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Acute toxicity and anticancer potential of knobweed (*Hyptis capitata*) ethanolic leaf extract and fraction

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Abstract

Ethanolic leaf extract of *Hyptis capitata* Jacq. showed a toxic effect on *Artemia salina* larvae[1]. The extract was simplified through fractionation using vacuum liquid chromatography to trace compounds with biological activity. The F1, F2, F3 and F4 fractions were used in the acute toxicity test on *A. salina* larvae. F1 showed the highest toxicity value of 196.77±2.7 µg/ml. As an initial screening for its anticancer potential, ethanolic leaf extract and F1 fraction were subjected to cytotoxicity test on T47D cells to obtain an IC_{50} value of 45.32±2.0 and >1000 µg/mL. The LC_{50} of the F1 fraction and its cytotoxicity level decreased compared to the ethanolic leaf extract. Based on GC-MS analysis, the ethanolic leaf extract and the F1 fraction contained neophytadiene, hexadecanoic acid, methyl ester, hexadecanoic acid, ethyl ester and heptadecanoic acid, 16-methyl-, methyl ester. Meanwhile, ϫ-sitosterol, docosanoic acid, ethyl ester and squalene are only found in the ethanolic leaf extract. The compounds contained in ethanolic leaf extract work synergistically. This implies the ethanolic leaf extract has the potential to be developed as an anticancer candidate.

Keywords

Anticancer, cytotoxicity, *Hyptis capitata*, GC-MS, toxicity

Introduction

Plants contain secondary metabolites that can be utilized for human benefit. In Indonesia, the cultural heritage of using many plants as medicinal ingredients is the basis for further exploration of the efficacy and safety of certain plants. For example, Knobweed or *Hyptis capitata* is one of the plants widely used by the community as a medicinal ingredient (1, 2). This plant is used to treat fever, open and internal wounds (1, 2) and also has opportunity to act as an antioxidant, antiviral, antibacterial and anticancer (3). However, the safety in its utilization and scientific potential still needs to be studied in depth to underlie its development in the future.

A previous research conducted an acute toxicity test of *H. capitata* ethanolic leaf extract using the Brine Shrimp Lethality Test Method on *Artemia salina* larvae and obtained LC_{50} 183.91 µg/ml (4) for the toxic category (4, 5). An early acute toxicity test can be the basis for the next stage (6). The initial screening methods determine the potential of a test material to be developed as an anticancer candidate (5). This research develops previous results comparing ethanolic leaf extract's toxicity level and fraction. It also associated the results of acute toxicity tests with their potential as anticancer based on cytotoxicity activity. The fractionation procedures simplify

and trace the secondary metabolite components in the ethanolic leaf extract and the toxicity activity using the Vacuum Liquid Chromatography method. The results were reused for acute toxicity tests using the Brine Shrimp Lethality Test method. The fraction that showed the highest activity was subjected to a cytotoxicity test on T47D breast cancer cells. A cytotoxicity test was carried out as an initial screening of the potential of the ethanolic leaf extract and its fraction as an anticancer candidate. Screening of metabolite compounds was also conducted using the Gas Chromatography-Mass Spectrometry Method.

Materials and Methods

The material used was *H. capitata* Jacq. (Fig. 1.) leaves taken from Bungapati village, Tana Lili Regency, North Luwu District, South Sulawesi. This plant has been identified in the plant systematics laboratory, Faculty of Biology, Universitas Gadjah Mada, with a certificate number of 014535/S.Tb/III/2019 (7).

Fig. 1. *Hyptis capitata*. Habit.

Extraction

The *H. capitata* leaves were taken in the morning, washed thoroughly with running water, covered with a black cloth and dried in the sun (until the dry weight was stable). The leaves were mashed with a grinder, filtered and then weighed. Subsequently, *H. capitata* leaf dry powder was extracted using absolute ethanol solvent in a ratio of 1:5.

Vacuum chromatography-mass spectrometry

A total of 2.5 g of ethanolic leaf extract was mixed with 5 g of silica gel 60 GF254 powder (Merck, Germany) to obtain a homogeneous mixture. The stationary phase used was 12 g of silica gel 60 GF254. Furthermore the eluent composition was n-hexane:chloroform 3:1(v/v), 2:2 (v/v), 1:3 (v/v), 100% chloroform, chloroform : ethanol 3:1 (v/v), 2:2 (v/v), 1:3 (v/v) and ethanol 100%. The obtained fraction was collected in an Erlenmeyer, poured into a porcelain dish and air-dried. VLC was followed by thin-layer chromatography to monitor the separation of the compound (8).

Thin-layer chromatography

The mobile and stationary phase used was ethyl acetate: methanol (7:3 v/v) and silica gel F254 (Merck, Germany). The chromatogram pattern was observed under UV light at 254 and 366 nm wavelengths and combined into one fraction.

A. salina hatching

A total of 0.5 g of artemia eggs were put in a plastic bottle filled with 1 liter of seawater and the hatchery was placed at room temperature. It was given an aerator and left for 36 hrs until the eggs hatched into larvae (4).

Brine shrimp lethality test

H. capitata leaf fraction was dissolved with Dimethylsulfoxide (DMSO) in a ratio of 1:5. Each fraction was made into a concentration series of 300; 150; 75; 37.5; 18.75 µg/ml. Meanwhile, the tube was filled with a volume of 20 ml and 10 larvae of 36 hrs old were put in the solution. The test was accompanied by a negative and solvent control (DMSO) and each concentration was made in 3 replications. There was also a negative control tube filled with seawater and 10 larvae. The tube was stored for 24 hrs without a cover and lamp irradiation. Furthermore, the number of live larvae was counted to determine those that died due to the treatment (4).

Cytotoxicity assay

A cytotoxicity test was performed on T47D cells cultured using 96 well-plates with a density of 10^4 cells/well in a complete RPMI culture medium (Gibco, Canada). In addition, 100 µl of cell suspension filled the well, and the cells were incubated for 24 hrs in a CO_2 incubator at 36 °C and 5% CO_2. These cells were treated when they reached 70-80% confluency. Ethanolic leaf extract and fraction 1 of 10 mg were weighed and dissolved in 100 µl DMSO (Merck, Germany). The series concentration was made in 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, and 12.5 µg/ml with 3 replications. The cultured cells were incubated for 24 hrs

in a CO_2 incubator. The conditions were observed and documented before adding MTT (Bio Basic, Canada) (5 mg/ml PBS) in a ratio of 1:9. Cells were incubated for 4 hrs in a CO_2 incubator and 100 µl Reagent Stop (SDS) (Merck, Germany) was added. The well-plate was wrapped in aluminum foil and incubated at room temperature overnight. The absorbance was measured with a microplate reader machine at 595 nm (9).

Gas chromatography-mass spectrometry (GC-MS)

Secondary metabolites were analyzed by the GC-MS method at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Yogyakarta, using an HP-5MS UI capillary column with a column length of 30 m, a diameter of 0.25 mm and thickness of 0.25 m. Furthermore, the sample was dissolved in pure ethanol and injected using a split technique. The initial conditions with the splitless model were as follows: split-flow 50 ml/min, with a front inflow of 1.00 ml/min. Helium was used as the carrier gas, and the injector temperature was programmed at 260 °C. The ion source temperature was 200 °C, while the mass spectrometry transfer line was 250 °C, with a speed of 5 °C/min. The gas saver time was 5 minutes, purge flow was 3 ml/min, and flow of gas saver was 5 ml/min. Meanwhile, the Mass spectrometry transfer line temperature was (MS) 250 °C and the detected peaks were analyzed descriptively (7).

Data analysis

The data obtained were analyzed quantitatively or qualitatively, and the LC_{50} value was determined using probit analysis. The IC_{50} value was determined by log concentration regression analysis. Furthermore, the data obtained were processed using SPSS 26 and analyzed with a one-way analysis of variance at a 95% confidence level and a significance value of $P < 0.05$.

Results

Fractionation with vacuum liquid chromatography

The results of the fractionation of the ethanolic leaf extract can be seen in Fig. 2. The obtained fractions were visualized by thin-layer chromatography, and the separation profile was observed under ultraviolet light. Based on the figure, the 8 fractions obtained were simplified in number by combining those with a similar pattern. The 8 fractions were simplified to 4 and used in an acute toxicity test on *A. salina*. The most toxic LC_{50} value was continued for the cytotoxicity test on T47D cells.

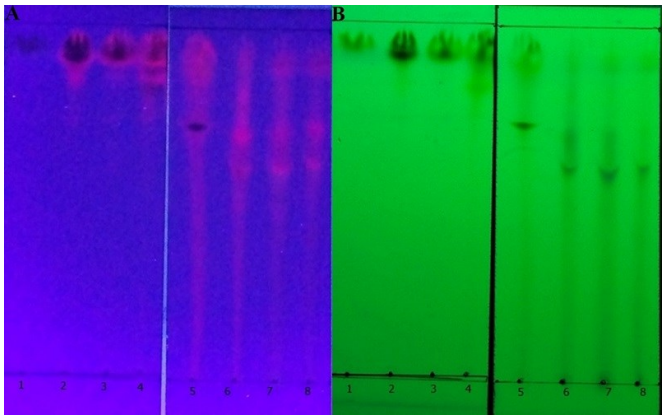


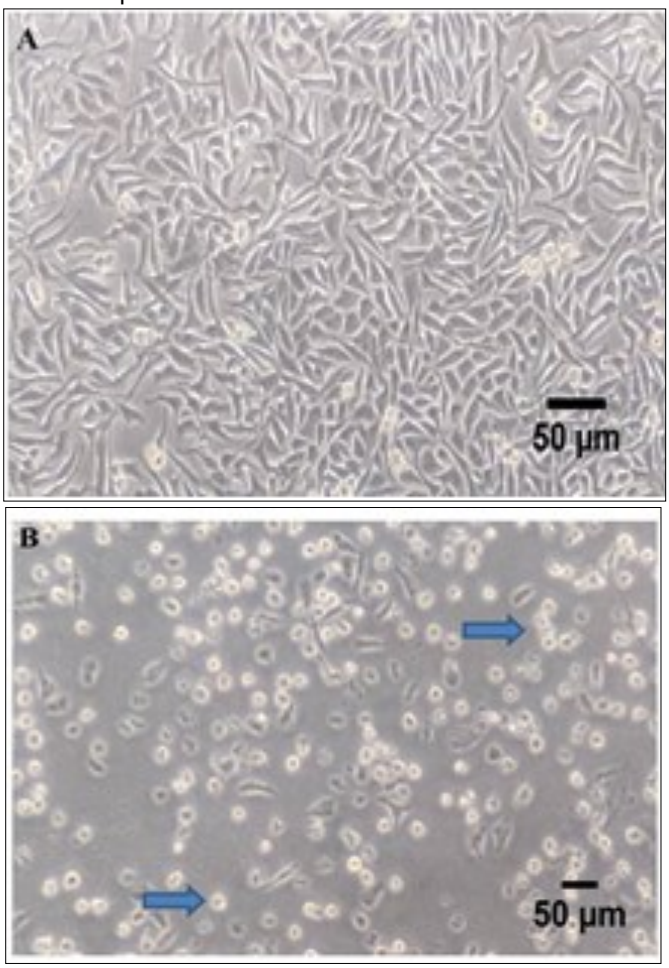
Fig. 2. Thin-Layer Chromatography Fraction leaves of *H. capitata*. **A**. Visualization with UV 365 nm; **B**. Visualization with UV 254 nm. 1 = n-hexane:chloroform fraction 3:1 (v/v); 2 = n-hexane:chloroform fraction 2:2 (v/v); 3 = n-hexane:chloroform fraction 1:3 v/v; 4 = 100% chloroform fraction; 5 = chloroform: ethanol fraction 3:1 v/v; 6 = chloroform:ethanol fraction 2:2 v/v; 7 =chloroform:etanol fraction 1:3 v/v; 8 = 100% etanol 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, 3, 4 were combined and were referred to as second fractions (F2); fraction 5 separately then referred to as the third fraction (F3) and fractions 6, 7 and 8 were combined and were referred to as the fourth fraction (F4).

Acute toxicity test and cytotoxicity

Based on the LC_{50} value (Table 1), the F1-F4 treatment was toxic to *A. salina*. The obtained LC_{50} value was <1000 µg/ml, which can be categorized as toxic (5). The treatment of the F1 fraction showed the highest acute toxicity value against *A. salina*. Meanwhile, the F1 cytotoxicity test on T47D cells showed a value of >1000 µg/ml (Tabel 1), hence, it is not cytotoxic. This is different from the IC_{50} value of ethanolic leaf extract treatment on T47D cells (Table 1), which was 45.32±2.0 µg/ml, classified as moderate. The morphology of cells exposed to ethanolic leaf extract and F1 fraction

Table 1. Acute toxicity and cytotoxicity of ethanolic leaf extract *H. capitata* and its fraction.

Treatment	LC_{50} Average±SD (µg/ml) on *A. salina*	IC_{50} Average±SD (µg/ml) on T47D cells
Ethanolic leaf extract	N.D	45.32±2.0
F1	196.77±2.7[a]	>1000
F2	246.15±5.8[b]	N.D
F3	286.93±10.23[b]	N.D
F4	390.275±32[c]	N.D



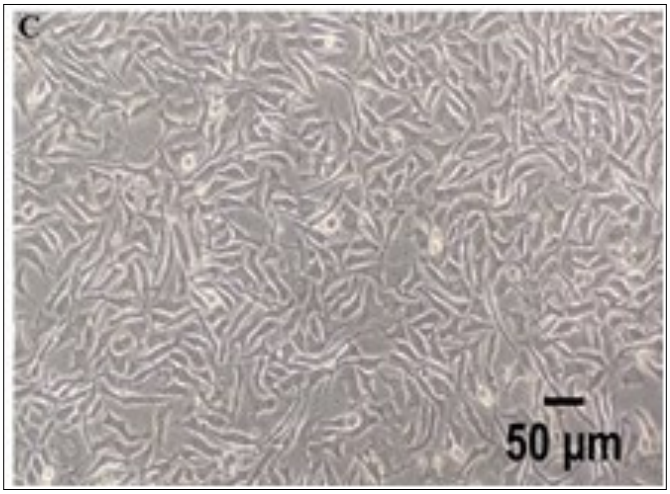


Fig. 3. Effect of Ethanolic leaves extracts and Fraction F1 treatment on T47D Cells using an inverted microscope. **A**. T47D cell control, **B**. ethanolic leaf extract treatment on T47D cells, **C.** Fraction F1 treatment on T47D cells.

can be seen in Fig. 3. Cells treated with the extract showed variations in morphology and the cytotoxicity effect was indicated by the change in the shape of the cells into small rounds. Different variables were shown in T47D cells exposed to the F1 fraction having the same morphology as control cells.

Identification of bioactive components

The peak chromatogram of the content of metabolite compounds in the ethanolic leaf extract and the F1 fraction can be seen in Fig. 4. It shows that 96 peaks were detected in the ethanolic leaf extract and F1 fraction 44 was detected. Meanwhile, the list of compounds with a similarity index value above 750 is presented in Table 2. In the ethanolic leaf extract, there were 27 components, while the F1 fraction had 12 compounds. The compounds contained in the extract and fraction F1 were neophytadiene, hexadecanoic acid, methyl ester, hexadecanoic ethyl ester, heptadecanoic acid and phytol.

Discussion

The acute toxicity and cytotoxicity test of the *H. capitata* Jacq. leaf fraction are presented in Table 1. It shows that the LC_{50} value of the F1 fraction treatment on *A. salina* larvae was 196.77±2.7 µg/ml, classified as toxic (5). The toxic effects of compounds can also be useful for treating several diseases (12). These are caused by the compounds contained in the extract. Based on statistical tests, these values are known to be significantly different from the LC_{50} of other fractions. Therefore, acute toxicity tests are conducted to evaluate the side effects after the organism is exposed to the test material within 24 hrs (6).

The LC_{50} value of the F1 fraction decreased when compared with ethanolic leaf extract in previous research (183.91 µg/ml). Meanwhile, the treatment of the extract on

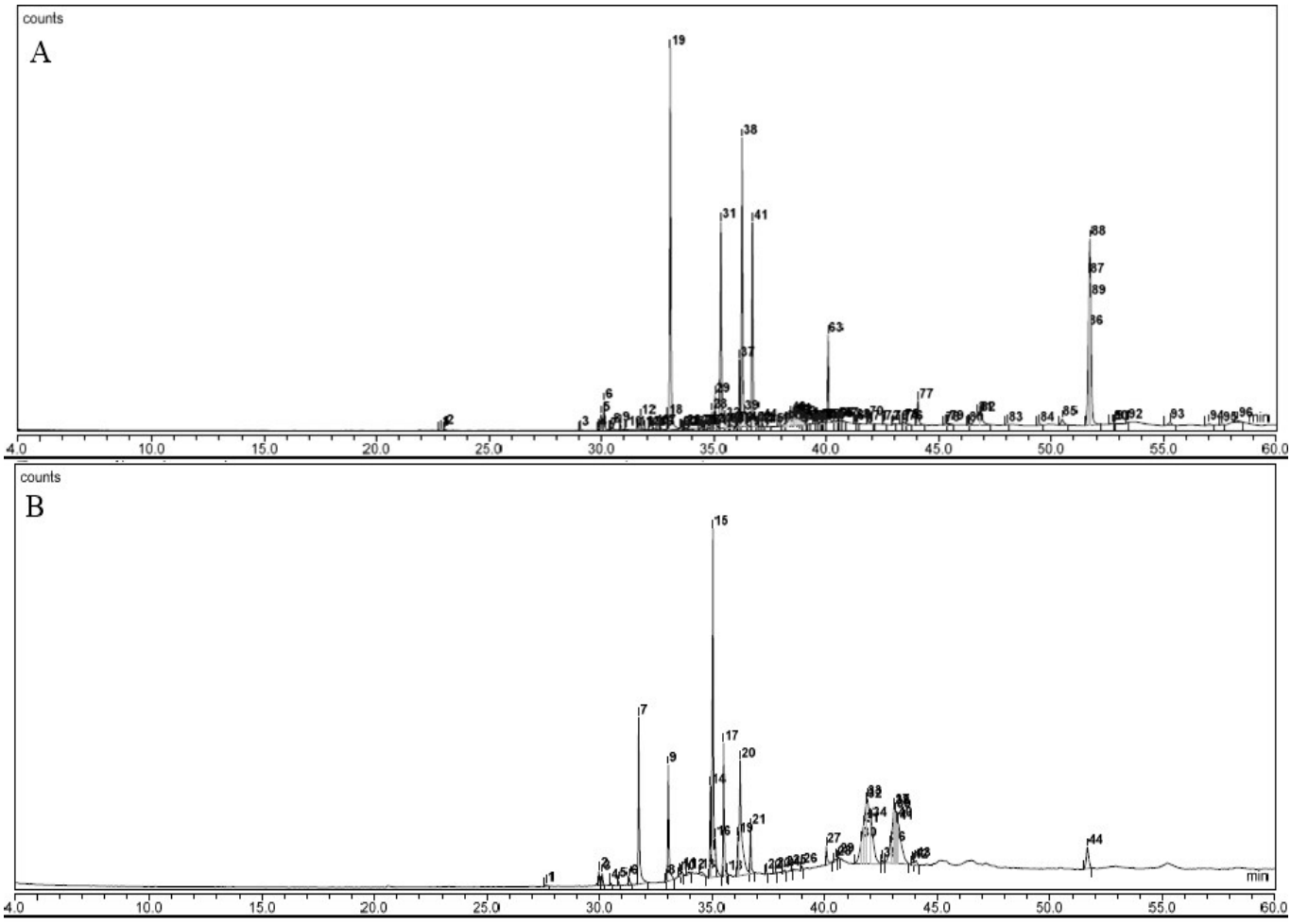


Fig. 4. Chromatogram of metabolite compounds from the leaves ethanolic extract and fraction F1. (A) Chromatogram of metabolite compounds of the *H. capitata* leaves ethanolic extract; 96 peaks were detected. (B) Chromatogram of the metabolite compounds fraction F1; 44 peaks were detected.

Table 2. Metabolite compounds of ethanolic leaf extract and fraction F1

Sl. No.	RT	%Area	Formula	Compound
Ethanolic leaf extract				
1	22.83	0.06	$C_{15}H_{24}$	ß-copaene
2	23.04	0.11	$C_{15}H_{24}$	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene
3	29.06	0.07	$C_{16}H_{32}O_2$	Tetradecanoic acid, ethyl ester
4	29.87	0.08	$C_{20}H_{40}$	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
5	29.97	0.65	$C_{20}H_{38}$	Neophytadiene
6	30.85	0.22	$C_{20}H_{40}O$	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	31.62	0.07	$C_{37}H_{76}O$	1-Heptatriacotanol
8	31.73	0.61	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
9	32.64	0.10	$C_{18}H_{34}O_2$	E-11-Hexadecenoic acid, ethyl ester
10	32.94	0.50	$C_{18}H_{34}O_2$	Ethyl 9-hexadecenoate
11	33.06	17.12	$C_{18}H_{36}O_2$	Hexadecanoic acid, ethyl ester
12	33.75	0.12	$C_{28}H_{48}O$	Cholestan-3-ol, 2-methylene-, (3ß,5a)-
13	34.92	0.66	$C_{19}H_{38}O_2$	Ethyl 14-methyl-hexadecanoate
14	35.04	1.47	$C_{19}H_{32}O_2$	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
15	35.30	11.17	$C_{20}H_{40}O$	Phytol
16	35.51	0.52	$C_{19}H_{38}O_2$	Heptadecanoic acid, 16-methyl-, methyl ester
17	36.13	2.62	$C_{20}H_{36}O_2$	9,12-Octadecadienoic acid, ethyl ester
18	36.26	12.34	$C_{20}H_{34}O_2$	Ethyl 9,12,15-octadecatrienoate
19	36.34	0.80	$C_{21}H_{36}O_4$	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)
20	36.17	8.18	$C_{20}H_{40}O_2$	Heptadecanoic acid, 15-methyl-, ethyl ester
21	37.14	0.17	$C_{20}H_{40}O_2$	Ethanol, 2-(9-octadecenyloxy)-, (Z)-
22	37.95	0.02	$C_{26}H_{44}O_5$	Ethyl iso-allocholate
22	38.72	0.55	$C_{29}H_{5}0O$	ɤ-sitosterol
23	40.08	3.89	$C_{22}H_{44}O_2$	Eicosanoic acid, ethyl ester
24	44.07	1.70	$C_{24}H_{48}O_2$	Docosanoic acid, ethyl ester
25	46.72	1.99	$C_{29}H_{50}O_2$	Vitamin E
26	51.72	9.89	$C_{30}H_{50}$	Squalene
27	58.30	0.17	$C_{30}H_{48}O_2$	Betulinaldehyde
F1				
1	29.97	0.55	$C_{20}H_{38}$	Neophytadiene
2	30.11	0.49	$C_{17}H_{36}O$	1-Hexadecanol, 2-methyl-
3	31.31	0.35	$C_{17}H_{32}O_2$	9-Hexadecenoic acid, methyl
4	31.73	7.98	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
5	33.05	5.23	$C_{18}H_{36}O_2$	Hexadecanoic acid, ethyl ester
6	34.92	3.49	$C_{19}H_{34}O_2$	Methyl 9-cis,11-trans-octadecadienoate
7	35.03	15.30	$C_{19}H_{36}O_2$	trans-13-Octadecenoic acid, methyl ester
8	35.51	4.98	$C_{19}H_{38}O_2$	Heptadecanoic acid, 16- methyl-, methyl ester
9	36.15	2.13	$C_{20}H_{40}O$	Phytol
10	41.88	6.17	$C_{30}H_{50}O$	A-amyrin
11	43.14	2.90	$C_{30}H_{48}O$	Lup-20(29)-en-3-one
12	2.11	51.67	$C_{30}H_{52}O$	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-heptadeca-3,7,11,15-

T47D cells showed a moderate level of cytotoxicity. Different variables were seen in the IC_{50} value of F1 fraction treatment on T47D cells. The level of cytotoxicity of the ethanolic leaf extract was moderate, indicating an opportunity to be developed as an anticancer candidate. On the other hand, the LC_{50} and IC_{50} values of the F1 fraction in T47D cells decreased compared to the crude extract. This is influenced by the composition of metabolite compounds in toxicity and cytotoxicity (13).

Based on the results of the GC-MS analysis, the ethanolic leaf extract contained several compounds which played a role in toxicity and cytotoxicity. The compounds are neophytadiene; hexadecanoic acid, methyl ester; hexa-

decanoic acid, ethyl ester; heptadecanoic acid, 16- methyl-, methyl ester; phytol; ϑ-sitosterol; docosanoic acid, ethyl ester; and squalene. They were also detected in the F1 fraction, except ϑ-sitosterol; docosanoic acid, ethyl ester; and squalene.

Neophytadiene and phytol are terpenoids with antimicrobial and anti-inflammatory properties (14). Neophytadiene is also one of the main components in *Acalypha segetalis* leaf essential oil, which play a role in the toxicity activity of *A. salina* larvae (15). Furthermore, the toxicity of this compound isolated from *Selenicereus hamatus* showed a high value for *A. salina* larvae, with an LC_{50} of 16.52 µg/ml (16). Meanwhile, phytol shows pharmacological activity and can be used as an anticancer (17, 18). Phytol enhances pro-carcinogens and induces genotoxicity as well as apoptosis in breast cancer cells (19). The content of phytol and, hexadecanoic acid, methyl esters in *Calotropis gigantea* leaves can also influence toxicity activity against *A. salina* larvae (20). In the ethanolic leaf extract and the fraction F1, these compounds had a toxic effect on *A. salina* larvae.

Hexadecanoic acid, methyl ester (palmitic acid, methyl ester) (21), hexadecanoid ethyl ester (palmitic acid, ethyl ester) (22), Heptadecanoic acid, 16- methyl-, methyl ester (methyl isostearat) (23) and docosanoic acid ethyl esters (Ethyl docosanoate) is a group of fatty acid ester compounds (24) with anticancer activity. Hexadecanoic acid can affect the growth of colon cancer cells (25), acts as a growth inhibitor, triggers apoptosis and inhibits the cell cycle in the G0/G1 phase (26). In addition, the ethyl ester derivative can inhibit DNA topoisomerase I and is an inducer of apoptosis in leukemia and neuroblastoma cells (27-29). Ethyl docosanoate has also been reported as a secondary metabolite in the cytotoxic activity of *Xetospongia testudinaria* isolate (30).

ϑ-sitosterol has anticancer activity and can inhibit the growth of MCF-7 and A549 cells. In addition, this compound triggers arrests in the G2/M phase of the cell cycle and reduces the expression of the transcription factor c-Myc in cell division (31).

In line with the previously described metabolites compounds, squalene also has cytotoxicity and antitumor potential against HeLa cells. In combination with the anticancer agent Adriamycin, it shows a synergistic effect (32). Furthermore, squalene has the potential as an anti-inflammatory and can inhibit inflammation by increasing the production of prostaglandin E2 during chemotherapy in tumor-bearing mice (33). The decreased level of cytotoxicity in the F1 was thought to be caused by the absence of ϑ-sitosterol, docosanoic acid ethyl esters and squalene.

Under certain circumstances, some compounds may exhibit a synergistic or positive effect. This is shown when the combination of many secondary metabolites is greater than the single effect (34). Therefore, the compounds detected in the ethanolic leaf extract work synergistically to provide a strengthening effect on toxicity and cytotoxicity. The reduction in the composition of compounds in the F1 fraction indicated a decrease in the acute toxicity and cytotoxicity effects. The absence of ϑ-sitosterol, docosanoic acid, ethyl ester and squalene in the F1 fraction greatly influenced the cytotoxicity level of the F1 fraction in T47D cells.

This study shows the opportunity of ethanolic leaf extract of *H. capitata* to be developed as an anticancer candidate. Several *Hyptis* species also have cytotoxic activity on cancer cell lines. These species include *H. suaveolens (*35, 36), *H. brevipes* (37), *H. martiusii* (38), *H. incana* (39) and *H. pectinata* (40).

Conclusion

The acute toxicity and cytotoxicity of ethanolic leaf extract are higher than the F1 fraction. The extract contained neophytadiene; hexadecanoic acid, methyl ester; hexadecanoic acid, ethyl ester; heptadecanoic acid, 16- methyl-, methyl ester; phytol; ϑ-sitosterol; docosanoic acid, ethyl ester; and squalene. These compounds can positively impact toxicity and cytotoxicity, working synergistically. Therefore, the ethanolic leaf extract of *H. capitata* can be developed as an anticancer candidate.

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Authors contributions

NT conducts research, processes data and manuscript writing. FZ contributed to provide important suggestions related to research and manuscript writing. WNJ conceived of the study and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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