

Identification of Chemicals in The Extract of *Merremia Vitifolia* and *Bidens pilosa* and The Histamine Inhibiting Assay

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Received September 12, 2022; Accepted February 06, 2023; Available online March 20, 2023

ABSTRACT. There are two species of plant that grow flourishing all around Indonesia including in Banten and West Java which are *Merremia vitifolia* and *Bidens pilosa*. In this study we evaluate how these plants could be potentially used as natural preserver of fish product especially *Auxis thazard*, to inhibit the histamine formation, and to find out how this activity correlates to the substances in polar extract of *B. pilosa* flowers and *M. vitifolia* leaves. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) were used to identify the substances of the plant's part extracts. There are 27 chemicals in *M. vitifolia* extract and 14 chemicals in *B. pilosa* extract that have been detected. A triglyceride has been detected, isolated, and characterized by FTIR, ¹H-NMR and ¹³C-NMR from n-hexane extract of *M. vitifolia* supported by LC-MS/MS data. Histamine formation in fish was determined after 30 min treatment with 4-hydroxybenzoic acid solution is around 40 – 51 mg / 100 g of fish, while treatment with *M. vitifolia* and *Bidens pilosa* extracts were less than 10 mg / 100 g of fish. This is the indication of high potential of both extract as preserver of fish products. Many of the identified substances have bioactivity like antimicrobial, anticancer, anti-inflammatory, antioxidant, and more, which influence the extracts' ability to inhibit the formation of histamine in fish.

Keywords: *Auxis thazard*, *B. pilosa*, *M. vitifolia*, Histamine Inhibition, Substances Identification

INTRODUCTION

Indonesia is an archipelago country and the highest fisheries producent country in South-east Asia. Indonesian consumed fish 47.34 kg/capita in 2017 and increasing every year (Firmansyah et al., 2019). Pelagic type of fish is widely obtained commercially in market at many cities especially in East Indonesia and it has high economic value for export trade including Frigate Tuna (*Auxis thazard*). Frigate Tuna is from the family of Scombridae that have red flesh. The damage of the fish by bacterial activity as well as enzymes can produce toxin called Scombrotxin. This toxin is histamine produced by fish because of improper refrigerated or preserved (Hattu et al., 2015). Scombrotxin poisoning is usually associated with consumption of fish containing high levels of histamine (Zare et al., 2015).

Histidine decarboxylase breaks down histidine into histamine in the skin, gut, and gills of the fish. There are wide range of bacteria that produce histidine decarboxylase including *Pseudomonas*, *Citrobacter*, *Clostridium*, *Klebsiella* and *Morganella morganii* (Pawul-Gruba & Osek, 2021). After histamine formed in the meat of fish, it will remain active even after

cooking, because histamine is heat stable. This is why, the formation of histamine should be prevented or inhibited to preserve the quality of fish. Histamine involved in human physiological functions such as local immune and inflammation responses, and neuromodulation if the body consumed high amount of histamine, it could pop up symptoms similar of allergic reaction. The typical observed symptoms are itching, nausea, vomiting, abdominal cramps, headaches, flushing and hypotension (Biji et al., 2016; Visciano et al., 2014).

Meanwhile, Indonesia is tropical country with redundant of medical plants and can be used as inhibitor of histamine production in fresh fish or any other biological activities. There are some reports on the plant's extract that possess antihistamine activity such as *Tamarindus indica* L. (Hattu et al., 2015), *Solanum nigrum* and *Ricinus communis* (Lomash et al., 2010), *Endophytic Fungi in Curcuma longa* L. (Lomash et al., 2010) and *E. prostrata* (Linn.) (Risfa et al., 2021). There are two species of plant that grow flourishing all around Indonesia including in Banten and West Java which are *Merremia vitifolia* and *Bidens pilosa*. Both plants are known traditionally to treat

several illnesses like headache, rheumatism, eye inflammation, dysentery, swollen glands, ear infections, and mouth ulcers (Akter et al., 2021; Xuan & Khanh, 2016).

The first plant is *M. vitifolia* which locally know in Indonesia as Bijalang Bulu or Akar Buluh, in recent years has been studied. It has immense potential as antioxidant, anti-arthritic and anti-nociceptive (Akter et al., 2021), as inhibitor of α -glucosidase (Tahya & Karnelasatri, 2021), and antibacterial (Hasanah et al., 2020). But very few reports about the chemical's composition of the extracts. Previously, we have studied the chemicals composition of n-hexane extract of *M. vitifolia* using GC-MS (Tahya & Karnelasatri, 2021), but in current research we study more on the chemicals composition of *M. vitifolia* extracts using LC-MS/MS and GC-MS. The study of chemicals compositions will open broader potential applications of this plant.

In contrast to *M. vitifolia*, *B. pilosa* is a plant that has been well studied. It is originally from South America but widely distributed in most tropical and subtropical areas of the world including Indonesia. It has many names all over the world, but in Indonesia, it is known as Ketul especially in Java. The major chemicals identified in *B. pilosa* are polyacetylenes, triterpenes, some essential oils, and flavonoids. These chemicals are considered as the most active substances that responsible for biological and pharmacological actions of the plant. But there are very few reports in chemicals constituents of *B. pilosa* grow in Indonesia, especially in west part of Java Island (Silalahi et al., 2021). In this study we evaluate how these plants could be potentially used as natural preserver of fish product especially *Auxis thazard* by inhibit the histamine formation. We try to find out how this activity correlates to the substances in polar extract of *B. pilosa*'s flowers and *M. vitifolia* leaves that harvested in Tangerang regency, Banten province, Indonesia. To identify the substances of the plants extract, we used liquid chromatography with tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS).

EXPERIMENTAL SECTION

Materials and Instruments

Fresh leaves of *Merremia vitifolia* and all part of the flowers of *Bidens pilosa* were collected in Panongan District, Tangerang Regency, Banten, Indonesia. For extraction process the solvents n-hexane (Merck), ethyl acetate (Merck), and methanol (Merck) were purchased from PT. Pasifik Kimia Indonesia. Chemicals used for histamine forming inhibition test are sulfanilic acid (Merck), sodium nitrite (Merck), NaCl (Merck), sodium sulfate anhydrate (Merck), sodium phosphate monohydrate (Merck), 1-butanol (Merck), Na₂CO₃ (Merck), hydrochloric acid (Merck),

histamine dihydrochloride (Merck), 4-hydroxybenzoic acid (Merck), TLC 60 aluminum plat F254 (Merck), distilled water, and filter paper.

Analytical Balance (Adventure Pro AV264C), Electric Heater (Cimarec 2), UV lamp, Centrifuge (Labofuge 200- Heraeus), UV-Vis Spectrophotometer (Apel PD-303S spectrophotometer), GC-MS instrument (Agilent GC 6890N 5975B MSD system), LC-MS/MS instrument (Waters, Acquity UPLC I-Class with Xevo G2-XF QToF), Agilent 500 MHz NMR spectrometer with DD2 console system and FTIR Alpha Bruker.

Samples Preparation and Extraction

Total of 500 g of leaves (*M. vitifolia*) sample and 500 g of flowers (*B. Pilosa*) sample were collected. Both samples were cleansed with distilled water, let it dry for 2 days at dark and room temperature. Chop each the samples into small parts and dried in oven at 80 °C for 36 hours. Samples were cut into smaller pieces with blender. As many as 80.48 g of dried rough powder of *M. vitifolia* leaf and 105.01 g of *B. pilosa* flower were obtained. Weight 20.0 g of the sample powder and added into 150 mL of solvent in 250 mL Erlenmeyer flask and tightly sealed. For *M. vitifolia*, sample was extracted with n-hexane and methanol, while *B. pilosa* sample was extracted with methanol only. Extraction took place for 24 hours in room temperature and dark condition. However, for flask containing extract of n-hexane of *M. vitifolia*, after 3 hours maceration was stop for string and let the mixture of liquid and solid be separated in 15 minutes. Took about 15 mL liquid extract of n-hexane of *M. vitifolia* and put into vial bottle and let solvent evaporate, and sample **1** ready to send for the first LC-MS/MS analysis. The flask containing the remain n-hexane extract was sealed again and continue maceration for 21 hours more. Flasks containing methanol extracts were continuing maceration process for 24 hours nonstop. After maceration, most solvents were evaporated using rotary evaporator. Samples were divided into several vial bottles. The samples contain methanol extract of *M. vitifolia* and *B. pilosa*, were designated as sample **2** and **3**, respectively (still containing small quantity of solvent) and send to analytical laboratory of ITB Bandung for GC-MS analysis. Other samples also contained methanol extract of *M. vitifolia* and *B. pilosa* were designated as sample **4** and **5** respectively, were sent to LC-MS/MS analysis. Sample **6** contained n-hexane extract of *M. vitifolia* was divided into 2 vial bottles (**6a** and **6b**) and let to dry (until free solvent) for 2 more days in dark place at room temperature. One dried sample (**6a**) was sent to LC-MS/MS analysis at Center of Chemistry Research, Indonesian Institute of Sciences, and the other dried sample (**6b**) was prepared for TLC preparative.

Isolation of Triglyceride

A dry and clean TLC 60 aluminum plat F254 size 20 x 20 cm 1 mm thick with based line was prepared. About 3 mL n-hexane solvent to dry extract in vial

bottle and mix it. Pipetted all the mixture of extract into the base line of TLC plat and let for air dry. Put TLC plat into chamber contain solvents mixture of n-hexane: ethyl acetate (8:1). After separation of substance in TLC plat, the yellow substance was scraped from the silica layer of TLC. The yellow power was dissolved in n-hexane 10 mL, and then filtered with Whatman 40. The yellow solution was dried for several hours until 1/3 volume remain. The solution was then spotted onto new, dry, and clean TLC 60 aluminum plat F254 size 5 x 10 cm at the base line, then run in the chamber with similar solvent. Any spots were observed under UV light. The isolated fraction was analysis with NMR and FTIR for structure identification.

Preparation of 4-Diazoniobenzenesulfonate Reagent

A mixture of 1.5 mL of 0.9% (w/v) sulfanilic acid in 4% HCl and 1.5 mL of 5% (w/v) NaNO₂ was immersed in ice water for 5 minutes. 6 mL of 5% NaNO₂ solution was added and allowed to stand for 5 minutes. The volume was adjusted to 50 mL with cold distilled water. Then, the reagents were kept in an ice bath for 15 minutes. Then let stand for 12 hours and ready to use (Hattu et al., 2015).

Histamine Standard Curve

Histamine dihydrochloride was dissolved in 100 mL of distilled water for concentration of 1000 ppm. The standard histamine solution of 1000 ppm was then diluted with distilled water to obtain a concentration of 100 ppm, which was then diluted to obtain concentrations of 5, 20, 40, 60, 80 and 100 ppm. A total of 1 mL of histamine standard solution. Every standard solution with concentrations of 5, 20, 40, 60, 80 and 100 ppm, was reacted with 4-diazoniobenzenesulfonate and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 496 nm. A curve of absorbance versus histamine concentration was made (Hattu et al., 2015).

Pre-treatment of Fish with Extracts

Powders of *M. vitifolia* and *B. pilosa*, and 4-hydroxybenzoic acid were weighed 5 g for 5% concentration, 10 g for 10% concentration, and 15 g for 15% concentration for each one of them, and then mixed into 100 mL distilled water at constant temperature of 60 °C and extracted for 1 hours. Then filtered with filter paper. The Frigate Tuna fish are washed, the dorsal part of the fish (without the skin) is taken from the fish body. Then it was sliced into small pieces, took 10 g of it, and soaked in aqueous extract of *M. vitifolia*, *B. pilosa*, and solution of 4-hydroxybenzoic acid at various concentrations for 30 minutes and then washed it with distilled water.

Histamine Extraction

Thin slices of Frigate Tuna fish meat weighed as many as 5.0 g. The sample was homogenized with 20

mL of 0.85% (w/v) NaCl solution for 2 minutes using a blender. Then it was put into a 75 mL centrifuge tube and centrifuged at 5300 rpm for 1 hour. The supernatant formed was made into 25 mL with 0.85% NaCl solution. The extract was used for further analysis. In a test tube, 1 mL of the extract was diluted to 2 mL with a solution of 0.85% NaCl and 0.5 g of a salt mixture (containing 6.25 g of anhydrous Na₂SO₄ to which 1 g of Na₃PO₄.H₂O), the solution was mixed evenly. Then 2 mL of n-butanol was added and shaken vigorously for 1 minute and allowed to stand for 2 minutes and homogenized so that the protein gel was damaged. The tube was then shaken for a few minutes and centrifuged at 3100 rpm for 10 minutes. Butanol located at the top (about 1 mL) was transferred into a clean and dry tube. Then evaporated to be completely dry. The residue was dissolved in 1 mL of distilled water and ready for next step (Hattu et al., 2015).

Histamine Content Analysis

A clean test tube containing 5 mL of 1.0% Na₂CO₃ solution was slowly added to 2 mL of 4-diazoniobenzenesulfonate reagent and mixed. Then added 1 mL of the residual solution obtained from the extraction process into the tube. The absorbance of the resulting color was measured immediately after 5 minutes at a wavelength of 496 nm using distilled water as a blank (Hattu et al., 2015). The increasing of histamine content was calculated by equation 1.

$$\Delta[\text{Histamine}](\text{mg}/100 \text{ g}) = [\text{Histamine}]_t - [\text{Histamine}]_0 \quad (1)$$

Where: $\Delta[\text{Histamine}]$ = the increasing of histamine concentration in mg/100 g of fish meat, $[\text{Histamine}]_t$ = histamine concentration after 30 minutes immersing treatment, $[\text{Histamine}]_0$ = histamine concentration at 0 minutes treatment.

RESULTS AND DISCUSSIONS

Chemicals Identification by LC-MS/MS in n-Hexane Extract of *M. vitifolia*

The LC-MS/MS analysis of n-hexane extract of *M. vitifolia* has been conducted. The chromatogram results for sample 1 and 6a are shown in Figure 1 and Figure 3. Table 1 dan 2 shows all the proposed compound that identified by LC-MS/MS analysis in n-hexane extract of *M. vitifolia* which are from sample 1 and 6a, respectively. From LC-MS/MS analysis, there are 12 compounds that has been identified in sample 1 and there are 4 compounds identified in sample 6a. The identification using Internal Library of the instrument (Waters), or based on the literature research, and based on the mass spectrum or exact mass of the compounds with higher similarity to the database of natural chemical products in NCBI PubChem. Total, we identified and proposed 16 chemical's names from n-hexane extract of *M. vitifolia*.

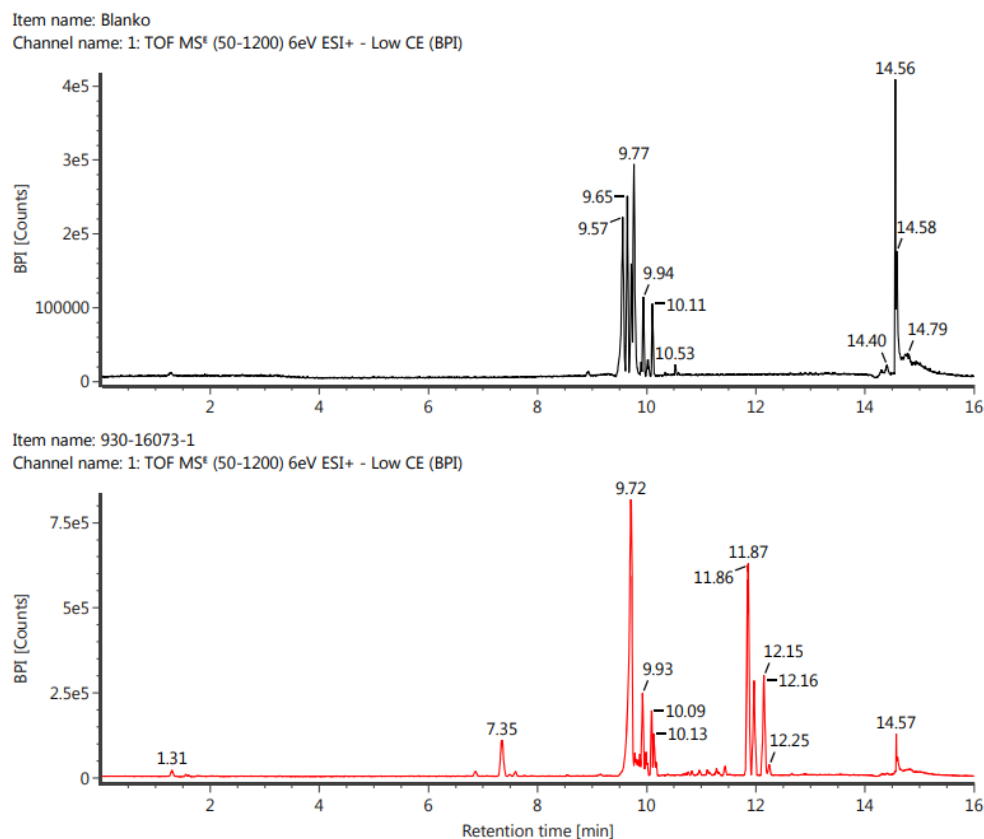


Figure 1. Chromatogram LC-MS/MS of n-hexane extract of *M. vitifolia* (sample **1**) in comparison with chromatogram of the blank.

Table 1. The proposed compounds that are identified by LC-MS/MS in n-hexane extract of *M. vitifolia* from sample **1**.

No	RT (Min)	Observed	Neutral Mass	Adducts	Mass	Proposed compound	Ref.
1	7.35	197.12	196.10994	H ⁺	0.8	Digiprolactone	Library Instrument
2	7.60	415.16	414.15796	H ⁺	-1.3	Gamma-sitosterol	(Tahya & Karnelasatri, 2021)
3	9.71	181.12	180.11503	H ⁺	1.0	Dihydroactinidiolide	PubChem CID 27209
4	9.93	277.22	276.20893	H ⁺	0.6	Stearidonic acid	Library Instrument
5	10.09	625.27	624.25706	H ⁺	1.4	Diglyceride + H ₂ O	(Duval et al., 2016)
6	10.14	593.28	592.26723	H ⁺	1.4	Pheophorbide a	PubChem CID 101690780
7	11.13	935.55	934.54424	H ⁺	1.4	Epigallocatechin gallate 4,4'-dipalmitate	PubChem CID 76958273
8	11.29	405.37	382.38108	Na ⁺	2.9	Pentacosanoic acid	Library Instrument
9	11.44	413.38	412.37052	H ⁺	0.4	Spinasterol	Library Instrument
10	11.86	903.56	902.55441	H ⁺	1.3	Polyunsaturated Triglyceride (C ₅₈ H ₉₂ O ₆) + H ₂ O	(Duval et al., 2016) (PubChem CID 131765888)
11	11.97	887.57	886.55950	H ⁺	1.1	Polyunsaturated Triglyceride (C ₅₇ H ₈₈ O ₆) + H ₂ O	(Duval et al., 2016) (PubChem CID 131765838)
12	12.15	871.57	870.56458	H ⁺	0.8	Polyunsaturated Triglyceride (C ₅₇ H ₉₀ O ₆)	(Duval et al., 2016) (PubChem CID 131765968)



Figure 2. One orange-yellowish spot was observed under UV light on TLC plat after the TLC preparative procedure was conducted.

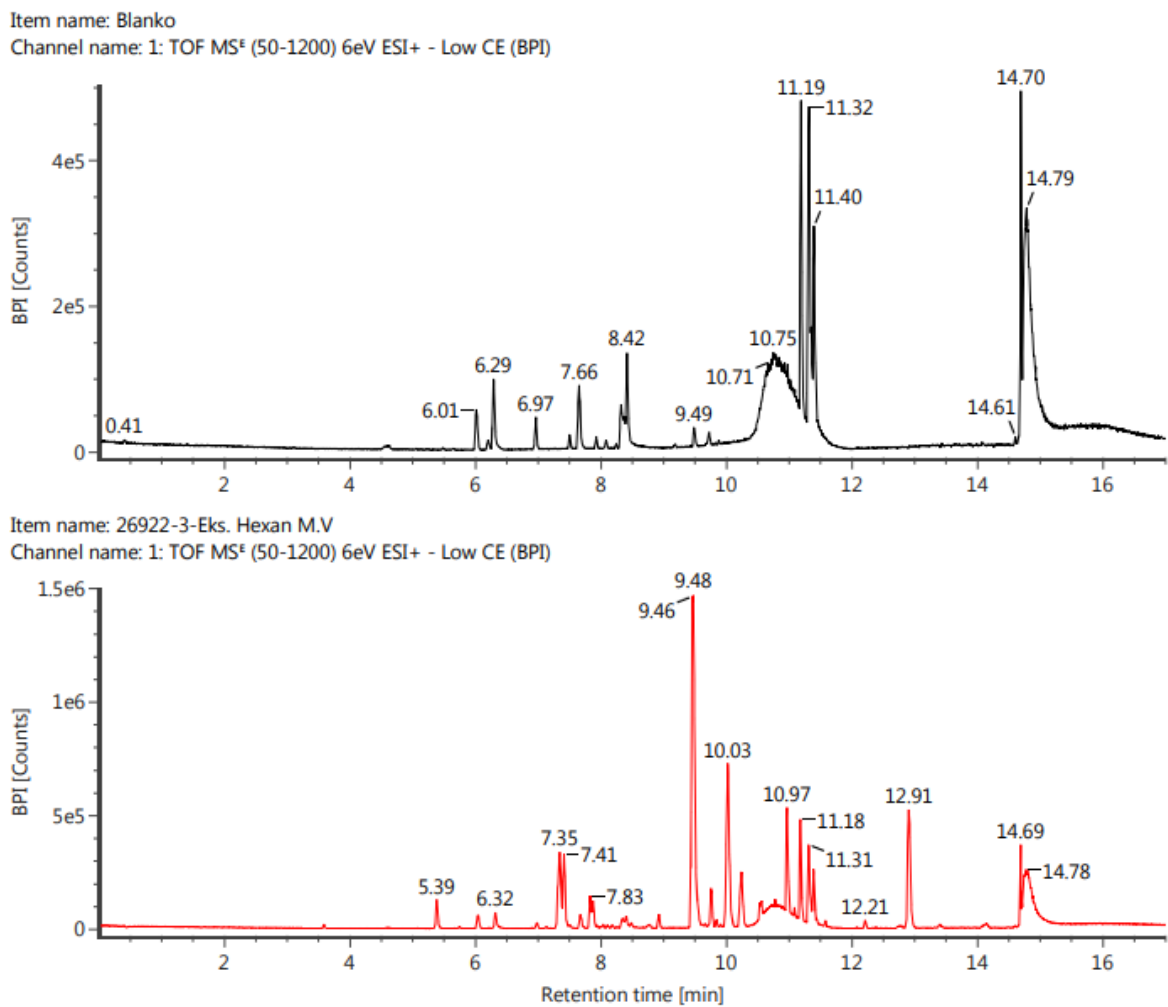


Figure 3. Chromatogram LC-MS/MS of n-hexane extract of *M. vitifolia* (sample **6a**) in comparison with chromatogram of the blank.

Table 2. The proposed compounds that are identified by LC-MS/MS in n-hexane extract of *M. vitifolia* from sample **6a**.

No	RT (Min)	Observed (m/z)	Neutral Mass (Da)	Adducts	Mass Error (mDa)	Proposed compound	Ref.
1	10.03	609.27	586.27780	H ⁺	4.5	Azedarachin C	Library Instrument
2	7.34	277.22	276.20893	H ⁺	-0.1	Stearidonic acid	Library Instrument
3	12.91	429.37	428.36543	H ⁺	0.2	Stigmastan-3,6-dione	Library Instrument
4	9.47	279.23	278.22458	H ⁺	0.6	Trichosanic acid	Library Instrument

Isolation and Identification of Triglycerides from n-Hexane Extract of *M. vitifolia*

TLC preparative result of the n-hexane extract of *M. vitifolia* shows only one spot under UV light. This isolation proses has high possibility to isolate a compound as only one spot observed in TLC plat as shown in **Figure 2**. This isolated fraction 1 was analyzed with FTIR, ¹H-NMR dan ¹³C-NMR to identify most possible compound. FTIR of the isolate fraction was shown in **Figure 4**. The FTIR spectral data shows vibration bands at 3011.07 cm⁻¹ (=C—H olefinic), 2923.69 cm⁻¹ (—CH₂— sym stretch), 2851.67 cm⁻¹ (—CH₂— asym stretch), 1742.42 cm⁻¹ (—C=O stretch), 1461.97 cm⁻¹ (—CH₂ deform), 1377.93 cm⁻¹ (—CH₃ deform), 1236.18 cm⁻¹ (—O—C=O), and 1168.24 cm⁻¹ (—CH—O stretch). Many of these vibration bands have similarity to the wave number of FTIR spectral of triglycerides (Harry-O'kuru et al., 2015).

The ¹H-NMR and ¹³C-NMR analysis were shown in **Table 3** exhibit characteristics of triglyceride (TG) (Sarpal et al., 2016). **Table 3** confirmed the triglyceride characteristics as we can compared to previous report. Sarpal et al., (2016) report the ¹H-

NMR signals at 4.38 to 5.2 ppm were assigned to protons of OCH₂ and OCH ester groups and ¹³C-NMR spectra display OCH₂ (sn-1, 3) and OCH (sn-2) of triglyceride at 62.05 and 68.87 ppm. The unsaturated carbons (—CH=CH) signal were between 126-132 ppm. The appearance of characteristics signals at 127.12 and 132.06 ppm due to C18:3 (Sarpal et al., 2016) while here we observed the single signal both at 127.25 and 132.11 ppm.

Mass spectrum and molecule fragmentation of triglyceride identified by LC-MS/MS in the n-hexane extract of *M. vitifolia*. This compound namely triglyceride 1 is second most abundance compound with retention time at 11.8618 min in the sample 1 based on the chromatogram in **Figure 1**.

An unusual feature of the mass spectra of triglycerides is an ion corresponding to the loss of water, the [M+H-18]⁺ peak. This fragmentation is unique among the mass spectra of esters (Hites, 1975). The [M+H]⁺ peak of Triglyceride 1 is m/z 855.55226. We can calculate the chemical formula of triglyceride 1 is C₅₈H₉₂O₆. The mass fragmentation of TG show specific fragment for the loss of RCOOH (fatty acid fragment) (Duval et al., 2016).

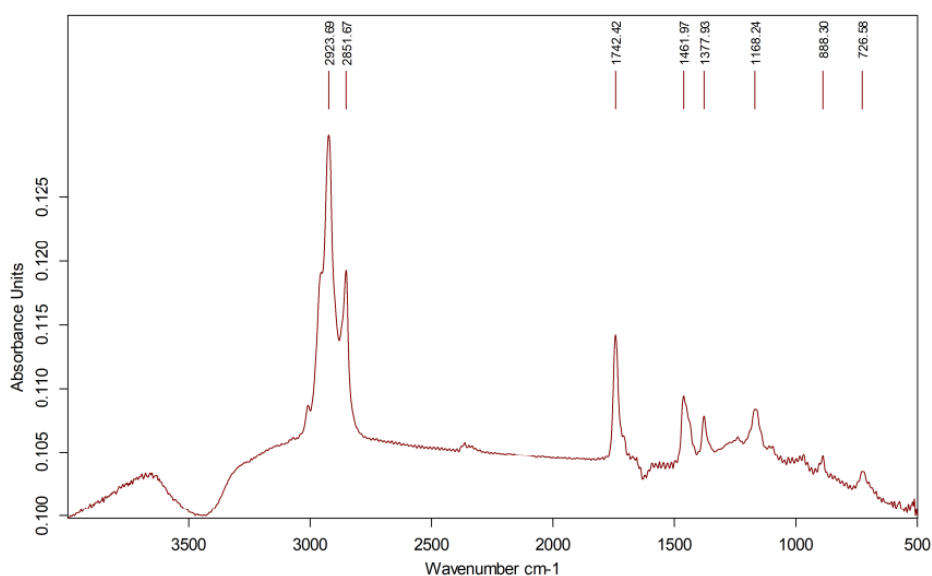
**Figure 4.** FTIR analysis of isolated fraction 1 of *M. vitifolia*

Table 3. Chemicals shift of $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ of isolated triglyceride from *M vitifolia* extract.

δ H (ppm), multiplicity	δ C (ppm)	Corresponding Functional group
4.1246 – 4.1602, dd	61.3492	$\text{CH}_2\text{—O}$ (Glyceryl)
4.2748 – 4.3071, dd	62.2476	$\text{CH}_2\text{—O}$ (Glyceryl)
4.5772 – 4.5914, d	69.0072	CH—O (Glyceryl)
-	173.0053 – 173.4249	O—C=O (Ester carbonyls)
5.0452 – 5.4067, m	124.1585 – 132.1081	$\text{HC=CH}_2\text{—}$ (olefinic)
6.3497 – 6.4065, m	113.2026 – 118.2671	HC=C—C=C
2.2774 – 2.3298, m	34.1695 – 34.5569	CH—C=O
2.1916 – 2.3298, m	25.6727 – 25.8548	CH—C=C
2.7544 – 2.8041, m	26.7387 – 26.9081	C=C—CH—C=C
0.8704 – 0.8911, m	14.2358 – 14.4378	Terminal —CH_3
1.2027 – 1.2507, m	29.5007 – 29.8554	$\text{—(CH}_2\text{)}_n\text{—}$

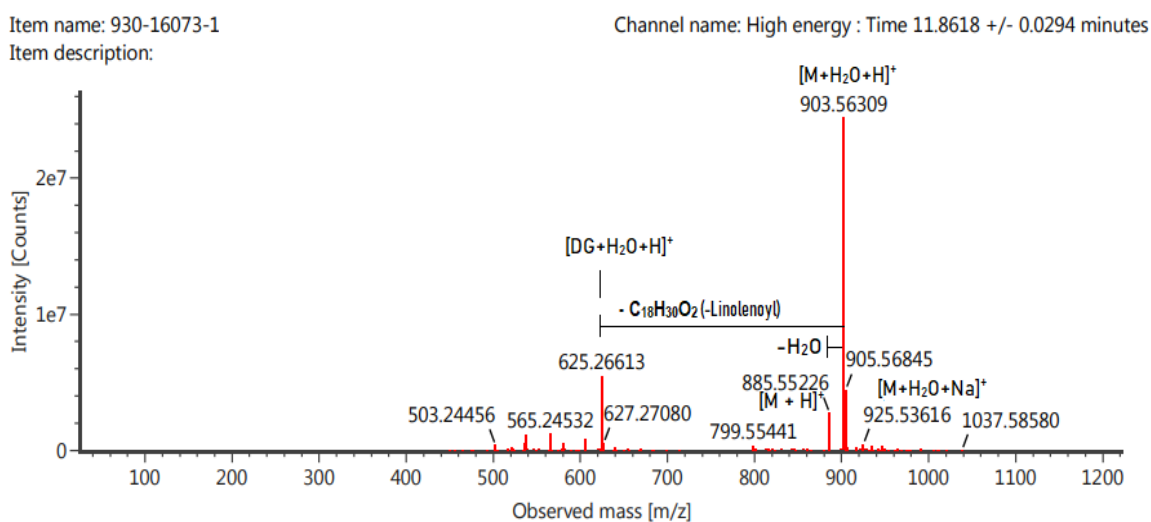
**Figure 5.** MS spectrum of triglyceride 1 identified in n-hexane extract of *M. vitifolia*

Figure 5 shows the loss mass fragment of linolenic or trichosanic acid ($\text{C}_{18}\text{H}_{30}\text{O}_2$) which means that the triglyceride 1 comprised of linolenoyl or trichosanoyl ($\text{C}_{18}:3$) unit as previously observed in $^{13}\text{C-NMR}$ signal.

Chemicals Identification by LC-MS/MS in Methanol Extract of *M. vitifolia*

The LC-MS/MS analysis of methanol extract of *M. vitifolia* has been conducted. The chromatogram results for sample 4 is shown in Figure 6. **Table 4** shows all the proposed compound that identified by LC-MS/MS analysis in methanol extract of *M. vitifolia* (sample 4). Blank chromatogram has been showed in **Figure 3**.

Chemicals Identification by GC-MS in Methanol Extract of *M. vitifolia*

The GC-MS analysis of methanol extract of *M. vitifolia* has been conducted. **Table 5** shows the compounds that identified by GC-MS analysis in methanol extract of *M. vitifolia* (sample 2). Identification of the substances used Library NIST17.L. The two most abundance compounds in this GC-MS

analysis of methanol extract of *M. vitifolia* are (Z)-15-octadecenoic acid methyl ester and n-hexadecanoic acid.

Chemicals Identification by LC-MS/MS in Methanol Extract of *B. pilosa*

The LC-MS/MS analysis of methanol extract of *B. pilosa* has been conducted. The chromatogram results for sample 5 is shown in **Figure 7**. **Table 5** shows all the proposed compound that identified by LC-MS/MS analysis in methanol extract of *B. pilosa* (sample 5). Blank chromatogram has been showed in **Figure 3**.

Chemicals Identification by GC-MS in Methanol Extract of *B. pilosa*

The GC-MS analysis of methanol extract of *B. pilosa* has been conducted. **Table 7** shows all the compounds that identified by GC-MS analysis in methanol extract of *B. pilosa* (sample 3). Identification of the substances used Library NIST17.L. The two most abundance compounds in this GC-MS analysis of methanol extract of *B. pilosa* are (E)-9-Octadecenoic acid and n-Hexadecanoic acid.

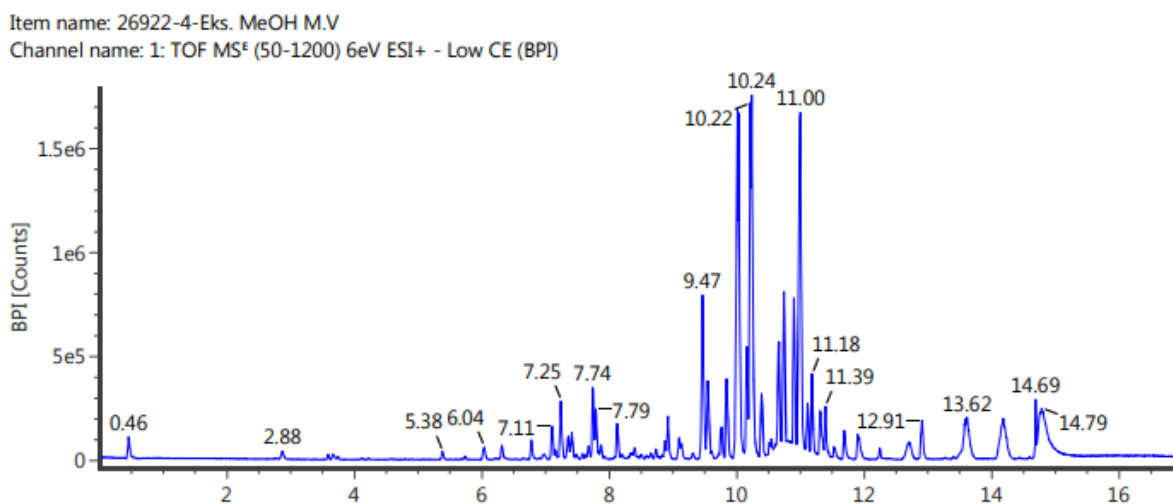


Figure 6. Chromatogram LC-MS/MS of methanol extract of *M. vitifolia* (sample 4).

Table 4. The five most abundance proposed compounds that are identified by LC-MS/MS in methanol extract of *M. vitifolia* from sample 4.

No	Observed m/z	Neutral Mass (Da)	Observed RT (min)	Proposed compound	Ref.
1	609.27	586.27780	10.03	Azedarachin C	Library Instrument
2	279.23	278.22458	9.47	Trichosanic acid	Library Instrument
3	593.28	592.26857	10.23	Pheophorbide A	PubChem CID 253193
4	607.29	606.28422	11	Methyl pheophorbide	PubChem CID 73074
5	653.30	652.28970	10.9	Pheopurpurin 7, trimethyl ester	PubChem CID 135441796

Table 5. Identified compounds in methanol extract of *M. vitifolia* using GC-MS analysis.

No	Name of compound	RT (Min)	% Area	SI (%)	Formula
1	2-(tert.-Butyldimethylsilyloxybenzylidene)acetophenone	7.495	4.638	70.03	C ₂₁ H ₂₆ O ₂ Si
2	Hexadecanoic acid, methyl ester	32.098	6.348	92.03	C ₁₇ H ₃₄ O ₂
3	n-Hexadecanoic acid	32.774	21.385	93.08	C ₁₆ H ₃₂ O ₂
4	9-Octadecenoic acid (Z)-, methyl ester	35.475	7.727	83.75	C ₁₉ H ₃₆ O ₂
5	2-Hexenal, (E)	35.694	3.286	82.5	C ₆ H ₁₀ O
6	Histamine, N-trifluoroacetyl	36.068	1.435	60.2	C ₇ H ₈ F ₃ N ₃ O
7	(Z)-15-Octadecenoic acid methyl ester	36.359	50.364	67.52	C ₁₉ H ₃₆ O ₂
8	2-tert-Butylphenol, tert-butyldimethylsilyl ether	50.735	4.019	53.29	C ₁₆ H ₂₈ OSi
9	3-Butoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	56.088	0.422	55.46	C ₁₃ H ₃₆ O ₄ Si ₄

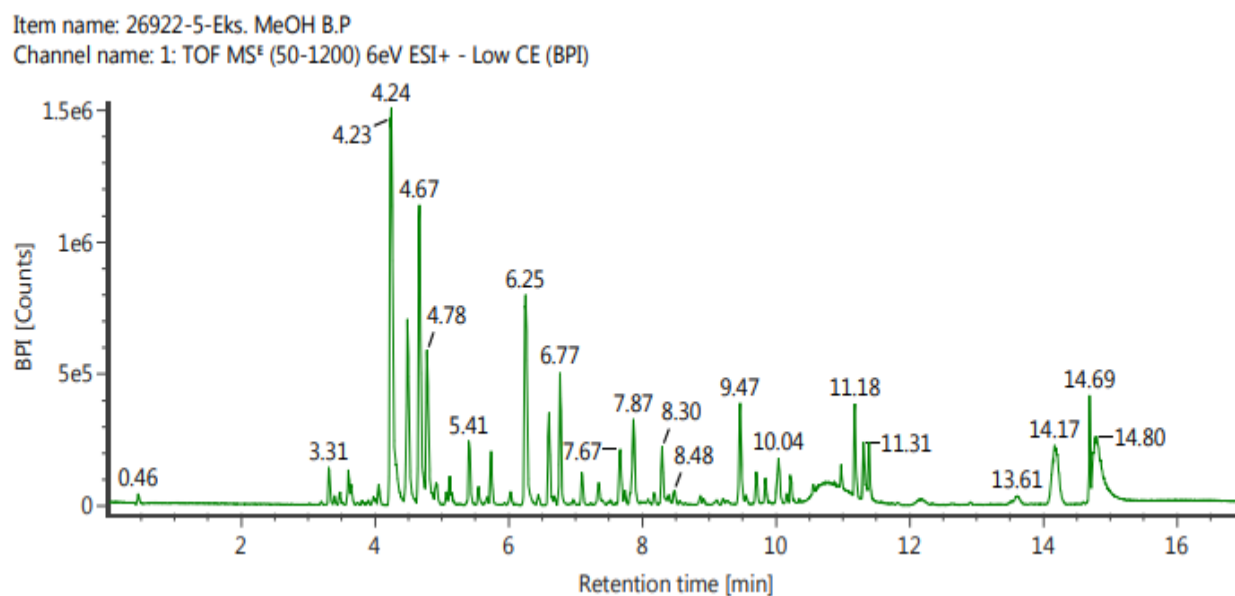


Figure 7. Chromatogram LC-MS/MS of methanol extract of *B. pilosa* (sample 5)

Table 6. The five most abundance proposed compounds that are identified by LC-MS/MS in methanol extract of *B. pilosa* from sample 5.

No	Observed m/z	Neutral Mass (Da)	Observed RT (Min)	Proposed compound	Ref.
1	287.05	286.04774	4.25	5,7,2',5'-Tetrahydroxy-flavone	Library Instrument
2	271.06	270.05282	4.67	Digitopurpone	Library Instrument
3	787.37	786.36286	4.79	N1,N5,N10,N14-Tetra-Trans-P-Coumaroylspermine	PubChem CID 9810941
4	584.28	583.26824	4.5	N1,N5,N10-tricoumaroyl spermidine	PubChem CID 14777879
5	185.10	184.08882	6.26	Isoquinaldonitrile, 3-methyl-, 2-oxide	GC-MS confirm

Table 7. Identified compounds in methanol extract of *B. pilosa* using GC-MS analysis

No	Name of compound	RT (Min)	% Area	SI (%)	Formula
1	1,1,1,3,5,5,5-Heptamethyltrisiloxane	0.165	1.466	60.32	C ₇ H ₂₂ O ₂ Si ₃
2	Isoquinaldonitrile, 3-methyl-, 2-oxide	28.59	0.703	87.83	C ₁₁ H ₈ N ₂ O
3	Hexadecanoic acid, methyl ester	32.097	1.222	91.5	C ₁₇ H ₃₄ O ₂
4	n-Hexadecanoic acid	32.851	21.746	97.28	C ₁₆ H ₃₂ O ₂
5	(Z)-15-Octadecenoic acid methyl ester	35.475	1.559	84.08	C ₁₉ H ₃₆ O ₂
6	9-Octadecenoic acid, (E)-	36.264	68.570	71.29	C ₁₈ H ₃₄ O ₂
7	Octadecanoic acid	36.602	2.630	65.05	C ₁₈ H ₃₆ O ₂
8	Glycidyl palmitate	38.917	0.727	63.97	C ₁₉ H ₃₆ O ₃
9	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	41.831	1.377	54.34	C ₂₁ H ₃₈ O ₃

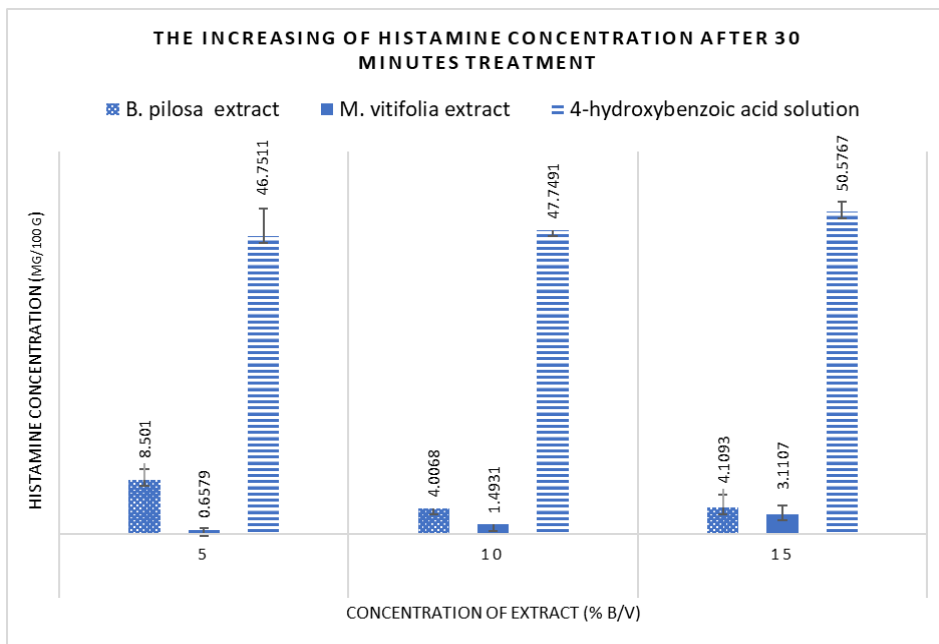


Figure 8. The increasing of histamine concentration (mg/100 g) in the fish meat after 30 minutes treatment with extracts and 4-hydroxybenzoic acid solution.

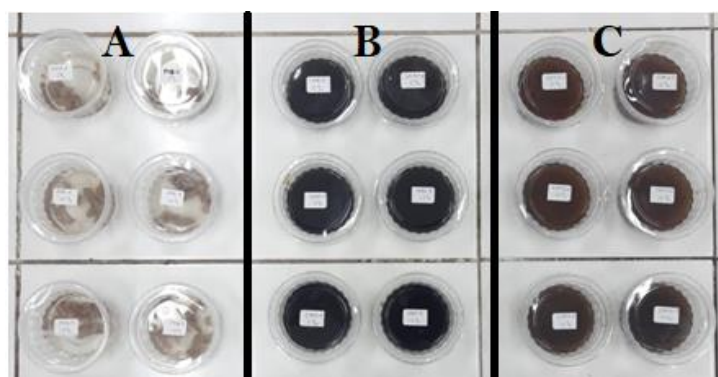


Figure 9. The immersing of fish meat in 4-hydroxybenzoic acid solution (A), in the extract of *B. pilosa* (B), and in the extract of *M. vitifolia* (C).

Histamine Inhibiting Test Result

Histamine inhibition test was a quantitative test to determine the concentration of histamine in the flesh of fish using spectrophotometer UV-Vis. For Histamine standard curve (Supplement Data), the variety of standard concentrations were made, and absorbances were measured at a wavelength of 496 nm. The increasing of histamine content in the meat of Frigate Tuna after treatment with various concentration of extracts and 4-hydroxybenzoic acid (4-HBA) has been shown in **Figure 8**. The histamine content was calculated using 4-diazoniobenzenesulfonate reagent and determined by spectrophotometer UV-Visible at wavelength of 496 nm in duplet (**Figure 9**).

We used 4-hydroxybenzoic acid (4-HBA) control standard because 4-HBA is known as antimicrobial substances capable of inhibit most of the Gram-positive and some Gram-negative bacteria (Cho et al., 1998; Kosova et al., 2015). The weakness of using 4-

HBA is the low solubility in water. We had to increase the temperature to dissolved 4-HBA, with vigorous stirring. Bacterial activity is the primary cause of histamine production in the fish. The average histamine concentration formed after treatment with *M. vitifolia* extract is smaller compared other treatments especially 4-HBA treatment. This means that *M. vitifolia* extract is better histamine formation inhibitor compared to *Bidens pilosa* extract or 4-HBA. This result has similar conclusion to previous reports (Bartolome et al., 2013; Hasanah et al., 2020; Xuan & Khanh, 2016) that polar extract of both plant's part could strongly inhibit the growth of many bacteria. Some lipids have potential as antimicrobial. The fatty acid is the precursors of triglyceride formation in cells. Chemicals like n-hexadecanoic acid, (E)-9-octadecenoic acid, hexadecanoic acid methyl ester, spinasterol have potent antimicrobial activities as shown in **Table 8** and **9**.

The activity of histidine decarboxylase is also important in the formation of histamine in fish. Potent inhibitor of histidine decarboxylase is 2-hydroxy-5-carbomethoxybenzoyloxyamine (Husztli & Sourkes, 1975). This chemical was a derivative of 4-hydroxybenzoic acid, so we supposed to observe any inhibition activity of 4-HBA. Histamine formation in fish after 30 min treatment with 4-HBA is around 40 – 51 mg / 100 g of fish. This value is more than 5 times bigger compared to treatment with the extracts, which means 4-HBA has less inhibitory activity. The European Commission Regulation set the maximum histamine content in fresh fish should equal to 20 mg per 100 g of fish. At this concentration no adverse effect caused by histamine to average human (Visciano et al., 2014). We observed that histamine concentration in fish was less than 10 mg / 100 g of fish after 30 min treatment with *M. vitifolia* and *B. pilosa* extracts. This value is smaller than other report of Tamarind extract (20% b/v) effect to histamine content in bullet Tuna after 30 min treatment by Hattu et al., (2015) which is 17.693 mg/100 g of fish. The

smaller the histamine content in fish means the higher the inhibitory effect the extract has. This data shows the preliminary indication of high potential of both extract as preserver of fish products. We also observed the small increasing of histamine as the *M. vitifolia* extract concentration increased. This phenomenon possibly due to the amount of histamine derivative that detected by GC-MS in the extract that has influenced UV-Vis measurements.

Bioactivity of The Identified Substances

The first aim of this study is to identify active substances in the extract of *M. vitifolia* dan *B. pilosa* using LC-MS/MS and GC-MS analysis. In LC-MS/MS analysis, chemicals identification based on mass spectrum, references, and exact neutral mass (Da) of the substance obtained from mass spectrum. The GC-MS analysis using library NIST.17.L to observe the highest similarity score of mass fragments spectrum. We identified 27 chemicals in *M. vitifolia* extract and 14 chemicals in *B. pilosa* extract. Some of those substances have biological activities as shown in **Table 8** and **9**.

Table 8. Bioactivity of some substances identified in *B. pilosa* extract

Name of compound	Bioactivity
Hexadecanoic acid, methyl ester	Anti-inflammatory (Igwe, 2014), antibacterial (Shaaban et al., 2021), antifungal (Abubacker & Deepalakshmi, 2013)
n-Hexadecanoic acid	Anti-inflammatory (Gopu et al., 2021), antibacterial (Johannes et al., 2016)
(E)-9-Octadecenoic acid	Antibacterial and antifungal (Ghavam et al., 2021)
Octadecanoic acid	Antibacterial (Pu et al., 2010)
5,7,2',5'-tetrahydroxy-flavone	Anticancer (Lian et al., 2021; Sonoda et al., 2004)
N1,N5,N10-tricoumaroyl spermidine	Hepatoprotective (Zhou et al., 2021), antioxidant (Negri et al., 2011)

Table 9. Bioactivity of some substances identified in *M. vitifolia* extract

Name of compound	Biological activity
Spinasterol	Antibacterial activity (Yang et al., 2017), anti-inflammatory, antidepressant, antioxidant and antinociceptive effects (Brusco et al., 2017), anticancer (Meneses-Sagrero et al., 2017), antifungal, and aphicidal (Ahmed et al., 2022)
Stigmastan-3,6-dione	Apoptosis induction of cancer cells (Duarte et al., 2009)
Trichosanic acid	Reduce platelet aggregation in human (Takenaga et al., 1988)
Pheophorbide a	Potent endothelin receptor antagonist (Ohshima et al., 1994), antiviral, anti-inflammatory, antioxidant and anti-proliferative effects in several human cancer cell (Saide et al., 2020)
Methyl pheophorbide	Antioxidative and anticarcinogenic (Das et al., 2016)

CONCLUSIONS

By using LC-MS/MS and GC-MS, 27 chemicals in *M. vitifolia* extract and 14 chemicals in *B. pilosa* extract have been detected and identified. A triglyceride has been isolated and identified by using FTIR, H-NMR and C-NMR supported by LC-MS/MS data. Histamine concentration in fish was less than 10 mg / 100 g of fish after 30 min treatment with *M. vitifolia* and *B. pilosa* extracts. Histamine formation in fish after 30 min treatment with 4-hydroxybenzoic acid is around 40 – 51 mg / 100 g of fish. This data shows the preliminary indication of high potential of both extract as preserver of fish products.

ACKNOWLEDGEMENTS

We thank Center of Research and Community Service (CRCS) UPH, for providing research fund with contract number P-005-FIP/II/2021.

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