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Research Article

Antioxidant and Cytotoxic Activities of Melinjo (*Gnetum gnemon* L.) Seed Fractions on HeLa Cell Line an *In Vitro*

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ABSTRACT

Introduction: Medicinal plants have been investigated for possible anti-cancer effects. One of them is *Gnetum gnemon* L (melinjo). This study aims determined *in vitro* antioxidant activity and the cytotoxic effects of polar, semipolar and non polar melinjo seed fractions againts HeLa cell line. **Methods:** The melinjo seed were extracted with ethanol as a solvent. Then, the fractionation was done using liquid-liquid extraction method with three different polarity solvent. Cytotoxic activity was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay in HeLa cell lines at concentrations ranging from 25 to 400 µg/mL. Antioxidant activity was determined by the diphenyl picryl hidrazil (DPPH) radical scavenging method. **Results:** Phytochemical screening indicated the presence of phyto-constituents like flavonoids, terpenoid and tanin. The DPPH scavenging activity by the melinjo seed aqueous, ethyl acetate and N-hexane fraction was 733,12 ± 18,95 µg/mL; 68,40 ± 1,9 µg/mL and 2035,70 ± 65,59 µg/mL, respectively. The cytotoxic activity of the melinjo seed fractions showed that the ethyl acetate was the most active fraction against HeLa cell line with IC_{50} value 45,27 µg/mL. **Conclusion:** In this study, we have observed that the melinjo seed fractions exhibited antioxidant and cytotoxic activity against HeLa cell lines. This is presumably due to the content of phytochemicals and stilbenoids such as resveratrol and gnetin C. Melinjo seeds are more potent as anticancer Compared with other plants that also contain RSV such as grape extract (*Vitis vinifera* L.) against lung cancer cells (A549). From the three fractions, the ethyl acetate fraction had the highest antioxidant and cytotoxic effect compared to the water and n-hexane fractions. *Gnetum gnemon* L. can be considered as a potential source of anticancer agents. However, more research is needed to determine the mechanism of action.

Key words: Antioxidant, Cytotoxic, Melinjo, Anti-cancer, HeLa.

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INTRODUCTION

The main causative factors in the induction of various chronic and degenerative diseases including cancer is oxidative stress.[1] Oxidative stress is imbalance condition between the formation and elimination of reactive oxygen species (ROS). There is an increase in ROS production and decreased antioxidant capability of the cell.[2,3] Reactive oxygen species (ROS) are chemically reactive molecules in cell and associated with various biological processes, including cell proliferation, differentiation and programmed cell death.[3] Antioxidants nutrients have been shown to be involved in ROS detoxification.[4] Antioxidants are divided into three groups, namely the phenol, the amin and the amino-phenols.[5]

Cervical cancer is one of the most common female cancers worldwide. It is caused by human papilloma virus (HPV) infection.[6] Based on the viruses ability to promote the proliferation of infected cells and lead to the malignant transformation, HPV can be subdivided into three classifications. There are low, intermediate and high risk oncogenic potentials.[7] Persistence of infection is more common with the high-risk oncogenic HPV types, causing cervical cancer cases in about 99,7%.[6,8]

Cancer treatment involves several approaches which include: surgical intervention, chemotherapy and radiation therapy or often a combination.[9] The chemotherapeutic drugs is severely limited because of their side effects. On the other hand, natural compounds have the potential to selectively exert cytotoxic effect on cancer cells without affecting normal cells.[10] Natural compound are complex chemical molecules present in varoius parts of plant. They have pharmacological or biological activities for the treatment of cancer and other diseases.[11] One of the plant that have natural compound is melinjo (*Gnetum gnemon* L.). It is known that melinjo seed extract (MSE) contains trans-resveratrol (tRV); isorhapontigenin; gnetin C, gnemonosides A, C, and D and gnetin L.[12] Melinjo seed extract (MSE) has been reported to have a broad spectrum of pharmacological effects such as anticancer,[10] inhibitory angiogenesis,[13] antibacteria,[14] antioxidant.[15] However, its possible effects of melinjo seed fraction against cervical cancer remain uncertain. The liquid-liquid extraction method aims to classify compounds based on the level of polarity.[16] This study will determine the antioxidant activity, cytotoxic and anti-proliferative of melinjo seed fractions on HeLa cervical cancer cell line. Antioxidant activity is associated with anti-proliferative activity (cytotoxic activity) on cancer cells[17] and calculated as half maximal inhibitory concentration (IC_{50}) value.

MATERIAL AND METHOD

Materials

Red melinjo seeds was collected from Tanjung Agung Village, Teluk Betung District, Pesawaran,

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Lampung, Indonesia in March 2021. HeLa cells are collection from the Cytogenetics and Cell Culture laboratory, Faculty of Medicine, Padjadjaran University, Bandung, Indonesia.

Determination and preparation of melinjo seed fraction

Plant identified has been done by Botanical Laboratory, Faculty of Mathematics and Natural Sciences, University of Lampung. Melinjo seed was sorted, washed, and drained, then the peel was separated from its seeds. The seeds were air-dried then extracted according to[18] with modified. Dried melinjo seeds were macerated with ethanol (Merck, New York, USA.) as an extraction solvent with ratio 1:3 w/v for 2 days. The extract was evaporated in 40°C, 50rpm with rotary evaporator. The MSE was further separated by liquid-liquid extraction. The thick extract was dissolved in warm water at a ratio of 1:10, then partitioned with n-hexane and ethyl acetate. The results that obtained ethyl acetate fraction and water fraction than evaporated in 40°C, 50rpm with rotary evaporator.

Phytochemical analysis

The qualitative study of phytochemicals aims to identify chemical constituents that have pharmacological activity, namely alkaloids, flavonoids, saponins, tannins and terpenoids. The test is based on visual observation between color changes and/or the formation of a precipitate after the addition of specific reagents.[19]

Antioxidant activity determination

Antioxidant activity was determined using spectrophotometer method modified from.[9] Two milliliter samples were mixed with 2 mL 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in ethanol. The mixture was incubated at room temperature (25 ± 2°C) in dark room for 30 min. Ascorbic acid was used as positive control. Absorbance was read at 516 nm using a spectrophotometer UV/VIS Perkin Elmer Lambda 25 and the percentage inhibition was calculated with following equation:

DPPH radical scavenging (%) = [(Abscontrol − Abssample)/ Abscontrol] × 100

Where Abs control is the absorbance of Ascorbic acid and Abssample is the absorbance of the melinjo seed fractions. IC_{50} value is the concentration of the melinjo seed fractions required to inhibit 50% of DPPH radical scavenging. A scatter graph was plotted to obtain IC_{50} value.

Cell culture and cytotoxicity assay

HeLa cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml of penicillin streptomycin. The cells were cultured at 37°C in an incubator with 5% CO_2. The cytotoxic effect of the melinjo seed fraction on the HeLa cells was assessed using MTT assay. Briefly, cells were seeded in a 96-multiwell plates (2×10^4 cells/well), and treated in triplicate with various concentrations of melinjo seed fractions (25~400 µg/ml) for 24 h and then incubated with MTT reagents for 2 h at 37°C. Absorbance was measured at 550 nm. The minimum concentration of fraction that was toxic to cancer cells was recorded as the effective drug concentration. The IC_{50} values, concentrations which reduce the absorbance of treated cells by 50%, were graphically obtained from the dose response curves.

Statistical analysis

All assays were carried out in triplicates and data presented as mean ± standard deviation. EXCEL package 2016 was used for the analyses of mean, standard deviation, and percentage inhibition while linear regression analysis was used to determine the IC_{50}. To determine the effect of concentration on viability cell between treatment analyzed using ANOVA followed by LSD test with a 95% confidence level.

RESULT

Total yield of melinjo seed fractions

The total yield of melinjo seed fraction by using the solvents, namely, aqueous, ethyl acetate and n-hexane were 0,6 g; 0,18 g; 0,18 g (weight/ weight), respectively with reference to the air dried plant material.

Phytochemical analysis

Preliminary screening of melinjo seed fractions aqueous, ethyl acetate and N-hexane showed the pressence of diversity of phytochemical constituents. The aqueous and ethyl acetate fraction has flavonoids, terpenoid and tanin. And the n-hexane fraction only has terpenoid.

Antioxidant activity

In this study, the different solvent for partition between aqueous, ethyl acetate and N-hexane seed fraction of *Gnetum gnemon* L. were subjected to DPPH free radical scavenging assay. The antioxidant capacity of the fraction was compared with ascorbic acid as the standard antioxidant. The IC_{50} value of ascorbic acid was 5.42 µg/mL with a correlation coefficient (R^2) 0.999. The DPPH scavenging activity by the *Gnetum gnemon* seed aqueous, ethyl acetate and N-hexane fraction is presented in (Figure 1). The results showed that there is significant difference (*p<0,05)* between the obtained DPPH scavenging activities of the three fractions based on one way Anova test. The ethyl acetate fraction has highest antioxidant activity compare to the aqueous and N-hexane fraction.

Effect of *Gnetum gnemon* L. seed fractions on viability HeLa cell line

The result of MTT assays revealed that the *Gnetum gnemon* L. seed fractions decreased the percent viability cells but different extent. Ethyl acetat was found more cytotoxic than the aqueous and N-hexana fractions toward HeLa cancer cell line. The IC_{50} values of aqueous, ethyl acetat and N-hexana of *Gnetum gnemon* L. seed fractions against HeLa cancer cell line are represented in (Table 1). The results show that the ethyl acetat fraction of melinjo seed has highest cytotoxic activity.

This fractions also revealed the morphological changes and shrinkage of cell leading to cell death (Figure 2). Some apoptotic cells indicated with shrinkage and irregular shape, while the HeLa cell shape was polygonal and attaching to the matrix.

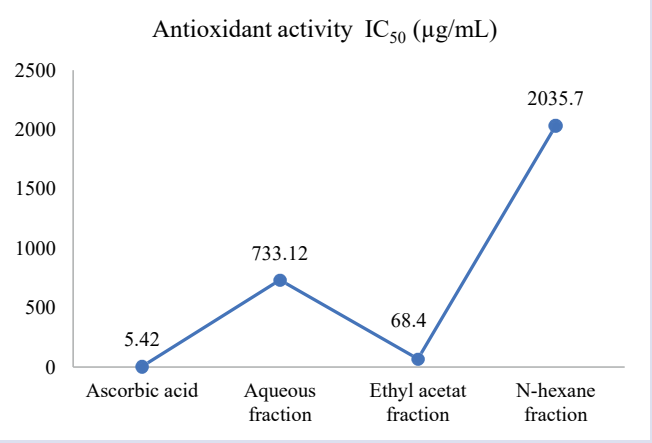

Figure 1: The values presented are mean ± standard deviation, n = 3. The results show that there is a significant difference based on the One Way Anova test at a confidence level of 5%.

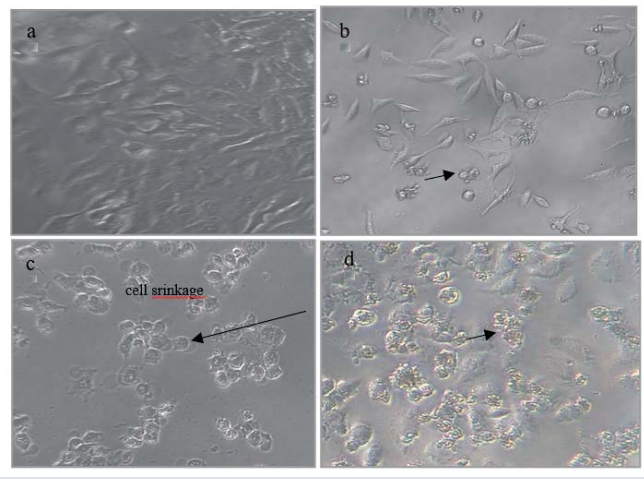

Figure 2: Morphological changes showing of HeLa cell line. (a) control cell; (b) treatment with aqueous fraction; (c) treatment with ethyl acetate fraction; (d). treatment with n-hexane fraction (magnification was 100x).

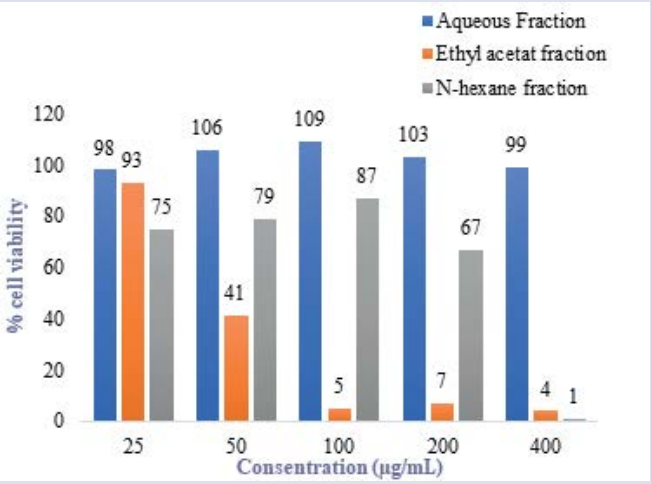

Figure 3: Cells viability after treatment with *Gnetum gnemon* L. seed fractions.

Table 1: IC_{50} value of *Gnetum gnemon* L. seed fractions against HeLa cell line (µg/mL).

Melinjo seed fraction	IC_{50} value (µg/mL)	
Aqueous fraction	No data	
Ethyl acetate fraction	45,27	$p<0,05$*
N-hexane fraction	209,06	
Doxorubicin	2,38	

The values presented are mean ± standard deviation, n = 3. The results show that there is a significant difference based on the One Way Anova test at a confidence level of 5%.

Reduction of cell viability depend on dose reduction was reported in cell treated with all fraction. The most potent in reduction cell viability was ethyl acetat fraction. Treated with *Gnetum gnemon* L. seed ethyl acetate fraction significantly enhanced of the mortality of cancer cells at the low concentration (50 µg/mL) (Figure 3).

DISCUSSION

The use of herbal medicines in the treatment of cancer due to its diversity phyto-metabolic concent has received attention.[20] Some active compounds including flavonoids, diterpenoids, triterpenoids and alkaloids are known to have anticancer effects.[21] Based on the results of phytochemical tests on the melinjo seed fraction found the chemical compounds including flavonoid, terpenoids and tannins. This compound can deactivate free radicals by donating hydrogen atoms to free radicals.[22] Hidrogen atom transfer is dominant to scavenge radical.[23] The role of antioxidants is the interactions depend on oxidative free radicals. The discoloration of DPPH indicates the antioxidant scavenging of the sample such as phenolic compounds, especially phenolic acids and flavonoids.[24] There is a relationship between ROS with oncogene function and suppression function. ROS Reduction activity will decrease MAPK activity, then decrease cFos and cJun activity. C-fos and cJun activities will have an impact decrease the activity of CDK 4 and 6 which in turn causes inhibition of the G1 phase which plays a major role in cell cycle proliferation. This can cause balance instability and predominately cause apoptosis in cells due to oncogene suppression.[25]

Based on the results of the cytotoxic test conducted on the melinjo seed fraction of HeLa cervical cancer cells, the three fractions (polar, semipolar and non-polar) were able to kill HeLa cells indicated by decrease in cell viability (%). The IC_{50} value of the ethyl acetat and n-hexane fractions obtained is <1000 µg/mL. Extracts that have IC_{50} values <1000 µg/mL have potential as anti-cancer.[26] If compared with previous studies that explored the anticancer potential of *Gnetum gnemon* L. in other cancer cells such as HT-29, Colon-26, MCF-7, DU145, PC-3 and PTEN-CaP8, the IC50 value was 35-39 g/mL.[10] This difference could be due to the content of secondary metabolites such as gnetin C (GC) and resveratrol (RSV) in the sample. The cytotoxic effects of plant are usually caused by the secondary metabolites contained in them. Several ingredients such as alkaloids, polyphenols, saponins, tannins, flavonoids and stilbenoids (GC and RSV) were found in melinjo (*Gnetum gnemon* L.).[27,28] Research with other plants that also contain RSV, such as in the study of[29] which explored grape extract (*Vitis vinifera* L.) against lung cancer cell line (A549), the IC_{50} value was 61,21±1.45µg/mL. It can be concluded that melinjo seeds have more potential as anti-cancer. As seen in the Table. 1, the ethyl acetate fraction had the highest cytoxicity effect. This possibility is because in the ethyl acetate fraction contain several active compounds such as flavonoids, triterpenoids, and alkaloids that have been shown to have anticancer effects.[21] The ethyl acetate fraction gave a higher cytotoxic effect due to the less polar flavonoid compounds. Ethyl acetate is able to dilute less polar flavonoid compounds such as isoflavones, flavonones, methylated flavones and flavonols.[22] In addition, in the melinjo seeds contain stilbenoid such as resveratrol and gnetin C. According to the research of,[30] the resveratrol content in ethyl acetate solvent was 686 mg/kg while in water and n-hexane solvent was not detected.

Melinjo seed fractions also be able to cell destruction based on their morphology (Figure 2). Normal HeLa cells are polygonal or round with extensive cytoplasm.[31] After being given the treatment, the cells became spherical and disconnected from each other and showed cellular shrinkage and nuclear condensation.[32] Cell shrinkage as a result of cell dehydration. Loss of intracellular fluid including K^+ and Cl^- causes apoptotic cell death under various inducers of apoptosis.[33] Figure 3 shows that the number of living cells decreases as the concentration and duration of exposure increase. This is presumably because the ethanol extract of melinjo seeds also contains resveratrol compounds, because resveratrol is a compound that is easily soluble in alcohol.[34] The study stated that the viability of HeLa cells treated with 20 mol/L resveratrol was reduced after 24 hours[34] and significantly reduced after 48 hours.[34]

Resveratrol can induce apoptosis through intrinsic and extrinsic apoptotic pathways. Based on research by,[35] after treatment with resveratrol, mitochondrial membrane disturbances and apoptosis-

related markers occurred, such as increase Bax/Bcl-2 ratio, and the form of caspase-8 and caspase-3. Resveratrol activates both Fas ligand-mediated and mitochondrial apoptosis in HL-60 cells by increasing Bax expression, release of cytochrome C into the cytosol.[36] This is in line with research conducted by[37] which stated that there was a decrease in mitochondrial membrane potential in HPV 16 positive cervical cancer cell lines. In addition, there was an increase in p53 expression leads to caspase-9 activation. Caspase-9 is a mitochondrial initiator in the intrinsic apoptotic pathway activated by septameric apoptosomes by mitochondrial release of cytochromes C, Apaf-1 and pro-caspase-9 and subsequent activation of caspase-3. Furthermore, activated caspase-9 can cleave and activate caspase-3. Caspase-3 as a caspase effector initiates the degradation process of apoptotic processes such as cell shrinkage, membrane blebbing, DNA fragmentation and the formation of apoptotic bodies (small cell units). Research by[38] proved an increase in caspase-3 and caspase-9 in DU145 prostate cancer cells treated with RSV. In addition to resveratrol, there is a secondary metabolite Gnetin C (GC), which is a component in the extract of melinjo seeds. It is known that the amount of GC in melinjo seeds is 28.0 mg/g.[39] Gnetin C has the ability to suppress endothelial cell function related to angiogenesis,[13] inhibit proliferation, migration.[40] Based on the *in-vivo* test, it was able to induce apoptosis through the caspase-3/7-independent mechanism (extrinsic pathway).[10]

Based on phytochemical tests, melinjo seed fraction contains tannin compounds that play role in inducing phosphorylation of the tumor suppressor p53. It can increase the expression of target genes such as p21 and BAX.[41] The inhibition of the G1/S phase transition is mainly dependent on the p21 and p27 levels. Tannins are able to inhibit the expression of p21 and p27.[42] Protein 27 is a protein that binds to cyclin and CDK so that there is an obstacle to the S phase. Tannins are able to inhibit the expression of cyclin D1, cyclin E and CDK-4.[41] On the other hand tannins can also change the expression of mitochondrial pore factors Bax, Bcl-2 and Bcl-XL.[42]

CONCLUSIONS

In the present study, we have observed that the melinjo seed fractions exhibited antioxidant and cytotoxic activity against HeLa cell lines. When compared with other plants that also contain RSV such as grape extract (*Vitis vinifera* L.) against lung cancer cells (A549), it can be concluded that melinjo seeds have more potential as anti-cancer. From the three fractions, the ethyl acetate fraction had the highest antioxidant and cytotoxic effect compared to the water and n-hexane fractions. Further studies are needed to determine the required mechanism of action.

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CONFLICTS OF INTEREST

None.

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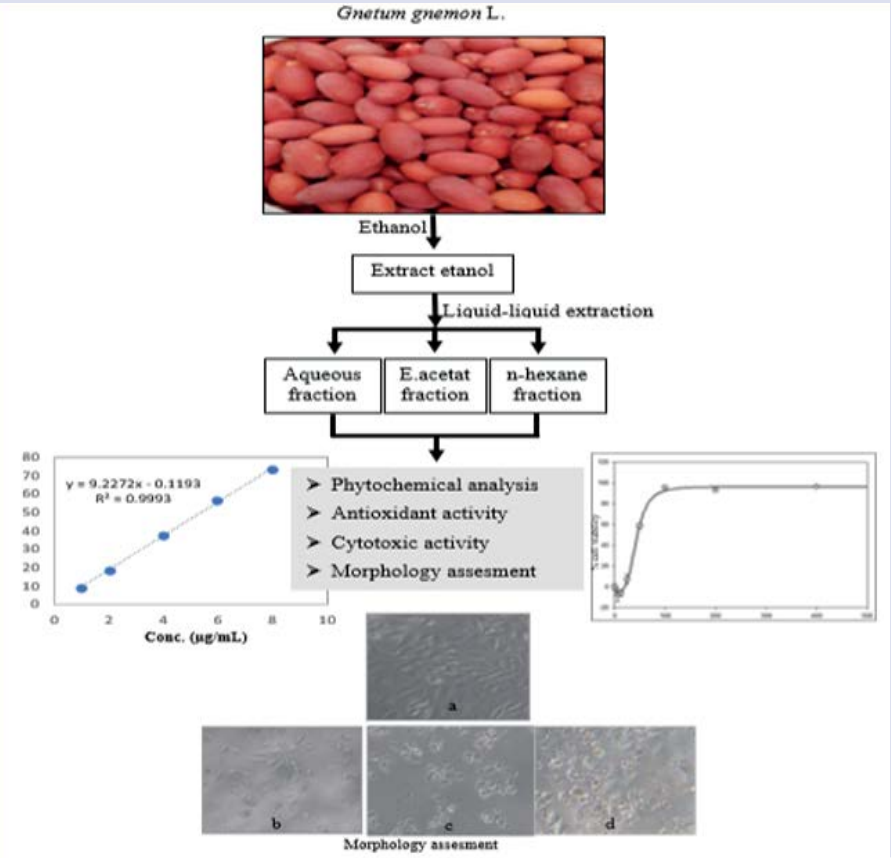
GRAPHICAL ABSTRACT



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