Master thesis for the degree of Master of Pharmacy

Isolation and characterization of antiviral compounds from marine organisms

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Summary

Metridium senile, Halocynthia pyriformis and *ParaParastichopus tremulus* were subjected to organic and aqueous extraction. The aqueous extract was made with water, while the organic was made with dichloromethane and methanol in 1:1 ratio. The aqueous and organic extracts were fractionated on reversed-phase HPLC and tested for antiviral activity against *Herpes simplex* virus-1. All the three organisms displayed anti-viral activity in the primary screening, but when retested their activity were lost. During the work with the thesis *Dendrodoa aggregata* was included.Screening of *D.aggregata* had shown that deoxytubastrine had antiviral activity in pilot studies. Tubasterine has shown antiviral activity in earlier studies so it was decided to purify and test deoxytubastrine against HSV-1. Analysis on LC-MS ESI-TOF in positive mode indicated the active compound with *m/z* 178.0906 amu which gave us the elemental composition $C_9H_{11}N_30$. The identity of deoxytubastrine was confirmed by LC-MS ESI-TOF in positive mode and ^{1}H-NMR. However, pure deoxytubastrine did not show any pronounced anti-viral activity.

Abbreviations

ACN	Acetonitrile
DCM	Dichloromethane
D-MEM	Dulbeccos Modified Eagles medium
DMSO	Dimethyl sulfoxide
ESI	Electrospray ionization
FBS	Fetal veal serum
HPLC	High performance liquid chromatography
m/z	mass to charge ratio
MeOH	Methanol
MS	Mass spectrometry
MTT	2H-Tetrazolium,2(4,5-dimethyl-2-tiazolyl)-3,5-diphenyl-,bromide
M09036	*Metridium senile*
M09037	*Parastichopus tremulus*
M09038	*Halocynthia pyriformis*
PBS	Phosphate buffered saline
RPMI	Rosell Park Memorial Institute medium
TOF	Time of flight
QqQ	Triple quadropole

1. Introduction

1.1 Bioprospecting

Humans have always been dependent of natures resources to obtain clothes, shelter and medicines. Chemical substances derived from animal, plants and microbes have been used for treating human diseases since the beginning of medicines history [1]. It is estimated that 60 % of the medicines people can get prescribed today can be traced back to nature [2]. However almost all of these drugs have arisen from terrestrial organisms, even though life in the sea has evolved with a very interesting and highly complex chemistry. Life in the sea appear in the strangest places and have produced the weirdest organisms and might have very big potential for treating human diseases [2]. The main reason there is less medicines arising from marine organisms has been the possibility to access the ocean depths and securing a reliable supply.

Marine organisms live under totally different environments than terrestrial. This makes life in the ocean unique and there are possibilities to find animals, plants and microbes very different than on land. The chemicals they make are very interesting for pharmaceuticals, since the possibility of finding lead-compounds never found before is high.

Marine bioprospecting is searching the ocean depths for these marine organisms and investigate whether they have any interesting molecules or genes which can be beneficial for humans. Since there has been little focus on the oceans as mentioned before, and that approximately 70 % of earths surface consists of water, the chances of finding novel interesting molecules or genes are very high. Especially when it comes to the areas along the Norwegian coast and the Barents Sea where there has been minimal exploration of the marine fauna for this purpose. Organisms living in cold environments develop different molecules than organisms living in warm. This is because it takes different characteristics and biochemical processes to live under extreme conditions such as low temperature, low pressure and little sunlight. Organisms living under these circumstances are called extremophiles, and can hold many interesting biologically active compounds.

1.2 MabCent SFI

MabCent –SFI is a Research based innovation centre which was started up in March 2007. It was established by the Research council of Norway and is hosted by the University of Tromsø.

Their field of research is through marine bioprospecting to analyze and characterize the bioactive compounds from Arctic and sub-Arctic marine organisms that have the potential for further research and commercialization.

Together with Marbio, which is were the high- throughput screening for bioactive compounds is performed, they purify, screen and identify compounds within several areas. The main focus is anti-bacterial activities, anti-cancer activities, anti-viral activity, anti-inflammatory activities and anti-diabetic activities[3].

1.3 Organisms

1.3.1 Cnidaria

The phylum Cnidaria includes the classes Scyphozoa, Cubozoa, Hydrozoa and Anthozoa. They are characterized by their radial or biradial symmetry. The basic structure is sac-like with the mouth as a single terminal opening, which also function as an anus. The mouth is surrounded by circles of tentacles. They only have two layers of cells in the body wall, ectoderm and endoderm, between which is the mesoglea. The mesoglea forms a thick fibrous layer in anemones. All cnidarians are characterized specially by the presence of stinging capsules on the tentacles of various types which are unique to the phylum. The cnidarians have two basic structures by which they are recognized from, a polyp (*M.senile*) which is sessile, more or less cylindrical and has the mouth at the free distal end. A medusa is free swimming, bell- to saucer shaped, with the surface upward and the tentacles and mouth on the abdominal side. The polyps may propagate asexually by fission or budding new polyps from themselves[4].

1.3.1.1 *Metridium Senile*

Metridium senile(sea anemone) belongs to the class Anthozoa from the phylum Cnidaria. They are very common from Scandinavia to Biscay. They can be found across the beach zone, overhangs, caves, beneath boulders on the lower shore and further down to 100 meters depth.

M.senile has a characteristic appearance with many, short tentacles which gives the anemone a soft expression. It can be as tall as 500 mm and are among the biggest sea anemones [4, 5].

Figure 1: Picture of *M.senile.* Published with permission from Sten-R Birkely, Marbank.

Despite the lack of efficient physical protection in a hostile environment, the Anthozoans survive mainly because of their chemical defense system by a series of secondary metabolites accumulating in their body or releasing to their surroundings. Some of the chemicals previously isolated from them are 5 cembrene-type diterpenes[6]. For example flexibilide, dihydroflexibilide, sinulariolide, epi-sinulariolide and epi-sinulariolide acetate from the coral *S.flexibilis* has showed remarkable antimicrobial activity, and have especially inhibited the growth of Gram-positive bacteria, such as *S.aureus.* Another substance, Briarellin Q, from the octocoral *B.polyanthes* has showed significant antiplasmodial activity *in vitro* against chloroquine-resistant *Plasmodium falciparum*, in addition it has showed strong activity against *Mycobacterium tuberculosis*[6].

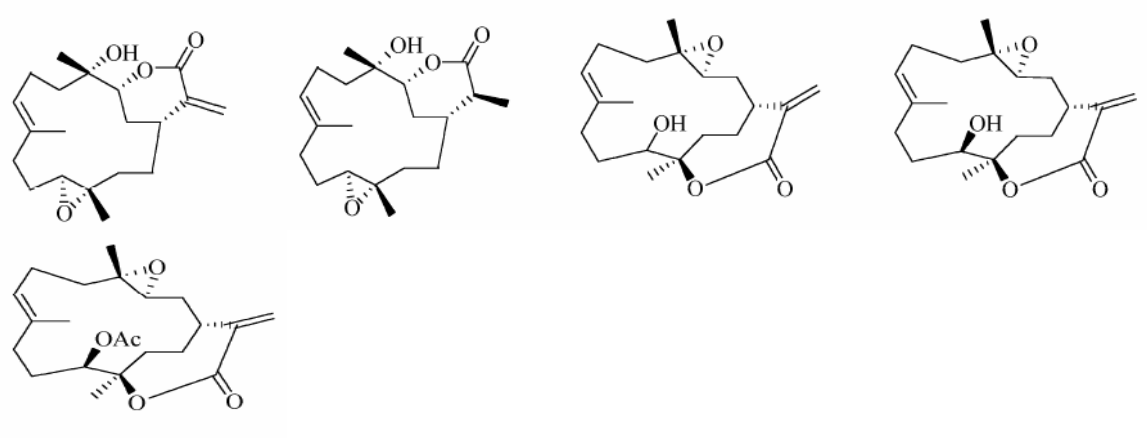


Figure 1: Diterpenes from *Sinularia flexibilis*

Metridoiolysin, a cytolytic and lethal protein from *M.senile* has been isolated[7]. It has been shown that metridiolysin possesses some distinctive properties in common with streptolysin O and other SH-activated cytolysins of bacterial origin. For example the lytic abilities for platelets and other mammalian cells[7].

In addition to *M.senile* this thesis included antiviral screening from *H.pyriformis* and *P.tremulus*. These were purified in two other parallel projects. Another marine organism *D.aggregata* was also included during the work with this thesis; this organism had been purified at MabCent earlier.

1.3.2 Chordata

The phylum Chordata includes the class Asidiacea. They separate from every other animal in four basic ways in which they are built up. That is their central nervous system placed on the animals back like a tube shaped string which goes over to their spinal marrow. Under the neural tube lies the embryos chorda dorsalis. Beneath the chorda dorsalis you can find the intestinal tract, with its frontal part designed as respiratorytract. Their heart lies on the abdominal side[8].

1.3.2.1 *Halocynthia pyriformis*

Halocynthia pyriformis belongs to the class Ascidiacea from the phylum Chordata. Although they have been observed as far south as Stavanger, they are especially common in northern Norway. They can be as tall as 100 mm. They normally live in low water and are usually red colored, but some examples in white has also been seen. *H.pyriformis* can be found both in areas

with strong streams and areas whit stagnant water and in any substrate from the tidal zone and down to 600 meters depth[5].

Figure 2: Picture of *H.pyriformis.* Published with permission from Sten-R Birkely, Marbank.

1.3.2.2 *Dendrodoa aggregata*

Dendrodoa aggregata also belongs to the class Ascidiacea from the phylum Chordata. It is very common in the northern parts of Norway. The specie lives both in colonies and separately. They are characterized by a spherical sack like shape, with two short siphons.

They can usually be found under rocks, empty sea shells and other shellfish. You can find them from the tidal zone and down to 600 meters depth[5].

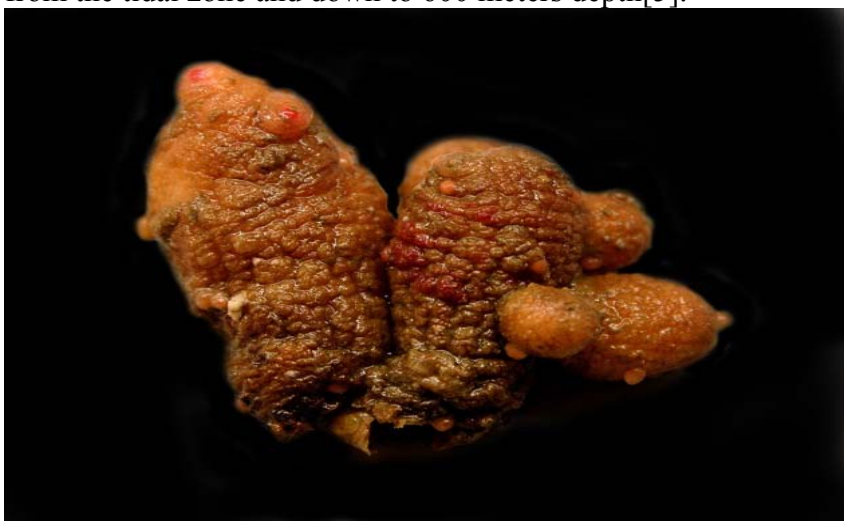


Figure 3: Picture of *D.aggregata.* Published with permission from Robert A. Johansen, Marbank.

1.3.3 Echinodermata

The phylum Echinodermata includes the classes Crinoidea, Asteroidea, Ophiuuroidea and Echinoidea. The phylum has a distinctive radial pattern which may take the form of five arms radiating from a central disc or a more globular or cylindrical shape. The typical echinoderm has an outer lay of calcareous skeletal plate. These may be joined together to form a complete shell. The echinoderms have a unique hydraulic water vascular system which controls the action of the tube feet. It consists of a ring around their throat and a radiating vessel internal to each ambulacrum. Echinoderms can live in a diversity of habitats. They are fully marine and barely penetrate estuaries. They can avoid desiccation by confining to rock pools and under boulders[4].

1.3.3.1 *Parastichopus tremulus*

Parastichopus tremulus belongs to the phylum Echinodermata. The specie has it habitat from northern Norway to Biscay. It is a big sea cucumber and can get as big as 50 cm long. They have a beautiful red colored back and are white on the abdominal side. Their body is covered with papillae which make it look like a wart. The tentacles around their mouth are very short. They are very common in the fjords from 20-1200 meters depth[5].

Figure 4: Picture of *P.tremulus.* Published with permission from Robert A. Johansen, Marbank.

1.4 Reversed phase HPLC

High Performance Liquid Chromatography is the most widely used method in analyzing pharmaceuticals. The method is based on having a sample injected in a mobile phase that is pumped through a column packed with materials that will retard and by that separate the molecules in the sample before they are detected by a detector. The pumps must be able to pump the mobile phase in a constant velocity against high pressure. The particles in the column give a counter pressure of 30-300 bar when the mobile phase is pumped through.

The column used in the HPLC in this thesis is reversed phase columns where the stationary phase is hydrophobic and the mobile phase aqueous solutions with acetonitrile and formic acid as organic modifiers. The sorbent in the columns are made by attaching hydrophobic groups, here C18-material, to silanol groups[9].

1.5 LC-MS

Mass spectrometry (MS) is a method that is based on the production of ions from molecules, which then are separated according to their mass-to-charge (*m/z*) ratio and detected in a detector. There are many different ion sources and mass separators, but in this thesis it will be focused on MS with electrospray ionization, triple quadrupole mass separator and time-of-flight mass separator[10].

In many applications that are measured with mass spectrometry, the sample that is to be analyzed is dissolved, preferably in an aqueous phase. The solution must flow into the front of the MS, but before it can provide a spectrum the solvent must be removed without loosing the sample. If this is not removed, its vaporization when entering the vacuum of the MS will produce a large increase in pressure and stop the instrument from working properly. Electrospray ionization is one of several ways of effecting the differentiation between the carrier solvent and the sample of interest. But unlike other methods introducing liquid into a MS, in addition of making ions a single charge electrospray also produces multiply charged ions that make accurate measurements of larger masses easier.[11]

Electrospray ionization takes place under atmospheric pressure making pseudo-molecular ions, these species are non-covalent complexes formed between an analyte of interest and any other components present in the ionized sample. The solution is pressed through a stainless capillary

and when applying a high voltage on the capillary, an aerosol is formed. This aerosol formation is assisted by using a nebulizer gas, often nitrogen, which flows around the capillary. The gas will also evaporate the charged droplets, which results in ionic species in the gas phase. These species are either positively or negatively charged depending on the potential applied to the capillary. This means that when a positive potential is applied, only positive charged species are made. Depending on what the solution has present, several species other than $[M+H]^+$ can be formed. These are often Na^+, K^+ and NH_4^+. Likewise when a negative potential is applied, only negative charged species are made. Also here other species than $[M-H]^-$ can be made depending on other components present in the sample[12].

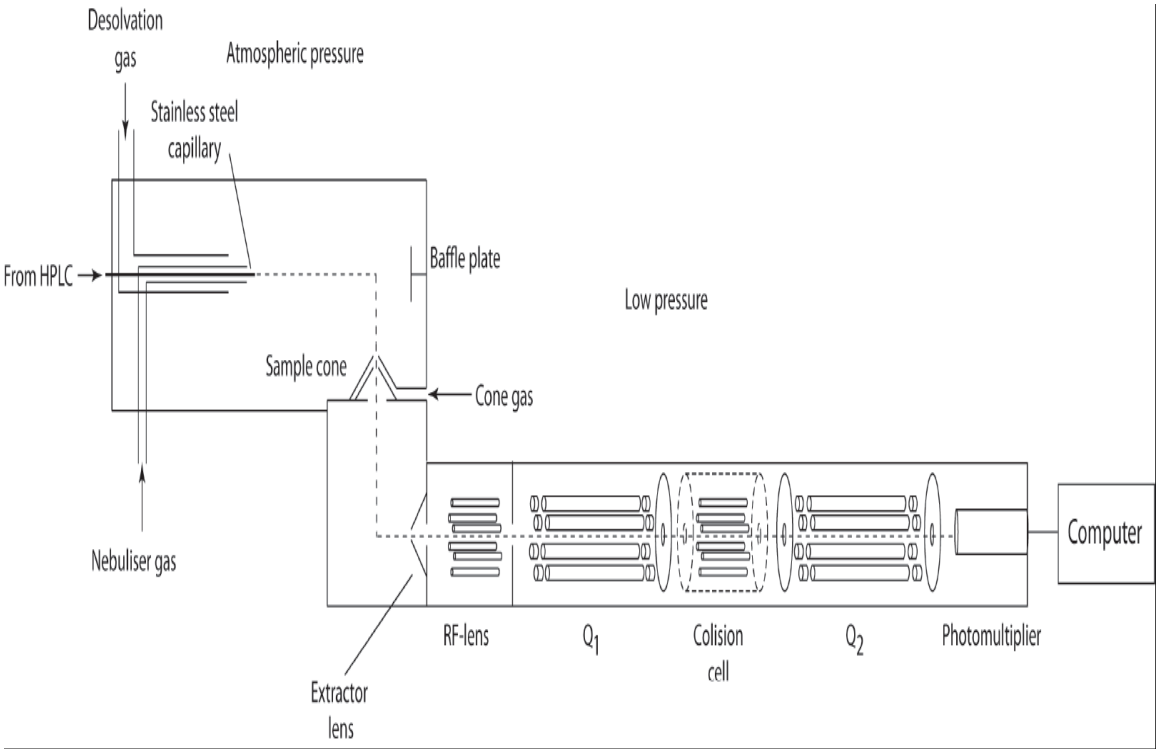


Figure 5: Schematic drawing of a Trippel quadrupole. Published with permission from Terje Vasskog.

The triple quadrupole (QqQ) has two quadrupoles with a hexapole or octapole in between. The quadrupole is made out of four parallel rods, where the pairs opposing each other are connected electrically. In the triple quadrupole Q_1 and Q_2 are the mass analyzers in which the ions are separated based on their *m/z*-ratio. The third quadrupole,which actually is a hexapole or octapole, is a collision cell. An radio frequency voltage is applied on the rods of the collision cell guiding

the ions through the cell without separating them. When an inert gas such as Ar, He or N_2 is introduced the collisions will be induced between the collision gas and the analyte ions.

If the collisions have high enough energy, the internal energy of the ions will increase and they can be fragmented and introduced to Q_2 and separated.

The mass spectrometer can be used as both a specific and a general detector. When used as a general detector the radio frequency voltage and direct current voltage are varied to scan the desired *m/z* interval.

As a specific detector the radio frequency voltage and direct current voltage is set with specific values so only ions with specific *m/z* gets through the quadrupole in a specific time interval [12].

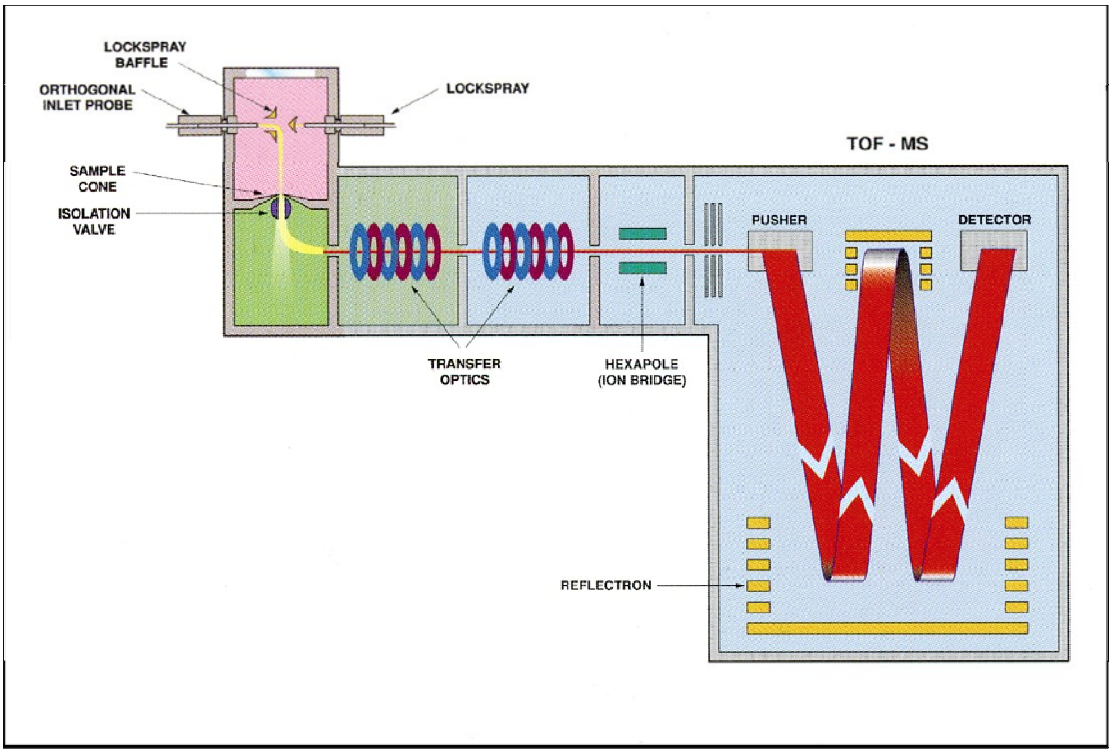


Figure 6: Schematic drawing of ESI-TOF MS with double reflectors. (www.waters.com)

The concept of Time of flight mass spectrometry (TOF) is that for a given population of ions which are all accelerated to the same kinetic energy, the ions which have a lower mass will have greater velocity when travelling and reach an end point sooner. Each ion with a particular *m/z* ratio has a unique flight time. The flight time begins when a high voltage pulse is applied to the back plate of the ion pulser and ends when the ion strikes the detector at a known distance[13].

One of the disadvantages of TOF is their restricted dynamic area, caused by the detectors need of time between every time it measures the ions (~ 5ns). If the sample is too concentrated, the detector will not manage to reset between each measurement since there is too many ions reaching the detector at the same time.

One of TOFs great advantages is that it calculates the molecule weight with great accuracy (below 5 ppm uncertainty). This is achieved by introducing a reference compound along with the sample. Since the ion source has two probes, one analyze probe and one reference probe, every 15 seconds there will be made a reference specter that is used to adjust the analyte spectra[14]. The instrument has almost an unlimited mass range, high analyzing speed and high resolution. Reflectors are used to balance the fact that the ions can have different kinetic energy. The ions with high kinetic energy will penetrate deeper in the reflector and use longer time before they turn than the ions with low kinetic energy. Several reflectors after each other will make the resolution even better.

1.6 Extraction

During the extraction the aim is to extract the compounds from the freeze-dried material over to the solution the extraction is made with. Since the theory says that like dissolves like the extraction is made in two stages. First the freeze-dried material was extracted with water in order to extract the polar molecules over to the aqueous phase. Then the apolar compounds that were left in the material were extracted with DCM and methanol. One can also upgrade the extraction by adjusting for example the pH in order to increase the selectivity of the extraction.

Since it is not know what kind of compounds the organism consist of, the best is to make an extraction that is as general as possible.

1.7 Virus

Viruses differ from all other infectious organisms in their structure and biology, especially in their reproduction. Although they carry conventional genetic information in their DNA or RNA, they do not have synthetic machinery that is needed for this information to be used to make new virus material. Virus itself is metabolically inert, and can only replicate after infecting a host cell and parasitize the hosts ability to transcribe or translate genetic information. They cause some of

the most common and some of the most deadly diseases in humans, exemplified with influenza and Ebola virus [15].

In this thesis *Herpes simplex* virus 1 (HSV-1) was used under the antiviral screening. Herpes simplex virus is a medium sized double-stranded DNA virus. There are actually two types of herpes simplex virus, HSV-1 and HSV-2. They cause a variety of clinical symptoms, with the basic lesion being an intraepithelial vesicle where the virus is shed. The infection usually transmits from the saliva or cold sores of other individuals. HSV-2 arose as a sexually transmitted variant of HSV-1, but lately the sites infected by the two types are less clearly distinct.

HSV is called a virulent virus and their lytic cycle is a six stage cycle. It starts at the penetration of the host cell. The viral acids will then form a circle, making the cell mistakenly copy viral DNA as its own. The viral DNA will then organize them selves as virus. Virus particles will then bud the cells and the viruses are free to infect other cells.

There is today over 40 compounds that are commercially available in pharmaceutical markets including alternative antiviral medicines [16]. Compounds extracted from algae have shown activity against a wide range of viruses, including HSV-1, HSV-2, HIV and others. Another compound, Fucoidan which is a sulfated polysaccharide isolated from *Fucus vesiculosus,* has shown inhibitory effect on the replication of DNA viruses.

But the most important contribution of marine organisms to antiviral medicines was the isolation and characterization of arabinosyl nucleosides from the sea sponge *Thethya cripta*. Semi synthetic modifications of arabinosyl nucleosides have resulted in producing cytarabine, acyclovir and azidothymidine which are in clinical use today [16].

1.8 Description of methods

The methods used in this thesis have all been taken from the methods that are used in Marbio which has established methods from sample preparation to fractionation and screening. The methods are designed is such manner that they can pick up as many compounds as possible, from small organic molecules to big macromolecules.

First all the organisms were cut to small pieces and freeze-dried. Sample materials were extracted in water and then a mixture of dichloromethane and methanol. These extractions are a simple way to separate the polar and apolar molecules from each other.

The organic and aqueous extracts where then fractionated by high performance liquid chromatography (HPLC). The fractions from the HPLC were tested for antiviral effects on HSV-1.

The antiviral screening is based on plaque- reduction assay (PRA-assay). The PRA-method measures viral efficiency in forming plaques in the presence of antiviral compounds. The cells are exposed with virus and a HPLC-fraction to see how many plaques is produced. Immonuglobuline (IgG) is also added to the media to prevent viruses to infect other cells when budding out. In this way only the cells that are infected the first time will produce plaques. Number of plaques formed in the wells that have been infected with HSV-1, is compared to the number of plaques formedin the wells that in addition has been exposed to test compounds. The cell line that is used for the antiviral screening is the African Green Monkey kidney cells (Vero cells).

Some active fractions were also tested with 2H-Tetrazolium,2-(4.5-dimethyl-2-3,-5 diphenyl-,bromide) assay (MTT). The MTT- method measure the toxicity of fractions against the test cells (Vero cells).

Fractions that show activity are further tested in different dilutions and screened again to confirm the activity. The fractions still having activity after the second screening will be purified and analyzed with mass spectrometer (MS). By using MS one can try to determine the exact mass and element composition of the active components. When element compositions are found they can be compared in different databases to find out if they are molecules previously discovered. This is called dereplication. Compounds that do not exist in databases are interesting for structure elucidation.

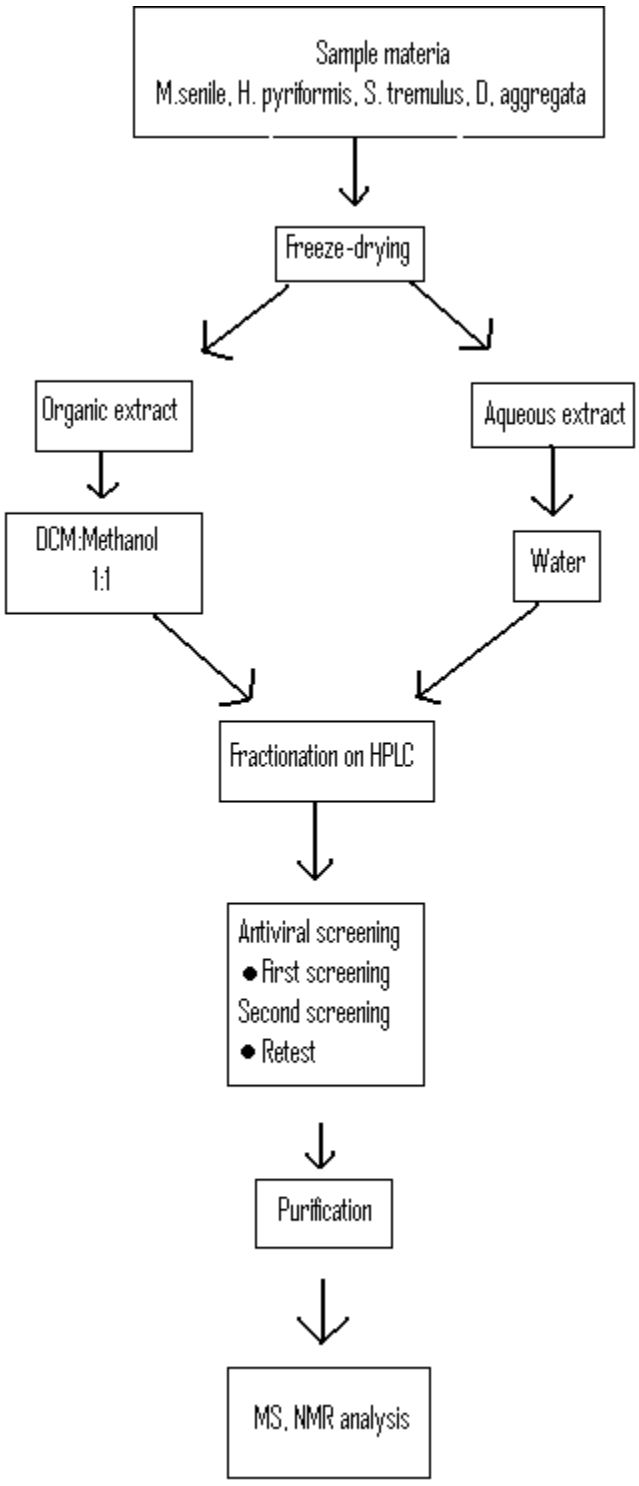


Figure 7: Sample flow from extraction until MS-analysis

1.9 Aim

The aim of this thesis is to try to find compounds with antiviral activity in *M.senile, H.pyriformis, P.tremulus* and *D.aggregata.* Two different extractions will be made, the first with water and the other a mixture of dichloromethane and methanol in 1:1 ratio, in order to find out if this has any effect on the screening result. Finally we will try to identify the known compounds by searching in databases. Any unknown compounds will also be structure elucidated as far as possible.

2. Materials and methods

2.1 Sampling and storage

Materials:

Heto Powerdry PL9000	Thermo (Waltman,USA)
Scale	Mettler Toledo (Greifensee, Switserland)
IKA A11 Basic	IKA Works (Staufen,Germany)

Metridium senile was collected 30th of May 2009 in Porsangerfjorden, Finnmark with Agazziz trawl at 62 meters depth.

ParaParastichopus tremulus was collected 6th of March 2009 in Tysfjorden with Agazziz trawl at 378 meters depth.

Halocynthia pyriformis was collected 29th of May 2009 in Porsangerfjorden, Finnmark by diving at 20 meters depth.

Dendrodoa aggregata was collected 5th of December 2006 in Skattøra, Tromsø at 35 meters depth. The organisms were then stored at -20^{o}C.

Frozen organisms were later taken out of the freezer, weighed and transferred to a wooden board. The frozen material was cut in 1 cm^3 pieces and transferred to a bowl. Aluminum foil with holes were used as cover and the bowls were then placed in Heto Power Dry PL 9000 for freezedrying.

2.2 Aqueous extract

Materials

Rotary evaporator, Laborata 4002	Heidolph (Nürnberg,Germany)
Heto Powerdry PL9000	Thermo
Scale	Mettler Toledo
Centrifuge	Multifuge 3 S-R (Heraeus)
Milli-Q water	Millipore (Billerica, MA, USA)
Ethanol	Merck (Darmstadt,Germany)
22 μm Millex GS filter	Millipore
Universal SM 30	Edmund Bühler GmBH (Hechingen,Germany)

The freeze-dried *Metridium senile* M09036-0-W01(153.54 g) was transferred to a mill and pulverized. The pulverized material was transferred in small doses to a Duran bottle by a funnel and Milli-Q water was poured in the bottle. Approximately 200 mL Milli-Q water was added at each addition of pulverized material and shaken until the amount of Milli-Q water corresponded to approximately 10x the weight of the material or got the consistency of a "soup". It was totally added 1.1 L Milli-Q water. The suspension was then stored at 5 ^{o}C in the refrigerator until the next day.

The suspension was centrifuged the next day for 30 min at 4000 rpm and 5^{o}C. The supernatant was decanted into 1 L flasks, and it was made sure that the fatty layer did not enter the flasks. Milli-Q water was added to the pellet, mixed properly and placed in the refrigerator for 30 min, before the process of centrifuging and decanting was repeated and the 2 supernatants were pooled. The supernatant was distributed to flasks (approximately 400 mL per flask) and placed in the freezer at a tilting angle and rotated every 20 min to ensure even distribution in the bottle. This is to make the freeze- drying easier and more efficient. The flasks were then placed in Heto Power Dry PL 9000 for freezedrying. The material was pulverized, scraped out and transferred to a bowl before it was stored at -20 °C.

It was weighed out 213 mg of the extract to a centrifugation tube and added 2 mL Milli-Q water. The mixture was shaken for 90 minutes, before 1 mL of it was transferred to another centrifugation tube. It was added 4 mL 96 % EtOH to the tubes and shaken well on a Universal SM 30 minishaker before being placed in the freezer over night. The next day the mixtures were thawed and then shaken vigorously and centrifuged at 4000 rpm for 30 min. The supernatant was transferred to a 25 ml rotary evaporation flask and vacuum evaporated at 40 ^{o}C down to approximately 2 mL. The mixture was placed in the freeze. The next day 2 mL of the mixture volume was divided to two centrifugation tubes containing 1 mL each and centrifuged at 13000 rpm for 30 min. The supernatant was filtered through 0.22 μm Millex GS filter over to HPLC tubes for fractionation.

2.3 Fractionation of aqueous extract

Materials

Waters 600 Controller	Waters (Milford,USA)
Waters 2996 Photodiode Array Detector	Waters
Waters 2767 Sample Manager	Waters
Atlantis Prep C-18 (10x250 mm; 10µm)	Waters
Acetinitrile	Merck
Milli-Q water	Millipore
Formic acid	
Waters 600E Multisolvent delivery System	Waters
Waters Prep Degasser	Waters
Waters 2767 Sample Manager, Injector and Collector	
Waters 2996 PDA Detector	
SpeedVac	

The aqueous extract from *Metridium senile* (M09036-0-L01) was now ready to be fractionated. From each tube 1000 µL of sample was injected in the HPLC column and fractionated with a flow rate of 6 mL/minutes and the eluent was collected over a time span of 40 minutes, resulting in 40 fractions. The gradient used in the process is shown in table 1. Each of the 40 fractions were distributed between 4 deep well plates and vacuum centrifuged for 1 hour and placed in the Heto Powerdry PL9000 for freeze drying.

The other organisms included in this thesis have undergone the same procedure for the aqueous extract.

Table 1: Mobile phase gradient for the fractionation of aqueous extracts.

Time (min)	Flow (ml/min)	Moblie phase A % w/ H_2O and 0.1 % formic acid	Moblie phase B % w/ ACN and 0.1 % formic acid	Curve
0	6	95	5	0
3	6	95	5	6
30	6	50	50	6
35	6	5	95	6
40	6	5	95	6
41	6	95	5	6

2.4 Organic extract

Materials

Rotary evaporator, Laborata 4002	Heidolph
Heto Powerdry PL9000	Thermo
Scale	Mettler Toledo
Centrifuge	Multifuge 3 S-R
Milli-Q water	Millipore
Whatman no 3 filter	Whatman (Maidstone, England)
Dichloromethane	Merck
Methanol	Merck
Universal SM 30	Edmund Bühler GmBH

The dry pellet remaining after the aqueous extract (75.42g) and stored in the freezer was freeze-dried and pulverized in a mill and transferred to a Duran bottle. A mixture of DCM and MeOH (1:1) was added to the pulverized material until it corresponded to

Approximately 10x the weight of the pellet and got a “soup” like consistency. The suspension was shaken vigorously and stored in the refrigerator until the next day. The suspension was vacuum filtrated the next day with Whatman nr 3 filter. When the suspension was filtrated, the pellet was reextracted in DCM/MeOH for 30 min and filtrated.

The pooled filtrate was transferred to a rotary evaporation flask and vacuum evaporated at 40 ^{o}C until there was 10-20 mL left. The concentrated filtrate was transferred to HPLC tubes and stored at -20 ^{o}C.

The organic extract was taken out from the freezer and 241.5 mg was transferred to one tube and 258.8 mg to another. It was added 3 mL hexane to each tube and shaken vigorously on a minishaker. Then 3 mL 90 % ACN was added to both and shaken vigorously on a minishaker.

The mixtures were centrifuged for 3 min at 3000 rpm before the ACN phase was transferred to a evaporation flask

3 mL 90 % ACN was added to the hexane phase again in both tubes and centrifuged, before the ACN phase was transferred to an evaporation flask.

The flask with the ACN phase was vacuum evaporated until there was approximately 2 mL left. The volume was distributed to two centrifugation tubes and the volume was adjusted to 1 mL with 90 % ACN before being placed in the freezer to the day after. The next day the 2 centrifugation tubes were thawed and shaken on a minishaker and centrifuged at 13000 rpm for 30 minutes. The mixture was transferred to 2 HPLC tubes and the volume was adjusted to 1 mL in each tube and it was ready for fractionation.

2.5 Fractionation of organic extract

Materials

Waters 600 Controller	Waters
Waters 2996 Photodiode Array Detector	Waters
Waters 2767 Sample Manager	Waters
XTerra® Prep RP_{18} (10x300 mm; 10µm)	Waters
Acetinitrile	Merck
Milli-Q water	Millipore
Formic acid	Merck
Waters 600E Multisolvent delivery System	Waters
Waters Prep Degasser	Waters
Waters 2767 Sample Manager, Injector and Collector	Waters
Waters 2996 PDA Detector	Waters

SpeedVac

The organic extracts were now ready to be fractionated. From each tube 1000 µL of sample was injected in the HPLC column and fractionated with a flow rate of 6 mL/minutes and the eluent was collected over a time span of 40 minutes, resulting in 40 fractions. The gradient used in the process can be seen in table 2. Each of the 40 fractions were then distributed between 4 deep well trays and vacuum centrifuged for 1 hour and placed in the Heto Powerdry PL9000 for freeze drying.

The other organisms included in this thesis have undergone the same procedure for the organic extract.

Table 2: mobile phase gradient for fractionation of organic extracts.

Time (min)	Flow (mL/min)	Mobile phase A % w/H_2O and 0.1 % formic acid	Mobile phase B % w/ ACN and 0.1 % formic acid	Curve
0	6	80	20	0
2	6	80	20	6
30	6	0	100	6
40	6	0	100	6
41	6	80	20	6

2.6 Dissolving HPLC-fractions

The organic HPLC-fractions in the deep well trays were added 7.5 µL DMSO and was shaken for 2.5 hours. Afterwards 750 µL D-MEM without FBS were added and the trays were shaken for another 30 minutes, before another 750 µL D-MEM without FBS were added and shaken for 5-6 minutes.

The aqueous HPLC-fractions in the trays were added 750 µL D-MEM without FBS and shaken for 2.5 hours. Afterwards another 750 µL D-MEM without FBS were added and the shaking procedure repeated for 30 minutes.

2.7 Plaque Reduction Assay

Materials and chemicals:

D-MEM	Invitrogen (Carlsbad, USA)
FBS, Fetal veal serum	Biochrom (Cambridge, UK)
Gammaglobuline 7 mg/mL	Sigma Aldrich (Munchen,Germany)
Crystal violet	Sigma Aldrich
Methanol	Merck
Milli-Q water	Millipore
Incubator, 37^{o}, 5 % CO_2	Samyo Electric

Virus:

HSV-1, ATCC VR-539

Cell line:

African Green Monkey kidney cells (Vero cells) ATCC no: CCL-81^{TM}

Day 1:

Vero cells were split up and cultivated in 24 well plates with a cell density of $1*10^5$ cells/well in 1 mL medium and incubated over night at 5% CO_2, 37^{o}C.

Day 2:

The next day a virus dilution giving 500 plaque forming units/mL was made in D-MEM w/FBS.The medium in each well were removed. It was added 100 µL HPLC- fractions and 100 µL virus solution to each well. The positive control was set to 100 µL virus solution and 100 µL D-MEM without FBS. The negative control was set to 100 µL D-MEM with FBS and 100 µL D-MEM without FBS.

The cells were then incubated for one hour at 37^{o}C, 5 % CO_2 to adsorb virus to the cells.

After one hour the medium in each well were removed and the cells were washed one time with PBS. 150 µL HPLC-fraction and 150 µL IgG solution were then added to each well and the cells were incubated for 48 hours at 37^{o}C, 5 % CO_2.

Day 4:

After 48 hours the medium in each well were removed and crystal violet in 5% methanol was added to each well and incubated for 20 minutes. Then the crystal violet was removed and the

fixed cells were washed carefully with Milli-Q water 3 times, before the wells were dried so the number of plaques could be counted under a loupe.

Table 3: HPLC fractions in plaque reduction assay. V= virus control/positive control, C= cell control/negative control

1	2	3	4	5	6
1	2	3	4	5	6
7	8	9	10	v	C
7	8	9	10	v	C

11	12	13	14	15	16
11	12	13	14	15	16
17	18	19	20	v	C
17	18	19	20	v	C

21	22	23	24	25	26
21	22	23	24	25	26
27	28	29	30	v	C
27	28	29	30	v	C

31	32	33	34	35	36
31	32	33	34	35	36
37	38	39	40	v	C
37	38	39	40	v	C

2.8 MTT assay

Materials and chemicals:

DMSO	Sigma Aldrich
RPMI-1640	Biochrom
D-MEM	Invitrogen
Aqueous One Solution Reagent (AQOS)	Promega
FBS, Fetal veal serum	Biochrom
DTM 880 Multimode Detector	Beckman Coulter
Plate station-SAGIAN	Beckman Coulter
Multimode detection software	Beckman Coulter
Micro titer tray	Nunc
Incubator, 37^{o}, 5 % CO_2	Samyo Electric
Multichannel pipette	Ependorf(Hamburg,Germany)

Cell line:

African Green Monkey kidney cells (Vero cells) ATCC no: CCL-81^{TM}

Day 1:

Vero cells were split up and cultivated in 96 well plates with a cell density of 1 x 10^4 cells/well medium and incubated over night at 5% CO_2, 37^{o}C.

Day 2:

The medium in each well were removed. It was added 50 µL RPMI-1640 with 10 % FBS and 50 µL of purified sample to each well. This was done in 3 parallels. The negative control was set to 50 µL RPMI-1640 with 10 % FBS and 50 µL RPMI-1640 without FBS. The plates were incubated for 3 days at 5 % CO_2, 37 ^{o}C.

Day 5:

It was added 10 µL AQOS to each well and incubated for 1 hour at 5 % CO_2, 37 ^{o}C. After 1 hour the absorbance at 485 nm was measured by DTM 880 Multimode Detector (AqueousOne485).

Table 4: Concentration of purified deoxytubastrine (see results for active compounds from *D. aggregate*) in MTT assay. N= Negative control

	Concentration (µg/mL)			Concentration (µg/mL)	Concentration (µg/mL)	Control	
	50	50	50			N	N
	40	40	40			N	N
	30	30	30			N	N
	25	25	25			N	N
	15	15	15				
	10	10	10				
	5	5	5				

2.9 Purification of active fractions

Materials and chemicals:

Waters 600 Controller	Waters
Waters 2996 Photodiode Array Detector	Waters
Waters 2767 Sample Manager	Waters
Atlantis Prep C-18 (10x250 mm; 10µm)	Waters
Acetonitrile	Merck
Milli-Q water	Millipore
Formic acid	Merck
Waters 600E Multisolvent delivery System	Waters
Waters Prep Degasser	Waters
Waters 2767 Sample Manager, Injector and Collector	Waters
Waters 2996 PDA Detector	Waters
Waters 3100 MS	Waters

The active fractions were purified by mass in a preparative HPLC-MS system. From the tube 1000 µL of sample was injected in the HPLC column and fractionated with a flow rate of 6 mL/minutes and the eluent was collected over a time span of 25 minutes. The gradient used in the process can be seen in table 5. The MS-conditions are listed in table 6.

Table 5: Mobile phase gradient for the purification of active fractions

Time (min)	Flow (mL/min)	Mobile phase A % w/H_2O and 0.1 % formic acid	Mobile phase B % w/ACN and 0.1 % formic acid	Curve
0	6	95	5	0
5	6	95	5	6
20	6	80	20	6
21	6	5	95	6
25	6	5	95	6

Table 6: Conditions of the MS-analysis from the purification of active fractions

Conditions	ES+
Capillary (kV)	3.0
Cone (V)	35
Source Temperature (^oC)	120
Desolvation Temperature (^oC)	300
Cone Gas Flow (L/Hr)	5
Desolvation Gas Flow (L/Hr)	550
Syringe Pump Flow (µL/min)	5

2.10 LC-MS ESI-QqQ

Materials:

Nano Acquity UPLC Waters

Quattro Premier XE Waters

Acetonitrile Merck

The purified fraction was dissolved in 50 % Acetonitrile and analyzed by being injected into the Tandem MS. The analyses were made in positive mode only. In table 7 the conditions of the MS is described closer.

Table 7: Conditions of the MS-analyze from active fractions having antiviral effect from *D.aggregata*.

Conditions	**ES+**
Capillary (kV)	3.30
Cone (V)	30.00
Source Temperature (^{o}C)	100
Desolvation Temperature (^{o}C)	250
Cone Gas Flow (L/Hr)	4
Desolvation Gas Flow (L/Hr)	49
Collision	18
Syringe Pump Flow (µL/min)	20.0

2.11 LC-MS ESI-TOF

Materials:

LCT Premier TOF MS	Waters
2795HT HPLC	Waters
XTerra® MS C_{18} (1.0x150 mm; 3.5 µm)	Waters
Acetonitrile	Merck
Formic acid	Sigma-Aldrich

The purified fraction with antiviral activity was subjected to HPLC- ESI-TOF. The analyses were made in positive mode only. The conditions of the HPLC and MS are listed in table 8 and 9.

Table 8: HPLC-conditions

Time (min)	Flow (µl/min)	Mobile phase A: % H_20 w/0.1 % formic acid	Mobile phase B: % ACN w/0.1 % formic acid
0	200	95	5
9	200	30	70
11	200	5	95
19	200	5	95

Table 9: Conditions of the MS-analysis from active fractions having antiviral effect from *D.aggregata*.

Conditions	ES+
Capillary (kV)	2.6
Cone (V)	35
Source Temperature (^{o}C)	120
Desolvation Temperature (^{o}C)	300
Cone Gas Flow (L/Hr)	5
Desolvation Gas Flow (L/Hr)	550
Syringe Pump Flow (µL/min)	5

2.12 NMR

Materials:

Varian Spectrometer 400 Mh_z	Varian (Palo Alto, USA)
Varian VnmrJ-software	Varian
Deuterated Methanol	Merck

The purified fraction was subjected to Varian 400 NMR spectrometer. The sample was dissolved in deuterated methanol and placed in the spectrometer and analyzed with ^{1}H-NMR specter.

3. Results

3.1 *Metridium senile*

3.1.1 Samples, extracts and fractions

The weight of the sea anemone *Metridium senile* (M09036) was 1500 grams and after freeze-drying the weight of the sample was 152.54 grams. The freeze-dried sample was subjected to both an aqueous and an organic extraction. The dry weight of the aqueous extract was 45.37 grams while the dry weight for the organic extract was 75.41 grams.

Aliquots of aqueous (213 mg) and the organic (241.5, 258.8 mg) extractions were the basis in which the fractionation were made by HPLC. The HPLC fractions were tested for bioactivity as described in the Materials and Methods section.

Subsequent fractions that showed bioactivity were retested in different dilutions to confirm the activity.

Figure 8 shows the UV-chromatogram from the primary fractionation of extracts from *M.senile.* The chromatogram is integrated for wavelength between 200 and 600 nm. As it can be seen, most components absorbs in the UV-area in the organic extract. The area where there was most absorption also showed activity when the fractions where screened for antiviral activity.

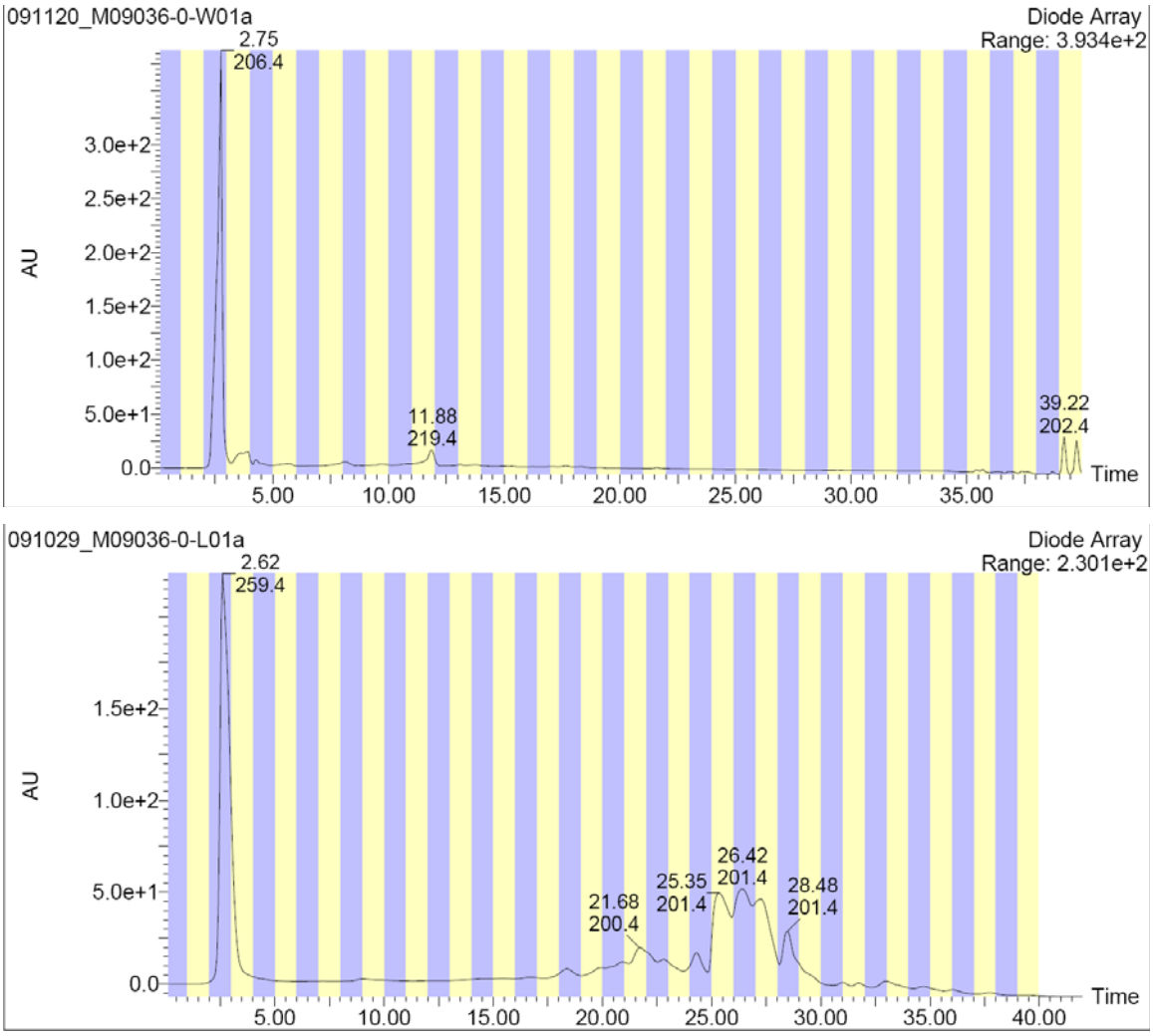


Figure 8: UV-chromatogram for the wavelength 200-600 nm of the aqueous (top) and organic fractionation (bottom) of *M.senile.*

3.1.2 Antiviral screening of *M.senile*

3.1.2.1 Organic fractions

Fraction number 26 (M09036-0-L01) displayed activity as showed in figure 9. The number of plaques was considerably less than the virus control. Fraction number 25 and 27 was also picked for a second screening even though they had many plaques compared to the virus control. But when comparing them with the control, the plaques size was smaller. Based on these characteristic plaques, it was decided to retest them to confirm the antiviral activity.

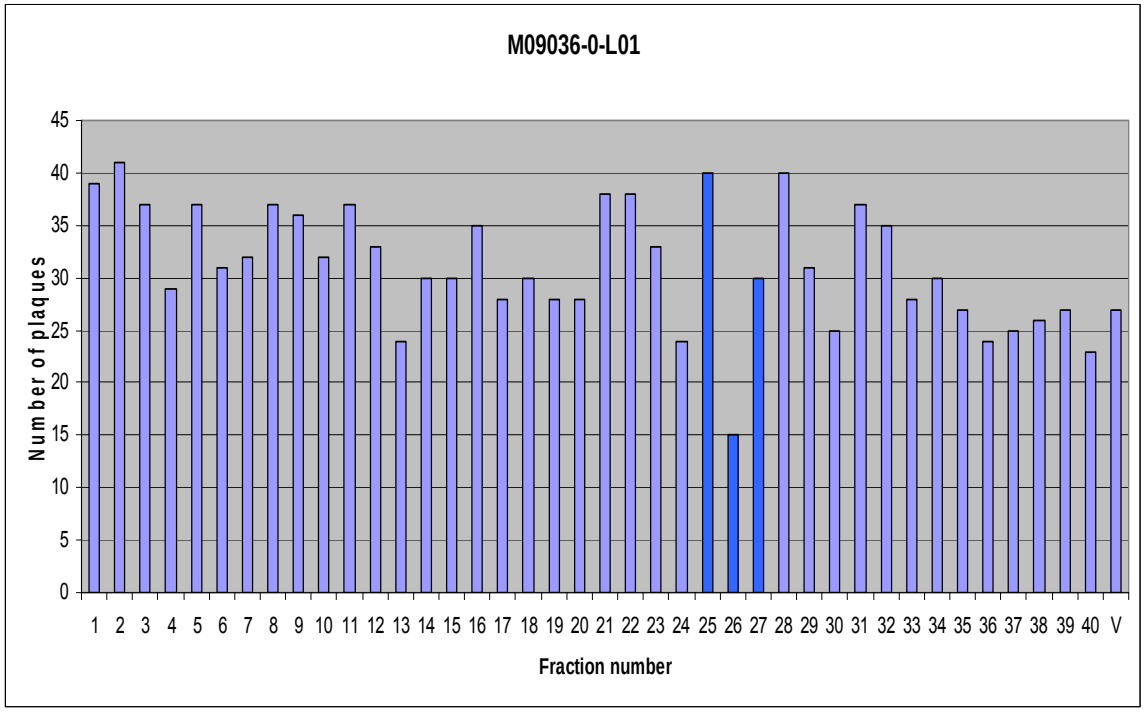


Figure 9: Antiviral activity of the 40 organic fractions from HPLC of extracts from *M. senile*.

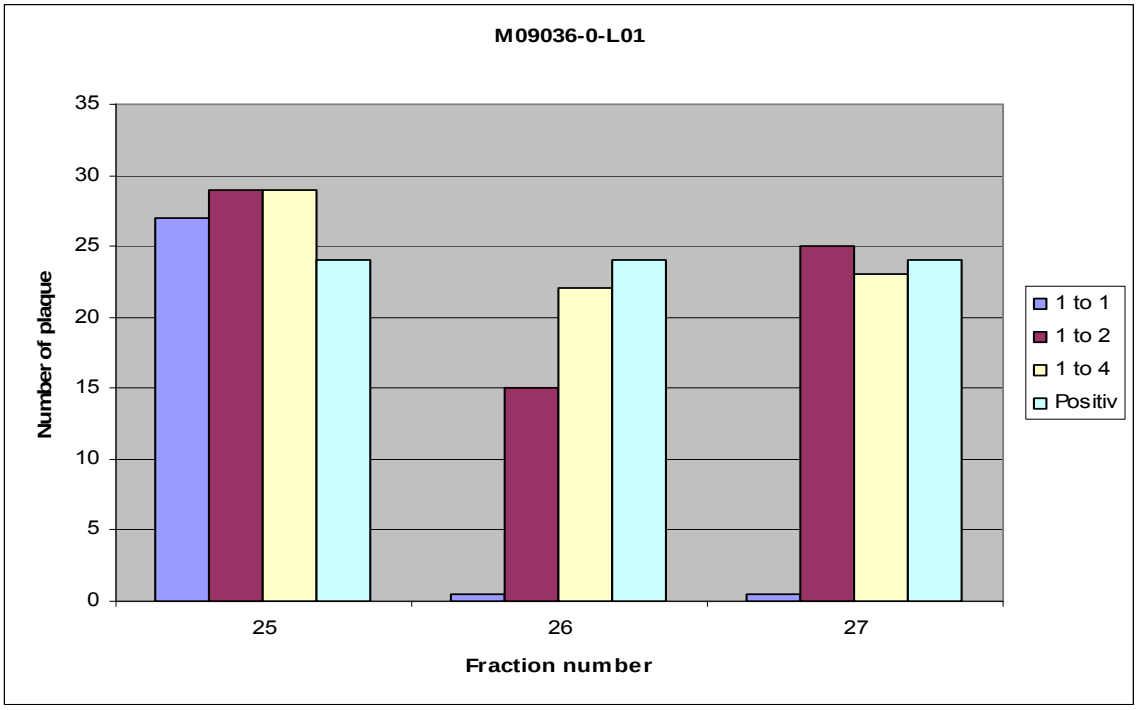


Figure 10: Antiviral activity of fraction 25, 26 and 27 when retested

Fractions 25, 26 and 27 were then retested in dilutions of 1:1, 1:2, 1:4to confirm the observations from the first screening. For fraction 26 as shown in figure 10, all the dilutions had more plaques than the control. The 1:1 dilution for fraction 26 and 27 has no plaques at all, but this activity disappeared when diluted further, so none of the fractions underwent further purification.

3.1.2.2 Aqueous fractions

The aqueous fractions of *M.senile* (M09036-0-W01) showed no antiviral activity as shown in figure 11.

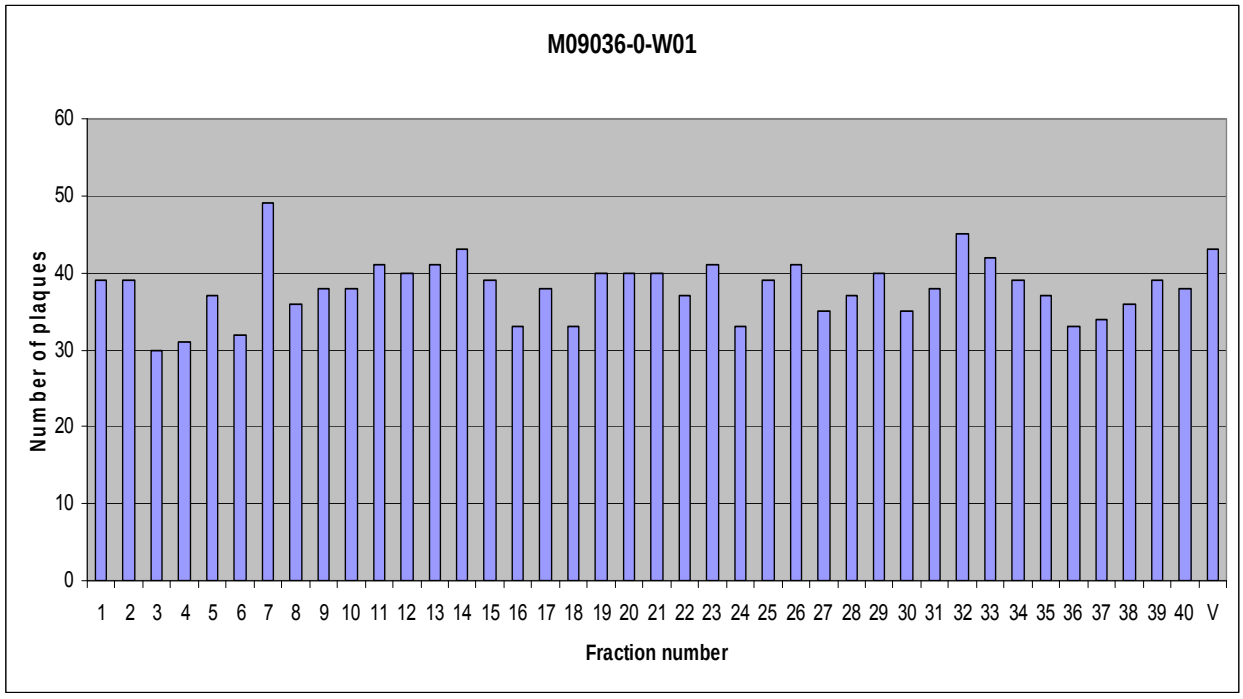


Figure 11: Antiviral activity of the 40 aqueous fractions of *M.senile.*

3.2 *Halocynthia pyriformis*

3.2.1 Samples, extracts and fractions

The weight of the sea anemone *Halocynthia pyriformis* (M09038) was 954.7 grams and after freeze-drying the weight of the sample was 104.6 grams. The freeze-dried sample was subjected to both an aqueous and an organic extraction. The dry weight of the aqueous extract was 51.93 grams while the dry weight of the organic extract was 52.33 grams.

Aliquots of the aqueous (211.8 mg) and the organic (288 mg and 293 mg) extractions were the basis in which the fractionation was made by HPLC. The HPLC-fractions were tested for bioactivity as described in the Materials and Methods section.

Subsequent fractions that showed bioactivity were retested in different dilutions to confirm the activity.

Figure 12 shows the UV-chromatogram from the primary fractionation of extracts from *H.pyriformis*. The chromatogram is integrated for wavelength between 200 and 600 nm. As it can be seen, most components also here absorbs in the UV-area in the organic extract. The area where there was most absorption also showed activity when the fractions where later screened for antiviral activity.

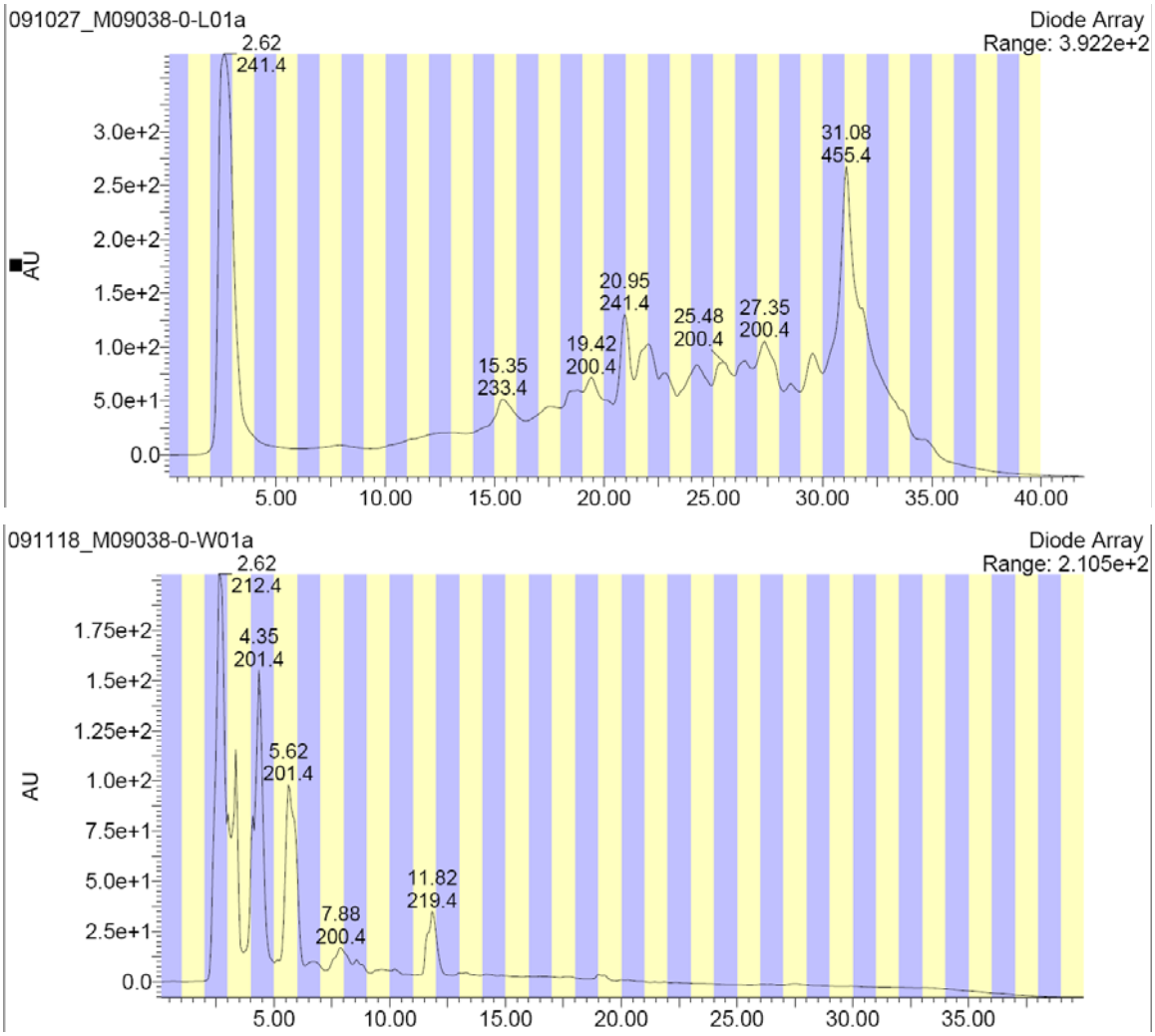


Figure 12: UV-chromatogram for the wavelength 200-600 nm of the aqueous and organic fractionation of *H.pyriformis.*

3.2.2 Antiviral screening of *H.pyriformis*

3.2.2.1 Organic fractions

Fractions 25, 28, 29, 30 and 31 (M09038-0-L01) displayed activity in the first screening as seen in figure 13.

Fraction 31 inhibited HSV-1 plaque formation completely, while 25, 28, 29 and 30 had some effect. Plaque size was also affected compared to the virus control. It was also decided to retest fraction 32, even though it had many plaques, but these showed very small characteristic plaques compared to the virus control. There were also observed cytotoxic effect on the Vero cells not caused by the virus in fraction 30, 31 and 32.

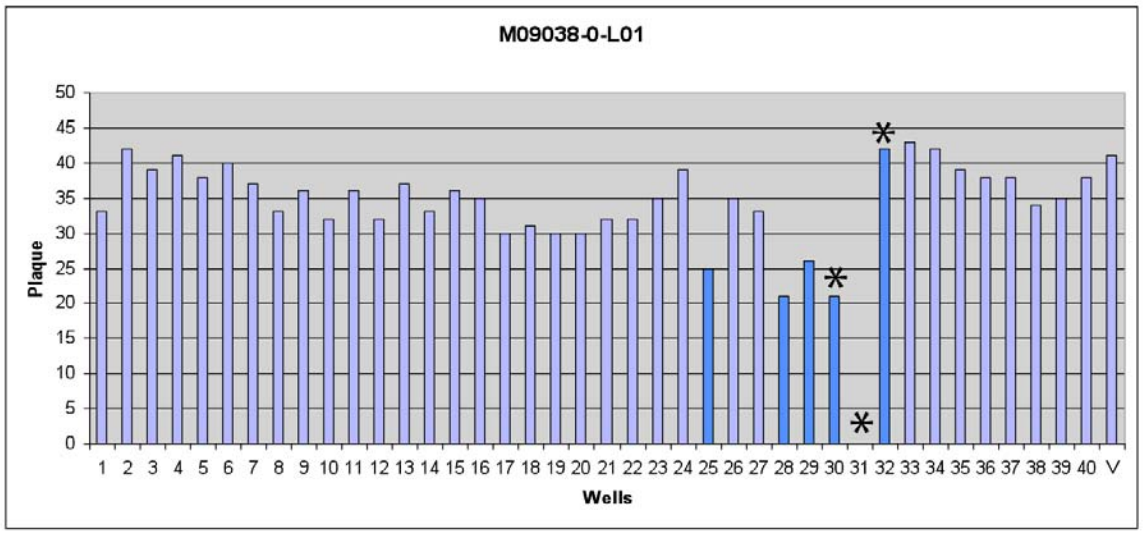


Figure 13: Antiviral activity of the 40 organic fractions from HPLC of extracts from *H.pyriformis.* The stars indicating the fractions with cytotoxic effects.

Fractions 25, 28, 29, 30, 31 and 32 were retested in dilutions of 1:1, 1:2 and 1:4 to confirm the activity as seen in figure 14 and 15. When diluted the fractions had little activity compared to observations in the first screening.

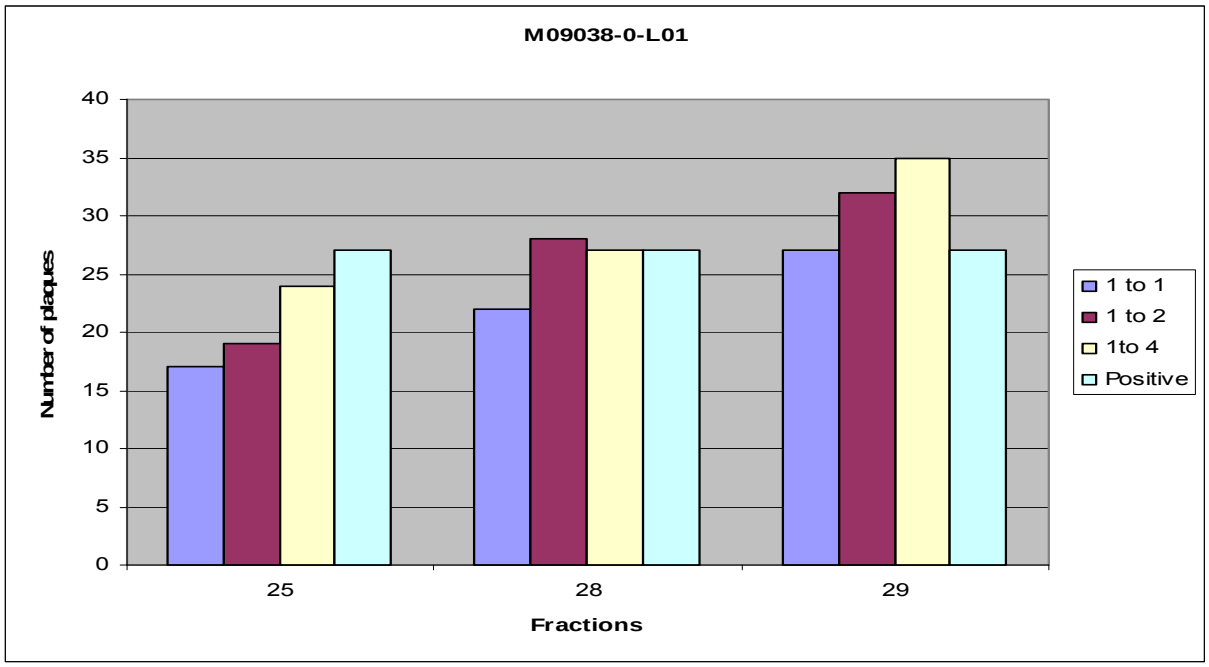


Figure 14: Antiviral activity of fraction 25, 28 and 29 when retested

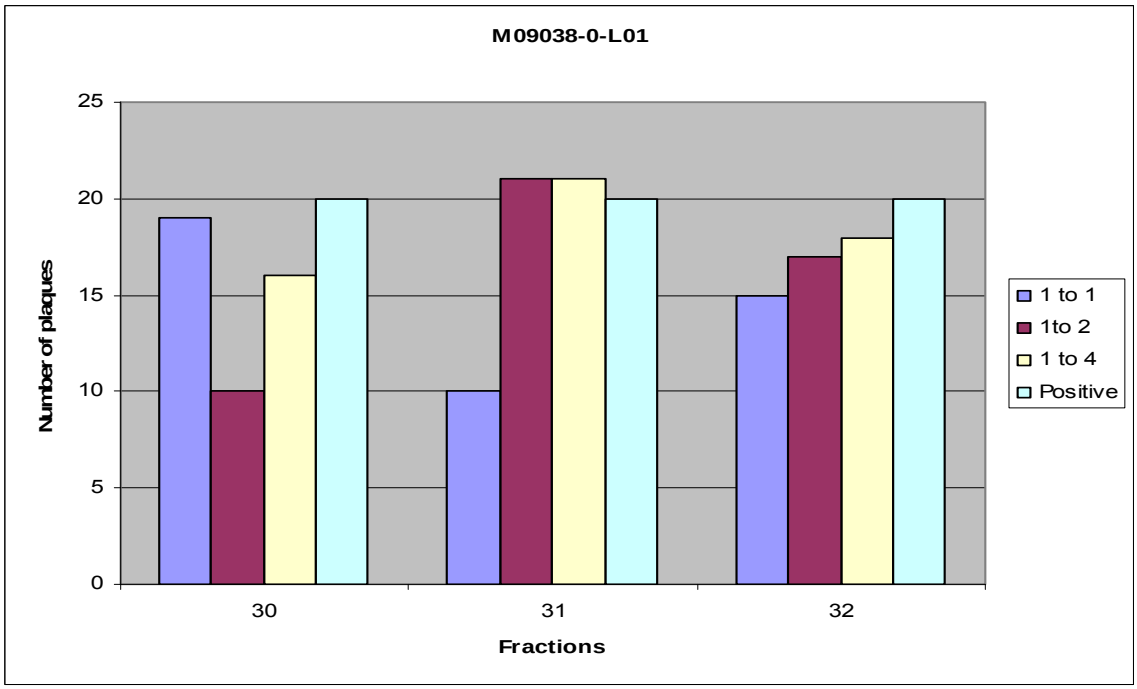


Figure 15: Antiviral activity of fraction 30, 31 and 32 when retested

3.2.2.2 Aqueous fractions

The primary fractionation (M09038-0-W01) did not show any clear inhibition of HSV-1 in any plaques as seen in figure 16. Fraction number 3 had affected the plaque size compared to the virus control, so it was decided to retest it.

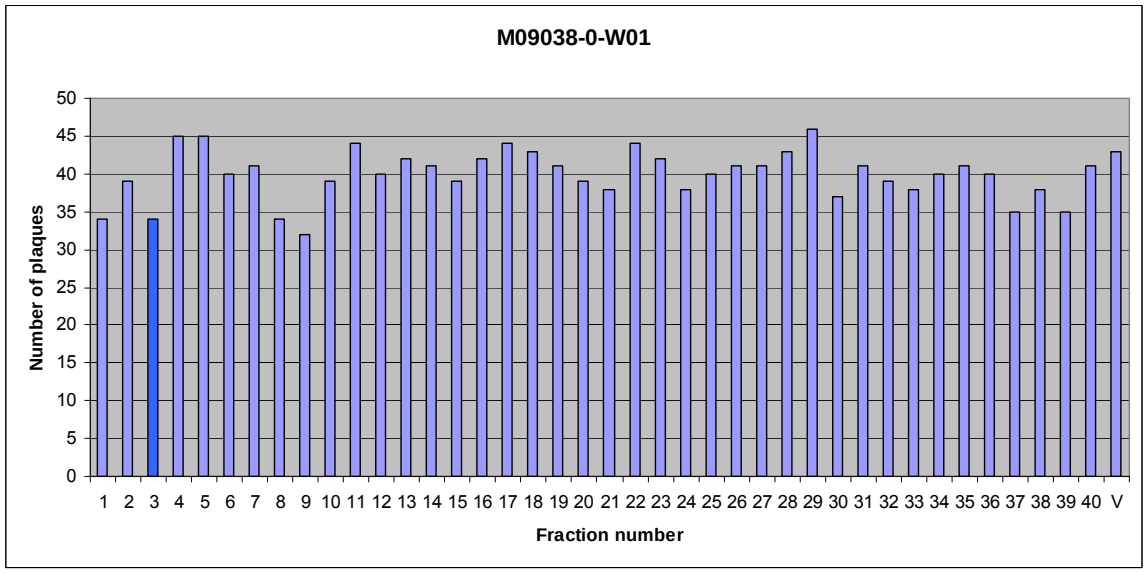


Figure 16: Antiviral activity of the 40 aqueous fractions from HPLC of extracts from *H.pyriformis*

Fraction 3 was retested in dilutions of 1:1, 1:2 and 1:4 to confirm the activity. Fraction number 3 did not show any activity in the re-test as seen in figure 17. Also there were no longer any distinctive differences in the plaque size compared to the control.

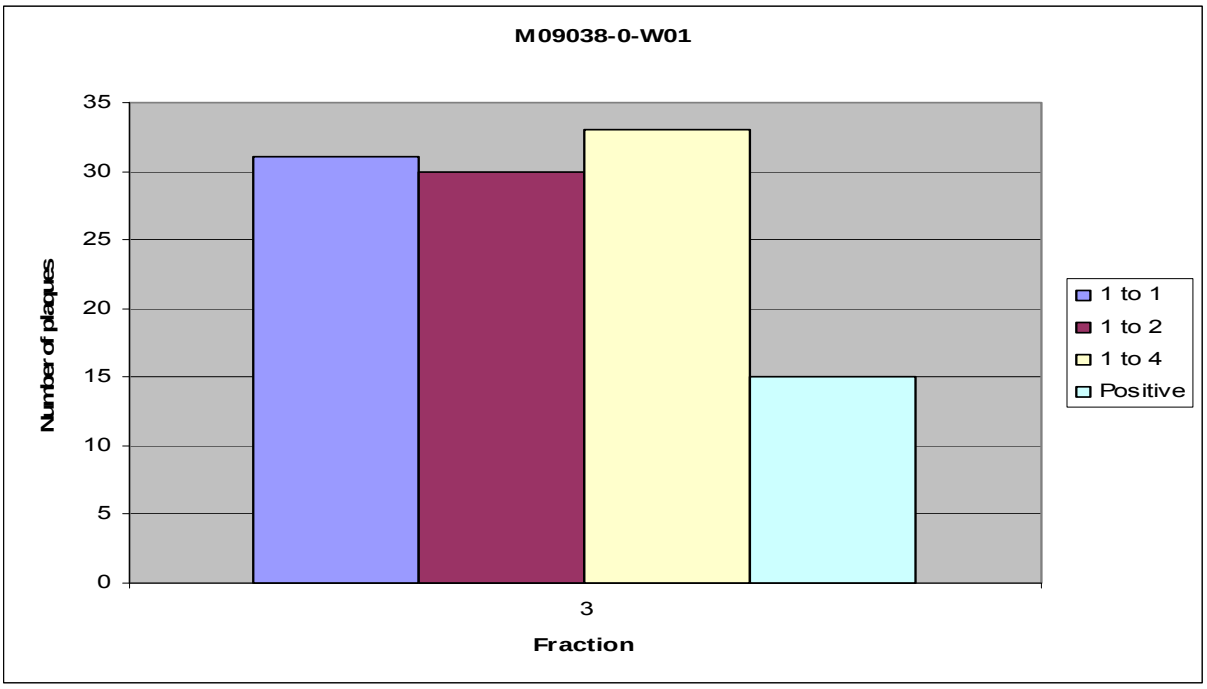


Figure 17: Antiviral activity of fraction 3 when retested

3.3 Parastichopus tremulus

3.3.1 Samples, extracts and fractions

The weight of *Parasitchopus tremulus* (M09037) was 995 grams and after freeze-drying the weight of the sample was 87.31 grams. The freeze-dried sample was subjected to both an aqueous and an organic extraction. The dry weight of the aqueous extract was 35.55 grams while the dry weight for the organic extract was 51.76 grams.

Aliquots of aqueous (209.9 mg) and the organic (297.5 and 295.8) extractions were the basis in which the fractionation was made by HPLC. The HPLC-fractions were tested for bioactivity as described in the Materials and Methods section.

Subsequent fractions that showed bioactivity were retested in different dilutions to confirm the activity.

Figure 18 shows the UV-chromatogram from the primary fractionation of extracts from *P.tremulus*. The chromatogram is integrated for wavelength between 200 and 600 nm. As it can be seen, most components also here absorbs in the UV-area in the organic extract. The area where there was most absorption also showed activity when the fractions where later screened for antiviral activity.

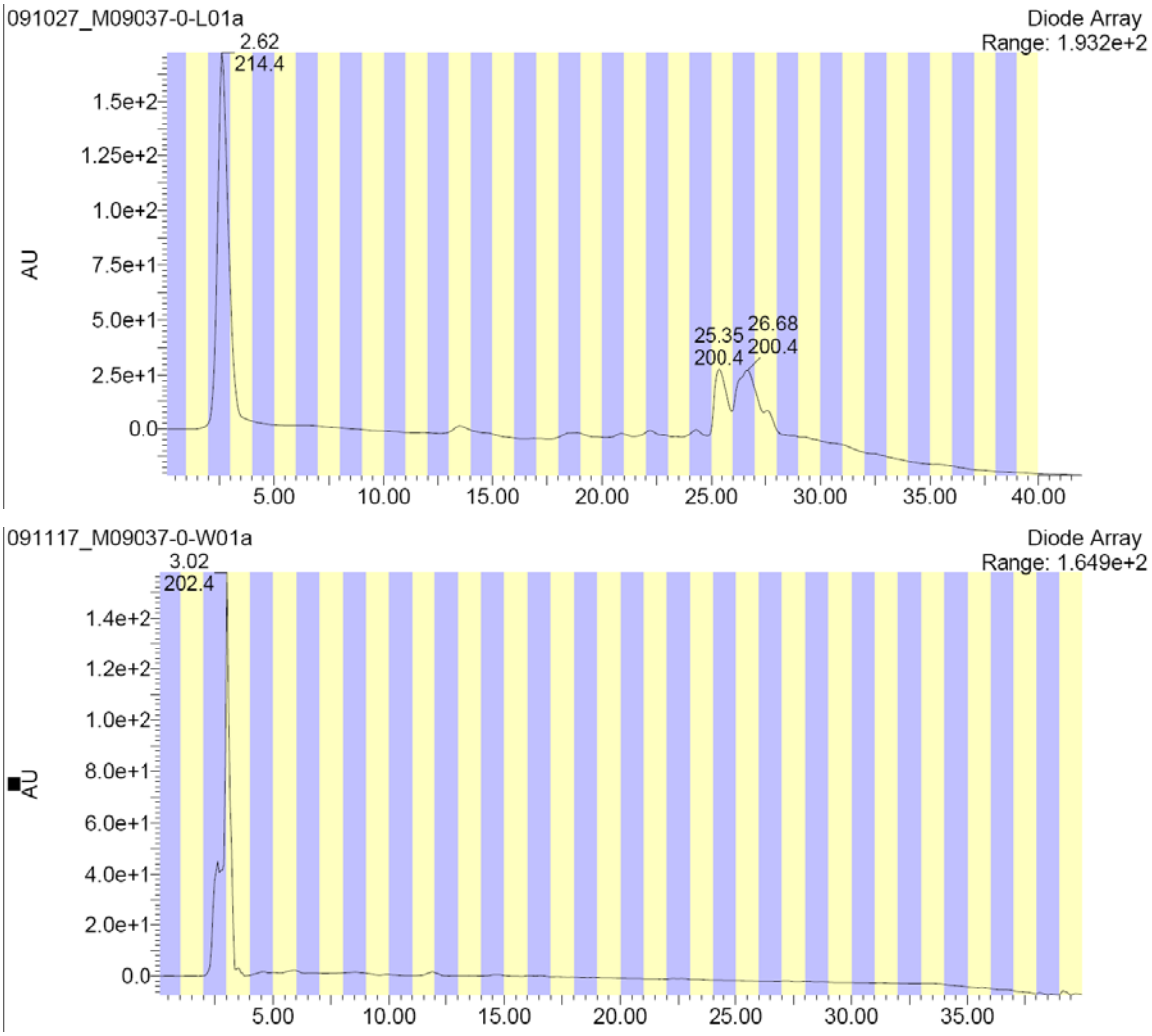


Figure 18: UV-chromatogram for the wavelength 200-600 nm of the organic fractionation (top) and aqueous (bottom) of *P.tremulus.*

3.3.2 Antiviral screening of *P.tremulus*

3.3.2.1 Organic fractions

The primary fraction showed activity against HSV-1 in fractions 15, 16, 24, 25 and 26 (M09037-0-L01) since they had much less plaques compared to the virus control. Fraction 23, 27 and 28 were also retested since the plaque size was affected compared to the virus control as seen in figure 19. There was not observed any toxicity on the Vero cells.

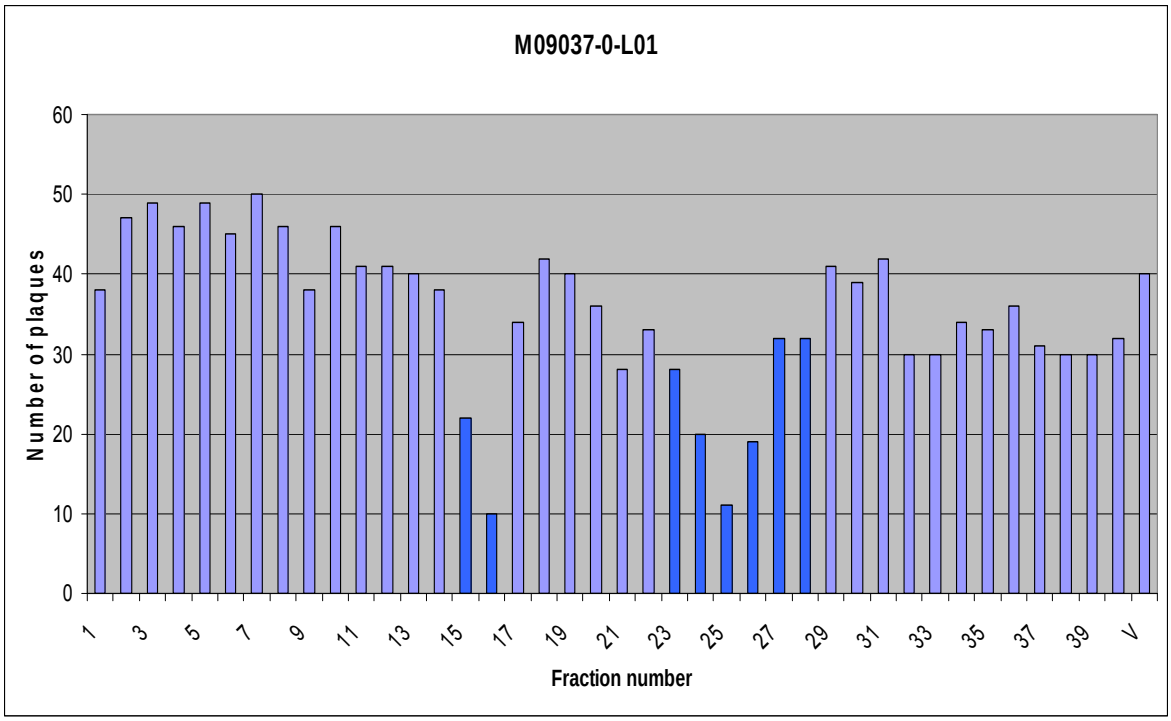


Figure 19: Antiviral activity of the 40 organic fractions from HPLC of extracts from *P.tremulus*

Fractions 15, 16, 23, 24, 25, 26, 27 and 28 were retested in dilutions of 1:1, 1:2, and 1:4 to confirm their activity as seen in figure 20, 21 and 22. In the second screening the activity could not be reproduced as seen in figure 10-12, so it was decided not to work any further with these fractions

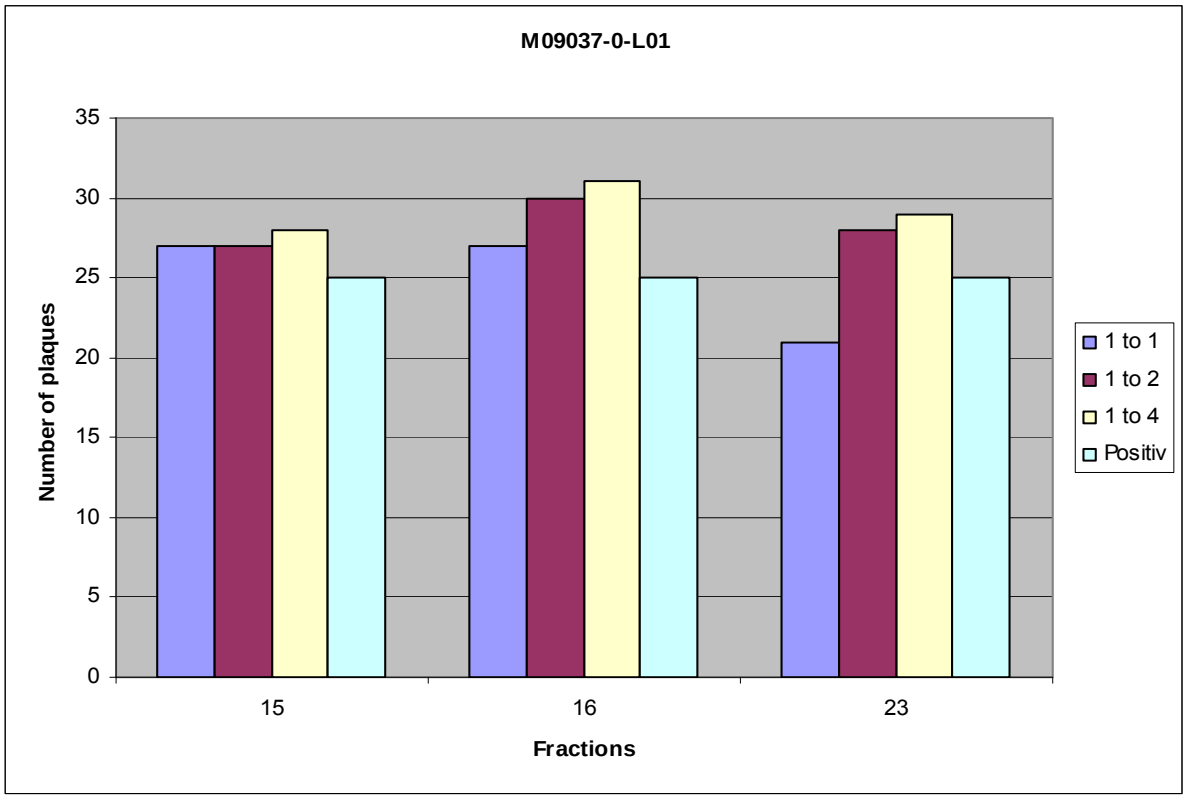


Figure 20: Antiviral activity of fraction 15, 16 and 23 when retested

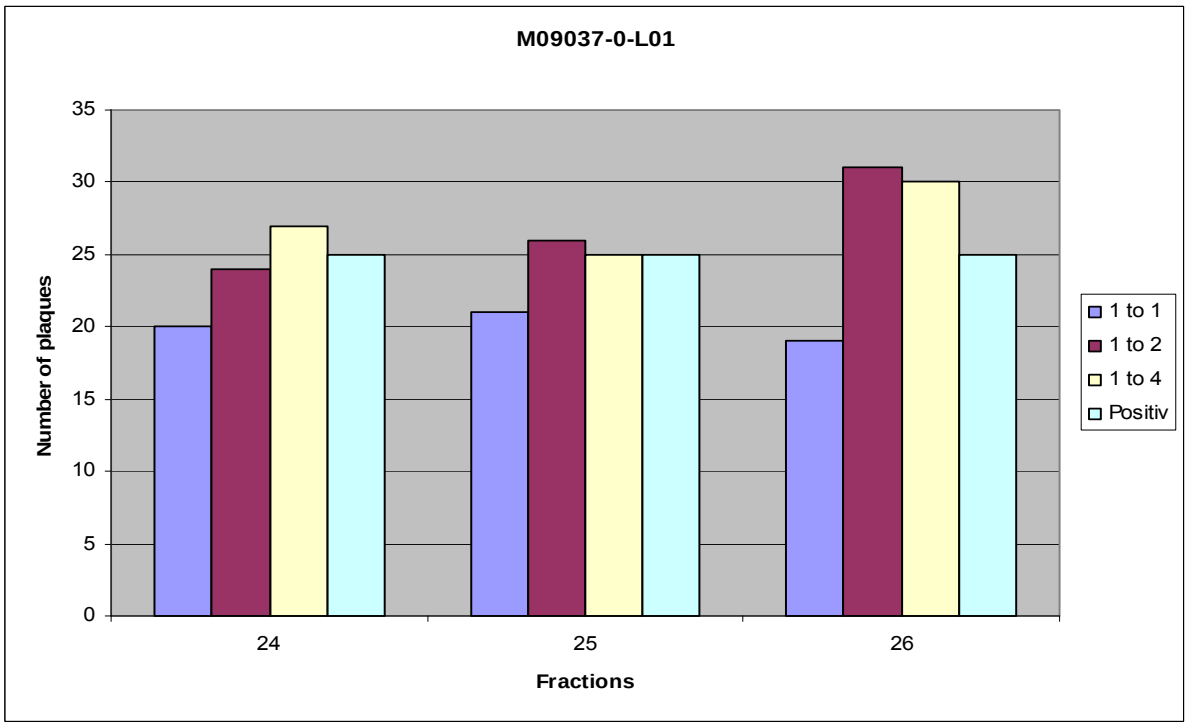


Figure 21: Antiviral activity of fraction 24, 25 and 26 when retested.

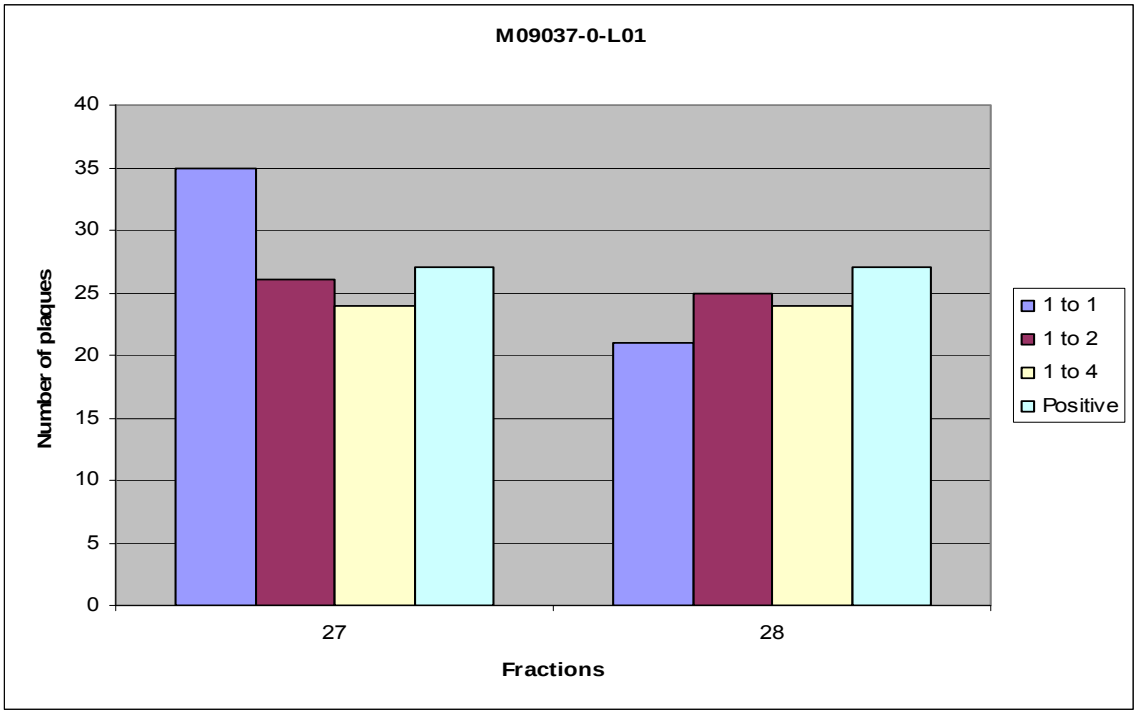


Figure 22: Antiviral activity of fraction 27 and 28 when retested.

3.3.2.2 Aqueous fractions:

The primary fraction displayed activity against HSV-1 on fractions 35, 36 and 37 (M09037-0-W01) as seen in figure 23. All the fractions had plaque size that was affected compared to the virus control. There was also observed cytotoxic effect on the Vero cells not caused by the virus in fractions 35, 36 and 37. It was decided to retest these fractions.

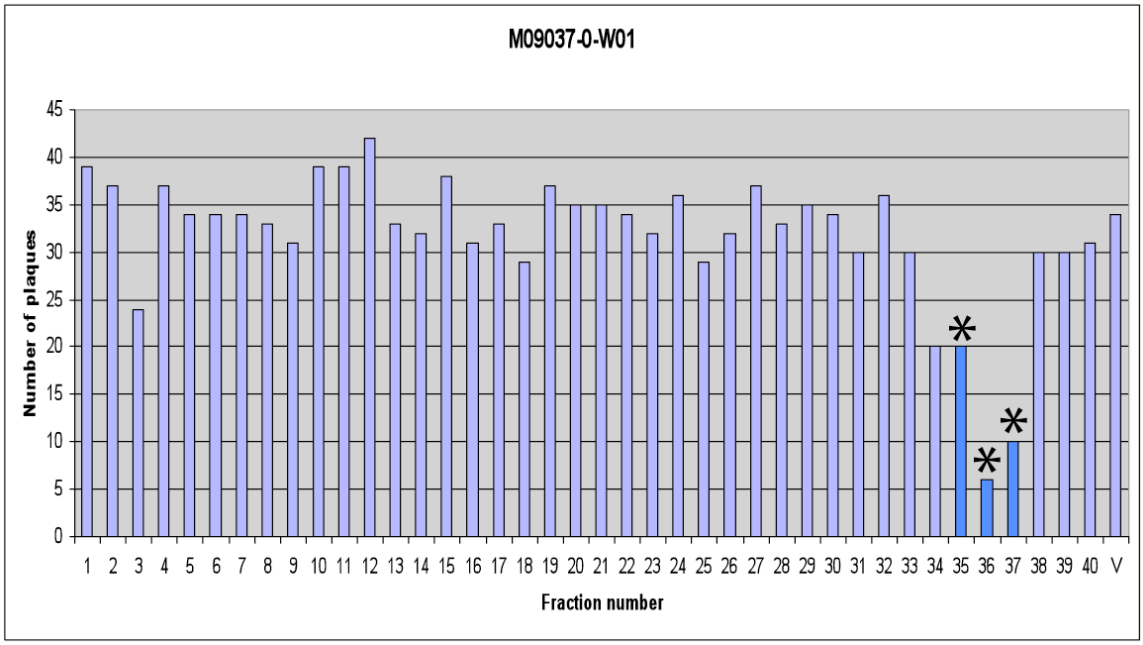


Figure 23: Antiviral activity of the 40 aqueous fractions from HPLC of extracts from *P.tremulus.*Stars indicate the fractions with cytotoxic effects.

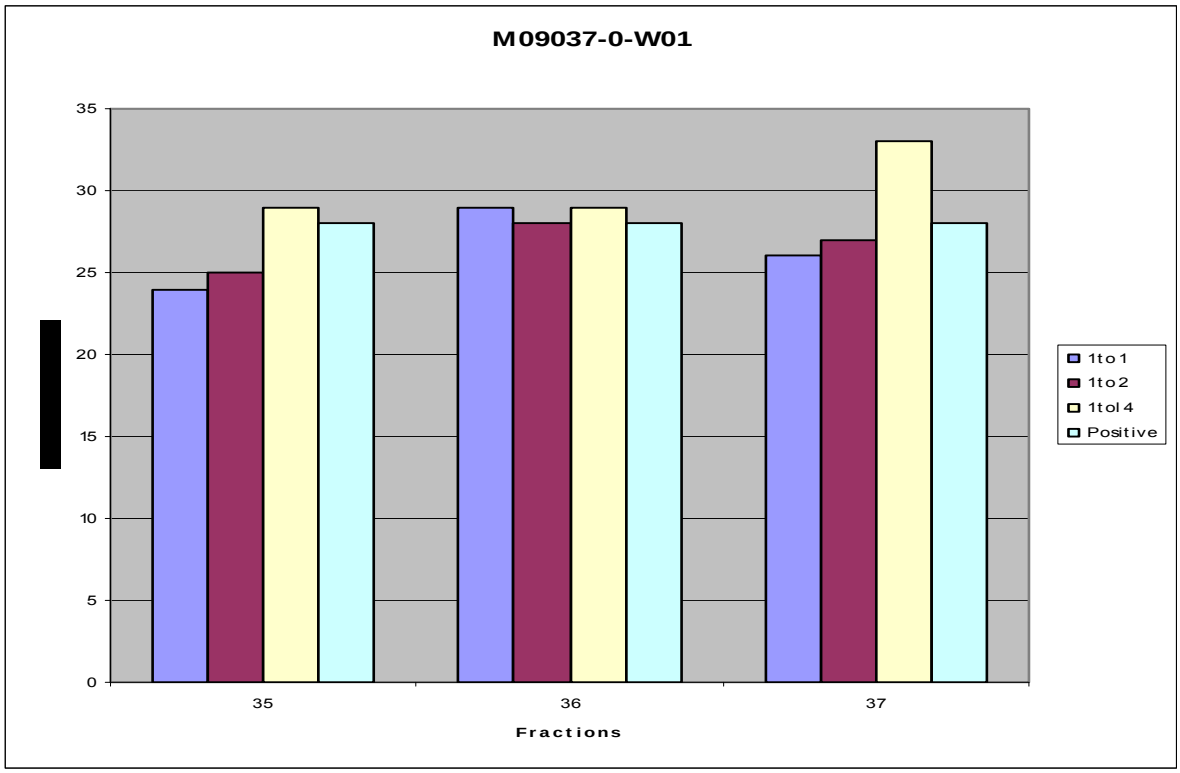


Figure 24: Antiviral activity of fractions 35, 36 and 37 when retested.

Fractions 35, 36 and 37 were retested in dilutions of 1:1, 1:2, and 1:4 to confirm their activity as seen in figure 24. In the second screening all the fractions showed no activity, and it was decided not to work any further with these fractions.

3.4 *Dendrodoa aggregata*

3.4.1 Samples, extracts and fractions

The weight of *Dendrodoa aggregata* (M06039) was 312.8 grams and after freeze-drying the weight of the sample was 45.68 grams. The freeze-dried sample was subjected to both an aqueous and an organic extraction. The dry weight of the aqueous extract was 6.64 grams while the dry weight for the organic extract was 3.94 grams.

Aliquots of the organic (250 mg) extractions were the basis in which the fractionation was made by HPLC. The HPLC-fractions were tested for bioactivity as described in the Materials and Methods section.

Figure 25 shows the UV-chromatogram from the primary fractionation of extracts from *D.aggragata*. The chromatogram is integrated for wavelength between 200 and 600 nm. The area where there was most absorption also showed activity when the fractions where later screened for antiviral activity.

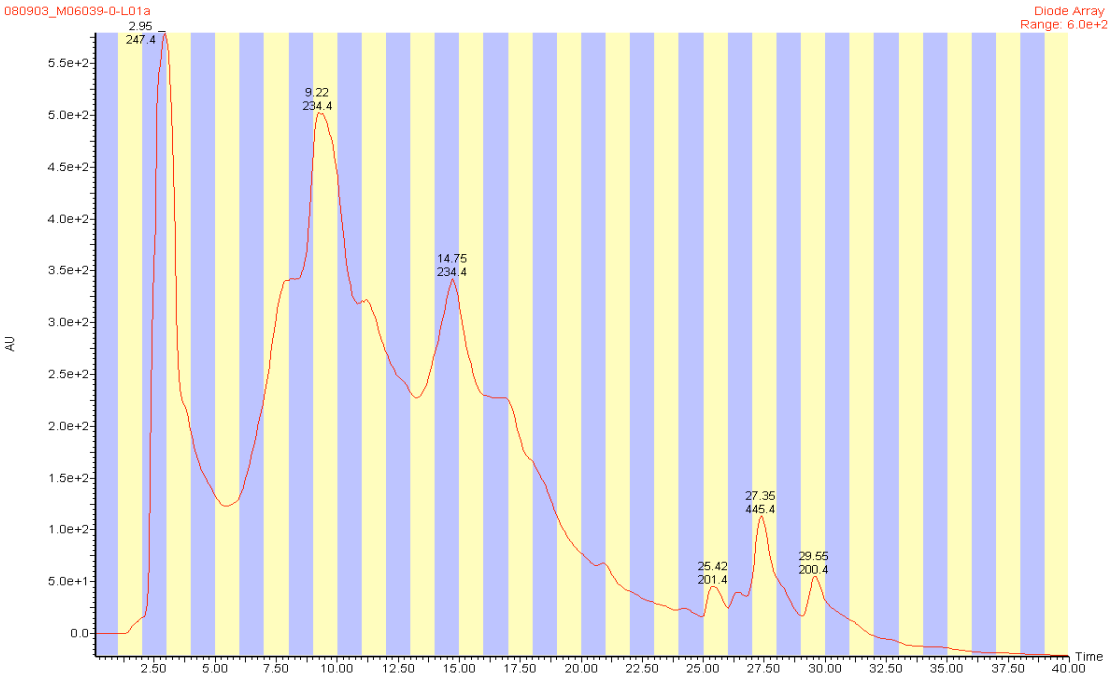


Figure 25: UV-chromatogram for the wavelength 200-600 nm of the organic fractionation of *D.aggregata.*

3.4.2 Antiviral screening of *Dendrodoa aggregata*

3.4.2.1 Organic fractions:

In the primary screening fractions 3 - 8, 12-17 and 29-31 showed small indications of plaques, the fractions from 9-11 had all inhibited HSV-1 as seen in figure 23. Previous pilot studies with deoxytubastrine had indicated some effect against HSV-1 (data not shown). In the literature it has been demonstrated that tubastrine has antiviral activity [17]. Studies of the fractions in figure 26 showed fraction 3 exerted antiviral activities. LC-MS of fraction 3 shows that deoxytubastrine is the dominating compound in fraction 3, and based on this it was decided to purify deoxytubastrine for further studies.

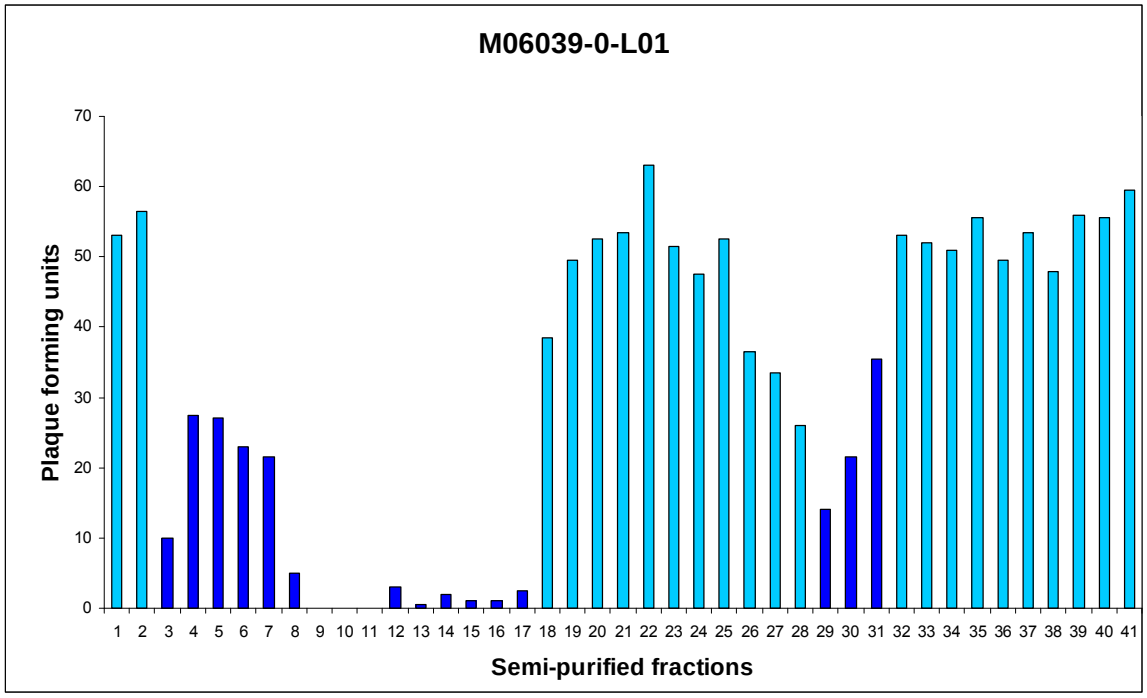


Figure 26: Antiviral activity of the 40 organic fractions from HPLC of extracts from *D.aggregata*

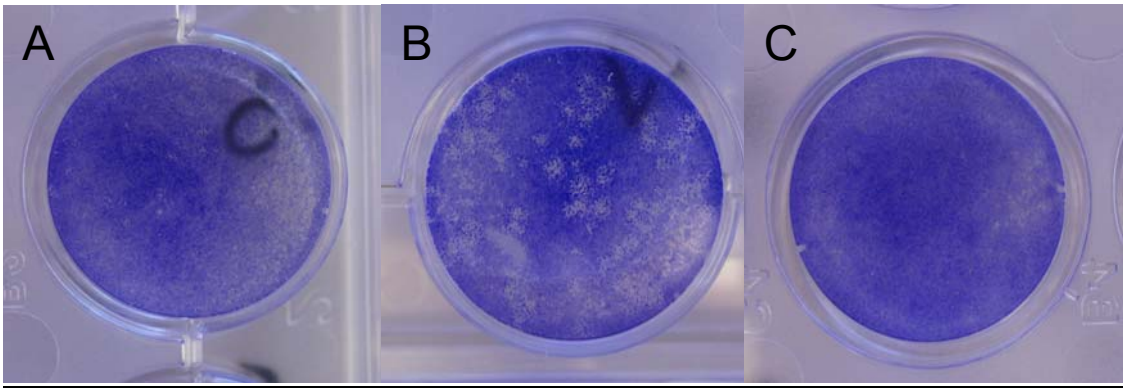


Figure 27: Pictures of plaques of the PRA from *D.aggregata.* A: Negative control B: positive control C: plaques from fraction 3.

Figure 27 shows pictures of the plaques from the primary screening of *D.aggregata*.

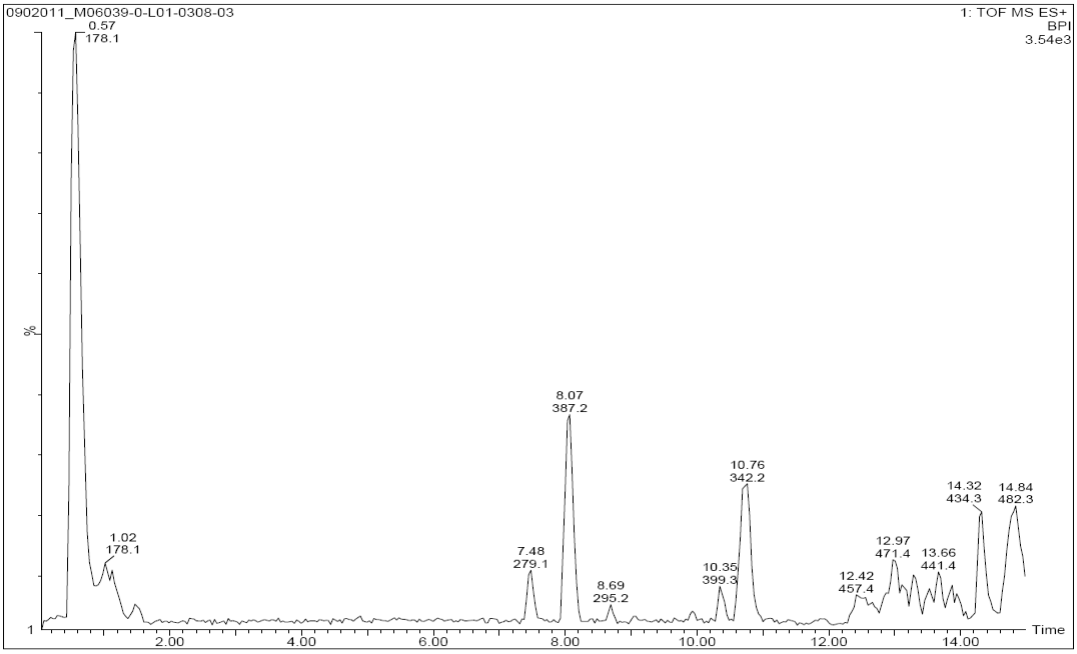


Figure 28: LC-MS ESI-TOF BPI ES+ from fraction 3 in *D.aggregata.*

Figure 28 shows the base peak index in LC-MS ESI-TOF in positive mode of fraction 3 from *D.aggregata*. The first peak of the specter is the base peak with *m/z* 178.0906.

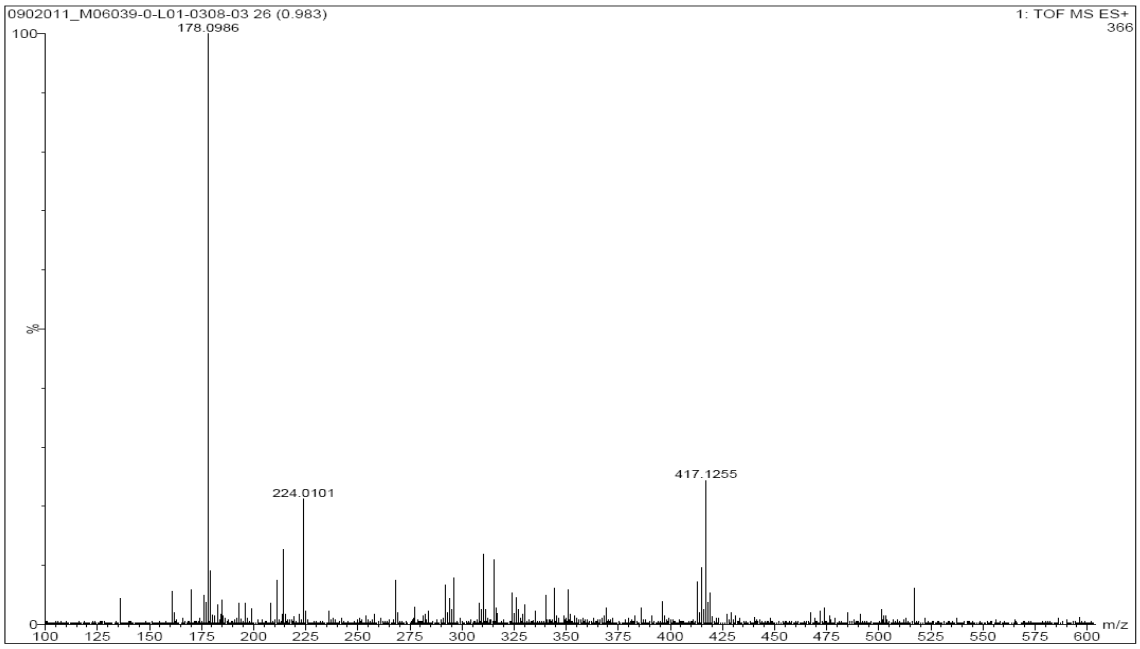


Figure 29: ESI+ MS spectra of the base peak from fraction number 3 *D.aggregata.*

Based on the base peak index, accurate mass and isotope distribution was made for fraction 3. Figure 29 shows LC-MS ESI-TOF specter with positive mode of fraction 3 from *D.aggregata.* The specter revealed one major peak of *m/z* 178.0906. The elemental composition analysis suggested the molecular formula to be $C_9H_{11}N_3O$. Searches on Dictionary of Marine Natural Products revealed the compound deoxytubastrine, shown in figure 30.

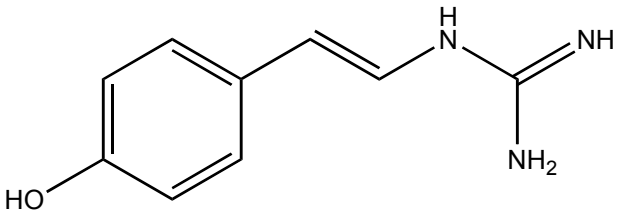


Figure 30: Structure of deoxytubastrine

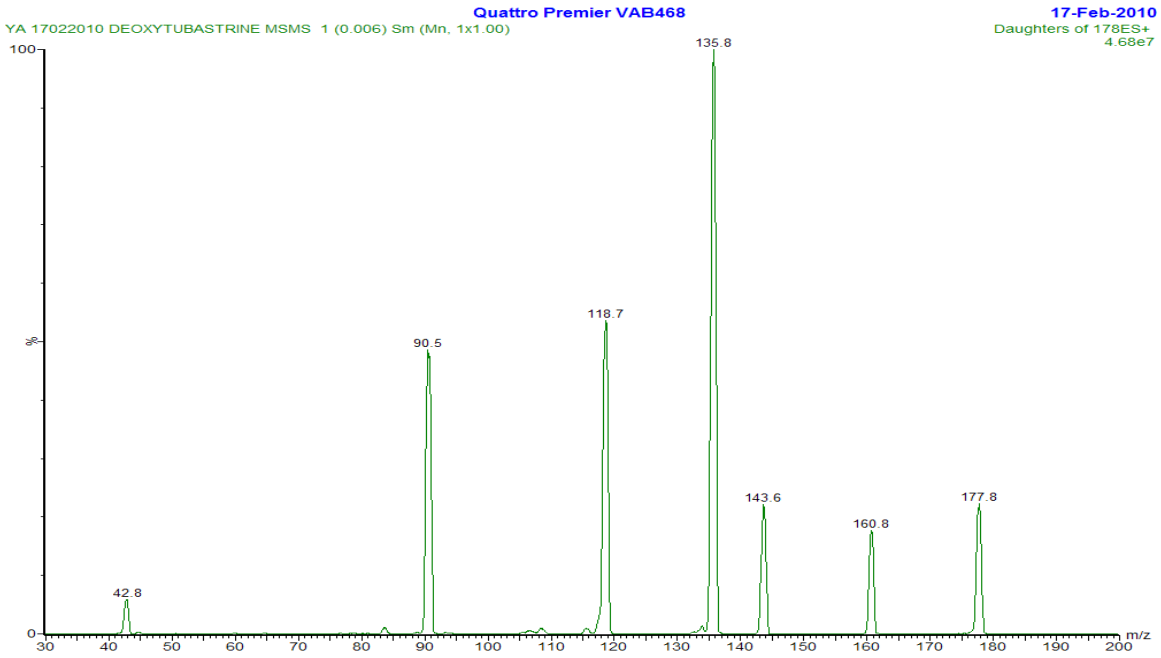


Figure 31: ESI+ MSMS daughter scan specter from deoxytubastrine

Figure 31 shows the fragmentation of *m/z* 177.8 in LC-MS ESI-QqQ in positive mode.

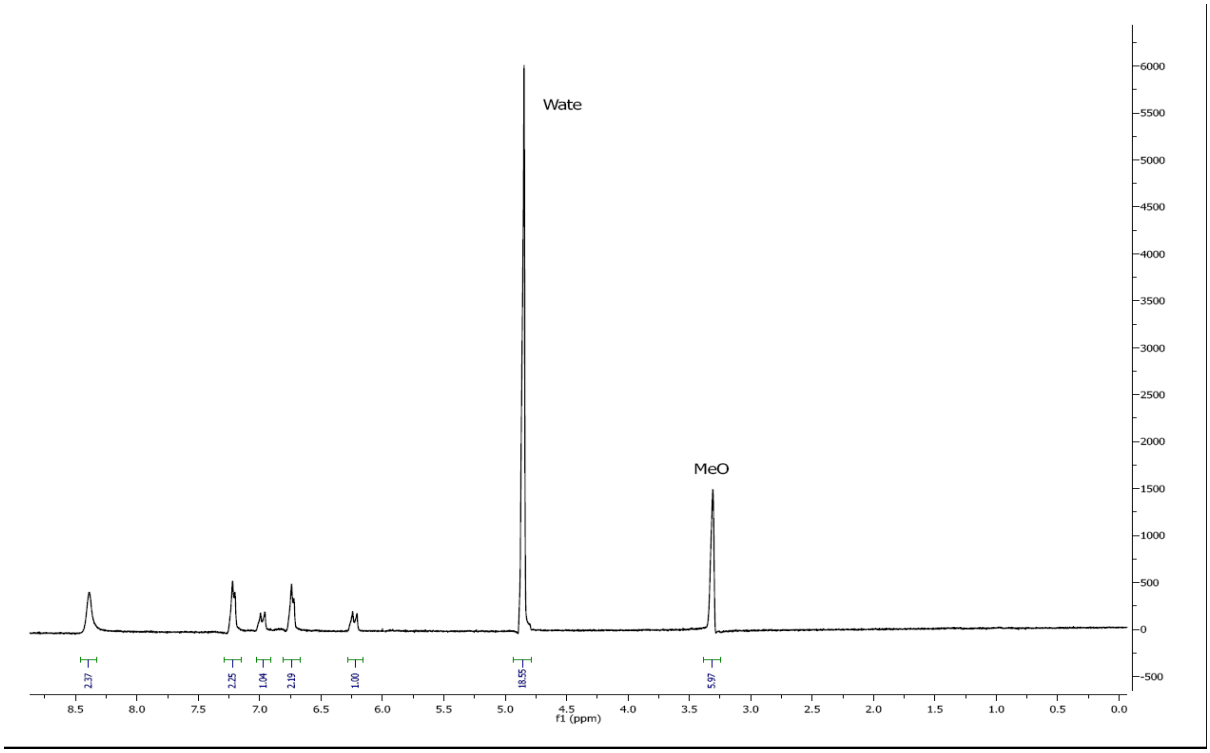


Figure 32: NMR specter of Deoxytubastrine.

The purified deoxytubastrine was also subjected to ^{1}H-NMR analysis. Figure 32 shows the ^{1}H-NMR specter. In the specter, the protons with chemical shift at 6.6 ppm and 7.5 ppm are characteristic to the protons on the benzene ring. The protons with chemical shift on 6.5 ppm and 7.0 are characteristic to the protons on the two carbon atoms on
–RC3H6N3 (R=C6H5O). The chemical shift on 8.5 ppm is probably the protons on one of the N-groups.

3.4.2.2 Screening

The organic extract from before HPLC-fractionation underwent a dose-response test to find out at which concentrations the plaques are formed and to check if any concentrations are toxic to the Vero cells. As shown in figure 33, the concentrations between 500-100 µg/mL there were no plaques at all. These concentrations were also cytotoxic to the Vero cells. There were no cells alive at 500 µg/mL, at 250 and 100 µg/mL some cells were alive. At 75 µg/mL there was observed some plaques, but the plaque size was affected compared to the virus control. First at 50 µg/mL there were a significant number of plaques, but also here the plaque size was affected compared to the control.

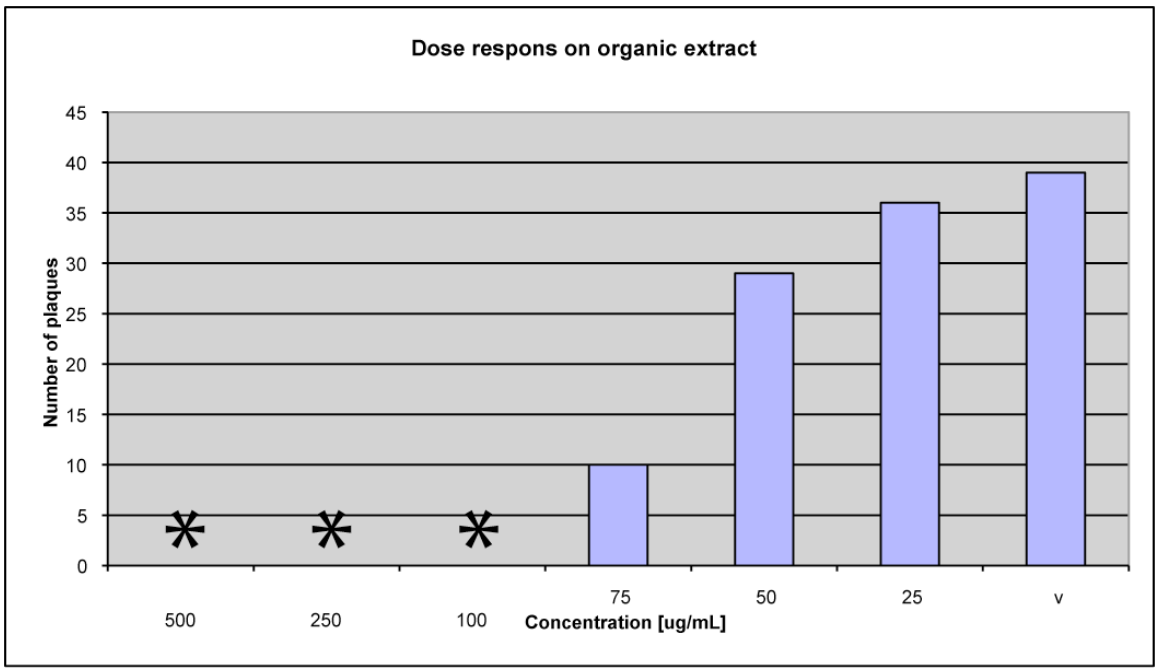


Figure 33: Dose respons for the organic extract. Stars indicate the cytotoxic concentrations.

Purified deoxytubastrine was tested for dose-response. The data showed that none of the concentrations inhibited plaque formation of HSV-1 as seen in figure 34. The concentrations between 50 µg/mL and 15 µg/mL had plaque sizes that were affected compared to the virus control.

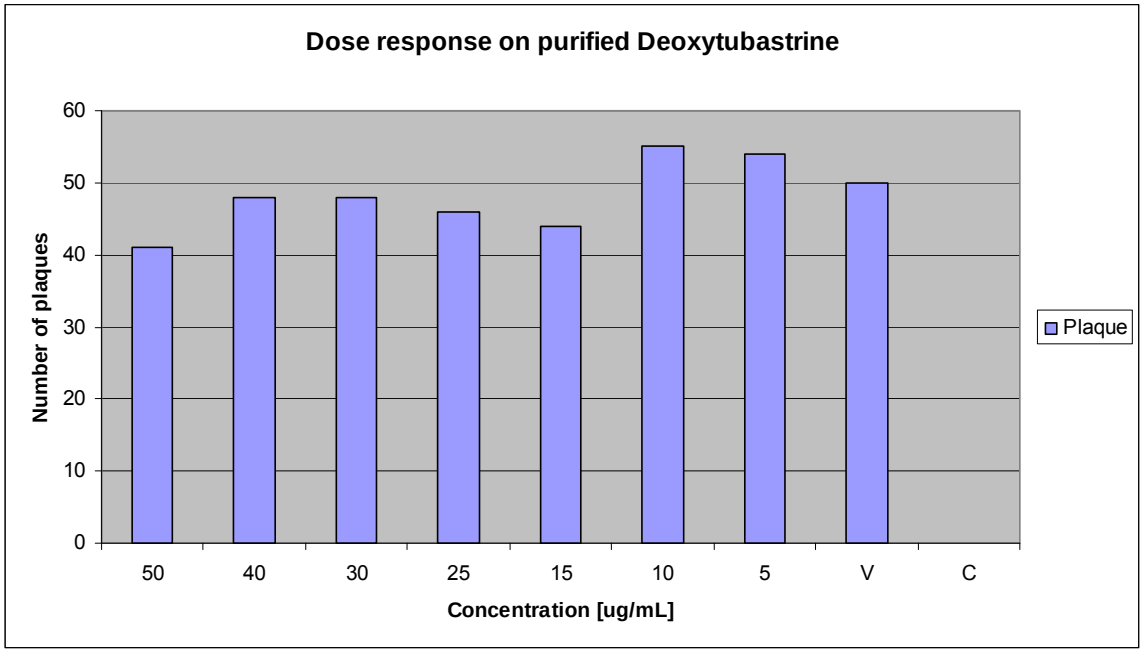


Figure 34: Dose response for purified Deoxytubastrine.

Purified deoxytubastrine was also tested for cytotxicity against Vero cells in the MTT- test. The results showed that deoxytubastrine is not cytotxic to Vero cells in the concentrations it was tested on, as seen in figure 35.

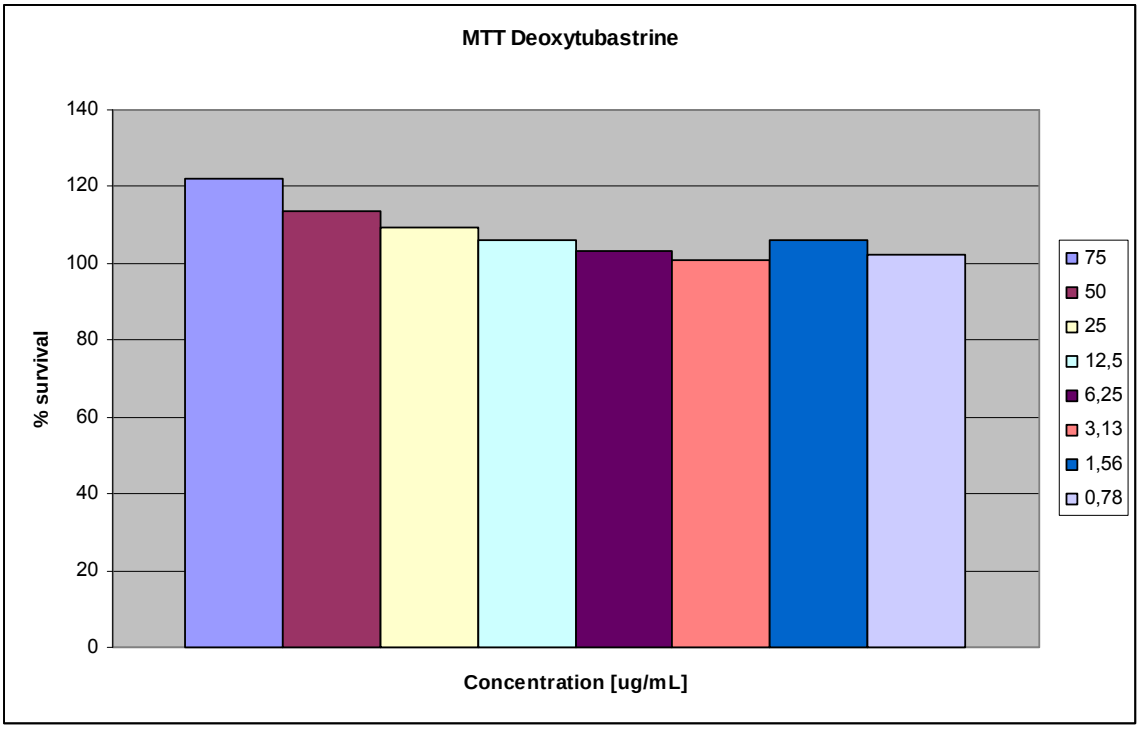


Figure 35: MTT for purified Deoxytubastrine.

4. Discussion

4.1 Antiviral screening

Since the organic and the aqueous extract are very complex, and can contain several components, fractionating them will distribute the compounds in the different fractions based on their chemical properties.

M.senile, H.pyriformis and *P.tremulus* were subjected to aqueous and organic fractionation. As it has been shown in the results most of the activity that were shown appeared in the organic fractions. Only one fraction (M09038-0-W01-3) in figure 16 from the aqueous fractions showed some activity. This might be because of the difficulties associated with the isolation and purification of water-soluble compounds. The abundance of salt from seawater into the water extracts makes the isolation of the compounds more perplexing[18].

Fraction 3 (M09038-0-W01) might have shown activity in the primary screening because of the presence of salts in the fraction. There are generally a lot of inorganic salts like NaCl in marine samples. The presence of these salts can influence the activity of the fractions. This can be the reason to the loss of the activity when diluted.

Retesting is made to verify if the activity from the primary fractions are true or false. The retest is made by testing the activity in different concentrations, in this way we can find out whether the activity is strong enough to be found when the compounds are further purified.

Retesting the active fractions from the primary screening in different dilutions showed no activity. Loss of activity is very common when it comes to retesting. The dilutions of the samples will also dilute the concentrations of the active compounds, which could be a possible reason for why the activity is lost. Improper calculations of the dilutions can also have an effect on the retested results. The PRA itself is a time consuming assay. It is not easily automated, and since the testing is done in 24 well plates and not in 96 well plates it means that a bigger volume of media and compounds are needed. This can make it more difficult to work with the assay and make room for more errors which could have an effect on the activity. Literature search did not yield any compounds with antiviral activity in the 3 organisms, so it was decided not to investigate further on these organisms.

4.2 Analysis

The MS-QqQ ESI in positive mode gave us several fragmentations of *m/z* 178.0906 (figure 31). The MS-QqQ has a slightly different *m/z* for deoxytubastrine than MS-TOF, since it is not as sensitive as the TOF when it comes to accurate mass. Based on the fragments a list of possible elemental compositions was made, as seen in table 10.

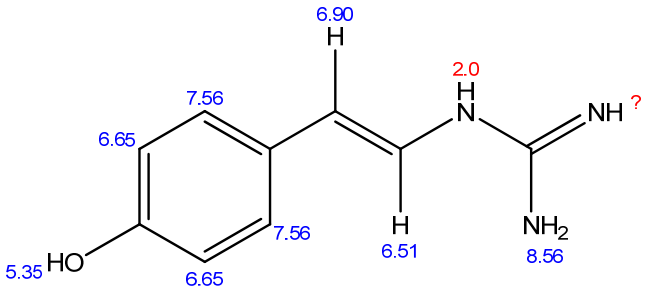
Table 10: Possible elemental compositions of *m/z* 178.0906 calculated based on LC-MS ESI-QqQ specter.

Daughter ion	Possible elemental composition
160.64	$C9H9N2O^+$ or $C9H10N3^+$
143.77	$C9H8N2^+$
135.77	$C8H9NO^+$
118.68	$C8H8O^+$
90.60	$C7H9^+$

The base peak index in LC-MS ESI-TOF in positive mode (figure 28) revealed one major peak of *m/z* 178.0906 after 0.57 minutes. When comparing this specter with the UV-chromatogram from the organic fraction of *D.aggregata* (figure 25) and the figure with the antiviral activity from the primary fractions in *D.aggregata* (figure 26), one can see in all the figures that after approximately 3 minutes in the primary fractionation there has been eluted compounds which later showed it contained deoxytubastrine.

Drawing the structure of deoxytubastrine (figure 31) in Chemdraw, gave us an estimated specter of ^{1}H-NMR of deoxytubastrine that can be seen in figure 36.

ChemNMR ^{1}H Estimation



Estimation quality is indicated by color: good, medium, rough

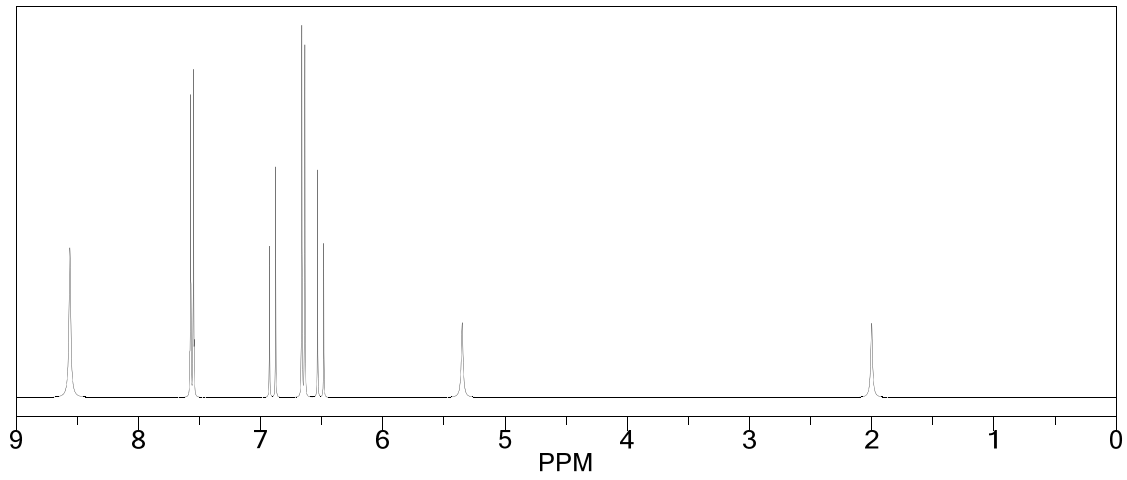


Figure 36: Estimated specter of deoxytubastrine from Chemdraw.

When we compare the estimated ^{1}H-NMR specter (figure 36) with the ^{1}H-NMR specter (figure 34) from the sample, we can see similarities between them suggesting they are the same compounds. Table 11 compares the chemical shifts from the estimated ^{1}H-NMR specter and the ^{1}H-NMR specter from deoxytubastrine.

Table 11: Comparison of the estimated chemical shifts and the actual chemical shifts of the protons bonded to C-atoms of deoxytubastrine.

Estimated ^{1}H-NMR	^{1}H-NMR from deoxytubastrine
6.51 ppm	6.5 ppm
6.90 ppm	7.0 ppm
7.56 ppm	7.5 ppm
7.56 ppm	7.5 ppm
6.65 ppm	6.6 ppm
6.65 ppm	6.6 ppm

5. Conclusion

Since tubastrine has shown antiviral activity earlier[17] it was decided to purify deoxytubastrine for further studies. The difference between tubasterine and deoxytubastrine are the loss of an OH-group in meta position in deoxytubastrine. When running dose-response on the extract (M06039-0-L01), the results showed clearly inhibition on HSV-1. But after the purification the dose-response test on deoxytubastrine showed no antiviral effect at all. The reason for this might be that deoxytubastrine alone does not have any antiviral effect, but might have synergetic effects with other compounds in fraction number 3, which gives it the antiviral effect. This synergetic effect might have been lost when deoxytubastrine was purified, and because of that the antiviral activity was no longer displayed.

Another reason for the lack of activity might be that we have purified the wrong compound in fraction 3, meaning that there actually is some other compounds in the fraction that has the antiviral activity. One possibility is to go back to fraction 3 and try to purify on other compounds to see if they have any antiviral activity.

Many different factors can influence the production of secondary metabolites from marine organisms. Geographical and environmental factors and seasonal variation could play an important role in producing secondary metabolites with pharmacological effects.

Since tubastrine has shown anti viral activity and deoxytubastrine did not, a theory could be whether it actually is the lost OH-group in deoxytubastrine that causes the antiviral activity. To confirm this conclusion further research is necessary.

6. Appendix

Table 12 : Conditions of the LC-MS ESI-Q

Conditions	ES +
Extractor (V)	4.00
RF Lens (V)	0.3
LM 1 Resolution	15.0
HM 1 Resolution	15.0
Ion Energy 1	1.0
Entrance	0
Exit	0
LM 2 Resolution	14.5
HM 2 Resolution	14.5
Ion Energy 2	1.0
Multiplier	600
Gas cell pirani Pressure (mbar)	1.65e-3

7. References

1. Koehn, F.E. and G.T. Carter, *The evolving role of natural products in drug discovery.* Nat Rev Drug Discov, 2005. **4**(3): p. 206-220.
2. C.Battershill, M.J., P.Long, *Marine biodiscovery:new drugs from the ocean depths.* Biologist, 2005. **52**: p. 8.
3. *About Mabcent-SFI*. 2010 01.05.2010]; Available from: http://www0.nfh.uit.no/mabcent/.
4. Ryland, J.S. and P.J. Hayward, *Handbook of the marine fauna of North-West Europe*. 1995, Oxford: Oxford University Press. X, 800 s.
5. Moen, F.E. and E. Svensen, *Dyreliv i havet: nordeuropeisk marin fauna*. 2004, Kristiansund: KOM forl. 608 s.
6. Changyun, W., et al., *Chemical defensive substances of soft corals and gorgonians.* Acta Ecologica Sinica, 2008. **28**(5): p. 2320-2328.
7. Bernheimer, A.W., L.S. Avigad, and K.-S. Kim, *Comparison of metridiolysin from the sea anemone with thiol-activated cytolysins from bacteria.* Toxicon, 1979. **17**(1): p. 69-71, 73-75.
8. Redaksjonen. *Ryggstrengdyr*. 2010 [cited 2010 07.05]; Available from: http://www.snl.no/ryggstrengdyr.
9. Pedersen-Bjergaard, S. and K.E. Rasmussen, *Legemiddelanalyse*. 2004, [Bergen]: Fagbokforl. 503 s.
10. Niessen, W.M.A., *Liquid chromatography-mass spectrometry*. 2006, Boca Raton, Fla.: Taylor & Francis. 608 s.
11. Herbert, C.G. and R.A.W. Johnstone, *Mass spectrometry basics*. 2003, Boca Raton, Fla.: CRC Press. 474 s.
12. Vasskog, T., *Occurrence of selected antidepressants in the Norwegian environment: pharmaceuticals in the environment*. 2008, Section of Medicinal Chemistry, Department of Pharmacy, Faculty of Medicine, University of Tromsø: [Tromsø]. p. 1 b. (flere pag.).
13. Ferrer, I. and E.M. Thurman, *Liquid chromatography time-of-flight mass spectrometry: principles, tools, and applications for accurate mass analysis*. 2009, Hoboken, N.J.: Wiley. XII, 261 s.
14. Guttormsen, K., *Isolering, rensing og karakterisering av antibakterielle-og anticancerforbindelser fra ekstrakter av Cucumaria frondosa (Holothuroidea)*. 2007, K. Guttormsen: Tromsø. p. 56 bl.
15. Mims, C.A., *Medical microbiology*. 2004, Edinburgh: Mosby. X, 660 s.
16. Yasuhara-Bell, J. and Y. Lu, *Marine compounds and their antiviral activities.* Antiviral Research. **86**(3): p. 231-240.
17. T.Higa, R.S.a., *Tubastrine, a new Guanidinostyrene from the Coral Tubastrea aurea.* CHEMISTRY LETTERS, 1987. **16**: p. 2.
18. Shimizu, Y., *Bioactive marine natural products, with emphasis on handling of water-soluble compounds.* J Nat Prod, 1985. **48**(2): p. 223-35.