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Effect of andrographis paniculata extract on mammary carcinoma in rats induced by dmba

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Abstract :

It has been well documented that chemical carcinogen, DMBA (7.12 dimethylbenz(a)anthracene), plays a role in the incidence and growth of mammary cancer. The present study was designed to investigate histochemical alteration of epithelial cells in mammary gland influenced by DMBA in the female Sprague-Dawley rat. DMBA-inducing mammary cancer is a useful model to investigate the changes of epithelial cells that occur during mammary cancer progression. Mammary cancer model was induced 10 times twice a week orally by DMBA, with 20 mg/kg body mass. Mammary cancer occurred in 90% for nine weeks after oral administration of DMBA, and it was represented with nodule on the mammary gland and the increasing of mammary gland volume compared with normal control ($F = 100.592$; $p < 0.001$). This study was also designed to investigate the preventive and curative effect of Andrographis paniculata extract on mammary carcinoma induced by DMBA. Preventive administration of three different doses of Andrographis paniculata (100 mg/kg, 300 mg/kg and 1000 mg/kg) was to maintain the volume of mammary gland as good as that in the normal control and significantly different with that in the DMBA model group ($F = 99.930$; $p < 0.001$). Andrographis paniculata has the preventive effect on limiting the frequency of cancer initiation and growth. However, the volume of mammary gland in the curative administration with three different doses of Andrographis paniculata (100 mg/kg, 300 mg/kg and 1000 mg/kg) was not statistically different from that in the DMBA model group ($F = 2.239$; $p > 0.05$). This data indicated that the extract of Andrographis paniculata had no significant effect on the treatment of DMBA-inducing mammary carcinoma. The Epithelial cells then were harvested on the 90th day and stained on routine histology staining and hematoxylineosin for morphological qualitative analysis. After that, the lesions were observed from the removed samples ranged widely from benign to malignant. The results then showed that DMBA induced cell proliferation, nuclear irregularities, numerous mitoses, and cell necrosis. Furthermore, the preventive treatment of Andrographis paniculata inhibited the progression of cell proliferation, while the curative treatment of Andrographis paniculata induced apoptosis in cancer cells. Finally, we hope that the further studies will provide some clues towards early detection and, hopefully, the prevention and the treatment of breast cancer.

Keyword :

DMBA, breast cancer, epithelial cells, Andrographis paniculata ,

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EFFECT OF ANDROGRAPHIS PANICULATA EXTRACT ON MAMMARY CARCINOMA IN RATS INDUCED BY DMBA

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ABSTRACT

It has been well documented that chemical carcinogen, DMBA (7.12 dimethylbenz(a)anthracene), plays a role in the incidence and growth of mammary cancer. The present study was designed to investigate histochemical alteration of epithelial cells in mammary gland influenced by DMBA in the female Sprague-Dawley rat. DMBA-inducing mammary cancer is a useful model to investigate the changes of epithelial cells that occur during mammary cancer progression. Mammary cancer model was induced 10 times twice a week orally by DMBA, with 20 mg/kg body mass. Mammary cancer occurred in 90% for nine weeks after oral administration of DMBA, and it was represented with nodule on the mammary gland and the increasing of mammary gland volume compared with normal control ($F = 100.592$; $p < 0.001$). This study was also designed to investigate the preventive and curative effect of Andrographis paniculata extract on mammary carcinoma induced by DMBA. Preventive administration of three different doses of Andrographis paniculata (100 mg/kg, 300 mg/kg and 1000 mg/kg) was to maintain the volume of mammary gland as good as that in the normal control and significantly different with that in the DMBA model group ($F = 99.930$; $p < 0.001$). Andrographis paniculata has the preventive effect on limiting the frequency of cancer initiation and growth. However, the volume of mammary gland in the curative administration with three different doses of Andrographis paniculata (100 mg/kg, 300 mg/kg and 1000 mg/kg) was not statistically different from that in the DMBA model group ($F = 2.239$; $p > 0.05$). This data indicated that the extract of Andrographis paniculata had no significant effect on the treatment of DMBA-inducing mammary carcinoma. The Epithelial cells then were harvested on the 90th day and stained on routine histology staining and hematoxylineosin for morphological qualitative analysis. After that, the lesions were observed from the removed samples ranged widely from benign to malignant. The results then showed that DMBA induced cell proliferation, nuclear irregularities, numerous mitoses, and cell necrosis. Furthermore, the preventive treatment of Andrographis paniculata inhibited the progression of cell proliferation, while the curative treatment of Andrographis paniculata induced apoptosis in cancer cells. Finally, we hope that the further studies will provide some clues towards early detection and, hopefully, the prevention and the treatment of breast cancer.

Keywords: *DMBA, breast cancer, epithelial cells, Andrographis paniculata*

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INTRODUCTION

Cancer is considered not only as the second main factor causing death in adults in developed countries, but also as one of the main factors causing death in children in the age of 1 to 14 years old. In 2007 in the United States of America, there were 1,444,920 new cases of cancer in which 559,650 people died with the ratio of mortality per 100,000 population total about 164.4 for men and 110.6 for women (NCI fact sheet, 2007). Meanwhile, in Indonesia cancer is considered as the sixth main factor causing death. Every year there are about 800,000 Indonesian people suffering with cancer (Semiono, 2007). However, the study on population migrating from one geographic area to another shows that this difference is caused by the difference of life style, not by ethnic factor, though both interact each other. In general, cancer causing lot of deaths in Europe and USA is cancer of lungs, colorectal, breast, and prostate (American Cancer Society Inc., 2006).

Moreover, cancer is a disease signed with the decreasing of controlling function of many mechanisms in managing process of life survival, proliferation, and cell differentiation. Thus, cells transforming to cancer usually express antigens, in cell surface that shows immaturity and abnormality of chromosomes, either in qualitative or quantitative, including many translocations and gene order appearances if compared with those in the normal condition. Many cells even have over fission and form local tumor which can press or attack normal structure (Katzung, 2007). One of the

effects is the disturbance or the failure of mechanism in managing multiplication and other homeostasis functions in multiceluler organisms (Ganiswarna, 1995). Specifically, breast cancer involves 32% of carcinoma types attacking women (Myths, 2005). Furthermore, the U.S. Centers for Disease Control and Prevention (CDC) reports that in the end of 2004, 215,990 of American women are diagnosed with breast cancer, and 40,580 of American women died due to the disease (CDC, 2005). Breast cancer actually can also attack men, but the possibility of attacking women is 100 times compared with men.

The progress of breast cancer, moreover, depends on the age (NCI, 2003). In breast cancer case, abnormal cell is formed in the tissue of breast, usually in the breast milk duct connecting with the nipple (ductal carcinoma in situ / DCIS) and in the lobule, a gland producing milk (lobular carcinoma in situ / LCIS) (Peeters et al, 2003). Breast cancer is also considered as malignant tumor, which is tumor cell invades tissues around or spread into another part of the body (metastasize), deriving from breast cells (National Breast Cancer Foundation, Inc. 2007).

In addition, breast cancer involves a number of disease units with many clinical symptoms (Jemal et al, 2006). Structure and homeostatic parenchyma of normal breast actually are maintained by dynamic interaction between epithelia cell of breast gland and related stroma. The elements of stroma are including vascularization, adiposity, immune cell, and fibroblast with its cellular products involving some growing matrix and extracellular matrix. Nevertheless, in breast cancer, the stroma is different from the normal one. The stroma of breast cancer is morphologically characterized with the increasing of accumulation, the changing of fibroblast, and the colagenization of extracellular matrix. It relates with the carcinoma related with fibroblast (carcinoma-associated fibroblasts/ CAF) that supports breast cancer and its growth.

Fibroblasts around ductal carcinoma in situ (DCIS) actually are growing more early in the spreading of carcinoma. And, in histology they are different from those in the normal breast tissue. The better understanding about the alteration occurred in fibroblast through carcinogenesis and its influence in the strategic growth and the epithelial cell behavior can become a strategic way to prevent and cure breast cancer (Sadlonova et al, 2004). Unfortunately, breast cancer usually treated by surgery, radiation, and chemotherapy with sitotoxic medicines which can damage DNA or acts as general inhibitor in cell proliferation. Therefore, some combinations of individual and regiment agents and chemotherapy are considered as an effective treatment for breast cancer, such as cyclophosphamide, doxorubicin, 5-fluorouracil (5-FU), and taxanes. Some cases of breast cancer even can respond the hormonal therapy, which can restrain the negative effects of estrogen that can support the growth of cancer cell (Wijayahadi et al., 2007).

However, the decision in choosing type of therapy is made based on the certain guideline, but not all patients got advantages from the therapy they took. One of efforts to solve the problem is by obtaining the cure pattern for each patient individually, known as personalized medicine (Thomadaki and Scorilas, 2006). However, this condition needs the accurate prognosis profile not only in determining which patient is supposed to have the systemic therapy (hormone/ chemotherapy) before and/or after operation, but also in determining what systemic treatment is suitable for patients. The improvement of microassay DNA and related technology nowadays even has created the possibilities in determining the progression and characteristics of tumor in more details and more individually (Jianjiang et al, 2007). This progress is not separated from the proteomic study of breast cancer that has successfully identified potential markers (such as molecular chaperone 14-3-3 sigma). And, the innovation of technology in big scale and the analysis have also been improved. The method of functional proteomics is also improved in order to analyze the line of intracellular signs (intracellular signaling pathways) guidelining the growth of breast cancer (Hondermarck et al, 2001). Though genomic approach is very promising to identify some specific cancer molecular markers, proteomic approach is also considered to be important in identifying the specific modification of cancer cells after the translation of protein (HPV research group, 2004). Thus, together with genomic approach, proteomic approach is also needed as a way to characterize the different molecular types of breast cancer. As a result, the new therapy strategy for treatment can be conducted in the future.

In order to show the complex interaction of cancer, moreover, the testing animal is needed for analyzing the mechanism of cancer due to the chemical induction (Russo and Russo, 1996). Therefore, the evaluation of cell proliferation in the tissue of testing animal is important not only for studying toxicology and carcinogen, but also for testing the efficiency of sitotoxic and chemopreventive medicines in the study of cancer (Dawson et al, 1993). Some models are improved to study the key elements in the growing of breast cancer. Nevertheless, no model is ideal, but the most useful model must be able to reflect the normal history and histopathology of diseases in humans, and to make the basic investigation possible for analyzing the

cellular and molecular mechanisms (Heppner et al, 2000).

Thus, rat is used as the model of breast cancer study for several reasons, for instance, its histology can be compared with humans'; the genital alterations can also be conducted in its mammary gland; it also has many mammary glands that make biopsy possible to be conducted in many places (rats have ten mammary glands); and it can purify the epithelial cells from lipid and then culture them (Cardiff and Wellings, 1999). The study on rats shows that this model is useful to analyze the initiation, promotion, and progress of each carcinogenic stage (Russo and Russo, 1996). The carcinogens that can be used to induce cancer in mammary gland of rats are including 3.4-benzo[a]piren, N-metil-N-nitrosourea (MNU), 3-metilkolantren (MCA), 2-asetilaminofluoren, etilnitrosourea, and butilnitrosourea, with DMBA and MNU are most commonly used (Russo and Russo, 1996).

The carcinogen process of DMBA, known as a multistep process, furthermore, involves the alteration of genotype (initiation) and proliferation of cells initiated into tumor (promotion) (Izzotti et al, 1999). The activation of DMBA into the active form of 3-4-dihidrodiol-1, 2-epoksida is catalyzed by sitokrom P-450 enzyme and epoksida hydrolyses (Jae et al, 2005). Enzymes (enzymes in Phase II) that have a role in detoxification and conjugation, such as glutathione S-transferase, and the role of the mechanism of DNA repairmen, are also important in determining the sustainability of tissues towards chemical carcinogens. Then, this active carcinogen can be bound with the nucleate acids and start the series of carcinogenic events (Gibson and Skett, 1991).

In addition, the side effect of the cure of cancer supports many people to take natural medicines (Sahu et al., 1984). One of the efforts is by searching and discovering the bioactive compound derived from Indonesian herbal medicines that have anti-cancer, especially anti-breast cancer. The result of these searching and discovering was andrografolida compounds from sambiloto (Andrographis paniculata Ness.) that has that activity (Sukardiman, 2005). In this study, female white SD rats (Sprague-Dawley) induced constantly by mammary cancer, DMBA, with 20 mg/kg BM dose for about three times, twice a week were observed for their mammary cancer. In this study, the preventive and curative effect of sambiloto extract (Andrographis paniculata Ness.) on mammary cancer in rat were also observed. The control group was given doxorubicin in order to compare the alterations occurred in the epithelial cell of mammary gland attacked by cancer, including in cells that are ready to grow; in cells that are already damaged (apoptosis); and in cells that got proliferation occurred in treated groups compared with the epithelial cell of mammary gland in the control group by using histochemical method.

The aim of this study, finally, was to prove the manifestation difference and morphological alteration among epithelial cells of mammary gland in female white SD rats (Sprague-Dawley) induced with DMBA in order to make them get mammary carcinoma and after treated not only with sambiloto extract therapy (Andrographis paniculata Ness.) with preventive and curative methods, but also with doxorubicin as the positive control in using histochemical method with hematoxylin-eosin.

MATERIALS AND METHODS

In this study, light microscopy tool (Olympus AX 70) was used with digital camera, microtome, laminar Air Flow Cabinet, glass tools, and rat cage. Chemical materials used in this study were DMBA, Carboxymethyl Cellulose Natrium (CMC Na), hematoxilyn eosin, NaH2PO4, Na2HPO4, and formaldehyde bought by Sigma Chemical Co. (St.Louis, MO); corn oil (Oleum maydis) obtained from Bratachem (Surabaya); and ethanol herbal sambiloto extract (Andrographis paniculata Nees) from BPPT.

In this study, the testing animals used were female Sprague-Dawley rats in the age of 25-30 days (with body mass about 60 – 150 grams) obtained from Laboratory of Animals, Faculty of Pharmacy, Airlangga University. The animals were taken care in controlled a room with the controlled lighting (12 hours of lighting and 12 hours of darkening) and the temperature of air about 30 ± 1°C. Food and drink were also given in ad libitum way.

The model of mammary carcinoma in rats was designed with inducing DMBA (inside oleum Zea mays) orally with 20 mg/kg BM dose twice a week for 5 weeks. The mammary carcinoma then occurred in the 8th week after the last induction. Andrographis paniculata Ness extract with 100 mg/kg, 300 mg/kg, and 1000 mg/kg doses was given to the testing animal with preventive and curative methods. In preventive method, the extract had given two weeks before the induction, and then it was continued until the testing animal was dead. Meanwhile, in curative method, the extract was given six weeks after the last induction. Besides that, there were also the mammary carcinoma control group and the control group obtaining a standard anti-cancer, doksorubisin. The body mass and the mammary carcinoma growth then would be routinely observed once a week. The

sacrifice of the testing animals was conducted 24 hours after the last giving test sample. The macroscopic and histophatology observations then were conducted by taking mammary tissue and cleaning it from skin and fat around it. Afterward, the volume and mass of the tumor were noted by using volumetric vessel. The tissue then was preparated by doing fiksasi using neutral bufered formalin. After that, cutting and staining with hematoxyllin eosin were conducted in Laboratory of Phatology and Anatomy, Medical Faculty -Unair-RSU Dr. Sutomo Surabaya. Analysis and photographing, finally, were conducted with blind test method.

RESULTS

The white female Sprague dawley rats were orally induced with 20 mg/ kg BM dose of DMBA for about 10 times in five weeks. After the period of maturation for about nine weeks, the testing animals then were sacrificed. Afterwards, both macroscopic observation, involving the volume of mammary gland, and histopathology observation were conducted. The prevalence of cancer could also be observed through the nodule occurred in each mammary gland. Specifically, after those animals were dead, the measuring of the volume of mammary gland in the cancer modeling group was compared with that in the normal group. The average of both the volume of the mammary gland and the prevalence of mammary cancer in those rats induced with DMBA could be seen in Table 1. The data shows that there was the difference of both the volume of mammary gland and the prevalence of mammary cancer between the normal group and the cancer modeling group. Based on the result of palpasi and the measuring of mammary gland volume, the prevalence of mammary cancer was 90 %. Based on the statistic analysis with one-way ANOVA test in the normal and cancer modeling (DMBA) groups, it is also known that there was a significant difference of the volume, $p < 0.001$.

Table 1. The average of mammary gland volume and prevalence of mammary cancer in rat induced with DMBA

Groups	Prevalence (%)	Gland Mammae Volume (ml) X + SD
Normal	0.0	0.13 + 0.02
Cancer Model (DMBA)	90.0	0.56 + 0.08

Moreover, as shown in Figure 1, it is also known that the DMBA induction did not influence the normal growth of rats. The Figure shows the normal growth of the body mass in the group of rats induced with DMBA compared with that in the normal group. Through one-way ANOVAs test analysis, then it is known that there was no significant difference (1.18) about the normal growth of the body mass between the normal group and the cancer modeling group, $p > 0.05$ ($p = 0.965$) and $F= 0.002$. It means that the induction of DMBA did not influence the normal growth of those rats. The decreasing of body mass in each group was possibly caused by the amount of food and drink consumed before the measuring, the condition of environment, and stress that they got because of the treatment.

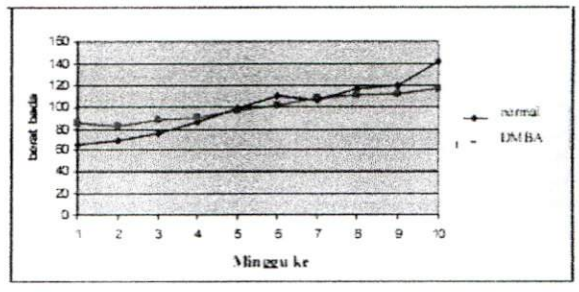

Figure 1. The normal growth of the body mass in the group of rats induced with DMBA compared with that in the normal group

In the treatment with the preventive method, the test sample was given before the oral induction of DMBA. The test sample then were orally given to those testing animals every day, in two weeks before the oral induction of DMBA, and in five weeks after the oral induction of DMBA (totally 12 weeks) with 100, 300, and 1000 mg/kg BM doses for each test sample. And, the oral induction of DMBA with 20 mg/kg BM dose was about twice a week in five weeks. The sacrifice of those testing animals then was conducted 48 hours after the last giving of the test sample.

Moreover, as seen in Table 2, it is known that there was the difference of the prevalence and the volume of mammary gland between the normal group and the DMBA modeling group, for each dose of preventive treatment. Based on the result of palpasi and the measuring of mammary gland volume, then, it is known that the prevalence of mammary cancer was 30 % for the group with 100 mg/ kg BM preventive dose, and 12.5% for the group with 300 mg/ kg BM preventive dose.

In addition, based on the statistic analysis with one-way ANOVAs test, it is also known that there was the significant difference between the group of the testing animals induced with DMBA and the control group, for each preventive dose. Based on the further statistic test with LSD, it is then known that there was also the difference of the volume of mammary gland between the normal group with each preventive dose and the

group of the testing animals induced with DMBA. Therefore, it indicates that the giving of sambiloto extract with preventive method influenced the decreasing of mammary gland in the rats induced with DMBA.

Table 2. Effect of preventive treatment of andrographis extract in the DMBA induced-mammary cancer

Groups	Prevalence (%)	Gland Mammae Volume (ml) X + SD
Normal	0.0	0.13 + 0.02
Cancer Model (DMBA	90.0	0.56 + 0.08
Preventive 100 mg/kg	30.0	0.17 + 0.07
Preventive 300 mg/kg	30.0	0.18 + 0.09
Preventive 1000 mg/kg	12.5	0.11 + 0.04

In this curative method, the test sample was given after the oral induction of DMBA with 20 mg/kg BM dose, twice a week for 5 weeks. After those animals were dead, it was known that the volume and the structure of mammary gland were different from those in the normal group. Thus, it could be used to analyze the prevalence of mammary cancer in each group. The prevalence of cancer and the volume of mammary gland in the normal group and in the group with each dose of curative treatment can be seen in Table 3.

Table 3. Effect of curative treatment of andrographis extract in the DMBA induced-mammary cancer

Groups	Prevalence (%)	Gland Mammae Volume (ml) X + SD
Normal	0.0	0.13 + 0.02
Cancer Model (DMBA	90.0	0.56 + 0.08
Preventive 100 mg/kg	87.5	1.07 + 1.13
Preventive 300 mg/kg	83.3	0.74 + 0.35
Preventive 1000 mg/kg	100.0	1.36 + 0.98

From Table 3, we can know that there was the difference of both the volume of mammary gland and the prevalence of mammary cancer between the normal group and the DMBA modeling group, for each curative dose. Based on the result of palpasi and the measuring of mammary gland volume, moreover, it is known that the prevalence of mammary cancer was 87.5 % for the group with 100 mg/ kg BM of curative dose, and 83.3 % for the group with 300 mg/ kg BM of curative dose. Based on the statistic analysis with one-way ANOVAs test continued with LSD, it is also known that there was the significant difference with reliability degree 0.05 between the group with 100 mg/kg BM and 1000 mg/kg BM doses of curative treatment and the normal group. Thus, it means that the giving of sambiloto extract with curative method did not influence the decreasing of mammary gland in the rats induced with DMBA.

In this positive control group, doxorubicin was given after the oral induction of DMBA with 20 mg/kg BM dose, twice a week in 5 weeks. Doxorubicin with 0.9 mg/ kg BM dose was given to the testing animals in intra-peritoneal way for about four series after the tumor nodule was formed. We then can see the volume of mammary gland in the normal group, in the DMBA modeling group, and in the positive control group (with doxorubicin). Based on that table, it can be seen that there was the difference of both the volume of mammary gland and the prevalence of mammary cancer between the normal group and the groups of DMBA modeling and positive control.

Furthermore, based on the statistic analysis with one-way ANOVAs test with reliability degree p=0.05, it is also known that there was the significant difference between the normal group and the positive control group. It indicates that the giving of doxorubicin did not influence the decreasing of mammary cancer in the rats induced with DMBA. Nevertheless, in order to identify the pathology description of the cell, the data then must be supported with the histology analysis for each of those testing animals. Thus, to analyze the morphological alteration, apoptosis, and proliferation in the epithelial cells of mammary gland, those rats must have surgery in order to take mammary gland of each group for being perpetrated in histochemical way with hematoxylin-eosin staining. Then, the difference of histopathology description of mammary gland between the normal group and the group of those testing animals induced with DMBA was treated with doxorubicin and sambiloto extract in each curative dose as seen in Figure 4.

The structure and homeostatic of normal mammary parenchyma were maintained by the dynamic interaction between mammary epithelial cells and related stroma. The elements of this stroma consist of vascularization, adiposity, immune cells, and fibroblast with their cellular products, including some growth matrixes and extracellular matrixes. In the slice of the normal mammary gland as seen in Figure 1 (A), there was a mammary formation looked like ductuses considered as mammary structural unit, known as TDLU (Terminal Ductal Lobular Unit). It means that there was a formation looked like glands with homogeny cells, or non pleumorphyc. In this normal slice, there were also fat gland and fibroblast bound tissue more than those in the animals induced with DMBA.

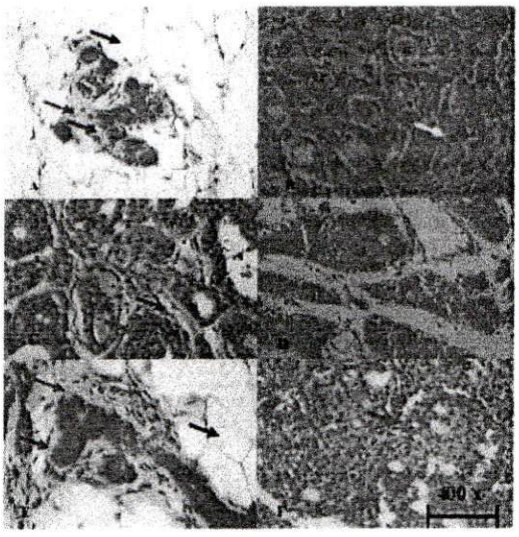

Figure 2. The Figure of the histopathology of mammary gland in SD rats with hematoxylin-eosin staining. (A) Normal. (B) DMBA. (C) Doxorubicin. (D) 100 mg/kg BM of sambiloto extract. (E) 300 mg/kg BM of sambiloto extract. (F) 1000 mg/kg BM of sambiloto extract (400 X zoom)

In cancer cells as seen in Figure 2 (B), there was a cellular alteration of 3 S (Size, Shape, and Stain), in which the size was getting bigger than the normal one with the various formation (pleumorfik) including round, oval, and polygonal with the irregular or rough edge of nucleus. During staining process, the more dark color would be emerged (hyperchromatine) in which the chromatins were not flat and the number of chromosome was also increasing. Mitosis was also improving since cells uncontrolly proliferate. Therefore, the number of cells was getting increasing and pleumorfik. In the term of tissue, there was also indication that cells jumped out from membrane, infiltrated into the tissue around, and formed multilayer cells. As a result, the adiposity tissue was disappeared (decreasing), and the mammary cells were not arranged in the gland formation. However, when doxorubicin was given to those testing animals as seen in Figure 2 (C), the bound tissue of fibroblast started to emerge among cells. As a result, the tissue started to make gland formation. It also could be seen that the cells were empty since they started to be abandoned by cancer cells due to either necrosis or apoptosis. Necrosis in those cells was signed with the existence of lymphocyte cells that were darker than the nucleus since the cytoplasm was damaged from outside and produced proteolysis enzymes. Thus, there was inflammation process before the nucleus was damaged. In that Figure, moreover, it can also be seen that there was apoptosis signed with the fragmentation of chromatin, and the nodule of nucleus surrounded by cytoplasm, so the formation looked like fractions called as apoptotic bodies. In cells that got apoptosis, furthermore, there was no lymphocyte since there was no inflammation caused by the programmed death of cells, known as suicide (program of cell suicide).

For animals treated with 100 mg/kg BM of sambiloto dose as seen in Figure 2 (D), the cytoplasm looked more red since there was fragmentation of nucleus due to apoptosis; the gland formation looked more clearly; the lipid cells started to emerge; and there was bound tissue among cell groups, so the tumor cells were localized. This condition could prevent the metastases and infiltration of tumor cells into other tissues. Meanwhile, for animals treated with 300 mg/ kg BM of sambiloto doses as seen in Figure 2 (E), the number of tumor cells was decreasing, and there was also bound tissue localizing the tumor cells. For animals treated with 1000 mg/ kg BM OF sambiloto doses as seen in Figure 2 (F), moreover, the number of tumor cells emerged was still high though many of the formation got fibrosis, which mean the tumor cells were dead and filled by bound tissue. In that Figure, necrosis was also emerged signed with the reddish color and the lack of cells. This necrosis possibly occured since the doses were too high, so it caused the over-dose reaction that could make many tissues dead.

In Figure 3 of the testing animal with the preventive dose of sambiloto extract, 100 mg/ kg BM, as shown in figure 3 (J), degenerative (non active) and localized tumor cells still could be seen. Thus, the gland formations were still emerged. There were also many cells that got apoptosis signed with the reddish color of cells. With 300 mg/ kg BM and 1000 mg/kg BM of preventive doses, there were still cancer cells growing, but they were not infiltrative. Though some of them looked severe, there was still bound with the tissue of fibroblast. As a result, they were still in the gland formation.

DISCUSSION

Mammary carcinoma has grown as the result of the combination between external factor and endogen factor, such as radiation exposure, food, social economy status, hormone, family or genetic factor. However, the specific agent that causes and is responsible with the initiation of this disease is still not identified. Thus, a study with a model for analyzing the interaction and examining some potential carcinogenic mechanism from chemical substances is required (Russo and Russo, 1996).

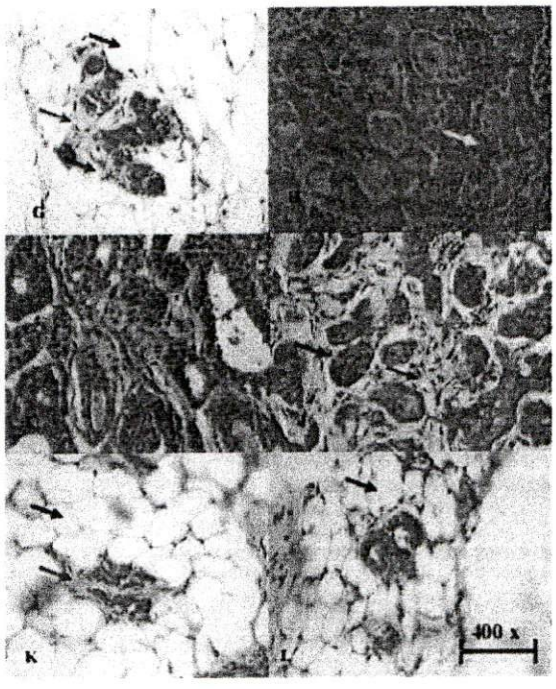

Figure 3. The Figure of the histopathology of mammary gland in SD rats with the hematoxylin-eosin staining, (G) Normal. (H) DMBA. (I) Doxorubicin. (J) 100 mg/kg BM of preventive sambiloto extract. (K) 300 mg/kg BM of preventive sambiloto extract. (L) 1000 mg/kg BM of preventive sambiloto extract (400 X zoom in).

In this study, thus, the mammary carcinoma was analyzed by using white female SD rats induced with DMBA cancer. The model of cancer in rat is considered as a good model since mammary cancer in this species is considered as a complex process in which the species can be gradually induced with chemical substances, radiation, virus, or genetics. This long term study of the rat model has proven that this model is useful to analyze the stages of carcinogenic initiation, promotion and progress. The sustainability of mammary gland of the rat towards the growth of neoplasm, furthermore, has also made this organ become a unique target in examining the potential carcinogen either from geotaxis chemical compound or from environmental materials (Russo and Russo, 1996). Besides that, the reason of using rat in the study of mammary cancer was because of many reasons, which were that its histology could be compared with humans; that the genetic alteration could be conducted in mammary gland; that it has many mammary glands which can make biopsy possible to be conducted in many areas (rat has ten mammary glands); and that it could purify epithelia cells from lipid and then culture them (Cardiff and Wellings, 1999).

Moreover, the comparative study of both the growth of the mammary gland growth and the pathogenic of mammary cancer in humans has also contributed in extrapolating from rat to human. Unfortunately, it still cannot be used directly to examine the risks in humans (Russo and Russo, 1996). However, it has been generally accepted that carcinogen induce the genetic damage through the formation of covalent bonds of DNA though this information is still not enough for carcinogenic initiation. Therefore, some studies have been conducted to examine the carcinogenic effects of DMBA on the formation of covalent bonds of DNA. Though it is not considered as natural substance, synthetic aromatic hydrocarbon compound of DMBA as tumor initiator has been widely used in model examining mammary cancer.

The maximal bonds of DNA in mammary gland of SD rats actually have been detected in 24-48 hours after inducing DMBA. With the ability of mammary gland in stimulating DMBA into reactive metabolite involving in carcinogens, DMBA could initiate cancer. Thus, this carcinogen could initiate cancer in target organs (epithelia cells of mammary gland) and non-target organ (liver) (Bayoumy, 2000).

Nevertheless, in this study the new tumor nodule was detected after the period of maturation more than two weeks, and the volume of cancer was also identified to be different from that of the normal mammary gland after having surgery. As seen in Table 1, the volume of cancer is statistically different from the normal mammary gland with $F(1.11) = 100.592$ and $p < 0.001$ through one-way ANOVAs test. Tumor of mammary gland induced by DMBA carcinogen usually causes adenocarsinoma depending on hormone. However, the number of tumor in every animal and the type of tumor suffered by every group of animal are influenced by age, reproducing records, and the condition of endocrine gland when exposed by carcinogen (Russo and Russo, 1996).

Based on Table 1, it can also be detected that DMBA in this study can induce 90 % of mammary cancer in animals. The reason is because that the treatment was firstly conducted in animals when they were in the age of 30-40 days. Mammary gland of the rat has grown rapidly in the age of 32-35 days, firstly as terminal end buds (TEB) which then grows into alveolar buds, even into terminal duct in the age of 40-60 days. In the normal condition, however, the most effective DMBA produces tumor during the period of transmission from TEB to alveolar buds (Banerjee et al, 2002).

Furthermore, the model of rat is actually considered as informative data that are difficult to be acquired in humans. Therefore, this model is used as information not only about the dose and route of administration used, but also about the optimal condition of host in responding the tumor. The dose of DMBA used in a study of mammary cancer, nevertheless, is usually various. There is a study using DMBA with 15 mg/ml dose in 120 days of observing period with the result of cancer about 94.7% (Fouad, 2005). In another study, with the same period of observing, the dose of DMBA used is about 20 mg/ml which also causes mammary tumor starting to be palpated in the 110th day after induction (Izzoti, 1999). In this study, DMBA was induced 10 times in 5 weeks which then can be able to induce 90 % of mammary tumor.

DMBA, however, could not influence the growth of normal rat. It can be seen in Table 2 showing that the growth of the normal rat was not different significantly from that of the control group. In this study, the control group of the normal rats was also conducted since the study of the growth of the normal mammary gland was still needed in order to know mammary carcinoma pathogenic induced by chemical substance. Therefore, it can clarify the role of differentiation in cancer initiation (Russo and Russo, 1996).

In addition, in this study sambiloto extract was also given with preventive and curative methods in order to analyze its effect on mammary cancer in rats since one of efforts in solving problem related with the cancer treatment is searching and discovering bioactive compound from Indonesian medical herbals possessing anticancer, especially for mammary cancer. And, andrografolida compound (Andrographis paniculata Nees) from sambiloto has finally been detected as anticancer that has the activation (Sukardiman, 2005).

Moreover, as seen in Table 3, through one-way ANOVAs test it is known that the giving of the extract with preventive method made the volume of mammary gland significantly different from that in the DMBA modeling group, with $F = 99.930$ and $p < 0.001$ (4.36), and significantly not different from that in the control group. The mammary cancer of rats with preventive method was about 30% for 100 mg/ kg BM and 300 mg/kg BM doses, and was about 12.5 % for 1000 mg/ kg BM dose. In other words, it indicates that the giving of sambiloto extract with preventive method has influenced the decreasing of mammary cancer in rats. Furthermore, the working mechanisms of the testing sample actually are to improve the elimination of DMBA, to restrain the working of DMBA, to recover cells attacked with tumor or to postpone the emerging of tumor nodule (BPPT, 2007). However, the reason of the difference among the control groups with different doses of treatment indicated by 20.372 and $p < 0.000$ with one-way ANOVAs analysis was actually because in this study the body mass of animals in the early stage of the study was various, F(3.12). Thus, the most influencing factor in this study was the age of animals in the early stage of the treatment (Lenoir et al, 2005).

Unlike preventive method, the result of curative method, as shown in Table 4 and Figure 4, indicated that there were no significant difference of the volume of mammary gland between the control group and the cancer modeling group. In other words, it indicates that the giving of sambiloto extract with curative method did not influence the decreasing of mammary cancer in rats induced with DMBA. The reason is because the sambiloto extract could decrease the initiation and promotion of carcinogen towards the mammary gland without restraining it progress. The similar mechanism is usually obtained from anti cancer deriving from natural extracts (Kathryn et al, 2001). Therefore, doxorubicin was also used as the positive control in analyzing the effect of chemotherapy compared with the giving of sambiloto extract. Doxorubicin then was given in intra-peritoneal way with conversion from dose usually used in humans, for about four times. Nevertheless, the interval of the giving was shorten (from twice or three times a week to once a week) since it is clinically supposed to be given in intra-vena way, not in intra-peritoneal way. Thus, doxorubicin given in intra-peritoneal way was expected to have the same effect with that given in intra-vena way. The reason of giving doxorubicin in intra-peritoneal way is because it is difficult to give doxorubicin in intra-vena way in rats.

Moreover, it is known that the volume of mammary gland in rats after the giving of doxorubicin statistically has no significant difference from that in the cancer modeling group. It indicates that doxorubicin did not influence the decreasing of mammary gland in rats. In this study, doxorubicin was given when the animal has already suffered with cancer. And, the observation of the effect was also short since the animal was examined one week after the giving of doxorubicin was stopped. It means that the giving of doxorubicin only can decrease the severity of cancer cell that was proven in histology way without decreasing the volume of mammary gland cancer in rats.

Therefore, in order to analyze the alteration of epithelial tissue due to mammary cancer, the surgery and preparation of mammary gland must be conducted in the control normal group, the DMBA modeling group, the group with curative method, the group with preventive method, and the group of those animals treated with

doxorubicin. The epithelial tissue then was observed for its morphological alteration. Thus, special staining was not needed, but hematoxylineosin staining was still needed considering as the routine staining of histology preparate (Fawcett, 2002). All moderate and severe tumors actually have two basic components: (1) parenchyma, consisting of cells transforming (neoplastic), and (2) non neoplastic that supports stroma, derived from the host and consisting of bound tissue and blood vessels supporting the growth of parenchyma cell and considered to be very important for the growth of neoplasm (Kumar et al, 2007).

Furthermore, the result of the alteration of the epithelial cell in mammary gland of SD rats can be seen in Figure 2 (B-F) and 2 (H-L). It is also known that there was the microscopic difference between the growth of moderate and severe tumor and the slice of normal mammary gland though the moderate tumor consisted of cells differentiating well and looked similar with the normal one as shown in Figure 2 (A) and 2 (G). In moderate tumor differentiating well, mitosis is seldom found and its configuration is also normal (Kumar et al, 2007). This condition can be found in rats treated with the preventive method as seen in Figure 2 (J-L). Meanwhile, the severe tumor was signed with the various differentiations of parenchyma cell, from the well differentiation to non differentiation, as occurred in the rats induced only with 20 mg/ kg BM dose of DMBA for about 10 times. It means that the anaplastic cell is considered to be the main sign of severity.

Anaplastic cells, as seen in Figure 2 (B) and (H), indicated pleomorfisme, which was the real variation in the terms of form and size. Generally, nucleus is very hypercromatic and big. The ratio of nucleus to cytoplasm even can reach 1:1 compared with in the normal condition about 1:4 or 1:6. In other words, it is still possible to have a giant cell that is bigger than other cells around, and to have a big nucleus or some nucleuses. Rough and colonized chromatins and much mitosis, moreover, are also possibly found and become atypical. And, mess bunches that sometimes look in tripolar and quadripolar form still can also be found. The reason is because, anaplastic cells usually do not form an organized orientation pattern among them (those cells are lost their normal polarity). Thus, tumor cell possibly grows in layers followed by the lost of communal structure, such as no gland formation (Kumar et al, 2007).

In addition, the microscopic Figure of mammary gland in SD rats given sambiloto with preventive method, as seen in Figure 2 (J-L), showed that there was fibrosis' capsule strictly limiting tumor and tissue around. This capsule possibly derived from the original stroma tissue since the parenchyma cell got atrophy due to the big tumor pressure. Though all moderate tumor has no capsule, there is still separating area with strict line around the lesion, so the tumor cell is localized and has no ability for infiltrating, invading, or spreading to the further areas (Kumar et al, 2007). Sambiloto component that has the effect of anti cancer is trepanned by lacto compound, which is andrographolide, not only restraining the effect of topoisomerase DNA and cell cycle by inducing protein that restrains the cell cycle, (p-27), but also decreasing the expression of cycline dependent kinase that possibly contributes on the effect of sitotoxic with DNA fragmentation and apoptosis induction (Sukardiman et al, 2007). Andrographolide also has some other mechanisms related with cancer, which is improving the TRAIL (Tumor necrosis factor–related apoptosis-inducing ligand) in some cancer cells. TRAIL is an important member of TNF sub family (Tumor Necrosis Factor) that has big potential in cancer therapy (Zhou et al, 2008). Andrographolide, furthermore, can also activate p53 through phosphorilation and stabilization of protein. Restraining and attacking v-Src action are conducted through improving the degradation of v-Src protein considered as viral oncogenes (Liang et al, 2008). Andrographolide blocks the cycle of G0-G1 cell phase through the induction of cell cycle inhibitor, p2, and then decreases the level of Cdk4. p27 binding with the complex Cdk4-cyclin D1 in preventing phosphorilation of Rb protein (retinoblastroma) and in releasing factor of E2F elongasi in order to prevent the transcription of some proteins needed by cells to enter the phase of 'S' (synthesis) of cell cycle (Satyanarayana et al, 2004). Besides that, andrographolide also restrain NF-B to be bound with DNA. NF-B is a transcription factor found in many immune cells participating in arranging genes involved in the process of cellular and physiology, such as growth and apoptosis. NF-B also manages apoptosis through the protein expression of anti apoptosis.

The fact that andrographolide restrains NF-B then can explain the effect of andrographolide on breast cancer cell (Hidalgo et al., 2004). The apoptosis description of mammary gland in SD rats given sambiloto with curative method as shown in Figure 3 (D-F) and with preventive method as shown in Figure 3 (J-L) shows that there were some cells got apoptosis signed with the reduction of cells or nucleus showing the lost of deformation and contact with neighborhood cells. The condensation of chromatin and the edge line on the nucleus membrane occurred and then was followed by the sprouting of plasma membrane, and the fragmentation of cells into the solid membrane with the closed structure, called as apoptotic bodies containing with sitosol, condensed chromatin, and organelles. This apoptotic bodies then is swallowed by macrophage and

moved into the tissue without causing inflammation respond (Birollo et al, 2005). In SD rats given sambiloto with 1000 mg/ kg BM dose and in rats given doxorubicin, cells got apoptosis and necrosis signed with the reddish color because of the damaged cytoplasm from outside followed by the damaged nucleus, and then producing proteolitik enzyme stimulating the process of inflammation. Therefore, 1000 mg/kg BM dose of sambiloto extract was possibly considered to be so high that caused more necrosis and death in those animals than that in the other treated groups.

CONCLUSION

Based on this result, it can be indicated that DMBA could induce rats with mammary cancer by concerning the age of the rats when being induced with the carcinogen. However, andrographolide compound in sambiloto extract with both preventive and curative methods had a good effect for recovering mammary cancer in rats. Nevertheless, the use of sambiloto extract with the preventive method was better and could decrease breast cancer in those rats through andrographolide mechanism contributing on the sitotoxic effect by DNA fragmentation and apoptosis induction. However, the further study concerned about the best administration doses and route, and the optimal condition of host for responding tumor due to DMBA induction in designing mammary cancer model is still needed. Besides that, the further study about the pharmacokinetics of andrographolide metabolism is also needed in order to determine the optimum effective doses of the photochemical components in constraining breast cancer in humans.

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