= 2934 cm^{-1} CH aliphatic and ν = 1688 cm^{-1} for C=O. The EIMS showed $[M]^+$ peak at *m/z* 718, analyzing for $C_{43}H_{50}N_4O_6$. The *m/z* 700 showed a loss of H_2O followed by retro Diels-Alder reaction to produce *m/z* 562 $[C_{35}H_{36}N_3O_4]^+$ (Fig. 2).

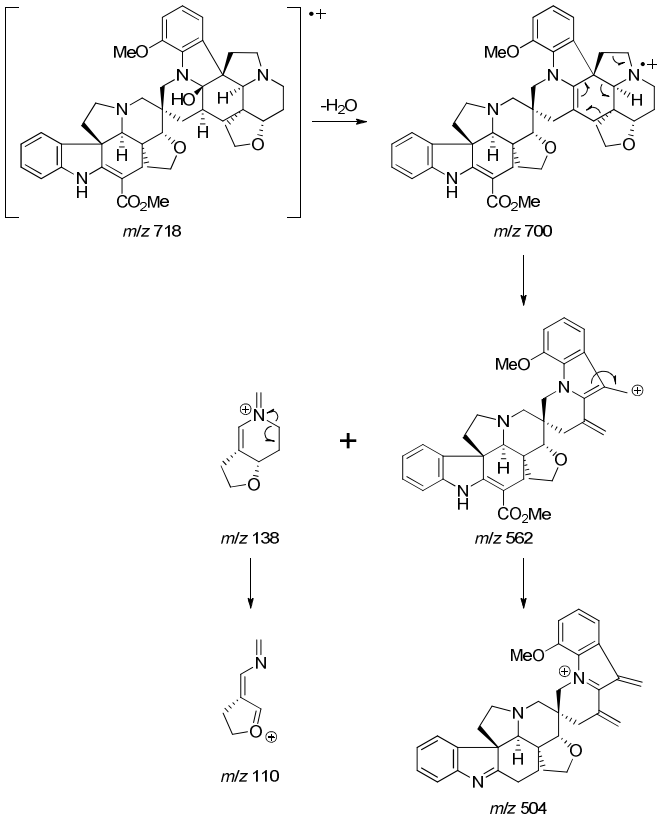
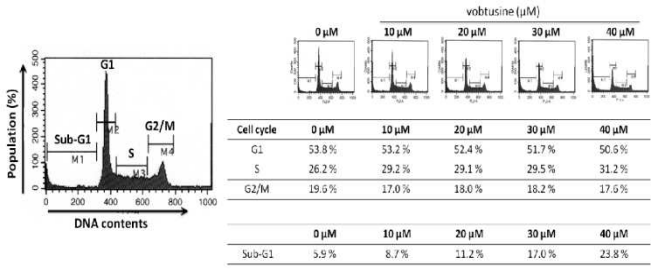


Fig. 2 Fragmentation of vobtusine (**1**)

The ^1H NMR spectrum in $CDCl_3$ indicated NH (δ_H 8.90) and multiplet at δ_H 6.6-7.3 ppm for 7 aromatic protons. The signal at δ_H 7.17 (1H, brd, 7.68 Hz) was assigned as H-11', and at δ_H 6.80 (brd, 7.68 Hz) was designated as H-9'. The signal at δ_H 6.88 (2H, brt) was assigned as H-10. The ^{13}C spectrum showed a similar chemical shift with vobtusine (**1**).

We evaluated for vobtusine cytotoxicity against HL-60 cell line. The cytotxic activity show that vobtusine have a moderate cytotoxic activity with IC_{50} 15.8 µM. The effect of vobtusine on the cell cycle was determined by the distribution of vobtusine-treated cells in the G_1, S, and G_2 / M phases detected by flow cytometry [21],[22]. The results of cell cycle analysis indicate the ratio of the number of cells in each cell cycle did not have significant differences depend on vobtusine concentration although cell number in G_1 phase have tendency to decrease according to the increasing of vobtusine (Fig. 3). Besides, the sub-G_1 phase population of cells treated with vobtusine was increased compared with that of cells without treatments. These results suggested that vobtusine induced apoptosis in HL-60 cells, which is initiated from G_1 phase.



Cell cycle	0 µM	10 µM	20 µM	30 µM	40 µM
G1	53.8 %	53.2 %	52.4 %	51.7 %	50.6 %
S	26.2 %	29.2 %	29.1 %	29.5 %	31.2 %
G2/M	19.6 %	17.0 %	18.0 %	18.2 %	17.6 %

	0 µM	10 µM	20 µM	30 µM	40 µM
Sub-G1	5.9 %	8.7 %	11.2 %	17.0 %	23.8 %

Fig. 3 The effects of vobtusine on the cell cycle of HL-60. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours, and added vobtusine/DMSO (concentrations of 40, 30, 20, and 10 µM). The negative control only added DMSO on HL-60 cells. Vobtusine is no concentration-dependent of G_1, S, and G_2/M phases whereas concentration-dependent of sub G_1 phase. The percentage of sub G_1 cells increased from 5.9% at 0 µM to 23.8% at 40 µM.

One of the apoptotic induction processes is DNA fragmentation in the nucleus, by examining DNA fragmentation from HL-60 cell lines treated with vobtusine. Showed that the occurrence of DNA fragmentation occurred at vobtusine concentrations of 20 µM or more. DNA fragmentation usually occurs during the final stages of the apoptotic process. These results indicate that vobtusine induces apoptosis, as evidenced by the presence of DNA fragmentation in HL-60 cell lines during treatment with vobtusine (Fig. 4).

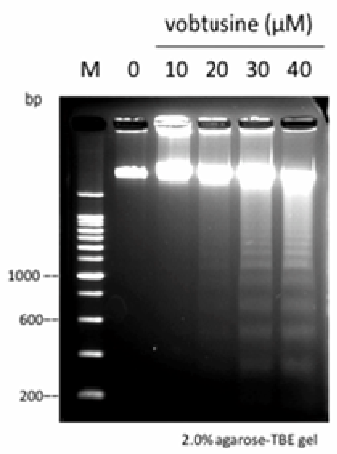


Fig. 4 Detection of apoptotic DNA ladder in vobtusine-treated HL-60 cells. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours, and added vobtusine/DMSO (concentrations of 40, 30, 20, and 10 µM) for 24 hours. Genomic DNA was extracted from HL-60 cells, which was separated by 2% agarose gel electrophoresis. Lane 1; marker size 200-bp, lane 2; DMSO as the negative control, lanes 3-6; vobtusine in various concentrations.

Apoptosis is mainly due to the continuous cell division and activation of the protein caspase, a cysteine protease directed by aspartate. Generally, caspase occurs in cells in the form of inactive pro-caspase, and when activated, it can trigger other pro-caspases, allowing the protease cascade's initiation. To confirm whether vobtusine can activate cascade of caspases that correlate with the enzymatic activity of caspase-3, -8, and -9, we investigated on HL-60 cells by observing at the expression of pro-caspase-3, -8, and -9 (Fig. 5). These detection by WB revealed that the three caspase precursors decreased depend on vobtusine concentration.

Furthermore, cleaved caspase-3 expression increased depend on vobtusine concentrations. These results indicate that the mechanism of action of vobtusine in inducing apoptosis by activating caspase-3.

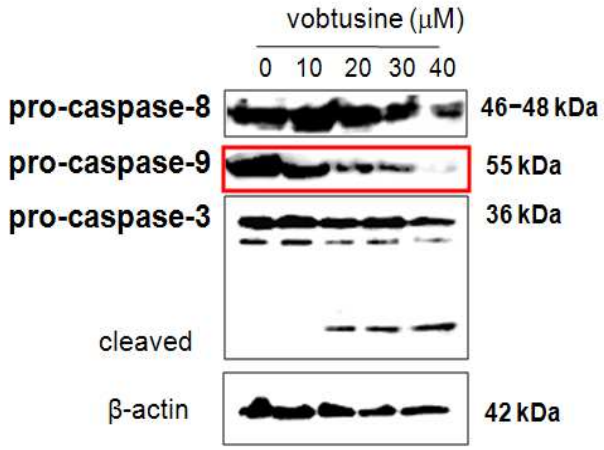


Fig. 5 Protein expression of pro-caspase-8, pro-caspase-9, and pro-caspase-3/caspase-3 in HL-60 cells treated with vobtusine at concentrations 0, 10, 20, 30, and 40 μM. HL-60 cells (1×10^5 cells/6 cm dish) incubated for 24 hours with vobtusine (10 - 40 μM). The resulting cell lysate was separated using SDS-PAGE (12.5%), and transferred to PVDF membrane. Dose-dependent decrease of pro-caspase-8, pro-caspase-9 and pro-caspase-3 was detected. Increase of cleaved caspase-3 were also detected in vobtusine-treated cell lysate (20-40 μM).

Vobtusine can induce apoptosis through caspase-3 activation (Fig. 6). When vobtusine combined with caspase-9 inhibitors, caspase-3 activation suppressed, this means that caspase-3 activity depends on caspase-9 activation. On the other hand, when vobtusine is combined with caspase-8 inhibitor slight decrease was observed. This shows that vobtusine activity in inducing apoptosis through caspase-3 activation is mainly depend on caspase-9 activation, not too dependent on caspase-8 activation. Because of the mechanism of vobtusine in inducing apoptosis through activation of caspase-9 without or a little activation of caspase-8, it suggested that the mechanism of induction apoptosis with vobtusine is through the intrinsic pathway and not through extrinsic pathway.

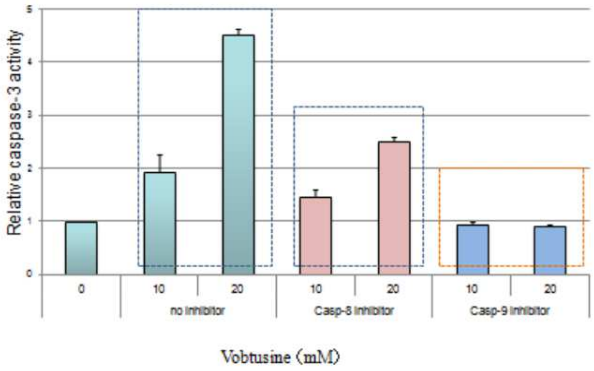


Fig 6 Effects of caspase-8 and caspase-9 inhibitor against caspase-3 activation. HL-60 cells (1×10^4 cells/well) in 96-well plate were incubated for 24 hours. Vobtusine (20 and 10 μM) dissolved in DMSO, respective 20 μM of caspase-8 inhibitor and caspase-9 inhibitor were co-added. Only DMSO was added to the negative control. The activation rate of caspase-3 was represented as the relative value as DMSO-treated sample is 1.0.

The mechanism of action of vobtusine in inducing apoptosis is through the intrinsic pathway involves the performance of the Bcl-2 family protein, namely pro-apoptotic proteins (Bid, Bax, Bad) and pro-survival proteins (Bcl-2, Mcl-1, Bcl-xL). To find out whether vobtusine works by increasing pro-apoptotic proteins expression or inhibiting pro-survival protein expression, an analysis of pro-apoptotic proteins expression and pro-survival protein was analyzed by western blotting. The results of this study indicated that the expression of Bid and Bcl-xL protein in vobtusine-treated HL-60 would reduce (Figure 5). Cleaved t-Bid increasing, is reported to activate Bax and Bak, is expected by the downregulation of Bid. Therefore, vobtusine is speculated to induce apoptosis via caspase-9 and regulated by upstreamed activities of Bcl-xL and Bid (Fig. 7).

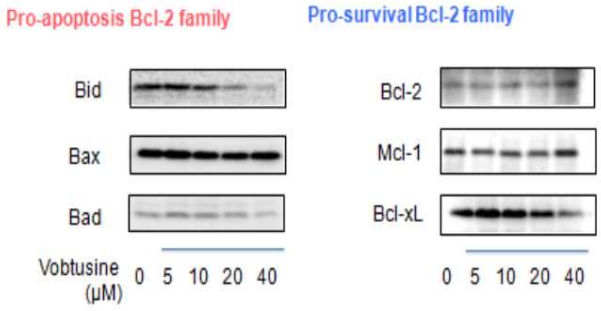


Fig. 7 Protein expression of Bcl-2 family in HL-60 cells treated with vobtusine. HL-60 cells (1×10^5 cells/6 cm dish) were incubated for 24 hours with vobtusine (5 - 40 μM). The resulting cell lysate was separated using SDS-PAGE (12.5%), and transferred to PVDF membrane. Dose dependent decrease of Bid and Bcl-xL were detected.

IV. Conclusions

The present study concluded that the mechanism action of vobtusine as anticancer drugs by inducing apoptotic cell death, through the mitochondrial pathway, including caspase-9, caspase-3, Bid activation, and Bcl-xL downregulation on HL-60 cell line.

Acknowledgment

This work was partially supported by Grand-AID for Scientific Research (C) Grant Number 19K07152, The Science Research Promotion Fund from The Promotion and Mutual Aid Corporation for Private Schools of Japan, Professor Grant Andalas University No. T/38/UN.16.17/PP.GK-KPR1GB/LPPM/2019 and Directorate General of Research Strengthening and Development, Ministry of Research, Technology and Higher Education for Doctoral Dissertation Research Grant with Contract Number 049/K10/ KM /contract-research / 2018.

References

[1] F. J. Lipp, L. M. Perry, and J. Metzger, "Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses.," *Brittonia*, 1981.

[2] H. Almehdar, H. M. Abdallah, A. M. M. Osman, and E. A. Abdel-Sattar, "In vitro cytotoxic screening of selected Saudi medicinal plants," *J. Nat. Med.*, 2012.

[3] B. S. Bhadane, M. P. Patil, V. L. Maheshwari, and R. H. Patil, "Ethnopharmacology, phytochemistry, and biotechnological advances of family Apocynaceae: A review," *Phytotherapy Research*. 2018.

[4] S. Hadi, B. Desy Ratnasari, M. Septiyana, S. Priyambodo, and I. M. Sudarma, "Antibacterial Assay and Alkaloid Lombine Distribution Study of Voacanga foetida (B.I)Rolfe from Lombok Island," *Orient. J. Chem.*, 2019.

[5] Y. Tang *et al.*, "Leucophyllinines A and B, bisindole alkaloids from Leuconotis eugeniifolia," *J. Nat. Med.*, vol. 73, no. 3, pp. 533–540, 2019.

[6] Prema *et al.*, "Three new quassinoids isolated from the wood of Picrasma javanica and their anti-Vpr activities," *J. Nat. Med.*, vol. 74, no. 3, pp. 571–578,2020.

[7] Y. Hirasawa *et al.*, "New vasorelaxant indole alkaloids, taberniacins A and B, from Tabernaemontana divaricata," *J. Nat. Med.*, vol. 73, no. 3, pp. 627–632, 2019.

[8] P. Amelia *et al.*, "Two new sarpagine-type indole alkaloids and antimalarial activity of 16-demethoxycarbonylvoacamine from Tabernaemontana macrocarpa Jack," *J. Nat. Med.*, vol. 73, no. 4, pp. 820–825, 2019.

[9] Dachriyanus, D. Arbain, D. P. Putra, M. V. Sargent, R. Susila, and F. S. Wahyuni, "Indole alkaloids from two species of Ophiorrhiza," *Aust. J. Chem.*, 2000.

[10] Dachriyanus, M. V. Sargent, and F. S. Wahyuni, "(+)-Isochimonanthine, a pyrrolidinoindole alkaloid from Argostemma yappii king," *Aust. J. Chem.*, vol. 53, no. 2, pp. 159–160, 2000.

[11] D. Arbain, A. L. T. Byrne, B. Dachriyanus, N. E. A, and M. V. S. B, "of Chemistry," *Aust. J. Chem.*, vol. 50, pp. 3–5, 1997.

[12] D. Arbain, L. T. Byrne, Dachriyanus, and M. V. Sargent, "Isomalindine-16-carboxylate, a zwitterionic alkaloid from Ophiorrhiza cf. communis," *Aust. J. Chem.*, 1997.

[13] A. Susanty, D. Dachriyanus, Y. Yanwirasti, F. S. Wahyuni, H. Fadhli, and P. A. Aswan, "Aktivitas Sitotoksik Ekstrak Etil Asetat Daun Tampa Badak (Voacanga foetida (Bl.)K.Schum) pada Kanker Kolon HTB-38," *JSFK (Jurnal Sains Farm. Klin.*, vol. 5, no. 2, pp. 142–146, 2018.

[14] A. Burlacu, "Regulation of apoptosis by Bcl-2 family proteins," *Journal of Cellular and Molecular Medicine*. 2003.

[15] R. Jan and G. e. S. Chaudhry, "Understanding apoptosis and apoptotic pathways targeted cancer therapeutics," *Advanced Pharmaceutical Bulletin*. 2019.

[16] S. Elmore, "Apoptosis: A Review of Programmed Cell Death," *Toxicol. Pathol.*, vol. 35, no. 4, pp. 495–516, 2007.

[17] P. Majtnerová and T. Roušar, "An overview of apoptosis assays detecting DNA fragmentation," *Mol. Biol. Rep.*, vol. 45, no. 5, pp. 1469–1478, 2018.

[18] D. Daddiouaissa, A. Amid, N. A. Kabbashi, F. A. A. Fuad, A. A. M. Elnour, and M. A. K. M. S. Epandy, "Antiproliferative activity of ionic liquid-graviola fruit extract against human breast cancer (MCF-7) cell lines using flow cytometry techniques," *J. Ethnopharmacol.*, vol. 236, no. December 2018, pp. 466–473, 2019.

[19] R. Singh, A. Letai, and K. Sarosiek, "Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins," *Nat. Rev. Mol. Cell Biol.*, vol. 20, no. 3, pp. 175–193, 2019.

[20] Z. Fu *et al.*, "Euphorbia lunulata extract acts on multidrug resistant gastric cancer cells to inhibit cell proliferation, migration and invasion, arrest cell cycle progression, and induce apoptosis," *J. Ethnopharmacol.*, 2018.

[21] K. Zaima *et al.*, "Biscarpamontamines A and B, an aspidosperma-iboga bisindole alkaloid and an aspidosperma-aspidosperma bisindole alkaloid, from Tabernaemontana sphaerocarpa," *J. Nat. Prod.*, vol. 72, no. 9, pp. 1686–1690, 2009.

[22] T. Kaneda, M. Matsumoto, Y. Sotozono, S. Fukami, and A. Eko, "Cycloartane triterpenoid (23 R , 24 E)- 23 - acetoxymangiferonic acid inhibited proliferation and migration in B16 - F10 melanoma via MITF downregulation caused by inhibition of both β - catenin and c - Raf – MEK1 – ERK signaling axis," *J. Nat. Med.*, vol. 74, no. 1, pp. 47–58, 2020.