Original article:

Anticancer Mechanism of *Melia azedarach,* Doxorubicin and Cyclosphamide Combination against Breast Cancer in Mice

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Abstract

***Background*:** Previous studies show that *Mindi* (*Melia azedarach*) has a potent cytotoxicity effect on MCF-7 via induction of apoptosis and cell cycle arrest. However its mechanism has not been established. This study was performed to determine the anti-cancer activity of *Melia azedarach,* doxorubicin, and cyclosphamide combination and to elucidate the underlying mechanism of this activity in mice. ***Study design and methods*:** This was an experimental study with a posttest control group design. Twenty four C3H mice inoculated with adenocarsinoma mammary were divided into four groups receiving one of the following treatment: Aquadest,combination of doxorubicin and cyclosphamide (dox-cyc); *Melia azedarach,* doxorubicin, cyclosphamide (MA-dox-cyc) combination; *Melia azedarach* alone (MA) respectively. BAX expression was determined by Immunohistochemistry. ***Results and conclusion*:** A significant decrease in tumor volume was found in combination group. This decrease might have been due to the significant increase is in BAX expression and decrease in AgNOR expression compared with that of control ($p < 0.05$). In conclusion, the combination of *Melia azedarach,* doxorubicin, cyclosphamide can decrease volume of adenocarsinoma mammary tumors in C3H mice via BAX expression increasing and decreasing AgNOR expression.

Keywords: Melia azedarach; tumor volume; BAX expression; cell proliferation

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Intoduction

Breast cancer is a global health concern and one of the leading causes of cancer death in women. According to the Surveillance Epidemiology and End Results (SEER) in 2012 it is estimated that 226,870 women were diagnosed with breast cancer in the United States, with a mortality rate of 39,920. Breast cancer therapies can have side effects with a low life expectancy. More than 60% of breast cancer patients used complementary and alternative medicine[1]. Anti-cancer properties of phytochemicals and extracts of medicinal plants have been studied for breast cancer in animal model. The anticancer activity has been previously reported in Mindi (*Melia azedarach*). Fruit of *Melia azedarach* has been shown to induce apoptosis leukemia cells (HL-60) in IC 50: 0.016 µM and cell culture gastric cancer (AZ 521) in the IC 50: 0.035 µM, with the method of Western Blot will increase the breakdown of caspase 3, 8 and 9[2]. Mindi leaves are known to stimulate the spleen cells to secrete TNFα (tumor Becrosis Factor α) and IFNγ *(*interferon γ*)* and increase the activity of NK cells *(*Natural Killer*)*[3]. *Mindi* is morphologically different from *Azadirachta indica*, but share almost the same phytochemical compounds including flavonoids, alkaloids, tannins, and other coumarin. Azadirachta indica leaves ethanol extract can inhibit the growth of MCF7 cells and HeLa cell culture and have a potential effect when combined with cisplatin[4]. Marker of cancer cell proliferation activity is NORs (Nuclear organizer regions) are black dots in nuclei stained with silver nitrate under light . . Suppression

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of cancer growth or decrese in volume of cancer is indicated by decrease in proliferation which can be seen from the decreased of black dots in the nuclei of cancer cells (AgNORs) and increased apoptosis indicated by suppression of proteins serving as antiapoptosis including the family of Bcl-2 or a protein that works to increase apoptosis. *Mindi* has been well studied, however a few studies have been conducted its effect on volume growth suppression of tumor in mice C3H mammary adenocarsinoma. The effectiveness of the ethanol extract of the *Mindi* leaves as breast anticancer candidate was determined by a decrease in black spots in the nucleus of cancer cells (AgNORs) which indicates a decrease in proliferation and increased Bax protein indicating increased apoptosis and a decrease in the volume of cancer in mice C3H mammary adenocarsinoma.

Method

Research design

In this Randomized study, 28 CH3 mice were divided into 4 groups according to the random number table: a control group; Group 2: Adriamycin and cyclosphamide; Group 3: Adriamycin, cyclosphamide and extract mindi; Group 4: extract mindi dose of 50 mg / KgBW . The independent variables in this study were of extract *mindi* and combinations of Adriamycin, cyclosphamide with scale ratio. While, the dependent variables of this research were AgNOR expression and increased expression of Bax protein with an interval scale. This research was carried out in the chemistry laboratory of Medical Faculty of Universitas Islam Sultan Agung and anatomical pathology of Faculty of Medicine, University of Indonesia for the treatment and painting of IHC, FK Unissula biology laboratory for the IHC slide observation.

Research procedure

licensing and research ethics

Licensing was related to the use of laboratory facilities for maintenance of animal biology. ethical research was needed considering that that this study usie animal to prevent misconduct during the study.

2. Tumor inoculation

Tumor inoculation is done by injecting a tumor cell suspension into the axilla C3H mice intraperitoneally (0.2 mL). Results inoculation approximately 1 week

3. treatment administration

Twenty four C3H mice inoculated with adenocarsinoma mammary were divided into four groups receiving one of the following treatment: Aquadest,combination of doxorubicin and cyclosphamide (dox-cyc); *Melia azedarach,* doxorubicin, cyclosphamide (MA-dox-cyc) combination; *Melia azedarach* alone (MA) respectively for 21days.

6. AgNOR measurement analysis and increase in Bax protein expression

the total number of black dots in the cell nuclei is calculated at a minimum of 100 cell nuclei and by dividing the total number of black spots by the number of cells observed, wearing a light microscope with a magnification 400x or 1000x. Calculation of Bax protein expression, the Hot Spot method**,** using a light microscope manually at 400x magnification on a zone of the field of view, at least 10 of the visual field by counting.

Data analysis

Research data presented in tabular form the mean and standard deviation of normality test is then performed to determine the distribution of the data. If the data is spread evenly then, the difference between the control and treatment was determined by parametric test ANOVA with 95% confidence intervals.

If the data normality test results show data is not evenly distributed then, to determine the difference between control and treatment groups Non-parametric tests were used Mann Whitney U with 95% confidence intervals.

Results and Discussion

This study has been approved by the Ethics Committee of the Faculty of Medicine Unissula and conducted in Labolatorium Biology, Faculty of Medicine, Universitas Islam Sultan Agung between April and May 2015. In this study, the extract obtained from the *Mindi* seeds obtained from Boyolali area. *Mindi* seeds were sorted according to size, degree of maturity (color). Selected seed were washed and dried in oven for 1 day. Soxchlet Extration was performed by dissolving 91.95 grams of seeds mindi in 95% ethanol solution. Extraction results resulted in 4.311 grams extract. The test results showed that active compounds of *mindi* extracts have a higher level of 438.23 ppm tannins and flavonoids quersetin of 63.50 ppm besides saponins.

Mindi single dose assessment against tumor volume

A total of 12 C3H mice successfully inoculated were divided into 2 groups: a control group and a treatment group *mindi* a single dose of 25 mg / KgBW. Monitoring Results of experimental animals conducted over three days, including measurement of weight C3H mice and tumor volume. The mean body weight of mice during treatment in the control group and did not increase significantly while after

a single dose treatment, mean body weight of mice in treated group tended to have increase but not significant. Graph of mean body weight of mice can be seen in Figure 1.

During the study the tumor volume was measured every 3 days using a caliper.

The result showed an increase in tumor volume in the control group and decrease in that of treatment group($p < 0.05$).

Assessment of effect of combination on tumor volume

Twenty four male C3H mice were divided into 4 groups including control (I), administration of adriamycin and cyclosphamide (II), the combination giving Adriamycin, cyclosphamide, and extract mindi (III), extract *mindi* single dose (IV). Measurement of the volume is done twice a week. The results of tumor volume measurements at the end of the treatment is presented in figure(3)

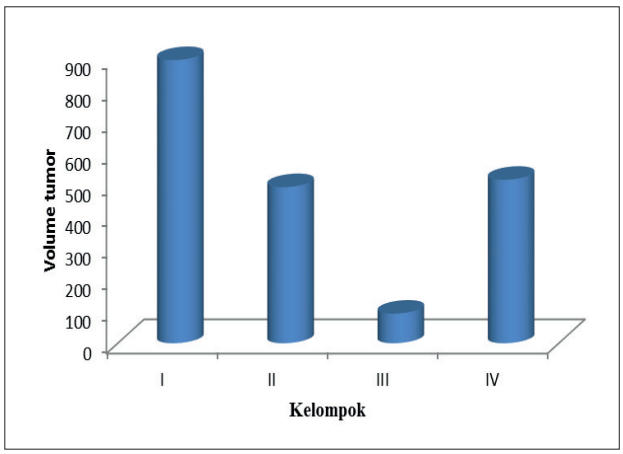

Figure 3. Tumor volume in each group. Measurements were made using calipers.

Based on Figure 3, there was a significant decrease in tumor volume in the combination group (III) compared with that of control (I) and adriamycin-cyclosphamide (II), as well as extract *mindi* single dose (IV) ($p < 0.05$). There was no significant difference in tumor volume ($p > 0.05$) between group II and IV.

Assessment of combination effect on expression of BAX

Evaluation of mechanisms of tumor volume decrease in combination (III) group is determined by apoptotic pathways. The administration combination significantly can increase of BAX expression compared with that of control, group II and IV. Mean of BAX expressions is presented in Figure 4.

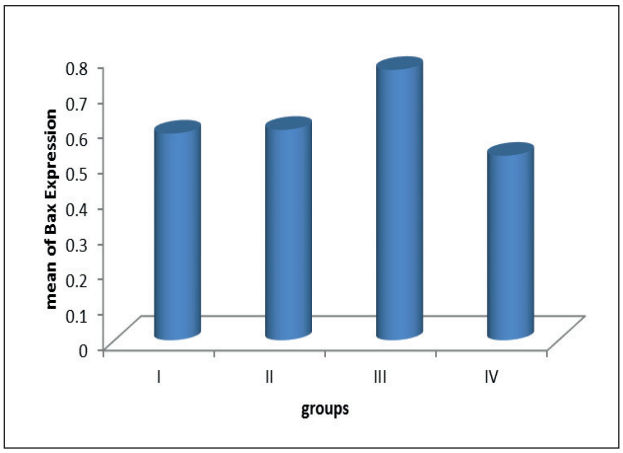

Figure 4. mean percentage of BAX expression in 4 groups

Figure 4 shows a significant increase in BAX expression in the combination group (III) compared with that of control (I) and adriamycin-cyclosphamide (II), as well as extract *mindi* single dose (IV) ($p < 0.05$). Among group I, II and IV there were no significant differences in mean percentage of BAX expression were significant ($p > 0.05$).

Evaluation of combination effect on the expression of AgNOR

AgNOR expression Observations were conducted to determine the mechanism of decrease in tumor activity via the inhibition of cell proliferation.

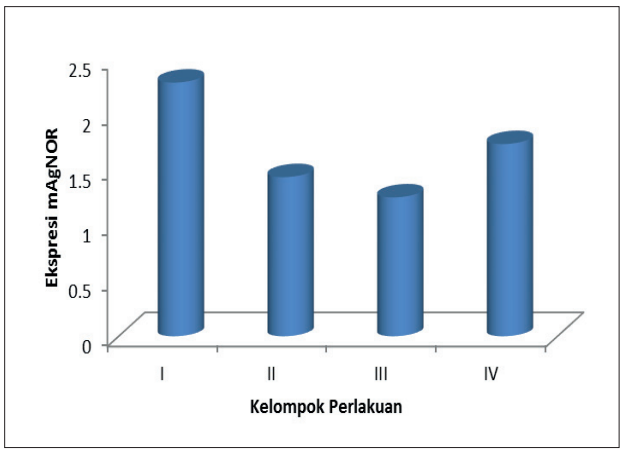

Figure 5. The mean percentage of AgNOR expressions

Figure 5 shows a significant increased expression of AgNOR in the combination group (III) compared with that of control (I) and adriamycin-cyclosphamide (II), as well as extract mindi single dose (IV) ($p < 0.05$). Between group II and IV there are no significant differences in mean percentage of AgNOR expression ($p > 0.05$).

AgNOR expression for all groups cell after the administration of treatment is presented can in Figure 1

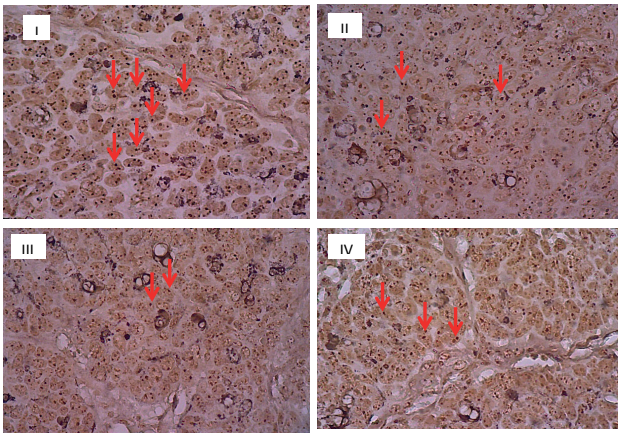

Figure 1. Expression of AgNOR after treatment. I (Control); II (Adriamycin-cyclosphamide); III (combination); IV (Extract Mindi). Preparation were observed at 400x magnification.

Discussion

The ethanol extract of Mindi showed some phytochemical compounds with the potential to inhibit the growth of cancer such as alkaloids, flavonoids and saponins by inhibiting the cell cycle and induction of apoptosis. Ahmed (2008) and Samudram *et al.* (2009) showed that the compounds of alkaloids, tannins, glikosidan and saponin extracts *Melia azedarach* dose of 50 mg / kg has antioxidant activity, by lowering lipid lowering activity of peroxidase and superoxide dismutase (SOD) and catalase, as well as reduce the amount of gluthation (GSH)[5,6]. Ethanol extract of the root bark of *Melia azedarach* showed cytotoxic activity by 1.7µg / mL against P388 leukemia cells in vitro[7].

Melianone active components of methanol extracts of *Melia azedarach* has cytotoxic effects with IC50 value of 3.6 ug/ml, while the compounds of 21-β-acetoxymelianone and 3-β-tigloylmelianol have a potent antiproliferative activity in lung cancer A549 by 100 and 91.8 ug / ml[7]. Wu *et al.* (2009) showed that steroid components isolated from leaves of *Melia azedarach* has a cytotoxic activity in some cancer cells (A549, H460, U251) in vitro with IC50 12.0 - 30.1 ug / ml[8]. Some studies showed that in addition to potentially toxic to cancer cells, active content isolated from the leaves of *M.azeradarach* also can inhibit the replication of HSV-1 and HSV-2 in vero cells in synergy with acyclovir administration without disturbing the biological activity of these cells[9].

Conclusion

Based on our results, The administration of the combination decreased tumor volume. The combination Increased BAX expression and decreased AgNOR exoression of breast cancer cell in Mice.

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