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Isolation and Cytotoxic Activity Test of Alkaloids from Dichloromethane Fraction of Bark of Tampa Badak (*Voacanga foetida* (Blume) Rolfe) Against T47D Cell Line

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Abstract

Cancer is a problem in the health sector because this disease has an increasing incidence of morbidity and mortality. While the treatment still leaves some drug side effects, which cause discomfort for cancers sufferers. The arises because most cancer drugs work non-selectively. Because of some of the problems above, it is necessary to encourage the search for alternative drugs treatment of cancer. *Voacanga foetida* (Blume) Rolfe (Apocynaceae) is a species of plant from the genus Voacanga known as the tampa badak from West Sumatra. This plant is known to be source of indole alkaloids and also bis-indole alkaloids potential as cancer drugs. Therefore, in this study we are interested to explore the potency of this plant as natural product sources with potential cytotoxic activity. The study was started from the isolation process and continued by characterization of the isolated compounds and then testing their cytotoxic activity T47D breast cancer cell line. The isolation process of alkaloid compounds were performed by acid-base extraction, liquid-liquid fractionation, and followed by separation using column chromatography. Then,the potency of cytotoxic activity of the isolates were screened through Brine Shrimp Lethality Test (BSLT) method and evaluated by MTT assay. The structure of three isolated alkaloids (VFB-DB1.1; VFB-DB2.1; and VFB-DB2.2) were charachterized using spectroscopic analyses, including UV and FT-IR. One isolated compound (VFB-DB1.1) was also characterized using ^{1}H NMR spectroscopy. Based on the similarity of ^{1}H NMR spectra of isolated compound when compared with the literature, compound VFB-DB1.1 was identified as voacangine. Then, the BSLT and MTT assay result showed that VFB-DB1.1 exhibited LC_{50} value of 15.8 µg/mL againt *Artemia salina* L.and IC_{50} value of 8.9 µg/mL against T47D breast cancer cells, respectively.

Keywords: voacangine, *V. foetida*; cytotoxic, MTT *Assay*

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1 Introduction

Alkaloid is one of secondary metabolites with biological activity and medicinal uses related to the cytotoxic activity [1]. Estimated more than 2000 compounds classified as alkaloids [2]. Two bis-indole alkaloids from *Voacanga foetida* (Blume) Rolfe have been reported to have cytotoxic activity against HL60 [3] and A549 cell lines [4]. Alkaloid compounds as anticancer agents work by inducing apoptosis, inhibiting metastasis, inhibiting the cell cycle, inducing autophagy, inhibiting angiogenesis, inducing necrosis, activating mitochondrial pathway, and playing a role in tumor development [5].

Voacanga foetida (Blume) Rolfe (Apocynaceae) is a species of plant from the genus Voacanga known as the tampa badak from West Sumatra. The genus is known to be the source of indole alkaloid as well as bis-indole alkaloid (dimeric alkaloid). Some isolated compounds from this plant such as voacristine, voacangine, lombine, and coronaridine were reported by Hadi to have antibacterial activity [6]. Our recent works also reported another alkaloids, i.e vobtusine and vobtusine lactone,two aspidosperma bis-indole alkaloids with potential cytotoxic activity [3], [4].

Ethanol extract of *V. foetida* leaves has been reported to exhibit *in vivo*analgesic activity [7], whereas the ethyl acetate extract of this plant leaves exhibited strong cytotoxic activity against HTB-38 colon cancer cells [8] and T47D breast cancer cells [9]. In addition, *n*-hexane, ethyl acetate, *n*-butanol fractions and several isolated compounds from *V. foetida* leaves were reported to have good cytotoxic activity against L1210 leukemia cell lines [10]. Then, one isolated compound from *n*-butanol fraction of *V. foetida* leaves also exhibited anti-proliferation activities against several cell lines including leukemia, lung, and cervical cancers [11].

Alkaloid can be isolated using acid-base extraction method [12]. The alkaloid from *V. foetida* leaves have been widely reported. However, the alkaloid content form the bark of this plant has not been explored. In this work, we are interested to isolate and explore the potency of cytotoxic activity of the isolated alkaloid against T47D breast cancer cell line

2 Experimental section

2.1 Apparatuses and Materials

The apparatuses used include distillation apparatus (Duran 50), rotary evaporator (Rotavapor R-210 Buchi), analytical balance (Shimadzu AUW-220 and Metler Toledo), TLC plate GF254 (Merck Millipore), UV lamp 254 nm and 366 nm (Camag®), Column Chromatography (Pyrex®), UV-Vis Spectrophotometer (Shimadzu®UV-1900i), IR Spectrophotometer (Shimadzu, IR Spirit, A224158), Spectrometer ^{1}H NMR (Bruker 400 MHz) melting point measuring device (Stuart SMP-30), a set of shrimp egg breeding equipment of Artemia salina Leach (dark container, aeration, lamp with light intensity

15W), flask culture (diameter 25 cm^2), 96 well-plate, laminar airflow, CO_2 incubator (HERA cell 150, Thermo electron corporation), Biological Safety Cabinet Class II Type A/B3 (Sanyo), Microplate Spectrometer (BIO-RAD), UV Ultra spec 2100 pro and NMR spectrometer (Brucker AV-400).

The materials used include bark of the plant *V. foetida*, silica gel 60 (40-63 m) (Merck), shrimp larvae of *Artemia salina* L. (Golden West), breast cancer cells T47D (Laboratory of Parasitology, Faculty of Medicine, University of Gajah Mada), RPMI 1640, penicillin and streptomycin were purchased form Technologies (Paisley, UK), trypsin EDTA was purchased form GIBCO (Auckland, New Zealand), fetal bovine serum was purchased form PAA Laboratories (Linz, Austria),propidium iodide (PI), phosphate buffered saline (PBS), and ribonuclease A (RNase A)were purchased form Sigma Chemicals (St. Louis, USA), dimethylsulfoxide (DMSO) was purchased form Merck (Hohenbrunn, Germany), and 3(4,5-dimethylthiazol-2-yl) diphenyltetrazolium bromide (MTT) was purchased form Phytotechnology Laboratories (Kansas, USA).

2.2 Sample Processing

The bark of *V. foetida* (12.5 kg) were dried for 15 days and then grinded to obtain dry bark powder (5.2 Kg).

2.3 Alkaloid Extraction and Fractionation

Dry powder (5.2 kg) was macerated with methanol (3×15 L) for five days each, and then filtered to obtain the macerate. The macerate was concentrated using vacuum rotary evaporator to obtain concentrated extract (1.15 L). The extract was acidified using H_2SO_4 2N to afford pH ± 1, then partitioned using dichloromethane (3×650 mL) to obtain the acidic dichloromethane fraction (VFB-DA, 15.5 g) and water fraction. The water fraction was added with natrium bicarbonate to afford pH ± 9 and then partitioned with dichloromethane (3×650 mL) to obtain basic dichloromethane fraction (VFB-DB, 5.6 g).

2.4 Alkaloid Phytochemical Screening

Each of fraction (0.5 g) puts into a test tube and then dissolved with chloroform (5 mL)and added withammonia chloroform 0.05 M (1:1). The mixture was stirred and then filtered into a new test tube. As much as1 mL of sulfuric acid 2N was added into filtrate, shaken for 1 minute, and allowed to form two layers. The acid layer (top) transfer to a new test tube and added by 1-2 drops of Mayer's reagent. The white precipitate indicates a positive alkaloid.

2.5 Alkaloid Separation and Purification

The basic dichloromethane fraction (VFB-DB, 4.5 g) was separated through column chromatography (d = 5 cm) using silica gel 60 GF_{254} as stationary phase and was eluted using a mixture of *n*-hexane and ethyl acetate (9:1) to methanol 100% using step gradient polarity (SGP) methodEluate in vial 1-33 was labelled as sub-fraction VFB-DB1 (1.1 g) and eluate in vial 34-94 was labelled as sub-fraction VFB-DB2 (0.6 g).The sub-fraction VFB-DB1 was purified by column chromatography (d = 2 cm) using silica gel 60 GF_{254} as stationary phase and was eluted with a mixture of *n*-hexane and ethyl acetate (9:1, 5:5) to ethyl acetate 100%. The isolate VFB-DB1.1 (65 mg) was obtained from eluate in vial 1-2. The sub-fraction VFB-DB2 was purified by column chromatography using a mixture of *n*-hexane and ethyl acetate (5:5) as mobile phase. The isolate VFB-DB2.1 (43 mg) was obtained from eluate in vial 1-37 and isolate VFB-DB2.2 (34 mg) was obtained from eluate in vial 38-77, after re-crystalization.

2.6 Characterization of Isolated Compounds

Each of isolated compound was analyzed by TLC using a mixture of organic solvent as mobile phase, the spots were observed under UV lamp and sprayed using Dragendorff's reagent, and the R_f values were determined. Characteristics of isolated compounds was dertermined through organoleptic examination, melting point determination, and spectroscopic analyses, including UV and IR. For isolate VFB-DB1.1, the measurement of ^{1}H NMR spectra was also performed.

2.7 Brine Shrimp Lethality Test

As much as 1 gram of larvae of *A. salina* hatched with 2 L of seawater in a container equipped with an aerator and lighting using a 15 watt incandescent lamp for 48 hours. Each compound was dissolved in chloroform and then was diluted to afford the concentrations of 100, 10, and 1 μg/mL in calibrated vials (5 mL). Each of concentration was made in triplicate and then the solvent was epavorated. In each vial containingthe compound, as much as 50 μL of DMSO was added, then about 2 mL of seawater and 10 shrimp larvae were added, and then the seawater was added again to reach the calibration limit (5 mL). After 24 hours, the observation was performed for counting the number of dead larvae. The number of dead larvae was compared to the total number of larvae to calculate the percentage of mortality using the table of probit value. Then, the IC_{50} was calculated.

2.8 MTT Assay

The T47D breast cancer cell line was seeded into 96-well microtiter plates at 10^4 cells per well. Cells were pre-incubated for 24 hours at 37 °C in an incubator with 5% CO_2. Various compounds (10 μL) were added to the cell cultures then incubated at 37 °C for 48 hours. On the third day, 15 μL MTT solution (5 mμg/mL) each cultured medium. After 2 hours of incubation, 100 μL of 10% SDS dissolved in 0.04 mol/L of HCl solution was added into each well to lyse the cells and solubilize the formazan crystals. The micropipette reader (Bio-Rad) is used to measure the optical density. We do three replicates of wells prepared for each sample. The difference in the absorbance between samples and controls to determine the living cells ratio, and then the IC_{50} value was calculated to determine the category of cytotoxicity of the tested compound

3 Results and Discussion

In this study, we succeeded in isolating three pure isolates labeled as VFB-DB1.1 (65 mg), VFB-DB2.1 (43 mg), and VFB-DB2.2 (34 mg), as depicted in Figure 1. All three isolates were characterized by TLC analysis using three types of mobile phases and all three TLC chromatograms showed single spots under UV 254 and 366 nm. The results of spraying the three TLC chromatograms with Dragendorf reagent also produced an orange color indicating that the three isolates were alkaloids.

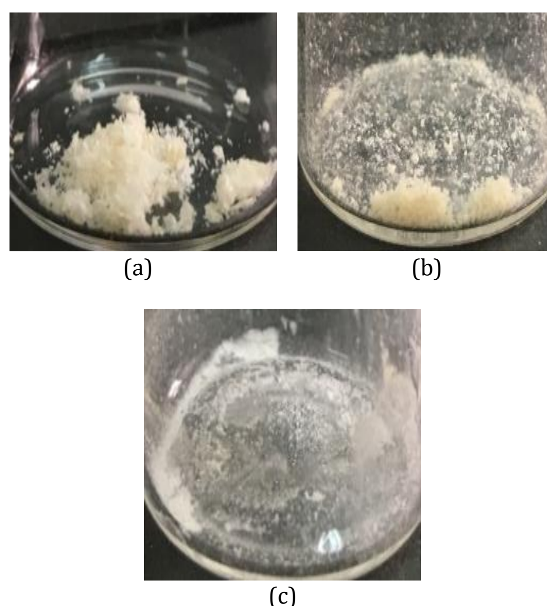

Figure 1. Three isolated compounds, (a) VFB-DB1.1, (b) VFB-DB2.1, and (c) VFB-DB2.2.

All three compounds have been characterized through UV and FT-IR spectroscopic analyses. However, only isolate VFB-DB1.1 continued to ^1H NMR analysis because it was obtained in the most amount compared to the other two isolates. Therefore, in this study we were only able to identify the structure of VFB-DB1.1 based on a comparison with the literature [13]. Meanwhile, the structure of the other two isolates has not been elucidated yet. However, in this study we have also carried out the primary screening for the three isolates by Brine Shrimp Lethalty Test (BSLT) and followed by a cytotoxicity test against T47D breast cancer cells.

Isolate VFB-DB1.1 was obtained in yellowish white crystal with melting point of 130-132 °C. The result of UV spectra measurement at concentration of 10 ppm showed that it absorbed UV light with maxima at 217 (ε=8.044), 240 (ε=14.280), and 284 (ε=10.184).

Then, the IR spectrum measurement was performed to ensure the presence of N-H group and the other functional group that characteristic for alkaloid. Based on the IR spectroscopy VFB-DB1.1, The broad absorption band with a spike at 3438 cm^{-1} indicated the presence of NH group. The absorption band at 1708 cm^{-1} and 1171 cm^{-1} indicated the presence of C=O and C-O of ester, respectively. In addition, the aromatic C=C and aliphatic C-H also appeared in the IR spectrum, as presented in table 1.

Table 1. Interpretation of the IR Spectrum of VFB-DB1.1

Wave Number (cm^{-1})	Type of Bond Vibration
3438	NH
2935	C-H aliphatic (asymmetric)
2860	C-H aliphatic (symmetric)
1708	C=O ester
1621	C=C alkene
1593	C=C aromatic
1224	C-N
1171	C-O

Based on the presence of the N-H group in the IR spectra, it can be predicted that the isolate VFB-DB1.1 is alkaloid compound. Then, the ^1H NMR spectra measurement was performed to ensure the prediction.The ^1H NMR spectrum of isolate VFB-DB1.1 in table 2 showed the typical proton signals for the indole alkaloid and its similar to the reported ^1H NMR spectrum of voacangine (13).

Table 2. Interpretation of ^1H NMR Spectrum of VFB-DB1.1

Proton	δ_H (ppm) of VFB-DB1.1[a]	δ_H (ppm) of Voacangine (13)[b]
NH	7.80 (bs, 1H)	7.67 (bs, 1H)
9	6.93 (*d*, 1H, *J* = 2.6 Hz)	6.92 (d, 1H, *J* = 2.4 Hz)
11	6.81 (*dd*, 1H, *J* = 8.6, 2.2 Hz)	6.80 (dd, 1H, *J* = 8.7, 2.4 Hz)
12	7.15 (*d*, 1H, *J* = 8.7 Hz)	7.13 (d, 1H, *J* = 8.7 Hz)
OCH_3	3.86 (*s*, 3H)	3.81 (s, 3H)
CO_2CH_3	3.72 (*s*, 3H)	3.71 (s, 3H)
3, 5, 6, 14, 15, 17, 18, 19, 20	3.57–0.89	3.54-0.90

[a] measured in $CDCl_3$ at 400 MHz
[b] measured in $CDCl_3$ at 300 MHz

Based on the ^1H NMR spectrum, a singlet signal at 7.80 ppm showed the presence of a proton bound to nitrogen atom in a heterocyclic indole moiety. The presence of this indole moiety of voacangine is strengthened by the appearance of three signals at 6.93; 6.81; and 7.15 ppm, where the signals are generated by protons H9, H11, and H12, respectively, characteristic of the substituted indole moiety of voacangine. The J_1 value (8.6 Hz) of the H11 signal is generated by the correlation between H11 and H12 which is in ortho position. Whereas the J_2 value (2.2 Hz) of the H11 signal is generated by the correlation between H11 and H9 which is in meta position. Furthermore, the two singlet signals at 3.86 and 3.72 ppm were indicated the presence of two methoxy groups, aromatic methoxy (Ph-OCH_3) and carboxymethoxy (-$COOCH_3$), respectively, as depicted in Figure 1. In addition, the signals at the range of 3.57 – 0.89 ppm were signals from the aliphatic protons 3, 5, 6, 14, 15, 17, 18, 19, and 20. These signals are very similar with previously reported literature that reported that the signals were appeared around 3.54 – 0.90 ppm [13].

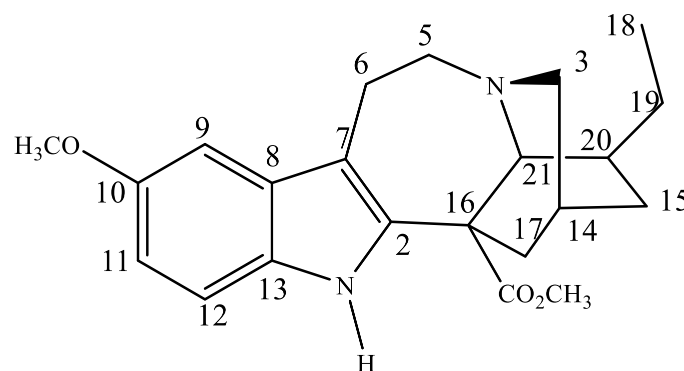

Figure 2. Structure of Isolate VFB-DB1.1 (Voacangine)

Isolate VFB-DB2.1 was obtained in yellowish crystal with melting point of 166-168 °C. The result of UV spectra measurement at concentration of 10 ppm showed that it absorbed UV light with maxima at 240 (ε=14.280). The IR spectrum measurement was also performed to to provide an overview of the structural characteristic of the isolate VFB-DB2.1. The functional groups or bond types were presented in table 3.

Table 3. Interpretation of IR spektrum of VFB-DB2.1

Wave Number (cm^{-1})	Type of Bond Vibration of VFB-DB2.1
3358	N-H
3302	N-H
3057	C-H aromatic
2945	C-H aliphatic
1712	C=O ester
1635	C=C alkena
1452	C=C aromatic
1233	C-N
1049	C-O

Isolate VFB-DB2.2 was obtained in yellowish crystal with melting point of 288-290 °C. The result of UV spectra measurement at concentration of 10 ppm showed that it absorbed UV light with maxima at 325 (ε=7.159). The IR spectrum measurement was also performed to to provide an overview of the structural characteristic of the isolate VFB-DB2.2. The functional groups or bond types were presented in table 4. Based on the IR spectra, this isolate showed slightly difference in structural characteristic when compared to other isolates. There is no N-H absorption band that indicates the presence of N-H bond around the wavelength of 3500-3000 cm^{-1}, it is suspected that this isolate is alkaloid with tertiary amine group.

Table 4. Interpretation of IR Spectrum of VFB-DB2.2

Wave Number (cm^{-1})	Type of Bond Vibrations of VFB-DB2.2
2934	C-H alifatis (asimetris)
2806	C-H alifatis (simetris)
1783	C=O ester
1664, 1603	C=C alkena
1461	C=C aromatis
1251	C-N
1100	C-O

The three isolates VFB-DB1.1, VFB-DB2.1, and VFB-DB2.2, and the fraction VFB-DB were tested for their cytotoxic activities using the BSLT method. The cytotoxic activity test using the BSLT method based on the toxic effect of the tested compounds on A. salina larvae by observing their immobilization in a vial containing the concentration of the tested compound solution. This immobilization is considered as death for larvae.

Based on the result of BSLT as presented in table 5, it can be observed that the higher the concentration of alkaloid solution, the greater the percentage of mortality of shrimp larvae produced. This is because the development of larvae is more sensitive to exposure to toxic compounds. Cytotoxic alkaloid compounds enter the body larvae orally and dermally which are then distributed throughout the larval body tissues and attack the cells, causing changes in concentrations inside and outside the cells. Functional and metabolic of larvae cells damaged. The effects occur quickly within 24 hours, 50% of larva deaths [14].

Table 5. The BSLT result

Samples	LC_{50} (μg/mL)
Fraction VFB-DB1	12.2
Isolate VFB-DB1.1	15.6
Isolate VFB-DB2.1	47.2
Isolate VFB-DB2.2	66.6

Based on probit analysis, the LC_{50} values of the fraction VFB-DB, isolates VFB-DB1.1, VFB-DB2.1, and VFB-DB2.2 are 12.2; 15.6; 47.2 and 66.6 μg/mL, respectively. Based on the results, the LC_{50} values of fraction and all three isolates are less than 200 μg/mL and can be categorized as very toxic according to Anderson [14] Furthermore, we test the cytotoxic activity using the MTT Assay method to determine the viability profile of breast cancer cells and determine the strength of the cytotoxic activity of the test compound through the IC_{50} value.

The activities cytotoxic with MTT assay method we found that the IC_{50} of the fraction VFB-DB, isolates VFB-DB 1.1, VFB-DB 2.1 and VFB-DB 2.2 were a dose-dependent manner, where it showed the IC_{50} values of 17.4; 8.9; 15.3 and 3.5 μg/mL respectively, as depicted in Figure 3.

Cytotoxic alkaloid abilities were as inhibitors of tubulin pathway; polymerization of microtubule proteins, the formation of the mitotic spindle, and the cell cycle at metaphase. Because they cannot perform cell division, the cell will undergo apoptosis. With the occurrence of apoptotic processes and inhibition of cell growth, the fraction and all isolate compounds potential as anticancer.

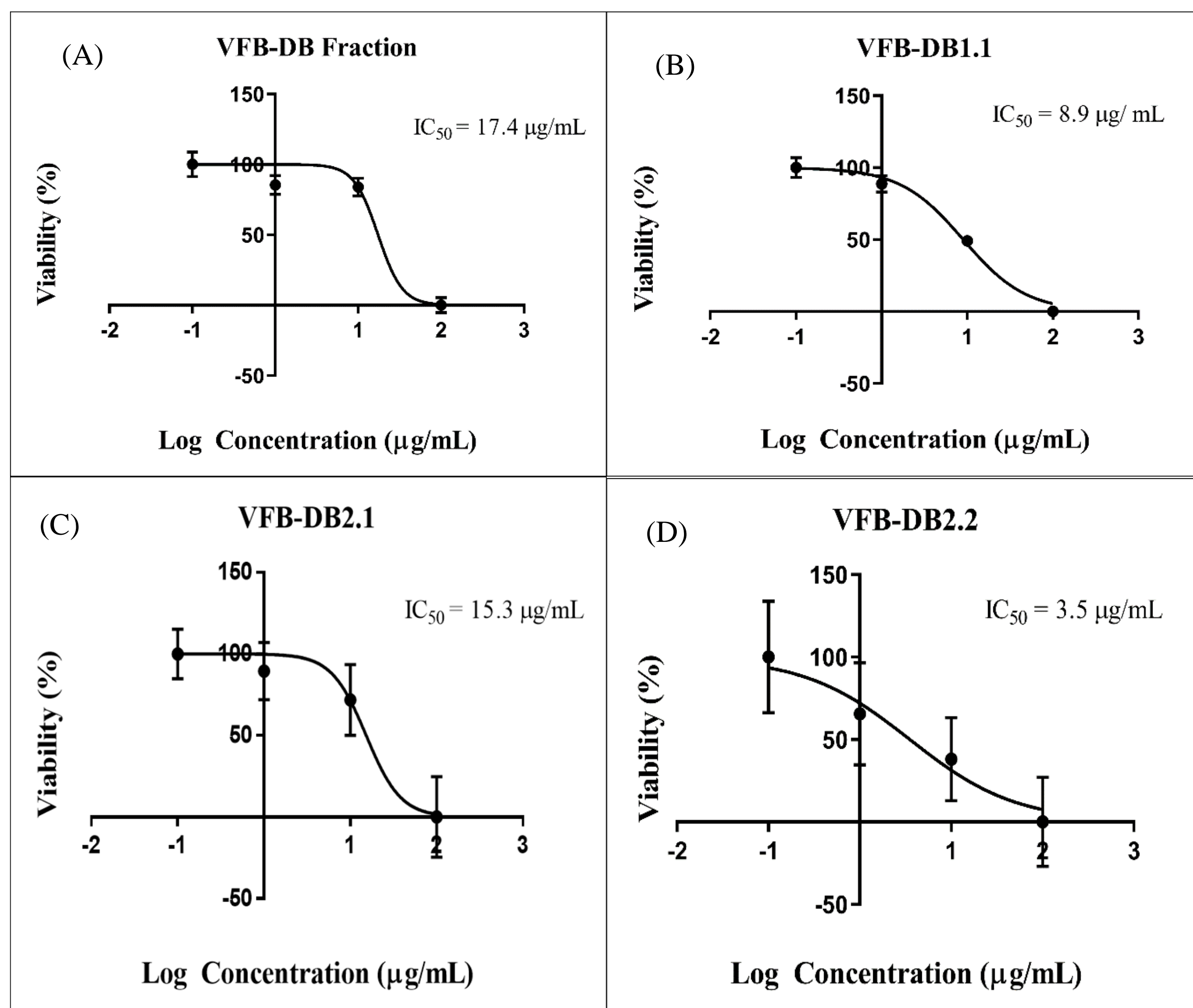

Figure 3. Viability Percentage and IC_{50} Value of (A) VFB-DB Fraction, (B) VFB-DB 1.1, (C) VFB-DB 2.1 and (D) VFB-DB 2.2

Compounds with cytotoxic activity are associated with anticancer because these compounds can kill cells whose growth and development are fast and uncontrolled, such as the growth and development of cancer cells. Compounds with cytotoxic activity can kill cells, including cancer cells [12]. Alkaloids have the main activity as cytotoxic, mutagenic, and carcinogenic [15]. The alkaloid mechanism acts as an anticancer is by inducing apoptosis through the activation of caspases that play an important role in programmed cell death. In addition, alkaloids inhibit the cell division cycle, especially in the G1, G2, and M phases involving cyc-A and cyc-B so that cancer cell division stops. Alkaloids also inhibit metastatic cancer cells and enable autophagy that leads to apoptosis [5].

The isolate VBF-DB 1.1 (voacangine) can kill T47D breast cancer cells in the cytotoxic pathway with an IC_{50} of 8.9 µg/mL, supported by the results of other researcher reported that vocangine triggered apoptosis associated with caspase-3 activation, Bax upregulation, and Bcl-2 suppression. Voacangine induces G2/M cell cycle arrest. In addition, the anticancer mechanism of action of voacangine on oral cancer cells is through the inhibition of the PI3K/AKT signaling cascade [16].

Based on this research, estimate the mechanism of action of the isolate VBF-DB1.1 (voacangine) on T47D breast cancer cells inhibits the cell cycle pathway, induction the apoptotic, and the PI3K/AKT pathway. Another compound isolated from the stem bark of *V. foetida* is vobtusine has an anticancer

mechanism of action inhibiting the cell cycle in the sub G1 phase and inducing apoptosis through internal pathways through caspase-3 activation and increasing BID expression, and decreasing Bcl-xL expression [3].

4 Conclusions

The isolation process from dichloromethane fraction of the stem bark of *V. foetida* resulted in three alkaloid isolates, namely VFB-DB 1.1 (65 mg), VFB-DB 2.1 (43 mg), and VFB-DB 2.2 (34 mg) with very high cytotoxic activity against T47D breast cancer cells.

5 Declarations

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5.2 Author Contributions

The names of the authors listed in this journal contributed to this research.

5.3 Funding Statement

This research was not supported by any funding sources.

5.4 Conflicts of Interest

The authors declare no conflict of interest.

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