International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.4, pp 782-789, 2015

Isolation and Structure Elucidation of Eumitrin A_1 from Lichen *Usnea blepharea* Motyka and Its Cytotoxic Activity

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Abstract: The purpose of this research was to isolate and elucidate structure of secondary metabolite compoundand to examine its cytotoxic activity of lichen *Usnea blepharea* Motyka obtained from Sulawesi – Indonesia. Powder of lichen was extracted using an acetone and continued separation by employing column chromatography (CC) with *n*-hexane and ethyl acetate solvents in a gradient elutionto produce a pure isolate. The structure was determined by UV-Vis, LC-MS, FTIR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR (HMBC, HMQC, COSY). The UV-Visspectrophotometer measurement was obtained maximumwavelength of 229 nm and 334 nm. The LC-MS data of this compound showed a molecular weight of680. The^{1}H-NMR, ^{13}C-NMR with DEPT spectrums showedthat this compound has 34 carbon atoms consisting of 2 methyl groups, 2 methoxy groups, 1 methyl group, 6 metin groups, 3 methylene groups, and 20 C quartener atoms. The2D-NMR (HMBC, HMQC,COSY) spectrums of analysis could be inferred that this compound is a Eumitrin A_1 with the formula structure $C_{34}H_{32}O_{15}$. The Eumitrin A_1 was examined its cytotoxic activityagainst Murine Leukemia P388 cells with IC_{50}4.5 µg/mL (very active).
Keywords: Eumitrin A_1, *Usnea blepharea* Motyka, Cytotoxic, Murine Leukemia P388 Cells.

Introduction

Lichen is a unique plant because it isformed from two different organism symbiosis through life together of mutual benefit between algae and fungi[1,2]. The products of lichen have been used as a traditional medicine in some countries and is still considered as an interesting alternative medicine[3]. In the past, native Americans used lichen for drugs, for examples:*U.longissima* as the smooth skin drug and *Letharea vulpine* as a yellow dye[4,5]. In India, the lichen was used to cure some diseases, i.e. asthma, bronchitis, stomach pain, allergy, the cracking of bones, liver and blood disorders[6,7]. Traditionally, the lichen has a number of uses depending on the species, such as *U. misaminensis*was used as anti-diare, influenza, and cough remedy. Lichen *Heterodermia boryi, Parmotrema stuppeum* (Taylor) Halye, *U. nilgirica, Pyine* sp. and *Parmotrema melanothix* (Mont.) Hale were utilizedas anti-microbial agent for*E.coli, Pseudomonas sp., Klebsiella sp., Staphylococcus aureus, Streptococcus viridians* and*Acinetobacter sp*[8,9]. *U. barbata* and *U. dasypoga*was used as anti-tuberculosis[10,11].

Based on empirical experience, *Usnea*sp. contain chemical compounds that have efficacy as a traditional medicine, stomach ailments such as diarrhea, bloody stools, thrush drug, cold drug, seizures, abdominal pain, difficult urination, menstrual disorders, hemorrhoids and headache[12,13]. The majority of *Usnea* plant contain a dominant chemical compound namely usnic acid[14]. Maulidiyah, et al. (2015) has reported that in the lichen *Usnea* sp. containing usnic acid compound that has activity to inhibit growth the Murine Leukemia

P388 cells[15]. *Usnea* is a genus of lichen that has been classified as a part of the family parmeliaceae, and spread throughout the world as forthe type of lichen about 20,000 species[16,17].

Lichen plant in Indonesia spread in various regions, i.e. Sulawesi, according to local communities that*U. blepharea* Motyka plant was used as a cure leprosy, cough medicine and drugs to eliminate the warts. Based on the above description the research on the chemical compounds contained in the lichen *U. blepharea* Motyka was carried out for obtaining the bioactivity. The isolation compound was tested the cytotoxic activity against Murine Leukemia P388 cells as an anti-cancer compound.

Experimental

1. Isolation and Purification

A 700 g powder of talus lichen *U. blepharea* Motyka was extracted by soxhlet uses acetone solvent for 8 hours in order to obtain crude extract of acetone. Then extract on acetone solvent was evaporated by using rotary evaporator vacuum to obtain dried extract.Further dried extract was eluted by using thin layer chromatography (TLC) to determine the composition of the solvent to get the best separation. TLC plates sprayed with a stain marker with 10% H_2SO_4 in methanol solution. The dried extract was separated by column chromatography (CC) in stationary phase of silica gel 60G that was eluted with a mixture *n*-hexane and ethyl acetate solvents by gradient elution. Pure isolates obtained then was purified and determined the molecular structure by using spectroscopy i.e. (^{1}H and ^{13}C) NMR, 2D-NMR, Infra Red (IR), Ultra Violet-Visible (UV-Vis), Liquid Chromatography-Mass Spectrometry (LC-MS). From the results of spectroscopic measurements, each was determined by comparing the molecular structure of each spectrum with data from literature and based on the basic theory of spectrometry.

2. Cytotoxic activity test against Murine Leukemia P388 Cells

Testing of cytotoxic activity against Murine Leukemia P388 cells was conducted by MTT Assay. In RPMI 1640 medium (with concentrations of >106 cells/mL) of a culture flask was put into 15 mL in a centrifuge tube then centrifuged at 1200 to 1300 rpm for 5 minutes at room temperature. Supernatant was separated by sterile Pasteur pipette and sediment (pellet cells) then was added 1 mL FBS (Fetal Bovine Serum) and 100 µL of DMSO, mixed gently, then transferred 2 mL into tube and the lid with paraffin. Furthermore, the dissolution of cells and centrifuged for separation process, further do materials testing subculture[15].

Results and Discussion

The compound has been isolated and shaped yellow crystals with a melting point = 263-264 with optical rotation $[\alpha]_D^{20}$ = -53° (CH_2Cl_2, c = 0,0005). Data from spectroscopy UV light that isolated compound (Fig. 1) has been measured at a wavelength of λ = 200-400 nm in dichloromethane solvent gives a maximum absorption at a wavelength of λ = 229 nm dan 334 nm. Absorption at λ_{max} = 229 nm showed the presence of double bonds conjugation C = C of benzene with the kind of transition $\pi \rightarrow \pi^*$. Absorption at 340 nm estimated λ_{max} is derived from the C=C chromofor group that are conjugated with C=O accompanied the extension of double bonds[18].

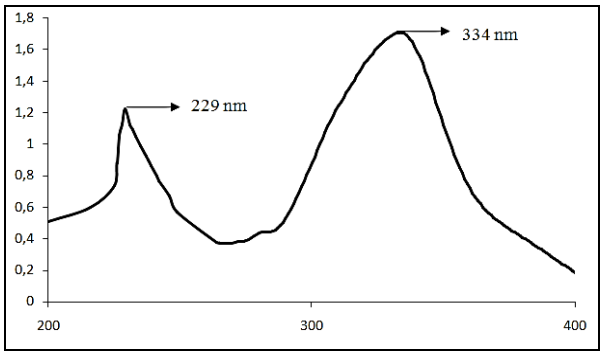

Fig. 1. Data measurement of UV Spectroscopy

Fig. 2 showed the result of LC-MS chromatogram of the molecular ion peaks visible on m/z = 680, this the isolated compound have molecular weight [M^+] = 680.

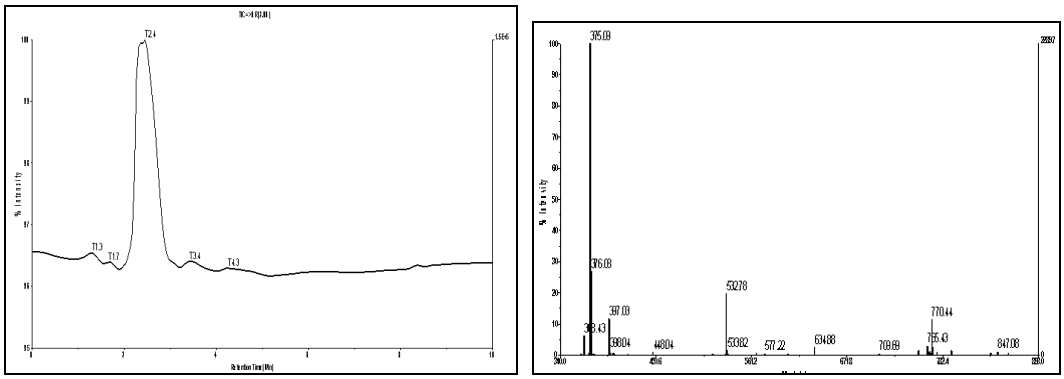

Fig 2. Data measurement of LC-MS Chromatogram

The IR spectra of the absorption bands in wavenumbers area at 3487 cm^{-1} is the stretching vibration of hydroxyl (OH) group. This is supported by the presence of bending vibration in areas of 1249 cm^{-1} for the hydroxyl (OH) group. The absorption band in the area of 2962 cm^{-1} is stretching asymmetric vibration of C-H bond from CH_3 group, supported by the existence of absorption band in wavenumber 1743 cm^{-1}that is stretching asymmetric vibration of C=O from ester group. Absorption band in the wavenumber 1612 $cm^{-1}$was indicated the presence of aromatic rings, while the absorption bands in wavenumber of 140 cm^{-1} and 1018 cm^{-1} have the binding vibration of C-O-C bonds, as can be seen in Fig. 3.

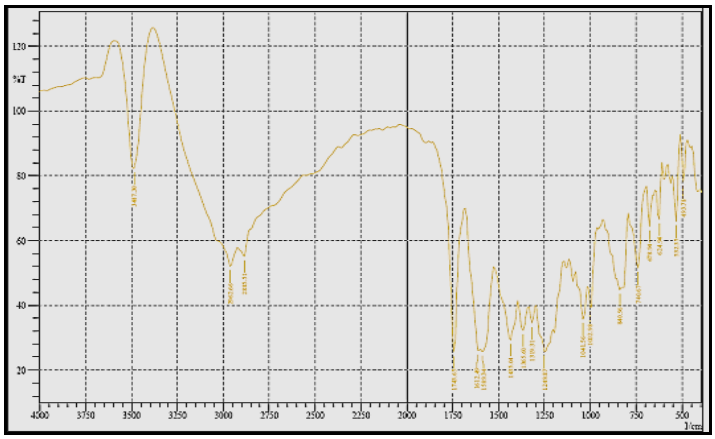

Fig. 3. Data measurement of Infra Red (IR) spectrum

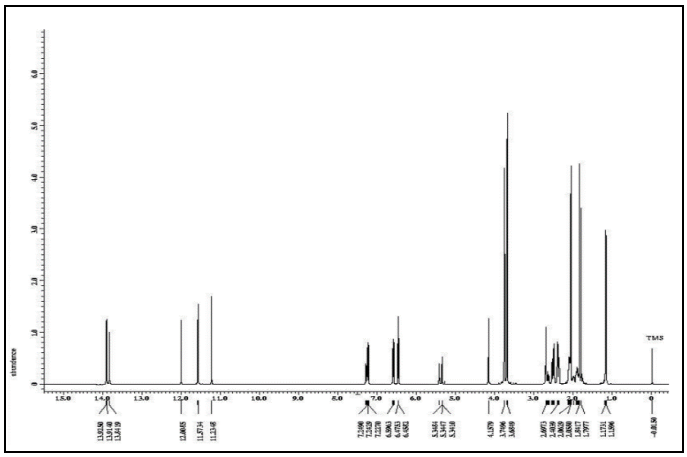

Fig. 4. Data measurement of ^{1}H-NMR spectrum

^{1}H-NMR spectrum on isolated compound showed a pair of aromatic protons with AB system on chemical shift δ_H 6.58 (1H d, J = 7,9 Hz) and δ_H 7.29 (1H d, *J* = 7,9 Hz). With a protons pair with *J* coupling 7.9 Hz means that the second proton is contiguous (ortho position). One other aromatic proton does not has a pair seen on δ_H 6.47 (1H, s). Two signals on δ_H = 3.76 (3H, s) and 3.68 (3H, s) showed a methoxy groups. An

acetyl group (CH_3CO-) indicated on δ_H 1.79 ppm (3H,s). This compound has five hydroxy group which forms hydrogen bonds with C=O group seen on δ_H (ppm) 13.92 (1H, s), 13.91 (1H, s), 12.00 (1H, s), 11.57 (1H, s), 11.23 (1H, s). Metin protons were indicated on δ_H (ppm) 5.34 (1H, s); 4.15 (1H, s); 6.58; 6. 47; 7.10; 2.69. The existence of the metin pair (CH) group with the chemical shift 2.63 (1H dd, J = 7,3 Hz; 4,9 Hz) with δ_H = 2.66 (1H dd, J = 7,3 Hz; 4,3 Hz) has predicted the existence of a methylene (CH_2) group but apparently one proton with one another are not identical, then occur germinal coupling with coupling constant of 7.3 Hz. The presence of methylene group was shown in 2.64 δ_H 2.40 (2H, m) and 2.5 (2H, m). Isolated compound similar to compound that has been reported by Maulidiyah(2011) the difference is the 18 atoms C quartener and 8 metin groups[19], while this compound obtained 20 atoms C quartener and 6 metin groups. The ^{1}H-NMR spectrum can be seen in Fig. 4.

^{13}C-NMR spectrum showed the carbonyl atoms from carbonyl at position δ_C = 188.05 ppm, 170.95 ppm, 169.07 ppm, 187.30 ppm and 184.86 ppm. Quaterner carbon atom that bind atoms O shown on position δ_C = 161.25 ppm; 177.48 ppm; 84.96 ppm; 149.93 ppm; 159.57 ppm; 128.96 ppm, 150.52 ppm and 116.04 ppm. The atom of C quartener are shown in the chemical shift δ_C = 100.3 ppm; 157.44 ppm; 111.29 ppm; 139.98 ppm; 117.71 ppm; 140.24 ppm and 100,65 ppm.

The results of ^{13}C-NMR and DEPT (Fig. 5 and 6) described an overview of this compound that has 34 carbon atoms consists of 2 methyl groups, 2 methoxy groups, 1 methyl group from acetyl group, 6 metin groups, 3 methylene groups and 20 C quartener atoms with the formula structure $C_{34}H_{32}O_{15}$. The results can be calculated and isolated compound hasa number of rings and double bonds as much 19 pieces. From spectroscopy data can be known that hydrogen deficiency indicate of 5 groups from C=O, 8 from C=C groups, and the remainder is 6 rings.

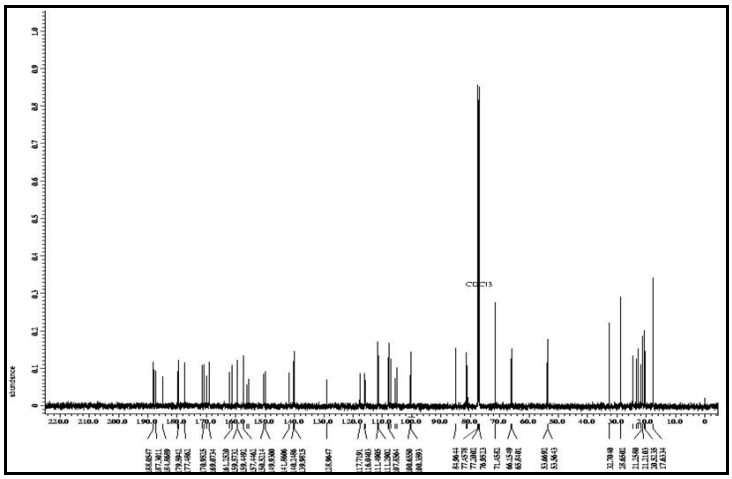

Fig. 5. Data measurement of ^{13}C-NMR spectrum

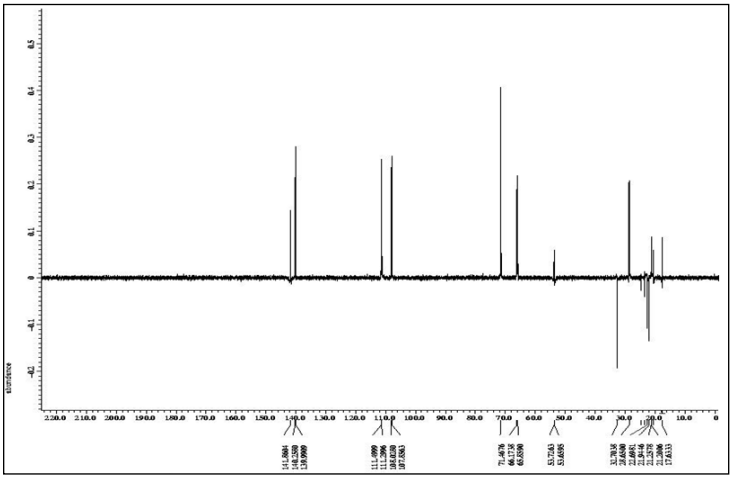

Fig. 6. Data measurement of DEPT spectrum

HMQC and HMBC (Fig. 7 and 8) can be determined proton pair that bonded with C atom, then the correlation between proton with C neighboring atom has distance between 2-3 bonds as may be seen in Fig. 8. From data shows (Fig. 7) the aromatic protons δ_H = 6.58 ppm bound on position C-4' (107.85 ppm), while the aromatic protons δ_H = 7.29 ppm bound on position C-3' (141.86 ppm). Other aromatic protons on δ_H = 6.47 ppm bound on position C-2' (111.49 ppm). Methoxy groups with δ_H = 3.68 ppm and δ_H = 3.76 ppm bound on position δ_C = 53.56 ppm and δ_C = 53.66 ppm. Acetyl group (CH_3CO-) on position δ_H = 1.79 ppm bound at a position δ_C = 21.25 ppm. Methyl group on δ_H = 2.06 ppm bound on position δ_C = 28.65 ppm, while the other methyl groups on δ_H = 1.17 ppm bound on position δ_C = 17.63 ppm. The methylene group on δ_H 2.40 ppm bound on δ_C= 21.21. Other methylene group on δ_H = 2.54 ppm bound on position δ_H = 22.68 ppm. Metin group on δ_H = 4.15 ppm, 5.34 ppm and 2.69 ppm bound on position δ_C = 71.45 ppm, 66.15 ppm and 20.52 ppm, respectively.

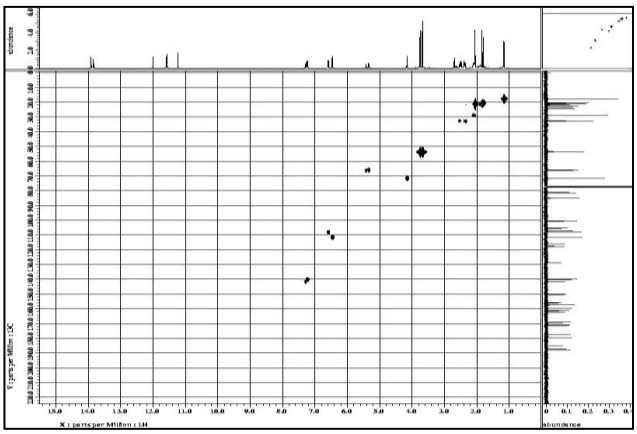

Fig. 7. Data measurement of HMQC Spectrum

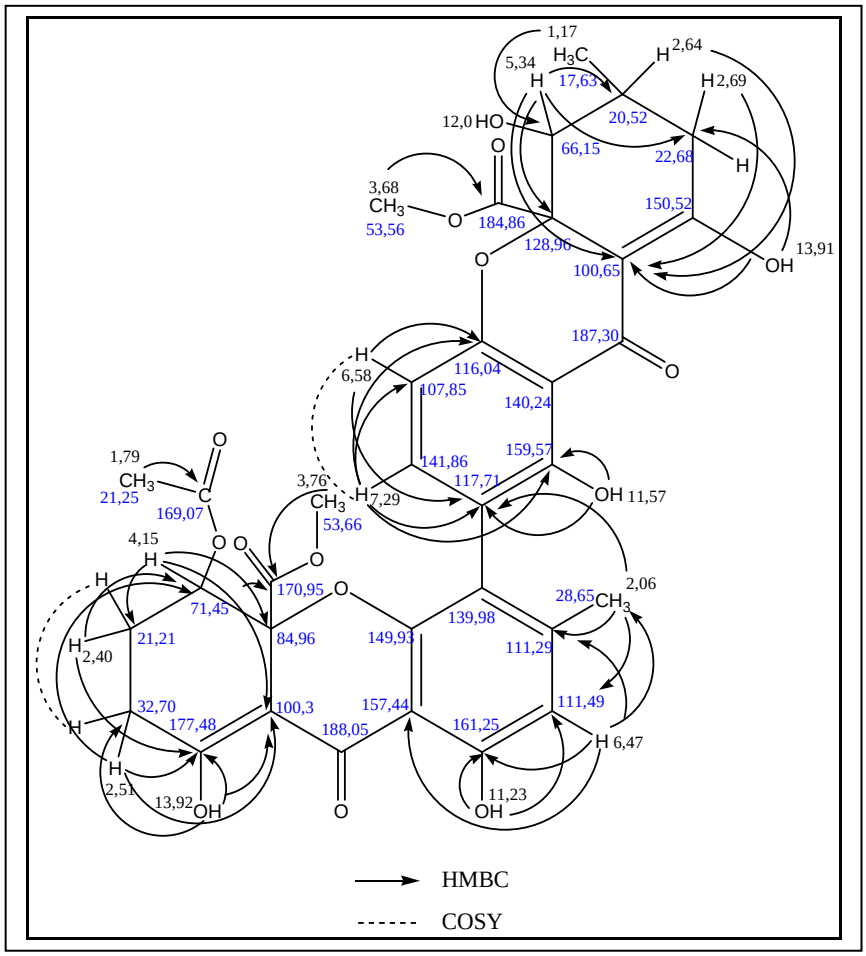

Fig. 8. The HMBC analysis of isolate compound

From the data of HMBCspectrum shows the resonance from one signals of aromaticproton on δ_H = 6.47 ppm have a correlation with carbons of C-1 (161.25 ppm), 3-CH_3 (28.65 ppm), C-3 (111.29 ppm) and C-1a (157.44 ppm). The aromatic proton on δ_H = 6.58 ppm has correlated with carbonsof C-2' (117.71 ppm) and C-4'a (116.04 ppm). Other aromatic proton on δ_H = 7.29 ppm has correlated with carbon proton of C-1' (159.57 ppm), C-2' (117.71 ppm) C-4' (107.85 ppm) and C-4'a (116.04 ppm). Signal of methoxy proton with δ_H = 3.68 ppm has correlated with carbon that has a chemical shift δ_C = 184.86 ppm, while other methoxy proton with δ_H = 3.76 ppm has corelated with carbon on δ_C = 170.95 ppm. The proton of hydroxy group with δ_H = 13.92 ppm has correlated with carbon on C-7 (32.70 ppm) and C-8 (177.48 ppm). C-9 (100.35 ppm) proton from hydroxy group δ_H = 11.23 ppm has correlated with carbon C-1 (161.25 ppm) and C-2' (111.49 ppm). Hydroxy protons at δ_H = 11.57 ppm has a correlated with carbon C-1' (159.57 ppm), and C-2' (117.71 ppm), while other hydroxy protons on δ_H = 13.91 ppm has correlated with carbon C-9' (100.65 ppm) and C-7' (22.68 ppm). Methylene protons at δ_H = 2.40 ppm has correlated with carbons C-5 (71.45 ppm) and carbon C-8 (177.48 ppm). Methylene protons at δ_H = 2.54 ppm has correlated with carbons C-5 (71.45 ppm), C-8 (177.48 ppm) and C-9 (100.3 ppm). The other metin protons at δ_H = 2.69 ppm has correlated with carbon C-9' (100.65 ppm). Proton of methyl group at δ_H = 2.06 ppm have correlated with carbons C-3 (111.29 ppm), C-2 (111.49 ppm) and C-2' (117.71 ppm). The other proton of methyl group at δ_H = 1.17 ppm has correlated with carbon C-5' (66.15 ppm). Proton of metin group at δ_H = 4.15 ppm has correlated with carbons C-5 (100.3 ppm), C-6 (21.21 ppm) dan C-10 (84.96 ppm). Metin proton at δ_H = 5.34 ppm has correlated with carbons C-10' (128.96 ppm), C-6' (20.52 ppm), C-7' (22.68 ppm) and C-9' (100.65 ppm). The geminal proton of metin at δ_H = 2.66 ppm has correlated with carbon C-9' (100.65). The methyl proton of acetyl group at δ_H = 1.79 ppm has correlated with carbon δ_C = 169.07 ppm. The analysis of HMBC from isolated compound can be seen at Fig. 9.

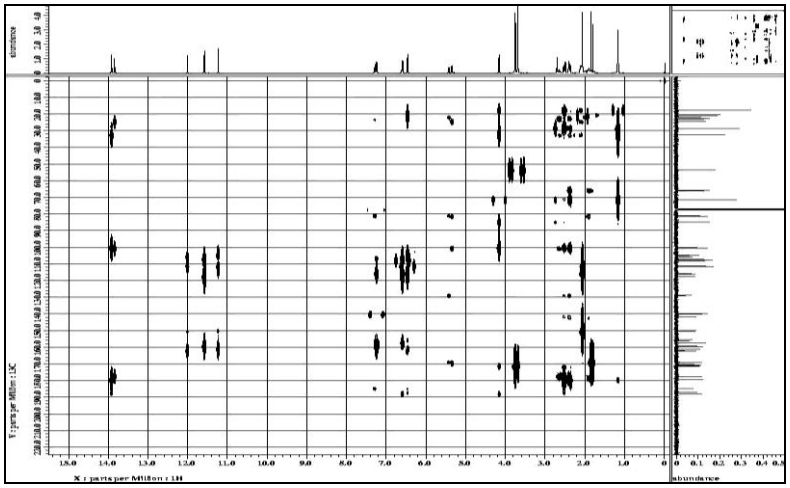

Fig. 9. Data measurement of HMBC spectrum

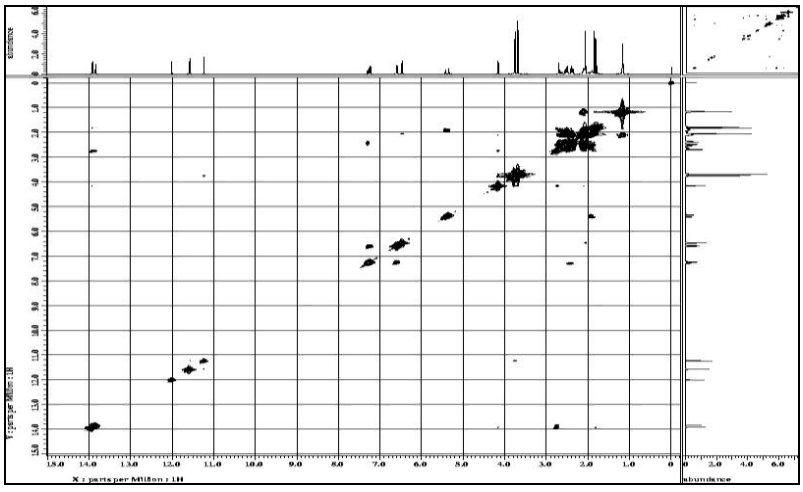

Fig. 10. Data measurement of COSY spectrum

Fig. 10 is an 2D-NMR COSY spectrum showed couples of proton spins i.e. H-4^1 6.59 ppm and H-3^1 7.2

ppm. Based on the results of analysis spectra by UV-Vis, FTIR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, it can be concluded that the isolated compound are Eumitrin A_1 compound with formula structure $C_{34}H_{32}O_{15}$. This is supported by results measurement of LC-MS which suggests these compounds have a molecule weight 680. According to Lee et al. the study of Eumitrin A_1 structure is very difficult to elucidate[20]. These compound has been found in previous lichen *Usnea bayleyi(Stirt.)* Zhlbr[12,21], and derivatives of species i.e. parmeliaceae *parmotrema cristiferum(taylor)* Hale[22], but it was discovered in *U. blepharea* Motyka and was carried out its cytotoxic activity test for the first time. The structure of Eumitrin A_1 can be seen in Fig. 11.

Cytotoxic activity test of Eumitrin A_1 against Murine Leukemia P388 cells based on MTT assay[23]. The IC_{50} specified by logarithmic equation. The results of measurement against cytotoxic activity shows the value of IC_{50} 4,509 µg/mL (Fig. 12). This done in triplo value with SD of 0.73. According to categories based on the study of cytotoxic cancer cells towards some of compounds stated very active if it has the value of IC_{50}< 5 µg/mL, active (IC_{50} 5-10 µg/mL), moderate (IC_{50} 11-30 µg/mL) and inactive (IC_{50}> 30 µg/mL)[15,24].

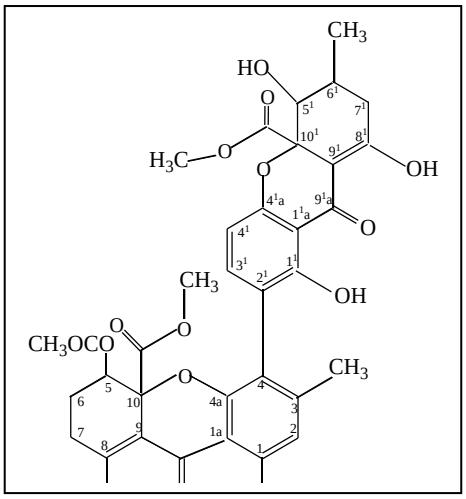

Fig. 11. Structure of isolated compound (Eumitrin A_1)

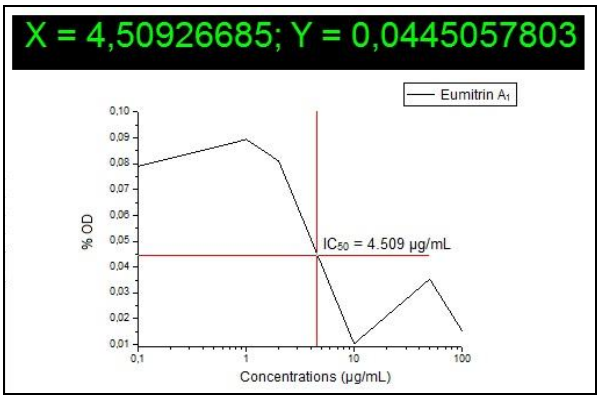

Fig. 12. Result measurement of Murine Leukemia P388 Cells

Conclusion

From the data analysis spectra by using UV-Vis, FTIR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR, it can be inferred the secondary metabolite compound has isolated from lichen *U. blepharea* Motyka plant is a Eumitrin A_1 with the formula structure of $C_{34}H_{32}O_{15}$ with molecule weight of 680. The cytotoxic activity test against Murine Leukemia P388 cells showed very active with IC_{50} values of 4.5 µg/mL.

Acknowledgements

Thank you for financial support of Ditlitabmas Dikti - Ministry of Research, Technology and Higher education, the Republic of Indonesia.

References

1. Yildrim E, Aslant A, Emsent B, Cakir A and Ercisli S. Insecticidal Effect of *Usnea longissima* (Parmeliaceae) Extract against Sitophilus granarius (Coleoptera: Curculionidae). Int. J. Agric. Biol.,2012, 14:303-306.
2. Brisdelli F, Perilli M, Sellitri D, Piovano M, Garbarino JA, Nicoletti M, Bozzi A, Amicosante G and Celenza G. Cytotoxic activity and antioxidant capacity of purified Lichen metabolites: An *In Vitro* study. Phytotherapy Research,2012, 27(3):431-437.
3. Ağar G, Aslan A, Sarioğlu EK, Alpsoy L, Ceker S. Protective activitty of the methanol extract of *Usnea longissima* against oxidative damage and genotoxicity caused by aflatoxin B_1*in vitro*.Turk J. Med. Sci., 2011, 41(6):1043-1049.
4. Rauf A, Latif A, Rehman S. and Afaq SH. In-vitro antibacterial screening of extracts of *Usnea*

longsissima lichen. Int. J. Bio and Pharma Tech.,2011, 2:14-18.
5. De Castro MEPC, Boufleur K, Soliza P, Nociti T, Melo JM, Amuda LK, Nahas R. Hypertrophic lichen planus-like atopic dermatitis: a case report. WAO Journal.,2015, 8(Suppl 1):A214.
6. Viviek MN, Yashoda K, Manasa M, Prashith Kekuda TR, Vinayaka KS. Radical scavenging and antibacterial activity of three Parmotrema species from Western Ghats of Karnataka, India. Journal of Applied Pharmaceutical Science.,2014, 4(3):086-091.
7. Singh S, Uperti DK, Lehri A and Paliwal AK. Quantification of Lichens commercially used in traditional perfumery industries of Uttar Pradesh, India. Indian Journal of Plant Sciences.,2015, 4(1):29-33.
8. Mitrović T, Stamenković S, Cvetković V, Tošić S, Stanković M, Radojević I, Stefanović O, Čomić L, Dačić D, Ćurčić M and Marković S. Antioxidant, Antimicrobial and antiproliferative activities of five Lichen species. Int. J. Mol. Sci.,2011, 12:5428-5448.
9. Prabhu SS and Sudha SS. Evaluation of the antibacterial properties of some Lichen species against human pathogens. Int. J. Adv. Res. Biol. Sci.,2015, 2(4):177-181.
10. Santiago KAA, Sangvichien E, Boonpragob K. and Dela Cruz TEE. Secondary metabolic profiling and antibacterial activities of different species of *Usnea* collected in Northern Philippines. Mycosphere.,2013, 4(2):267-280.
11. Kosanić M. and Ranković B. Screening of antimicrobial activity of some lichen speies in vitro. Kragujevac J. Sci.,2010, 32:65-72.
12. Huneck S. and Yoshimura I. Identification of Lichen Substances, 2nd ed. Berlin, New York-Tokyo: E-Publishing Springer, 1996,4.
13. Shrestha G. and Larry LStC. Anti-microbial activity of extracts from two Lichens-*Ramalina menziesii* and *Usnea lapponica.* Bulletin of the California Lichen Society.,2013, 20(1):5-10.
14. Shukla P, Uperti DK. and Tewari LM. Secondary metabolite variability in lichen genus *Usnea* in India: A potential source for bioprospection. G-Journal of Environmental Science and Technology.,2015, 2(4):44-55.
15. Maulidiyah, Azis T, Sabarwati SH, Nurdin M. Cytotoxic activity of (-)-usnic acid compound from lichen *Usnea* sp.on *Murine Leukemia* P388 Cells. JIFI.,2015, 13(1).
16. Thippeswamy B, Naveenkumar KJ, Bodharthi GJ. and Shivaprasad SR. Antimicrobial activity of ethanolic extract of *Usnea longissima*. Journal of Experimental Science., 2011, 2(12):01-03.
17. Toma N, Ghetea L, Nitu R, Cordol DI. Progress and perspectives in the biotechnology of lichens. Roum. Biotechnol. Lett.,2001, 6:1-15.
18. Gupta P, Barun S, Roy S. Spectrophotometric analysis of some reactive AZO dyes used in textile industries. Indian Journal of Applied Research.,2014, 4(4):37-39.
19. Maulidiyah, Cahyana AH, and Suwarso WP. A new phenolic compound from acetone extract of lichen *Usnea.* Indonesian Journal of Chemistry., 2011, 11(3):290-294
20. Lee MY, Park HM, Son GH. and Lee CH. Liquid Chromatography-Mass Spsectrometry-based chemotaxonomic classification of *Aspergillus* spp. and eluation of the biologocal activity of its unique metabolite, neosartorin. J. Microbiol. Biotechnol.,2013, 23(7):932-941.
21. Laily BD, Zakaria Z, Samsudin MW, Elix JA. Chemical Profile of Compounds from Lichens of Bukit Larut, Peninsular Malaysia. Sains Malaysiana.,2010, 39(6):901-908.
22. Louwhoff HJJS. and Elix JA. The lichens of Raratonga, cook islands, south pacific ocean II: *Parmeliaceae.* Lichenologist.,2000, 32(1):49-55.
23. Patel JR, Choubisa, Dholakiya BZ. Plant Derived compounds having activity against P388 and L1210 Leukemia cells. Chemical Sciences Journal.,2011, CSJ-33:1-16.
24. Sahid A, Pandiangan D, Siahaan P, Rumondor MJ, Uji sitotoksisitas ekstrak metanol daun sisik naga (Drymoglossum piloselloides Presl.) terhadap sel leukemia P388. *Jurnal MIPA UNSRAT Online,* 2013, 2(2):94-99.

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