



Biological and chemical characterization of ianthelline isolated from a marine sponge

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Preface

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Abstract

Previous studies of sponges have led to the discovery of the commercially available drugs cytarabin and vidarabin. The arctic sponge *Stryphnus fortis* showed antibacterial and anticancer activity in the primary screening in the MabCent screening program; most likely from more than one compound.

Dereplication with TOF-MS suggested the presence of ianthelline as one of the active compounds. In order to purify large amounts of the ianthelline, the extract was fractionated using flash chromatography and the fractions found to contain the active compound were combined and submitted to semi-preparative HPLC for further purification.

TOF-MS, MS/MS and ^1H , ^{13}C , HMBC and HSQC NMR confirmed the presence of ianthelline in the product from the semi-preparative HPLC. Ianthelline was tested for antibacterial activity, antioxidant activity and anticancer activity. Ianthelline had a dose-response relationship antibacterial activity against *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus* and MRSA, the activity was highest for *S. aureus* and MRSA. Ianthelline showed only a slight antioxidant activity compared to curcumin. Ianthelline showed anticancer activity against A2058 (melanoma), MCF7 (breast carcinoma) and HT29, but also killed normal lung fibroblasts (MRC5), indicating a general toxicity. Anticancer activity occurred after a minimum of 24 hours exposure of ianthelline against A2058 and normal lung fibroblast (MRC5), and after a minimum of 48 hours exposure of ianthelline against HT29.

Abbreviations

Abl	Abelson Kinase
CD ₃ OD	Deuterium methanol
CFU	Colony Forming Units
COSY	Correlation Spectroscopy
DMEM	Dulbecco's Modified Media
DMSO	Dimethyl Sulfoxide
<i>E. coli</i>	<i>Escherichia coli</i>
EMEM	Eagle Minimal Essential Media
<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
ESI+	Positive mode ElectroSpray ionization
ESI	ElectroSpray Ionization
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Ability of Plasma
HMBC	Heteronuclear Multiple-Bond Correlation
HPLC	High Pressure Liquid Chromatography
HSQC	Heteronuclear Single-Quantum Correlation
PKA	Protein Kinase A
MIC	Minimum Inhibitory Concentration
MIT	Minimum Intensity Treshold
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MS	Mass Spectroscopy
MS/MS	triple quadropol Mass Spectroscopy
MTS tetrazolium reagent	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-tetrazolium reagent
<i>m/z</i>	Mass-to-charge
NCI	National Cancer Institute in USA
NMR	Nuclear Magnetic Resonance
NOEL	No-Observed-Effect Level
NEAA	Non-Essential Amino Acid

P. aeruginosa *Pseudomonas aeruginosa*

PBS Dulbecco's Phosphate Buffered Saline

rpm Rotation Per Minute

RPMI-1640 Roswell Park Memorial Institute -1640 cell media

S. aureus *Staphylococcus aureus*

TOF-MS Time-Of-Flight Mass Spectrometry

TPTZ 2,4,6-TriPyridyl-sTriazine

Trolox 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid

UPLC Ultra Performance Liquid Chromatography

1. Introduction

1.1 The MabCent-SFI program:

The MabCent-SFI program was founded in 2007, by the Research Council of Norway. In the MabCent program, Marbank collects Arctic marine organisms, performs taxonomy studies, produces extracts from the marine organisms and performs bioassay guided purifications. Marbio screen the extract from Marbank for bioactive compounds in the MabCent program. The Marbio bioactivity screening consists of automated steps and bioactivity for antibacterial, anticancer, antioxidant, antidiabetic, antiviral, immunostimulants, antiinflammatory activity, and samples are analyzed in a high throughput screening manner. In a high throughput screening program, compounds or natural product extracts can be analyzed for many different bioactivities in a relatively short time [1].

1.2 Bioprospecting.

Since before the invention of the written language, humans have used nature as a source for drugs. The earliest known documentation is from 2735 B.C. in China. The western world started to document remedies from herbs 500 years ago. The laboratory study of drugs from natural origin began when Serfürner isolated morphine from opium in 1803 [2]. Today 57 % of all new drugs approved are natural, natural derived, biological or is a natural product mimic [3].

A. Douglas Kinghorn wrote in the Foye's Principles of Medical Chemistry: "The greater phylogenetic diversity, the greater the resultant chemical diversity" [2]. The National Cancer Institute (NCI) of USA started screening plants for antitumor compounds in the 1950s and the project has been continued since [4]. However the main focus has been on terrestrial plants and animals, while the marine area has been neglected in comparison. This has led to rediscovering of already known compounds [5]. Of the many animal phyla existing, only one is solely terrestrial (onychophora), while eight of the major phyla only exist in water [2]. The Barents Sea with its diversity of unique psychrophilic organisms may harbour unique secondary metabolites with bioactivity against human diseases.

1.3 Anticancer drug discovery:

In 2008, 190 000 persons had one of the 200 different cancer diagnoses in Norway. Colon cancer, breast cancer and melanoma are all on the top 10 list of most common cancer types [6, 7].

23 % of the reported cancer incidence for women was breast cancer in 2008 in Norway. There are several options of adjuvant treatment depending on whether the cancer is estrogen dependent or not.

10 % of the reported cancer incidences were colon cancers in 2008 in Norway [6]. Colon cancer responds slightly to moderately to antineoplastic drugs. Fluorouracil alone or in different combinations with folinat, oksaiplatin and/or kapecitabin is used as adjuvant treatment to surgery and radiation of colon cancers [8].

The incidence rate of melanoma in Norway in 2008 was 17.6 per 100 000 person-year. The only present cure for melanoma is surgery alone or in combination with radio therapy. Only a few cytostatics have a slight effect on melanoma and no cytostatics are used for treatment of melanoma[8]. This makes melanoma cells an important drug target in the search for new drugs.

Over 60 % of the commercially available cancer drugs are either directly a natural compound or derived from a natural compound[3, 4]. Some anticancer drugs have marine origin. Cytarabine was the first marine derived drug to reach the commercial market. It acts as a cytidine analogue and causes formation of ceramid, which in turn causes apoptosis. It is used for treatment of leukemia and is the most effective drug against acute myelocytic leukemia [9, 10].”

Trabectedin was isolated and biocharacterized from a tunicate in 1969, but didn't get approved by EMEA before 2009 (not approved by U.S Food and Drug Administration) [11, 12]. Trabectedin has a novel DNA binding mechanism and is a third line therapy for soft tissue sarcoma and platina resistant ovarian cancer [12, 13].

Protein kinases play key roles in the regulation of important cellular processes including proliferation, differentiation, cell growth, cell death, and survival.

Mutations or epigenetic changes leading to deregulated activity of a wide variety of kinases are associated with serious diseases like cancer [14]. Constant activation of Abl is associated with tumor invasiveness and inhibition of PKA induces apoptosis in pancreatic cancer [15, 16].

1.4 Antibiotics drug discovery:

Infectious diseases are one of the major death causes in the developing countries. The spread of multiresistant bacteria makes it necessary to search for compounds with completely new mechanisms of action [17]. Newly discovered antibiotics have taken a shift from novel targets and structures to alterations of already known compounds [9]; at the same time antibiotic resistance incidence from 1990 to 2000 has increased 20 times for *S. aureus* in England [2]. *S. aureus* (Gram+), MRSA (Gram+), *E. faecalis* (Gram+), *P. aeruginosa* (Gram-) and *E. Coli* (Gram-) are some of the main causes of hospital infections [18].

1.5 Sponges:



Figure 1: Frozen specimen of *Stryphnus fortis*. Photo: Robert A. Johansen, Marbank

Since sponges are filter feeders, they are constantly exposed to bacteria in the water. In addition sponges are sessile and have no immune cells; this makes secondary metabolites their key defence against bacteria and predator attacks. Sponges may live in symbiosis with bacteria and chemical means to control and nurse the bacteria are thus required. Any natural product the sponge releases into the water must be extremely potent, since the substance will be quickly diluted by the water [5, 19, 20].

Previous studies of sponges have led to the discovery of cytarabin and vidarabin which are now commercial available and several other compounds from sponge origin are currently in clinical trials [10]. Vidarabine, the second commercially available marine derived pharmaceutical from a natural product was the first antiherpes virus drug. Because of toxicity it is only used for life threatening herpes simplex virus encephalitis [2, 9, 10]. *Stryphnus fortis* belongs to the astrophorida order of demosponges. The astrophorida order are widely distributed around the world and usually lives at deep waters in the Arctic but lives in shallow waters in the tropical seas [21]. *Stryphnus fortis* was collected northwest of Spitsbergen.

1.6 lanthelline:

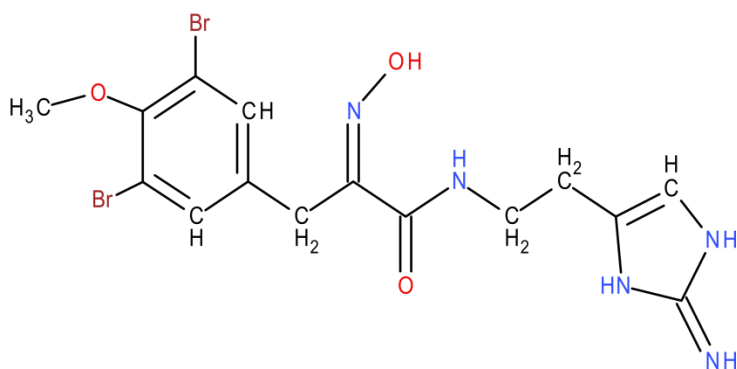


Figure 2: The molecular structure of ianthelline

lanthelline is a bromotyrosine secondary metabolite first discovered in 1986 by Litaudon in the sponge *lanthella Ardis* at the coast of Bahamas [22, 23]. According to the Dictionary of Marine Natural Products lanthelline have an accurate mass of 472.9698 Da and a molecular structure as shown in figure 1. lanthelline has shown activity against cancer, bacteria and fungi[24]. S. R Kelly et. al. in 2005 proposed a possible mechanism for its activity against bacteria. They suggested that preventing attachment of the bacteria to the sponge was a defence mechanism used by the sponge[20]. lanthelline has previously been identified from the sponge *Aplysilla sp.* by MabCent [25].

1.7 Selection of methods:

The extraction method used in this project is based on the US National Cancer Institute (NCI) extraction method for marine organisms. The NCI method used, focus on extracting as broad as possible small molecules. Other extraction methods exist for macromolecules, proteins etc.

Preparative HPLC and flash chromatography are often used to purify compounds from complex matrices such as marine organisms. The flash offers a higher loading capacity than the preparative HPLC, but with a lower resolution. Hence, samples purified on a flash chromatography system will more often need a second purification step to obtain a pure compound.

Both preparative HPLC and flash chromatography might be used in combination with bioassay guided purification. Bioassay purification uses bioactivity results to isolate compounds from extracts. Different types of activity can be selected based on what one is looking for. In this project anticancer and antibacterial activity were selected.

Dereplication is a method used to recognize already known compounds with bioactivity from an extract, before spending time isolating them [2]. High resolution MS can be used to obtain the accurate mass and isotope distribution. The elemental composition calculated from the accurate mass and isotope distribution, can be used to search databases for known compounds. Several such databases exist, and among them is the Dictionary of Marine Natural Products that is an edited database with more than 35 000 compounds of marine origin.

2. Aim of the study

The aim of this study was to isolate ianthelline from a sponge extract. This should be achieved through bioassay guided purification. It was important to map the bioactivity to make sure that other bioactive compounds in the extracts were not discarded. Even though ianthelline have previously been described, little information about its bioactivity profile is available. It was therefore important to characterize ianthelline biologically.

3. Materials and Methods:

3.1 Extraction and HPLC fractionating of *Stryphnus fortis*:

3.1.1 Retrieving the sponge:

The sponge, *Stryphnus fortis* was collected in September 2007 northwest of Spitsbergen 79° 33'.30" N 8° 53'.00 E. It was dredge trawled at 333 m depth. The biomass sample was stored in the freezer (-22 °C).

3.1.2 Extraction of *Stryphnus fortis*:

Materials used:

Dichloromethane (Merck kgea, Darmstadt Germany)

Methanol (sigma-aldrich, München Germany)

Formic acid (Merck maursyre > 98 % pro analysi)

MilliQ water (Millipore, Billerica, MA, USA)

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

IKA® A11 basic grinder (IKA works, Staufen Germany)

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

Rotavapor: Heidolph Laborata 4002 (Nürnberg, Germany)

Whatman filter paper 125 Ø (Springfield Mill, England)

Preparation of the samples:

2.001 kg frozen material of *Stryphnus fortis* was cut into 2 cm cubes without the sponge melting and freeze dried for 2 days in 1/3 filled pyrex bowls covered with perforated aluminum foil. This is done to maximize the surface area so that the freeze drying becomes less time consuming. The perforated aluminum was used to prevent potential foam created during boiling, from enter into the freeze drier.

Aqueous extraction:

The freeze-dried cubes of *Stryphnus fortis* were grinded with an IKA® grinder and the powder was transferred to 4 tara 1 L Duran bottles (300 mL in each). The mass of the powder was measured. 300 mL MilliQ water was added to each bottle (total 1.2 L water) and the bottles were shaken until its content looked like porridge. The porridge was allowed to swell at 5 °C for 30 minutes. Afterwards 200 mL MilliQ water was added to each bottle (total 800 mL) and the bottles were shaken. The “soup” was extracted at 5 °C for 20 hours. The “sponge-soup” was transferred to centrifugal beakers and the remains in the bottles were washed to the centrifugal beakers with approximately 20 mL of water. The samples were then centrifuged at 4000 rpm, 5 °C for 30 minutes. The water supernatant was collected in bottles and stored at - 22 °C for 3 days. The pellet in the centrifugal beakers was resuspended with 250 mL MilliQ water (total 1.5 L water) and extracted once more for 30 minutes. Afterwards the samples were centrifuged at 4000 rpm, 5 °C for 30 minutes. The water supernatant was collected in bottles and stored at - 22 °C for 3 days. The pellet was distributed over the largest area possible in pyrex bowls, frozen to - 22 °C and continued to the organic extraction.

The frozen supernatant was melted and transferred to round flasks compatible with the freeze dryer. The supernatant was then frozen at - 22 °C while turning the bottles every 20 minutes to ensure as large as possible surface area of the ice. The supernatant was then freeze-dried. The freeze-dried material was grinded with mortar and pistil and transferred to tara 45 mL VWR tubes with caps. The tubes were marked as M10037-0-W01 and stored at - 22 °C. The freeze drying ensures concentration of compounds and dry powder is more stable than wet material.

Organic extraction:

The frozen pellet from the water extraction was freeze-dried for 2 days. The freeze-dried pellet was grinded by use of an IKA grinder to powder and filled to 3 1 L Duran bottles (250 mL powder in each bottle). 600 mL dichloromethane and methanol 1:1 was added to each of the bottles (total 1.8 L dichloromethane and methanol) and shaken. The pellet was extracted for 20 hours at 5 °C. The bottles were then shaken and the content was filtered through a Whatman Ø125mm filter with a Büchner flask. The substance that did not pass the filter was extracted once more with 300 mL dichloromethane and methanol 1:1 in each bottle (total

900 mL dichloromethane and methanol) for 30 minutes. The samples were filtrated again trough a Whatman Ø125mm filter.

Filtrate from the first and second extraction was mixed and evaporated on a rotavapor until it was viscous as syrup and transferred to glass tubes and stored at - 22 °C. The tubes were marked M10037-0-L01.

3.1.3 Preparation of the extracts from *Stryphnus fortis* before HPLC fractionating

Material used:

Ethanol 96 % (Sigma-aldrich)

MilliQ water (Millipore)

n-hexane AnaIR NORMAPUR (VWR international)

Acetonitril HPLC grade (Merck)

Centrifuge: Biofuge Pico (Heraeus)

Minishaker (VWR international, Radnor, Pennsylvania, USA)

Millex GS filter 0.22 µm (Millipore)

Rotavapor: Heidolph Laborata 4002

Edmund Bühler GmbH 5m-30 control shaker (Hechingen, Germany)

Preparation of the aqueous extract:

206.6 mg dry powder marked M10037-0-W01 was transferred to 14 mL polystyrene centrifugal tubes and 2000 µL MilliQ water was added. The tube was placed horizontally on an Edmund Bühler GmbH 5M -30 Control shaker for 90 minutes. 1 mL sample from the centrifugal tube was distributed evenly to two centrifugal tubes and each tube was added 4 mL 96% ethanol. The centrifugal tubes were shaken on a minishaker before placed in the freezer at -22 °C for 20 hours. The samples were collected from the freezer and shaken on a minishaker once more. The samples were then centrifuged at 5°C at 4000 rpm for 30 minutes. The supernatant was transferred to a 25 mL flask and evaporated on a rotavapor until the sample had a volume of less than 2 mL. The samples were distributed to two plastic micro centrifugal tubes and the tubes were adjusted with MilliQ water until it was 1 mL in each tube. The samples were centrifuged at 4000 rpm at 5 °C for 30 minutes.

A syringe was used to suck up the supernatant and press it trough a 0.22 µm Millex GS filter to a glass tube. The filtered sample was distributed between two tubes and the volume was

adjusted to 1.3 mL in each tube with MilliQ water. The two tubes were used in the preparative HPLC fractionation.

Preparation of the organic extract:

334.0 mg and 355.7 mg organic extract marked M10037-0-L01 were added to two glass tubes with 3 mL of hexane in each. 3 mL of 90% acetonitrile was added to each of the tubes and the tubes were shaken. The tubes were then centrifuged for 5 minutes at 4000 rpm. The hexane phases were transferred to two new tubes. The acetonitrile phases were kept. 3 mL 90 % acetonitrile was added to the hexane tubes and the tubes were stirred with a minishaker. The tubes were centrifuged and the hexane phases were discarded. All acetonitrile phases were transferred to a 25 mL flask and evaporated with a rotavapor until it was only approximately 1.5 mL left in the flask. The content in the 25 mL flask was divided between two micro plastic centrifugal tubes and the flask was washed with acetonitril until it was 1 mL in each micro plastic centrifugal tube. The two micro plastic centrifugal tubes were centrifuged at 13000 rpm for 30 minutes and the supernatant was transferred to two glass tubes and the amount in the tubes were adjusted to 1 mL in each with 90 % acetonitril. In order to homogenize the samples, the samples were mixed together and then divided between the two tubes again. The two tubes were used in the preparative HPLC fractionation.

3.1.4 HPLC fractionation of the extracts from *Stryphnus fortis*

Equipment:

Waters 600 controller (Milford, MA, USA)

Waters 2767 sample manager

Waters prep degasser

Waters 515 HPLC pump

Waters 2996 photodiode array detector

Waters 3100 mass detector

Waters flow splitter

SC250 Express SpeedVac Concentrator (Thermo Fisher Scientific)

RTV4104 Refrigerated Trap (Thermo Fisher Scientific)

Software:

Waters MassLynx 4.1; Waters OpenLynx 3.5; Waters FractionLynx 3.5

Mobile Phases:

A: 0.1 % formic acid (Merck formic acid pro analysi) in MilliQ water

B: 0.1 % formic acid in acetonitrile (Merck acetonitrile HPLC grade)

Aqueous and organic extracts fractionation:

Column used for aqueous extract fractionation: Atlantis prep α C18 10 μ m 10*250 mm (Waters).

Column used for organic extract fractionation: xTerra prep RP18 10 μ m 10*300 mm (Waters)

Semi-preparative HPLC was used to fractionate the extracts. One fraction was equivalent to one minute elution from the column. A linear gradient was selected and the gradient used for the aqueous extract is shown in table 1, and the gradient used for the organic extract is shown in table 2.

The 40 fractions were distributed to eight deep well plates (96-well); 1.4 mL fraction in each well; the first fraction was in A1 of the deep well plates, the second fraction was in B1 of the deep well plates and so on. The deep well plates with fractions from the aqueous extract were marked M10037-0-W01. The deep well plates with fractions from the organic extract were marked M10037-0-L01. The deep well plates were dried in a speedvac for removing the acetonitrile and then freeze dried in order to remove the water.

Table 1: Gradient used for the aqueous extract fractionation:

Step	Time (min)	Flow (mL/min)	% A	% B
1	Initial	6	95	5
2	3	6	95	5
3	30	6	50	50
4	35	6	5	95
5	40	6	5	95

Table 2: HPLC gradient for fractionation of the organic extract

Step	Time	Flow	% A	% B
1	Initial	6	80	20
2	2	6	80	20
3	30	6	0	100
4	40	6	0	100

3.2 General antibacterial assay with *E. Coli*, *P. aeruginosa*, *E. Faecalis*, MRSA and *S. Aureus*.

Material used:

Brain hearth infusion (Oxoid, Cambridge, England)

Müller Hinton bullion (Merck)

Gentamicyn (Biochrome)

Blood agar plates (University Hospital of North-Norway)

ISA plates (University Hospital of North-Norway)

Heidolph Incubator 1000

Victor 3 Multilabel Counter (PerkinElmer, Waltham, MA, USA)

Software: WorkOut 2.5 (dazdaq, Brighton, England)

Preparation of the bacteria:

The bacteria species (see table 3) were plated from freeze stock (-80 °C) to blood agar over night (+20 hours). 10 µL plastic inoculating loops were used to seed the different bacteria from the blood agar to a 8 mL fitting growth media (see table 3). The bacteria were incubated for 20 hours at 37 °C.

Table 3: Preferential growth media for each strain of bacteria:

Bacteria	Growth media	Bacteria density in well
<i>Staphylococcus aureus</i> ATCC 25923	Mueller Hinton bullion	$0.5-3 \times 10^5$ CFU/mL
<i>Escherichia coli</i> ATCC 25922	Mueller Hinton bullion	$0.5-3 \times 10^5$ CFU/mL
<i>Enterococcus faecalis</i> ATCC 29212	Brain Heart Infusion	$0.5-3 \times 10^5$ CFU/mL
<i>Pseudomonas aeruginosa</i> ATCC 27853	Mueller Hinton Bullion	$3-7 \times 10^4$ CFU/mL
Methicillin resistant <i>Staphylococcus aureus</i> (MRSA) ATCC 33591	Mueller Hinton	$0.5-3 \times 10^5$ CFU/mL

2 mL of the bacteria suspension were transferred to 25 mL new growth media. *E. coli* and *E. faecalis* were incubated for 1.5 hour, while *S. aureus*, *P. aeruginosa* and MRSA were incubated for 2.5 hour.

100 μ L of the bacteria suspension which had been incubated for 1.5 – 2.5 hours, were diluted in fresh growth media by a factor of 100. 1.00 mL of this media was diluted further by a factor of 10. In total the bacteria suspension was diluted 1000 times.

The diluted suspension was added to the microtiter plates immediately after the control counting was performed.

Control counting: Bacteria suspension that had been incubating for 1.5 – 2.5 hours was diluted in 0.9 % NaCl autoclaved MilliQ water by a factor of 1 000 000. 100 μ L of the NaCl in MilliQ water diluted bacteria suspension was seeded on ISA plates and incubated. After 18 hours the number of bacteria colonies on the ISA plates was counted.

Gentamicyn control: Gentamicyn 10 mg/mL was diluted in MilliQ water to concentrations: 0.025 μ g/mL, 0.06 μ g/mL, 0.125 μ g/mL, 0.25 μ g/mL, 0.5 μ g/mL, 1 μ g/mL, 2 μ g/mL, 4 μ g/mL, 8 μ g/mL, 20 μ g/mL and 30 μ g/mL. The gentamicyn control was diluted by factor 2 in the microtiter plates with bacteria suspension. The gentamicyn control was incubated for 20 hours at 37 °C.

Microtiter plate setup:

Microtiter plates were marked with sample name and bacteria name (one plate for each bacterium).

50 μ L sample (HPLC fractions or ianthelline) was added to the microtiter plate in two parallels for each bacterium specie. 50 μ L bacteria suspension were added to the wells in the microtiter plate, except for row 1, where the negative control was.

Negative control: 50 μ L sterile appropriate growth media was added to row 1 with 50 μ L autoclaved MilliQ water.

Positive control: 50 μ L bacteria suspension were added to row 12 with 50 μ L autoclaved MilliQ water.

The plates were incubated for 20 hours at 37 °C.

Antibacterial analysis:

Bacterial growth renders the culture media opaque. Light absorbance determines the percentage survival using a positive and negative growth control.

Sample microtiter plates: The plates were visually inspected and visible growth inhibition was noted. The plates were also analyzed with Victor multilabel counter to measure light absorbance at 600nm.

Gentamicyn control: The plates were visually inspected and the minimum inhibitory concentration (MIC) for gentamicyn was noted. The result was compared to the expected MIC for gentamicyn.

Control counting: Numbers of colony forming units (CFU) were counted and concentration in the stock solution was calculated. The CFU counted were controlled that it corresponded with the number CFU intended for plating.

3.3 General anticancer assay:

Material used:

Fetal bovine serum (FBS) (Biochrom, Berlin, Germany)

Dulbecco's Phosphate Buffered Saline (PBS) (Biochrom)

RPMI-1640, (Biochrom)

Dulbecco's modified media (DMEM) (Invitrogen, California. USA)

Eagle minimal essential media (EMEM), (Biochrome)

Gentamicyn 10 mg/mL (Biochrom)

Non-essential amino acids (NEAA), (Biochrom)

L-alanyl-L-Glutamin 200 μ M (Biochrome)

Sodium Puryvat 100 μ M (Biochrom)

Cell Titer 96[®] Aqueous One Solution Reagent (Promega, USA)

DTX multimode detector (Beckman Coulter, INC CA92821 USA)

Trypsin (Invitrogen)

Viability of the cancer cells was measured using MTS tetrazolium reagent which would be converted to formazan if metabolic active cells were present. Formazan has a peak absorbance of 490 nm, which can be used along with a negative and positive control to quantify the percentage live cells [26].

All cancer cell lines used in these experiments were adherent cancer cell, which means that they adhere to the bottom of the cell culture flask and in the microtiter plates.

Splitting of cancer cells:

Adherent cancer cells were split in order to maintain them in monolayer and plate the cells evenly in all the microtiter wells.

The cells were inspected with a light microscope that their morphology looked normal and they were in monolayer on the bottom of the cell culture flask. Cell culture media were poured out. The cell culture flask was washed with 10 mL heated (37 °C) Dulbecco's Phosphate Buffered Saline (PBS). The cells were split into individual cells using 5 mL 2.5 % trypsin in PBS. The trypsin also detach the cells from the bottom of the flask. Trypsin act on the cell walls and too long treatment with trypsin would cause the cells to die. Trypsin were poured out after approximately 5-6 seconds, and the flask were placed in an incubator until

the cells could visually be seen sliding of the bottom of the flask (approximately 3 minutes). The serum in the media inhibits the trypsin activity. 10 mL preheated fresh media were added to the cells (see table4).

Table 4: Optimal cell media for the different cell lines

Cell line	Optimum cell media
A2058 (human melanoma), ATCC CRL-11147	10 % Fetal bovine serum (FBS) and 0.1 % gentamicyn in D-MEM.
HT29 (human colon carcinoma), ATCC HTB-38	10 % FBS and 0.1 % gentamicyn in RPMI-1640
MRC5 (normal lung fibroblast), ATCC CCL-171	10 % FBS, 1 % NEAA, 1 % Na Pyruvat, 1 % L-glutamin and 0.1 % gentamicyn in E-MEM
MCF7 (human breast carcinoma), ATCC HTB-22	10 % FBS, 1 % NEAA, 1 % Na Pyruvat, 1 % L-glutamin and 0.1 % gentamicyn in E-MEM

A fitting amount of media with cells were transferred to a new cell culture flask. (depending on the concentration of the cells established using the microscope previously and the cell line) and added fresh media (see table 4) until it was 10 -15 mL media in the flask. The new flask was kept at 37 °C with 5 % CO₂ until it needed to be maintained again.

500 µL from the old cell culture flask was added to 500 µL trypan blue. Cells with disrupted cellwalls would become blue with trypan blue, while intact cells would be non-stained. 10 µL of the mixture was added to a hemacytometer (counting chamber) and the normal looking non-stained cells were counted. 2000 cells in 100 µL media (mixture of media with cells and fresh media) per well were seeded in the microtiter plate. The plate was stored at 37 °C with 5 % CO₂ overnight unless indicated differently.

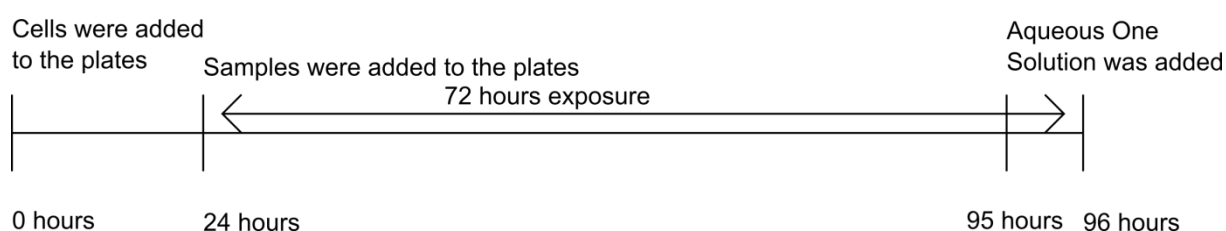


Figure 3: Standard cancer assay time-line. After 96 hours absorbance at 485 nm was measured.

Adding of samples:

After the cells in the plates had been incubated overnight the cells were inspected in microscope to check that they were alive and evenly distributed to all the wells. Media from the plates was removed by turning the plates up-side-down on paper and tapping them. Since the cells were adherent they stick to the bottom of the wells.

Fresh 50 μ L RPMI- 1640 with 10% FBS and 0.1 % gentamicyn were added to the wells immediately after removing old media. 50 μ L sample (flash fractions, HPLC fractions or ianthelline in RPMI-1640 with 0.1 % gentamicyn but without 10 % FBS) were added to the cells in three parallel wells. Whenever possible the outer wells were avoided to prevent drying and subsequently false positives.

The cells were then exposed to the test samples for 72 hours in a standard procedure (see figure 3). 10 μ L Aqueous One Solution Reagent were added to each well and the plates were incubated for 1 hour at 37 °C in 5 % CO₂. Afterwards the microtiter plates were tapped gently in order to “distribute” the color evenly in the wells. The microtiter lids were swiped with paper towels in order to remove water droplets which would otherwise prevent the robot to remove the lids.

The plates were analyzed by measuring light absorbance at 485 nm with DTX Multimode Detector. Percentage survival was calculated using a negative standard for each of the cell lines measured previously by MabCent and the positive controls (cells + 100 μ L 5 % FBS in RPMI-1640) row 11 and 12 in the plates.

3.4 Primary screening of the HPLC fractions from *Stryphnus fortis*

3.4.1 Primary screening of HPLC fractions of the extracts from *Stryphnus fortis* against

bacteria:

Material:

Dimethyl sulfoxide (DMSO), (Sigma-Aldrich)

Gentamicyn (biochrom)

7.5 μ L dimethyl sulfoxide was added to each of the dried aqueous and organic HPLC fractions (deep well plates marked M10037-0-L01 and deep well plates marked M10037-0-W01) and the plates were set on shaker for 10 min for the aqueous extract and 1 hour for the organic extract. 750 μ L MilliQ water was added to each fraction and the deep well plates were on shaker for 2 hours for the water extract and 4 hours for the organic extract.

The fractions were added to microtiter plates as described in section 3.2 and as shown in figure 4:

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	1	1	9	9	17	17	25	25	33	33	P
B	N	2	2	10	10	18	18	26	26	34	34	P
C	N	3	3	11	11	19	19	27	27	35	35	P
D	N	4	4	12	12	20	20	28	28	36	36	P
E	N	5	5	13	13	21	21	29	29	37	37	P
F	N	6	6	14	14	22	22	30	30	38	38	P
G	N	7	7	15	15	23	23	31	31	39	39	P
H	N	8	8	16	16	24	24	32	32	40	40	P

Figure 4: Microtiter plate setup.

The numbers indicate fraction number (bold number is row number). N is negative control. P is positive control.

3.4.2 Anticancer screening of HPLC fractions of the extracts of *Stryphnus fortis*:

Material:

DMSO (sigma-aldrich) and the rest as described in section 3.3

To the organic HPLC deep well plate M10037-0-L01 7.5 μ L dimethyl sulfoxide (DMSO) was added to each well and was placed on shaker for 1.5 hour. Heated (37°C) 750 μ L RPMI-1640 were added to the fractions

The aqueous HPLC deep well plate M10037-0-W01 was added heated (37°C) 750 μ L RPMI-1640 without FBS to the fractions and was placed on shaker for 2 hours.

Melanoma A2058 were plated in microtiter plates and the general cancer assay described in 3.3 was followed. HPLC fractions were added as shown in figure 5.

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A		1	1	1	8	8	8	15	15	15	P	P
B		2	2	2	9	9	9	16	16	16	P	P
C		3	3	3	10	10	10	17	17	17	P	P
D		4	4	4	11	11	11	18	18	18	P	P
E		5	5	5	12	12	12	19	19	19	P	P
F		6	6	6	13	13	13	20	20	20	P	P
G		7	7	7	14	14	14				P	P
H											P	P

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A		21	21	21	28	28	28	35	35	35	P	P
B		22	22	22	29	29	29	36	36	36	P	P
C		23	23	23	30	30	30	37	37	37	P	P
D		24	24	24	31	31	31	38	38	38	P	P
E		25	25	25	32	32	32	39	39	39	P	P
F		26	26	26	33	33	33	40	40	40	P	P
G		27	27	27	34	34	34				P	P
H											P	P

Figure 5: Two microtiter plates were needed for screening HPLC fractions. P stands for positive control. The numbers indicate the different HPLC fractions.

3.5 Dereplication of active HPLC fractions from the organic extract of *Stryphnus fortis*:

Waters Acquity UPLC

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

MS: Waters LCT Premier

Software: MassLynx 4.1

Mobile Phases:

A: 0.1 % formic acid (Merk formic acid pro analysi) in MilliQ water

B: 0.1 % formic acid (Merk formic acid pro analysi) in acetonitril (Merk acetonitril HPLC grade)

Standard ESI+ conditions used for TOF-MS:

Capillary: 2.6 kV

Cone: 35 V

Source temperature: 120 °C

Desolvation temperature: 300 °C

Cone gas flow: 5 L/hour

Desolvation gas flow: 550 L/hour

Syringe pump flow: 5 µL/min

Table 5 : UPLC gradient used for dereplication. The gradient was linear

Step	Time (min)	Flow (mL/min)	% A	% B
1	Initial	0.300	90	10
2	3.50	0.300	0	100

Bioactive fractions were analyzed using UPLC-ESI+ TOF-MS to obtain the accurate mass of the active compounds. ESI was set in positive mode. Masslynx predicted the elemental composition of the active compound from the accurate m/z and isotope distribution. The acquired elemental composition was used to search the Dictionary of Marine Products

database for natural products. This is normally done to prevent wasting of time on already known compounds, however in this project it was done to confirm the presence of ianthelline.

3.6 Isolation of ianthelline from the extract from *Stryphnus fortis*

The organic extract from *Stryphnus fortis* was first fractionated by flash chromatography, since the flash system used could take up to 2 g sample in one column, at the cost of lower resolution. Cancer screening and MS of the flash fractions from the extract from *Stryphnus fortis* was done to identify which fractions would proceed to the semi-preparative HPLC.

3.6.1 Flash chromatography of the organic extract from *Stryphnus fortis*:

Materials:

SC250 Express SpeedVac Concentrator (Thermo)

RVT4104 Refrigerated Vapor Trap (Thermo)

Flash system: Biotage SP4 (Uppsala, Sweden)

Acetone (Sigma)

Methanol Prolabo HiPerSolv for HPLC isocratic grade (VWR)

Flash stationary phase: Diaion HP20SS Supeclo (Sigma-aldrich)

Flash was used two times. The first time flash was used it was done to identify the active compounds with MS and it required only one column for the organic extract. The second time flash was used, it was used as a step to isolate ianthelline from the organic extract and 6 SNAP columns were used for the organic extract.

Preparation of Snap column (Biotage):

6 g Diaion HP20SS Supelco column material was added into a 100 mL Erlenmeyer flask.

Diaion HP20SS Supelco column material has styrene-divinylbenzene as adsorbent.

Conditioning of the stationary phase: Approximately 300 mL methanol was poured over the column material and the flask was swirled. The flask was left for 15 minutes and then the

methanol was poured out. Then approximately 300 mL water was added and the flask was left for another 25 minutes. A Vacuum manifold was used to packing the biotage column with the packaging material.

Preparation of the organic extract before flash:

80 mL hexane was added to a flask with 2 g organic extract M10037-0-L01 and the flask was swirled. The sample was liquid-liquid extracted twice with 40 mL 90 % methanol. The methanol phases were kept, while the hexane phase was discarded. The methanol phases were evaporated on a rotavapor until it was less than 10 mL left. The sample was transferred to a glass tube and the flask was washed with 100 % methanol until it was approximately 5 mL in the tube. The sample in the tube was divided between two tubes and 1 g diaion HP20SS was added to each of the tubes. The tubes were vacuum centrifuged (SC250 Express SpeedVac Concentrator and RVT4104 Refrigerated Vapor Trap).

Flash fractionation:

The two tubes with samples were added to the top of the stationary phase in the column and the gradient in table 6 was used (stepwise gradient):

Table 6: Flash gradient

Time (minutes)	% Water	% Methanol	% Acetone
1-6	95	5	
7-12	75	25	
13-18	50	50	
19-24	25	75	
25-30		100	
31-34		50	50
35-44			100

The flow rate was 12 mL/min and each fraction was 24 mL with a total of 27 fractions. The fractions were distributed into deep well plates in the same manner as for the preparative HPLC fractions (see chapter 3.1.4).

3.6.2 Anticancer screening on the flash fractions from the organic extract of *Stryphnus*

fortis:

Material used:

Dimethyl sulfoxide (DMSO), (Sigma-Aldrich) and as described in section 3.3

7.5 μ L DMSO was added to each of the well of the flash fractions of the organic extract in the deep well plate. The deep well plate was placed on a shaker for 1.5 hour.

Deep well plate with organic flash fractions was added 7.5 μ L DMSO to each well and were placed on shaker for 1.5 hour. Heated (37°C) 750 μ L RPMI-1640 was added to the fractions

The general cancer assay method described in section 3.3 was followed. Flash fractions were added as shown in figure 6.

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A		1	1	1	8	8	8	15	15	15	P	P
B		2	2	2	9	9	9	16	16	16	P	P
C		3	3	3	10	10	10	17	17	17	P	P
D		4	4	4	11	11	11	18	18	18	P	P
E		5	5	5	12	12	12	19	19	19	P	P
F		6	6	6	13	13	13	20	20	20	P	P
G		7	7	7	14	14	14				P	P
H											P	P

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A		21	21	21							P	P
B		22	22	22							P	P
C		23	23	23							P	P
D		24	24	24							P	P
E		25	25	25							P	P
F		26	26	26							P	P
G		27	27	27							P	P
H											P	P

Figure 6: Flash fraction cancer cell plate set up. Two microtiter plates were used. P stands for positive control. The numbers indicate the different flash fractions.

3.6.3 ESI+ MS of selected flash fractions of the organic extract from *Stryphnus fortis*:

Equipment:

Waters 600 controller

Waters 2767 sample manager

Waters pep degasser

Waters 515 HPLC pump

Waters 2996 photodiode array detector

Waters flow splitter

Waters 3100 mass detector

ESI+ mode

Capillary: 3 kV

Cone: 35 kV

Source temperature: 120 °C

Desolvation temperature: 300 °C

Cone Gas: 5 L/hour

Desolvation gas: 600 L/hour

Software:

MassLynx 4.1; OpenLynx 3.5; FractionLynx 3.5

Mobile Phases:

A: 0.1 % formic acid (Merck formic acid pro analysi) in MilliQ water

B: 0.1 % formic acid in acetonitrile (Merck acetonitrile HPLC grade)

Flash fractions 2, 6, 8, 10, 11, 12, 14, 16, 18 and 20 were selected to be analyzed by MS, based on the bioactivity profile shown in figure 18. Flash fraction 8, 10, 11, 12, and 14 had the largest amount of ianthelline and flash fractions 8-15 were used for further purification.

3.6.4 Isolation of ianthelline from flash fractions of the organic extract from *Stryphnus fortis* using HPLC-MS:

Material used:

Rotavapor: Heidolph Laborata 4002

Centrifuge: Heraeus Biofuge Pico

Methanol HPLC grade (Merck)

n-hexane AnaIR Nomrapur (VWR)

The same MS equipment, software and HPLC mobile phases described in section 3.6.3 were used for this experiment.

Preparation of the flash fractions from the organic extract from *Stryphnus fortis*:

Flash fractions 8-15 were evaporated on a rotavapor until it was as syrup. 400 μ L sample was mixed with 300 μ L methanol and 300 μ L hexane. The samples were centrifuged at 13000 rpm for 3 minutes. The methanol phase was used to the HPLC isolation of ianthelline, while the hexane phase was discarded.

HPLC-MS setup:

Column: X-terra prep RP18 column 10*300mm

The HPLC-MS was programmed to keep the sample which yielded a threshold over 5 millions area units at 473.9 m/z .

Table 7: The gradient used for isolation of ianthelline. A linear gradient was selected

Step	Time (minutes)	Flow (mL/min)	% A	% B
1		6	70	30
2	10	6	50	50
3	11	6	0	100
4	15	6	0	100
5	16	6	0	100

3.7 Confirming that the purified compound from *Stryphnus fortis* was ianthelline

Fractions containing ianthelline from HPLC-MS isolation were evaporated using a rotavapor.

3.7.1 Confirming the presence of ianthelline using MS-TOF

The same method as the dereplication described in section 3.5 was used. Accurate m/z and isotope distribution of ianthelline were used to calculate the elemental composition by Masslynx.

3.7.2 Confirming the presence of ianthelline using MS-MS.

Waters Quattro Premier XE

Collision gas: Argon 4.0 (Yara, Oslo, Norway)

Polarity: ESI+

Capillary: 3.50kV

Cone: 30.00 V

Source temperature: 100 °C

Desolvation temperature: 250 °C

Nebulizator gas flow: 21 L/Hour

Desolvation gas flow: 98 L/hour

Collision energy: 20

Syringe pump flow: 20.0 μ L/min

Argon was used as collision gas. Electro Spray ionization in positive mode was used and Quadrupole 1 was set to let m/z of 473.80 into the collision cell. The collision cell energy was set to 20 and Quadrupole 2 was set to scan. Ianthelline was infused directly into the MS with the built-in syringe pump.

3.7.3 Confirming the presence of ianthelline by NMR

Material used:

Varian Spectrometer 400 Mhz (Varian, Palo Alto, USA)

Deuterated DMSO (Sigma)

Ianthelline was dissolved in deuterated DMSO and ^1H NMR, ^{13}C NMR, COSY (CORrelation Spectroscopy), HMBC (HETRONuclear Multiple-bond CORrelation) and HSQC (HETRONuclear Single-Quantum CORrelation) were recorded.

3.8 Biological characterization of ianthelline

Isolated ianthelline was dissolved in DMSO (sigma-aldrich) to a concentration of 20 mg/mL.

Ianthelline was stored in DMSO at 5 °C.

3.8.1 Dose-response of ianthelline against *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus* and MRSA:

Material:

MilliQ water (Millipore)

Dimethyl Sulphoxide (Sigma-Aldrich)

Gentamicin

Ianthelline 20 mg/mL in DMSO was mixed with autoclaved MilliQ water in the concentration of: 500 µg/mL, 250 µg/mL, 150 µg/mL, 100 µg/mL and 50 µg/mL. In the microtiter plates ianthelline was diluted by a factor of 2.

DMSO positive controls were made in the concentrations: 2.5 % DMSO, 1 % DMSO and 0.5 % DMSO in autoclaved MilliQ water. The DMSO was diluted by a factor of 2 in the microtiter wells. DMSO positive controls were made to make sure the activity of ianthelline was not caused by DMSO.

The antibacterial assay was done as described in section 3.2 and the microtiter plate set up as shown as in figure 7.

	1	2	3	4	5	6	7	8	9	10	11	12
A	N	250 µg/mL	250 µg/mL									P
B	N	100 µg/mL	100 µg/mL									P
C	N	75 µg/mL	75 µg/mL									P
D	N	50 µg/mL	50 µg/mL									P
E	N	25 µg/mL	25 µg/mL									P
F	N	1.25 % DMSO	1.25 % DMSO									P
G	N	0.5 % DMSO	0.5 % DMSO									P
H	N	0.25 % DMSO										P

Figure 7: Microtiter plate setup.

N is negative control. P is positive control. Ianthelline were placed in the concentrations indicated in red. The % DMSO indicates the concentration of DMSO in the control wells.

3.8.2 Antioxidant activity of ianthelline, dose-response:

Material used:

Curcumin (Sigma)

Dimethyl sulphoxide (DMSO), (Sigma)

MilliQ water

6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid 97 % (TROLOX) (sigma-aldrich)

Sodium acetat trihydrate (Natrium acetat trihydrat pro analysi, Merck)

37 % HCl

2,4,6-tripyridyl-s-triazine (TPTZ), (Fluka Sigma-aldrich)

Antioxidant activity was measured using a ferric reducing ability of plasma (FRAP) assay. The FRAP assay detects the amount ferric-tripyridyltriazine reduced to Fe(II) by antioxidants [27].

Ianthelline 20 mg/mL was mixed with MilliQ water to the concentrations: 2125 µg/mL, 850 µg/mL, 425 µg/mL and 212.5 µg/mL. The samples were diluted by a factor of 8.5 in the microtiter plate.

Curcumin was dissolved in DMSO MilliQ water 1:1 in the concentration 20 mg/mL. The curcumin was further mixed with MilliQ water to the concentrations: 2125 µg/mL, 850 µg/mL, 425 µg/mL and 212.5 µg/mL. The samples were diluted by a factor of 8.5 in the microtiter plate.

31.25 mg TROLOX was dissolved in 1.25 mL Methanol. The solution was then mixed with water to the concentrations: 250 µM, 125 µM, 62.5 µM, 31.25 µM and 15.625 µM. The TROLOX was diluted by a factor of 8.5 in the microtiter plate. TROLOX is a vitamin E analogue which was used as a reference for the ferric reducing ability of ianthelline and curcumin.

Ferric reducing ability of plasma (FRAP) –reagent: 25.7 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 5 mL MilliQ water, 15.6 mg 2,4,6-tripiridyl-s-triazine, 5 mL 40 mM HCl, 50 mL acetate buffer pH 3.6 (3.01 g acetate (Merck) + 16 mL acetic acid + MilliQ water to 1000 mL.). The FRAP reagent was heated in incubator (37 °C) for 30 minutes.

20 µL sample and TROLOX were transferred into separate wells on a microtiter plate (see figure 8). 20 µL DMSO was added to some of the wells to control that the ferric reducing ability was not caused by DMSO. In the wells H11 and H12 170 µL MilliQ water was added as a blank sample. 150 µL FRAP-reagent was added to the wells except for H11 and H12. The plate was incubated for 30 minutes at 37 °C and then shaken to evenly distribute the blue color in case of a reaction. Light absorbance at 593 nm was measured using DTX multimode detector.

	1	2	3	4	5	6	7	8	9	10	11	12
A	250 µg/mL	250 µg/mL									0 µM (20 µL water)	0 µM (20 µL water)
B	100 µg/mL	100 µg/mL									1.83 µM	1.83 µM
C	50 µg/mL	50 µg/mL									3.68 µM	3.68 µM
D	25 µg/mL	25 µg/mL			20 µL DMSO	20 µL DMSO					7.35 µM	7.35 µM
E	250 µg/mL	250 µg/mL			20 µL DMSO	20 µL DMSO					14.71 µM	14.71 µM
F	100 µg/mL	100 µg/mL			20 µL water	20 µL water					29.41 µM	29.41 µM
G	50 µg/mL	50 µg/mL			20 µL water	20 µL water						
H	25 µg/mL	25 µg/mL									170 µL water	170 µL water

Figure 8: Microtiter plate set up for ianthelline dose-response antioxidant activity. Ianthelline is in the concentration marked in red on the plate. Curcumin is in the wells marked with green. Trolox is in the wells marked with blue.

3.8.3 Ianthelline dose-response Cancer:

Material used:

See section 3.3

Ianthelline 20 mg/mL in DMSO was added to RPMI-1640 with 0.1 % gentamicyn in the following concentrations: 500 µg/mL, 200 µg/mL, 150 µg/mL, 100 µg/mL and 50 µg/mL.

Upon adding the samples to the plates the samples were diluted by a factor of 2.

Positive control: To make sure DMSO were not the cause of activity, controls were added DMSO in the concentrations: 2.5 %, 1 %, 0.5 % and 0 %. Upon adding the samples to the plates the samples were diluted by a factor of 2.

A2058 (melanoma), HT29 (colon carcinoma), MCF7 (breast carcinoma) and MRC5 (normal lung fibroblasts) were plated in separate microtiter plates and incubated for 24 hours at 37 °C in 5 % CO₂. Afterwards ianthelline samples were added as described in 3.3 as shown in

figure 9. After 72 of hours of exposure Aqueous One Solution was added and the plates were read.

	1	2	3	4	5	6	7	8	9	10	11	12
A											0.5 % DMSO	0.5 % DMSO
B		250 µg/mL	250 µg/mL	250 µg/mL	1.25 % DMSO	1.25 % DMSO	1.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.5 % DMSO	0.5 % DMSO
C		100 µg/mL	100 µg/mL	100 µg/mL	1.25 % DMSO	1.25 % DMSO	1.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.5 % DMSO	0.5 % DMSO
D		75 µg/mL	75 µg/mL	75 µg/mL	1.25 % DMSO	1.25 % DMSO	1.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.5 % DMSO	0.5 % DMSO
E		50 µg/mL	50 µg/mL	50 µg/mL	1.25 % DMSO	1.25 % DMSO	1.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.25 % DMSO	0.5 % DMSO	0.5 % DMSO
F		25 µg/mL	25 µg/mL	25 µg/mL	0 % DMSO	0 % DMSO	0 % DMSO	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
G											0.5 % DMSO	0.5 % DMSO
H											0.5 % DMSO	0.5 % DMSO

Figure 9: lanthelline dose-response cancer plate set up. lanthelline were placed in the final concentrations indicated in red.

The % DMSO indicates the concentration of DMSO in the control wells.

3.8.4 Effect of time of exposure of ianthelline against cancer:

Material used:

See section 3.3

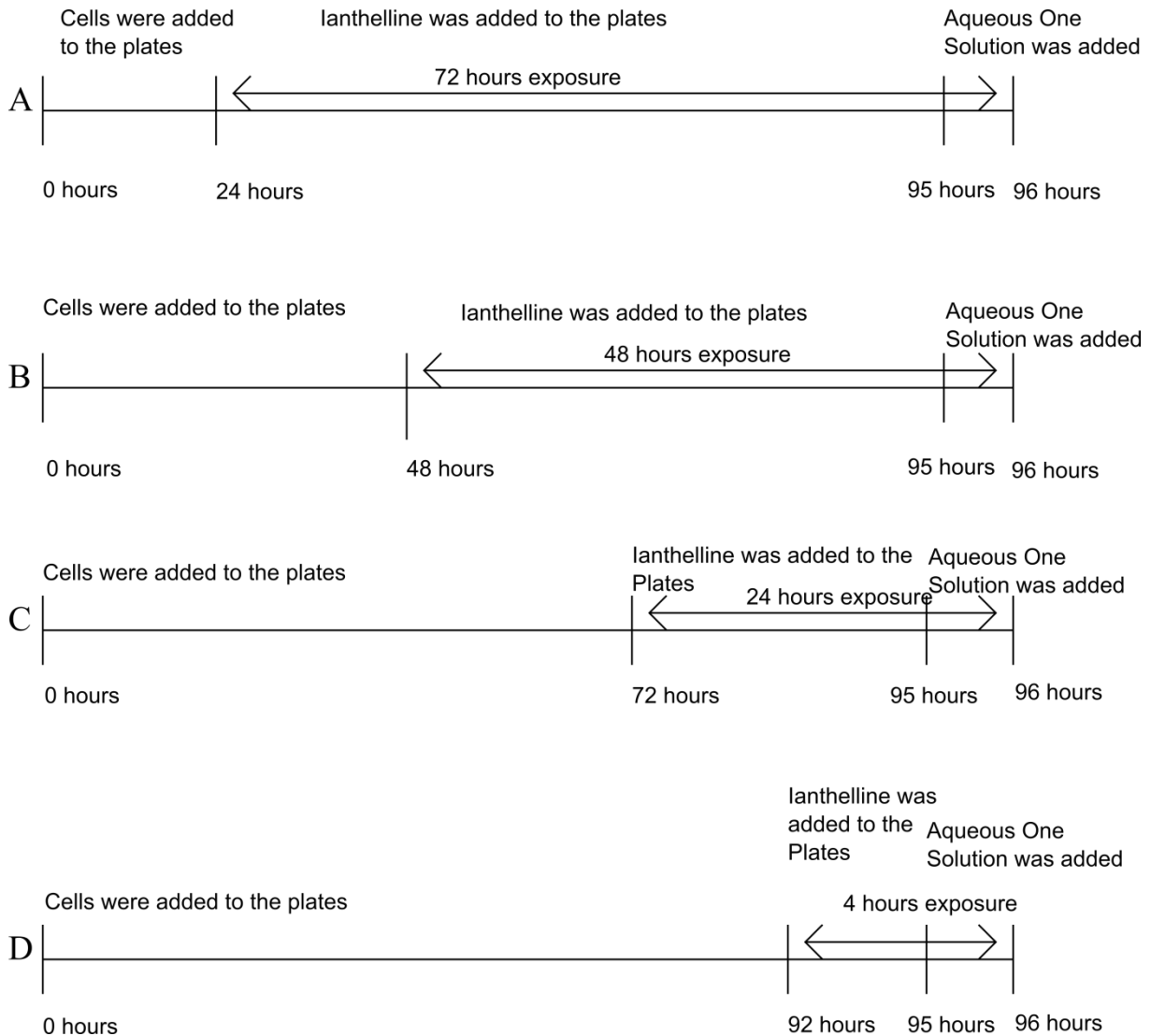


Figure 10: Cancer assay time-line for ianthelline dose-response-time. After 96 hours light absorbance at 485 nm was measured. A shows the time-line for 72 hours exposure to ianthelline. B shows the time-line for 48 hours exposure to ianthelline. C shows the time-line for 24 hours exposure to ianthelline. D shows the time-line for 4 hours exposure to ianthelline

Ianthelline 20 µg/mL in DMSO were added to RPMI-1640 with 0.1 % gentamicin in the following concentrations: 200 µg/mL, 180 µg/mL, 160 µg/mL, 140 µg/mL, 120 µg/mL, 100 µg/mL, 80 µg/mL, 60 µg/mL and 40 µg/mL. Upon adding the samples to the plates the samples were diluted by a factor of 2.

Positive control: To make sure DMSO was not the cause of activity, controls were added DMSO in the concentrations: 2 %, 1 % and 0 %. Upon adding the samples to the plates the samples were diluted by a factor of 2.

A2058 (melanoma), HT29 (colon carcinoma), MCF7 (breast carcinoma) and MRC5 (normal lung fibroblasts) were plated in separate microtiter plates as described in section 3.3 and the microtiter plates were incubated. The cancer cells were exposed to ianthelline either for 72 hours, 48 hours, 24 hours or 4 hours. See figure 10 for experimental design and figure 11 for microtiter layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A											0.5 % DMSO	0.5 % DMSO
B		100 µg/mL	100 µg/mL	100 µg/mL	40 µg/mL	40 µg/mL	40 µg/mL	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
C		90 µg/mL	90 µg/mL	90 µg/mL	30 µg/mL	30 µg/mL	30 µg/mL	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
D		80 µg/mL	80 µg/mL	80 µg/mL	20 µg/mL	20 µg/mL	20 µg/mL	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
E		70 µg/mL	70 µg/mL	70 µg/mL	1 % DMSO	1 % DMSO	1 % DMSO	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
F		60 µg/mL	60 µg/mL	60 µg/mL	1 % DMSO	1 % DMSO	1 % DMSO	0 % DMSO	0 % DMSO	0 % DMSO	0.5 % DMSO	0.5 % DMSO
G		50 µg/mL	50 µg/mL	50 µg/mL	1 % DMSO	1 % DMSO	1 % DMSO				0.5 % DMSO	0.5 % DMSO
H											0.5 % DMSO	0.5 % DMSO

Figure 11: Ianthelline dose-response-time cancer plate set up. Ianthelline were placed in the concentrations indicated in red. The % DMSO indicates the concentration of DMSO in the control wells.

3.8.5 PKA and Abl inhibition of purified ianthelline from *Stryphnus fortis*:

Material used:

Luminescence reader: Perkin Elmer Envision

White 384 well microtiter plate: OptiPlate-384 (Perkin Elmer)

Kinase RR-Kit (Biothema, Stockholm, Sweden)

ATPReagent SL

Diluent C

ATP standard 5 μ M

Kinase RR buffer

DMSO (sigma-aldrich)

MilliQ water (Millipore)

Staurosporin (Biomol International, Plymouth Meeting, PA)

DL-dithiothreitol (DTT) (Sigma-aldrich)

AbLTID EK-12 (GL Biochem, Shanghai, China¹)

Kemptide (GL Biochem)

Abl enzyme (Millipore)

PKA enzyme (Millipore)

Ianthelline in 20 mg/mL DMSO was diluted with MilliQ water to the concentrations: 500 μ g/mL ianthelline, 375 μ g/mL ianthelline, 250 μ g/mL ianthelline and 125 μ g/mL ianthelline. In the assay ianthelline was diluted by a factor of 5.

Preparation of the reagent mixture and blank mixture:

ATPReagent SL (Luciferase and luciferin) was dissolved in 4 mL Diluent C. ATPReagent SL in Diluent C, Kinase RR buffer (Protein stabilizers), PKA/Abl enzyme, MilliQ water, DL-dithiothreitol (reduces disulphide bridges in proteins) and Kemptid/AbLTide (protein substrate) were mixed together according to table 8

Table 8: Reagent mixture and blank mixture composition.

Active PKA mix	Blank PKA mix	Active Abl mix	Blank Abl mix
7500 μ L Kinase RR buffer	117.5 μ L active PKA mix without Kemptide	7500 μ L Kinase RR buffer	7500 μ L Kinase RR buffer
1200 μ L ATPReagent SL in Diluent C (Konsentrasjon av luciferase og luciferin?)	2.5 μ L MilliQ water	1200 μ L ATPReagent SL in Diluent C (Konsentrasjon av luciferase og luciferin?)	1200 μ L ATPReagent SL in Diluent C (Konsentrasjon av luciferase og luciferin?)
37.5 μ L 0.4 M DTT		37.5 μ L 0.4 M DTT	
74.9 μ L MilliQ water		74.9 μ L MilliQ water	74.9 μ L MilliQ water
7.8 μ L PKA enzyme (Konsentrasjon?)		7.8 μ L Abl enzyme (Konsentrasjon?)	7.8 μ L PKA enzyme (Konsentrasjon?)
185 μ L 2mM Kemptide		185 μ L 2mM Kemptide	185 μ L 2mM Kemptide

5 μ L of the ianthelline solutions was mixed with 15 μ L active mix. The assay was made with two parallels for each ianthelline solution in the white microtiter plate. The positive control was made from 5 μ L staurosporin and 15 μ L active. The positive control had three parallels in the white microtiter plate. The control was made of 2.5 % DMSO in MilliQ water and 15 μ L active mix. The control had three parallels. Blank control was made of 5 μ L 2.5 % DMSO in MilliQ water and 15 μ L blank mix. The blank control had three parallels in the white microtiter plate. 5 μ L 5 μ M ATP standard was added to all the microtiter wells.

The luminescence was measured right after adding of the ATP standard and one hour after the ATP standard was added. Blank represent 0 % activation of the kinases and control represent the 100 % activation.

4. Results

4.1 Extraction and fractionation of *Stryphnus fortis*:

4.1.1 Extraction yield:

The wet biomass weight of *Stryphnus fortis* used for the extraction was 2001.34 g. After freeze drying and grinding the biomass weight was 420.38 g, which proceeded to the aqueous extraction. The aqueous extraction yield was 104.81 g. The biomass used for the organic extraction after the aqueous extraction was 298.79 g. The organic extract with residues of dichloromethane and methanol was 34.62 g.

4.1.2 Fractionation of the sponge extracts:

The organic and aqueous extracts were fractionated separately. The UV detector measured the absorbance between 200 -600 nm. Most of the organic extract compounds eluted between 2.5 minutes and 31 minutes (se figure 12). There were five larger peak absorbances at 2.62 minutes (the largest peak), 8.88 minutes, 13.18 minutes, 19.47 minutes and 27.98 minutes (se figure 12).

For the aqueous extract, most of the compounds eluted between 2.40 minutes and 26 minutes (se figure 13). The aqueous extract appeared to be more complex than the organic extract.

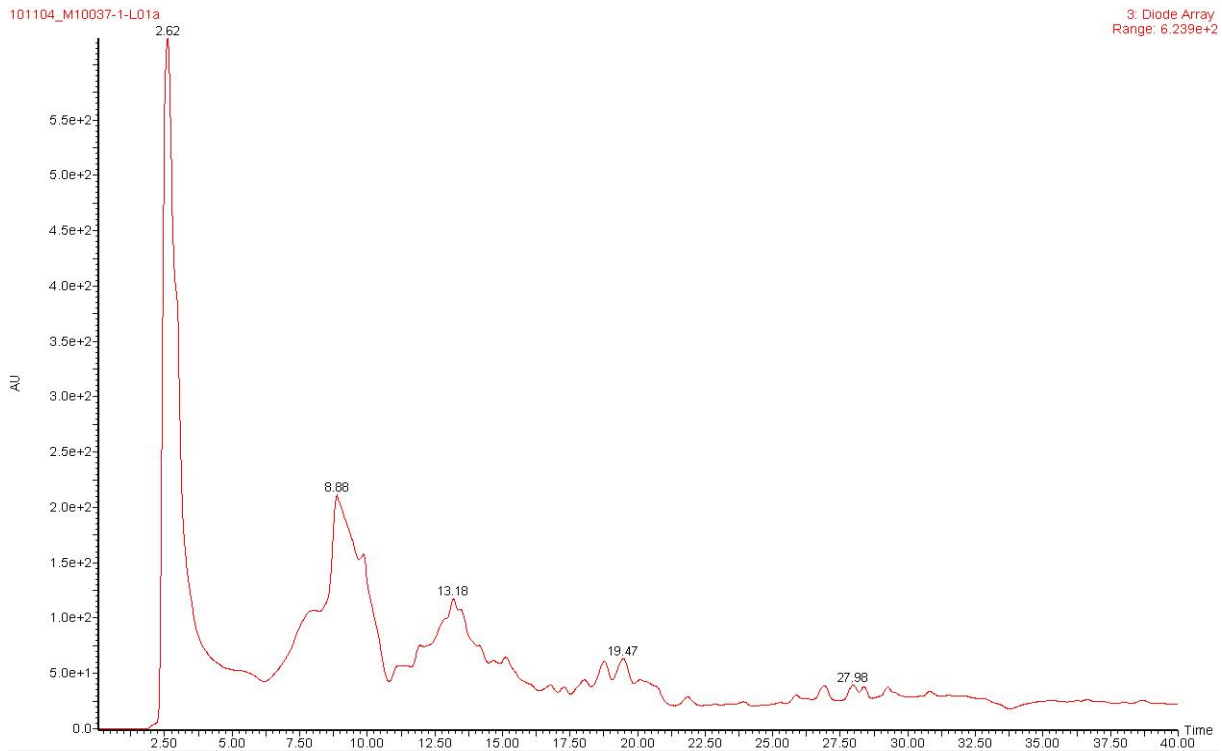


Figure 12: Chromatogram of the fractionation of the organic extract of *Stryphnus fortis*.

One minute represent one fraction, a total of 40 fractions. Time is on the x-axis and absorbance between 200 and 600 nm is on the Y-axis.

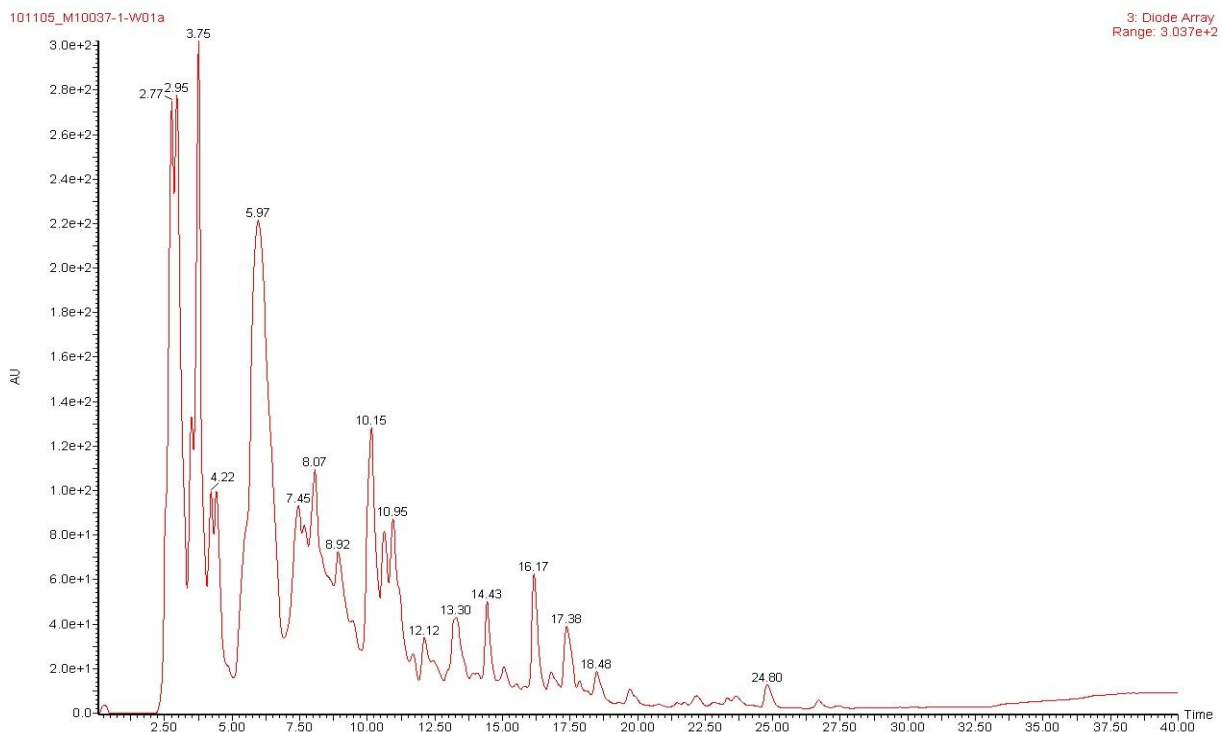


Figure 13: Chromatogram of the fractionation of the aqueous extract of *Stryphnus fortis*.

One minute represent one fraction, a total of 40 fractions. Time is on the x-axis and the absorbance between 200 and 600 nm is on the Y-axis.

4.2 Primary bioactivity screening of the HPLC fractionated extracts of *Stryphnus fortis*

The primary bioactivity screening of the fractionated extract of *Stryphnus fortis* showed that the organic extract had several fractions with activity against both bacteria and cancer (see figure 15 and 16).

The aqueous extract had only one fraction with activity against cancer and none of the fractions had any activity against any of the bacteria (*E. Coli*, *E. faecalis*, *P. aeruginosa*, *S. aureus* and MRSA) (see figure 14 and 16).

4.2.1 Primary antibacterial screening of the HPLC fractionated extracts of *Stryphnus fortis*:

In the MabCent screening program they have defined cut-off values for active and inactive fractions. When the absorbance at 600 nm was less than 0.05 the fractions were considered active and if the absorbance was less than 0.09 the fractions were considered questionable with regard to antibacterial activity. Cut-off values are not needed for this project.

Here the percentage survival for the HPLC fraction of the organic extract of *Stryphnus fortis* has been calculated using a negative sterile control and a positive control. Fractions which gave low survival of the cancer cells were considered active.

The HPLC fractions of the aqueous extract of *Stryphnus fortis* did not kill the bacteria. Fraction 3 actually gave higher growth of *P. aeruginosa* and *E. coli* (see figure 14).

The HPLC fractions of the organic extract of *Stryphnus fortis* had three different areas where the bacteria growth were inhibited (see figure 15). Fractions 3 and 8-10 were active against all the bacteria species. Fractions 4, 11 and 12 were only active against *S. aureus*. Fractions 27 and 29 were active against the gram positive bacteria *E. faecalis*, *S. aureus* and MRSA, but not the gram negative bacteria *E. coli* or *P. aeruginosa*. Fraction 28 was active against *E. faecalis* and *S. aureus* but not *E. coli*, *P. aeruginosa* or MRSA.

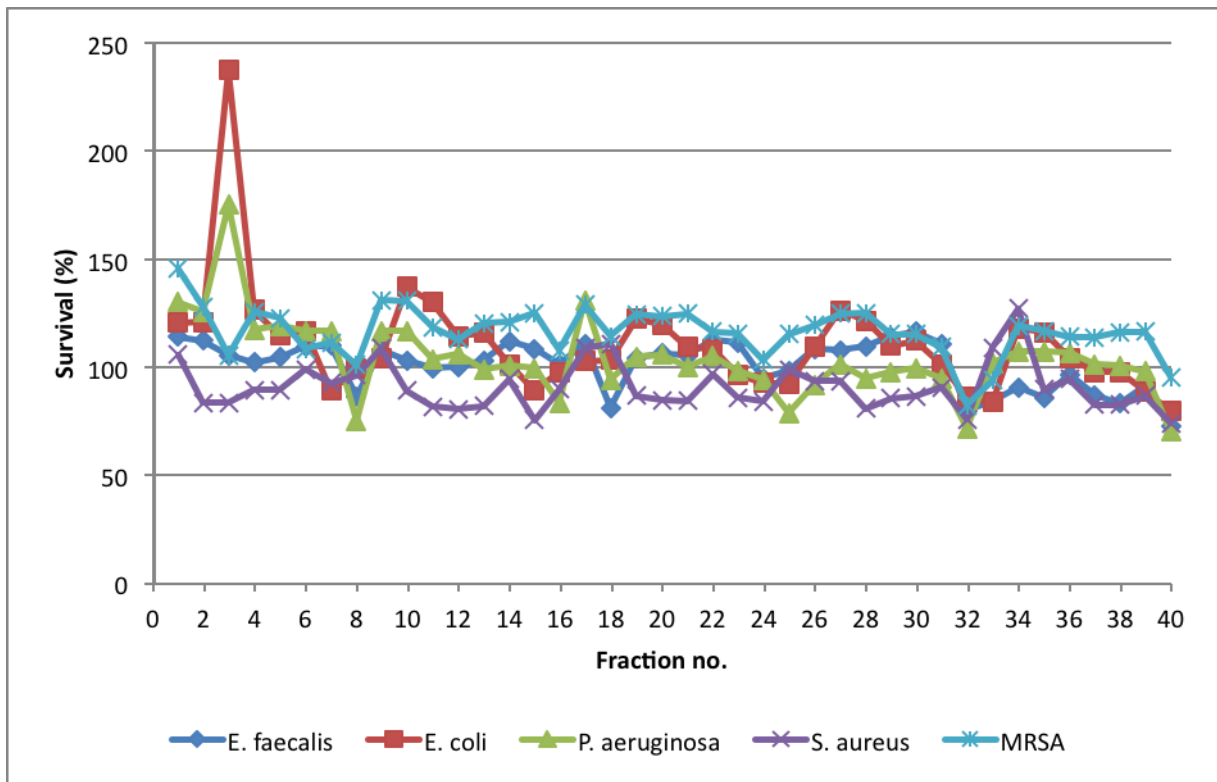


Figure 14: Antibacterial activity of the HPLC fractions of the aqueous extract of *Stryphnus fortis* (M10037-0-W01). Each graph represent the survival of the bacteria: *S. aureus* (lilac), MRSA (bright blue) and *E. faecalis* (dark blue), *P.aeruginosa* (green) and *E.coli* (red) after 24 hours of exposure to HPLC fractions from the aqueous extracts of *Stryphnus fortis*.

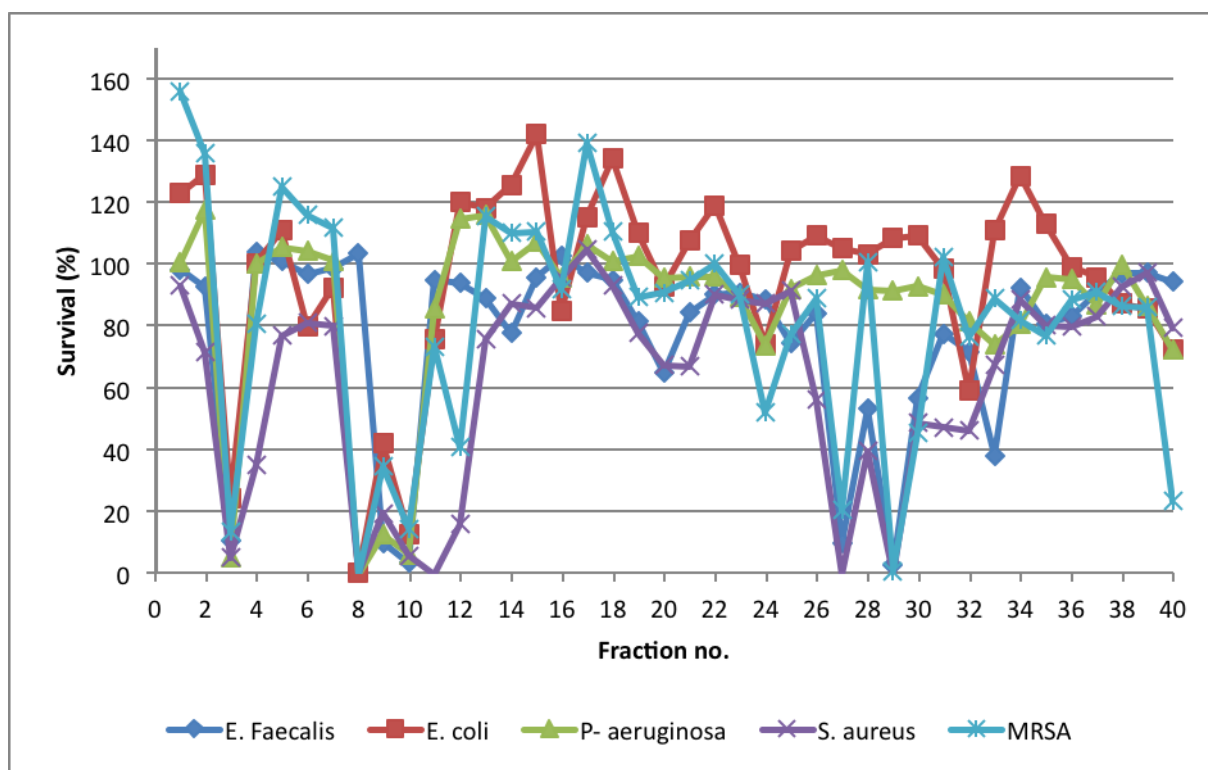


Figure 15: Antibacterial activity of the HPLC fractions from the organic extract of *Stryphnus fortis* (M10037-0-L01) Each graph represent the survival of the bacteria: *S. aureus* (lilac), MRSA (bright blue) and *E. faecalis* (dark blue), *P.aeruginosa* (green) and *E.coli* (red) after 24 hours of exposure to HPLC fractions from the organic extracts of *Stryphnus fortis*.

4.2.2 Primary anticancer screening of the HPLC fractionated extracts of *Stryphnus fortis*:

Fractions which gave less than 50 % survival of the cancer cells were considered active against A2058 cells in the MabCent screening program. Fractions that gave less than 60 % survival of the cancer cells were considered questionable against A2058 cells in the MabCent screening program. This is used for high throughput screening where several thousands of extracts is examined; a cut-off value is needed. Cut-off values are only used as a guidance for this project.

The HPLC fractions of the aqueous extract of *Stryphnus fortis* (M10037-0-W01) did not exert any anticancer activity, except for fraction 3 (see figure 16). Several HPLC fractions of the organic extract of *Stryphnus fortis* (M10037-0-L01) showed anticancer activity against A2058 melanoma cells (see figure 16). HPLC Fractions 3, 9-10, 13-15, 18-23 and 26-30 of the organic extract of *Stryphnus fortis* were considered active against A2058 melanoma cells.

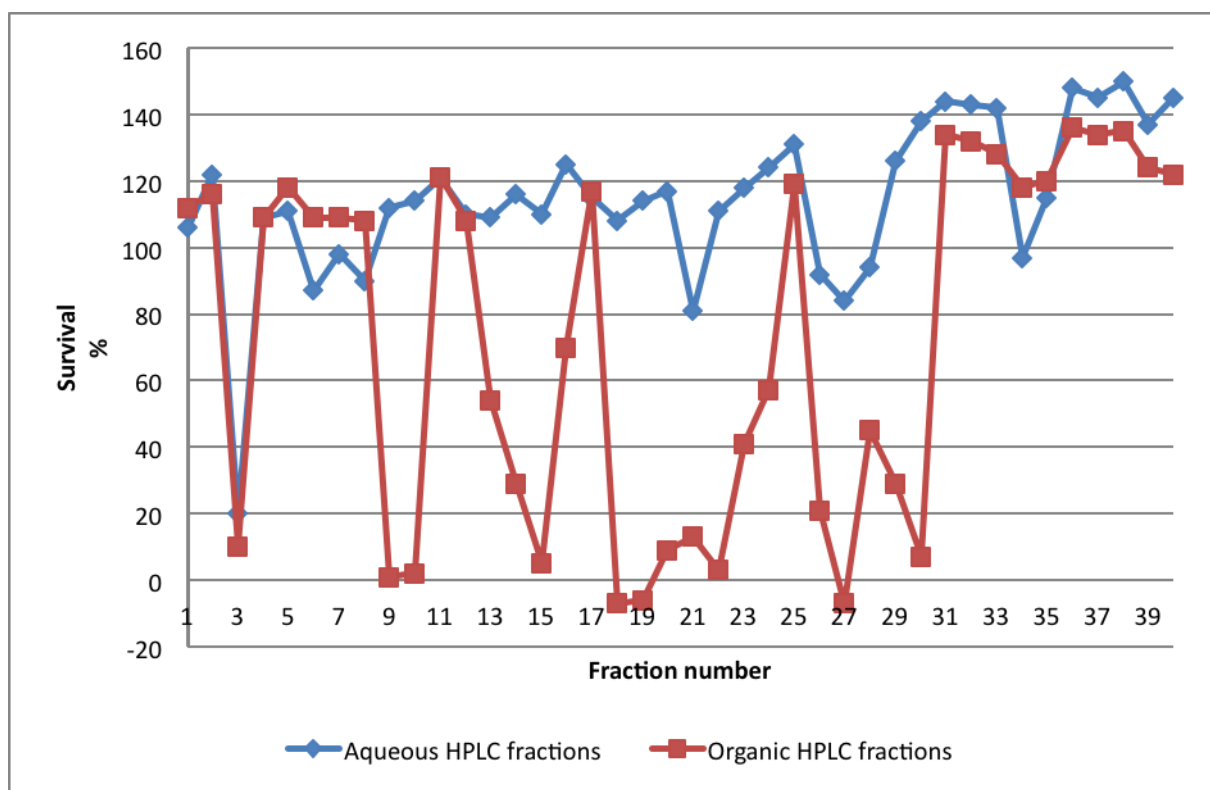


Figure 16: A2058 (Melanoma) cell survival after 72 hours exposure to the HPLC fractions of the organic (red) and aqueous (blue) extract of *Stryphnus fortis* (M10037-0-L01 and M10037-0-W01).

4.3 Dereplication of the active compound of *Stryphnus fortis*:

The dereplication of the active fractions from the primary screening showed that ianthelline was present in the organic extract M10037-0-L01. The ESI+ TOF-MS measured the protonated mass to be m/z 473.9779 and the isotope pattern (1:2:1) indicate the presence of two bromines. The MS software calculated the elemental composition of the active compound to be $C_{15}H_{17}N_5O_3Br_2$ (in ESI+ mode the compound will have one extra H) (see figure 17). The database search gave ianthelline as a possible candidate for the activity in the primary screening.

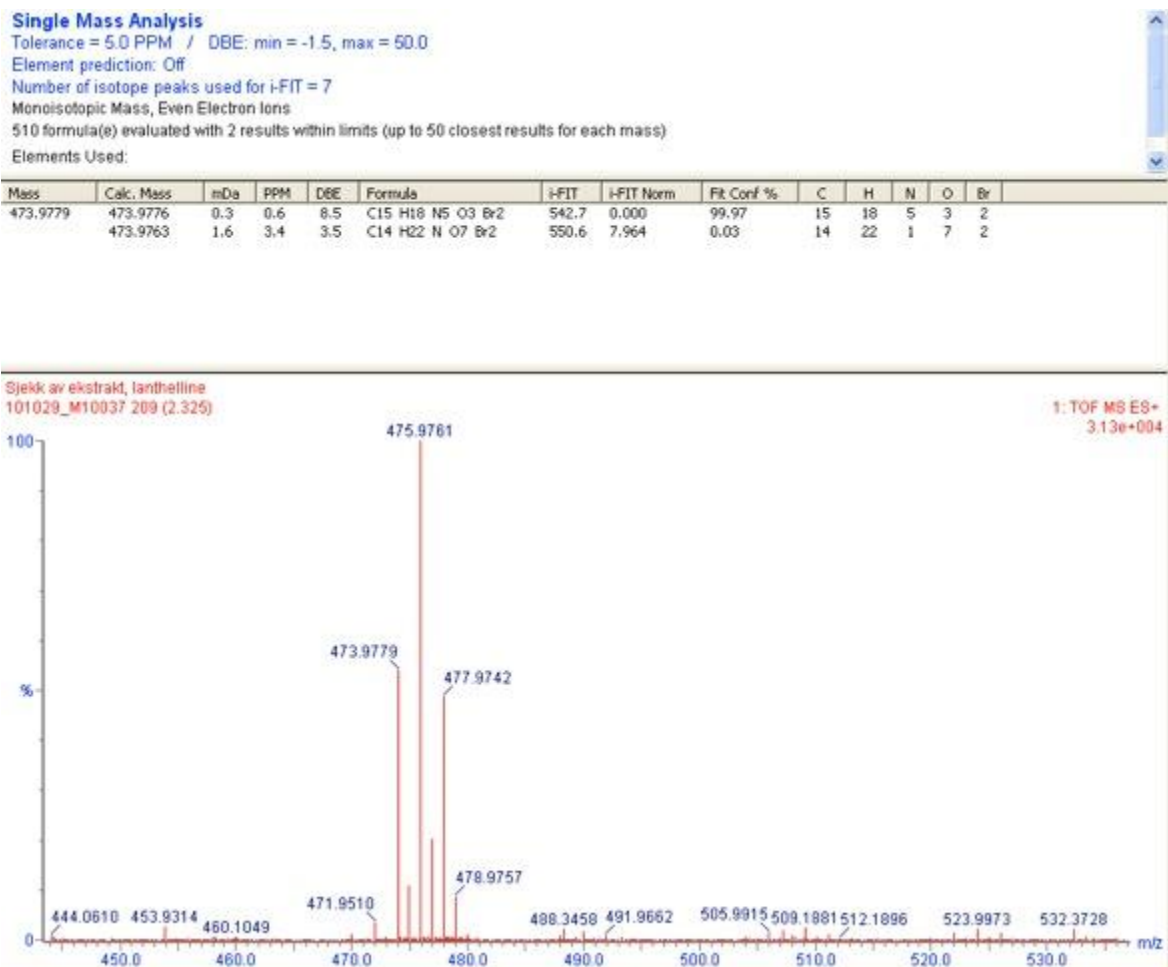


Figure 17: Elemental composition report at the top and the TOF-MS spectra at the bottom of the active compound.

4.4 Isolation of ianthelline from the organic extract of *Stryphnus fortis*:

After the flash fractionation of the organic and aqueous extract from *Stryphnus fortis*, the fractions were screened for anticancer activity to map the bioactivity and as a part of the bioassay guided purification. The ESI+ MS determined where ianthelline had eluted during the flash screening; those fractions were selected for further purification by semi-preparative HPLC.

The organic flash fractions 2 (not active), 6 (active), 8 (active), 10 (active), 11 (active), 12 (active), 14 (not active), 16 (active), 18 (active) and 20 (active) were checked for the presence of m/z 473.9 (protonated) (see figure 18). Ianthelline was found in all the fractions checked, but ianthelline was most abundant in fraction 8, 10, 11, 12 and 14. Fractions 8-15 were used to purify ianthelline.

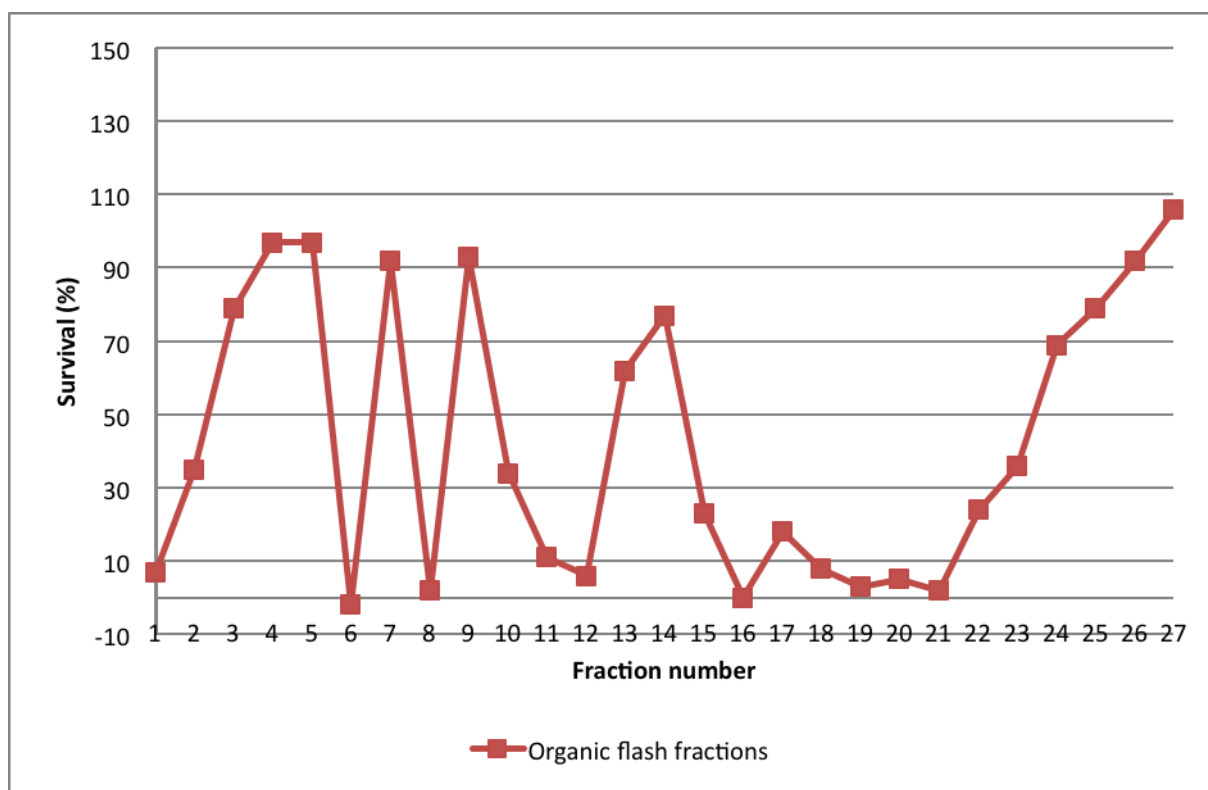


Figure 18: A2058 (Melanoma cell) survival after 72 hours exposure to flash fractions from the organic extract from *Stryphnus fortis*.

4.4.1 Isolation of ianthelline from the organic extract of *Stryphnus fortis* by use of HPLC after flash fractionation:

The selected flash fractions of the organic extract from *Stryphnus fortis* were evaporated on a rotavapor and dissolved in acetonitril and injected to the semi-preparative HPLC coupled with an ESI+ MS. The semi-preparative HPLC coupled with an ESI+ MS collected the protonated m/z 473.9 with a minimum intensity threshold (MIT) of 5 000 000 area units.

As seen in figure 19 and 20, the HPLC successfully separated ianthelline from the other compounds. The final yield of the purified compound after 7 rounds with flash fractionating (not all dried flash fractions were used) and 73 injections on the semi-preparative HPLC was 45.3 mg.

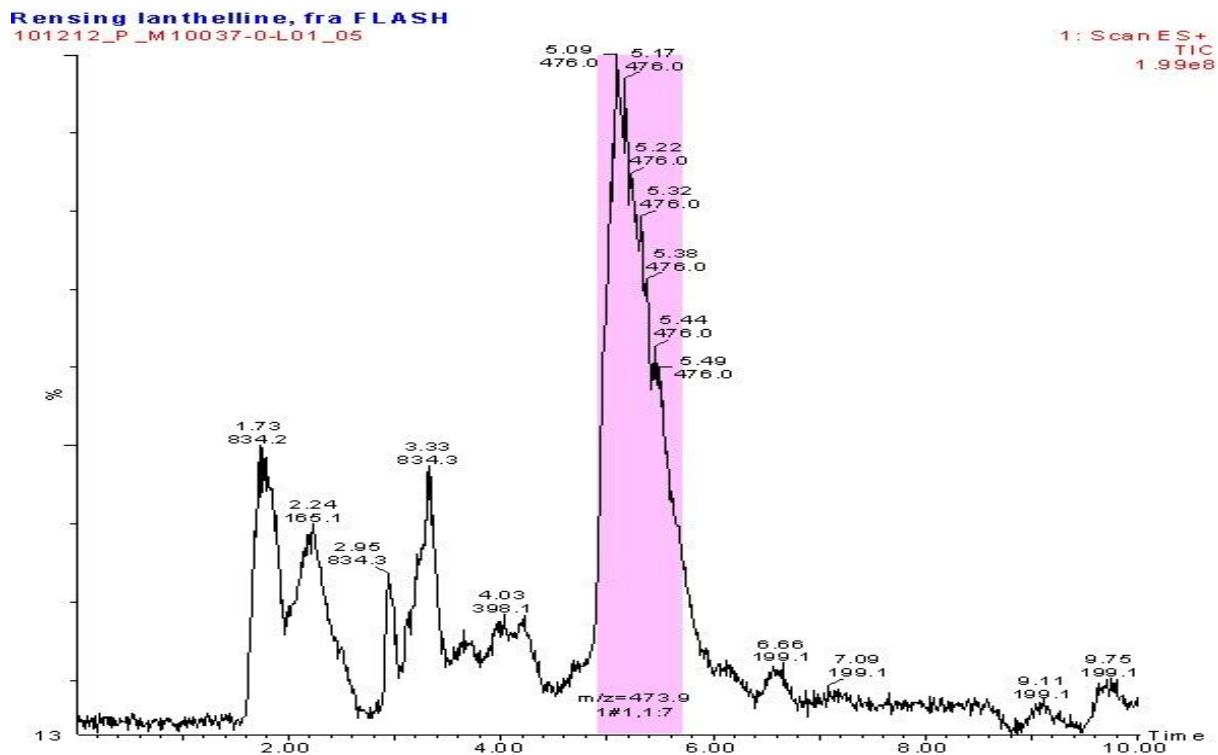


Figure 19: Chromatogram of the isolation of ianthelline by using HPLC. The time is on the x-axis and the peak intensity is on the y-axis. The purple section is where the protonated compound with m/z 473.9 was collected from the flow from the HPLC column.

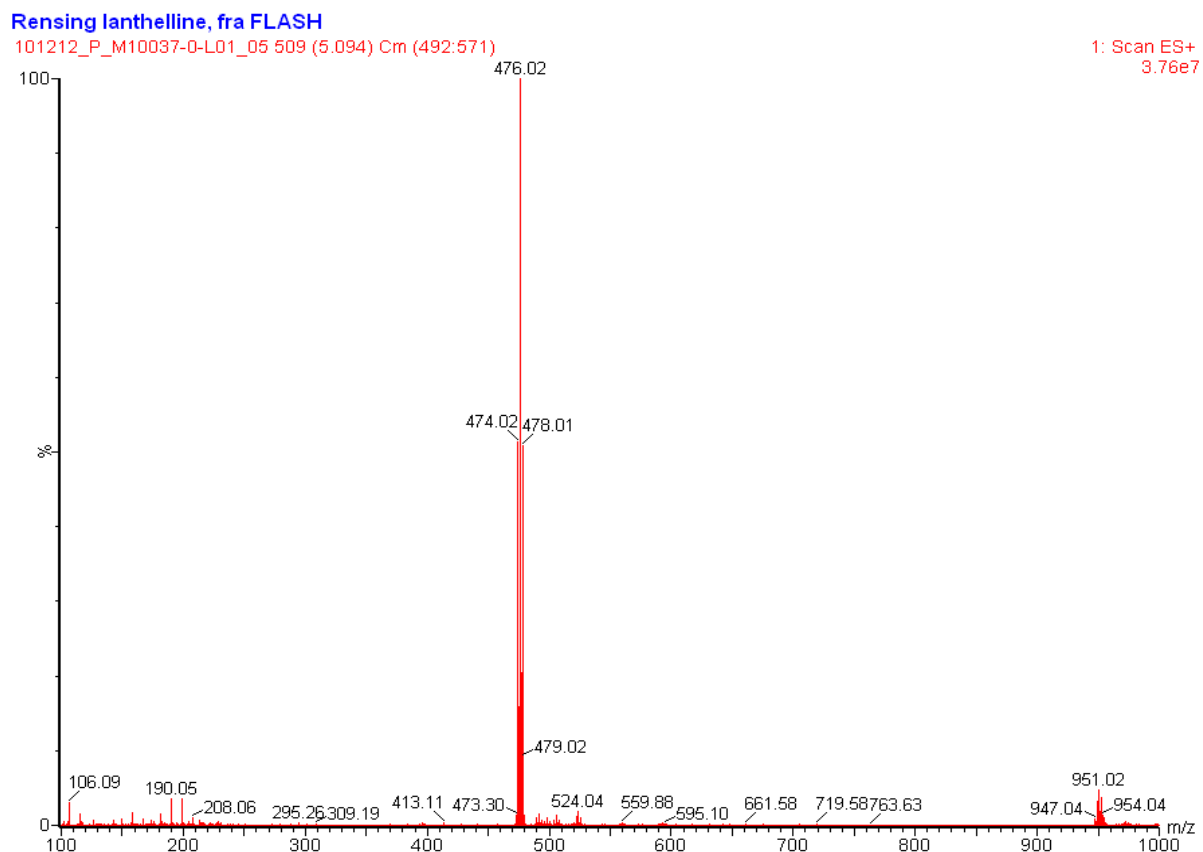


Figure 20: MS specter of the purple section of figure 19 of the purified compound.

4.5 Confirmation of the presence of ianthelline in the purified compound from *Stryphnus fortis*:

4.5.1 ESI+ TOF-MS of the purified compound from *Stryphnus fortis*:

As seen in figure 21, the m/z 473.942 (+1 proton from the ESI) correspond to the mass of ianthelline. The 1:2:1 peak intensity pattern confirms the presence of two bromines. The smaller peak in the chromatogram (m/z 946.9606) is a dimer of ianthelline. The MS software calculated the elemental composition to be $C_{15}H_{17}N_5Br_2$ (in the ESI+ the compound had an extra proton).

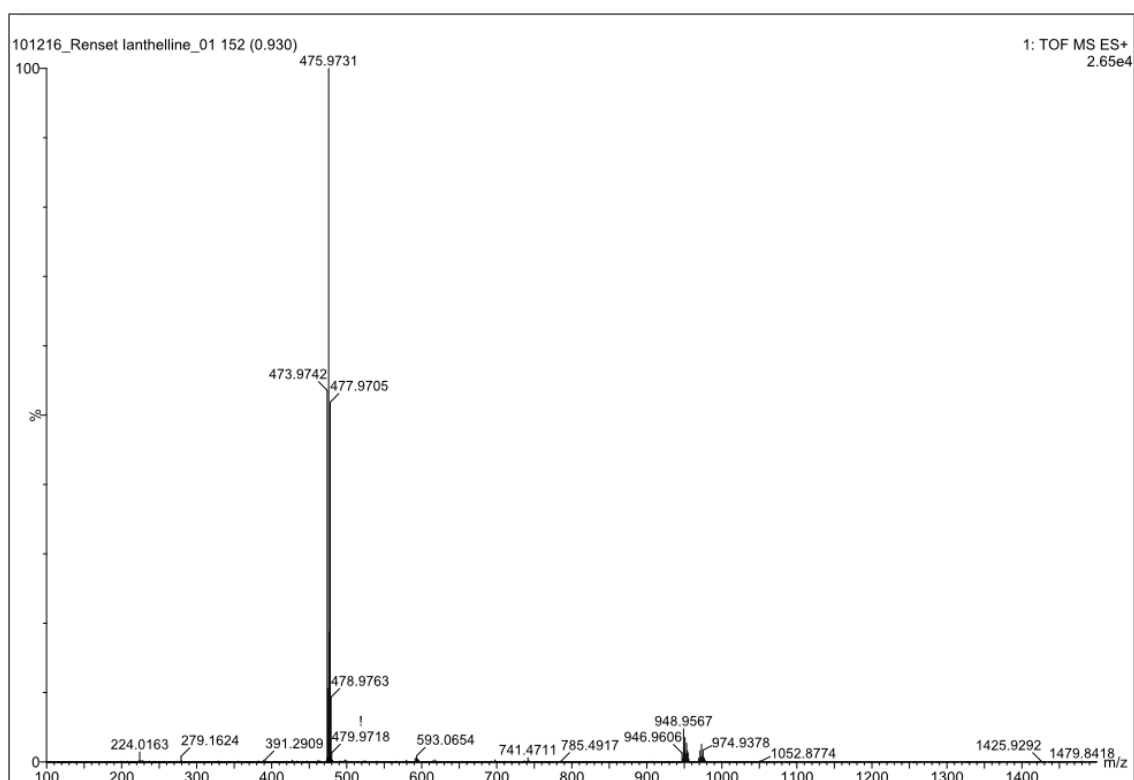


Figure 21: ESI+ MS-TOF Specter of the purified compound at m/z 473.9742 (protonated). The peak at m/z 946.9606 is a protonated dimer of ianthelline.

4.5.2 ESI+ MS/MS of the purified compound from *Stryphnus fortis*:

A daughter ion scan of the m/z 473.9 (protonated) was performed. As seen in figure 22, the major fragment ions were m/z 110.1, m/z 127.2, m/z 153.2 and m/z 276.9. Note that sections in figure 22 have been magnified 186, 82 and 16 times.

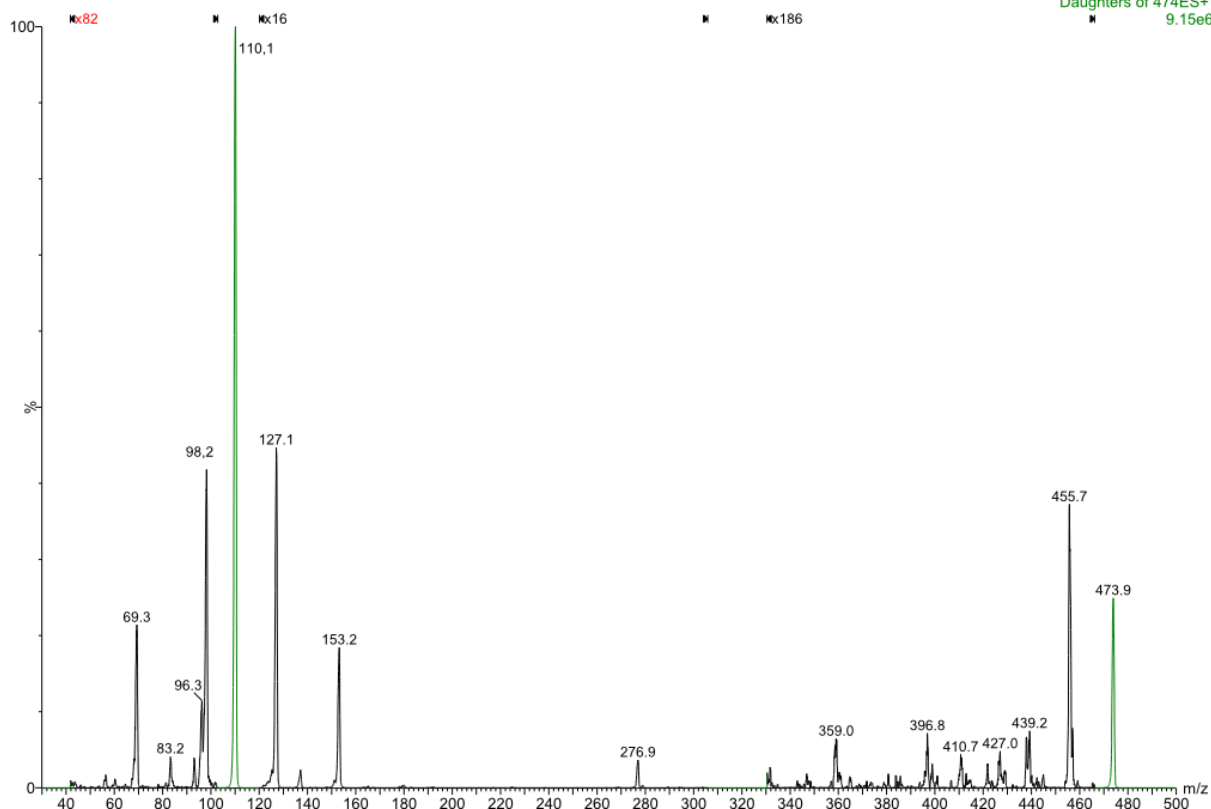


Figure 22: ESI+ MS/MS Daughter ion scan spectrum of the purified compound at m/z 473.9. Areas have been magnified according to the X values above the spectrum. The green peaks have not been magnified. Base peak is at m/z 110.1.

4.5.3 NMR of the purified compound from *Stryphnus fortis*:

The DMSO ^1H NMR shifts of the purified compound, matches almost perfectly with Litaudon's DMSO ^1H NMR shifts (see table 9). The DMSO ^{13}C NMR shifts of the purified compound match fairly well with Litaudon's CD_3OD ^{13}C NMR (see table 9).

The HSQC of the purified compound was indistinct, but with the exception of H-13, all the H-C correlations were revealed (see Appendix C). COSY correlation of the purified compound shows that H-9, H-10 and H-11 are chained neighbors to each other (see Appendix C). HMBC and COSY correlation is shown in figure 25.

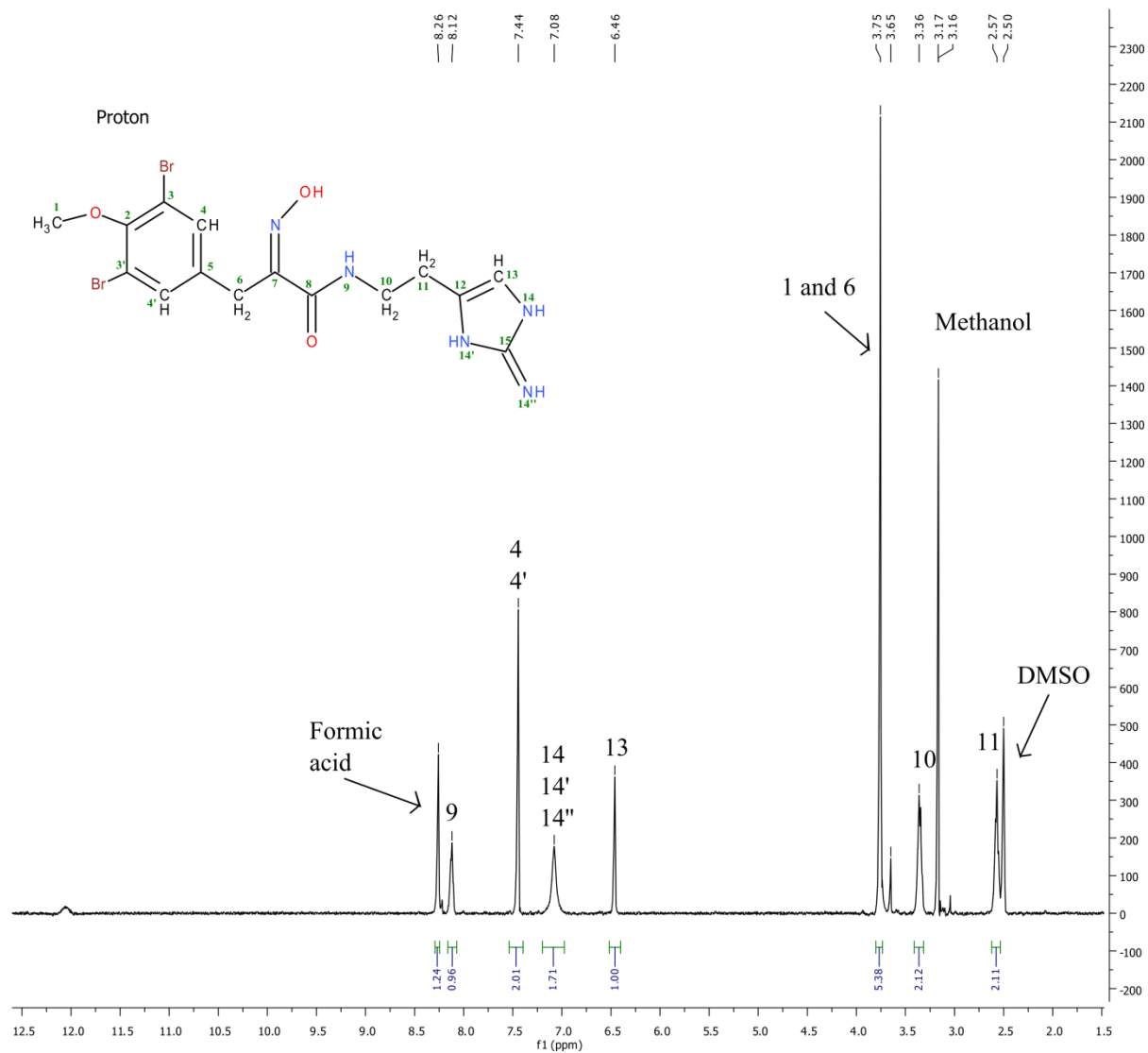


Figure 23: ¹H NMR spectrum of the purified compound. The peaks are numbered according to the structure of ianthelline and the solvents are indicated. Chemical shift (ppm) is on the x-axis and peak intensity is on the y-axis.

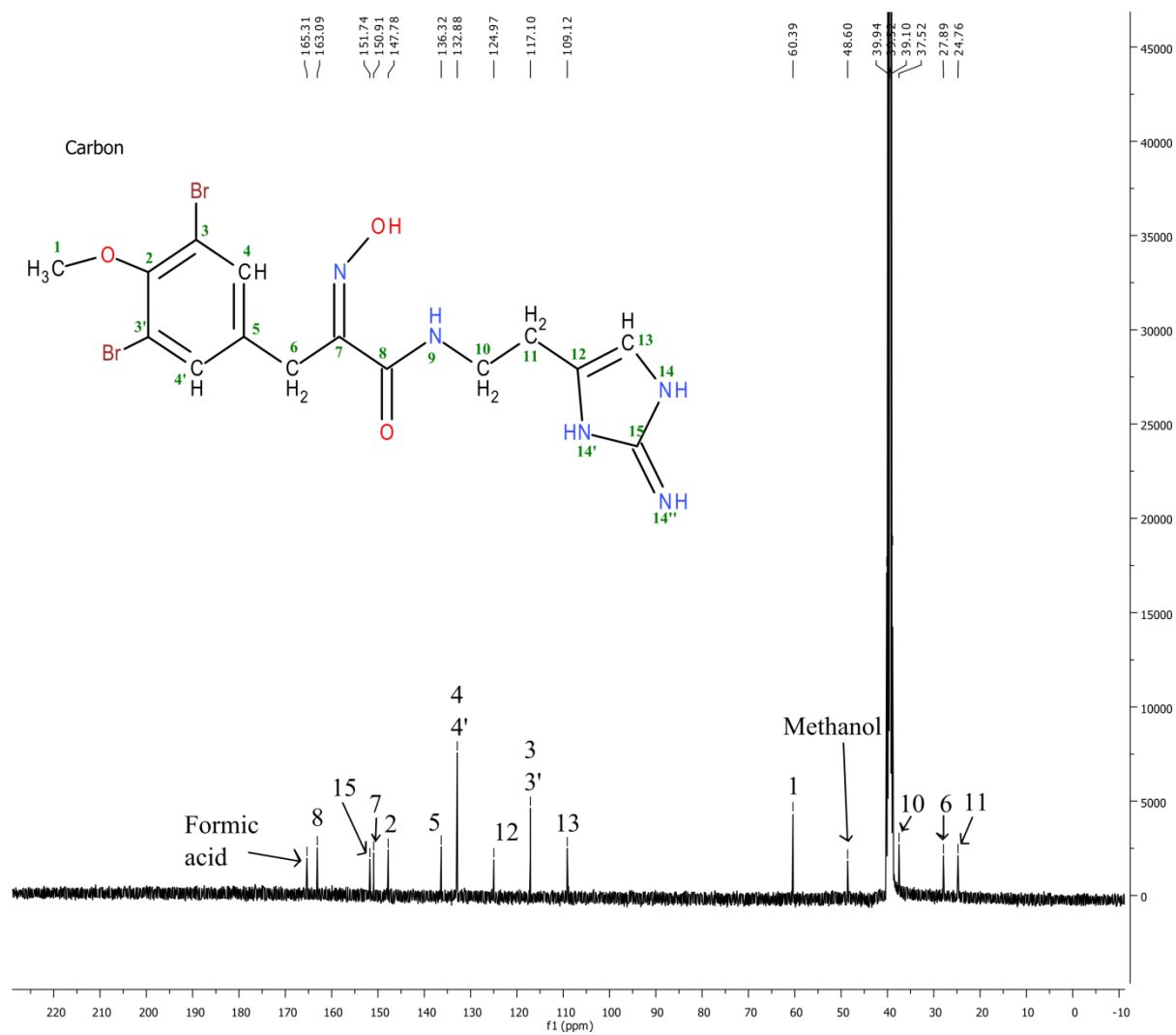


Figure 24: ^{13}C NMR spectrum of the purified compound. The peaks are numbered according to the structure of ianthelline and the solvents are indicated. Chemical shift (ppm) is on the x-axis and peak intensity is on the y-axis.

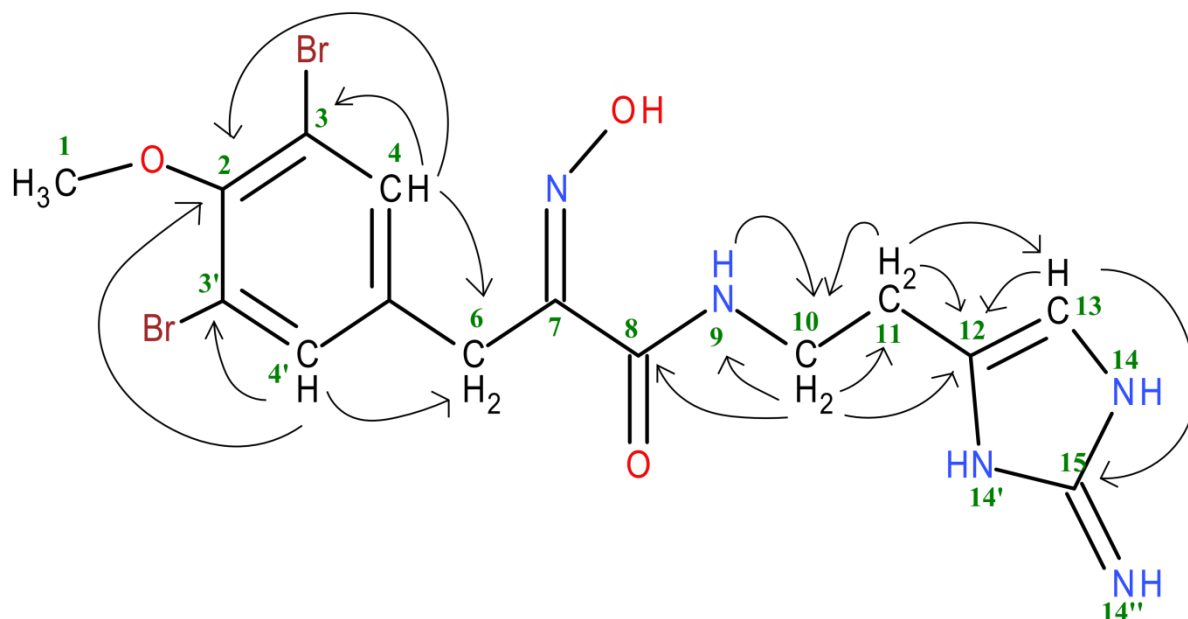


Figure 25: lanthellines structure with COSY and HMBC NMR correlation indicated.

Table 9: ^1H NMR and ^{13}C NMR assignments of the purified compound, compared to Litaudon's results. HMBC and COSY correlation for the purified compound.

Atom no.	δ_{H} Purified compound	δ_{H} DMSO Litaudon [22]	δ_{C} Purified compound	δ_{C} CD_3OD Litaudon[22]	HMBC ^1H - ^{13}C	COSY
1	3.75 (s)	3.75	60.39	61.0		
2			147.78	148.6		
3/3'			117.10	118.5		
4/4'	7.44 (s)	7.44	132.88	134.4	2, 5, 6	
5			136.32	126.0		
6	3.75 (s)	3.75	27.89	28.8		
7			150.91	152.1		
8			163.09	165.6		
9	8.12 (t)	8.09				10
10	3.36 (q)	3.36	37.52	38.9	8, 11, 12	9, 11
11	2.57 (t)	2.58	24.76	25.7	10, 12, 13	10
12			124.97	137.2		
13	6.46 (s)	6.50	109.12	110.7	12, 15	
14/14'/14''	7.08 (s)	7.37				
15			151.74	153.8		

4.6 Biological characterization of ianthelline purified from *Stryphnus fortis*:

4.6.1 Antibacterial activity of ianthelline purified from *Stryphnus fortis*:

MIC (minimum inhibitory concentration) is the lowest concentration which gives no visible growth of the bacteria [18].

Purified ianthelline from *Stryphnus fortis* was tested against *E. faecalis*, *E. coli*, *P. aeruginosa*, *S. aureus* and MRSA.

The gram positive bacteria *S. aureus* and MRSA in this test were more sensitive to ianthelline than the gram positive bacteria *E. faecalis* and the gram negative bacteria *E. coli* and *P. aeruginosa* (see figure 26). Purified ianthelline gave MIC values: 50 µg/mL for *S. aureus*, 75 µg/mL for MRSA, 100 µg/mL for *E. faecalis* and 250 µg/mL for *E. coli* and *P. aeruginosa*.

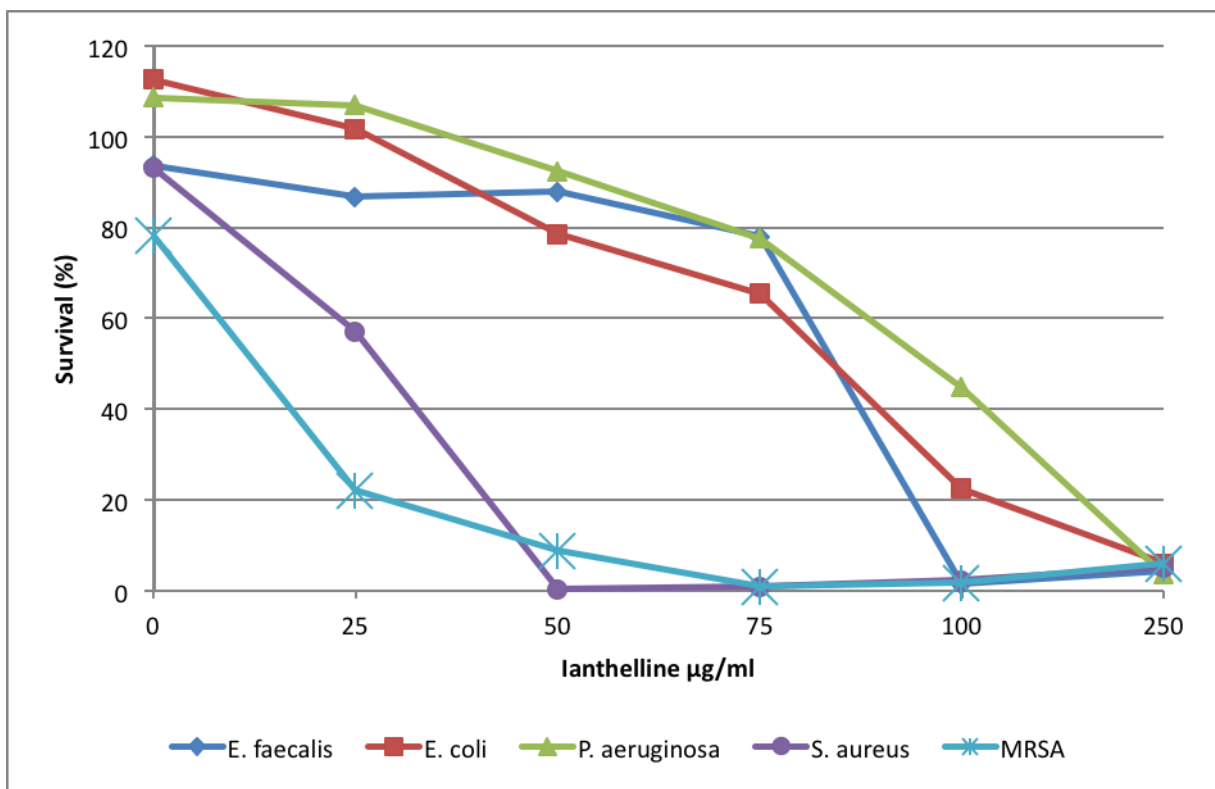


Figure 26: Antibacterial activity of ianthelline after 24-hour exposure against five different bacteria: *E. faecalis* (dark blue), *E. coli* (red), *P. aeruginosa* (green), *S. aureus* (lilac) and methicillin resistant *S. aureus* (MRSA) (bright blue).

4.6.2 Antioxidant activity of ianthelline purified from *Stryphnus fortis*.

Ianthelline showed dose dependent activity in the FRAP assay but the activity was not high compared to curcumin, a known antioxidant (see figure 27). Ferric reducing ability was measured by using light absorbance at 593 nm (Fe(II) maximum absorbance), and a standard curve for trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) was measured and the activity was expressed as trolox equivalents (μM).

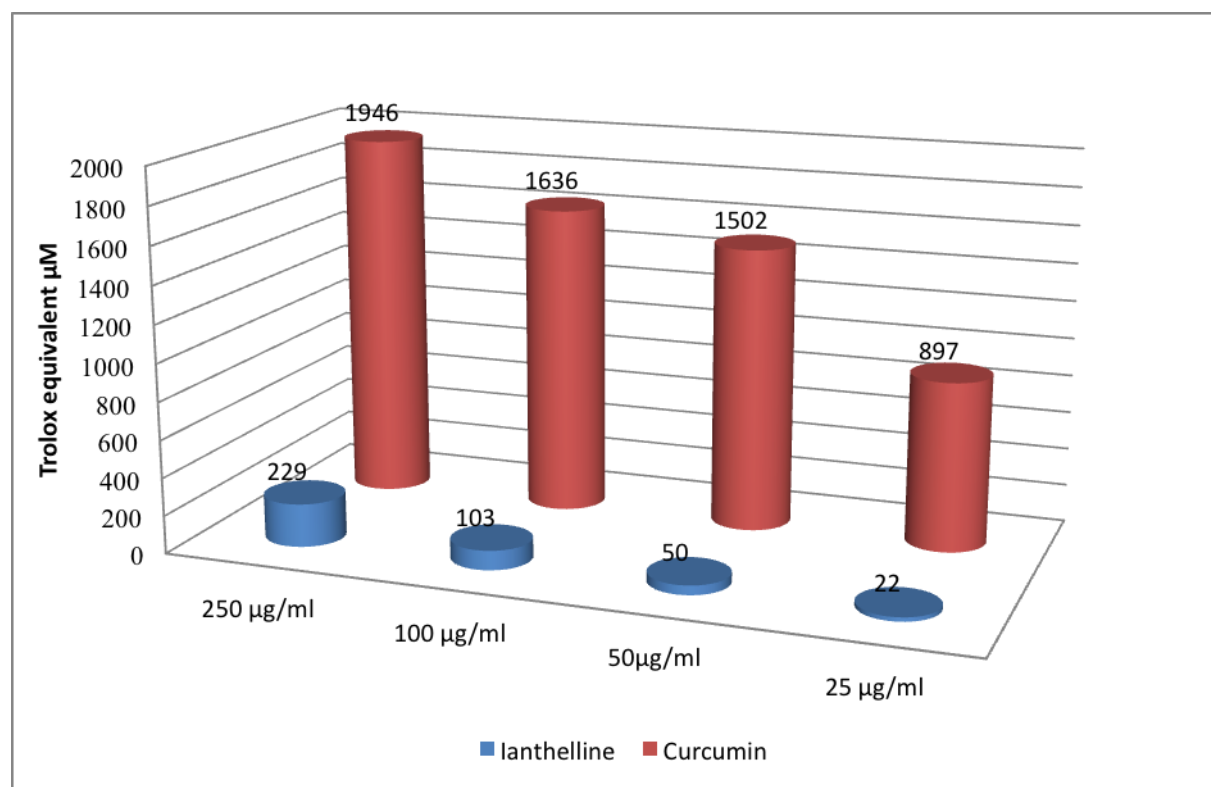


Figure 27: Antioxidant activity of ianthelline. Ianthelline (blue) was compared with curcumin (red) in the concentration denoted below the columns; activity was expressed in μM trolox equivalent.

4.6.3 Comparison of ianthelline's activity against the different cancer cell lines and normal lung fibroblasts (MRC5):

Purified ianthelline from *Stryphnus fortis* was tested against A2058 (human melanoma), HT29 (human colon carcinoma) and MCF7 (human breast cancer). MRC5 (normal lung fibroblast) was used to obtain toxicity data.

At 75 $\mu\text{g/ml}$ ianthelline, A2058, MRC5 and MCF7 were the most susceptible cell lines. HT29 were least affected by ianthelline at 75 $\mu\text{g/ml}$ (see figure 28).

In addition to use the Aqueous One Solution Reagent for cell viability; light microscopy was used to inspect the cells. In the wells exposed to the highest concentration, very few cells were present. The few cells present were not stained by trypan blue.

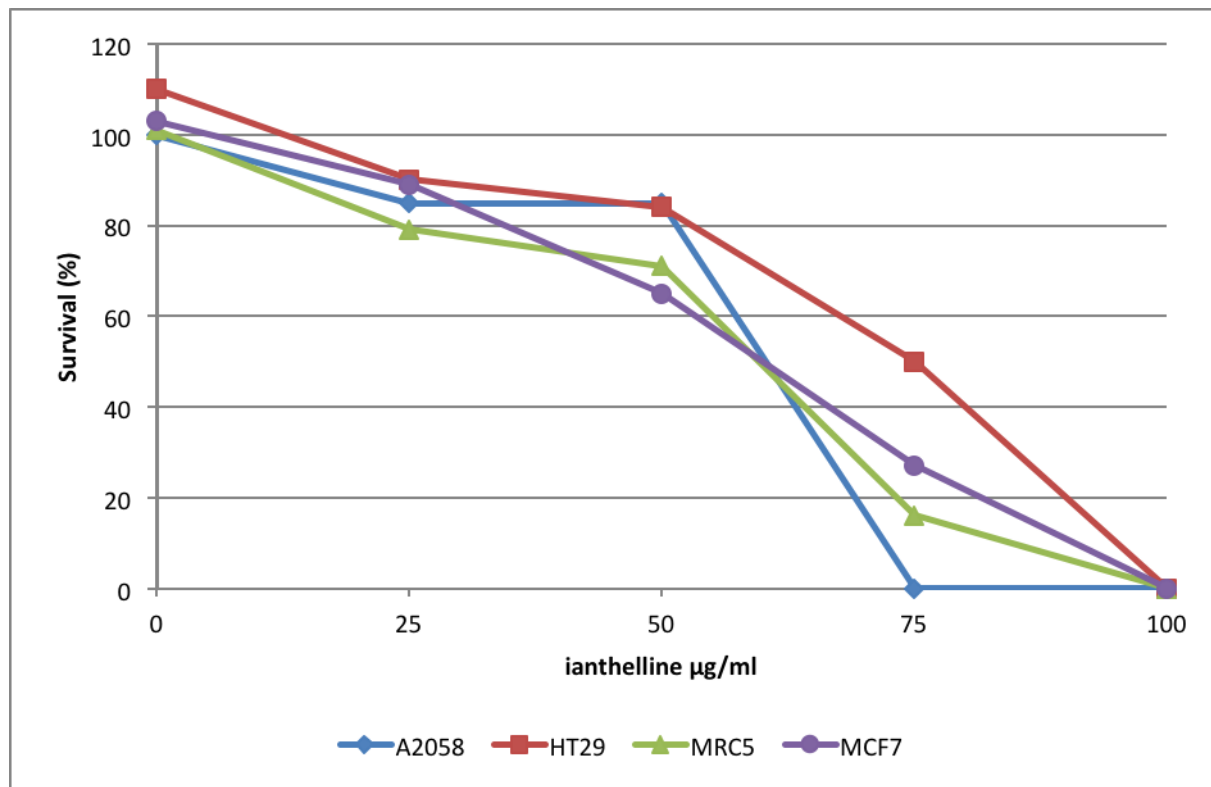


Figure 28: Anticancer activity of ianthelline after 72 hours of exposure. Each graph represent one cancer cell line; A2058 melanoma (blue), HT29 colon carcinoma (red), MRC5 normal lung fibroblast (green) and MCF7 breast carcinoma (lilac).

4.6.4 Time of exposure effects of ianthelline against cancer:

Different exposure times of ianthelline to the cells was used to investigate if the activity a direct immediate effect on the cells or if it had a more slow activity. A2058, MCF7 and HT29 cancer cell lines were exposed to isolated ianthelline from *Stryphnus fortis* for 4-, 24-, 48- and 72-hours. Normal lung fibroblasts (MRC5) was used as a control for general toxicity. The results from the MCF7 cells were discarded because of low survival of the positive control.

The survival of the A2058 cells was not affected after 4 hours of exposure. After 24 hours 80 µg/mL ianthelline was needed to inhibit A2058, in contrast to 60 µg/mL ianthelline at 48 and 72 hours exposure (see figure 29).

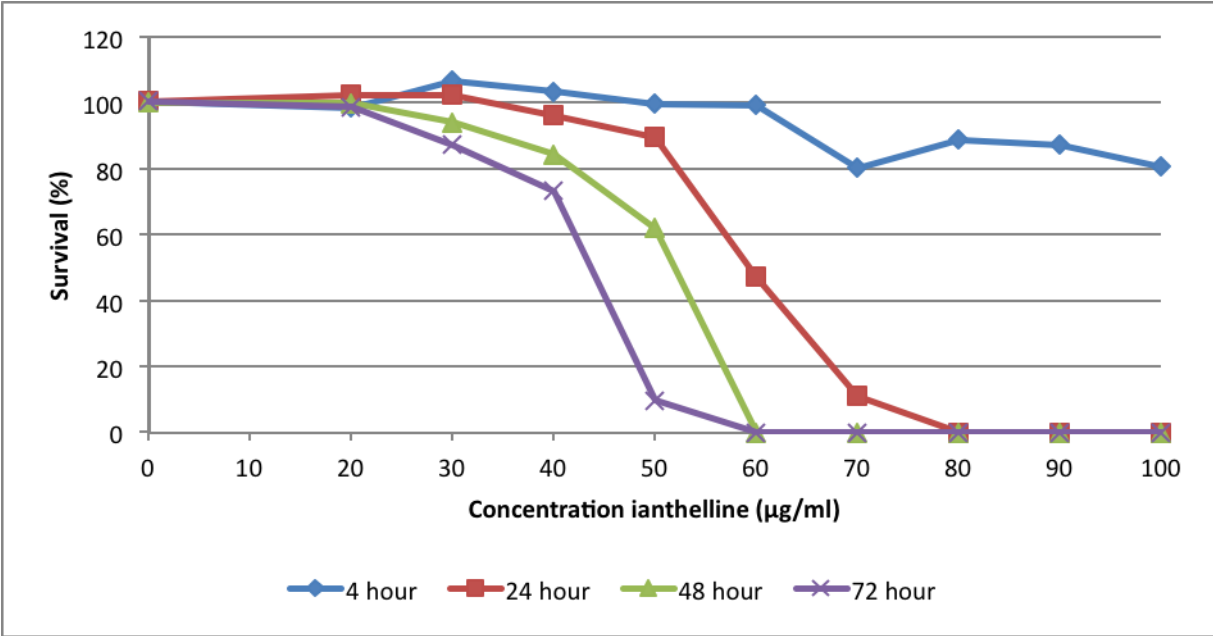


Figure 29: Dose-response-time of ianthelline against A2058 (melanoma). Each graph represents different exposure time of ianthelline; 4 hours of exposure to ianthelline (blue), 24 hours of exposure to ianthelline (red), 48 hours of exposure to ianthelline (green) and 72 hours of exposure to ianthelline (lilac).

Neither 4 hours nor 24 hours exposure to ianthelline affected HT29 cells. After 48 hours ianthelline showed a slight effect against HT29 cells at the ianthelline concentrations tested. Ianthelline needed to be present for at least 72 hours to effectively inhibit HT29 cells at 90 µg/mL (see figure 30).

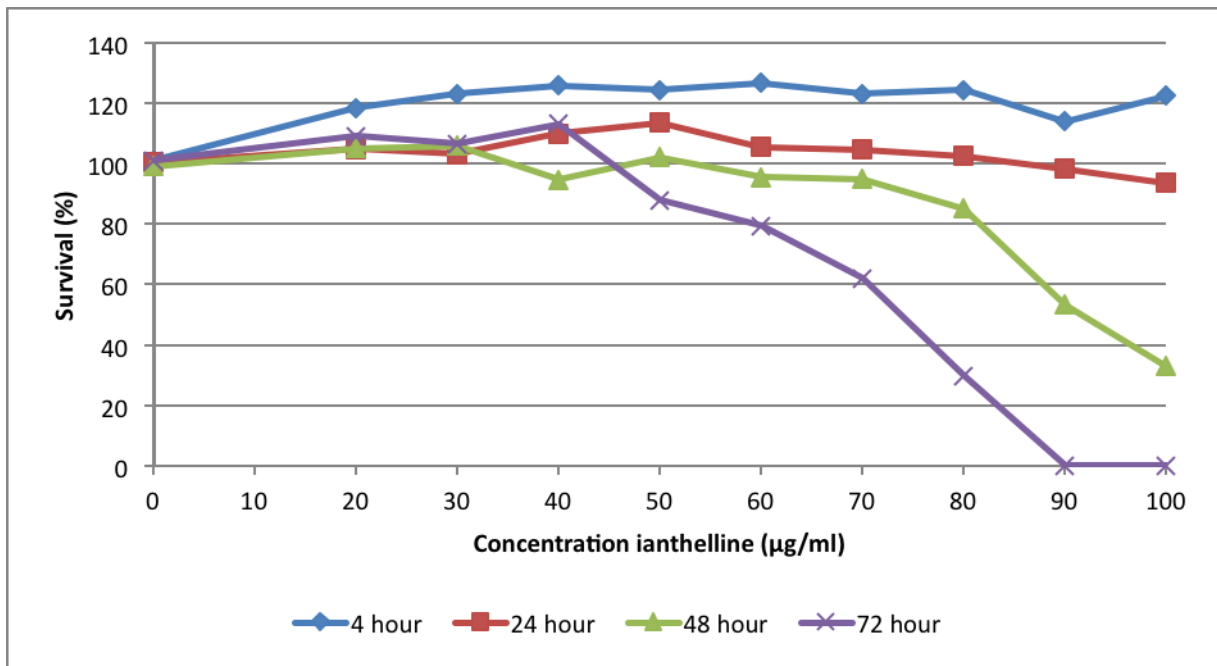


Figure 30: Dose-response-time curve of ianthelline against HT29 (colon carcinoma). Each graph represents different exposure time of ianthelline; 4 hours of exposure to ianthelline (blue), 24 hours of exposure to ianthelline (red), 48 hours of exposure to ianthelline (green) and 72 hours of exposure to ianthelline (lilac).

ianthelline showed no effects on normal lung fibroblasts (MRC5) after 4 hours. After 24 hours ianthelline was active against normal lung fibroblasts (MRC5). The effects increased with the time of exposure (see figure 31).

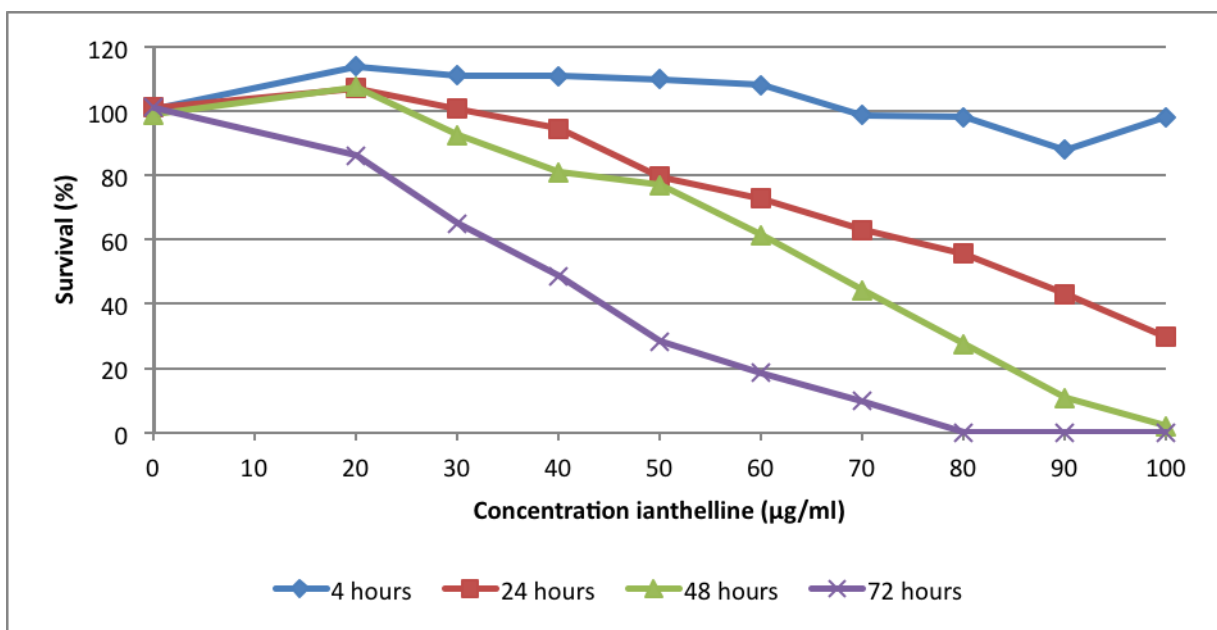


Figure 31: Dose-response-time of ianthelline against MRC5 (normal lung fibroblasts). Each graph represents different exposure time of ianthelline; 4 hours of exposure to ianthelline (blue), 24 hours of exposure to ianthelline (red), 48 hours of exposure to ianthelline (green) and 72 hours of exposure to ianthelline (lilac).

4.6.5: PKA and Abl dose-response for ianthelline purified from *Stryphnus fortis*:

Ianthelline had a slight dose-response inhibitory relationship against PKA, and no inhibitory effects against ABL (see figure 32).

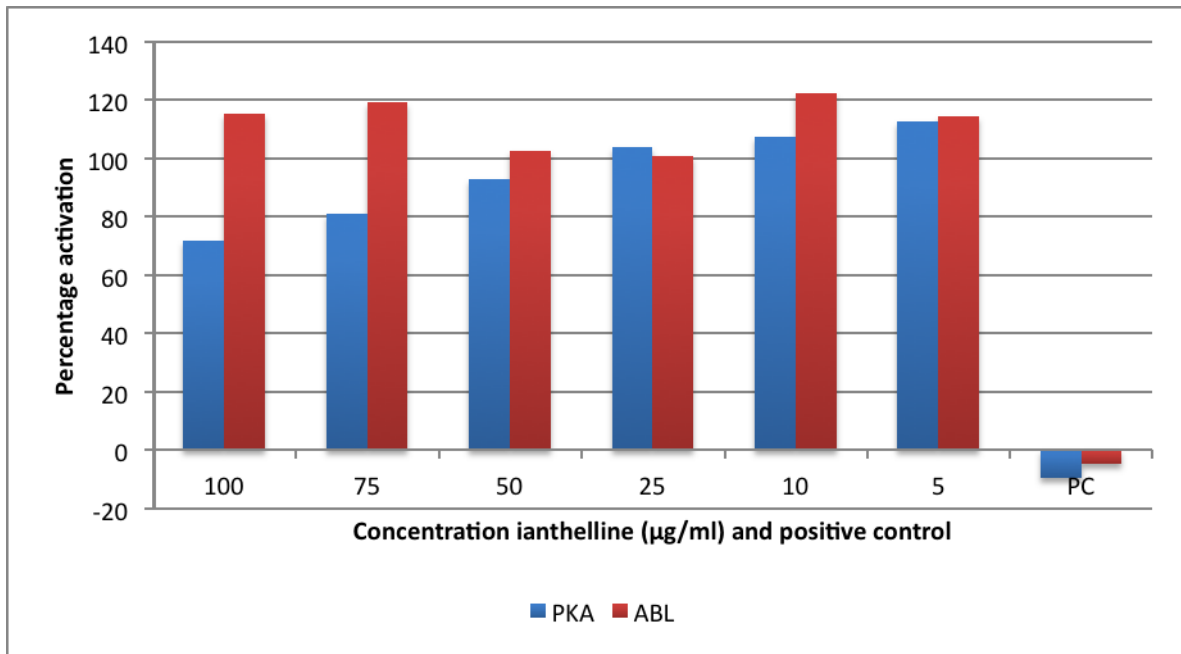


Figure 32: Dose-response ianthelline inhibitory effects against PKA (blue) and Abl (red) compared to positive control (PC) expressed in percentage activation.

5. Discussion

5.1 Extraction and HPLC fractionation of *Stryphnus fortis*:

Freeze drying contributes to the breaking of cell walls and subsequently making the extraction yield larger. The grinding process ensures homogenization and larger surface area for extraction [28]. The reason for extracting first with water and then with dichloromethane is first of all because the other way around doesn't have any benefits to extraction yield, and secondly could potentially expose lab workers to toxic dichloromethane residues during aqueous extraction, thirdly because according to USA National Cancer Institute (NCI) it would turn the freeze dried grinded biomass into a gel that would be difficult to work with [28]. The organic solvents (dichloromethane mixed with methanol 1:1) chosen are the ones that have proven to give the greatest extraction yield by NCI [28]. The cold slow extraction is used instead of hot short extraction to prevent degrading of active compounds, although the water could potentially hydrolyze the compounds and harbor microorganisms capable of producing endotoxins which might produce false positives later on [28, 29]. Before fractionation the organic extract is dissolved in acetonitrile and liquid-liquid extracted with hexane. The hexane is afterwards discarded, the reason for discarding the hexane phase is that compounds preferring the hexane phase would be so non-polar that they would use extremely long time in the HPLC column and interfere with future analysis on the column, difficult to do bioactivity screening and be only to small extent available in blood as drug candidates.

5.2 Primary bioactivity screening of the HPLC fractionated extracts of *Stryphnus fortis*:

The HPLC fractions of the aqueous extract from *Stryphnus fortis* showed neither activity against melanoma A2058 cancer cells nor any activity in the bacteria screening. A part from the obvious reason that there might not be any active compounds present, this can be due to rapid degradation of compounds of the water extract or too low concentration to detect any activity.

The organic extract had several HPLC fractions with activity against bacteria and melanoma. Because there are fractions without activity between the fractions with activity, there is a high probability that there are more than one compound present that is active against cancer and bacteria.

5.2.1 Antibacterial activity of the HPLC fractions from the organic and aqueous extract of *Stryphnus fortis*:

The HPLC fraction 3 of the aqueous extract of *Stryphnus fortis* gave a higher survival of *P. aeruginosa* and *E. coli* (see figure 14). If it hadn't been for normal survival in *E. faecalis*, *S. aureus* and MRSA, a hypothesis could have been that the fraction 3 absorbed light. Both *E. coli* and *P. aeruginosa* are gram negative bacteria, whereas the others are gram positive. There could actually be a compound in *Stryphnus fortis* beneficial for gram negative bacteria or light absorbing metabolites is formed by *E. coli* and *P. aeruginosa* from a compound in fraction 3 (however no odd color was seen before light absorbance was measured). This is not in focus in this project.

The HPLC fraction of the organic extract of *Stryphnus fortis* had several areas with activity; there is probably more than one compound with antibacterial activity.

5.2.2 Anticancer activity of the HPLC fractions from the organic and aqueous extract of *Stryphnus fortis*:

The linear range for the correlation between living cells and absorbance measured is according to the producer 0.3 -0.8 Absorbance at 490 nm [26]. In this study we used half the amount of Aqueous One Solution recommended by the producer, but the results from this

project is still valid, since positive control results with lower absorbance than 0.3 was discarded and half the amount of Aqueous One Solution Reagent have shown to be fine in previous studies at MabCent [26]. False negatives can be obtained by compounds which reduces MTS tetrazolium to the 490 nm absorbing formazan product [26]. This can be prevented by inspecting every single well using a microscope, however in a high throughput screening program such as MabCent this would be very time consuming and thus impractical.

A2058 (human melanoma) cells were chosen for the primary screening because the A2058 cells were fast growing and robust cell line.

5.3 Dereplication of the active compound of *Stryphnus fortis*:

Ianthelline had previously been identified in another sample of *Aplysilla sp.*, and it was worked after the hypothesis that it would be present in this sample as well. Usually dereplication is used to exclude further analysis of already known compounds, but as mentioned earlier dereplication in this project was used to confirm ianthellines presence.

5.4 Isolation of ianthelline from the organic extract of *Stryphnus fortis*:

5.4.1 Flash chromatography of the organic extract from *Stryphnus fortis*:

As semi-preparative HPLC cannot isolate large amount of compounds, a flash system was used instead to roughly separate ianthelline from the rest. Then semi-preparative HPLC was performed for further purification of the cleaner flash fractions.

5.4.2 Melanoma screening of the flash fractions from the organic extract of *Stryphnus fortis*:

The melanoma screening of the flash fractions from the extracts determines the bioassay guided purification in which flash fractions are to be analyzed for active compounds in the MS, and further purified by the semi-preparative HPLC.

The melanoma screening of the flash fractions from the organic extract were as mentioned earlier, used to identify which fractions were to be purified further.

All the fractions checked had m/z 473.9 (protonated) present, but because fraction 8, 10, 11, 12 and 14 had the largest intensity of m/z 473.9, fractions 8-15 were used to purify ianthelline.

As the primary screening of ianthelline showed that there was a high probability of being more than one compound in the organic extract of *Stryphnus fortis*, the flash fractions 1-7 were kept for further analysis by MabCent, flash fractions 8-15 (the ones with the highest content of ianthelline) were used to purify ianthelline, flash fractions 16-22 were kept for further analysis by MabCent, and flash fraction 23-27 were discarded because of low activity.

5.4.3 Semi-preparative HPLC of the dried flash fractions of the organic extract of *Stryphnus fortis*:

As there was so much ianthelline present in the sample, a threshold value of 5 000 000 area units could be chosen, to obtain purer compounds. Selecting a high threshold value would mean to sacrifice some ianthelline to get a purer result. There was no problem later on with too little ianthelline.

5.5 Confirmation of the presence of ianthelline in the purified compound from *Stryphnus fortis*:

We used the hypothesis that our active compound was ianthelline because it had previously been identified in a sample of *Aplysilla sp.*

5.5.1 MS-TOF of the purified compound from *Stryphnus fortis*

The MS-TOF revealed a peak triplet with intensity of 1:2:1 with m/z values of 473.9742 475.9731 and 477.9705 which correspond to the predicted mass of ianthelline (472.9698) plus one proton. The peak intensity of 1:2:1 indicates the presence of two bromines. The MS-TOF software also predicted the atom composition of the triplet to be $C_{15} H_{17} N_5 Br_2$ which corresponds to the atom composition of ianthelline.

5.5.2 MS-MS of the purified compound from *Stryphnus fortis*

The MS-MS product ion scan of the m/z 473.9 showed ions that can be explained by fragmentation of ianthelline. The parent ion at m/z 473.9 and the base peak ion at m/z 110.1 and m/z 127.1 and m/z 153.2 were the most abundant peaks in the spectrum (see figure 22). The m/z 110.1, m/z 127.1, m/z 276.9 and m/z 153.2 fragments have all fragmented next to a heteroatom, which is common in collision induced fragmentation (see figure 33). The peak at m/z 127.1 could have been formed by two different fragments (see figure), the first is most likely responsible for the fragment and involves a proton transfer in addition to the second proton responsible for the charge on the fragment. The second possibility involves transfer of a $-OH$ group which is not as common as proton transfer. Fragment m/z 98.2 is most likely formed by proton transfer and fragment m/z 69.3 most likely involves formation of a new ring structure.

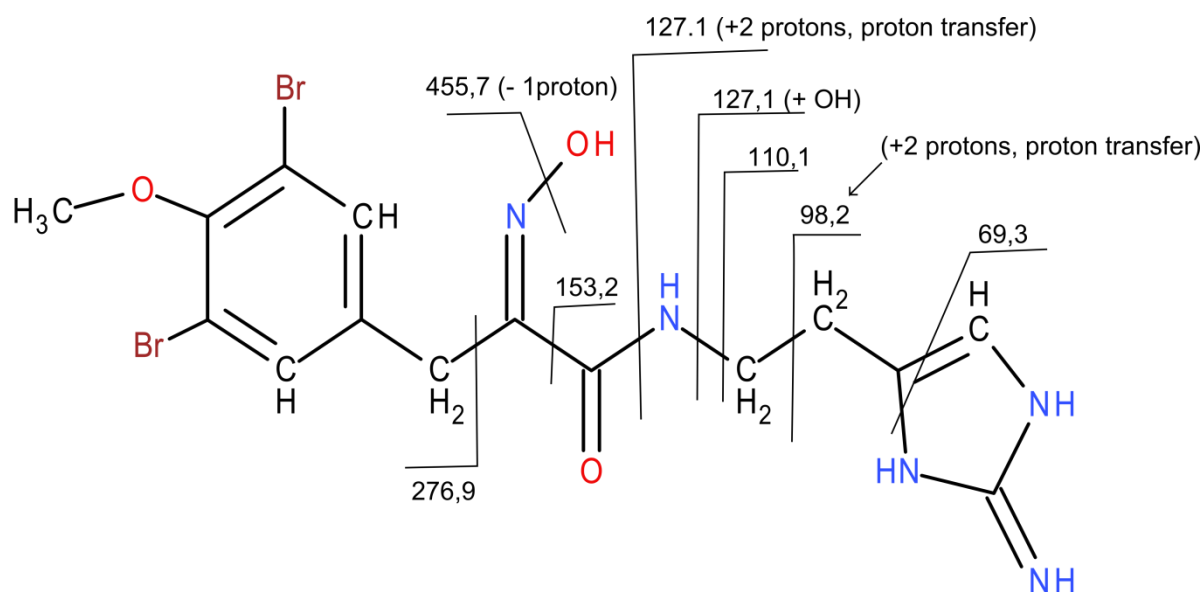


Figure 33: Fragmentation of ianthelline in the MS-MS product ion scan.

5.5.3 NMR of the purified compound from *Stryphnus fortis*:

The ^1H NMR of the purified compounds matches Litaudon's of ianthelline so well that there is no doubt that the purified compound is ianthelline. But the ^{13}C NMR had two major differences; The HMBC shows that the 5 shift and 12 shift have opposite placement. In Litaudon's article ianthelline is not run through any 2D NMR, but used literature search to find the position of the carbons.

5.6 Biological characterization of ianthelline

5.6.1 Antibacterial activity of ianthelline:

As expected ianthelline showed antibacterial activity. Ianthelline have previously shown activity against *S. aureus* and the fungus *Candida albicans*. Ianthelline showed a dose-response activity against all the bacteria tested (see figure 26). Ianthelline had lowest MIC against the *S. aureus* and MRSA.

5.6.2 Antioxidant activity of ianthelline isolated from *Stryphnus fortis*:

Ianthelline showed weak antioxidant properties, but they were low compared to the strong known antioxidant curcumin. The activity is so small that ianthelline is not a candidate as a antioxidant dietary supplement.

5.6.3 Anticancer activity of ianthelline isolated from *Stryphnus fortis*:

Ianthelline showed dose-response anticancer activity; however ianthelline also affected normal lung fibroblasts (MRC5), reflecting a general toxicity. To investigate the potential mode of action for ianthelline, target-based analysis against two different kinases was performed. Neither PKA nor Abl were inhibited, indicating that these are not the target for ianthelline.

A time study to investigate whether ianthelline affected the cells by lysis of the membrane, which usually is a rapid process, was performed. As expected the cell viability was reduced the longer the cells were exposed to ianthelline. None of the cell lines tested against ianthelline was affected after 4 hours with exposure to ianthelline. Ianthelline needed to be present for at least 24-48 hours to show effect and all cell lines was affected after 72 hours with exposure to ianthelline. Trypan didn't stain the remaining cells after 72 hours of exposure to ianthelline. The long time needed for ianthelline to have an effect and that the cells were not stained by trypan, indicate that ianthelline might promote apoptosis. A possible further study could be a caspase assay, which detects the caspase enzyme activity formed during apoptosis [30].

6. Conclusion and further perspectives:

Ianthelline was successfully isolated from *Stryphnus fortis* by flash chromatography and bioassay guided semi-preparative HPLC, after the dereplication suggested the presence of ianthelline in the extract. TOF-MS, MS/MS and NMR confirmed that the compound isolated was ianthelline.

The primary screening of *Stryphnus fortis* indicated that there might be more than one compound responsible for the bioactivity. Further research could include identification, isolation and characterization of these other compounds.

Ianthelline showed an anticancer and antibacterial activity, but also killed normal lung fibroblasts (MRC5), indicating a general toxicity.

Ianthelline shows promising effects both against different bacteria and cancer cells even though it displays a general toxicity. Regarding this, ianthelline might not be a very good drug candidate as it is, but in future studies it might be used as a model for synthesis of new bioactive compounds where the synthesis strives to maintain or increase the activity while the toxicity should be minimized.

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Appendix A: Elemental Calculation by Masslynx of ianthelline

Elemental Composition Report

Single Mass Analysis

Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0

Element prediction: Off

Number of isotope peaks used for i-FIT = 7

Monoisotopic Mass, Even Electron Ions

1514 formula(e) evaluated with 26 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 5-50 H: 0-100 N: 0-10 O: 0-10 Br: 0-2

Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
473.9742	473.9776	-3.4	-7.2	8.5	547.9	0.5	C15 H18 N5 O3 Br2
	473.9664	7.8	16.5	8.5	548.4	1.0	C16 H18 N3 O4 Br2
	473.9763	-2.1	-4.4	3.5	552.8	5.4	C14 H22 N O7 Br2
	473.9677	6.5	13.7	13.5	554.4	7.0	C17 H14 N7 Br2
	473.9817	-7.5	-15.8	12.5	558.0	10.6	C20 H18 N3 O Br2
	473.9736	0.6	1.3	4.5	558.2	10.8	C10 H18 N7 O5 Br2
	473.9704	3.8	8.0	12.5	558.2	10.8	C21 H18 N O2 Br2
	473.9723	1.9	4.0	-0.5	560.7	13.3	C9 H22 N3 O9 Br2
	473.9835	-9.3	-19.6	-0.5	562.4	15.0	C8 H22 N5 O8 Br2
	473.9696	4.6	9.7	0.5	565.3	18.0	C5 H18 N9 O7 Br2
	473.9757	-1.5	-3.2	11.5	578.7	31.3	C11 H9 N9 O8 Br
	473.9798	-5.6	-11.8	15.5	579.3	31.9	C16 H9 N7 O6 Br
	473.9699	4.3	9.1	20.5	579.3	32.0	C18 H5 N9 O3 Br
	473.9784	-4.2	-8.9	10.5	579.4	32.0	C15 H13 N3 O10 Br
	473.9685	5.7	12.0	15.5	579.5	32.1	C17 H9 N5 O7 Br
	473.9825	-8.3	-17.5	14.5	580.1	32.7	C20 H13 N O8 Br
	473.9739	0.3	0.6	24.5	580.2	32.8	C23 H5 N7 O Br
	473.9726	1.6	3.4	19.5	580.2	32.8	C22 H9 N3 O5 Br
	473.9766	-2.4	-5.1	23.5	581.0	33.6	C27 H9 N O3 Br
	473.9667	7.5	15.8	28.5	581.3	33.9	C29 H5 N3 Br
	473.9819	-7.7	-16.2	22.5	586.9	39.5	C17 N9 O9
	473.9707	3.5	7.4	22.5	587.1	39.7	C18 N7 O10
	473.9747	-0.5	-1.1	26.5	587.6	40.2	C23 N5 O8
	473.9787	-4.5	-9.5	30.5	588.2	40.8	C28 N3 O6
	473.9675	6.7	14.1	30.5	588.3	40.9	C29 N O7
	473.9827	-8.5	-17.9	34.5	588.8	41.4	C33 N O4

Appendix B: Supplementary information of the MS/MS parent ion scan done on the purified compound:

Calibration: Dynamic 2

Extractor: 3.00 V

RF lens: 0.3 V

LM 1 resolution: 15.0

HM 1 resolution: 15.0

Entrance: 0

Exit: 0

LM 2 resolution: 14.5

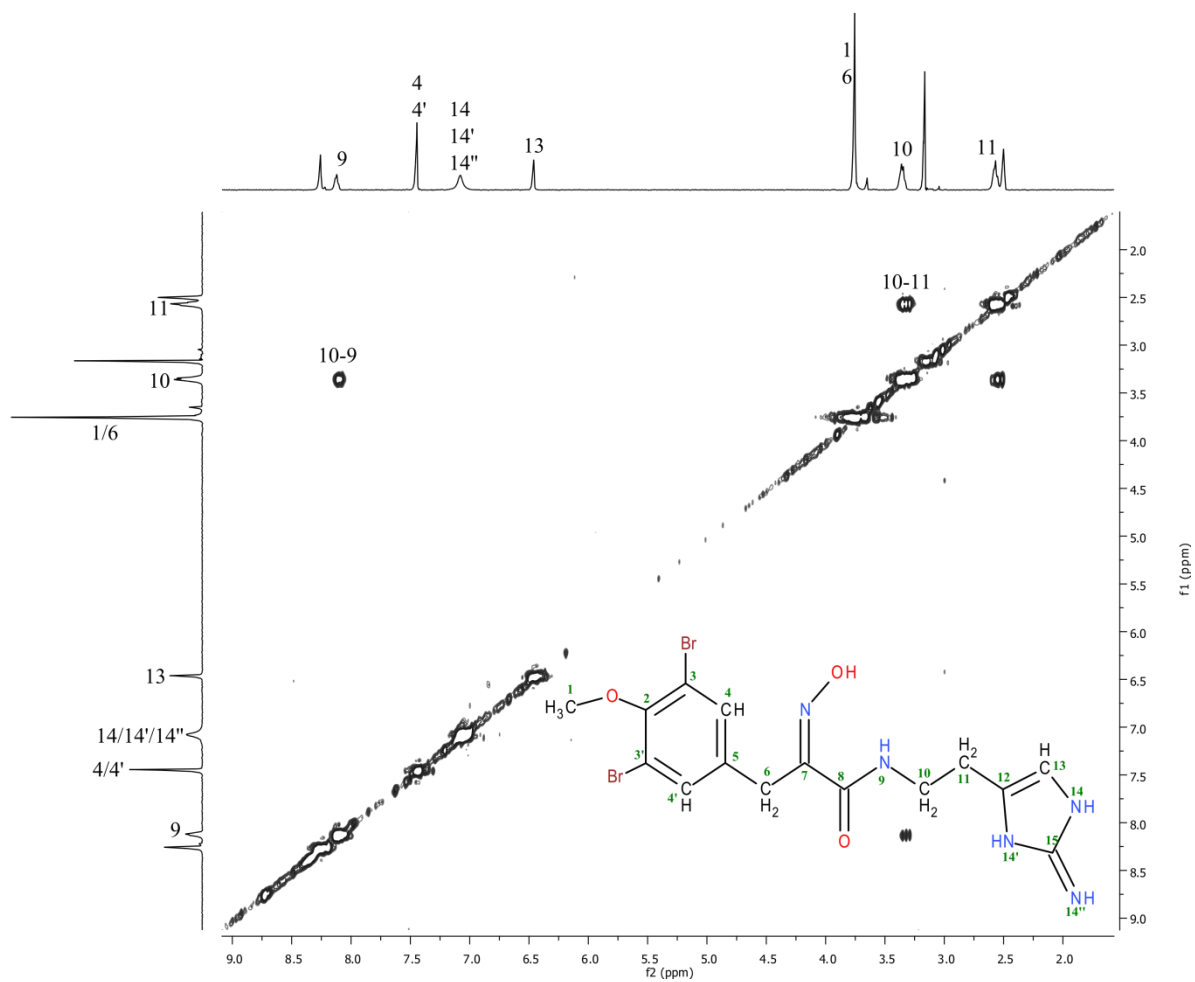
HM 2 resolution: 14.5

Multiplier: 514 V

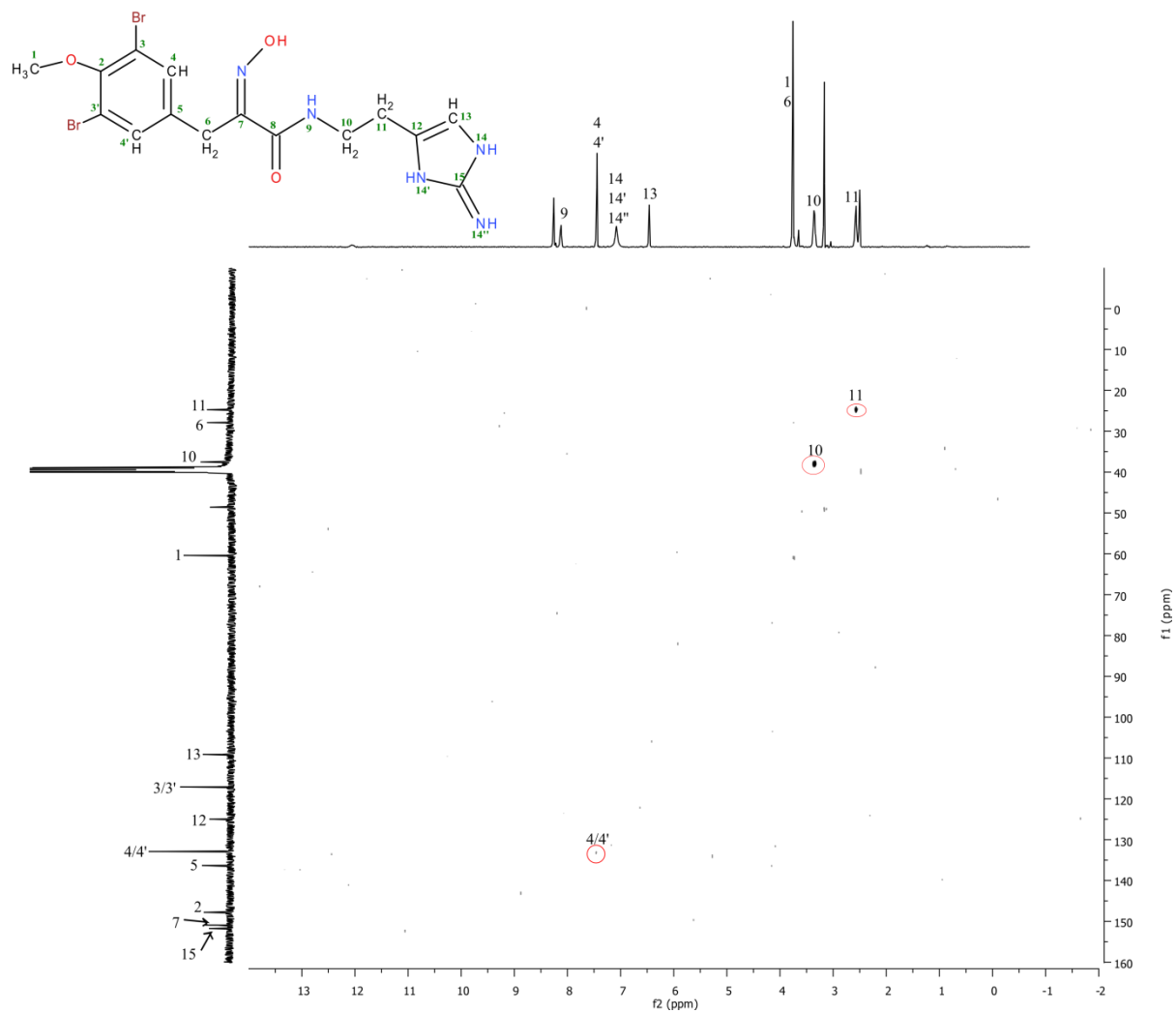
Gas cell pirani pressure: 1.85e-3 mbar

Appendix C: NMR spectra

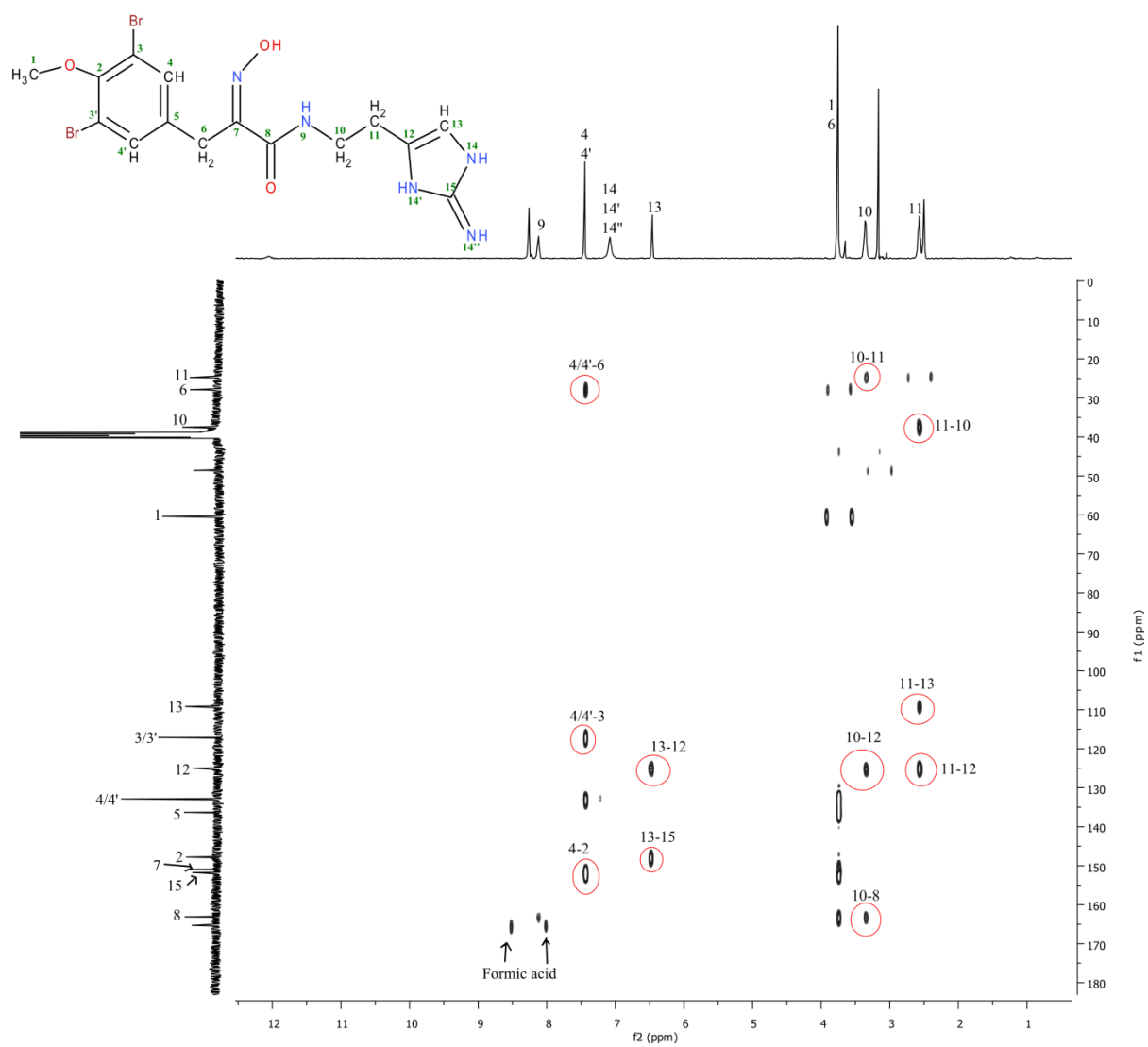
COSY NMR specter of ianthelline purified from *Stryphnus fortis*:



HSQC NMR specter of ianthelline purified from *Stryphnus fortis*:

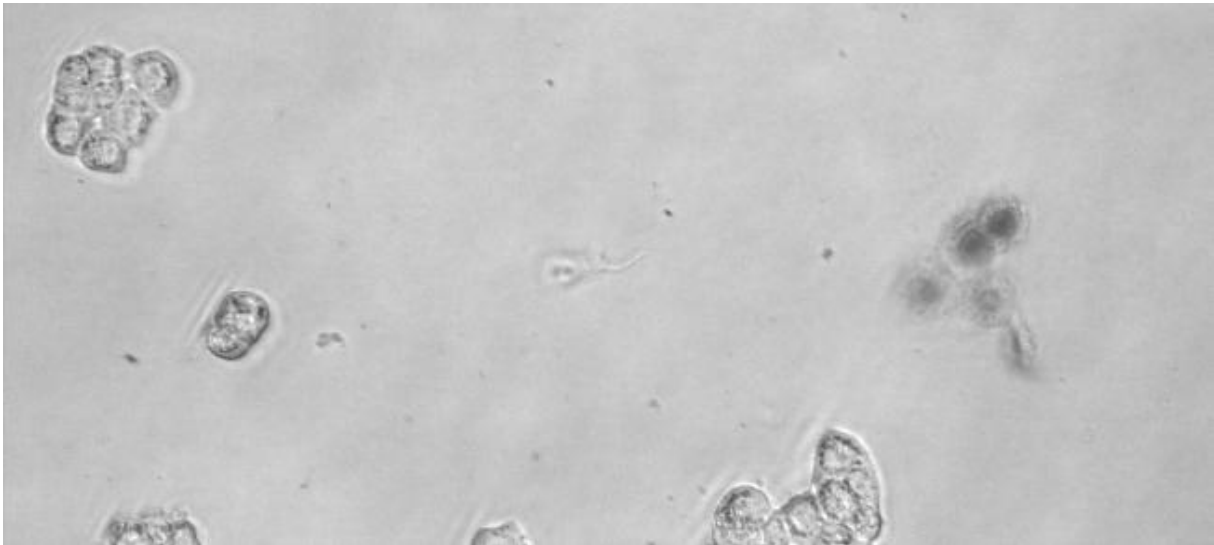


HMBC NMR specter of ianthelline purified from *Stryphnus fortis*:



Appendix C: Normal and trypan stained HT29 cells

Light microscope picture. The clear cells are normal HT29 cells while the blurry darker cluster of cells to the right is trypan stained dead cells. These cells were not exposed to ianthelline.



Appendix E: Poster shown at Biopros 2011

UNIVERSITETET I TROMSØ UiT

MabCent-SFI

sfi Centre for Research-based Innovation



ISOLATION AND CHARACTERISATION OF IANTHELLINE FROM AN ARCTIC SPONGE

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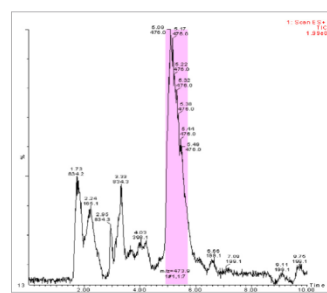
INTRODUCTION

A large number of natural products exerting a wide range of pharmacological effects including anti-inflammatory-, antibacterial-, and anticancer, have been isolated from marine sponges. An Arctic sponge extract showed antibacterial and anticancer activity in the primary screening in the MabCent screening program. In order to purify large amounts of the active compound, the extract was fractionated using FLASH chromatography and the fractions found to contain the active compound were combined and submitted to preparative HPLC for purification.

ToF-MS analysis identified ianthelline as the molecule responsible for the observed bioactivity. After analysis and structure confirmation using UPLC-MS/MS and 1H-NMR, ianthelline was tested for anticancer, antibacterial, and antioxidant activity.

PURIFICATION

Preparative HPLC-MS purification



To purify ianthelline from the selected FLASH fractions, a preparative HPLC-MS system was used.

Figure 1: Positive electrospray TIC (total ion current) chromatogram obtained from preparative HPLC-MS purification of ianthelline. Fraction collected is indicated by the marked area. The m/z-value 473.9 represents protonated ianthelline. Ianthelline were found in rich amounts in the extract, resulting in high quantity of pure compound.

MOLECULAR STRUCTURE

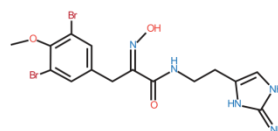
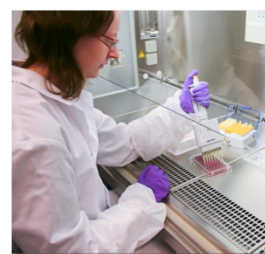


Illustration 1: Molecular structure of ianthelline.



ANALYSIS OF PURIFIED COMPOUND

Purified ianthelline was submitted to UPLC-ToF MS, UPLC-MS/MS and 1H-NMR analysis for structure confirmation and purity determination.

UPLC-ToF MS

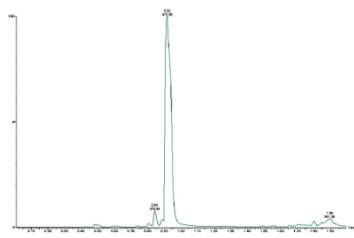


Figure 2: Positive electrospray BP (base peak intensity) chromatogram obtained from analysis of purified ianthelline. The compound shows an isotopic pattern with 1:2:1 intensity ratio of the m/z-values 473.9:475.9:477.9, confirming that the molecule is dibrominated. The peak with m/z-value 475.97 represents the most abundant isotope form, containing one Br-79 and one Br-81 atom. This peak is by far most abundant, indicating a high degree of purity. Using Mass Lynx software, element composition of the compound was found to be C15H18N5O3Br2. This matches the protonated molecular formula of ianthelline.

UPLC-MS/MS

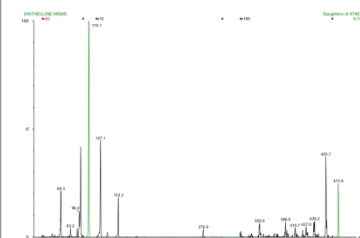


Figure 3: Positive electrospray MS/MS product ion scan spectrum of ianthelline. The chromatogram shows the molecular ions m/z-value of 473.9. Parts of the chromatogram are enlarged to give visible peaks. Degree of enlargement is indicated at the top of the chromatogram. Argon was used as collision gas.

1H-NMR

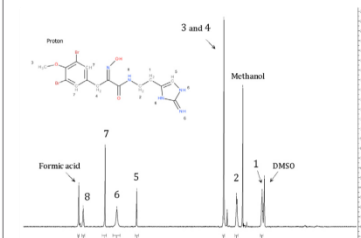


Figure 4: The 1H-NMR spectrum of ianthelline dissolved in d6-DMSO analysed on a 400 MHz spectrometer. Atomic labelling is shown in the illustration.

BIOACTIVITY TESTING: Purified ianthelline was tested for anticancer, antibacterial and antioxidant activity.

Anticancer activity

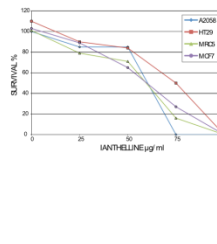


Figure 5: Anticancer activity of ianthelline affected the cell viability of the cancer cell lines A2058 (human melanoma), HT29 (human colon carcinoma) and MCF7 (human breast carcinoma) at the highest test concentrations (75 and 100 µg/ml), but it also affected the normal lung fibroblasts (MRC5) at these concentrations.

Antibacterial activity

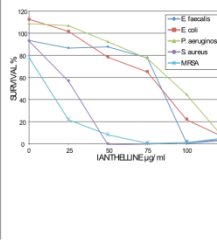


Figure 6: Antibacterial activity of ianthelline against five different bacteria (E. faecalis, E. coli, P. aeruginosa, S. aureus, methicillin resistant S. aureus (MRSA)). Ianthelline was more active against S. aureus and MRSA, but exerted only modest effect against E. faecalis and the gram-negative strains (P. aeruginosa and E. coli). Absorbance measured at 600 nm was compared to a positive control and negative control to calculate % survival.

Antioxidant activity

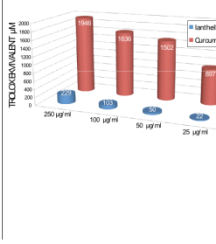


Figure 7: Ianthelline showed dose dependent activity in the FRAP assay but the activity was not high compared to curcumin. Ferric reducing ability was measured by using light absorbance at 593 nm (Fe(II) maximum absorbance), and a standard curve for trolox (6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid) was measured and the activity were expressed as trolox equivalents (µM).

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