**Naphthalene Derivatives from Bawang Tiwai Bulb *Eleutherine bulbosa* in Borneo**

**Supriatno Salam1,3, Baso Didik Hikmawan1, Erwin Samsul1, Herman2, Islamudin Ahmad2, Laode Rijai2, Mohd Azlan Nafiah3,\*.**

1Faculty of Pharmacy, Universitas Mulawarman, Samarinda 75119, Kalimantan Timur, Indonesia. supriatno.salam@unmul.ac.id; didikhikmawan@farmasi.unmul.ac.id, erwinsamsul@farmasi.unmul.ac.id

2Pharmaceuticals Research and Development Laboratory of Pharmaca Tropics, Faculty of Pharmacy, Universitas Mulawarman, Samarinda 75119, Kalimantan Timur, Indonesia. herman.mulawarman@gmail.com; islamudinahmad@farmasi.unmul.ac.id, laode@farmasi.unmul.ac.id

3Department of Chemistry, Faculty of Science and Mathematics, Sultan Idris Education Universiti, Tg Malim 35900, Perak, Malaysia. supriatno.salam@fsmt.upsi.edu.my, azlan@fsmt.upsi.edu.my

Corresponding authors. E-mail: azlan@fsmt.upsi.edu.my

**Abstract**

Naphthalene derivative compounds named Eleutherol (**1**) and Eleutherol C (**2**) have been isolated from Bawang Tiwai Bulb *Eleutherine bulbosa*. After isolating compounds **1** and **2** from the EtOH extract using conventional chromatography methods, their cytotoxic activity was tested against T47D breast cancer cells in vitro. Their chemical structures were elucidated based on spectroscopic analysis including IR, HR-TOFMS, 1D, and 2D NMR, and by comparison to those related spectra previously reported. Compounds **1** and **2** were tested for their cytotoxic effects against T47D breast cancer cells and showed moderate cytotoxicity against T47D breast cancer cells with IC50 values 117.15 and 80.21 µM, respectively, compared with cisplatin 24.07 µM.

**Keywords**: *Eleutherine bulbosa,* Eleutherol, Eleutherol C, T47D breast cancer, East Kalimantan.

**Introduction**

*E. bulbosa* is an herbaceous plant belonging to the Iridaceae family which is distributed mainly in South Africa, South America, and Southeast Asia and grows mostly in sulfur areas, 600–2000 m above sea level [1]. In Indonesia, *E. tubers* are widely consumed by local tribal communities in Kalimantan, namely the Dayak tribe, and are traditionally used as a medicinal plant to increase breast milk production and treat diabetes, breast cancer, stroke, hypertension, and sexual disorders [2]. Research related to the search for compounds that play a role in the various properties of *E. bulbosa* tubers has been carried out. Naphthalene, anthraquinone, and naphthoquinone are the main constituents of *E. bulbosa* [3,4] which exhibit various pharmacological properties such as anti-diabetic [2], anti-inflammatory [5], anti-microbial [6,16], anti-melanogenesis [7], anti-MRSA [15], Antifungal [18] and anti-cancer [8]. As an anti-cancer, much research has been carried out on isolated compounds from *E. bulbosa*. For example, the compounds Eleutherinoside C and Isoeleutherin showed selective cytotoxic activity and inhibited TCF/β-catecin transcription in SW480 cancer cells in a dose-dependent manner [8], the compound isoeleutherol showed potent activity in both cancer cell lines against HeLa and MCF-7 cells with LC50 values of 35.4 and 23.8 ppm respectively [9,11]. The activity of inhibit the cell cycle and induct apoptosis in HeLa cancer cell with a combination of Sabrang onion (*E. palmifolia* (L.) Merr) and Starfruit Mistletoe (*Macrosolen cochinchinensis* (Lour.) [17]. This study is a report on naphthalene derivative compounds that were isolated from the ethanolic extract of *E. bulbosa.* Eleutherol (**1**) and Eleutherol C (**2**) which are derivatives of naphthalene compounds have been isolated from *E. bulbosa,* along with their cytotoxic activity against T47D breast cancer cells *in vitro.* Activity tests were carried out on T47D because this naphthalene group has very good activity against cytotoxics, especially breast cancer cells.

**Material and Methode**

**General experimental procedures**

The UV spectrum was measured using a TECAN Infinite M200 pro (Mannedorf, Switzerland) with MeOH. The IR spectra were recorded on SHIMADZU IR Prestige-21 in KBr (Kyoto, Japan). The high-resolution of time-of-flight mass spectrometry (HR-TOFMS) data was recorded with Water Xevo QTOF MS (Milford, Massachusetts, USA). NMR spectrum was evaluated using JEOL ECZ-500 (Tokyo, Japan) at 500 MHz for 1H and 125 MHz for 13C using tetramethylsilane (TMS) as the internal standard. In addition, the chemical shifts are expressed in ppm, concerning the CDCl3 (δH 7.26/δC 77.2) signals. Column chromatography (CC) was performed using silica gel (70-230 mesh) (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed with silica gel 60. TLC plates were precoated with silica gel GF254 (Merck, 0,25 mm, Darmstadt, Germany) using various solvent systems. Compounds were visualized under UV light (254 and 365 nm) or by spraying the heated silica gel plates with 10% H2SO4 in EtOH.

**Plant material**

The bulbs of *E. bulbosa* were collected from Batu Cermin, Samarinda, East Kalimantan, Indonesia. The plant was identified at the Laboratory of Ecology of Tropical Forest Biodiversity, Faculty of Forestry, Mulawarman University, Samarinda, East Kalimantan. Indonesia. The plant was shade-dried (<45°C), coarsely powdered, and stored in an airtight container.

**Extraction and isolation**

The dried *E. bulbosa* (200 g) was extracted with distilled ethanol at room temperature for 4 days (4×2.5 L) and concentrated using a vacuum rotary evaporator, yielding a concentrated extract (24 g). About 20 g of ethanol was chromatographed using silica gel column CC (70-230 mesh) with *n*-hexane: ethyl acetate as eluent (5% stepwise) and obtained 5 combined fractions (Fraction A-E).

Fraction A in the isocratic column chromatography and eluted using *n*-hexane: ethyl acetate (9:1) obtained fractions A1-A2. The A2 fraction was purified by recrystallization to obtain compound **1** (7.5 mg).

Fraction B was separated by silica gel column chromatography (70-230 mesh) with a gradient of 2.5% using *n*-hexane: ethyl acetate (9:1-7:3) eluent to produce 5 fractions (Fraction B1-B5). Then fraction B3 was selected to be purified using silica gel column chromatography with *n*-hexane: ethyl acetate (8.5:1.5) as eluent to obtain compound **1** (16.3 mg).

Fraction C was chromatographed on a silica gel column and eluted using *n*-hexane: ethyl acetate with a gradient of 2.5% (9:1-6:4) to produce 9 fractions (Fraction C1-C9). The C4 fraction was selected to be purified using column chromatography. Silica gel was eluted isocratically using *n*-hexane: ethyl acetate (7.5:2.5) and obtained compound **2** (14.2 mg).

*Eleutherol* (**1**) a needle-shaped white crystal (MeOH); IR (KBr) *Vmax* 3471, 1664, 1382, 1320, 1243 cm-1; 1H-NMR and 13C-NMR data see Table 1; HR-TOFMS [M + H]+ *m/z* 245.1351 (calcd. C14H13O4 *m/z* 245.1361).

*Eleutherol C* (**2**) a yellow amorphous powder (MeOH); IR (KBr) *Vmax* 3471, 1664, 1382, 1320, 1243 cm-1; 1H-NMR and 13C-NMR data see Table 1; HR-TOFMS [M + H]+ *m/z* 245.1351 (calcd. C14H13O4 *m/z* 245.1361).

**Bioassays for cytotoxic activity**

The T47D cells were seeded into 96-well plates at a density of 3 x 104 cells/well and incubated in a humidified CO2 incubator for 24 h. Varying concentrations of the test compound were dissolved in DMSO, followed by six desirable concentrations prepared using PBS (phosphoric buffer solution, pH 7.30-7.65). each concentration of the compound was added to the wells in triplicate and incubated in a humidified CO2 incubator for 48 h, the negative control wells received only DMSO, and cisplatin was used as the positive control. After an incubation period, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added to all test and control well and the incubation was continued for another 4 h. The MTT-stop solution containing SDS (sodium dodecyl sulphate) was added to wells and incubated again for another 24 h. The absorbance values were read using a microplate reader at a wavelength of 550 nm. IC50 values were determined by the linear regression method using Microsoft Excel software. The IC50 is the concentration required for 50% growth inhibition [12].

**Results and discussion**

The ethanol extract of *E. bulbosa* was isolated using conventional column chromatography (CC) packed with silica gel 60 with gradient and isocratic elution and spectroscopic analysis to obtain the naphthalene derivatives, Eleutherol (**1**) and Eleutherol C (**2**) (Fig. 1).

Compound (**1**) was obtained as a needle-shaped white crystal soluble in methanol. The molecular formula was C14H12O4 (Fig. 2), based on the high-resolution time-of-flight (HR-TOFMS) spectrum with a [M + H]+ ion peak at *m/z* 245.1351 (calcd. C14H13O4 *m/z* 245.1361) and obtained Nine degrees of unsaturation. The Infrared (IR) spectrum (Fig. 3) showed the presence of hydroxyl groups (*Vmax* 3471 cm-1), lactone functional groups (*Vmax* 1664 cm-1), and Aromatic carbon (*Vmax* 1382, 1320, 1243 cm-1). The Proton nuclear magnetic resonance (1H-NMR)spectrum (Fig. 4, Table 1) showed one methyl proton signal at δH 1.725 (3H, d, *J* = 6 Hz, CH3-1՛), one proton methine signal at δH 5.72 (1H, q, *J* = 6.5 Hz, H-1), one hydroxyl chelate signal at δH 9.63 (1H, s, OH-9), one oxygenated methyl proton signal at δH 4.09 (3H, s, OCH3-2՛), one isolated aromatic proton signal at δH 7.83 (1H, s, H-4) and three aromatic proton signals at δH 6.91 (1H, d, *J* = 7.5 Hz, H-7), δH 7.39 (1H, t, *J* = 8.5 Hz, H-6) δH 7.54 (1H, d, *J* = 8.5 Hz, H-5) which implied the presence of a benzene ring in (**1**). Carbon nuclear magnetic resonance (13C-NMR) along with distortions enhancement by polarization transfer (DEPT) (Fig. 5) showed the presence of fourteen carbon signals consisting of five quaternary carbons *sp2* at δC 117.5 (C-8a), 125.9 (C-9a), 127.9 (C-3a) and 137.2 (C-4a). Two quaternary carbons are oxygenated at δC 149.2 (C-9) and 156.6 (C-8). Four carbons of methine at δC 106.3 (C-7), 116.5 (C-4), 123.7 (C-5) and 126.7 (C-6). One carbon of methine is oxygenated at δC 77.5 (C-1). One methyl at δC 19.2 (C-1՛), one oxygenated methyl at δC 56.5 (C-2՛), and carbon lactone at δC 170.7 (C-3). These functionalities accounted for six out of the total nine degrees of unsaturation. The remaining three degrees of unsaturation were consistent with a naphthalene skeleton. The IR spectra as well as the NMR data indicated (**1**) is a substituted naphthalene derivative [10] which was further confirmed by 2D NMR spectra HMQC (Fig. 6) and HMBC (Fig. 7). The connectivity of (**1**) was established mainly by proton multiple bond connectivity (HMBC). Fig. 9 for the HMBC spectrum showed the signal aromatic at δH 7.39 (1H, t, *J* = 8.5 Hz, H-6) was correlated to δC 106.3 (C-7) and 123.7 (C-5) which suggested that the aromatic proton was located at C-6. The methoxyl proton at δH 4.09 (3H, s, OCH3-2՛) was correlated to δC 156.6 (C-8) which indicated that the methoxy group was located at C-8, respectively, the hydroxyl proton at δH 9.63 (1H, s, OH-9) was correlated to δC 149.2 (C-9), 125.9 (C-9a) and 117.5 (C-8a), and the aromatic proton at δH 7.83 (1H, s, H-4) was correlated to δC 127.9 (C-3a) and 137.2 (C-4a), which indicated that the hydroxyl group and aromatic proton were located at C-9 and C-4. The methyl proton at δH 1.725 (3H, d, *J* = 6 Hz, CH3-1՛) was correlated to δC 77.5 (C-1) and 127.9 (C-3a), proving the presence of a methyl group located at C-1. The signal of proton methine at δH 5.72 (1H, q, *J* = 6.5 Hz, H-1) was correlated to the carbonyl lactone at δC 170.7 (C-3), which indicated that the lactone ring was formed between C-3a, C-3, and C-1. Based on the analysis of the spectra IR, 1D, and 2D NMR and compared with the previously reported literature, the structure of (**1**) was similar to the known compound Eleutherol, thus the structure of (**1**) was elucidated as shown and named Eleutherol.



Fig. 1. Structures of Compounds **1** and **2**

Compound (**2)** was obtained as a yellow amorphous powder soluble in methanol. The molecular formula was C14H12O4, based on the high-resolution time-of-flight (HR-TOFMS) spectrum with a [M + H]+ ion peak at *m/z* 245.1351 (calcd. C14H13O4 *m/z* 245.1361) and obtained Nine degrees of unsaturation. The Infrared (IR) spectrum showed the presence of hydroxyl groups (*Vmax* 3471 cm-1), lactone functional groups (*Vmax* 1664 cm-1), and Aromatic carbon (*Vmax* 1382, 1320, 1243 cm-1). The Proton nuclear magnetic resonance (1H-NMR)spectrum showed one methyl proton signal at δH 1.725 (3H, d, *J* = 6 Hz, CH3-1՛), one proton methine signal at δH 5.72 (1H, q, *J* = 6.5 Hz, H-1), one hydroxyl chelate signal at δH 9.63 (1H, s, OH-9), one oxygenated methyl proton signal at δH 4.09 (3H, s, OCH3-2՛), one isolated aromatic proton signal at δH 7.83 (1H, s, H-4) and three aromatic proton signals at δH 6.91 (1H, d, *J* = 7.5 Hz, H-7), δH 7.39 (1H, t, *J* = 8.5 Hz, H-6) δH 7.54 (1H, d, *J* = 8.5 Hz, H-5). The 1H-NMR and 13C-NMR spectra seemed identical to those of (**1**) (Table 1). However, the HMBC spectrum of (**2**) (Fig. 8) displayed the hydroxyl proton at δH 9.63 (1H, s, OH-9) was correlated to δC 156.6 (C-8), which indicated that the hydroxy group was located at C-8 (Fig. 8). The methoxyl proton at δH 4.09 (3H, s, OCH3-2՛) was correlated to δC 149.2 (C-9), 125.9 (C-9a) proving the presence of methoxy group located at C-9., respectively. Based on these descriptions and comparing the respective spectroscopic evidence with existing publications, the structure of (**3**) was elucidated and determined as a known compound as Eleutherol C. [13].



Fig. 2. HR-TOFMS spectra of Compound **1**.

Compounds **1** and **2** were tested for cytotoxic activity against T47D breast cancer cells using the method previously described and cisplatin was used as a positive control. Compounds **1** and **2** showed moderate cytotoxicity with IC50 values of 117.15 and 80.21 µM, respectively. This indicates that the location of the hydroxyl group can increase cytotoxic activity [14], whereas in compound **2** the hydroxyl group is located at C-8 and is not blocked by other groups which causes the IC50 to be lower than compound **1**.

Table 1 NMR spectral data for **1** and **2** (500 MHz for 1H and 125 MHz for 13C in CDCl3)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Position Carbon | 1 |  |  | 2 |  |
| 13C-NMRδC (mult,) | 1H-NMRδH [(∑H, mult, *J*(Hz)] |  | 13C-NMRδC (mult,) | 1H-NMRδH [(∑H, mult, *J*(Hz)] |
| 1 | 77.5 (s) | 5.72 (1H, q, 6.5) |  | 77.5 (s) | 5.72 (1H, q, 6.5) |
| 3 | 170.7 (q) | - |  | 170.7 (q) | - |
| 3a | 127.9 (q) | - |  | 127.9 (q) | - |
| 4 | 116.5 (s) | 7.83 (1H, s) |  | 116.5 (s) | 7.83 (1H, s) |
| 4a | 137.2 (q) | - |  | 137.2 (q) | - |
| 5 | 123.7 (s) | 7.54 (1H, d, 8.5) |  | 123.7 (s) | 7.54 (1H, d, 8.5) |
| 6 | 126.7 (s) | 7.39 (1H, t, 8,5) |  | 126.7 (s) | 7.39 (1H, t, 8,5) |
| 7 | 106.3 (s) | 6.91 (1H, d, 7.5) |  | 106.3 (s) | 6.91 (1H, d, 7.5) |
| 8 | 156.6 (q) | - |  | 156.6 (q) | - |
| 8a | 117.5 (q) | - |  | 117.5 (q) | - |
| 9 | 149.2 (q) | - |  | 149.2 (q) | - |
| 9a | 125.9 (q) | - |  | 125.9 (q) | - |
| 1՛-OCH3 | 19.2 (t) | 1.72 (3H, d, 6) |  | 19.2 (t) | 1.72 (3H, d, 6) |
| 2՛-CH3 | 56.5 (t) | 4.09 (3H, s) |  | 56.5 (t) | 4.09 (3H, s) |
| 9-OH | - | 9.63 (1H, s) |  | - | - |
| 8-OH | - | - |  | - | 9.63 (1H, s) |



Fig. 3. IR spectra of Compound **1** (KBr).



Fig. 4. 1H-NMR spectra of Compound **1** (500 MHz, CDCl3).



Fig. 5. 13C-NMR and DEPT 135o spectra of Compound **1** (125 MHz, CDCl3).



Fig. 6. HMQC spectra of Compound **1**.



Fig. 7. HMBC spectra of Compound **1**.



Fig. 8. HMBC spectra of Compound **2**.



Fig. 9. (a) selected HMBC correlation of **1** (b) selected HMBC correlation of **2**

**Conclusions**

Naphthalene derivatives, namely Eleutherol (**1**) and Eleutherol C (**2**) were isolated from the bulbs of *E. bulbosa.* The cytotoxic activity was evaluated against the T47D breast cancer cell line *in vitro*, Compounds **1** and **2** showed weak and no cytotoxic activity with IC50 values of 117.15 and 80.21 µM, respectively compared with cisplatin 24.07 µM. Suggesting that the location of the hydroxyl group can increase cytotoxic activity.

**Acknowledgments**

We thank to the Tropical Pharmacy Research and Development Laboratory at Mulawarman University and the Department of Chemistry, Faculty of Science and Mathematics, Sultan Idris Education Universiti, Tg Malim 35900, Perak, Malaysia.

**Conflicts of Interest**

The authors have declared no conflict of interest.

**References**

1. Insanu, M.; Kusmardiyani, S.; Hartati, R. Recent studies on phytochemicals and pharmacological effects of *Eleutherine americana* Merr. Procedia Chem. 2014, 13, 221–228
2. Ieyama, T.; Gunawan-Puteri, M.D.; Kawabata, J. α-Glucosidase inhibitors from the bulb of *Eleutherine americana.* *Food Chem. 2011, 128, 308–311.*
3. Chen Z, Huang H, Wang C, Li Y, Ding J, Ushio S, Hiroshi N, Yoichi I. Hong-conin, a new naphthalene derivative from Hong-Cong the rhizome of *Eleutherine americana* Merr and Heyne (Iridaceae). *Chem Pharm Bull 1986; 34: 2743–2746*
4. Hara H, Maruyama N, Yamashita S, Hayashi Y, Lee KH, Bastow FK, Chirul, Marumoto N, Imakura Y. Elecanacin, a novel new naphthoquinone from the bulb of *Eleutherine americana. Chem Pharm Bull 1997; 45: 1714– 1716*
5. Paramita, S.; Nuryanto, M.K. Anti-inflammatory activity of Bawang Dayak (*Eleutherine bulbosa* (Mill. Urb.)) ethanol bulb extracts*. J. Vocation. Health Stud*. 2018, 2, 51–55.
6. Jiang, H.; Man, W.J.; Hou, A.J.; Yang, L.; Xing, X.D.; Yan, M.L.; Guo, X.Y.; Yang, L. The chemical constituents from the active fractions of *Eleutherine bulbosa* with their anti-microbial activity. Nat. *Prod. Res. 202*0, 34, 1743–1749.
7. Arung, E.T.; Kusuma, I.W.; Christy, E.O.; Shimizu, K.; Kondo, R. Evaluation of medicinal plants from Central Kalimantan for antimelanogenesis. *J. Nat. Med*. 2009, 63, 473–480.
8. Li, X., Ohtsuki, T., Koyano, T., Kowithayakorn, T., & Ishibashi, M. 2009. New Wnt/ beta-catenin signaling inhibitors isolated from *Eleutherine palmifolia*. Chemistry– An Asian Journal*, 4, 540–547*
9. Manjula, K. K.; Bindu, R.; Nair. Isolation of isoeleutherol from microwave assisted methanol extract of *eleutherine bulbosa* bulbs, its crystal structure elucidation and cytotoxicity evaluation. *Journal of molecular structure.* 2022, 1247, 131376.
10. Chen, D.L.; Hu, M.G.; Liu, Y.Y.; Li, R.T.; Yu, M.; Xu, X.D.; Ma, G.X. New naphthalene derivatives from the bulbs of *Eleutherine americana* with their protective effect on the injury of HUVECs. *Molecules 2018, 23, 2111.*
11. Almeida, M.; Salam, S.; Rahmadani, A.; Helmi.; Narsa, A. C.; Kusuma, S. A. F.; Sriwidodo. [The Potency of the Genus *Uncaria* from East Borneo for Herbal Medicine Purposes: A Mini review](https://jtpc.farmasi.unmul.ac.id/index.php/jtpc/article/view/457). *Journal of Tropical Pharmacy and Chemistry* 2022, 6, 167-176.
12. Supriatno; Nurlelasari; Herlina, T.; Harneti, D.; Maharani, R.; Hidayat, A.T.; Mayanti, T.; Supratman, U.; Azmi, M.N.; Shiono, Y. A. New Limonoid from Stem Bark of *Chisocheton pentandrus* (Meliaceae). Nat. Prod. Res. 2018, 32, 2610–2616.
13. Alves TMA, H. K. 2003. *Eelutherinone A Fungitoxic Naphtoquinone from Eleutherine Bulbosa (Iridiceae).* Memorias do Instituto Oswaldo Cruz.
14. Salam, S.; Harneti, D.; Maharani, R.; Safari, A.; Hidayat, A. T.; Lesmana, R,; Nafiah, M. A.; Supratman, U.; Prescott, T. A. K.; Shiono, Y. [Cytotoxic triterpenoids from *Chisocheton pentandrus*](https://www.sciencedirect.com/science/article/pii/S0031942221001084). Phytochemistry 2021. 187, 112759.
15. Chen, D.; Sun, Z.; Liu, Y.; Li, Z.; Liang, H.; Chen, L.; Xu, X.; Yang, J.; Ma, G.; Huo, X. 2020. Eleucanainones A and B: Two dimeric structures from the bulbs of Eleutherine americana with anti-MRSA activity. *Org. Lett. 22, 3449–3453.*
16. Ifesan BOT, Ibrahim D, Voravuthikunchai SP, 2010. Antimicrobial activity of crude ethanolic extract from *Eleutherine americana*, *Journal of Food, Agriculture & Environment*, 8(3&4), 1233-1236
17. Mutiah, R.; Listiyana, A.; Suryadinata, A.; Annisa, R.; Hakim, A.; Anggraini, W.; Susilowati, R. 2019. Activity of inhibit the cell cycle and induct apoptosis in HeLa cancer cell with combination of Sabrang onion (*Eleutherine palmifolia* (L.) Merr) and Starfruit Mistletoe (*Macrosolen cochinchinensis* (Lour.) Tiegh). J. Appl. *Pharm. Sci. 8, 122–128.*
18. Xu, J. Z.; Qiu, F.; Qu, G. X; Wang, N. L; Yao, X.s. 2006 Studies on the Antifungal Constituens Isolated from *Eleutherine americana. Chin. J. Med. Chem. 15, 157-161.*