

Anticancer Activity of Bioactive Compounds from *Kaempferia rotunda* Rhizome Against Human Breast Cancer

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ABSTRACT

Kaempferia rotunda known as kunci pepet or kunir putih in Indonesia, has been traditionally used in as abdominal pain, sputum laxative, wounds, and diarrhea colic disorder. This study was conducted to determine anticancer activity of bioactive compounds from *Kaempferia rotunda* rhizome against human breast cancer *in vitro* and *in vivo*. The isolation of bioactive compounds from methanol extract *K. rotunda* was carried out by chromatographic method, and the structure was elucidated based on spectroscopy method. The *in vitro* cytotoxicity test was done on human breast cancer T47D cell lines by MTT ([3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. *In vivo* activity assay was done by xenografting female Balb C mice with human breast cancer T47D cell implantation into the mammae fat-pad. *In vitro* cytotoxicity assay against human breast cancer of chloroform extract of *K. rotunda* and pinostrobin (1) showing their IC₅₀ were 41.72 µg/ mL and 59.8 µg/ mL respectively. *In vivo* assay of chloroform extract of *K. rotunda* and pinostrobin (1) by xenografting female Balb C mice showed the average of cancer incidence for each group is 50-100%. The growth of cancer volume from each groups appear on the fourth day, and reach a maximum cancer volume after the seventh day. The fastest cancer volume decrease occurred in group treatment with chloroform extract of *K. rotunda* in the dose of 500 mg/Kg bw, and group treatment with pinostrobin (1) in the dose of 20 mg/Kg bw. The bioactive compounds can repair breast tissue histopathology, and suppress *c-Myc* expression on mice with T47D breast cancer xenograft. These findings proved that *K. rotunda* rhizome is potential to be developed as breast cancer chemotherapeutic agent.

Keywords: *Kaempferia rotunda*; human breast cancer; T47D cell line; Xenograft method

INTRODUCTION

Cancer and tumor are the dangerous diseases nowadays. Cancer is the uncontrolled growth of cells, followed by cells invasion into the surrounding tissue and metastasized to other body parts. The main character of cancer cells is continuous proliferation, causing an imbalance between living cells and dead cells. According to the world health organization (WHO)¹, it is estimated by the year of 2010 cancer ranked as the second major cause of death worldwide after heart disease and will be the first ranks in the 2030. In 2005, cancer killed about 206,000 people in Indonesia, 135,000 of them were below 70 years. Breast cancer is a cancer that invades the breast tissue. According to available data, 22.9% of cancer cases number is breast cancer. Therefore, the appropriate treatment is necessary to improve the quality of life of cancer patients. Cancer treatment is usually based on the removal of the cancerous tissue, kill cancer cells, and minimize the effect on the surrounding of normal cells. Currently, the cancer therapies that usually done are surgery, radiotherapy, and chemotherapy, but each type of these treatments have their own risks. Operation can be managed in several cancers that have been growing, but it is difficult to treat the cancer in the early stages of metastasis. Treatment with radiation

capable of killing cancer not only locally, but also kills the surrounding normal cells. Most of chemotherapy drugs such as taxol, 5-fluorouracil (5-FU), and adriamycin have a target on cell division, but on the another hand chemotherapy can cause diarrhea and hair loss. Some of chemotherapeutics agents are also not effective for cells undergoing p53 mutation. So it is necessary to develop new agents for cancer therapy safer²⁻⁷.

Kaempferia genus is perennial member of the Zingiberaceae family and is cultivated in Indonesia and other parts of Southeast Asia. Number of studies had been conducted, providing information related to *Kaempferia* as chemopreventive agent. *Kaempferia parviflora* and *Kaempferia pandurata* have been reported for anticancer. Research conducted by Leardkamolkarn⁸ showed that the methanol extract of *K. parviflora* have high cytotoxic activity against human cholangiocarcinoma (HuCCA-1 and RMCCA-1).

Kaempferia rotunda known as kunci pepet or kunir putih in Indonesia, has been traditionally used in as abdominal pain, sputum laxative, wounds, and diarrhea colic disorder. The plant is used in the folk medicinal system of Bangladesh for treatment of high blood sugar levels as commonly observed in diabetic patients, as well as for

Table 1. The cytotoxic activity (IC₅₀) of crude extracts of *K. rotunda* rhizome and pure compounds against breast cancer T47D

No	Crude extract and pure compounds	IC ₅₀ (µg/mL)
1	Methanol extract	71,60 ± 2.03
2	Heksane extract	175,87 ± 3.40
3	Chloroform extract	41,72 ± 0.97
4	5-hydroxy-7-methoxyflavanone (pinostrobin)	59,38 ± 1.77
5	7-hydroxy-5-methoxyflavanone	> 1000
6	5,7-dihydroxyflavanone	122,71 ± 9.20
7	Crotopoxide	> 1000
8	Doxorobusin (positive control)	76,21 ± 3.40

Table 2. Percentage of Mean activity cancer decreased treatment with chloroform extract of *K. rotunda* (ECK)

Groups	Number mice life	Cancer volume maximum (mm ³)	Cancer volume after treatment (mm ³)	Percentage of activity decreased (%)	Mean cancer
I (Control)	3	271.87 ± 89.88	80.45 ± 99.77	32.09 ± 38.15	
II (ECK Dose 250 mg/Kg BB)	4	129.65 ± 131.18	33.88 ± 60.83	82.61 ± 25.15	
III (ECK Dose 500 mg/Kg BB)	4	304.90 ± 273.04	6.16 ± 1.5	91.99 ± 16.59	
IV (ECK Dose 750 mg/Kg BB)	5	307.38 ± 167.05	31.37 ± 56.64	66.51 ± 48.75	

Table 3. Percentage of Mean activity cancer decreased treatment with pinostrobin(1) (PN)

Groups	Number mice life	Cancer volume maximum (mm ³)	Cancer volume after treatment (mm ³)	Percentage of activity decreased (%)	Mean cancer
I (Control)	4	227.63 ± 149.24	107.10 ± 87.07	45.37 ± 47.34	
II (PN Dose 10 mg/Kg bw)	5	403.45 ± 333.82	111.91 ± 108.97	55.42 ± 41.13	
III (PN Dose 20 mg/Kg bw)	4	236.62 ± 154.02	88.39 ± 103.45	77.30 ± 20.64	
IV (PN Dose 40 mg/Kgbw)	5	272.251 ± 240.04	118.63 ± 41.65	55.05 ± 41.65	

treatment of pain. This plant is considered as an important medicinal plant in the ancient system of traditional medicine Ayurveda in India. The Ayurvedic drug, hallakam, containing tubers of the plant is considered as stomachic, antiinflammatory to wounds and bruises, and useful for treatment of mental disorders and insomnia⁹⁻¹¹. Phytochemically, the plant has been attributed to contain flavonoids, crotopoxide, chalcones, quercetin, flavonols, β-sitosterol, stigmasterol, syringic acid, protocatechuic acid, and some hydrocarbons which have been previously reported¹⁰⁻¹¹. Researchers also have reported the antioxidant potential of methanol extract of *K. rotunda*, and showed the presence of compounds flavonoids and phenolic derivatives^{11,12}. The presence of compounds that show potential as an inhibitor of lipid peroxidation, indicates that the plant can be useful in diseases such as myocardial infarction, diabetes mellitus, hepatic injury, atherosclerosis, rheumatoid arthritis, and cancer. In the previous studies, we have reported that the methanol extracts and flavanone compounds isolated from *K. rotunda* showed as antimutagenic¹³. Flavanones compounds which have been isolated from the chloroform extract of *K. rotunda*, namely 5-hydroxy-7-methoxyflavanone (1), 7-hydroxy-5-methoxyflavanone (2) and 5, 7- dihydroxyflavanone (3) are shown in Figure

1. As a continuation of the study, we have isolated a compound contained in a relatively polar chloroform fraction of *K. rotunda*, and determine its activity against human breast cancer *in vitro* and *in vivo*.

The *in vitro* cytotoxicity test was done on human breast cancer T47D cell line by MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay¹⁴. Extract and pure compound showed cytotoxic activity continued to test *in vivo*. *In vivo* activity was done by xenografting female Balb C mice, with T47D breast cancer cell implantation into the mammae fat-pad (total suspension of 6x10⁶ cells in 300 mL FBS). Xenograft method is a method that is widely used to create a model of the cancer. Application of human xenograft models have many benefits, because it can increase our understanding in the development of malignant and may useful in the development and exploration of new therapy¹⁵⁻¹⁷.

Human breast cancer xenografts have been widely used to study the development of breast cancer, gene expression effect on cancerogenicity as an acquisition of antiestrogen resistance, and to screen new endocrine and cytotoxic agents. This method also provides an opportunity to learn a variety of important interactions between the cancer and host tissues, including endocrinologic, immunological, and cancer-stroma interactions¹⁸. In this method, a model

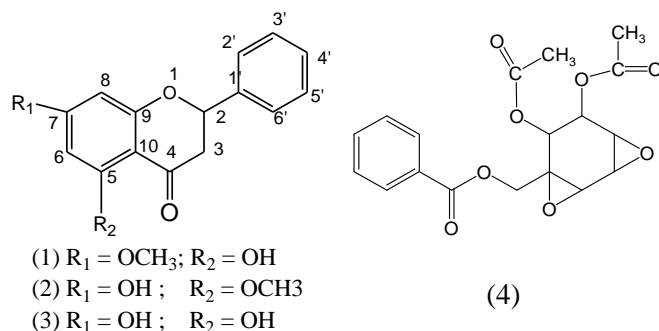
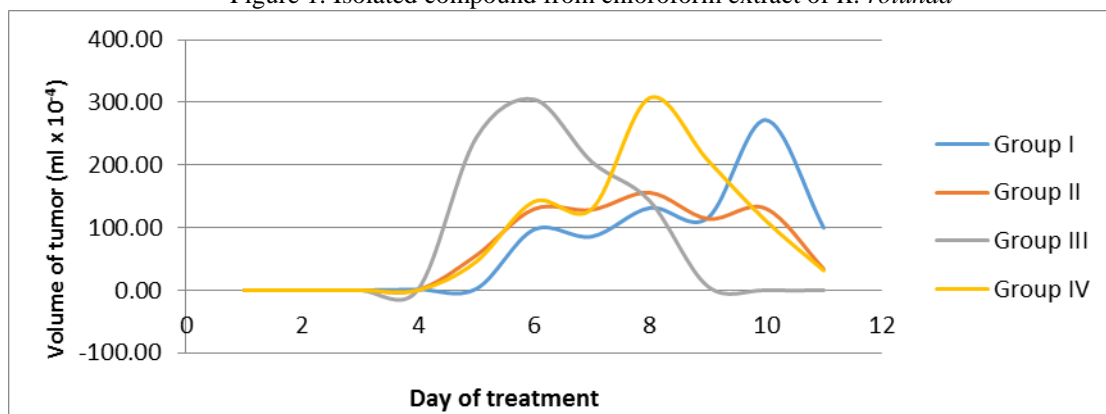
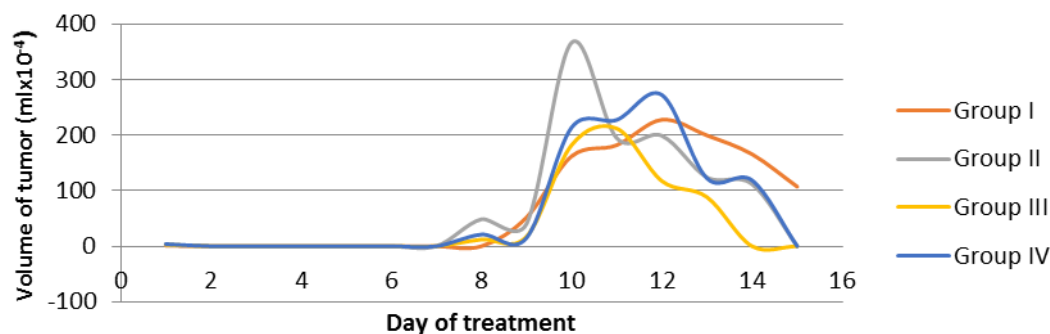
Figure 1. Isolated compound from chloroform extract of *K. rotunda*Figure 2. Graph of Mean cancer volume growth of the control group (I) and chloroform extract of *K. rotunda* treatment

Figure 3. Graph of Mean cancer volume growth of the control group and Pinostrobin (1) treatment

of human cancers implanted under the skin or in an organ in which the cancer originated. Cancer cells injected in rats or mice and the cancer growth will cause a lump that can be observed about 1-8 weeks later. The advantage of using this method is that this cancer models of genetic and epigenetic which approach the condition of cancer that occurs in humans, so this method could be used in the study of the molecular mechanism. Some cancer drugs have been successfully used for treating patient clinically. One is herceptin, which also use the xenograft method in its preclinical trial¹⁹.

MATERIAL AND METHOD

Apparatus and reagent

Glassware, syringe injection, camera, counter, desk glass, eppendorf, object glass, surgical instrument, analytical balance, caliper, and light microscope Olympus Bx51, U-TVO, 5XC-2, 360586 Japan. UV and IR spectra were measured with UV-2400PC Series and Shimadzu FTIR

Prestige 21, respectively. ¹H and ¹³C NMR spectra were recorded with NMR Agilent 400 spectrometers, operating at 400.0 MHz (¹H) and 100.0 MHz (¹³C) using residual and deuterated solvent peaks as internal standards. Evaporator Buchi Rotavapor R-114, vacuum liquid chromatography (VLC) was carried out using Si-gel Merck 60 GF254 (230–400 mesh), column chromatography using Si-gel Merck 60 (200–400 mesh), and TLC analysis on precoated Si gel plates Merck Kieselgel 60 F254 0.25 mm, 20 x 20 cm.

Extract of *K. rotunda* and flavanones isolated compounds. Full details of the isolation and identification structure are given in previous study¹³, T47D cell culture was obtained from Parasitology laboratory, Gadjah Mada University, Indonesia, and grew in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with fetal bovine serum 10% (FBS; Gibco), dan 1% Penicillin-Streptomycin (Gibco) at temperature 37°C and with a flow of CO₂ 5% (Heraeus). *c-Myc* monoclonal antibody, 10% formalin buffer, haematoxylin and eosin (HE), ketamine-HCl,

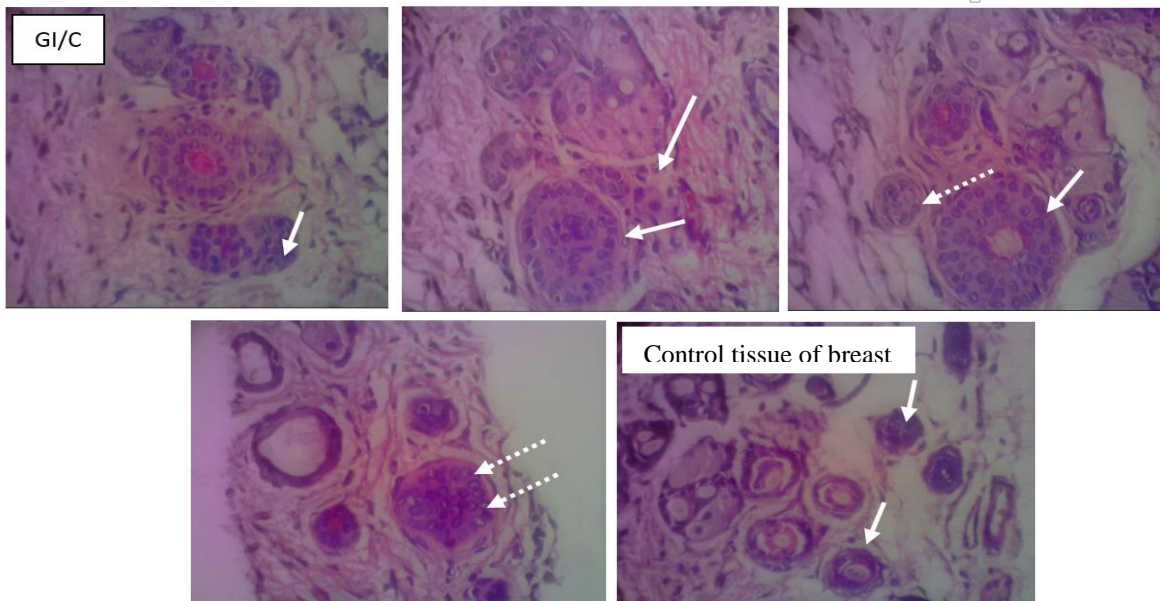


Figure 4: c-Myc expression based immunohistochemistry assay (IHC) for each group control and treatment with chloroform extract of *K. rotunda*

(The cancer tissue showed as the mamae ductus became multilayer (→), and the administration of bioactive compounds could maintain the single layer mamae ductus (---->)

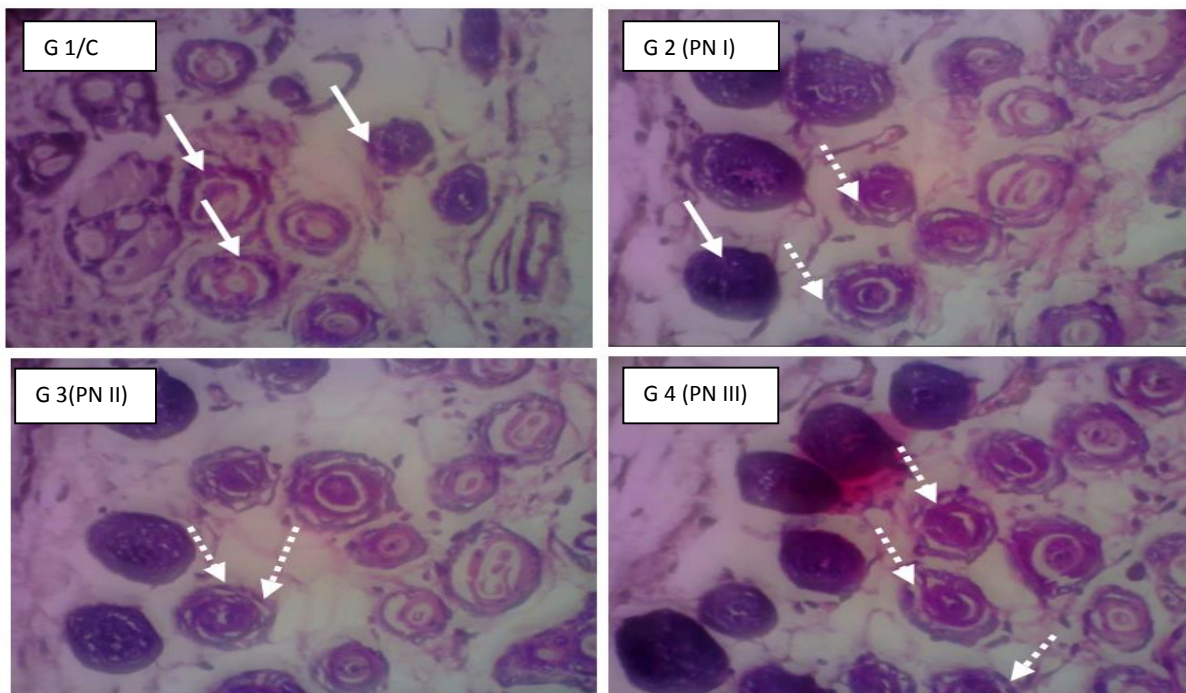


Figure 5: C-Myc expression based immunohistochemistry assay (IHC) for each group control and treatment with pinostrombin

(The cancer tissue showed as the mamae ductus became multilayer (→), and the administration of bioactive compounds could maintain the single layer mamae ductus (---->)

estradiol (Sigma), trypsin (Sigma), DAB (*Diaminobenzidine tetrachloride*) (Sigma), MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma), DMSO (Dimethyl sulfoxide), SDS (Sodium dodecyl sulphate) 10%, and 0.01 N chloride acid.

Animal test

The experiments were carried out on adult female Balb-c mice obtained from LPPT, Gadjah Mada University, Indonesia. All mice, 3-4 week old, weighed between 22,5 - 25 g and were kept under a stable environmental conditions with 12:12 light-dark cycle, at 23-25 °C room temperature. The animals were fed standard granulated

chow (pellets 789) and had an access to drinking water *ad libitum*. Animal experiment was done in accordance with Institutional Protocols of animal care.

Extraction and isolation

Extraction and isolation of three flavanones from *K. rotunda* had been described in previous study¹³. The continuation of the isolation from relatively polar chloroform fraction of *K. rotunda*, as many as crude fraction (5 g) was purified by column chromatography gravitation using Si-gel Merck 60 (200–400 mesh), (ϕ : 2.0 cm, t = 15 cm) eluted with hexane-ethyl acetate (6 :4) as solvent to give 52 fractions. Fractions (18-30) were combined and evaporated to give a white crystal of crotepoxide (140 mg) (4). The structure of this compound (4) was established on the basis of their spectral data, including UV, IR and NMR one and two dimension HMQC and HMBC.

In vitro test

The *in vitro* cytotoxicity test was investigated using 96 wells plate with cell density 2×10^4 cells per mL. Into each well was added with 100 μ L cells in culture medium Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with fetal bovine serum 10% (FBS; Gibco), dan 1% Penicillin-Streptomycin (Gibco) at temperature 37°C and with a flow of CO₂ 5% (Heraeus). Each sample was dissolved in culture medium containing 0.05% DMSO, and 100 μ L of each sample in the different concentrations was added into each well in triplicate and was then incubated in CO₂ incubator for 12- 24 hours at 37°C. MTT solution (10 μ L per 100 μ L medium) was added to all wells of an assay, and plates were incubated for 4 hours at 37°C in CO₂ incubator. As much as 100 μ L formazon (10% SDS and 0.01 N HCl) was added into each well and mixed on a shaker for 5 minutes. The wells were incubated in the dark room for 12- 24 hours at room temperature. The absorbance was measured using multiwell scanning spectrophotometers (ELISA reader) at wavelength 595 nm. The absorbance is directly proportional to the number of living cells. Therefore the dead cell could be calculated to determine LC₅₀¹⁴.

In vivo test

T47D cell culture preparation for xenograft

T47D cell was cultured in DMEM. After the cell was confluent, the medium was removed and cells were washed using FBS (Sigma). Cells were detached using trypsin and centrifuged at 1500 rpm for 2-5 minutes. Cells were washed twice using FBS and resuspended in FBS. The total cells are 6×10^6 cells per 300 μ L per injection.

Implantation of T47D cell culture to mice by Xenograft

The animal tested were 48 female Balb-c mice aged 3-4 week old. Mice were divided into control and treated group and were adapted for 1 week. The prepared T47D cells were taken using syringe. Before the implantation mice were anesthetized with ketamine HCL (0,02 mL/mice). Mice were laid on flat board with lamp. The area for injection was cleaned and sterilized using ethanol. Cells suspension (6×10^6 cells in 300 μ L FBS) was inoculated subcutantly in mammary fat-pad tissue after right hip of the mice using TB 26 gauge syringe. As many as 300 μ L cell suspension was injected slowly for more than 5

minutes. Area around the injection point was washed with warm FBS. Body temperature, breath, and heartbeat were monitored.

Treatment of chloroform extract *K. rotunda* (CEK) on xenografted mice

The mice were grouped into four groups, each group 6 mice as follow: Xenograft T47D + Estradiol (I); Xenograft T47D cells + Estradiol + CEK 250 mg/ Kg bw (II); Xenograft T47D cells + Estradiol + CEK 500 mg/Kg bw (III); Xenograft T47D cells + Estradiol + CEK 750 mg/Kg bw (IV). Three days after T47D cells were implanted, the CEK was administrated in 12 days. The estradiol for the control group and the treatment groups were administered every two days.

Treatment of pinostrobin (1) on xenografted mice

The mice were grouped into four groups as follow: Xenograft T47D + Estradiol (I); Xenograft T47D + Estradiol + pinostrobin (1) 10 mg/Kg bw (II); Xenograft T47D + Estradiol + pinostrobin (1) 20 mg/Kg bw (III); Xenograft T47D + Estradiol + pinostrobin (1) 40 mg/Kg bw (IV). Three days after T47D cells were implanted, the pinostrobin (1) was administered in 12 days. The estradiol for the control group and the treatment groups were administered every two days.

Cancer observation

All mice's breast cancer development was monitored. Cancer diameter and body weight were measured every day. Cancer mass was measured vertically and horizontally using caliper. Cancer volume calculated using Carlsson's equation

$$V = (axb^2)/2 \quad (1)$$

V = cancer volume

a = cancer's longest diameter

b = cancer's shortest diameter

Calculation of activity due to a decrease in cancer volume of chloroform extract *K. rotunda* and pinostrobin (1) =

$$\frac{(\text{Volume maximum cancer} - \text{Volume cancer after treatment}) \times 100 \%}{\text{Volume maximum cancer}} \quad (2)$$

Result from control and treatment groups were expressed as mean \pm SD %.

Histopathology profile of breast cancer tissue

Mice's breast cancer tissue was cleaned and washed using ice-cold physiological saline then submerged in 10% formalin buffer at room temperature. Breast cancer tissue was attached on paraffin block then sliced and placed on object glass. The tissue were stained with haematoxylin and eosin (HE) and observed using light microscope.

c-Myc expression on breast cancer tissue using immunohistochemistry (IHC)

The mice cancer tissue were obtained and submerged in peroxidase blocking solution at room temperature for 10 minutes, incubated in prediluted blocking serum 25°C for 10 minutes. Each prepare were added with *c-Myc* monoclonal antibody, washed with PBS for 5 minutes. Prepare were added with DAB (*Diaminobenzidine tetrachloride*) for 10 minutes, incubated with haematoxylin eosin for 3 minutes, and then washed with aquadest. Prepare were cleaned, dropped with mounting

media, and covered with coverslip. *c-Myc* expression was observed using light microscope Olympus Bx51, U-TVO, 5XC-2, 360586 Japan. The preparate was observed qualitatively. The tissue expressed *c-Myc* will be seen as brown colour.

Statistical analysis

The data of all experiments were represented as Mean \pm SD and were analyzed with SPSS 13.0 statistic software was used for analysis. Differences were considered significant at $p < 0.05$.

RESULTS

Isolation

In previous research, flavanones compounds have been isolated from the chloroform extract of *K. rotunda*, namely 5-hydroxy-7-methoxyflavanone (pinostrobin) (1), 7-hydroxy-5-methoxyflavanone (2) and 5, 7-dihydroxyflavanone (3) [13]. Continuing isolation of compounds from relatively polar chloroform fraction of *K. rotunda*, resulted one compound namely crotepoxide (4). Crotepoxide (4) was obtained as a white crystal. The UV (in methanol solvent) λ_{\max} : 273; 238 nm. The IR (KBr pellet) ν_{\max} : 3467; 2955; 1764; 1725; 1282; 1235; 1121; and 720 cm^{-1} . ^1H NMR (400 MHz, Acetone- d_6): δ 2.93 (3H, s); 2.99 (3H, s); 3.09 (1H, dd; 2 Hz; 8 Hz); 3.44 (1H, dd, 3 Hz; 8 Hz); 3.65 (1H, d, 3 Hz); 4.23 (1H, d, 13); 4.56 (1H, d, 13); 4.97 (1H, dd, 2Hz; 8Hz); 5.69 (1H, d, 8); 7.45 (2H, t, 8 Hz); 7.58 (1H, t, 8 Hz); 8.01 (2H, d, 8.0); ^{13}C NMR (100 MHz, Acetone- d_6): δ 20.59; 20.63; 48.04; 52.58; 53.78; 59.37; 62.43; 69.42; 70.35; 128.53 (2C); 129.10; 129.72 (2C); 133.51; 165.75; 169.70; 170.01 ppm. Further support for the structure 4 was obtained from HMQC and HMBC measurement. This compound has the similar NMR data with those reported in the literature [10]. Structure of crotepoxide (4) shown in the figure 1.

In vitro cytotoxicity

All *K. rotunda* rhizomes extracts and isolated compounds were subjected to cytotoxic activity screening against human breast cancer T47D cell line. Cytotoxic properties against T47D cells were evaluated according to the method of MTT assay of crude extract and pure compounds from *K. rotunda* are presented in Table 1. All of sample demonstrated not significant cytotoxicity against human breast cancer T47D cells with IC_{50} values more than 10 $\mu\text{g}/\text{mL}$ [20]. Nonetheless, chloroform extract and pinostrobin demonstrated IC_{50} value less than doxorubicin (positive control). Chloroform extract of *K. rotunda* and pinostrobin (1) showing their IC_{50} were 41.72 $\mu\text{g}/\text{mL}$ and 59.8 $\mu\text{g}/\text{mL}$ respectively. Doxorubicin has been known as a cure for cancer, but in this study showed IC_{50} value 76.21 $\mu\text{g}/\text{mL}$.

In vivo test

The Effects of chloroform extract of *K. rotunda* and pinostrombin on breast cancer growth

On xenografted mice, implantation of T47D breast cancer cells in the breast tissue of mice (6×10^6 cells in suspension as much as 300 μL) showed different percentage of cancer volume in each group. Cancer incidence for each group showed average 50-100 %, it is the parameter whether the xenograft is done successfully. The mean of cancer volume for 12 days were calculated using Carlsson's equation.

Graph of mean of cancer volume growth of the control group and chloroform extract of *K. rotunda* treatment is showed in figure 2, while the graph of mean of cancer volume growth of the control group and pinostrobin (1) treatment is showed in figure 3.

Based on this result, it can be seen that the growth of cancer volume from each group began to appear on the fourth day, and reaching a maximum cancer volume after the seventh day. The fastest cancer volume decrease occurred in group treatment with chloroform extract of *K. rotunda* in the dose of 500 mg/Kg bw, and group treatment with pinostrobin (1) in the dose of 20 mg/Kg bw. The calculation of activity due to a decrease in cancer volume of chloroform extract of *K. rotunda* and pinostrobin (1) can be shown in Table 2 and 3. From the data showed that the chloroform extract of *K. rotunda* and pinostrobin (1) can reduce the volume of the breast cancer on mice. The optimum dose of chloroform extract of *K. rotunda* 500 mg/Kg bw, while pinostrobin (1) optimum dose of 20 mg/Kg bw.

The effect of chloroform extract of *K. rotunda* (CEK) and pinostrobin (1) on *c-Myc* expression of breast cancer tissue

c-Myc is usually found in excessive amounts in the case of breast cancer, because this protein plays a major role in the carcinogenesis of breast cancer [21]. *c-Myc* is a regulator gene that codes for a transcription factor. The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation.

Furthermore, the mechanism of chloroform extract *K. rotunda* and pinostrobin (1) in the inhibition of breast cancer can be seen from the data immunocytochemistry. Immunocytochemistry was done in order to observe whether of chloroform extract of *K. rotunda* and pinostrobin (1) suppress the expression of *c-Myc* on breast cancer. The observation showed difference colour between groups treated with bioactive compounds compared to control groups. In the control group (Figure 4 and 5) cancer tissue looks brown because of the interaction between *c-Myc* antibody and *c-Myc* protein in tissues, as an indication of expression of *c-Myc*. From the observation, several preparations showed thickening of the cancer cell nodules of tissue preparation and treatment control group (Fig.4 & 5). Observations of the expression of *c-Myc* in this study can only be done qualitatively, because it is very difficult to count the number of cells of each sample. In the case of breast cancer, it is generally found the excessive expression of *c-Myc*. On the treatment groups with chloroform extract of *K. rotunda* and pinostrobin (1) showed that these compounds are able to decrease the expression of *c-Myc*. Figure 4 and 5 showed preparations of cancer tissue which can be seen as the multilayer mamae ductus (\longrightarrow). In the breast cancer case, carcinogenesis is indicated by the number of mamae duct which became multilayer. Meanwhile the administration of bioactive compounds could maintain the single layer mamae ductus ($\cdots\cdots\longrightarrow$). Inhibition of *c-Myc* protein expression plays an important role in reducing the cancer nodule formation and tissue repair. The observation

associated with haematoxylin eosin staining which showed that the treatment by chloroform extract of *K. rotunda* and pinostrobin (1) are capable to maintain epithelial tissues as a single layer of the mammary ducts which indicates that the network is back to normal.

DISCUSSION

Phytochemicals investigation on rhizomes of *K. rotunda* has afforded three flavanones, namely 5-hydroxy-7-methoxyflavanone (pinostrobin) (1), 7-hydroxy-5-methoxyflavanone (2) and 5, 7- dihydroxyflavanone (3), and one lacton is crotepoxide (4). Based on this study all crude extracts and isolated compounds exhibited low cytotoxic activity against human breast cancer T47D cell lines. Chloroform extract of *K. rotunda* and pinostrobin (1) showing their IC₅₀ were 41.72 µg/ mL and 59.8 µg/ mL respectively. Doxorubicin has been known as a cure for cancer, but in this study showed IC₅₀ value 76.21 µg/ mL. However, chloroform extract of *K. rotunda* and pinostrobin (1) have IC₅₀ smaller than doxorubicin, so both showed relatively better activity. The results also showed that chloroform extract and pinostrobin (1) more active than other extracts and pure compounds from *K. rotunda* against breast cancer cells T47D. The in vitro test in accordance with the in vivo test which the chloroform extract of *K. rotunda* a dose of 500 mg/Kg bw can reduce cancer volume of 91.99 ± 16.59%, while pinostrobin (1) at a dose of 20 mg/Kg bw can reduce volume cancers of 77.30 ± 20.64%.

Previous cytotoxic investigations on the extracts or isolate compounds of the plant have revealed different ranges of activity against several cancer cell lines. The extracts and isolated compounds which exhibit cytotoxic index IC₅₀ less than 10 µg/mL were considered to have significant cytotoxic activity²⁰. Furthermore, pinostrobin (1) showed an anticancer activity in cell culture of human mammary carcinoma. Another study showed the effect of pinostrobin (1) on herpes simplex virus-1. Herpes simplex virus-1 could be inhibited by the tested flavonoid. All these previous researches showed that the flavonoid pinostrobin (1) has various effects in different cells. It has also been proven that pinostrobin (1) has a strong antiproliferative and cytotoxic effect in several cell types. The study pinostrobin (1) showed antiproliferative activity on HUVEC and become an important part in atherosclerosis treatment²². However, on cytotoxic evaluation against murine leukemia P-388 cells using MTT assay of pinostrobin (1) was inactive²³.

Pinostrobin (1) is a flavanone major compound which can be isolated from chloroform extract of *K. rotunda*. Flavonoids are found in several medical plants. Herbal remedies containing flavonoids have been used in folk medicine around the world. Epidemiological studies have provided data that high dietary intake of flavonoids with fruits and vegetables could be associated with a low of prevalence cancer in human. This is supported by a multitude of *in vitro* and *in vivo* studies, which show that flavonoids may inhibit various stages in the carcinogenesis process, such as cancer initiation, promotion, and progression²⁴.

Several studies have been reported¹⁵⁻¹⁷, that xenograft method are usually using nude mice homozygous as recipients of cancer xenografts, but this study were carried out on adult female Balb-c mice. However, in order to accelerate the growth of cancers so that the cancers can be observed in a shorter time, each control and treatment groups of mice were injected by estradiol (0.28 mg/ mice once every 4 days). The research showed that the number of live mice from each group ranged from 50 - 100% with an incidence of cancers per group 100%. Thus we also conducted an *in vivo* experiment on mice with T47D cell-xenografted cancer for 12 and 14 days. The results showed that the cancers were suppressed by chloroform extract of *K. rotunda* and pinostrobin (1) treatment but did not shrink in a dose-dependent manner.

Human cancer xenografts on immunodeficient animal models provided a mean to evaluate potential anticancer drugs in preclinical studies and are applicable for studying many different types of human malignancies¹⁵⁻¹⁷. In conclusion, *in vitro* cytotoxicity assay chloroform extract of *K. rotunda* and pinostrobin (1) showed low activity, but *in vivo* study we found that the chloroform extract of *K. rotunda* and pinostrobin (1) can reduce the volume of the breast cancer on mice by xenograft method. The optimum dose of chloroform extract of *K. rotunda* 500 mg/Kg bw, while pinostrobin (1) optimum dose of 20 mg/Kg bw. The bioactive compounds can repair breast tissue histopathology, and suppress c-Myc expression on mice with T47D breast cancer xenograft. These findings proved that *K. rotunda* rhizome is potential to be developed as breast cancer chemotherapeutic agent.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

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