (Kb; Kilobases) of the fragments corresponding to the molecular weight marker is indicated DNA Ladder 1Kb (New England Biolabs). The intensity of the fluorescence in the gel determines the amount of genomic DNA obtained for each of the indicated samples.*

The genomic DNA obtained was quantified using image analysis software Free GelAnalizer (http://www.gelanalyzer.com) depending on the amount of fluorescence detected in the gel and by comparing the fluorescence emitted by the bands of the marker, which contain a known amount of DNA.

5.7.2 AMPLIFICATION OF REGIONS RDNA 16S OR ITS1-2

In the case of bacteria, identification is carried out by amplification and sequencing of the region of the genome that codes for 16S ribosomal RNA. In this work, primers 27F (5'-TAGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were selected.

In the case of fungi and yeasts, identification at the species level is carried out by amplification and sequencing of certain regions of the genome called Internal Transcribed Spacer (ITS) 1 and 2. In this work, ITS1 primers were selected (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3').

For amplification, the AmpONE Taq DNA polymerase (GeneAll) PCR kit was used. First, dilutions of the previously purified genomic DNA were prepared, at a final concentration of 100-200 ng/µL, and 1 µl of said solutions was used as a template for the PCR reactions. The PCR reactions were prepared in a final volume of 30 µL, in 0.2 mL Eppendorf tubes, following the manufacturer's instructions. The following in Table 6 was included in each reaction.

Table 6. PCR mix

Reaction component of PCR	**Volume (µL)**
Taq DNA polymerase (2.5U/µl)	0.3
10X Taq Buffer	3.0
dNTPs mix (2.5 mM each)	2.4
First FW (10 µM)	1.2
First RV (10 µM)	1.2
DNA template (100-200 ng/µL)	one
H_2O	20.9

Once the PCR reactions were prepared, they were placed in an iCycler–IQ thermocycler (BioRad) and subjected to the following thermal cycle program descripted in Table 7.

Table 7. Thermal cycle program

Temperature (ºC)	***Time***	***Cycles***
95	2'	1
95	20''	35
50	30''	
72	1'	
72	5'	1
16	5'	1

After developing the program, 2 µL of each amplification product was mixed with 2 µL of 2X Loading Dye (Geneall) and subjected to 1% agarose gel electrophoresis (Figure 44). The electrophoresis conditions were identical to those described in the previous section.

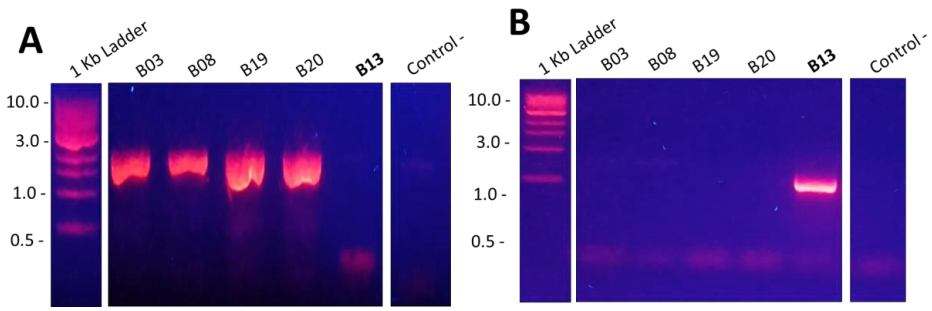


Figure 44. **Result of the amplification of the 16S and ITS1-2 region by PCR**. To the left of each image is the size of the fragments (Kilobases; Kb) corresponding to the DNA Ladder 1Kb molecular weight marker (New England Biolabs). A control amplification without template DNA has been included to determine the absence of contamination. A) Amplification of the 16S rDNA region. B) Amplification of the ITS1-2 region.

5.7.3 PURIFICATION OF PCR PRODUCTS AND SEQUENCING

The purification of the PCR products is essential, since it allows to eliminate the excess of primers and nucleotides that have not been used during the amplification.

For this, the EXO-SAP-IT kit (Affimetrix-USB) was used, following the manufacturer's instructions. Once purified, each PCR product was used to prepare the sequencing reaction, using the BigDye ™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), following the manufacturer's instructions. To obtain a reading confirmation, sequencing from both ends of each amplicon was performed, using the same primers described above for PCR amplification.

Next, the sequencing reactions were precipitated following the procedure described by the manufacturer of the sequencing kit (precipitation with EDTA + Ethanol) and resuspended in 10 µL of HiDi Formamide (Thermo Fisher Scientific) prior to injection into the 3500 Series Genetic sequencer Analyzer (Thermo Fisher Scientific).

Capillary electrophoresis was performed using a standard 50 cm capillary (Thermo Fisher Scientific) and POP7 polymer (Thermo Fisher Scientific).

The reference protocol was used for these conditions (FastSeq50_POP7).

The phylogenetic characterization of the CAL 114 isolate was performed by Savoca et al (2019) where the investigation methods are reported.

6. RESULTS

6.1 ANTIBACTERIAL ACTIVITY

The outcome of the cross-streak test was, in greater percentage, negative. The tests showed the inhibition of the growth of some test strains in the confluence area of some indicator strains; unlike for other test strains, the indicator strains did not exert any inhibition, which could lead to a sort of resistance against the pathogenic strain.

6.2 ANTIVIRAL ACTIVITY

The results obtained showed for most of the samples no difference between the negative control and the viruses treated with the supernatant of the strains under examination.

On average, 92.5% of the tested strains showed no antiviral activity (-); about 6.2%, on average, showed a slight difference between the negative control and the treated virus, translatable as a possible inhibition of viral replication (+/-); only 1.3% of the strains tested against the 3 viruses were able to inhibit their replication (+), (Table 8).

Table 8. Response to antiviral activity through plaque assay

STRAIN	*Herpes simplex*	*Poliomavirus 3*	*Zika virus*	*STRAIN*	*Herpes simplex*	*Poliomavirus 3*	*Zika virus*
ID1	-	-	-	**ID25**	-	-	-
ID2	-	-	-	**ID26**	-	-	-
ID3	-	-	-	**ID27**	-	-	-
ID4	-	+/-	-	**ID28**	-	-	-
ID5	-	-	-	**ID29**	-	-	-
ID6	-	-	-	**ID30**	-	-	-
ID7	-	-	-	**ID31**	-	-	-
ID8	-	-	-	**ID32**	-	-	-
ID9	-	-	-	**ID33**	-	-	-
ID10	-	-	-	**ID34**	-	-	-
ID11	+/-	+/-	-	**ID35**	-	-	-
ID12	-	-	-	**ID36**	-	-	-
ID13	-	-	-	**ID37**	-	-	-
ID14	-	-	-	**ID38**	-	-	-
ID15	-	-	-	**ID39**	-	-	-
ID16	-	+/-	-	**ID40**	-	-	-
ID17	-	-	-	**ID41**	-	-	-
ID18	-	-	-	**ID42**	-	-	-
ID19	-	-	-	**ID43**	-	-	-
ID20	+/-	+/-	-	**ID44**	-	-	-
ID21	-	+	-	**ID45**	-	-	-
ID22	-	-	-	**ID46**	-	-	-
ID23	-	+	-	**ID47**	-	-	-
ID24	-	-	-	**ID48**	-	-	-

6.3 ANTIPROLIFERATIVE ACTIVITY

Among the totality of the tested strains, in order to identify the molecules responsible for the anti-proliferative activity, it was decided to proceed with the subsequent investigation methods (CC; TLC; NMR) on only 2 samples of the total tested; ID-37 belonging to the *Firmicutes* genus (associated with the mollusc *Margaritifera margaritifera*) and CAL 114 belonging to the genus *Pseudoalteromonas* (associated with the *Haliclona dancoi* sponge).

Preliminary tests carried out through the MTT test showed for the CAL 114 strain a mortality of the A549 cell line equal to about 51% with 5 µL of supernatant of the inoculated strain and about 64% with a quantity of supernatant of 10 µL (Figure 45).

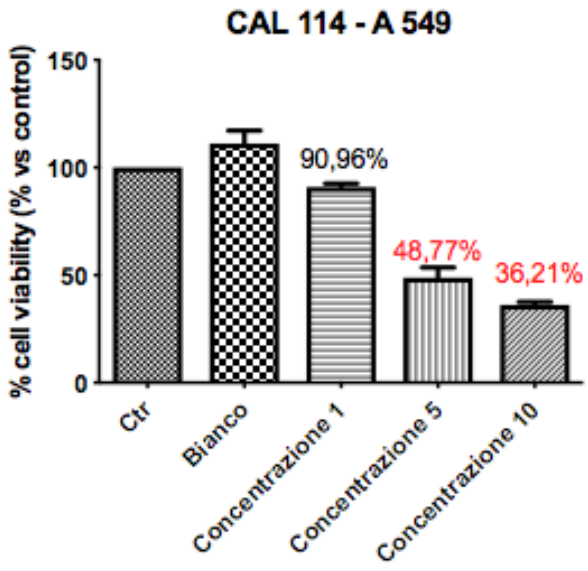


Figure 45. Mortality of CAL 114-induced A 549 cell line

For the ID-37 strain, mortality was appreciated on all three cell lines tested in screening phases.

In the A172 cell line the mortality obtained was just over 50%, with an inoculation of 5 µL of the supernatant and about 60% with an inoculum of 10 µL (Figure 46).

In A549 the mortality was just under 49% inoculating 5 μL of the supernatant and about 63% increasing the quantity to 10 μL(Figure 47).

For C6 a significant mortality was recorded inoculating 10 μL, obtaining a value of approximately 72% (Figure 48).

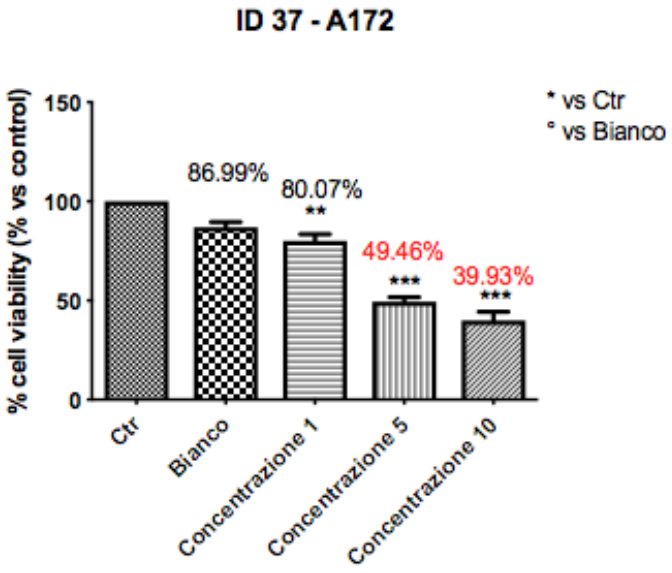


Figure 46. Post-inoculation viability of A172 cell line

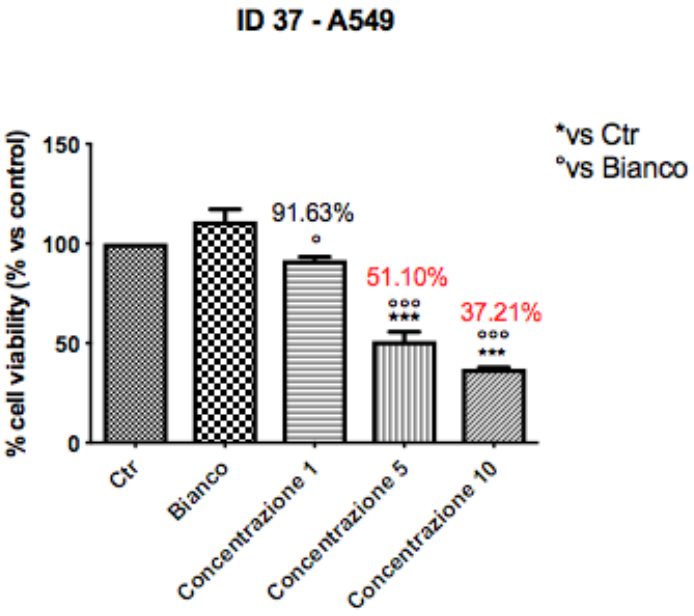


Figure 47.Post-inoculation viability of A549 cell line

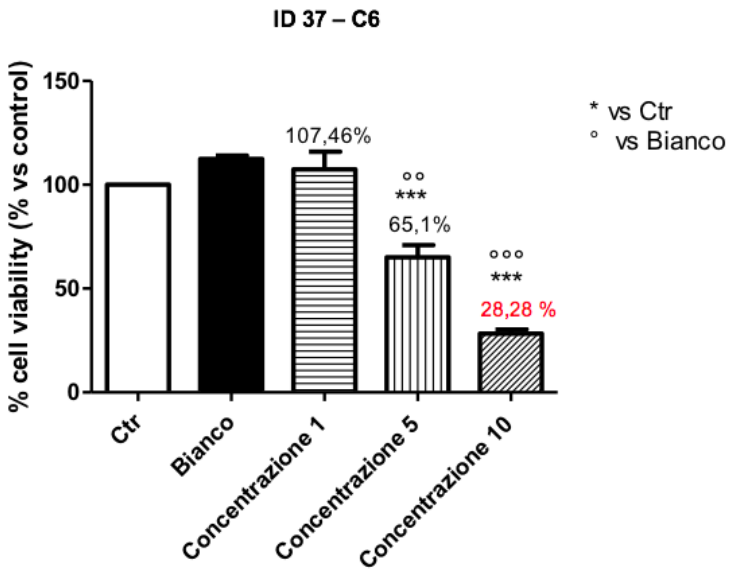


Figure 48. Post-inoculation viability of C6 cell line

Through the use of TLC and NMR it was therefore possible to highlight the differences between the pellet and supernatant fractions of the sample, to identify those that are chemically similar, thus obtaining a smaller and more heterogeneous number of samples on which to concentrate (Table 9, 10) (Figures 49, 50, 51).

Table 9. ID37 pellet fractions

SAMPLE	FRACTION	WEIGHT (g)
ID-37 PELLET	1-7	0.1628
	8-9	0.0958
	10-11	0.2974
	12	0.1258
	13-14	0.1097
	15	0.0359

Table 10. ID37 supernatant fractions

SAMPLE	FRACTION	WEIGHT (g)
ID-37 SUPERNATANT	1-3	4.7999
	4-5	4.2972
	6	0.9813
	7	0.5906
	8	1.0303
	9	0.6448
	10	0.3894

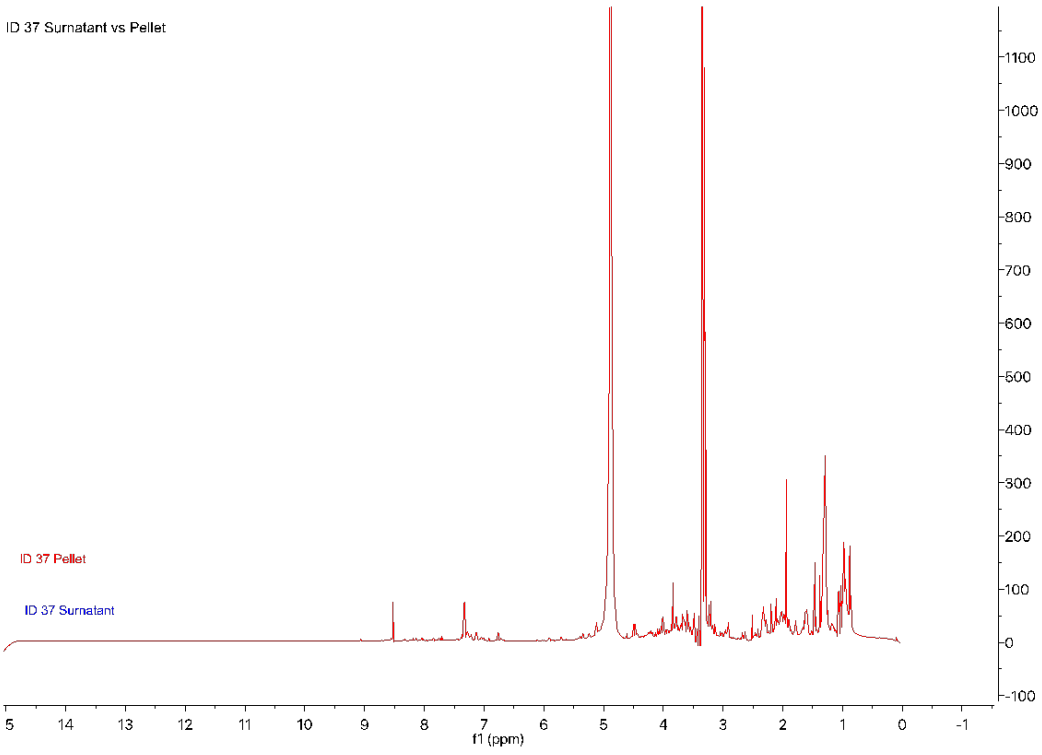


Figure 49. NMR spectrum comparison between ID37 supernatant-pellet

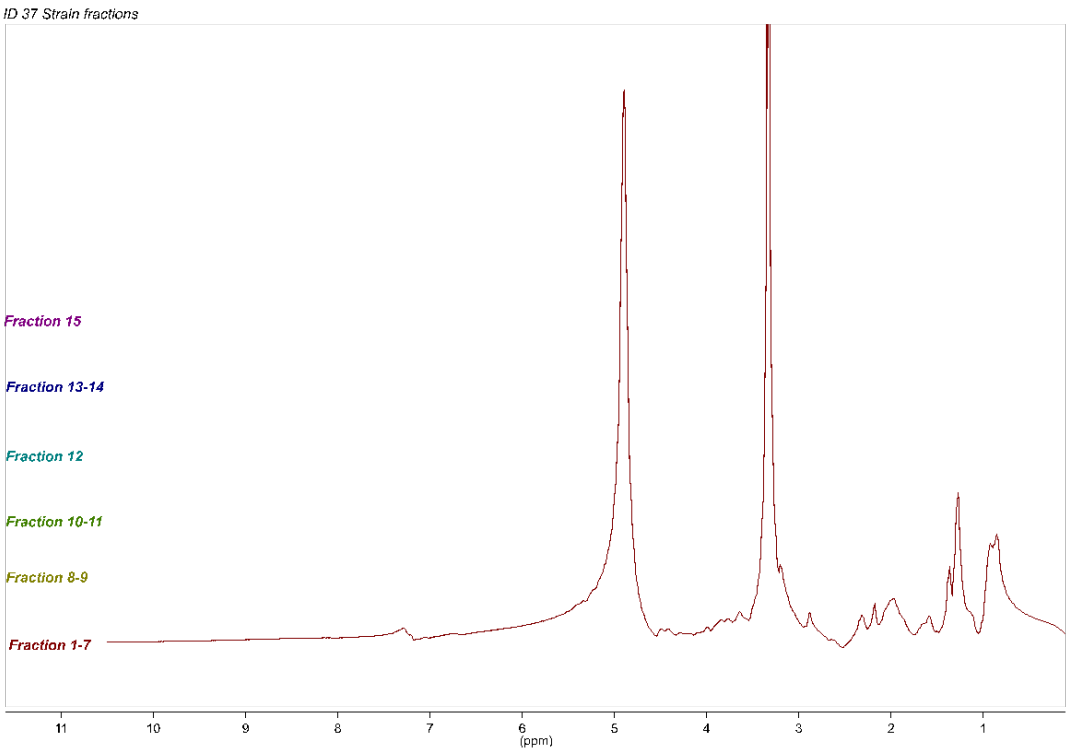


Figure 50. ID37 strain fractions

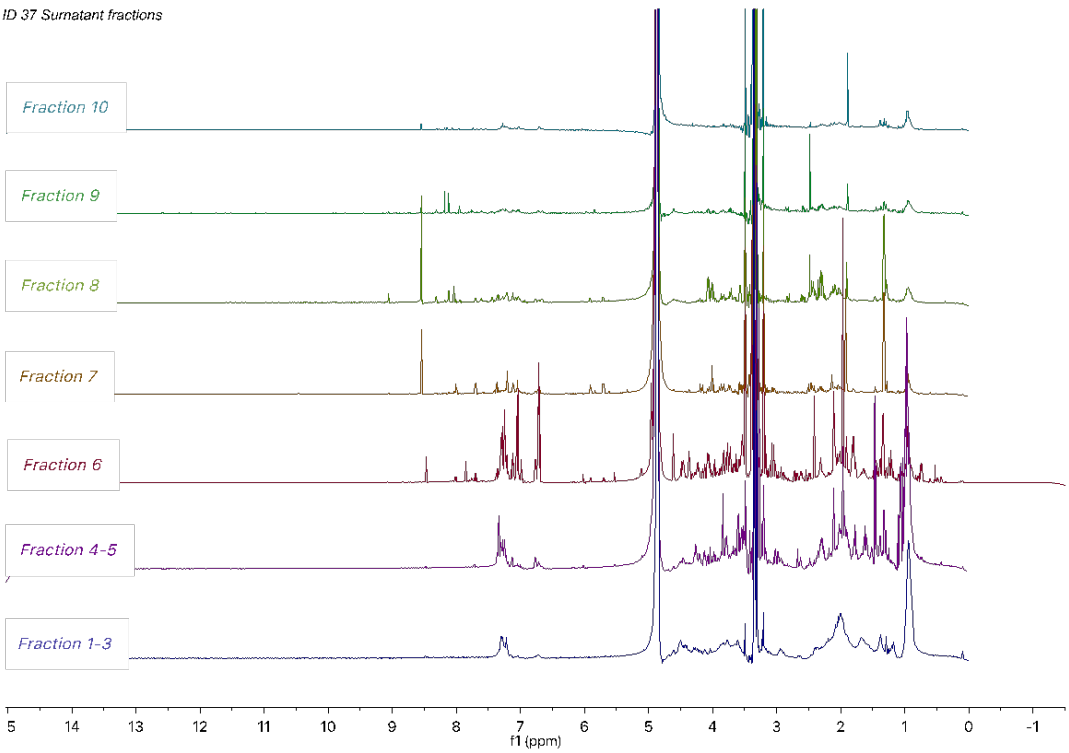


Figure 51. ID37 Supernatant fractions

Once the individual fractions constituting the pellet and the supernatant of the sample were obtained, these were subjected to a further anti-proliferative test to determine which fraction contained the active compound. The detection method applied for the antiproliferative tests was the Sulforhodamine B Assay (SRB Assay).

The tests carried out on the fractions of the supernatant of the ID-37 strain showed a reduction in global growth of 50% (GI50) positive on 3 of the 7 fractions obtained from the chromatographic column (Table 11); in particular the fraction 1-3 showed interesting GI50 values on all the 6 cell lines tested, therefore applying to subsequent tests.

Table 11. ID37 supernatant fractions with anti-proliferative activity (GI50 values)

Supernatant Fractions	**Antiproliferative Activity (µg/mL) - Cmax 250 µg/mL time 48h**					**Exp.**
	Cell Line Tested					
	A459	**HBL-100**	**HeLa**	**SW1583**	**T47D**	**WiDr**
1-3	**3,7**	91	**67**	**11**	166	120
4-5	221	250	250	250	250	250
6	**69**	250	250	250	250	250
7	250	250	250	250	250	250
8	250	250	250	250	250	250
9	250	250	250	250	250	250
10	**66**	250	250	250	250	250

Similarly, for the fractions obtained from the pellet, also, in this case, different fractions showed important GI50 values (Table 12); with the same criterion applied for the fractions of the supernatant, it was decided to proceed with the subsequent tests only on the fraction 1-7.

Table 12. ID37 pellet fractions with anti-proliferative activity (GI50 values)

Pellet Fractions	**Antiproliferative Activity (µg/mL) - Cmax 250 µg/mL time 48h**					**Exp.**
	Cell Line Tested					
	A459	**HBL-100**	**HeLa**	**SW1583**	**T47D**	**WiDr**
1-7	**<2.5**	**41**	**26**	**7.1**	**65**	**36**
8-9	135	250	250	160	250	250
10-11	89	250	250	84	250	250
12	178	250	250	250	250	250
13-14	164	250	250	250	250	250
15	87	250	250	108	250	250

On fractions 1-3 of the supernatant, a further fractionation was carried out through a size-exclusion chromatographic column, set up with Sephadex LH-20.

The 110 fractions obtained were reduced in number through the use of TLC and the NMR-based examination which allowed the chemically similar fractions to be joined together (Figure 52) obtaining a final quantity of 5 fractions (Table 13).

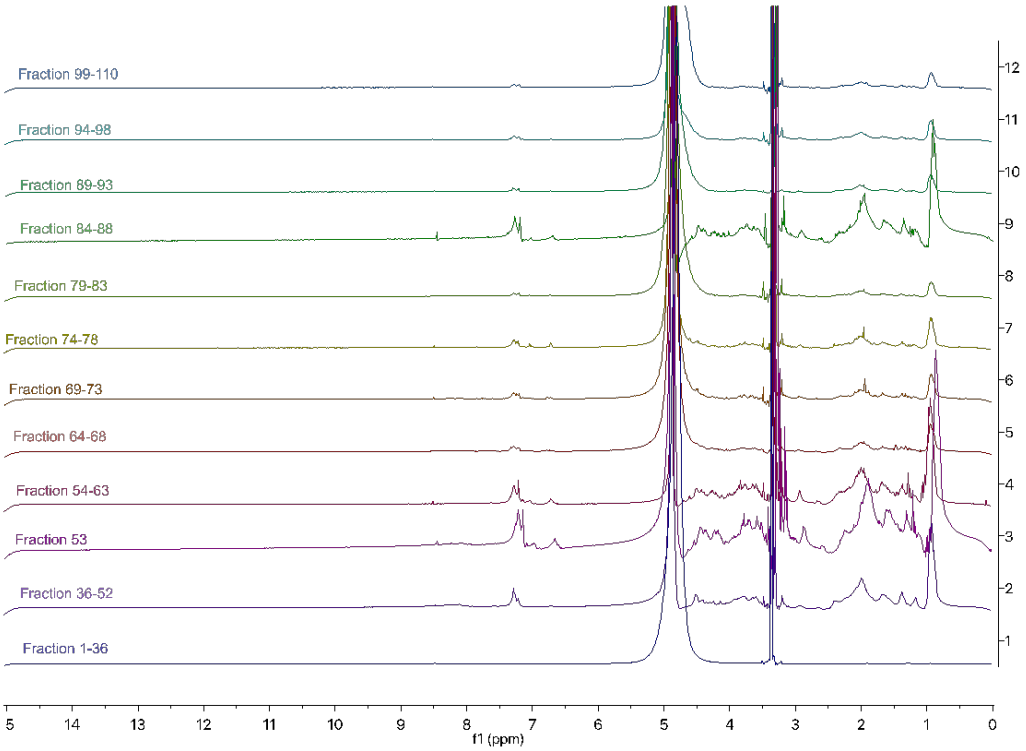


Figure 52. NMR spectra of ID37 Surnatant fractions

Table 13. ID37 surnatant fractions

SAMPLE	FRACTION	WEIGHT (g)
ID37 SUPERNATANT FRACTIONS	1	1.1966
	2	1.3799
	3	0.1167
	4	0.0466
	5	0.1185

The fractions obtained were then again subjected to the Sulforhodamine B assay to determine the fraction with antiproliferative activity (Table 14).

Table 14. GI50 values of 1-3 supernatant fractions (ID37)

1-3 Supernatant Fractions Cell Line	Antiproliferative Activity (μg/mL) - Cmax 250 μg/mL Exp. time 48h					
	Cell Line Tested					
	A459	HBL-100	HeLa	SW1583	T47D	WiDr
1	250	250	250	250	250	250
2	250	250	250	250	250	250
3	250	250	250	250	250	250
4	96	250	250	92	250	250
5	**8.1**	238	**91**	**17**	**158**	**112**

Fraction 5 showed excellent results in most of the tested cell lines. This fraction will be candidate to continue the characterization studies to identify the molecules presents through NMR experiments.

Simultaneously with the investigations that led to obtaining the fraction 5 of the supernatant ID37 as an active fraction, the same procedure was performed on the pellet fraction (Table 15).

Table 15. ID37 pellet fractions

SAMPLE	*FRACTION*	*WEIGHT (g)*
ID37 PELLET FRACTIONS	*1-7*	*0.1628*
	8-9	*0.0958*
	10-11	*0.2974*
	12	*0.1258*
	13-14	*0.1097*
	15	*0.0359*

On the six fractions tested with the SRB assay, some of them showed a positive outcome, but only fractions 1-7 showed interesting values on all the cell lines tested (Table 16). Concerning this, further investigation was carried out on fractions 1-7.

Table 16. GI50 values of 1-3 pellet fractions (ID37)

1-3 Pellet Fractions	Antiproliferative Activity (μg/mL) - Cmax 250 μg/mL Exp. time 48h					
	Cell Line Tested					
	A459	HBL-100	HeLa	SW1583	T47D	WiDr
1-7	**<2,5**	**41**	**36**	**7,1**	**65**	**36**
8-9	135	250	250	160	250	250
10-11	89	250	250	84	250	250
12	178	250	250	92	250	250
13-14	164	238	91	17	158	112
15	87	250	250	108	250	250

Comparison of the ^{1}H NMR and TLC at different conditions of the active subfraction 5 of the fractions 1-3 of the supernatant of ID37 and fractions 1-7 of the pellet, showed that both samples possess similar polarity and chemical structure and additional purification will be required in order to determine their chemical structure by spectroscopic methods. Due to the complexity of these samples, this part of research is still ongoing.

Concerning the CAL 114 strain, maintaining the same investigation procedure, 5 fractions were obtained from the supernatant and 7 fractions from the pellet, as shown below, in Tables 17 and 18 and Figures 53 and 54.

Table 17. CAL 114 supernatant fractions

SAMPLE	FRACTION	WEIGHT (mg)
CAL 114 SUPERNATANT FRACTIONS	1-2	63.57
	3-5	180.55
	6-7	47.55
	8-10	123.53

Table18. CAL 114 pellet fractions

SAMPLE	FRACTION	WEIGHT (mg)
CAL 114 PELLET FRACTIONS	1	1.74
	2	11.86
	3	155.88
	4	54.45
	5	33.68
	6-8	77.57
	9-10	55.85

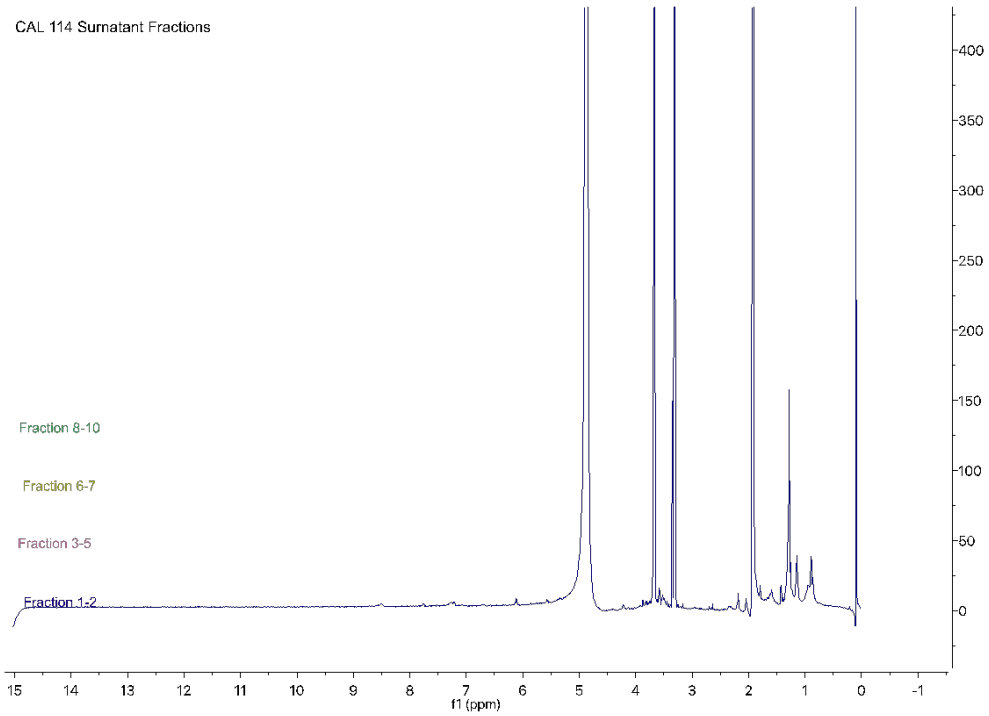


Figure 53. NMR spectra of CAL 114 supernatant fractions

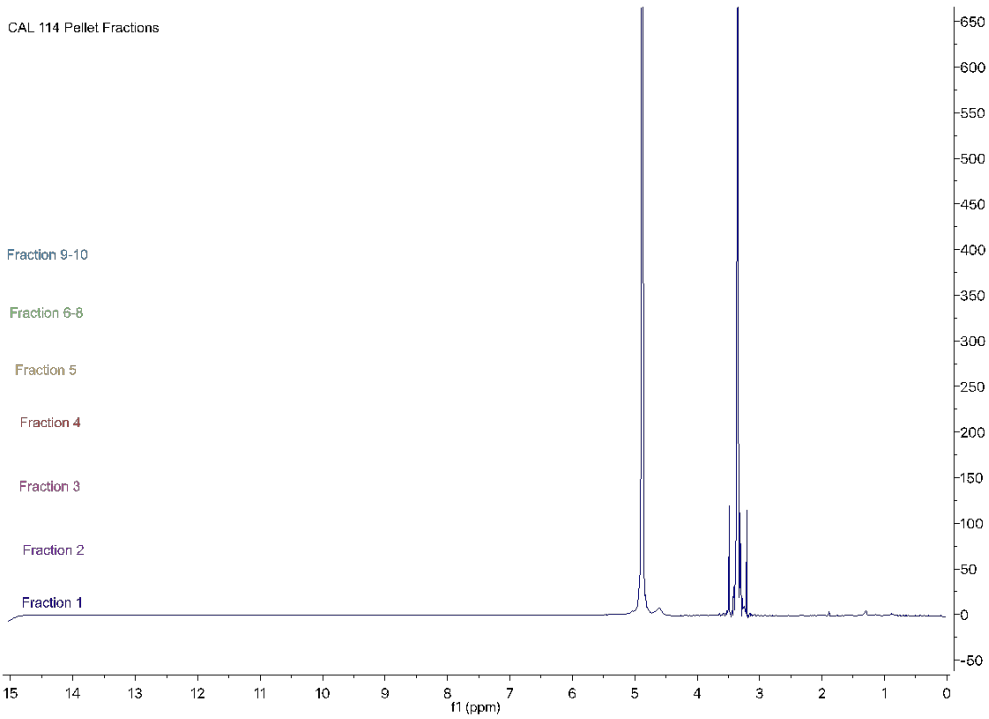


Figure 54. NMR spectra of CAL 114 pellet fractions

Testing the fractions obtained with SRB assay, two fractions with interesting positivity were obtained, such as to continue the planned investigations on them (Table 19, 20).

Table 19. GI50 value of CAL 114 supernatant fractions

CAL 114Surnatant Fractions	Antiproliferative Activity (μg/mL) - Cmax 250 μg/mL Exp. time 48h					
	Cell Line Tested					
	A459	HBL-100	HeLa	SW1583	T47D	WiDr
1-2	**<2,5**	**37**	**15**	**6,6**	**10**	**10**
3-5	150	250	250	198	250	250
6-7	250	250	250	250	250	250
8-10	250	250	250	250	250	250

Table 20. GI50 value of CAL 114 pellet fractions

CAL 114 Pellet Fractions	Antiproliferative Activity (μg/mL) - Cmax 250 μg/mL Exp. time 48h					
	Cell Line Tested					
	A459	HBL-100	HeLa	SW1583	T47D	WiDr
1	250	250	250	250	250	250
2	250	250	166	102	250	250
3	250	250	250	250	250	250
4	250	250	250	250	250	250
5	250	250	250	250	250	250
6-8	48	250	250	91	250	250
9-10	**12**	234	**71**	**23**	**43**	**78**

6.4 ANTIPARASITIC ACTIVITY

The results obtained showed that only three fractions out of six of the ID37 pellet possessed biological activity against the parasite *Leishmania amazonensis* and only 2 fractions for *L. donovani.*

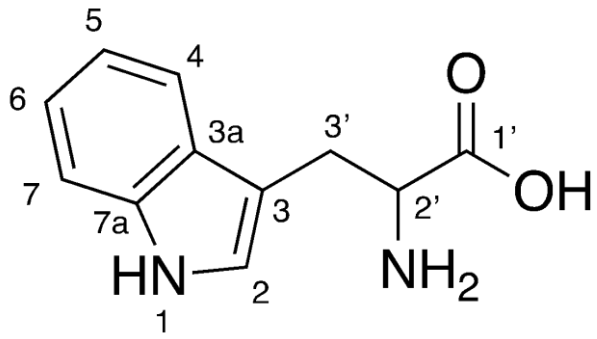
Unlike the fractions of the supernatant of ID37, all showed activity towards *Acanthamoeba castellanii* whereas none against the kinetoplastid parasites: *Leishmania amazonensis, L. donovani* and *Trypanosoma cruzi.*

For this reason, it was decided to continue the investigation on the chemical characterization of compounds contained in the fraction of the supernatant of ID37 which has shown to be more promising, fraction 7. The subsequent chromatographic steps that led to a further fractioning of the fraction 7 into 8 subfractions identified the subfraction 4 as an active fraction.

The analysis of the spectroscopic data (^{1}H, ^{13}C, COSY, HSQC and HMBC) allowed to distinguish the presence of two molecules in the sample. The compounds were identified as the amino acid, tryptophan (1) (Figure 55), and the anticancer nucleoside, cytarabine (2) (Figure 56). The NMR data of 1 and 2 (Table 21, 22) were confirmed with those reported in the scientific literature (Marion, 2013; Lorenzo-Morales et al., 2013).

Table 21. ^{1}H and ^{13}C NMR data of tryptophan (1) in CD3OD (600 MHz, 298 K)

Tryptophan (1)		
Position	δ_H (*J* in Hz)	δ_C, type
2	7.20, s	125.2, CH
3	-	109.5, C
3a	-	128.5, C
4	7.70, ddd (8.0, 1.0, 1.0)	119.3, CH
5	7.12, ddd (8.2, 7.0, 1.2)	122.7, CH
6	7.05, ddd (8.0, 7.0, 1.0)	120.0, CH
7	7.36, ddd (8.2, 0.9, 0.9)	112.4, CH
7a	-	138.4, C
1'	-	174.4, C
2'	3.88, dd (9.4, 4.1)	56.6, CH
3'	3.52, ddd(15.3, 4.1, 1.0) 3.16, dd (15.2, 9.4)	28.4, CH_2

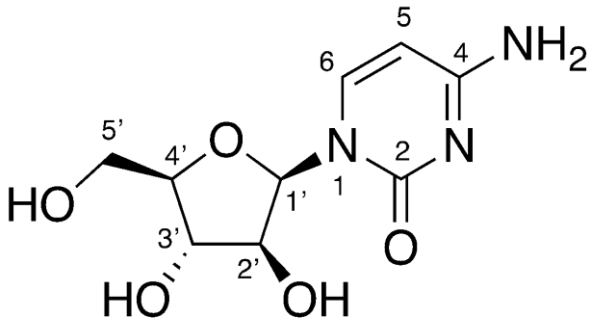


tryptophan (**1**)

Figure 55. Tryptophan

Table 22. ^{1}H and ^{13}C NMR data of cytarabine (2) in CD3OD (600 MHz, 298 K)

Cytarabine, Ara-C (2)		
Position	δ_H (*J* in Hz)	δ_C, type
2	-	166.2, C
4	-	152.4, C
5	5.70, d (8.1)	102.6, CH
6	8.01, d (8.1)	142.7, CH
1'	5.90, d (4.7)	90.7, CH
2'	4.20, dd (5.1, 5.1)	75.9, CH
3'	4.17, dd (5.1, 4.6)	71.3, CH
4'	4.01, ddd (4.6, 2.9, 2.9)	86.4, C
5'	3.84, dd (12.2, 2.7) 3.73, dd (12.2, 3.1)	62.2, C



cytarabine (Ara-C) (**2**)

Figure 56. cytarabine (Ara-C)

Additionally, the analysis of the 2D NOESY experiment allowed to confirm the relative configuration of the sugar moiety of compound 2 as shown in Figure 48. NOE correlations of the anomeric proton H-1 'with H-2', H-4 'and H -6, situated the aromatic ring, 2'-OH group and the hydroxy methylene at C-5 'on the same face of the molecule. The location of the 3-OH on the opposite site was confirmed with the spatial correlation observed between H-3 'and methylene H2-5' (Figure 57).

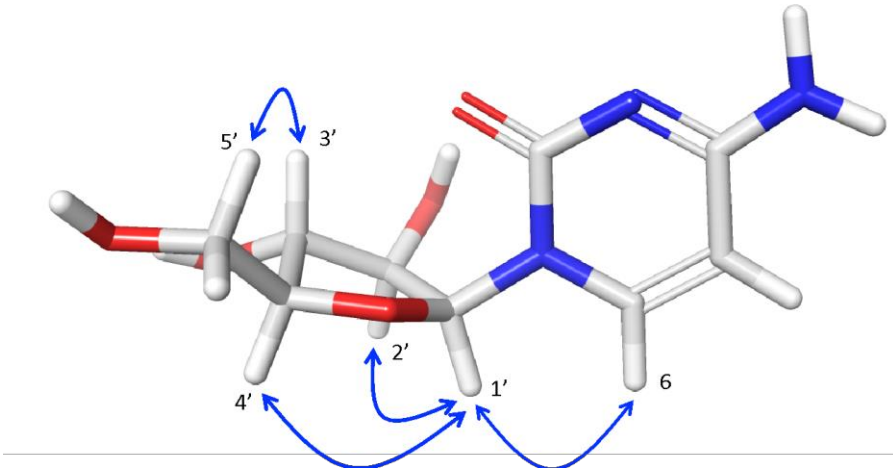


Figure 57. Selected key NOE correlations of cytarabine.

6.5 PHYLOGENETIC CHARACTERIZATION OF POSITIVE ISOLATES

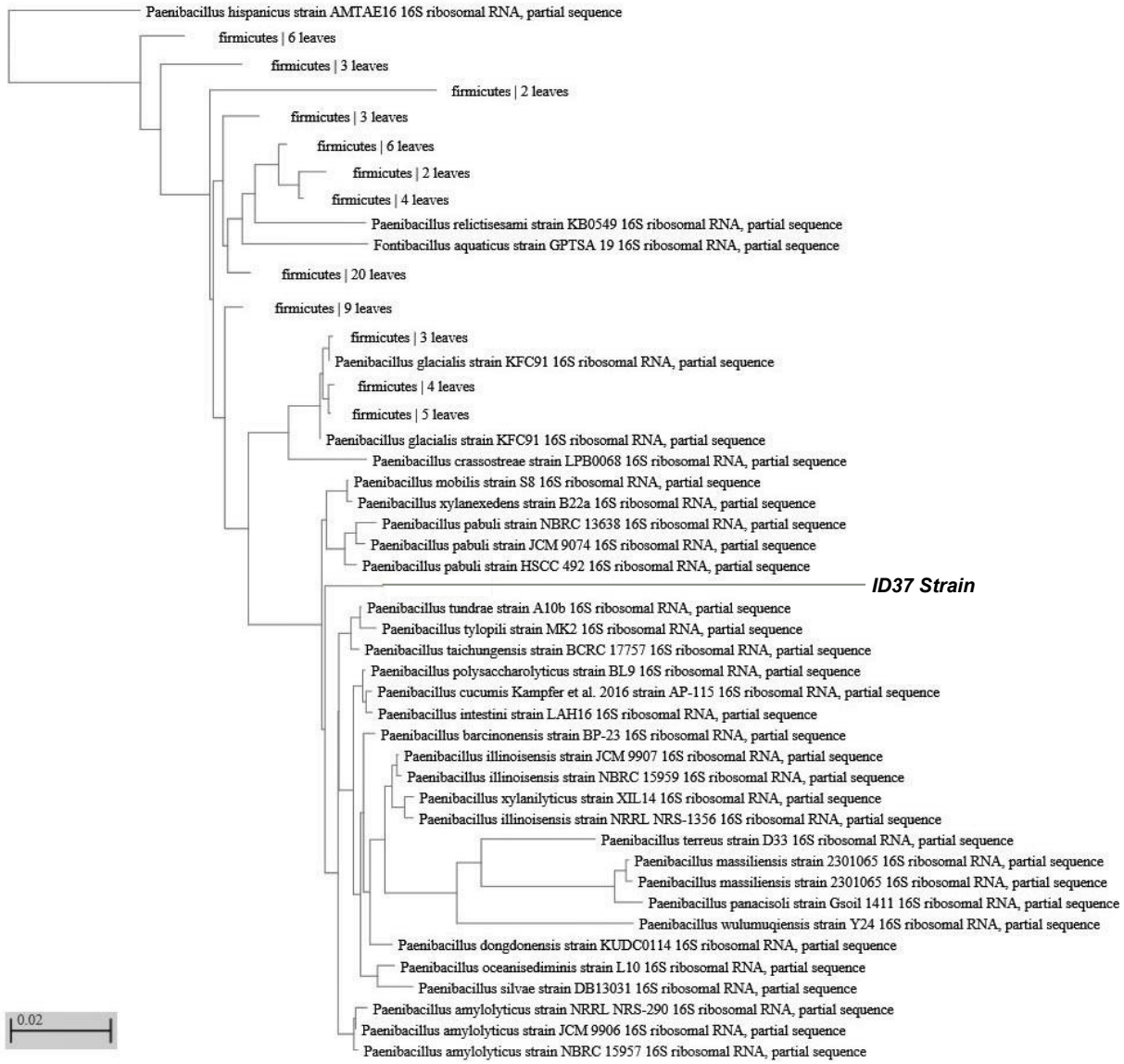


Figura 58. Phylogenetic characterization of ID37 strain

The phylogenetic investigation showed the belonging of the ID37 strain (Figure 58) to the phylum of the gram positive Firmicutes, and in particular to the *Paenibacillus* genus. Relative to the CAL 114 strain, Savoca et al. (in press) have detected the belonging of the strain to the phylum of the gram negative Proteobacteria, and in particular to the *Pseudoalteromonas* genus.

7. DISCUSSIONS

The study carried out in the context of this research examined the microbial communities associated with bivalve molluscs (*Margaritifera margaritifera*) sampled in Arctic Norway and microorganisms associated with Porifera (*Calyx arcuaria, Haliclona virens, Haliclona (Rhizoniera) dancoi, Haliclona rudis*) championships during the XX Antarctic expedition, with the aim of identifying the production of bioactive molecules and their antibacterial, antiviral and antiproliferative activity.

Regarding the antibacterial activity, the tested polar strains did not express activity against the target pathogenic strains used.

The causes of this outcome may be due to various factors such as the thermolability of the product or the non-sensitivity of the target to the metabolite. Another probable cause could be attributed to minimal production of the product such as not to inhibit the growth of the pathogen.

About the antiviral activity, preliminary tests carried out on the supernatants showed positive results on the part of a limited number of investigated strains, around 12%.

The investigations on the anti-proliferative activity have shown themselves to be the most articulated and complex for the other investigations carried out but at the same time the most exhaustive as they allowed to identify the bioactive molecules responsible for the inhibition of the tested cancer cell lines.

The preventive investigations, carried out on a first battery of strains through the application of the MTT assay, immediately showed two samples that presented positivity on the different cell lines tested and on which it was decided to investigate in more detail; in parallel, the aforementioned methodology was applied to the remaining strains, which however showed a modest positivity. The techniques adopted in the following phase, such as chromatography (column and TLC) and Nuclear Magnetic Resonance, allowed to separate the mix of compounds that constituted the entirety of the

samples in different fractions, thus obtaining the final fraction containing the molecule responsible for the antiproliferative activity.

The continuous and conspicuous division of the extracts obtained during the search for anti-proliferative activity and the desire to create research workgroups by the Instituto Universitario de Bio-Orgánica "Antonio González" and the Instituto Universitario de Enfermedades Tropicales de Canarias of the Universidad de La Laguna allowed the execution of further investigations, not planned in the initial research project, such as the studies carried out on the possible existence of anti-parasitic activity.

These analyses made it possible to obtain excellent results, thus enriching the knowledge on the potential of metabolites extracted from isolated strains.During antiparasitic test, the Cytarabine molecule (Ara-C) was found to be responsible for the inhibitory activity.

9. CONCLUSIONS

Although the tested strains have not demonstrated antimicrobial activity, the scientific literature documents the ability of cold-adapted organisms to carry out this activity.

Therefore further in-depth analyses are necessary.

About the antiviral activity, having previously evaluated only the supernants, it would be interesting to carry out a new analysis cycle associated with chromatographic investigations to obtain extract fractions from positive strains, on which NMR spectroscopy experiments will be performed to characterize any molecules responsible for the aforementioned activity.

Concerning the antiproliferative activity, the analyses necessary for the final characterization of the molecules responsible for this activity, required research and verification steps throughout the investigation, thus determining an extended and articulated execution schedule, which did not allow the ultimate characterization of the positive molecules.

Considering also the structural complexity of the functional groups revealed through spectroscopic analysis, it was not possible to characterize the molecules in their entirety, also limiting the search for further positive fractions on a reduced number of samples compared to the one fixed in the design phase of the study.

The results obtained, although partial, were encouraging, thus highlighting the need to complete the research carried out

About antiparasitaric activity, today Cytarabine molecule is mostly used in antineoplastic treatments for the treatment of acute myeloid leukaemia and test carried out in the past have also shown antiviral properties of the molecule against *Herpes zooster* and *Cytomegalovirus*.

However, the same research has shown a therapeutic index (therapeutic:toxic) very close to a unit value, thus demonstrating the

inefficiency of the molecule for this treatment because it is toxic to tissue cells and not classifying it as a possible antiviral agent.

In the bibliography, there is no evidence of the use of the molecule as an antiparasitic agent.

Based on these results, the research deserves to be studied in depth with additional in vitro and/or in vivo tests to further prove this property.

In conclusion, with this research work, it is evident once again how the marine environment and its resources reveal their potential, and specifically how extremophilic microorganisms constitute an important source of active metabolites, whose properties represent an important resource in the biotechnological field.

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