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MASS SPECTROMETRY (MS) PROFILING ON POTENT ANTICANCER FRACTIONS OF *Calthropella* sp.

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ABSTRACT

This study aims to analyze the Mass Spectrometry(MS) profile of potent anticancer fractions of marine sponge *Calthropella* sp. The two potent fractions (F1 and F2) were analyzed by High Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS) to identify the compounds exist in each fractions. The LC-MS analyses were conducted on the bestelution system, isocratic elution of 80% MeCN (with 0.1% formic acid) + 20% milliQ H2O (with 0.1%formic acid). Based on LC-MS analyses, it is strongly predicted that F1 consist of 4'-N-methyl-5'- hydroxystaurosporine, 24-methyleneergost-4-ene-3-one, and one new compound. F2 is predicted to consist bengamide Q, clavepictine A, 4'-N-methyl-5'-hydroxystaurosporine, biemnic acid, carteriofenoneA, and one new compound

Keywords: Mass Spectrometry, *Calthropella* sp., marine sponge, anticancer activity

ABSTRAK

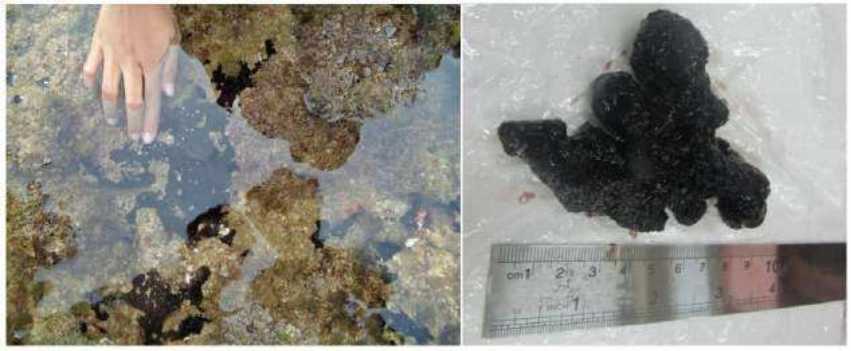
Penelitian ini bertujuan untuk menganalisis profil Mass Spectrometry (MS) dari fraksi toksik spons *Caltropella* sp. Dua fraksi toksik (F1 dan F2) dianalisis menggunakan High Resolution Liquid *Chromatography-Mass Spectrometry* (HR LC-MS) untuk mengidentifikasi senyawa di tiap-tiap fraksi.Analisis LC-MS dilakukan pada sistem elusi terbaik 80% MeCN (dengan 0.1% asam format) + 20% milliQH_2O (dengan 0.1% asam format). Analisis LC-MS mengindikasi kuat bahwa senyawa hasil isolasi dari fraksi toksik F1 terdiri dari 4'-N-metil-5'-hidroksistaurosporin, 24-metileneergos-4-en-3-on, dan satu senyawa baru. Fraksi toksik F2 diprediksi kuat mengandung bengamida Q, klafepiktin A, 4'-N-metil-5'- hidroksistaurosporin, asam biemnik, karteriofenon A, dan satu senyawa baru.

Kata Kunci: Mass Spectrometry, *Calthropella* sp., spons laut, aktivitas antikanker

1. INTRODUCTION

A cytotoxicity-guided isolation has led to the finding of toxic fractions of *Calthropella* sp. marine sponge.[1] The fractionation and cytotoxic assay were done on the previous study. [2] The ethyl acetate extract was suspended in 1 mL of ethyl acetate and subjected to column chromatography. The elution solvent system was a series of gradient polarity eluents: 100% n-hexane followed by (n-hexane/ethyl acetate) (98:2), (95:5), (9:1), (8:2) and (1:1) (v/v) the separation continued by same gradient of (ethyl acetate/methanol) and 100% methanol. The elution was driven through the column by applying pressure. Fractions of a standard volume were collected and then combined based on the spots similarity using TLC.[1] The two potent fractions (F1 and F2) showed promising cytotoxicity on human MCF7 breast cancer cells with IC_{50}< 30 µg/mL by MTT cytotoxic assay. Notably, F1 exhibited cytotoxic activity toward MCF7 cancer cell lines (IC_{50} =22.84 µg/mL) and F2 exhibited even higher cytotoxicity with IC_{50} =1.92 µg/mL.[2]

Marine sponges are a rich source of novel pharmacological (bioactive) compounds.[3,4,5] In particular, *Calthropella* sp. (family of *Calthropellidae*). *Calthropella* sp. was found encrusting in intertidal zone of Krakal Beach, the southern coastal area of Gunungkidul (see Figure 1). This type of sponge is originally a deep sea sponge, living in a still and steady marine environment in Atlantic Ocean.[6] Such different environmental condition mostly affects on the metabolic substances produced by the sponge.[7] The same-family of sponge, *Pachastrissa* sp. (family of *Calthropellidae*) reported several chemical constituents, namely bengamides, bengazoles,[8] kabiramides.[9] All previous isolated compounds showed potent biological activities *e.g.* cytotoxicity, antimicrobial, and antimalarial activity.



(a) (b)

Figure 1. Marine sponge *Calthropella* sp. (a) underwater, and (b) after freezing

Liquid chromatography (LC) separates the sample components and then introduces them to the mass spectrometer (MS). The LC-MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components. Electrospray Ionization- Mass Spectrometry (ESI- MS) is based on the determination of the mass-to-charge (m/z) ratio of an analyte. ESI-MS is well known as a "soft" ionization technique, which provides primarily molecular ions that occur at relatively high values of mass-to-charge ratio (m/z), rather than fragment ions which occur at relatively low m/z values. In ESI, positive ions formed when the components in gas phase accept protons $[M+nH]^+$ or other cation $[M+Na]^+$. Negative ions may also be formed by loss of protons $[M-nH]^-$ in ESI-MS.[10]

General classification of genus in one family is based on morphologycally, genetically, and chemically (chemotaxonomy). Chemotaxonomy or the classification of an organism according to its natural products may assist in dealing with unknown genera related species may produce related secondary metabolites.[11] With this in mind, this present study analyze the MS profile of potent anticancer fractions of marine sponge *Calthropella* sp. and understanding its chemotaxonomy.

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2. METHODS

Equipment and Tools

The solvent used for general purposes were commercial grade; Merck pro-analysis solvent e.g. methanol (MeOH), milliQ water, HPLC grade acetonitrile (MeCN), formic acid (HCOOH). Laboratory glassware (Iwaki Pyrex), analytical balance (Nettler Toledo AB204-S), Ultrasonic bath SIBATA, vortex mixer (Thermoline), MicrOTOF Agilent Bruker Daltonics 1100 LC-HRESIMS.

Determine the Best Elution System

The active fractions (F1 and F2) were analyzed by HR LC-MS to identify the compounds exist in each fractions. The LC-MS analyses were conducted on an Agilent 1100 Series HPLC system coupled with a Bruker Daltonics micrOTOF-HS mass spectrometer (ESI). The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 µm, 25°C, 0.2 mL/min). Each analysis was conducted for 40 minutes running.

MS-Profiling and Structure Identification

The MS software predicted the elemental composition of the compound from the accurate m/z and isotope distribution. The mass data were used in conjunction with the taxonomy data described above to conduct a search of the MarinLit database.[12] Since a given nominal mass may correspond to several molecular formulas, lists of such requirements are especially useful when evaluating the possibility structure of a compound. There are two ways one can go about determining the posssibility structure of compound, the nitrogen rule and degree of unsaturation.

The nitrogen rule is a general principle which may prove useful when attempting to solve organic mass spectrometry structures. The rule stated, if the molecular mass of an unknown compound to the nearest integer value is an odd number, the compound contains an odd number of nitrogens in its molecular formula. Correspondingly, if the molecular mass is an even number, the compound contains zero or an even number of nitrogens in its molecular formula. This rule is derived from the fact that, perhaps coincidentally, for the most common chemical elements in neutral organic compounds (hydrogen, carbon, nitrogen, oxygen, silicon, phosphorus, sulfur, and the halogens), elements with even numbered nominal masses form even numbers of covalent bonds, while elements with odd numbered nominal masses form odd numbers of covalent bonds, with the exception of nitrogen, which has a nominal (or integer) mass of fourteen, but has a valency of three.

Calculating the degrees of unsaturation (DoU) is also useful information since knowing the DoU make it easier to figure out the molecular structure; it helps to double-check the number of π bonds and/or cyclic rings. Unsaturated molecules contain double bond(s), triple bond(s) and/or ring(s). For common organic compounds, CaHbNcOdXe, the DoU can be calculated as:

$$DoU = ½ (2+(2a)–b+c−e)$$

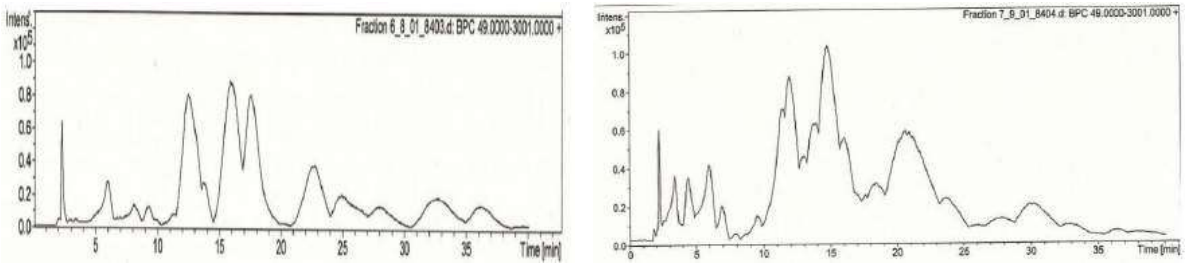
with X as the number of halogens (F, Cl, Br, I).

One degree of unsaturation is equivalent to 1 ring or 1 double bond (1π bond). Two degrees of unsaturation is equivalent to 2 double bonds, 1 ring and 1 double bond, 2 rings, or 1 triple bond (2 π bonds). The DoU only gives the sum of double bonds, triple bonds and/or rings. It does not give the exact number of rings or double or triple bonds, but rather the sum of the number of rings and double bonds plus twice the number of triple bonds. Knowing the DoU in a molecule is quite useful when trying to determine a structure, because it tells the number of rings, double or triple bonds which are present in the compound.

3. RESULTS AND DISCUSSION

Best Elution System

The analysis was done for the most potent fractions (F1 and F2) of *Calthropella* sp. which showed strong cytotoxic value. It was analyzed by Liquid Chromatography–Mass Spectrometry (LC-MS) to obtain the accurate mass and identify the possible structure of compound. The best elution system was obtained with isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in milliQ H_2O for 40 minutes. The shown chromatogram was not perfectly separated, especially after 10 minutes separation. This probably due to the eluent system which was not appropriate yet. Another possibility was due to an easy-degraded properties of marine compounds.[13] Most of the compounds were eluted after 10 minutes separation. There were larger peaks with high intensity eluted between 10 minutes and 25 minutes (see Figure 2). This is indicated that most compounds which are exist in F1 and F2 are relatively nonpolar compounds.



(a) (b)

Figure 2. LC Chromatogram with 80% MeCN: 20% H_2O (v/v): (a) F1 and (b) F2

MS-Profiling and Structure Identification of F1

There are 13 peaks detected in total, 10 of them detected as impurities. Those three peaks were detected at the retention time (RT) 6.0; 17.5; and 36.1 minute. The overall detected peaks are listed on Table 1.

Tabel 1. Predicted Identified Compound of F1 by MS-Profiling

Retention Time (RT)	MS Measured mass (amu)	Molecular formula of theoretical mass (amu)	Predicted Compound
6.0	497.2316 amu $[M+H]^+$	$C_{29}H_{28}N_4O_4$ (496.2110 amu)	4'-N-methyl-5'-hydroxystaurosporine (18 DoU)
17.5	347.3235 amu $[M+H]^+$	$C_{19}H_{42}N_2O_3$ (346.2872 amu)	New compound
36.1	397.3392 amu $[M+H]^+$	$C_{28}H_{44}O+H^+$, (397.3392 amu)	24-methyleneergost-4-ene-3-one

The HR-ESIMS spectrum peak at the minute 6.0, as shown in Figure 3, shows some positive ion peaks at m/z 497.2316 $[M+H]^+$, m/z 514.2597 $[M+H_2O]^+$, and m/z 519.2148 $[M+Na]^+$. HR-MS provided the molecular formula $C_{28}H_{28}N_6O_3$, requiring 18 sites of unsaturation. The molecular formula indicates to compound with theoretical molecular mass of 496.2223. Previously, there is no related study for compounds with molecular formula of $C_{28}H_{28}N_6O_3$ isolated from marine organism. However, compound with molecular mass of 496 amu with the closest theoretical mass as 496.2223 (Δ mass=28.05 ppm) had been successfully isolated. The new indolocarbazole alkaloid, 4'-N-methyl-5'-hydroxystaurosporine was isolated from the culture broth of a marine sponge *Clathrina coriacea* collected on the coast of Fuerteventura Island in the Canary Islands archipelago.[14] The molecular formula was established as $C_{29}H_{28}N_4O_4$ (18 DoU) with theoretical mass of 496.2110 amu. The compound showed cytotoxicity against P388, A549, HT-29, and SK-MEL-28 cell lines.

This new alkaloid compound is predicted to be the most likely compound since it has the nearest theoretical mass and the same predicted number of unsaturation degree (18 DoU). Eight rings contribute for 8 DoU, 9 π bonds (9 DoU) in total of 3 benzenes, and 1 π bond (1 DoU) attributed to a carbonyl ketone.

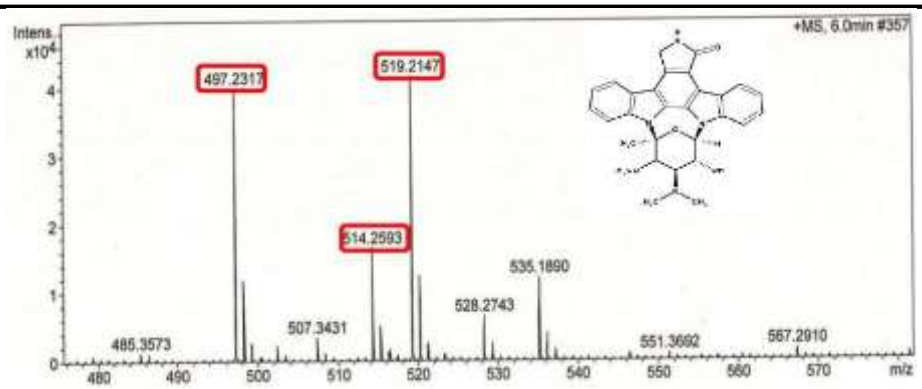


Figure 3. The HR-ESIMS spectrum peak of F1 at the minute 6.0

The HR-ESIMS spectrum peak at the minute 17.5 has $[M+H]^+$ at 347.3235 and $[M+Na]^+$ signal at m/z 369.3065, suggesting the molecular formula $C_{19}H_{42}N_2O_3$ (calculated $C_{19}H_{42}N_2O_3$+Na, 369.3065). This molecular formula indicates to compound with theoretical molecular mass of 346.3195. The suggessted formula is in accordance with the nitrogen rule as it has even number of nitrogen atom. Previously, there is no related study for compounds with molecular formula of $C_{19}H_{42}N_2O_3$ isolated from marine organism. The closest compound with molecular mass of 346 amu is $C_{23}H_{38}O_2$, theoreticalmass of 346.2872. However, the mass difference is up to 93.28 ppm. Thus, it is predicted to be a new compound.

The HR-ESIMS spectrum peak at the minute 36.1 has [M+H], signal at m/z 397.3392, suggesting the molecular formula $C_{28}H_{44}O$ (calculated $C_{28}H_{44}O$+H, 397.3392). This molecular formula indicates to compound with theoretical molecular mass of 396.3392. The compound with molecular formula of $C_{28}H_{44}O$ has previously been isolated as 24-methyleneergost-4-ene-3-one, a marine sterol of Sinularia soft corals *Sinularia dissecta*. This novel sterol was isolated together with four new sterols from the same soft coral species.[15] To date, there is no biological activity reported from this compound.

MS-Profiling and Structure Identification of F2

The MS chromatogram profile of F2 is as similar as F1 (see Figure 2), nonetheless it's rather complex in F2. In result, there are 15 peaks detected in total, 9 of them detected as impurities. The overall detected peaks are listed on Table 3.

Tabel 3. Predicted Identified Compound of F2 by MS-Profiling

Retention Time (RT)	MS Measured mass (amu)	Molecular formula of theoretical mass (amu)	Predicted Compound
4.4	582.4256 $[M]^+$	$C_{32}H_{58}N_2O_7$ (582.4244 amu)	bengamide Q
6.0	497.2316 $[M+H]^+$	$C_{28}H_{29}N_6O_3$ (497.2223 amu)	4'-N-methyl-5'-hydroxystaurosprine
7.0	348.2991 $[M+H]^+$	$C_{21}H_{38}N_3O$ (348.2991 amu)	clavepictine A (5 DoU)
9.6	363.3193 $[M+H]^+$	$C_{20}H_{38}N_6$ (363.3145 amu)	biemnic acid
12.9	540.4622 $[M+H]^+$	$C_{32}H_{62}NO_5$ (539.4549 amu)	new compound
13.6	501.3893 $[M+H]^+$	$C_{33}H_{48}N_4$ (500.3866 amu)	carteriofenone A (7 DoU)

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The HR-ESIMS spectrum peak at the minute 4.4 has the positive ion peaks at m/z 605.4738 $[M+Na]^+$ and m/z 582.4256 $[M]^+$ with suggesting molecular formula $C_{32}H_{58}N_2O_7$. Based on literature study, the molecular formula refers to bengamide Q ($C_{32}H_{58}N_2O_7$) with theoretical mass of 582.4244 amu. The classes compound of bengamides were earlier isolated from marine sponge *Pachastrissa* sp. collected along the Djibouti coast, Africa.[7] This type of sponge is in one family as *Calthropella* sp. family of *Calthropellidae*. In the view of bioactivity, bengamide Q exhibited a potent antifungal activity against *Candida albicans* (MIC=7 µg/mL).

The HR-ESIMS spectrum peak at the minute 6.0, shows some positive ion peaks at m/z 497.2316 $[M+H]^+$, m/z 514.2597 $[M+H_2O]^+$, and m/z 519.2148 $[M+Na]^+$ suggesting molecular formula of $C_{28}H_{28}N_6O_3$ requiring 18 sites of unsaturation. This molecular formula indicates to compound with theoretical molecular mass of 496.2223. In this case, the new indolocarbazole alkaloid, 4'-N-methyl-5'-hydroxystaurosporine which also being detected in F1 is the most likely compound, since it is also being eluted at the same retention time (see Figure 3).

The HR-ESIMS spectrum peak at the minute 7.0, shows positive ion peaks at m/z 348.2991 $[M+H]^+$, m/z 349.2991 $[M+2H]^+$ and $[M+Na]^+$ ion peak at m/z 370.2857 with suggesting molecular formula $C_{21}H_{37}N_3O$ corresponding to 5 DoU. The suggested molecular formula is in accordance with the nitrogen rule as it has odd number of nitrogen atom. Other peaks which show moderate intensity i.e. m/z 419.2893; 463.3160; 507.3441 were detected as triton, common impurities in ESI positive mode. The suggested molecular formula indicates to compound with theoretical molecular mass of 347.2937 amu. Previously, there is no related study for compounds with molecular formula of $C_{21}H_{37}N_3O$ isolated from marine organism. However, compound with molecular mass of 347 amu with the closest theoretical mass as 347.2937 (Δ mass=32.25 ppm) was earlier identified as clavepictine A (see Figure 4). The molecular formula was established as $C_{22}H_{37}NO_2$ with theoretical mass of 347.2824 amu. Clavepictine A was first isolated from the Bermudian tunicate *Clavelina picta*.[16] Raub and Cardellina (1991) reported clavepictine A inhibit growth of murine leukemia and human solid tumor cell lines (P-388, A-549, and U-251) at concentrations less than 9 µg/mL (IC_{50}=1.8-8.5 µg/mL) and effectively kill each cell line at less than 25 µg/mL (LC_{50} = 10.1-24.7 µg/mL). This compound is predicted to be the most likely compound, since it has the nearest theoretical mass, satisfy the nitrogen rule, and the same predicted number of unsaturation degree (5 DoU). One was readily attributed to an acetate ester, two more comprised a conjugated diene, leaving two rings to be placed in the molecule.

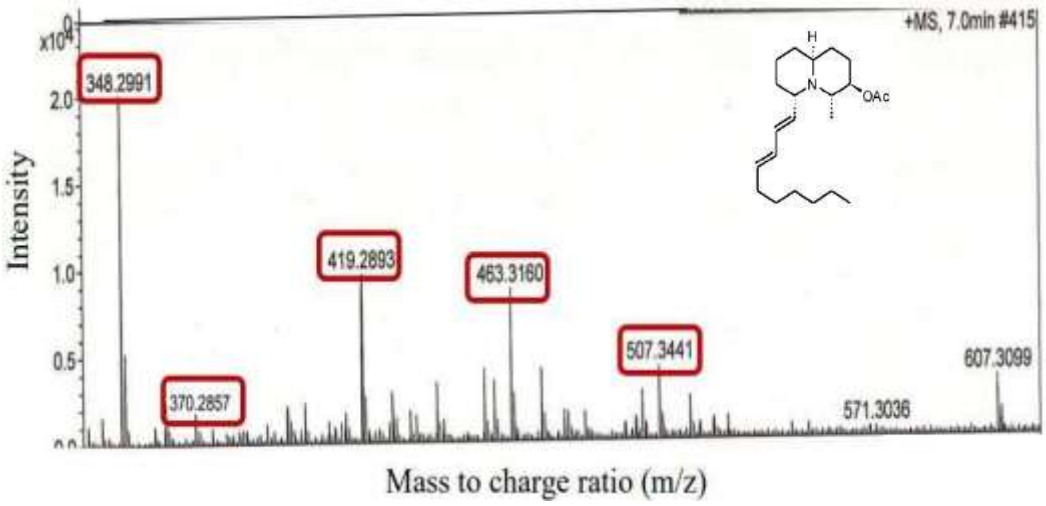

Figure 4. The HR-ESIMS spectrum peak of F2 at the minute 7.0

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The HR-ESIMS spectrum peak at the minute 9.6, has positive ion peaks at m/z 363.3193 $[M+H]^+$ and m/z 385.3037 $[M+Na]^+$ with molecular formula of $C_{20}H_{38}N_6$ (calculated $C_{20}H_{38}N_6$+Na, 383.3037). This spectrum indicates to compound with theoretical molecular mass of 362.3158 amu. Based on literature study, there is no marine isolated compound with molecular formula of $C_{20}H_{38}N_6$. The closest compound is biemnic acid, a new 24-acetylenic acid, molecular formula of $C_{24}H_{42}O_2$ with theoretical mass of 362.3185 (Δ mass=11.04 ppm). Biemnic acid was isolated from Red sea sponge *Biemna ehrenbergi* together with one new sterol ehrenasterol.[17] Nonetheless, instead of 5, biemnic acid posses 4 sites of unsaturation.

The HR-ESIMS spectrum peak at the minute 12.9, has positive ion peaks at m/z 540.4622 $[M+H]^+$, suggesting molecular formula of $C_{32}H_{61}NO_5$ with correspond to 6 DoU. This spectrum indicates to compound with theoretical molecular mass of 539.4549 amu. The suggested molecular formula is in accordance with the nitrogen rule as it has odd number of nitrogen atom. Previously, there is no related study for compounds with molecular formula of $C_{32}H_{61}NO_5$ isolated from marine organism. The closest compound with molecular mass of 539 amu is $C_{39}H_{49}NO_5$ (539.3610 amu), difference mass value up to 174.095 ppm. Thus, it is predicted to be a new compound.

The HR-ESIMS spectrum peak at the minute 13.6, shows base peaks: m/z 501.3893 $[M+H]^+$, and peak 523.4344 $[M+Na]^+$ with suggesting molecular formula of $C_{33}H_{48}N_4$ with 7 sites of unsaturation. This spectrum indicates to compound with molecular mass of 500.3878 amu. The suggested molecular formula is in accordance with the nitrogen rule as it has odd number of nitrogen atom. The compound carteriofenone A (see Figure 5), $C_{32}H_{52}O_4$, theoretical mass of 500.3866 (Δ mass=2.398 ppm) is the most likely compound as it has the same number of DoU as predicted compound. Two carbonyl ketone contribute to 2 DoU, 4 rings as 4 DoU, and 1 π bond correspond to 1 DoU, resulted 7 DoU in total. This new scalarane sesterterpenoid was obtained from the marine sponge *Carteriospongia foliascens* collected from the South China Sea, showed cytotoxicity against the mouse lymphocytic leukemia cell line (P388) with an IC_{50} value of 0.96 μM.[18]

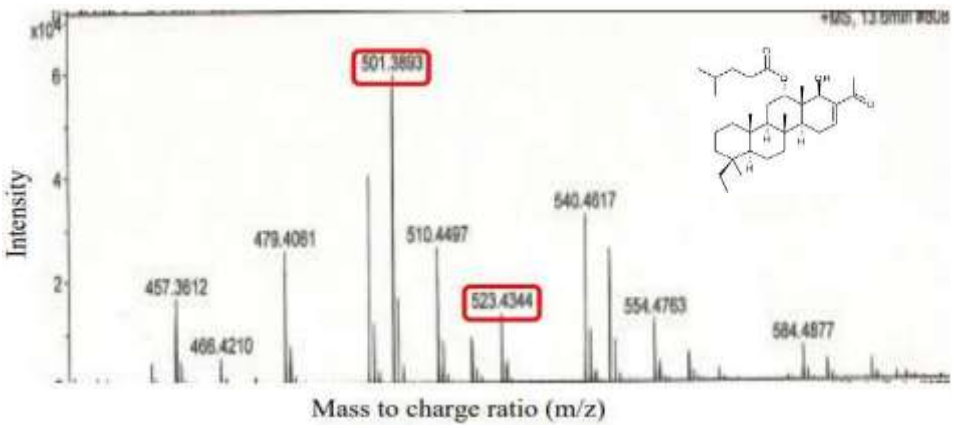

Figure 5. The HR-ESIMS spectrum peak of F2 at the minute 13.6

4. SUMMARY

The compounds which are predicted to exist in F1 are known compounds 4'-N-methyl-5'-hydroxystaurosporine, 24-methyleneergost-4-ene-3- one, and one new compound. The compounds which are predicted to exist in F2 are known compounds 4'-N-methyl-5'-hydroxystaurosporine, biemnic acid, bengamide Q, clavepictine A, carteriofenone A, and one new compound. Several known compounds which already have biological activities predicted to contribute for the cytotoxic activity of each fraction, specially 4'-N-methyl-5'- hydroxystaurosporine which exist in both active fractions. The predicted existence of bengamide Q proves the chemotaxonomy between *Calthropella* sp and *Pachastrissa* sp.

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