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Research Article

The anticancer activity of ethanol extract of *Chromolaena odorata* leaves in 7,12-Dimethylbenz[a]anthracene in (DMBA) induced breast cancer Wistar rats (*Rattus novergicus*)

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Abstract

Background: Breast cancer chemotherapy with standard drugs such as doxorubicin will induce cardiotoxicity. Therefore, this study aims to evaluate the anticancer activity of *C. odorata* leaves extract in DMBA induced breast cancer on rats.

Methods: Seven groups of *Rattus novergicus* were used: Four treatment groups of *C. odorata* extract (500, 1000, 2000, and 4000 mg/kg BW), normal control, breast cancer control, and doxorubicin treatment group. The number, volume, and weight of the nodule and the rats' body weight were compared among groups. Data was analyzed using paired t-test or one-way ANOVA with post hoc analysis as appropriate.

Results: Significant decline of the number, volume, and weight of cancer nodules was observed in the treatment group ($p < 0.001$). The weight of the cancer nodule at week 16th was also significantly reduced in G_{Co2000} compared to G_{doxo} ($p < 0.0001$). A significant increase in body weight was also dose-dependent, especially at week 11th ($p < 0.05$ in all comparisons) and week 16th ($p < 0.001$ in all comparisons).

Conclusion: This study suggested that the ethanol extract of *C. odorata* leaves has anticancer and antiproliferative activity.

Keywords

breast cancer, *Chromolaena odorata*, DMBA, doxorubicin

Introduction

Chromolaena odorata (L.) King and Robinson, formerly known as *Eupatorium odoratum*, is a perennial wild shrub native to North America (Ekos 2011). This plant is regarded as an invasive weed that causes a serious threat to diversity in the natural ecosystem. Despite that, the traditional use of this plant in the community has

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shown its potential as herbal medicine (Matawali et al. 2019). Some compounds of this plant has been extensively studied, such as phenolic acid (Phan et al. 2001), flavonoid (Hung et al. 2011; Omokhua-Uyi et al. 2020), pentacyclic triterpenoid (Prabhu 2012), L-asparaginase enzyme (Yusriadi et al. 2019) and phytosterol (Ikewuchi et al. 2013).

The biological properties of *C. odorata* that has been investigated included anticancer (Adedapo et al. 2016), antioxidant (Boudjeko et al. 2015), antibiotics (Irobi 1997; Omokhua-Uyi et al. 2020), anti-inflammatory (Owoyele et al. 2005), antidiabetic (Marianne et al. 2014; Yusuf et al. 2020c) and wound healing (Sirinthipaporn and Jiraungkoorskul 2017). As an anticancer, part of this plant has been studied in recent research for its cytotoxicity effect on various cancer cell lines, such as HT29 (lung cancer) (Adedapo et al. 2016), MCF-7 and T4D7 (breast cancer) (Harun et al. 2012; Yusuf et al. 2020b), HeLa (cervical cancer) (Nath et al. 2015), A431(skin cancer) and HepG2 (hepatocellular cancer).

The cancer inhibitory mechanism of this plant was also investigated. The antioxidant activity of the *C. odorata* leaves was better than ascorbic acid with 1.68 gr and 1.6 gr (in hexane and ethyl acetate extracts, respectively) (Yajarla et al. 2014). The antioxidant property of this plant might potentially reduce the oxidative damage caused by reactive oxygen species (ROS) and prevent free radical-mediated damage to cells (Vijayaraghavan et al. 2017; Putri and Fatmawati 2019). The ethanol extract of the leaves was proved to induce apoptosis and growth inhibition in breast cancer cells (Yusuf et al. 2020a). Kaempferide, a compound found in the leaves of *C. odorata*, have a cytotoxicity ability by inducing caspase-dependent apoptosis which led to the cleavage of DNA repair enzyme PARP (Poly ADP-Ribose polymerase) (Nath et al. 2015).

The anticancer activity of *C. odorata* mentioned above will be beneficial in discovering new compounds to be used as single or co-chemotherapy for cancer in humans, especially breast cancer. The anticancer activity of *C. odorata* on breast cancer cell lines has been previously studied (Harun et al. 2012; Kouamé et al. 2013; Yusuf et al. 2020a). However, to the best of author knowledge, no study has been performed to analyze the effect of *C. odorata* on breast cancer *in vivo.* Therefore, this study aims to investigate the cytotoxic activity and the anticancer mechanism of ethanol extract of *C. odorata in vivo* on 7,12-dimethylbenz[a]anthracene (DMBA) induced breast cancer Wistar rats.

Materials and methods

Ethical approval

This study obtained ethical approval from The Ethical Committee of Medical Research, Faculty of Medicine, The University of Lambung Mangkurat with No. 240/KEPK-FK UNLAM/EC/VII/2020

Experimental animals and study setting

Forty-two female healthy Wistar rats (*Rattus novergicus*), weighing 120–170 grams and age of 45 days, were purchased from Animal Breeding House Unit, University of Gadjah Mada, Yogyakarta, Indonesia. The animals were acclimatized to the laboratory conditions for 7 days and maintained under 12 hours light and 12 hours dark conditions. The animals were kept in polypropylene cages in the Animal House of Center for Food and Nutrition Studies at room temperature 22 °C±3 °C with free access to standard rat pellets and water *ad libitum*.

Experimental animals were divided into seven groups with each group consisting of six animals: three control groups, namely G_{normal} (non-cancer control group/normal control), G_{cancer} (cancer without treatment group) and G_{doxo} (cancer with standard doxorubicin treatment group), and four treatment with *C. odorata* extract with a dose of 500, 1000, 2000 and 4000 mg/kg body weight (BW), assigned as $G_{Co500,}$ G_{Co1000} $G_{Co2000,}$ and $G_{Co4000.}$

This range of doses was selected based on previous acute toxicity studies. Although other study reported acute toxicity on 2700 mg/kg BW (Ijioma et al. 2014), many other studies found that the ethanol extract of *C. odorata* leaves was well-tolerated by adult mice between 10–5000 mg/kg BW (Haji Jasnie 2009; Aba et al. 2015; Asomugha 2015). Another guideline provides the use of *in vitro* cytotoxicity IC50 (concentration at which cell viability is inhibited by 50%) values to estimate acute *in vivo* toxicity LD50 (the dose that produces lethality in 50% of the animals tested) values using Registry of Cytotoxicity (RC) prediction model (NTP 2001). However, the guideline was based on known chemicals and cannot be extrapolated to the crude extract as in our study.

After acclimatization, the body weight of all experimental animals was measured and followed by breast cancer induction by DMBA in all groups, except G_{normal} (assigned as time 1, T1). The induction was performed by feeding 20 mg/kg BW of DMBA suspended in CMC-Na 0.5% orally three times per week for 5 weeks. At the end of T5, breast palpation was performed to calculate the number, diameter, and volume of the nodules formed. The administration of doxorubicin and *C. odorata* extract was started on T6. Doxorubicin was given for 11 weeks with a dose of 15 mg/kg BW once a week intraparenterally for G_{doxo} group, while the treatment group received the ethanol extract of *C. odorata* leaves every day according to the dose, orally for 11 weeks. Data on the body weight, breast palpation, and nodule volume measurement were collected on T11 and T17 (completion of the experiment). On T17, after all data was collected, the animal was euthanized by injecting 2 mg/kg BW ketamine to the experimental animal, followed by nodule weight measurement and breast tissue collection.

Preparation of *C. odorata* leaves extract

C. odorata leaves was carefully identified at Biology Research Center of the Indonesian Institute of Sciences "Herbarium Bogoriense" or the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Syiah Kuala

University, Banda Aceh. Fresh twenty-five kilograms of *C. odorata* leaves were collected, washed with running water three times, dried for two weeks, and then grounded using a grinder mill. Ten kilograms of *C. odorata* grounded leaves were then extracted using 80% ethanol with frequent stirring for 24 hours. The liquid extract was then filtered using Whatman filter paper and the residue was re-extracted three times with fresh solvent every 24 hours. The extract was filtered and concentrated by using a rotary evaporator at 40 °C. The final extract (220 gr) was then stored in a wide-mouthed and tightly closed bottle at 4 °C until used.

The phytochemical analysis of ethanol extract of *C. odorata*

The phytochemical analysis of the ethanol extract of *C. odorata* used in this study had been previously conducted by using Gas chromatography–mass spectrometry (GC-MS) and published in different study (Yusuf et al. 2021). The result was presented in Suppl. material 1: Table S1.

The number of nodules after induction and after treatment

The tumor nodule was calculated by breast palpation. The breast palpation was performed three times: after induction (at the end of T5), and at the end of T11 and T17 (at the end of the experiment).

The measurement of nodule volume and weight

The number of nodules was measure by breast palpation of the Wistar rats on T11 and T17. The diameter of the cancer nodule was measured by using callipers with an accuracy of 0.05 cm. This nodule diameter data is used to calculate the nodule volume using the following formula (Kubatka et al. 2014): $V = \pi . (S_1)^2 . S_2/12$ (S_1, S_2 are tumor diameters; $S_1 < S_2$). The nodule weight was measured on T17 after euthanasia by weighing the nodule after surgery on each group.

Histopathological evaluation

Tumors were removed from euthanized rats, washed with 0.9% NaCl and fixed in 10% formalin fixative for 24 h. The tissues were then dehydrated in ascending series of alcohol (from 70% to absolute alcohol), cleared with xylol and embedded in paraffin wax with a melting point of 56–58 °C. The blocks were cut to obtain 4- to 5-µm-thick serial sections using a rotary microtome, stained with hematoxylin-eosin, and observed under a light microscope with 10–40 × magnification (BX51, Olympus company, Japan).

AgNOR staining and counting

AgNOR staining was performed according to the guidelines (Aubele et al. 1994; Ofner et al. 1994). Each section was soaked in sodium citrate buffer (at pH 6.0) and incubated in an autoclave at 120 °C for 20 minutes. The slide was allowed to cool down to 37 °C followed by soaking the slide in a freshly prepared silver staining solution containing one part by volume of 0.5% gelatine in 1% formic acid and two parts of 50% aqueous silver nitrate solution, incubated at 37 °C for 11 minutes. The reaction was stopped by washing the slides with double-distilled deionized water. All stained sections were dehydrated in increasing grades of concentration of ethanol and then clarified in xylene.

For each AgNOR stained slide, at least 100 nuclei per microscopic field was calculated for three microscopic fields. The observation was performed under a light microscope with 1000 × magnification (BX51, Olympus company, Japanese). The AgNOR counting was performed by dividing the total number of silver-stained dots per cell by the total of cells observed. We also calculated the average silver-stained dot in the treatment group with G_{cancer} and G_{doxo}.

Statistical Analysis

Results are presented as means ± standard deviation (SD). Data was analyzed using the one-way analysis of variance (ANOVA) with 95% confidence interval (05% CI). Multiple comparisons were carried out with the Least Significant Difference (LSD) test. Statistical significance of differences was considered at a p-value < 0.05.

Result

The number of nodules after induction and after treatment

The success of breast cancer induction was indicated by palpation of the mass during breast examination of the rats. The number of breast nodules was decreased in all groups, except animals in Gcancer, which continued to increase each week (Fig. 1). After week 6th, the number of nodules in G_{Co2000} and G_{Co4000} decreased significantly when compa-

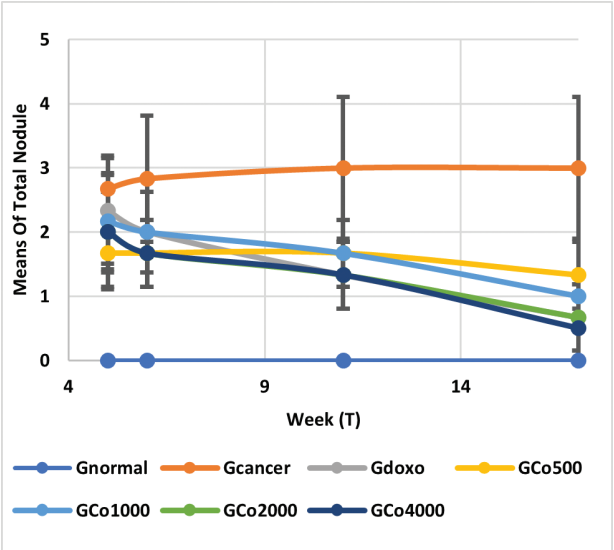

Figure 1. The means of total nodules observed on T5, T6, T11, and T17.

red to G_{cancer} ($p < 0.05$). A decrease of tumor nodules was also observed at week 11th\in all treatment groups (except G_{Co500}) when compared with G_{normal} and G_{cancer} ($p < 0.05$ in all comparisons). At the end of treatment (week 17th), the number of nodules in the treatment group ($G_{Co500,}$ G_{Co1000} $G_{Co2000,}$ and $G_{Co4000.}$) also significantly decreased when compared to G_{cancer} ($p < 0.001$). However, there was no statistically significant decrease in the number of nodules between the G_{doxo} and the treatment group. There was also no statistically significant reduction in the number of nodules with different doses among all treatment groups.

The volume of breast cancer nodule

The volume of cancer nodules at week 6th in all treatment groups decreased significantly when compared with G_{cancer} ($p < 0.0001$) except G_{Co2000} (Fig. 2). Meanwhile, when compared with G_{doxo}, a significant reduction in the nodule volume was observed only at G_{Co1000} and G_{Co4000} ($p < 0.05$). At week 11th, the nodule volume decreased significantly only in G_{Co2000} when compared to G_{doxo} ($p < 0.0001$).

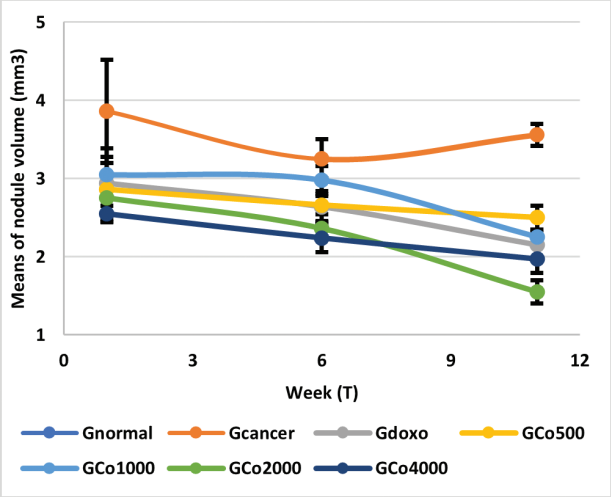

Figure 2. The means of nodules volume observed on T1, T6, and T1.

The weight of the cancer nodule

The weight of the cancer nodule at week 16th was also significantly reduced in G_{Co2000} when compared to G_{doxo} ($P < 0.0001$). The weight of cancer nodules also differed significantly when comparing G_{Co500} with G_{Co1000}, G_{Co2000} and G_{Co4000} ($p < 0.001$). However, there was no statistically significant decline of nodule weight among all treatment groups with different extract doses (Fig. 3). The most significant decrease in the mean weight of cancer nodule was observed in G_{Co2000}.

The body weight of experimental animals

On weight observation at week 6th, G_{Co2000} and G_{Co4000} showed significant weight gain compared to G_{doxo} ($p < 0.001$ in both comparisons). Whereas at week 11th, body

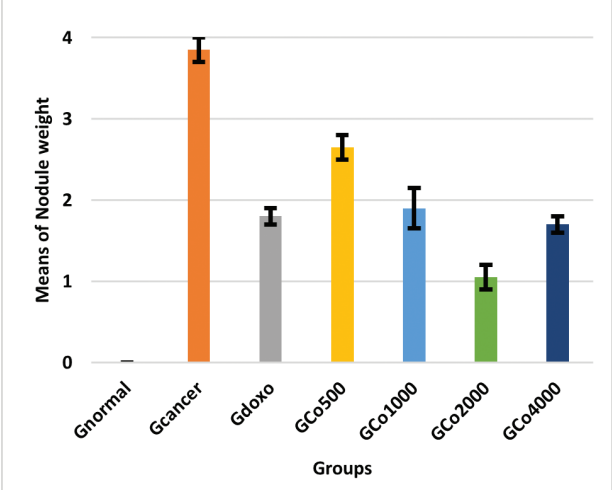

Figure 3. The means of nodules weight observed on week 16th.

weight of all treatment groups increased significantly compared to G_{doxo} ($p < 0.001$), except G_{Co500}. At the end of the treatment, the body weight of the treatment group increased significantly compared to G_{doxo}, where $p < 0.05$ for G_{Co500} & G_{Co1000} and $p < 0.001$ for G_{Co2000} and G_{Co4000} (Fig. 4). A significant increase in body weight was also observed among the treatment groups with the increasing dose of *C. odorata* extract, the body weight of the experimental animals increased significantly, especially at week 11th ($p < 0.05$ in all comparisons) and week 16th ($p < 0.001$ in all comparisons).

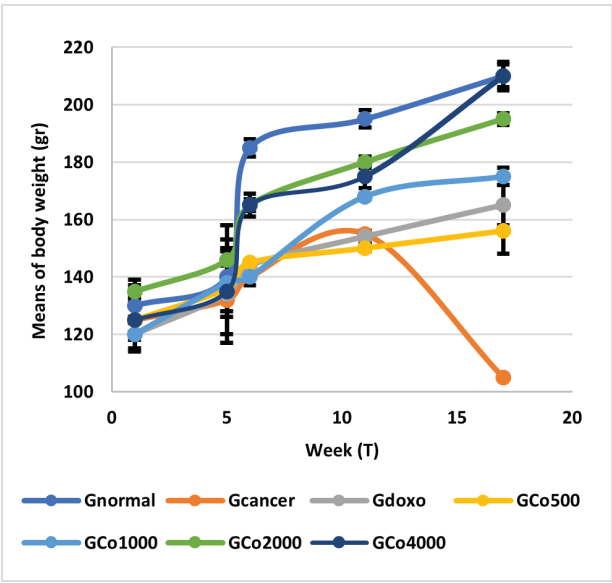

Figure 4. The means of body weight of experimental animals observed during the experiment.

The average number of AgNOR points on breast cancer cells

There was a significant reduction in the AgNOR point in breast cancer cells in all treatment groups when compared with G_{cancer} ($p < 0.05$ in all comparisons). Among the

treatment groups, there was a significant difference of the AgNOR points at G_{Co1000}, $G_{Co2000,}$ and G_{Co4000} when compared to G_{Co500} ($p < 0.001$). AgNOR points in cancer cells of experimental animals in G_{Co4000} (group with maximum *C. odorata* extract dose) decreased although not statistically significant when compared with G_{Co1000} & G_{Co2000}. The highest decrease in AgNOR point was observed in G_{Co2000}, although not statistically significant when compared to G_{doxo} (Fig. 5).

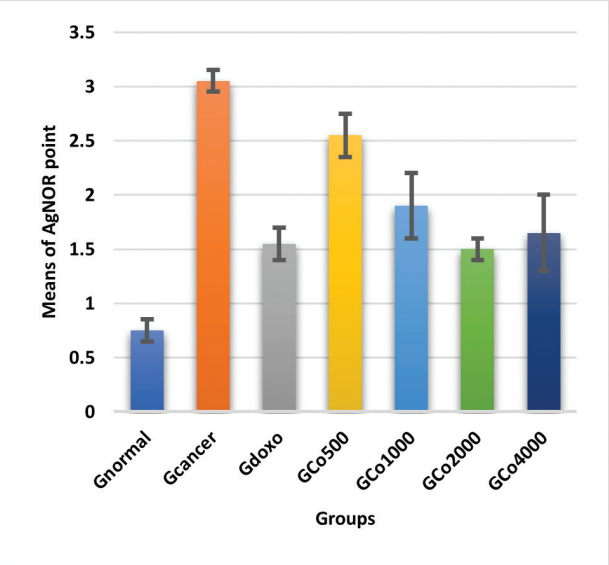

Figure 5. The means of AgNOR point on breast cancer tissue of experimental animals.

Discussion

In this study, breast cancer was induced by oral feeding of DMBA to the experimental animals. DMBA follows a series of mechanism in inducing breast cancer, starting from metabolic activation in the mammary gland (Lin et al. 2012), then the carcinogenic metabolites interact with rapidly proliferating cells in the terminal end buds (Russo et al. 1982) to form DNA adducts and mutations which resulted in malignant cells transformation (Lee et al. 2008). In our study, breast cancer was successfully induced after 5 weeks of 20 mg/kg oral feeding of DMBA three times a week. This result aligns with another study which induces mammary tumor by multiple low oral doses of DMBA (Qing et al. 1997).

Our result also showed that the number of nodules in all treatment groups declined significantly compared cancer group at the end of the experiment ($p < 0.001$). However, there was no statistically significant decrease in the number of nodules between the treatment group and the group treated with doxorubicin. The effect of *C. odorata* extract has also been previously studied in breast cancer cell line which showed that *C. odorata* inhibit breast cancer cell growth and induced apoptosis by reducing the expression of Bcl-2 proteins (Yusuf et al. 2020a).

The volume (at week 11th) and the weight of cancer nodules (at week 16th) in group treated with 2000 mg/kg BW of *C. odorata* extract declined significantly when compared to group treated with doxorubicin ($p < 0.0001$). Selvanathan et al. (2020) investigated the IC_{50} of *C. odorata* on breast cancer cells (MCF-7) and colon cancer (HCT116) were 70 µg/mL and 1.100 µg/mL, respectively (Selvanathan and Sundaresan 2020). However, to the best of author knowledge, no IC50 study on experimental animal has been previously carried out. No significant difference was observed in the volume and the tumor's weight with different doses of *C. odorata* extract in vivo. In contrast, different dose of extract influence the apoptotic stage and Bcl-2 protein expression on breast cancer cell (Yusuf et al. 2020a). Meanwhile, dose-dependent increase was also observed in the body weight of experimental animals.

By identifying the AgNOR point in mouse breast cancer tissue, cell proliferation activity can be observed in experimental animals. A significant reduction in the number of AgNOR points was observed in all treatment groups compared to the cancer group. The highest decrease in AgNOR point was observed in groups treated with 2000 mg/kg BW of C. odorata extract, although not statistically significant compared to the group treated with doxorubicin. AgNOR point in group treated with 4000 mg/kg BW was also declined. This means that treatment with *C. odorata* extract effectively reduces the epithelial cells' proliferation rate in the breast glands. AgNOR has a positive correlation with proliferation in breast cancer and a significant association with tumor prognosis(Ceccarelli et al. 2000).

Conclusion

This study provides a preliminary data on the cytotoxicity effect of the ethanol extract of *Chromolaena odorata* leaves against DMBA induced breast cancer Wistar rats. Further research on the active component of *C. odorata* leaves, its chemotherapeutic properties and the mechanism of cytotoxic activity are warranted.

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Supplementary material 1

Table S1

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Data type: table (docx. file)

Explanation note: GCMS evaluation of chemical compound of *C. odorata* ethanol extract.

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