(*S*)-MIMOSINE FROM THE INDONESIAN SENSITIVE PLANT (*Mimosa invisa* Colla) AS AN ANTI-MELANOGENESIS AGENT
(*S*)-MIMOSINA DARI TUMBUHAN SIKEJUT BESAR (*Mimosa invisa* Colla) SEBAGAI ANTI-MELANOGENESIS

Adiwarna[1], ,Suminar Setiati Achmadi[1], Anggia Murni[2], Novriyandi Hanif[1]*

[1] *Department of Chemistry, IPB University, Jl. Tanjung, IPB Campus Dramaga, Bogor 16680, Indonesia*

[2] *Pusat Studi Biofarmaka Tropika, Gedung CRC lt.2 Kampus IPB Taman Kencana, Jl. Taman Kencana no.3, Bogor 16128, Indonesia*

* *Department of Chemistry, IPB University, Jl. Tanjung, IPB Campus Dramaga, Bogor 16680, Indonesia; nhanif@apps.ipb.ac.id*

Abstrak

Ekstrak dari daun sikejut besar atau *Mimosa invisa* Colla (Fabaceae) telah diuji aktivitas anti melanogenesis menggunakan sel melanoma $B_{16}F_{10}$. Fraksi aktif diperoleh dari pemurnian menggunakan kromatografi cair vakum yang teridentifikasi kaya akan senyawa mimosina. Kandungan mimosina pada fraksi ini dikonfirmasi menggunakan metode kromatografi dan spektroskopi. Gugus 3-hidroksipiridina terdeteksi dengan spektrum ultraviolet (UV) pada panjang gelombang 204,6 dan 273,8 nm, sedangkan pada spektrum infra merah (IR) gugus asam amino terdeteksi adanya absorpsi pada panjang gelombang 1655, 2850 dan 3300^{cm-1}. Pada pengukuran spektrum NMR fraksi aktif yang dilarutkan dalam larutan D_2O memberikan sinyal yang muncul pada medan rendah 8.43, 7.60, 7.51 dan 3.47 ppm yang menunjukkan bagian dari 3-hidroksipiridina. Data Kromatografi cair SM/SM membuktikan bahwa bobot molekul mimosina adalah 198.0641 mmu ($C_8H_{10}N_2O_4$)

yang terkonfirmasi dengan analisis ion fragmen. Pada uji aktivitas fraksi tersebut menunujukkan aktivitas antimelanogenesis pada hambatan 32 µg/mL menggunakan sel melanoma $B_{16}F_{10}$.

Kata Kunci: anti-melanogenesis, ESIMS, *Mimosa invisa* Colla, mimosina

Abstract

Giant sikejut or *Mimosa invisa* Colla (Fabaceae) leaf extract was evaluated for its anti-melanogenesis activity using $B_{16}F_{10}$ melanoma cells. An active fraction was obtained using vacuum liquid chromatography which is known to be rich in mimosine compounds. The presence of (*S*)-mimosine in the fraction was confirmed by chromatography and spectroscopy studies. The 3-hydroxypyridine group was proved by the ultraviolet spectrum that appeared at λ_{max} 204.6 and 273.8 nm, while the infrared spectrum showed the presence of amino acids based on of absorption peaks at 1655, 2850, and 3300 cm^{-1}. The NMR spectrum of the fraction dissolved in D_2O showed signals at the lower field at 8.43, 7.60, 7.51, and 3.47 ppm evidenced for the 3-hydroxypyridine. The relative configuration of mimosine is *S* based on the NMR study and comparison with that of related literatures. The ESIMS data proved that the principal fraction contained mimosine with 198.0641 mmu ($C_8H_{10}N_2O_4$), which was also confirmed by its fragment ions. On the activity, *M. invisa* leaf extract was evaluated $B_{16}F_{10}$ melanoma cells and exhibited anti-melanogenesis activity at a concentration of 32 µg/mL.

Keywords: anti-melanogenesis, ESIMS, *Mimosa invisa* Colla, mimosine

INTRODUCTION

Melanogenesis is a biochemical process producing melanin pigment which plays an important role in the protection of skin against UV light, but abnormal accumulation of this pigment causes unaesthetic hyperpigmentation (Choi and Shin, 2016; Pillayar *at al*., 2015). It has been reported that melanogenesis inhibitors act mainly through the downregulation of tyrosinase activity (Kim and Uyama, 2005). Additionally, (*S*)-mimosine is a known inhibitor of tyrosinase. Therefore, mimosine may be an antimelanogenesis agent (Kim and Uyama, 2005; Nguyen Nguyen and Tawata, 2015; Nguyen Nguyen and Tawata, 2016). Mimosine is a non-protein amino acid containing an alanine side chain bound to a pyridone ring. It is a very polar compound. Mimosine has been reported to exhibit various activities such as anticancer, antiinflammation, antifibrosis, antimicrobial, antiviral, and pesticidal activities (Patro *et al*., 2016). Just recently, mimosine dipeptide was reported as an improved inhibitor against melanogenesis and cyclooxygenase (Pillayar *at al*., 2015).

Giant *sikejut* or sensitive plant [*Mimosa invisa* (Fabaceae)] is a subshrub with multi-branched vines that grows widely in the open land of Indonesia. The root and the whole plant are used as folk medicine to treat various ailments such as convulsions, alopecia, diarrhea, dysentery, insomnia, tumors, and snake bite (Ueda and Yamamura, 1999). In addition, the plant is unique for its physiological activity (Vepsäläinen *et al*., 2005). It has very rapid movement of the leaves when they are stimulated by touch or heating. Moreover, a variety of chemical constituents, including alkaloids (Vepsäläinen *et al*., 2005; León *et al*., 2004), flavonoids, polyketides (Ohsaki *et al*., 2006), and terpenoids (Boonpisuttinant *et al*., 2013) have been identified in this species. Azmi *et al.* (2011) described that the plant *M. invisa* or giant *sikejut* was able to use as herbal medicine to

72 treat some diseases, but the activity of the plant toward melanogenesis has not been
73 reported yet. L-Mimosine is a signature molecule of *Mimosaceae* plants (Xuan *et al.* 2013).
74 Therefore, the molecule is expected to contain in the collected plant *M. invisa* or giant
75 *sikejut*. In 2014, Vijayan and Joseph showed that the leaves of giant *sikejut* contained L-
76 mimosine (0.04%) determined by high performance liquid chromatography (HPLC) [mobile
77 phase H_3PO_4 (1%)]. The molecule was then characterized to have retention time (t_R) 4.1
78 minutes. Thin layer chromatography (TLC) analysis of L-mimosine using CH_3OH:$CHCl_3$
79 (15:85) showed that retention factor (*Rf*) 0.50. In addition, Champanerkar *et al.* (2010)
80 characterized L-mimosine using LC-MS/MS to have the molecular ion *m/z* 197.70 and
81 fragmen ions 162.80 (*base peak*), 160.80, 144.80, 111.10, 109.90 mmu.
82 Because of its abundance and its unique properties both chemistry and biology, we
83 investigated chemical constituent of *M. invisa* and evaluated its anti-melanogenesis
84 activity.
85
86 **METHODS**
87
88 **Materials**
89 The sensitive plant (*M. invisa* Colla) was obtained from Cikondang village, Leuwiliang,
90 Bogor. The specimen was verified at the Herbarium Bogoriense, Research Center for
91 Biology, Indonesian Institute of Sciences (LIPI), Indonesia. Silica gel 60 (0.063-0.20 mm,
92 MERCK) was used for column chromatography. Analytical TLC was employed using
93 commercial silica gel 60 F254 with monitoring under UV light at λ 254 and 366 nm. Murine
94 melanoma $B_{16}F_{10}$ cells were obtained from ATCC® CRL 6475™ and maintained as

monolayer culture in inoculation medium consisted of Dulbecco's modified Eagle medium (DMEM) (SIGMA-ALDRICH, St Louis, MO), 10% fetal bovine serum (FBS) (SIGMA-ALDRICH, St Louis, MO), 100 □g/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B 37 °C with 5% CO_2.

Instrumentation

^{1}H NMR spectra were recorded on a JEOL 500 FTNMR operating at 500 MHz. Chemical shifts (δ) were referenced to D_2O signal and are given in ppm. The coupling constants (*J*) are in Hz. ESIMS data were obtained on ultra-performance liquid chromatography-mass spectrometry (UPLCMS) Waters Acquity Xevo G2-LC-MS/MS QtoF. The UPLCMS was conducted using an ACQUITY UPLC® BEHC18 1.7 μm column (2.1 × 50 mm) eluted with a gradient of MeOH-H_2O-HCO_2H, a flow rate of 0.3 mL/min and with monitoring at λ 254 nm. Detection was achieved using electrospray ionization in positive mode (ESI positive mode). Ultraviolet spectra were recorded on Pharmaspec UV-1700 SHIMADZU spectrophotometer, while infrared spectra were documented using an Agilent Technologies Cary 630 FT-IR. Anti-melanogenesis activity was determined on BIORAD USA 32i spot plate reader.

Procedures

Extraction and isolation of active fraction

The leaves of *M*. *invisa* (200 g) were extracted exhaustively with MeOH. The combined extracts were concentrated under reduced pressure. The resulting extract (10 g) was fractionated using vacuum liquid chromatography with gradient elution of $CHCl_3$-MeOH to give 15 fractions. A small portion of MeOH extract was tested for alkaloid content using Dragendorff, Wagner, and Meyer reagents. Each fraction was profiled by TLC (monitored at

366 nm). The alkaloid mimosine fraction was then characterized on the TLC plate. The mimosine-rich fraction (9.3 mg, 0.0047%) was isolated using a preparative TLC, which was further elucidated using chromatography (TLC) and a variety of spectroscopic methods (UV, IR, NMR, and ESIMS).

Anti-melanogenesis assay

The mimosine-rich fraction was tested against $B_{16}F_{10}$ melanoma cells to evaluate its melanogenesis inhibitory activity. The melanogenesis assay was performed according to the method of Boonpisuttinant and coworkers (Vijayan and Joeph, 2014) with some modifications. The cells were seeded at density of 10^5 cells per plate and incubated overnight. Subsequently a mimosine-rich fraction was added at concentrations ranging from 0 to 32 μg/mL. Vitamin C was used as a positive control. After 48-hour incubation, the cells were washed with PBS and dissolved in 500 μL of 10% NaOH (SIGMA-ALDRICH, USA). The isolated melanin was then incubated for 1 hour at 60 °C. The amount of melanin in the solution was determined spectrophotometrically by measuring the absorbance at 450 nm. The total melanin content was estimated using a microplate reader (BiORAD type iMark, Japan). The cell viability assay was also performed with some modifications on the previous protocol (Vijayan and Joseph, 2014).

RESULTS AND DISCUSSION

The isolated mimosine-rich fraction

To a small portion of the concentrated MeOH extract, followed with checking for the presence of alkaloids using three relevant reagents,the fraction showed positive results. The residue (10 g) was then purified using VLC silica with gradient elution $CHCl_3$-MeOH to

give 15 fractions in which the 7th fraction (116.89 g) was selected since it contained mimosine in relative abundance as indicated by the spot on TLC plate. Further purification of the 7th fraction was performed using a preparative TLC (silica) with eluent $CHCl_3$-MeOH 85:15 to give a mimosine-rich fraction (9.3 mg, 0.0047 %).

Chemical structure

The TLC profile of the target fraction showed similar R_f of 0.44 (MeOH-$CHCl_3$ 85:15, UV detection λ 366 nm) as reported by Vijayan and Joseph which was consistent with the existence of mimosine (Pretsch *et al*., 2009). The UV spectrum of a mimosine-rich fraction exhibited peaks on λ_{max} (MeOH) of 204.6, 224.2, 273.8, and 325.0 nm, which are in a good agreement with the presence of chromophore hydroxypyridone (enol/enamine form, **1**) having an equilibrium with the tautomer form of its keto/iminium molecule, **2**. This caused the λ_{max} of the keto molecule to shift towards a higher λ_{max} (red shift) (Figure 1).

The presence of amino and carboxylic acid in the form of zwitter ion as in **3** or **4** was confirmed by IR data which showed *v* 2850, 2917, and 1650 cm^{-1}. The iminium functional group conjugated with a carbonyl as in **4** was evidenced by IR data which showed a weak absorption of 1650 cm^{-1} (Peng *et al*., 2013) concluding that the mimosine was actually in the tautomeric form as in **3** and **4** (Figure 2). The broad absorption of *v* 3300 cm^{-1} was due to enol and enamine functional groups possessing the tautomeric properties of the keto form (Peng *et al*., 2013; Sakai *et al*., 1997).

The ^1H NMR data of the target fraction measured in D_2O disclosed the presence of the-$CH=NR_2^+$proton δ 8.43 (s, 1H) as in **4**. Further support was derived from observation of two alkenic proton δ 7.50 (d, *J* = 5.2 Hz, 1H) and 7.61 (d, *J* = 5.2 Hz, 1H). This suggested the

161 geometry of alkene was in *Z* form. The methine proton attached to NH_3^+ functional group
162 showed δ 3.47 (dd, *J* = 11.2, 5.0 Hz, 1H) and was consistent with having as *S* configuration
163 (δ 3.47, Δ δ = 0), which measured in the same NMR solvent as reported by Sakai and co-
164 workers. The ABX proton bearing to heterocylic ring showed a resonance at δ 2.03 (dd, *J* =
165 11.2, 7.3 Hz, 1H) and 3.05 (t, *J* = 1.5 Hz, 1H) confirming the proton δ 3.47 should be anti (θ
166 180°) to the proton δ 2.03 and the proton 3.05 should be in gauche orientation (θ 60°-90°).
167 The relative stereochemistry of C2 mimosine was proposed on the basis of coupling
168 constant analysis and comparison the chemical shift and conformation with dysherbaine as
169 shown in Figure 3 (Sakai *et al*., 1997). All data are consistent with the conformer/tautomer
170 **4** measured in D_2O.

171 The molecular formula of the principal fraction was mainly established on the basis
172 of the mass spectral data. (*S*)-Mimosine gave a peak for molecular ion at *m/z* 198.0641
173 mmu on the positive-ion ESI run with MeOH-H_2O-HCO_2H. The molecular ion was consistent
174 with the molecular formula $C_8H_{10}N_2O_4$. To secure the analysis, we checked the key
175 fragment ions of the target fraction and confirmed them as mimosine. The proposed
176 fragmentation mechanism of the mimosine rich-fraction was presented in Figure 4.

177 **Anti-melanogenesis activity**

178 The fraction 7.1 (mimosine-rich fraction) showed a weak activity against melanoma
179 $B_{16}F_{10}$ cells with 33.90% inhibition at 32 µg/mL (Table 1). In addition, our hypothesis used
180 melanoma $B_{16}F_{10}$ cells to investigate anti-melanogenesis activity was also supported by
181 Boonpisuttinant and coworkers in 2013 who used $B_{16}F_{10}$ cells on the ethanol extract of
182 *Hypoxis aurea* Lour (leaves). The ethanol extract showed inhibition 57.95% (100 µg/mL),

while vitamin C as positive control showed lower inhibition at 54.42%. Loizzo *et al.* 2012 in their review on tyrosinase inhibitor reported that mimosine had IC_{50} 3.70 μM (0.73 μg/mL). The weak activity could be caused by the presence of impurities on the target sample.

CONCLUSIONS

The mimosine-rich fraction has been isolated from the *Mimosa invisa* Colla leaves which showed anti-melanogenesis activity against murine melanoma $B_{16}F_{10}$ cells at 32 μg/mL. As mimosine is very polar and undergoes tautomerization, this presented challenges in purification and structure elucidation. We determined the planar structure of mimosine including stereochemistry using NMR, UV, IR, and ESIMS spectroscopy data as well as comparison to the literature values. This is the first report of the Indonesian *Mimosa invisa* Colla containing (*S*)-mimosine, the unique molecule possessing tautomer keto/enol-enamine/iminium system.

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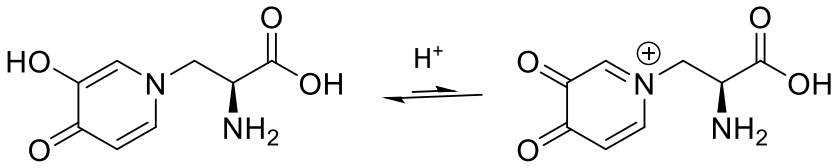
251

Table 1. Viability dan inhibition of 7. 1 fraction againts melanoma $B_{16}F_{10}$ cells

F7.1	OD	Viability	Inhibition

µg/mL		%	%
0.00	0.65	100.00	0.00
0.25	0.55	84.05	15.95
0.50	0.66	101.84	-1.84
1.00	0.62	95.09	4.91
2.00	0.65	99.08	0.92
4.00	0.64	97.39	2.61
8.00	0.60	92.64	7.36
16.00	0.59	90.95	9.05
32.00	0.43	66.10	33.90

OD = Optical Density



1

λ_{max} 204.6 nm

λ_{max} 273.8 nm

2

λ_{max} 224.2 nm

λ_{max} 325.0 nm

Figure 1. The observed λ_{max} on the fraction justified the presence of hydroxypyridone chromophore as in **1**.

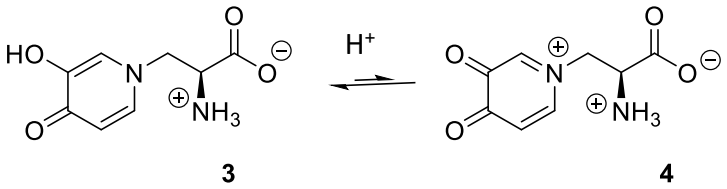


Figure 2. The presence of amino acid in the form of zwitter ion as in evidenced by IR spectroscopy data.

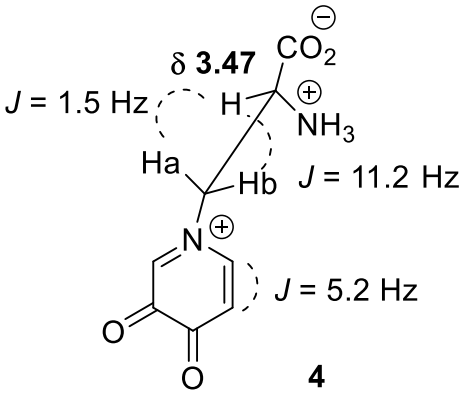
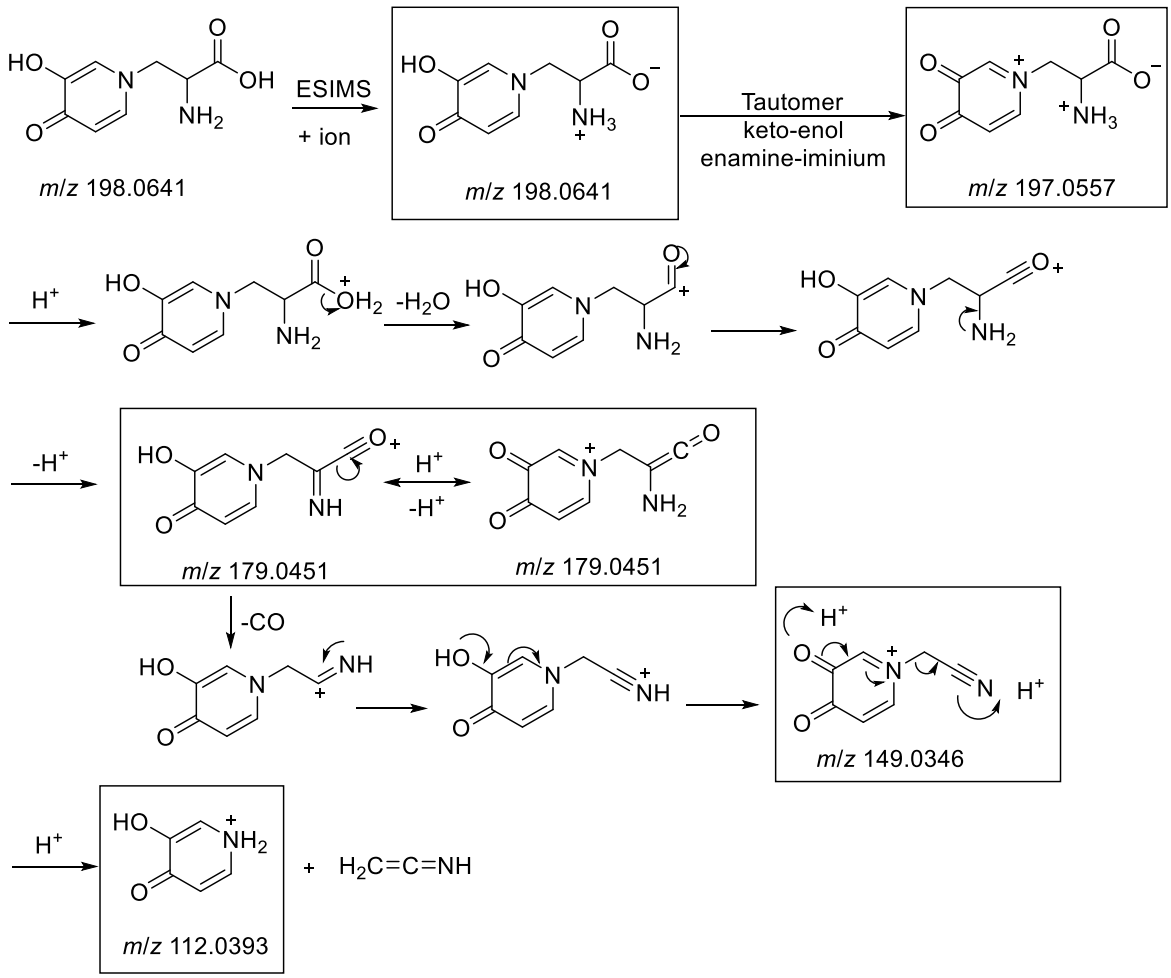


Figure 3. The possible major conformer of (*S*)-mimosine with δH_2 3.47 measured in D_2O.



307

308 **Figure 4.** The proposed-fragmentation mechanism of ion fragments observed in the ESIMS

309 measurement (MeOH-H_2O-HCO_2H) confirmed the presence of mimosine.

310