Journal of Advances in Medical and Pharmaceutical Sciences
5(4): 1-9, 2016, Article no.JAMPS.22301
ISSN: 2394-1111

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Antiproliferative Activity by Ethanolic Extract of Red *Alpinia galanga* (L) Willd in Inoculated Breast Carcinoma Cells of C3H Mice

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Authors' contributions

This work was carried out in collaboration among both authors Author AA designed the study with author SW, performed the statistical analysis and wrote the first draft of the manuscript. Author SW performed the treatment and literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMPS/2016/22301
Editor(s):
(1) Palmiro Poltronieri, National Research Council of Italy, CNR-ISPA, Italy and Institute of Sciences of Food Productions, Operative Unit in Lecce, Italy.
Reviewers:
(1) Gerhard Hamilton, Medical University of Vienna, Austria.
(2) Anonymous, University of Napoli Federico II, Italy.
Complete Peer review History: http://sciencedomain.org/review-history/12542

Original Research Article

Received 27th September 2015
Accepted 20th November 2015
Published 3rd December 2015

ABSTRACT

Background: The objective of this study is to investigate the anticancer activity of ethanolic extract of *Alpinia galanga* Willd (galangal) on breast adenocarcinoma cells transplanted in C3H mice. In some previous studies ethanolic extract of galangal containing active substance i.e 1'-acetoxychavicol acetate (ACA), is used as anticancer by various mechanisms such as induction of apoptosis, inhibiting cell proliferation, antiinflammation and antioxidant.
Methods: This experimental study was designed by post test only controlled design group, using 32 C3H mice. The C3H mice were inoculated with tumor cells and then divided into 4 groups: control group (K) and three treatment groups (T1, T2 and T3) given graded doses of ethanolic extract of galangal (225 mg/kobo/day, 450 mg/kobo/day and 675 mg/kobo/day) for 2 weeks. After all mice were terminated, we count the tumor volume of each mice. Ki-67 immunostaining was performed to analyse cell proliferative activity. All the data were analyzed with Pearson Chi

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Square and OneWay Anova.
Results: This study found that there is marked differences of tumor volume between groups, with control group has the highest tumor volume and treatment group 3 is the lowest. The immunoexpression of Ki-67 has highest mean score at control group and the lowest at T3 group. There were statistically significant correlation between tumor volume and dose of galangal extract, and significant differences among groups of Ki-67 immunoexpression.
Conclusion: Oral administration of ethanolic extract of *Alpinia galanga* Willd at the graded doses has anticancer activity on C3H breast adenocarcinoma by inhibition of cell proliferative activity and growth of tumor volume. The best result of anticancer activity is found in group with the highest dose of galangal extract (675 mg/kgBW).

Keywords: Antiproliferative activity; inhibition of tumor growth; breast adecarcinoma cells; ethanolic extract of red galangal; Ki-67 immunostaining.

1. BACKGROUND

According to data from the Agency for Cancer Registration, Indonesian Association of Pathologist (IAPI) in 2006, breast cancer is the second most common cancer in Indonesian women, but the first common cancer in Padang, West Sumatera. Most drugs currently available for cancer treatment have limited potential because they are toxic with adverse effects, ineffective or expensive. Hence, the identification of novel, efficient and less toxic anticancer drugs remains a challenging task. Use of plants extract could be the effective methods to solve this problem [1,2].

Plants extract has chemopreventive potential by many mechanism. Indonesia has the second biggest biodiversity in the world expressed by a high number of medicinal plants, such as ginger, and galangal (also known as lengkuas). Such galangal is generally found in red and white. The first is widely used as spice in Indonesian cuisine whereas the second employ to treat gastric disorders, act as anti-fungal, anti-itching and to increase appetite [3-5]. According to Rusmarilin [6], red galangal has more anticancer active compounds than white galangal. Thus, galangal kills cancer cells and protects healthy cells from malignant transformation, dual benefit that is rare among chemopreventive agents.

The major phytoconstituents which have been isolated from the galangal is I-Acetoxychavicol Acetate (ACA) and hydroxychavicol acetate (HCA). The extracts of galangal showed acetylcholinesterase-inhibitory, platelet-activating factor (PAF)-inhibitory, antimicrobial, antibacterial, antifungal, antioxidant and apoptosis activities. It has been reported that extract of galangal also showed anti-inflammatory, immunostimulating, antinociceptive, hepatoprotective, cytotoxic and anticancer activities [7-10]. Rusmarilin [6] studied ethyl acetate extracts of galangal inhibit the proliferation of cancer cells in vitro and in vivo. The result indicated that the highest concentrations of ACA present in rhizome extract of red *A. galanga* causing a significant reduction growth of lung carcinoma cells (A 549 cell line), and suppress the proliferation of serous ovarian papillary adenocarcinoma. Galangal extract also inhibit the growth of transplanted breast carcinoma in mice, it may have an apoptosis stimulating effects indicated by wide necrosis area in microscopic features [6].

Several studies reported the mechanism of anticancer activity of galangal. Rusmarilin stated that galangal extract induce the secretion of IFN-γ. Galangal is one of the herbs that have anti-inflammatory effects by inhibitory pathway of NFκB, inhibit COX-2 and production of nitric oxide. Study of ACA effect on apoptosis found that ACA inhibit mitogen-activated protein kinase (MAPK) that lead to decrease of intracellular polyamine. Decrease of polyamine will activate caspase-3 pathway and lead to apoptosis [4,6,11,12].

In the present study, we aimed to assess the anticancer activity of ethanolic extract of galangal on the breast adenocarcinoma cells transplanted in female strain C3H mice by doing evaluation the tumor growth and Ki-67 immunoexpression as proliferative activity marker.

2. MATERIALS AND METHODS

This study was an experimental study using post test only controlled group design. Thirty two female strain C3H mice (treatment groups) and donor mice strain C3H, obtained from Anatomical Pathology Laboratory of the Faculty of Medicine,

University of Indonesia were used in the experiments. Inclusion criterias for the experimental mice are 3 – 4 months, and body weight 15 – 25 grams. All experimental animals were maintained under standard laboratory condition with free access to food and water. All animals procedures were approved by Research Ethics Committee, Faculty of Medicine, Andalas University (Ethical Clearance No.: 149/KEP/FK/2012).

2.1 Preparation of the Galangal Extract

The *Alpinia galanga* roots, harvested in 9 months old, were collected from Laboratory of Natural Products and Chemical Synthesis, Diponegoro University. The fresh red galangal roots were cut into small pieces, oven-dried at 50℃ and grinded into powder. The dried powder (40 g) were extracted with ethanol in a soxhlet apparatus about 10 times cycles. The ethanol from extract was allowed to evaporated with a rotary vacuum evaporator to yield a crude extract. The ethanolic extract of galangal at doses of 225 mg/kg BW, 450 g/kg BW and 675 mg/kgBW was prepared by dissolved in aquabidest to get 0,2 ml solution.

2.2 Tumor Inoculation and Administration of Ethanolic Extract of Galangal

Tumor transplantation was done on day 0 (2 weeks before treatment). The mammary tumor cells were obtained from donor mice. Donor mice was terminated by ether to remove the tumor tissue. After the tumor pulp was made, NaCl solution was added in equal volume with tumor volume. Tumor inoculation is done by injecting tumor pulp subcutaneously in the right axillar region of recipient mice at a dose 0.2 ml using insulin syringe, containing approximately 10^6 living tumor cells. After tumor growth, we measure the tumor volume and terminated one mouse of each groups to morphological examination of tumors.

Thirty two strains C3H female mice were divided into 4 groups: control group (C), treatment 1 (T1), treatment 2 (T2). treatment 3 (T3), each group consist of 8 mice. All control and treatment mice were treated as follows : control group was given standard diet and did not receive ethanolic extract of galangal, T1 group (standard diet, ethanolic extract of galangal at a dose 225 mg/kgBW/day, T2 (standard diet, ethanolic extract of galangal 450 mg/kgBW/day), T3 (standard diet, ethanolic extract of galangal 675 mg/kgBW/day). Ethanolic extract of galangal was given to the mice for 2 weeks, orally. Body weight of mice was counted at the start of the study, subsequent to the 1st week, the beginning of 2nd week and at the end of the 2nd week (before termination).

2.3 Antiproliferative Activity Test

Measurement of tumor volume started 1 week after inoculation, the second week of the beginning and the end of the second week before termination. After the treatment period ended, all the mice were terminated remove the tumor tissue and measure the tumor volume using the ruler (in mm^3).

The tumor tissues were proceeded with common histopathological technique to get paraffin blocks. For the evaluation of cell proliferative activity, we used Ki-67 stain (Monoclonal Rat Anti-Mouse Ki-67 Antigen by DakoCytomation, code No. M 7249). Interpretation of Ki-67 staining was carry out by Allred scoring (Fig. 1), which was reported as number of positive cells to total cells (proportion score) and intensity of staining (intensity score), in 5 microscopic 40 x fields [13]. Statistical analysis of the results was performed in SPSS software using the Pearson Chi Square and OneWay Anova.

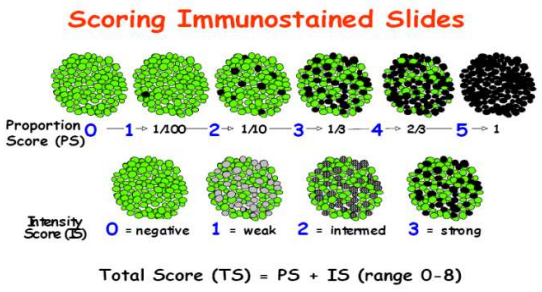


Fig. 1. Allred scoring

3. RESULTS

3.1 Morphological Examination

At the beginning of the first week after inoculation, the tumor mass was in fact grow in the site of inoculation. One mouse of each groups were randomly terminated and the tumors were cut out to undertake a morphological examination of tumor with hematoxylin and eosin staining (Fig. 2).

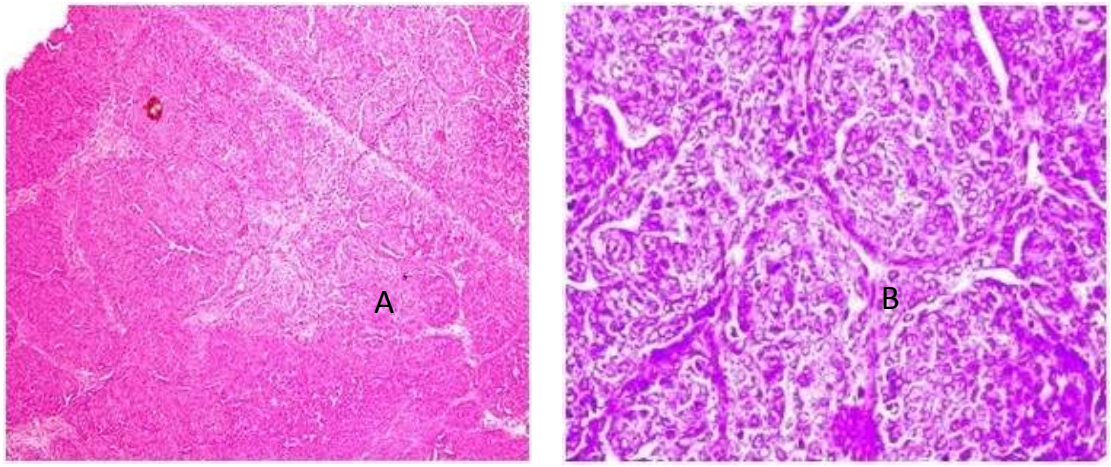


Fig. 2. Microscopic features of breast carcinoma of C3H mice (Hematoxylin and eosin stain)

A. Breast carcinoma, one week after inoculation (Hematoxylin and eosin stain, x 100).
B. Breast carcinoma one week after inoculation (Hematoxylin and eosin stain, x 400).

3.2 Measurement of Tumor Volume

Before the termination phase, we measured the weight of all bodies of mice. The weight of the smallest mice before final termination is 18,4 g and largest is 25,8 g. After that, tumor mass was removed and measured the tumor volume. As a result, tumor volume of the smallest mice was found in T3 group (12.6 $mm^{3)}$ and the largest was in the control group (216.4 mm^3) (Fig. 3 and Table 1).

Table 1 shows that the mean tumor volume of the control and treatment groups on week 1 and week 2 is not too different but the mean tumor volume after termination shows marked differences. Statistical analysis by Pearson (p = 0,000) shows that there is a significant relationship between the dose of galangal with a reduction in tumor volume.

Fig. 4 shows the Ki-67 immunostaining, with decrease of score between groups. The highest mean of Ki-67 expression was found in control group (5,00±0,25) and the lowest in T 3 group (2,22±0,69). Statistical analysis of Ki-67 score by OneWay Anova, there has a differences among the groups (p=0,001) (Table 3).

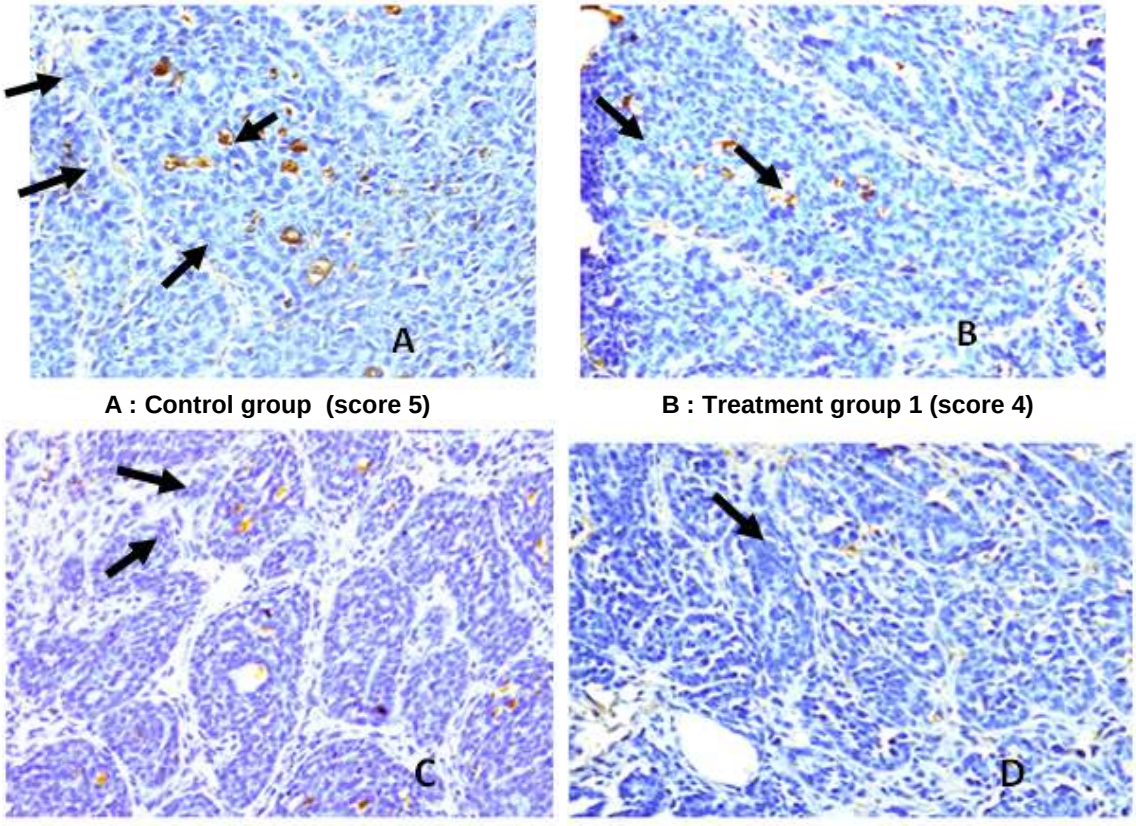
Fig. 3. Tumor volume of each groups

Table 1. Body weight and tumor volume of each groups

Groups	Body weight (gr)				Tumor volume (gr)		
	H0 (tumor inoculation)	**M1**	**M2**	**Pre termination**	**M1**	**M2**	**After termination**
C 1	22,8	23,9	24	24,1	26,6	49,2	182,5
C 2	22,9	22,7	22,8	22,9	16,6	12,9	192,5
C 3	22,9	23,8	24,1	24,3	19,7	39,5	161,8
C 4	24	25	24,8	22,2	23,8	25,1	187,4
C 5	21,2	22	22,3	21	25,8	25,1	216,4
C 6	20,1	20,5	21	21,9	19,8	23,2	149,7
C 7	21,3	23,1	21,8	24,7	30,1	59,2	142,0
C 8	24	24,4	-	-	22,8	-	-
					Mean = 23,2	Mean = 33,46	
T 1.1	22,3	24,1	24,2	24,7	17,4	15,0	17,1
T.1.2	21,4	21,8	21,5	21,6	20,1	61,8	93,6
T 1.3	24,4	26,1	26,1	26	16,2	35,7	47,8
T 1.4	22,8	22,2	22,3	22,6	17,8	34,1	52,8
T 1.5	20,2	21,3	21,2	21	32,5	48,9	61,3
T 1.6	20,4	22,2	21,8	21,6	15,2	27,7	45,7
T 1.7	22,1	23,5	23,8	23	33,7	33,7	42,4
T 1.8	23,2	24,1	-	-	20,2	-	-
					Mean = 21,84	Mean = 36,7	
T 2.1	20,1	21,6	21,6	20	25,8	60,5	65,4
T 2.2	21,1	20,6	20,5	20,5	20,9	19,9	33,1
T 2.3	22,5	23	23,2	22,3	24,2	32,1	53,6
T 2.4	25,5	24,5	24,7	25,8	27,6	33,8	51,5
T 2.5	20,2	21	21,2	20	29,1	53,9	43,6
T 2.6	20,9	21,2	21,5	20,5	30,9	31,4	63,9
T 2.7	20	21	21	19,6	20,4	34,9	63,9
T 2.8	19,9	21,6	-	-	29,4	-	-
					Mean = 25,56	Mean = 38,07	
T 3.1	20	18	18	19,1	17,6	18,3	54,1
T 3.2	21,2	21,2	21,5	22,2	22,3	19,7	25,8
T 3.3	19,4	17,2	17,2	18,4	18,8	25,3	12,6
T 3.4	20,5	20,5	20,2	20,2	18,9	40,4	71,6
T 3.5	21,2	22	22	21,4	29,4	44,0	42,1
T 3.6	18	18	20,9	21,4	20,8	30,1	59,2
T 3.7	20,5	20,7	18,2	18,5	29,0	31,7	49,0
T 3.8	17	17	-	-	18,4	-	-
					Mean = 22,4	Mean = 29,93	

Table 2. Mean of tumor volume between groups (After termination)

Groups	Mean ± SD	Median
C	176,04±26,22	182,500
T 1	51,53±23,03	47,800
T 2	53,57±12,08	56,600
T 3	44,91±20,14	49,000
Pearson chi square (p < 0,001)	p = 0,000	



A : Control group (score 5)

B : Treatment group 1 (score 4)

C : Treatment group 2 (score 3)

D : Treatment group 3 (score 2)

Fig. 4. Ki-67 immunostaining, x 400

Table 3. Multivariate analysis of Ki-67 immunoexpression

Groups	Mean±SD	Median
Control	5,00±0,25	**5,0**
T1	3,46±0,47	3,4
T2	3,20±0,86	3,0
T3	2,23±0,69	2,0

Sig. p = 0,001

4. DISCUSSION

Medicinal plants are widely used in traditional cultures and they are becoming increasingly popular in modern society as alternatives to synthetic drugs. Thousand of plants are being used as herbal medicine since long ago. Indonesia, as a tropical country has so many medicinal plants that widely used with or without science facts (known as “Jamu”, meaning traditional medicine from plants).Scientific approach is essential to further develop rational use and mechanism of jamu. *Alpinia galangal* (L) Willd known as Lengkuas, is used in medication, culinary and cosmetics aims. The researchers found that white galangal is used for spice in Indonesian cuisine but red galangal has potential role as medicinal plant. Rusmarilin [6] has proved that red galangal containt more active compounds of phyto-pharmacological properties. Chudiwal et al. [9] stated that galangal rhizome is used to improve appetite, aphrodisiac, tonic, diuretic, expectorant and useful in headache,

rheumatic pains, sore throat, pain in chest, diabetes, and diseases of the kidney [5,6,9,14].

The plant derived from anticancer agents enacted for inhibiting cell proliferative activity and induction of apoptosis. *A. galanga*, grouped into the Zingiberaceae family, has been studied and numbers of chemical constituent have been isolated. *A. galanga* has active compound such as 1'S'-1'-acetoxychavicol acetate (ACA), 1 'S'-1'-acetoxyeugenol acetate, 1'S'--1'-hydroxychavicol acetate (HCA), trans-p-hydroxycinnamaldehyde, flavonoids (kaemperol, kaempferide, galangin, alpinin) and so on [9,15]. 1'S'-1'-acetoxychavicol acetate (ACA) has various pharmacological functions of antitumor, antiinflammatory, antifungal, antioxidative activity. Galangin (3,5,7-tryhydroxyflavone), flavonoid originally found in galangal root, is considered to play a role as antimicrobial, potent anticancer effect, specifically through inhibition of enzyme CYP1A1 and modulation of aryl hydrocarbon receptor [6,9].

We used a strain C3H mice in this study because this mice types have a tendency to malignant transformation through genetic susceptibility and transmission of virus MMTV (Mouse Mammary Tumor Virus). By using the technique of inoculation, the transplanted tumor cells will grow to tumor mass, and such growing took place in the first week after inoculation until the termination. In the control group tumor volume tends to increase in size, while in the treatment group tumor volume tends to shrink due to the effects of galangal extract. In this case Turusov and Mohr [16] underlined that the growth of mammary tumors in mice C3H strain could reach 2.4 mm/week.

Table 1 shows the mean of tumor volume for the control and treatment groups. The mean of the highest is found in the control group and the lowest in the treatment group 3 (T3). It can be concluded that the ethanolic extract of galangal at a dose of 225 mg/kgBW/day could inhibit proliferative activity and when the dose was increased up to 675 mg/kgBW/day, the tumor volume is shrank.

Galangal (*Alpinia galanga*) with ACA and HCA as active substance has the potential to reduce the incidence or mortality of cancer by reducing the activity of proliferation and induction of apoptosis. Polyamine, an organic compounds are synthesized in cells via highly regulated pathway. Their actual function is not entirely clear but if cellular polyamine synthesis is inhibited, cells stop growing or severely retarded. Polyamine metabolism is also known involve in apoptotic process. In experimental animal studies that examined the effect of ACA on chemically induced carcinogenesis, a reduction in polyamine concentration correlated with reduced incidences of neoplasm and tumor formation. The exact mechanism of ACA in modulate polyamine metabolism is not known but several studies state that ACA reduced polymine activity by inhibiting MAPK-dependent pathway. Decreased polyamine intracellular level stimulate caspase-3 activity, that is involved in initiation phase of apoptosis [8,10,12,17]. HCA isolated from *A. galanga* has cytotoxic effect to human leukemic HL 60 and U 937 cell lines [18]. Induction of apoptosis in tumor cells lead to increase of cell death and shrinkage of tumor mass [6].

From this study, we found that Ki-67 immunoexpression score was highest in control group and lowest in T3 group. Statistical analysis by OneWay Anova shows the significant differences between groups. Thus, we concluded : 1) by using the dose 225 mg/kgBW/day, the ethanolic extract of galangal inhibit the cell proliferative activity; 2) experimental mice group that has been given higher dose of ethanolic extract of galangal shows more higher inhibition of cell prolifertive activity.

Investigation using rat showed that ACA could inhibit development of azoxymethane induced colonic aberrant crypt foci through its suppression of cell proliferation. 1'S'-1'-acetoxychavicol acetate (ACA) also acts as antioxidant, which specifically blocks xanthine oxidase, and NADPH oxidating systems generating O_2. Antioxidant activity of ACA also achieved by strong superoxide anion scavenging activity and as lipoxygenase inhibitor. Previous mechanism will inhibit tumor promotion during tumorigenesis [19]. 1'S'-1'-acetoxychavicol acetate (ACA) isolated from *A. galanga* responsible for antiinflammatory acitivity by inhibit nitric oxide (NO) and Cyclooxygenase-2 (COX-2). Red galangal containing ACA also has a role inhibiting NFkB through the inhibition of cell cycle regulatory proteins cyclin D1 which results in barriers to the transition phase of the cell cycle from G1 to S, this activity decreases cell proliferation and tumor cells do not grow [3,4,10,20].

Several previous studies have using galangal as an alternative therapy for cancer because galangal also has cytotoxic activity. Suja S [15] in his study revealed that ethanolic extract of

Alpinia galanga and Alpinia officinarum could reduce the growth and multiplication of the PC-3 cell lines. Hartono [4] which using ethyl acetate extract of galangal as well as research of Rusmarilin [6] have proved that the ethyl acetate extract of galangal could inhibit the proliferation of cancer cells in culture and transplanted model [5,6,7]. Similarly, Lee and Houghton [21] and Chudiwal et al. [9] also have proved the efficacy of anti-cancer ACA against MCF7 (Michigan Cancer Foundation 7) on breast cancer cells and COR L23 in lung cancer. He [22] in his research on *Alpinia oxyphylla* found that this herbal medicine also containt antiangiogenic potential, *in vitro* and *in vivo*, by inhibition of neovascularisation [15,22].

5. CONCLUSION

Oral administration of ethanolic extract of *Alpinia galanga* Willd at the dose 225 mg/kgBW/day, 450 mg/kgBW/day and 675 mg/kgBW/day for 2 weeks after tumor transplant and tumor growth has shown anticancer activity on C3H breast adenocarcinoma by inhibition of cell proliferative activity and the growth of tumor volume. The best result of anticancer activity is found in the group using the highest dose of galangal extract (675 mg/kgBW). Ethanolic extract of red galangal containing higher ACA components has potential anticancer activity that have various mechanisms including induction of apoptosis by activating caspase-3 pathway, antiinflammatory effects by inhibition of NF-ƙB, NO and COX-2, antioxidant effects by blocking xanthine oxidase, and NADPH oxidating systems generating O_2, strong superoxide anion scavenging activity and as lipoxygenase inhibitor. In sum, ACA also has cytotoxic activity and antiangiogenic potential.

CONSENT

All authors declare that this research use only experimental animals. No patients involved in this study.

ETHICAL APPROVAL

The authors have obtained ethical approval from Ethical Commision, Faculty of Medicine, Andalas University, Padang, Indonesia.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12542