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Study of the Taxonomic Diversity and Bioactive Potential of Actinomycetota Associated with *Ruta graveolens*

Sílvia Filipa da Silva Ferreira

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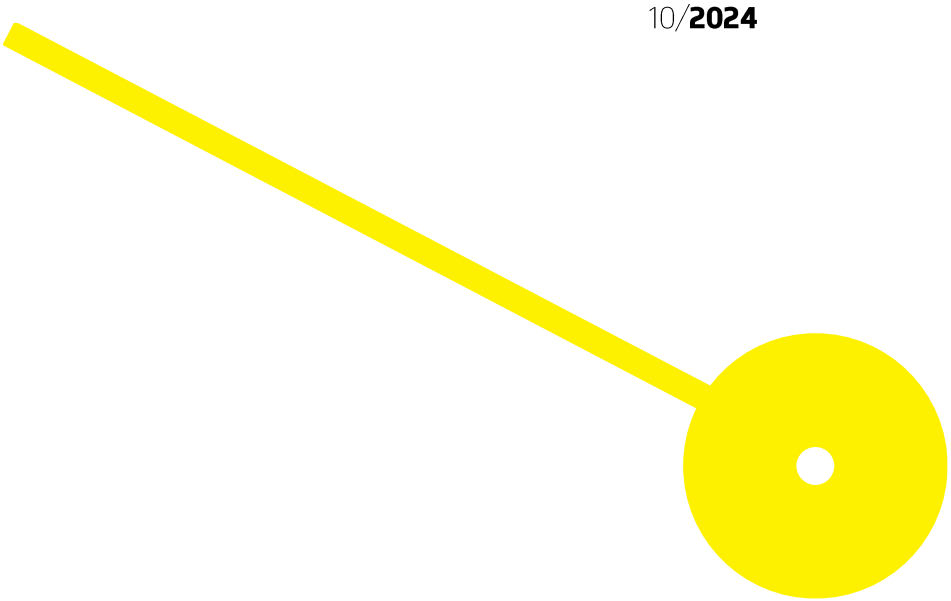

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Study of the Taxonomic Diversity and Bioactive Potential of Actinomycetota Associated with *Ruta graveolens*

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Publications

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Ferreira , S. ., Ribeiro , I., Oliveira , R. S., & Carvalho , M. F. (2024). "Exploring Actinobacterial Diversity in *Ruta graveolens*: Phylogenetic Identification and Bioactive Potential Investigation." IJUP- Young Research. 8-10 May 2024. Faculty of Economics at the University of Porto.

Ferreira , S. ., Ribeiro , I., Oliveira , R. S., & Carvalho , M. F. (2024). "Actinobacteria from *Ruta graveolens*: A Dual Source of Anticancer Activity and Biosurfactants Production." 6th MEETING ON MEDICINAL BIOTECHNOLOGY. 17 May 2024. School of Health of Porto.

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Resumo

A planta medicinal, *Ruta graveolens*, possui diversas propriedades bioativas, como propriedades antibacterianas, anti-inflamatórias e citotóxicas. As bactérias do filo Actinomycetota são bem conhecidas pela sua excelente capacidade de produção de compostos bioativos. Elas podem viver em associação com uma vasta diversidade de organismos, incluindo plantas, e exibem um potencial significativo para a bioprospecção de novos compostos com aplicações biomédicas e industriais, incluindo compostos citotóxicos e biosurfactantes. Os biosurfactantes bacterianos são compostos com propriedades tensioativas e emulsificantes, caracterizados por grupos hidrofílicos e hidrofóbicos que reduzem a tensão superficial entre duas fases. A descoberta de novos compostos é um fator importante também para o desenvolvimento de novos fármacos. O objetivo deste trabalho foi explorar as atividades citotóxicas e antimicrobianas e a produção de biosurfactantes em estirpes de Actinomycetota isoladas de *R. graveolens*. Nos ensaios de atividade antimicrobiana, as estirpes de Actinomycetota não apresentaram atividade contra os microrganismos testados: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli, Salmonella typhimurium* e *Candida albicans.* Trinta e seis estirpes de Actinomycetota foram avaliadas quanto às suas propriedades citotóxicas contra a linha celular cancerígena de fígado humano (HepG2) e a linha celular endotelial cerebral não cancerígena (hCMEC/D3). Adicionalmente, estas estirpes foram também avaliadas quanto à sua capacidade de produção de biosurfactantes através de ensaios de emulsificação, dispersão, colapso da gota e tensão interfacial. Nove extratos brutos exibiram capacidade de reduzir a viabilidade celular da linha cancerígena HepG2 (cancro do fígado), após 48 h de exposição, com reduções entre 20–30%, e um extrato com 60% de redução. Um extrato foi o mais promissor, mostrando não ter toxicidade na linha celular não tumoral, mas reduzindo a viabilidade na linha celular cancerígena. Em relação à produção de biosurfactantes, 29 extratos demonstraram ter potencial de produção nos diferentes ensaios. Dentro destes, nove apresentaram alta atividade biosurfactante, oito exibiram atividade moderada e 12 mostraram atividade baixa a moderada. Na análise de desreplicação dos extratos de Actinomycetota verificou-se a presença de alguns metabolitos associados a produtos naturais conhecidos, mas também mostrou a presença de quatro possíveis novas moléculas. Para além disso, através da rede molecular foi possível verificar a presença de dois clusters que estão associados a compostos conhecidos, como as surfactinas e amfibactinas, ambas moléculas com propriedades anfifílicas mas nunca anteriormente reportadas em associação com estirpes Actinomycetota.

Palavras–chave: Actinomycetota; Bioatividade; Endofíticos; Biosurfactantes; *Ruta graveolens*

Abstract

The medicinal plant, *Ruta graveolens*, possesses various bioactive properties, including antibacterial, anti-inflammatory, and cytotoxic activities. Bacteria from the phylum Actinomycetota are well-known for their excellent ability to produce bioactive compounds. They can live in association with a wide diversity of organisms, including plants, and display significant potential for the bioprospecting of new compounds with biomedical and industrial applications, including cytotoxic compounds and biosurfactants. Bacterial biosurfactants are compounds with surfactant and emulsifying properties, characterised by hydrophilic and hydrophobic groups that reduce surface tension between two phases. The discovery of new compounds is an important factor in the development of new drugs. This work aimed to explore the cytotoxic and antimicrobial activity and biosurfactant production in Actinomycetota strains isolated from *R. graveolens.* In antimicrobial activity tests, Actinomycetota strains did not show activity against the microorganisms tested: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium* and one yeast, *Candida albicans*. Thirty-six Actinomycetota strains were evaluated for their cytotoxic properties against the human liver cancer cell line (HepG2) and the non-cancerous brain endothelial cell line (hCMEC/D3). Additionally, these strains were also assessed for their biosurfactant production capabilities through emulsification, dispersion, drop collapse, and interfacial tension assays. Nine crude extracts showed the ability to reduce the viability of the HepG2 cancer cell line (liver cancer) after 48 h of exposure, with reductions between 20–30%, and one extract with a 60% reduction. One extract was the most promising, showing no toxicity to the non-tumour cell line but reducing viability in the cancer cell line. Regarding biosurfactant production, 29 extracts demonstrated potential in various assays. Among these, nine showed high biosurfactant activity, eight exhibited moderate activity, and 12 showed low to moderate activity. In the dereplication analysis of the Actinomycetota extracts, the presence of some metabolites associated with known natural products was observed, but four potential new molecules were also identified. Furthermore, through molecular networking, the presence of two clusters associated with known compounds such as surfactins and amphibactins was confirmed, both amphiphilic molecules that had never before been reported in association with Actinomycetota strains.

Keywords: Actinomycetota; Bioactivity; Endophytes; Biosurfactant; *Ruta graveolens*

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List of Abbreviations

16S rRNA– 16S ribosomal RNA

AIA– Actinomycete Isolation Agar

BLAST– Basic Local Alignment Search Tool

BP– Base pair

BS– Biosurfactants

$CaCO_3$ – calcium carbonate

CMC– Critical micelle concentration

DMEM– Dubelco's Modified Eagle Medium

DMSO– Dimethyl sulfoxide

GNPS– Global Natural Products Social Molecular Networking

hCMEC/D3– human brain capillary endothelial cells

HepG2– liver hepatocellular carcinoma

LC–HRESIMS/MS– liquid chromatography–high resolution electrospray ionization tandem mass spectrometry

MTT– (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

MSAC– Microbial surface-active compounds

NaHCO3– hydrogen carbonate

NCBI– National Center for Biotechnology Information

NPs– Natural products

OSMAC– One strain many compounds

PAHs– Polycyclic aromatic hydrocarbons

PBS– phosphate-buffered saline

PCA– Plate Count Agar

PCR– Polymerase Chain Reaction

RGEA– *Ruta graveolens* Extract Agar

RL– Rhamnolipid

Ruta graveolens– *R. graveolens*

SCN– Starch Casein Agar

SL– Similar sophorolipid

1. Introduction

There is evidence that microorganisms play a crucial role in ecosystem stability. They are the oldest living organisms in the biosphere and are responsible for revitalising dead matter converting it into nutrients and making it available for other organisms to grow (1, 2). The microbial diversity in the environment is so vast that most microbes remain underexplored, along with their effect on human health (2). While they could be beneficial to life on Earth, they can also be pathogenic to humans (2). The search for novel bioactive compounds is an effective approach to combat this problem, with the natural environment still being the major source of such molecules (3, 4). The antibiotics era started between the 1950s and 1970s when the most known important classes of antibiotics were discovered (5). These bioactive compounds are naturally produced by a variety of fungi and bacteria, but the most appealing phylum of microorganisms capable of producing these secondary metabolites is Actinomycetota, particularly Actinomycetes (6). Actinomycetota is a rich source of compounds, exhibiting diverse biological activities and potential therapeutic applications, requiring a comprehensive understanding of their biological roles (7). They produce antibiotics like streptomycin, terramycin, and aureomycin and reproduce through binary fission or spore formation (8). Actinomycetes play an important vital role in the production of bio-emulsifiers (9). Biosurfactants produced by bacteria are of particular interest due to their antibacterial, antifungal, and antiviral properties with therapeutic and biomedical potential (10). Biosurfactants have numerous advantages due to their specificity, biodegradability, and less toxicity. They are surface-active molecules produced mainly by microorganisms. The term refers to compounds having some influence on interfaces. Medicinal plants and their components have been utilized in traditional medicine for centuries and have significantly influenced the development of modern medicine (11). These plants are considered suitable for the human body and produce fewer side effects than pharmaceuticals, making them effective, safe, and low-cost for treating various diseases (12). *Ruta graveolens*, a Rutaceae medicinal plant, native to Mediterranean and tropical regions, is used for its antispasmodic and analgesic effects, as well as its antirheumatic activity (13). Research on *R. graveolens* extract has shown its antibacterial properties, potentially aiding in the development of drugs for infectious disease treatment, paving the way for new herbal medicine innovations (14). *Ruta graveolens*, when homeopathically potentiated induces cell death in brain cancer cells through telomere dynamics, mitotic catastrophe, and apoptosis (15). Studies suggest that *R. graveolens* may have

potential benefits, but the evidence is limited, with no randomized trial demonstrating a clear improvement in cancer symptoms or treatment toxicity (16). However, no study has yet been carried out on the endophytic actinomycetes of *R. graveolens* (16).

1.1. *Ruta graveolens*

Rutaceae, also known as the citrus family, is a vast group with approximately 150 genera and 1600 species of shrubs and small trees, largely found in the old and new world's temperate regions (17). Among the most common medicinal plants in this family is *R. graveolens*, commonly referred to as "Arruda" in Portuguese, "Aruvada" in Tamil, and "Rue or Sudab" in Hindi (17, 18). It is native to Europe, particularly the Mediterranean area, and is widely distributed in temperate and tropical climes worldwide (17, 19).

Rue, is a perennial herbaceous plant, that typically reaches about one meter in height. It is characterized by its greyish-green colour and strong odour, and it is highly regarded for its therapeutic properties. The leaves are small, rectangular, deeply divided, and pinnate, with glandular spots (Figure 1 A-B) (17). Traditionally, Rue has been used medicinally for its antifungal (20), antibacterial (21), anti-inflammatory (22), and cytotoxic effects (23).

Moreover, it has also been demonstrated to be useful in treating gliomas (24) because of its capacity to cause apoptosis (25).

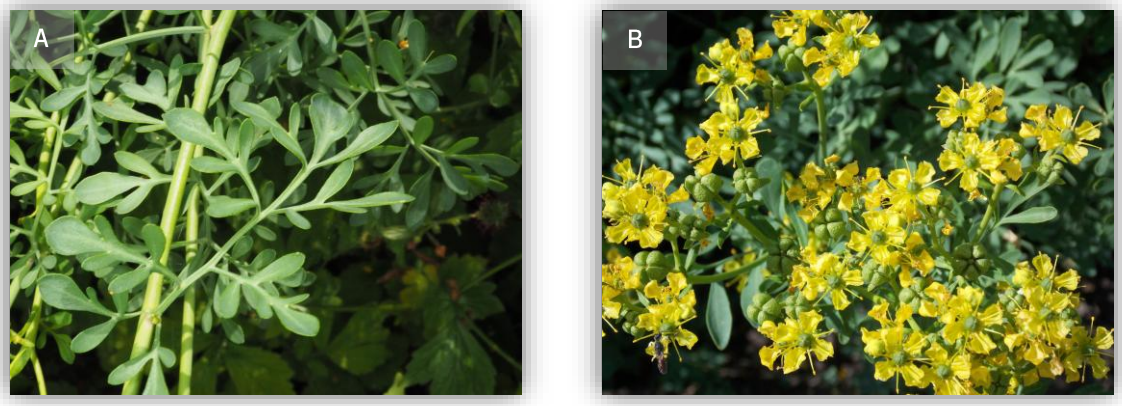


Figure 1. (A) Detailed view of the leaves of Ruta graveolens; **(B)** Full view of the plant *Ruta graveolens* (adapted from Kew Science).

R. graveolens plant provides a high amount of bioactive compounds. Previous chemical analyses on Rue found different subgroups of coumarins including simple coumarins, psoralen-type furanocoumarins, dihydrofuranocoumarins, and coumarin dimers (26). It also found alkaloids,

volatile oils, flavonoids, and phenolic acids in the plant (27). Coumarin is a crucial component that successfully recovers vitiligo (28). However, the bioactive components of *R. graveolens* have not been fully described, limiting their widespread application in modern medicine (29). As a result, further research is necessary to *clarify R. graveolens* chemical composition and biological activity.

1.2. Endophytic Bacteria

According to De Bary (30), the word endophytic means "in the plant" and is derived from the Greek words "endon" meaning within, and "phyton" meaning plant, and refers to the existence of microbes inside infected plant tissues without causing harm to the host plant (31). Various microbial populations, including bacteria and fungi, have been discovered residing within the internal tissues of plants as endophytes (32). Microbes produce metabolites that promote plant growth, repel insects and pests, act as antimicrobials against plant pathogens, and provide protection under stress conditions (33) (34) (35) (36). Bacterial endophytes benefit plant defence by releasing antimicrobial compounds, creating siderophores, competing for resources, and influencing the plant's defence mechanisms (37). Endophytes mainly inhabit spaces between cells, which contain high levels of carbohydrates, amino acids, and inorganic nutrients, and are present in different parts of plants such as roots, leaves, stems, flowers, and seeds (37). Within the rhizosphere, which is the area where soil meets roots, plants release significant quantities of exudates that impact microbial populations and enable intricate interactions between plants and microbes (37). Different types of endophytic bacteria use various mechanisms to colonize plants. Obligate endophytes rely on plant metabolism for survival and are transmitted by seed, spreading vertically or through vectors (38). Facultative endophytes live freely in the soil before colonizing plants internally through the rhizosphere. Passive endophytes enter plants through root hair wounds, adhering to the root surface as associative bacteria (38). Rhizobacteria living in the rhizosphere are closely associated with plant roots. Roots serve as the main entry point for endophytic bacteria, with high frequencies found in this area. Enzymatic activity aids the penetration of these microorganisms into plants, which can also be transmitted through seeds (38) (Figure 2).

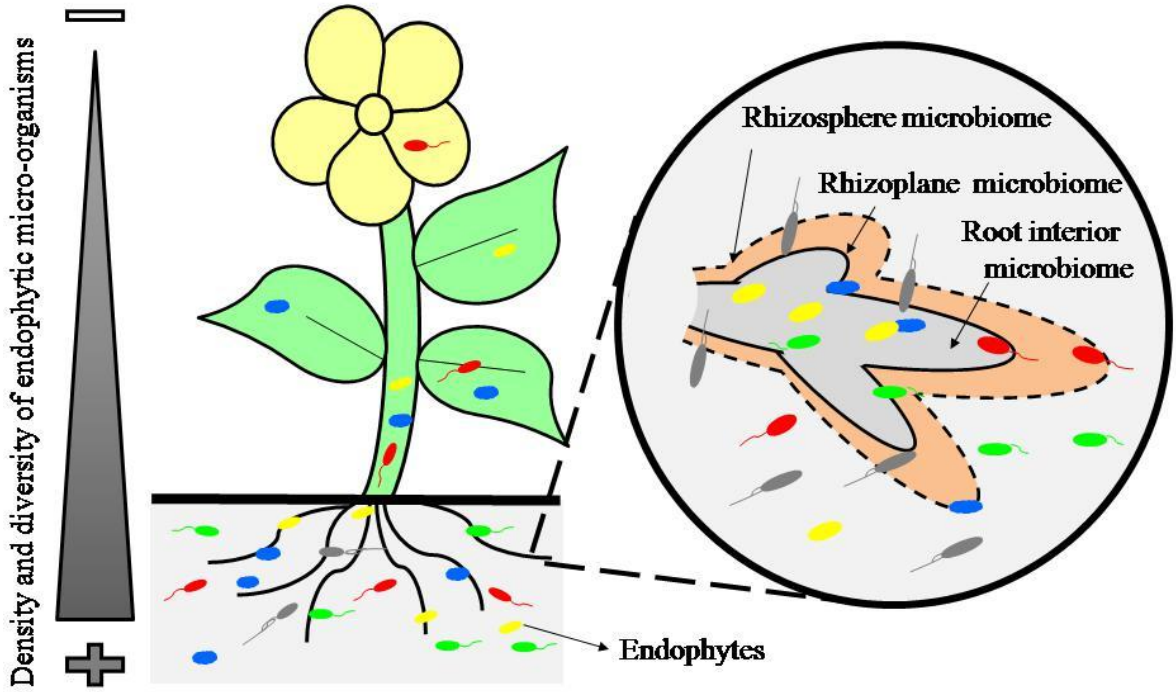


Figure 2. Representation of the presence of endophytic microbes in the above-ground and underground parts of plants from the rhizosphere, rhizoplane, and internal root tissue. (Adapted from (38))

The host plant genotype and the native soil composition are both seen as crucial in attracting bacterial endophytes to the plant (37). Research on root endophytes of *Arabidopsis* plants in varying soils suggests that the type of soil may impact the makeup of the bacterial endophyte community in the plant roots. This suggests that diverse types of soil can have varying bacterial populations acting as the initial source of microorganisms (39). Kandelet al. (2017) also demonstrated that factors like soil quality, water content, temperature, as well as characteristics of the host plant like genotype and age, play a significant role in determining the composition of bacterial communities in roots and leaves (37). The significant relationships between endophytic actinobacteria and plants are considered a crucial area of study. Many researchers are studying the bioactivities and industrial uses of these endophytic actinobacteria, which are a promising source of unique bioactive compounds (32).

1.3. Actinomycetota Phylum

The phylum *Actinomycetota* (formerly, *Actinobacteria*) is one of the biggest taxonomic groups in the domain Bacteria, consisting of 5 subclasses and 14 suborders among the 18 major families

(40). Actinomycetes are gram-positive bacteria that form spores aerobically, with a genome high in guanine-cytosine (57-75%) and are classified in the Actinomycetales order known for their growth of substrate (41). The phylum Actinomycetota is supported by analysis of 16S rRNA (16S ribosomal RNA) genes, conserved insertions and deletions (indels) in specific proteins, and specific gene rearrangements (42). Most Actinobacteria do not possess a clear cell wall, instead, they form a non-septate and thin mycelium, reproducing through various methods such as binary fission, conidia, and sporulation. The usual appearance of actinobacteria includes two distinct areas known as aerial mycelium and substrate mycelium (43). The former extends outward or protrudes and are mainly for reproduction, while the latter are roots growing in the agar/substrate medium for nutrition. Actinobacteria are crucial for the ecosystem because they help break down organic matter, participate in bioremediation and bio weathering, and produce numerous secondary metabolites that have many therapeutic uses (43). Actinomycetota are well-known for their ability to prosper in various environments, earning them the designation of extremophiles due to their resilience in extreme conditions such as high temperatures in volcanic regions and freezing climates in the Antarctic and Arctic (43). They are also found in a variety of soils, freshwater, and marine ecosystems, as well as within plants and animals, which host diverse Actinomycetota (44). Furthermore, they can survive in other challenging habitats, such as the deep ocean, where they play a crucial role in nutrient cycling under harsh conditions (44). They are found in higher quantities in soil compared to other environments, especially in alkaline or organic-rich soils, where they make up a significant portion of the microbial community (45). Actinobacteria growth is influenced by factors such as temperature, pH, and soil moisture. They grow well in temperatures between 25-30ºC, while thermophilic varieties can live in temperatures of 50-60ºC (46). Actinobacteria grow well in soil with a pH range of 6-9, with their peak growth preference being towards neutral pH levels (45). Typical actinomycete colonies have distinct granular characteristics including filamentous mycelium-like fungus and spore-forming qualities (for example, genera *Streptomyces*, *Microbispora*, *Streptosporangium*, and *Microbispora*) (47). The majority of Actinomycetales strains in soil are derived from *Streptomyces*, making up more than 95% of the community (48). Actinobacteria are known for producing secondary metabolites and bioactive substances like antibiotics (49). They are valuable for various industries due to their production of enzymes (50). These microbes have biotechnological uses, including manufacturing essential enzymes like protease, lipase and amylase for making recombinant proteins (51). In the field of agriculture, actinobacteria in soil

synthesize various compounds crucial for soil processes, such as antifungal agents and enzymes that break down fungal cell walls. These compounds can be used to produce herbicides as an alternative to expensive and environmentally harmful chemical pesticides (52). Actinomycetes are also valuable for producing antibiotics on a large scale, and important in biopharmaceuticals for creating treatments like antibacterial, antifungal, anti-inflammatory, and anticancer medications (53).

1.4. Bioactive Potential of Actinomycetota

Actinomycetota are the most common group of microorganisms that produce bioactive compounds. They make up over two-thirds of all naturally derived antibiotics used today in medicine, veterinary practice, and agriculture (54). Recently, there has been a rise in the worldwide need for new chemotherapeutic compounds and antibiotics that are highly effective against dangerous microorganisms and resistant agents from viruses, bacteria, and fungi (55). One traditional method to discover new bioactive compounds, especially those with unique chemical structures and biological significance, is to collect different microbes from a range of, sometimes isolated, environments. Actinomycetota produces a variety of secondary metabolites that have significant biotechnological importance, and optimizing fermentation conditions can increase their production (56). These metabolites are only produced when the growth of the microorganisms slows down or stops, after primary metabolism. Factors like nutrient availability, pH, temperature, and the presence of certain substances like precursors or inhibitors influence the synthesis of these metabolites (50). The production of secondary metabolites by Actinomycetota is linked to different growth phases, starting with an adaptation phase where protein production is induced, followed by exponential growth with limited metabolite synthesis, a transition phase with intensified enzyme synthesis leading to metabolite production, and ultimately a production phase where maximal metabolite production occurs (50, 57, 58). The production of these metabolites is carried out by large enzymatic complexes, an example of biosynthetic pathways, like polyketide synthases and non-ribosomal peptide synthases, which are modified by tailoring enzymes to produce bioactive compounds (59). The adaptable Table 1 shows various types of secondary metabolites produced by Actinomycetota, which have different types of bioactivities (60). Among these, antimicrobial and anticancer activities are highlighted, as they are one of the aims of this study.

1.4.1. Antimicrobial Activity

Even with advancements in antibiotic research and production, infectious diseases remain the leading causes of death globally, with bacterial infections causing approximately 17 million deaths annually, mostly affecting children and the elderly (61). Infections caused by multiresistant organisms significantly increase morbidity, mortality, and health care costs (62). Because of the rise in antibiotic resistance to pathogens, it is important to find new antimicrobial drugs (63). Soil contains multiple ecological niches, and its microorganisms create a variety of biomolecules that are biologically active against an extensive range of pathogens (64). Numerous Actinomycetota synthesize secondary metabolites, which consist of antimicrobial compounds (65). This bacterial phylum has proven extremely useful to the pharmaceutical industry due to its almost limitless ability to create secondary metabolites with different biological functions and chemical structures (66). Actinomycetes possess the capability to produce antimicrobial compounds like beta-lactams, polyketides, glycopeptides, macrolides, tetracycline, aminoglycosides, polyenes, and actinomycins (67). *Streptomyces* represents roughly 50% of Actinomycetota and 75% of commercially relevant antibiotics (49). *Streptomyces*, part of the Actinomyceaceae family, produce a variety of secondary metabolites like antibiotics, anticancer, antioxidants, antimicrobials, and immunosuppressants. With over 800 species, *Streptomyces* is globally recognized as a rich source of antibiotics for human therapy, responsible for around half of effective antibiotics (64). Each *Streptomyces* strain can produce an average of 30 secondary metabolites, including valuable compounds for biotechnological uses in cold environments (64). Various well-known antibiotics such as erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin are found in these species (68). In contrast, rare Actinomycetota are not well-known for generating bioactive compounds because they are less frequently isolated in the laboratory. Therefore, the potential of rare Actinomycetota to produce antibiotics remains mostly unexplored (68).

1.4.2. Anticancer Activity

Cancer is a major public health issue in developed and developing countries. Despite the extensive attempts to create therapies, there are still no effective medications accessible, and natural product extracts remain the most hopeful source of new cancer medications (69). Actinomycetes are a rich source of natural products with a wide range of biological activities (70).

They are closely associated with the natural cancer prevention ability because they can easily adapt and thrive in various areas of the body (43). Actinomycetota are recognized for their ability to produce various compounds that have uses in various fields. They create useful antitumor medications like anthracyclines, glycopeptides, aureolic acids, enediynes, antimetabolites, carzinophilin, mitomycins, and more (71). After the launch of recombinant DNA technology, several antitumor biosynthetic gene clusters have been found and studied in actinomycetes (71). Particularly *Streptomyces* species from extreme places are seen as a remarkable source of new biosynthetic gene clusters that could be used to create anti-cancer medications (70).

Table 1. Examples of secondary metabolites produced by Actinomycetota (adapted from (60)).

Bioactive Compounds	Biological Activity	Source	References
6-Prenylindole	Antifungal	*Streptomyces* sp. TP-A0595	(72)
Anicemycin	Antitumor (anchorage-independent growth inhibitor)	*Streptomyces thermoviolaceus* TP-A0648	(73)
Kakadumycin A	**Antibacterial, Antimalaria**	***Streptomyces* sp. NRRL 30566**	**(74)**
Diketopiperazine, Gancidin W	Low toxic, Antimalarial agent	*Streptomyces* sp. SUK10	(75)
1-Acetyl-β-carboline, Indole-3-carbaldehyde, 3-(Hydroxyacetyl)-Indole, Brevianamide F, and Cyclo-(L-Pro-L-Phe)	**Antibacterial**	***Aeromicrobium ponti* LGMB491**	**(76)**
Lansai A-D	**Antifungal and anticancer**	***Streptomyces* sp. SUC1**	**(77)**
3-Acetonylidene-7-Prenylindolin-2-one and 7-Isoprenylindole-3-carboxylic acid	Antifungal	*Streptomyces* sp. neau-D50	(78)
2-(furan-2-yl)-6-(2S,3S,4-trihydroxybutyl) pyrazine	Antiviral	*Jishengella endophytica* 161111	(79)
Pterocidin	**Anticancer**	***Streptomyces hygroscopicus* TP-A0451**	**(80)**

Linfuranone	**Antimicrobial, non-cytotoxic**	***Microbispora* sp. GMKU 363**	**(81)**
Clethramycin	Antifungal	*Streptomyces hygroscopicus* TP-A0623	(82)
Ansamitocin	**Antibacterial and antitumor**	***Actinosynnema pretiosum***	**(83, 84)**
Demethylnovobiocins	**Antimicrobial**	***Streptomyces* sp. TPA0556**	(73)
Cedarmycin A and B	**Antibacterial, anti-Candida**	***Streptomyces* sp. TP-A0456**	**(85)**
Xiamycin	Antiviral	*Streptomyces* sp.GT2002/1503	(86)
Saadamycin	Antifungal agent	*Streptomyces* sp. Hedaya 48	(87)
Kaempferol, Isoscutellarin, Umbelliferone and Cichoriin	Antioxidants	*Streptomyces* sp. Tc052	(88)
Alnumycin	**Antibacterial**	***Streptomyces* sp.DSM 1175**	**(89)**
Celastramycins A and B	**Antimycobacterial, Antibacterial**	***Streptomyces setonii*, sp. Q21, *Streptomyces sampsonii*, QuH- 8**	**(90)**
Lupinacidin C	Antitumor	*Micromonospora lupini Lupac* 08	(91, 92)
Naphtomycin A	Antitumor	*Streptomyces* sp. CS	(93)
Streptol	Anti-fungal	*Dactylosporangiumsp. strain* SANK 61299	(94)
Actinomycin X2	**Antimicrobial**	***Streptomyces* sp. R-5**	**(95)**
Munumbicins A, B, C and D	**Antimicrobial, Antimalarial, Antitumor**	***Streptomyces* sp. NRRL 30562**	**(96)**
Coronamycin	Antifungal, Antimalarial	*Streptomyces.* sp. MSU-2110	(97)
Munumbicins E-4 and E-5	**Antimalarial, antibacterial**	***Streptomyces* sp. NRRL 30562**	**(98)**
S-adenosyl-Nacetylhomocysteine	Antioxidant, Neuroprotection	*Micromonospora* sp. PC1052.	(99)
Proximicin	**Antibacterial, Antitumor**	***Verrucosispora maris* AB-18-032.**	**(100) (101) (102)**
6-alkalysalicilic acids, salaceyins A and B	**Anticancer**	***Streptomyces laceyi* MS53**	**(103)**
7-Octadecenamide	**Antimicrobial**	***Nocardia caishijiensis***	**(104)**
9, 12-Octadecadienamide (Linoleamide)	**Antimicrobial**	***Pseudonocardia carboxydivorans* SORS 64b**	**(104)**

1.5. Biosurfactants

Chemical surfactants have been included in numerous commercial products since the 1930s (105). Due to their structural variations, these molecules contain both hydrophilic and hydrophobic parts in their structure, allowing for diverse uses in environmental and industrial fields (105). The primary physical and chemical effect of surfactants is to decrease the tension at the boundaries between different liquids, solids, and gases, facilitating the blending and interaction of separate phases, typically water and oil (105, 106). They accomplish this due to being amphiphilic, containing both hydrophilic and hydrophobic parts (106). Despite their clear benefits, the main disadvantages of chemical surfactants are their lack of sustainability (as they come from fossil fuels) and problems with biodegradability (107).

Biosurfactants (BS), which are surface-active compounds derived from living organisms, have been synthesized by microorganisms for millions of years, with documentation of their presence coming more recently. Saponins have been recognized for over one hundred years (106), however the glycolipid BS rhamnolipid (RL) from *Pseudomonas* was officially documented in 1949 (108), and the similar sophorolipid (SL) from the yeast *Candida apicola* came in 1961 (109). Surfactin found in Bacillus subtilis in 1968, and serrawettins from Serratia marcescens in 1986 are two lipopeptides that were identified (110). Biosurfactants are substances produced by microorganisms that show significant abilities in interacting with surfaces and forming emulsions (111). The effectiveness of biosurfactants relies on the level of surface-active components until reaching the critical micelle concentration (CMC). Above the CMC, biosurfactant molecules come together to create micelles, bilayers, and vesicles (112) (Figure 3). Various chemical structures comprise these compounds, including glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids (111). A prior assessment by Parkinson in 1985 (113) effectively summarized important elements of the BS field that remain unchanged. He pointed out the main categories of biosurfactants and the difficulties in improving growth conditions for biosurfactant production, affirming that biosurfactants can be just as effective or even more effective than traditional synthetic surfactants, and their future use may be influenced by production economics. Potential benefits may involve decreased toxicity, biodegradability, suitability for human skin, resilience under extreme conditions (pH, temperature, and salinity), and manufacturing from cost-effective and sustainable sources (114–117). Due to these factors, biosurfactants have garnered notable interest in various sectors such as food, environmental

protection, textile, oil, agriculture, cosmetic, medical, and pharmaceutical industries in recent years (118).

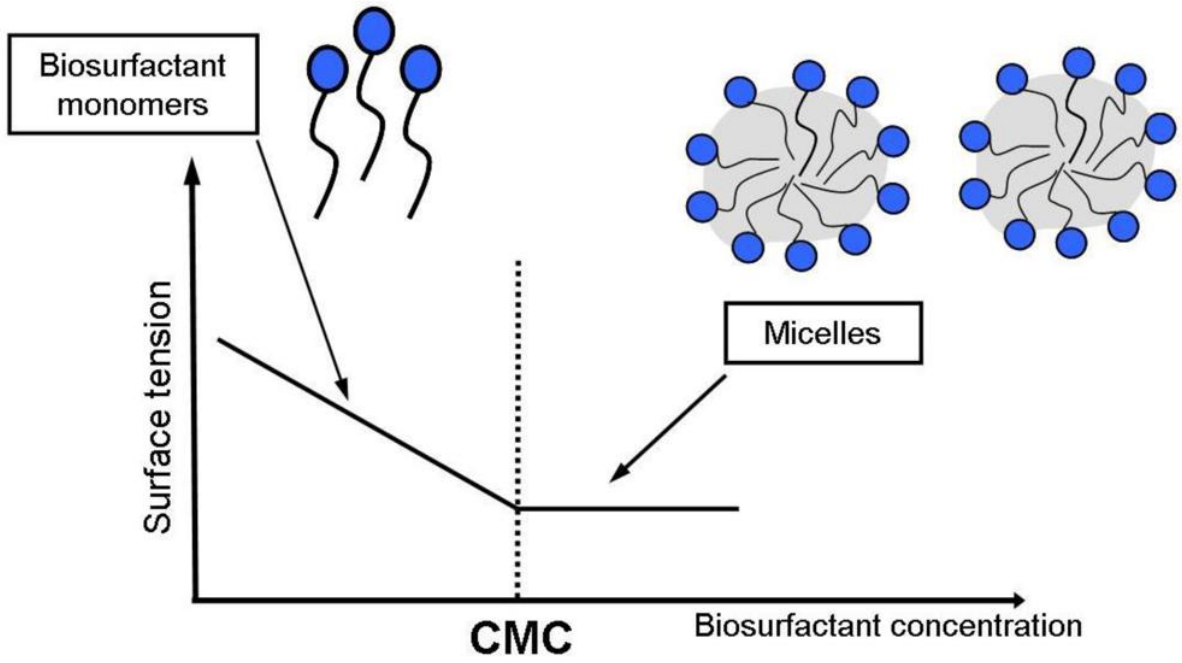


Figure 3. The correlation among the concentration of biosurfactant, surface tension, and micelle formation. (Adapted from (112))

1.5.1. Biosurfactants and Actinomycetota

Different types of microorganisms, such as actinobacteria, have demonstrated the ability to biodegrade petroleum hydrocarbons. Different genera of actinobacteria, including *Actinomyces*, *Streptomyces*, *Rhodococcus*, *Gordonia*, *Micrococcus*, and *Nocardia*, have demonstrated the capability to break down these hydrocarbons (119). Furthermore, actinobacteria produce biosurfactants that improve the availability of different pollutants, making it easier to break down these hydrocarbons (120). The biosurfactant-producing *Brachybacterium paraconglomeratum* MSA21, a marine Actinomycetota found in sponges, was discovered. Researchers found that the biosurfactant created by these bacteria has the potential to serve as a sustainable option instead of chemical surfactants (121). The *Streptomyces* genus is also identified for its capability to produce biosurfactants. The species known as *Streptomyces* sp. VITDDK3, obtained from the coastal region, has exhibited capabilities in producing biosurfactants, as well as tolerating heavy metals and performing biodegradation. It was mentioned that using this strain could bring great advantages for different industrial processes and bioremediation (122).

1.5.2. Therapeutic Potential of Medical Biosurfactants

The possibilities of biosurfactants in medicine have revealed significant prospects and obstacles but are still in their initial phases (123). They provide a possible alternative for artificial drugs and antimicrobial agents and can be applied as safe and efficient therapeutic agents (111). Biosurfactants may also include signalling molecules that interact with host and/or bacterial cells, inhibiting infections (111). Moreover, they support the assertion of possible participation in preventing microbial attachment and their potential for developing anti-adhesion coatings for implant materials (111). More opportunities for healthcare advancement are being created by the potential roles of carriers in delivering drugs, anti-bacterial substances, anti-viral substances, and therapeutic substances for treating tumours (123). Biosurfactants improve drug delivery and therapeutic benefits by interacting with cell membranes to target specific cells and increase treatment effectiveness (124). Lipopeptide biosurfactants from bacteria are widely used in the antibacterial field due to their inherent antibacterial properties. They disrupt microbial cell membranes to achieve antibacterial effects, dehydrate phospholipid bilayers, impact membrane function, and hinder microbial attachment to surfaces and tissues (123). Table 2 shows the applications and functions of biological biosurfactants in various fields of medicine. This unique inhibitory mechanism makes biosurfactants valuable in the pharmaceutical industry for their ability to synergistically work with medications to boost therapeutic benefits. Their multifunctionality enhances treatment efficacy and makes them a valuable tool in combating bacterial infections (123). Biosurfactants offer promising benefits in medicine, yet face challenges like stability issues linked to environmental factors. Microbial growth conditions may affect their characteristics and production, impacting their suitability for medical use (123). More research is required to ensure biosurfactant safety for humans, especially those with weak immune systems, considering they are microbial metabolites with potential risks (123).

Table 2. The application and function of biosurfactants in various fields of medicine. (Adapted from (123))

Application	Function	Reference
Drug Carrier Components	Enhance drug delivery capabilities and bioavailability	(125–127)
	Activate enzyme pathway	(128, 129)

Inducing Tumor Cell Death and Differentiation	Effect mitochondria pathway	(130–132)
	Regulation cell cycle	(133–135)
Antibacterial Activity	Disruption of cell membranes and proteins responsible for essential function	(136, 137)
	Changing the external environment of bacteria	(138, 139)
	Effect on the mitochondria pathway	(140, 141)
Antiviral Activity	Damage the viral envelope and hinder the virus's ability to penetrate host cells	(142)
Immunomodulatory Effects	Direct interaction with immune cells Promoting antigen presentation Modulating the activation state of immune cells	(143–145)

1.5.3. Biosurfactants in Industrial Applications

Increased industrial activities and natural resource exploitation have caused significant environmental issues. Petroleum is widely used, leading to pollution like increased biological and chemical oxygen demands in soil and groundwater. Oil tank spills worldwide have damaged ecologically preserved areas (146). Natural alternatives to synthetic surfactants are needed, with microbial surface-active compounds (MSAC) emerging as potential options. MSAC can dissolve hydrocarbons, oils, and heavy metals, functioning as foam generators and emulsifiers (147). They help process and remove environmental contaminants like petroleum byproducts and polycyclic aromatic hydrocarbons (PAHs) (148, 149). Incorporating MSAC into environmentally friendly practices is crucial for mitigating pollution from industrial activities and resource exploitation (150). The environmental uses of biosurfactants rely on two main interaction mechanisms. Biosurfactants enhance substrate accessibility and, conversely, improve contact with the cell membrane by boosting its hydrophobic nature, enabling hydrophobic substances to engage with bacteria cells (151). Around 60–90% of petroleum substances can be broken down by bacteria, although this decomposition process may take a long time. Biosurfactants are utilized to improve the bioavailability of pollutants, a crucial factor in oil remediation efforts (152).

2. Aim and outline of this thesis

The lack of efficient medicines is growing and there is an urgent need to identify new ways to treat various health problems, such as infections caused by multi-resistant microorganisms and to combat numerous forms of cancer. The search for natural products is increasing, with Actinomycetota standing out for their ability to produce secondary metabolites with potential therapeutic applications. Biosurfactants, also natural compounds, have numerous applications in biomedicine, pharmaceuticals and also in industry, such as water treatments.

The purpose of this master's thesis was to explore the potential to produce bioactive compounds with antimicrobial and anticancer activity, and biosurfactants by Actinomycetota isolated from *R. graveolens*, which is well-known for its therapeutic uses. The specific objectives of this thesis are:

- **Phylogenetic identification of Actinomycetota strains isolated from *R. graveolens*:** Growth and taxonomic identification of strains previously isolated from different parts (leaves, stems, and roots) of *R. graveolens*.
- **Screening for antimicrobial activity:** Evaluation of the antimicrobial potential of isolated actinobacterial strains against Gram-positive and Gram-negative bacteria and a yeast.
- **Screening for anticancer activity:** Identification of Actinomycetota strains with potential anticancer properties by determining the cytotoxicity of the extracts against human cancer cell lines (HepG2) and non-cancerous cell lines (hCMEC/D3), using the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)) assay.
- **Screening for biosurfactant production:** Investigate the potential of the isolated strains to produce biosurfactants, using assays such as emulsification activity, drop collapse assay, oil spreading test, and interfacial tension measurements.
- **Dereplication of bioactive extracts and exploration of potential new compounds candidates:** Evaluation of chemical compositions in active extracts through LC/MS and molecular networking for potential new compound candidates. Combine results from assays on antimicrobial and anticancer activity and biosurfactant production with chemical analysis in order to identify potential new actinobacterial metabolites with medicinal/industrial value.

The thesis is organized into seven main sections. The introduction gives a comprehensive overview of the importance of Actinomycetota in drug discovery, with a focus on their association with medicinal plants. The aim and outline of this thesis contain the main goals and specific objectives of the study. The materials and methods section details the procedures for isolating, characterization and testing of actinobacterial strains. The results section presents the data obtained from antimicrobial, anticancer, and biosurfactants, as well as chemical analyses. The discussion interprets the results in the context of their potential applications in drug discovery. The conclusions summarize the key findings, and finally, the future perspectives suggest directions for further research based on the obtained results.

3. Materials and Methods

3.1. Sample and isolation of endophytic Actinomycetota

This work of this thesis is a continuation of the work previously started as part of the final project for the Bachelor's degree in Environmental Health, which led to the isolation of 56 bacterial strains. For better contextualization, a brief description of the procedure of isolation of these strains is included here.

A specimen of the plant *R. graveolens* was collected in May 2019 from a private garden in Vila Nova de Gaia, Portugal. The plant was dissected into three parts: stem, roots and leaves. Each part was then treated with distilled water and sonicated, and every damaged tissue or soil particle was excluded. The surface of the tissues was sterilised with 70% ethanol to eliminate epiphytic microorganisms. To prevent fungal growth, the tissue fragments were also placed in a 10% sodium hydrogen carbonate ($NaHCO_3$) and washed three times with distilled water. The surface sterilization's efficiency was assessed by culturing 100 μL of all final wash water and sterilized plant pieces on Plate Count Agar (PCA) and three selective cultured media for Actinomycetota isolation: Starch Casein Agar (SCN), Actinomycete Isolation Agar (AIA), and *R. graveolens* Extract Agar (RGEA). The three parts of the plant were also subjected to a second pre-treatment, consisting of the addition of 0.05 g calcium carbonate ($CaCO_3$) during maceration. After that, all the samples were plated into three media selective for Actinomycetota: AIA (per liter of distilled water): 4 g of sodium propionate, 0.5 g of K_2HPO_4, 0.2 g of Na_2CO_3, 0.366 g of $FeSO_4$, 0.1 g of L-Arginine, 0.4 g of $MgSO_4$ and 17 g of agar; SNC (per liter of distilled water): 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$,

0.01 g of $FeSO_4.7H_2O$ and 17 g of agar; RGEA (per liter of distilled water): 100 mL of plant and soil extract, obtained by washing plant fragments and soil with 500 mL of distilled water and 16 g of agar. All were supplemented with cycloheximide, nystatin, and nalidixic acid, to inhibit the growth of fungi and Gram-negative bacteria. The plates were incubated for 4 weeks at 28 ºC. During that period, the plates were regularly inspected through visual observation and each isolate was cryopreserved at -80 °C in 30% (*v/v*) glycerol. All the sample strains initially isolated on RGEA medium were subsequently cultured in AIA in order to obtain more biomass. Colonies exhibiting different morphological characteristics were carefully selected and repeatedly re-streaked on the same agar medium until pure colonies were obtained. Each morphologically distinct isolate on the culture plates was carefully observed, and its characteristics—including color, shape, size, presence of spores, mycelium formation, and pigment production—were recorded. The objective was to identify key actinobacterial traits, such as spore formation and mycelium production.

3.2. Taxonomic Identification of the Isolates

For taxonomic identification of the isolates, genomic DNA was extracted from all isolated microorganisms through the E.Z.N.A. bacterial DNA KIT (Omega Biotek, Norcross, GA). PCR amplification was performed with universal primers 27F (5'- GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-30') (153). The PCR mixture (total volume of 10 µL) contained: 5 µL of MYTaq Mix (Bioline,UK), 1 µL of primer 27F (2 µM), 1 µL of primer 1492R (2 µM) and 3 µL of DNA template. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s, 72 °C for 2 min and a final extension at 72 °C for 10 min.

A second approach was conducted for the isolates that did not perform well with MYTaq Mix (Bioline, UK) by using the Taq PCR Master Mix (Qiagen, Valencia, CA). In this case, the PCR conditions were initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s, 72 °C for 90 s and a final extension at 72 ºC for 10 min.

PCR products were separated on a 1.5% agarose gel containing SYBR Safe (ThermoFisher Scientific, USA), at 150 V for 30 min. The resulting 16S rRNA sequences were analyzed using Geneious software (version 11.1.4) and the consensus sequences were compared against the databases: 16S ribosomal RNA (Bacteria and Archaea) and nucleotide collection (nr/nt), both from NCBI BLAST, using the blastn algorithm. To complement the taxonomic evaluation of the isolates, a phylogenetic tree was elaborated. According to BLAST results, the three closest

neighbour sequences for each isolate were selected, choosing only organisms belonging to different species. Once the sequences were obtained for all isolates, a Geneious alignment was performed resulting in an alignment with 1400 bp. The phylogenic tree was made using the Maximum Likelihood method with 1000 bootstraps based on the Tamura-Nei model (154). The tree was constructed using the Molecular Evolutionary Genetics Analysis Program Version 11.0 (MEGA11).

3.3. Preparation of crude extracts

To prepare crude extracts for bioactivity assays, namely antimicrobial and anticancer activities, all Actinomycetota isolates were grown on their respective agar medium. The plates were incubated for 2 weeks at 28 ºC. A loopful of each isolate was used to inoculate 30 mL of the respective culture medium in 100 mL Erlenmeyer flasks. The flasks were incubated at 28 °C, 100 rpm, in the dark for 5 days. After this period, 0.5 g of Amberlite® XAD16N resin was added to the cultures and incubated for an additional 3 days to adsorb the compounds released into the medium (Figure 4-A). The biomass and resin were centrifuged at 3046 g for 5 min, washed three times with distilled water, and freeze-dried.

For chemical extraction, the freeze-dried biomass and resin were then extracted twice with a 1:1 ratio (v/v) mixture of methanol and acetone. The mixture was agitated for 30 min, and the liquid phase was collected through Whatman No. 1 filter paper (Figure 4-B). The organic extracts were dried in a rotary evaporator, dissolved in DMSO (dimethyl sulfoxide) to obtain stock solutions with final concentrations of 10 and 1 mg/mL, and used for bioactivity assays.

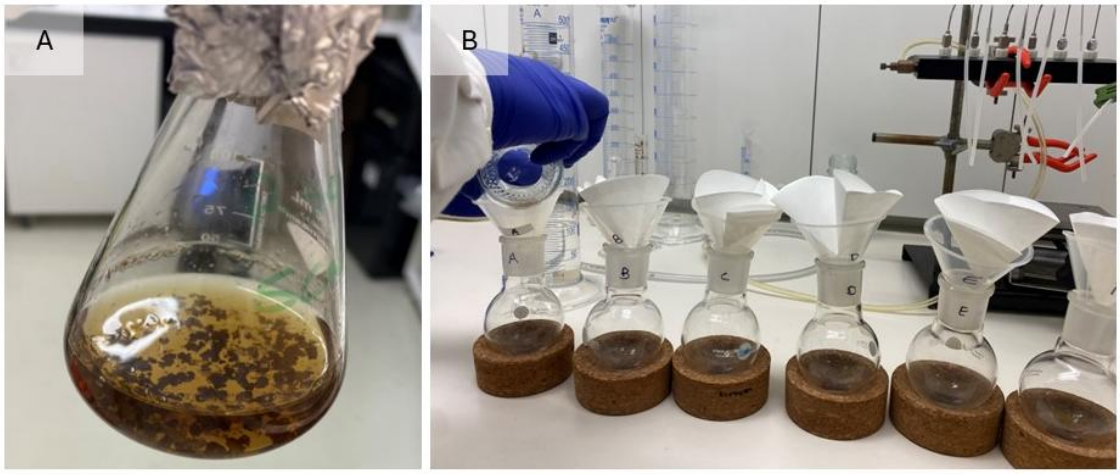


Figure 4. (A) Liquid cultures of selected actinobacterial isolates showing bacterial growth in Erlenmeyer flasks with the addition of Amberlite® XAD16N resin to the cultures.
(B) Preparation of crude extracts, illustrating the separation between the liquid phase and the biomass.

3.4. Bioactivity assays

3.4.1. Antimicrobial activity

The antimicrobial activity of the Actinomycetota extracts was determined using the agar-based disk diffusion method. Five reference microorganisms were used: two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Bacillus subtilis* (ATCC 6633); two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Salmonella typhimurium* (ATCC 25241); and one yeast, *Candida albicans* (ATCC 10231). The bacterial strains were cultured on Mueller-Hinton agar (MH; BioKar Diagnostics) and the yeast on Sabouraud Dextrose agar (SD; BioKar Diagnostics). Each microorganism was suspended in the corresponding liquid medium, and the turbidity was adjusted to 0.5 McFarland standard (OD_{625} = 0.08–0.13). The suspensions were then used to seed the agar plates (MH and SD) by evenly streaking them with a swab dipped in the inoculum cultures.

Sterile paper discs (6 mm diameter; Oxoid) were placed on the inoculated agar plates and loaded with 15 µL of each Actinomycetota extract at a concentration of 1 mg/mL. Negative controls consisted in discs filled with 15 µL of DMSO (1 mg/mL), while positive controls included enrofloxacin (1 mg/mL) for bacteria and nystatin (1 mg/mL) for yeast. The plates were incubated at 37 °C for 24 h, and the diameter of the halos was measured to determine a zone of growth inhibition. Each extract was evaluated in duplicate in independent assays.

3.4.2. Cytotoxic activity

Two cell lines were selected for this study (Figure 5): the liver hepatocellular carcinoma (HepG2) (Sigma-Aldrich (St. Louis, MO, United States)) cancer cell line and the non-cancer line human brain capillary endothelial cells (hCMEC/D3) (Sigma-Aldrich (St. Louis, MO, United States)). The two cell lines were cultured using routine procedures and exposed to Actinomycetota extracts for cytotoxicity evaluation. Cells were cultured in Dubelco's Modified Eagle Medium (DMEM) from Gibco (Thermo Fischer Scientific, Waltham, Massachusetts, USA) supplemented with 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 1% (v/v) penicillin/streptomycin (Biochrom) at 100 IU/mL and 10 mg/mL, respectively, and 0.1% (v/v) amphotericin (GE 23 Healthcare, Little Chafont, United Kingdom) The cells were incubated at 37 °C in a humidified atmosphere containing 5% of CO_2. A cell passage was performed every week when the cells hit 80-90% confluence.

The first procedure involved passaging the cells by removing the previous media and washing them with 2 mL of warm phosphate-buffered saline (PBS) (2x) (Gibco, Germany). Then, 1 mL of TrypLE express enzyme (1x) (Gibco, Denmark) was added to detach cells from the flask wall and incubated for 5 min. Following incubation, 5 mL of medium was added to deactivate the enzyme. The cell suspension was then transferred to a Falcon tube and centrifuged for 5 minutes at 1500 rpm. The medium was removed, and the pellet was resuspended in 1 mL of fresh medium. 20 µL of cell suspension were transferred to new culture flasks containing medium and incubated as previously described.

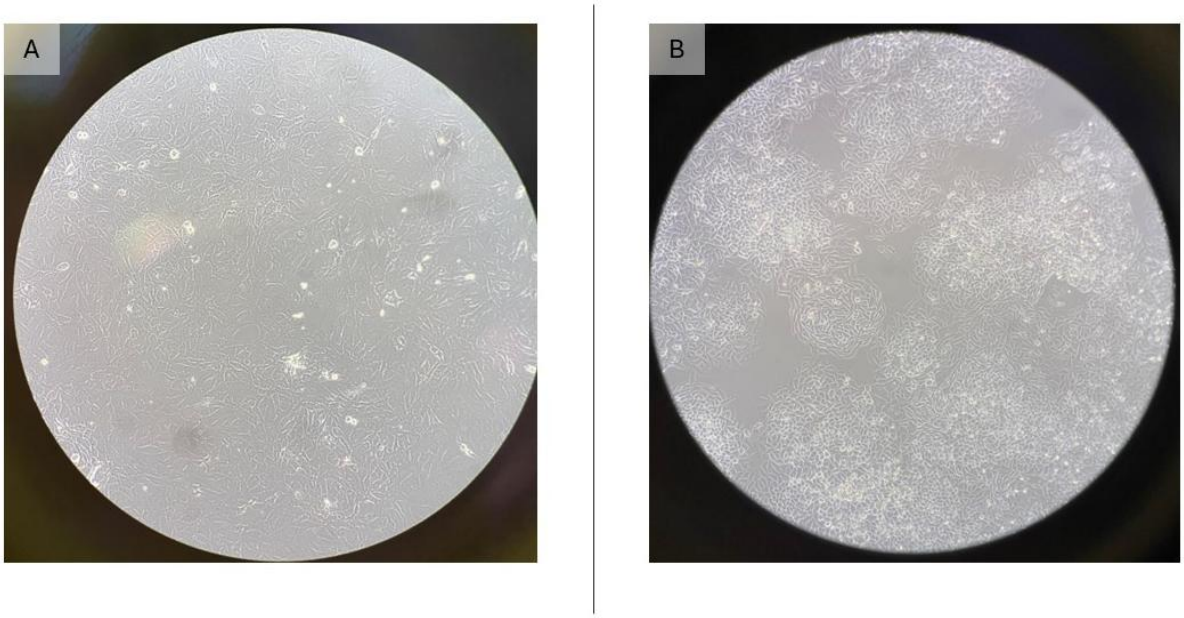


Figure 5. (A) Microscopic image of the HCMEC cell line at 40x magnification; **(B)** Microscopic image of the HELPG2 cell line at 40x magnification.

For the cell viability assay (Figure 6), cells were seeded in 96-well culture plates at a density of $3.3x10^4$ cells/mL. After 24h of incubation, the cells were treated with 100 μL of fresh media supplemented with the crude extracts at a final concentration of 15 μg/mL. Positive controls consisted of 20% DMSO while 5% DMSO was used as the negative control. Cellular viability was assessed after 48 h of exposure by adding 20 μL of MTT (Sigma-Aldrich, MO, USA) to each well at a final concentration of 0.2 mg/mL. After 3-4 h of incubation, the medium was carefully aspirated, and the insoluble formazan salts were dissolved in 100 μL DMSO. Absorbance was measured at 550 nm (Synergy HT, Biotek, USA) Cellular viability was expressed as a percentage relative to the negative control. The assays were performed in triplicate.

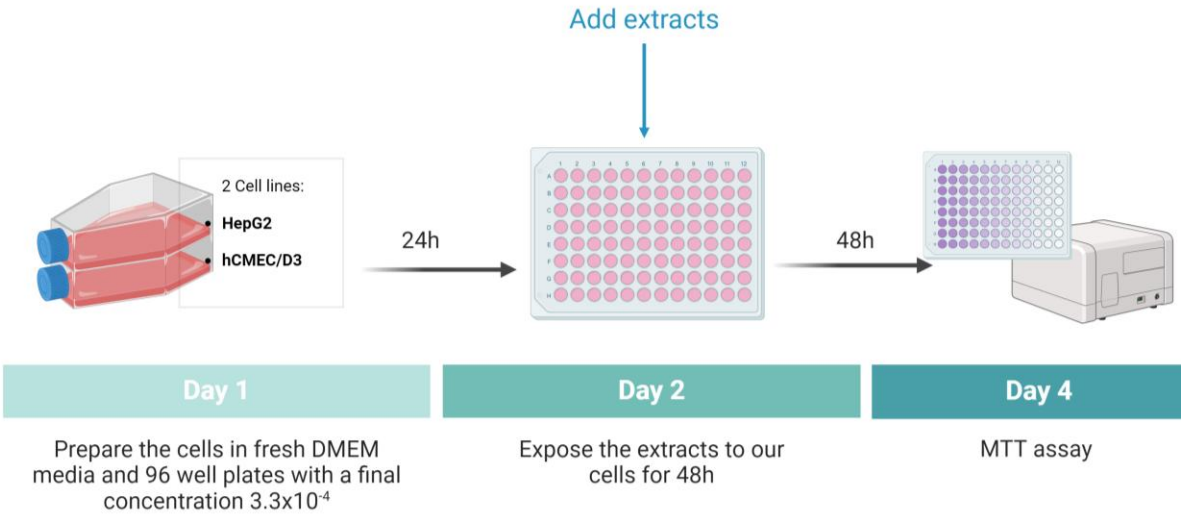


Figure 6. Illustration of the cell viability assay performed on two cell lines, HepG2 and hCMEC/D3, using the MTT assay.

3.4.3. Statistical analysis

Results from the cytotoxic assays were statistically analysed by comparing the results obtained in the extracts and the solvent control. Firstly, the Kolmogorov–Smirnov test was used to verify the normality distribution of each data, and Bartlett's test for equal variances. For parametric data, one-way ANOVA was applied. Since none of the data followed normality, nonparametric tests were performed. For nonparametric data, the Kruskal–Wallis test was used followed by Dunn's multiple comparison test. The significance level established for all tests was at $p < 0.05$.

3.5. Quantification and detection of biosurfactants

3.5.1. Growth of Actinomycetota strains for biosurfactants screening

The previously isolated Actinomycetota strains were cultured in their respective agar media. The growth conditions were optimized for biosurfactant production using Kim's broth, which contained the following components per liter of distilled water: 1 g $NaNO_3$, 0.1 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, and 0.2 g yeast extract. Each isolate was inoculated into 100 mL Erlenmeyer flasks containing 30 mL of Kim's medium supplemented with 3% filtered olive oil as a hydrophobic carbon source. The flasks were incubated at 28 °C and 100 rpm in the dark for two weeks. On the same day, 3 mL samples were taken from each flask and stored at -20 °C to compare the initial values with the final values on the day of culture collection. After the

incubation period (Figure 7), the cultures were centrifuged at 6500 rpm for 5 min and the supernatants were transferred to new falcon tubes and stored at -20 °C.

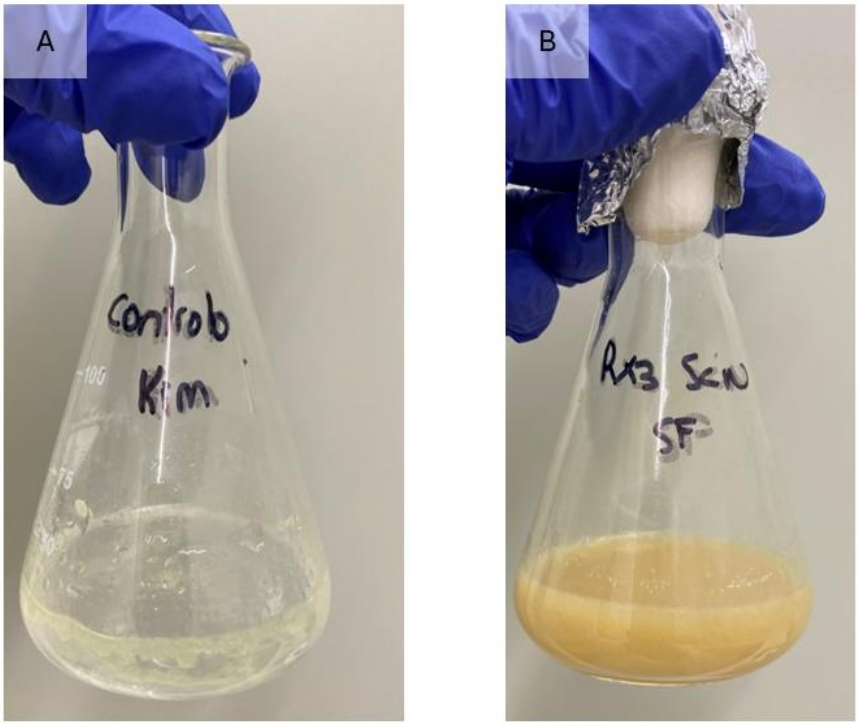


Figure 7. (A) Positive control: Kim's broth with 3% filtered olive oil. **(B)** Growth of the Actinomycetota strain RX3 with 3% filtered olive oil after two weeks of incubation at 28 °C.

3.5.2. Emulsification activity assay (E24)

The assay was conducted in test tubes containing 2 mL of olive oil and 2 mL of each sample. For positive and negative controls, the sample was substituted by 2 mL of Triton X-100 (Liofilchem, Italy) solution at a concentration of 1 mg/mL, a known chemical surfactant, and Kim's medium, respectively. The contents of the tubes were then vortexed at high speed for 2 min to achieve complete mixing and emulsification. After vortexing, the test tubes were kept undisturbed at room temperature for 24 h to allow the emulsified layers to settle (Figure 8).

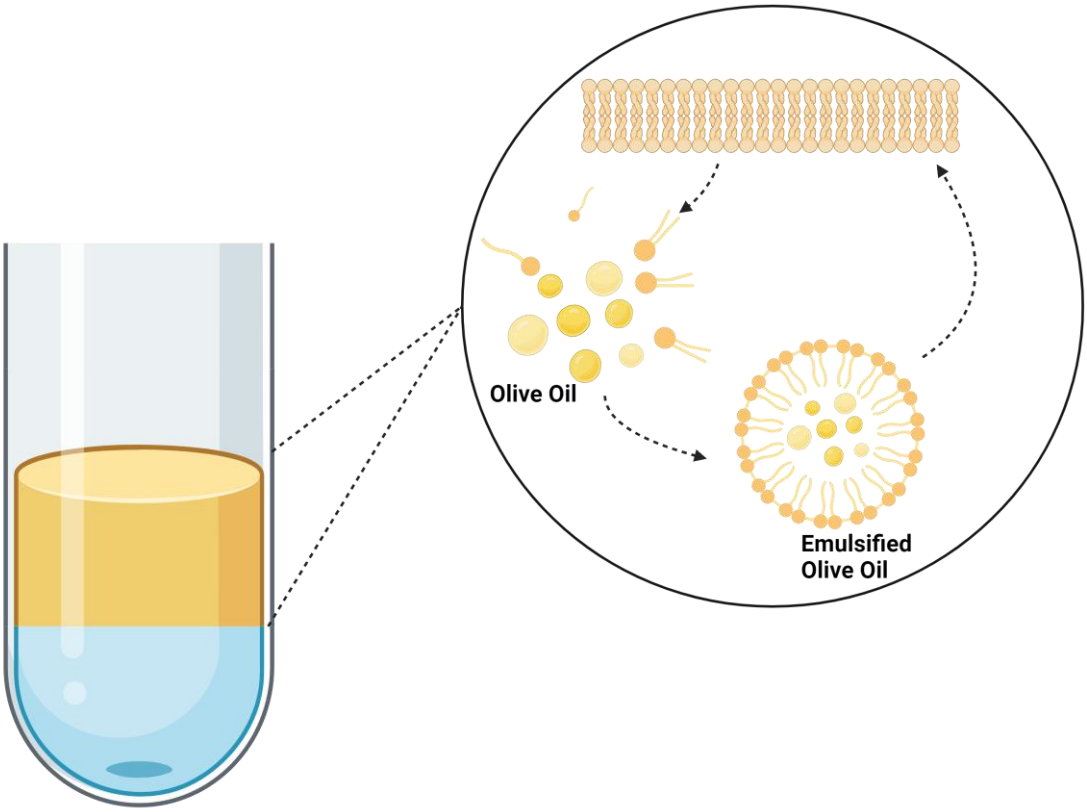


Figure 8. Illustration of the emulsification activity assay. The process shows how olive oil is captured, leading to the formation of an emulsion.

After this period, the height of the emulsified layer and the total height of the liquid in each test tube were carefully measured. This calculation provided a percentage number indicating the emulsification activity for each sample and control. The emulsification activity was estimated using the formula:

$$\text{Emulsification Activity (\%)} = \frac{\text{Height of the Emulsified Layer}}{\text{Total Height of the Liquid Column}} \times 100$$

3.5.3. Oil spreading test

The bottom of a Petri dish was initially filled with distilled water. A drop of olive oil, dyed with Sudan Black B (Biochem, France) for improving visualization of the result, was then slowly dripped on the surface of the water. Next, a drop of cell-free supernatant was dropped on the oil's surface. A positive result (cell-free culture supernatant with biosurfactant activity) consisted of the displacement of the oil and the formation of an oil-free halo, with the diameter of this halo reflecting the biosurfactant's activity (Figure 9-A). Triton X-100 was used as a positive control and Kim's broth as a negative control.

3.5.4. Drop collapse method

A drop of paraffin (Sigma-Aldrich, MO, USA) was first added to the lid of a 96-well plate, followed by a drop of cell-free culture supernatant placed on top of the paraffin drop. After allowing the drops to sit for 1 min, the shape of the supernatant droplet was carefully observed using a magnifying glass. The results were considered positive for biosurfactant production if the supernatant droplet collapsed (Figure 9-B). To validate the assay, Triton X-100 (Liofilchem, Italy) solution at a concentration of 1 mg/mL, was used as a positive control, while Kim's broth was used as a negative control.

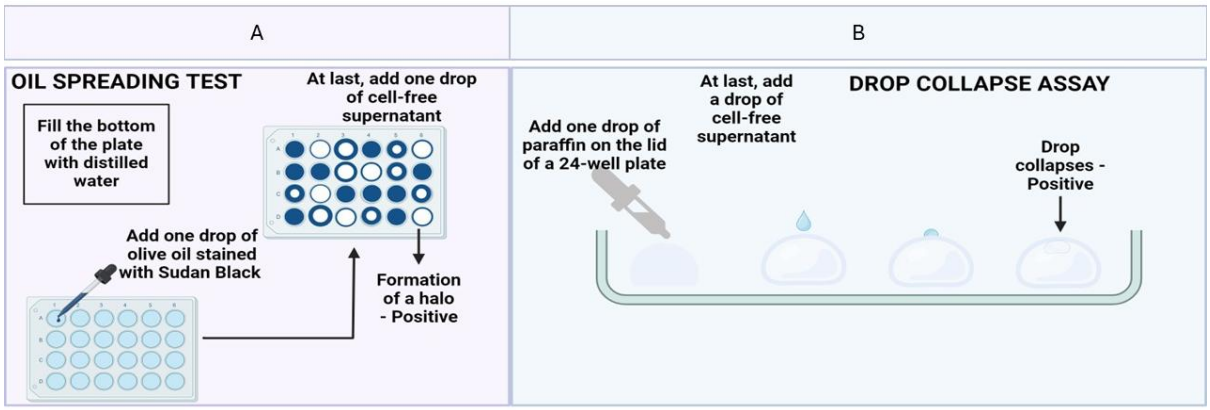


Figure 9. (A) Illustration of the oil spreading test. **(B)** Illustration of the drop collapse assay.

3.5.5. Interfacial tension test

In addition to the assays conducted in this study, the interfacial tension test was also performed as further evidence of biosurfactant production. Due to the requirement for specialized equipment, this test was carried out at i3S, in the platform Biointerfaces and Nanotechnology.
The test was performed using a video-based optical contact angle measurement device OCA 15 plus, provided with an electronic syringe unit (Dataphysics Instruments GmbH, Germany). The measurements were carried out around 20 ºC. The samples used for the studies were kept at 5 ºC before analysis. The liquid surface tension of the samples was measured by the pendent drop method with droplets of 4 mL of the sample being dispensed and video imaged. At least three drops were made per sample type. Liquid surface tension was calculated using SCA20 software (Dataphysics, version 2.0).

3.5.6. Biosurfactant liquid–liquid extraction

Before the biosurfactant extraction, the strains were grown in 30 mL of Kim's medium supplemented with 3% filtered olive oil as a hydrophobic source. The flasks were incubated at 28 °C and 100 rpm in the dark for two weeks. Following culture extraction, the supernatant was separated and used for surfactant extraction. To extract the biosurfactants, the pH of the cell-free supernatant was adjusted to 2 using a 6 N HCl solution (CHEMLAB-ANALYTICAL bvba, Belgium) and refrigerated at 4 °C overnight. The biosurfactant was extracted using a 2:1 solution of chloroform and methanol, with a final concentration of 1 mg/mL (155, 156). The chloroform-methanol solution was added to the cell-free broth in equal parts and aggressively agitated for 10 min. The mixture was then left undisturbed for 20 min to allow the phases to settle. After settling, the upper phase (aqueous/methanol phase) was carefully pipetted into a new beaker for disposal, while the lower phase containing the chloroform, and the compound of interest was retained. This extraction procedure was repeated three times. The collected organic extracts were then dried using a rotary evaporator.

3.5.7. Dereplication analysis

The Actinomycetota extracts exhibiting cytotoxicity activity and biosurfactant production were submitted to mass spectrometry MS-based dereplication and molecular networking analysis. Bioactive extracts were dissolved in methanol at a final concentration of 2 mg/mL, while the biosurfactant-producing extracts were dissolved in isopropanol at the same concentration. Both samples were analysed using liquid chromatography-high resolution electrospray ionization tandem mass spectrometry (LC-HRESIMS/MS) to identify any new bioactive compounds in the crude extracts. The chromatographic step was conducted under the same conditions as described by Ribeiro et al. (157). The raw data obtained from MS-based dereplication was converted to mzML format and submitted to the Global Natural Products Social Molecular Networking (GNPS) platform for analysis to determine if the extracts displayed any significant matches that could elucidate the observed activities (158). Three dereplication analyses were conducted using the Insilico Peptidic Natural Product Dereplicator, Dereplicator VarQuest, and Dereplicator+, with default parameters, except for setting ion mass precursor tolerance and fragment ion mass tolerance to 0.005 Da. A p-value threshold of $\leq 10^{-10}$ was applied for both DEREPLICATOR and DEREPLICATOR VarQuest, along with a rigorous value of 15 for

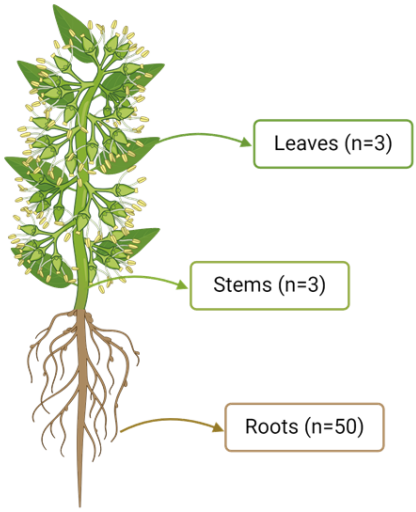
DEREPLICATOR+, which was implemented to reduce false matches. All these matches were manually checked to see if they were associated with compounds produced by Actinomycetota or plant sources, and any that did not meet these criteria were discarded (159, 160).

In addition, a molecular network was constructed using the GNPS data analysis workflow, applying default parameters (except for the precursor ion mass tolerance and fragment ion mass tolerance, which were set to 0.02 Da). The molecular network was imported into Cytoscape v3.8.2 to visualize sets of spectra from related molecules and the distribution of m/z clusters among Actinomycetota extracts. For this analysis, we focused exclusively on unique single-strain m/z clusters and manually inspected the corresponding LC-HRESIMS chromatograms to determine the mass of each target metabolite, including the parent mass and adducts. The calculated masses based on m/z values of main compounds were entered into the Dictionary of NP (version 27.1, CRC Press, Abingdon, UK) to verify if they matched with compounds previously identified from Actinomycetota.

4. Results

4.1. Taxonomic identification of Actinomycetota isolated from Ruta graveolens

Under the scope of a previous final project for the Bachelor's degree in Environmental Health, 56 strains were isolated from the medicinal plant *R. graveolens*, using three selective media for Actinomycetota. Most of the isolates were obtained from the roots (n=50), followed by the leaves (n=3), and stems (n=3) (Figure 10). Many isolates exhibited diverse morphological characteristics, with several strains displaying typical traits of Actinomycetota, such as slow growth, growth inside the agar, and the production of spores and pigments (Figure 11). The incubation period for these isolates ranged from four to six weeks, depending on their origin.



Isolates From *Ruta graveolens*					
LEAVES	STEMS	ROOTS			
F7	C6	R1	R43	R77	R101
Fx1	Cx1	R4	R51	R78	R102
Fx2	Cx2	R7	R57	R79	R109
		R8	R58	R81	R111
		R9	R60	R83	R113
		R17	R62	R84	REP12
		R18	R63	R85	RX3
		R22	R65	R86	RX4
		R24	R66	R87	RX5
		R32	R67	R88	RX9
		R35	R69	R89	RX10
		R38	R71	R92	
		R39	R72	R95	

Figure 10. Illustration of all isolates found in the leaves, stems and roots of *Ruta graveolens.* The accompanying table provides the names of each isolate.

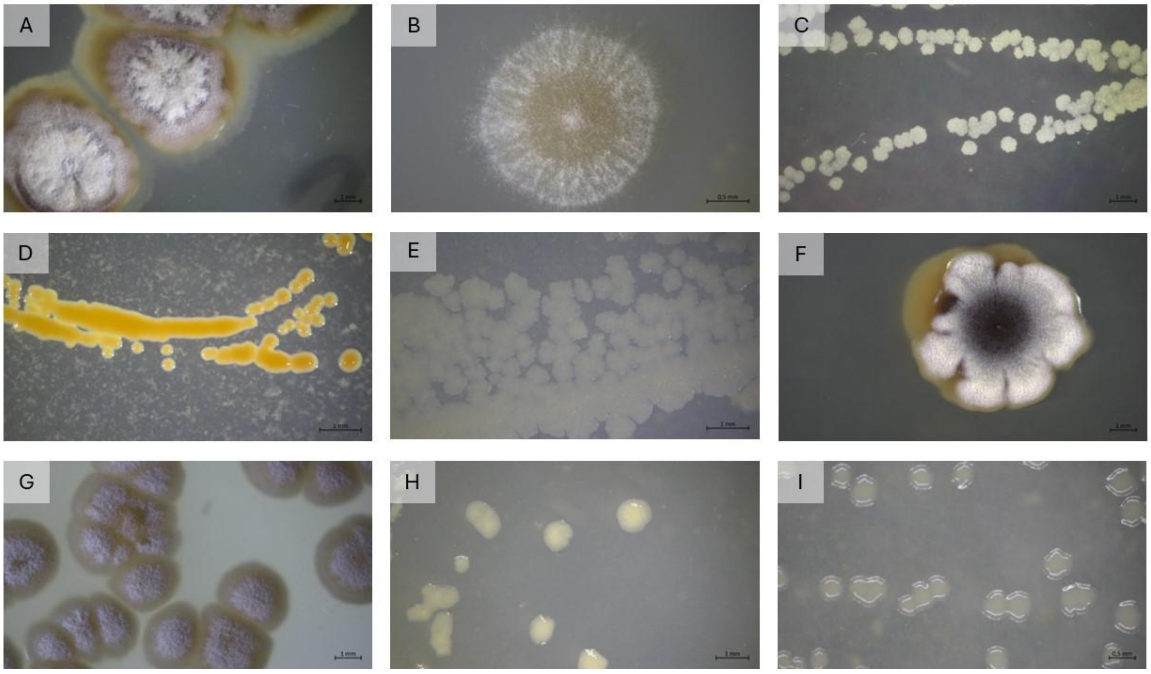


Figure 11. Morphological diversity of some actinobacterial strains isolated from *R. graveolens*: **(A)** Strain R43, **(B)** Strain R95, **(C)** Strain R85, **(D)** Strain R78, **(E)** Strain R51, **(F)** Strain R39, **(G)** Strain R8, **(H)** Strain R92 and **(I)** Strain R66.

Of the isolated strains, 36 were taxonomically identified, through 16S rRNA gene sequencing, as belonging to the Actinomycetota phylum (Table 3). The remaining strains were classified within the phyla Pseudomonadota and Bacteroidota.

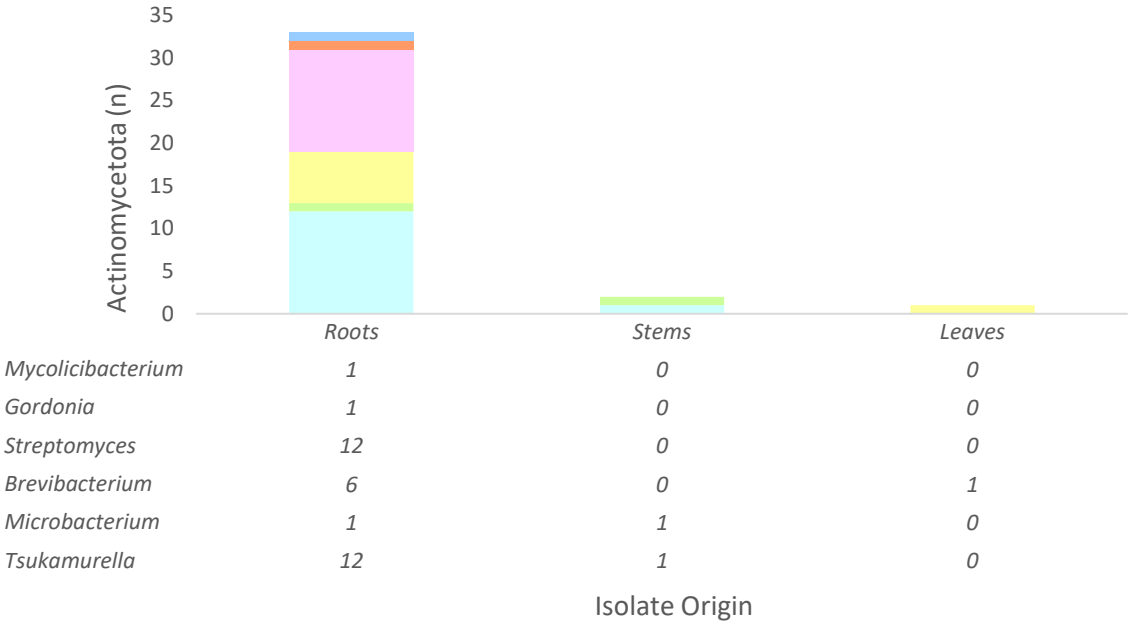
Table 3. Taxonomic identification of the isolates recovered from the *Ruta graveolens*. Different colours in the Table refer to microorganisms belonging to different species.

ISOLATE	ISOLATION MEDIUM	PHYLUM	CLOSEST IDENTIFICATION	SIMILARITY % *
R17	AIA	Actinomycetota	*Tsukamurella* sp.	92.26
R24	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.78
R1	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.86
R67	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.86
RX5	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.78
R85	SCN	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.93
R51	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.93
RX4	AIA	Actinomycetota	*Tsukamurella ocularis*	99.86
RX3	SCN	Actinomycetota	*Tsukamurella ocularis*	99.78
R71	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.86
CX1	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.93
R109	AIA	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.98
R84	SCN	Actinomycetota	*Tsukamurella tyrosinosolvens*	99.57
R83	SCN	Actinomycetota	*Microbacterium ginsengiterrae*	98.21
C6	AIA	Actinomycetota	*Microbacterium ginsengiterrae*	97.53
R77	AIA	Actinomycetota	*Brevibacterium sediminis*	99.78
R4	AIA	Actinomycetota	*Brevibacterium sediminis*	99.79
R65	AIA	Actinomycetota	*Brevibacterium sediminis*	99.86
R92	SCN	Actinomycetota	*Brevibacterium sediminis*	99.86
FX2	AIA	Actinomycetota	*Brevibacterium sediminis*	99.71
R9	AIA	Actinomycetota	*Brevibacterium sediminis*	99.86
R88	AIA	Actinomycetota	*Brevibacterium sediminis*	99.86
R39	SCN	Actinomycetota	*Streptomyces umbrinus*	99.50
R43	SCN	Actinomycetota	*Streptomyces umbrinus*	99.86
R62	AIA	Actinomycetota	*Streptomyces mayteni*	99.86
R95	SCN	Actinomycetota	*Streptomyces novaecaesareae*	99.21
R8	AIA	Actinomycetota	*Streptomyces umbrinus*	99.43
R7	AIA	Actinomycetota	*Streptomyces umbrinus*	99.50
R79	AIA	Actinomycetota	*Streptomyces umbrinus*	99.36
R57	AIA	Actinomycetota	*Streptomyces canus*	99.78
R38	AIA	Actinomycetota	*Streptomyces aldersoniae*	99.28
R18	AIA	Actinomycetota	*Streptomyces umbrinus*	99.07
R60	AIA	Actinomycetota	*Streptomyces umbrinus*	99.57
R22	AIA	Actinomycetota	*Streptomyces umbrinus*	99.50
R78	AIA	Actinomycetota	*Gordonia hydrophobica*	97.98
R66	AIA	Actinomycetota	*Mycolicibacterium llatzerense*	99.85

FX1	AIA	Pseudomonadota	*Advenella kashmirensis* subsp. *methylica*	99.72
R32	AIA	Pseudomonadota	*Advenella kashmirensis* subsp. *methylica*	99.79
R86	SCN	Pseudomonadota	*Advenella kashmirensis* subsp. *methylica*	99.72
R69	AIA	Pseudomonadota	*Advenella kashmirensis* subsp. *methylica*	99.79
R87	AIA	Pseudomonadota	*Methylobacterium* sp. BJ001	99.56
CX2	AIA	Pseudomonadota	*Methylobacterium* sp. BJ001	99.63
R102	SCN	Pseudomonadota	*Bosea vestrisii* 34635	99.70
R45	SCN	Pseudomonadota	*Bosea vestrisii* 34635	99.78
R58	AIA	Pseudomonadota	*Bosea vestrisii* 34635	99.70
RX10	AIA	Pseudomonadota	*Bosea vestrisii* 34635	99.78
R63	AIA	Pseudomonadota	*Variovorax beijingensis*	99.93
R81	AIA	Pseudomonadota	*Inquilinus ginsengisoli*	98.82
R113	AIA	Pseudomonadota	*Inquilinus ginsengisoli*	98.88
F7	AIA	Pseudomonadota	*Phyllobacterium loti*	98.40
R72	AIA	Pseudomonadota	*Rhizobium giardinii*	93.58
R35	AIA	Pseudomonadota	*Rhizobium herbae*	99.77
R101	SCN	Bacteroidota	*Sinomicrobium oceani*	99.64
RX9	SCN	Bacteroidota	*Sinomicrobium pectinilyticum*	96.27
R89	SCN	Bacteroidota	*Sinomicrobium oceani*	99.63

*The similarity values were obtained using BLAST with the 16S gene from the NCBI database

The recovered isolates were distributed by six Actinomycetota genera: *Microbacterium, Brevibacterium, Streptomyces, Gordonia, Mycolicibacterium* and *Tsukamurella* with the largest fraction of isolates being assigned to the last genus. The majority of the isolates were obtained from the roots of *R. graveolens* (Figure 12).



	Roots	Stems	Leaves
Mycolicibacterium	1	0	0
Gordonia	1	0	0
Streptomyces	12	0	0
Brevibacterium	6	0	1
Microbacterium	1	1	0
Tsukamurella	12	1	0

Figure 2. Correlation of *Actinomycetota* species obtained (36) with the origin of *Ruta graveolens*, including roots, stems, and leaves.

In addition, the obtained isolates consistently clustered with species already described in the literature (Figure 13). The analysis of the 16S rRNA gene showed similarity values that fell below 98.7%, which is the threshold for identifying a new species (161). This limit is determined by the idea that species with less than 98.7% similarity in their 16S rRNA gene sequences are sufficiently genetically different to be classified as distinct species. Therefore, these results indicate that some strains have a percentage of similarity below the threshold (R17, R83, C6, R78 and R72), where the sequencing results need to be confirmed. However, other results indicate that strains R81, R113, F7 and Rx9 could potentially be classified as new species, as they exhibit less than the designated similarity threshold. In the phylogenetic tree of Figure 13, strains R83 and C6 cluster with a bootstrap value of 100%, showing a close evolutionary relationship. This supports the idea that they belong to the same species in the genus *Microbacterium*.

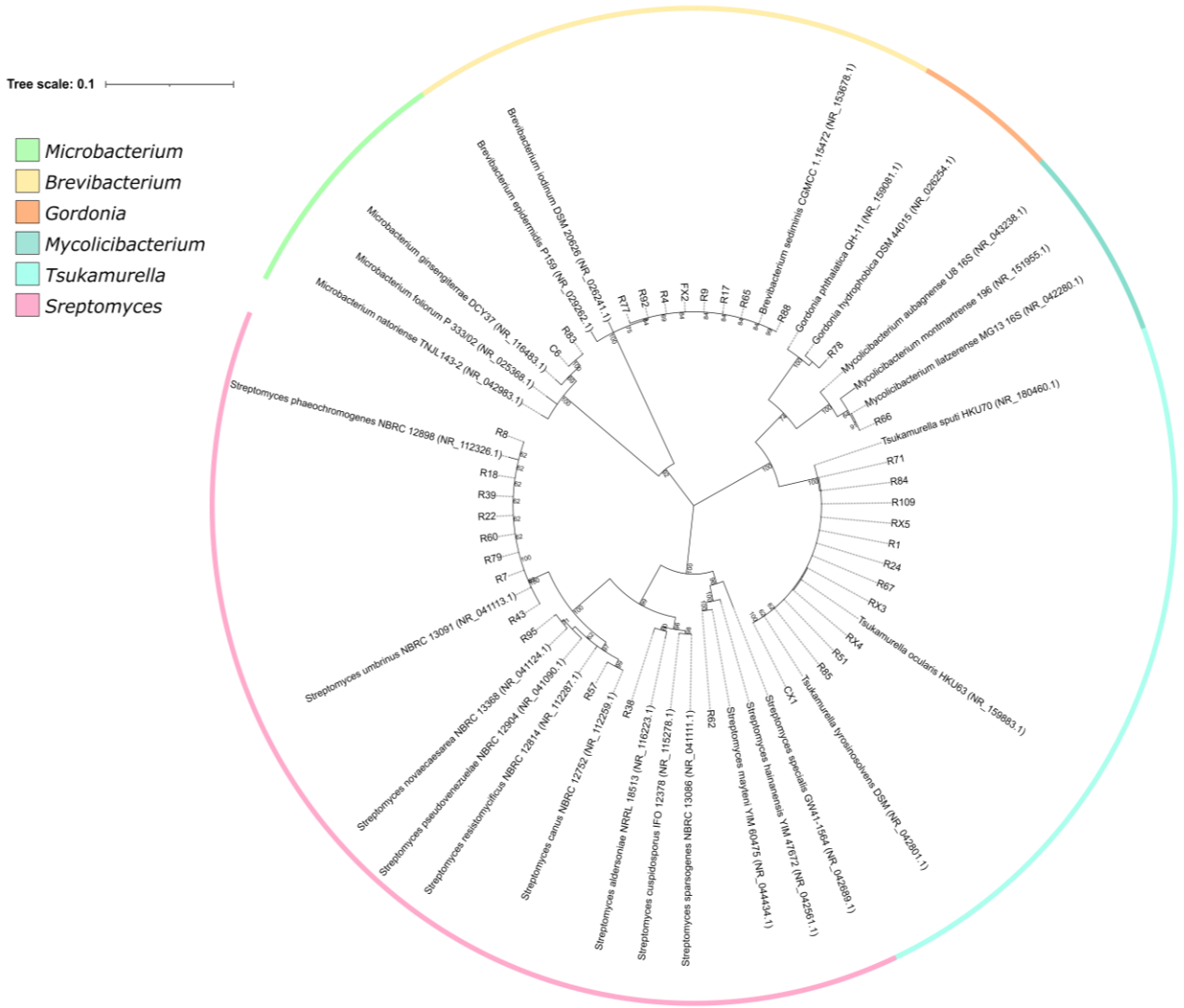


Figure 13. Phylogenetic relationship of the 36 Actinomycetota isolates recovered from *Ruta graveolens*, based on 16S rRNA gene homology with their GenBank nearest neighbours.

4.2. Screening of the bioactive potential of the isolated Actinomycetota strains

The bioactive potential of all Actinomycetota strains isolated from *R. graveolens* was screened by preparing crude extracts from liquid cultures of these strains and testing them for their antimicrobial and anticancer activities and biosurfactant production.

4.2.1. Antimicrobial activity

The potential of all Actinomycetota strains isolated in this study to produce compounds with antimicrobial activity was investigated using five reference strains: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Candida albicans*. However, none of the 36 extracts tested showed antimicrobial activity against these strains.

4.2.2. Cytotoxic activity

For the investigation of anticancer activity, the percentage of cell viability after exposure to Actinomycetota crude extracts (15 µg/mL) for 48 h was measured in two cell lines. The results showed that the crude extracts of 11 actinobacterial isolates presented cytotoxic activity against at least one of the cell lines tested (Annex 1 and 2).

Strains R24, R9, R8, R4, Rx5, Cx1, R84, R38, and R51 exhibited notable cytotoxic activity, reducing cell viability by 20–30% in the HePG2 cancer cell line. Among these strains, R24, Rx5, Cx1, R84, and R51 belong to the genus *Tsukamurella*, R9 and R4 to the genus *Brevibacterium*, and R8 and R38 to the genus *Streptomyces*. Strain R84 was the most promising, showing no inhibition in the normal cell line, but some inhibition in the cancer cell line. Additionally, the strain R95, from the genus *Streptomyces*, demonstrated a significant cytotoxic effect by reducing cell viability by 60% in the HepG2 cancer cell line, suggesting a strong antitumor activity compared to other strains tested (Figure 14–A).

In the non-cancer hCMEC/D3 cell line, three strains (R9, Rx5 and R18) showed a moderate reduction in cell viability by 25–30%. The strain R18 belongs to the genus *Streptomyces*. The strains R51 and R95 demonstrated stronger cytotoxic effects in the non-cancer hCMEC/D3 cell line, reducing cell viability by 40 and 60%, respectively (Figure 14–B).

The high cytotoxic activity released by strain R95 displays strong cytotoxic properties on both cancerous and non-cancerous cells, suggesting promising advantages for cancer therapy.

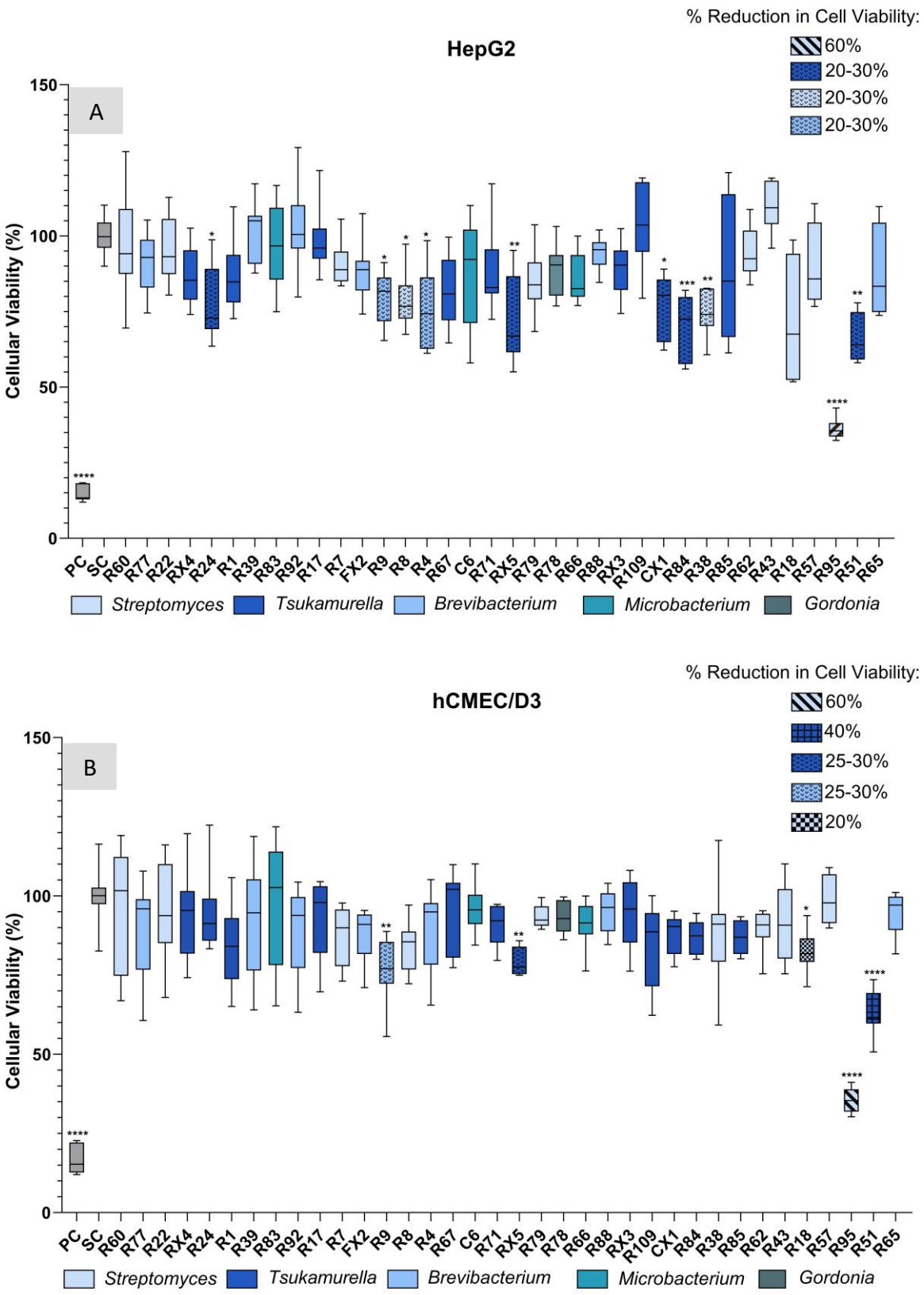


Figure 14. Actinomycetota crude extracts tested against cell lines HepG2 **(A)** and hCMEC/D3 **(B)**. SC and PC indicate solvent and positive control, respectively. Values are presented as mean ± standard deviation from two independent assays conducted in triplicate and significant

differences compared to the solvent control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

4.3. Screening of Biosurfactants Production

4.3.1. Emulsification activity assay (E24)

Sixteen out of the 36 Actinomycetota strains showed emulsification activity. All results were compared with a positive control consisting of Triton X100 reagent (1 mg/ml) and a negative control consisting of Kim's broth. The emulsification activity was estimated using the previously mentioned E_{24} formula. The results revealed varied levels of emulsification activity, with 8 samples displaying high emulsification activity (>35%), 7 showing moderate activity (25–35%) and one showing low/moderate activity (<25%) (Figure 15). The majority of the strains that revealed positive activity were affiliated with the genera *Tsukamurella* (10 strains) and *Streptomyces* (5 strains), with one strain 20 belonging to the genus *Microbacterium*.

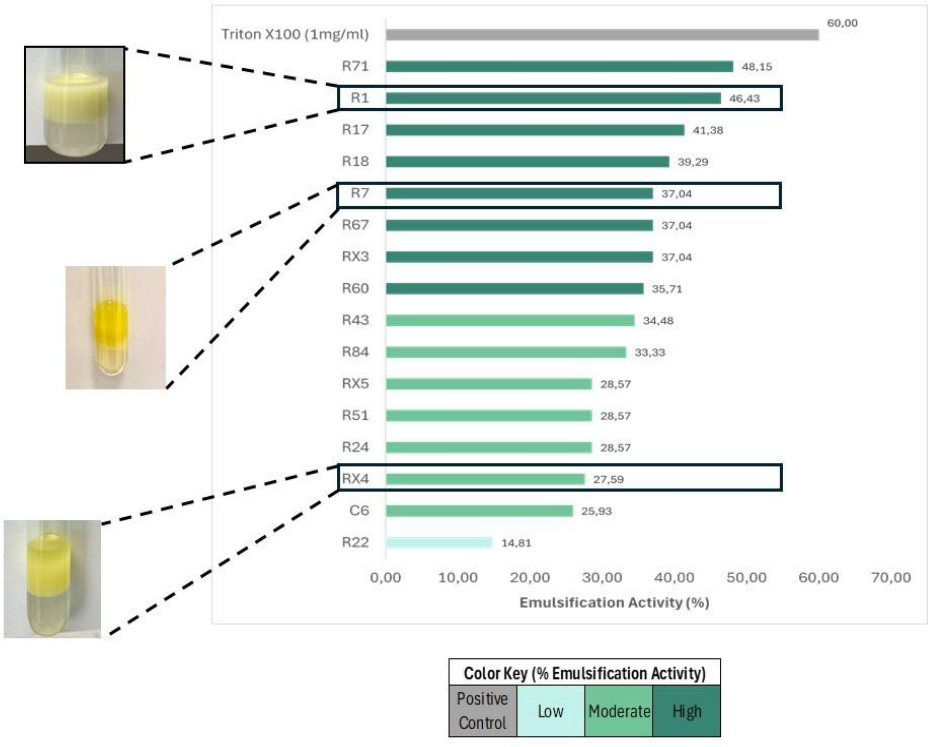


Figure 15. Emulsification activity of the 16 Actinomycetota strains isolated from *Ruta graveolens* that showed a positive result.

4.3.2. Drop collapse method

In the drop collapse assay, 18 isolates showed positive results, indicating that these isolates have the potential to produce biosurfactants. The supernatants of the isolates R71, R1, R17, R67, Rx3, R84, Rx5, R51, R24, and Rx4, showed a very strong drop collapse effect, similar to the positive control Triton X100, indicating that these strains may be producing extracellular biosurfactants (Figure 16). Distilled water, which served as the negative control, showed no drop collapse effect. Six supernatants exhibited moderate activity (R43, R109, R85, Cx1, R66 and R79) and two supernatants showed low activity (C6 and R78).

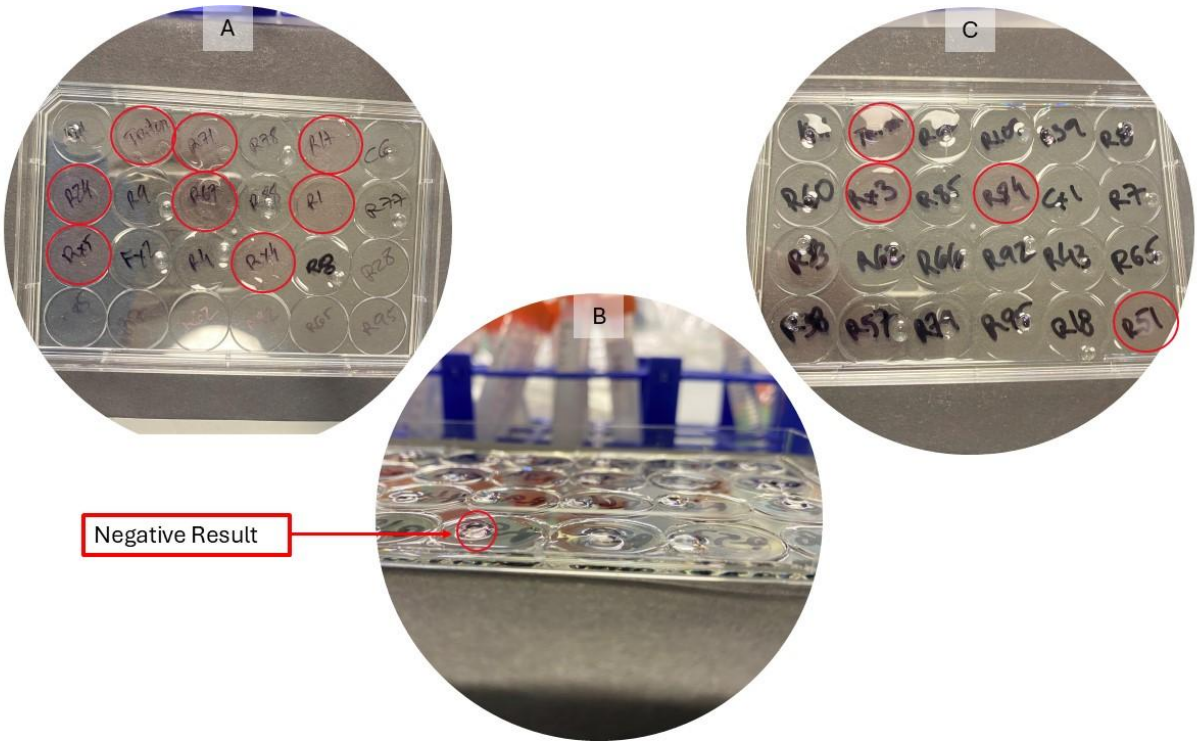


Figure 16. Demonstration of results from the drop collapse method, comparing with two controls: a positive control (Triton X-100) and a negative control (Kim's broth). Images A and C are examples of positive results marked in red, indicating the drop collapsed on paraffin. B- negative results, where the drop remains visible.

The strains that exhibited positive activity were affiliated with the Actinomycetota genera *Tsukamurella* (13 strains), *Streptomyces* (two strains), *Microbacterium* (one strain), *Gordonia* (one strain), and *Mycolicibacterium* (one strain).

4.3.3. Oil spreading test

The biosurfactant addition expanded the droplet, breaking the oil film to create an oil-free zone. The dispersion measured indicated efficacy, with high activity showing large spreading, moderate activity with limited spreading, and low activity with little spreading. Results showed high activity in 12 Actinomycetota supernatants, moderate activity in four supernatants, and low activity in 11. Strains Rx3, R84, R43, R79, R18, R51, R71, R17, R24, R67, Rx5 and Rx4 showed high activity, producing results similar to the positive control Triton X100 (Figure 17). The strains R66, R88, R77 and R4 showed moderate activity. The strains that showed low activity (R22, R109, R39, R85, Cx1, R7, R95, C6, R9, R1 and Fx2) produced an effect closer to the negative control (Kim's broth), but despite appearing inactive, the halos they formed were larger than those in the positive control.

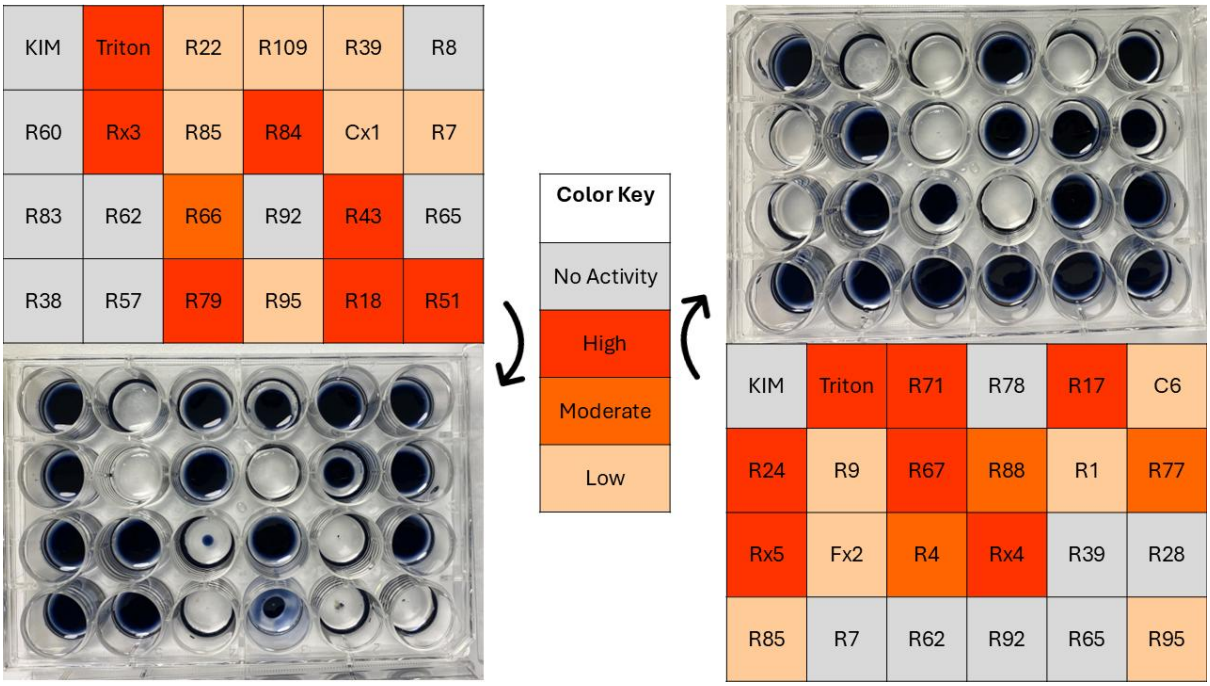


Figure 17. Results of the oil spreading test, with a colour key indicating activity levels: high, moderate, and low.

The majority of the Actinomycetota strains exhibiting positive results were associated with the genera *Tsukamurella* sp. (13 strains), *Streptomyces* (seven strains), *Brevibacterium* sp. (five strains), *Microbacterium* (one strain) and *Mycolicibacterium* (one strain).

4.3.4. Interfacial tension test

The lower the interfacial tension of a biosurfactant, the greater its surfactant activity, indicating a stronger capacity to decrease the cohesive forces at the interface between immiscible phases. In the interfacial tension test, six isolates (R1, R17, R71, R67, R84 and Rx5) were able to reduce the interfacial tension to a value below 30 mN/m, demonstrating high activity in reducing the tension between the two immiscible liquids. Three other isolates (Rx3, R51 and R66) reduced the interfacial tension to a value below 40 mN/m. Although this indicates less efficacy than those strains with <30 mN/m, it is still considered high activity. (Figure 18). The culture medium KIM showed an interfacial tension value of 70 mN/m, which is very close to the normal value of water (71 mN/m), indicating the absence of biosurfactants.

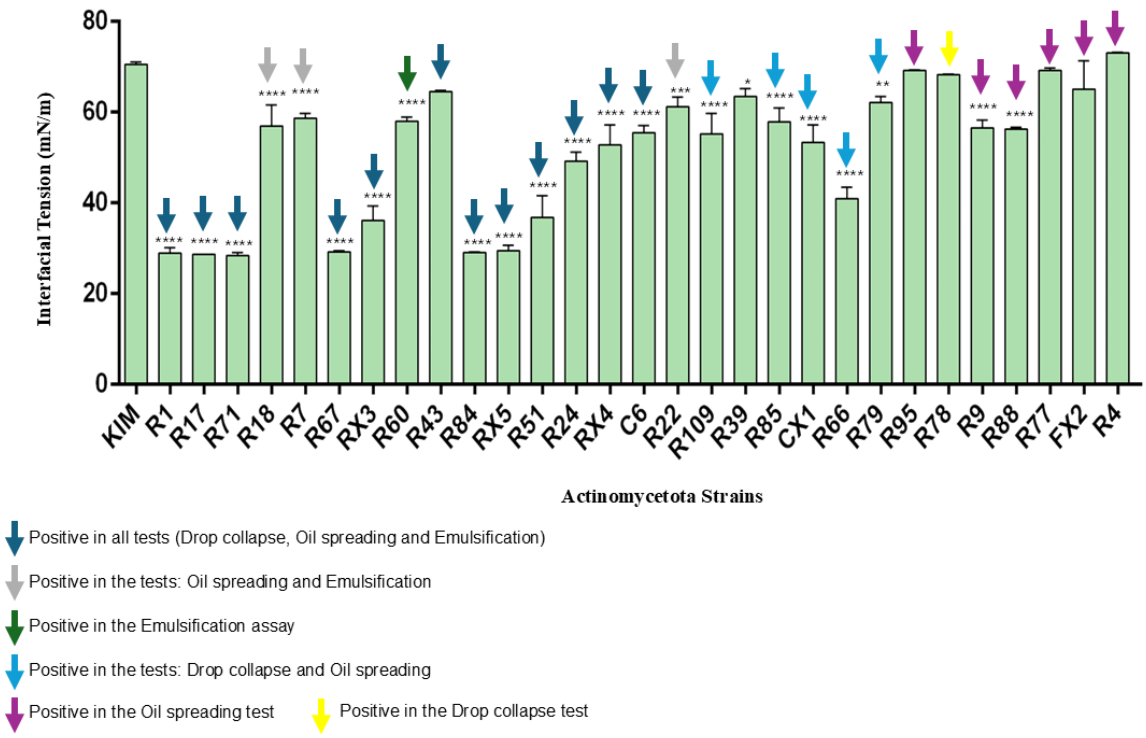


Figure 18. Interfacial tension measured in 29 actinobacterial strains isolated from *Ruta graveolens*. The arrows represent the three different assays used to test the biosurfactant activity (Drop collapse, Oil spreading and Emulsification activity). Values are presented as mean ± standard deviation from one independent assay conducted in triplicate and significant differences compared to the positive control are annotated with asterisks in the graphs (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Almost all isolates that exhibited high activity with interfacial tension values below 40 mN/m also yielded positive results in other biosurfactant production assays, such as the drop collapse, oil spreading and emulsification tests. In contrast, the R66 isolate only exhibits activity in drop collapse and oil spreading assays.

4.4. Dereplication analysis

A set of 19 actinobacterial crude extracts was selected for dereplication analysis. These extracts were chosen according to their performance in bioactivity screening, capability to decrease at least one cancer cell line viability (10 actinobacterial extracts) and capacity to reduce the interfacial tension to values below 40 mN/m (9 actinobacterial extracts), respectively.

According to the dereplication data of bioactive extracts in cytotoxic activity, for the DEREPLICATOR, the results were not considered due to the score of all extracts being under a threshold *p*-value ≤ 10^{-10}. When using DEREPLICATOR with the VarQuest algorithm, 10 matches were obtained for four actinobacterial extracts (R8, Rx5, R84 and R95), all isolated from the roots of *R. graveolens* (Annex 3). This analysis allowed the annotation of analogues of anticancer compounds. Nevertheless, only three matches were considered to have cytotoxic activity and, none of them corresponded to known Actinomycetota-derived compounds. Using DEREPLICATOR + *in silico* tool, it was possible to annotate various secondary metabolites in ten bioactive actinobacterial extracts, resulting in 42 matches (Annex 3). However, only for the extract R9, it was possible to relate the cytotoxic activity with the hit from an antitumor Actinomycetota-derived compound. The rest of them did not show matches with any secondary metabolite that could explain the cytotoxic activity observed in these extracts. To complement these data, a molecular network was additionally constructed using GNPS (Annex 4). We identified a total of 13 single-strain clusters belonging to different actinobacterial extracts. This criterion was selected to increase the likelihood that each cluster corresponds to an unknown compound. Among the clusters, six were associated with extract R95, and four with extract R8, both of the genus *Streptomyces*. Additionally, two clusters were associated with extract R51, and one cluster was linked to extract R84, both belonging to the genus *Tsukamurella*. The extracted ion chromatogram (EIC) for each m/z value was analysed for single-strain clusters to assess relative abundance and identify common adduct ions (such as hydrogen, sodium, and ammonia). Seven clusters were selected, and the most abundant m/z values were used to calculate accurate masses for querying the Dictionary of NP (version 27.1) and NP Atlas database. For five

clusters from the extracts *Streptomyces novaecaesareae* of R95 (major compounds m/z 669.302, m/z 301.118 and m/z 520.313), *Streptomyces umbrinus* of R8 (major compound m/z 399.274) and *Tsukamurella tyrosinosolvens* of R51 (major compound m/z 399.274), hits from Actinomycetota-derived compounds were found that correspond to the accurate masses of known natural products (Neoantimycin A 1-Ketone, *N*-deformyl; Illicinone E 12-Deoxy, 12-chloro, 1α,2-dihydro; Antibiotic PA 133B; Valylvalylargininal *N*-Ac; and Gibboside, respectively). For the two remaining clusters from the extract *Streptomyces novaecaesareae* of R95 (major compound m/z 366.150 and m/z 1052.45), no compounds were identified in the dereplication, suggesting they may contain new metabolites. The chromatographic peaks correspond to the major compounds present in each cluster, which appear to be produced in sufficient quantities for future analysis (Figure 19).

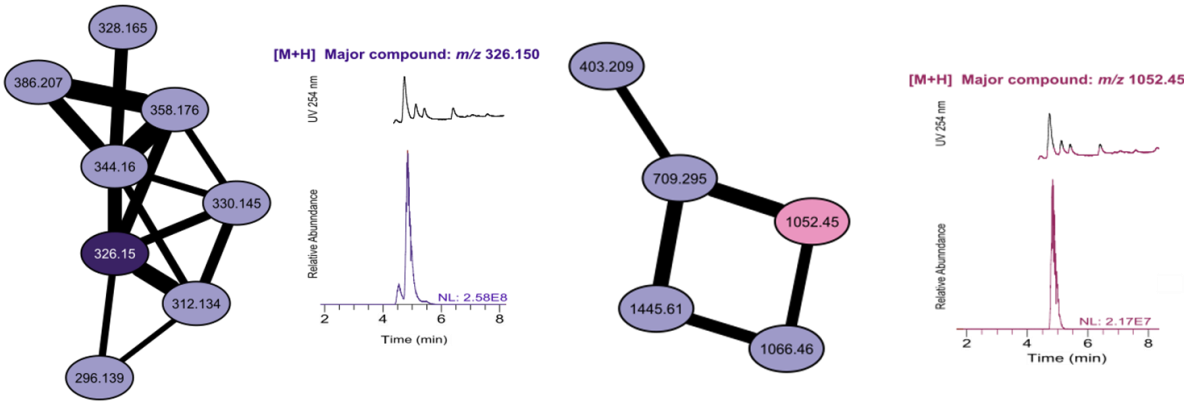


Figure 19. Analysis of the GNPS-generated molecular network LC-HRESI MS/MS of Actinomycetota crude extracts from R95, which may contain new metabolites. Single-strain clusters represented by connected ellipses annotated with the corresponding m/z values. The major compound is estimated from the UV chromatogram and EICs and represented with a different colour from the remaining cluster nodes. The NL (normalization level) value corresponds to the base peak intensity of the m/z value when compared with the mass composition of the Actinomycetota crude extract.

Regarding biosurfactant production, the dereplication analyses using DEREPLICATOR revealed the presence of hits for only one actinobacterial extract (R66), resulting in nine matches (Annex 3). The results showed the presence of biosurfactant compounds in all matches, but none were associated with Actinomycetota. Similarly, when using DEREPLICATOR with the VarQuest algorithm on the same extract (R66), 11 matches were identified, all with biosurfactant properties, but without any association with Actinomycetota to confirm the biosurfactant production (Annex

3). Using DEREPLICATOR + *in silico* tool, it was possible to annotate various secondary metabolites in four bioactive actinobacterial extracts (R66, R67, Rx5 and Rx3) with 16 matches (Annex 3). Only six were associated with plants or Actinomycetota but with no association with biosurfactant activity. In molecular networking analysis (Annex 5), we identified a total of 12 single-strain clusters, demonstrating the diversity of metabolites present in the extracts. Among these, six are associated with the strain *Mycolicibacterium* sp. R66. Additionally, three clusters are linked to Rx3, while two clusters with R51 and one cluster with R71, all belonging to the genus *Tsukamurela*. After discarding the clusters whose m/z values did not follow the selection criteria, the presence of common adduct ions and a high relative abundance, only four clusters from extract R66 remained.

In two of these clusters (major compound m/z 583.443 and m/z 797.329), no hits were found in the dereplication analysis related to Actinomycetota-derived compounds, indicating that these clusters may be new metabolites. In figure 20 shows the chromatographic peaks of the major compounds present in each cluster and their relative abundance.

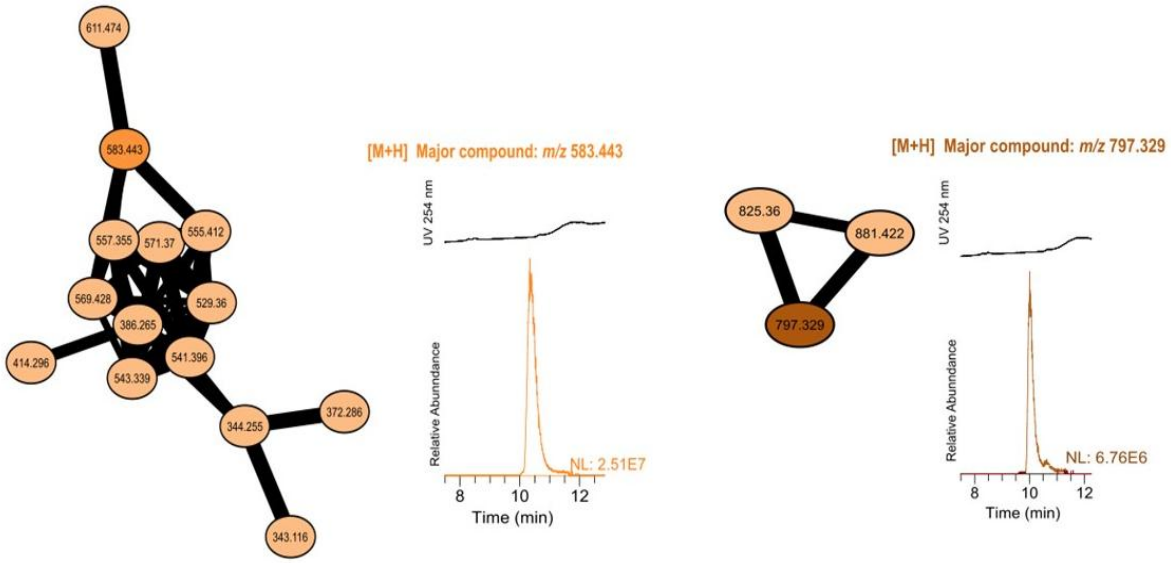
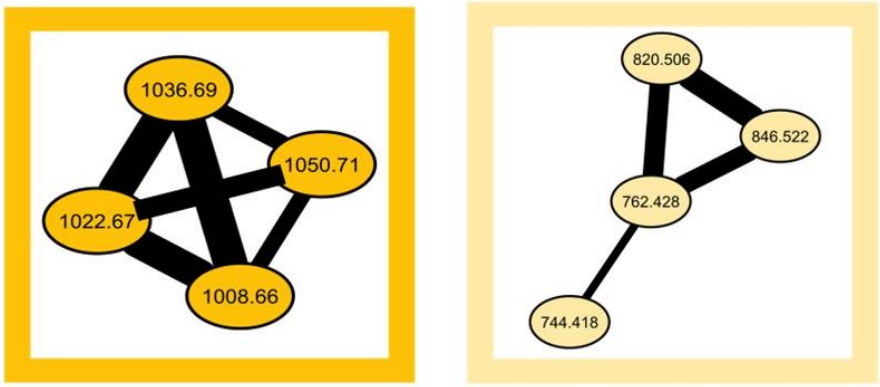


Figure 20. Analysis of the GNPS-generated molecular network LC-HRESI MS/MS of Actinomycetota crude extracts from R66 which may contain new metabolites. Single-strain clusters represented by connected ellipses and annotated with the corresponding m/z values. The major compound is estimated from the UV chromatogram and EICs and represented with a different colour from the remaining cluster nodes. The NL (normalization level) value corresponds to the base peak intensity of the m/z value when compared with the mass composition of the Actinomycetota crude extract.

Furthermore, the other two clusters are associated with compounds known as surfactins and amfibactins, both molecules with amphiphilic properties but never previously reported in association with Actinomycetota strains (Figure 21).



Powerful microbial surfactants originally derived from *Bacillus subtilis*

Amphiphilic siderophores derived from *Vibrio cyclitrophicus*1F53

Figure 21. Analysis of the GNPS-generated molecular network LC-HRESI MS/MS of Actinomycetota crude extract from the strain *Mycolicibacterium llatzerense*, R66, Single-strain clusters represented by connected ellipses and annotated with the corresponding m/z values. The m/z values are related to surfactins and amphibactins families, both with no association with Actinomycetota or plants.

5. Discussion

Natural products (NPs) have become one of the most important sources in drug discovery, especially for infectious diseases and cancer (162).

In this study, several bacterial isolates were obtained from *R. graveolens* belonging to the phylum Actinomycetota, suggesting that these microorganisms live in symbiosis with this plant, as the study targeted endophytic bacteria. A total of 56 isolates were obtained, of which 36 were identified as Actinomycetota. Most isolates were obtained from the roots of *R. graveolens*, which serve as the main entry point for endophytic bacteria (32, 38). The majority of the Actinomycetota isolates were recovered from the AIA culture medium, which is selective for isolating actinobacteria from the soil and water. SCN medium is also a highly sensitive and specific medium for the growth of actinobacteria, using sodium nitrate and casein as a nitrogen source. For instance, studies have successfully isolated Actinomycetota from different environments such as soil, rhizosphere, and even extreme locations using selective media, including AIA (163).

Analysis of the 16S rRNA gene sequencing showed that the majority of the isolated bacteria (22%) belonged to the *Tsukamurella* genus. *Tsukamurella* is a non-motile, aerobic, Gram-positive bacterial genus commonly found in soil, arthropods, water, and sponges. It does not form spores (164). Despite the limited information about this genus and its classification as an environmental saprophyte, it has been recognised as the cause of opportunistic human infections (165). Nevertheless, recent research has identified *Tsukamurella* as an important area of exploration because of its potential for discovering new antimicrobial compounds through biosynthesis and bioactivity (166). Following this genus, the *Streptomyces* genus accounted for 21% of the isolates. The isolation of *Streptomyces* strains from *R. graveolens* is particularly promising, as this genus is recognised for being a major source of bioactive molecules. Previous studies indicated that *Streptomyces* is the most prolific source of new metabolites with biological activities in human health or other fields like agriculture (167–169).

Nonetheless, the experimental approach adopted in the present study also allowed the isolation of strains affiliated with other actinobacterial genera – *Brevibacterium*, *Microbacterium*, *Gordonia* and *Mycolicibacterium*. For *Gordonia* and *Mycolicibacterium*, only one isolate from each genus was obtained, and no association between these genera with *R. graveolens* was described before. *Mycolicibacterium* is a relatively recent genus, officially validated in 2018. This species previously belonged to the *Mycobacterium* genera, but studies indicated the need for separate classification. They were originally included in the larger group, which held both pathogenic and non-pathogenic species (170). Although *Mycolicibacterium* is relatively new to intensive study, its genetic and ecological diversity suggests significant bioactive potential that remains largely unexplored. Comparing with our results, Qin et al. 2012, also isolated from a medicinal plant (*Maytenus austroyunnanensis*), more than 300 Actinomycetota distributed into 21 genera, including *Streptomyces*, *Gordonia*, *Microbacterium* and *Tsukamurella* (171). The treatment methods and the selective media used in this study also allowed for the isolation of bacterial genera belonging to phyla other than Actinomycetota. These bacterial genera were found to belong to the phyla Pseudomonadota (seven genera identified) and Bacteroidota (one genus identified). This finding is consistent with other studies that have isolated Actinomycetota from plants or soil environments, where diverse microbial communities are often co-isolated due to environmental pressures, reinforcing the broad-spectrum nature of these environments (172).

In the bioactivity assays, all Actinomycetota isolates obtained were screened for their antimicrobial and cytotoxic activities. The antimicrobial activity tested against *Staphylococcus*

aureus, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhimurium*, and *Candida albicans* showed no efficacy against any strain. While some research shows that both *R. graveolens* and Actinomycetota have antimicrobial properties (20, 173), the extracts of the Actinomycetota strains obtained in this study did not show any ability to inhibit the growth of bacteria or yeast. The cultivation conditions used to obtain the crude extracts may not have been ideal for inducing the production of antimicrobial compounds. Moreover, the study could have been limited by the small variety of reference microorganisms used in the assay and the number of available extracts.

In contrast, the cytotoxic activity of the actinobacterial extracts showed more promising results in the bioprospection of Actinomycetota from *R. graveolens*. Among the 36 actinobacteria isolates, 10 extracts demonstrated the capacity to decrease the viability in the cancer cell line, following 48 h of exposure. Most of the active extracts showed cytotoxicity towards the non-cancerous cell line, demonstrating general cytotoxicity. One extract, R18, exhibited cytotoxic effects on non-cancerous cells but lacked activity against HepG2, suggesting it is toxic to healthy cells but not harmful to the cancerous HepG2 line. Nevertheless, extract R84 (belonging to *Tsukamurella* genus) exhibited promising anti-cancer effects while causing minimal harm to non-cancerous cells. There is no evidence that associates the genus *Tsukamurella* to anticancer activity. Additionally, strain R95, demonstrated a significant cytotoxic effect by reducing cell viability by 60% in the two cell lines, suggesting a strong anticancer activity compared to other strains tested. Belonging to the *Streptomyces* genus, strain R95 belongs to the *Streptomyces* genus, which is recognized for its strong anticancer activity and for producing some well-known anticancer drugs, such as doxorubicin and bleomycin (174, 175). These findings indicate that certain isolates may have the potential to produce secondary metabolites that demonstrate potent cytotoxic effects on cancer cells, warranting further investigation in future studies.

In the present work, biosurfactant producers were explored in all Actinomycetota strains. The isolates were analysed using different screening methods including spreading oil assay, drop collapse method, interfacial tension and emulsification indices, since previous reports have recommended that the use of multiple techniques to screen for biosurfactant-producing strains is more efficient (Annex 6) (176, 177). In the emulsification activity assay, the more stable the emulsion formed, the more efficiency is shown by the biosurfactant. Of the 36 actinobacteria isolates, 16 strains showed emulsification activity, compared against both a positive control (Triton X100) and a negative control (Kim broth). Most of the active strains are phylogenetically

related to *Tsukamurella* (10/16) and *Streptomyces* (5/16), with one strain belonging to *Microbacterium* genus. The prevalence of *Tsukamurella* sp. within the positive strains indicates that this genus may hold potential for new biosurfactants, but no prior literature has mentioned anything about it. While the results of *Streptomyces* are more expected since this species has been known for its ability to produce bioactive compounds, such as biosurfactants (178). Some strains (R71, R1 and R17) showed very high performance, with emulsification levels very approximated with Triton X100 when compared to the positive control. This emphasizes the potential of actinobacteria as natural providers of emulsifying substances, presenting safer and more environmentally friendly options to synthetic surfactants such as Triton X100 (179).

In the drop collapse test, the supernatant is deposited on top of a solid surface with paraffin. A positive result, indicated by the collapse of the drop, is an indication of the presence of biosurfactants. A total of 18 isolates were found to be positive in the drop collapse test. The results were compared to the positive control (Triton X100) and the negative control (Kim broth). In the absence of surfactants, the hydrophobic surface repels polar water molecules, keeping the drops stable (180). If the liquid includes surfactants, the drops can spread out or even collapse due to the decrease in force or interfacial tension between the liquid drop and the hydrophobic surface. Drop stability relies on the concentration of surfactant and is associated with both surface and interfacial tension (180). In some cases, the collapse was partial, resulting in reduced activity. One significant observation is that *Tsukamurella* species were the most prevalent among the active isolates, representing 13 out of the 18. This finding supports the idea that *Tsukamurella* could be a promising source of biosurfactants. The presence of two *Streptomyces* strains, along with isolates from *Microbacterium*, *Gordonia*, and *Mycolicibacterium*, highlights the broader taxonomic diversity of Actinomycetota capable of producing biosurfactants.

The oil spreading assay results revealed a visible range of activities across the tested strains, with 12 showing high activity, four displaying moderate activity, and 11 demonstrating low activity. The strains with high activity produced halos like the positive control (Triton X100), indicating strong biosurfactant production, as their supernatants were able to effectively displace the dyed olive oil. The strains showing moderate and low activity were still able to form halos larger than those seen in negative control (Kim broth). Unlike many studies that quantitatively measure halo diameters (181), in this case, we evaluated the results visually, which provided a general but informative understanding of the activity levels. While this qualitative approach gave us an indication of biosurfactant presence, it limits the ability to draw precise comparisons between the

strains. The taxonomic analysis further increases the association between Actinomycetota and biosurfactant production, with the majority of high-activity strains belonging to *Tsukamurella* sp. and *Streptomyces* sp. This reinforces previous findings that these genera are rich biosurfactant producers.

The results for interfacial tension showed that six isolates (R1, R17, R71, R67, R84, and Rx5) had high biosurfactant activity, reducing interfacial tension to below 30 mN/m. Additionally, three isolates (Rx3, R51, and R66) lowered interfacial tension to below 40 mN/m, indicating high biosurfactant activity but slightly less efficacy compared to the first mentioned. Such significant reductions in interfacial tension are characteristic of highly active biosurfactants and suggest that these isolates produce potent compounds capable of reducing the surface tension between the immiscible phases. The culture medium Kim, which served as the negative control, showed an interfacial tension value of 70 mN/m—close to the standard value for water (71 mN/m)—confirming the absence of biosurfactant production. Almost all the isolates that exhibited high activity in the interfacial tension test (values below 40 mN/m) also showed positive results in other biosurfactant production assays, such as the drop collapse, oil spreading, and emulsification tests. This consistency across multiple assays strengthens the validity of the results and confirms the ability of these strains to produce effective biosurfactants. When comparing these results to values reported for biosurfactants in similar studies (155), it becomes evident that the biosurfactants from these isolates show promising activity, even though the compounds remain in their crude form and have not yet been purified. However, the isolate R66, which reduced interfacial tension to just below 40 mN/m, only demonstrated activity in the drop collapse and oil spreading assays. This discrepancy could indicate that R66 produces a biosurfactant that performs well in certain contexts but may not have the same versatility as those produced by the other isolates.

Identifying the bioactive compounds of interest may pose a challenge, requiring the use of dereplication tools to prevent the rediscovery of known compounds (162). Metabolomic profiling offers a unique approach to discovering new natural products with biotechnological interest (182). In this study, we investigated the metabolic profile of all the bioactive extracts of the Actinomycetota isolated from the *R. graveolens*. To obtain an overall knowledge of the metabolic profile, three dereplicator tools were employed on the 19 selected Actinomycetota crude extracts. Regarding anticancer activity, dereplication using the DEREPLICATOR tool initially did not yield any significant hits due to all extracts having scores below the threshold. However, when using

the VarQuest algorithm, four extracts (R8, Rx5, R84, and R95) were found to contain analogues of known anticancer compounds. These matches, though analogues, did not correspond to compounds previously described in Actinomycetota, which suggests that these strains may produce novel variants of cytotoxic compounds. Nevertheless, only three of the matches were confirmed to exhibit cytotoxic activity, leaving most of the observed activity unexplained by known metabolites. The additional use of DEREPLICATOR+ allowed for the annotation of various secondary metabolites, with a total of 42 matches across 10 bioactive extracts. Interestingly, only the extract of *Brevibacterium sediminis* R9 yielded a hit that corroborates its cytotoxic activity: the antitumor macrolactone Atacamycin C produced by *Streptomyces* sp. C38 (183). For the remaining crude extracts, no hits were found related to known cytotoxic compounds. This suggests that many of the bioactive extracts could contain new molecules responsible for their observed activity, reinforcing the need for further investigation into these unexplained activities. As the GNPS platform provides annotation comparing fragmentation patterns, it is possible to detect not only compounds present in a sample but also their analogues that have similar fragments in the mass spectrum (184). Therefore, the construction of a molecular network allows grouping sets of spectra of related molecules to form clusters, even when the spectra do not correspond to any known compound (158). Molecular networking analysis using GNPS revealed 13 single-strain clusters from different extracts, increasing the probability of discovering unknown compounds. After discarding the clusters whose m/z values did not meet the selection criteria (presence of common adduct ions and a high relative abundance), seven clusters remained: three associated with *Streptomyces novaecaesareae* from extract R95, one with *Streptomyces umbrinus* from extract R8, and one with *Tsukamurella tyrosinosolvens* from extract R51, which corresponded to the accurate masses of known natural products. The remaining two clusters found in the extract R95 (major compounds m/z 326.15 and m/z 1052.45) did not result in any matches with natural products present in the databases, so it probably corresponds to new natural products.

In the dereplication analyses of biosurfactant production, only extract R66 yielded hits. These hits included the widely studied lipopeptide biosurfactants belonging to the two main families: surfactins (Surfactins A-D, Bamylocin) and fengycins (Plipastatin A-B), both typically produced by *Bacillus* species and never reported in association with the Actinomycetota phylum (185), which suggests that the biosurfactants identified may be also produced by actinobacteria or represent novel biosurfactants that have yet to be fully characterized.

The molecular networking analysis for biosurfactant-producing extracts identified a total of 12 clusters, with the majority associated with extract R66 from *Mycolicibacterium*. The remaining clusters were linked to other extracts, such as Rx3, R51, and R71, from the genus *Tsukamurella*. Among the clusters associated with R66, four were selected for further analysis based on their high abundance and the presence of common adducts ions, yet no known compounds could explain the biosurfactant activity in two of these clusters. This opens the possibility that these clusters may contain novel molecules with surface-active properties. Two of the clusters were associated with compounds like surfactins and amfibactins—well-known biosurfactants—although they have not previously been reported in association with Actinomycetota strains. Furthermore, as only single-strain clusters were analysed in detail, there might be additional chemical novelty when analysing the set of crude extracts.

Moreover, it is important to highlight that dereplication was only conducted on compounds that showed anticancer activity and biosurfactant production. This means that we did not evaluate extracts that lacked these activities, and these extracts may possess other bioactive characteristics that remain undiscovered.

In the dereplication analysis of anticancer compounds, it is important to acknowledge the possibility that some samples might possess antimicrobial properties that were not detected during the assays. In VarQuest, about six hits were found related to antimicrobial activity in strains Rx5, R84 (belonging to the species *Tsukamurella tyrosinosolvens)* and R95 (*Streptomyces novaecaesareae*) indicating that these strains can possibly produce antimicrobial compounds. In DEREPLICATOR +, also six hits corresponded to antimicrobial activity in strains R9 (*Brevibacterium sediminis*) and R8 (*Streptomyces umbrinus*). This could be due to several factors. Firstly, the crude nature of the extracts may have masked any antimicrobial activity since the compounds were not fractionated. Fractionation can help isolate active components, which might be diluted or inhibited in crude mixtures. Additionally, it is possible that the experimental conditions we used were not optimal for certain compounds to express their activity (186). Biosynthetic pathways or specific environmental factors could be required for some compounds to exhibit their full potential, which may not have been provided in our assays (186). The OSMAC (one strain many compounds) approach could be another strategy to avoid this problem since it aims to unlock a wide range of metabolites that a single strain can produce and different growth conditions, including modifying selected physical (culture support, growth duration) or chemical parameters (growth medium composition, pH) (187, 188).

Another potential explanation is that the concentration of active antimicrobial compounds in these crude extracts might have been too low to produce measurable effects that are detected during the dereplication analysis (189).

6. Conclusion

This research focuses on the potential of Actinomycetota strains associated with *R. graveolens* to produce bioactive compounds, particularly with cytotoxic and biosurfactant activities. Out of the 36 Actinomycetota strains screened, 10 displayed cytotoxic activity on the HepG2 liver cancer cell line, with a specific extract showcasing selective behaviour through a substantial decrease in cancer cell viability without harming non-cancer cells. These results indicate that the bioactive compounds found in these strains may be promising new natural products.

Additionally, the discovery of biosurfactant production in various strains contributes to the increasing amount of confirmation that Actinomycetota could provide new biosurfactants for both industrial and biomedical uses. The discovery of six possible new compounds, in addition to two of them being associated with known biosurfactants, such as surfactins and amphibactins, reveals the metabolic variety available in Actinomycetota. Notably, this represents the first time these compounds have been linked to this group, opening new avenues for studying their mechanisms of action and potential applications.

In conclusion, this research increases our knowledge of the bioactive abilities of Actinomycetota linked to *R. graveolens*, highlighting the importance of plant-microbe interactions in the search for novel natural products.

7. Future perspectives

Genomic analysis will greatly increase future research on biosurfactants, offering a more in-depth understanding of their biosynthetic pathways. By uncovering the genetic basis of biosurfactant synthesis, we can identify new types of biosurfactants and better understand their mechanisms of action. Additionally, the promising activity seen in anticancer assays creates interesting possibilities for further investigation. Large-scale cultivation of the most promising Actinomycetota strains will enable fractionation to identify the specific compounds responsible for the observed bioactivity.

The next steps could be to determine the isolation and chemical structure elucidation of new biosurfactant molecules and test them for different bioactivities, specifically focusing on their

antimicrobial effects. Running thorough bioactivity tests will allow us to evaluate their effectiveness in different uses and possibly discover new medicinal substances.

Our strains also have the potential to belong to new species within other phyla beyond Actinomycetota, such as Pseudomonadota and Bacteroidota. So, is crucial to sequence the genome of these strains to confirm if they are novel species and to validate them taxonomically. Confirming these results, it is necessary to do a polyphasic approach of bacterial classification.

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9. Annexes

Annex 1. Percentage of cell viability from cytotoxic assays of actinobacterial extracts in triplicate across three independent experiments on the HepG2 cell line.

	% CELL VBLT								
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
PC	13,32533013	12,96518607	16,56662665	18,21001329	18,3429331	18,21001329	11,93415638	12,75720165	13,16872428
SC	99,03961585	98,67947179	102,2809124	106,6016837	93,44262295	99,95569348	99,79423868	90,02057613	110,1851852
R60	94,11764706	90,63625452	96,99879952	84,2711564	112,5830749	93,84138237	105,2469136	69,5473251	127,8806584
R77	74,54981993	90,87635054	78,99159664	94,24014178	105,2724856	95,30350022	92,90123457	86,83127572	102,1604938
R22	80,43217287	88,47539016	86,31452581	93,17678334	106,0700044	95,83517944	105,1440329	92,28395062	112,7572016
Rx4	74,06962785	79,59183673	79,59183673	78,28976518	102,6140895	89,58794864	90,53497942	85,39094651	100
R24	73,82953181	66,86674671	72,62905162	63,53566681	72,84005317	71,51085512	90,74074074	87,55144033	98,76543211
R1	88,47539016	78,39135654	72,62905162	84,80283562	109,6588392	98,49357554	77,57201646	82,81893004	88,99176955
R39	89,91596639	91,59663866	98,07923169	87,72707133	117,2352681	105,0066464	105,5555556	106,0699588	107,4074074
R83	96,75870348	83,31332533	87,75510204	96,10101905	116,7035888	74,96677005	111,8312757	105,6584362	106,8930041

						100,4873726			95,16460905
R92	97,4789916	119,0876351	79,83193277	129,1980505	101,2848914		100,5144033	96,50205761	
		104,8019208	85,47418968		93,17678334	99,82277359	95,98765432		93,41563786
R17	100,120048			121,6216216				91,76954733	
	88,83553421	96,75870348	87,87515006	105,5383252	83,47363757	92,91094373			83,74485597
R7							89,81481481	86,21399177	
	88,83553421		74,18967587		89,32210899	86,13203367	85,80246914		78,08641975
Fx2		94,35774317		107,3992025				89,30041152	
		86,43457383		85,06867523		65,39654409	73,04526749		
R92	77,91116447		91,23649463		81,74568011			86,11111111	70,57613169
	74,54981993		74,06962785		85,20159504	82,14443952			
R8		76,71068427		97,2972973			67,48971193	81,48148148	71,19341564
	65,90636255	86,55462185	98,43937575			85,73327426	63,27160494		62,44855967
R4				103,0128489	82,67611874			61,21399177	
	67,34693878	76,83073229	81,63265306	84,80283562	99,42401418	79,88480284		99,58847737	80,86419753
R67							64,6090535		
	93,99759904	110,0840336	81,39255702		92,24634471				58,02469136
C6				102,4811697		101,6836509	68,62139918	73,7654321	
			72,38895558	82,94195835			81,89300412		
R71	81,272509	82,95318127			117,2352681	101,4178113		80,76131687	89,71193416
	95,19807923	63,50540216	55,10204082			83,87239699	59,56790123	65,74074074	66,87242798
Rx5				78,9543642	89,5879486				
	88,47539016	84,87394958	68,42737095				82,71604938	83,84773663	
R79				94,10722198	103,6774479	83,34071777			75,41152263
	88,59543818	78,99159664	90,39615846	76,82764732			90,94650206		90,63786008
R78					81,4798405	103,1457687		96,50205761	
			76,95078031	82,54319894		83,20779796			80,65843621
R66	79,83193277	91,95678271			99,9556934		79,9382716	95,47325103	
	99,51980792		95,43817527	84,66991582		88,52459016			92,79835391
R88		95,67827131			101,9494905		92,48971193	96,39917695	

R109	102,4009604	83,67346939	80,67226891	86,39787328	90,38546743	96,63269827	93,51851852	93,82716049	74,38271605
Rx3	103,6014406	119,2076831	116,6866747	107,6650421	101,4178113	95,70225964	93,72427984	79,42386831	118,9300412
Cx1	62,66506603	84,27370948	71,54861945	97,69605671	85,60035445	85,60035445	76,44032922	62,24279835	88,99176955
R84	61,34453782	71,54861945	73,58943577	82,01151972	73,37173239	81,87859991	55,96707819	56,37860082	87,24279835
R38	60,74429772	80,07202881	70,22809124	101,4178113	82,67611874	94,24014178	74,07407407	71,09053498	82,61316872
R85	81,03241297	84,75390156	85,35414166	120,9570226	120,4253434	140,2303943	61,72839506	61,31687243	94,03292181
R62	89,79591837	96,99879952	88,59543818	106,6016837	95,56933983	92,51218432	83,84773663	87,96296296	108,744856
R43	121,6926100	80,25918000	182,3930000	110,4029000	106,6146000	95,97114000	118,0050000	108,2183000	119,1343000
R18	114,6887200	79,66926100	147,3735400	54,29946000	50,33073000	51,77390000	98,61982000	80,74028000	93,24789000
R57	209,8249000	146,4980500	147,3735400	85,68851000	85,86891000	76,66867000	110.6650000	99.98455000	112,6321500
R95	94,26070000	67,41245100	102,7237400	36,25977000	34,09501000	34,81660000	36,51192000	32,37139000	43,09912000
R51	207,1984400	180,3501900	245,1361900	65,66446000	58,08779000	62,41732000	77,91719000	87,52397000	91,54896000
R65	324,2217900	300,8754900	363,3268500	88,57486000	78,11185000	73,78232000	109,7240000	115,6932500	121,5632800
	1st assay			2nd assay			3rd assay		

HepG2

Annex 2. Percentage of cell viability from cytotoxic assays of actinobacterial extracts in triplicate across three independent experiments on the hCMEC/D3 cell line.

	% CELL VBLT								
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
PC	15,0382328	15,2931181	15,42056075	12,57240204	12,77683135	12,06132879	21,69754931	22,59414226	22,77346085
SC	101,0620221	82,58283772	116,3551402	95,67291312	100,0681431	104,2589438	100,7770472	99,16317992	100,0597729
R60	106,0322855	118,6491079	97,11129992	66,95059625	72,06132879	77,58091993	119,0675433	101,6736402	105,7979677
R77	107,8164826	99,91503823	95,96431606	60,71550256	75,63884157	77,78534923	98,08726838	97,36999402	92,7077107
R22	115,4630416	116,1002549	104,6304163	84,02044293	67,97274276	86,16695063	98,80454274	93,78362224	91,0938434
Rx4	119,6686491	109,6006797	102,8462192	74,20783646	91,07325383	78,70528109	97,72863127	96,65271967	94,14225941
R24	122,3449448	107,6890399	100,5522515	83,30494037	91,07325383	84,32708688	95,03885236	91,45248057	90,37656904
R1	105,7774002	102,3364486	91,37638063	65,11073254	75,53662692	73,18568995	93,60430365	85,71428571	82,48655111
R39	118,7765506	112,9141886	92,90569244	80,03407155	75,33219761	63,98637138	107,2325164	99,34249851	96,47340108
R83	121,8351742	121,0705183	99,91503823	77,98977853	78,80749574	65,31516184	115,8398087	108,4877466	105,4393305
R92	99,15038233	112,786746	104,375531	63,27086882	89,94889267	73,79897785	99,88045427	87,6867902	97,72863127
R17	102,0815633	103,9932031	104,5029737	69,71039182	81,36286201	82,69165247	97,90794979	96,1147639	98,26658697

							86,96951584	92,88702929	
R7	97,74851317	94,56244696	106,7969414	73,0834753	82,48722317	76,25212947			96,11476395
		88,95497026			80,85178876	84,12265758	93,06634788	95,39748954	93,96294082
Fx2	94,18011895		109,6006797	71,03918228					
	74,29906542		86,91588785	55,60477002	72,26575809				75,33886491
R92		81,81818182				72,36797274	84,10041841	88,76270173	
	88,95497026		102,2090059	72,26575809	78,60306644		88,22474577		
R8		97,11129992				76,14991482		85,17632995	85,89360431
			93,41546304		82,18057922	76,96763203	96,47340108		97,90794979
R4	105,1401869	114,0611725		65,51959114				97,19067543	
				82,18057922	78,80749574	77,37649063			104,7220562
R67	101,0620221	102,0815633	109,855565				102,3909145	103,6461447	
			84,49447749	89,74446337	92,50425894		98,08726838		
C6	102,7187766	110,1104503				93,11754685		95,57680813	97,72863127
	93,67034834			89,74446337	84,63373083	85,96252129	92,16975493	96,29408249	97,36999402
R71		97,36618522	79,65165675						
	74,09929663			77,98977853	75,22998296	75,53662692			80,51404662
Rx5		85,13169074	77,10280374				85,89360431	74,95517035	
			92,39592184	90,45996593	99,14821124	92,70868825	90,73520622	89,47997609	92,16975493
R79	94,30756161	99,53271028							
	92,26847918	98,25828377			98,84156729	92,09540034			
R78			86,15123195	93,42419089			87,68679021	107,9497908	99,70113568
				94,44633731		99,96592845		86,96951584	91,45248057
R66	98,51316907	76,33814783	89,71962617		95,16183986		88,76270173		
		96,34664401		84,63373083		89,43781942	97,36999402	93,60430365	98,98386133
R88	103,9932031		102,7187766		88,21124361				
				84,94037479		86,26916525	103,8254632		
R109	108,0713679	91,12149533	100,5522515		76,25212947			104,5427376	117,6329946
	62,31945624			89,33560477	88,00681431	86,88245315	100,0597729	89,83861327	66,34787806
Rx3		96,21920136	145,1571793						

Cx1	78,88700085	90,61172472	95,19966015	84,42930153	84,83816014	90,35775128	90,73520622	94,68021518	77,64494919
R84	80,03398471	92,39592184	116,6100255	82,48722317	81,05621806	89,64224872	94,50089659	89,65929468	85,17632995
R38	59,26083263	128,3347494	117,502124	88,82453152	94,65076661	93,4241908	92,34907352	89,83861327	76,03108189
R85	86,40611725	89,71962617	103,6108751	81,36286201	82,58943782	87,59795571	93,24566647	93,42498506	80,15540944
R62	93,54290569	75,44604928	95,3271028	89,33560477	90,86882453	89,64224872	95,21817095	91,27316198	84,45905559
R43	80,19303000	75,40915000	81,57784000	137,6104000	139,4805000	102,8959000	100,0000000	110,0905000	100,0000000
R18	79,68947000	71,38061000	83,84389000	86,18182000	86,80519000	79,01299000	79,01299000	93,80090000	79,68967000
R57	97,81788000	89,88670000	91,27151000	114,2338000	108,9351000	91,48052000	105,8824000	107,7828000	102,0814000
R95	32,10239000	31,22115000	34,74612000	41,14286000	37,55844000	38,49351000	36,10860000	30.27179000	40,31674000
R51	60,17625000	50,73437000	58,28787000	70,28571000	63,11688000	69,03896000	68,55204000	60,13575000	73,57466000
R65	101,0911000	90,64205000	87,87243000	97,09091000	97,87013000	94,12987000	99,77376000	81,71946000	99,72568000
	1st assay			2nd assay			3rd assay		

hCMEC/D3

Annex 3. Dereplication results for all extracts with hits from actinobacteria or plant-associated sources in both biosurfactant and cytotoxicity assays, using Dereplicator, Dereplicator VarQuest, and Dereplicator+.

DEREPLICATOR

Strain ID	Compound Name	Strain Activity	Score	*p*-Value	Peptide Mass	Spectrum Mass	Adduct	Peptide FDR %
R66	SNA 60-367-13	Biosurfactant	24	8,90E-44	1504,84	753,429	M+2H	0
	Surfactin D	Biosurfactant	18	5,20E-38	1049,7	1050,71	M+H	0
	SNA 60-367-2'-Deoxy	Biosurfactant	21	1,00E-37	1488,85	745,431	M+2H	0
	Surfactin C1	Biosurfactant	17	1,00E-36	1035,68	1036,69	M+H	0
	SNA 60-367-6	Biosurfactant	20	1,80E-35	1476,81	739,413	M+2H	0
	Surfactin B1	Biosurfactant	16	1,70E-34	1021,67	1022,67	M+H	0
	Surfactin A1	Biosurfactant	16	2,10E-34	1007,65	1008,66	M+H	0
	Esperin	Biosurfactant	14	1,00E-31	1035,68	1036,69	M+H	0
	SNA 60-367-7	Biosurfactant	17	1,30E-29	1490,83	746,421	M+2H	0

DEREPLICATOR
VARQUEST

Strain ID	Compound Name	Strain Activity	Score	*p*–Value	Peptide Mass	Spectrum Mass	Adduct	Peptide FDR %
Rx5	Xenobovid A	Citotoxic	11	4,70E-28	765,5	655,404	M+H	0
	Actinomycin G1	Citotoxic	6	4,70E-15	1272,6	583,33	M+2H	0
R95	Peptidolipin F	Citotoxic	9	1,00E-22	781,459	712,388	M+H	0
	Stenothricin	Citotoxic	6	5,40E-14	907,407	479,782	M+2H	0
R84	Antibiotic A 54145-3-Demethoxy, 3'''-deoxy	Citotoxic	9	2,30E-17	1625,79	479,782	M+2H	0
	Discodermin A	Citotoxic	7	9,10E-15	1704,83	903,983	M+2H	0
	Surugamide D	Citotoxic	6	4,90E-15	897,605	395,367	M+2H	0
R8	Destruxins N^{Val}-De-Me	Citotoxic	7	1,00E-16	563,332	281,266	M+2H	0
R66	SNA 60-367-9	Biosurfactant	25	4,90E-46	1490,83	753,429	M+2H	0
	SNA 60-367-13	Biosurfactant	24	1,90E-43	1504,84	753,429	M+2H	0
	Esperin	Biosurfactant	18	8,30E-43	1035,68	1022,67	M+H	0
	Bacillus amyloliquefaciens Surfactin 1	Biosurfactant	19	1,50E-40	1035,68	1050,71	M+H	0
	Surfactin 1-Me_ester	Biosurfactant	18	4,70E-39	1049,7	1036,69	M+H	0

Surfactin 7-L-Valine_analogue, 1-Me_ester	Biosurfactant	19	7,20E-39	1035,68	1050,71	M+H	0
SNA 60-367_2'-Deoxy	Biosurfactant	21	1,00E-37	1488,85	745,431	M+2H	0
Surfactin C_{13}ai- monomethyl ester	Biosurfactant	17	1,00E-36	1021,67	1008,66	M+H	0
SNA_60-367-6	Biosurfactant	20	1,80E-35	1476,81	739,413	M+2H	0
Bamylocin A	Biosurfactant	15	1,80E-31	1021,65	1008,66	M+H	0

DEREPLICATOR +

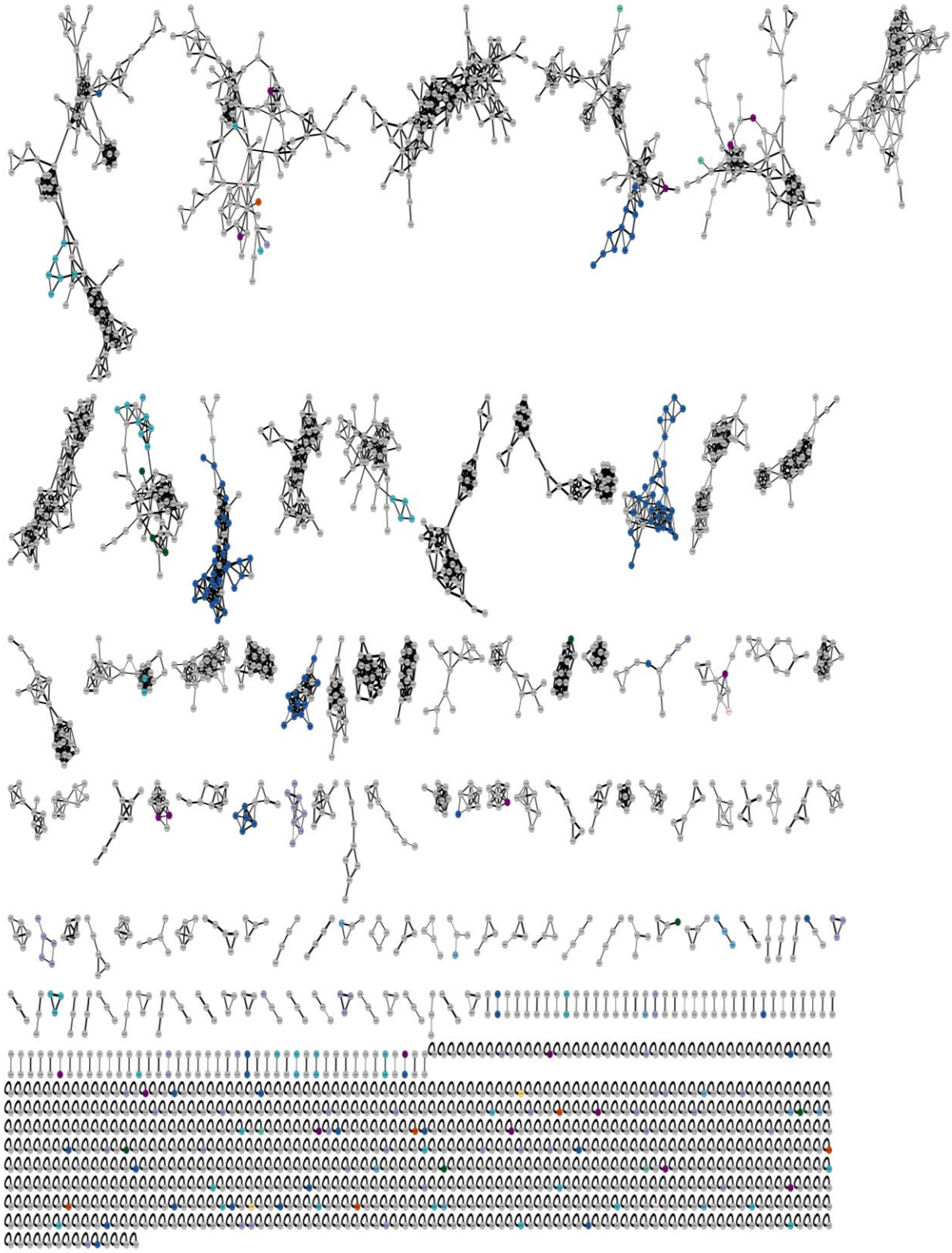
Strain ID	Compound Name	Strain Activity	Score	p-Value	Metabolite Mass	Spectrum MZ	Adduct	Metabolite FDR %
R8	20(29)-Lupene-3,11-diol 3-Ketone	Citotoxic	24	NA	440,365	441,372	M+H	0
	2-Amino-8-octadecene-1,3,4-triol, N-(2R-Hydroxydocosanoyl), 1-O-β-D-glucopyranoside	Citotoxic	21	NA	815,649	816,657	M+H	0
	6,26-Dihydroxydammara-20,24-dien-3-one	Citotoxic	21	NA	456,36	457,367	M+H	0
	12-Ursene-3,11-diol 11-Ketone,_3-hexadecanoyl	Citotoxic	20	NA	678,595	679,602	M+H	0
	Monensin M1	Citotoxic	20	NA	672,445	673,449	M+H	0
	Pamamycin 621C	Citotoxic	16	NA	621,46	622,467	M+H	0
	Antibiotic MS 282B	Citotoxic	15	NA	635,476	636,483	M+H	0
	Tylosin, 8CI, 9CI, BAN, INN 2^A-Deoxy, 12α,13α-epoxide	Citotoxic	15	NA	915,519	916,525	M+H	0

Cx1	20(29)-Lupene-3,11-diol 1,2-Didehydro, 3-ketone	Citotoxic	25	NA	438,35	439,357	M+H	0
	Cryptophorine 7',8'-Dihydro,_Me_ether	Citotoxic	24	NA	277,241	278,245	M+H	0
	20,28-Cyclostigmasta-5,9(11)-dien-3-ol	Citotoxic	24	NA	410,355	411,362	M+H	0
	15-Norerythromycin A 6,12-Dideoxy, 3''-O-de-Me	Citotoxic	19	NA	673,44	674,445	M+H	0
R38	16,21-Pachysanadiene-3,28-diol	Citotoxic	23	NA	440,365	441,372	M+H	0
	Chaxalactin B 14-Deoxy	Citotoxic	17	NA	440,293	441,298	M+H	0
	15-Norerythromycin A 6-Deoxy, 3''-O-de-Me	Citotoxic	15	NA	689,435	690,438	M+H	0
R4	20(29)-Lupene-3,6-diol_3-Ketone	Citotoxic	23	NA	440,365	441,372	M+H	0
	2-Hydroxy-22(29)-hopen-23-oic acid	Citotoxic	22	NA	456,36	457,367	M+H	0
	2-(7,9-Pentacosadiynyl)furan	Citotoxic	19	NA	410,355	411,362	M+H	0
R9	Cucurbita-1(10),5,22,24-tetraen-3-ol	Citotoxic	22	NA	422,355	423,362	M+H	0
	29-Norlanosta-8,24-diene-1,2,3-triol	Citotoxic	18	NA	444,36	445,367	M+H	0
	15-Norerythromycin A 6,12-Dideoxy	Citotoxic	17	NA	687,456	688,46	M+H	0
	3-(3,4-Dihydroxyphenyl)-1-propanol 1-Eicosanoyl	Citotoxic	15	NA	462,371	463,378	M+H	0
	Atacamycin C	Citotoxic	17	NA	440,293	441,297	M+H	0
R24	Monensin M2	Citotoxic	19	NA	688,44	689,449	M+H	0
	Antibiotic H 668	Citotoxic	18	NA	688,44	689,45	M+H	0
	Concanamycin A 23-O-Deglycosyl	Citotoxic	18	NA	692,45	693,455	M+H	0
	Roxaticin	Citotoxic	17	NA	606,377	607,384	M+H	0
	Antibiotic AB 023A	Citotoxic	15	NA	550,351	551,354	M+H	0
Rx5	6-Hydroxy-3(15),7(14)-caryophylladien-8-one 12-Acetoxy, Me ether	Citotoxic	16	NA	306,183	307,19	M+H	0

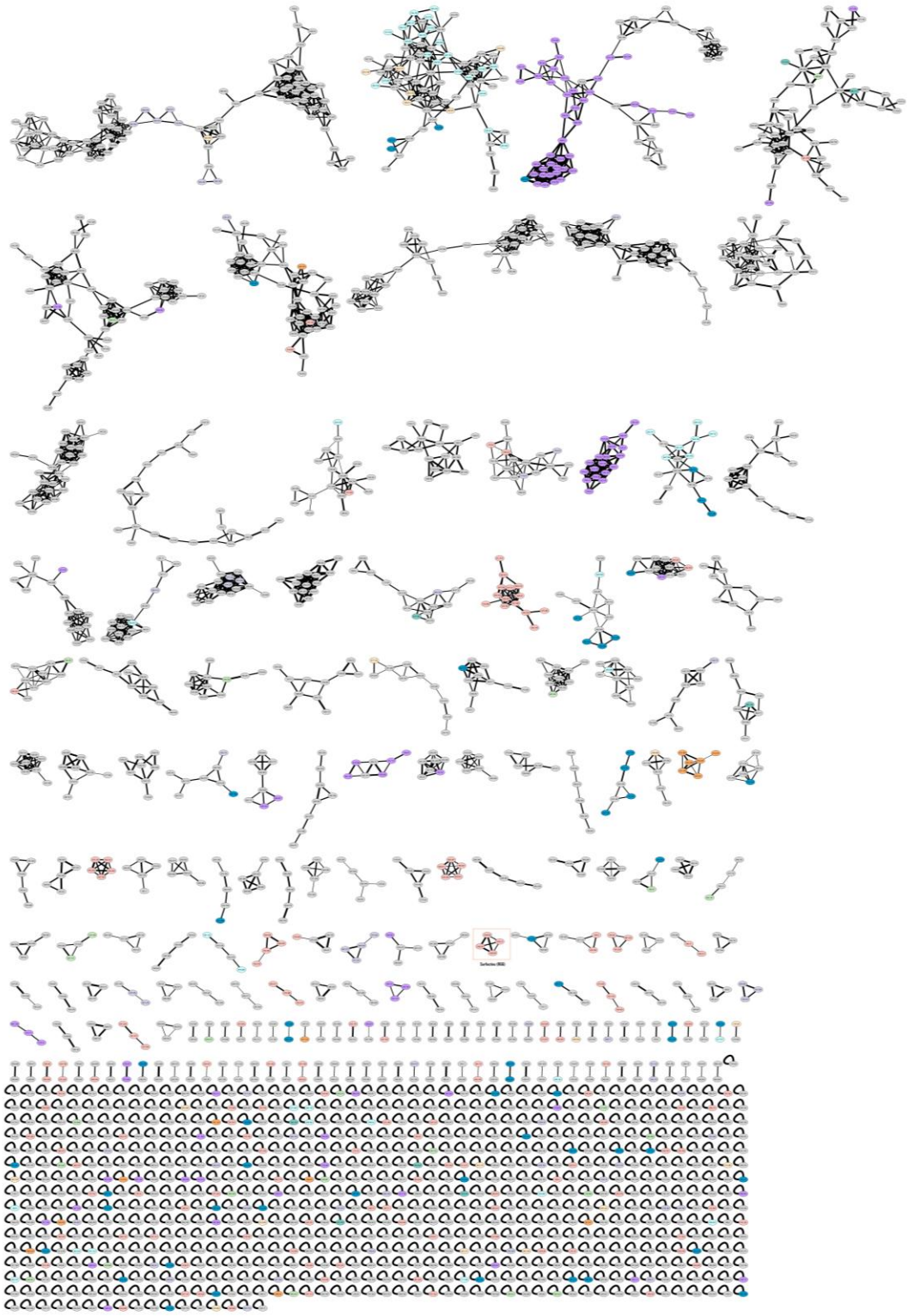
	Oasomycin_A	Citotoxic	16	NA	1026,65	514,335	M+2H	0
	Chlorobactene 1',2'-Dihydro, 1'-hydroxy	Citotoxic	15	NA	550,417	551,424	M+H	0
	Landomycin A 5E-Epimer, 11-deoxy, 3E-deglycosyloxy	Citotoxic	15	NA	940,409	941,419	M+H	0
	Dodecylbenzene	Biosurfactant	20	NA	246,235	274,242	M+H	0
	Irnigaine N-Me	Biosurfactant	15	NA	331,288	332,292	M+H	0
R51	Kitasamycin	Citotoxic	21	NA	1268,57	635,291	M+2H	0
	2-Amino-6,8-octadecadiene-1,3,4-triol N-(2R-Hydroxytetracosanoyl), 1-O-β-D-glucopyranoside	Citotoxic	19	NA	841,664	842,672	M+H	0
	Ajugol 6-O-(8-O-Arabinosyloxy-2,6-dimethyl-2-octenoyl)	Citotoxic	18	NA	648,299	649,307	M+H	0
	3,12,14,17-Tetrahydroxypregn-5-en-20-one 12-Ac, 3-O-[α-L-cymaropyranosyl-(1→4)-β-D-digitoxopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranoside]	Citotoxic	16	NA	1256,69	629,35	M+2H	0
	Nomilinic acid Ring D hydroxyacid, 17-O-β-D-glucopyranoside	Citotoxic	16	NA	670,284	671,289	M+H	0
	Terracinolide A 6-Debenzoyl, 3-de-Ac, 6-Ac	Citotoxic	16	NA	738,31	739,315	M+H	0
R84	Dodecylbenzene	Citotoxic	20	NA	246,235	247,242	M+H	0
	20-Hydroxystigmasta-4,6-dien-3-one	Citotoxic	18	NA	426,35	427,357	M+H	0
	6'-Apo-ψ-caroten-6'-al 6'-Carboxylic acid, Me ester	Citotoxic	18	NA	472,334	473,345	M+H	0

	3-(4-Hydroxyphenyl)-2-propenoic acid, 9CI Hexacosyl ester	Citotoxic	16	NA	528,454	529,461	M+H	0
R95	14,17-Epoxy-3,12,20,24,25-malabaricanepentol 3-O-β-D-Glucopyranoside	Citotoxic	16	NA	672,445	673,451	M+H	0
R66	Bacircine 1	Biosurfactant	35	NA	1007,65	1008,66	M+H	0
	Bacircine 2	Biosurfactant	40	NA	1021,67	1022,67	M+H	0
	Bacircine 4	Biosurfactant	39	NA	1035,68	1036,69	M+H	0
	Chaxalactin B 15-Me ether	Biosurfactant	15	NA	470,303	471,311	M+H	0
	fungichromin B	Biosurfactant	20	NA	684,408	685,416	M+H	0
	Esperin	Biosurfactant	40	NA	1035,68	1036,69	M+H	0
	Mycobactin S	Biosurfactant	16	NA	827,504	828,511	M+H	0
	SNA 60-367-2'-Deoxy	Biosurfactant	77	NA	1488,85	745,431	M+2H	0
	SNA 60-367-12	Biosurfactant	81	NA	1504,84	753,429	M+2H	0
	SNA 60-367-6	Biosurfactant	63	NA	1476,81	739,413	M+2H	0
	SNA 60-367-7	Biosurfactant	63	NA	1490,83	746,421	M+2H	0
	Surfactin D	Biosurfactant	40	NA	1049,7	1050,71	M+H	0
R67	bergenin-8-O-alpha-L-rhamnoside	Biosurfactant	16	NA	490,132	491,138	M+H	0
Rx3	Roflamycoin	Biosurfactant	15	NA	738,455	739,46	M+H	0

Annex 4. Complete molecular networking developed using MS/MS data from the extracts exhibiting cytotoxic activity.



Annex 5. Complete molecular networking developed using MS/MS data from the biosurfactant-active extracts.



Annex 6. Biosurfactant activity for each strain associated with its respective phylum, assessed through the Drop Collapse Assay, Oil Spreading Test, Interfacial Tension (mN/m), and Emulsification Activity (%). Activity levels are categorized as +++ (high activity), ++ (moderate activity), and + (low activity).

Strain ID	Phylum	Drop Collapse Assay	Oil Spreading Test	Interfacial Tension (mN/m)	Emulsification Activity (%)
C6	*Microbacterium ginsengiterrae*	+	+	55,45	25,93
CX1	*Tsukamurella tyrosinosolvens*	++	+	53,28	-
FX2	*Brevibacterium sediminis*	-	+	72,54	-
R1	*Tsukamurella tyrosinosolvens*	+++	+	28,87	46,43
R109	*Tsukamurella tyrosinosolvens*	++	+	55,16	-
R17	*Tsukamurella sp.*	+++	+++	28,62	41,38
R18	*Streptomyces umbrinus*	-	+++	56,99	39,29
R22	*Streptomyces umbrinus*	-	+	61,21	14,81
R24	*Tsukamurella tyrosinosolvens*	+++	+++	49,21	28,57
R39	*Streptomyces umbrinus*	-	+	63,50	-
R4	*Brevibacterium sediminis*	-	++	65,02	-
R43	*Streptomyces umbrinus*	++	+++	64,58	34,48
R51	*Tsukamurella tyrosinosolvens*	+++	+++	36,82	28,57
R60	*Streptomyces umbrinus*	-	-	58,10	35,71
R66	*Mycolicibacterium llatzerense*	++	++	40,88	-
R67	*Tsukamurella tyrosinosolvens*	+++	+++	29,14	37,04
R71	*Tsukamurella tyrosinosolvens*	+++	+++	28,34	48,15
R77	*Brevibacterium sediminis*	-	++	69,27	-
R78	*Gordonia hydrophobica*	+	-	68,23	-
R79	*Streptomyces umbrinus*	++	+++	62,17	-
R84	*Tsukamurella tyrosinosolvens*	+++	+++	29,07	33,33
R85	*Tsukamurella tyrosinosolvens*	++	+	57,87	-
R88	*Brevibacterium sediminis*	-	++	56,22	-
R9	*Brevibacterium sediminis*	-	+	56,58	-

R95	*Streptomyces novaecaesareae*	-	+	69,14	-
Rx3	*Tsukamurella ocularis*	+++	+++	36,14	37,04
RX4	*Tsukamurella ocularis*	+++	+++	52,74	27,59
RX5	*Tsukamurella tyrosinosolvens*	+++	+++	29,52	28,57