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**Isolation of bioactive metabolites from the red
alga *Sphaerococcus coronopifolius***

DIPLOMA THESIS

Committed to University of Zagreb, Faculty of Pharmacy and Biochemistry

Zagreb, 2015.

This diploma thesis is reported at the Department of Pharmacognosy, University of Zagreb, Faculty of Pharmacy and Biochemistry and is implemented in the Department of Pharmacognosy and Chemistry of Natural Products, Faculty of Pharmacy, National and Kapodistrian University of Athens under the professional supervision of Associate Professor Marijana Zovko Končić in cooperation with Assistant Professor Efstathia Ioannou and Professor Vassilios Roussis.

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1. INTRODUCTION

1.1. POTENTIAL OF MARINE ORGANISMS AS SOURCES OF NATURAL PRODUCTS

Almost three quarters (79%) of the earth's surface is covered by water, but still most of the pharmacognostic studies are focused on terrestrial plants and medicines that can be derived from these plants. Other organisms are also considered to have pharmacognostic potential, in particular different microbes and since recently various marine organisms, such as algae, sponges, ascidians and marine-derived bacteria and fungi. Marine organisms live in a much different environment than terrestrial organisms. That is the reason to assume that secondary metabolites will vary significantly from the ones that terrestrial plants produce. This area of marine organisms is considered to be uncharted territory if we talk about their pharmacognostic potential. Since 1940s with the development of SCUBA diving and open-circuit self-contained underwater breathing apparatus there has been a tremendous growth of marine pharmacognosy researches. Also the need for more new bioactive compounds is growing from day to day. In the last six decades many different marine organisms have been explored for bioactive compounds (Brahmachari, 2012; Jimeno et al., 2004; Newman et al., 2004).

The compounds from marine organisms that are being investigated are chemical compounds that organisms produce as part of their normal metabolic activities. Metabolic products are generally divided in two big groups: primary metabolites and secondary metabolites. The biggest number of compounds of natural origin is secondary metabolites, species-specific chemical agents that can be grouped into various categories. Natural products are often synonyms for secondary metabolites. Those metabolites are usually relatively small molecules with structural diversity and a molecular weight under 3,000 Daltons. They are called secondary metabolites because they are not biosynthesized by the general metabolic pathways. Also, secondary metabolites don't have a primary function which is directly involved in the normal growth, development or reproduction of an organism (Kliebenstein, 2004).

For the last two decades researchers are in constant search for potential pharmaceuticals and natural products are considered to be one of the major sources for development of new drugs. To find a single bioactive molecule that can find its place in pharmacy and medicine is

an extremely long and complicated process. However, a number of natural products isolated from marine organisms have already become active ingredients of pharmaceutical preparations because they showed remarkable biological activities, while many others are still in various stages of clinical trials (Brahmachari, 2012; Koehn et al., 2005).

Various methods for the separation and isolation and also instrumentation methods for the structural characterization, such as nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS) and X-ray diffraction analysis, have helped researchers to get bioactive compounds in pure form with clearly defined structures (Martins et al., 2014).

1.2. ALGAE IN GENERAL

Algae are broad group of mostly aquatic, photosynthetic microorganisms (from unicellular to multicellular). They look like plants known as phytoplankton, but better known as a living organism plant without roots, leaves or flowers. It is estimated that there are more than 25.000 species of algae. Most of them are marine algae in the oceans and the rest are freshwater algae. Water flowers, water moss, seaweed or kelp - these are all forms of algae. Algae can come in different sizes, from tiny picoplankton which must be 1.000 times increased that we could see it all the way to giant grass in the oceans that can grow to 50 meters in length.

They belong to the groups of prokaryotes and eukaryotes. These groups can be further divided in classes shown in Table 1 (<http://botany.si.edu/projects/algae/introduction.htm>).

Table 1. Classification of algae in the groups pf eukaryotes and prokaryotes

EUKARYOTES	PROKARYOTES
Bacilliarophyceae Chlorophyceae (green algae) Chrysophyceae (golden algae) Dinophyceae (dinoflagellates) Phaeophyceae (brown algae) Rhodophyceae (red algae)	Class Cyanophyceae (blue green algae)

A common characteristic of all algae is performing photosynthesis in which they produce oxygen as a by-product (unlike some photosynthetic bacteria). With the exception of blue-green algae, algae are eukaryotes what means that their cells contain organelles- nucleus and mitochondria membrane separated from the cytoplasm. Eukaryotic algae also contain chloroplasts in which are located pigments which are absorbing solar energy during the process of photosynthesis. For most algae, along with other pigments which are giving them a distinctive coloration (phycoerythrin - red, phycocyanin and allophycocyanin - blue, fucoxanthin - brown, violaxanthin - violet, etc.), the primary pigment is chlorophyll - green pigment.

Although they have many similarities to land plants, algae are not considered to be real plants because they lack a specialized vascular system (conduction system of fluid and nutrients) roots, stems, leaves (taking nutrients, liquids and gases directly from the water column) and an indoor reproductive organs (flower or cone). Algae need only minerals and sunlight for their bloom, and life in water to prevent its drying out.

1.3. THE RED ALGA *SPHAEROCOCCUS CORONOPIFOLIUS*

Red algae (Rhodophyceae, from the Greek words $\rho\acute{o}\delta\omicron\nu$ = red and $\phi\upsilon\tau\acute{o}\nu$ = plant) are a large group of mostly multicellular, marine algae, including many seaweeds. There are between 5,000 and 6,000 living species, while other sources say that there are more than 10,000 species of algae. *Sphaerococcus coronopifolius* is a species also known as *Haematocelis fissurata* Crouan.frat or in English as Berry Wart Cress. First chemical analysis on this alga was done on 1976 and since then great number of brominated cyclic diterpenes has been isolated (Woelkerling, 1990). On figure 1 is shown how *S. coronopifolius* looks like. The taxonomic classification of *S. coronopifolius* is presented in Table 2 (<http://www.algaebase.org>).



Figure 1. The red alga *Sphaerococcus coronopifolius* (from skaphandrus.com website)

Table 2. Taxonomic classification of *Sphaerococcus coronopifolius*

Empire:	Eukaryota
Kingdom:	Plantae
Phylum:	Rhodophyta
Subphylum:	Eurhodophytina
Class:	Florideophyceae
Subclass:	Rhodymeniophycidae
Order:	Gigartinales
Family:	Sphaerococcaceae
Genus:	<i>Sphaerococcus</i>

1.3.1. DESCRIPTION OF *SPHAEROCOCCUS CORONOPIFOLIUS*

This alga grows from a discoid holdfast to form an erect, compressed, two edged, cartilaginous, thallus with scarlet fronds and dark brown-red main axes long to 300 mm. It has a lot of branches which are made as distichous, subdichotomous or alternative. Small final branchlets are often fringed with short marginal proliferations. The tetrasporic phase is crustose and was previously referred to as *H. fissurata* Crouan.frat. *Plocamium cartilagineum* is similar, but *Plocamium* shows a different pattern of branching which has not been seen in *Sphaerococcus* (<http://www.habitas.org.uk/marinelife/>).

1.3.2. DISTRIBUTION, HABITAT AND ECOLOGY

The gametangial phase of *S. coronopifolius* is located mostly in the south-western coast of England, Wales, around Ireland and western coasts of Scotland.

The tetrasporic phase has been recorded from Ireland and Scotland. Europe: Mediterranean, Azores, Portugal, Atlantic coasts of Spain and France. It can also be found around Canary Islands. Geographical coordinates on which *S. coronopifolius* can grow are showed in Table 3.

Table 3. Geographical coordinates on which *Sphaerococcus coronopifolius* can grow

Latitude (degrees):	40.12 – 59.25
Longitude (degrees):	-10.30 – 18.51

Frequent habitat of this alga is rocks in shallow areas with the depth up to 25.6 m maximum. Depth range was based on 95 specimens in one taxon. Water temperature and chemistry ranges were based on 52 samples. Environmental ranges in which *S. coronopifolius* can grow are shown in Table 4 (<http://www.iobis.org/mapper>).

Table 4. Environmental ranges in which *Sphaerococcus coronopifolius* can grow

Depth range (m):	0 – 25.6
Temperature range (Celsius degrees):	11.845 – 16.269
Nitrate ($\mu\text{mol/l}$):	1.692 – 4.757
Salinity (PPS):	35.008 – 37.969
Oxygen (ml/l):	5.382 – 6.346
Phosphate ($\mu\text{mol/l}$):	0.229 – 0.335
Silicate ($\mu\text{mol/l}$):	1.778 – 3.727

1.3.3. REPRODUCTION

Red algae can reproduce themselves asexually, sexually or by exchanging these two mechanisms (this is called exchange of generations). The cycle of reproduction can be caused by various factors, such as day length. They have no moving cells; spores and gametes are fixed and the algae rely on water as a carrier of cells in organs. Vegetative reproduction is achieved by simple partitioning (at unicellular forms), by breaking colonies or only with some broken parts of the talus. There is also a reproduction with spores which results with certain number of different types of spores (monospore, tetraspore and polyspore). Sexual reproduction is presented by atypical oogamy (Lee, 2008). On Figure 2 is shown typical red algae life cycle.

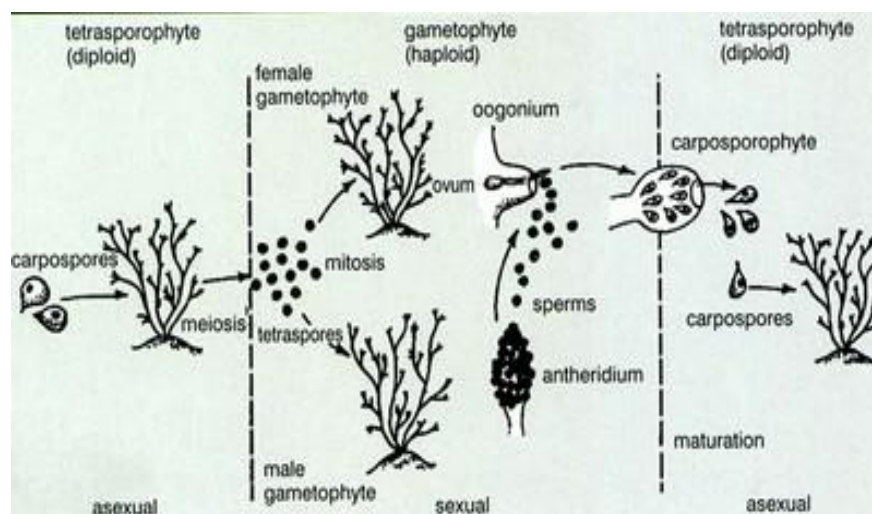


Figure 2. Outline of a typical red algae life cycle. After Wallace et al. (1986) in Fosså and Nilsen (1996) (from advancedaquarist.com website)

2. AIM OF PROJECT

The ultimate goal of the research associated with the chemistry of natural products is the isolation of secondary metabolites, which will show biological activity or some other worthwhile application.

Mediterranean sea shows wide biodiversity and therefore wide pharmacological potential. In this project it has been studied chemical composition of the one cosmopolitan bright-red alga *S. coronopifolius*. Since now it has been known that this red alga has in its own composition many interesting brominated diterpenes even though they are much more common in brown algae. For some of isolated metabolites it has been proven that they have certain antibacterial, insecticidal, antifungal and antiviral activity. Cafieri et al. in 1982 and 1988 isolated the diterpenes sphaeopyrane and 12*S*-hydroxybromosphaerol from this alga; later in 2001 Etahiri et al. isolated two new bromoditerpenes which showed antibacterial activity against Gram (+) bacteria. Need for new antimicrobial agents is getting bigger every day if it is considered that new sorts of bacteria are getting more and more resistant to all existing drugs.

The aim of this study was to isolate and then identify some of the compounds in the sample of red alga *S. coronopifolius* collected in Liapades bay at Corfu Island, Greece, in October of 2014.

3. MATERIALS AND METHODS

3.1. MATERIAL PREPARATION

Specimens of the red alga *S. coronopifolius* were collected by SCUBA diving in Liapades bay at Corfu Island, Greece, at a depth of 8 – 15 m in October of 2014. A voucher specimen is kept at the Herbarium of the Department of Pharmacognosy and Chemistry of Natural Products, University of Athens (ATPH/MP0485). *S. coronopifolius* was initially air-dried (0.85 kg dry weight). The dried algal biomass was extracted with 100% MeOH at room temperature for one day and the obtained extract was filtered and evaporated under vacuum until total dryness. A second and a third cycle of extraction were performed with mixtures of DCM /MeOH (3:1). After two days of samples being extracted in mentioned solvents, extracts were again filtered and evaporated under vacuum. The final weight of the dark green oily residue that was obtained from the combined extracts was 23.64 g.

3.2. METHODS

3.2.1. CHROMATOGRAPHY

Chromatography (from Greek χρώμα *chroma* "color" and γράφειν *graphein* "to write") is one term that describes set of laboratory techniques for the separation of mixtures. For this method mixture has to be dissolved in a fluid called the mobile phase, which carries it through other part of chromatography system which is holding another material called the stationary phase. The various compounds of the mixture travel with different speeds, and that way they are being separated. There are couple of ways to divide existing chromatography methods, but most common division is the one based on physical state of mobile phase (Ettre, 1993).

3.2.1.1. LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a method for separation of substances based on their interaction between a stationary solid phase and a liquid mobile phase. There are two subtypes of this chromatography: traditional and high performance LC (HPLC). Traditional LC is being performed in different sized columns depending on the weight of the sample that needs to be analyzed using this method. Columns are being packed with porous material, usually silica gel. The liquid mobile phase goes through the column by gravity, so the flow can be pretty slow and this method is not particularly effective in that case. The principle of gravity column chromatography (GCC) is based on differential adsorption of substances by the adsorbent. The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of the adsorbent and the polarity of the solvent. If the activity of the adsorbent is very high and the polarity of the solvent is very low, then the separation is very slow but gives a good separation. On the other hand, if the activity of the adsorbent is low and the polarity of the solvent is high, the separation is rapid but gives only a poor separation. This method can be improved and accelerated by using suction from an aspirator that creates a partial vacuum in the flask. Vacuum liquid chromatography (VLC) became one of the most common methods for separation of mixtures because it is a pretty simple and inexpensive method that is capable of giving good results in a short time period (Ettre, 1993).

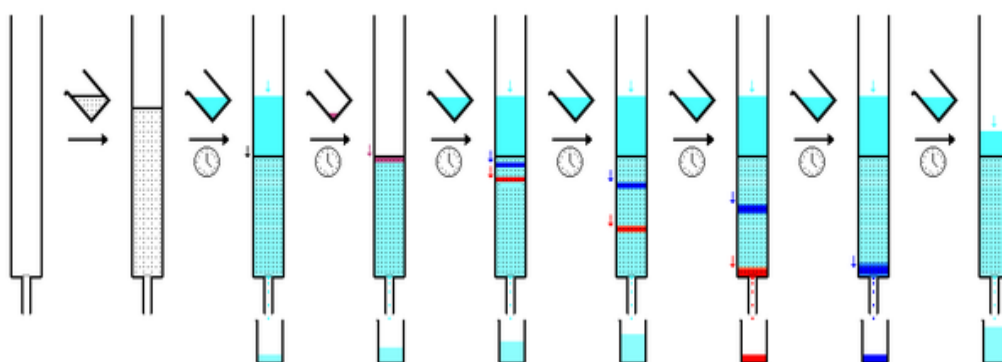


Figure 3. Gravity column chromatography shown in seven steps (from wikipedia.org website)

Gravity column chromatography showed in seven steps (Figure 3):

1. Column is being loaded with silica or some other column medium
2. Eluting solvent is added to compact silica layer and to remove air bubbles
3. Purple mixture as a thin layer is added to top of silica layer
4. Eluting solvent is added and eluted (purple layer separates into a red and blue layer)
5. Eluting solvent is added and eluted (red and blue layers separate further)
6. Red layer is being collected (the faster moving layer)
7. Blue layer is being collected (the slower moving layer)
8. No more compounds to be eluted, so process ended here

In HPLC the sample is forced to be moved by an eluent at high pressure (the mobile phase) through a column. Column is filled with a stationary phase. Basic HPLC scheme is shown on Figure 4. HPLC is divided into two different subtypes based on the polarity of the mobile and stationary phases. Methods in which the stationary phase is more polar than the mobile phase are named normal phase liquid chromatography and the opposite is named reversed phase liquid chromatography (Ettre, 1993).

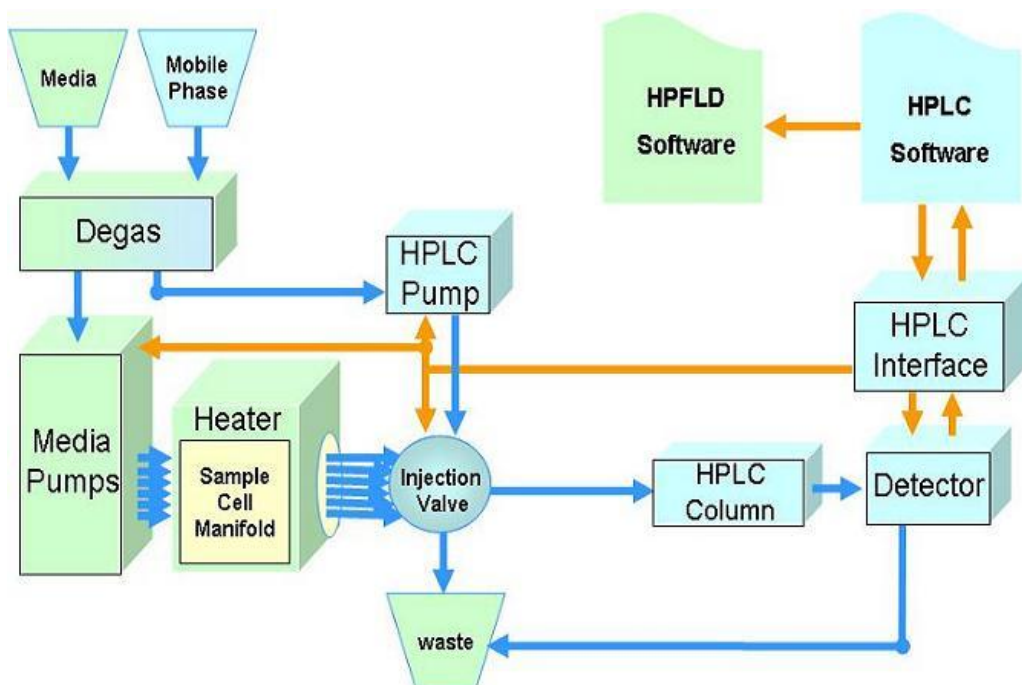


Figure 4.HPLC scheme (from pharmaanalytic.com website)

3.2.1.2. THIN LAYER CHROMATOGRAPHY

Based on physical state of mobile phase TLC is also one kind of liquid chromatography. If we consider chromatographic bed shape, then TLC is planar type of chromatography. This method is similar to paper chromatography with a difference in stationary phase- it is a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. After sample application on the TLC plate, a plate needs to be located with its small part under the place with applied samples in solvent or solvent mixture (mobile phase). Capillary action takes different analytes up on the TLC plate at different rates and that way separation is achieved (Figure 5). Sometimes compounds being separated with this method may be colorless, so for visualization of the spots where compounds ended their journey on TLC plate is has been made reagent of MeOH, glacial acetic acid, sulphuric acid (H_2SO_4) and p-anisaldehyde. During this project TLC method was mostly used to identify compounds present in a given mixture and determine the purity of substances (Ettre, 1993).

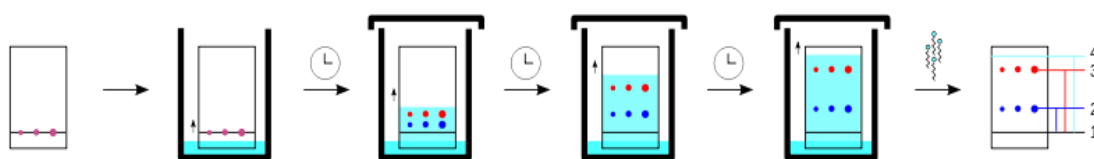


Figure 5.Development of a TLC plate, a purple spot separates into a red and blue spot (from drgpinstitute.wordpress.com website)

3.2.1.3. 1H NMR

1H NMR spectroscopy (proton nuclear magnetic resonance) detects signals of chemically non-equivalent protons in analyzed compounds. The number of protons is estimated based on the ratio of the integral area under individual signals. Together with ^{13}C NMR, 1H NMR is a one of most common tools for molecular structure characterization.

3.2.1.4. GAS CHROMATOGRAPHY – MASS SPECTROMETRY

GC-MS make an effective combination for chemical analysis. Gas chromatography part is there to separate components in the sample. Each of those separated components ideally produces a specific spectral peak which can be recorded on a paper chart or electronically. Through the very narrow column flows gas carrier which is actually mobile phase. Carrier gases are usually argon, helium, hydrogen, nitrogen, or hydrogen because those are inert gases and they do not react with stationary phase or components of sample. Compounds in gas steam exit the column with different time (retention time) based on their various chemical and physical characteristics and their interaction with a specific column filling, stationary phase. The GC instrument uses usually MS detector to measure the compound's mass spectrum on their exit from column (Figure 6). Compound's mass spectrum is then easily used for qualitative identification (<http://www.scientific.org/tutorials/articles/gcms.html>).

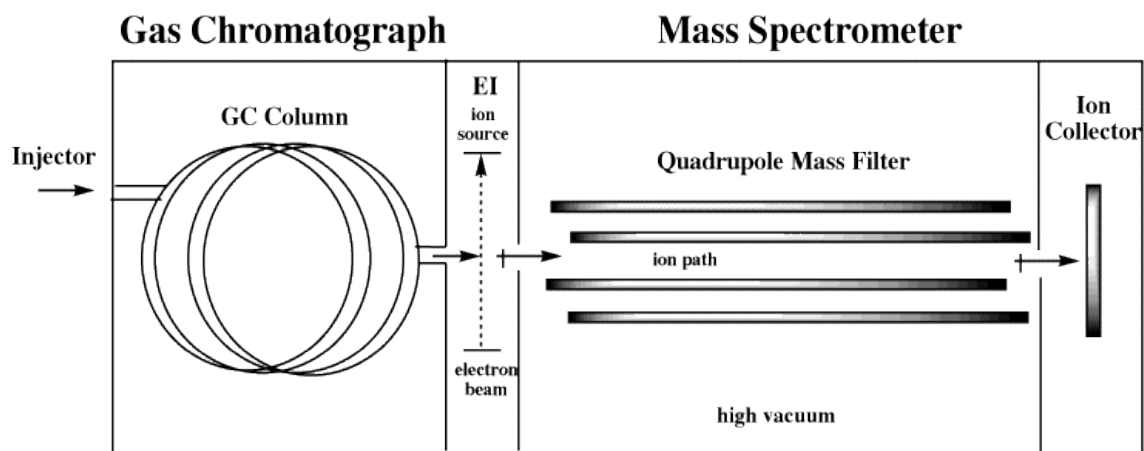


Figure 6. Simplified scheme of GC – MS (from orgchem.colorado.edu website)

3.3. INSTRUMENTATION

NMR spectra were recorded using CDCl₃ (Aldrich-Sigma Chemical Company) on Bruker AC 200 and Bruker DRX 400 spectrometers. Chemical shifts are given on a δ (ppm) scale using TMS as internal standard.

Low resolution EI mass spectra were measured on a Hewlett Packard 5973 mass spectrometer. Low-resolution CI mass spectra were measured in positive mode on a Thermo Electron Corporation DSQ mass spectrometer using a Direct-Exposure Probe and methane as the CI reagent gas.

Column chromatography separations were performed with Kieselgel 60 (Merck). HPLC separations were conducted using a CECIL 1100 Series liquid chromatography pump equipped with a GBC LC-1240 refractive index detector, using a Techsil100DS (Wellington House, 25 cm x 22 mm) column. TLC were performed with Kieselgel 60 F₂₅₄ (Merck aluminum support plates) and spots were detected after spraying with 15% H₂SO₄ in MeOH reagent and heating at 100 °C for 1 min.

Evaporation of the solvents under vacuum was conducted using a BÜCHI rotavapor R-114 and also BÜCHI waterbath B-480 at 38°C.

All solvents used were of analytical grade (LAB-SCAN Analytical Sciences), which were distilled prior to use. All solvents used for HPLC separations were filtered and degassed prior to use.

3.4. SEPARATION AND ISOLATION OF BIOACTIVE METABOLITES

The isolation procedure of bioactive metabolites from the initial residue of *S. coronopifolius* marked with code name SCF is shown diagrammatically in Figure 7.

Vacuum liquid chromatography on silica gel normal phase column was the first action done on initial residue (23.64 g). The system of eluting solvents was composed of mixtures cHex / EtOAc and EtOAc / MeOH in increasing polarity systems. Result of this vacuum liquid chromatography was in total 27 fractions (Table 5), which were then tested chromatographically with TLC. For fractions from number 1 to 12 used solvent system was 70 % cHex : 30 % EtOAc ; for fractions from 12 to 22 it was used 30 % cHex : 70 % EtOAc and for samples from 23 to 27 it was used 95 % EtOAc : 5 % MeOH. Fractions that exhibited similar chromatographic characteristics were combined, so the final number of obtained fractions after this vacuum liquid chromatography was 14 (Table 6). This table also shows weights of final 14 samples.

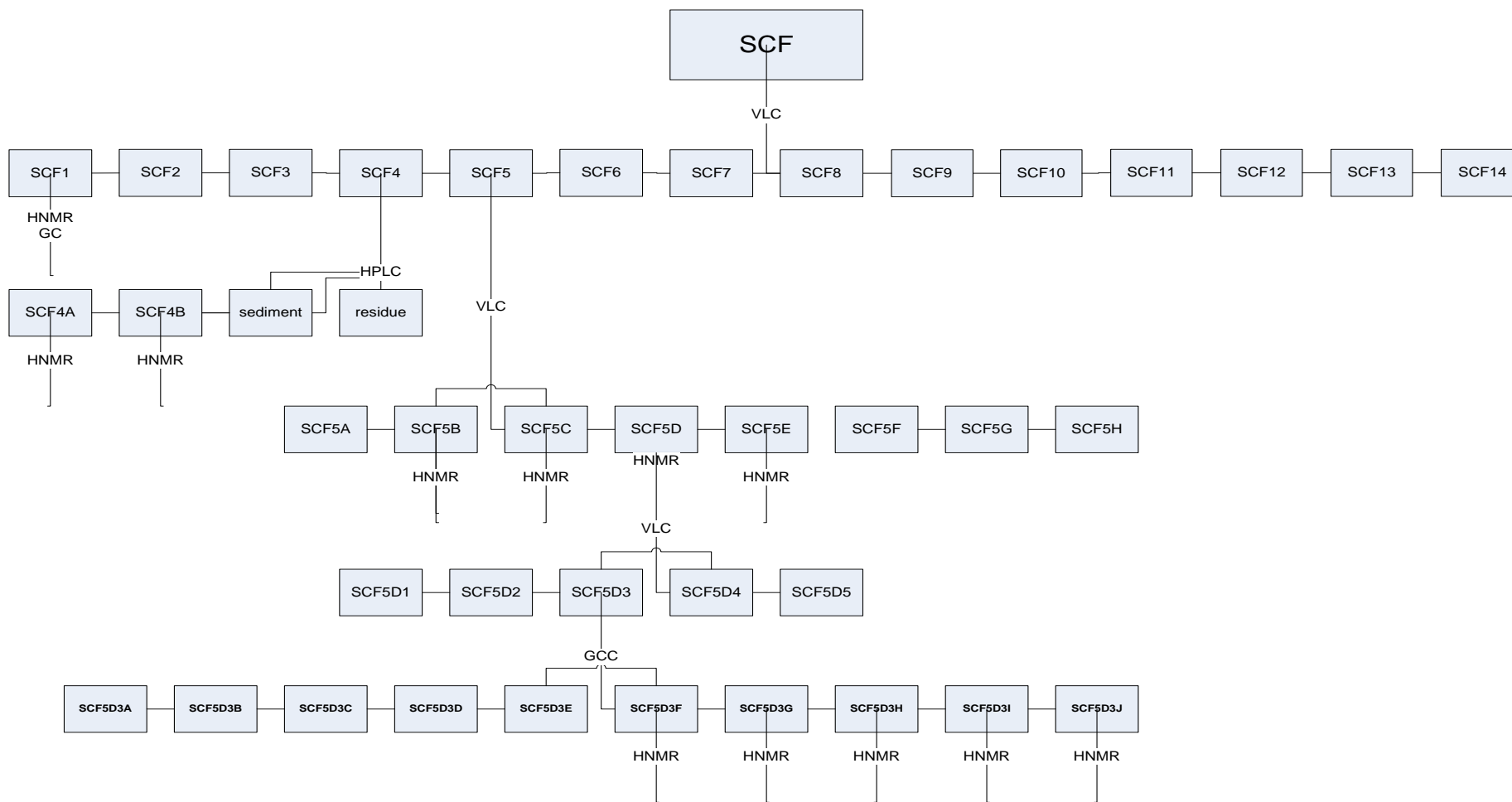


Figure 7. Scheme of isolation of bioactive metabolites from sample of *Sphaerococcus coronopifolius*

Table 5. Elution solvent systems, fractions collected after using mentioned solvent systems and volumes of collected fractions (ml)

Solvent of eluation	Fraction	Volume (ml)
100 % cHex	1 and 2	250
95 % cHex : 5 % EtOAc	3 and 4	250
90 % cHex : 10 % EtOAc	5 and 6	250
85 % cHex : 15 % EtOAc	7 and 8	250
80 % cHex : 20 % EtOAc	9 and 10	250
70 % cHex : 30 % EtOAc	11 and 12	250
60 % cHex : 40 % EtOAc	13 and 14	250
50 % cHex : 50 % EtOAc	15 and 16	250
30 % cHex : 70 % EtOAc	17 and 18	250
10 % cHex : 90 % EtOAc	19 and 20	250
100 % EtOAc	21 and 22	250
90 % EtOAc : 10 % MeOH	23 and 24	250
75 % EtOAc : 25 % MeOH	25 and 26	250
100 % MeOH	27	500

Samples with code name SCF1 and SCF2 were identified by analysing their spectroscopic data (GC- MS) and comparing them with literature data. Samples with code names SCF1 and SCF5 were also tested by ¹H-NMR.

Table 6. Fractions obtained after combining, code names and final weights (g)

Combined fractions	Code name	Weight (g)
1+2+3	SCF1	1.574
4+5+6+7	SCF2	0.04
8	SCF3	0.287
9+10	SCF4	10.262
11	SCF5	2.008
12	SCF6	0.409
13+14	SCF7	1.409
15+16+17	SCF8	1.281
18	SCF9	0.258
19+20	SCF10	0.503
21+22	SCF11	0.362
23+24	SCF12	0.1280
25	SCF13	0.0633
26+27	SCF14	1.873

The SCF4 residue (10.262 g) was diluted approximately five times. Diluted SCF4 residue (2.5355 g) was subjected to HPLC with isocratic system 100 % MeOH as eluent (Table 7). Received two fractions, SCF4A and SCF4B, were tested spectroscopically by ¹H-NMR. That identification method showed that these two substances are purified substances (Table 8).

Also, during this HPLC there has been isolated sediment that couldn't be dissolved in eluent MeOH. Mentioned sediment (0.0643 g) was removed in another vial and eventually dissolved in dichloromethane. Residue weight was 1.1609 g.

Table 7. Chromatography conditions for HPLC procedure done on sample SCF4

CHROMATOGRAPHY CONDITIONS	
Column	Techsil10ODS, 25 cm x 22 mm
Eluent	100% MeOH
Eluation flow	5 ml/min
Detector	GBC LC 1240 R.I. detector

Table 8. Code names, weights (g) and elution times (min) for two compounds separated with HPLC from sample SCF4

Code name	Weight (g)	Elution time (min)
SCF4A	0.1994	16.437
SCF4B	1.0226	18.617

Sample SCF5 was first tested with couple of different solvent systems for TLC that included cHex, EtOAc and DCM while trying to find the best one for separation of its components.

Next step was vacuum liquid chromatography on silica gel normal phase column done on initial residue SCF5 (2.008 g). The system of eluting solvents was composed of mixtures cHex / DCM and DCM / EtOAc in increasing polarity systems. Result of this vacuum liquid chromatography was in total 8 fractions (from A to H) (Table 9), which were tested chromatographically with TLC. Solvent system that has been used for TLC was 80 % cHex : 20 % EtOAc. This table also shows weights of final 14 samples. TLC with solvent system 95 % n-pentane : 5 % acetone and 95 % n-pentane : 5 % EtOAc was done on sample SCF5D.

Table 9. Code names, elution solvent systems, collected fractions (ml) and weights (g) after VLC on sample SCF5

Code name	Solvent system	Volume (ml)	Weight (g)
SCF5A	80% cHex : 20% DCM	150	0.0084
SCF5B	50% cHex: 50% DCM	150	0.352
SCF5C	20% cHex : 80% DCM	150	1.1664
SCF5D	100% DCM	150	0.3012
SCF5E	90% DCM : 10% EtOAc	150	0.109
SCF5F	70% DCM: 30% EtOAc	150	0.0664
SCF5G	50% DCM : 50% EtOAc	150	0.018
SCF5H	100% EtOAc	150	0.0075

Next step for this sample (0.3012 g) was vacuum liquid chromatography on silica gel normal phase column. The system of eluting solvents was composed of mixtures n-pentane and EtOAc in increasing polarity systems. Result of this vacuum liquid chromatography was 9 fractions. Some of mentioned nine fractions that exhibited similar chromatographic characteristics were combined, so the final number of obtained fractions was five (Table 10 and Table 11). Samples SCF5D3 and SCF5D4 were also tested spectroscopically by ¹H-NMR.

Table 10. Elution solvent systems, number of fractions and volumes (ml) of collected fractions after VLC on sample SCF5D

Solvent system	Fraction	Volume (ml)
100% n-pentane	1	50
95% n-pentane : 5% EtOAc	2	50
90% n-pentane : 10% EtOAc	3 and 4	50
85% n-pentane : 15% EtOAc	5 and 6	50
80% n-pentane : 20% EtOAc	7 and 8	50
100% EtOAc	9	50

Table 11. Code names, fractions obtained after combining and final weights (mg) after VLC on sample SCF5D

Code name	Fractions combined	Weight (mg)
SCF5D1	1+2+3	4.4
SCF5D2	4	13.8
SCF5D3	5	214.4
SCF5D4	6	36.8
SCF5D5	7+8+9	6.7

Next step for sample SCF5D3 (0.2144 g) was gravity column chromatography on silica gel normal phase column (Table 12). Based on dimensions of column, it was possible to calculate approximate volume of eluent needed for chromatography ($V = \pi \times d^2 \times h$).

Table 12. Chromatography conditions for sample SCF5D3

CHROMATOGRAPHY CONDITIONS	
Height of column (cm)	12.5
Radius of column (cm)	3
Eluent system	95% cHex : 5% EtOAc (for samples 1-35)
Eluent system	90% cHex : 10% EtOAc (for samples 35-44)
Eluent system	100% EtOAc (sample 45)

Result of this gravity column chromatography was in total 45 fractions, from which first 35 fractions were received after using 95 % cHex : 5 % EtOAc as eluent. Next 10 fractions were collected with 90 % cHex : 10 % EtOAc used as eluent. All 45 fractions were tested chromatographically with TLC where 90 % cHex : 10 % EtOAc was the solvent system. Fractions that showed similar chromatographic characteristics were combined, so the final number of fractions after GCC on SCF5D3 sample was 10 (from A to J) (Table 13). Samples SCF5D3F, SCF5D3G, SCF5D3H and SCF5D3I were tested spectroscopically by ¹H-NMR.

Table 13. Code names, fractions obtained after combining and final weights (mg) of sample SCF5D3

Code name	Fraction number	Weight (mg)
SCF5D3A	1-4	1.4
SCF5D3B	5	0.6
SCF5D3C	6	0.6
SCF5D3D	7	0.5
SCF5D3E	8-15	2.2
SCF5D3F	16-19	49.8
SCF5D3G	20-23	25.1
SCF5D3H	24-35	104.4
SCF5D3I	36-44	14.8
SCF5D3J	45	7.6

4. RESULTS AND DISSCUSION

Specimens of *S. coronopifolius* were collected in Liapades bay at Corfu Island, Greece, in October of 2014. The dried algal thalli were exhaustively extracted at room temperature with 100 % MeOH and mixtures of DCM /MeOH (3:1). The obtained organic extract was subjected to a series of chromatographic separations that resulted in the isolation of three secondary metabolites (1–3).

The structure elucidation of the isolated compounds was based on analysis of their spectroscopic data (NMR and MS) and comparison of their spectroscopic and physical characteristics with those reported in the literature.

4.1. COMPOUND 1

Compound 1, (code name SCF1), isolated as a colorless not-oily compound, had a molecular formula of $C_{15}H_{24}$, as deduced from its MS and ^{13}C NMR spectra.

In particular, compound 1 displayed in the mass spectrum (Figure 8) the molecular ion $[M]^+$ at m/z 204.

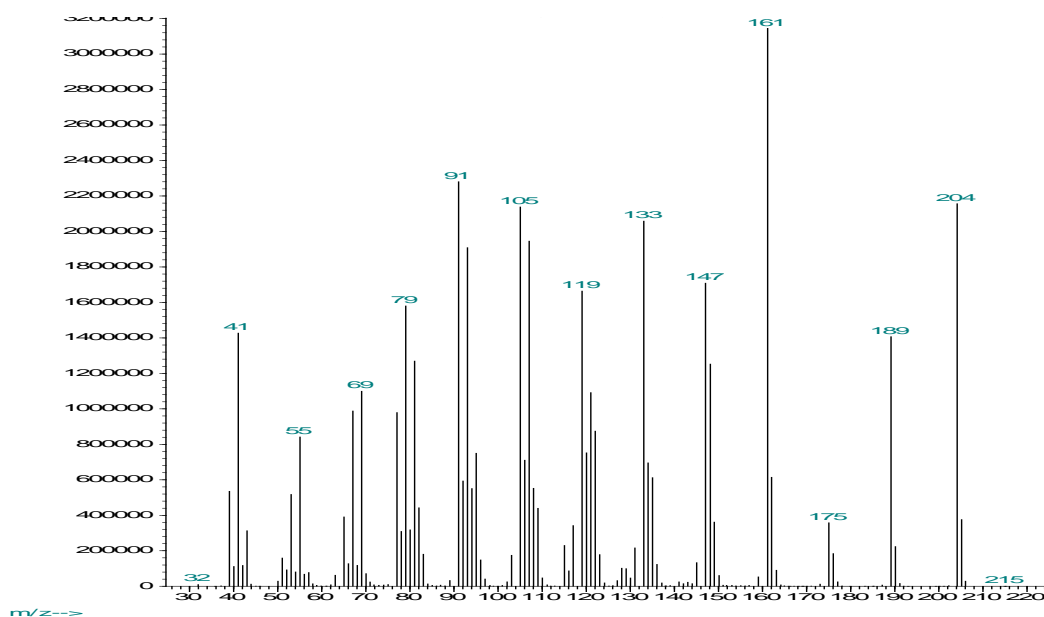


Figure 8. Mass spectrum (EI-MS) of compound 1

The ^{13}C NMR spectrum of **1** (Figure 9) revealed 15 carbon signals. Among them, evident were two olefinic carbons resonating at δ 109.7 and 152.1.

The ^1H NMR spectrum of **1** (Figure 10) included signals for an exomethylene group (δ 4.70 and 4.73) and three methyls on quaternary carbon atoms (δ 0.94, 0.96 and 1.00).

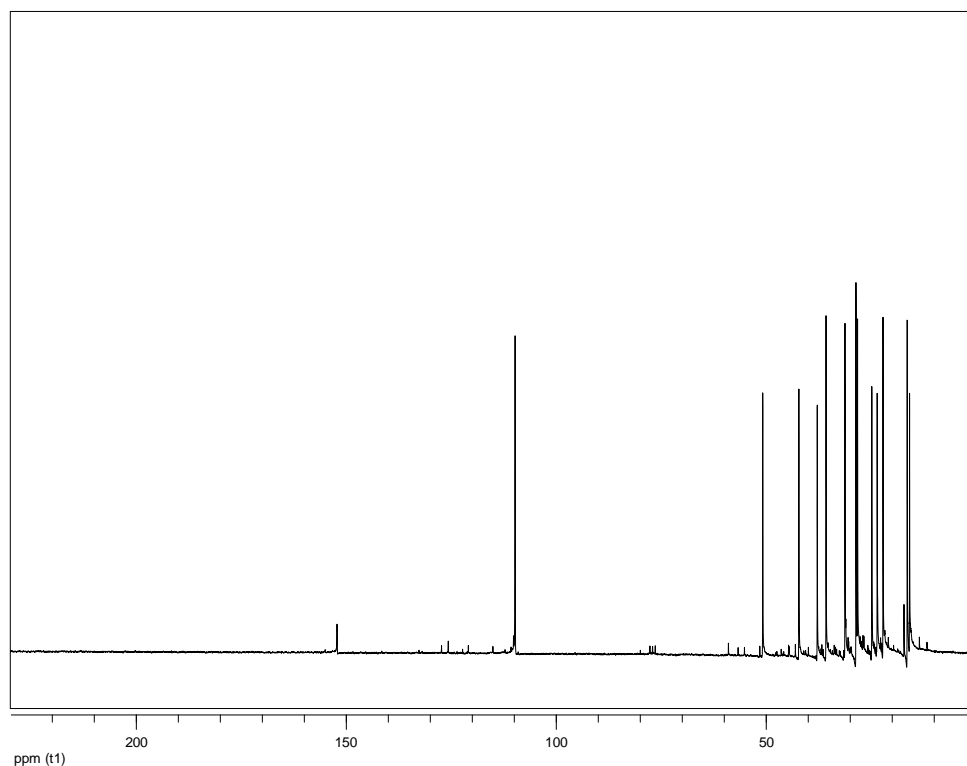


Figure 9. ^{13}C NMR spectrum of compound **1**

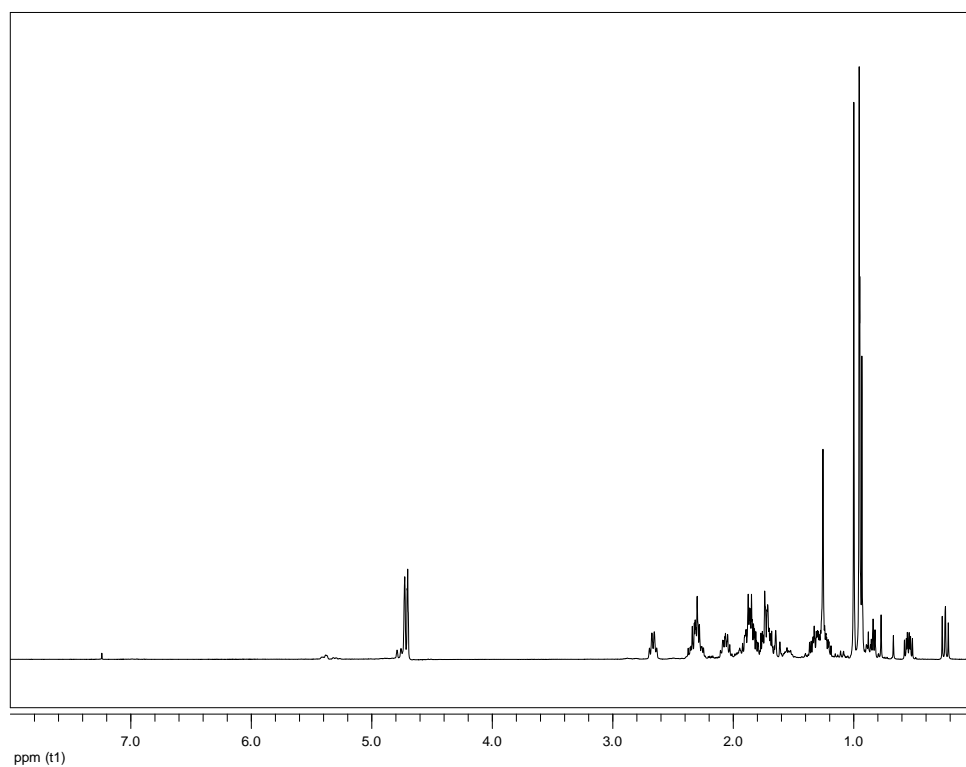


Figure 10. ¹H NMR spectrum of compound **1**

Comparison of the spectroscopic data of compound **1** (Table 14) with those reported in the literature for relevant molecules allowed for the identification of **1** as *allo*-aromadendrene (Figure 11) (De Rosa et al., 1988; Faure et al., 1991).

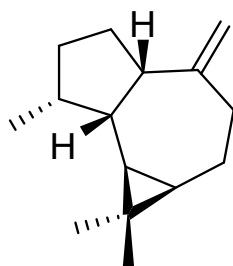


Figure 11. Structure of compound **1**

allo-Aromadendrene is common compound of many plants and it has been isolated in the past. One research done on mixed-type *Cinnamomum osmophloeum* leaves showed that *allo*-aromadendrene in it has protective activity against oxidative stress but also it prolonged the lifespan of *Caenorhabditis elegans* what indicated on its ability to delay aging (Yu et al., 2014).

Table 14. NMR data of compound **1** in CDCl₃ (δ in ppm, multiplicity, *J* in Hz)

Position	δ_C	δ_H
1	50.8	2.66 (brq 8.2)
2	28.2	1.89 (m), 1.73 (m)
3	31.2	1.73 (m), 1.32 (m)
4	37.8	2.07 (brdhept 2.8, 7.0)
5	42.2	1.86 (m)
6	23.5	0.24 (dd 9.4, 11.0)
7	24.8	0.55 (ddd 5.9, 9.4, 11.0)
8	22.1	1.83 (m), 1.24 (m)
9	35.7	2.31 (m)
10	152.1	
11	17.1	
12	28.6	1.00 (s)
13	15.8	0.96 (s)
14	16.4	0.94 (d 7.0)
15	109.7	4.73 (brs), 4.70 (brs)

4.2. COMPOUND 2

Compound **2**, (code name SCF4B), isolated as a brown-yellow oily compound, had a molecular formula of $C_{20}H_{32}Br_2O$, as deduced from its MS and ^{13}C NMR spectra.

In particular, compound **2** displayed in the mass spectrum (Figure 12) the fragment ion $[M+H-H_2O]^+$ at m/z 429 with isotopic peaks at m/z 431 and 433 with a relative intensity of 1:2:1, which indicated the presence of one hydroxyl group and two bromine atoms in the molecule.

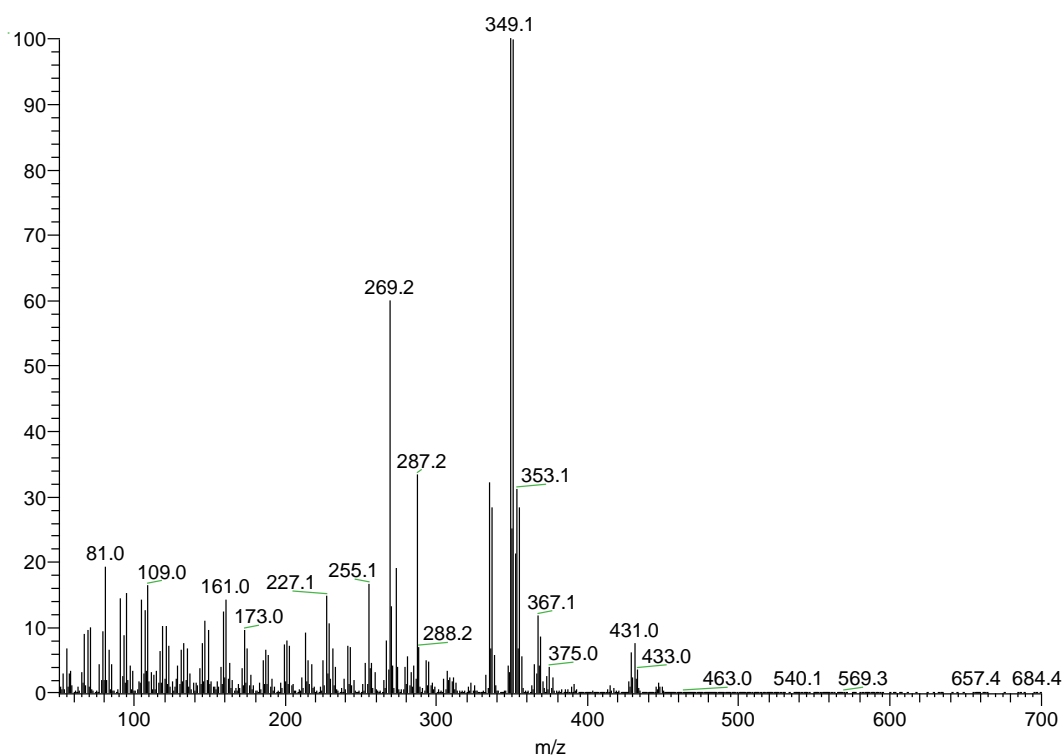


Figure 12. Mass spectrum (PCI-MS) of compound **2**

The ^{13}C NMR spectrum of **2** (Figure 13) revealed 20 carbon signals. Among them, evident were one oxygenated carbon resonating at δ 72.3, two olefinic carbons resonating at δ 126.9 and 128.6 and two halogenated carbons resonating at δ 40.4 and 68.9.

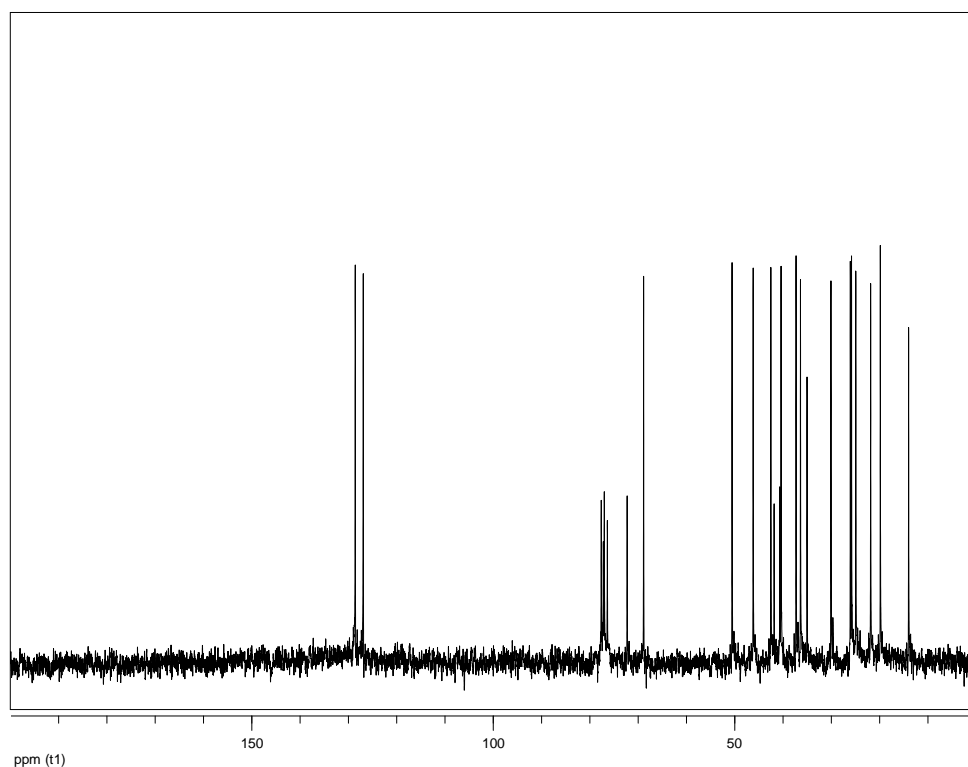


Figure 13. ¹³C NMR spectrum of compound **2**

The ¹H NMR spectrum of **2** (Figure 14) included signals for two olefinic methines (δ 5.67 and 5.99), one halomethine (δ 3.97), one halogenated methylene (δ 3.59 and 3.91), two methyls on tertiary carbon atoms (δ 0.88 and 0.95) and two methyls on quaternary carbon atoms (δ 1.28 and 1.36).

Comparison of the spectroscopic data of compound **2** (Table 15) with those reported in the literature for relevant molecules allowed for the identification of **2** as bromosphaerol (Figure 15) (Fattorusso et al., 1976; De Rosa et al., 1988; Cafieri et al., 1990).

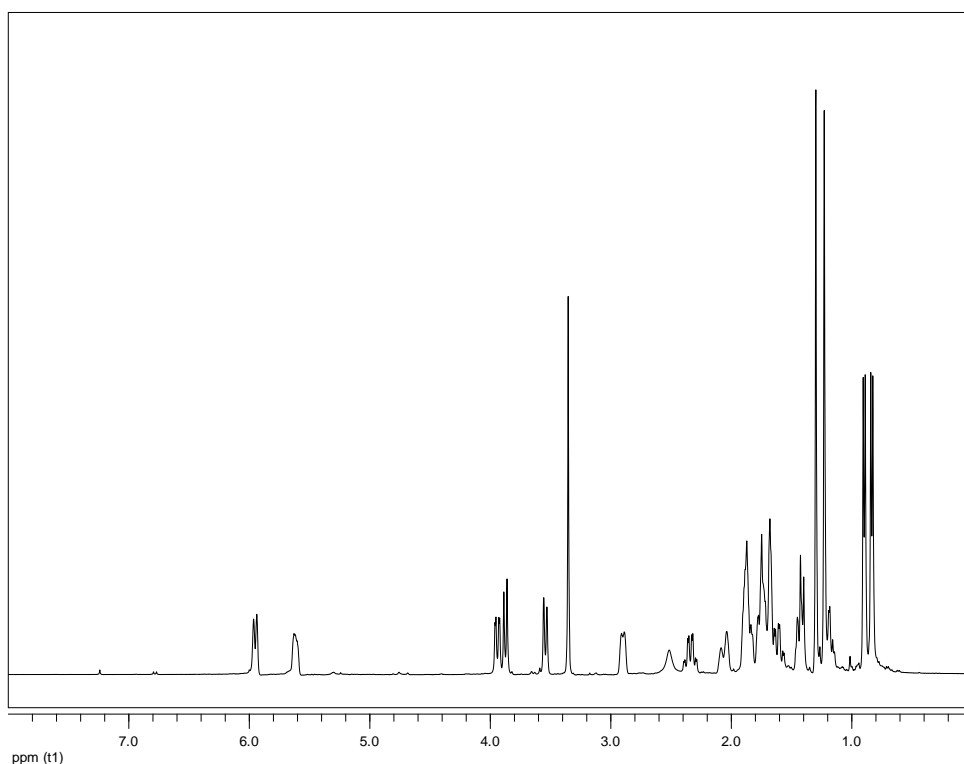


Figure 14. ^1H NMR spectrum of compound **2**

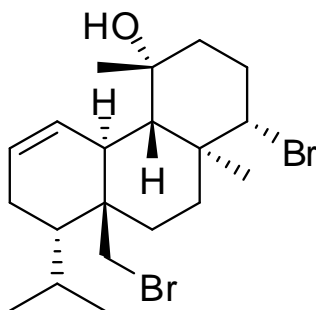


Figure 15. Structure of compound **2**

Bromosphaerol has been isolated before from this alga by the group of researchers from France and Portugal. In mentioned research they proved that all isolated compounds (Sphaerodactylomelol, Bromosphaerol, 12S-hydroxybromosphaerol, 12R-hydroxybromosphaerol and Sphaerococcenol A) have anti-proliferative activity on HepG-2 cells (in vitro carcinoma model of a human hepatocellular cancer) at sub-toxic concentrations. About its antimicrobial activity- the highest growth inhibition against *Staphylococcus aureus* was obtained exactly for Bromosphaerol (Rodrigues et al., 2015).

Table 15. NMR data of compound **2** in CDCl₃ (δ in ppm, multiplicity, J in Hz)

Position	δ_C	δ_H
1	128.6	5.99 (brd 10.6)
2	126.9	5.67 (dm 10.6)
3	21.8	2.12 (m), 1.92 (m)
4	42.5	1.72 (m)
5	41.8	
6	24.9	1.66 (brddd 14.5, 13.7, 4.7), 1.50 (m)
7	36.4	1.83 (ddd 13.7, 4.7, 2.7), 1.22 (m)
8	40.7	
9	50.5	1.46 (d 11.0)
10	37.3	2.96 (dm 11.0)
11	72.3	
12	46.2	1.72 (m), 1.62 (m)
13	30.1	2.39 (tdd 12.9, 12.5, 4.3), 1.96 (m)
14	68.9	3.97 (dd 12.5, 3.5)
15	14.0	1.28 (s)
16	35.0	1.36 (s)
17	40.4	3.91 (d 10.6), 3.59 (dd 10.6, 2.0)
18	26.0	1.98 (m)
19	25.8	0.88 (d 7.0)
20	19.8	0.95 (d 7.0)

4.3. COMPOUND 3

Compound **3**, (code name SCF4A), isolated as a light green not-oily compound, had a molecular formula of $C_{20}H_{29}BrO_2$, as deduced from its MS and ^{13}C NMR spectra.

In particular, compound **3** displayed in the mass spectrum (Figure 16) the molecular ion $[M]^+$ at m/z 380 with an isotopic peak at m/z 382 with a relative intensity of 1:1, which indicated the presence of one bromine atom in the molecule. The characteristic fragment ion $[M-OH]^+$ at m/z 362 with an isotopic peak at m/z 364 with a relative intensity of 1:1 indicated the presence of a hydroxyl group in the molecule.

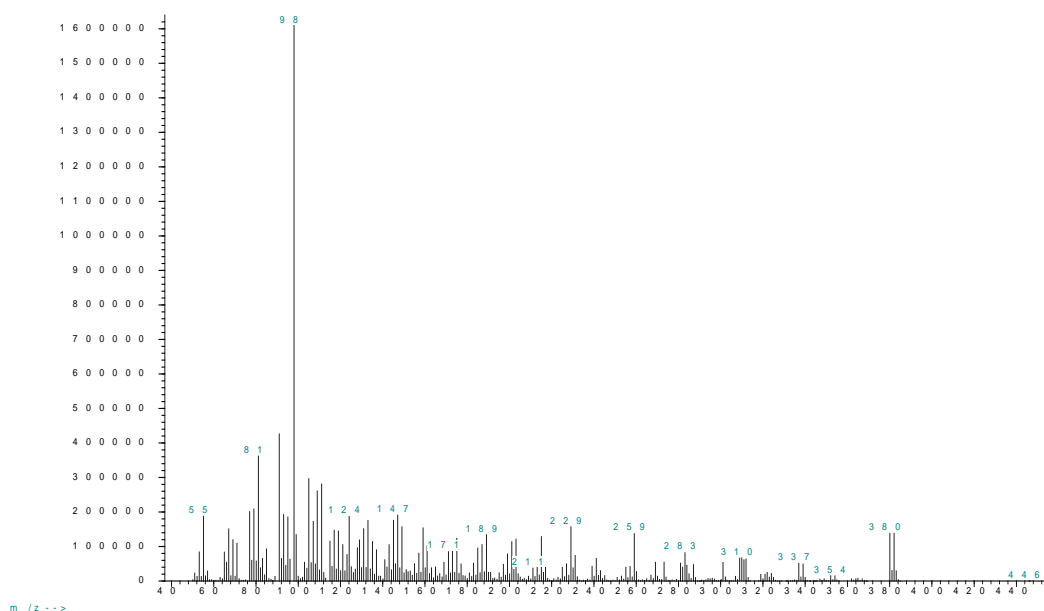


Figure 16. Mass spectrum (EI-MS) of compound **3**

The ^{13}C NMR spectrum of **3** (Figure 17) revealed 20 carbon signals. Among them, evident were one carbonyl resonating at δ 203.4, one oxygenated carbon resonating at δ 75.3, four olefinic carbons resonating at δ 124.4, 127.7, 128.2 and 162.0 and one halogenated carbon resonating at δ 39.8.

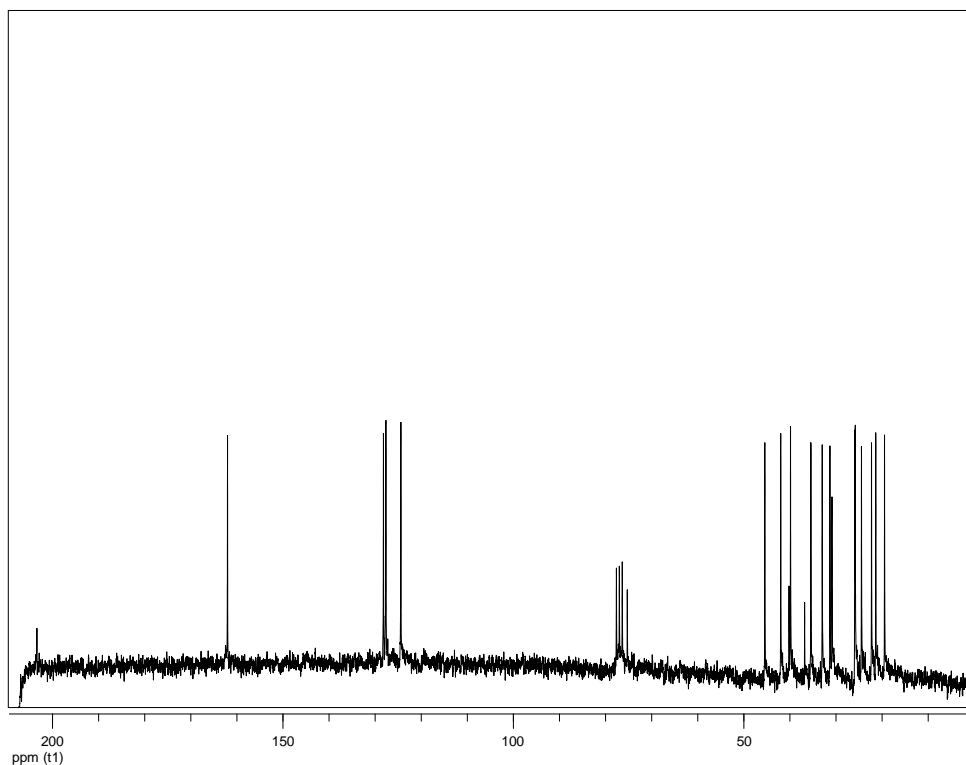


Figure 17. ^{13}C NMR spectrum of compound **3**

The ^1H NMR spectrum of **3** (Figure 18) included signals for an isolated AB system of an α,β -unsaturated ketone moiety (δ 6.05 and 6.81), two olefinic methines (δ 5.73 and 6.03), one halogenated methylene (δ 3.70 and 3.88), two methyls on tertiary carbon atoms (δ 0.90 and 0.96) and two methyls on quaternary carbon atoms (δ 1.07 and 1.32).

Comparison of the spectroscopic data of compound **3** (Table 16) with those reported in the literature for relevant molecules allowed for the identification of **3** as sphaerococcenol A (Figure 19) (Fenical et al., 1976; De Rosa et al., 1988).

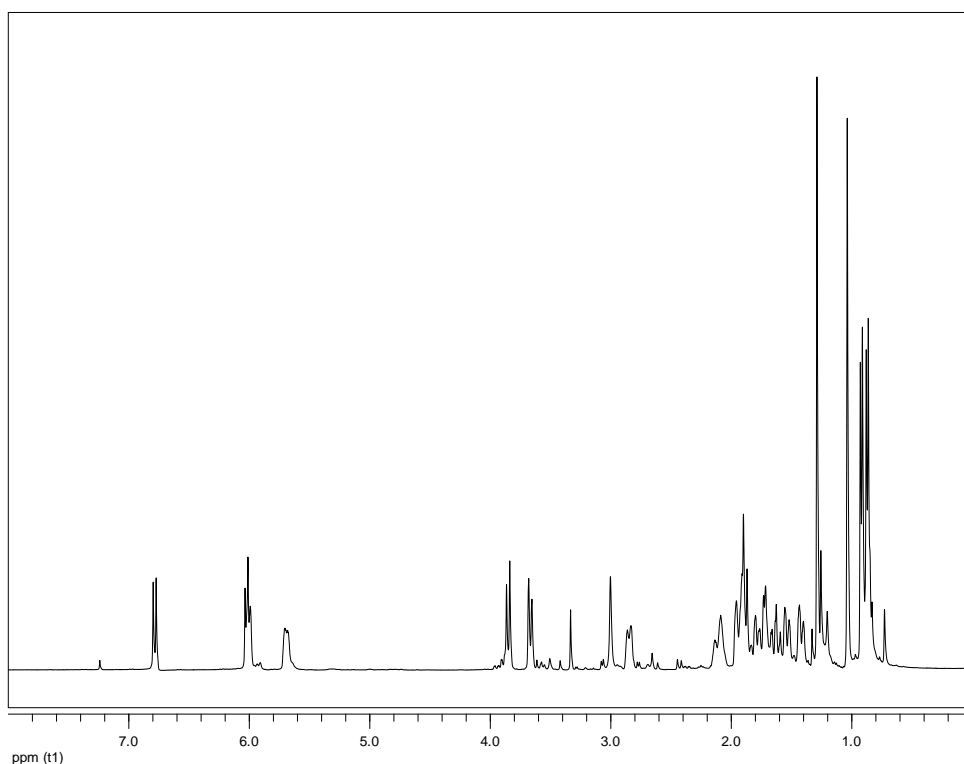


Figure 18. ^1H NMR spectrum of compound **3**

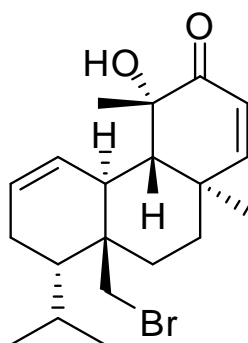


Figure 19. Structure of compound **3**

Sphaerococcenol A has been isolated in the past from the red alga *S. coronopifolius*, collected on the Atlantic coast of Morocco. In that research Sphaerococcenol A showed antimalarial activity against the chloroquine resistant *Plasmodium falciparum* FCB1 strains with an IC_{50} of $1\ \mu\text{M}$ (Etahiri et al., 2001).

Also Smyrniotopoulos et al. in 2010 were testing in vitro antitumor activity evaluation of brominated diterpenes from the same alga. Results showed that compounds like Sphaerococcenol A with the sphaerane carbon skeleton exhibit increased antitumor activity.

Group of these researchers went one step further by using computer assisted phase-contrast microscopy (quantitative videomicroscopy). This way they tried to find the mechanisms of action of most active compounds under study, among which was also Sphaerococcenol A, on the apoptosis-resistant U373 glioblastoma cell line. It showed that Sphaerococcenol A is cytostatic compound that delay U373 GBM cell growth through marked decreases in mitosis entry for this compound.

Table 16. NMR data of compound **3** in CDCl₃ (δ in ppm, multiplicity, *J* in Hz)

Position	δ_C	δ_H
1	128.19	6.03 (brd 10.4)
2	127.66	5.73 (ddt 10.4, 5.0, 2.5)
3	22.27	2.15 (ddd 19.5, 7.0, 5.0, 2.5), 1.98 (m)
4	41.98	1.76 (brd 7.0)
5	40.17	
6	32.97	1.83 (dddd 13.7, 13.2, 4.6, 2.5), 1.57 (ddd 13.7, 2.7, 2.5)
7	24.45	1.66 (ddd 13.2, 13.2, 3.7), 1.45 (ddd 13.2, 4.6, 2.5)
8	36.79	
9	45.43	1.91 (d 12.4)
10	35.43	2.88 (ddq 12.4, 5.0, 2.5)
11	75.27	
12	203.40	
13	124.41	6.05 (d 10.0)
14	162.03	6.81 (d 10.0)
15	21.35	1.07 (s)
16	31.30	1.32 (s)
17	39.85	3.88 (d 10.4), 3.70 (dd 10.4, 2.5)
18	25.79	1.95 (m)
19	25.92	0.96 (d 6.6)
20	19.45	0.90 (d 6.6)

5. CONCLUSION

The present work focused on the chemical composition of the organic extract of the red alga *S. coronopifolius*. Algal specimens were collected in Liapades bay at Corfu Island, Greece, in October of 2014. The extract was subjected to chromatographic separations resulting in the isolation and identification with spectroscopic methods (NMR and MS) of three secondary metabolites.

The isolated metabolites were identified as *allo*-aromadendrane, bromosphaerol and sphaerococcenol A.

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7. SUMMARY

Marine organisms have been underinvestigated for many years when compared to most terrestrial plants regarding the isolation of their metabolites and their evaluation for different medical purposes. In last six decades that scientists turned their attention on the marine environment, they discovered wealth of marine metabolites for a wide array of applications.

The red alga *Sphaerococcus coronopifolius* is marine plant that has been investigated in the past for its chemistry. Many of its isolated compounds which belong in the group of bromoditerpenes showed antitumor and antimicrobial effects. Those discoveries are good motivation for marine chemists to continue exploring this red alga. The main objective of this diploma thesis was to isolate and identify bioactive compounds from the red alga *S. coronopifolius*.

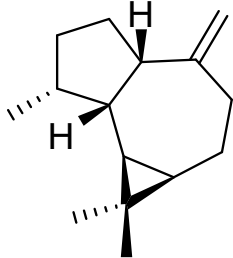
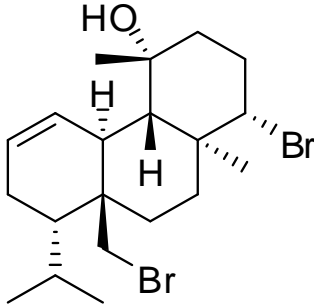
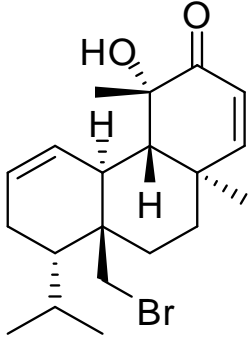
The isolation of the bioactive compounds from *S. coronopifolius* started with vacuum liquid chromatography of the initial organic extract, which yielded a number of fractions. The first of these fractions was identified as *allo*-aromadendrene. The fourth fraction was subjected to HPLC analysis, which resulted in the isolation of two pure compounds. These compounds were identified as bromosphaerol and sphaerococcenol A.

8. SUPPLEMENTS

8.1. ABBREVIATIONS

^1H NMR	One dimension nuclear magnetic resonance experiment with respect to hydrogen-1-nuclei
^{13}C NMR	One dimension nuclear magnetic resonance experiment with respect to carbon-13-nuclei
CDCl_3	Deuterated chloroform
COSY	CORrelated SpectroscopY
δ	Chemical shift of the absorption of the core relative to tetramethylsilane (in ppm) (NMR)
GC	Gas Chromatography
GCC	Gravity Column Chromatography
d	doublet (NMR)
DCM	DiChloroMethane
EtOAc	Ethyl acetate
CHex	Cyclohexane
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
m	Multiplet (NMR)
Me_2CO	Acetone
MeOH	Methanol
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
q	quartet (NMR)
s	singlet (NMR)
t	triplet (NMR)
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography

8.2. ISOLATED METABOLITES AND THEIR STRUCTURES

Metabolite	Structure
<p data-bbox="295 450 568 539">1 <i>allo</i>-Aromadendrane</p>	 <p>The structure of <i>allo</i>-Aromadendrane is a complex polycyclic sesquiterpene. It features a decalin core with a fused cyclohexane ring and a five-membered ring containing a double bond and a methyl group. The stereochemistry is indicated with wedged and dashed bonds.</p>
<p data-bbox="331 808 533 898">2 Bromosphaerol</p>	 <p>The structure of Bromosphaerol is a tricyclic sesquiterpene. It consists of a decalin core with a fused cyclohexane ring. It has a hydroxyl group (HO) and a bromine atom (Br) on the top ring, and another bromine atom (Br) on the bottom ring. Stereochemistry is shown with wedged and dashed bonds.</p>
<p data-bbox="304 1144 560 1234">3 Sphaerococcenol A</p>	 <p>The structure of Sphaerococcenol A is a tricyclic sesquiterpene. It features a decalin core with a fused cyclohexane ring. It has a hydroxyl group (HO) and a carbonyl group (O) on the top ring, and a bromine atom (Br) on the bottom ring. Stereochemistry is shown with wedged and dashed bonds.</p>

PROŠIRENI SAŽETAK

UVOD

Gotovo tri četvrtine ili 79% Zemljine površine pokriveno je vodom, ali još uvijek većina farmakoloških studija usredotočena je na kopnene biljke i lijekove koji mogu biti dobiveni iz njih. Za ostale organizme se također smatra da imaju farmakološki potencijal. To su posebice različiti mikrobi, te od nedavno prepoznati i priznati i drugi različiti morski organizmi kao što su ribe, školjke, alge, bakterije i gljivice. Morski organizmi žive u mnogo drugačijem okruženju od kopnenih organizama. To je glavni razlog za pretpostavku da će se njihovi sekundarni metaboliti značajno razlikovati od onih sekundarnih metabolita koje kopnene biljke proizvode.

Komponente morskih organizama koji su istraživane su kemijski spojevi koje organizmi proizvode kao dio svojih normalnih metaboličkih aktivnosti. Metabolički produkti općenito su podijeljeni u dvije velike skupine: primarne metabolite i sekundarne metabolite. Najveći broj spojeva prirodnog podrijetla su upravo sekundarni metaboliti, kemijski spojevi specifični za svaku vrstu koji se mogu rasporediti u različite kategorije. Ti metaboliti su obično relativno male molekule s molekulskom masom ispod 3000 daltona te se međusobno strukturalno razlikuju. Oni se nazivaju sekundarni metaboliti jer nisu nastali sintezom u organizmu glavnim metaboličkim putovima.

Pronalazak jedne bioaktivne molekule koja može pronaći svoje mjesto u farmaciji i medicini je vrlo dug i složen proces. No mnogi prirodni produkti izolirani iz morskih organizama su već postali aktivni sastojci farmaceutskih pripravaka jer su pokazali široki spektar bioloških aktivnosti, dok su mnogi drugi metabolički produkti još uvijek u različitim fazama kliničkih ispitivanja.

U ovom diplomskom radu analiziran je kemijski sastav kozmopolitske svijetlo-crvene alge *Sphaerococcus coronopifolius*. Dosad je poznato da ova crvena alga u svom sastavu ima mnogo zanimljivih bromiranih diterpena, iako su spomenute kemijske strukture mnogo češće u smeđim algama. Cilj ovog istraživanja bio je izolirati te potom identificirati što više spojeva iz uzorka crvene alge *Sphaerococcus coronopifolius* prikupljenog u uvali Liapades na otoku Krfu, u Grčkoj, u listopadu 2014. godine.

MATERIJALI I METODE

Uzorak crvene alge *Sphaerococcus coronopifolius* prikupljen je ronjenjem u uvali Liapades na otoku Krfu u Grčkoj, na dubini od 8-15 metara u listopadu 2014. Referentni uzorak se čuva u herbariju Zavoda za farmakognoziju i kemiju prirodnih proizvoda, Sveučilišta u Ateni (ATPH / MP0485). *Sphaerococcus coronopifolius* je početno osušena smrzavanjem (0.85 kg suhe mase). Prva ekstrakcija svježeg uzorka je učinjena s 100 %- tnim MeOH na sobnoj temperaturi tijekom jednog dana, a dobiveni ekstrakt se filtrirao i upario u vakuumu do suha. Drugi i treći ciklus ekstrakcije vršio se s DCM : MeOH = 3 : 1. Nakon dva dana ekstrakcije uzorka spomenutim otapalima, ekstrakti su se filtrirali i uparili pod vakuumom na isti način kao i nakon prve ekstrakcije. Konačna masa tamnozelenog ostatka koji je nastao kombinacijom skupljenih ekstrakata nakon sva tri ciklusa ekstrakcije i filtracije je 23,64 grama.

Razdvajanja kromatografijama na koloni su provedena s Kieselgel 60 (Merck). HPLC razdvajanja su provedena uz korištenje Cecil 1100 Series pumpe za tekućinsku kromatografiju opremljenog s GBC LC-1240 detektorom indeksa loma, te korištenjem Techsil100DS (Wellington House, 25 cm x 22 mm) stupaca. TLC su provedene s Kieselgel 60 F₂₅₄ (Merck aluminijske pločice), a sastavnice razdvojene na TLC pločici su detektirane špricanjem reagensom 15% -tne H₂SO₄ u MeOH te zagrijavanjem na 100 °C tijekom jedne minute. Isparavanje otapala pod vakuumom je provedeno pomoću Buchi rotacijskog isparivača R-114 i vodene kupelji Buchi B-480 na 38 °C.

NMR spektri su snimljeni korištenjem CDCL₃ (Aldrich-Sigma Chemical Company) na Bruker AC 200 i 400 spektrometrima Brukeru DRX. Kemijski pomaci su prikazani na δ (ppm) skali upotrebom TMS kao unutarnjeg standarda. Niskorezolucijski EI maseni spektri su mjereni na Hewlett Packard 5973 masenom spektrometrom. Niskorezolucijski CI maseni spektri su mjereni u pozitivnom stanju na Termo Electron Corporation DSQ masenom spektrometru pomoću Direct-Exposure Probe i metana kao CI reakcijskog plina.

Sva otapala koja su se koristila su analitički odobrena (LAB-SCAN Analytical Sciences), te su destilirana prije upotrebe. Sva otapala koja su se koristila za HPLC razdvajanja su filtrirana i otplinjena prije upotrebe.

REZULTATI I RASPRAVA

Izolacija bioaktivnih spojeva crvene alge *Sphaerococcus cornipifolius* provedena je kromatografskim metodama: vakuumskom tekućinskom kromatografijom, tekućinskom kromatografijom na stupcu i tekućinskom kromatografijom visoke učinkovitosti. Otkrivanje kemijskih struktura ovdje izoliranih spojeva provedeno je na osnovu analize njihovih spektroskopskih podataka (MS i NMR spektri), te usporedbom sa spektroskopskim i fizičkim karakteristikama već ranije navedenim u literaturi.

Prva od izoliranih supstancija je identificirana kao *allo*-aromadendren, dok su druge dvije supstancije identificirane kao bromosferol i sferokocenol A.

Temeljna dokumentacijska kartica

Sveučilište u Zagrebu
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Diplomski rad

IZOLACIJA BIOAKTIVNIH METABOLITA IZ CRVENE ALGE *SPHAEROCOCCUS CORONOPIFOLIUS*

Anamarija Tomić

SAŽETAK

Kada govorimo o izolaciji bioaktivnih metabolita i komponenti za različite medicinske svrhe, morski organizmi su godinama nedovoljno istraživani u odnosu na većinu kopnenih biljaka. U proteklih nekoliko desetljeća otkad su znanstvenici počeli više istraživati morski okoliš, otkrili su bogatstvo morskih metabolita za široki spektar primjene. Crvena alga *Sphaerococcus coronopifolius* je morska biljka koja je i ranije često istraživana zbog svojeg kemijskog sastava. Mnogi od njenih izoliranih spojeva, koji spadaju u skupinu bromoditerpena, su pokazali antitumorski i antimikrobni učinak. Ta otkrića su dobra motivacija za znanstvenike da nastave istraživati ovu crvenu algu. Cilj ovog diplomskog rada je bio izolirati i identificirati bioaktivne komponente crvene alge *S. coronopifolius*. Izolacija bioaktivnih komponenti *S. coronopifolius* počela je vakuum tekućinskom kromatografijom na početnom organskom ekstraktu, što je rezultiralo većim brojem frakcija. Prva od spomenutih frakcija je identificirana kao *allo*-aromadendren. Četvrta frakcija je podvrgnuta HPLC analizi, što je rezultiralo izolacijom dviju čistih bioaktivnih komponenti identificiranih kao bromosferol i sferokocenol A.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 40 stranica, 19 grafičkih prikaza, 16 tablica i 29 literaturni navod. Izvornik je na engleskom jeziku.

Ključne riječi: *Sphaerococcus coronopifolius*, metaboliti, *allo*-aromadendren, bromosferol, sferokocenol A

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Basic documentation card

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Diploma thesis

ISOLATION OF BIOACTIVE METABOLITES FROM THE RED ALGA *SPHAEROCOCCUS CORONOPIFOLIUS*

Anamarija Tomić

SUMMARY

For many years marine organisms have been underinvestigated when compared to most terrestrial plants regarding the isolation of their metabolites and their evaluation for different medical purposes. In last couple of decades that scientists turned their attention on the marine environment, they discovered wealth of marine metabolites for a wide array of applications. The red alga *Sphaerococcus coronopifolius* is marine plant that has been investigated in the past for its chemistry. Many of its isolated compounds which belong in group of bromoditerpenes showed antitumor and antimicrobial effects. Those discoveries are good motivation for scientists to continue exploring this red alga. The main objective of this diploma thesis was to isolate and identify bioactive compounds of red alga *S. coronopifolius*. The isolation of the bioactive compounds from *S. coronopifolius* started with vacuum liquid chromatography of the initial organic extract, which yielded a number of fractions. The first of these fractions was identified as *allo-aromadendrene*. The fourth fraction was objected to HPLC analyses, which resulted in the isolation of two pure compounds identified as bromosphaerol and sphaerococcenol A.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 40 pages, 19 figures, 16 tables and 29 references. Original is on English language.

Keywords: *Sphaerococcus coronopifolius*, metabolites, *allo-aromadendrene*, bromosphaerol, sphaerococcenol A

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