Research Article

Essential Oil and Ethanolic Extract of Curcuma Inhibits Mammae Tumors in Rats Induction by DMBA

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ABSTRACT

Curcuminoids and xanthorizol are active components that may act as anticancer in the rhizome of Curcuma xanthorrhiza. The purpose of this study was to determine the content of the class of compounds in curcuma extract and essential oil using the phytochemical screening test method, the Folin-Ciocalteu method to determine the total phenolic content and the AlCl3 method for the total fentuconoid content. In addition, the activity of curcuma in inhibiting the influence of DMBA on the growth of breast cancer in rats was determined. Curcuma extract used is the result of extraction using 70% ethanol on an industrial scale. Total phenolic content and flavonoid extract of curcuma were determined by spectrophotometer. Inhibition testing of tumors formed due to DMBA induction was carried out by administering temulawak extract at doses of 35, 70, 140, 210 and 280 mg/kg body weight. Based on the phytochemical analysis, the ethanol extract of curcuma contained terpenoids, alkaloids, phenolic compounds and flavonoids. The results of the analysis of the ethanol extract of curcuma contained total phenols and flavonoids with concentrations of 42.96 mg GAE/g and 3.96 mg QE/g, respectively. The group that was given curcuma extract as the control group (p<0.05) had a significant difference. Based on the Mann-Whitney analysis, the third group with a dose of 140 mg/kg BW had the highest effect on tumor inhibition. The hope is that the development of curcuma extract into a standardized herbal medicine so that it can be used for the treatment of breast cancer can use the results of this research.

Keywords: Curcuma xanthorrhiza, DMBA, Breast Cancer, Curcuminoids, Xanthorizol
I. INTRODUCTION

Cancer is an abnormal cell growth caused by various factors that can change gene expression and cause dysregulation between cell proliferation and cell death. Uncontrolled cell proliferation develops into a population of cells that invades tissues and metastasizes to other organs (Muscaritoli et al., 2021). Two causes of cancer, namely endogenous and exogenous factors. Endogenous factors include genes and certain gene products, hormones, and enzymes. Exogenous factors include radiation, chemical carcinogenic compounds, and viruses (Wu et al., 2018). One of the chemical carcinogenic compounds is 7,12-dimethylbenz[a]anthracene (DMBA). DMBA has four bonded aromatic rings and two substitutional methyl groups located on the C7 and C12 atoms. DMBA can cause lesions in the mammary glands (Hamza et al., 2022). There are eight risk factors that have been proven to cause cancer, including obesity, low intake of fruit and vegetables, physical inactivity, smoking, alcohol consumption, unsafe sex, air pollution, and aging (Muscaritoli et al., 2021).

Breast cancer is a malignant tumor that attacks the breast tissue. The breast tissue consists of mammary glands (milk-producing glands), glandular ducts (milk ducts), and breast-supporting tissue. Breast cancer does not attack the skin of the breast which serves as a wrapper. Breast cancer causes cells and breast tissue to change shape to become abnormal and multiply uncontrollably. Breast cancer is the second most common cancer in women after cervical cancer in Indonesia. Breast cancer generally attacks women aged more than 40 years old, however young women can also develop breast cancer (Shumway et al., 2020).

Globocan, International Agency for Research on Cancer (IARC) in 2012 estimated breast cancer as the cancer with the highest percentage of new cases (43.3%) and the highest percentage of deaths (12.9%) in women globally. Based on the 2013 Basic Health Research data, the prevalence of breast cancer in Indonesia reached 1 per 2000 women (Ministry of Health, 2018). It was the highest type of cancer case in outpatients and inpatients, reaching 12,014 patients (28.7%) according to the Hospital Information System in 2010 (Ministry of Health of the Republic of Indonesia, 2015).

Chemotherapy is one of the breakthroughs in cancer control. Despite the successful outcomes, the toxicity and side effects of chemotherapy are substantial. Side effects of chemotherapy in breast cancer patients include alopecia, nausea, vomiting, myalgia, neuropathy, increase susceptibility to infection,
stomatitis, diarrhea, and thrombocytopenia (Faisel et al., 2013). The cost of chemotherapy is generally quite high, alternatively many people choose traditional treatments from natural ingredients that have been empirically believed to have anticancer activity. One of the natural ingredients that has been studied and was shown to have antitumor activity is Curcuma (Curcuma xanthorrhiza Roxb.) (Fitria et al., 2019).

Several pharmacological studies showed that Curcuma is efficacious as an antimicrobial, anticancer, antifungal, antioxidant, hypolipidemic, antihyperglycemic (Syamsudin et al., 2019). The toxicity test of the rhizome extract of Artemia salina shrimp larvae using the Brine Shrimp Lethality Test (BSLT) at 96% ethanol by (Prasetyorini et al., 2011), gave the best results on the maceration method. reported extracts of ethyl acetate fraction, and isolates of Curcuma rhizome had antiproliferative activity against T47D breast cancer cells with IC₅₀ concentrations of 19.15 g/mL, 17.07 g/mL, and 19.22 g/mL, respectively. Curcuminoids are some of the main bioactive compounds of Curcuma that give the rhizome a yellow color. Studies have shown the pharmacological potential of curcuminoid compounds as antioxidants, anti-inflammatory, antitumor, and anti-allergic properties (Hewlings & Kalman, 2017). Xanthorrhizol, a component of Curcuma, has anticancer activity, especially against breast cancer (Anggakusuma et al., 2009).

In vitro study using a combination of Curcuma and propolis (Hasan et al., 2019) has been reported, however, there was no in vivo study that has been conducted. The potential of curcuma as an anticancer in vivo using Curcuma extract produced on a pilot scale has never been done. This study aims to investigate the activity of Curcuma extract in inhibiting the growth of DMBA-induced rat mammae tumor in Sprague Dawley rats.

II. METHODS
A. Sample Preparation

Sample preparation was carried out according to Hasan et al. (2019) with modifications. Standardized of Curcuma extract was obtained from PT Phytochemindo Reksa using ethanol as a solvent containing curcuminoid compounds of 6.06% and xanthorrhizol of 7.72%. The finished extract was then stored in bottles at cold storage. The essential oil samples were obtained from PT Phytochemindo Reksa using hexane as a solvent extraction method with a curcuminoid content of 1.93% and a xanthorrhizol content of 17.60%.
B. Phytochemical Qualitative Test
Phytochemical Qualitative Test was carried out based on references (Harborne (1998) and Widjajakusuma et al. (2019) with modification of the materials used. Phytochemical testing was carried out to identify the chemical content in the ethanolic extract of curcuma rhizomes as a first step to determine the type of active component (phenolics, flavonoids, alkaloids, saponins, tannins, steroids and terpenoids).

C. Total Phenolic Content
Determination of total phenolic content using standard gallic acid was adapted from Javanmardi et al. (2003). A total of 0.2 mL of sample was added with 2.5 mL of 10% Folin-Ciocalteu reagent and incubated at 25 °C for 8 minutes. 2 mL of 7.5% Na$_2$CO$_3$ was added and incubated again at 25 °C for 30 minutes. The absorbance was measured using a spectrophotometer at a wavelength of 765 nm. Total phenolic value was calculated with the following equation:

$$C = c \frac{V}{m}$$

Note:
C = Total phenolic content (mg GAE/g)
c = sample concentration (mg/L)
V = sample volume (L)
m = sample weight (g)

D. Total Flavonoid Content
Determination of flavonoids content using standard Quercetin was adapted from Kawiji et al. (2011). A total of 0.2 mL of sample was added with 3 mL ethanol, 0.2 mL 10% AlCl$_3$, 0.2 mL Potassium acetate 1 M, and filled up to 10 mL with distilled water. The solution was incubated for 30 minutes at room temperature and its absorbance was measured on UV-Vis spectrophotometry at a wavelength of 376 nm. Determination of total flavonoids was expressed in quercetin equivalent (mg QE/g extract).

$$\text{Flavonoid QE (C)} = c \frac{V}{m}$$

Note:
C = Total phenolic content (mg QE/g)
c = sample concentration (mg/L)
V = sample volume (L)
m = sample weight (g)

E. Induction of 7,12-dimetilbenz(a)antrasen (DMBA) (Modified)
DMBA induction was carried out using a modification of Asyura et al. (2017). DMBA solution in olive oil was prepared at a dose of 20 mg/kg BW. The DMBA solution was stored in a glass bottle. Injection was done subcutaneously into the third mammary from below on the left side of the rat's body. The induction process was carried out twice with a span of 1 week from the first injection. ACUC number is 174-2020 IPB.
1. Treatment of rats

The treatment of rats followed the combination of Hasan et al. (2016) by making modifications. The number of samples for each treatment was determined using the Federer formula (Federer, 1966):

\[(n-1) (r-1) \geq 15\]

Description:
\( n = \) number of treatment groups
\( r = \) number of samples per treatment group

A total of 32 rats were divided into 8 groups. The group consisted of 5 treatments groups with different dose of Curcuma extract, 1 treatment group with essential oil, 1 tumor control group induced by DMBA, and 1 normal control group. The rats, except those from the healthy control group, were induced by DMBA through subcutaneous injection in mammary tissue. The 5 treatments groups with Curcuma extract, each contained 4 rats, were given the Curcuma extract orally with the following doses.

- **Group T1**: Curcuma extract dose of 35 mg/kg BW.
- **Group T2**: Curcuma extract dose of 70 mg/kg BW.
- **Group T3**: Curcuma extract dose of 140 mg/kg BW.
- **Group T4**: Curcuma extract dose of 210 mg/kg BW.
- **Group T5**: Curcuma extract dose of 280 mg/kg BW.
- **KT group**: tumor control group.
- **EO group**: 140 mg/kg essential oil treatment.
- **Normal Group**: normal controls not induced by DMBA.

The Curcuma extract and essential oil were given orally every day for 8 weeks, after the DMBA induction. Both groups were necropsied after 75 days.

2. Tumor palpation

The position of the tumor is detected macroscopically by palpation and diameter once a week starting after induction of DMBA (Hasan et al., 2016). The growth of tumor diameter was calculated to determine the volume of the tumor formed using the following formula.

\[\text{Volume tumor (V)} = \frac{ab^2}{2}\]

Note:
\( a = \) diameter of the longest tumor
\( b = \) shortest tumor diameter

3. Euthanization of test animals

The rats were euthanized after the last treatment with ketamine:xylazine at a dose of 100 mg/mL:20 mg/mL (10:1) intraperitoneally (Mustofa et al., 2019). The rats were exsanguinated via cardiac puncture and the mammary tissues were collected for histopathological analysis.

4. Histopathological preparations

Making histopathological preparations following the guidelines in the research of Hasan et al. (2016). The mammary glands of rats were fixed in 10% BNF solution and cut into thin tissue and placed in a tissue cassette followed with dehydration process by immersion in
graded alcohol (ethanol 70%, 80%, 90%, 96%, absolute I, and absolute II). The remaining ethanol was clarified with xylol I, II, and III. Afterward was infiltration process with liquid paraffin at 60 °C for 30 minutes for 4 times. Prior to use, the mold was washed with a mixture of 96% ethanol, xylol, and distilled water. Hot paraffin was poured into the molding block until half the volume of the mold. This was the embedding stage where the tissue was carefully implanted into a mold containing liquid paraffin. The process was carried out carefully to prevent the tissue from touching the bottom of the mold which later would be covered with liquid paraffin. The paraffin-embedded tissue was cut with a microtome of 4-5 m thickness and the pieces were put into warm water at 40 °C to melt the paraffin to allow the pieces to unfold. Finally, the tissue preparation was placed on a slide and dried in an incubator at 60 °C for one night.

5. Hematoxylin-Eosin staining

The preparation slide was processed with deparaffination using xylol twice with a time of 2 minutes each and rehydrated in a series of graded alcohols (Hasan et al., 2016). Rehydration started with absolute alcohol for 2 minutes followed with 95% and 80% ethanol for 1 minute each. Afterward, the preparation was rinsed with running water for 10 minutes. The staining process was a repeated staining and rinsing process with two dyes. First, hematoxylin (Mayer's dye) was used to stain the tissue for 8 minutes before it was rinsed with running water. The stained tissue was soaked in a lithium carbonate solution for 30 seconds and rinsed with running water. Second, the tissue preparation was stained with eosin dye for 2-3 minutes and again rinsed with running water. For re-dehydration, graded alcohols were used, 95% ethanol and absolute ethanol I for 10 times each, and with absolute ethanol II for 2 minutes. Xylol I was added for 1 minute and continued with xylol II for 2 min. Permount adhesive and a cover slip was put on the hematoxylin-eosin-stained preparation. It was further dried and kept for microscope observation.

6. Histopathological analysis

Mammary tissue from hematoxylin-eosin (HE) staining was performed under a light microscope with a magnification of 40 times (Hasan et al., 2016). The general description and histopathological changes of mammary tissue were recorded descriptively.

7. Analysis of Data

Statistical analysis for quantitative data from the measurements of tumor weight and volume were done using the IBM SPSS Statistics version 2.2
The data was tested using the Saphiro-Wilk test for normality, and the Levene test for homogeneity. Nonparametric values were analyzed using Mann-Whitney and Kruskall-Wallis test. If the data is significantly different at $p<0.05$, the data is considered significant.

III. RESULTS AND DISCUSSION

The chemical contents in the ethanolic extract of Curcuma rhizome were identified through phytochemical testing and the type of active component were determined. Phytochemical qualitative tests were carried out, among others, on alkaloids, flavonoids, phenolics, saponins, steroids, triterpenoids, and tannins. Table I shows that Curcuma extract contains phenolic, while Curcuma essential oil does not contain phenolic.

Other compounds found in Curcuma extract include flavonoids, alkaloids, and triterpenoids. Saponins, tannins, and steroids were not found in Curcuma extract. Similarly, the Curcuma essential oil contains flavonoids, alkaloids, and triterpenoids, whereas saponins, tannins, and triterpenoids were not found in essential oils.

The results obtained in this study, phenolic compounds, flavonoids, alkaloids, and triterpenoids are found in the ethanolic extract of Curcuma rhizome. It is in accordance with the results of Yurleni (2018) analyzing with various of methods, extraction, both maceration, Soxhlet, and reflux, but did not detect the presence of steroids. Contrary, Prasetyorini et al. (2011), reported that the ethanolic extract of Curcuma was also positive for steroids.

Environment and genetics can affect the content of secondary metabolites in plants. Curcuma grown at different altitude may have different environmental temperature and can affect the biochemical processes found in the curcuma (Khumaida et al., 2019). Secondary metabolite compounds are also influenced by soil texture of the growing site and the biosynthetic precursors of secondary metabolites (Khumaida et al., 2019). The Curcuma and EO samples used in this study originated from Sukabumi and were extracted on a development scale at PT...
**Phytochemindo Reksa.** In Yurleni (2018) research, the sample used was from the *Angso duo* Market Jambi, while Prasetyorini et al. (2011) used sample from the Experimental Garden of the Industrial Plant Research Institute in Sukamulya Sukabumi.

From Table I, alkaloids are found in the essential oils. According to Syamsudin et al. (2019) this result does not match. It can be caused by differences in the extraction method used. The EO used in this study was extracted using the solvent extraction method or the so-called solvent extraction. The working principle of this method is separation based on differences in solubility. Some solids can be dissolved by a certain solvent enabling the desired dissolved material to be obtained in the solvent (Seager et al., 2018). Solvent extraction technique is a technique where the two immiscible liquids move in opposite directions in continuous contact with each other, this causes the separation of solutes (Patel et al., 2019).

According to Habli et al. (2017), there are several mechanisms of alkaloids as anticancer, among others, to damage breast cancer cell DNA which is characterized by the upregulation of H2AX as a marker of DNA damage, inhibition of cell growth by induction of growth inhibition in the G2/M phase, activation of the p38 MAPK pathway which leads to cytotoxicity against breast cancer and inhibits the activation of the NF-kB pathway thereby the cancer development pathway is inhibited, to target HER2 cancer cell death, inhibition of p-glycoprotein (P-gp) as a member of ABCB1 of the ABC protein so as to prevent multidrug resistance.

Many pharmacological activities own by terpenoids (Kohnen-Johannsen & Kayser, 2019). It can activate the immune system by enhancing cell-mediated and humoral immune responses. Due to its anticancer immunity, terpenoids also has the potential as anticancer and antimitastases. Through the activation of intracellular signaling cascades, terpenoids can induce apoptosis in various cancer cells. Furthermore, terpenoids showed inhibition effect to various oncogenic expressions and various signaling cascades involved in tumor and cancer development (Kuttan et al., 2011).

Phenolics are a class of secondary metabolites found in plants. The total phenolic content was determined spectrophotometrically at a wavelength of 765 nm. In the measurement of total phenolic content, the concentration of extract used was 3000 ppm. Gallic acid equivalent (GAE) with units of mg GAE/g extract is used to express the total phenolic value, wherein 1 gram of sample extract contains 1 mg of the compound equivalent.
to the standard (gallic acid). In this study, Follin-Ciocalteu reagent was used to determine the total phenolic analysis. The standard equation obtained was $y = 0.0033x + 0.0206$ with $R^2$ value of 0.9922. From the analysis, the total phenolic content in the Curcuma extract was 42.96 mg GAE/g Curcuma ethanol extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Phenolic (mg GAE/g)</th>
<th>Total Flavanoid (mg QE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This Research</td>
<td>42.96</td>
<td>3.96</td>
</tr>
<tr>
<td>Qader et al. (2011)</td>
<td>88.0</td>
<td></td>
</tr>
</tbody>
</table>

Based on research Table II, the smaller amount compared to the results of Qader et al. (2011) may be influenced by differences in the cultivation site of the sample and the method used. Research by Qader et al. (2011) carried out the extraction using a laboratory scale maceration method, while in this study the extraction method on a development scale was used. According to Ramayani, et al. (2016), the extraction method had a significant effect on the total phenolic content of the measured extract.

Several mechanisms of phenolics as anticancer breasts including gallic acid is through inhibition of cell proliferation by halting the cells in the G2/M phase. Benzoic acid can halt the G2/M phase cell cycle in MDA-MB-231 and MCF-7 breast cancer cells, also benzoic acid increases caspase-3. Ferulic acid can suppress metastasis in breast cancer cell lines by replacing the mesenchymal transitional epithelium (EMT). Epidermal Growth Factor (EGF), which is induced by migration and chemotaxis of breast cancer cell lines can be inhibited by 4-methyl-3-nitrobenzoate derivatives (Anantharaju et al., 2016).

Flavonoids are a group of secondary metabolites which are included in the phenolic compound group. The total flavonoid content was determined spectrophotometrically at a wavelength of 376 nm. Curcuma extract 3000 ppm was used to measure the total flavonoid levels. The total value of flavonoids was expressed in the form of quercetin equivalent (QE) with units of mg QE/g extract, wherein 1 gram of sample extract contains 1 mg of the compound equivalent to the standard (quercetin). AlCl$_3$ reagent and a quercetin standard series were used to determine the total flavonoids. The standard curve equation obtained was $y = 0.0033x + 0.0158$ with an $R^2$ value of 0.9946. From the equation, the total flavonoid content was 3.96 mg QE/g ethanol extract of Curcuma.

Role of flavonoids is to protect plants from biotic and abiotic stresses, as signaling molecules, allelopathic compounds, phytoalexins, as detoxifying
agents, and as defense compounds against microbes (Panche et al., 2016). The total content of flavonoids in the ethanol extract (Table II) is smaller compared to the report by Pistelli et al. (2012), in which the total flavonoid content in methanol extract was 11.8 g QE/mg extract. Again, it can be influenced by differences of the cultivation site, the solvent used, and the method used. Differences in solvents can cause differences in the total flavonoid content of a sample because a compound would dissolve in solvents that have the same polarity. Flavonoid compounds are divided into several types that have different polarities. The solubility of flavonoids in solvents is affected by the level of polarity which is depending to the number and position of the hydroxyl groups of each type of flavonoid which affected (Verdiana et al., 2018). Methanol has fewer C atoms hence a higher level of polarity than ethanol. Compounds bound by methanol and ethanol solvents would have different levels of polarity (Ruslan, 2018).

There are several mechanisms of flavonoids as breast anticancer, including inhibition of proliferation by acting antagonistically with hydrogen peroxisa (H₂O₂), induction of apoptosis by arresting the cell cycle in MCF-7 and TNBC cell lines through inhibition of PI₃K activation and increase of FOXO₃, increase of caspase 3 activity and caspase 9 that induce apoptosis, inhibition of iNOS (inducible Nitric Oxide Synthase) and inactivation of the JAK2/STAT3 signaling pathway, inhibition of growth and migration through the lymphatic endothelium barrier, suppression of the antiapoptotic pathway regulated by NFkB (Sudhakaran et al., 2019).

Rat BW was measured during the acclimatization, DMBA induction, extract administration, and follow up. All treatment groups experienced an increase in BW during the acclimatization period (M-2 and M-1). M0 was the first week of DMBA induction, while M1 to M16 were the week after induction and receiving treatment (Figure 1). Prior to the study, the rats had varied BW, however at M-2 and M-1 the BW were not significantly different (p>0.05) between the groups. In general, the rats experienced an increase BW until the 16th week, except for the EO group which showed a decrease up to 41.58% at the 5th to the 16th week after treatment. The highest increase in BW was in the T2 group with 42.81% of increase, while the lowest increase was observed in the T5 group, with only 1.46% of increase. The increases in BW of T1, T3, T4, KT, and the normal group were 21.49%, 25.88%, 21.02%, 38.84% and 39.32%, respectively.

Normality test is used to determine whether the data is normally or not normally distributed. Homogeneity test is a
test used to determine whether the research data group has the same variance or not. The Shapiro Wilk test found that the BW data of rats were not normally distributed in the T1, T3, T4, tumor, and EO groups (p < 0.05) and on the contrary in the T2, T5, and normal groups (p > 0.05). From the Levene homogeneity test, the BW data showed an inhomogeneous variation (p< 0.05).

Figure 1. Sprague Dawley rat body weight.

The results of the normality and homogeneity test showed that the data distribution was not normal and inhomogeneous. The Kruskal-Wallis nonparametric analysis test was carried out to see the difference in BW between the treatment groups, followed by Post hoc analysis using the Mann-Whitney test to find out which groups had significant differences. A significant difference was found in BW of the rats between the experimental groups based on the Kruskal-Wallis’s test, p value = 0.000 (p<0.05 was considered as significant).

The results of the Mann-Whitney test (Table III) showed variations in BW of rats between treatment groups. A significant difference was seen in T2, T5,
and tumor control groups BW compared to the normal rat group with p-values of 0.000, 0.003, and 0.000, respectively (p<0.05 was considered as significant). On the other hand, the BW of the T1, T3, and EO groups did not have a significant difference with the normal group with p-values of 0.535, 0.877, and 0.058, respectively.

Rats induced with DMBA generally decreased body weight (Kusnul et al. 2019), so that it can be seen whether the curcuma extract can improve the body weight of the rats. Data on body weight of the rats showed that the average weight growth increased at the end of the week, except for the EO group which experienced a decrease. The groups that had significant differences in weight with the normal group were only T2, T5, and KT groups. It is not in line with the research results of Kusnul et al. (2019), that reported a significant difference in body weight between the DMBA-induced group and the non-DMBA-induced group. The group that was not induced by DMBA experienced a stable increase, while the group of rats induced by severe DMBA increased steadily.

Rats in the Curcuma extract treatment group experienced increase in body weight compared to the initial weight. It indicates that Curcuma extract may improve body weight. Curcuma is often used in rural areas to increase appetite. Feed consumption and weight of white rats are increased by Curcuma ethanol extract treatment (Rahardjo, 2010). For experimental animals, Curcuma is given and can be used for smooth work, to accelerate gastric emptying. It causes a feeling of hunger and thus increases appetite (Widjajakusuma et al., 2019).

The decrease in body weight in the EO group could affect the effect of cachectic. Cachectic is a tumor necrosis factor (TNF) which has a function to decompose fat and to reduce enzymes that play a role in the production and storage of fat. Cachectic can cause a condition called cancer cachexia, or weight loss due to cancer. The development of body weight can also be influenced by stress conditions due to DMBA induction and the administration of the test extract, so that it can reduce feed consumption or can affect the metabolism of experimental animals (Wibowo et al., 2011). In a study (Darmawan and Pramono 2016), giving EO can reduce body weight in rat. This is because essential oil of C. xanthorrhiza decreases the appetite of rats.

Measurement of tumor volume was carried out after the mice were induced with DMBA, measurements were made once a week in the treatment groups T1, T2, T3, T4, T5, KT, and the essential oil group. Overall, all groups experienced an increase in tumor volume until the 6th week, in exception for the T3 group (Figure 2). Each
The tumor volume in the Curcuma treatment group had a significant difference compared to KT, however the tumor volume in the EO group did not have a significant difference with KT. This shows that all the treatment groups that were given Curcuma extract had inhibition effect on tumor development. The T3 treatment group showed the largest reduction of tumor size compared to the other Curcuma treatment groups, therefore the T3 treatment group was the group that had the highest effect on the inhibition of rat mammary tumor volume. In contrast, the KT group had the largest increase in tumor size, showing that DMBA induction increased tumor progression (Wuyung, 2016).

The highest delta value was found in KT with a delta value of 9.255 cm³ while in the T1, T2 and EO groups the delta values were 1.867 cm³, 6.584 cm³ and 0.638 cm³, respectively. KT, T1, T2, and EO showed that the volume at the end of the week were larger than the base line. In contrast, the tumor volume at the end of the 16th week in the T3, T4, and T5 groups was smaller than the initial volume. The observed delta values in groups T3, T4 and T5 were -1.019 cm³, -0.574 cm³ and -0.503 cm³, respectively.

The delta value in the EO group was relatively small, it showed that EO has the
effect to reduce tumor progression. Fitria et al. (2019) showed that essential oil from Curcuma has an LC$_{50}$ of 83.6 g/mL and has antiproliferative activity against MCF-7 cancer cells with an IC$_{50}$ value of 139.8 g/mL. LC$_{50}$ is the concentration that can cause death by 50% in the test organism. Curcuma essential oil has toxic potential because it has an LC$_{50}$ value of less than 1000 g/mL. It was able to inhibit the proliferation of MCF-7 cancer cells by 59.1% at a concentration of 170 g/mL.

**Figure 2.** Effect of treatment on the development of breast tumor volume in Sprague Dawley rats.

- **T1** (dose group 1), **T2** (dose group 2), **T3** (dose group 3), **T4** (dose group 4), **T5** (dose group 5), **EO** (Essential Oil dose group), **KT** (tumor control)

**Table IV.** Results of the Mann Whitney analysis of tumor volume between treatment groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>KT</th>
<th>EO</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>0.748</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>0.072</td>
<td>0.047(*)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>0.167</td>
<td>0.080</td>
<td>0.541</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>0.96</td>
<td>0.079</td>
<td>0.886</td>
<td>0.638</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KT</td>
<td>0.027(*)</td>
<td>0.043(*)</td>
<td>0.002(*)</td>
<td>0.010(*)</td>
<td>0.030(*)</td>
<td>KT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EO</td>
<td>0.613</td>
<td>0.818</td>
<td>0.030(*)</td>
<td>0.66</td>
<td>0.072</td>
<td>0.060</td>
<td>EO</td>
<td></td>
</tr>
</tbody>
</table>

Description:

- (*) = Significantly different
- T1 = Curcuma group with a dose of 35 mg/kg
- T2 = Curcuma group with a dose of 70 mg/kg
- T3 = Curcuma group with a dose of 140 mg/kg
- T4 = Curcuma group with a dose of 210 mg/kg
- T5 = Curcuma group dose of 280 mg/kg
- EO = 140 mg/kg essential oil treatment group
- Normal = Group not induced by DMBA
- KT = Tumor control without test extract

According to Wang et al. (2016), curcumin showed an effect on the growth and invasion of breast cancer through the downregulation of the NF-κB signaling...
pathway. Curcumin can also induce breast cancer apoptosis by regulating the expression of apoptosis-related genes through the extrinsic pathway by Bcl-2, by increasing the antiapoptotic protein Bcl-2 and decreasing the proapoptotic protein Bax, thereby increasing the Bax/Bcl-2 ratio.

The mechanism of curcumin as an anticancer can also be through the termination of the cell cycle in the G2M phase and the final phase in MCF-7. Curcumin can prevent the loss of T cells and can inhibit immune-suppressing cytokines. In inhibiting the growth of breast cancer, the mechanism can also be through metastatic factors. First, it can inhibit angiogenesis, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in ER-negative breast cancer cells. Second, it can influence metastatic factors through invasion. Curcumin can also inhibit oncogenes, such as the gene erbB2/neu (HER2) which encodes p185neu tyrosine kinase. Overexpression of these genes causes breast cancer (Wang et al., 2016). The combination of xanthorrhizol with curcumin showed synergism in inhibiting the growth of cancer cells compared to xanthorrhizol alone (Oon et al., 2015).

Histopathological appearance of all treated groups shows a different morphology from the normal group (Figure 3). Microscopic appearance in the normal group shows the presence of lactiferous ducts lined with 1-2 layers of cuboidal epithelium. The epithelial layer was neat and tight, and there was normal connective tissue. A widening of the lumen of the male duct was seen in the microscopic picture of T1, T4, and T5 treatment groups, in addition to T1 and T4 groups there were inflammatory cells in the lumen of the lactiferous duct. Irregular microscopic appearance of cells was spotted in T2 group, which consisted of elongated and round cells. Moreover, in T2 group it appeared that the epithelial cells had proliferated towards the ductal lumen. In T3 group, there was an elongated cell shape that would likely to develop into fibrosarcoma. KT microscopic results shows that the lactiferous ducts underwent a change in shape to become tortuous. The epithelial cells surrounding the lactiferous ducts were composed of more than 2 layers and were irregular. In the EO group, the lactiferous ducts were surrounded by more than 4 layers of irregularly shaped cuboidal epithelium. In addition, the lactiferous ducts were also found to have a narrower lumen, or the lumen diameter was getting smaller.
Figure 3. Histopathology of the mammary glands: a. mammary gland of normal group; as a normal tissue formation composed of mammary glands and adipose tissue; b. mammary glands in T1 group reveals proliferation and enlargement of mammary gland (adenoma); c. mammary glands of T2 group shows abundant tumor cells with consist of mammary epithelial/Ep and spindle cells; d. T3 treated group reveals development of tumor (epithelial and spindle) cells; e. T4 group indicates a suppressed development of tumor cells, which companied by appearance of adipose tissue; f. T5 group shows that proliferation of epithelial cells are still presence with adipose tissue; g. tumor group which indicates irregular form of mammary gland surrounding the lumina (Lu) with proliferation of epithelial cells (Ep); h. EO treated group shows a thickening and proliferation of mammary gland epithelial cells, these cells are still abnormally form combined with adipose tissue.

Histopathological features of the mammary glands of rat showed the development of an abnormal shape in DMBA-induced rats. Indicators of changes that can be observed include epithelial layer thickness, ductal diameter, and ductal lumen diameter. The mean epithelial thickness in the Curcuma group had fewer layers than KT and EO groups. Epithelial cells in the EO group that were stacked more than 4 layers showed that cell repair had not occurred. In EO group, the volume delta value was 0.638 cm$^3$, which is relatively lower compared to T1 and T2 groups. It shows that EO has healing potential but low repairing ability.
Changes in the thickness of the ductal epithelial layer in the group given Curcuma extract showed a change towards improvement, which was marked by a decrease in epithelial thickness due to decreased cell proliferation activity, although it was not close to the normal group. The decrease in the mean thickness of the ductal epithelium would affect the average diameter of the ductal lumen, like the diameter of the ductal lumen would widen as the average thickness of the epithelium decreases. Cell repair is indicated by an increase in the mean ductal lumen (Larasati et al., 2019).

According to Nansi et al. (2015), inhibition of cell proliferation and induction of apoptosis in mammary gland epithelial cells is occurred due to the action of curcumin compounds. Curcumin can counteract free radicals and act as an antioxidant to inhibit the metabolic process of carcinogens into active forms in the body. It can act as a cyclooxygenase inhibitor to prevent excessive proteinoid production. The occurrence of inflammatory reactions and cell proliferation can be reduced by Curcumin, and it can stimulate apoptosis. Curcumin can inhibit the expression of c-myc which is a proliferative gene. In addition, it can increase the activity of the p53 gene, which is a tumor suppressor and regulatory gene that is activated if there is damage to DNA or certain stress on cells.

Antiproliferative activity can also be inhibited by xanthorrhizol by inducing apoptosis through regulation of Bcl-2, p53, and PARP-1 (Lim 2016). According to Akhtar & Swamy (2018), in an in vivo study, xanthorrhizol can inhibit tumor formation and its development by reducing ornithine decarboxylase (ODC), COX-2, and Nf-kB signaling activity. Ornithine decarboxylase is a rate limiting enzyme in the polyamine formation pathway and has an important role in several biological functions including embryonic development, cell cycle, and proliferation. Overexpression of ODC will increase tumorigenesis in vivo. The reduced ODC activity would reduce cancer progression (Wu et al., 2011).

The histopathological results of the T3 mammary glands have the potential to develop into fibrosarcoma. It can also be affected due to the formation of sores near the leg bones. Fibrosarcoma is a malignant tumor originating from mesenchymal cells, usually located in deep soft tissue or adjacent to bone. It is a form of pathological transformation of spindle-shaped fibroblast cells. Breast fibrosarcoma is a rare tumor and vary in size, from less than 1 cm to more than 40 cm. Primary breast sarcomas can spread by hematogenous metastases or by direct invasion (Lee et al., 2011).
The place where DMBA undergoes activation of an active metabolite, such as DNA adduct, is in the epithelial cells of the mammary glands. Production of reactive oxygen species (ROS) occurs during metabolic activation of DMBA. The DMBA metabolite will cause DNA adducts (complexes formed on certain parts of DNA that are covalently bound to mutagen compounds) with guanine bases in DNA, causing oxidative damage to the structure and function of DNA, proteins, and lipids. Mutations may occur due to the DMBA metabolite interacting with electron-rich DNA centers. The interaction that occurs between DMBA and DNA in a cell is the initial stage of chemical carcinogenesis (Larasati et al., 2019). According to Wuyung (2016), the type of tumor that appears can be influenced by age, reproductive history, hormonal environment of the rat at the time of exposure to carcinogens, diet, and the dose of carcinogen given.

IV. CONCLUSION

Curcuma ethanol extract contains phenolic compounds, flavonoids, alkaloids, and terpenoids that have the potential to suppress the development of mammary tumors. The total phenolic and total flavonoid content of Curcuma ethanol extract were 42.96 mg GAE/g extract and 3.96 mg QE/g extract, respectively. The highest activity in inhibiting the growth of mammary tumors was at the dose of 140 mg/kg BW with a delta value of -1,019 cm$^3$.

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REFERENCES


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