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Antitumor Activities of *Typhonium flagelliforme* Extract Combined with Natural Interferons of Canine and Feline in Mice with *DMBA* Induced Skin Tumors

(AKTIVITAS ANTITUMOR KOMBINASI EKSTRAK *Typhonium flagelliforme* DENGAN INTERFERON ALAMI ANJING DAN KUCING PADA MENCIT DENGAN TUMOR KULIT YANG DIINDUKSI *DMBA*)

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ABSTRACT

Indonesia has various plants with potential activities and uses as antitumor agents, one of which is *Typhonium flagelliforme*. This study aimed to examine *in vivo* the effects of combination therapy of *T. flagelliforme* leaf-ethanolic extract and natural interferon of canine or feline as an antitumor agent. In this study, 18 female mice of ddY strain induced with 7.12-dimethyl-benz(α)anthracene (DMBA) carcinogenic substance were divided into six treatment groups. All tumor tissues formed were collected and made for histopathological slides. The slides were stained with Hematoxylin-Eosin (HE) and immunohistochemistry (IHC) procedure using antibodies for proliferating cell nuclear antigen (PCNA) and Caspase-3. The macroscopic observations showed that the skin surface of the mice groups with tumors had alopecia with a reddish color. The microscopic observations showed three tumors types: anaplastic carcinoma, fibrosarcoma, and squamous cell carcinoma. The mitotic, angiogenesis, PCNA and Caspase-3 tests showed significant differences among the groups administered with the single and combination test materials. The combination therapy of *T. flagelliforme* leaf-ethanolic extract and natural interferon can stabilize mice's body weight, inhibit tumor growth, reduce mitosis and angiogenesis, and reduce PCNA and Caspase-3 expression.

Keywords: combination; interferon; *Typhonium flagelliforme* ethanolic extract; tumor.

ABSTRAK

Indonesia memiliki beragam jenis tanaman dengan aktivitas potensial dan kegunaan sebagai agen antitumor, salah satunya adalah keladi tikus (*Typhonium flagelliforme*). Penelitian ini bertujuan mengevaluasi pengaruh terapi kombinasi ekstrak etanol daun keladi tikus dan interferon alami anjing atau kucing sebagai agen antitumor secara *in vivo*. Dalam penelitian ini, 18 ekor mencit betina galur ddY yang diinduksi dengan zat karsinogen 7,12-dimetilbenz(α) antrasen (DMBA) dibagi menjadi enam kelompok perlakuan. Semua jaringan tumor yang terbentuk dikoleksi dan dibuatkan slide histopatologi. Slide diwarnai dengan Hematoxylin-Eosin (HE) dan prosedur imunohistokimia (IHC) menggunakan antibodi terhadap *proliferating cell nuclear antigen* (PCNA) dan Caspase-3. Hasil pengamatan makroskopis menunjukkan permukaan kulit kelompok mencit penderita tumor mengalami alopecia dengan warna kemerahan. Pengamatan mikroskopis menunjukkan tiga jenis tumor yang terbentuk, yaitu karsinoma anaplastik, fibrosarkoma, dan karsinoma sel skuamosa. Hasil uji mitosis dan angiogenesis maupun uji PCNA dan Caspase-3 menunjukkan perbedaan yang nyata antar kelompok kontrol dengan kelompok yang diberi perlakuan bahan uji serta antar kelompok yang diberi bahan uji tunggal dan kombinasi. Terapi kombinasi ekstrak etanol daun *T. flagelliforme* dan interferon alami dapat menstabilkan bobot badan mencit, menghambat pertumbuhan tumor, mengurangi mitosis dan angiogenesis, serta menurunkan ekspresi PCNA dan Caspase-3.

Kata-kata kunci: ekstrak etanol daun keladi tikus; interferon; kombinasi; tumor

INTRODUCTION

Tumor is a pathological growth disorder characterized by excessive and abnormal cell proliferation (Sinha, 2018). A high tumor prevalence (42.1%) is found in carnivorous animals such as domestic dogs and cats. These animals also have a high tumor incidence rate (Madsen *et al*., 2017). Control of tumors is usually done by surgery, chemotherapy, and radiotherapy strategies. These treatments, however, pose problems, namely an inability to select tumor target cells and an emergence of tumor cell resistance that can reduce the optimation of the treatment. Almost all treatments used in chemotherapy have adverse side effects on normal tissues and organs (Abdoon *et al*., 2016).

Another treatment for tumor therapy is the use of herbs and biological substances (Priosoeryanto *et al*., 2020). Indonesia is one of the countries with the world's most enormous flora and fauna biodiversity. The country has more than 2,500 species of medicinal plants widely used by the locals (Rahayu*et al*., 2020). One of them that has potential activity as an antitumor agent is *Typhonium flagelliforme*. An antiproliferative activity on MCA-B1 and MCM-B2 cells from using *T. Flagelliforme* leaf-ethanolic extract in an *in vivo* study was reported (Priosoeryanto *et al*., 2020).While an *in vitro* study, it showed a high potential to be used for breast cancer therapy (Zakaria *et al*., 2018).

Interferon (IFN) is a protein in the cytokine group produced by the body as a product of viral or other microbial infections (Franco *et al*., 2017). As a biological substance potentially used for tumor therapy, interferon has antiviral, antiproliferative, anti-inflammatory, and immuno-modulatory functions (Setthawongsin *et al*., 2019). The use of omega cat interferon recombinant (rFeIFN-ω) inhibited tumor cell growth in various dogs and cats tested *in vitro* (Villaverde *et al*., 2016).

There are currently no reports on the use of a combination of *T. flagelliforme* extract and natural canine or feline interferon tested *in vivo* as antitumor therapy. This study aimed to examine *in vivo* the effects of combination therapy of *T.*

flagelliforme leaf-ethanolic extract and natural canine interferon (nCaIFN) or natural feline interferon (nFeIFN) as an antitumor agent.

RESEARCH METHODS

This research was approved by the Veterinary Ethics Commission of the Faculty of Veterinary Medicine, IPB University number 153/KEH/SKE/X/2019.

Typhonium flagelliforme Extract

The leaf-ethanolic extract of *T. flagelliforme* used in this study was obtained from the previous study (Priosoeryanto *et al*., 2020).

Interferon

In this study, the natural canine interferon (nCaIFN) and feline interferon (nFeIFN) used were obtained from the previous study (Priosoeryanto *et al*., 2014).

Experimental Animal

The experimental animals of this study consisted of eighteen female mice of ddY strain aged five weeks and weighed 20-25 g. The mice were stood in plastic containers of 35x20x15 cm^3 covered with woven wire each with three mice. They are kept in a cool room of the Animal Unit Laboratory, Animal Education Hospital IPB University. The mice were acclimatized for two weeks, fed twice daily, and drank *ad libitum*.

Tumor Induction

Tumor induction was done by administration of *7.12-dimethylbenz(α)-anthracene* (DMBA) at a dose of 25 µg/0.05 mL/head (Muti'ah *et al*., 2016). DMBA was dissolved in acetone solvent to get the desired dose. The mice were induced subcutaneously on the back twice a week for four weeks, and observations were made until tumors developed (at week 13^{th}).

Administration of the Test Material

The mice that previously been induced with tumors divided into six treatment groups. The grouping of mice is presenting in Table 1.

Interferon was given at a dose of 102 U/head, while *T. flagelliforme* leaf-ethanolic extract was given at a dose of 120 mg/kg BW (Priosoeryanto *et al*., 2020). The tumor mass was injected with the test material intratumorally for three consecutive days every week for four weeks (Sandriya *et al*., 2021).

Table 1. Division of the therapy groups

Group	Treatment Substances
Group 1	Control (placebo)
Group 2	*T. flagelliforme* leaf-ethanolic extract
Group 3	nCaIFN
Group 4	nFeIFN
Group 5	nCaIFN + *T. flagelliforme* leaf-ethanolic extract
Group 6	nFeIFN + *T. flagelliforme* leaf-ethanolic extract

Macroscopic Observation

The macroscopic representation of the tumor mass tissue examined includes skin and hair abnormalities (alopecia, wounds, scabs, and color of the tumor mass), which were observed by looking at the tumor mass and parts around the tumor mass, tumor diameter measured weekly at vertical and horizontal directions; shape of the tumor mass (round or cauliflower-like); weight of mice (weighed every week using digital scales); and presence of the tumor mass in other parts of the body. Observations were done from day first to seventh after the therapy.

Termination of Experimental Animals

After the therapy and observation period were over, all mice were sacrificed by euthanasia using a dose three times higher than the lethal anaesthetic dose. The anaesthetic used is a combination of 300 mg/kg BW Ketamine and 15-30 mg/kg BW Xylazine (AVMA, 2013).

Sampling and Histopathological Preparations

All mice were necropsied after being sacrificed and the tumor masses were collected. The tumor masses were then fixed, and histopathological slides were made by staining with Haematoxylin-Eosin (HE) or an immunohistochemical (IHC) procedure.

Immunohistochemistry

Immunohistochemical staining was based on the Abcam DAB staining kit series ab64261 protocol (Abcam,Cambridge, UK). In this study, primary antibodies in the form of proliferative cell nuclear antigen (PCNA) and Caspase 3 were used. The final step after the staining process was the addition of mounting medium (Entellan®, Merck, New Jersey, USA) and the slides were covered with cover glass.

Microscopic Observation

The microscopic representations observed were tumor cell structure, differentiation, growth rate, metastasis, mitotic index, and angiogenesis. The degree of tumors differentiation was classified into (a) high differentiation if the cells formed resembled the original cells that were visible, bridges between cells were transparent, and mitosis was slow and did not undergo metastasis; (b) moderate differentiation if the cell shape was less clear, arranged atypically, the growth rate was relatively faster, bridges between cells were not prominent, mitosis was relatively large, and size and shape vary; (c) low differentiation, if it showed poor cell characteristics, no bridges between cells, and high mitosis occurred: (d) undifferentiated if it showed indefinite cell characteristics or primitive or abnormal cell shape (Singh, 2014).

The mitotic index was obtained by counting the number of cells undergoing mitosis (division) in ten microscope fields of view with 40x10 magnification and then averaged (Mirzaiian *et al.,* 2020). Angiogenesis was observed by counting the number of blood vessels in five microscope fields of view with 40x10 magnification twice in a row and then averaged (Ullah *et al.,* 2013).

Immunohistochemical

Observations to determine the positivity and color intensity by measuring the grayscale value using the ImageJ program (Yao *et al.,* 2018). The positivity was the percentage of positive area calculated in 10 fields of view with 40x10 magnification (Li *et al.,* 2019), while the color intensity was measured in 10 fields of view with 40x10 magnification (Sun *et al.,* 2018). The darker area showed a lower intensity value, indicating an inverse correlation between the antigens number and their numerical value. The equalization of the antigen numbers and their numerical values was done using the reciprocal intensity method, where the maximum value of the intensity test was subtracted by the intensity value obtained so that the antigen number would be proportional to the numerical value (Nguyen *et al.,* 2013).

Data Analysis

All quantitative data obtained from the macroscopic and microscopic observations were statistically analyzed using one-way Analysis of Variance, and further analysis using Duncan's Multiple Range Test (DMRT) was performed for the treatment that gave significant effect, while qualitative data was presented descriptively.

RESULTS AND DISCUSSION

Macroscopic Lesions

In parts of the skin of the DMBA-induced mice, the tumor mass grew with reddish skin or alopecia. The results of weekly observations on differences in tumor diameter growth are presented in Table 2. In the first week, the increase in tumor diameter in the control group (I) did not show any significant difference ($p>0.05$) compared to the groups given the test material (II, III, IV, V, and VI). Meanwhile, the groups given the combined test materials of *T. flagelliforme* ethanolic extract and nCaIFN (V) or nFeIFN (VI) showed a decreasing trend of tumor diameter. In the second week, there were significant differences ($p<0.05$) among the control group (I) and the extract group (II) compared to the interferon groups (III and IV) and the groups given the combination of extract and interferon (V and VI). In the third week, there were significant differences ($p<0.05$) among the control group (I) and the extract group (II) compared to the interferon groups (III and IV) and the groups given the combination of extract and interferon (V and VI), and among the interferon groups (III and IV) and the extract and nFeIFN combination group (VI). The group given nCaIFN therapy showed a decreasing trend of tumor diameter in the third week. The results in the fourth week showed significant differences ($p<0.05$) among the control group (I) and the extract group (II) compared to the groups of interferon (III and IV) and combination of extract and interferon (V and VI), in which significant differences ($p<0.05$) were found among the interferon groups (III and IV) and the extract and interferon combination groups (V and VI).

Table 2. Comparison of the increase in tumor diameter between weeks in the control group and the groups were given the test materials

Group	Comparison of increase in tumor diameter (mm)			
	Week 1 and 0	Week 2 dan 0	Week 3 and 0	Week 4 and 0
I	0.097±0.35^{a}	0.193±0.06^{a}	0.247±0.04^{a}	0.337±0.08^{a}
II	0.060±0.07^{a}	0.187±0.07^{a}	0.333±0.07^{a}	0.310±0.10^{a}
III	0.003±0.13^{a}	0.007±0.11^{b}	-0.003±0.11^{b}	-0.013±0.12^{b}
IV	0.083±0.08^{a}	0.033±0.08^{b}	0.027±0.12^{b}	-0.007±0.14^{b}
V	-0.040±0.10^{a}	-0.073±0.13^{b}	-0.100±0.12^{bc}	-0.203±0.05^{c}
VI	-0.080±0.13^{a}	-0.077±0.03^{b}	-0.170±0.05^{c}	-0.213±0.07^{c}

Different letters in the same column indicated significantly different ($p<0.05$)

The results of the comparing of the weight gain in the control group and the groups given the test material are presented in Table 3. In the first week, the increase in tumor diameter in the control group (I) did not show any significant difference ($p>0.05$) compared to the groups given the test materials (II, III, IV, V, and VI). In the second week, there were significant differences ($p<0.05$) among the control group (I) compared to the groups given the test material nFeIFN (IV), a combination of extract and nCaIFN (V), and combination of extract and nFeIFN (VI). In the third week, there were significant differences ($p<0.05$) among the control group (I) compared to all groups given the test

materials. The test results in the fourth week showed significant differences ($p<0.05$) among the control group (I) compared to all groups given the test materials and also among the interferon groups (III and IV) compared to the extract and interferon combination groups (V and VI).

Table 3. Comparison of the mice weight gain in the control group and the groups given the test materials

Group	Comparison of mice weight gain (g)			
	Week 1 and 0	Week 2 dan 0	Week 3 and 0	Week 4 and 0
I	-0.667±0.58^{a}	-1.667±1.53^{a}	-3.667±0.58^{a}	-4.333±1.15^{a}
II	0.333±0.58^{a}	0.667±0.58^{ab}	1.333±1.15^{b}	1.667±0.58^{b}
III	-0.333.±2.08^{a}	0.333±3.21^{ab}	0.667±1.15^{b}	1.667±0.58^{b}
IV	1.333±1.15^{a}	2.000±1.00^{b}	1.667±0.58^{b}	2.333±0.58^{b}
V	1.333±1.53^{a}	1.667±0.58^{b}	2.333±1.15^{bc}	4.000±1.00^{c}
VI	0.667±0.58^{a}	2.000±1.00^{b}	3.667±0.58^{c}	4.333±0.58^{c}

Different letters in the same column indicated significantly different ($p<0.05$)

Microscopic Representations

The results of histological observations on anaplastic carcinoma, Fibrosarcoma, and squamous cell carcinoma are presented in Figure 1.

Figure 1. Microscopic representation of tumor, 1: *Anaplastic carcinoma*; 2: *Fibro-sarcoma*; 3:

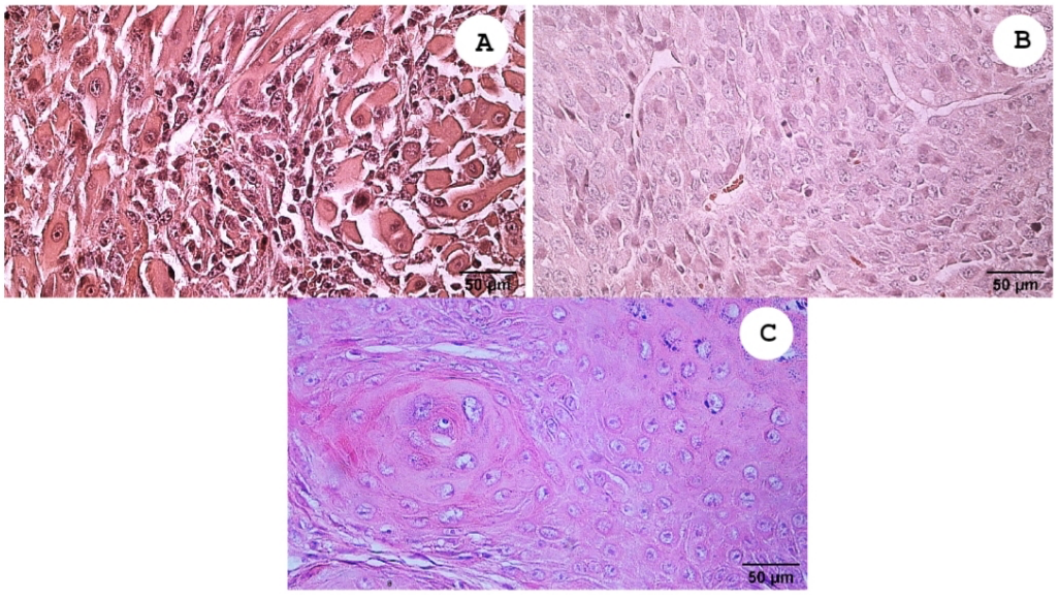

Figure 1. *Squamous cell carcinoma*

The results of microscopic observations on mitotic and angiogenesis are presented in Table 5. The results showed significant differences ($p<0.05$) in the control group compared to all groups given the test materials. The means mitotic index and angiogenesis value of the groups were in the order of the control group (I) > the nCaIFN group (III) > the *T. Flagelliforme* leaf- ethanolic extract group (II) > the nFeIFN group (IV) > the *T. flagelliforme* leaf-ethanolic extract and nCaIFN combination group (V) > the *T. flagelliforme* leaf-ethanolic extract and nFeIFN combination group (VI).

Table 4 Results of DMRT on the index of mitosis and angiogenesis

Group	Mitosis Index	Angiogenesis
I	4.9±0.4[a]	4.7±0.3[a]
II	3.3±0.6[b]	3,1±0.5[b]
III	3.4±0.3[b]	3.2±0.7[b]
IV	3.0±0.7[b]	2.9±0.5[b]
V	1.5±0.3[c]	1.5±0.5[c]
VI	1.5±0.4[c]	1.2±0.5[c]

Different letter in the same column indicated significantly different ($p<0.05$)

The results of PCNA expression on immunohistochemical staining showed significant differences among the groups and are presented in Figure 2. The positivity and intensity of PCNA expression results were further tested using the DMRT, and the results are given in Table 6. The control group showed significant differences ($p<0.05$) compared to all groups given the test materials. Among the groups given the single test material, namely *T. flagelliforme* leaf-ethanolic extract, nCaIFN, and nFeIFN, there were significant differences ($p<0.05$) compared to the groups given the combination of *T. flagelliforme* leaf-ethanolic extract and nCaIFN or nFeIFN. The latter groups resulted in lower PCNA expressions than those given the test materials in a single administration.

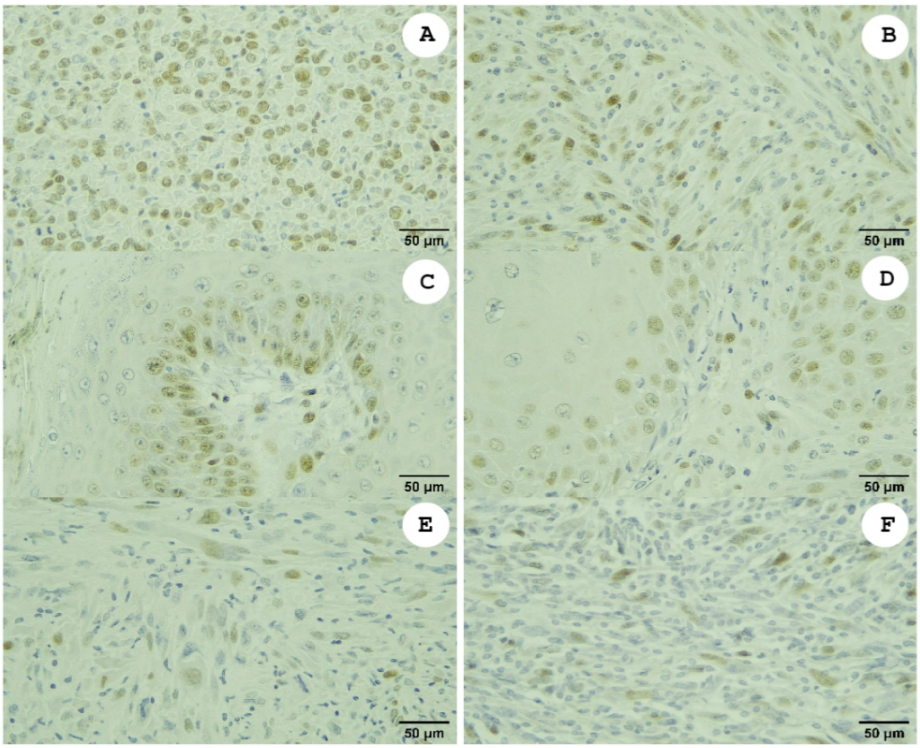

Figure 2 PCNA expression in tumor tissues. The brown color indicates PCNA expression. A: Control group; B: Leaf-ethanolic extract of *T. Flagelliforme* group; C: nCaIFN group; D: nFeIFN group; E: Combination of nCaIFN and leaf-ethanolic extract of *T. Flagelliforme* group; F: Combination of nFeIFN and leaf-ethanolic extract of *T. Flagelliforme* group.

Table 5. Results of DMRT on the PCNA expression in the control group and the groups given the test materials

Group	Positivity (% Area)	Intensity (AU)
Control	25.6±4.0[a]	190.7±1.2[a]
Extract	17.4±0.9[b]	181.2±2.1[b]
nCaIFN	16.5±1.0[b]	179.1±3.4[bc]
nFeIFN	15.1±1.2[b]	174.9±2.0[c]
nCaIFN+Extract	11.1±1.9[c]	157.6±5.8[d]
nFeIFN+Extract	9.5±2.0[c]	156.1±5.0[d]

Different letter in the same column indicated significantly different ($p<0.05$)

The results of observation on the Caspase-3 expression on immuno-histochemical staining are presented in Figure 3. The positivity and intensity of Caspase-3 expression were significantly different among the mice groups. Therefore data analysis proceeded to Duncan's test with the results presented in Table 7. The control group was significantly different ($p<0.05$) from all groups given the test materials. The single administration of the test material resulted in significantly different expressions ($p<0.05$) compared to the groups given the test materials in combination. The combination of *T. flagelliforme* leaf-ethanolic extract and nCaIFN or nFeIFN treatments gave lower positivity and intensity than the single administration.

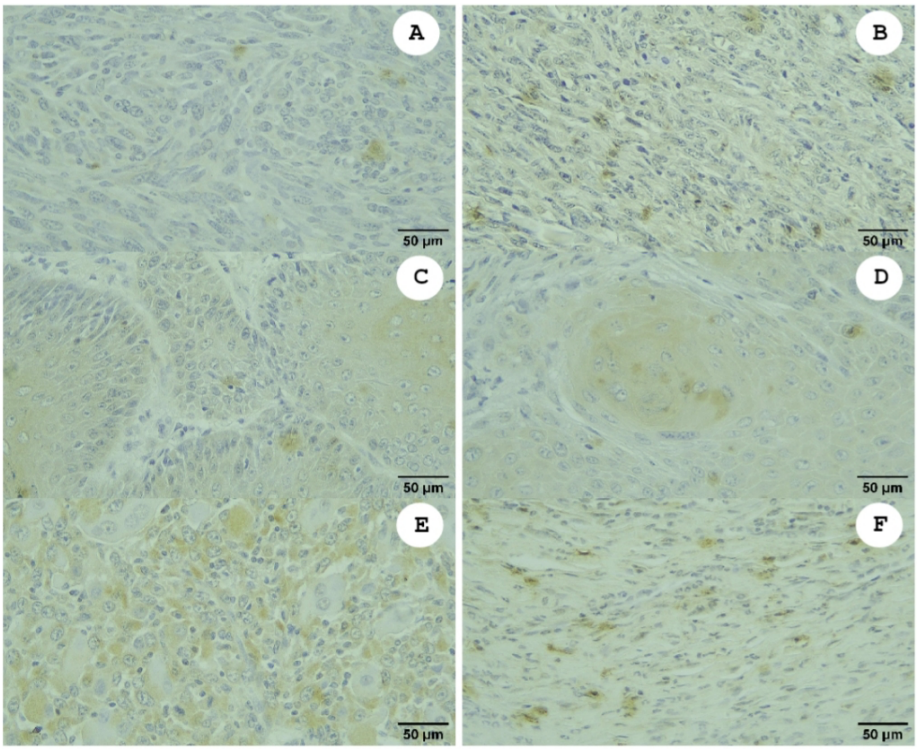

Figure 3 Caspase-3 expression in tumor tissues. The brown color indicates Caspase-3 expression. A: Control group; B: Leaf-ethanolic extract of *T. Flagelliforme* group; C: nCaIFN group; D: nFeIFN group; E: Combination of nCaIFN and leaf-ethanolic extract of *T. Flagelliforme* group; F: Combination of nFeIFN and leaf-ethanolic extract of *T. Flagelliforme* group.

Table 6. Results of DMRT on the Caspase-3 expression in the control group and the groups given the test materials

Group	Positivity (% Area)	Intensity (AU)
Control	11.7±0.9[a]	160.7±5.7[a]
Extract	15.3±0.8[b]	179.5±4.6[b]
nCaIFN	15.4±1.4[b]	177.9±3.6[b]
nFeIFN	14.4±0.7[b]	181.1±2.9[b]
nCaIFN+Extract	18.2±0.3[c]	192.3±4.9[c]
nFeIFN+Extract	20.2±2.4[d]	193.5±1.7[c]

Different letter in the same column indicated significantly different ($p<0.05$)

Based on the results of microscopic observations, it was confirmed three types of tumors, namely fibrosarcoma, squamous cell carcinoma, and anaplastic carcinoma. The fibrosarcoma was a microscopically found as a solid, soft mass with necrosis, ulceration, or bleeding resulting from tumor malignancy (Sofyan *et al.,* 2020). In this study, the skin was overgrown with fibrosarcoma tumors and showed alopecia, ulcers, and tumors in the form of solid masses with a reddish color. Squamous cell carcinoma is macroscopically a reddish and in forms of a prominent mass with a scab on its surface (Kopecki *et al.,* 2015). In this study, the mouse skin with squamous cell carcinoma also showed alopecia, a reddish color, and scabs on the surface of the tumor. Macroscopically, anaplastic carcinoma is a solid mass that is large and the surface is necrotic and expands rapidly (Kuhn *et al.,* 2019). Histologically, anaplastic carcinoma looked biphasic, composed of malignant and mesenchymal epithelium, and had a fast-growing character and low prognostic rate. Fibrosarcoma is a malignant tumor composed of pathologically altered fibroblast spindles with a very high development rate (Ishida *et al.,* 2019). Squamous cell carcinoma was composed of squamous epithelial cells and keratin pearls.

The results of the delta measurement of the tumor diameter showed different values among the groups. In the extract group (II), the results were not significantly different from the control, but there was a decrease in the tumor diameter. Administration of the leaf-ethanolic extract of *T. flagelliforme* can inhibit tumor development. This extract contains flavonoids, alkaloids, saponins, steroids, terpenoids, and glycosides (Sianipar *et al.,* 2016).Terpenoids and flavonoids can inhibit tumor growth and reduce tumor volume (Chodidjah *et al.,* 2017). Using interferon can decrease tumor cell growth by inducing cell cycle arrest, apoptosis, and necroptosis (Ni & Lu, 2018).

The results of the measurement showed that the mice in group I were experiencing weight loss week by week. Weight loss in mice is due to metabolic changes and lipogenesis due to tumors (Yang *et al.,* 2019). Flavonoids in the leaf-ethanolic extract of *T. flagelliforme* can increase the body weight of tumor sufferers (Feng *et al.,* 2014). Flavonoids can directly capture and prevent the regeneration of Reactive Oxygen Species (ROS) and indirectly increase the antioxidant activity of cellular antioxidant enzymes. The formation prevention of ROS by flavonoids occurs in several ways, namely by inhibiting the work of xanthine oxidase and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase enzymes and preventing redox reactions that can produce free radicals (Hardiningtyas *et al.,* 2014). Flavonoids can reduce lipid peroxidation due to ROS (Henneberg *et al.,* 2013). The use of interferon in the therapy did not significantly affect the changes in mice body weight (Hamana *et al*., 2016).

Based on mitotic and angiogenesis indices, the leaf-ethanolic extract of *T. flagelliforme* combined with interferon gave better results than the single administration. Mitosis is cell reproduction that produces new and genetically identical cells. The

calculation of the mitotic index can be used as a basis for determining tumor size, proliferation rate, and aggressiveness (Augsburger *et al.,* 2017). After the fractionation and isolation processes, it can be identified that the flavonoid contained in *T. flagelliforme* leaf-extract was 6C-glucosyl apigenin, which is also known as isovitexin (Sohail *et al.,* 2021).

Isovitexin from *T. flagelliforme* leaf extract can increase apoptosis. The apoptotic process increases because isovitexin increases levels of the miR-34a and Bax proteins and decreases the Bcl-2 and Mcl-1 proteins (Farida *et al*., 2012). The leaf extract of *T. flagelliforme* can also reduce COX-2 production, decrease COX-2, and increase the occurrence of apoptosis (Xu *et al.,* 2020). Interferon (IFN) can suppress the production of K562 cells, which can induce apoptosis by increasing the expression of Fas and FasL proteins (Setiawati *et al.,* 2016). Interferon can decrease tumor cell growth through cell cycle arrest in the G1 phase via the miR-29a/b pathway (Xis *et al.,* 2017). The combination of *T. flagelliforme* leaf extract and IFN in this study showed a better reduction in the rate of mitosis than the single administration.

Angiogenesis is the process of forming new blood vessels. This process is essential to bring nutrients and oxygen to the tumor area. Angiogenesis is also needed in the process of tumor development and metastasis (Ni and Lu, 2018). Isovitexin in *T. flagelliforme* leaf extract can inhibit HIF-1α so that it can reduce the gene encoding hypoxia that subsequently encodes transformation of the growth factor beta 1 (TGF-α1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGFα), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase 1, collagen III, and aldolase A that leads to a decreased metastatic potential and angiogenesis (Sandriya *et al.,* 2021).

Administration of interferon can inhibit the process of angiogenesis. Interferon can stimulate antitumor pathways through the sorafenib signalling (multi-kinase inhibitor) pathway, which also has angiogenic effects (Rajabi & Mousa, 2017). The use of IFN-α in tumor therapy can inhibit endothelial cell development by inhibiting the activity of angiogenic substances such as bFGF, IL-8, and MMP-9 (Enomoto *et al.,* 2017). Interferon-inducible protein 27 (IFI27) also exhibits anti-angiogenic activity by decreasing the secretion of vascular endothelial growth factor (VEGF-A), which then down-regulates AP-1 and AP-2 (c-jun and c-fos) (Chiang *et al.,* 2019). The use of nCaIFN and nFeIFN has antiangiogenic effects, similar to other interferons. In this study, the combined use of IFN with *T. flagelliforme* leaf extract could reduce the occurrence of angiogenesis better than the single administration.

Proliferating cell nuclear antigen (PCNA) is one of the proteins required for tumor development. The isovitexin content of *T. flagelliforme* leaf extract can reduce apoptosis and decrease PCNA expression (Liang *et al.,* 2019). The decrease in PCNA expression is due to the improvement in the expression of the p53 gene, which is a transcription factor for p21 expression. Increased p21 expression can inhibit DNA synthesis by inactivating PCNA (Ganesan &d Xu, 2017). The use of IFN as a therapeutic agent can inhibit PCNA expression. However, the use of IFNγ in several cases of oral squamous cell carcinoma inhibited PCNA expression (Gkouveris *et al*., 2018). Increased levels of IFNβ in tumor tissue can decrease PCNA expression (Bakrania *et al.,* 2017). This study found that the combination of *T. flagelliforme* leaf extract and interferon gave better results than the single administration.

Caspases belong to the cysteine proteases group that play an essential role in the proliferation and inflammation processes. Caspase-3 is the primary regulator for the occurrence of apoptosis, which it can cut functional proteins in cells that often cause apoptosis (Zhou *et al.,* 2018).

Isovitexin in *T. flagelliforme* leaf extract can significantly increase Bax and caspase-3 levels (Zhu *et al.,* 2021). The content of linoleic acid in *T. flagelliforme* can binds to the peroxisome proliferator-activated receptor (PPARγ) and will activate caspase-9 and caspase-3 (Putra *et al.,* 2020). The IFNγ can induce and activate caspase-8, which will stimulate the formation of caspase-3, where this signalling begins with the activation of the Fas regulator (Jimbo *et al.,* 2020). IFNβ155 in melanoma can increase caspase-3 activity up to ten times higher than without it (Borden 2019). The combination therapy of *T. flagelliforme* with IFN gave higher caspase-3 values than the single administration.

CONCLUSION

The combination of *T. Flagelliforme* leaf-ethanolic extract and natural interferon can maintain the mice body weight stability, inhibit tumor growth, inhibit mitosis, inhibit angiogenesis, reduce PCNA expression, and increase caspase-3 expression. This combination treatment gave better results than the single administration.

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