BIODIVERSITAS
Volume 23, Number 1, January 2022
Pages: 572-580

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230162

Cytotoxicity extract and fraction of knobweed (*Hyptis capitata*) and its effect on migration and apoptosis of T47D cells

NELSIANI TO'BUNGAN[1,4], RARASTOETI PRATIWI[2], SITARINA WIDYARINI[3], LAURENTIUS HARTANTO NUGROHO[2♥]

[1]Doctoral Program in Tropical Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia
[2]Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia. Tel.: +62-271-580839, Fax.: +62-271-6492355, ♥email: hartantonugroho2005@ugm.ac.id
[3]Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Jl. Fauna 2, Karangmalang, Sleman 55281, Yogyakarta, Indonesia
[4]Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta. Jl. Babarsari, Sleman 55281, Yogyakarta, Indonesia

Manuscript received: 13 October 2021. Revision accepted: 31 December 2021.

Abstract. *To'bungan N, Pratiwi R, Widyarini S, Nugroho LH. 2021. Cytotoxicity extract and fraction of knobweed (*Hyptis capitata*) and its effect on migration and apoptosis of T47D cells. Biodiversitas 23: 572-580. Hyptis capitata* Jacq. has an ethnobotanical history. The leaves and stems of *H. capitata* have been reported to have potential anticancer properties. This study was conducted to determine the anticancer potential of the roots, stems, leaves, and flowers of *H. capitata*. Organs of *H. capitata* were extracted in stages using chloroform and continued with methanol to obtain eight extracts. Crude extracts were tested for cytotoxicity on T47D and WiDr cells with the MTT method. Potential inhibition of cell migration by potential fraction was tested with scratch wound healing assay and the content of secondary metabolites was analyzed with gas chromatography-mass spectrometry (GC-MS). An Annexin V-Pl apoptosis test was performed to determine the number of living cells, necrosis, and apoptosis. The results showed that the root chloroform extract showed the highest cytotoxic activity against T47D cells (34.90 ± 4.78µg/mL) and WiDr (44.65 ± 12.07 µg/mL), and it was referred to as a potential extract. Fractionation of the root chloroform extract resulted in four fractions (F1, F2, F3, and F4). Fraction F2 had the highest cytotoxic activity (13.8 ± 0.65 µg/mL) and was selective against T47D cells (selectivity index 3.71). Fraction F2 showed its antimetastatic and apoptotic potential against T47D cells. Gas chromatography-mass spectrometry analyses combined with library showed that the root chloroform extract and fraction F2 contain ferruginol, campesterol, and stigmasterol.

Keywords: Apoptosis, cytotoxicity, knobweed, metastasis, T47D

INTRODUCTION

The number of cancer deaths reached 9.8 million in 2018 (Anonymous$_a$ 2009; Bray et al. 2018). Breast cancer is the most common type of cancer in women. There are 2.1 million women affected every year and at the same time, it is the biggest cause of cancer death in women (Anonymous$_b$ 2018). Subsequently, colorectal cancer ranks as the second most common type of cancer in women (Sudarmawan et al. 2010) and the third most common type of cancer in men after lung and prostate cancer (Anonymous$_a$ 2009; Bray et al. 2018).

Chemotherapy is one of the therapies used in the treatment of cancer. However, difficulties in choosing the dose, lack of drug specificity, fast metabolism, drug resistance, and side effects resulted in the success of chemotherapy being much limited (Nurgali et al. 2018). It becomes a challenge to obtain a new healing method that has high potency and specificity with low side effects at an affordable cost.

In Indonesia, plant usage as traditional medicine is one of the important ways to improve public health (Nurrani 2013). The role of plant bioactive compounds that have pharmacological activity has received much attention as new treatment agents because of their strong pharmacological activity, low toxicity, and affordability (Mondal et al. 2014). *Hyptis capitata* Jacq. (Figure 1) is one of the plants that have anticancer potential. Yamagishi et al. (1988) reported that *H. capitata*, which grows in Taiwan, contains triterpene acids, two of which are A-hyptatic acid and 2α-hydroxyursolic acid. A-hyptatic acid and 2α-hydroxyursolic acid showed high cytotoxic activity against HCT-8 colon cancer cells, with ED_{50} values of 4.2 µg/mL and 2.7 µg/mL, respectively. Haryanti and Widiastuti (2017) also reported that leaf infusion of *H. capitata* has a moderate level of cytotoxicity against MCF-7 cells with an IC_{50} value of 248.6 µg/mL.

In this study, *H. capitata* was extracted by graded maceration using chloroform and methanol as solvents. The extract obtained was then used for a cytotoxicity test with the MTT method on T47D and WiDr cells to determine the potential extract. The potential extract was tested for cytotoxicity on Vero cells as non-target cells. The potential extract was fractionated. The obtained fraction was tested for cytotoxicity on T47D cells and Vero cells to determine the potential fraction. Potential fractions were tested for antimetastatic activity with the scratch wound healing test and tested for apoptosis activity with Anexin V-PI Flowcytometry. The content of secondary metabolites in the extract and potential fractions were analyzed by gas chromatography-mass spectrometry analysis.

Figure 1. *Hyptis capitata* Jacq. A. Aerial Part, B. Flower, and C. Leaf (Setyawati et al. 2015)

MATERIALS AND METHODS

The material used in this research was *H. capitata*, which was taken from Bungapati village, Tana Lili subdistrict, North Luwu district, South Sulawesi, Indonesia (2°34'36.3"S 120°36'03.6"E). *H. capitata* was identified based on the study of Setyawati et al. (2015). All parts of *H. capitata* were separated. Roots, stems, leaves, and flowers were observed and characterized. The roots were brown, the stems were juicy green when young and became hard-brown when old. Green leaves were ovate to elliptical-ovate in shape. The flowers were dense globular heads, green in color, and turned brown as they matured. *H. capitata* has been determined manually by the specialist in the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia with certificate number 014535/S.Tb/III/2019. Herbarium of *H. capitata* was stored in the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada. Extraction and fractionation were conducted at the Biochemistry Laboratory, Faculty of Biology, Universitas Gadjah Mada. Meanwhile, screening cytotoxicity, cell migration, and apoptosis assay were conducted at Parasitology Laboratory and Clinical Pathology Laboratory Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada This research was approved by the Medical and Health Researches Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, with certificate number KE/FK/0303/EC/2020.

Extraction of *Hyptis capitata*

Roots, stems, leaves, and flowers of *H. capitata* were separated from each other and washed with clean water, drained, covered with a black cloth, and then dried under the sun to dry (stable dry weight). Roots, stems, leaves, and dried flowers were ground separately using a grinder and then filtered using a 40-mesh sieve. The powder obtained was extracted by graded maceration, starting with chloroform solvent and then followed by methanol. The ratio of simplicia and solvent is 1:5 (w/v).

Cytotoxicity test

The cytotoxicity test was performed on T47D, WiDr, and Vero cells as nontarget cells. Cells were cultured using 96 well plates with a density of 10^4 cells/well for T47D and WiDr cells, in an RPMI complete culture medium (Gibco, Canada). Vero cells were cultured at a density of 12×10^3 cells/well in a DMEM complete culture medium (Gibco, Canada). Each well is filled with 100 uL. Cells were incubated in a CO_2 incubator at 36°C, 5% of CO_2 for 24 hours. Cells that showed 70%–80% confluency were then treated. The sample extract/fraction (10 mg) was weighed and then dissolved in 100 uL of DMSO (Merck, Germany). The samples were made in series with concentrations of 200 µg/mL, 100µg/mL, 50 µg/mL, 25 µg/mL, and 12.5 µg/mL. Each concentration was made with three replications (Triplo). The treated cells were incubated in a CO_2 incubator for 24 hours. The condition of the cells was observed, and then MTT (Bio Basic, Canada) (5 mg/mL PBS) was added in a ratio of 1:9, 1 ml MTT plus 9 mL of complete medium. Cells were incubated for 4 hours in a CO_2 incubator. Then the Stop reagent (SDS) (Merck, Germany) was added. The well-plate was wrapped in aluminum foil and then incubated overnight. The absorbance was measured with a microplate reader machine at a wavelength of 595 nm. The data obtained were analyzed by log concentration regression analysis to determine the concentration that inhibits cell growth by 50 % (IC_{50}) (Anonymous$_c$ 2009; Mahardika et al. 2016).

Fractionation with Vacuum Liquid Chromatography (VLC)

The stationary phase used for VLC contained 12 g of silica gel 60 GF254 (Merck, Germany). A total of 2.5 g of the chloroform extract of the roots of *H. capitata* was mixed with 5 g of silica gel 60 GF254 until the solution becomes homogeneous. The mobile phase has eight compositions, namely a mixture of n-hexane:chloroform 3:1 (v/v), 2:2 (v/v), 1:3 (v/v), 100% chloroform, chloroform:methanol 3:1 (v /v), 2:2 (v/v),1:3 (v/v) and methanol 100%. The eluent obtained was accommodated in an Erlenmeyer flask followed by thin-layer chromatography (Dewatisari et al. 2021).

Thin-Layer Chromatography (TLC)

Thin-layer chromatography was performed with silica gel F254 (Merck, Germany) as the stationary phase. The mobile phase used was n-hexane:ethyl acetate 7:3 (v/v). Chromatograms were detected using ultraviolet light at wavelengths of 254 and 366 nm. The same pattern in the result of TLC analysis, combined into one fraction.

Scratch wound healing assay

T47D cells were cultured using 24 well-plates (density 125×10^3 cells/well), with a volume of 500 uL/well in RPMI complete culture medium. Cells were incubated at 36°C, 5% of CO_2 for 24 hours. Cells were observed under a microscope, when the cells reached 80% confluency. The complete RPMI culture medium was then replaced with RPMI starvation medium. Cells were incubated again for 24 hours at 36°C, 5% of CO_2. 10 mg of fraction F2 was

weighed and then mixed with 100 uL DMSO. Next, a series of concentrations of 0.4, 0.2, and 0.1 µg/mL were made in an RPMI starvation medium. Cells were scratched using a Yellow-tip. Cells were washed with 500 uL PBS (Invitrogen, Camarillo) to remove loose cells. Cells were treated with each variation of the concentration of fraction F2 and there was also control of T47D cells. The volume of starvation medium added after treatment was 1000 uL. Cells were observed and documented as 0-48 hour observations. Then the cells were incubated at 36°C, 5% of CO_2. Cells were observed and documented at 24 and 48 hours. The data obtained were analyzed with ImageJ (Anonymous$_c$ 2009).

Apoptosis Test with the annexin V-PI method

T47D cells were harvested and distributed at a density of 5×10^5 cells/well in a complete culture medium of 2000 µL/well on a 6-well-plate. Cells were incubated in an incubator at 36°C with 5% of CO_2 flow for 24 hours. The medium on the microplate was discarded and the cells were washed with PBS (Invitrogen, Camarillo). The test sample was added as much as ½ IC_{50} dan IC_{50} in 2000 µL of RPMI complete culture medium. The microplate was incubated at 36°C with 5% of CO_2 flow for 24 hours. After 24 hours, the state of the cells was documented. Then the cells were harvested. The medium from each well was accommodated into a conical tube. Cells were washed with 1000 µL PBS/well; PBS was collected into conical tubes beforehand. In each well, 150 µL of trypsin-EDTA 0.25% (Gibco, Canada) was added and incubated for 3 minutes in an incubator at 36°C. The complete culture medium (1000 µL) was added to each well and cells were resuspended. The culture medium was taken from each well and accommodated in each conical tube and centrifuged at 2000 rpm for 3 minutes. The supernatant was discarded and the conical tube was wrapped with aluminum foil. A total of 100 µL of Annexin V-PI reagent and 350 µL of buffer were added to each conical tube. It was then incubated at room temperature and in the dark for 10 minutes. The cell suspension was transferred to a flow cyto-tube for injection into the FACS-Calibur flow cytometer. Flow cytometry was performed with a light beam of 488 nm at a medium speed of 500 cells/second. The test results were analyzed descriptively (Nuriliani et al. 2013).

Identification of secondary metabolites by Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry analysis was carried out in The Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada Yogyakarta (capillary column using HP-5MS UI). The carrier gas was helium. The injector temperature programmed was 260°C. Initial conditions with the splitless model were as follows: split flow 50 ml/minute, with front inlet flow 1.00 ml/minute. Ion source temperature 200°C and mass spectrometry transfer line temperature 250°C. Purge flow 3 ml/minute, gas saver flow 5 mL/minutes, and gas saver time 5 minutes. The sample was dissolved in pure chloroform and injected using the split technique. The peaks formed were analyzed descriptively.

Data analysis

The results obtained were analyzed qualitatively and quantitatively. The data were processed using SPSS 26 and analyzed using one-way analysis of variance at a confidence level of 95% and the significance value is $P < 0.05$.

RESULTS AND DISCUSSION

Cytotoxicity extract and fraction of *Hyptis capitata*

The first stage of the in vitro cytotoxicity test was carried out in chloroform and methanol extracts of the roots, stems, leaves, and flowers of *H. capitata* against T47D and WiDr cells. Based on the IC_{50} values in Table 1, it is known that the chloroform extract of *H. capitata* root showed a high level of cytotoxicity against T47D cells. The root chloroform extract is therefore called the potential extract. The potential extract was an extract that is continued for the fractionation stage.

The *H. capitata* root chloroform extract, which was a potential extract, was taken for the fractionation stage. Based on the visualization of the fractions using thin-layer chromatography in Figure 2, it can be seen that the fractions are grouped based on similar profiles. From the eight fractions obtained, fractions with similar profiles were combined to obtain four fractions. The four fractions were continued for the cytotoxicity test on T47D cells. The results of the cytotoxicity test of fractions 1-4 against T47D cells (Table 1) showed that fraction 2 (F2) was had a high level of cytotoxicity against T47D cells. This fraction was then referred to as the potential fraction.

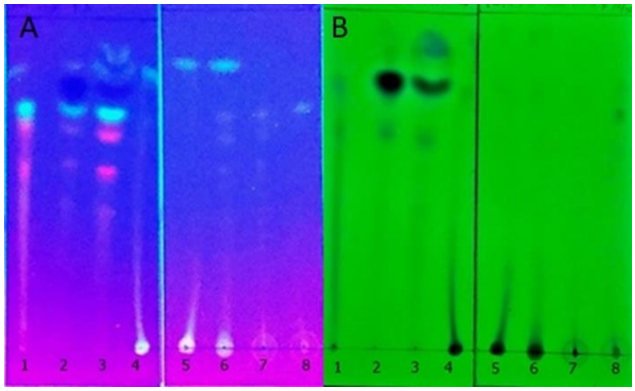

Figure 2. Thin-Layer Chromatography Fraction of *Hyptis capitata*. A. Visualization with UV 365nm; B. Visualization with UV 254 nm. 1 = n-hexane:chloroform fraction 3:1 (v/v); 2 = n-hexane:chloroform fraction 2:2 (v/v); 3 = n-hexane:chloroform fraction 1:3 v/v; 4 = 100% chloroform fraction; 5 = chloroform: methanol fraction 3:1 v/v; 6 = chloroform:methanol fraction 2:2 v/v; 7 =chloroform:methanolfraction 1:3 v/v; 8 = 100% methanol fraction. Based on the visualization, the fractions were grouped into four, namely fraction 1 separately, then referred to as the first fraction (F1); fractions 2 and 3 were combined and were referred to as second fractions (F2); fraction 4-6 were combined and were referred to as the third fraction (F3) and fractions 7 and 8 were combined and were referred to as the fourth fraction (F4)

Table 1. Cytotoxicity of extracts and fraction of *Hyptis capitata* against T47D, WiDr, and vero cells

Treatment	IC50 Average±SD (µg/mL)			Selectivity index
	T47D	WiDr	Vero	
Extract *H. capitata*				
Root chloroform extract	34.90 ± 4.78^{b}	44.65 ± 12.07^{b}	42.72 ± 7.51^{b}	1.22
Stem chloroform extract	260.84 ± 9.58^{c}	>1000	-	-
Leaf chloroform extract	199.48 ± 17.10^{b}	268.17 ± 29.31^{c}	-	-
Flower chloroform extract	>1000	>1000	-	-
Root methanol extract	99.27 ± 2^{b}	41.70 ± 2.19^{b}	-	-
Stem methanol extract	251.87 ± 50.04^{c}	>1000	-	-
Leaf methanol extract	>1000	>1000	-	-
Flower methanol extract	441 ± 151.77^{c}	>1000	-	-
Fraction *H. capitata*				
F1	41.31 ± 0.82^{b}		-	-
F2	13.8 ± 0.65^{a}		51.26 ± 5.56^{b}	3.71
F3	49.24 ± 1.1^{b}		-	-
F4	>1000		-	-
Control				
Doxorubicin	0.6 ± 0.23^{a}	11.74 ± 3.01^{a}	-	-
DMSO	>1000	>1000	-	-

Note: The IC_{50} value is represented by mean ± standard error of three replications. Different superscript alphabetic letters were significantly different at $p < 0.05$ by the Tukey test. Cytotoxicity categories based on the US National Cancer Institute (Hameed 2012; Srisawat et al. 2013): very toxic $IC_{50} \leq 20$ µg/mL, moderate/toxic IC_{50} 21–200 µg/mL, weak IC_{50} 201–500 µg/mL, and non-toxic $IC_{50} \geq$ 500 µg/mL. High selectivity if the selectivity index > 3.

Selectivity index

The selectivity index was obtained by comparing the IC_{50} value of the extract and potential fraction (T47D cells) to the IC_{50} value of non-target cells (Vero cells). From Table 1, it can be seen that the selectivity index of the root chloroform extract was low, while the selectivity index of fraction F2 was high.

T47D cell migration

Fraction F2 treatment of T47D cells showed an inhibitory effect on cell migration. The greatest inhibition of cell migration was at a concentration of 0.4 µg/mL (Table 2). The gap closure area in each treatment and control of T47D cells at 24 and 48 hours of observations can be seen in Figure 2.

Apoptosis T47D

The effect of fraction F2 treatment on T47D cell apoptosis based on Annexin V flow cytometry analysis can be seen in Figure 3. The treatment of ½ IC_{50} and IC_{50} fractions affected the percentage of apoptotic cells (Table 3).

Analysis of Annexin V-PI in T47D cells after 24 hours of incubation of fraction F2 ½ IC_{50}; IC_{50} and Doxorubicin ½ IC_{50} treatments, namely 6.9 g/mL, 13.8 g/mL, and 0.3µg/mL. The diagram can be divided into four regions defined as follows: percentage of necrotic cells (Q1); percentage of late apoptotic cells (Q2); percentage of living cells (Q3); and percentage of early apoptotic cells (Q4). The percentage of cell conditions in the control cells were Q1: 1.3%; Q2: 3.3%; Q3: 93.3%; and Q4:4.1%.

Table 2. Percentage Gap Closure of Fraction F2 treatment

Treatment	Gap Closure (%) ± SD	
	24 Hours	48 Hours
F2 0.1 µg/mL	3.7 ± 0.77^{a}	29.17 ± 5.3^{b}
F2 0.2 µg/mL	13.42 ± 3.9^{a}	24.24 ± 9.13^{b}
F2 0.4 µg/mL	4.42 ± 3.39^{a}	9.54 ± 5.18^{a}
Control	14.96 ± 7.19^{a}	59.34 ± 11.93^{b}

Note: The closing percentage value is represented by the mean ± standard error of three replications. Different superscript alphabetic letters were significantly different at $p < 0.05$ by the Tukey test

Table 3. Percentage of T47D cell condition after exposure fraction F2 and doxorubicin by annexin V-PI flowcytometry analysis

Treatment	1/2 IC_{50} (%)				IC_{50} (%)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
F2	8.4	27.4	46.3	17.9	3.9	33.2	12.7	50.2
Doxorubicin	13.4	27.8	43	15.8	N.D	N.D	N.D	N.D

Note: N.D. = not determined

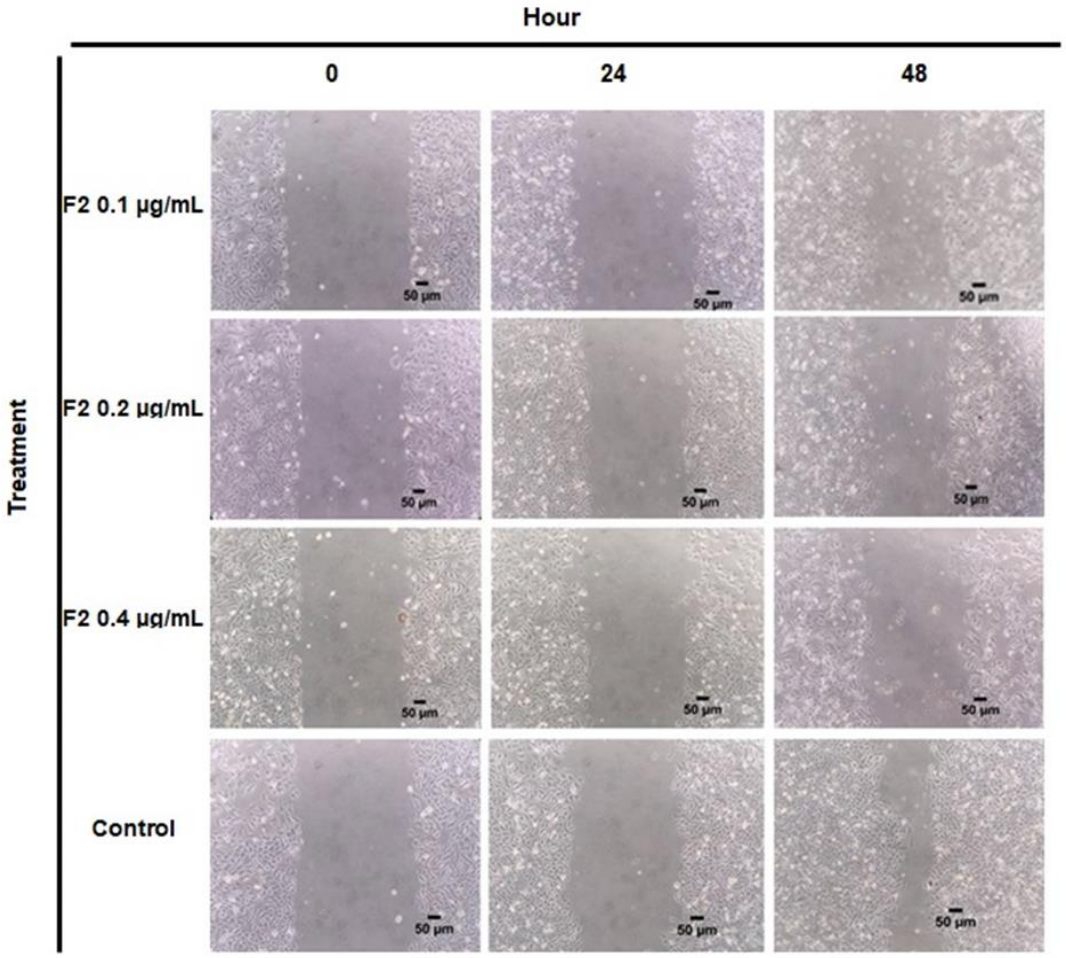

Figure 3. Effect of Fraction F2 on Cell Migration Morphology of Treated T47D cells using an Inverted Microscope (100×). The appearance of etched T47D cells at 0, 24, and 48 hours after fraction F2 treatment and cell control. Fraction F2 demonstrated an inhibitory effect on gap closure

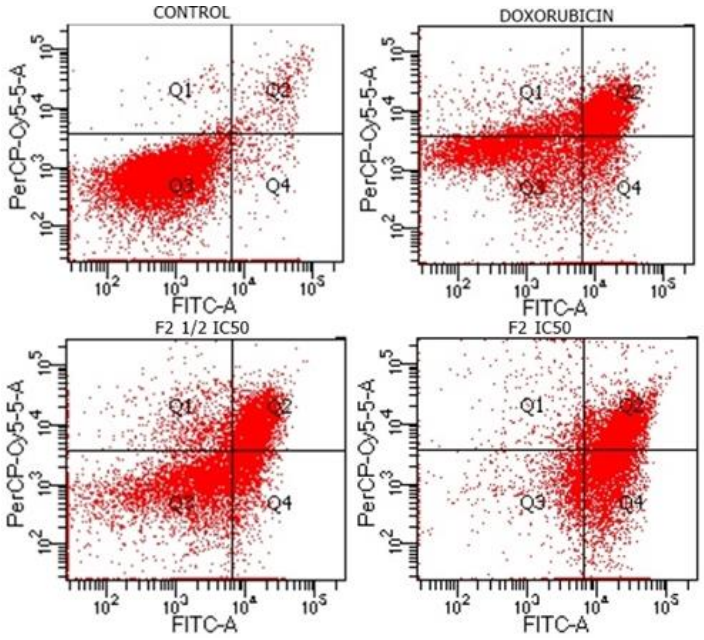

Figure 4. Detection of early apoptosis and late apoptosis by annexin V-PI flowcytometry

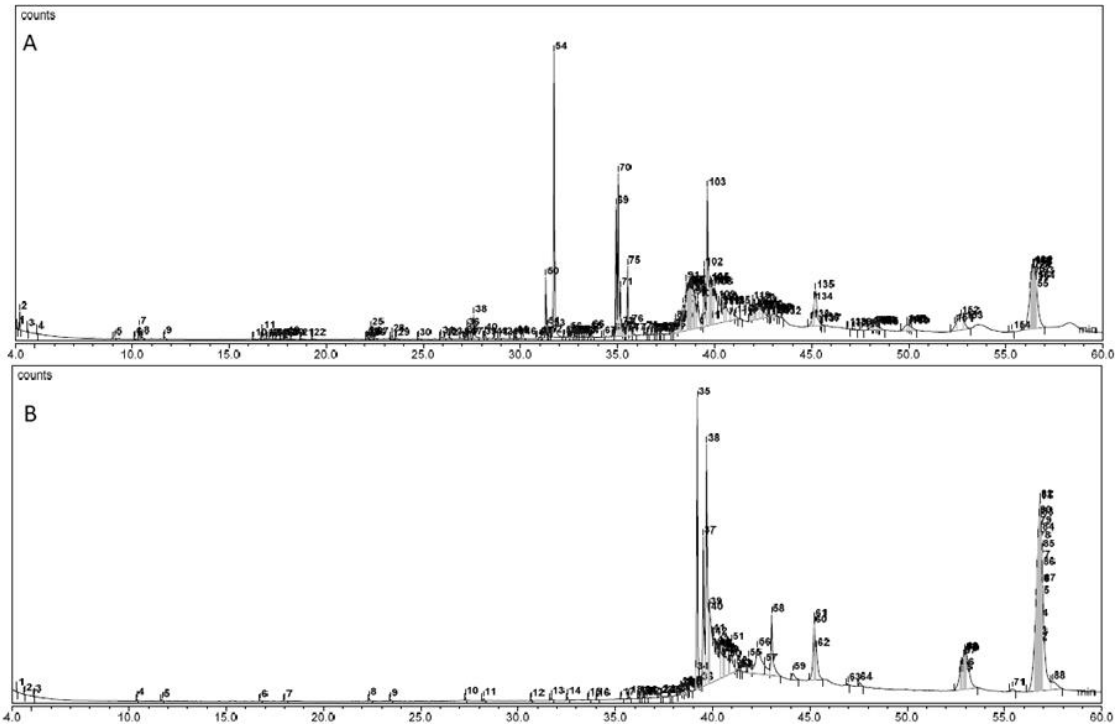

Figure 5. Chromatogram of bioactive components from the root chloroform extract and fraction F2. (A) Chromatogram of bioactive components of the *Hyptis capitata* root chloroform extract; 164 peaks were detected. (B) Chromatogram of the bioactive component fraction F2; 88 peaks were detected

Table 4. Bioactive compounds of the root chloroform extract and fraction F2

No.	RT	%Area	Formula	Compounds
Root chloroform extract				
	10.37	0.27	C11H24	Undecane
	11.67	0.06	C10H22	Octane, 3,5-dimethyl-
	16.72	0.29	C15H32	Dodecane, 2,7,10-trimethyl-
	22.3	0.27	C21H44	Heptadecane, 2,6,10,15-tetramethyl-
	29.53	0.05	C20H40O2	Ethanol, 2-(9-octadecenyloxy)-, (Z)-
	29.79	0.05	C18H54O9Si9	Cyclononasiloxane, octadecamethyl-
	31.08	0.03	C23H38O2	6,9,12,15-Docosatetraenoic acid, methyl ester
	31.31	1.83	C17H32O2	9-Hexadecenoic acid, methyl ester,
	31.5	0.07	C16H30O2	9-Hexadecenoic acid
	31.65	0.2	C17H36	Tetradecane, 2,6,10-trimethyl-
	31.73	8.17	C17H34O2	Hexadecanoic acid, methyl ester
	34.92	3.67	C19H34O2	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
	35.04	4.9	C19H36O2	trans-13-Octadecenoic acid, methyl ester
	35.5	0.25	C25H42O2	Cyclopropanebutanoic acid
	35.51	2.06	C19H38O2	Methyl stearate
	36.79	0.04	C26H44O5	Ethyl iso-allocholate
	37.96	0.55	C20H30O2	cis-5,8,11,14,17-Eicosapentaenoic acid
	38.69	2.07	C29H50O	γ-sitosterol
	39.14	1.84	C20H30O	Ferruginol
	39.46	2.23	C21H30O2	Retinoic acid
	39.78	1.14	C21H32O2	Pregn-5-en-20-one, 3-hydroxy-, (3ß,17a)-
	49.92	0.3	C30H30O	9,19-Cyclolanostane-3,7-diol
	52.67	2.26	C28H48O	Campesterol
	56.42	1.28	C29H48O	Stigmasterol
F2				
	22.35	0.09	C17H36	Tetradecane, 2,6,10-trimethyl-
	39.13	0.26	C20H30O2	Podocarpa-8,11,13-triene-7ß,13-diol, 14-isopropyl-
	39.21	7.52	C20H30O	Ferruginol
	39.52	4.56	C21H30O2	Dronabinol
	39.81	1.64	C21H32O2	Pregn-5-en-20-one, 3-hydroxy-, (3ß,17a)-
	52.98	0.62	C28H48O	Campesterol
	56.86	2.89	C29H48O	Stigmasterol

Identification of bioactive components

The chromatogram of the root chloroform extract and fraction F2 bioactive components can be seen in Figure 4. The bioactive components of the root chloroform extract and F2 based on GC-MS analysis are presented in Table 4. In the root chloroform extract, there are 23 bioactive compounds that have a similarity index above 750, while in F2, there are seven compounds (Table 4). Bioactive compounds found in both are ferruginol, campesterol, and stigmasterol.

Discussion

From Table 1, it is known that the root chloroform extracts of *H. capitata* was cytotoxic to T47D and WiDr cells. The level of cytotoxicity of the root chloroform extract was moderate. However, the stem, leaf, and flower extracts have not shown a promising level of cytotoxicity as anticancer candidates. Some plants, one of which is *H. capitata*, contain secondary metabolites that have the potential to be developed as anticancer substances (Yamagishi et al. 1988; Haryanti and Widiastuti 2017). The IC_{50} value based on *in vitro* cytotoxicity tests is a parameter of a material that has the potential to be developed as an anticancer candidate or vice versa (Hazekawa et al. 2019). The difference in the level of cytotoxicity can be caused by differences in the content of secondary metabolites in each organ because some secondary metabolites are stored in special compartments, either in the form of specialized organs or cells (Pagare et al. 2015; Anggraito et al. 2018).

The results of the cytotoxicity test of four fractions (F1, F2, F3, and F4) showed that fraction F2 has the highest cytotoxicity level against T47D cells. Slightly different from the cytotoxicity level of the root chloroform extract, the level cytotoxicity of fraction F2 is classified as very toxic (Hameed 2012; Srisawat et al. 2013). The high-level cytotoxicity indicates an opportunity to be developed as an anticancer candidate (Ellithey and Ahmed 2018)

Comparing the IC_{50} values of the root chloroform extract and fraction F2, treatment in T47D and Vero cells was also carried out to determine the selectivity of the test compound. The selectivity index of the root chloroform extract was low at 1.22, while the fraction F2 selectivity index was 3.71, which was high. A selectivity index above 3 refers to high selectivity (Mahavorasirikul et al. 2010). So fraction F2 is selective against T47D. A high selectivity index is important because it shows that the test compound has the potential to kill cancer cells greater than normal cells (Meiyanto et al. 2010). A promising anticancer candidate is expected to have a minimal negative impact on normal cells but has a high ability to kill cancer cells (Morley et al. 2007). WiDr cells were no longer included in the selectivity test and further tests because based on the IC_{50} value, the root chloroform extract was more selective against T47D cells.

It is necessary to study the mechanism of action of an anticancer substance to determine the effect of the substance on the characteristics of cancer cells. Therefore, in this study, the effect of the test compound on cell migration and apoptosis was studied. Fraction F2 treatment on T47D cells showed inhibition of cell migration. The percentage of gap closure decreases with an increasing dose of fraction F2 administered. The smallest percentage of gap closure was in the fraction F2 treatment of 0.4 µg/mL, which was 9.54 ± 5.18%. Meanwhile, the percentage of gap closure in control cells was 59.34 ± 11.93% (Table 4). This result shows that fraction F2 has the potential as an inhibitor of cell migration or antimetastatic activity. Metastasis is one of the characteristics of cancer cells (Hanahan and Weinberg 2011). Therefore, it becomes one of the targeted factors in cancer therapy.

An apoptotic test of T47D cells using the Annexin V-PI method after exposure to fraction F2 ½ IC_{50} dan IC_{50} showed that cells undergoing apoptosis increased with the addition of fraction F2 dose. The percentage of apoptotic cells in the ½ IC_{50} treatment was 45.3%, and in the IC_{50} treatment, it reached 83.4%. The percentage of necrotic cells was less in the fraction F2 IC_{50} treatment. Thus, it can be said that fraction F2 treatment triggers apoptosis in T47D cells. The ability of fraction F2 to trigger apoptosis can suppress one of the characteristics of cancer cells' ability to avoid apoptosis (Hanahan and Weinberg 2011; Idikio 2011). Thus, the anticancer activity of fraction F2 was observed via its ability to induce apoptosis of T47D cells.

The results of the GC-MS analysis of the root chloroform extract and fraction F2 in Table 4 summarize the compounds identified in both, but only library matches with a direct matching factor (SI) above 750 were included. The anticancer effect of both is probably due to the presence of ferruginol, campesterol, and stigmasterol. Ferruginol belongs to the abietane diterpenoid group, characterized by cyclic structures, having the abietane (C20) carbon framework and an aromatic ring C (Gonzales, 2012). Meanwhile, campesterol and stigmasterol are phytosterol groups. Stigmasterol is characterized by a steroid skeleton with a hydroxyl group in position C-3, unsaturated bonds in positions 5-6 of the B ring, and positions 22-23 in the alkyl substituent (National Center for Biotechnology Information$_a$ 2021). Campesterol is a simple sterol and is characterized by a steroid skeleton with a hydroxyl group in C-3, saturated bonds throughout the sterol structure, exception of 5-6 double bond in the B ring (National Center for Biotechnology Information$_b$ 2021). If it is related to the visualization using thin-layer chromatography in Figure 2, there are six spots in F2, with racing factor (*Rf*) values from bottom to top being 0.42, 0.47, 0.63, 0.75, 0.8, and 0.86 respectively. An *Rf* value of 0.47 indicates the presence of terpenoid, while *Rf* values of 0.75, 0.8, and 0.86 indicate the presence of a steroid group in fraction F2 (Fasya et al. 2020; Husnah and Wijayanti 2019).

A study reported that ferruginol, which is an abietane diterpenoid, is one of the bioactive compounds of plants that can suppress the proliferation of human malignant melanoma cells SK-Mel-28 (Jia et al. 2019). Furthermore, ferruginol triggers phosphorylation of key proteins such as p38 and translocation of NF-B into the nucleus to execute apoptosis (Jia et al. 2019). Ferruginol also plays an important role to inhibit the growth of thyroid cancer cells

via induction of mitochondrial apoptosis (Luo et al. 2019). Xiong et al. (2017) investigated the effect of ferruginol on human ovarian cancer cells and stated that ferruginol showed anticancer effects by inducing apoptosis, inhibiting migration, and inducing the G2/M phase in OVCAR-3 cells. Costa-Lotufo et al. (2004) reported that Carnasol and 11,14-dihydroxy-8,11,13-abietatrien-7-one, which are abietane diterpenoids isolated from hexane roots extract of *Hyptis brevipes*, show high cytotoxicity against cancer cells HL-60, MCF-7, and HCT-8.

Campesterol and stigmasterol are phytosterols, which are common in the diet (Awad and Fink 2000). Kazłowska et al. (2013) stated that *Porphyra dentata* sterol fraction consisting of cholesterol, β-sitosterol, and campesterol can significantly induce apoptosis and inhibit 4T1 *in vitro* cell growth. In addition, intraperitoneal injection of this sterol fraction into 4T1 tumor cells implanted in BALC/c mice significantly inhibited tumor nodule growth and increased the mice survival rate. However, stigmasterol can suppress cell viability. *In vivo*, stigmasterol interferes with tumor angiogenesis and it also has anti-inflammatory properties (Ghosh et al. 2011; Kangsamaksin et al. 2017). In addition, stigmasterol can inhibit development and induce apoptosis in human ovarian cancer cells (OV90) (Bae et al. 2020).

Phytosterols are able to suppress reactive oxygen species production and trigger apoptosis (Wayengo et al. 2019). Phytosterols can also reduce the growth of positive and negative estrogen receptors. This has an impact on de novo activation of ceramide synthesis by triggering serine palmitoyl transferase activity and will impact on mediates anti-tumor activity (Ramprasath and Awad 2015; Rath et al. 2018). In addition, another important thing is the ability of phytosterols to stimulate sphingomyelin turnover. The presence of sphingomyelin on the surface membrane triggers the failure of cells to respond to growth-suppressing signals, cells become resistant, resulting in immune escape. Decreased sphingomyelin by phytosterols has an effect on suppressing tumorigenesis and metastasis (Tallima et al. 2021).

In conclusion, fraction F2 of the chloroform extract of *H. capitata* can be developed as a promising anticancer substance because it is selective to T47D cells and has the ability to induce apoptosis and antimigration activity. This fraction contains ferruginol, campesterol, and stigmasterol. These components play an important role as anticancer agents by inducing apoptosis and inhibiting cell migration.

ACKNOWLEDGEMENTS

We would like to thank Universitas Gadjah Mada, Yogyakarta, Indonesia for supporting this research through RTA grants 2021. The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

Anggraito YU, Susanti R, Iswari RS, Yuniastuti A, Lisdiana WH, Nugrahaningsih, Habibah NA, Bintari SH. 2018. Metabolit Sekunder dari Tanaman. [Thesis]. Fakultas Matematika dan Ilmu Pengetahuan Alam, Universitas Negeri Semarang, Semarang. [Indonesian]

Anonymous$_a$. 2009. Worldwide Cancer Data. American Institute of Cancer Research. https://www.wcrf.org/dietandcancer/worldwide-cancer-data/

Anonymous$_b$. 2018. Cancer. World Health Organization. www.int/news.room/fact-sheets/detail/cancer.

Anonymous$_c$. 2009. In Vitro Test Protocol. Cancer Chemoprevention Researches Center (CCRC) Universitas Gadjah Mada. http://ccrc.farmasi.ugm.ac.id/en/?page_id=240.

Awad AB, Fink CS. 2000. Phytosterols as anticancer dietary components: Evidence and mechanism of action. J Nutr 130 (9): 2127-2130. DOI: 10.1093/jn/130.9.2127.

Bae H, Song G, Lim W. 2020. Stigmasterol causes ovarian cancer cell apoptosis by inducing endoplasmic reticulum and mitochondrial dysfunction. Pharmaceutics 12 (6): 488. DOI: 10.3390/pharmaceutics12060488.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer J Clin 68 (6): 394-424. DOI: 10.3322/caac.21492.

Costa-Lotufo LV, Araújo ECC, Lima MAS, Moraes MEA, Pessoa C, Silviera ER, Moraes MO. 2004. Antiproliferative effects of abietane diterpenoids isolated from *Hyptis martiusii* Benth (Labiatae). Pharmazie 59 (1): 78-79.

Dewatisari WF, Nugroho LH, Retnaningrum E, Purwestri YA. 2021. The potency of Sansevieria trifasciata and S. Cylindrica leaves extracts as an antibacterial against pseudomonas aeruginosa. Biodiversitas 22 (1): 408-415. DOI: 10.13057/biodiv/d220150.

Ellithey MS, Ahmed HH. 2018. Bioactive marine-derived compounds as potential anticancer candidates. Asian J Pharm Clin Res 11 (11): 464-466. DOI: 10.22159/ajpcr.2018.v11i11.28954.

Fasya AG, Amalia S, Megawati DS, Salima F, Kusuma VA, Purwantoro B. 2020. Isolation, identification, and bioactivity of steroids isolates from *Hydrilla verticillata* petroleum ether fraction. IOP Conf Ser Earth Environ Sci 456 (1): 1-8. DOI: 10.1088/1755-1315/456/1/012009.

Ghosh T, Maity TK, Singh, J. 2011. Evaluation of antitumor activity of stigmasterol, a constituent isolated from B*acopa monnieri* Linn aerial parts against Ehrlich Ascites Carcinoma in mice. Orient Pharm Exp Med 11 (1): 41-49. DOI: 10.1007/s13596-011-0001-y.

Hameed E. 2012. Phytochemical studies and evaluation of antioxidant, anticancer and antimicrobial properties of *Conocarpus erectus* L. growing in Taif, Saudi Arabia. Eur J Med Plants 2 (2): 93-112. DOI: 10.9734/ejmp/2012/1040.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: The next generation. Cell 144 (5): 646-674. DOI: 10.1016/j.cell.2011.02.013.

Haryanti S, Widiastuti Y. 2017. Aktivitas sitotoksik pada Sel MCF-7 dari tumbuhan Indonesia untuk pengobatan tradisional kanker payudara. Media Litbangkes 27 (4): 247-254. DOI: 10.22435/mpk.v27i4.5010.247-254. [Indonesian]

Hazekawa M, Nishinakagawa T, Kawakubo-Yasukochi T, Nakashima M. 2019. Evaluation of IC_{50} levels immediately after treatment with anticancer reagents using a real-time cell monitoring device. Exp Ther Med 18 (4): 3197-3205. DOI: 10.3892/etm.2019.7876.

Husnah YA, Wijayanti N. 2019. Aktivitas fagositosis makrofag mencit (*Mus musculus* L.) yang diinduksi ekstrak daun jeruk purut (*Citrus hystrix* D . C .). Jurnal Kajian Ilmu Kesehatan dan Teknologi 1 (2): 26-33. [Indonesian]

Idikio HA. 2011. Human cancer classification: A systems biology-based model integrating morphology, cancer stem cells, proteomics, and genomics. J Cancer 2 (1): 107-115. DOI: 10.7150/jca.2.107.

Jia Y, Wu C, Zhang B, Zhang Y, Li J. 2019. Ferruginol induced apoptosis on SK-Mel-28 human malignant melanoma cells mediated through P-p38 and NF-κB. Hum Exp Toxicol 38 (2): 227-238. DOI: 10.1177/0960327118792050.

Kangsamaksin T, Chaithongyot S, Wootthichairangsan C, Hanchaina R, Tangshewinsirikul C, Svasti J. 2017. Lupeol and stigmasterol suppress tumor angiogenesis and inhibit cholangiocarcinoma growth in mice via downregulation of tumor necrosis factor-a. PLoS ONE 12 (12): e0189628. DOI: 10.1371/journal.pone.0189628.

Kazłowska K, Lin HTV, Chang SH, Tsai GJ. 2013. In vitro and in vivo anticancer effects of sterol fraction from red algae porphyra dentata. Evidence-Based Complement Altern Med 2013: 11-13. DOI: 10.1155/2013/493869.

Luo G, Zhou J, Li G, Hu N, Xia X, Zhou H. 2019. Retraction: Ferruginol diterpenoid selectively inhibits human thyroid cancer growth by inducing mitochondrial dependent apoptosis, endogenous reactive oxygen species (ros) production, mitochondrial membrane potential loss and suppression of Mitogen-Activa. Med Sci Monit 25: 2935-2942. DOI: 10.12659/MSM.932341.

Mahardika AB, Wahyuono S, Wahyuningsih MSH. 2016. Sitotoksisitas senyawa hasil isolasi daun *Tithonia diversifolia* (hemsley) a. gray) terhadap sel t47d, mcf7 dan evsa-t. Majalah Farmaseutika 12 (2): 401-410. [Indonesian]

Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. 2010. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. BMC Complement Altern Med 10: 4-11. DOI: 10.1186/1472-6882-10-55.

Meiyanto E, Susidarti RA, Jenie RI, Utomo RY, Novitasari D, Wulandari F, Kirihata M. 2010. Synthesis of new boron containing compound (CCB-2) based on curcumin structure and its cytotoxic effect against cancer cells. J Appl Pharm Sci 10 (2): 60-66. DOI: 10.7324/JAPS.2020.102010.

Mondal J, Panigrahi AK, Khuda-Bukhsh AR. 2014. Conventional chemotherapy: problems and scope for combined therapies with certain herbal products and dietary supplements. Austin J Mol Cell Biol 1 (1): 1-10.

Morley KL, Ferguson PJ, Koropatnick J. 2007. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. Cancer Lett 251 (1): 168-178. DOI: 10.1016/j.canlet.2006.11.016.

National Center for Biotechnology Information$_a$. 2021. PubChem Compound Summary for CID 5280794, Stigmasterol. https://pubchem.ncbi.nlm.nih.gov/compound/Stigmasterol.

National Center for Biotechnology Information$_b$. 2021. PubChem Compound Summary for CID 173183, Campesterol. https://pubchem.ncbi.nlm.nih.gov/compound/Campesterol.

Nurgali K, Jagoe RT, Abalo R. 2018. Editorial: Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequelae? Front Pharmacol 9: 1-3. DOI: 10.3389/fphar.2018.00245.

Nuriliani A, Ariyanto IA, Santi MR, Mahnedra A, Dewi NWES, Huda AL, Wijayanti N. 2013. Aktivitas sitotoksik dan apoptosis ekstrak spons spesies A anggota ordo Astroporida terhadap sel hela (Cervical Cancer Cell Line). Biota 18 (1): 45-53. DOI: 10.24002/biota.v18i1.263.

Nurrani L. 2013. Pemanfaatan tradisional tumbuhan alam berkhasiat obat oleh masyarakat di sekitar cagar alam tangale. Info BPK Manado 3 (1): 1-22. DOI: 10.1103/PhysRevA.86.060301. [Indonesian]

Pagare S, Bhatia M, Tripathi N, Pagare S, Bansal YK. 2015. Secondary metabolites of plants and their role: Overview. Curr Trends Biotechnol Pharm 9 (3): 293-304. DOI: 10.5958/2249-0035.2015.00016.9.

Ramprasath VR, Awad AB. 2015. Role of phytosterols in cancer prevention and treatment. J AOAC Intl 98 (3): 735-738. DOI: 10.5740/jaoacint.SGERamprasath.

Rath G, Christophe S, Benoit L, Hervé S, Hamid M, Hassan ELB, Stephane D, Laurent M. 2009. De novo ceramide synthesis is responsible for the anti-tumor properties of camptothecin and doxorubicin in follicular thyroid carcinoma. Intl J Biochem Cell Biol 41 (5): 1165-1172. DOI: 10.1016/j.biocel.2008.10.021.

Setyawati T, Narulita S, Bahri IP, Raharjo GT. 2015. A Guide Book to Invasive Alien Plant Spesies in Indonesia. Research, Development and Innovation Agency Ministry of Environment and Forestry Republic of Indonesia, Indonesia.

Srisawat T, Chumkaew P, Heed-Chim W, Sukpondma Y, Kanokwiroon K. 2013. Phytochemical screening and cytotoxicity of crude extracts of *Vatica diospyroides* Symington type LS. Trop J Pharm Res 12 (1): 71-76. DOI: 10.4314/tjpr.v12i1.12.

Sudarmawan IH, Dlilir D, Mudigdo A, Budiani DR. 2010. The effect of ethanolic and petroleum ether fractions of bawang dayak (*Eleutherine palmifolia*) bulb extract on expression of p53 mutant in breast cancer cell line T47D. Biofarmasi 8: 17-26. DOI: 10.13057/biofar/f080103.

Tallima H, Azzazy HME, El Ridi R. 2021. Cell surface sphingomyelin: key role in cancer initiation, progression, and immune evasion. Lipids Health Dis 20 (1): 1-12. DOI: 10.1186/s12944-021-01581-y.

Woyengo TA, Ramprasath VR, Jones PJH. 2009. Anticancer effects of phytosterols. Eur J Clin Nutr 63 (7): 813-820. DOI: 10.1038/ejcn.2009.29.

Xiong WD, Gong J, Xing C. 2017. Ferruginol exhibits anticancer effects in OVCAR-3 human ovary cancer cells by inducing apoptosis, inhibition of cancer cell migration and G2/M phase cell cycle arrest. Mol Med Rep 16 (5): 7013-7017. DOI: 10.3892/mmr.2017.7484.

Yamagishi T, Zhang DC, Chang JJ, McPhail DR, McPhail AT, Lee KH. 1988. The cytotoxic principles of *Hyptis capitata* and the structures of the new triterpenes hyptatic acid-A and -B. Phytochemistry 27 (10): 3213-3216. DOI: 10.1016/0031-9422(88)80028-1.