



Treatment of *Thyponium Flageliforme* in Combination with *Curcuma Zedoaria*, and *Phyllanthus Niruri* Synergistically Enhances Apoptotic and Anti-Proliferative Effect on Breast Cancer

Chodidjah¹, Eni Widayati^{2*} and Taufiqurrachman Nasihun³

¹Department of Anatomy and Master Program of Biomedical Sciences of Sultan Agung Islamic University, Semarang, Indonesia; eniwidayanti@unissula.ac.id

²Department of Chemistry, Medical faculty of Sultan Agung Islamic University, Semarang, Indonesia

³Department of Biochemistry, and Master Program of Biomedical Sciences of Sultan Agung Islamic University, Semarang, Indonesia

Abstract

Introduction: Breast cancer is the most common cancer and the second leading cause of cancer death in women. Combinations of *Thyponium flageliforme*, *Curcuma zedoaria* and *Phyllanthus niruri* (TCP) have been proven as immunomodulator and retarded tumor progression in vivo. **Objective:** to investigate the apoptotic and anti-proliferative effect of TCP in C3H mice suffered from adenocarcinoma cells. **Methods:** 18 mice inoculated with one million cells of breast adenocarcinoma per mouse were assigned into three groups, TCP with dosage 85 and 125 mg were treated for 21 days to evaluate tumor volume, expression of Ki-67 and Capase3. Expression of Ki-67 and Capase3 were measured by immunohistochemistry staining, whilst cancer volume was assessed using calipers. **Results:** Mann Whitney analysis indicated that tumor volume in TCP groups were lower, otherwise for expression of caspase₃, $p < 0.05$ respectively. Whereas expression of Ki-67 in TCP groups was lower, but not significant, $p > 0.05$. **Conclusion:** TCP treatment with 85 and 125 mg dosage capable of reducing tumor volume, increasing caspase₃ and lowering Ki-67 expression.

Keywords: Apoptosis, Breast Cancer, *Thyponium flageliforme*, Tumor Volume

1. Introduction

Breast cancer is the most common malignant tumor and the second leading cause of cancer death in women worldwide¹. The incidence of the mammary gland cancer highest is in North America and Northern Europe

and lowest in Asia and Africa^{2,3}. The incidence rate of breast cancer in Asia Pacific is about 43 per 100.000 populations. Of that China is the highest incidence (46%), followed by Japan (14%) and Indonesia (12%)⁴. So far, the treatments of breast cancer consisting of surgery particularly in primary tumor, and radiotherapy,

*Author for correspondence

Email: eniwidayanti@unissula.ac.id

as well as chemotherapy; unfortunately it was not effective, even left various side effects such as hair loss, nail changes, infertility, and premature menopause⁵. In Indonesia *Thyponium flageliforme* (TF), *Curcuma zedoaria* (CZ), and *Phyllanthus niruri* (PN), abbreviated as TCP are known as traditional plants that capable of enhancing the immune response or retarded the breast cancer cells progression in vivo⁶. However, the underline effect mechanism of TCP treatment on breast cancer in vivo remains inconclusive.

The previous studies demonstrated that administration of PN alone has been proven capable of increasing the expression caspase3 and 7 and indeed apoptosis in cell line cancer^{7,8}. Another evidence also indicated that treatment of PN extract was able to inhibits cell cancer metastasis mediated by tumor cell invasion and blocking migration in the culture of Human Lung (A549) and breast cancer cells (MCF, -7) in vitro respectively⁹. Treatment with CZ alone at the dose of 300 mg/kg and 750 mg/kg Body Weight (BW) has been proven able to inhibit expression of p53 and H-ras in mutant mammary gland cells. Moreover, administration of CZ also able to reduces the incidence of mammary gland tumors in rats¹⁰. In addition, treatment of TF orally at the dose of 200, 400, and 800 mg/kg BW in BALB/c mice leukemia during 28 days was evidenced reduces the number of immature granulocyte cells in the peripheral blood circulation¹¹. Furthermore, my previous study demonstrated that administration of TF syrup at the dose of 40 and 80 mg/ml once daily for 25 days in C3H mice were inoculated with breast cancer cells, albeit capable of lowering expression of Her2neu protein, but did not reduce the tumor volume. The active substance that mediates PN, CZ, and TF action on tumor including expression of p53, caspase 3 and 7, and reducing immature granulocyte in peripheral blood circulation is polyphenols, curcumin, and flavonoids respectively. Refer to the previous study aforementioned, administration of TCP alone on breast cancer both in vitro and in vivo did not effective. Therefore in the present study treatment with combination of TCP on breast cancer in vivo, will synergistically reduce tumor volume mediated by enhancing of tumor cells apoptosis and debilitate cells tumor proliferation.

In principal, the breast cancer progression is determined in one hand by uncontrollable cellular

proliferation due to DNA mutation, such as BRCA1 and BRCA2 genes mutation. In normal condition these genes prevent cancer by encode protein synthesis to keep the cells from growing abnormally. By these mutation genes cannot block growth abnormally, and that lead to cancer formation⁵. On the other hand, despite the cellular proliferation is normal, however, its progression continuously persisting caused by impairment of gen suppressor tumor such as p53. Protein p53 produced by P53 gene is a genome guard, by which abnormal cell growth can be controlled. Inherited mutation of this gene cause Li-Fraumeni Syndrome and people with this syndrome have an increased risk of breast cancer⁵. In addition, gene mutations will induce expression of p53 and lead cells into G1 arrest for repairing or apoptosis dependent on the scale of mutation. In certain extent mutation and double strand gen damages can be repaired by the available mechanism in the body hence prevent cancerogenesis. However, in large extent genes damage may induce unrepaired genes and mutation and result in cancer due to uncontrollable proliferation and impairment of apoptosis. Therefore, concern of pharmacological treatment in cancer to date is addressed to induce the cells cancer apoptosis and suppress cells proliferation. The growing evidences indicated that flavonoids is one of the most popular plant (including TCP) constituent and commonly used by human worldwide. Flavonoids, however have various pharmacological properties^{12,13}, including anti-inflammatory, antioxidant, and cytotoxic anti-tumour activities^{12,14}. Objective of the present study to proof the treatment of TCP combination is synergistically able to reduce tumor volume mediated by increase in apoptosis and debilitate cells tumor proliferation. Increase in apoptosis characterized by elevation of caspase3 expression, and debilitated cells proliferation marked by decrease in expression of Ki-67.

2. Methods

In experimental study with Post test only control group design, a total of 18 female mice C3H (3 month old and 50 gram body weight) were used, obtained from the laboratory of animal of Indonesian University medical college. All mice have been inoculated with breast cancer cells taken from a donor mouse and assigned into

3 groups, six mice of each. (1) Control Group (C-G), mice were treated with distilled water. (2) 85 mg TCP group (TCP-85), mice got 85 mg/BW TCP. (3) 125 mg TCP group (TCP-125), all mice were treated with TCP 125 mg/BW. All treatments were given orally in 0.2 ml solution once daily for 21 days. All mice were kept in acclimatization for one week with environmental controlled temperature (20–24 °C), constant humidity (55-60 %), and controlled photoperiod (12 h light and 12 h dark) properties, with food and water ad libitum before start of the experiment. At the end of the study (day 22), the size of the tumors was measured manually (length, width, height in mm) using calipers. Furthermore, all rats were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar) and a tissue cancer sample was taken to form histopathology slides preparation to determine Ki-67 and caspase3 expression. All slides preparations were stained with immunohistochemistry method. All animal protocols were approved by the Research Ethics Committee of Sultan Agung Islamic University Medical Faculty, Semarang, Central Java, Indonesia.

2.1 TCP Extract

TCP was obtain from the market and extracted by Soxhlet methods from the whole plants. The extracts were prepared in the laboratory of Sultan Agung Islamic University Medical Faculty, Semarang, Central Java Indonesia

2.2 Breast Adenocarcinoma Cells Inoculation

Following mice were acclimated, one million cells of breast cancer was inoculated by subcutaneous injection of 0.2 ml tumor pulp into fat pad of mice. The tumor pulp was prepared from a tumor (breast cancer) taken from a donor mouse that had been sacrificed by ether anesthesia. The tumor was powdered, then physiologic saline was added in a 1:1 ratio.

2.3 Histopathology Slides Preparation

The histopathology slides were prepared by deparaffining, i.e., immersion of the tissue sections in xylol for 2 minutes, then in absolute ethanol, 95% ethanol, 80% ethanol, and 70% ethanol, respectively, for 1 minute, and

then running water for 10-15 minutes. After immersed in 3% H₂O₂ during 30 minutes, the slides washed three times in PBS for 2 minutes each, placed in 0.25% trypsin for 6 minutes at 37⁰ C, and then washed again in PBS three times for 2 minutes each. After that, the slides were immersed in the solutions of monoclonal antibody anti-caspase 3 and anti Ki67 for 30 minutes. Subsequently the slides washed three times in PBS for 2 minutes of each, then 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 washed in distilled water. Lastly the slides were immersed in Mayer Hematoxylin stain for 6 minutes, washed in running water, and dehydrated, cleared, and mounted. 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, one drop of DAB stock solution; xylol, absolute ethanol, and ethanol at concentrations of 70%, 80%, and 95%; anti caspase3; and anti-Ki67.

2.4 Assessment of Ki-67 and Caspase3 Expression

Ki67 and caspase3 expression as indicator of cellular proliferation and apoptosis respectively, were determined by Alred Score methods. The number of Ki-67 and caspase3 expressions was identified by light microscopy at 400 x magnifications in 10 fields of view of each slide. Two hundred cells in each field were counted and determined the number of Ki-67 and caspase3 expression. The expression of Ki-67 characterized by the brown colored simply in nucleus, whereas caspase3 marked by the brown colored in both cytoplasm and nucleus.

2.5 Statistical Analysis

The total number of Ki-67 and caspase3 expression were calculated and presented as the mean ± SEM. Differences between groups were analyzed for statistical significance, using a Kruskal Wallis and Man Withney (SPSS 22.0). A level of p < 0.05 was considered as statistically significant.

3. Results

In all mice, a palpable tumor have grown and seen at the size of about 2 mm x 2 mm x 1mm following one week adenocarcinoma inoculation. Therefore further

treatment of TCP on mice suffer from cancer can be performed. Following treatment of TCP for 21 days on those mice, the assesment result of tumor volume, expression of Ki-67 and caspase3 can be seen in table 1.

Table 1: Tumor volume, expression of Ki-67 and Caspase3 after treatment of TCP

Variables	Groups			P Kruskal Wallis
	C-G (n = 6)	TCP-85 (n = 6)	TCP-125 (n = 6)	
Tumor vol. Before treatment (mm ³)	41.00	47.07	33.64	-
Tumor vol. after treatment (mm ³)	2772.58	50.99	336.14	-
Δ between before and after treatment (mm ³)	2306.98 (+1151.30)	3.92 (+ 38.26)	302.50 (+266.64)	< 0.05
Ki-67 Expression (%)	3.8 (+ 0.37)	2.8 (+ 0.80)	3.6 (+ 0.67)	> 0.05
Caspase3 Expression (%)	6.2 (+ 0.2)	8.4 (+ 0.40)	10.2 (+ 0.48)	< 0.05

3.1 Tumor Volume after Inoculation of Adenocarcinoma Cells

The result of the study as shown in table 1 indicate that after inoculation of adenocarcinoma cells on mice, the highest Δ between tumor volume before and after treatment was in C-G, followed by TCP-125, and the lowest was in TCP-85. Kurskal Wallis analysis on tumor volume displayed that there was significant difference amongst groups, p < 0.05. Mann Whitney analysis showed that tumor volume in C-G was significant higher compared to that of TCP-85

and TCP-125, p < 0.05. In contrary, tumor volume in TCP-85 was significant lower compared to that of TCP-185, p < 0.05 (Figure 1).

3.2 Expression of Ki-67

Expression of Ki-67 was highest in C-G, followed by TCP-125, and the lowest was in TCP-85. However, Kruskal Wallis analysis on expression of Ki-67 amongst groups showed no significant difference, p > 0.05 (Figure 2).

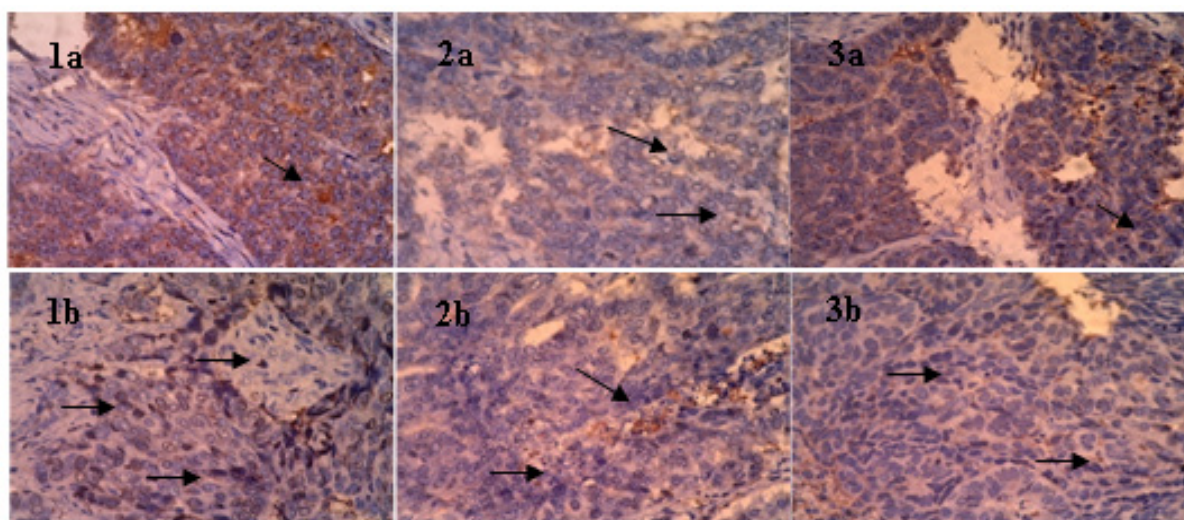


Fig. 1. Expression of caspase3 in C-G (1a); expression of caspase3 in TCP85 (2a); expression of caspase3 in TCP 125 (3a). Expression of Ki-67 in C-G (1b); expression of Ki-67 in TCP 85 (2b); expression of Ki-67 in TCP 125 (3b).

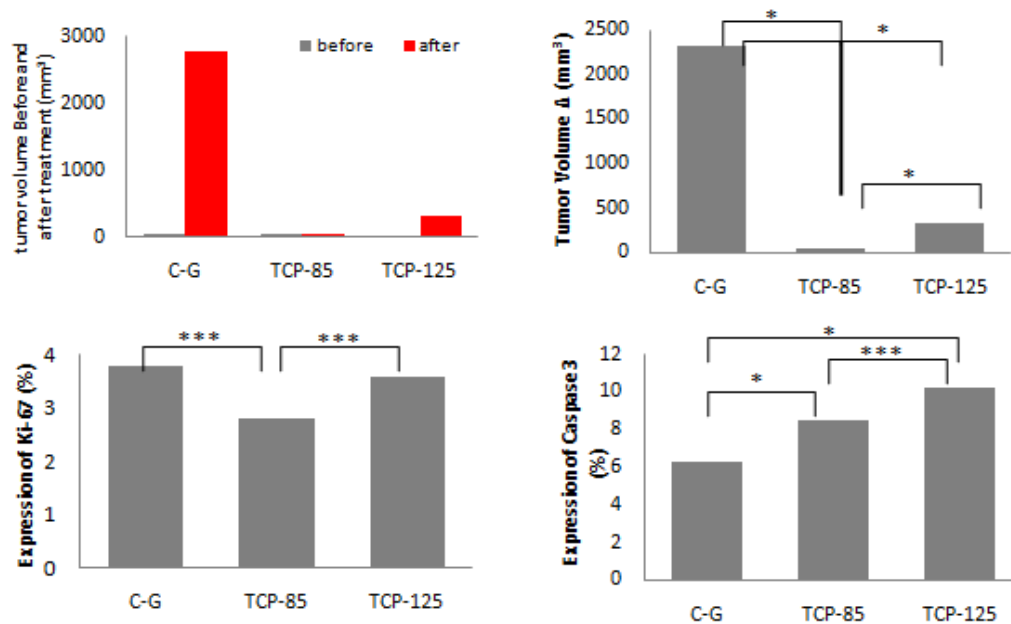


Fig. 2. Tumor volume before and after treatment, The Δ (differences) of Tumor Volume, Expression of Ki-67 and Caspase3 amongst groups. Mann Withney Analysis: * $p < 0.05$; ** $p < 0.05$; *** $p > 0.05$.

3.3 Expression of Caspase3

Expression of caspase3 was highest in TCP-125, followed by TCP-85, and the lowest was in C-G groups. Kruskal Wallis analysis on caspase3 amongst groups showed significant difference, $p < 0.05$. Moreover, Mann Whitney analysis demonstrated that the expression of caspase3 in TCP-85 and TCP-125 were higher compared to that of C-G, $p < 0.05$. Otherwise, expression of caspase3 in TCP-125 compared to that of TCP-85, were not difference significantly, $p > 0.05$ (Figure 2).

4. Discussion

In the present study to develop breast cancer was used breast adenocarcinoma cells inoculation on fat pad of mice. The result indicated that inoculation of adenocarcinoma cell was proved capable of growing carcinoma in a week. This result in line with the study was reported by Sousa and coworkers that orthotropic inoculation of breast carcinoma cell on normal mice capable of inducing growth, development, and even metastasis. After tumor inoculation within 7 days, involvement of positive lymph node with micro metastases was observed in 85% of the animals in ipsilateral sentinel lymph node (SLN; right sub iliac node) and contra lateral lymph node (LN; left

sub iliac node). However, in the group of 14 days, there were metastatic deposits in the ipsilateral SLN and contra lateral LN in 100% and 50% of animals, respectively¹⁵. The Study of Gregoreo and coworkers also demonstrated that inoculation of breast cancer cells 4T1 was able to induce primary tumor growth and metastasis in mice¹⁶. Another study reported by Neudert at al. also pointed out that inoculation of breast cancer into femoral artery was able to stimulate multiple osteolytic lesions in the distal femoral and proximal tibiae within 18 days after inoculation, with a success rate of 95-100 % and no additional co morbidity¹⁷. Lastly, inoculation of tumor cells isolated from human suffer from breast cancer into mammary fat pad of nude mice was significantly induce breast cancer develop and metastasis to multiple mouse organs¹⁸. Nevertheless, in the present study the tumor volume resulted from breast cancer inoculation in all groups were not similar (comparable) in size. The highest volume occurred in TCP-85, followed by C-G, and the lowest was in TCP-125 (Figure 2). Accordingly, to evade the information bias in all groups, tumor volume were measured following treatment with TCP is the Δ (difference) tumor volume between before and after treatment. After treatment of TCP the highest Δ of tumor volume was in C-G, followed by TCP-125, and the

lowest in TCP-85, suggesting that the most effective dose of TCP was TCP 85 and TCP 125 later on.

In addition to reduction of tumor volume, TCP treatment was also proven increase expression of caspase3, particularly in TCP-125 and TCP-85. Considering caspase3 is an executor enzyme in apoptosis mechanism, therefore increase in expression of caspase3 suggesting that the rate of cellular apoptosis of tumor volume was also increased. Based on this result the tumor volume reduction in TCP-125 should be lower compared to that of TCP-85. Instead, in this study the tumor volume reduction in TCP-85 was higher compared to that of TCP-125. Eventhough, the tumor volume reduction in TCP-85 and TCP-125 was no significant difference, however this result is remain inconsistence, since the major constituent of TCP is flavonoids. There are various evidences that the proapoptosis effect of flavonoids on tumor cell was in the dose-effect dependent manner^{19,20}. Moreover, in the view point of molecular biology this incosistence result possibly associated with DNA unfragmentation and chromatin uncondensation of tumor cells. It is possible since in some extent action of caspase3 to dismantle cells and induce formation of apoptotic bodies needs prior chromatin condensation and DNA fragmentation²¹. Therefore, to induce chromatin condensation and DNA fragmentation, the dose of TCP may be increased twice or trice fold, considering poliphenol and flavonoids capable of inducing apoptosis on tumor cells were in suprphysiologis dose, however further study is needed. On the other hand, expression of Ki-67 as a marker of cellular proliferation during mitosis, were lowest in TCP-85 followed by TCP-125, and C-G later on. Eventhough, the expression of Ki-67 in TCP-85, TCP-125, and CG was no significant difference, however this result was consistent with tumor volume reduction in TCP-85, TCP-125, and C-G. Taken together, this result imply that the tumor volume reduction was prominently associated with apoptosis and supression of proliferation induced by the high expression of caspase3 and lower expression of Ki-67 (Figure 2).

The tumor volume reduction after treatment of TCP is attributable to the constituent of TCP consisting of terpenoids, phenylpropanoids, flavonoids, sesquiterpenes, curserenon, arismol, and curcumin. There are various evidences indicated that administration

of these compounds (terpenoids, phenylpropanoids, flavonoids, and sesquiterpenes) were able to debilitate tumor progression and so called as anti-tumor⁶. Study was reported by Rachman and coworker showed that treatment with curserenon and arismol were extracted from CZ by hexan solvent capable of increasing expression of caspase3 and apoptosis²². Moreover, in vitro study reported by Shin, et al. displayed that administration of curcumin in ovarian cancer cells line (Siha cells) was able to activate cytochrome c and caspase3 and reduce up to 73% of ovarian cancer cell viability²³. Mankaran and coworker also reported that administration of TP in cell cultures of human lung carcinoma (NCL-H23) was able to increase apoptosis detected by TUNNEL method²⁴. Study was reported by Fuida showed that CZ oil has cytotoxic effects on NSCLC cell cultures characterized by apoptosis²⁵. In addition, CZ have also been proven increases the activity of caspase9, 8, and 3, and poly (ADPribose) polymerase (PARP). Otherwise, CZ decreased Bcl-2 and Bcl-xl protein in one side and increase Bax in another side, leading to increase Bax to Bcl2 ratio. Moreover, CZ also inhibit secretions of AIF and cytochrome c, Endo G to the cytoplasm as well as increase the expression P53 protein in H1229 cell culture²⁵. CZ also inhibit proliferation of H1229 cells line through the AKT and NF-kb (Chen, 2013).

5. Conclusion

Based on the result of the present study and aforementioned discussion conclusion can be inferred that TCP treatment with 85 and 125 mg dosage capable of reducing breast tumor volume, increasing caspase₃ and lowering Ki-67 expression.

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7. Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

8. References

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