Effects of Papaya Leaf Extract (Carica papaya L.) on Cellular Proliferation

by Yuly Peristiowati

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Effects of Papaya Leaf Extract (*Carica papaya* L.) on Cellular Proliferation and Apoptosis in Cervical Cancer Mice Model

Effets de l'extrait de feuilles de papaye (*Carica papaya* L.) sur la prolifération et l'apoptose cellulaires chez le modèle de souris atteintes d'un cancer du col utérin

Y. Peristiowati · Y. Puspitasari · Indasah

Abstract This study is aimed at analyzing the anticancer properties of papaya leaf extract, specifically the inhibition of cell proliferation and apoptotic induction through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and p53 pathways. Twenty-five mice (Mus musculus), aged 2 months and weighing 20–30 g, was injected with 0.5 mg dexamethasone for 7 days. The mice were then injected intracutaneously with 1 ml of HeLa cells (8×10^6 HeLa cells/microliter). The mice were divided into five groups (5 each): negative control (P1) (5% CMC-Na, sodium carboxymethyl cellulose), treatment II (225 mg/kg BW (body weight) papaya leaves methanol extract), treatment III (450 mg/kg BW), treatment IV (750 mg/kg BW), and treatment PV (2 mg alcohol anticancer drug). Papaya leaf extract treatments were applied for 2 weeks. Then, the tumor tissue was isolated for hematoxylin and eosin staining. Immunohistochemical imaging was used to detect Ki-67, caspase-3, NF-κB, and p53 expression. Further analysis was undertaken using the ImmunoRatio software program. The results indicated that administration of papaya leaf methanol extract significantly increased the expression of NF-κB and p53 at a dose of 450 mg/kg BW. Our results also showed that the mice treated with 450 mg of papaya leaf extract per kg of BW (P3) had the largest increase of caspase-3 expression compared to the negative control group. Papaya leaf ethanol extract decreased the cancer cell proliferation index and increased apoptosis of cancer cells in animal models of cervical cancer; it may also work to increase NF-kB expression and expression of the p53 gene.

Keywords Cervical cancer · *Carica papaya* L. · NF-κB · *p53*

Résumé Cette étude visait à analyser les propriétés anticancéreuses de l'extrait de feuilles de papaye, en particulier l'inhibition de la prolifération cellulaire et l'induction de l'apoptose par le facteur nucléaire amplificateur de chaînes légères kappa des lymphocytes B activés (NF-kB) et des voies p53. Vingt-cinq souris (Mus musculus), âgées de deux mois, pesant 20 à 30 g, ont reçu une injection de 0,5 mg de dexaméthasone pendant sept jours. Les souris ont reçu une injection intracutanée de 1 ml de cellules HeLa (8×10^6 cellules HeLa/microlitre). Les souris ont été réparties en cinq groupes (chacun comprenant cinq souris), comme suit : témoin négatif (P1) [CMC-Na à 5 %], traitement II (225 mg/kg de poids corporel d'extrait de méthanol de feuilles de papaye), traitement III (450 mg/kg de poids corporel), traitement IV (750 mg/kg de poids corporel) et traitement PV (2 mg de médicament anticancer à base d'alcool). Les traitements à base d'extrait de feuilles de papaye ont été administrés pendant deux semaines. Ensuite, le tissu tumoral a été isolé en vue de sa coloration à l'hématoxyline et à l'éosine. Un test immunohistochimique a été utilisé pour détecter l'expression de l'antigène Ki-67, de la caspase-3, du facteur transcriptionnel NF-kB et du gène p53. Une analyse plus poussée a été réalisée à l'aide du logiciel ImmunoRatio. Les résultats ont indiqué que l'administration d'extrait de méthanol de feuilles de papaye stimulait considérablement l'expression du facteur transcriptionnel NF-kB et du gène p53 à une dose de 450 mg/kg de poids corporel. Nos résultats ont également montré que les souris traitées avec 450 mg d'extrait de

Y. Peristiowati (*) · Y. Puspitasari
Nursery Program Study, Surya Mitra Husada Institute of Health Science, Kediri, East Java, Indonesia. e-mail: yulystikes@gmail.com

Indasah
Health Science Study Program, Surya Mitra Husada Institute of Health Science, Kediri, East Java, Indonesia

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feuilles de papaye par kilogramme de poids corporel (P3) présentaient la plus forte augmentation de l'expression de la caspase-3 par rapport au groupe témoin négatif. L'extrait d'éthanol de feuilles de papaye a entraîné une diminution du taux de prolifération des cellules cancéreuses ainsi qu'une augmentation de l'apoptose des cellules cancéreuses chez des animaux de laboratoire atteints d'un cancer du col utérin. Il pourrait également contribuer à augmenter l'expression du facteur NF-kB et du gène p53.

Mots clés Cancer du col utérin · *Carica papaya* L. · Facteur transcriptionnel NF-κB · gène *p53*

Introduction

Cancer is the second leading cause of death in human beings after heart disease. Cancer leads to the death of between 100 and 350 per 100,000 people worldwide each year [1,2]. Cervical cancer is primary cancer that originates in the cervix (cervical canal and/or porsio [2]. In Indonesia, it is reported that the number of new cervical cancer diagnoses is 100 per 100,000 people per year. There are 180,000 new cases in women aged 45–54 years; cervical cancer is ranked among the top 10 most frequently diagnosed cancers in women [3,4]. Tumor growth may occur due to uncontrolled cell proliferation and reduced apoptosis. The impaired balance between apoptosis and cell proliferation is an important factor for the development and progression of the tumor, specifically, and of cancer more generally. Assessment of apoptosis and proliferation is important for the evaluation of tumor growth or reduction [5–7].

Carica papaya L., or papaya, is one of the many plants known to have anticancer properties. It acts by increasing apoptosis and inhibiting cell proliferation. Papaya leaf methanol extract has inhibitory activities against the DNA enzyme topoisomerase II, an enzyme that plays an important role in the process of DNA replication, transcription, recombination, and cell proliferation. The inhibition of enzyme activity causes a bond between the enzyme and substrate; this bond forms a protein-linked DNA brakes (PLDB) that leads to apoptotic cell death. This study is aimed at observing the potential of papaya leaf extract to act as an anticancer agent by inhibiting cell proliferation and by enhancing apoptotic induction [8,9,10].

Materials and methods

Experimental design

This experimental study was performed in a laboratory using a post-test controlled group design. The study used 24 female C3H mice that were aged 2 months and that had a body weight (BW) of 20–30 g. The mice were obtained from the Integrated Research Center (LPPT) at Gadjah Mada University, Yogyakarta, Indonesia. This research has passed the test of ethical innovation by the Research Ethics Commission of the Faculty of Veterinary Medicine at Airlangga University (Number: 408-KE).

Preparation of papaya leaf chloroform fraction

The papaya leaf test material (Carica papaya L.) was obtained from the Indonesian Science Center (LIPI UPT), Conservation Center Plant of Purwodadi Botanical Garden Pasuruan East Java. Papaya leaves were extracted in the Laboratory of Nutrition and Drugs, Faculty of Pharmacy, Airlangga University.

For this study, 350 g of papaya leaf powder (*Carica papaya* L.) was macerated with hexane solvent to remove the fat content (defatted). Maceration was done until the extract had a clear color. The residue was macerated with hexane and further re-macerated with a combination of acidic methanol (pH: 3) and 1% tartrate; the maceration was repeated until the extract had a clear color. The pH of the methanolic extract was increased from 3 to 9 using NH_4OH 5%. The aim of this process was to hydrolyze the alkaloids from the salt form into the base form so that it could be degraded by organic solvents like chloroform. The chloroform fraction that was obtained was then evaporated with a rotavapor to obtain a chloroform fraction [8,9].

Animal models of cervical cancer

HeLa cells were implanted into mice whose immune system had been suppressed by an injection of 0.5 mg of dexamethasone per kg of body weight for 7 days. The mice received an intracutaneous injection of HeLa cells (8×10^6 cells/ microlite). They were observed for the formation of nodules at the injection site. Mice were divided into five groups (5 mice each): negative control (P1) with treatment of 5% sodium carboxymethyl cellulose (CMC-Na), treatment II (dose of 22[illegible] g/kg BW papaya leaves methanol extract), treatment III (dose of 450 mg/kg BW), treatment IV (dose of 750 mg/kg BW), and treatment PV (2 mg alcohol anticancer drug).

After all of the groups were inoculated with cancerous tumor cells and nodules (1–2 weeks) were formed at the injection site, the following treatments were given: K(−), standard diet without papaya leaf extract; K(+), standard diet with anticancer drugs; P1, standard diet and 225 mg of papaya leaf extract per kg of body weight; P2, standard diet and 450 mg of papaya leaf extract per kg of body weight; P3 standard diet and 750 mg of papaya leaf extract per kg of body weight. All animals were treated for 2 weeks. After the end of the treatment period, the mice were sacrificed with ether anesthesia and the tumor tissue was removed. The tumor tissue was processed into paraffin blocks and then prepared for histopathologic examination using hematoxylin and eosin staining. Further, Ki-67 immunohistochemical imaging was performed to detect cellular proliferation, and caspase-3 was examined to detect apoptosis [11].

Preparation of tumor tissue in paraffin blocks

Paraffin blocks containing cancer biopsy tissue were cut into 4 µm slices using a microtome and then dehydrated with xylol. It rehydrated with various concentrations of ethanol (from high to low, respectively), followed by phosphate buffer saline (PBS) 3 × 5 min. The tissue preparation was incubated in the DAKOR buffer antigen retrieval solution at 98 °C for 20 min and then chilled for 20 min. The tissue was washed three times with PBS for a period of 5 min per wash; it was then incubated in a peroxidase block (NovocastraR). The preparations were re-washed three times with PBS for a period of 5 min per wash; they were then incubated overnight with MIB-1 antibody 19 °C. The next step was to wash the tissue samples again three times with PBS for a period of 5 min for each wash. The tissue was again incubated with a second antibody of 61 volink horseradish peroxidase (HRP) (Novocastra R) for 60 min at room temperature. After each wash, it was stained with hematoxylin (NovocastraR). The preparations were dehydrated using various concentrations of ethanol (low to high, respectively), followed by xylol clearance. The final step was to mount the tissue samples [12,13].

Immunohistochemical response of caspase-3 and KI-67

Microscopic preparation of tumor tissue included depopulating the tissue with xylol, rehydrating it with various concentrations of 19 anol (from high to low, respectively), and washing it three times in PBS for a period of 5 min per time. The tissue preparation was then incubated in the DAKOR buffer retriev 34 ntigen at 98 °C temperature for 10 min and chilled for 20 min at room temperature. After washing three times with a combination of PBS and Tween for a period of 3 min per wash, the tissue preparation was incubated on Quench peroxidase cell signaling R and rewashed two times with a combination of PBS and Tween for 3 min per wash.

The tissue was inc 42 ted in a blocking solution for 60 min, followed by the primary antibody (apoptosis) at 4 °C overnight. The tissue was then washed 57 e times with a combination of PBS and Tween 52 5 min per wash, incubated with a secondary antibody for 30 min, re-washed three times with a combination of PBS and Tween for 5 min per wash, and washed with diaminobenzidine (DAB) chromogen for 2–10 min. The tissues were stained using hematoxylin and eosin staining, dehydrated with ethanol, purified with xylol, and mounted [13,14].

Apoptotic staining was performed using SignalStain cleaved caspase-3 (Asp175; an apoptosis marker) immunohistochem 9 try detection kit. KI-67 staining was performed using rabbit anti-KI-67 (a marker of cellular proliferation) polyclonal antibody (100 Ul-Bioss). The caspase-3 apoptosis index, which is a positive percentage of cancerous tissue, was evaluated randon 33 to avoid biased results. The KI-67 proliferation antigen was identified as a nuclear antigen associated with cell proliferation. Detailed cell cycle analysis of Kl-67 has shown that the antigen was present in the cell nucleus at all phases, including in the mitotic phase, while the resting phase (G0) did not express KI-67 [13,14].

Microscopic observations were performed on three randomly selected fields that contained at least 1,000 cells (using M = 400). To minimize the variation, the data variation value used was not m 28 than 5%. Using the caspase-3 apoptotic index, results above 0.02 was classified as high apoptosis and levels below 0.02 were classified as low apoptosis (18). Microscopy results were also calculated using the apoptosis index to detect the presence of brown color with the ImmunoRatio online program [13,14].

Immunohistochemical reactions of NF-κB

The 24 paraffin blocks were collected; hematoxyline and eosin staining and immu 31 istochemistry staining were performed. Expression of nuclear factor kappalight-chain-enhancer of a 8 ivated B cells (NF-κB) gave positive results based on the brown color of the cytoplasm and the nucleus of the tumor cells [13]. The immunoactivity of the cell was calculated using ImmunoRatio software pro 16 m. Immunohistochemical staining of NF-κB using rabbit polyclonal antibody to NF-κB p65-CHIP Grade (ab7970) (abcam), each with a manual procedure was followed by scanning using an InteliPATH FLX automated slide staining

machine. The collected paraffin blocks were cut into 4 µm slices using a microtome and then placed onto coated glass surfaces. The paraffin cuts were stained using an avidin–biotin method of immunohistochemical staining.

The paraffin cuts were depolarized three times with xylol (5 min per time); rehydrated three times with ethanol for 5 min; rehydrated with 90%, 80%, and then 70% alcohol for 5 min at each concentration; rinsed with running water for 5 min; and soaked in 3% hydrogen peroxidase solution for 5 min. The cuts were rinsed with distilled water, and then an unmasking retrieval antigen was applied two times with boiling citrate for 5 min per time. Next, the cuts were chilled at room temperature for 15 min, incubated with 0.2% O_2H_2 in methanol for 10 min, rinsed with PBS, dropped with serum blocking 1.5%, and then incubated for 5[illegible] min. A primary rabbit polyclonal antibody called anti-NF-κB p65 antibody-ChIP Grade (ab 7970; abcam) was applied at a dilution of 1:200; the cuts were then incubated for 60 min. They were then rinsed three times with PBS (5 min per time), dripped with a secondary antibody called TrekAvidin universal link (Biocare Medical), and incubated at room temperature for 20 min. Chromogen was then added to the samples, and the samples incubated for 5–10 min. After rinsing with running water for 5 min, the samples were counterstained with Mayer's hematoxylin for 2 min and then washed with running water. After dehydration with alcohol at concentrations of 70%, 80%, and 90% and ethanol for 3 min, the samples were incubated in xylol and then mounted. Finally, NF-κB immunoexpression was assessed by categorizing the percentage of brown gallbladder mucosal cells with varying intensity in the nucleus.

Immunohistochemistry of p53

The presence of p53 was measured using immunohistochemical techniques. The tissue was cut into 4 µm slices and attached to a coated glass object by poly-L-lysine. The samples were incubated in an oven overnight at 37 °C; deparaffinated with xylen[illegible]ree times (for 3 min each time); and rehydrated using 100% ethanol for 2 min, 95% ethanol for 2 min, 70% ethanol for [illegible] min, and water for 1 min. The tissue was soaked in peroxidase blocking solution at room temperature for 10 min. It was incubated in a prediluted blocking serum at 25 °C for 10 min and then soaked in the anti-p53 monoclonal antibody at 25 °C for 10 min. It was washed with PBS for 5 min, incubated with secondary antibody (conjugated to horseradish peroxidase) at 25 °C for 10 min, washed with PBS for 5 min, incubated with peroxidase at 25 °C for 10 min, and then washed with PBS for 5 min. After that, it was incubated with DAB chromogen at 25 °C for 10 min, incubated in hematoxyline and eosin for 3 min, and washed with running water. The tissue preparations were cleaned with mounting media and placed on the cover glass.

After immunohistochemical treatment with p53 or pH monoclonal antibody (Santa Cruz), the preparations were interpreted on the basis of three conditions:

- the interpreter of p53 was blinded to the clinical and pathological data of each case;
- the calculation of p53 expression was a semi-quantitative evaluation. The calculation of the percentage of malignant cells was recorded as positive for 200 cells using an online software called ImmunoRatio;
- staining was only considered positive if the cell membrane appeared to be brown.

The expression percentages are presented based on the results of the ImmunoRatio analysis.

Results

An analysis of variance (Anova) test examined the treatment using a significant value $P < 0.05$ means to observe the significant differences between the experimental treatments (P1, P2, P3, P4, P5) that affected caspase-3, KI-67, NF-κB, and p53. Those treatments influenced caspase-3, KI-67, NFκB, and p53 as much as 93.8%, 96.2%, 83%, and 99.5%, respectively.

The following results were obtained from the Dami regression test.

Dependent variable: caspase-3

The treatment of P2, P3, P4, and P5 had a significant value < 0.05. The greatest influence (from P1 to P5) was by P5 because the largest p-value of treatment was obtained with P5 using the anticancer drug, while the treatment with papaya leaf extracts was the most influential for the P3 treatment with a dose 450 mg/kg of body weight (Figs 1, 2).

Dependent variable: KI-67

The treatment of P2, P3, P4, and P5 had a significant value < 0.05. The greatest influence (from P1 to P5) was by P5 because the largest p-value was obtained using the Alkiri anticancer drug, while treatment with the papaya extract that was most influential was the P3 treatment with a dose of 450 mg/kg of body weight (Figs 3, 4).

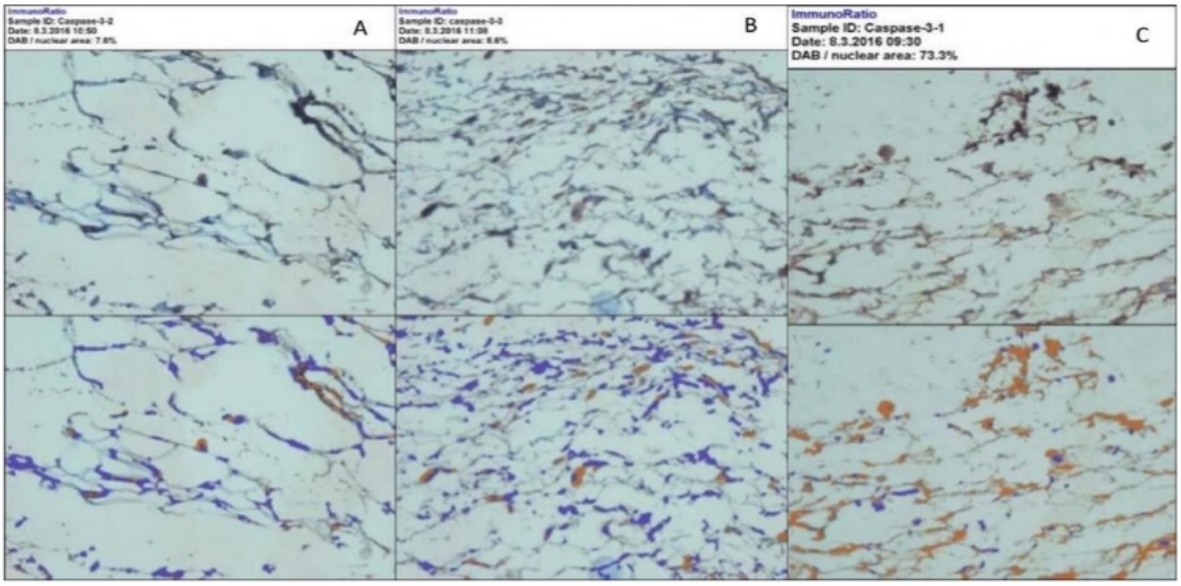

Fig. 1 Immunohistochemical results of cancer cell apoptosis using the ImmunoRatio analysis program to examine e caspase-3 antibody, shown by the presence of a brownish color, in the following treatment groups: CMC-Na 0.5% (Fig. 1A), 2 mg/kg of alkeran (an anticancer drug) with apoptotic index 8.5% (Fig. 1B), and 450 mg papaya leaf extract per kg of body weight with apoptosis index of 73.3% (Fig. 1C)

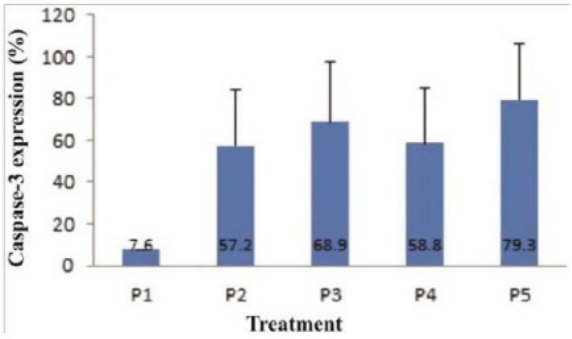

Fig. 2 Based on the immunohistochemical immunization results of the ImmunoRatio analysis; the treatment with 450 mg of papaya leaf extract per kg of body weight per day (P3) in mice with cervical cancer showed the highest increase of caspase-3 expression (cell apoptosis), with a mean value of 68.9% in the cancer tissue of the negative control group that was treated with CMC-Na 5% (P1). However, P3 had a lower result than treatment (P5), wherein the rats are treated with an anticancer drug (alkarene) at a dose of 2 mg

Dependent variable: NF-κB

The treatment of P2, P3, P4, and P5 had a significant value < 0.05. The greatest influence (from P1 to P3) was by P3 because of the largest B-value with the treatment using papaya leaf extract at a dose of 450 mg/kg of body weight (Figs 5, 6).

Dependent variable: p53

The treatment of P2, P3, P4, and P5 had a significant value < 0.05. The greatest influence (from P1 to P3) was by P3 because of the largest B-value with the treatment using papaya leaf extract at a dose of 450 mg/kg of body weight (Figs 7, 8).

Discussion

Identifying the expression of apoptosis of cancer cells with caspase-3 antibodies

From the results of the immunohistochemical examination using ImmunoRatio analysis, the treatment group of 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the highest increase of caspase-3 expression (cell apoptosis), with a mean of 68.9% in cancer tissue compared to the negative control group (P1) in mice with cervical cancer. However, the P3 level was lower than with the treatment (P5) using 2 mg of the anticancer drug alkarene. In the Sheridan study results, the apoptotic index in cervical

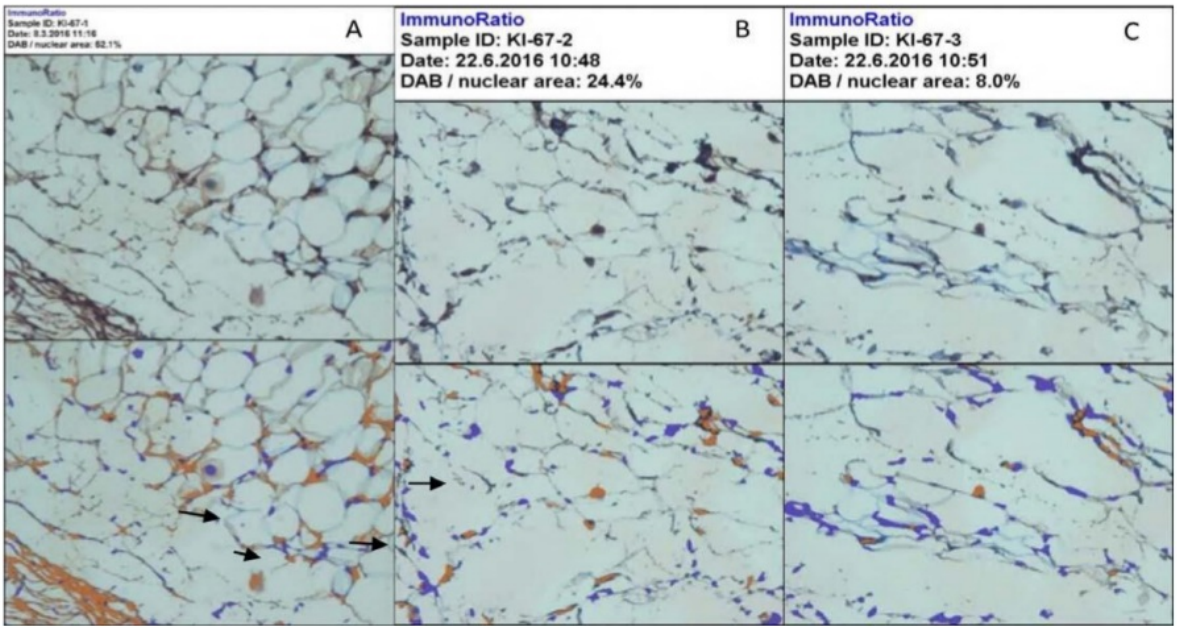

Fig. 3 Immunohistochemical results of cancer cell proliferation using the ImmunoRatio analysis program to examine KI-67 antibody, shown by the presence of a brownish color, in the following treatment groups: CMC-Na 0.5% with a proliferation activity of 24.4% (Fig. 3A), 2 mg of alkarene with a proliferation activity of 57.4% (Fig. 3B), and 450 mg of papaya leaf extract per kg of body weight with a proliferation activity of 8.0% (Fig. 3C)

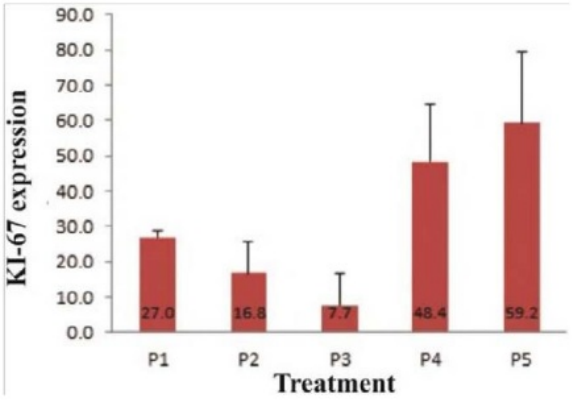

Fig. 4 Based on the immunohistochemistry results from the ImmunoRatio analysis; the group of mice with cervical cancer treated with 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the lowest yield of expression of KI-67 (cell proliferation) with a mean of 7.7% in the cancer tissue of the negative control group that was treated with CMC-Na 5% (P1)

cancer was observed by the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) method and ranged from 0.01 to 0.08. Apoptosis detected by caspase-3 was associated with cell death and tumor cell repopulation. Cancer cell apoptosis, detected by caspase-3, occurred as a result of mitotic failure; this observation was made in vitro using HeLa cells. Papaya leaf extract can serve as a pro-apoptosis that could cause an increase in the expression of apoptosis in cervical cancer cells. In the apoptotic process, either at the embryogenic or pathological stages, a peptide from a protein cysteine protease group called caspase has an important function. During apoptosis, the group plays a role in the inflammatory process. Caspase-3 is the most important caspase group in the apoptotic process and, in this study, was observed as a brown color in the nucleus [15,16].

Identifying the inhibition of proliferation of cancer cells with KI-67 antibodies

From the results of the immunohistochemical examination using ImmunoRatio analysis, the group that used 450 mg of papaya leaf extract dose per kg of body weight per day (P3) to treat mice with cervical cancer showed the lowest decrease in the expression of KI-67 (cell proliferation), with a mean of 7.7% in cancer tissues, compared to the negative control group (that is, the group treated with CMC-Na 5%; P1).

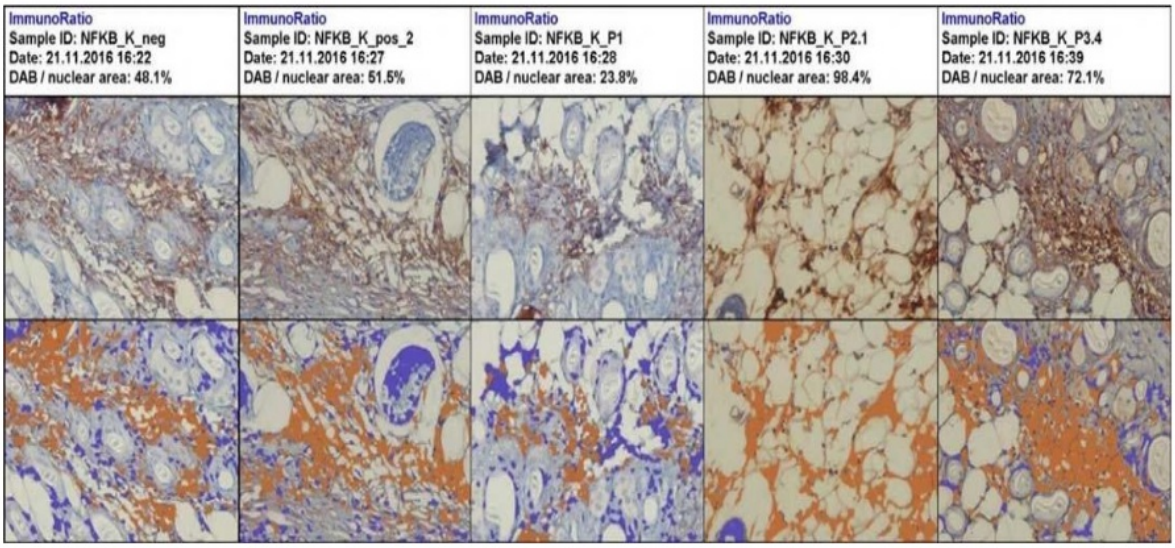

Fig. 5 Immunohistochemical results using the ImmunoRatio analysis program to examine the expression of NF-κB expression, shown by the presence of a brownish color, in the following treatment groups: CMC-Na 0.5% with NF-κB expression of 48.1% (Fig. 3A), 2 mg of alkaline with NF-κB expression of 51.5% (Fig. 3B), 225 mg of papaya leaf extract per kg per kg of body weight per day with 23.8% NF-κB expression (Fig. 3C), 450 mg of papaya leaf extract per kg of body weight per day with 98.4% NF-κB expression, and 750 mg of papaya leaf extract per kg of body weight per day with 72.1% NF-κB expression

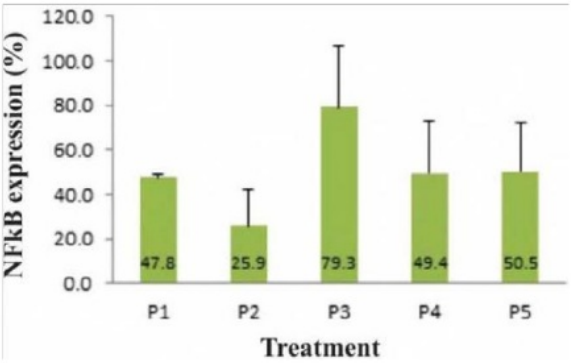

Fig. 6 Based on the immunohistochemical immunization results using the ImmunoRatio analysis, the treatment using 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the highest increase of NF-κB expression, with a mean of 79.3% in cancer tissue, compared to the negative control group that was treated with CMC-Na 5% (P1) and compared to the group that was treated with 2 mg of the anticancer drug alkaren (P5)

KI-67 proliferation antigens were identified as nuclear antigens associated with cell proliferation. Detailed cell cycle analyses of KI-67 have shown that the antigen is present in the cell nucleus at all phases, including the mitotic phase; while the resting phase (G0) does not express KI67. Although the structure and properties of this protein are understood, its functional role is still not fully known [17].

The KI-67, BRCA, and p53 genes are three protooncogene and tumor suppressor genes that normally function to control cell growth; however, if mutations occur in these three genes, uncontrolled cell growth and hyperproliferation occurs. The p53 gene is a tumor suppressor gene and an effector phase checkpoint molecule (at phases G1, G2, S and M which denote the growth phases 1 and 2, the synthetic phase, and the mitotic phase respectively) that acts by inducing the p21 cell cycle inhibitor, which causes cessation of the cell cycle. Mutations in the p53 gene result in uncontrollable cell proliferation. BRCA is also a tumor suppressor gene that plays an important role in repair of double-chain DNA damage; a reduction in BRCA mutations results in gene instability, which causes tumor cell growth [18].

KI-67 is a non-histone core protein that has two isoforms with molecular weights of 359kD and 320kD. This gene is located on the 11q25 chromosome, and is found in the nucleolus cortex and in the solid fibrin component in the nucleolus during the interphase of the cell cycle. The half-life of KI-67 ranges from 1 to 1.5 hours. Healthy breast tissue expresses KI-67 at low levels (< 3%) [19,20].

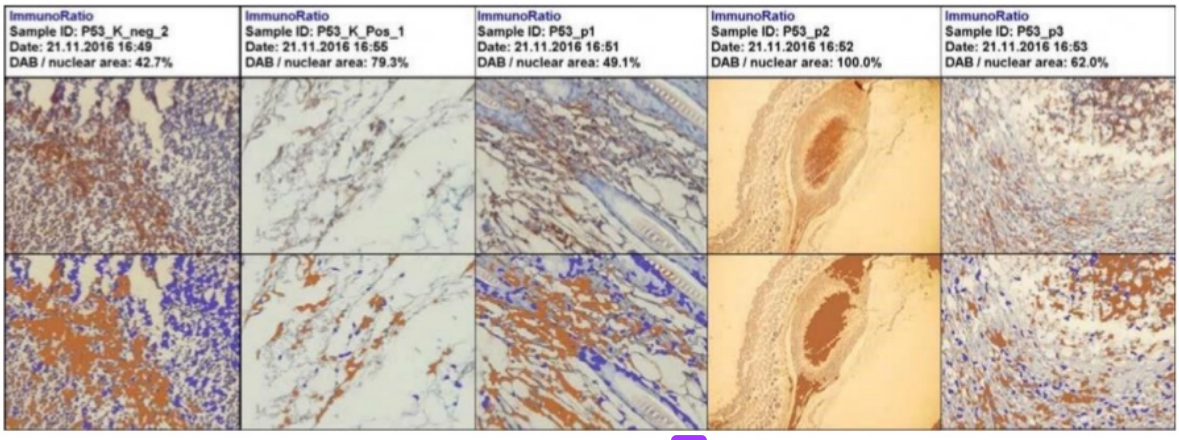

Fig. 7 Immunohistochemical results using the ImmunoRatio analysis program to examine the expression of p53 in cancer cells, shown by the presence of a brownish color, in the following treatment groups: CMC-Na 5% with 42.7% NF-κB expression (Fig. 7A), 2 mg of alkaline with 79.3% NF-κB expression (Fig. 7B), 225 mg of papaya leaf extract per kg of body weight per day with 49.1% NF-κB expression (Fig. 7C), 450 mg of papaya leaf extract per kg of body weight per day with 100% NF-κB expression, and 750 mg of papaya leaf extract per kg of body weight per day with 62.0% NF-κB expression

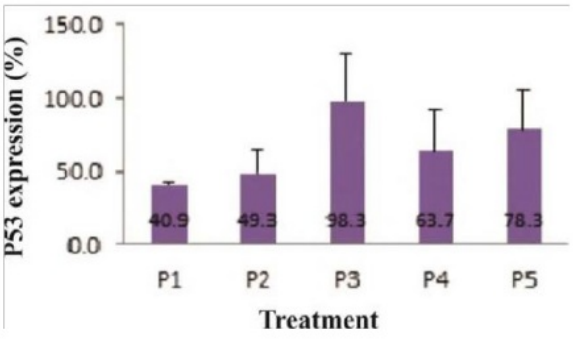

Fig. 8 Based on the immunohistochemistry results using ImmunoRatio analysis, the treatment of 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the highest result of increase in p53 expression, with a mean of 98.3% in cancer tissue compared to the negative control group that was given CMCNa 5% (P1) and compared with the group that was treated with 2 mg of the anticancer drug alkaren (P5)

The expression of KI-67 varies according to the cell cycle phase. Cells express this antigen during the growth phase 1 (G1), the synthetic phase (S), growth phase 2 (G2), and the mitotic phase (M); but it is not expressed during the resting phase (G0). The levels of KI-67 in the G1 and S phases begin to rise until they reach the highest level during the mitotic phase; in the anaphase and telophase phases there is a sharp decrease in expression. This protein has an important role in the process of cell division, but until now its exact function is unknown [17].

KI-67 is a biological marker that can reflect the state of cell proliferation. Data show that KI-67 is a prognostic factor in carcinoma mammae [17,18]. KI-67 is a protein in the dividing tissue; it plays an important role as a marker of cell division. Increased levels are followed by lesions with high grading and microinvasion; therefore, it is not surprising that KI-67 is a predictor of recurrence of in situ ductal carcinoma (DCIS) [19].

Cell proliferation is the division and growth of cells, whose mechanisms and arrangements are based on cell cycles. Cell proliferation activity can also be detected by immunohistochemical KI-67. KI-67 is expressed in cells that proliferate during G1, S, G2, and M phases, but not in the G0 phase of the cell cycle [17].

Cells normally undergo mitotic cleavage during the cell cycle; mitotic cleavage functions to produce new cells that are useful for regeneration and to repair damage. The cell cycle is regulated by the DNA sequence in each cell. Cells have genes called proto-oncogene (such as the KI-67 gene) that regulate cell proliferation; they also have genes that serve to regulate the cessation or inhibition of cell proliferation called gene suppressors (such as p53). These genes act as controls; when these genes are mutated, the associated proteins are not well formed and there is cell division that should not occur [16].

Mutation of KI-67 genes in the mitotic phase leads to uncontrolled cell division, which results in the movement of cells that divide and which induces tumor cells to pass through the damaged or lysed basement membrane and into the blood circulation system (bloodstream). The tumor cells

that make up this clot will spread hematologically and will eventually enter the blood vessels where they can directly invade the veins through the vena cava and be detected in the blood [18,19,20].

KI-67 is a cell proliferation marker that is useful for determining growth fraction in tumor cells during the active phase of the cell cycle [16,21]. The expression of KI-67 in different grades is based on three groups: low-grade expression of Ki67 (< 15%), intermediate-grade expression (16–30%), and high-grade expression (> 30%) [17,22].

Identification of NF-κB expression in cancer cells with NF-κB antibody (Abcam)

From the results of the immunohistochemical examination using ImmunoRatio analysis, the group treated with 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the highest increase in the expression of NF-κB, with a mean of 79.3% in cancer tissue, compared to the negative control group that was treated with CMCNa 5% (P1) and compared with the group treated with 2 mg of the alkaline anticancer drug (P5).

NF-κB regulates the expression of genes involved in various processes that have a role in the development and progression of cancers, such as cellular proliferation, migration, and apoptosis. NF-κB is not a single gene, but a family of genes that are related to transcription factors, including NFκB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rail, and RelB [14,15]. NF-κB is distributed in all activated cells. It regulates the expression of various target genes that promote cell proliferation, regimens, and inflammatory responses, and it contributes to the pathogenesis of various diseases, including malignancy. The NF-κB signaling pathway is regulated by the kappa B inhibitor (IkB) family. NFκB is widely present in the cytoplasm. It binds to IKB, which holds NF-κB in the organoplasm. Activation of the NF-κB pathway is one of the key survival mechanisms for different types of malignancies [14,23,24].

The NF-κB protein family is an important family of transcription factors for the regulation of gene expression associated with biological functions, such as immune and inflammatory responses, cell growth and proliferation and cell defense against stress (for example, ultraviolet rays, irradiation, oxidants, and DNA damage). NF-κB is a dimer that can be either a heterodimer or homodimer; it can be formed between five types of proteins, namely p50, p52, p65 (RelA), Rel-B, and c-rails [15,25].

As we know, NF-κB may play a role in cell cycle activation for cell proliferation and in the inhibition of apoptosis. NF-κB may also play a role in cell cycle inhibition. However, the core of this IKK/NF-κB pathway activation is that it remains the same for "cell survival". The activity of IKK/NF-κB that inhibits the cell cycle is also related to the cell defense system (that is, the system needed to survive in response to stress, which in this case is primarily DNA damage) [17]. Cells with high proliferative activity, such as fibroblast cells and cancer cells, will always enter the cell cycle. Exposure to a DNA-damaging compound can induce cells to activate NF-κB for the cessation of the cell cycle. This is one of the emergency responses of the cell to sustain its lifespan. NF-κB has physiological functions as a transcription factor [14,26].

The death of myeloma cancer cells is due to the effect of the papaya leaf chloroform fraction (*Carica papaya* L.). The main content alkaloids is suspected to act by inhibiting DNA enzyme topoisomerase II. With the inhibition of DNA enzyme activity topoisomerase, the binding process between enzymes and cancer cell DNA can continue for a longer period of time. Hence it leads to the formation of PLDBs, which is the result of fragmentation or damage to cancer cell DNA, and will subsequently affect the process of replication of cancer cells.

Identification of p53 expression in cancer cells with p53 antibody (Santa Cruz)

The result of the immunohistochemistry examination using ImmunoRatio analysis showed that treatment with 450 mg of papaya leaf extract per kg of body weight per day (P3) showed the highest increase of P53 expression, with a mean of 98.3% in cancer tissue, compared to the negative control group (P1) and (P5).

The p53 gene (as a tumor suppressor gene) will accumulate, stopping DNA replication at the check point and allowing DNA to repair itself. If the repair process fails, p53 will stimulate the mitochondria to release cytochrome c to the cytosol and, in this case, it will be blocked by the antiapoptic member of the Bcl-2 gene. In the cytochrome, cytosol c together with apoptosis protease activating factor1 (Apaf-1) and pro-caspase-9, form caspase-9; this complex is called apopto-some. As the initial caspace, caspase-9 will activate the executioner caspase (that is, caspase-3, 6 and 7), this can cause apoptosis [27].

Research by Rumiyati et al. [28] is in line with research by Sukardiman et al. [8] from the Faculty of Pharmacy at Airlangga University. Sismindari et al. extracted 350 g of papaya leaf powder with a chloroform solvent. The extract

was then used to incubate mouse myeloma cancer cells in an incubator at 37 °C. The following tiered doses were used: 25 µg, 50 µg, 100 µg, 150 µg, 200 µg, and 300 µg per ml. After 24 hours, the viability or survival rate of cancer cells was observed. The results showed that the viability of myeloma cells decreased with the increasing dose of papaya leaf extract. At a dose of 25 µg, the life rate of the myeloma cells reached 81.5%, whereas at a dose of 300 µg, viability fell to 4.77%. Sukardiman et al. [8] stated that the value of IC_{50} (concentration of inhibitor when response is reduced by half) of the chloroform extract of the papaya leaf was a dose of 104.4 µg/ml.

The papaya leaf has anticancer abilities because it contains ribosome inactivating protein (RIP). This protein is able to kill or suppress the development of cancer cells. RIP is a toxic protein that is capable of inhibiting protein synthesis by inactivating the ribosome, which is the site of protein synthesis; hence, proteins fail to form. When the process of protein synthesis is impaired, the development of cancer cells is also hampered [24] Sukardiman et al. [8] supported the results of the study by Duke et al. [23], which mentioned that the content of secondary metabolites in papaya leaves is alkaloid carpaine, a pseudocarpaine that belongs to a class of piperidine alkaloids. An alkaloid that belongs to piperidine has anticancer abilities. The semisynthesis product between alkaloid piperidine and alkaloid flavonoid is flavopiridol, which induces apoptosis.

Huda et al. [24] has compared the potential anticancer activity of the papaya leaf chloroform fraction and the activity of methanol extract of the papaya leaf to the cytotoxic activity in cell cultures of myeloma. Gomez and Santoz [24] mentioned that RIP protein is able to cut RNA and double strand supercoil DNA into nick circulars, which are pseudo-circles, and linear strings. This inference is in line with Rumiyati et al. [28], who mentioned that a 6 mg/ml dose of papaya leaf protein is capable of cutting supercoil DNA into a pseudo-linear circle. The ability of papaya to cut increases with the addition of protein concentration used.

In addition, the protein, which is contained in the leaf plant of the family Caricaceae, can trigger the apoptosis pathways and suppress cancer development. Rumiyati et al. [28] found that papaya leaf protein extracts can increase p53 protein expression. Remarkably, papaya leaf extract can also enhance Bcl2 protein concentration. Protein P53 regulates apoptosis in cancer cells, while Bcl2 is an antiapoptosis protein or inhibitor of the cell suicide program.

According to Sukardiman et al. [8], papaya leaf extract can inhibit the activity of topoisomerase II. Topoisomerase is an enzyme that cancer cells need to proliferate. When enzyme activity is inhibited, the cancer cell DNA will be damaged and cannot replicate. Thus, cancer cells will be unable to develop. Inhibition of topoisomerase activity II also affects the increased expression of p53 protein and triggers apoptosis. In the chloroform extract assay, the presence of apoptosis also increased with increasing concentration of extract. The highest levels of apoptosis occurred in the group that was administered a dose of 300 µg/ml, which obtained 46.86% apoptosis, while the lowest levels of apoptosis occurred in the group administered a dose of 25 µg/ml, which obtained only 3.76%.

In our research, papaya leaf ethanol extract was analyzed by high-performance liquid chromatography (HPLC) using the MS (Quasi Protanasi) method. The result of the total chromatogram ion detected in the papaya leaf extract was a carpain compound with a molecular weight (BM) of 479.000. Carpain compounds in papaya leaves are thought to have anticancer properties.

The results of mouse models with cervical cancer caused by HeLa cell insertion indicated that a dose of 450 mg of papaya leaf extract per kg of body weight can increase the expression of NF-κB, with a mean value of 79%. This was detected in cancer tissue following immunohistochemical examination with antibody NF-κB (abcam), while the expression of P53 using immunohistochemical examination with antibody P53 (Santa Cruz) showed a significant increase at a dose of 450 mg per kg of body weight.

Although it has been proven in stage-one studies that papaya leaf ethanol extract can decrease the cancer cell proliferation index and increase apoptosis of cancer cells in animal models of cervical cancer, it may also work to increase NF-κB expression and expression of the p53 gene. However, further research about the toxicity of papaya leaf ethanol extract is needed. Toxicity assays are needed to determine the maximum dose that can be used in animal models of cervical cancer. After the toxicity test, it would be necessary to conduct pre-clinical studies using placebos before papaya leaf extract can be used in a clinical setting.

Conclusion

Papaya leaf ethanol extract decreased the cancer cell proliferation index and increased apoptosis of cancer cells in animal models of cervical cancer; it may also work to increase NF-κB expression and expression of the p53 gene.

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