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Investigation of Antioxidant and Anticancer Activity againts MCF-7 and HeLa Cancer Cells of Melinjo (*Gnetum gnemon* L.)

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One of the major causes of death in the world is cancer. The cancer frequently affects in women especially breast cancer and cervical cancer. Many anticancer drugs have been developing throughout time due to the side effect of cancer treatments. Current study, plants have been extensively explore for their bioactive compound that is effective as anticancer drug candidates. Gnetum gnemon L. plant contains a bioactive compound that is beneficial for health and can be developed as an anticancer agent. The aim of this study was to investigate the potential of Gnetum gnemon L. seed extract as an antioxidant and anticancer in two cells line, MCF-7 and HeLa cells. Methods: The antioxidant evaluated through the DPPH (2,2-Diphenyl-1-picrylhydrazyl) and MTT (3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide) assays conducted for cytotoxicity. Phytochemical qualitative screening identified the flavonoids, tannins, and terpenoids. The result of the DPPH assay was 543.19 ± 11.43 μg/mL and the MTT assay with IC50 value 316 ± 45.76 μg/mL, 489.57 ± 4.03 μg/mL on MCF-7 and HeLa cells respectively. Based on these findings, antioxidant activity of the Gnetum gnemon L. seed extract against MCF-7 and HeLa cancer cells line defined as moderate cytotoxicity. The percentage (%) cell viability of MCF-7 and HeLa cells decreased as the concentration of the extracts increased. Further investigation is needed to make a quantitative analysis of these compounds and their mechanism of action as anticancer activities.

Keywords: Anti-cancer; Cytotoxicity; DPPH assay; Gnetum gnemon L.

Cancer remains one of the leading causes of morbidity and mortality globally. There are many diseases caused by oxidative stress such as cancer [1] . It occurs from a multiple-step process which includes initiation, promotion, and progression stages, called carcinogenesis [2.] The incidence rate of cancer rises to 18.1 million new cases and 9.6 million cancer deaths in 2018. From 36 different types of cancer, cancer mainly affects women in the form of breast and cervical cancer [3.] In present, various treatments i.e chemotherapy, radiotherapy, surgery, and chemically derived drugs are used as cancer treatment [4]. However, those modality treatments have some side effects [5]. Therefore, discovery and development studies were still required to find new and effective anticancer drugs [6].

Generally, plants have been used widely to prevent and treat cancer for a long period [7]. Plants medicine has been recognized as a source of potential therapeutic due to their biologically active compounds. Thus, many anticancer drugs

and antioxidants are derived from natural sources [8]. Over 60% of chemotherapeutic drugs are identified and isolated from plants or their synthetic derivatives [6]. Plant-derived agents have an important role in the treatment of cancer due to producing a wide range of chemical constituents called a secondary metabolite (9). For instance, among the many plants, *Gnetum gnemon* L. (*G. gnemon*) local name: Melinjo has been identified with the presence of secondary metabolite and polyphenols [10,11]. Melinjo (*G. gnemon*) is a gymnospermae plant native to Southeast Asia whose seeds and fruits are commonly used in Indonesian cuisine [11]. The seed flour of melinjo was evaluated for nutritional composition, antioxidant activity and functional properties. It consisted primary utilitarian groups such as: amines, amides, amino acids, polysaccharides, carboxylic acids, esters and lipids [12].

Data from preclinical studies show the potential of the melinjo seeds as antioxidants, decreasing uric acid, antimicrobial, anti-obesity, and anti-cancer [13,14,11,15]. Therefore, extensive investigation of the potential of the seeds as anticancer agents is necessary. Thus, thoroughly evaluating cytotoxic activity and screening raw extracts of the seeds would confirm these effects.

MATERIAL AND METHODS

Chemical and Reagents

The antioxidant was performed by colorimetric through DPPH assay and MTT assay were conducted for cytotoxicity. The DPPH and MTT kit was purchased from Sigma-Aldrich. All chemicals and solvents were analytical grades and obtained from commercial sources.

Materials Collection and Identification

The edible parts of *G.gnemon* were selected because they are widely consumed. *G.gnemon* seeds were harvested in March 2021 from the Pesawaran Regency of Lampung Province, Indonesia. The botanical identification of *G.gnemon* was performed at the Botanical Laboratory, Faculty of Mathematics and Natural Sciences, University of Lampung, Lampung, Indonesia.

Preparation and Extraction

Based on our previous study [16], seeds were air-dried in air shade at room temperature, then ground to a uniform powder. The powder of dry seeds G. *gnemon* (300 g) was macerated in 1.2 L of ethanol for three days and then filtered. The thick extracts were obtained by concentrating with a rotary evaporator at 40°C and stored at 4°C until being used. For qualitative phytochemical analysis, the residue was reconstituted in a solvent before testing.

Qualitative Phytochemical Analysis

The ethanol seed extract was subjected to the qualitative phytochemicals screening of some chemical compounds i.e. alkaloids, flavonoids, saponins, tannins, and triterpenoids. Mayer test for identifying alkaloids, Shinoda test, Foam test, Braemer's test, and Salkowski test for flavonoids, saponin, tannin and terpenoid respectively [17,18].

DPPH Antioxidant Activity Determination

The DPPH was used to determine the free radical scavenging activity [19, 20.] A total 2 mL of DPPH (50 ppm in ethanol) was mixed with 2 mL extract. This was incubated for 30 min at room temperature (23-25°C) and protected from light. The absorbance was measured with a spectrophotometer at 517 nm [16] then the IC_{50} value (µg/mL) determination was followed. Experiments were conducted in triplicates.

Cell Culture

MCF-7 and HeLa cell lines were used during the experiment. Cells were cultured in a complete growth medium: Roswell Park Memorial Institute (RPMI 1640) media supplemented with 10% fetal bovine serum (FBS), antibiotics (100 I.U/mL penicillin and 100 µg/mL streptomycin) at 37°C, 5% CO_2 incubator humidity. Cells were cultured in healthy conditions and exponentially growing cells (-80% confluency) were used for experiments.

Cytotoxicity Assay

The cytotoxicity of the ethanol extract on MCF-7 and HeLa cells was measured using an MTT assay. The cells were seeded in 96-microwell plates a density of the cell $2x10^4$ cells/well then incubated for 24 hours (h). A total 100 µL amount of extract was added to test wells and the microplates were incubated for 24 h. In addition, removing the supernatant and add 20 ìL of MTT solution to each well and incubated for 3 h. The supernatant was added with 100 ìl DMSO and dissolved formazan crystals. The amount of formazan crystal was measured at wavelength 570 nm [21]. The percentage

of the cell viability of the cells treated with ethanol extract was calculated according to the formula:

$$\% \text{ Cell viability} = (Abs_{sample} - Abs_{control})/ Abs_{control} x100\%$$

The inhibitory concentration (IC) 50 value was calculated from a graph plotting % cell viability against the concentration of the sample.

Observation of Morphological Changes

MCF-7 and HeLa cells were seeded in 96-microwell plates at the density of the cell $2x10^4$ cells/well then incubated for 24 h. They were then treated with the extract and cell morphology was observed and analyzed after 24 h incubation.

Statisctic Analysis

All data analysis were performed using Microsoft Excel software 2016 and the result was presented as mean ± SD with triplicate (n=3).

Tabel 1. Qualitative Phytochemical screening of *G. gnemon* L. seed extracts

Constituents	Test	Result
Alkaloid	Mayer test	-
Flavonoid	Shinoda test	+
Saponin	Frothing test / Foam test	-
Tanin	Braemer's test	+
Terpenoid	Salkowski test	+

Abbreviations: (+), present;("), absence; N, Not indicated.

RESULTS

Sample extraction

The *G.gnemon* seeds extract was obtained by evaporation of ethanol as a solvent and the percentage by weight yield was estimated at 0.55% based on the solvent used. The qualitative phytochemical investigation result found flavonoid, tannin, and terpenoid (Table 1).

DPPH Antioxidant Activity Determination

The most common method to determine the antioxidant activity of various compounds is the DPPH assay [22]. This assay was measured by changing the purple ethanol solution of DPPH. The antioxidant agents can convert DPPH into *1-1 diphenyl-2-picryl hydrazine* in the yellow molecule, by transferring electrons or hydrogen [8]. The result of antioxidant activity as mean ± SD was 543.19±11.43 µg/mL in 3 replications (Figure 1). The ascorbic acid as a standard with a coefficient correlation (R^2) of 0.9993 has IC_{50} 5.42 ± 0.009 µg/mL.

Cytotoxicity assay

The cell viability on MCF-7 and HeLa cells was assessed in vitro through MTT assay. The extract is revealed as moderate cytotoxicity (Figure. 2). According to ISSO: 10993-5 standard, percentages of cell viability over 80% are considered as non-cytotoxicity; within 80%–60% weak; 60%–40% moderate and underneath 40% strong cytotoxicity respectively following above

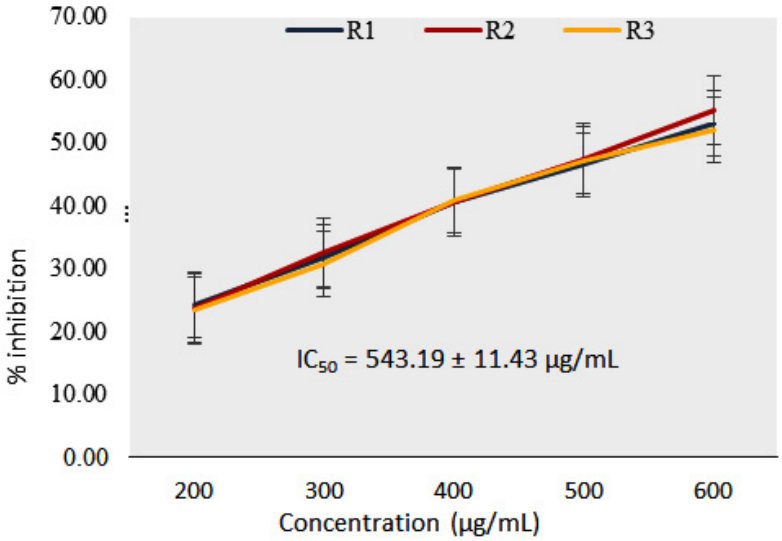

Fig. 1. The IC_{50} of ethanol *G.gnemon* L. seeds extract in DPPH assay (mean ± SD)

80% as non-cytotoxicity, within 80-60% as a weak, 60-40% moderate and below 40% strong cytotoxicity [23]. Base on Figure. 2 indicated that after being treated with the extract for 24 h, significantly decreased the % cell viability ($p<0.05$) at the final concentration, up to 47.55% and 62% for MCF-7 and HeLa cells respectively.

The cytotoxicity of the extract (IC_{50}) was determined in comparison to the anticancer drug doxorubicin (Table.2). The IC_{50} values obtained refer to 50% of cells inhibited by extracts. Based on Table 2. Shown that *G.gnemon* extract has IC_{50} 300-400 µg/mL for both cancer cells. Moreover, the extract indicates moderate cytotoxic (100-1000 µg/mL) [24].

Observation of Morphological Changes

We evaluated the morphology changes of both breast cancer and HeLa cells with microscopically examined after 24 hours given extract treatment. A major change in cell morphology was observed in cell shrinkage, cell wall blebbing (Figure.3)

Morphological changes were revealed after MCF-7 and HeLa were treated with *G.gnemon* seeds extract compared to untreated cells. Figure (A) shows that untreated MCF-7 cells appear healthy, normal, and confluent cells while treated cell (B) it appeared that cell vacuolization was formed, and the cells appeared to be smaller and rounded. Crude extract of *G.gnemon* also induced cell death in HeLa cells. HeLa cells without treatment showed a flat polygonal shape with good permeability, complete nucleoli, clear and adherence (C) while in treated cells (D), the cell was observed shrinkage, the shape became fusiform cells or long circles, and some cells had started to undergo apoptosis.

DISCUSSION

There are many anticancer drugs that have been developed over time. However due to side effects of cancer treatments, a new anti-cancer substance that is more effective and less side effects

Table 2. Result of cytotoxicity against MCF-7 and HeLa cell lines

	Cytotoxic activity (IC_{50}, µg/mL)	
	MCF-7(mean ± SD)	HeLa(mean ± SD)
G.gnemon extract	316.19 ± 45.76	489.57 ± 4.03
Doxorubicin	0.14 ± 0.12	2.38 ± 3.5

The IC_{50} in MTT assay (mean ± SD) of three independent experiments, n=3

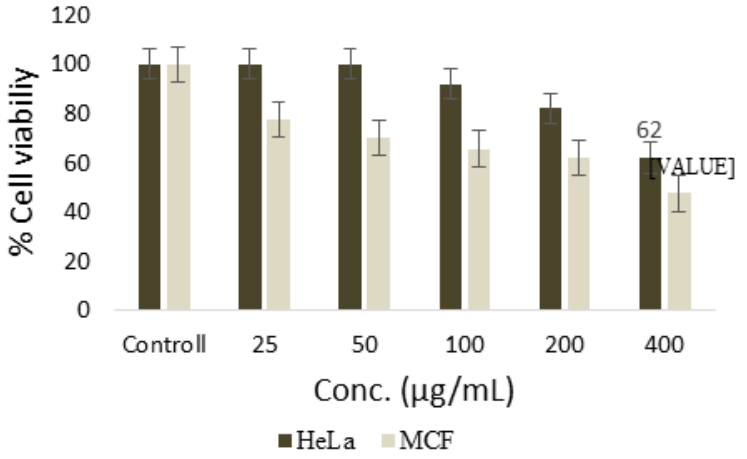

Fig. 2. Decreased % cell viability of MCF-7 and HeLa cancer cell line by *G.gnemon* extrats

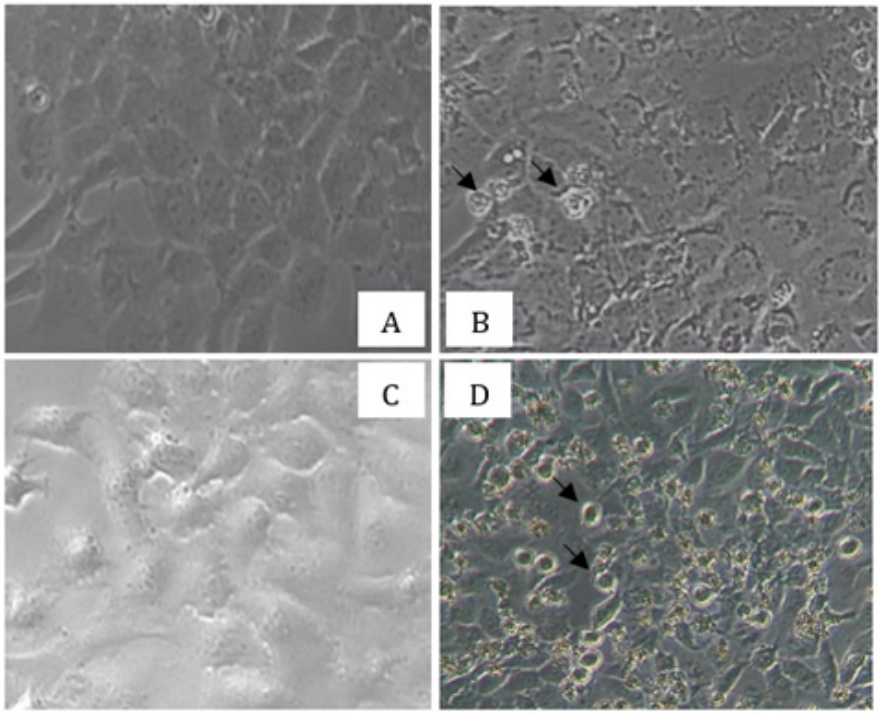

Fig. 3. Microscopic observations. *G.gnemon* seeds extract effect on MCF-7 and HeLa cells.
(A) MCF-7 untreated cells, (B) MCF-7 treated cells, (C) HeLa untreated cells, and (D) HeLa treated cells. Observation of cells using an inverted microscope (200x magnification).
The arrow indicates that the cell is apoptosis.

is needed. A large number of plants are well known as alternative medicine that considered less side effects. Our previous study has been conducted several plant medicines including robusta coffee [25]**,** *Polygonum pulchrum* [26]**,** *Annona muricata* Linn [27]**,** *Jatropha gossypifolia* [28]**.** In the recent study, a part of species *G.gnemon* L was used to evaluate for its antioxidant and anticancer activity. These plants had been intensively studied for their therapeutic properties. We observed the presence of various phytochemicals (Table 1) and these phytochemicals showed antioxidant and anticancer activities. Phytochemical compounds of natural products as antioxidants have a main role as radical scavenging that produce certain diseases**.** The reactive oxygen species (ROS) is a chemically reactive molecule in cells and associated with a variety of biological processes, such as cell proliferation, differentiation and programmed cell death ROS has a relationship with the oncogene function and suppression function [29]. Concisely, ROS will results in G1 phase inhibition leading to a significant influence on cell proliferation [16]. Antioxidant presence deactivates free radicals by donating hydrogen atoms to free radicals that are dominant to scavenge radicals [30]. Those are explanations of how antioxidant prosperity toward on underlying cancer mechanism. An uncontrolled increase in cell proliferation and decreased cellular apoptosis are characteristics of cancer. Approaches for treating cancer should be apoptosis induction and growth inhibition of tumor cells [31].

Some parts of *G. gnemon* plant are known to have anticancer activity, especially in leaves and seeds. Recent studies there were several reports the extract of *G. gnemon* leaves was indicated have high IC_{50} values. The ethanol extract of *G. gnemon* leaves on previous studied showing the IC_{50} values was at high potential toxicity [32]. Moreover, Bioactivity assay of *gnetumal* and *p-coumaric acid* as a bioactive compound of this plant indicated possessed more potent tyrosinase inhibitory activity, with IC_{50} values of 31.6 and 2.3 mM, respectively, than that of a positive control

kojic acid [33]. In our study presented *G.gnemon* seed extract have anticancer activity as moderate IC_{50} values. A different result was shown by Narayan et al. which presented IC_{50} value as potential toxicity [34]. We presumably it could cause the different type and specification of sample testing, in which the amount of compound was known. The preparation sample testing would expressed a different result. The specific isolation compound of plants can understand clearly the mechanism and further investigation on anticancer candidates. Such as like our studied that performed the bioactive from Robusta Coffee such as caffeine and chlorogenic acid against on cell line Hep-G2 [35,36].

The antioxidant activity were carried out to assess the capacity of plant extracts to scavenge free radicals. Compare with the previous study regarding the antioxidant activity of melinjo seed have been reported by Supriyadi *et al*. tested with the *2,22 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid* (ABTS) assay, this study shown that the melinjo seed has very weak category for antioxidant activity [37]. This may be due to differences in the method of the assay. Extracts showing low antioxidant that measured use one method should not be discarded as poor sources of antioxidant without having been compared with other methods [38]. Phenol compounds in the melinjo seed is the main component to have the potential for antioxidant activity [39]. High levels of phenol could be considered as a good source of antioxidants that will act in the prevention of many diseases e.g carcinomas [40].

According to the literature, mechanism of action flavonoids are anti-cell proliferation, induced apoptosis and cell cycle arrest, inhibition angiogenesis and suppression of metastasis [41, 42]. Terpenoid through induced apoptosis intrinsic pathway [43]. Tannins induced G1 arrest, phosphorylation of the tumor suppressor protein p53 [44]. As known *G. gnemon* has several anticancer activities with a certain mechanism such as inhibits endothelial senescence [45]. In our report studied that fraction of *G. gnemon* seed showed antioxidant and cytotoxic activity against HeLa cell lines [16]. In vivo studies exhibited *Gnetin C* as another bioactive compound that has lead activity over the stilbenoid. *Gnetin C*-treated tumors showed reduced mitotic activity and angiogenesis and a significant increase in apoptosis compared to all the other groups. The data suggest that *Gnetin C* is more vigorous in declining tumor progression in prostate cancer xenografts than Res or Pter [46]. Beside phytochemical compounds, these plants are rich in resveratrol and gnetin C content [10]. Resveratrol is a natural polyphenolic phytoalexin that has been shown to have antioxidant activity and anticancer properties. Resveratrol suppresses cell proliferation and induced apoptosis through mitochondrial and p53 signaling pathways in human cervical carcinoma [47]. In a previous study, *gnetin C* induced apoptosis through inhibits the mTOR and MAPK pathway in acute myeloid leukemia (AML) [48]. Morphological changes of the cancer cell are beginning event during the induction of apoptosis i.e. cell blebbing, shrinkage, nuclear fragmentation, chromatin condensation, and so on [21, 49,] in this study presented in Figure 3.

Recently, some phytochemical compounds were identified by qualitative methods and the anticancer activity of the extract was classified as moderate cytotoxicity. These studies exhibit that *G. gnemon* is a natural source with potent activity as anticancer candidates. Many bioactivities of this plant need to be investigated and developed with in vitro and in vivo assay. Further investigation needs to be performed quantitative analysis and mechanism of action as anti-cancer of these plant compounds.

CONCLUSION

In the light of these findings, it is apparent that *G. gnemon* or melinjo seeds could be considered as a vital source of antioxidants. It is observed that extract of *G. gnemon* had significant anticancer activity in different cancer cell lines using in vitro models. We investigated the phytochemical, antioxidant and cytotoxicity of the *G. gnemon* seed extract against the MCF-7 and HeLa cells line. Our findings revealed that the extract was of moderate cytotoxicity. The % cell viability of MCF-7 and HeLa cells decreased as the concentration of the extracts increased.

Conflict of Interest

There is no conflict of interest.

Funding Source

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