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RESEARCH ARTICLE

Antiproliferative and apoptotic effect of Knobweed (*Hyptis capitata*) root methanol extract on WiDr colorectal cancer cells

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Abstract

Methanol extract of Knobweed (*Hyptis capitata*) root has cytotoxic activity on WiDr cells, but the mechanism of its anticancer action as well as the secondary metabolite contents have not been further explored. This study aimed to extract the roots of *H. capitata* by a graded maceration, using absolute chloroform, and absolute methanol. Methanol extract was used for subsequent assays to trace the mechanism of anti-cancer action. Tests carried out include a doubling time test with the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide, as well as an apoptosis test with Annexin V-PI Flow cytometry method. The secondary metabolite content was identified by the Gas Chromatography-Mass Spectrometry (GC-MS). The results showed that, *H. capitata* root methanol extract treatment caused morphological changes on WiDr cells. Moreover, the treatment suppressed the cellular proliferation, indicating that it can provide an anti-proliferation effect. Furthermore, the extract treatment at IC_{50} and $2IC_{50}$ caused 65.45% and 97.6% of apoptosis cells, respectively. These results proved that *H. capitata* root methanolic extract has anticancer activity with anti-proliferation properties, and the ability to trigger apoptosis on WiDr cells. Phytochemical compounds that might play a role as anti-cancer, among others include chatecol, 9-hexadecanoic acid, hexadecanoid acid-ethyl ester, methyl stearate, ferruginol, retinoic acid, campesterol, stigmasterol, and γ-sitosterol.

Keywords

anti-cancer; ethnobotany; GC-MS; doubling time; annexin V-PI

Introduction

Knobweed (*Hyptis capitata* Jacq.) is a Hyptis plant that is widely used in traditional medicine (1) and its *in-vitro* anticancer potential has been reported (2,3). According to a previous study, the root methanol extract has cytotoxicity activity against WiDr cells, with an IC_{50} value of 41.70 ± 2.19 µg/mL (4). The anticancer mechanism tracing of *H. capitata's* root methanol extract in WiDr cells has not been explored. In addition, the content of phytochemical compounds that plays a role in cytotoxic activity is also unknown.

Some of the characteristics of cancer cells targeted during treatment are associated with the ability to proliferate indefinitely and avoid apoptosis (5,6). This ability causes cancer cells to develop rapidly in a short time. The tracing of *H. capitata* root methanol extract treatment effect on apoptosis activity and WiDr cells proliferation is expected to form the basis for its development as an anti-cancer candidate in the future.

In this study, *H. capitata* root methanol extract was tested for its anti-proliferation properties by the colorimetric method with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), as well as doubling time parameters. Meanwhile, its ability to induce apoptosis in WiDr cells was tested using the Annexin V-PI flow cytometry method. The identification of phytochemical compounds in the extract was analyzed by the GC-MS (Gas Chromatogphy-Mass Spectrometry) method.

Materials and Methods

H. capitata was obtained from Bungapati Village, North Luwu District, South Sulawesi, Indonesia. The plant was identified at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, with certificate number 014535/S.Tb/III/2019. The method of identification, harvesting, drying, and powdering of the simplicia has been published previously (4).

Extraction

The powder of *H. capitata* root was extracted using the graded maceration method, which has been published previously (4). Graded maceration using chloroform as the first solvent. After the chloroform extract was obtained, the remaining chloroform in the simplicia was evaporated. The simpliciawas subjected to continued maseration with methanol.

Annexin V-PI flow cytometry assay

WiDr cells were cultured with a density of $5x10^5$ cells/well in a complete medium of RPMI (Gibco, Canada) and then subsequently in a 6-well plate. Each well was filled with 2000 µl of suspense cells and incubated for 24 hours in a CO_2 incubator, at a temperature of 36 °C, CO_2 5%. Furthermore, the cells were observed, and documented, while the medium was removed and was washed with PBS (Invitrogen, Camarillo). The treatment was carried out using the extract concentration of about IC_{50} and $2IC_{50}$, while the concentration of doxorubicin used in positive controls was 6 µg/mL. The cells were re-incubated for 24 hours in a CO_2 incubator, at a temperature of 35ºC, CO_2 5%, then they were observed, documented, and harvested. The medium of each well was accommodated into a conical tube and the cells were washed with PBS 1000 µL/well. Each well was added with 150 µL of 0.25% trypsin-EDTA and incubated for 3 min inside a 36°C incubator. About 1 mL of complete culture medium was added to each well, and the cells were resuspended. Furthermore, the culture media was taken from each well, accommodated in each conical tube, and centrifuged at a speed of 2000 rpm for 3 minutes. The cells were washed with cold PBS (done by 2x) and suspended with a 1X Binding Buffer at a concentration of 1×10^6 cells/mL. A total of 100 µL of cells suspension was transferred into a 5 mL culture tube, then 5 µL of FITC Anexin V and 5 µL of PI reagent were added, and the mixture was homogenized with vortex. The cells were incubated for 15 minutes at room temperature, in a dark state. A total of 400 µL 1X Binding Buffer was added and analysis was performed with a flow cytometry machine (4,7).

Antiproliferation assay (doubling time)

WiDr cells were cultured using 96 well plates specifically 4 plates with a density of 5×10^3/well in a complete culture medium of RPMI 1640. Each well was filled with 100 µL medium, and the cells were incubated inside a CO_2 incubator with a temperature of 36°C for 24 hours. When the cells have shown 70-80% confluent, methanol extract of *H. capitata* root in concentration series of 35, 17.5, 8.75, 4.38, and 2.19 µg/mL was further used for treatment. About 0.25 µg/mL Doxorubicin was used as a positive control and DMSO as a solvent control. Each plate was incubated at 0, 24, 48, and 72 hours, while each concentration was triplicated.

GC-MS analysis

The secondary metabolite content in the root methanol of *H. capitata* extract was analyzed using the GC-MS method conducted at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Yogyakarta. The GC-MS test uses an HP-5MS UI capillary column with a thickness of 0.25 µm, a column length of 30 m, and a diameter of 0.25 mm. The GC-MS initial condition with splitless model and mass list range (amu) 40-500. The sample volume injected was 1 µl. GC-MS method used has been published previously (3).

Data analysis

Annexin V-PI flow cytometry apoptotic induction and GC-MS data were analyzed descriptively. Identification of compounds from GC-MS analysis was carried out by comparing the spectra of the sample mass with the internal library search report. Meanwhile, antiproliferation data in the form of absorbance in each treatment at each incubation hour was used to determine the doubling time. The doubling time were processed using SPSS 26 and analyzed using one-way analysis of variance at a 95% confidence level and a significance value of $P < 0.05$.

Results

The morphological characteristics of WiDr cells exposed to *H. capitata* root methanol extract are presented in Fig. 1. The morphology of the treated cells appears to be smaller in size than the control. Based on the results, the treatment with the extract exerted a toxic effect on cells as demonstrated by the changes in the morphology.

Anti-proliferative assay

The effect of *H. capitata* root methanol extract with various concentrations on the doubling time of WiDr cells is shown in Fig. 2. Based on these images, the treatment extended the doubling time of the cells compared to the control. This suggests that the extract can slow down cells division, thereby suppressing the proliferation of WiDr cells.

Annexin V-PI flow cytometry assay

Based on the annexin V-PI flow cytometry assay in Table 1, there were variations in the percentage of necrosis and apoptosis cells in the IC_{50} and $2IC_{50}$ root methanolic extract of *H. capitata* treatments as shown in Fig. 3. The number of

apoptosis cells increased with the addition of root methanolic extract concentrations, while the number of necrotic cells reduced.

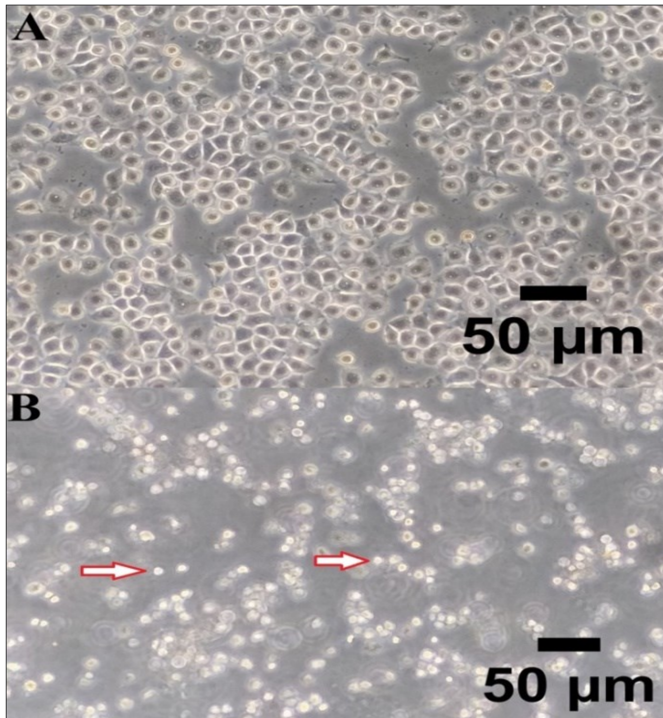

Figure 1. Effect of *H. capitata* root methanol extract treatment on WiDr cells under an inverted microscope. **A**. WiDr cells control, **B**. WiDr cells treated with *H. capitata* root methanol extract IC_{50}.

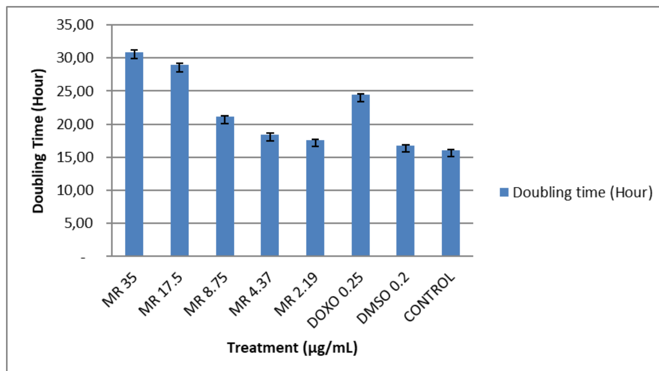

Figure 2. Effect of treatment with *H. capitata* root methanol extract at various concentrations on the doubling time of WiDr cells. The treatment appeared to slow down the proliferation of WiDr cells and showed a dose-dependent effect. The treatments of *H. capitata* root methanol extract at concentrations of 35, 17.7, 8.75 and 4.37 µg/mL were significantly different from each other, and also significantly different from doxorubicin, cells control and DMSO treatments.

Table 1. Percentage of WiDr cells conditions after treatment with *H. capitata* root methanol extract and doxorubicin

No	Treatment	Percentage (%)			
		Q1	Q2	Q3	Q4
1	IC_{50} (41.70 µg/mL)	9.55± 0.45	23.25± 2.9	25±4. 4	42.2± 0.4
2	2 IC_{50} (82.40 µg/mL)	0.3± 0.1	22.8± 0.95	2.1±0. 5	74.75± 0.35
3	Doxorubicin (6 µg/mL)	2.35± 0.65	46.4±1 1.8	20.5± 18.1	30.75± 6.95
4	Cells control	4.8± 2.0	5.25± 1.35	90±3. 4	0.0

Note: **Q1**: necrotic, **Q2**: late apoptotic, **Q3**: Viabel, **Q4**: early apoptotic

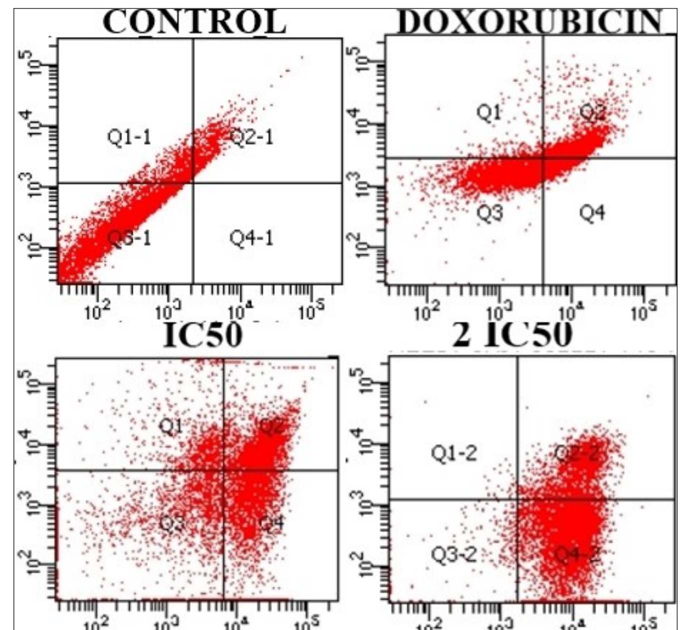

Figure 3. Detection of early apoptosis and late apoptosis in WiDr cells with annexin V-PI Flow cytometry.

Identification of phytochemical compounds based on GC-MS

Analysis of the phytochemical compound in the methanol extract of *H. capitata* root showed that about 147 peaks were detected as shown by the chromatogram in Fig. 4. The compounds with the highest similarity index value are presented in Table 2 which shows that several compounds are contained in the methanol extract of *H. capitata* root. These compounds are suspected to play a role in the induction of apoptosis and also act as antiproliferatives. They include chatecol, 9-hexadecanoic acid, hexadecanoid acid-ethyl ester, methyl stearate, ferruginol, retinoic acid, campesterol, stigmasterol, and γ-sitosterol.

Discussion

The use of *H. capitata* root methanol extract on WiDr cells exhibited a cytotoxic effect which affected their morphological form. WiDr cells became smaller, rounded, and had decreased contact with one another. The cytotoxicity effect of a test material can affect the stability of the cells' structure (3,8).

Based on doubling time tests, the treatment with *H. capitata* root methanol extract can inhibit cells proliferation. This was shown by the extended doubling time in the treatment group compared to the control. The extended doubling time increases with a rise in the extract concentration, indicating a dose-dependent effect. The decrease in WiDr cells proliferation after treatment showed that the test material has anti-proliferation activity, hence, the time required by cancer cells to divide into doubles became extended (9).

In line with the doubling time assay, the annexin V-PI flow cytometry apoptosis assay showed that the treatment of *H. capitata* root methanol extract at IC_{50} and $2IC_{50}$ caused most of the cells to undergo apoptosis. Total apoptosis and necrosis cells at IC_{50} treatment were 65.45%

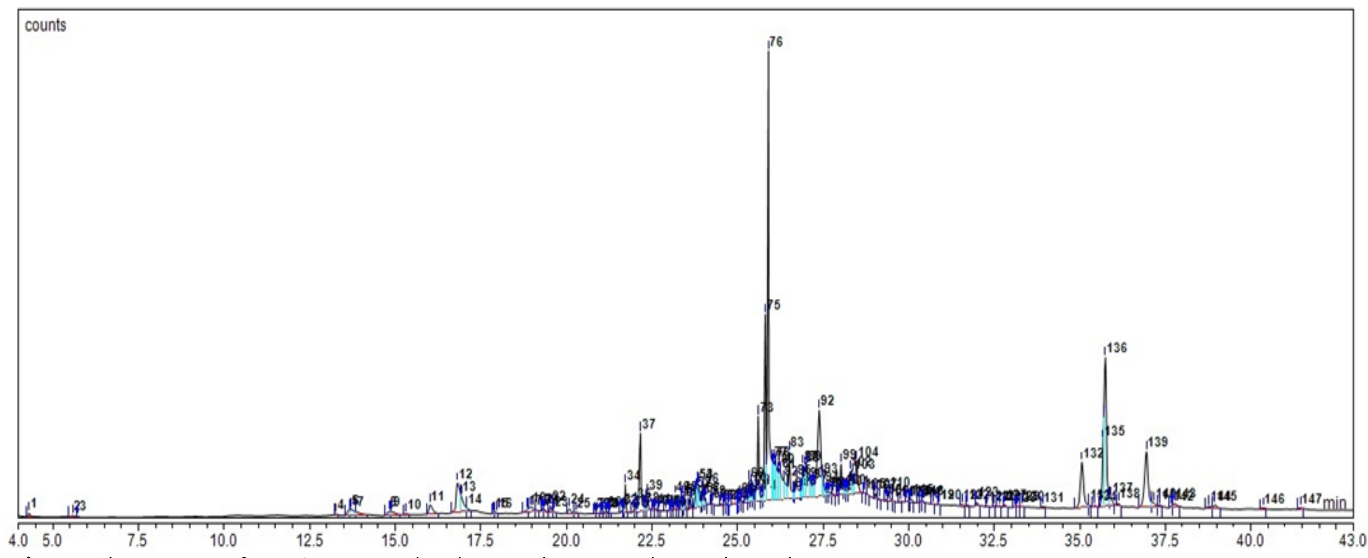

Figure 4. Chromatogram of *H. capitata root methanol extract.* About 147 peaks were detected

Table 2. Compounds suspected to be contained in *H. capitata* root methanol extract based on GC-MS analysis

No	RT	Compound	Chemical Formula	Similarity Index	Area (%)
1	13.67	Catechol	C6H6O2	752	0.29
2	16.02	4-Ethylcatechol	C8H10O2	802	0.86
3	19.54	Dodecanoic acid, 3-hydroxy-	C12H24O3	757	0.23
4	20.84	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3ß,5Z,7E)	C27H44O3	752	0.02
5	22.17	9-Hexadecenoic acid	C16H30O2	872	3.51
6	22.29	Estra-1,3,5(10)-trien-17ß-ol	C18H24O	760	0.18
7	22.38	Hexadecanoic acid, ethyl ester	C18H36O2	822	0.42
8	23.35	9,12-Octadecadienoic acid, methyl ester, (E,E)-	C19H34O2	838	0.23
9	23.45	Methyl stearate	C19H38O2	766	0.23
10	23.37	13-Heptadecyn-1-ol	C17H32O	802	0.65
11	23.83	cis-13-Octadecenoic acid	C18H34O2	853	0.59
12	25.06	cis-13-Eicosenoic acid	C20H38O2	768	0.16
13	25.44	1-Heptatriacotanol	C37H76O	801	0.25
14	25.60	Ferruginol	C20H30O	889	3.03
15	25.81	Retinoic acid	C21H30O2	749	5.92
16	26.02	Pregn-5-en-20-one, 3-hydroxy	C21H32O2	761	1.21
17	26.91	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl este	C35H68O5	780	0.93
18	28.11	Quassin	C22H28O6	729	0.96
19	29.75	4HCyclopropa[5',6']benz[1',2':7,8]azuleno[5,6-b]oxiren-4-on	C22H30O8	759	0.01
20	35.06	Campesterol	C28H48O	899	3.65
21	35.75	Stigmasterol	C29H48O	939	8.73
22	36.94	γ-sitosterol	C29H50O	912	4,93

and 9.55%, respectively. Meanwhile, in the 2IC$_{50}$ treatment, there was an increase in total apoptosis cells and a decrease in necrosis cells, with percentages of 97.6% and 0.3% respectively. A rise in the extract concentration increased apoptosis but reduced necrosis as expected in the case of cancer. Excessive necrosis triggers an inflammatory response which has tumor potential as well as cancer cells proliferation (10,11). Based on this assay, the methanol extract of *H. capitata* root can trigger apoptosis cells. Meanwhile, cells death through apoptosis is highly expected, because the ability to avoid apoptosis is one of the hallmarks of cancer (12).

Various natural ingredients can act as anti-cancer agents (13, 14), while the cytotoxicity, antiproliferation, and apoptosis-inducing effects possessed by the methanol extract of *H. capitata* root are influenced by the phytochemicals content. Phytochemical compounds such as chatecol, 9-hexadecanoic acid, hexadecanoid acid-ethyl ester, methyl stearate, ferruginol, retinoic acid, quassin,

campesterol, stigmasterol, and γ-sitosterol are predicted to contribute as anticancer.

Chatecol is one of the several secondary metabolites in plants, which is a benzenediol (15). Natural catechol treatment in lung cancer cells triggers arrest in the G1 phase of the cells cycle, while suppressing the expression of proteins that play a role in G1-S progression (16). It can also provide an anti-proliferation effect on Panc-1 pancreas cancer cells (17). Therefore, the presence of catechol in the root methanolic extract of *H. capitata* is suspected to be involved in its anti-proliferation ability in WiDr cells.

Furthermore, 9-hexadecanoic acid and hexadecanoid acid-ethyl ester are fatty acid groups (18). They are also known as palmitelaidic acid (18), and ethyl palmitate (19), respectively. Palmitelaidic acid is similarly contained in the chloroform extract of *Aquilarria malacensis* leaves, and is thought to play a role in anti-cancer activity (20). Ethyl palmitate has also been found in the ethanol extract of *H. capitata* leaves and is thought to contribute to its cytotoxic activity in T47D breast cancer cells (3). Hexadecanoid acid-ethyl ester isolated from *Arisaema flavum* (Forssk.) Schott showed anti-cancer activity on MCF-7 breast cancer cells through inhibition of cells growth (21). Therefore, the content of this phytochemical compound in the methanolic root extract of *H. capitata* can contribute to the inhibitory of WiDr cells growth.

Ferruginol, campesterol, stigmasterol, and γ-sitosterol are phytochemical compounds reportedly contained in chloroform root extract and fraction of *H. capitata* which are thought to play a role in the induction of apoptosis and anti-metastasis in T47D breast cancer cells (4). The presence of all the three compounds in methanolic root extract can contribute as an apoptosis inducer in WiDr cells. Furthermore, ferruginol, which is a group of phenol compounds, can act as an antineoplastic (22) and induce apoptosis of PC3 prostate cancer cells, through the activation of caspase (23). Compounds such as campesterol, stigmasterol, and γ-sitosterol are plant sterols (24-27). A study reported that the intake of enriched nutrients with plant sterols can prevent the risk of breast, colon, and cervical cancer (28). The presence of plant sterols tends to also induce apoptosis and trigger a decrease in the number of reactive oxygen species (29). Campesterol and stigmasterol together with beta-sitosterol are commonly found in foods consumed by humans. Plant sterols have great potential as antiproliferative agents and can also induce apoptosis (24, 28, 30). Similarly, γ-sitosterol treatment in Caco-2 colon cancer cells and HepG2 liver cancer cells showed cytotoxic effects, with the mechanism of apoptosis induction and decreased regulation of c-myc expression (31).

Methyl stearate is a fatty acid methyl ester (32), and its role independently as an anticancer has not been reported. However, it is one of the main components identified in the methanolic extract of *Mentha spicata* L., with a cytotoxic effect on HepG2 and HCT-116 cells (33).

Retinoic acid is a derivative of retinol with the ability to control cells division and differentiation (34, 35). It tends to target one of the hallmarks of cancer, namely the ability of the cells to avoid differentiation (12). Its presence as a regulator of cells division reportedly plays a role in the inhibition of WiDr cells proliferation.

Conclusion

H. capitata root methanol extract has anti-cancer activity with anti-proliferation ability and triggers apoptosis in WiDr cells. The compounds chatecol, 9-hexadecanoic acid, hexadecanoid acid-ethyl ester, methyl stearate, ferruginol, retinoic acid, campesterol, stigmasterol, and γ-sitosterol, are thought to contribute to its anti-cancer activity. However, the cellular mechanism of the anti-proliferative and apoptotic effect should be further confirmed in future studies as well as other types of cancer cells models and *in vivo* investigations.

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Compliance with ethical standards

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

Ethical issues: None.

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