Original article

The effect of the ethanol extract of *Typhonium flagelliforme* on apoptosis adenocarcinoma mamma cells in C3H mice

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Abstract: Malignancy cause high impact in mortality in worldwide. *Typhonium Flagelliforme* have been known to improve the immune system. The aims of this study was to determine the effects of *Typhonium Flagelliforme* on the tumor necrosis factor alpha (TNF-α) level and percentage of apoptotic cells in mice tumor The post test study design with randomized control group of 24 C3H mice with tumor were divided into 4 groups: I (control), 2 ; 3; and 4 (treatment). All treatment were given 0.2 cc of extract at the dose of 200, 400 and 800 mg/kgBW respectively for 30 days. TNF-α level was from tumor tissue were measured using ELISA methods, and percentage of apoptotic cells from tumor tissue were determined using immunohistochemistry assay with TUNEL antibody. The mean difference of TNF-α expression was tested using Anova at the significant level of 0.05. Apoptotic cells was analyzed by *Mann Whitney.* The result indicate that average of TNF α level in the treated groups were higher than those of control group ($p<0.05$). The percentage of apoptotic cells at the dose 200 mg/KgBW was higher than that of control group, groups 2 and 3($P<0.05$). In conclusion, *Typhonium Flagelliforme* can increase in the percentage of apoptosis cells but not in the levels of TNF α.

Keywords: *Thyponium flagelliforme,* Tumor Necrosis Factor-α, apoptotic cells

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Introduction:

Typhonium flagelliforme has been shown to increased apoptotic of Human Oral tongue cancer cells.[1] Tumor growth inhibition occurs due to a decrease in proliferation and an increase in apoptosis[2] Both of these effects as a result of the effects of bioactive flavonoids, alkaloids, linoleic acid, asubstance found in *Typhonium flagelliforme*[1] Tumor growth inhibition due to the effects of *Typhonium flagelliforme* has not been established. Dichloromethane extract of roddent tuber *(Typhonium flagelliforme)*, semipolar, containing linoleic acid*,* hexadecanoic acid*,* octadecanoic acid in has been shown to induce apoptosis in Human T4-limphoblastoid cell line CEMss.[2-3]

Flavonoids from other plant as immunomodulator can increase the levels of IFNγ and induce apoptosis. IFNγ can enhance macrophage activity resulting in tumor necrosis factor α (TNFα), and may play a role in apoptosis pathway. Ethanol extract of Roddent tuber mice contains flavonoid, fatty acid. There has been few studies conducted on the in vivo effect of ethanolic extract of roddent tuber to stimulate immune system, increase apoptosis, and as anti inflammatory. Pinpinella Alpina treatment with 100-150 mgdaily dose for 15 days capable of improving oxidative stress marked by increase in GPx activity and decrease in XO activity and inhibit apoptosis characterized by decrease in the expression of Bax and caspase3 mRNA. There were strong negative correlations between antioxidant activityand apoptosis on Sprague male rats after UVB irradiation.[4]

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4-Phenylbutyrate acid (4-PBA) is a short chain fatty acid with a low r molecular weight,can reduce levels of endothelial cells apoptosis which were exposed to proinflammatory cytokine TNF-α.[5]

This present study aimed to determine the in vivo effect of roddent tuber extract on TNFαin mice with tumors can not be explained in stimulating the immune stem cells, as well as increased apoptosis.

In 2019, an estimated 268,600 new cases of invasive breast cancer will be diagnosed among women and approximately 2,670 cases will be diagnosed in men. In addition, an estimated 48,100 cases of DCIS will be diagnosed among women. Approximately 41,760 women and 500 men are expected to die from breast cancer in 2019[6]. Breast cancer has a high incidence and mortality rate and is among the most common cancer in the world. According to World Health Organization (WHO) it is necessary to have a health system with high capacity which requires the development of knowledge.Breast cancer treatment including surgery, radiation and immunotherapy, has not been effective dueto a wide variety of side effects often occur, especially radiation an chemotherapy. without solusion, the resources and has minimal side effects.

Typhonium flagelliforme (TF) ethanol extract contains flavonoids/ quercetin, alkaloids, phenols, triterpenoids. Administration of *Typhonium flagelliforme* syrup was able to decrease expression of the Her 2neu receptor and the BCL2 protein in mice, but did not decrease breast cancer volume.[7-8] The dietary constituent fisetin (flavonoid) induces apoptosis and inhibits multiple signaling pathways that are involved incolon cancer growth , that fisetin inhibits expression of COX2 in HT29.[8] Dichlorometanolic extract of of *Typhonium flagelliforme* increased caspase-3 expression of MCF7 breast cancer cell line with caspase-3 gen deletion.[9]

Typhonium flagelliforme ethanolic extract given in C3H mice with adenocarcinoma mamma has been shown to stimulate the production of IFN γ (enhancement of the immune system) / IFN γ activate macrophages, thus generate the multiple cytokine, among others. Tumor necrosis factor (TNFα), play a role in apoptosis and cell proliferation. This study evaluate the paraameters of immune system and apoptosis in C3H mice inoculated with adenocarcinoma mammae tumors and were treated with ethanolicextract of rodent tuber at a dose of 200, 400, and 800 mg /kg, in vivo. The parameters of the immune system evaluated was levels TNF α. Apoptotic cells was evaluated using TUNEL staining.

Material and Method:

This was a post test research using randomized control group design. *Typhonium flagelliforme* plants obtained from the road area Sri Rejeki West Semarang with one year old of plant life, the part used is the tubers of plants. ethanol extract of *Typhonium flagelliforme* was obtained from Drug Development Center of Advanced Research Institute for Natural Product Diponegoro University, Semarang. The content of *Typhonium flagelliforme* extract was examined at the laboratory of Biology of Gadjah Mada University Yogyakarta.

The C3H mice aged 10 weeks, weighing 19- 20 grams was obtained from laboratory Pathology Anatomy Experimental University of Indonesia. Treatment was done at laboratory Pathology Anatomy Experimental University of Indonesia. The level of TNF α was evaluated in the laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, University of Indonesia. The apoptotic cells from tumor tissue were assessment using TUNNEL was conducted in Prof. Dr. Sardjito Hospital Yogyakarta.

A total of 24 C3H mice after being adapted for one week, inoculated with tumor cell mixture of adenokarsinoma mamma. On day 10, the tumor was palpable in all mice. The mice were randomly devided into four groups of 6 mice each: group C (control), T 1 group (Treatment 1: 200mg/kg), T2 group (400 mg/kg) and T3 group (800mg/kg). The treatmen was goven for 30 days. On day 31, mice tumor tissue was taken and subjected to TNF-level assay using ELISA. The sample of tumor tissue was preparaed in paraffin blocks and stainned using painting TUNEL to observe the cells undergoing apoptosis by light microscopy (Niclon Eclipse E400) using Hot Spot method in which is a cell painted in brown in the 5 field of view than 1000 cells so that each field of view had 200 cells.

Data on TNF α levels were tested for normality using *Kolmogorov Smirnov* and tested with *Levene homogeneity test*resulting in normal data distribution and homogeneous followed by *One*

Way Anova resulting in $p > 0.05$. TUNEL data was not normal and not homogeneous thus it was followed by *Kruskall Wallis* test ($p<0.05$)the differences between groups were tested by the *Mann Whitney test.*

Ethical Clearance:

The study protocol was approved by the Commission of Heath Research Ethics, Faculty of Medicine , Diponegoro University and dr Karyadi Central General Hospital, Semarang.

Preparation of *Typhonium flagelliforme* ethanol extract (TFEE)

One years old of *Typhonium flagelliferme* were obtained from the area aroun Sri Rejeki Street, West Semarang , foe utilization of theit tuber. TFEE was prepared at the Center for Drug Development from Natural Substances, Diponegoro. Weighing 1 kg were washed in running water, cut into small pieces and left in a dryng chamber at 40^0C ^{for} 48 hours. The dried material was ground to powder in a mill grinder. The powder was extracted in a soxlet apparatus with ethanol as a solvent. Then evaporated to a concentrate in a rotary vacuum evaporator at 70^0C. The concentrate was diluted with water to yield 20 mg/ml, 40 mg/ml and 80 mg/ml, respectively.

Preparation of tumor tissue samples for homogenization:

From each tumor sample , 50 mg was weighed, lysed and dissolved in 250 µl extraction buffer in a test tube. Each sample was mixed at 4^0C for 3 minutes in a homogenizer at 1000 rpm to yield soluble material (the test tubes with the tissue samples were put in a jar containing ice cubes). The insoluble tissue remmants at the bottom of the tube were transferred to a different test tube. All samples were centrifuged at 1000 g for 10 minutes, then stored at – 80^0C until required for determination of TNF α levels.

ELISA assay for TNF α

Prepare tissue sample solution was diluted more than five –fold with mouse tnf alpha sultion (Genway Biotech, San Diego). Add 100 µl of each standard and sample into apropiate well, cover well and incubate for 2,5 hours at room temperature with gentle shaking. Discard the solution and wash 4 times with 1x wash solution, Wash by filling each well with 300 µl wash buffer using a multi – channel pippete. Complete removal of liquid at each step . After the last wash , remove any remaining Wash Buffer by aspirating. Invert the plate and blot it agains clean paper towel. Add 100 µl of 1x prepared biotinylated antibody to each well. Incubate for 1 hour at room temperature with gentle shaking. Discard the solution, repeat the wash . Add 100 µl of prepared Streptavidin solution . Incubated for 45 minutes at room temperature with gentle shaking. Discard the solution, repeat wash. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with genle shaking. Add 50 µl stop solution to each well. Read at 450 nm immediately.

Terminal deoxynucleotidyl transferase mediated X-dUTP nick end labeling (TUNEL) staining Protocol[10]

Dewax paraffin embedded section in xylene and rehydrate in graded ethanol series to water. Use cuolin Jars. Xylene 5 minutes, Xylene 5 minutes, absolute ethanol 5 minutes, 95% ethanol 5 minutes, 70% ethanol 3 minutes, 30% ethanol 3 minutes. Rinse with PBS , then wash in Coplin jars with PBS 2 times for 3 minutes. Put 50 µl of TUNEL cocktail on test sections on other slides. Cover with parafilm pieces, Be careful that there are no bubles trapped underneath the parafilm, and that the parafilm pieces covering test and control sections do not touch. Incubated in humidified chamber for 60 minutes at 37^0C in dark (cover water bath with alumunium foil). Removed parafilm coverslip off the section by pipeting PBS around the edge of parafilm until it floats, then lift it with tweezers. Wash 3 times in PBS for 3 minutes in Coplin Jars.

Measurement the apoptotic cells by Hot Spot Method

The apoptotic cells was assessed under a Nikon Eclipse E 400 light microscope which uses 5 high power fields containing 1000 adenocarcinoma cells, so that each high power field contain 200 cells. Assesement of apoptotic cells at Sardjito Hospital Yogyakarta.

Results and Discussion:

Mean levels of TNF α is presented in at the table 1 showing that the highest mean levels of TNF was found in the control group, followed by group T 1 (200 mg / kg), group T3 (800 mg / kg), and group T 2(400mg / kg).

Table 1. mean and standard deviation of TNF α levels, and apoptotic cells by TUNEL Staining

Variables	Group	Mean	N	standard deviation
TNF α	Control	21.31	6	4.52
	Treatment 1	17.85	6	1.49
	Treatment 2	15.82	6	3.95
	Treatment 3	17.11	6	3.26
Apoptotic cells	Control	5.00	6	0.89
	Treatment 1	33.50	6	2.25
	Treatment 2	6.50	6	0.83
	Treatment 3	6.16	6	0.98

The test results One way ANOVA p> 0.05)showed that there was no difference between control group and treatment group 1,2,3. Based on this results,stating that rodent tuber ethanol extract at the dose of 200 mg/ kg, 400 mg / kg and 800mg / kg orally can not increased the levels of TNF α C3H mice inoculated with adenocarcinoma mamma.

The apoptoticcell number with TUNELstaining in Table 1 shows that the highest number of apoptosis cells was found in treatment group 1 (200 mg / kg), followed by treatment group 2 (at a dose of 400 mg / kg), 3 treatment groups (800 mg / kg), and the control group.

Table 2. The *Mann Whitney*test of apoptotic cells by TUNEL among the groups

No.	Group	Control	Treatment 1	Treatment 2	Treatment 3
1.	Control		0.002	0.026	0.093
2.	Treatment 1			0.002	0.002
3.	Treatment 2				0.699
4.	Treatment 3				

Mann Whitney test results showed a difference between control group and the treatment group 1. There was no difference control group and treatment group 3 (dose 800 mg). The treatment group 1 was different from the treatment groups 2 and 3. There was no difference between group 2 and 3. Based on these results the that the effect of the ethanol extract of rodent tuber 200mg / kg orally increase the number of apoptosis tumor cells in mice C3H recorded with TUNEL inoculated with adenocarcinoma mamma cells was accepted.

Discussion:

There were no dead laboratory animals during the study. The laboratory animal were C3H mice with the same weight, age, and gender, maintenance as well as tumor bulb injection treatment. The observation of the dependent variable was considered the same in each mouse. The results of inoculation of tumor bulb in experimental animals can be observed macroscopically and microscopically. Inoculation of tumor bulp was done by considering the genetics of experimental animals between the donor and the recipients. In C3H mice when inoculated withtumor cells of adenocarcinoma mamma from C3H mice donor, 90% will grow tumor.On the day ten, tumor was observed in all the experimentall animals.[11] TNF-α levels in all groups were equal. The highestnumber of apoptotic cells with TUNEL staining was found at a dose of 200 mg /kg BW showing that this group had the highest number of apoptotic cells. In theory TNF α play a role in the process of apoptosis, but there were no significant difference in the levels of TNF-α among control group, group 1 (200mg / kgBW), group 2 (400 mg / kgBW) and group 3 (800 mg / kg BW). This is likely bioactive substances of the *Typhonium flagelliforme* acts inhibit the TNF α level. The quercetin significantly inhibited TNF-α production and gene expression in a dose-dependent manner[11]

Increased in the number of apoptotic cells in group of 200 mg / kg BW was likely through a different apoptotic pathways. Bioactive substances contained in in the ethanol extract of the *Typhonium flagelliforme* acts as a ligand, binds to membrane receptors of tumor cells, activate *Fas Asosiated Death Domain* (FADD), activate protease in the cytoplasm (caspase 8). Caspase 8 is active through apoptotic signal brought by FADD, then caspase 8 will split in the form of proenzyme. Protease causes the expression ofthe protein from the outside and the inside of the mitochondria namely cytochrome c and some caspaseeffectors. Cytochrome binds to a pro-enzyme which is an effector of caspase and stimulate proteolytic through caspase 8. In the process of apoptosis, caspase 9 damage pores of the nucleus of cells andcaspase 3 gets into the nucleus of cellsto break *Inhibitor of Caspase Activated DNAse* (ICAD). The absence of *Caspase Activated DNase* (CAD) from the complex molecules would result

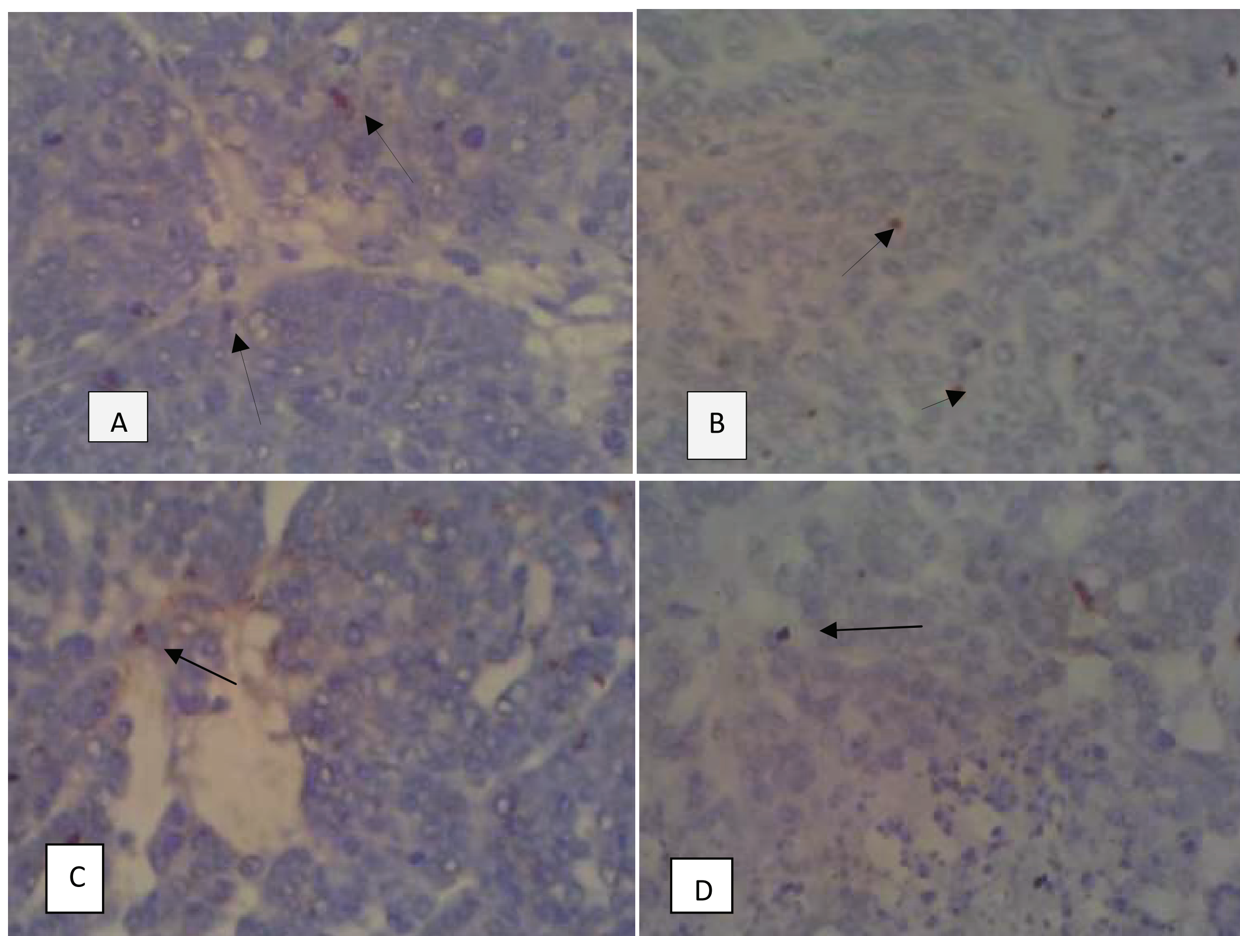

Figure 1. Tumor cells apoptotic by tunnel staining at 400x magnification. A controls group, B dose group 1 (T1), C dose group 2 (PT2) D 3 dose group (T 3). The arrows indicate apoptotic cells

in degradation of DNA .[12] resulting in cell apoptosis observed with TUNEL staining.

At doses of 400mg/ kgBW and 800 mg /kgBW the number of apoptotic cell decreased, because substances in *Typhonium flagelliforme* ethanol extract depends on the molecular weight, solubility, PH, the stability of the substances, transport through the intestinal barrier, absorption, distribution,metabolism, as well as the bonding agent with the target cell receptors.[13]. TNFα is likely to play a role in the process of proliferation.

Interaction of TNF-α and *TNF Receptor-1* (TNFR-1) and TNFR-2 activate various signal transduction. TNFR-1 contributes to the process of apoptosis and proliferation of tumor cells. TNF α and TNFR-1 bonding will bind *TNF-associated death domain* (TRADD) and recruit the adapter protein receptor interacting protein (RIP), TNFR -associated factor 2 (TRAF-2), and *Fas Associated Death Domain.* TRAF-2 inhibits apoptosis through *cytoplasmic protein inhibitor of apoptosis* (cIAP). The association of TRAF-2 and cIAP, cytoplasmic protein occurring and activating cFos/ cJun transcription factor through cascade mitogen activated caspase (MAPK) and cJun N terminal kinase (JNK). TRAF-2 and RIP adapter proteins activate transcription factor nuclear factor kappa B (NF-kB) through NF-kB inducing kinase (NIK) and inhibitor of kB kinase (IKK). NF-kB transcription factors, cFos/cJun induce antiapoptotic gene transcription,resulting in tumor cell proliferation.[14] *Typhonium. flagelliforme* tuber extracts possess compounds with significant antibacterial and antioxidant effect.[15] Mangosteen peel (*Garcinia mangostana Linn.*) contains xanthanknown as an antioxidant. Administration of 12% dose ethyl acetate extract of mangosteen peel cream is proven to significantly lower, TNF-α levels and Caspase-3

levels in the epidermal tissues of guinea pig skin exposed to ultraviolet B light.[16]

Conclusion:

- the administration of *Typhonium flagelliforme* ethanoic extract at the dose of 200. 400 and 800 mg / kg BWcan not increase the levels of TNF α of adenocarcinoma mamma in C3H mice
- Ethanol extract at the dose of 200 mg / kg BW increase apoptotic cells with TUNEL staining from adenocarcinoma mamma mice C3H.

the administration of Ethanol extract of *Typhonium flagelliforme* at dose of 400 mg / kg and 800 mg / kg BW may not increase apoptotic cells with TUNEL staining from adenocarcinoma mamma C3H mice

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