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Anticancer Activity of The Ethanol Fraction of *Gnetum gnemon* L. Seeds on HeLa Cell Lines

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Abstract: Cervical cancer results from abnormal cell development in the cervix and is caused by the Human Papillomavirus (HPV). Cancer treatment with chemotherapy drugs has dangerous side effects because it can attack normal cells. *Gnetum gnemon* L. seeds are known to possess anticancer potential. Therefore, natural ingredients are necessary to develop new cancer drug strategies with minimal side effects. This study aims to determine the anticancer activity of the ethanol fraction of *Gnetum gnemon L.* Seeds (EFGS) against HeLa cells. The research method used in this study was an identification test to detect compounds from EFGS using the TLC method and the HeLa cell cytotoxic test using the MTT Assay method. The results showed that EFGS contained stilbenoid group compounds based on TLC-Densitometry, with an Rf value of 0.22 cm. The cytotoxic test results on HeLa cells indicated an IC_{50} value of 784 µg/mL, classifying it as having the potential as a moderate category anticancer agent. Therefore, this study indicates that EFGS can be utilized as a chemopreventive agent.

Keywords: *Gnetum gnemon* L; cervical cancer; HeLa cells; anticancer

INTRODUCTION

Cancer is still one of the major health problems in the world, including in Indonesia. Cancer is a non-communicable disease characterized by abnormal cell growth that cannot be controlled, caused by cell DNA mutations.[1] One of the most common types of cancer in women is cervical cancer. Cervical cancer is an abnormal cell development in the cervix caused by the Human Papilloma Virus.[2] Based on Global Burden Cancer (GLOBOCAN) data, it is estimated that there are 604,000 new cases of cervical cancer and 342,000 deaths worldwide in 2020.[3] Various strategies have been carried out to handle and treat cancer, namely surgery, chemotherapy, and radiotherapy. Treatment using chemotherapy has drawbacks related to its side effects. The effects of chemotherapy drugs are very strong, killing cancer cells and attacking normal cells, especially cells that rapidly divide, such as bone marrow cells, causing a decrease in white blood cells.[4] Based on these reasons, it requires special attention related to the development of new cancer drugs using natural ingredients that are thought to have minimal side effects.

Natural ingredients from Indonesian plants that have the potential to be developed as an anticancer is *Gnetum gnemon* L. One of the common ingredients in *Gnetum gnemon* L. is a stilbenoid. The most commonly found stilbenoid derivatives are resveratrol.[5] Besides that, there are several other types of stilbenoid compounds named gnetin C, gnemonoside L, gnemonoside M, gnemonoside D, gnetin E, and isorhapontigenin.[6] Stilbenoid compounds are known to have anticancer potential by inducing apoptosis of cancer cells(7). Previous research conducted by Walidah et al, 2017[8] regarding the cytotoxic test of *Gnetum gnemon* L. extract on MCF7/HER2 breast cancer cells showed that *Gnetum gnemon* L. extract had anticancer potential with an IC_{50} value of 90 µg/mL. The cytotoxic potential of *Gnetum gnemon* L. extract was explored in previous studies. Researchers further investigated this potential by using fractions from *Gnetum gnemon*

L. seeds. These fractions were then tested on HeLa cells to determine their cytotoxic activity against HeLa cancer cells.

Cervical cancer drug development with in vitro study was conducted on HeLa cells using the MTT Assay method. HeLa cells are continuous cell lines derived from cervical cancer epithelial cells (cervix) of a woman named Henrietta Lacks. HeLa was the first human cell line successfully cultured. HeLa cells were central in linking the human papillomavirus to cervical cancer. HeLa cells are one of the cells that are quite safe and are commonly used for cell culture research.[9] The cytotoxic test was performed using an in vitro toxicity test with a cell culture process to detect a compound's antineoplastic ability. The cytotoxic test using the MTT Assay method is a colorimetric method based on measuring the formed formazan crystals. Dead cells lose the ability to convert MTT into purple formazan. The purple formazan crystals result from a reaction between the MTT salt and the tetrazolium succinate reductase system, which is present in the mitochondria of living cells. The formation of color serves as a useful marker to identify the amount of cell viability.[10] Based on this background, this study explored the anticancer activity of the ethanol fraction of *Gnetum gnemon* L. seeds (EFGS) against HeLa cervical cancer cells with in vitro study. This research is expected to support previous research in developing and tracing the efficacy of *Gnetum gnemon* L. as an anticancer drug.

MATERIAL AND METHOD

This study used a laboratory experimental research design consisting of identifying compounds using the TLC method and in vitro using the MTT Assay method. This research was conducted at the UMY Research Laboratory, Cells Culture Laboratory, and Pharmaceutical Technology Laboratory.

Research Instrument

Analytical balance (Sartorius), aluminum foil (Diamond), glassware (Pyrex), UV-Vis spectrophotometer (SHIMADZU), 254 nm and 366 nm UV lamps, chamber (GG), Densitometry, separating funnel (HERMA), autoclave, Laminar Air Flow (Labconco), rotary evaporator (IKA RV10), blender (Cosmos), 96-well plate (Nunc), ELISA reader (Bio-Rad), micropipette (Gilson), cell counter (Brand), Tissue Culture Flask, capillary tube (Pyrex), porcelain cup (Pyrex), CO_2 incubator, water bath (Memmert), yellow tip, inverted microscope (Zeiss), and silica gel GF_{254}.

Research Materials

Gnetum gnemon L. seeds, 70% ethanol, HeLa cervical cancer cells, ethyl acetate, chloroform, formic acid, methanol, PBS Phosphate Buffer Salin washing solution, Foetal Bovine Serum, Roswell Park Memorial Institute (RPMI) containing Fetal Bovine Serum (FBS) 15% (v/v), Penicillin-streptomycin 1.5 % (v/v), Dimethyl sulfoxide (DMSO), MTT 5 mg/ml in culture medium, Sodium dodecyl sulfate (SDS) stopper reagent in 0.1% HCL, Trypsin-EDTA, Dulbecco's Modified Eagle's Medium (DMEM), fungizone 0.25 %.

Research procedure

Extraction and Fractionation

Gnetum gnemon L. seeds are washed and dried in the sun for 5-10 days. The dry seeds were blended and sifted into fine powder. 2500 g of the powder was macerated for 5 days and re-macerated for 2 days using 70% ethanol (1:10). The extract was fractionated using the liquid-liquid method with ethyl acetate (1:1) using a separatory funnel. The part dissolved in the ethanol fraction was taken and concentrated with a rotary evaporator at 100 rpm at 60℃, followed by condensation with a water bath and the yield value was measured.

Identification test of the compound with the TLC Method

The EFGS test sample was dissolved in methanol and then spotted using a capillary tube on a GF254 silica gel plate. In a closed vessel, the mobile phase was eluted using chloroform: ethyl acetate: formic acid (5:4:1). The TLC plate was inserted into the chamber until the mobile phase was completely eluted. After that, the plate was dried and observed under visible light, UV 254, 366 nm, using a densitometry tool to calculate the Rf value.

Cytotoxic Test with MTT Assay Method

The tool was sterilized in an autoclave for 20 minutes at 121˚C with a pressure of 15 lb. The complete media consisted of RPMI, 15% FBS, 0.25% Fungizone, and 1.5% penicillin-streptomycin. HeLa cells were prepared in tissue culture flasks until confluent, then harvested and counted using a hemocytometer. The EFGS test solution was prepared at a concentration of DMSO <0.2%. Furthermore, a series of concentrations (62.5, 125, 250, 500, and 1000 µg/mL) in culture media was prepared. Cells and samples were subjected to the MTT Assay cytotoxic assay. The cells were distributed into 96-well plates, incubated for 48 hours, and then washed and incubated using reagents. Live cells reacted with MTT to form purple formazan crystals. After 4 hours, 100 µL of SDS stopper solution in 0.1% HCl was added to dissolve the formazan crystals. The mixture was left for several hours or overnight and then read with an ELISA reader at a wavelength of 595 nm.

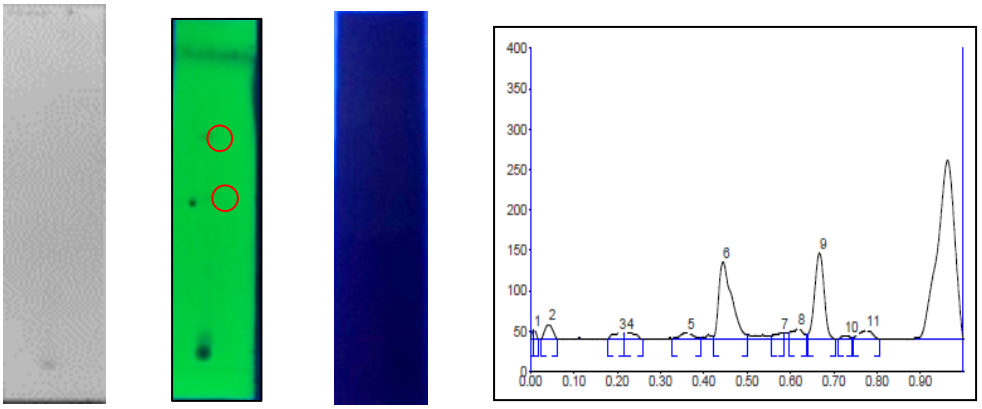
RESULT

Extraction and Fractionation

The maceration and re-maceration processes were carried out by soaking 2500 g of *Gnetum gnemon* L. seed powder using a polar solvent, 70% ethanol, in as much as 7500 mL. The extract obtained was then liquid: liquid fractionated using a semi-polar solvent, ethyl acetate (1:1), to obtain the volume of ethanol fraction as much as 5650 mL. The fraction results were then evaporated using a rotary evaporator, and a volume of 1375 mL was obtained. The evaporated fraction was thickened using a water bath, and 61.02 g of EFGS was obtained with a dark brown color and a yield value of 6.6%.

Thin Layer Chromatography

Identification of the stilbenoid group compounds contained in EFGS was carried out by the TLC method. The EFGS test stock solution was prepared using methanol solvent at 50 mg/mL concentration. TLC analysis of the extract was carried out by spotting the test solution on a TLC plate which had been eluted with the mobile phase of chloroform: ethyl acetate: formic acid in a ratio of 5:4:1 and using silica gel GF254 as the stationary phase. Silica gel GF254 is an adsorbent with universal acids, bases, or neutral properties.[11] The TLC plate was observed under UV light 254 and 366 nm. The results of the TLC-densitometry test can be seen in Figure 1. EFGS was suspected to be positive for containing Stilbenoid compounds, Gnetin C, and Resveratrol with Rf values obtained in this study of 0.22, 0.67, and 0.73 (seen in Table 1).



(a) (b) (c) (d)

Figure 1. Thin layer chromatography profile of EFGS (a) in visible light, (b) in UV 254 nm light, (c) in UV 366 nm light, and (d) Chromatogram of Densitometry

Table 1. Standard Rf values and EFGS compounds based on TLC - Densitometry

Rf Value densitometry of EFGS	Standard Rf Value	Peak Number Chromatogram of Densitometry	Compound	Information
0.22	0.25	4	Stilbenoid	+
0.67	0.67	9	Gnetin C	+
0.73	0.72	10	Resveratrol	+

Cytotoxic activity using MTT Assay

The percentage of cell viability to the EFGS treatment with various concentrations can be seen in Table 2. Table 2 shows a correlation between EFGS and the cytotoxic effect, revealing a dose-dependent pattern. The higher the EFGS concentration is, the smaller the percentage of cell viability will be.

Table 2. EFGS cytotoxic activity test results against HeLa cells

Concentration (µg/mL)	% Cell Viability
62.5	96.20
125	90.86
250	90.53
500	78.28
1000	29.70

The linear regression between cell viability and EFGS concentration can be seen in Figure 2. The equation y = -0.0698x + 104.16 with an R2 of 0.9492 was obtained. Based on this equation, the IC_{50} value was calculated. The IC50 value was obtained at a concentration of 784 µg/mL. These results illustrated that EFGS had moderate cytotoxic potential in inhibiting HeLa cervical cancer cells.

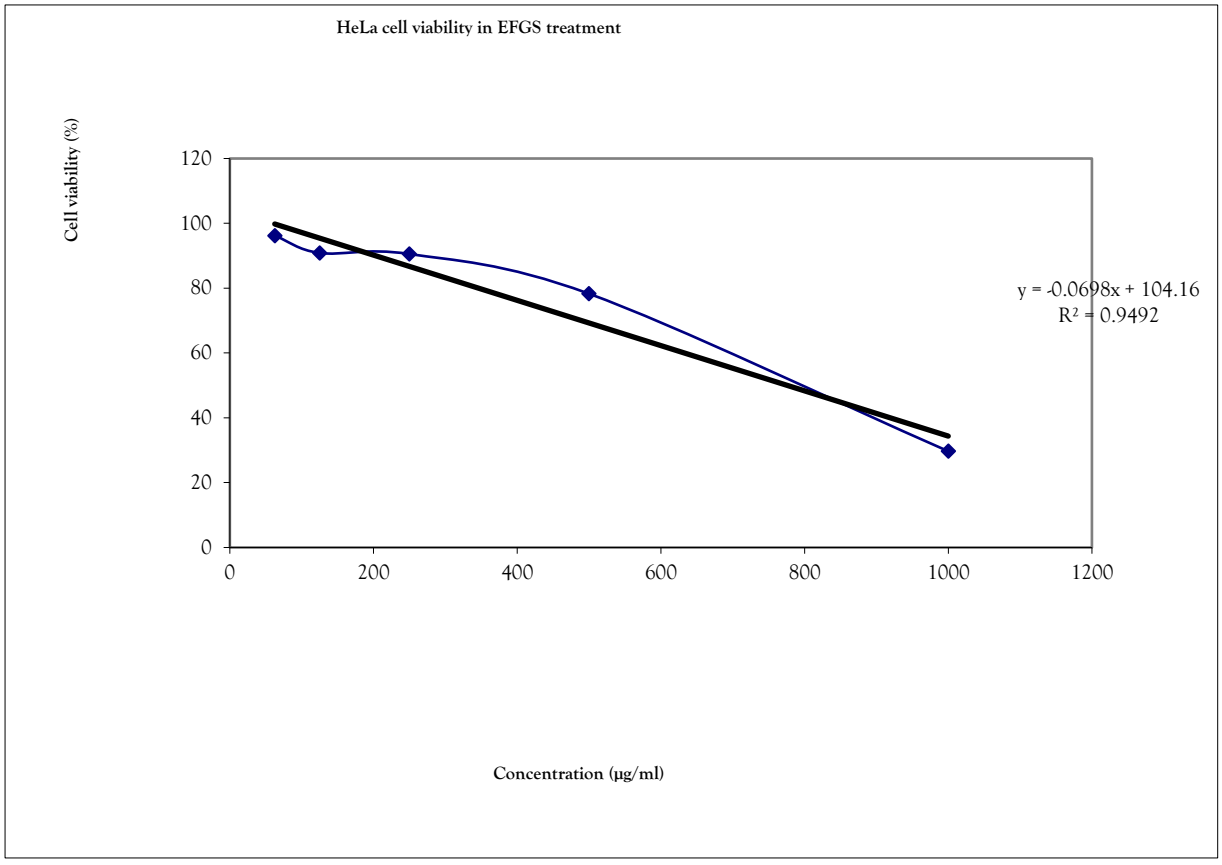
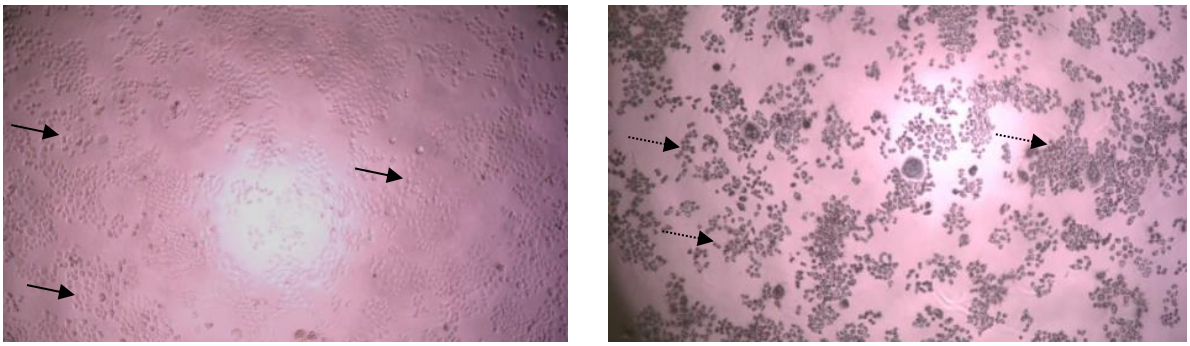


Figure 2. Graph of Cell Viability Percentage in EFGS Treatment



(a) (b)

Figure 3. Morphological Changes in HeLa Cells in Treatment with EFGS (a) Before being treated (b) After being given treatment and incubation and addition of MTT reagent

DISCUSSION

To discover new anticancer agents that may have fewer side effects or minimize resistance to current anticancer drugs, a study regarding *Gnetum gnemon* L. was conducted. Based on the data above, this study revealed the anticancer activity of EFGS on HeLa cell lines. Research begins with the extraction process using the maceration method. Maceration was carried out for 5 days and 2 days for re-maceration, aiming to maximize the active compounds' withdrawal process in *Gnetum gnemon* L. seed powder. The extracts obtained from the maceration and remaceration stages were then fractionated. Fractionation is a method of separating liquids from liquids based on their polarity. The fractionation principle with solvents depends on the distribution of solutes and a certain ratio between the two solvents that do not mix.[12] The resulting fraction was then concentrated, and dark brown-colored EFGS was obtained.

The stilbenoid group compounds contained in EFGS were identified by thin-layer chromatography (TLC). The TLC plate that has been spotted was then eluted with the mobile phase and observed under UV light with a wavelength of 254 and 366 nm. The plate would be fluorescent in 254 nm UV light, while the sample would be dark-colored. It could happen because, at 254 nm UV light, there was an interaction between UV light and the fluorescence indicator found on the plate or the stationary phase. Meanwhile, the stain would fluoresce at 366 nm UV light, and the plate was dark colored. It happened because, at 366 nm UV light, there was an interaction force between UV light and the chromophore groups bound by autochrome groups in the stains. In addition, a densitometry instrument equipped with a spectrophotometer with a light emission with a regulated wavelength of 200 - 700 nm was used. Based on the observation of TLC-densitometry results, it was suspected that EFGS positively contained stilbenoid compounds, gnetin c and resveratrol. These results refer to research conducted by Pratiwi, 2019,[13] which used the gnetin C standard with an Rf value of 0.67 cm. The resveratrol standard was 0.72 cm, and the stilbenoid group showed an Rf value of Visible Light UV Light 254 nm UV light 366 nm Densitometry by 0.25 cm.

Cytotoxicity test is a test to determine the ability of a fraction to have a toxic effect on cells at certain concentrations. One of the common methods that can be used to test cytotoxicity in vitro is the MTT assay. MTT assay is a colorimetric test to measure the metabolism activity of living cells by adding samples to each well and then incubating it for 4 hours. At the time of incubation, the yellow MTT with the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes reduce MTT so that the cells turn into purple formazan crystals. Therefore this test was used to measure the viability of cells regarding reductive activity as the enzymatic conversion of tetrazolium compounds to crystalline water formazan, which was insoluble by dehydrogenase that occurs in mitochondria.[14] In this research, the EFGS sample was tested for its cytotoxic effect on HeLa cervical cancer cells by looking at IC_{50} as a parameter describing the concentration needed to inhibit cell growth by 50%. The higher or greater the IC_{50} value is, the lower or non-toxic the compound's toxicity will be.[15] The IC_{50} value can be calculated using a linear regression equation for the relationship between concentration and percentage of cell viability. Meanwhile, the percentage of cell viability can be converted from the absorbance obtained from the measurement results at a wavelength of 595 nm. The intensity of the purple color formed is proportional to the number of surviving cancer cells. The results of the EFGS cytotoxic test on HeLa cells showed that the IC_{50} value was at a concentration of 784 µg/mL. These results indicated that EFGS had moderate cytotoxic potential in inhibiting

HeLa cervical cancer cells. There are 3 levels of cytotoxicity based on IC_{50} values, namely IC_{50} < 100 µg/mL (potential cytotoxic), IC_{50} 100-1000 µg/mL (moderate cytotoxic), and IC_{50} > 1000 µg/mL (non-toxic).[16] A compound can be considered an anticancer agent if it exhibits potential cytotoxicity; however, if the levels show moderate cytotoxicity, it can function as a chemopreventive agent, capable of preventing and inhibiting the growth of cancer cells.[17] Previous research using the ethyl acetate fraction of *Gnetum gnemon* L. seeds against HeLa cervical cancer cells demonstrated strong cytotoxic results with an IC_{50} value of 21.69 µg/ml.[18] Based on the findings of this study and previous research, it was evidenced that Genetum gnemon L. seeds had the potential to be utilized as an anticancer agent.

The shape of cell morphology is one of the characteristics that can be seen when cells experience toxicity when there is treatment by the presence of compounds or chemicals.[19] The cytotoxic activity of HeLa cancer cells can also be observed microscopically to see its morphology. The results of observing HeLa cells can be seen in Figure 3, where there are morphological differences in the cells before and after the addition of EFGS and MTT reagents. Figure 3(A) represents the morphology of HeLa cells before sample administration. In contrast, figure 3(B) shows the morphology of HeLa cells after sample administration, addition of MTT reagent, and incubation. Furthermore, the cell morphological changes may also indicate the occurrence of apoptosis.[20]

CONCLUSION

Based on the identification test of the ethanol fraction of *Gnetum gnemon* L. seed (EFGS), it was suspected that EFGS contained stilbenoid, resveratrol and gnetin C group compounds based on the TLC-Densitometry test. Furthermore, for the in vitro test, the IC_{50} value was 784 µg/mL, so EFGS was considered to have moderate cytotoxic potential in inhibiting HeLa cervical cancer cells as the IC_{50} value was in the class of 100-1000 µg/mL (moderate cytotoxic).

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CONFLICT OF INTEREST

We stated that there is no conflict of interest between the authors.

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