Cytotoxic and Antiproliferative Activities of *Melia azedarach* Leaves Ethanolic Extract on A549 Human Lung Cancer Cells

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ABSTRACT

Melia azedarach (Meliaceae) is known locally as *mindi, gringging, renceh,* or *cakra-cikri* is known to have pharmacological properties. The leaves contain flavonoids such as kaempferol and quercetin that have anticancer activity. The objective of this research is to evaluate the potency of *Melia azedarach* leaves ethanolic extract as anticancer by inhibition of cancer cell proliferation. Cytotoxic effect was analyzed by Brine Shrimp Lethality Test (BSLT) and cell viability (MTT) methods using Chang human normal liver cells and A549 human lung cancer cells. Antiproliferative effect of the extract was analyzed by cell direct calculation method using hemacytometer. The leaves were extracted with ethanol 96% by maceration method. Phytochemical investigation showed that the extract contains flavonoids, alkaloids, tannins, saponins, and steroids. Assay of BSLT showed that crude ethanolic extract of *M. azedarach* has a cytotoxic effect with LC_{50} value of 63.98 µg/mL, which is an indication for very potential bioactive compund as anticancer. Consistent with BSLT assay, cell viability (MTT) assay showed that this extract was able to reduce cell viability with IC_{50} values of 299.22 µg/mL on Chang cells and 130.56 µg/mL on A549 cancer cells. As control, *Curcuma zedoaria* extract at 75 µg/mL reduced A549 cells viability to 38.8%, which was equivalent to the effect of *M. azedarach* extract at 50 µg/mL. These data suggest that *Melia azedarach* extract is potentially more bioactive than *Curcuma zedoaria* extract. This result is supported by the ability of the extract (5 µg/mL) to inhibit A549 cells proliferation as much as 73.53%.

INTRODUCTION

Cancer is a malignant tumor which is characterized by the abnormal body cells growth with the potential to invade or spread to other body parts (Vij et al. 2014). WHO (2014) reported that 8.2 million people worldwide died of cancer in 2012. Lung cancer ranked first about 19%, followed by liver cancer 9%, stomach cancer 8%, colon cancer 7%, and breast cancer 6%. About 90-95% cases of cancer are caused by environmental factors and 5-10 % due to genetic factors. Environmental factors which could increase the risk of developing cancer are smoking, diet, alcohol, infections, radiation, stress,

lack of physical activity, obesity, and pollutants (Anand et al. 2008).

One of the alternatives for cancer therapy is by utilizing the medicinal plants which have anticancer activities. Exploration of anticancer herbal with high effectiveness and minimum side effects need to be developed. One of the plants that potentially contains anticancer compounds is *Melia azedarach* (Pan et al. 2014). This plant is versatile because it can be used for various purposes including health remedy (Ahmed et al. 2012). The Chinese often use it as a raw material of traditional medicine for treatment of hypertension and anthelmintic (Asadujjaman et al. 2013). According to Azam et al. (2013) and Bhargava (2013), all parts of the plant which includes the root, bark, leaf, fruit, and seeds contain a variety of active compounds that are thought can be potential as cardioprotective, antioxidant, antimicrobial, antiulcer, analgesic, anticancer, antipyretic, antiplasmodial, and anti-inflammatory.

Aoudia et al. (2013) reported that *M. azedarach* leaves ethanolic extract contained kaempferol and quercetin. Both of these compounds were the flavonoids that have anticancer activity (Kuldeep and Eisha 2014). These compounds prevented oxidative damage in the body's cells, lipids, and DNA that can increase the risk of developing cancer (Calderon et al. 2011). According to Cui et al. 2008, consumption of kaempferol was correlated with a decrease in the incidence of lung cancer of the smokers as well as non-smokers. This compound inhibited the proliferation of lung cancer cells and caused naturally programmed cell death. Quercetin inhibited the proliferation of various cancer cell lines, one of which was A549 cells (Ren et al. 2003).

Although, *M. azedarach* leaves have been known to contain bioactive compounds, the scientific information on the benefits of *M. azedarach* leaves as anticancer is still limited. The objective of this research is to evaluate the in vitro potency of *M. azedarach* leaves extract as anticancer on human lung cancer cell lines. This paper reported the cytotoxic and anti-proliferative activities of *M. azedarach* leaves extract on A549 human lung cancer cells.

METHODS

Materials

M. azedarach leaves were obtained from Biopharmaca Research Center, Bogor Agricultural University, Bogor. Cysts of *Artemia salina* Leach (*A. salina* Leach) and sea water were obtained from Faculty of Fisher and Marine Science, Bogor Agricultural University. A549 human lung cancer cells (ATCC-CCL185) and Chang human normal liver cells (ATCC-CCL13) were obtained from ATCC (American Type Culture Collection). *Curcuma zedoaria* extract as positive control of cytotoxicity assay by viability (MTT) method were obtained from UD. Rachma Sari. DMSO, $NaHCO_3$, trypsin, tripan blue, penicillin-streptomycin, FBS (fetal bovine serum), PBS (phosphate buffered saline), DMEM (Modifed Dulbecco's Eagle Medium), and tetrazolium (MTT) were obtained from Sigma Aldrich. The other materials were ethanol 96%, methanol, H_2SO_4, HCL, chloroform, ammonia, Dragendrof, Mayer, and Wagner reactans, acetic acid anhydride, $FeCl_3$, diethyl ether, , aluminum foil, and Whatmann filter paper No. 1.

The tools which were used were oven, blender, micropipette, serological pipette, digital balance, rotary evaporator, water bath, spectrophotometer UV-Vis (Genesis 10v), lamp, aquarium, aerator, lup, BSLT plate, microplate 96 (Bio-Rad), plate 12 (Bio-Rad), Biosafety cabinet class II, sentrifuse (Flexpin), incubator CO_2, flask, microplate reader (Bio-Rad), hemacytometer, and microscope (Nikon).

Preparation of Extract (BPOM 2005)

The leaves were collected, washed, and air-dried for seven days at room temperaturre. The dried leaves were stored in an oven at 40°C until the constant weight was obtained. Subsequently, the simplicia was made into powder using a blender. The powdered simplicia were extracted with ethanol 96% by maceration method in volume rasio of 1:10 (v/v) for two days at room temperature. The filtrate was evaporated using a rotary evaporator at 50°C.

Phytochemical Assay (Seth and Sarin 2010)

Flavonoids. 0.1 g of extract was mixed with 2 mL of methanol and was heated for 5 minutes at 80°C. The filtrate was mixed with 0.01 g of $Mg.3H_2O$, and then mixed with 1 mL of H_2SO_4 and 1 mL of amylalcohol. The formation of red or orange colour on lining of amylalcohol showed the presence of flavonoids.

Alkaloids. 0.5 g of extract was mixed with 10 mL of chloroform and 2 drops of ammonia. Chloroform fraction was separated and acidified with 1 ml of H_2SO_4. The fraction of H_2SO_4 was taken and added with

Dragendorf, Meyer, or Wagner reagent. Alkaloids presence was characterized by precipitates of the following colours: white by Meyer, red by Dragendorf, and brown by Wagner reagents.

Tannins. 0.1 g of extract was mixed with 5 mL of water, heated for 5 minutes at 80°C, and then mixed with 3 drops of $FeCl_3$. The formation of green indicated the presence of tannins.

Saponins. 0.1 g of extract was mixed with 5 mL of water, heated for 5 minutes at 80°C, and then shaked until foamy. The existence of a stable foam for 15 minutes showed the presence of saponins.

Steroids and terpenoids. 0.5 g of extract mixed with 25 mL of ethanol, heated for 5 minutes at 80°C, and then filtered. Filtrate was evaporated and mixed with 1 mL of diethyl ether. Diethyl eter layer was mixed with Lieberman-Buchard reagent. The formation of blue or green indicated the presence of steroids and red or purple indicated the presence of terpenoids.

Brine Shrimp Lethality Test (BSLT) method (Syafii et al. 2014)

Cysts of *A. salina* Leach were weighed about 50 mg, put in an aerated container which contained sea water and then incubated for 48 hours under artificial light. Cysts of *A. salina* Leach were hatched into larva after 24 hours. Larva of 48 hours old *A. salina* Leach were used for this assay. About 10 of *A. salina* Leach larva were put into the vial containing sea water and extract were added in final concentrations of 9, 22.5, 45, 90, 180, 360 µg/mL, and 0 µg/mL (control). Observation was made after 24 hours of incubation. The numbers of larva which lived were counted and expressed as % mortality value using the formula:

$$\% \text{ Mortality} = \frac{\text{Lcontrol} - \text{Lsample}}{\text{Lcontrol}} \times 100\%$$

L = The numbers of larva which lived

Cell Culture (Doyle and Griffiths 1999)

The growth medium was made by preparing DMEM powder in sterile bottles and adding 3.7 g of $NaHCO_3$, penicillin-streptomycin 1 % , and FBS 10%, homogenized and the volume was made into 1000 mL with double-distilled water. Chang human normal liver cells and A549 human lung cancer cells were grown using flask in DMEM growth medium until confluence in air/CO2 (95%/5%) incubator at 37°C. The cells were subcultured using trypsination method. Briefly, the medium was discarded and rinsed with PBS solution, then 5 mL of trypsin was added and followed by incubation at 37C for 5 minutes. The reaction was stopped by addition of 4 mL medium. The suspension was centrifuged for 5 minutes at 700 g. The supernatant was discarded and the pellet (cells) was mixed with 5 mL of medium. Subsequently, the number of cells were calculated using a hemocytometer; for cell viability assay 5000 cells/well were added each in 100 µL volume into a 96 well-microplate and 100,000 cells/well each in 2000 µL volume into a 12 well-microplate for antiproliferative assay. All cell cultures were incubated for 24 hours in a 5% CO_2 incubator at 37°C before the experiment.

Viability (MTT) Assay (CCRC 2009)

In this assay, both Chang and A549 cells were incubated with or without extracts at final concentrations of: 0, 5 10, 15, 25, 50, 100, 250, 500, 800, and 1000 µg/mL in total volume of 100 µL each well. The *Curcuma zedoaria* extract was added at 75 mg/mL as positive control. All cell cultures was incubated for 48 hours in a 5% CO_2 incubator at 37°C. Subsequently, tetrazolium (MTT) 5 mg/mL was added in aliquots of 10 µL/well which turned the cell medium into yellow colour. All cells were incubated for 4 hours in 5% CO_2 incubator at 37 ° C. After incubation, the cell medium was discarded and 100 µl of ethanol 96% were added into each wells. The absorbance values was measured using a spectrophotometer at 595 nm wavelength. The results were expressed as % inhibition value using the formula:

$$\% \text{ Inhibition} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100\%$$

A = Absorbance

Antiproliferative Assay (CCRC 2009)

Following 24 hours incubation, medium of A549 cell cultures were discarded, and the cells were incubated with extract at concentration of 5 µg/mL (based on the results of cytotoxicity assay on Chang and A549 cells). This experiment was carried out for 24, 48, and 72 hours. At the end of experiment, the cells were harvested by trypsination method as mentioned earlier. The cell suspension was centrifuged for 5 minutes at 700 g. The supernatant was discarded and the cell pellet was mixed with 5 mL of medium. The

number of cells were calculated using a hemacytometer.

Statistical Analysis

Results are expressed as means of triplicate dishes at each point±SEM. Statistical significance was analyzed with one-way ANOVA.

RESULTS AND DISCUSSION

The M. *azedarach* leaves ethanolic extract yield was 26.96 ± 0.12 %. Results on the qualitative analysis of phytochemical constituents of *M. azedarach* leaves extract were showed in Table 1. The extract contained flavonoids, alkaloids, tannins, saponins, and steroids but did not contain terpenoids. One of the phytochemical constituents of plants which have been known as antiproliferative agent is flavonoids. Flavonoids is one of the kinds of polyphenols which was contained in plants (Doughari et al. 2009). Aoudia et al. (2013) reported flavonoids which contained in *M. azedarach* leaves ethanol extract were: kaempferol-3-O-β-rutinosida, quercetin-3-O-β-D-glycoside, quercetin-C, kaempferol-C, and rutin.

The BSLT cytotoxicity assay showed that the extract was cytotoxic with LC_{50} value of 63.98 µg/mL (Figure 1). This is an indication for very potential bioactive compund anticancer because of the very small LC_{50}<1000 µg/mL (Mudi and Salisu 2009, Emrizal et al. 2014). This test was done as early screening for anticancer prior to in vitro assay using cell cultures (Oktaviani et al. 2013). Syafii et al. (2014) and Jafari et al. (2013) reported that BSLT test correlated with cytotoxic and antiproliferative activities on cell cultures. The lower LC_{50} value of the extract, the higher the cytotoxicity and antiproliferative effects on cell cultures.

Table 1. Phytochemical constituents of extract

No.	Constituents	Extract
1	Flavonoids	+
2	Alkaloids	+
3	Tannins	+
4	Saponins	+
5	Steroids	+
6	Terpenoids	-

+ (The presence of phytochemical constituents) and
- (The absence of phytochemical constituents)

The cytotoxicity assay using cell viability (MTT) method is shown in Figure 2. The *M. azedarach* extract showed a cytotoxic effect on both normal Chang and cancer A549 cells at all concentrations Both types of cells decreased their viability upon incubation with this extract. The IC_{50} value of the extract were 299.22 µg/mL on Chang cells and 130.56 µg/mL on A549 cells. This result shows that both type of cells are good cell model for bioactive potency of this extract. However, the A549 lung cancer cells were more susceptible than the Chang normal liver cells as showed by smaller IC_{50} value. The similar observation had been reported by Schnablegger (2010) on his study using some cancerous cell lines and normal peripheral blood monocytes. The susceptibility of the cancer cell lines to bioactive components was due to the higher proliferative activity.

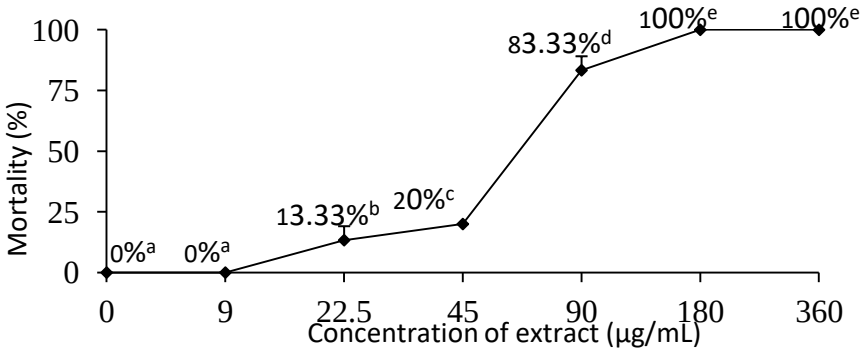


Figure 1. Result on the BSLT assay of extract. Asterisks illustrate statiscally significant differences between concentrations and mortality

On the contrary, extract of *C. zedoaria* which served as positive control (K+) only bioactive to A549 cancer cells. At 75 µg/mL, this extract reduced A549 cells viability to 38.78 %, which was equivalent to the effect of *M. azedarach* extract at 50 µg/mL. These results suggested that *M. azedarach* extract was potentially more effective than *C. zedoaria* extract as anticancer. This is consistent with report by Lakshmi et al. (2011) on the effect of isolated isocurcumenol from *Curcuma zedoaria* rhizomes on human and murine cancer cells. *Curcuma zedoaria* was found to inhibit the cancer cells without inducing significant effect to the normal cells. This lack of bioactive effect on normal cells could be due to the absent of the mechanism(s) that were otherwise present only in cancer cells.

Figure 3 shows the results of antiproliferative assay. The extract at 5 µg/mL could inhibit A549 cells proliferation as much as 73.53% after 72 hour incubation. This inhibition was quite significant since it was caused by crude extract. The inhibition of A549 cells growth by mindi extract could be due to the flavonoids content of the extract which decreased the cell proliferation ability. Flavonoids extract from *Gymnostemma pentaphyllum* was reported to inhibit A549 cells proliferation by 92% (Tsui et al. 2014). They reported that flavonoids from *Gymnostemma pentaphyllum* inhibited A549 cells proliferation by cell cycle arrest and induction of apoptosis. Flavonoids

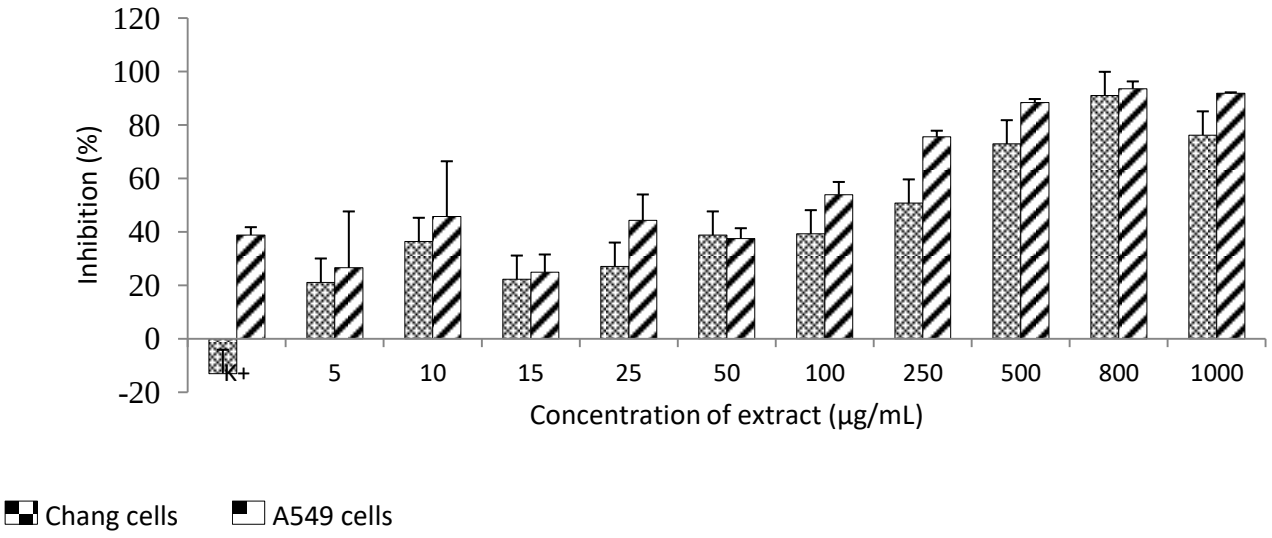


Figure 2. Result on MTT assay of extract on Chang and A549 cells viability. Asterisks illustrate statiscally significant differences between concentrations and inhibition activity

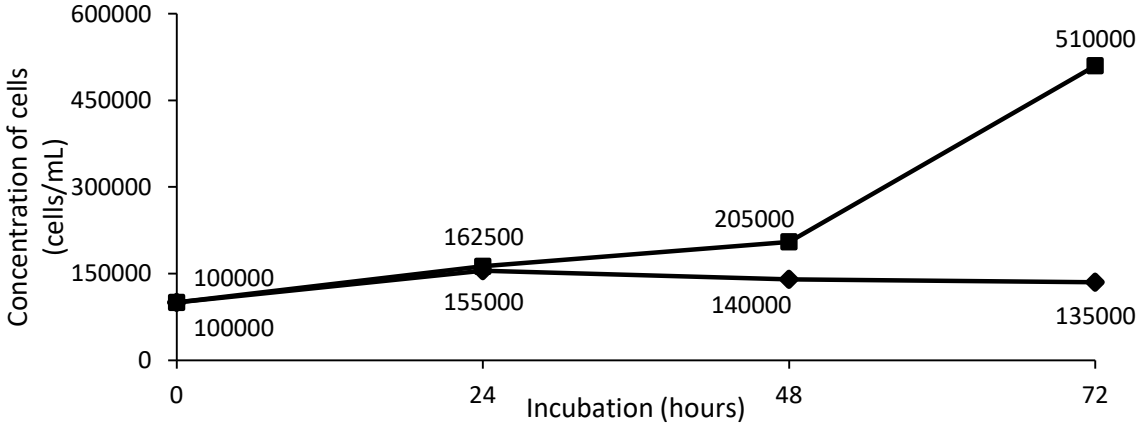
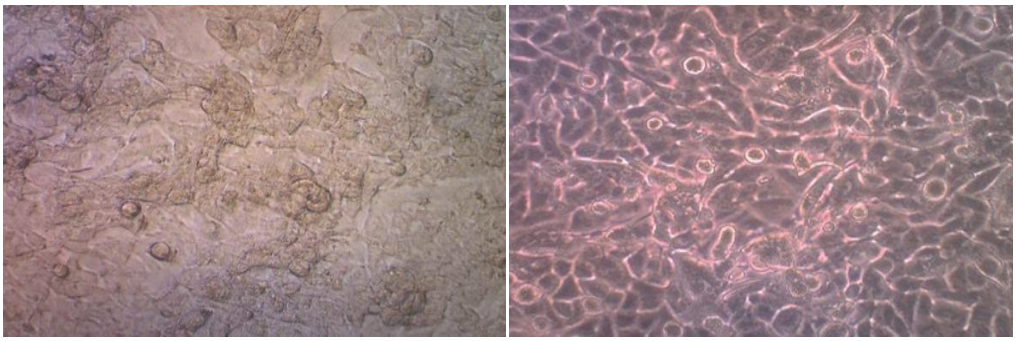


Figure 3. A549 cells proliferative cell (—◆—) with extract of 5 µg/mL and (—■—) without extract



(a) (b)

Figure 4. (a) A549 cells with extract at 5 µg/mL dan (b) A549 cells without any extract

inhibited the cyclin dependent kinase (Cdk) which is the regulator of the cell cycle (Rao et al. 2012). The action point of flavonoids were inhibition of CAK (Cdk-Activating Kinase) enzyme so inhibited the formation of Cyclin-Cdk complex. This caused a series of signal transduction to be stopped and the cell cycle was stopped at G1 phase (post-mitosis) or S phase (DNA synthesis) (Meiyanto and Septisetyani 2005).

The morphology of A549 cells with extract did not show a good shape and did not colonize (Figure 4a), while A549 cells without extract showed good cell shape and the cells grew in colony (Figure 4b). Faried et al. (2007) reported similar observation on the effect of gallic acid isolated from Phaleria macrocarpa (Scheff) on human cancer cell lines morphology. They reported that flavonoids induced apoptosis by reducing the regulation of antiapoptosis protein, like tyrosine kinase. Flavonoids inhibited the signal transduction process of growth factors by acting as a competitive inhibitor which prevented the phosphorylation of tyrosine kinase receptor (Lee et al. 2001). Inhibition of proliferation and induction of apoptosis also could be increased through the proapoptosis proteins regulation, like BAX (Bcl-2-associated X protein), BAD (Bcl-2-associated death promoter), BAK (Bcl-2 homologous antagonist killer), P21, P27, and P53 (Utami 2011, Tsui et al. 2014).

CONCLUSION

The result of the present study showed that the *Melia azedarach* leaves ethanolic extract is very potential bioactive compund as anticancer. Extract has a LC_{50} value of 63.98 µg/mL and IC_{50} values of 299.22 µg/mL on Chang cells and 130.56 µg/mL on A549 cells. *M. azedarach* extract was more effective compared to *Curcuma zedoaria* extract in inhibit cancer cells proliferation. *Curcuma zedoaria* extract at 75 µg/mL only reduce A549 cells viability of 38.78 %, which is equivalent to *M. azedarach* extract at 50 µg/mL. This is supported by antiproliferative assay which showed that extract at 5 µg/mL could inhibit A549 cells proliferation as much as 73.53 %.

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