



Isolation and Characterization of Anti-inflammatory Compounds from Marine Organisms: *Eucratea loricata* and *Echinus esculentus*

Minh-Anh Thuy Do

Master thesis for the degree Master of Pharmacy

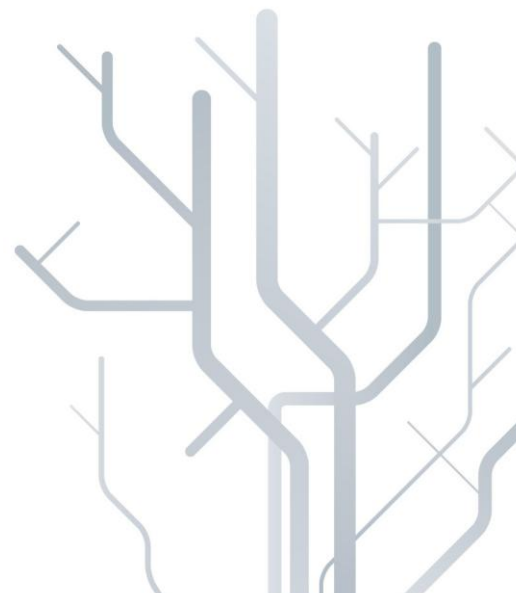
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Supervisors:

Terje Vasskog

Jeanette Hammer Andersen

Espen Hansen



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“Đằng sau sự thành công của con là tình yêu của Ba Mẹ”

My deepest gratitude goes to my Ba, Ma and my sisters, as well as my Bo, Me and my brothers. Last but not least my fiancé Thong Ngo, thank you for everything.

Finally, thanks to all my friends with whom I have shared precious memories with.

Tromsø, May 2012

Minh-Anh Thuy Do

Abstract

It is globally accepted that natural products play a crucial role in drug discovery. In the last decade, the investigation of marine natural products has resulted in a remarkable number of compounds with promising biological activities. Marine natural products have been shown to display antibacterial, antifungal, anticancer, antiviral, antiparasitic, anti-inflammatory activity and several other pharmacological activities of benefit to humankind.

In this project, an investigation of the anti-inflammatory and immunostimulatory activities of extracts from two Arctic marine invertebrate species; a bryozoan, *Eucratea loricata*, and a sea urchin, *Echinus esculentus*, is presented. Anti-inflammatory activity was analyzed by using the monocyte/macrophage cell lines: THP-1 (accurate monocytic leukemia) and U937 (leukemic monocyte lymphoma). Immunostimulatory activity was analyzed by using THP-1 cell line. Effects on cell culture were monitored as reduced NF κ B reporter activity in transfected cell line, and as reduced or increased production of the cytokines TNF- α and IL-1 β .

Bioassay-guided fractionation of the extracts revealed the presence of anti-inflammatory activity in the *Echinus esculentus* extracts. The structure of the target compound was partly elucidated using high resolution mass spectrometry, mass spectrometric fragmentation, and nuclear magnetic resonance spectroscopy.

In addition, this present project provides background information about natural product research and current anti-inflammatory investigations of marine invertebrate species. Furthermore, the potential of Arctic and sub-Arctic marine invertebrates as sources of structurally novel, bioactive agents is demonstrated.

Content

Acknowledgement	i
Abstract	ii
Content	iii
Abbreviation list	v
1 Introduction	1
1.1 MabCent-SFI	1
1.2 Marine bioprospecting	1
1.3 Inflammation	3
1.4 Anti-inflammatory drug discovery	4
1.5 Sea urchin	6
1.6 Bryozoans	7
1.7 Natural products drug discovery	9
1.8 The aim of thesis	12
2 Material and methods	13
2.1 Sampling and storage	13
2.2 Extraction of <i>Eucratea loricata</i>	13
2.3 Preparation of the extracts before HPLC fractionating.....	14
2.4 HPLC fractionation of the aqueous and organic extracts.....	15
2.5 Flash chromatography of the organics extracts.....	16
2.6 Dissolving HPLC and flash fractions for bioactivities screening	17
2.7 Thawing and splitting of U937 and THP-1 cells	18
2.8 Anti-inflammatory assay with U937 cell lines	19
2.9 Anti-inflammatory and immunostimulatory assay with THP-1 cell lines	22
2.10 Dereplication of the active HPLC fraction from the organic extracts of <i>Echinus esculentus</i>	26
2.11 Purification of the target compound from the organic extracts of <i>Echinus esculentus</i>	27
2.12 Structure elucidation of the target compound	28
2.13 Testing of the anti-inflammatory activity of the target compound	29
3 Results	30
3.1 Extraction yield of <i>Eucratea loricata</i> and <i>Echinus esculentus</i>	30
3.2 HPLC fractionation of <i>Eucratea loricata</i> and <i>Echinus esculentus</i>	30
3.3 Flash fractionation of the organic extracts	33
3.4 Anti-inflammatory and immunostimulatory bioscreening	34
3.4.1 Anti-inflammatory screening of the aqueous extract of <i>Eucratea loricata</i>	34
3.4.2 Immunostimulatory screening of the aqueous extract of <i>Eucratea loricata</i>	35
3.4.3 Anti-inflammatory screening of the organic extract of <i>Eucratea loricata</i>	36
3.4.4 Immunostimulatory screening of the organic extract of <i>Eucratea loricata</i>	38

3.4.5	Anti-inflammatory screening of the <i>Echinus esculentus</i> gonad extract	39
3.4.6	Immunostimulatory screening of the <i>Echinus esculentus</i> gonad extract	41
3.4.7	Anti-inflammatory screening of the <i>Echinus esculentus</i> intestines extract	42
3.4.8	Immunostimulatory screening of the <i>Echinus esculentus</i> intestines extract	44
3.5	Dereplication of the active HPLC fraction from the <i>Echinus esculentus</i> gonad extract	45
3.6	Purification of the target compound from the <i>Echinus esculentus</i> gonad extract	46
3.7	Structure elucidation of the target compound	48
3.8	Testing of the anti-inflammatory activity of the target compound	52
4	Discussion	53
5	Conclusion and future perspective	58
	References	59

Abbreviation list

ACN	Acetonitril
amu	atomic mass unit
BPI	Base Peak Intensity
BSA	Bovine Serum Albumin
Da	Dalton
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
g	gravity
gCOSY	gradient COrrrelation SpectroscopY
gHMBCAD	gradient Heteronuclear Multiple-Bond Correlation with ADiabatic pulse
gHSQCAD	gradient Heteronuclear Single-Quantum Correlation with ADiabatic pulse
kV	Kilovolts
HPLC-MS	High Pressure Liquid Chromatography Mass Spectrometry
HR-MS	High-Resolution Mass Spectrometry
LPS 055:B5	Lipopolysaccharides from <i>Escherichia coli</i> 055:B5
MgCl ₂	Magnesium Chloride
MeOH	Methanol
<i>m/z</i>	mass-to-charge ratio
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PMA	Phorbol 12-Myristate 13-Acetate
pNPP	p-NitroPhenyl Phosphate
ppm	parts per million
ROESYAD	Rotating-frame Overhauser Effect SpectroscopY with ADiabatic pulse
rpm	revolutions per minute
RPMI 1640	Rosell Park Memorial Institute medium
THP-1	Human acute monocytic leukemia cell line
TBS	Tris Buffered Saline
ToF-MS	Time-of-Flight Mass Spectrometry
U937	Human leukemic monocyte lymphoma cell line
WET1D	Water Eliminated though Transverse gradients one-Dimensional

1 Introduction

1.1 MabCent-SFI

The MabCent-SFI is a Centre for Research-based Innovation on Marine Bioactives and Drug Discovery. In the MabCent-SFI program, Marbank collects and stores Arctic and sub-Arctic marine organisms for further taxonomy studies and extract production. Bioassay guided purification, screening and identification of bioactive compounds are performed by the analytical platform Marbio. By using high-throughput assays, Marbio screen for novel bioactivities against bacteria, viruses and tumors as well as antioxidant, anti-diabetics, immunostimulants and anti-inflammatory activities.

In the period 2007-2011, MabCent has performed more than 260 000 screening events and identified several “hits”, and some of these hits may become valuable drug “leads”. In cooperation with four commercial partners, MabCent-SFI has identified and characterized the structure of over 40 bioactive molecules, and based on this MabCent has two patents [1].

1.2 Marine bioprospecting

Throughout the ages, natural products have always been the mainstay of disease therapy, and are still considered to play an important role in modern medicine. Almost half of the drugs approved since 1994 are based on natural products. It is well known that plants, microorganisms, marine organisms, vertebrates and invertebrates are important sources where natural product medicines have derived from [2-5].

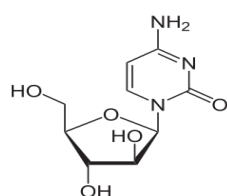
In the last 30 years, the interest in marine bioprospecting has increased among researchers in the whole world. The marine environment differs from land-based ecosystems and offers a great chemical diversity and high biochemical specificity. Relatively low octanol-water partition coefficient, more rotatable bonds and stereogenic centres are some of the chemical properties of small-molecule natural products that make them favorable as lead structures for drugs discovery [6, 7]. Marine organisms are therefore considered as treasures that remain a relatively unexplored source for novel bioactive compounds that could eventually be developed into therapeutics.

However, as a consequence of the complex molecular structures of natural products, pharmaceutical companies have lately shifted to using synthetic chemical libraries. Clinical trials for new natural therapeutic products was reduced with 30 % between 2001-2008 [8]. In addition to structural complexity, drug discovery from natural products faces many other challenges, such as difficulty in collection process, limited sample quantity and problems associated with purification and identification of active agents. Once identified, the molecules are often complex which may be

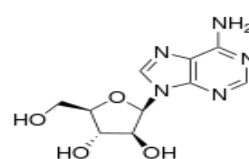
difficult and expensive to produce synthetically. Besides, crude fractions of biological materials are not as amenable to high throughput screening (HTS) as libraries of pure synthetic compounds [6, 9]. Despite this decline, the utility of natural products as sources for novel structures in drug discovery, without a doubt, is still in progress. From around the 1940s to date, of the 175 small molecules related to cancer research, approximately 50 % are actually either natural products or natural product derivatives. The current success rate of drug discovery from the marine environment is approximately 1.7- to 3.3-fold better than the industry average [7, 9, 10].

Currently, the Food and Drug Administration (FDA) in the United States have approved three marine-derived drugs, namely Cytarabine (**1**) (Cytosar-U[®], discovered in sponges), Vidarabine (**2**) (Vira-A[®], discovered in sponges), and Ziconotide (**3**) (Prialt[®], discovered in cone snails). The disease area for these drugs are cancer, antiviral and pain, respectively [11]. In 2007 the European Union approved a marine-derived anticancer drug, Trabectedin (**4**) (Yondelis[®]). In addition, 13 marine-derived compounds are either in phase I, phase II or phase III clinical trials, and several hundred novel marine compounds are in the preclinical pharmaceutical pipeline [11, 12]. As mentioned, marine-derived drugs have shown anticancer and antiviral activities. However, it has also been discovered marine compounds with antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, and antituberculosis effects, in addition to a number of other pharmacological activities which is necessary in treatment and prevention of diseases [11-14].

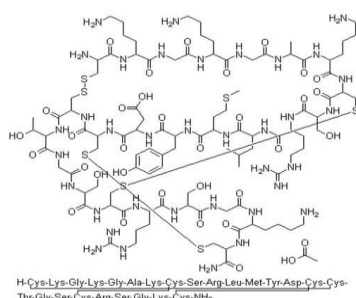
With several agents approved as drugs, and the rich pipeline of agents in clinical trial and preclinical evaluation, the marine environment has performed exceptionally well in yielding new drugs. This suggests that the value of natural products in new drug discovery will continue to be significant in the years to come.



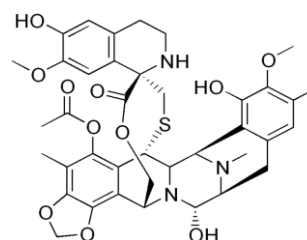
Cytarabine (1)
Cryptotheca crypta



Vidarabine (2)
Tethya crypta



Ziconotide (3)
Conus mangus



Trabectedin (4)
Ecteinascidia turbinata

1.3 Inflammation

Inflammation is a defensive response triggered when the body is threatened by for example pathogens, damaged cells or irritants. These responses are essential for humans in combating infections and for promoting healing and restoration to normal function in the event of injury. Unfortunately, these defensive responses can occasionally go wrong, leading to different inflammatory diseases.

Inflammatory diseases include rheumatoid arthritis, atherosclerosis, Alzheimer's, asthma, psoriasis, multiple sclerosis, and inflammatory bowel diseases, and many of these inflammatory diseases are becoming common throughout the world [15, 16]. According to World Health Organization (WHO) estimates, about 235 million people suffer from asthma and it is the most common chronic disease among children. Approximately 0.3–1.0% of the general population is affected of rheumatoid arthritis. It has been estimated that rheumatoid arthritis affects approximately 1.5 million people in the United States and the prevalence in Norway is estimated to be about 0.5 %. Like other inflammatory diseases, asthma and rheumatic conditions continue to be a large and growing public health problem [17-19].

The three major groups of drugs used in treatment of inflammatory diseases are corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying antirheumatoid drugs (DMARDs), which include biological agents. These drugs are widely used and are effective in treating many inflammatory diseases. Corticosteroids also play a major role in the therapy of organ transplantation because of their anti-inflammatory and immunosuppressive effects. Unfortunately, they are also associated with several serious side effects, while the biological agents are expensive in use. Corticosteroids are well known for causing Cushing's syndrome, in addition to other adverse effects such as hyperglycemia, increased susceptibility to infection, psychiatric disturbances, etc. Long term use of NSAIDs causes gastrointestinal ulceration and bleeding and platelet dysfunction [16, 20].

Inflammation is a complex process involving a multifactorial network of chemical signals to mediate the action. The primary anti-inflammatory targets include cyclo-oxygenase (COX)-1 and 2 enzymes, cytokines such as tumor necrosis factor (TNF)- α and interleukins (IL-1 β , IL-6), and transcription factor as nuclear factor (NF)- κ B and several more. TNF- α and ILs have been identified to play a central role in the pathogenesis of many inflammation diseases, especially asthma and rheumatoid arthritis. TNF- α and ILs are intercellular signal proteins released by immune cells, and have many functions in promotion and resolution of inflammation. The transcription factor NF- κ B is a main regulator of the expression of several genes involved in the activation of inflammation. NF- κ B has been described to have a major role in pathogenesis of inflammatory bowel diseases and also of rheumatic diseases. It is well established that the excessive production of pro-inflammatory mediators is implicated in

several inflammatory diseases. Therefore, inhibition of the overproduction of these mediators is a crucial, exciting target in treatment of these conditions [15, 16, 20].

1.4 Anti-inflammatory drug discovery

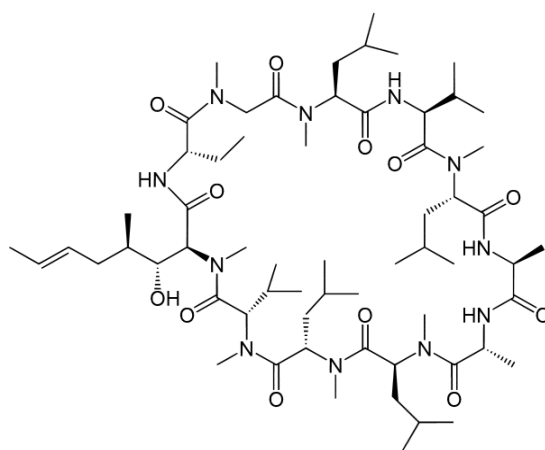
It is now globally accepted that natural products play a dominant role in the discovery of leads for the development of drugs for treating human diseases. Thus, natural products offer great hope in the identification of bioactive compounds and their development into drugs for the treatment of inflammatory diseases.

Cyclosporine (**5**) is a natural product which has been successfully developed as first-line immunosuppressive medicine in treatment of transplant rejection. This compound was discovered by accident in 1972 and isolated from the fungus *Tolypocladium inflatum* from Hardangervidda, Norway. Cyclosporine was approved for use in 1983 and has since been an important medication, apart from in transplants, in the treatment of inflammatory diseases like rheumatoid arthritis and psoriasis. The drug has numerous actions related to immunosuppressive activity, but the main action is a selective inhibitory effect on IL-2 and IL-4 gene transcription [16, 21]. This example highlights the significant role of natural products as a source of drug discovery.

Recently, there have been identified numerous anti-inflammatory pharmacologically active compounds from marine organisms. These compounds have been purified from many different marine sources including sponges, molluscs, bryozoans, sea combs, algae, echinoderms, ascidians and bacteria [13, 15, 22-26]. The Red alga *Gracilaria verrucosa* have possessed anti-inflammatory activities by inhibition of lipopolysaccharide (LPS)-induced nitric oxide (NO) production, TNF- α and IL-6 [27]. Earlier studies have suggested that lipid extract of the blue-green alga *Nostoc commune* repressed expression of pro-inflammatory mediators, such as TNF- α , COX-2, IL-6 and IL-1 β , by inhibiting the activation of NF- κ B pathway in RAW 264.7 macrophages [28]. Astaxanthin, the main carotenoid pigment found in the marine world of algae and aquatic animals, have shown anti-inflammatory properties. Astaxanthin have exhibited anti-inflammatory activities by suppressing the NF- κ B activation. It inhibited the production of pro-inflammatory mediators such as TNF- α and IL-1 β [29]. Hymenialdisine alkaloids isolated from marine sponges, such as *Acanthella aurantica* and *Stylissa massa*, are considered as potential anti-inflammatory agents. Its anti-inflammatory properties achieved through interfering with NF κ B, in addition to the ability to decrease the IL-8 and IL-1 β production [30-32].

Several reviews have been published on natural anti-inflammatory compounds with other particular targets like COX-1 and -2, lipoxygenases (LOXs), nitric oxide (NO), phospholipases A₂ (PLA₂s) pathway etc. These pro-inflammatory mediators have also been considered as potential targets in anti-

inflammatory drug discovery. Many new anti-inflammatory cembranolides have recently been identified from different soft coral *Lobophytum*. These cembranolides inhibited the expression of inducible nitric oxid synthase (iNOS) and COX-2 [13, 33, 34]. Several marine organisms, especially corals, snails and sponges, have shown to inhibit the enzymatic activity of PLA₂ [15, 22]. Although these active compounds are not drugs per se, they have exhibited anti-inflammatory activity through various molecular mechanisms, and they are potential candidates for anti-inflammatory drug development.



Cyclosporine (5)
Tolypocladium inflatum

1.5 Sea urchin



Figure 1: Sea urchins, *Echinus esculentus*. Photo: © S-R. Birkely, MARBANK

There are over 6000 species of the phylum Echinodermata, and the sea urchins are included in this phylum. The body of a sea urchin is spherical, hard-shelled and covered with movable spines. Their normal size is about 3-10 cm in diameter [35]. Sea urchins feed mainly on algae and encrusting animals. Along with four or five gonads, the digestive tract takes up most of the internal space of the sea urchin. The gonads of sea urchins are eaten in several places around the world, including the United Kingdom and Portugal. Sea urchins can be found all over the world in all oceans. Approximately 15 species of sea urchin have been detected along the Norwegian coast [36]. Currently, several publications have suggested that sea urchins are a rich source for bioactive compounds, and in 2008 the first antimicrobial peptides (AMPs) isolated from sea urchins were reported. These were Strongylocins 1 and 2, and were isolated from coelomocyte extracts of the green sea urchins, *Strongylocentrotus droebachiensis*. These sea urchins were collected off the coast of Tromsø, Norway, and displayed potent activities against Gram-positive and Gram-negative bacteria [37]. Similarly, two novel AMPs, named Centrocins 1 and 2, were presented by Li et al. in 2010. These peptides were isolated and characterized from a coelomocyte extract of the same specie, and also showed potent antibacterial activity [38]. In a previous study, done by Haug et al., antibacterial activity was also detected in different body parts of the same species of sea urchins. The highest antibacterial activity was found in the shell and in the coelomocytes [39]. Sheean et al. have detected anti-inflammatory activity in extracts from gonadal tissue of the Australian purple sea

urchin *Heliocidaris erythrogramma*. The extracts have possessed different levels of anti-inflammatory activity by inhibition of COX-1 and 2 [40].

Even though further investigation is required, several studies have already suggested that sea urchins are a potential source for the discovery of novel drugs.

1.6 Bryozoans



Figure 2: The picture shows a colony of *Eucratea loricata*. Photo: ©Robert Andre Johansen, MARBANK

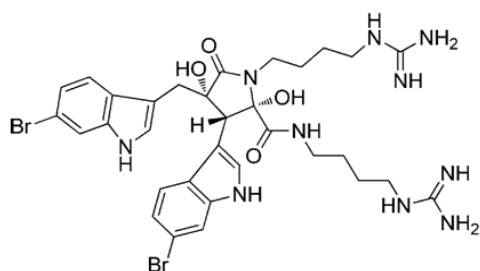
Bryozoans are small (<1 mm), aquatic and benthic invertebrates that live in colonies. These animals have also been called for living fossils; they have existed on earth for about 600 million years and have not changed appreciably over the last 300 million years [35, 36]. The bryozoan body consists of a polyp, protected by a gelatinous or calcareous box. Bryozoans are sessile filter-feeders and food is collected via a circle of ciliated tentacles [35]. Bryozoans are found in both freshwater and marine environments, but no biologically active compounds have been identified from freshwater bryozoans. There have been described more than 8000 species of bryozoans, thereof more than 400 have been verified in Norwegian waters [35, 36]. Due to the diversity, complexity and the sessile nature of the colonies, the bryozoans have been regarded as a promising source for bioactive compounds.

Approximately 200 different compounds from 32 species have already been identified, and it has been found compounds with potential commercial value in 14 of these species [41]. Bryostatin 1, amathaspiramides, alternamides and perfragilin are compounds from bryozoans that have shown cytotoxicity against human cancer cells [41-43]. Among the 13 agents of marine origin currently in

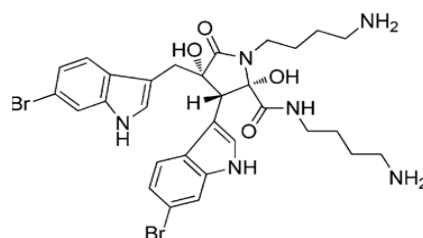
clinical trials, bryostatin 1 is in phase I. Other compounds from bryozoans, convolutamine H 27 and convolutindole A 33, have also shown an inhibitory effect on the development of the larval stage of *Haemonchus contortus*, a type of sheep parasites. This discovery has potential clinical use as the potency of these two compounds are greater than commercially available products [41].

Recently, MabCent in cooperation with other universities has isolated four compounds from the Arctic bryozoan *Tegella cf. spitzbergensis*. The ent-eusynstyelamide B (6) and three new derivatives, eusynstyelamides D (7), E (8) and F (9), all four compounds have revealed antibacterial activities. In addition, eusynstyelamides D and E have also shown weak cytotoxic activity against the human melanoma A 2058 cell line [44].

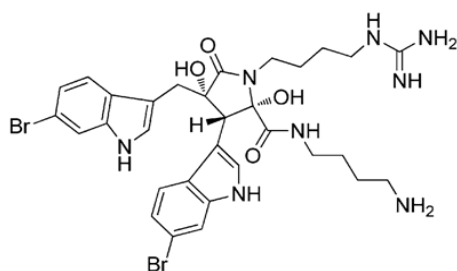
Considering the great diversity of bioactive compounds found in relatively few species of the manifold bryozoan world, searching for pharmacologically active compounds from bryozoans appear to be very promising.



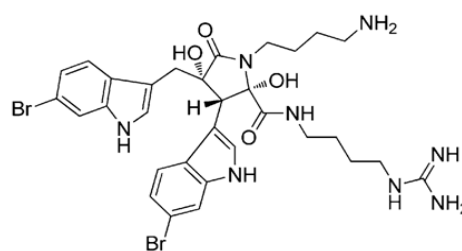
Ent-eusynstyelamide B
(6)



Eusynstyelamide D
(7)



Eusynstyelamide E
(8)



Eusynstyelamide F
(9)

1.7 Natural products drug discovery

There are two main strategies for research in the area of natural products; older- and modern strategy [45]. In the older strategy, the chemistry of compounds is in focus and selection of natural sources is based mainly on ethnopharmacological information as well as traditional uses. Isolation and identification of compounds are performed before biological activity testing (primarily *in vivo*). The modern strategy, so-called bioactivity-guided isolation is, as the name indicates, more focused on bioactivity. Biological assays (mainly *in vitro*) are used to target the isolation of bioactive compounds. Selection of organisms is based on ethnopharmacological information and traditional use, but might also be randomly selected. In modern strategy natural marine sources are particularly utilized.

In this project, the bioassay-guided isolation strategy was applied. Below is a short overview of the processes used in this project (fig. 3). The steps required for the preparation of the material prior to extraction includes collection, taxonomic determination and storage of the samples.

There are various extraction methods available in the field of marine natural products drug discovery. The choice of extraction method depends on the nature of the source material as well as the aim of the isolation. Extraction is a process of obtaining one or more components from a solid material or withdrawal of a solute from a liquid to another liquid. Solid samples are usually cut into small pieces or ground into fine particles to facilitate solvent penetration. Stirring or shaking can be applied to increase the diffusion rate. In this project, the extraction aims at getting as many new compounds as possible to introduce them in further biological assays. Thus, a water extraction and an organic extraction were performed. The water extraction isolates hydrophilic compounds, while the organic extraction is used to isolate lipophilic substances (fig. 3; step 1).

The crude extracts are complex mixture of compounds, and are therefore not suitable for instant bioactivity screening. Thus, the crude extracts need to be fractionated prior to bioactivity screening (fig. 3; step 2). There are several techniques available for fractionation of crude extracts. Here, HPLC and flash chromatography was applied. Fractionation is a procedure used to separate mixtures into fractions, which have compositions consistent with the gradient. HPLC is a high performance method using small particle media, suited to the purification of complex samples where high purity is required. Flash is a useful technique for rapid separation and offers a higher loading capacity than the preparative HPLC, but with a lower resolution. Consequently, samples purified on a flash chromatography system will more than often need a second purification step to obtain a pure compound.

The HPLC and flash fractions were further investigated by biological assays to pinpoint possible active compound(s) (fig. 3; step 3). A bioassay can be defined as the use of a biological system to detect properties, e.g. antibacterial, anticancer, anti-inflammatory etc., of fractions under controlled

conditions. In this project, *in vitro* bioassays were carried out by two cell lines; THP-1 and U937, to identify anti-inflammatory and immunostimulatory properties. Fractions that were considered to be active in the primary screening were re-tested to eliminate the possibility of getting false positives. Once the bioactive fractions were identified after the secondary screening, further analysis was done by high-resolution mass spectrometry (HR-MS). This technique permits characterization of active compounds based on molecular weight and elemental composition. Subsequently, those compounds suspected to provide activity were identified, and the HR-MS data was used for a search query in different databases of natural products to ascertain whether the molecules are previously known, the so-called dereplication process (fig. 3; step 4).

Further purification and isolation of active compounds was achieved by using preparative HPLC-MS (fig. 3; step 5). This technique allows the isolation of compounds based on their mass-to-charge (m/z) ratio.

The next step was structure elucidation of the isolated compounds (fig. 3; step 6). There are different spectroscopic techniques for structure determination of natural products. In this project structure elucidation was carried out by using high resolution mass spectrometry, tandem mass spectrometry (MS/MS) and nuclear magnetic resonance spectroscopy (NMR). MS/MS gives information about fragmentation pattern. Electrospray ionization was used to produce ions from molecules. NMR spectrum provides information about the number of protons and carbons as well as presence of other elements such as nitrogen and bromide, and the binding orientation of these atoms.

With data from HR-MS, MS/MS, and NMR, the molecular structure of active compounds might be determined with quite high reliability.

In this project, the purified compound was further dissolved in DMSO and bioscreened in a dilution series to confirm their activity (fig. 3; step 7).

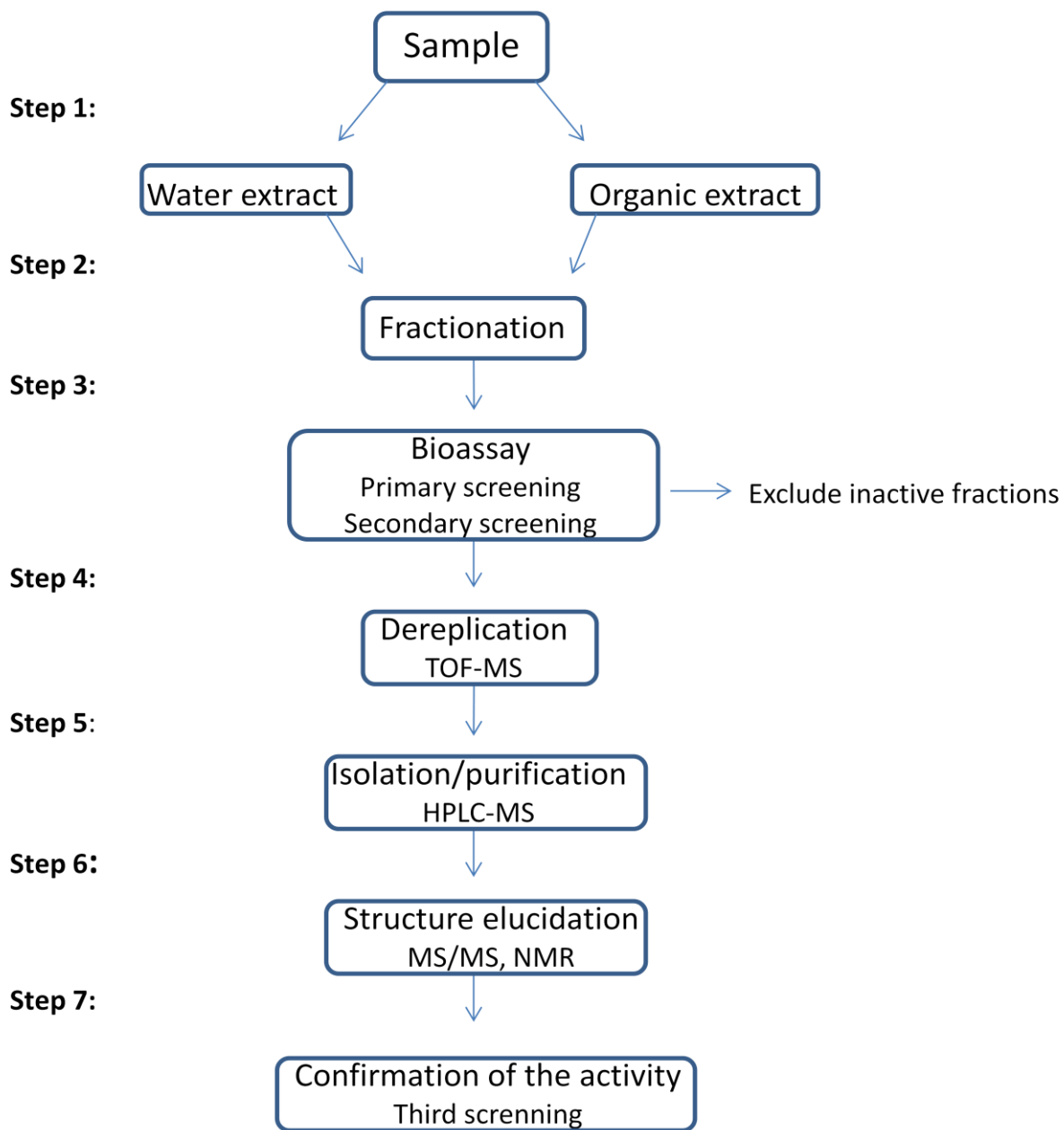


Figure 3: A general overview of the processes involved in natural products drug discovery.

1.8 The aim of thesis

The aim of this project was to investigate extracts from a bryozoan, *Eucratea loricata*, and a sea urchin, *Echinus esculentus* for anti-inflammatory activities. If possible, the molecular structure of the active compounds would be determined using high resolution mass spectrometry, mass spectrometric fragmentation, and nuclear magnetic resonance spectroscopy.

2 Material and methods

2.1 Sampling and storage

The bryozoan, *Eucratea loricata*, identification number M08004, was obtained in January 2007 outside Bear Island (Bjørnøya) located in the western part of the Barents Sea. It was found at 30 m depth. The biomass sample was stored at -22 °C in the dark.

The Sea urchin, *Echinus esculentus*, identification number M10027, was collected in May 2010, at 56 m depth, outside Hammerfest, Bondøya in Finnmark. The organism was stored at -22 °C in the dark. Further, different body parts were extracted separately, and the extracts were stored at -22 °C in the dark. In this project, extracts M10027-1; gonad, and M10027-2; intestines, were examined.

2.2 Extraction of *Eucratea loricata*

Materials used:

DCM (99.8 %, Merck, Darmstadt, Germany)

MeOH (99.9 %, Sigma-Aldrich, St.Louis, MO, USA)

MilliQ water (Millipore, Billerica, MA, USA)

Centrifuge: Heraeus Multifuge 3 S-R (Hanau, Germany)

Freeze-drier: Heto Power Dry PL9000 (Thermo Fisher Scientific, Waltman, MA, USA)

A11 Basic grinder (IKA Works, Staufen, Germany)

Rotary evaporator: Heidolph Laborata 4002 (Nürnberg, Germany)

Scale: Mettler Toledo PB3002-S Fact (Greifensee, Switzerland)

Whatman filter paper 125 Ø (no. 3) (Springfield Mill, England)

Preparation of the samples:

The material was cut into approximately 1 cm³ pieces while still frozen, then transferred to Pyrex dishes, covered with perforated aluminum foil and freeze dried for 2 days.

Aqueous extraction:

The freeze-dried material was pulverized and transferred to tara 1 L Duran bottles. MilliQ water was added, approximately ten times the dry weight of the material (a total of 1.7 L water), and shaken until the suspension got a slurry consistency. The suspension was extracted at 5 °C for 20 hours. Extraction was performed at 5 °C to minimize the risk of bacterial decomposition of the compounds of interest and to maintain the sample stability.

The suspension was centrifuged at 3400 g, 5 °C for 30 minutes. The water supernatant was collected and the pellet re-extracted with MilliQ water (a total of 1.0 L) for 30 minutes. After centrifugation, the supernatant was collected and stored at 5 °C for 20 hours. The pellet was transferred to Pyrex dishes and stored at -22 °C for the organic extraction.

The supernatant was transferred to 1 L round flasks (approximately 200 mL per flask) and stored at -22 °C. The flasks were turned every 30 minutes during freezing to get as large surface area of the ice as possible. This was done to reduce the time of freeze-drying. The supernatant was then freeze-dried for 3 days, before the material was grinded with mortar and pestle, transferred to 50 mL centrifuge tubes and stored at -22 °C in the dark.

Organic extraction:

The freeze-dried pellet was grinded and collected in tared 1 L Duran bottles, (about 30-40 g powder per bottle). The material was then extracted with the solvent mixture DCM-MeOH, (1:1, v/v). Approximately 10x of the material's weight of the extractive solvent was added (1.4 L), until the suspension got a slurry consistency. The suspension was extracted at 5 °C for 20 hours before it was vacuum filtered with Whatman no. 3 filters. The extraction was repeated one more time with DCM-MeOH solvent (0.6 L), for 30 minutes and then filtered. In total 2.0 L of DCM-MeOH was used in the organic extraction.

The filtrate was then concentrated to 10-20 mL using a rotavapor before transferred to 13 mm test tubes and stored at -22 °C in the dark.

2.3 Preparation of the extracts before HPLC fractionating

Material used:

ACN (99.9 %, Merck)

EtOH (99.9 %, Sigma-Aldrich)

Hexane (97.0 %, Sigma-Aldrich)

MilliQ water (Millipore)

Centrifuge: Biofuge Pico (Heraeus)

Centrifuge: Heraeus Multifuge 3 S-R

Millex GS filter 0.22 µm (Millipore)

Minishaker: VWR international, Radnor

Rotary evaporator: Heidolph Laborata 4002

Shaking apparatus: Edmund Bühler GmbH VWR international SM 30 (Germany)

Preparation of the aqueous extract:

200 mg of the extract was transferred to 13 mL centrifuge tubes and 2 mL MilliQ water was added. The tubes were placed horizontally on the shaking apparatus, and shaken for 90 minutes at 160 rpm/minute. Afterwards, 1 mL of the mixture was mixed with 4 mL 96% EtOH, the mixture shaken on the minishaker and then stored at -22 °C for 20 hours.

Next day the mixture was placed in room temperature (approximately 23 °C) for 5 minutes before shaken on the minishaker, and centrifuged at 3400 g, at 5 °C for 30 minutes. The supernatant was collected and evaporated with a rotavapor down to less than 2.0 mL. The volume was then distributed to two eppendorf tubes and filled up to 1 mL with MilliQ water, before centrifuged on the centrifuge Biofuge Pico at 16200 g, 5 °C, for 30 minutes. The supernatant was filtrated with 0.22 µm Millex GC filter. The filtrate was collected in two 12 mm HPLC tubes, and filled up with MilliQ water to 1 mL.

Preparation of the organic extract:

Approximately 300 mg of the extract was dissolved in 3 mL hexane and shaken 4 times on the minishaker with a 1 minute break between the shaking. 3 mL 90 % ACN was added and mixed on the minishaker before the sample was centrifuged at 20 g for 3 minutes. The hexane phase was removed and extracted once more with 3 mL 90 % ACN. The ACN phases were concentrated with a rotavapor until there was less than 1 mL left before the sample was stored at 5 °C for 20 hours.

Next day, the sample was transferred to a 1 mL centrifuge tube and the volume adjusted to 1 mL using 90 % ACN. The sample was centrifuged at 16200 g, at 5 °C, for 30 minutes. The supernatant was transferred to a 12 mm HPLC tube, and the volume adjusted to 1 mL with 90 % ACN.

2.4 HPLC fractionation of the aqueous and organic extracts

Material used:

Column: xTerra prep RP18, 10*300 mm, particle size 10 µm (Waters, Milford, MA, USA)

Degasser: Waters prep

Detector: Waters 2996 Photodiode Array

Fraction collector: Waters 2767 sample manger

Injector: Waters 600 controller

Pump: Waters 515 HPLC

RVT4104 Refrigerated Trap (Thermo Fisher Scientific)

SC250 Express SpeedVac Concentrator (Thermo Fisher Scientific)

Software: Waters MassLynx 4.1; Waters Openlynx 3.5; Waters fractionLynx 3.5

Mobile phase A: MilliQ water (Millipore) with 0.1 % formic acid (98.0 %, Merck)

Mobile phase B: ACN (99.9 %, Merck) with 0.1 % formic acid (Merck)

900 µl of the sample was injected. A linear gradient was selected and the gradient used for the aqueous extract and organic extracts can be found in table 5 and 6, respectively. In total, 40 fractions were collected for each sample. Each of the 40 fractions was distributed in 4 deep well plates. The plates were vacuum centrifuged for 3 hours to remove organic solvents and then freeze-dried for 24 hours to remove the remaining water.

Table 1: HPLC gradient used for the aqueous extract fractionation

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
(Initial)	6	95	5
2	6	95	5
30	6	50	50
40	6	5	95

Table 2: HPLC gradient used for the organic extracts fractionation

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
(Initial)	6	80	20
2	6	80	20
30	6	0	100
40	6	0	100

2.5 Flash chromatography of the organic extracts

Material used:

Acetone (99.8 %, Sigma-Aldrich)

MeOH (99.9 %, Sigma-Aldrich)

MilliQ water (Millipore)

Flash system: Biotage SP4 (Uppsala, Sweden)

Flash stationary phase: Diaion HP20SS, particle size 75-150 µm (Sigma-Aldrich)

SC250 Express SpeedVac Concentrator (Thermo)

RVT4104 Refrigerated Trap (Thermo)

Preparation of the organic extracts before flash fractionation:

2 g extract was dissolved in hexane (40 mL/g), and subsequently liquid-liquid extracted twice with 40 mL 90 % MeOH. The MeOH phases were collected and concentrated to approximately 2 mL. The sample was then divided in two 13 mm centrifuge tubes, and 1 g diaion HP20SS was added to each tube. Further, the samples were vacuum centrifuged until dryness.

Flash fractionation:

The samples were added to the top of the column with stationary phase (approximately 6 g), and a stepwise gradient was selected and used (table 3). The fractionations were operated with a flowrate of 12mL/min and gave 24 mL in each fraction. This resulted in 27 fractions of each sample. Further, each fraction was concentrated to approximately 1 mL, and transferred to tara 13 mm tubes, before vacuum centrifuged to dryness. The dry mass of each flash fraction was then weighed.

Table 3: Gradient used for the organic extracts flash fractionation

Time (min)	% water	% MeOH	% Acetone	Fraction number
0-6	95	5		1-3
6-12	75	25		4-6
12-18	50	50		7-9
18-24	25	75		10-12
24-36		100		13-18
36-40		50	50	19-20
40-54			100	21-27

2.6 Dissolving HPLC and flash fractions for bioactivities screening

Material used:

DMSO (99.5 %, Sigma-Aldrich)

FBS (Biochrom)

Gensumycin (Biochrom)

Hygromycin B (Sigma-Aldrich)

L-glutamine (Sigma-Aldrich, G7513)

MilliQ water (Millipore)

Penicillin/Streptomycin (Sigma-Aldrich, P4458)

RPMI 1640 (Biochrom, FG1385)

Shaking apparatus: Edmund Bühler GmbH SM 30

Dissolving HPLC fractions:

Assay media for THP-1: RPMI-1640 with 10 µg/mL Gensumycin

Assay media for U937: 49 mL RPMI-1640, 1 mL penicillin/streptomycin, 74 µL hygromycin B, 1 mL FBS and 500 µL L-glutamine

Aqueous fractions: dissolved with 750 µL assay media and shaken for 3 hours.

Organic fractions: dissolved with 7.5 µL DMSO and shaken for 2.5 hours, subsequently, 750 µL assay media was added and the fractions shaken for 30 minutes.

Dissolving flash fractions:

Each flash fraction was dissolved in DMSO to give a concentration of 40 mg/mL. The samples were then shaken for 30 minutes, before 25 µL of each fraction was transferred to a deep well plate, and filled up to 1 mL with MilliQ water to give a concentration 1 mg/mL.

2.7 Thawing and splitting of U937 and THP-1 cells

Material used:

Centrifuge: Heraeus Multifuge 3 S-R (Hanau, Germany)

FBS (Biochrom)

Gensumycin 10mg/mL (Biochrom)

Hygromycin B (Sigma-Aldrich)

Incubator, 37 °C, 5 % CO₂

L-glutamine 200 µM (Sigma-Aldrich)

Penicillin/Streptomycin (Sigma-Aldrich)

RPMI-1640 (Biochrom)

Sodium pyruvat 100 µM (Biochrom)

Table 4: The composition of growth media and assay media in U937 and THP-1 cell lines.

Cell line	U937	THP-1
Growth media	45 mL RPMI-1640, 1 mL penicillin/streptomycin, 75 µL hygromycin B, 500 µL L-glutamine, 5 mL FBS	50 mL RPMI-1640, 50 µL gensumycin, 10 nM sodium pyruvat, 5 mL FBS
Assay media	49 mL RPMI-1640, 1 mL penicillin/streptomycin, 74 µL hygromycin B, 500 µL L-glutamine, 1 mL FBS	RPMI-1640 with 10 µg/mL Gensumycin

A sterile environment must be kept when working with the cells. This was done by aseptic technique and all equipment and reagents must be sterile before use (e.g. by autoclaved or washed with 70 % ethanol). Especially for the THP-1 cell line, media must be endotoxin free and all equipments have to be pyrogen free.

Thawing cells from the nitrogen tank:

A cryotube with cells was taken out from a nitrogen tank and immediately placed into a 37 °C water bath for thawing. The thawed cells were then transferred to a 50 mL centrifuge tube and 10 mL of pre-warmed growth media (37 °C) was added dropwise, slowly at the beginning and then faster, over a period of two minutes. Afterwards the cell suspension was centrifuged at 200 g for 7 minutes. The

supernatant was removed and 10 mL growth media was added. The cell suspension was transferred to a culture flask and incubated at 37 °C, with 5 % CO₂.

Cell splitting:

Before splitting, the cells were inspected in microscope to check for cell density, if the cells appeared to be normal and confluent, then they were split.

The cell culture was transferred to a 50 mL centrifuge tube and centrifuged at 200 g for 7 minutes.

The supernatant was then poured out and the cell pellet was resuspended in new pre-warmed growth media.

For U937: 10 µL cell suspensions were transferred to an eppendorf tube and 90 µL growth media was added, subsequently 10 µL cell dilution was transferred to a counting chamber for cell counting in a light microscope. The U937 cells were split on Monday and Wednesday 1:3 and Friday 1:5 in order to maintain the cell density between 0.1×10^6 and 2.0×10^6 cells/mL.

For THP-1: 0.5 mL cell suspensions were transferred to a falcon tube and 0.5 mL trypan blue was added to count how many cells that were alive. After approximately 1 minute, 10 µL of the mix was transferred to a counting chamber for cell counting. The THP-1 cells were split twice a week with a cell density of approximately $2-3 \times 10^5$ cells/mL.

2.8 Anti-inflammatory assay with U937 cell lines

Material used:

DMSO (99.5 %, Sigma-Aldrich)

EnVision plate reader (Perkin Elmer)

FBS (Biochrom)

LPS 055:B5 (Sigma-Aldrich, L2880)

One-Glo Luciferase assay buffer (Promega, E605)

Assay media (table 4)

Growth media (table 4)

Negative control: assay media with 0.1 % DMSO

Positive control: assay media with 1 µg/mL LPS

On the experiment day, approximately 40 000 cells/well were seeded (8×10^5 cells/mL) in 50 µL assay media. The plates were stored at 37 °C, with 5 % CO₂ for 3 hours. After incubation, the cells were inspected in microscope to check that they were alive and evenly distributed to all the wells, before

25 μ L HPLC or 5 μ L flash fraction and negative control were added (an overview of the complete process is shown in figure 4, while the plate setup is shown in figure 5).

After 30 minutes incubation, assay media with 1 μ g/mL LPS (positive control) was added to every well except the negative control wells. The plates were incubated over night at 37 °C, with 5 % CO₂. Next day, 30 μ L OneGlo-substrate was added to every well. The plates were then shaken at 400 rpm for 3-5 minutes in the dark.

The plates were analyzed by monitoring NF κ B activity in the cells by measuring relative light units (RLU) using an EnVision plate reader. Percentage activity was calculated by using negative controls and positive controls, which were included within every plate.

MabCent have set different cut-off values for active and inactive fractions. Fractions were defined as active when NF κ B activity was <50 %, questionable when fractions showed 50-75 % activity and inactive if NF κ B activity was >75 %, compared to the LPS-treated cells.

Active fractions were then identified and subsequently retested in a dilution series. The secondary screening was performed in the same manner as the primary screening, but with difference in plate setup and fraction concentration. Figure 6 and 7 show plate setup for HPLC and Flash fractions, respectively. HPLC fractions were tested undiluted, diluted 1:2 and 1:4. Flash fractions were tested at the concentrations 50, 25, 10 and 1 μ g/mL. All fractions were tested in duplicates.

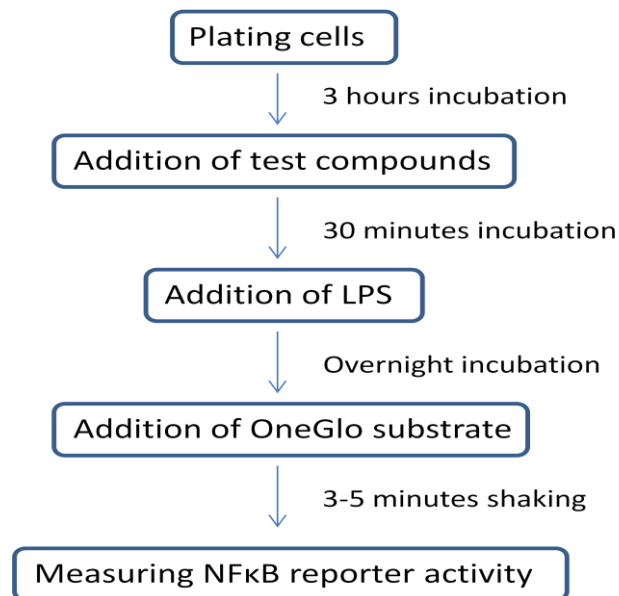


Figure 4: Overview of the anti-inflammatory NF κ B reporter assay in U937 cell lines

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		17	18	19	20	21	22	23	24	P	P	
C		17	18	19	20	21	22	23	24	P	P	
D		9	10	11	12	13	14	14	16	P	P	
E		9	10	11	12	13	14	14	16	N	N	
F		1	2	3	4	5	6	7	8	N	N	
G		1	2	3	4	5	6	7	8	N	N	
H												

Figure 5: Placement of fractions (1-24) in a microtiter plate in primary anti-inflammatory screening with U937 cell line. P: positive control, N: negative control.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		A(u)	A(u)	C(u)	C(u)	E(u)	E(u)	G(u)	G(u)	P	P	
C		A(1:2)	A(1:2)	C(1:2)	C(1:2)	E(1:2)	E(1:2)	G(1:2)	G(1:2)	P	P	
D		A(1:4)	A(1:4)	C(1:4)	C(1:4)	E(1:4)	E(1:4)	G(1:4)	G(1:4)	P	P	
E		B(u)	B(u)	D(u)	D(u)	F(u)	F(u)	H(u)	H(u)	N	N	
F		B(1:2)	B(1:2)	D(1:2)	D(1:2)	F(1:2)	F(1:2)	H(1:2)	H(1:2)	N	N	
G		B(1:4)	B(1:4)	D(1:4)	D(1:4)	F(1:4)	F(1:4)	H(1:4)	H(1:4)	N	N	
H												

Figure 6: Placement of HPLC fractions in a microtiter plate in secondary anti-inflammatory screening with U937 cell line. P: positive control, N: negative control, u: undiluted, diluted 1:2 and 1:4.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		A50	A25	A10	A1	D50	D25	D10	D1	P	P	
C		A50	A25	A10	A1	D50	D25	D10	D1	P	P	
D		B50	B25	B10	B1	E50	E25	E10	E1	P	P	
E		B50	B25	B10	B1	E50	E25	E10	E1	N	N	
F		C50	C25	C10	C1	F50	F25	F10	F1	N	N	
G		C50	C25	C10	C1	F50	F25	F10	F1	N	N	
H												

Figure 7: Placement of Flash fractions (with concentration: 50, 25, 10 and 1 µg/mL) in a microtiter plate in secondary anti-inflammatory screening with U937 cell line. P: positive control, N: negative control.

2.9 Anti-inflammatory and immunostimulatory assay with THP-1 cell lines

Material used:

Anti-Human IL-1 β Purified (eBioscience)
Anti-Human TNF- α Purified (eBioscience)
Biotin Anti-Human IL-1 β (eBioscience)
Biotin Anti-Human TNF- α (eBioscience)
BSA (Sigma-Aldrich)
Diethanolamine (99.0 %, Sigma-Aldrich)
Extravidin alkaline phosphatase (Sigma-Aldrich)
LPS 1 mg/mL (Sigma-Aldrich)
MgCl₂ (VWR)
NaCl (VWR)
Nunc Maxisorp 96F-well ELISA plate (VWR)
Dulbecco's PBS (Invitrogen)
PMA, stock solution 1 mg/mL (Sigma-Aldrich)
pNPP substrate (Sigma-Aldrich)
Recombinant human IL-1 β (Biolegend)
Recombinant human TNF- α (Biolegend)
Tris (Sigma-Aldrich)
Tween-20 (VWR)
ELx405 plate washer (Bio-tek)
DTX 880 plate reader (Beckman Coulter)

An overview of the entire procedure (preparation and ELISA assay) is given in figure 8.

Preparation before ELISA:

Day 1: A cell suspension with a concentration of 10⁶ cells/mL was made, followed by the addition of 50ng/mL PMA to make the monocytes differentiated to macrophages. 100 μ L cell suspensions was transferred to each well in a 96-wells microtiter plate, and incubated at 37 °C, with 5 % CO₂ for 48 hours.

Day 3: The cells were inspected in microscope to confirm that the monocytes had differentiated to macrophages and that cells were evenly distributed in every well. Then the old media was removed, the cells were washed with PBS (90 μ L/well), and new growth media was added. The plates were incubated at 37 °C, with 5 % CO₂ for 24 hours.

Day 4: The cell density was checked before the old growth media was removed, and new growth media was added, (90 μ L/well). Following this 10 μ L of the HPLC fraction was added. All fractions were tested in duplicates.

Immunostimulatory assay: 10 μ L of 50 ng/mL LPS solution was added to positive controls. The plates were incubated at 37 °C, with 5 % CO₂ for 6 hours, and then kept at -80 °C until ELISA.

Anti-inflammatory assay: the plates were incubated at 37 °C, with 5 % CO₂ for 1 hour before 10 μ L of 50 ng/mL LPS solution was added to every well except the cell controls (negative controls). The plates were incubated at 37 °C, with 5 % CO₂ for 6 hours, and afterwards stored at -80 °C until ELISA.

Reagent preparation before ELISA:

(All reagents were prepared immediately prior to use)

TBS: 1.21 g Tris and 8.8 g NaCl per liter MilliQ water, pH 7.4

Anti-Human antibodies solution: the antibodies were diluted in 10 nM TBS to give a concentration of 2 μ g/mL

Wash buffer: TBS with 0.05 % Tween-20

Blocking buffer: TBS with 2 % BSA, pH 7.4

Assay diluents: TBS with 1 % BSA, pH 7.4

Biotin Anti-Human IL-1 β solution: Anti-Human IL-1 β biotin dilutes in the assay diluents to give a concentration of 0.5 μ g/mL

Biotin Anti-Human TNF- α solution: Anti-Human TNF- α biotin dilutes in the assay diluents to give a concentration of 3 μ g/mL

Extravidin alkaline phosphatase solution: Extravidin alkaline phosphatase dilutes 1:10000 in the assay diluents

Diethanolamine buffer: 100 mg MgCl₂ and 97 mL diethanolamine per liter MilliQ water, pH 9.8

pNPP substrate solution: 1 mg/mL pNPP substrate in 1 M diethanolamine

One day prior to running ELISA, Nunc Maxisorp 96F-well ELISA plates were coated with 100 μ L antibody solution of "Anti-Human TNF- α Purified" (for TNF- α ELISA) or "Anti-Human IL-1 β Purified" (for IL-1 β ELISA). The plates were incubated overnight at 37 °C, with 5 % CO₂.

ELISA assay:

The sample plates were taken out from the freezer for thawing, while the pre-coated plates were washed with Wash buffer by using “Wash ELISA” program (300 μ L Wash buffer/well, wash 4 times) in the ELx405 plate washer (all subsequent washes were performed similarly). Following this, 200 μ L blocking buffer was added to all wells, and the plates were incubated at room temperature for 1 hour while shaking. The plates were washed before assay diluents were added, subsequently, samples or standards were added. The total volume in each well should be 100 μ L. See figure 9 for plate setup.

For TNF- α ELISA: samples containing LPS were diluted 1:10 while samples without LPS were diluted 1:2. A serial dilution of the 1000 pg/mL top standard was performed within each plate. Thus, the human TNF- α standard concentrations were 500, 250, 125, 62.5, 31.3, 15.6 pg/mL. Assay diluents served as the zero standard (0 pg/mL). In addition, cell controls and LPS controls (positive controls) were also included within every plate.

For IL-1 β ELISA: samples containing LPS were diluted 1:4, samples without LPS were diluted 1:2. Similarly, a serial standard dilution of human IL-1 β was also prepared as previously described in TNF- α ELISA. Cell controls and LPS controls were also included within the plates.

The plates were then incubated at room temperature for 2 hours while shaking. Afterwards, the plates were washed by “Wash ELISA” program before 100 μ L Anti-Human TNF- α Biotin solution (for TNF- α ELISA) and Anti-Human IL-1 β Biotin solution (for IL-1 β ELISA) was added to all wells. The plates were shaken at room temperature for 1 hour followed by washing and addition of 100 μ L Extravidin-alkaline phosphatase solution to all wells. The plates were incubated at room temperature for 30 minutes with shaking before they were washed by “Soak wash ELISA” program (300 μ L Wash buffer/well, soaking for 30 seconds per wash, wash 5 times). Next, 100 μ L pNPP substrate solution was added to all wells, and the plates were incubated at room temperature for 45 minutes.

Subsequently, the absorbance at 405 nm was determined for each well by using DTX 880 plate reader. The amount of TNF- α (for TNF- α ELISA) and IL-1 β (for IL-1 β ELISA) was calculated by using negative and positive controls which were included within each plate and expressed as percent.

In the MabCent anti-inflammatory screening program with THP-1 cells, fractions with >50 % inhibition compared to LPS-treated cells were defined as active. Fractions displaying 40-50 % inhibition were considered to be questionable, while those showing <40 % inhibition were inactive.

In immunostimulatory screening, fractions possessing >10 % stimulation compared to LPS-treated cells were identified as immunostimulatory fractions. Fractions showing <5 % stimulation were defined as inactive, and questionable if displaying 5-10 % stimulation.

Active fractions were identified and retested in a dilution series. Secondary screening follows almost the same method as primary screening. However, the test concentrations used in this case were undiluted and diluted concentration with a ratio of 1:2 and 1:4.

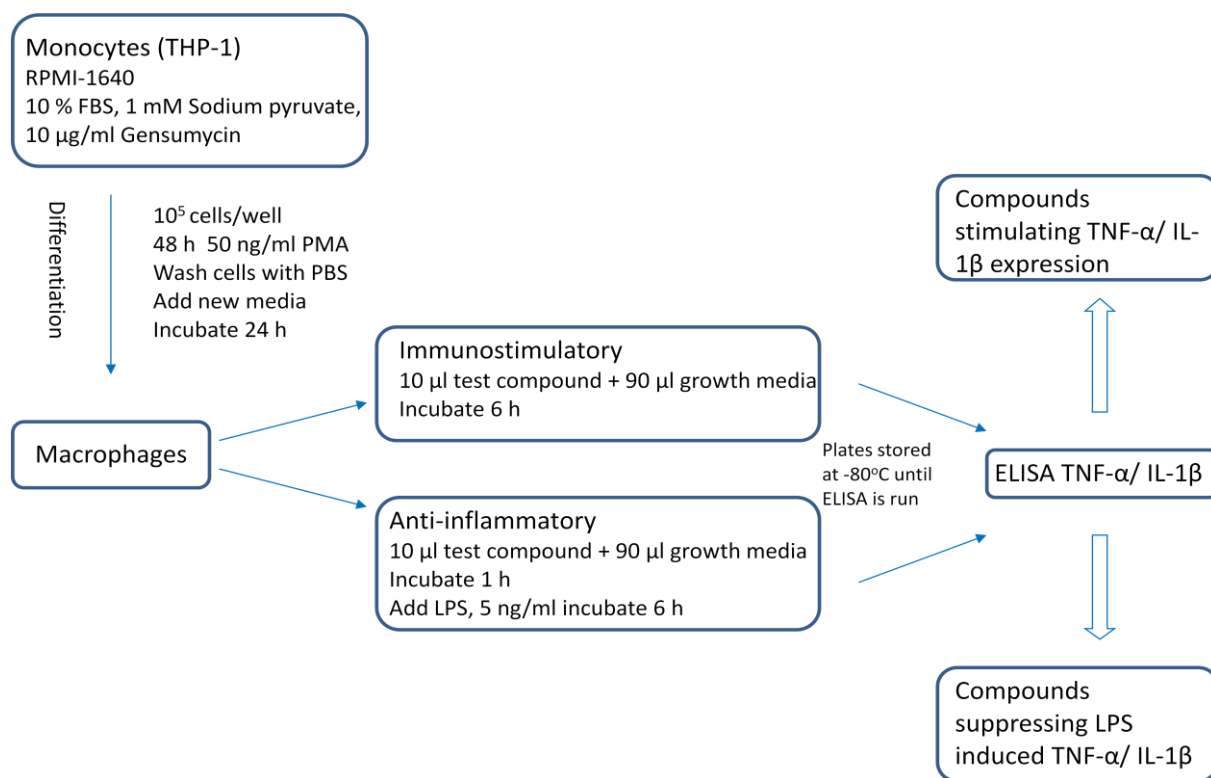


Figure 8: Overview of the anti-inflammatory and immunostimulatory assay TNF- α /IL-1 β with THP-1 cell lines

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	1	5	5	9	9	13	13	17	17	cell controls	Serial standard: 1000-15.6 pg/mL
B	2	2	6	6	10	10	14	14	18	18		
C	3	3	7	7	11	11	15	15	19	19	LPS controls	
D	4	4	8	8	12	12	16	16	20	20		
E	21	21	25	25	29	29	33	33	37	37	Cell controls	
F	22	22	26	26	30	30	34	34	38	38		
G	23	23	27	27	31	31	35	35	39	39	LPS control	
H	24	24	28	28	32	32	36	36	40	40		

Figure 9: Placement of fractions in a 96-well ELISA plate in primary anti-inflammatory screening with THP-1 cell line.

2.10 Dereplication of the active HPLC fraction from the organic extracts of *Echinus esculentus*

Based on the primary and secondary screening, HPLC fraction 27 from the organic extract M10027-1-L01, and HPLC fraction 27 from the organic extract M10027-2-L01 were identified as active.

Unfortunately, the amount of the crude organic extract M10027-2-L01 would not be enough to carry through isolation and the other following processes. Therefore HPLC fraction number 27 was not further investigated. However, HPLC fraction 27 of the organic extract M10027-1-L01 was analyzed by using ToF-MS to identify compound(s) that caused the observed anti-inflammatory activity. The analysis was carried out in both positive and negative electrospray mode.

Material used:

Waters Acquity UPLC

Column: Acquity UPLC® BEH C18, 2.1*50 mm, particle size 1.7 µm

MS: Waters LCT Premier

Software MassLynx 4.1

Mobile phase A: 0.1 % formic acid (Merck formic acid pro analyse) in MilliQ water

Mobile phase B: 0.1 % formic acid (Merck formic acid pro analyse) in ACN (Merck ACN (99.9 %) HPLC grade)

Standard ESI+/- conditions and UPLC gradient used for ToF-MS are listed in table 5 and 6, respectively.

Table 5: ToF-MS instrument parameters used to analyze the accurate mass of the active compound

Polarity	ES +	ES -
Capillary	2.60 kV	2.90 kV
Cone	50 V	50 V
Source temperature	120 °C	120 °C
Desolvation temperature	300 °C	300 °C
Cone gas flow	5 L/h	5 L/h
Desolvation gas flow	500 L/h	650 L/h
Syringe pump flow	5 µL/min	5 µL/min

Table 6: The UPLC linear gradient used to analyze the accurate mass of the active compound

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
(Initial)	0.35	80	20
3.5	0.35	0	100
5.0	0.35	0	100

In addition to the HPLC fraction 27, which displayed activity, the inactive HPLC fractions 25, 26 and 28 were also analyzed. From these ToF-MS data, based on the chromatograms and spectra, a compound with m/z 568.3 was detected in the active fraction, and not in the inactive fractions. Consequently, this compound, with m/z 568.3, was surmised to cause the anti-inflammatory activity in cell screening. Predicting the elemental composition of this compound was achieved by using the MassLynx software.

2.11 Purification of the target compound from the organic extracts of *Echinus esculentus*

Material used:

ACN (99.9 %, Sigma-Aldrich)

Formic acid (98.0 %, Merck)

Hexane (97.0 %, Sigma-Aldrich)

MeOH (99.9 %, Sigma-Aldrich)

Rotary evaporator: Heidolph Laborata 4002

HPLC-MS equipments (see section 2.4)

SC250 Express SpeedVac Concentrator (Thermo)

Preparation of the crude organic extract before purification:

The active fraction was extracted and purified from the crude organic extract of M10027-1-L01. 3.0 g crude extract was extracted twice with 50 mL of 90 % (v/v) ACN and 50 mL hexane. The ACN phase was collected and concentrated to approximately 3 mL using a rotavapor.

Purification of the active fraction:

Isolation of the active compound was carried out by using the same HPLC-MS equipment, software and mobile phases as described in section 2.4. A X-Select CSH prep Fluoro-Phenyl 5 μ m 10*250 mm column (Waters). The injection volume was set to 200 μ L and the HPLC-MS was programmed to collect the compound with a mass-to-charge ratio of 568.3. The gradient used for purification of this compound is shown in table 7. This mobile phase gradient was selected by a “trial-and-error” approach.

Table 7: The mobile phase gradient used for purification of the target fraction

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
(Initial)	6	56	44
20	6	54	46
21	6	0	100
25	6	0	100

Estimation of the purity of the isolated compound:

Fractions containing *m/z* 568.3 from HPLC-MS purification were collected and concentrated down to 1 mL by using speedVac. Furthermore, the sample was dissolved in 50 % (v/v) ACN and analyzed by UPLC-ToF-MS to estimate the purity of the isolated compound. The analysis was made in both positive and negative electrospray mode. The conditions used in ToF-MS are mentioned in table 5 and the used gradient can be seen in table 8. Mobile phase A: MilliQ water with 0.1 % formic acid (98.0 %, Merck). Mobile phase B: ACN (99.9 %, Merck) with 0.1 % formic acid (Merck).

Table 8: UPLC gradient using in UPLC-ToF-MS

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
(Initial)	0.35	90	10
10	0.35	0	100
12	0.35	0	100

2.12 Structure elucidation of the target compound

The structure of the active compound was determined using NMR and tandem quadrupole MS/MS.

Structure elucidation by using NMR:

An Agilent Inova 600 MHz NMR spectrometer equipped with an inverse detection HCN cryprobe was used in the NMR analysis. 1.4 mg of the active compound was dissolved in 500 μ L methanol- d_4 and DMSO- d_6 respectively. For both solvents, 1D 1 H NMR, COSY, HSQC and HMBC were recorded using WET solvent suppression on the residual solvent peaks as well as the water peaks, gradient selection and adiabatic pulses where applicable. All NMR data was acquired at 298 K.

Structure elucidation by using tandem quadrupole MS/MS:

A Waters Xevo TQ-MS was applied for MS analysis of the purified compound. Nitrogen (from generator, Peak Scientific, Glasgow, UK) was used as cone gas and desolvation gas, and Argon 4.0 (Aga, Oslo, Norway) was used as collision gas. The sample was infused directly into the MS with the built-in syringe pump. Cone voltage was optimized in order to get the highest intensity of the protonated molecular ion, and the collision energy was optimized during the infusion to produce an

optimal daughter ion spectra. The data were acquired and processed using MassLynx version 4.1 software.

2.13 Testing of the anti-inflammatory activity of the target compound

0.8 mg of the isolated compound was dissolved in 16 μ L DMSO (99.8 %, Sigma-Aldrich) and 24 μ L MilliQ water to achieve a stock solution with a concentration of 20 mg/mL. A dilution series was prepared from this stock solution in the concentrations 750, 500, 250, 100, 50, and 25 μ g/mL. In the microtiterplate the target compound was diluted by a factor of 10. In this experiment it was taken into account that the THP-1 cells, based on previous experiences, tolerated only up to approximately 0.1 % DMSO. In the presence of higher concentration of DMSO, the viability of THP-1 cell may be affected, consequently false positive might be brought about.

The anti-inflammatory assay was performed as described in section 2.9. In addition to cell controls and LPS controls (positive controls) which were included within the plate, DMSO cell controls and DMSO LPS controls were also included. The DMSO concentration in the cell controls and the LPS controls was 0.15 %. This was done to make sure that DMSO did not cause the activity. All fractions were tested in duplicates.

3 Results

3.1 Extraction yield of *Eucratea loricata* and *Echinus esculentus*

The biomass wet weight of *Eucratea loricata* (M08004) was 1728.4 g, and after freeze-drying the biomass weight was 291.3 g. The biomass weight used for the aqueous extraction was 291.3 g, and the extraction yield 51.4 g. The material weight used for the organic extraction was 227.4 g, and the extraction yield 27.4 g.

The wet-weight of *Echinus esculentus* gonad (M10027-1) was 1038.0 g and the dry-weight was 150.7 g. This dry-weight of material was used for the aqueous extraction and yielded 54.1 g extract. The material weight used for the organic extraction was 80.1 g, and the extraction yield 33.1 g.

The wet-weight of intestines of *Echinus esculentus* (M10027-2) was 1000.0 g and dry-weight was 116.2 g. The weight used for aqueous extraction was 116.2 g and the yield 28.2 g. 78.5 g material was used for organic extraction, and this yielded 6.3 g.

3.2 HPLC fractionation of *Eucratea loricata* and *Echinus esculentus*

The aqueous and organic extracts were fractionated separately. The UV-chromatograms were obtained with wavelengths between 200-600 nm, and positive and negative electrospray data were acquired over an m/z range of 150-1500.

As the UV-chromatogram shows in figure 10, most of the components of the aqueous extract of *Eucratea loricata* (M08004-0-W01) eluted between 2.5-7.0 minutes. In this interval, four larger peaks were detected at 2.76 (the injection peak), 3.95, 5.03 and 6.58 minutes. In the next 33 minutes a smaller peak appears at 10.56 but other than that nothing remarkable was registered.

For the organic extract of *Eucratea loricata* (M08004-0-L01), most of the compounds were detected between the retention times of 8-24 minutes (fig. 11), with an injection peak which eluted at 2.55 minutes. The UV-chromatogram shows many small and indistinct peaks. Both the UV- and the ion chromatograms of the organic extracts indicated that no components eluted after 25 minutes.

The UV-chromatogram of the organic extract *Echinus esculentus* (M10027-1-L01) shows the injection peak at about 2.53 minutes (fig. 12). As the ion chromatograms reveals, the rest of components were detected at a retention time of 15-25 minutes. In this interval, several large and distinct peaks were observed.

Most of the compounds from the organic extract of *Echinus esculentus* (M10027-2-L01) eluted between 10-25 minutes (fig. 13) and during this retention time, several distinct peaks were detected especially in the ES+ chromatogram.

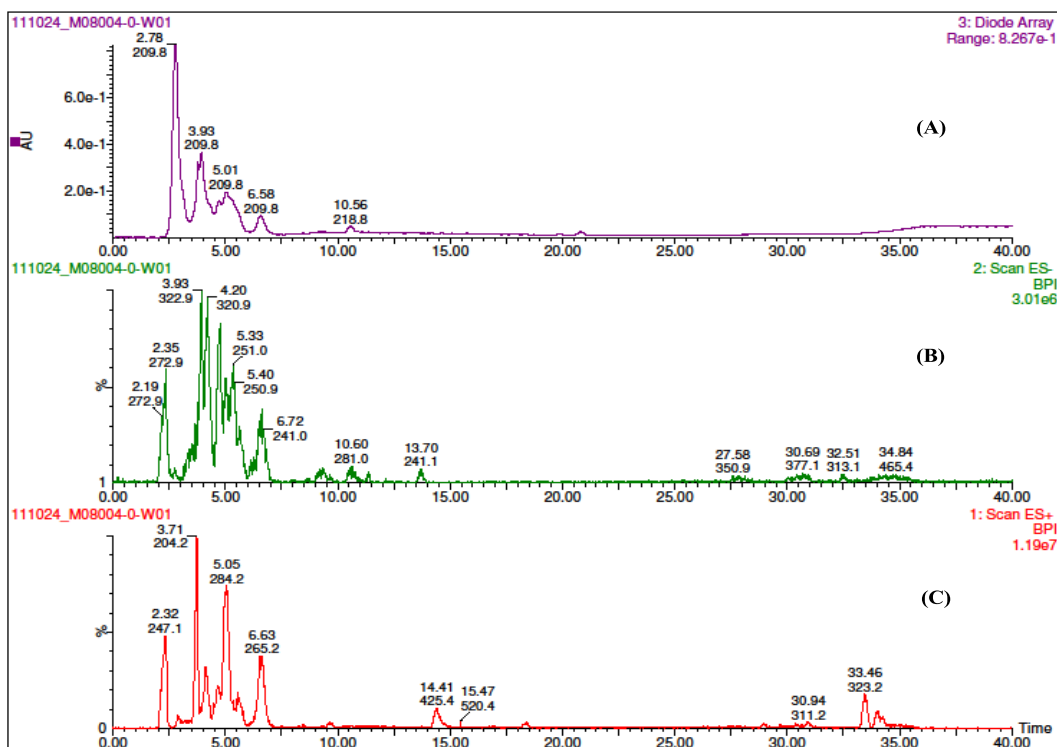


Figure 10: UV- and MS chromatograms of M08004-0-W01. A) UV-chromatogram at absorbance 200-600 nm, and B) negative and C) positive electrospray in the m/z range 150-1500. All the peaks are annotated with retention time (top) and UV-absorbance or m/z (bottom).

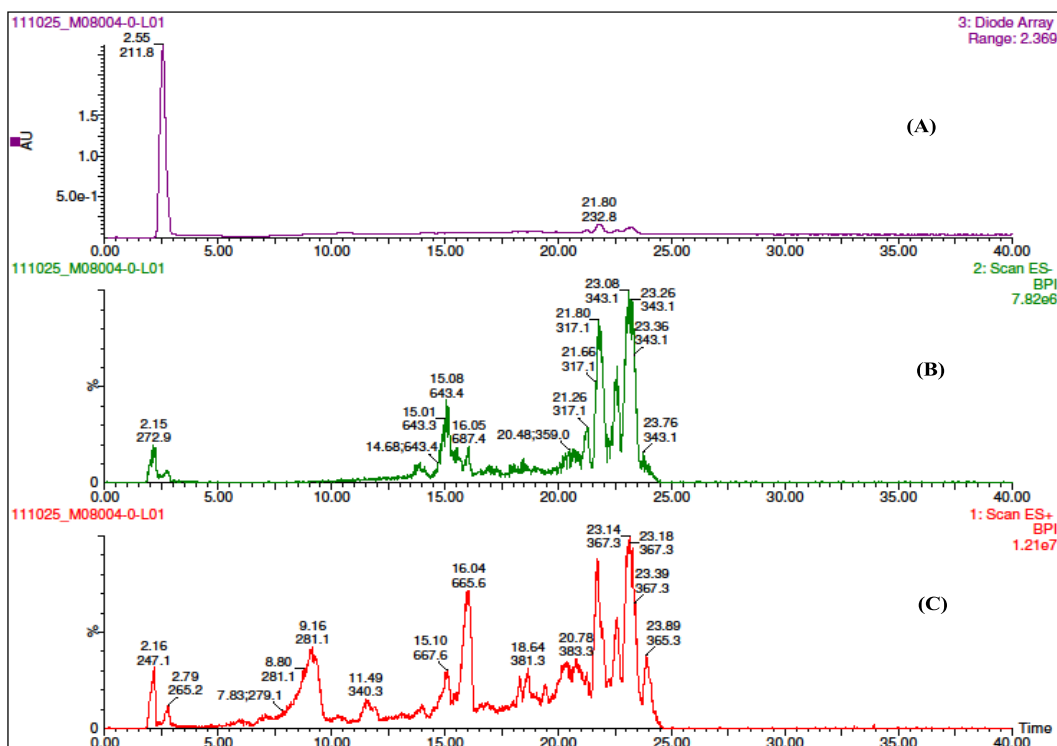


Figure 11: UV- and MS chromatograms of M08004-0-L01. A) UV-chromatogram at absorbance 200-600 nm, and B) negative and C) positive electrospray in the m/z range 150-1500. All the peaks are annotated with retention time (top) and UV-absorbance or m/z (bottom).

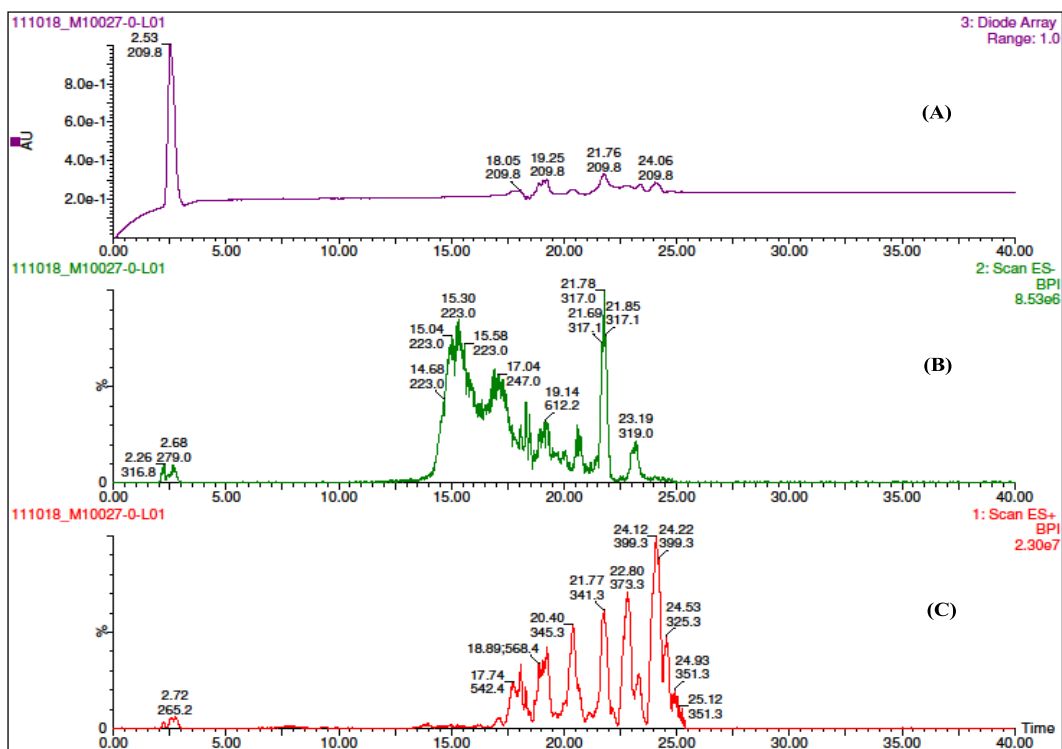


Figure 12: UV- and MS chromatograms of M10027-1-L01. A) UV-chromatogram at absorbance 200-600 nm, and B) negative and C) positive electrospray in the m/z range 150-1500. All the peaks are annotated with retention time (top) and UV-absorbance or m/z (bottom).

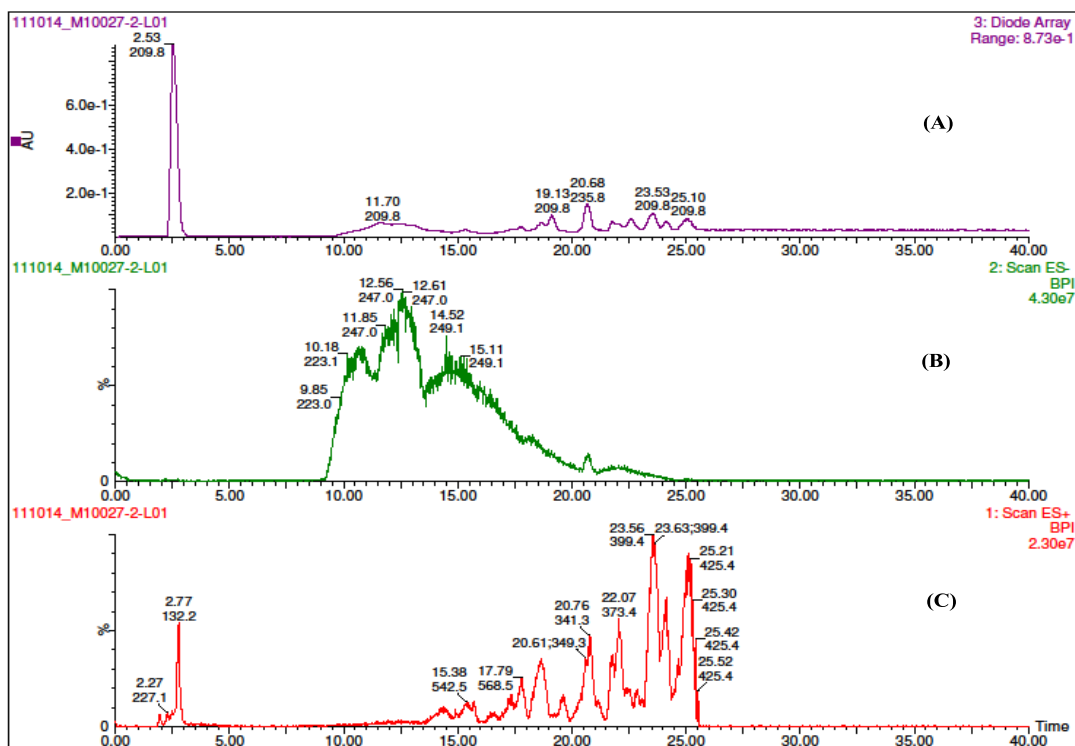


Figure 13: UV- and MS chromatograms of M10027-2-L01. A) UV-chromatogram at absorbance 200-600 nm, and B) negative and C) positive electrospray in the m/z range 150-1500. All the peaks are annotated with retention time (top) and UV-absorbance or m/z (bottom).

3.3 Flash fractionation of the organic extracts

All three organic crude extracts; M08004-0-L01, M10027-1-L01 and M10027-2-L01, were fractionated by flash chromatography. As it shows in table 9, all the three samples contained a high amount of compounds that eluted in the first fraction. Fraction numbers 13-16 contained a relatively high amount of extract compared to fractions 2-12 and 17-27. Sample M10027-1-L01, fraction number 7, differed from the rest in that it contained as much as 170 mg of the extract.

Table 9: List over the weight of the extract of different samples yield after flash fractionation.

	M08004-0-L01	M10027-1-L01	M10027-2-L01
Fraction number	Weight of the extract (mg)	Weight of the extract (mg)	Weight of the extract (mg)
1	119.1	55.2	108.2
2	68.1	4.1	6.2
3	6.3	2.1	5.7
4	4.6	1.4	3.5
5	5.9	2.1	9.8
6	2.9	1.8	5.8
7	4	170.5	8.6
8	7.4	4.4	26.5
9	3	1.7	6
10	2.4	39.8	4.1
11	7.4	17.7	34.5
12	6.5	2.1	3.5
13	50.5	3.3	4
14	149.3	142	151.9
15	40.7	53	79.6
16	16.5	32.4	23.7
17	10.2	15.9	13
18	6.7	8.1	8.6
19	8.3	5.1	6.8
20	17.6	7.8	7.6
21	5.2	0.7	1.4
22	5.2	3.4	2.6
23	2	1.4	1.3
24	1.5	1.3	1.3
25	1.5	1.3	1.6
26	1	1.2	1.1
27	1	1.3	0.9

3.4 Anti-inflammatory and immunostimulatory bioscreening

As described in section 2.8 and 2.9, MabCent have defined different cut-off values for active and inactive fractions. A short overview is listed in table 10.

Table 10: Cut-off value for NFκB, IL-1β and TNF-α in anti-inflammatory screening (AIF), and for IL-1β and TNF-α in immunostimulatory screening (IS)

Cell line	Active	Questionable	Inactive
NFκB in U937, AIF screening	< 50 % activity	50-75% activity	>75% activity
IL-1β, TNF-α in THP-1, AIF screening	>50 % inhibition	40-50 % inhibition	<40 % inhibition
IL-1β, TNF-α in THP-1, IS screening	>10 % stimulation	5-10 % stimulation	<5 % stimulation

3.4.1 Anti-inflammatory screening of the aqueous extract of *Eucratea loricata*

As it shows in figure 14, no HPLC fractions of the aqueous extract of *Eucratea loricata* reduced the NFκB reporter activity.

Fractions 39 and 40 (fig. 15) are shown to have a weak anti-inflammatory activity, with an inhibitory effect on IL- production slightly over 50 % compared to LPS-treated cells. However, these fractions displayed no inhibitory activity on TNF-α production. Nevertheless, fractions 39 and 40 were retested for both IL-1β and TNF-α, but no inhibitory activity was observed (figure not shown).

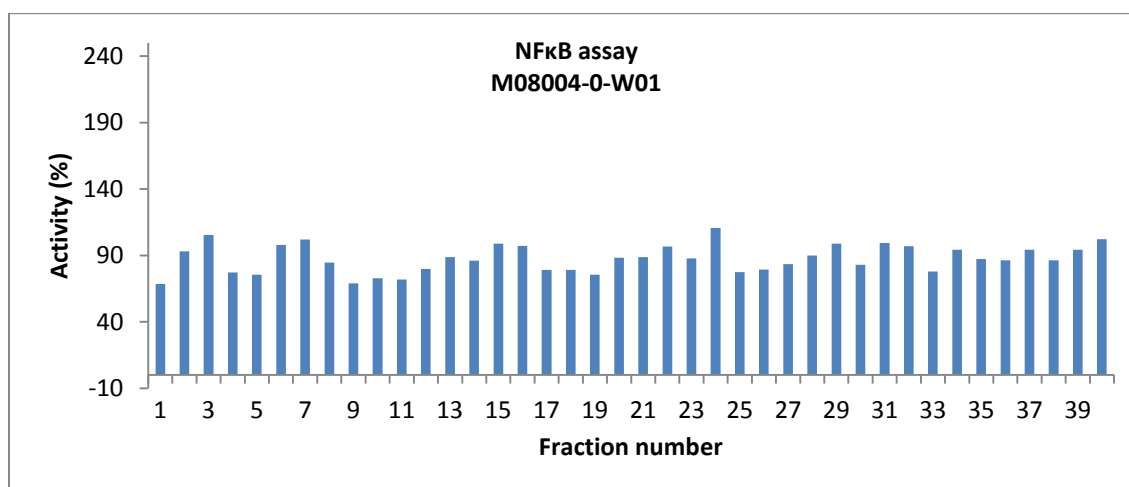


Figure 14: Primary anti-inflammatory screening of 40 different HPLC fractions from the aqueous extracts of *Eucratea loricata* (M08004-0-W01). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the HPLC fractions.

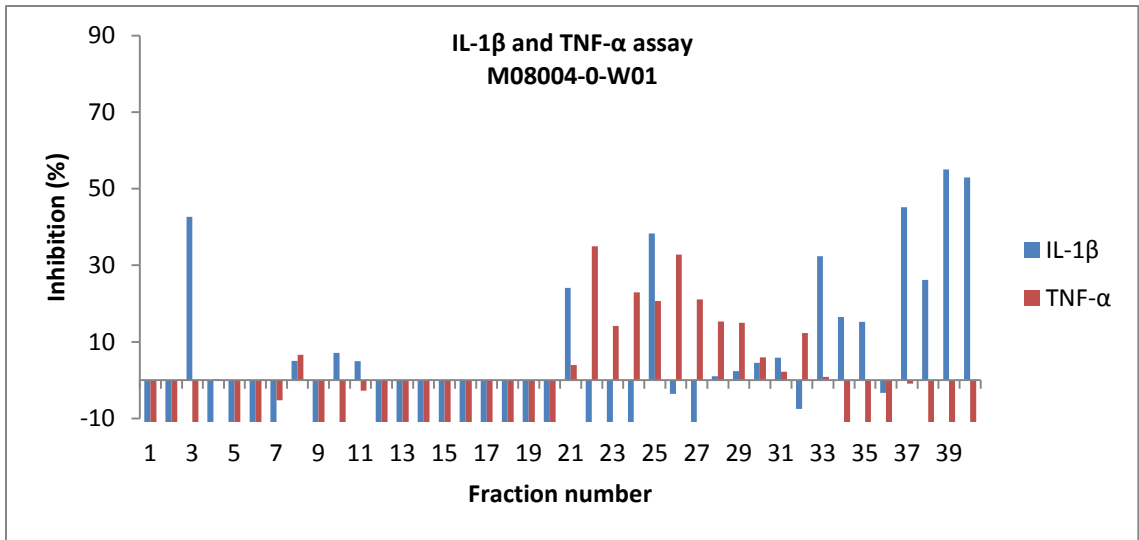


Figure 15: Primary anti-inflammatory screening of 40 different HPLC fractions from the aqueous extracts of *Eucratea loricata* (M08004-0-W01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions.

3.4.2 Immunostimulatory screening of the aqueous extract of *Eucratea loricata*

The aqueous extracts of *Eucratea loricata* did not exert any immunostimulatory effect in the TNF- α assay (fig. 16). In the IL-1 β assay, fraction number 5 possessed the highest immunostimulatory activity with only 6 % stimulation. The activity was considered as questionable, but this was not further investigated.

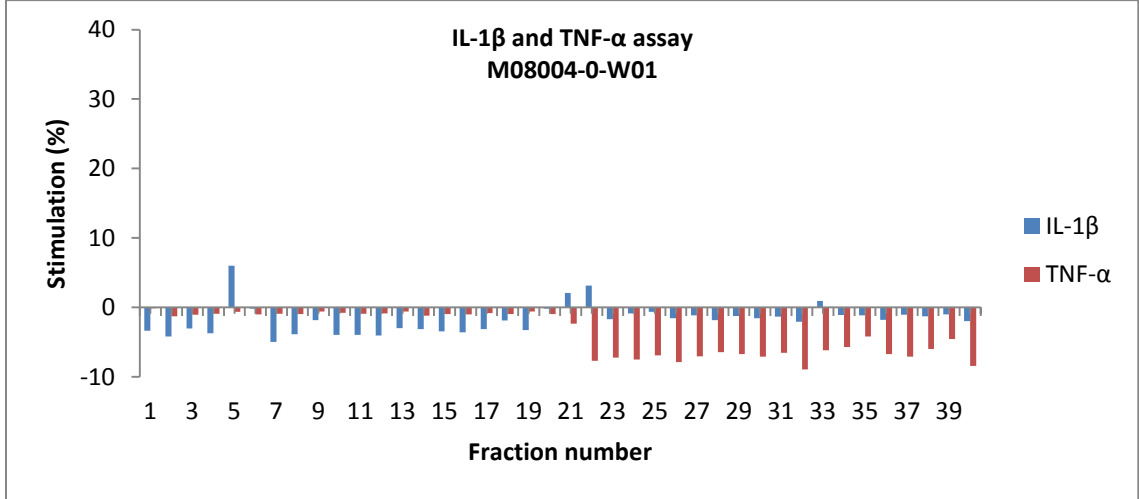


Figure 16: Primary immunostimulatory screening of 40 different HPLC fractions from the aqueous extracts of *Eucratea loricata* (M08004-0-W01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions.

3.4.3 Anti-inflammatory screening of the organic extract of *Eucratea loricata*

The HPLC fractions of the organic extract of *Eucratea loricata* revealed anti-inflammatory activity in NFκB, TNF-α and IL-1β screening. Fractions 19-31 and 33 reduced the NFκB activity with more than 50 % compared to LPS-treated cells (fig. 17). These fractions were identified as active and were retested. In the retesting (results shown in figure 18), fractions 22-28, 30 and 33 retained the anti-inflammatory activity at undiluted concentration, but no anti-inflammatory activity was observed when diluted 1:2 and 1:4.

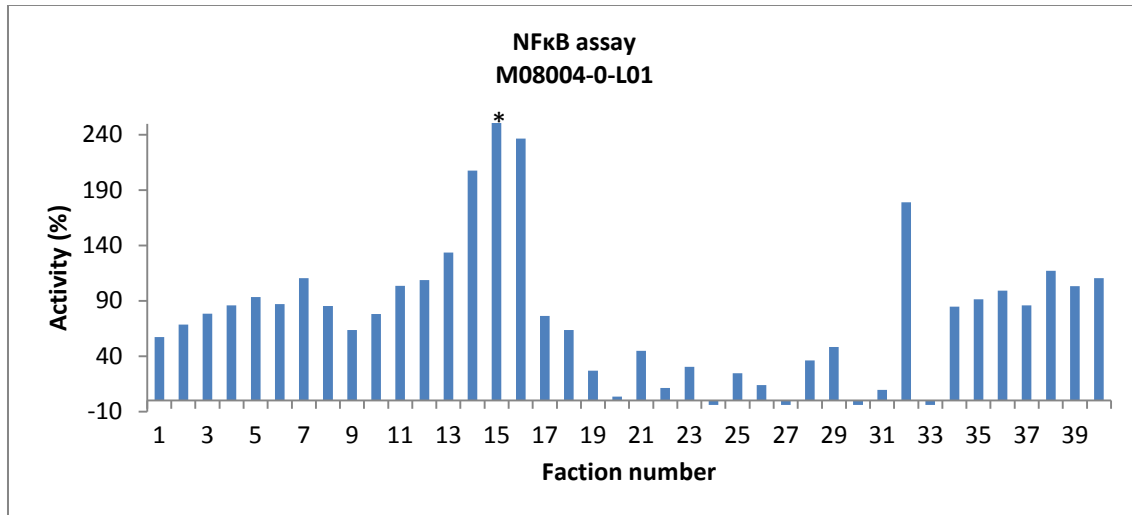


Figure 17: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts of *Eucratea loricata* (M08004-0-L01). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the HPLC fractions. *Activity exceeds 240 % activity.

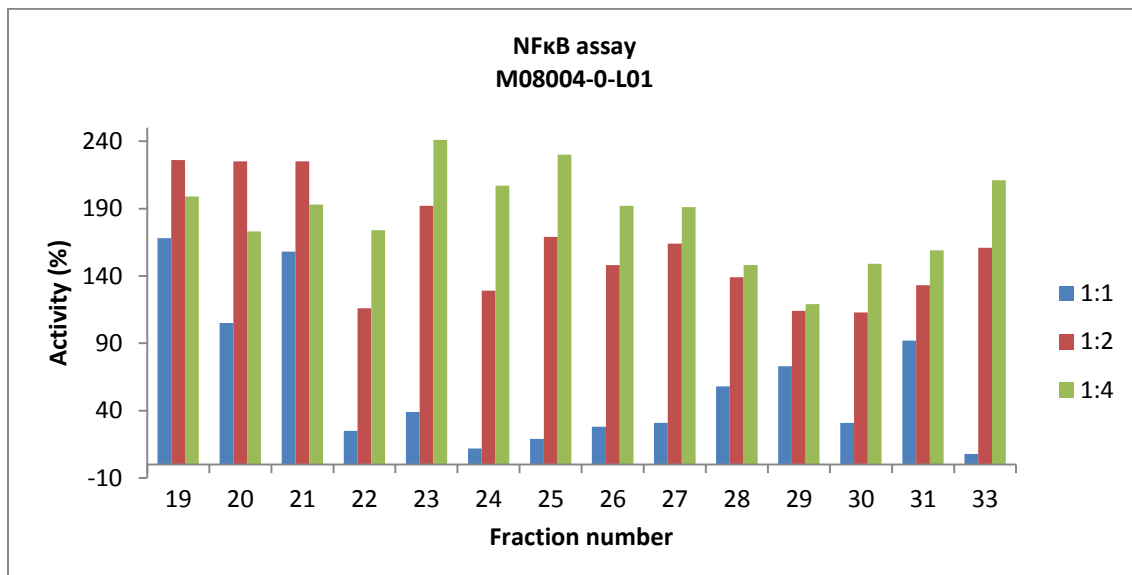


Figure 18: Secondary anti-inflammatory screening of the active HPLC fractions from the organic extracts of *Eucratea loricata* (M08004-0-L01). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

As is shown in figure 19, fraction 25 displayed a weak inhibitory effect on IL-1 β production in THP-1 cells, while fraction 33 revealed an inhibitory effect on TNF- α production. Nevertheless, these two fractions (25 and 33) were retested for both IL-1 β and TNF- α but no inhibitory activity was observed neither at undiluted nor diluted concentrations (figure not shown).

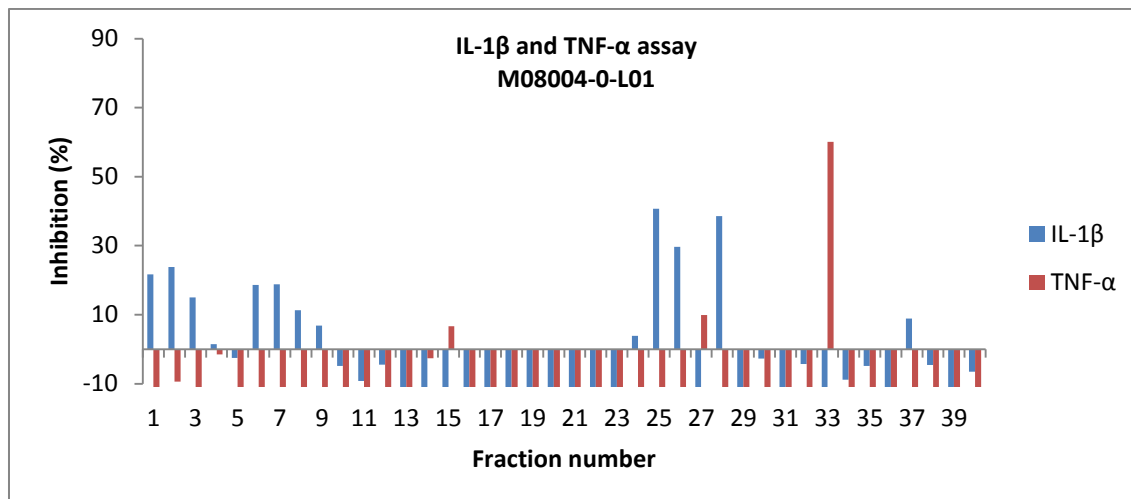


Figure 19: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts of *Eucratea loricata* (M08004-0-L01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions.

In addition to the screening of the HPLC fractions of *Eucratea loricata*, screening of the organic flash fractions of this organism was also conducted. At the concentration of 50 $\mu\text{g}/\text{mL}$, the flash fractions 10-12 of the organic extract of *Eucratea loricata* displayed anti-inflammatory activity in the NF κ B assay in the U937 cell lines (fig. 20). These three fractions were retested in a dilution series (fig. 21). Fractions 11 and 12 still kept the anti-inflammatory activity at 50 $\mu\text{g}/\text{mL}$, and fraction 12 also at 25 $\mu\text{g}/\text{mL}$, but the anti-inflammatory activity disappeared at further dilution.

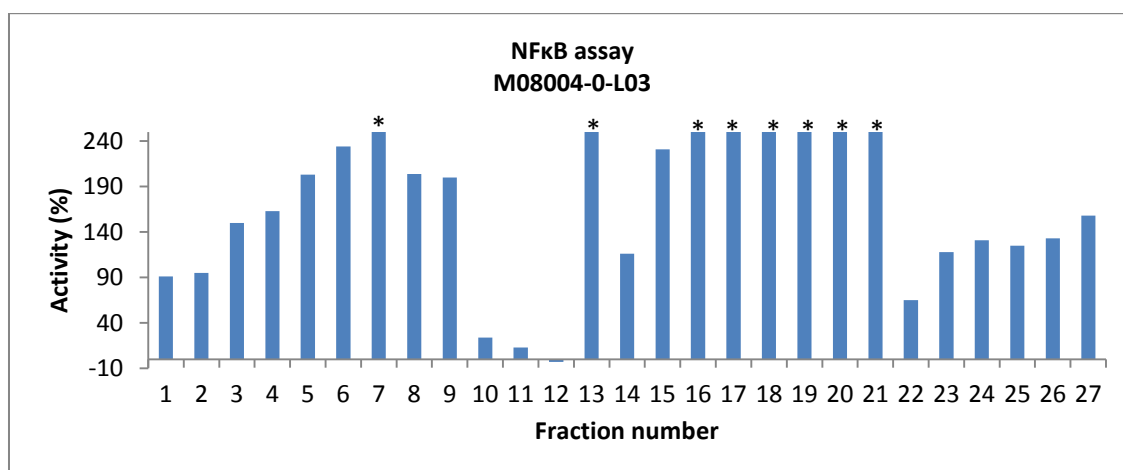


Figure 20: Primary anti-inflammatory screening of 27 different flash fractions from the organic extracts of *Eucratea loricata* (M08004-0-L03). Each column represents the percent of the LPS-induced NF κ B activity in the U937 cells after 24 hours of exposure to the flash fractions. *Activity exceeds 240 % activity.

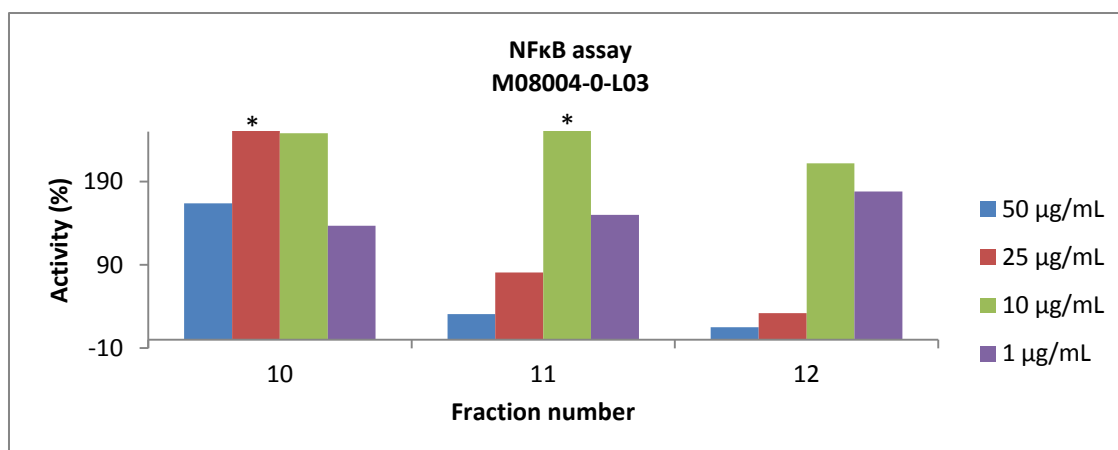


Figure 21: Secondary anti-inflammatory screening of the active flash fractions from the extracts of *Eucratea loricata* (M08004-0-L03). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the flash fractions. *Activity exceeds 240 % activity.

3.4.4 Immunostimulatory screening of the organic extract of *Eucratea loricata*

As figure 22 shows, the organic extracts of *Eucratea loricata* displayed immunostimulatory effect in both IL-1β and TNF-α screening. The fractions 1, 13, 15-17, 19-24, 28 and 29 produced more than 10 % of IL-1β compared to LPS-treated cells. These fractions were considered as active, especially fraction 17, 19, and 29 with a stimulatory effect of 147 %, 55 % and 53 % in IL-1β screening, respectively. Fractions 17, 21, 28 and 29 were also considered as active in the TNF-α screening. There is a higher probability of finding active compounds in those fractions showing activity in both IL-1β and TNF-α screening. Based on this, fractions 17, 21, 28 and 29 were retested.

The results from the secondary screening revealed that only fraction 29, at undiluted concentration, displayed stimulatory effect slightly above 10 % compared to LPS-treated cells in IL-1β screening. The rest did not show any remarkable stimulatory effect at any concentrations in both IL-1β and TNF-α screening (figure not shown).

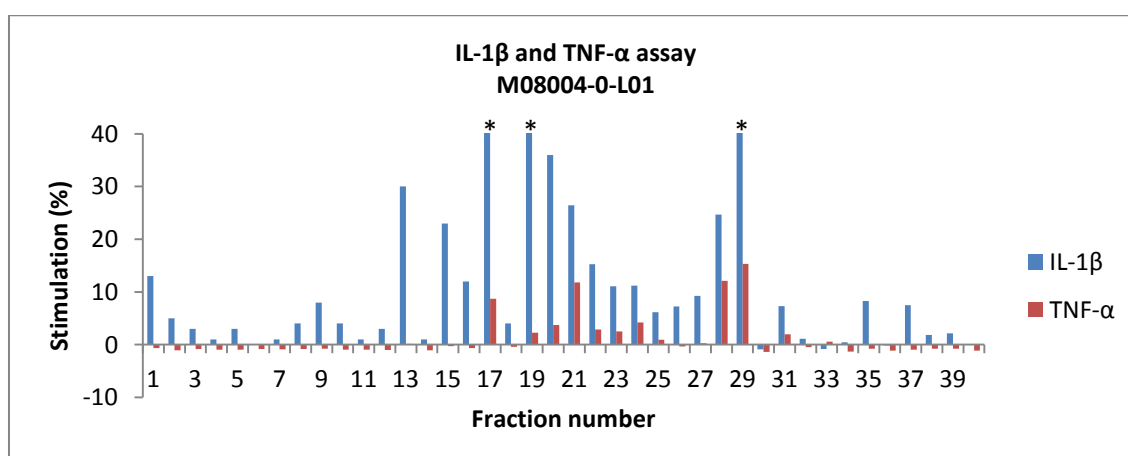


Figure 22: Primary immunostimulatory screening of 40 different HPLC fractions from the organic extracts of *Eucratea loricata* (M08004-0-L01). Each column represents the percent of IL-1β (blue) or TNF-α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions. *Activity exceeds 40 % activity.

3.4.5 Anti-inflammatory screening of the *Echinus esculentus* gonad extract

The HPLC fractions of the organic extract, *Echinus esculentus* (M10027-1-L01), exhibit anti-inflammatory activity on U937 cell lines (fig. 23). In this case fraction 27 possessed strong anti-inflammatory activity. The same fraction also displayed activity in both IL-1 β and TNF- α screening (fig. 24). Since fraction 27 was shown to be potent in NF κ B, IL-1 β and TNF- α assays, there is an increased likelihood of finding active compounds and therefore it was retested.

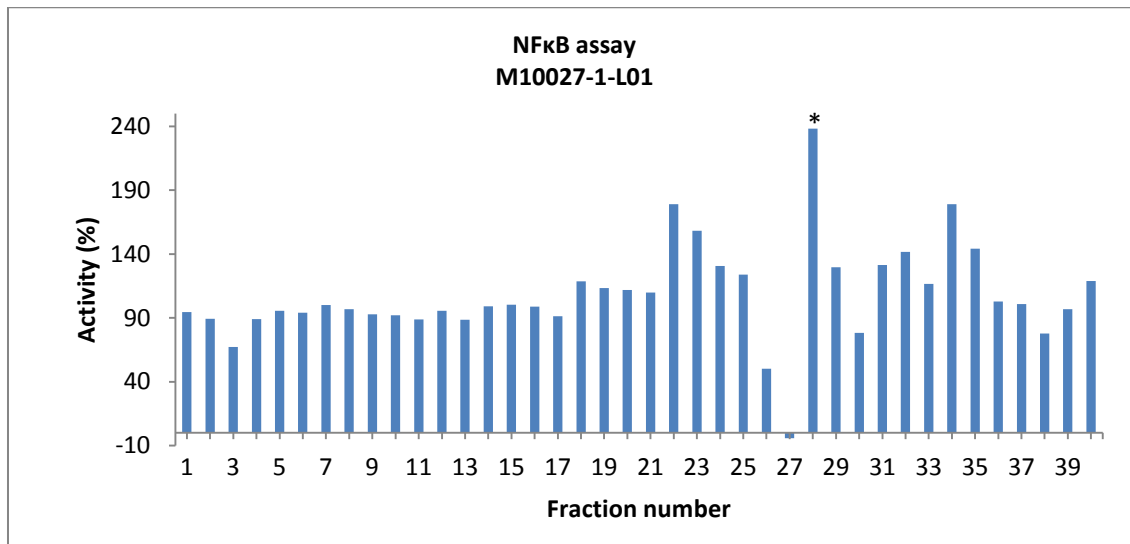


Figure 23: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts *Echinus esculentus* (M10027-1-L01). Each column represents the percent of the LPS-induced NF κ B activity in the U937 cells after 24 hours of exposure to the HPLC fractions. *Activity exceeds 240 % activity.

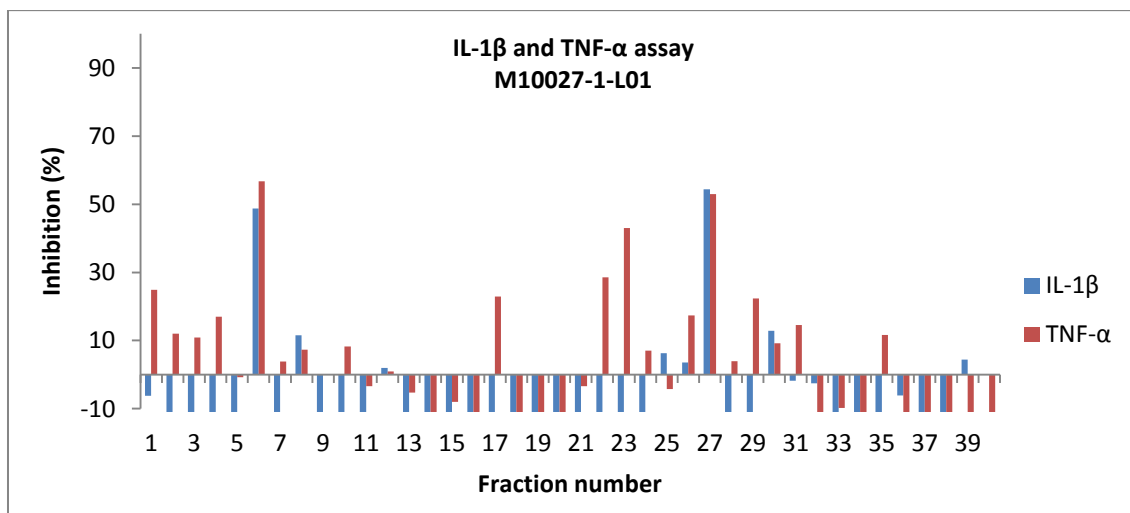


Figure 24: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts of *Echinus esculentus* (M10027-1-L01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions.

The result from the retesting of fraction 27 with U937 revealed no activity (figure not shown). In TNF- α retesting, the inhibitory effect was not high enough to be considered active (fig. 26). The undiluted concentration of fraction 27 retained the anti-inflammatory activity in IL-1 β screening, but this effect was lost in further dilution (fig. 25). Nevertheless, fraction 27 was a candidate for further investigation.

In THP-1 cells, the inhibitory effect of fraction 6 is questionable with a value slightly below 50 % in IL-1 β screening, and 57 % in TNF- α screening (fig. 25 and 26). This fraction was retested, but no anti-inflammatory activity was observed.

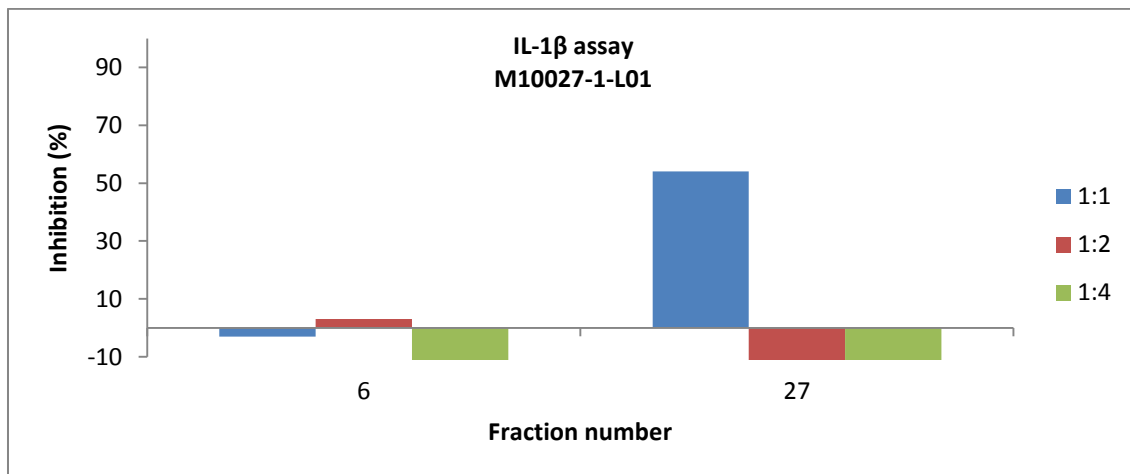


Figure 25: Secondary anti-inflammatory screening of the active HPLC fractions from the extracts of *Echinus esculentus* (M10027-1-L01). Each column represents the percent of IL-1 β production by the THP-1 cells after 7 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

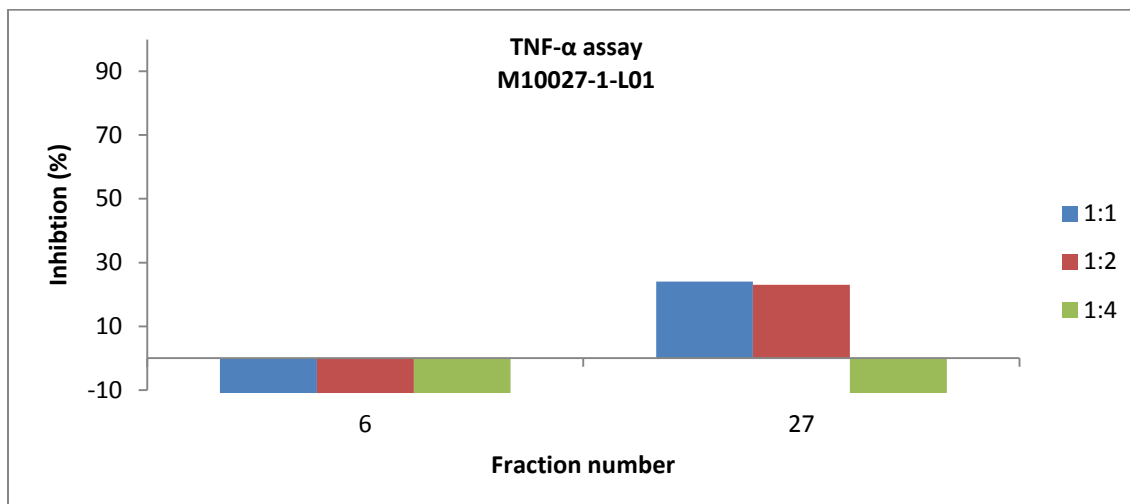


Figure 26: Secondary anti-inflammatory screening of the active HPLC fractions from the extracts of *Echinus esculentus* (M10027-1-L01). Each column represents the percent of TNF- α production by the THP-1 cells after 7 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

In addition to the screening of the HPLC fractions of the *Echinus esculentus* gonad (M10027-1-L01), the flash fractions of this sample were also screened against NFκB activity in U937. At 50 μg/mL, the flash fractions 8, 9, and 11-14 were considered to be active (fig. 27), but in retesting, the anti-inflammatory activity was not observed in any of these fractions (fig. 28).

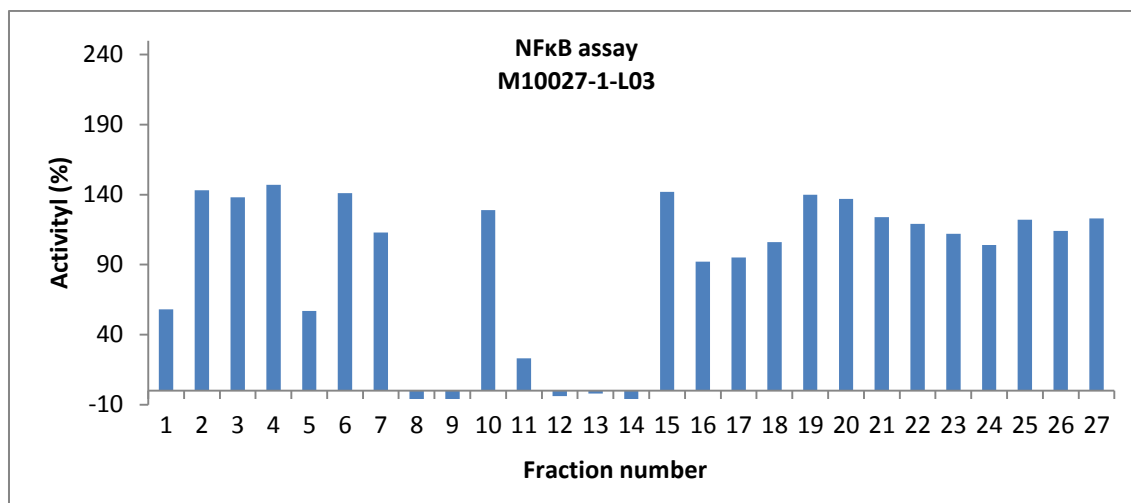


Figure 27: Primary anti-inflammatory screening of 27 different flash fractions from the organic extracts of *Echinus esculentus* (M10027-1-L03). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the flash fractions.

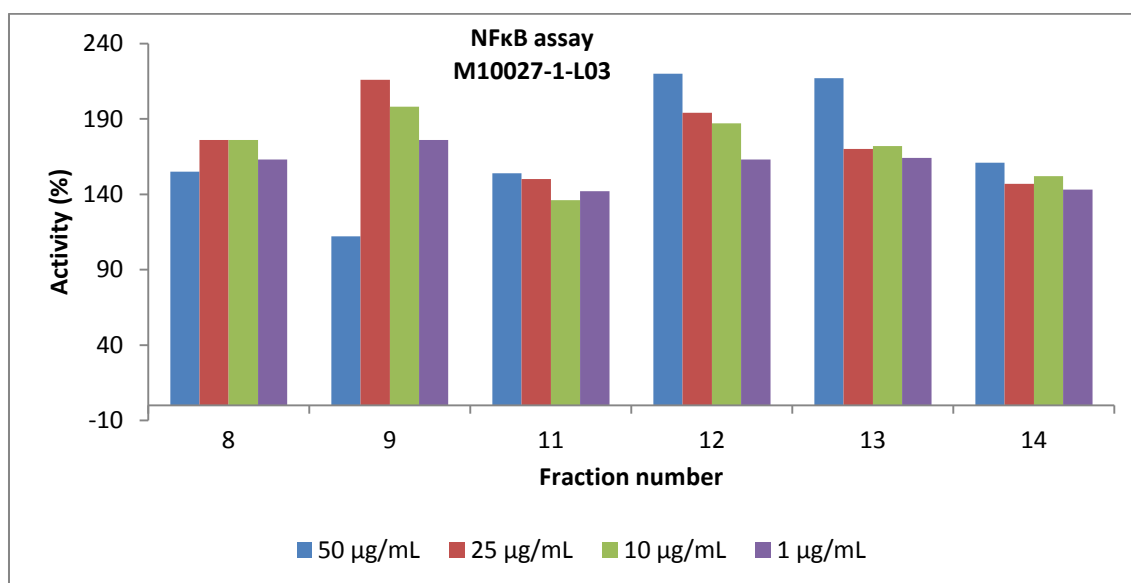


Figure 28: Secondary anti-inflammatory screening of the active flash fractions from the extracts of *Echinus esculentus* (M10027-1-L03). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the flash fractions.

3.4.6 Immunostimulatory screening of the *Echinus esculentus* gonad extract

In the immunostimulatory screening of the *Echinus esculentus* gonad extract, no HPLC fractions were active, neither in IL-1β nor TNF-α screening (figure not shown).

3.4.7 Anti-inflammatory screening of the *Echinus esculentus* intestines extract

HPLC fractions 26 and 27 from *Echinus esculentus* intestines were shown to have anti-inflammatory activity in NFκB assay in the U937 (fig. 29). In retesting, as figure 30 shows, fraction 26 kept the activity at undiluted concentration, but the activity diminished at further dilution. For fraction 27, activity was observed in both undiluted and dilute concentration 1:2. However at 1:4 dilution, the anti-inflammatory activity became very weak.

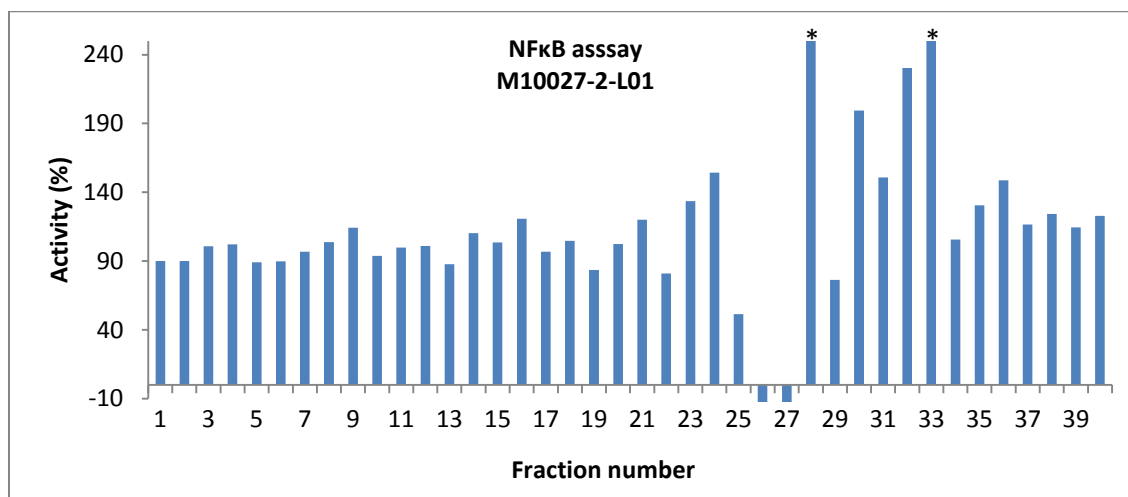


Figure 29: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts of *Echinus esculentus* (M10027-2-L01). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the HPLC fractions. *Activity exceeds 240 % activity.

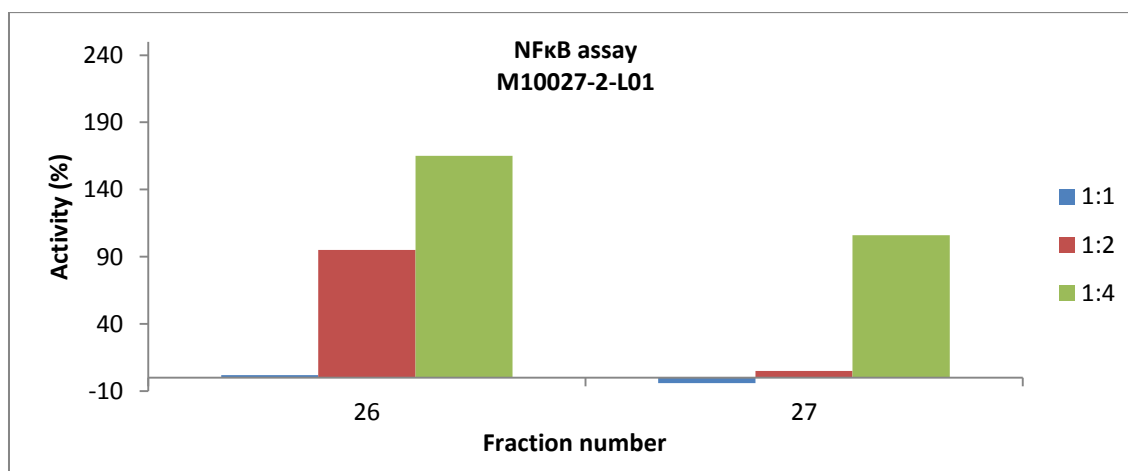


Figure 30: Secondary anti-inflammatory screening of the active HPLC fractions from the extracts of *Echinus esculentus* (M10027-2-L01). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

In the THP-1 cells assay (fig. 31), fraction 27 was identified to be active in IL-1β screening, while fraction 7, 8, 12, 16, 21, 27 and 28 were active in TNF-α screening. Since the results from both IL-1β and TNF-α screening were consistent with each other in that the fraction 27 revealed anti-inflammatory activity, only this fraction was retested. In both IL-1β and TNF-α screening this fraction still possessed anti-inflammatory activity at undiluted concentration, but further dilution lead to loss

of activity (fig. 32 and 33). However, as figure 33 shows, the fraction 27 possessed a dose-response relationship in the TNF- α assay.

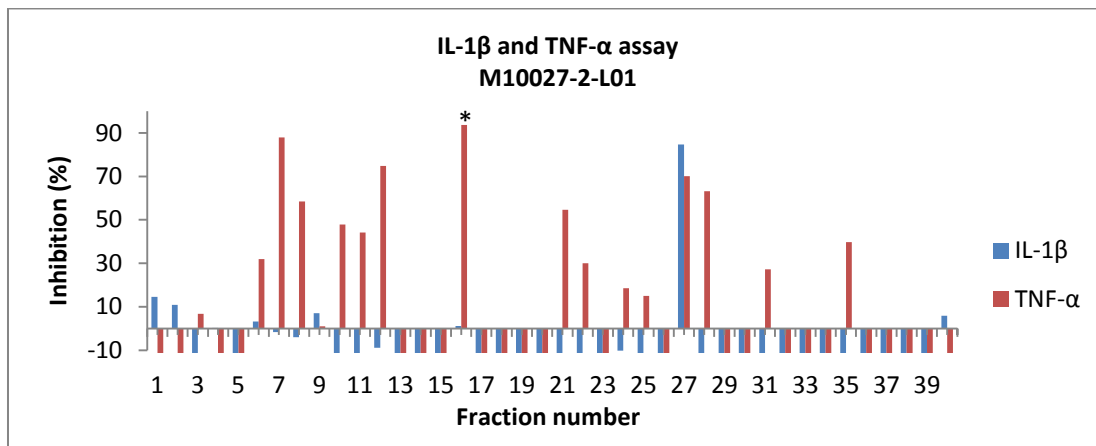


Figure 31: Primary anti-inflammatory screening of 40 different HPLC fractions from the organic extracts of *Echinus esculentus* (M10027-2-L01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the HPLC fractions. *Activity (%) exceeds 90 % activity.

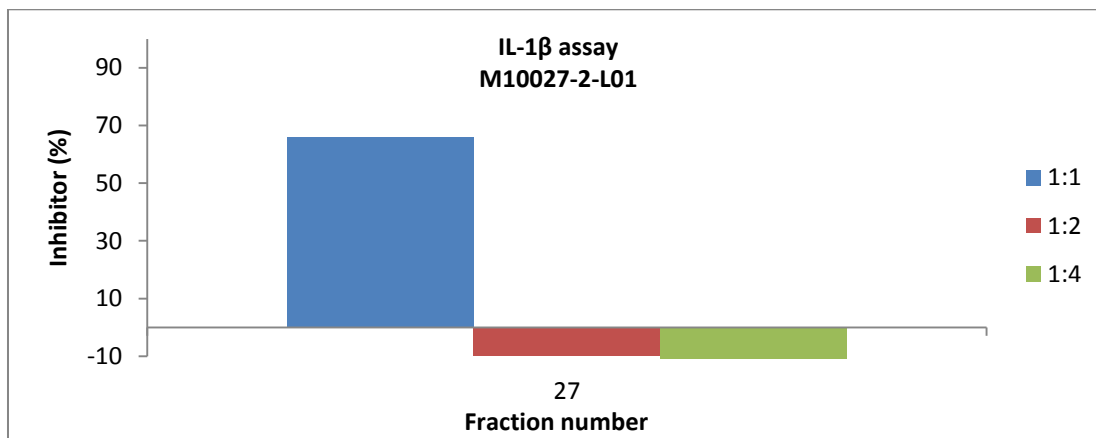


Figure 32: Secondary anti-inflammatory screening of the active HPLC fractions from the extracts of *Echinus esculentus* (M10027-2-L01). Each column represents the percent of IL-1 β production by the THP-1 cells after 7 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

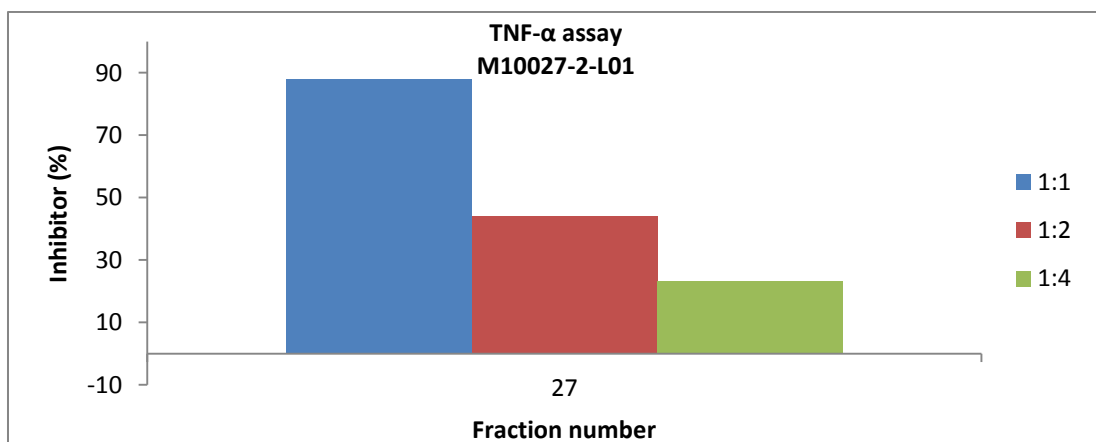


Figure 33: Secondary anti-inflammatory screening of the active HPLC fractions from the extracts of *Echinus esculentus* (M10027-2-L01). Each column represents the percent of TNF- α production by the THP-1 cells after 7 hours of exposure to the HPLC fractions (undiluted, diluted 1:2 and 1:4).

In addition to the screening of the HPLC fractions of the *Eucratea loricata* intestines (M10027-2-L01), screening of the organic flash fractions (50 µg/mL) of this organ was also conducted. The flash fractions 8-17 of this extract displayed anti-inflammatory activity in the NFκB assay in the U937 cell lines (fig. 34). These fractions were identified to be active and were retested. As figure 35 shows, only fractions 16 and 17 retained activity at 50 µg/mL, and fraction 16 also at 25µg/mL, but the activity declined in further dilution. However, as the columns show, both fractions revealed a dose-response relationship. Fraction 15 also showed a dose-response relationship, but even at the highest concentration, the activity was over the cut-off value. The other fractions did not exhibit significant activity at any concentration.

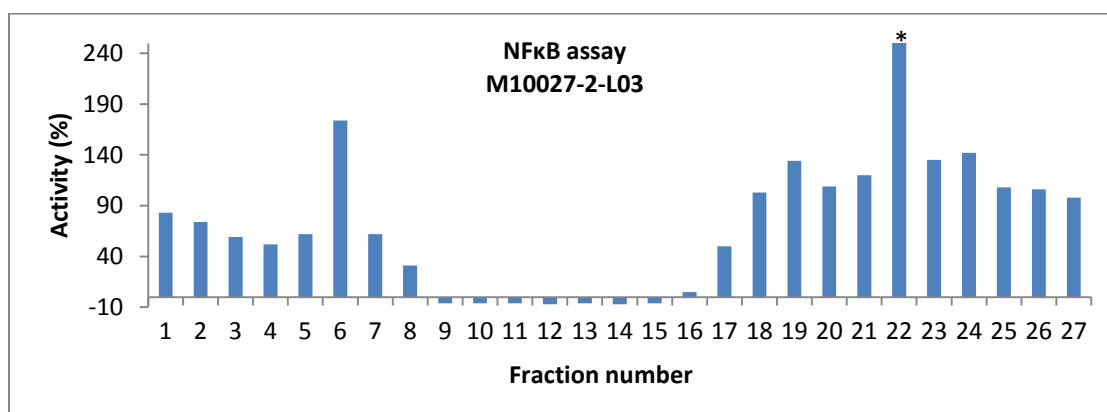


Figure 34: Primary anti-inflammatory screening of 27 different flash fractions from the organic extracts of *Echinus esculentus* (M10027-2-L03). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the flash fractions. *Activity exceeds 240 % activity.

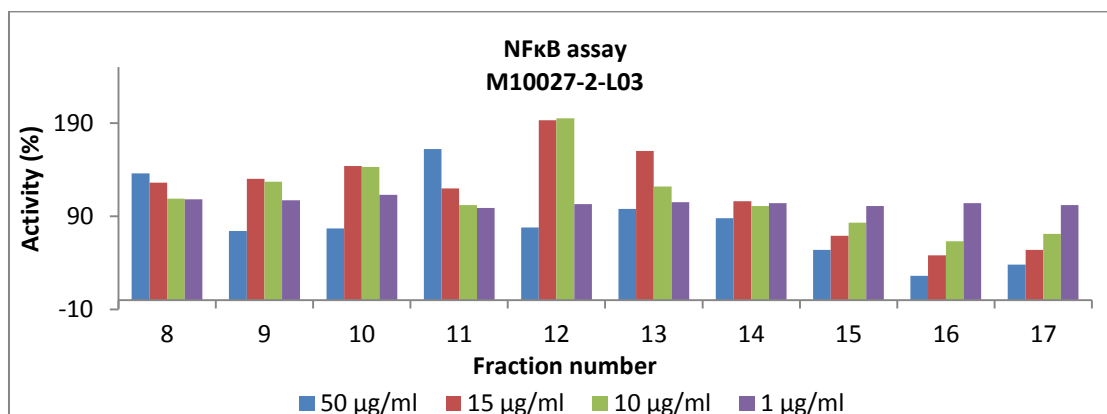


Figure 35: Secondary anti-inflammatory screening of the active flash fractions from the extracts of *Echinus esculentus* (M10027-2-L03). Each column represents the percent of the LPS-induced NFκB activity in the U937 cells after 24 hours of exposure to the flash fractions.

3.4.8 Immunostimulatory screening of the *Echinus esculentus* intestines extract

HPLC fractions of the *Echinus esculentus* intestines did not exhibit any stimulatory activity in the TNF-α screening. In the IL-1β screening, a weak stimulatory activity was observed in some of the fractions, but the activity was below the defined cut off value to be considered as active (figure not shown).

3.5 Dereplication of the active HPLC fraction from the *Echinus esculentus* gonad extract

After the primary and secondary screening, the bioactivity data showed that the HPLC fraction 27 of the *Echinus esculentus* intestines was active. Since the results from all three NFκB, IL-1β, and TNF-α screening were consistent with each other, this fraction was considered to contain the compound(s) that caused the observed anti-inflammatory activity. The fraction revealed a dose-response relationship in all three different assays (fig. 30, 32 and 33). This fraction was therefore a candidate for further investigation. Unfortunately, as mentioned in section 2.10, the amount of the crude organic extract M10027-2-L01 was too minuscule to any further purification and characterization. However, the bioactivity data revealed that the HPLC fraction 27 of the *Echinus esculentus* gonad also possessed anti-inflammatory activity in all three primary NFκB, IL-1β, and TNF-α screening. A weak dose-response relationship was also detected in the secondary screening of TNF-α. Both fractions 27 from the M10027-1-L01 and the M10027-2-L01 extracts were further analyzed by ToF-MS, and the data indicated that these fractions contained the same compounds as the ToF-MS chromatograms of these fractions were quite similar. Hence, the HPLC fraction 27 of the *Echinus esculentus* gonad was further investigated.

To identify the compound(s) that possibly caused the observed anti-inflammatory activity in the bioactivity screening, the active fraction 27 and the inactive fractions 25, 26 and 28, were analyzed by UPLC-ToF-MS. Possible active compound(s) were determined by the comparison of the chromatograms and the spectra of the active fraction and the inactive fractions. The compound with m/z 568.3 was detected in the active fraction but not in the inactive fractions, therefore the molecule with mass 567.3417 amu was selected as a target for isolation.

The dereplication of the target compound in fraction 27 from the organic extract M10027-1-L01, as is shown in figure 36, gave the protonated mass of the compound to be 568.3417 amu. From the isotope pattern and the accurate mass, the MS software calculated the elemental composition of the target compound to be $C_{30}H_{50}NO_7P$ (in ESI+ mode the compound will have one extra H), with a mass accuracy of -0.5 ppm and i-Fit value of 267.5 (fig. 36). The accurate mass and the elemental composition were also used for a search query in the database Dictionary of Marine Natural Products, but no items were found.

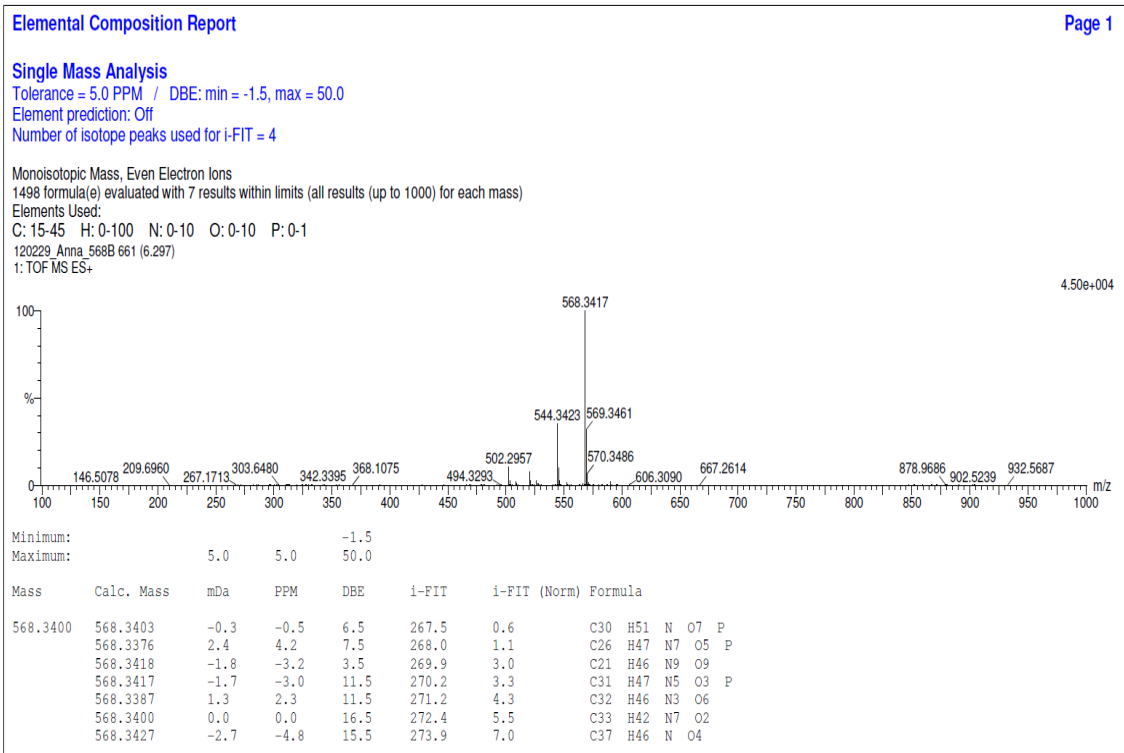


Figure 36: Elemental composition report and the ESI+ ToF-MS spectra of the target compound. All the signals are annotated with m/z .

3.6 Purification of the target compound from the *Echinus esculentus* gonad extract

An up-scaled purification of the target compound of the crude extract M10027-1-L01 was performed to provide sufficient material for structure elucidation and the studies of the biological mode of action. The purification of the target compound was achieved by using preparative HPLC coupled with fraction triggering ESI+ MS. Several gradients of mobile phases were tested to obtain an optimized gradient in order to improve isolation of the compound. The preparative HPLC-MS was set to collect the protonated mass 568.3 amu with a minimum intensity threshold (MIT) of 1 000 000. As seen in figure 37, the HPLC-MS separated the target compound, 568.3 amu (protonated mass), from the other compounds. The compound eluted after approximately 10 minutes with the selected gradient. A total of approximately 2.5 mL extract was injected, (13 injections), and the yield was 1.40 mg dry sample.

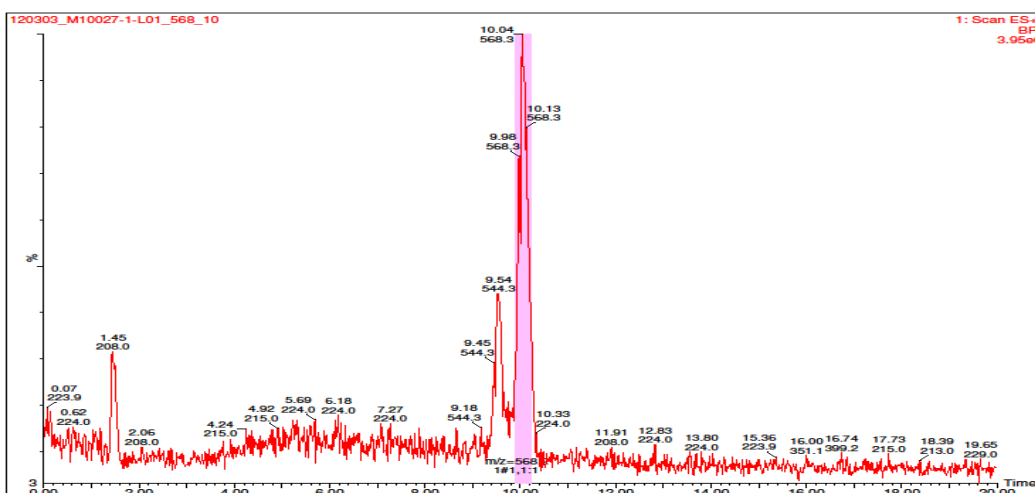


Figure 37: Chromatogram of the target compound by using HPLC-ESI+ MS. The time is on the X-axis and the peak intensity is on the Y-axis. The purple section is where the protonated compound with mass 568.3 amu was collected from the flow from the HPLC column. All the peaks are annotated with retention time (top) and m/z (bottom).

UPLC-ToF-MS of the purified compound from *Echinus esculentus*:

To estimate the purity of the isolated compound it was analyzed by UPLC-ToF-MS in positive and negative mode. As seen in figure 38, the MS chromatograms indicated the successful purification of the target compound in the preparative HPLC-MS. From the ESI+ chromatogram (fig. 38, A), the compound with m/z 568.3164 $[M+H]^+$ is by far the dominating signal indicating few impurities. Two smaller peaks were detected right before the compound eluted at 6.28 min. Similarly, the ESI- chromatogram (fig. 38, B) showing the dominating signal of the m/z 612.3312 $[M+FA-H]^-$ compound. A smaller peak was observed right before the compound eluted at 6.27 min, a smaller peak was also detected at 6.69 min.

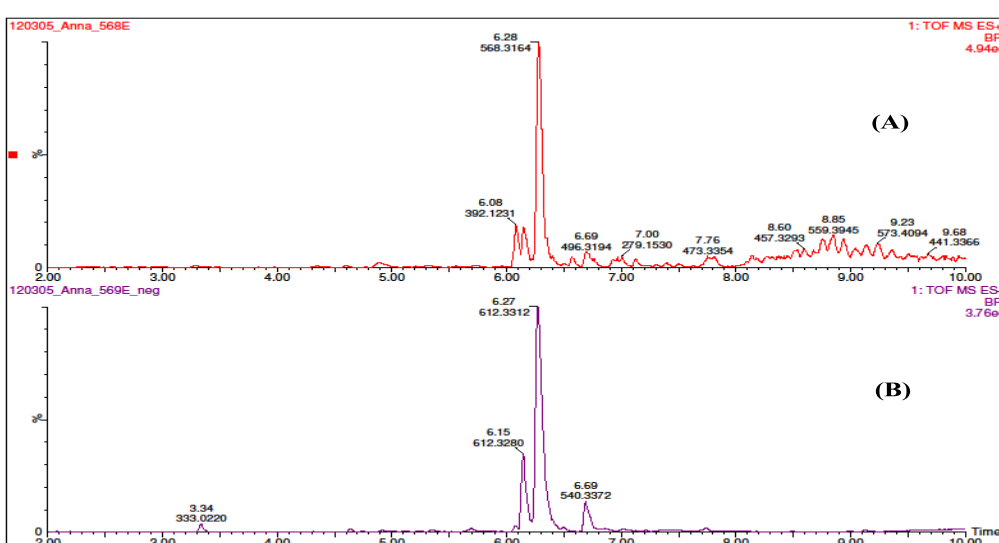


Figure 38: UPLC-ToF-MS chromatogram of the purified compound. A) positive and B) negative electro spray in the m/z range 150-1500. All the peaks are annotated with retention time (top) and m/z (bottom).

3.7 Structure elucidation of the target compound

The elemental composition of the isolated compound ($C_{30}H_{50}NO_7P$) was established using ToF-MS during dereplication. In order to determine the actual structure of the compound it was analyzed by NMR and tandem quadrupole MS/MS.

NMR of the purified compound from *Echinus esculentus*:

In attempt to achieve a full structure elucidation, the purified compound was also analyzed by NMR spectroscopy, and 1D 1H NMR, COSY, HSQC and HMBC were recorded.

As seen in figure 39, the 1H NMR spectrum showed resonances in the aliphatic region (1-2ppm), this has also been confirmed by the 2D COSY spectrum (fig. 40) and the HSQC+HMBC spectrum (fig. 41). Both spectra revealed a peak at around 0.6 ppm (peak w). There were no aromatic resonances in the spectrum (7-8 ppm).

The HSQC spectroscopy for carbon-proton interactions revealed a hydroxyl group with hydrogen shift at around 3.3 and carbon shift at approximately 65 (fig. 41, peak E) and ether group with hydrogen shift at approximately 3.6 and carbon shift at around 67 (fig. 41, peak D). This was also observed in the 2D COSY spectrum (fig. 40, peak E and D).

The HSQC+HMBC spectrum and the 2D COSY analysis for proton interactions of the sample displayed resonances in the trimethylamine region. As figure 41 shows, the trimethylamine group has hydrogen shift at around 3.1 (f1) and carbon shift at approximately 55 (f2). The carbon at 65 ppm was found to be bearing two hydrogen atoms (4.0 ppm) and one nitrogen atom (fig. 41, peak H). The carbon at 60 ppm was shown to be attached to two hydrogen atoms and a phosphate group (fig. 41, peak G).

With the elemental composition ($C_{30}H_{50}NO_7P$) calculated by MS software and the NMR results, it was suggested that the molecule structure consist of a phosphocholine group with unknown side chain(s).

However, since the sample amount was too small and not pure enough to provide clear signals, identification of the structure with high reliability was challenging. Moreover, some signals were unidentifiable and from the elemental composition, $C_{30}H_{50}NO_7P$, an oxygen atom still remained unidentified.

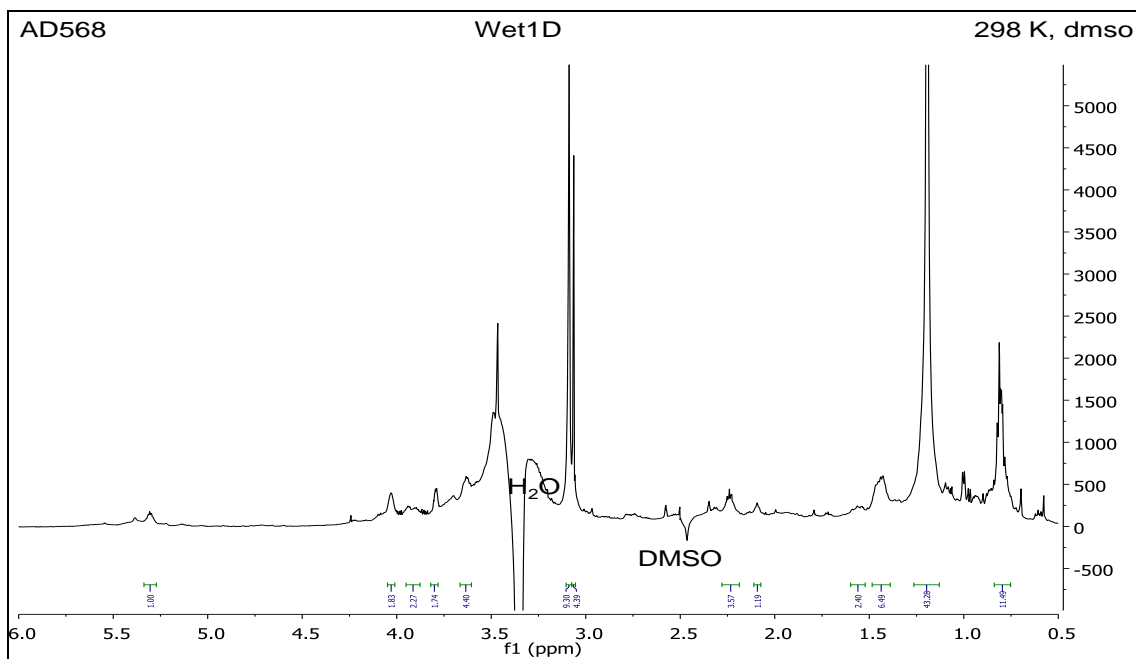


Figure 39: ^1H NMR specter of the purified compound. The large peak at 1.25 ppm implied alkane chain, and the peaks at 3.0-3.5 ppm might indicate ether and hydroxyl group. Chemical shift (ppm) is on the x-axis and peak intensity is on the y-axis.

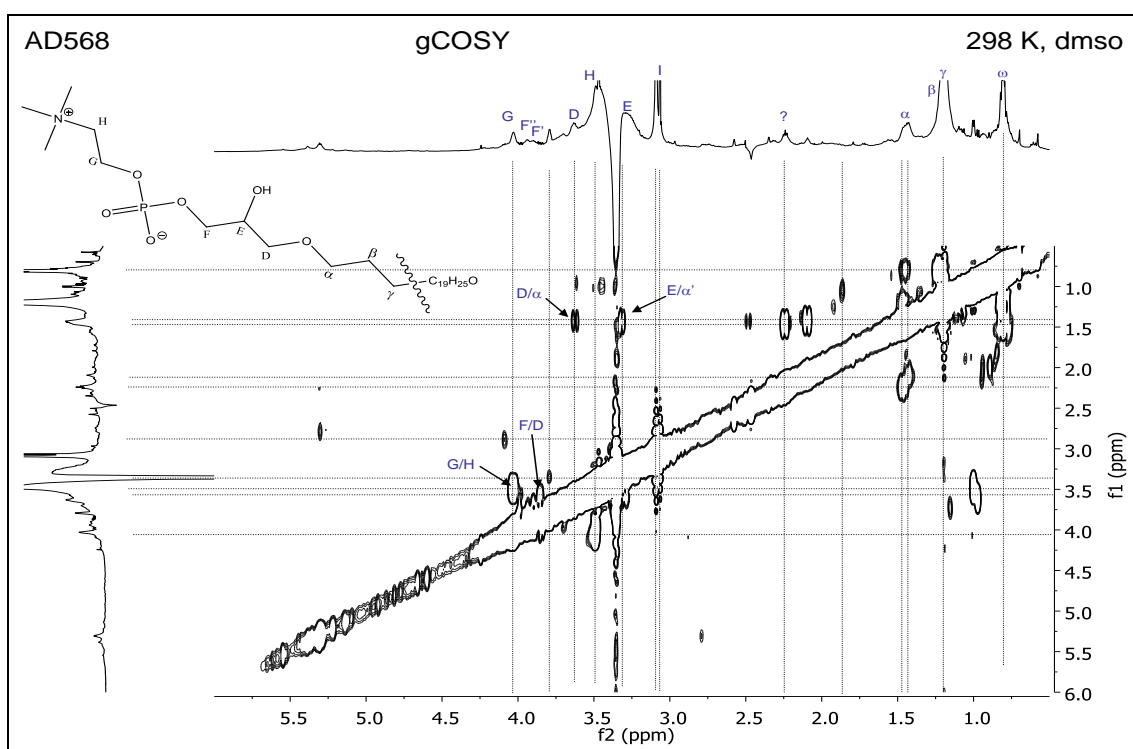


Figure 40: 2D COSY specter of the purified compound. The peaks are signed according to the structure of the purified compound. The peak I at approximately 3.1 ppm (f2) implied the trimethylamine group, hydroxyl group revealed a shift at 3.3 (peak E), and ether group at approximately 3.6 ppm (peak D).

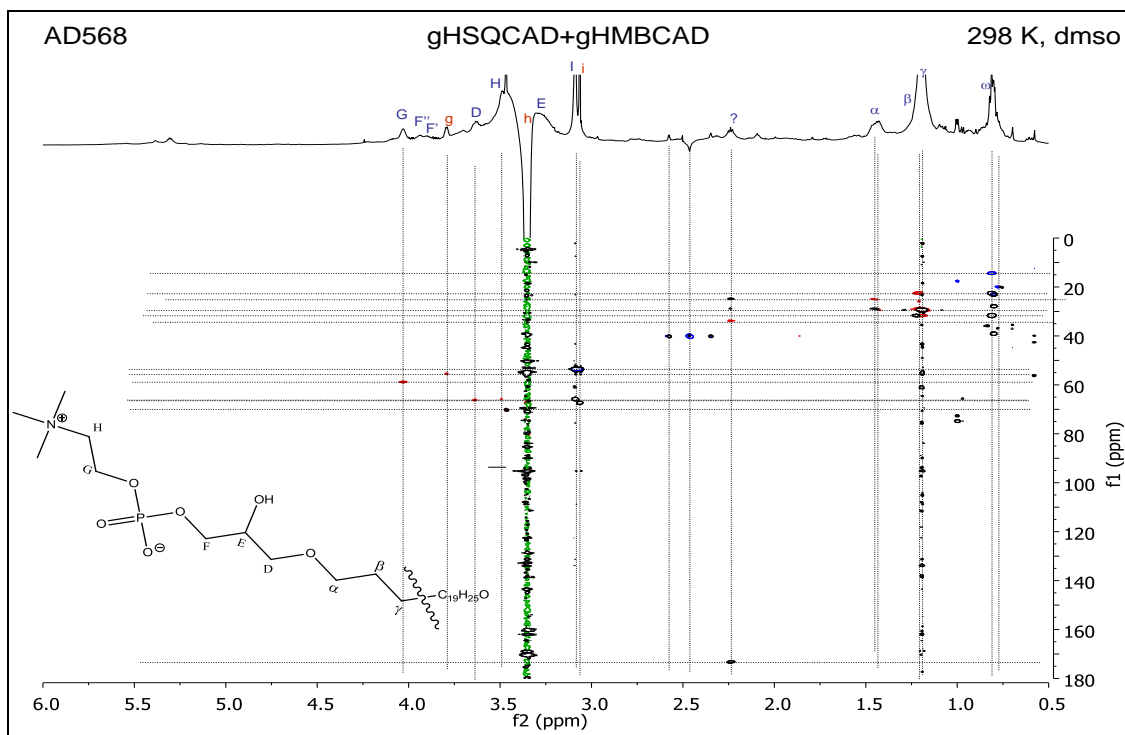


Figure 41: HSQC+HMBC specter of the purified compound. The peaks are signed according to the structure of the purified compound. The trimethylamine group show hydrogen shift (f2) at approximately 3.1 (peak I) and carbon shift at approximately 51. Hydroxyl group revealed carbon shift at about 65 (peak E), and ether group with carbon shift at approximately 6.7 (peak D). Hydrogen shift (ppm) is on the x-axis (f2), and carbon shift (ppm) is on the y-axis.

Tandem quadrupole MS/MS of the purified compound from *Echinus esculentus*:

The information provided from the UPLC-ToF-MS and NMR analysis implied that the purified compound consist a phosphocholine group with an unknown side chain. To confirm the phosphocholine group presence in the compound, analysis by a tandem quadrupole MS/MS was performed. A positive ion daughter scan of the m/z 568.3 ($[M+H]^+$) was performed. The daughter ion specter is seen in figure 42, and some of the most intense fragment ions are explained in figure 43 (and also in figure 44). The phosphocholine fragment ion revealed the mass-to-charge ratio at 184. The fragment ion with m/z 551 is consistent with the mass of the molecule (568.3 amu) when it loses a hydroxyl group. However, some intense fragment ions could not be explained from the surmised phosphocholine molecular structure show in figure 43, especially the fragment ion m/z 301.12. As seen in figure 43, the fragment ion m/z 285 could be identified if the oxygen atom was assumed to be placed in the unidentified chain, but the intense fragment ion m/z 301 could not be explained. However, the fragment ion m/z 301 can be explained by the molecular structure suggested in figure 44, but this can not be confirmed by NMR data.

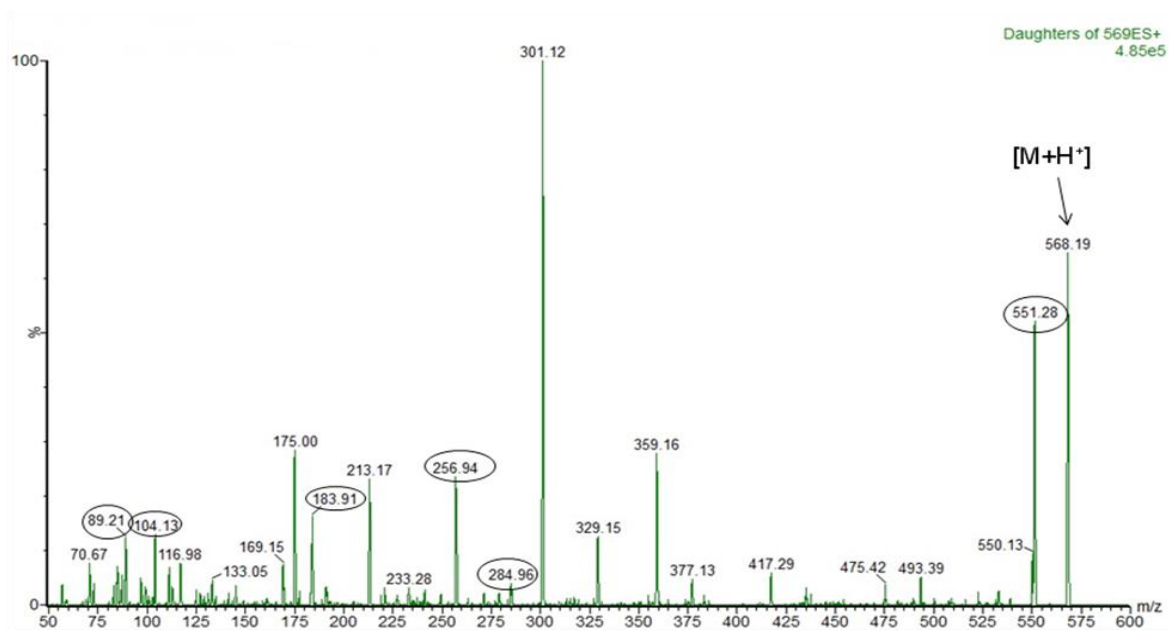


Figure 42: ES+ MS/MS daughter ion scan spectrum of the purified compound at m/z 568.3. All the fragments are annotated with m/z . The fragments marked with a circle correspond with the mass identified in the molecule shown below (fig. 43 and 44).

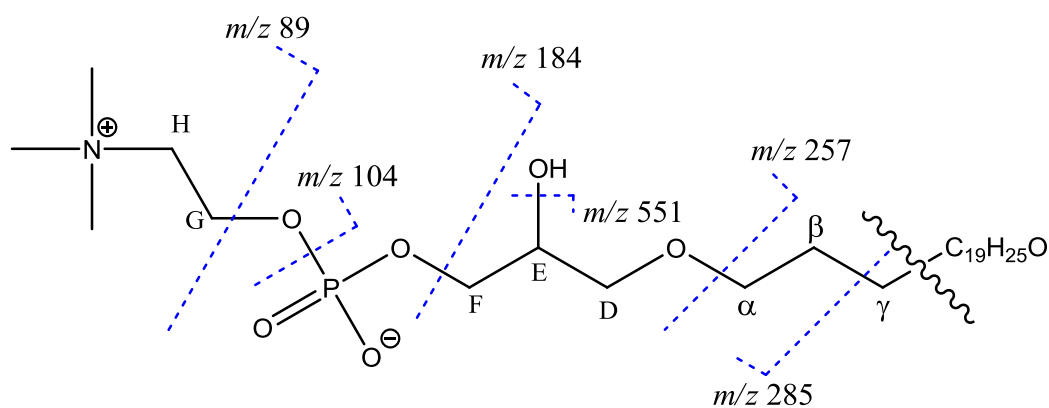


Figure 43: Structure of phosphocholine showing the fragments with an unidentified side chain, which was presumed to contain an oxygen atom.

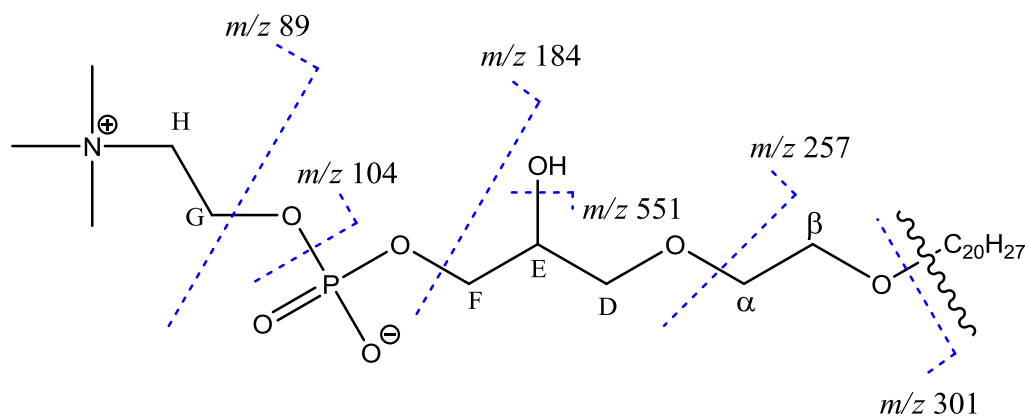


Figure 44: Structure of phosphocholine showing the fragments with an unidentified side chain. The fragments shown here are corresponded to the fragments marked with a circle revealed in the figure 42, in addition to the intense fragment m/z 301.

3.8 Testing of the anti-inflammatory activity of the target compound

After dereplication, purification, isolation and structure elucidation of the target compound, it was retested to confirm the anti-inflammatory activity observed in the primary and secondary screening with THP-1 cells. As figure 45 shows, the target compound did not exert anti-inflammatory activity at any concentrations tested neither in the IL-1 β nor the TNF- α screening.

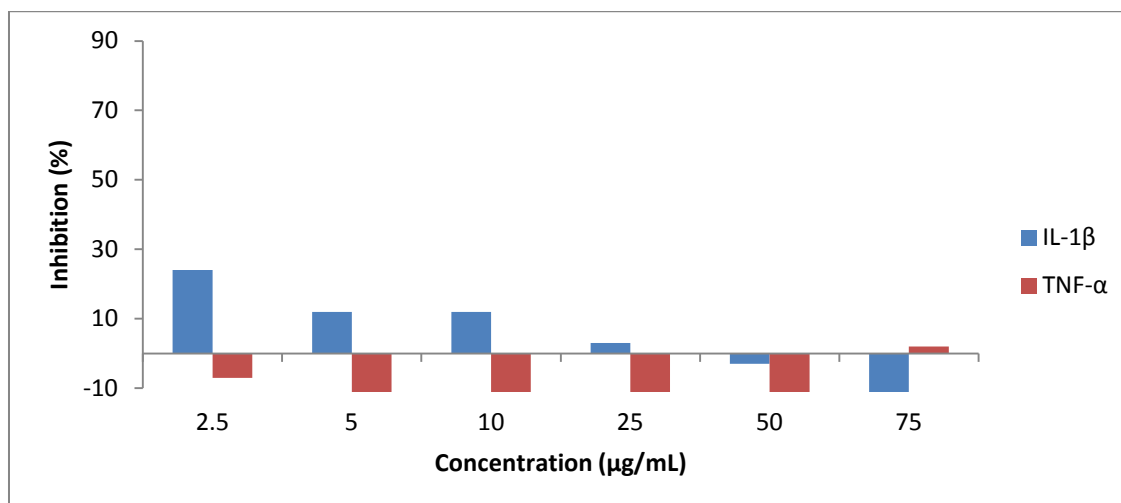


Figure 45: Anti-inflammatory screening of the target compound from the extracts of *Echinus esculentus* (M10027-1-L01). Each column represents the percent of IL-1 β (blue) or TNF- α (red) production by the THP-1 cells after 7 hours of exposure to the target compound.

4 Discussion

Over the last years, the marine environment has provided a remarkable proportion of compounds with anti-inflammatory activities. The need for novel anti-inflammatory drugs is increasing due to issues concerning the serious side effects of the traditional anti-inflammatory drugs. The aim of this project has been to investigate for anti-inflammatory and immunostimulatory activities of extracts from two Arctic marine invertebrate species; a bryozoan, *Eucratea loricata*, and a sea urchin, *Echinus esculentus*. Further, an attempt to elucidate the structure of the purified compound was carried out. Although bioassay-guided fractionation has become a powerful tool in natural products drug discovery, cell-based assays of natural product extracts are complicated. One of the challenges is associated with the complexity of the biological system. The anti-inflammatory NF κ B assay with U937 cells is relying on a reporter gene, and due to signal amplification of cell-signaling cascades, reporter gene assays are very sensitive immune systems. Thus, all foreign substances that could not be recognized by the cells might trigger the cells to response. These non-specific interferences may also occur in THP-1 cells and trigger the production of IL-1 β and TNF- α , or it may be as simple as the concentrations of the substances present in the extract are too high so that they interact nonspecifically with a receptor in the cells. In addition, unfortunately, natural products extracts are typically complex mixtures and the concentrations of different substances in crude extracts are unknown.

Since the screening for compounds exerting anti-inflammatory or immunostimulatory activities was not always easy owing to the complexity of cellular interactions involved during an inflammatory response, the same fractions were tested against several targets. The data generated from various assays made it possible to compare whether the results were consistent with each other. If the same fractions revealed activities in at least two of three assays, the fractions was considered as a potential candidate for further investigation. By this way, it provided the opportunity to sort out, in an early stage, whether the observed anti-inflammatory was due to non-specific effects or specific anti-inflammatory effects.

It was prepared an aqueous extract and an organic extract of the bryozoan. The screening results show that more anti-inflammatory activity was found in the HPLC organic fractions than in the HPLC aqueous fractions. There are many possible explanations for this. The most common problem associated with water extracts is bacterial and fungal growth and these microorganisms might degrade active components present in the extract. Moreover, water extracts normally contain ionic and polar substances, which are not cell membrane permeable and therefore unable to reach a potential cellular target.

Because no HPLC fractions of the aqueous extract reduced the NF κ B reporter activity, moreover, only fractions 39 and 40 possessed weak activity (55% and 53%, respectively) in the IL-1 β assay but not in the TNF- α assay, the aqueous extract was not fractionated by flash chromatography. In addition, due to the experience obtained at MabCent, the organic solvent extracts are relatively enriched in smaller, moderately polar and non-polar molecules. These small molecules are the ones most likely to have drug-like properties, so it is believed that there is a greater likelihood of detecting biologically active substances in the organic extracts. The crude extracts from the organic extractions were therefore fractionated by both HPLC and flash chromatography. The dry weight of each fraction after flash fractionation (table 9) was dissolved in DMSO to give a concentration of 40mg/mL. In this manner, fractionation by flash chromatography provides the opportunity to have control over the test concentration as early as possible in the primary screening.

Although 15 (fractions 19-31 and 33) of the 40 HPLC fractions of the bryozoan organic extract displayed anti-inflammatory activity in the primary NF κ B screening, only fraction 25 showed a weak activity in the IL-1 β testing, and only fraction 33 revealed activity in the TNF- α screening. The results from NF κ B, IL-1 β and TNF- α assays were not consistent with each other. In NF κ B activity retesting, the anti-inflammatory activities in fractions 22-27, 30 and 33 were reproducible at undiluted concentration, but not at diluted concentrations. For fractions 25 and 33, the activity could neither be replicated in IL-1 β or TNF- α assay. Similarly, the flash fractions 10, 11 and 12 displayed activity in NF κ B assay, but in the retest, the activity could not be observed at dilute concentrations. This might be explained by the non-specific effects, the activity could be observed when there was high enough amount of substances present in the fractions, and once the fractions were diluted, the activity could not be detected. The same reason might also explain why the HPLC fractions 17, 21, 28 and 29 exerted the immunostimulatory activity in the primary IL-1 β or TNF- α assay, but not in the retest. In addition, because of the high sensitivity of the assays, any foreign substances might provoke the cells to respond.

The HPLC fraction 6 of the gonad extract from the *Echinus esculentus* (M10027-1-L01) revealed anti-inflammatory activities in both IL-1 β and TNF- α assay. And the HPLC fraction 27 displayed activity in all three assays. In retesting of the fractions 6 and 27, only fraction 27 retained the activity in the IL-1 β assay at undiluted concentration. The flash fractions 8, 9 and 11-14 from the same extract showed anti-inflammatory activity in NF κ B assay, but the activity could not be replicated in the retest. As discussed above, the anti-inflammatory activity observed in the primary testing might owe to the non-specific response in the cells.

In the screening of *Echinus esculentus* intestinal (M10027-2-L01), the HPLC fraction 27 exerted strong anti-inflammatory activities in all three NF κ B, IL-1 β and TNF- α assays. In NF κ B retesting, the fraction retained the activity at undiluted and diluted concentration 1:2. In both IL-1 β and TNF- α retesting,

the fraction possessed activity only at undiluted concentration. However, both NF κ B and TNF- α assays gave a good dose-response relationship. Similarly, the flash fractions 16 and 17 also showed a dose-response relationship in NF κ B retesting. This might indicate that the observed anti-inflammatory activities were truly caused by the specific responses from cells since the activities declined corresponded to the concentrations of the fractions.

It was suspected that the HPLC fraction 27 and the flash fractions 16 and 17 might consist of similar substances, since the polarity of the mobile phases at the time the HPLC fraction 27 eluted was similar to the mobile phases polarity at the time flash fractions 16 and 17 eluted. This could be confirmed by using ToF-MS to analyze these fractions. Comparison of ToF-MS chromatograms and spectra of these fractions might reveal whether the same substances were present in these fractions. Nevertheless, this was not prioritized due to the insufficiency of crude M10027-2-L01 extract for further investigation.

Bioactivity data was used in order to guide the identification of the target compound for structure elucidation. The results from ToF-MS analysis of the HPLC fraction 27 from both gonad and intestinal extracts of the *Echinus esculentus*, indicated that both fractions most likely consisted of the same substances. Therefore, it was decided to dereplicate and isolate compounds from fraction 27 from the gonad extract.

During the dereplication, the compounds m/z 341.2, 345.1 and 568.3 were discovered in fraction 27 but not in the nearby inactive fractions. In addition, since the concentration of these compounds was correlated with the activity exerted by fraction 27, these compounds, with m/z 341.2, 345.1, and 568.3, were suspected to be the compounds that might cause the observed anti-inflammatory activity. However, after repeated isolation attempts of m/z 341.2 and 345.1, these two compounds could not be detected in ToF-MS even though isolation by the preparative HPLC-MS seemed to be successful. Earlier it has been shown that this is not entirely uncommon. Natural products are well known for their chemical complexity, and marine extracts may contain extremely labile compounds. Decomposition of these compounds may occur at any step during the purification process. Heat, light, air and pH are among other factors that may lead to the degradation of compounds. Materials used for separation may also activate some reactions.

Nevertheless, isolation of the compound with m/z 568.3 was successful and structure elucidation of this compound was carried out using MS and NMR. From the ToF-MS data, the empirical formula of the compound was calculated to be C₃₀H₅₁NO₇P. Analysis of the MS and NMR data indicated that the compound consisted of a phosphocholine group with an unknown side chain. Because of the splitting in the peak I on the 2D COSY and the HSQC+HMBC spectrum (fig. 40 and 41), as well as some unidentified fragments in MS-MS specter (fig. 42), it was suggested that the purified extract might contain at least two different kinds of phosphocholine molecules. This might be possible to confirm,

but it is challenging due to the requirement of higher amount and higher purity of the sample to provide better MS and NMR spectra.

Elucidation of the unknown side chain might be possible by using GC-MS. Because of the higher energy transfer during ionization compared to the LC-MS technique, GC-MS is capable to break carbon-carbon bonds, and hence detect differences in the carbon side chain. Due to a limited time perspective in this project, this was not performed at this stage. Moreover, since the results from the testing of the purified compound did not show any anti-inflammatory activity, it was logical to accept that the compound was no "hit" as a lead compound, and therefore further investigation was not carried out.

On the other hand, the molecular formula generated by using ToF-MS resulted in several alternatives, and the inconsistency between the HR-MS and NMR data might be due to that the molecular formula, $C_{30}H_{51}NO_7P$, was not the correct one.

In testing of the purified compound, no activities could be detected at any concentrations. There are several reasons for why the activity could not be reproduced after purification. The active compound might be unstable in the selected conditions for the isolation process, leading to loss or reduction of the activities detected in primary screening and retesting. There is also the possibility that the activities were caused by other compounds than the purified compound. To confirm this, every compound present in the fraction need to be isolated and tested, which is too time-consuming and resource demanding to carry out in this project. The activity might also arise from a synergic effect from several compounds present in the fraction. Moreover, since the isolation of the compounds m/z 341.2 and 345.1 could not be achieved, the possibility that it was these compounds that were bioactive could not be excluded.

In this study only the gonads and intestine, collected the same day, were investigated for bioactivity. There is still a great amount of work that could be done, for instance looking at differences between male and female extracts, seasonal variation in extract composition and differences due to geographical location, diet, pollution and other environmental factors. There is also the possibility of screening other organs and tissues of *Echinus esculentus*, including the shell. Similarly for bryozoans, varying environmental factors and geographical locations may cause a difference in metabolite production between colonies. It has also been speculated that several natural products may not actually originate from bryozoans themselves, but from endosymbiotic microorganisms unique to bryozoans [41].

The results from bioactivity testing revealed the *Echinus esculentus* intestinal extract (M10027-2-L01) as an extract with high possibility of containing active compounds. However, the amount of the crude extract was too minuscule to attempt any further purification and characterization. In order to pursue the bioactive constituents, it is likely that a recollection of the organism will be needed in

order to have sufficient extract to purify. This will hopefully lead to sufficient quantities of purified bioactive compound(s) to permit subsequent characterization. On the other hand, the processes involved from the sample collection to structure elucidation are time-consuming and resource demanding.

Although no novel scaffolds were characterized from *Eucratea loricata* and *Echinus esculentus*, this project has demonstrated the complete marine bioprospecting process as a starting point in the drug discovery process. Most of natural product screening is focusing on novel anticancer compounds. This project has contributed in amplifying the knowledge about screening of natural product with anti-inflammatory and immunostimulatory activities. In addition, this project provides background information about marine natural product research and current anti-inflammatory investigations of marine organisms. Furthermore, the project has also highlighted that marine organisms contain interesting substances, and that the marine environments still might be an important source for novel bioactive compounds. The present project is the first report on the isolation of phosphocholines from the sea urchin *Echinus Esculentus*, and hopefully, this work might also assist MabCent to recognize phosphocholines in samples analyzed in the future, which can be both time and resources saving.

5 Conclusion and future perspective

The aim of this project was to investigate extracts from the bryozoan *Eucratea loricata* and the sea urchin *Echinus esculentus* for anti-inflammatory and immunostimulatory activities, and if possible determine the molecular structure of the active compounds. A total of four extracts: M08004-0-W01 (aqueous extract of the bryozoan), M08004-0-L01 (organic extract of the bryozoan), M10027-1-L01 (organic extract of the sea urchin gonad), and M10027-2-L01 (organic extract of the sea urchin testines) were screened against NF κ B, IL-1 β and TNF- α . No significant immunostimulatory activities were detected in any of these extracts. The results from the anti-inflammatory screenings indicated that fraction 27 from both M10027-1 and M10027-2 extracts were active. Since the amount of the crude extract M10027-2 was not high enough to carry through the isolation and structure elucidation process, only fraction 27 of the extract M10027-1 was further investigated. After the dereplication, the molecule with 567.3 amu, with the elemental composition C₃₀H₅₀NO₇P, was chosen to be the target compound. The results from structure elucidation of the molecule indicated that the target compound was a phosphocholine with an unknown side chain. Testing this target molecule for anti-inflammatory activity revealed that it had no effect. Therefore it was concluded that the compound was not the active agent.

The present project has shown that the sea urchin *Echinus esculentus* intestines contain bioactive constituents. Recollection of the organism is necessary in order to pursue any further investigation for bioactive constituents, and this might hopefully lead to the discovery of interesting compounds with anti-inflammatory activity. In addition, further isolation of the remaining compounds present in fraction 27 may hopefully provide active agents. Moreover, further investigation of the structure of the target compound may lead to the discovery of a new molecule. There are many important and useful results from the NMR and MS/MS analysis. Nevertheless, further structure elucidation will demand better purity of the compound for NMR analysis, while GC-MS is an option for elucidating the side chain.

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