

Typhonium flagelliforme decreases protein expression in murine breast cancer

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ABSTRACT

BACKGROUND

Breast cancer treatment is still ineffective, having also various side effects. Breast cancer growth is affected by human epidermal growth factor receptor 2 (HER2/neu) and B cell lymphoma 2 (BCL2) expression. In vitro studies on continuous culture of continuous culture of human lymphoblasts (CEMs) showed that *Typhonium flagelliforme* (TF) increases apoptosis. The objective of this study was to evaluate whether TF syrup (TFS) could decrease HER2/neu and BCL2 expression as well as breast cancer volume (BCV) in mice.

METHODS

An experimental post-test only control group design was conducted on 18 C3H mice with breast cancers, randomly allocated to 3 groups of 6. Group 1 received 0.2 ml of distilled water. Group 2 and 3 animals were each given 0.2 ml of 40 mg/ml and 80 mg/ml TFS, respectively. The treatment was given orally once daily for 25 days. Assessment of HER2/neu and BCL2 expression was by immunohistochemistry, whereas BCV was measured by caliper. Anova and LSD were used for data analysis.

RESULTS

There was a significant difference in HER2/neu and BCL2 expression as well as in BCV among the treatment groups. LSD analysis showed that HER2/neu and BCL2 expression in group 3 (51.60%; 24.60%) was significantly lower than in group 1 (245.40%; 114.40%) as well as group 2 (235.50%; 54.20%) ($p=0.000$). BCV in group 3 (4392.33 mm^3) was significantly greater than BCV in group 1 (253.87 mm^3) ($p=0.002$), but was not significantly different from BCV in group 2 (3667.16 mm^3) ($p=0.306$).

CONCLUSION

Supplementation with TFS decreases HER2/neu and BCL2 expression. TF appears to be a promising plant demonstrating anti cancer activity.

Keywords: Breast cancer, BCL2, HER2/neu, apoptosis, mice

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***Typhonium flagelliforme* menurunkan ekspresi protein pada kanker payudara mencit**

ABSTRAK

PENDAHULUAN

Pengobatan kanker payudara (KP) belum efektif, masih menyisakan efek samping. Perkembangan KP dipengaruhi oleh ekspresi human epidermal growth factor receptor 2 (HER2)/neu dan B cell lymphoma 2 (BCL2). Penelitian pada sel continuous culture of human lymphoblasts (CEMs) menunjukkan, umbi keladi tikus (UKT), meningkatkan sekresi cytochrome c, ekspresi caspase3, fragmentasi DNA, dan apoptosis. Tujuan penelitian ini untuk membuktikan, sirup UKT mampu menurunkan ekspresi HER2/neu, BCL2, dan volume kanker payudara.

METODE

Rancangan eksperimen *post test only control group design* mengikut sertakan 18 mencit C3H dengan KP. Mereka secara random alokasi sederhana dibagi dalam 3 kelompok (6 mencit setiap kelompok). Kelompok 1, mendapat aqua 0.2 ml. Kelompok 2 dan kelompok 3, diberi 0.2 ml (40 mg/ml) dan 0,2 ml (80 mg/ml) sirup UKT. Perlakuan dilakukan sekali sehari secara oral selama 25 hari. Pemeriksaan ekspresi HER2/neu dan BCL2 dilakukan dengan immunohistokimia, sedangkan volume kanker diukur dengan kaliper. Analisis statistik menggunakan oneway ANOVA dan LSD, dengan tingkat kemaknaan $<0,05$.

HASIL

Terdapat perbedaan ekspresi Her2neu, BCL2, dan volume KP yang bermakna antara ketiga kelompok perlakuan ($p=0,000$). Analisis LSD menunjukkan ekspresi HER2/neu dan BCL2 (51,60%; 24,60%) pada kelompok 3 lebih rendah bermakna dibanding kelompok 1 (245,40%; 114,40%) ($p=0,000$). Demikian pula dibanding dengan kelompok 2 (235,50%; 54,20%) ($p=0,000$). Volume KP pada kelompok 3 (4392,33 mm³) lebih tinggi bermakna dibanding kelompok 1 (2563,87 mm³) ($p=0,002$), dibanding dengan kelompok 2 (366,16 mm³) tidak menunjukkan perbedaan bermakna ($p=0,306$).

KESIMPULAN

Pemberian sirup UKT mampu menurunkan ekspresi HER2/neu dan BCL2. Umbi keladi tikus nampaknya mempunyai aktivitas anti kanker.

Kata kunci: Kanker payudara, BCL2, HER2/neu, apoptosis, mencit

INTRODUCTION

Breast cancer (BC) is a complex cancer that is most frequent among women after cancer of the cervix, in Indonesia and Asia, as well as globally.^(1,2) Several factors, genetic as well as environmental, play a role in BC pathogenesis. Among the various types of BC, invasive ductal carcinoma is the most frequent.⁽³⁾ The modern modes of treatment of BC still rely on surgery, irradiation, and cytostatics. These treatment modes, besides requiring substantial expenditures, also do not yet give satisfactory

results, and even leave various harmful side effects, such as hair loss and skin hyperpigmentation. In view of this situation, it is essential to search for alternative treatments of BC that are more effective, more affordable, and safer, particularly treatments using natural or herbal substances. Traditionally, communities have used *Typhonium flagelliforme* [TF] (rodent tuber, *umbi keladi tikus*) for treatment of BC. On the other hand, there has been no definitive scientific evidence for the curative action of TF on BC. The curative effect of TF on BC needs to be proven, in view of the increasing numbers

of BC patients. It is estimated on the basis of global data that there are more than 1 million women with the diagnosis of BC, with the number of deaths amounting to more than 410.000.^(4,5)

So far, there have been only a few studies on TF. One of these is the *in vitro* study on continuous culture of human lymphoblasts (CEMs) treated with 10 and 20 µg/ml of TF. The results of this study showed that TF could increase cytochrome c secretion, caspase 9 and caspase 3 expression, DNA fragmentation, and apoptosis.⁽⁶⁾ An *in vivo* study on oral administration of TF to BALB/c mice with induced leukemia found that TF was able to reduce the numbers of immature granulocytes. The treatment with TF was carried out for 28 days, using doses of 200, 400, and 800 mg/kg BW/day.⁽⁷⁾ The abovementioned results of increased cytochrome c secretion, caspase 9 and caspase 3 expression, DNA fragmentation, and apoptosis, may provide clues to the curative action of TF on BC.

Traditionally, TF has been used by communities for the treatment of cancers. The compounds present in TF that are considered to have curative effects on cancer are the flavonoids. Epidemiological studies of lung cancer have shown that flavonoids are beneficial for its treatment.⁽⁸⁾ The study conducted by Sghaire et al.⁽⁹⁾ also showed that flavonoid-containing plants are capable of inducing apoptosis and inhibiting the proliferation of human leukemia cells. Furthermore, a study using the flavonoids genkwanin and chrysin, in combination with the enzyme CYP1 (polypeptide 1, subfamily A, family 1, of the cytochrome P450 group), that plays a role in the metabolism of xenobiotics, showed an increased antiproliferative effect on BC cells.⁽¹⁰⁾ On the other hand, trastuzumab, an anti-BC agent that has been widely and effectively used as chemotherapeutic adjuvant, being a monoclonal antibody derived from humanized recombinant DNA, is able to bind to the extracellular domain of the human epidermal growth factor receptor-2 (HER2)/neu. This binding prevents the binding of the natural ligand

to the receptor and reduces the number of receptors by a process of endocytosis.⁽¹¹⁾ In addition, trastuzumab is also able to directly inhibit the activity of tyrosine kinase as messenger protein. In consequence, these two processes lead to decreased proliferation of BC cells.⁽¹²⁾ With reference to this mechanism, trastuzumab is also effectively used in the treatment of BC metastases and expresses HER2/neu.⁽¹³⁻¹⁵⁾ The flavonoid-containing TF, which has been shown to induce apoptosis and to be antiproliferative, is expected to be able to reduce BC volume, by a mechanism similar to that of trastuzumab, using decreased HER2/neu and BCL2 expression as indicators. Therefore the present study was conducted with the objective to evaluate the effect of administration of TF syrup against BC, with as indicators the reduction in expression of HER2/neu and BCL2, and BC volume in female mice.

METHODS

Study design

The study design used was the experimental post test only control group design. The study was conducted from March to April 2013 in the Experimental Laboratory, Faculty of Medicine, University of Indonesia, Jakarta, for the intervention and preparation of paraffin blocks. Immunohistochemical assays for HER2/neu and BCL2 expression were done in the Laboratory of Pathologic Anatomy, Prof Dr Sardjito Hospital, Yogyakarta.

Experimental animals

The sample used in this study comprised 3 month-old female CH3 mice, weighing around 20-25 grams, obtained from the Experimental Laboratory, Faculty of Medicine, University of Indonesia, Jakarta. The sample size was determined according to WHO guidelines to be 6 mice per group. The mice were adapted for one week, after which the tumor pulp was transplanted by subcutaneous injection of 0.2 ml tumor pulp into each mouse (to a total of 18

mice). The tumor pulp was prepared from a tumor taken from a donor mouse that had been killed by ether anesthesia. The tumor was cleaned from blood vessels and necrotic tissue, minced, then physiologic saline was added in a 1 : 1 ratio. One week after transplantation, \pm 2 mm x 2 mm x 1 mm palpable tumors had grown in all mice.

Intervention

After the growth of the tumors, the mice were randomly allocated to three groups of 6 mice each. Group 1 was given 0.2 ml distilled water, group 2 was given 0.2 ml of 40 mg/ml TF syrup, and group 3 was given 0.2 ml of 80 mg/ml TF syrup. All interventions were given orally, once daily, for 25 days.

Preparation of *Typhonium flagelliforme* syrup

The fresh TF rhizomes were washed in running water, then sliced. Six kilograms of TF rhizome was then extracted by maceration using 60 l of 90% ethanol as solvent (1:10), the resulting extract weighing 50 g. Simple syrup was prepared from saccharose 130 g and Nipagin 0.25% b/v. The mixture was stirred on a low flame until homogenous, then hot distilled water was added to a volume of 200 ml. The 40 mg/ml TF syrup (TFS) was made by adding 100 ml of simple syrup to 4 g TF extract, while the 80 mg/ml TFS was prepared from 8 g TF extract plus 100 ml simple syrup.

The TF syrup was prepared in the Laboratory of Chemistry, Faculty of Medicine, Sultan Agung Islamic University, Semarang, from TF rhizomes obtained from West Semarang, Central Java.

Measurement of breast cancer volume

At the end of the study (day 26), the size of the tumors was measured (length, width, height in mm) using calipers.

Determination of Bcl-2 and HER2/neu expression

After the tumor had been measured, a tissue sample was taken for preparation of

histopathology slides, which were stained immunohistochemically to determine HER2/neu and BCL2 expression. The reagents used for preparation of the histopathology slides comprised: H₂O₂ 3%; trypsin 0.25% in phosphate buffered saline (PBS); 3,3'-diaminobenzidine (DAB) solution (as color indicator) prepared from 1 ml distilled water, 50 drops of H₂O₂ buffered substrate, 1 drop of DAB stock solution; xylol, absolute ethanol, and ethanol at concentrations of 70%, 80%, and 95%; anti-Her2/neu/ERBB2 (US Biological); and anti-Bcl-2. The histopathology slides were prepared by deparaffining, i.e. immersion of the tissue sections consecutively for 2 minutes in xylol, then in absolute ethanol, 95% ethanol, 80% ethanol, and 70% ethanol, respectively, for 1 minute, and running water for 10-15 minutes. The slides were then immersed in 3% H₂O₂ for 30 minutes, washed three times in PBS for 2 minutes each, placed in 0.25% trypsin for 6 minutes at 37°C, then again washed three times in PBS for 2 minutes each. After this the slides were placed in the monoclonal antibody solutions of anti Bcl-2/anti HER2/neu for 30 minutes, washed three times in PBS for 2 minutes each, placed in peroxidase-labeled secondary antibody solution, washed three times in PBS for 2 minutes each, placed in chromogen substrate solution for 5 minutes, washed three times in PBS for 2 minutes each, then rinsed in distilled water. The slides were then immersed in Mayer Hematoxylin stain for 6 minutes, washed in running water, and dehydrated, cleared, and mounted. BCL-2 and HER2/neu expression was determined by light microscopy at 400x magnification, using the hot spot area method in 10 fields of view. In each field 100 cells were examined, and the numbers counted of the cells expressing Her2/neu (brown colored cell membrane), and BCL2 (brown colored cytoplasm).⁽²³⁾

Statistical analysis

Statistical analysis was by one-way anova followed by LSD, using a significance level of 0.05.

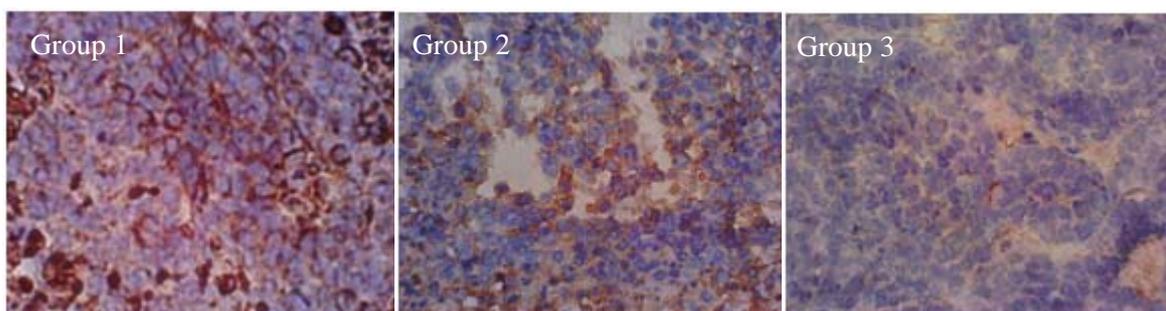


Figure 1. HER2/neu expression (brown colored cell membrane) in the treatment groups

Ethical clearance

The study was conducted after ethical clearance from Medical/Health Bioethics Commission, Faculty of Medicine, Sultan Agung Islamic University, Semarang.

RESULTS

During the intervention, one mouse from group 2 (the 40 mg/ml group) died on day 15 and one mouse from the 80 mg/ml group died on day 21. Up to the end of the study, the number of mice in the control group was still complete (6 animals), whereas in groups 2 and 3 there only remained 5 animals in each. The death of the animals is believed to be caused by lowered immune defenses as a result of tumor growth.

After administration of the intervention for 25 days, the C3H mice with BC showed differences in HER2/neu expression between the three groups (Figure 1). The lowest HER2/neu expression was in group 3 (40 mg/ml), followed by group 2 (20 mg/ml), and the highest expression was in group 1 (controls). Results of Anova indicated that the differences were

significant ($p=0.000$). Furthermore, LSD analysis showed that HER2/neu expression in group 3 was significantly lower than that in group 1 ($p=0.000$). In comparison with group 2, HER2/neu expression in group 3 was also significantly lower ($p=0.000$). This was also the case with HER2/neu expression in group 2, which was significantly lower than that in group 1 ($p=0.011$) (Table 1).

The BCL2 protein assay results also found differences in expression between the 3 groups (Figure 2). BCL2 expression was lowest in group 3 (40 mg/ml), followed by group 2 (20 mg/ml), while the highest expression was in group 1 (controls). Results of anova indicated that the differences were significant ($p=0.000$). LSD statistical analysis yielded results indicating that BCL2 expression in group 3 was significantly far lower than that in group 1 ($p=0.000$). In comparison with group 2, BCL2 expression in group 3 was also significantly lower ($p=0.000$). This was also the case with BCL2 expression in group 2 which was significantly lower than that in group 1 ($p=0.000$) (Table 1).

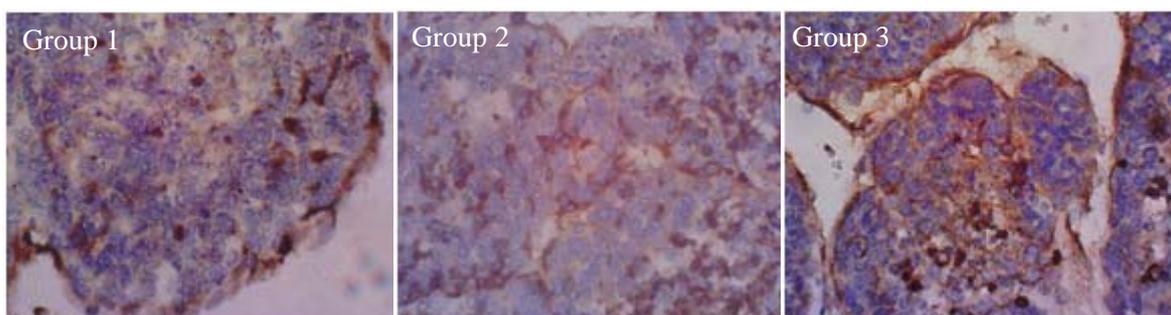


Figure 2. BCL2 protein expression (brown colored cytoplasm) in the treatment groups

Table 1. Mean HER2/neu and BCL2 expression and mean breast cancer volume

Variable	Group 1	Group 2	Group 3	P	
	Mean ± SD	Mean ± SD	Mean ± SD	Anova	L SD
HER2/neu (%)	245.40 ¹ ± 3.57	235.50 ² ± 7.66	51.60 ³ ± 2.88	0.000	0.000 ^{1,2,3} 0.011 ^{1,2} 0.000 ^{2,3} 0.000 ^{1,2,3}
BCL2 (%)	114.40 ¹ ± 2.60	54.20 ² ± 4.02	24.6 ³ ± 1.81	0.000	0.000 ^{1,2} 0.000 ^{2,3} 0.002 ^{1,2,3}
BCV (mm ³)	2563.86 ¹ ± 332.74	3667.15 ² ± 739.56	4392.33 ³ ± 774.48	0.000	0.059 ^{1,2} 0.306 ^{2,3}

HER=human epidermal growth factor; BCL=B cell lymphoma; BCV= breast cancer volume

DISCUSSION

The results of this study showed that administration of TFS at doses of 20 and 40 mg/ml for 25 days was able to significantly reduce HER2/neu expression in breast cancer cells in comparison with the control group. These results are in accord with the results of Chen's study, proving that flavonoids are able to effect degradation of HER2neu receptors, in spite of the unexplained mechanism of signal transduction.⁽¹⁷⁾ The results are also in agreement with other studies, providing evidence that flavonoids, compounds frequently found in plants, are able to inhibit the in vivo proliferation of both hematopoietic cancer cells and BC cells.^(9,10) This proliferation-inhibiting effect of flavonoids on cancer cells is strongly believed to be similar to that of trastuzumab or tamoxifen. The results of a study involving 3 randomized controlled trials (RCTs) showed that the addition of trastuzumab, a monoclonal antibody against the HER2/neu receptor, to anthracyclin and taxan used as chemotherapeutic adjuvants, was found to be of benefit in improving the survival rate of women with HER2/neu-expressing BCs.⁽¹³⁾ In addition, administration of the anti-estrogen tamoxifen as chemotherapeutic adjuvant in postmenopausal patients with phase 1 BC expressing estrogen receptors, was also of substantial benefit, and could even reduce the risk of recurrences by 35–50% 15 years after surgery.⁽¹³⁾ Both these facts illustrate how HER2/

neu and estrogen receptors in BC cells may be manipulated with monoclonal antibodies as well as receptor agonists to cure cancer. In the reduction of HER2/neu expression by flavonoids, these compounds do not involve monoclonal antibodies, but function as agonists of the HER/neu receptor. The activity of flavonoids as HER2/neu agonists causes a decrease in the expression of HER2/neu receptors, so that no cell proliferation takes place. Furthermore, flavonoids are strongly believed to be able to play the role of estrogen receptor agonists in cancer cells. They are thought to cause a decrease in the numbers of estrogen receptors, so leading to cessation of cell proliferation. There is evidence that the flavonoid genistein is a strong estrogen agonist and acts to inhibit tumor cell proliferation in vitro.^(18,19) Referring to these various facts, it may be deduced that flavonoids contained in TF can act as agonists of HER2/neu as well as estrogen receptors, although this requires further studies.

The results of the present study also prove that administration of TF at a dose of 0.2 ml (40 mg and 80 mg/ml) was capable of reducing BCL2 expression in BC cells. These study results are consistent with those of a study by Mohan,⁽⁷⁾ showing that administration of TF was able to decrease BCL2 expression, DNA fragmentation, and apoptosis. BCL2 is an anti-apoptotic protein, involved in the survival and proliferation of cancer cells. BCL2 acts as anti-apoptotic protein by preventing cytochrome c and Diablo/smac

release from the mitochondria. Diablo/smac is a protein that plays an important role in binding to the inhibitor of apoptosis protein (IAP), so that once released by the mitochondria, it will bind to IAP, inducing the cell to undergo apoptosis, resulting in tumor regression.⁽²⁰⁾

With reference to the roles of BCL2 and the HER2/neu receptor as mentioned above, the reduction in cancer volume is a result of decreased expression of the BCL2 protein and the HER2/neu receptor in breast cancer cells, through the mechanisms of apoptosis and antiproliferation. However, the present study was shown to yield the opposite result, because an increase in BC volume was found after administration of TF. This contradictory result is strongly believed to be caused by the type of flavonoids contained in TF. Flavonoids have in general positive roles in human health. Various studies show that flavonoids have anti-ischemic, anti-platelet, anti-inflammatory, and anti-lipoperoxidant effects.⁽⁹⁾ In addition, flavonoids have also been shown to inhibit the activities of various enzymes in the oxidation system, such as lipoxygenases, cyclooxygenases, monoxygenases, and xanthine oxidases. The activities of these enzymes are closely related to the antioxidant properties of flavonoids. The antioxidant effect of flavonoids is due to neutralization of ROS, which initiates lipid peroxidation and lipid peroxides by binding metal ions and the system of enzymes that is responsible for the formation of free radicals. On the other hand, flavonoids, particularly quercetins, in addition to possessing antioxidant functions, also have mutagenic and carcinogenic effects.^(21,22)

With reference to this fact, the increase in BCV after receiving TF is strongly thought to be related to the mutagenic and carcinogenic properties of flavonoids. These properties are substantially the result of the pro-oxidant activity of flavonoids (quercetin) that is related to that of catechol or pyrogallol, which may produce free radicals in the presence of metal ions, such as Fe and Zn.⁽⁹⁾ In consequence, although there is a reduction in HER2/neu receptor and BCL2

protein expression, the cells do not undergo apoptosis, and there is even an increase in proliferation, so that the volume of the cancer is increased. The mutagenic and carcinogenic properties are not found in all flavonoids. An analysis of TF content by a study team before starting their study, showed that the majority of flavonoids present in TF is of the quercetin type. This agrees with the evidence reported by Sghaire et al.,⁽⁹⁾ who showed that flavonoids with mutagenic and carcinogenic properties are found in the quercetin group.

In connection with this fact, one limitation of the present study is the omission to measure the Fe and Zn concentrations, that may promote the mutagenic effect of the quercetin flavonoids on breast cancer growth. Therefore further studies are required, where in addition to measurement of Fe and Zn concentrations during the administration of flavonoids, it should be determined which flavonoids have mutagenic and carcinogenic properties, so that only safe flavonoids will be consumed by the community. Another limitation of this study was that it did not measure other variables that play a role in proliferation or apoptosis.

CONCLUSION

Administration of *Typhonium flagelliforme* syrup was able to decrease expression of the Her2neu receptor and the BCL2 protein in mice, but did not decrease breast cancer volume.

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