Evaluation Report

Final Decision: Reconsider for Evaluation after Modifications and Clarifications

Article No.: 101171-IJCR-AJ
Article Type: Research Article

Figures Available: 1 Figure Cited: 1
Table Available: 4 Tables cited: 4

Manuscript falls in the scope of the journal? Yes No
My observations/comments about this article are:

No.	Part	⁾ Comments	Author Response
1	**Cover letter**	⁾ Overall Ok	
2	**Write up**	⁾ Overall Ok	
3	**Title**	⁾ Main title is the major component of a research article to attract the readers and increase the readership that's why title of research article should be effective, simple and concise (within 14 words). It should indicate accurately the purpose of the study. Author is requested to rewrite an attractive main title.	Anticancer activity of Linamarin from Cassava Leaves (Manihot esculenta Cranz) on Raji Cells
4	**Running Title**	⁾ Overall Ok	
5	**Author's Information**	⁾ Overall Ok	
6	**Author's Contribution**	⁾ Overall Ok	
7	**Abstract**	⁾ Overall Ok	
8	**Keywords**	⁾ Overall Ok	
9	**Introduction**	⁾ Overall Ok	
10	**Materials and Methods**	⁾ Please indicate both the manufacturer's name and location (including city, state, and country) for all specialized equipments, kits, software, incubators, instruments, pH meter,	MTT reagent (Sigma Aldrich, Darmstadt, Germany), laminar air flow (Nuarie), incubator (Nuarie), phase contrast microscope (Olympus, Japan), electric scales (Sartorius),

		and reagents used in the experiment.	micropipette (Socorex), centrifuge (B. Braun biotech Internasional), vortex (Genie), waterbath (Labec), pH meter (TOA), Tissue Culture Flask (TCF) 25 cm2 (Nunclone), microplate 96 (Nunclone), hemocytometer (Neubauer), filter millipore 0,2 um (Labec), fluorescence microscope (Olympus, Japan), glass object (Sigma Aldrich), deck glass (Sigma Aldrich), light microscope (Olympus, Japan).
11	**Results**	Overall Ok	
12	**Figures**	Author is advised to label the figure 1 correctly by which a reader can easily understand the results obtained in figure 1.	Ok
13	**Tables**	This value is not present in table 1. Author is advised to provide the correct value in table 1, in its description and discussion.	Ok
14	**Discussion**	Overall Ok	
15	**Conclusion**	Overall Ok	
16	**Acknowledgement**	Overall Ok	
17	**Significance Statement**	Overall Ok	
18	**References**	Author is advised to Provide DOI or URL of all listed references.	Ok

Guidelines to attend the Comments:

- Author is requested to please highlight the amended portion in the manuscript. It will be more helpful for us in cross checking of suggested modifications.
- Please give your response in the Evaluation report as well under the column **"Author Response"** for all the parts of the manuscript.
- Incorporate all the recommended modifications in their respective sections throughout the manuscript.

Plagiarism checked
101171-IJCR-AJ

Research Article

Anticancer Activity of Linamarin from Cassava Leaves (*Manihot esculenta* Cranz) on Raji Cells

Commented [WU1]: Main title is the major component of a research article to attract the readers and increase the readership that's why title of research article should be effective, simple and concise (within 14 words). It should indicate accurately the purpose of the study. Author is requested to rewrite an attractive main title.

Dwi Sutiningsih[1*], Mohamad Arie Wuryanto[2], Henry Setyawan Susanto[3], Sujud Hariyadi[4], Mustofa[5]

1. Department of Epidemiology and Tropical Disease**,** Faculty of Public Health, Diponegoro University, Semarang, Indonesia, E-mail: dwisuti98@gmail.com
2. Department of Epidemiology and Tropical Disease**,** Faculty of Public Health, Diponegoro University, Semarang, Indonesia, E-mail: arie.epid@gmail.com
3. Department of Epidemiology and Tropical Disease**,** Faculty of Public Health, Diponegoro University, Semarang, Indonesia, E-mail: henrysmg@gmail.com
4. Department of Epidemiology and Tropical Disease**,** Faculty of Public Health, Diponegoro University, Semarang, Indonesia, E-mail:hariantosudjut@yahoo.com
5. Department of Pharmacology, Faculty of Medicine, University of Gadjah Mada, Yogyakarta, Indonesia, E-mail : mustofafk@ugm.ac.id

***Correspondence:** Dwi Sutiningsih, Department of Epidemiology and Tropical Disease Faculty of Public Health, Diponegoro University, Semarang, Indonesia**,** Phone / Fax: +62 247460044, Email: dwisuti98@gmail.com

LiveDNA*: 62.16151

Author contributions

Dwi Sutiningsih: Performed literature review, developed research proposal, conducted experiments and data analysis, and wrote manuscript.

Mohamad Arie Wuryanto: Participated in research design and manuscript writing.

Henry Setyawan Susanto: Reviewed research proposal and contributed tocytotoxic examination and data analysis.

Sujud Hariyadi: Participated in data analysis and contributed to manuscript writing.

Mustofa: Conducted the experiments and wrote the manuscript.

ABSTRACT

Background and Objective: Linamarin is a active compound isolated from the leaves of cassava (*Manihot esculenta*Cranz) that has a cytotoxic effects on HT-29, MCF-7, and HL-60 cells. This study aims to determine the cytotoxic and antiproliferation activity and induction of p53 protein in Raji cells after administration of various concentrations of linamarin from cassava leaves (*Manihot esculenta* Cranz).

Materials and Methods: Linamarin was isolated from cassava leaves (*Manihot esculenta* Cranz) using a multilevel purification method. Linamarin cytotoxicity was tested on Raji cells using the MTT method, while antiproliferation activity was tested using a *doubling time* test. P53 protein expression was observed by immunocytochemical tests. The cytotoxic activity of Raji cells was expressed by the value of concentration 50. The *doubling time* was calculated by comparing the *slope* values of the log graphs of the number of cells at various times. Raji cells that were positive for p53 protein showed brown painted nuclei or cytoplasm.

Results: Linamarin from cassava leaves can inhibit cytotoxic activity and proliferation on Raji cells. The higher the linamarin concentration, the longer the doubling time of Raji cells. The expression of p53 protein on Raji cells after linamarin administration was higher than the control. P53 protein expression was found in the nuclei (91.05%) and cytoplasm (8.95%).

Conclusions: Given those findings, linamarin from cassava leaves has the potential to be developed as an anticancer agent.

Keywords: Linamarin**,** *Manihot esculenta* Cranz, cytotoxic, antiproliferative, p53 protein, Raji cells

INTRODUCTION

Obstacles and side effects caused by various cancer treatments have necessitated the discovery of highly effective alternatives with minimal side effects. One such effort is the development of drugs from plants that contain anticancer compounds. The development of cancer drugs from plants has several advantages, among which are their low cost, availability, and relatively few side effects[1].

In Indonesia, cassava has considerable economic value compared to other tubers. Not only is cassava (*Manihot esculenta* Cranz) one of the world's principal food staples after grains and corn, [1] their leaves, widely consumed in Indonesia and elsewhere, are rich in vitamins A, C, K, among others, and minerals, including iron, calcium, and phosphorus. The energy content of cassava leaves is greater than most other green vegetables [2]. Cassava also contains cyanogenic glucoside compounds, which consist of linamarin and lotaustrain at a ratio of 10:1 [3,4] . Linamarin has potential use as an anti-neoplastic compound[5,6]. The mechanism of linamarin in the treatment of cancer using linamarase gene therapy has been investigated by Cortes in 2002.

Meanwhile the Idibie [6] study states linamarin in root tubers has been proven in vitroto have cytotoxic effects on HT-29, MCF-7, and HL-60 cells. From the results of this study, Inhibitor Concentration 50 (IC_{50}) was obtained in the amounts of > 300 µg/ml, 235.96 ± 9.87 µg/ml, and 246.51 ± 10.12 µg/ml after incubation for 48 hours. In this study, linamarin was obtained from cassava leaf extracted with methanol. The study of Yusuf *et al.* [7] using linamarin isolated from cassava leaves also showed cytotoxic effects on Caov-3 cells and Hela cells. The IC_{50} value of the two cell lines is 38 µg/ml and 57 µg/ml respectively. Cancer cell death has been caused by the linamarin content found in cassava plants[8-11] . Carotene and vitamin C compounds found in cassava leaves are thought to have anticancer properties[12-15] . Research by Enger *et al.*[16], stated that carotene is protective toward colon adenoma rather than

other carotenoids in the early stages of tumor formation. Kontek *et al.*[17] stated that vitamin C had a positive effect on the damage level of oxidative DNA in colon cancer cells.

The benefits of cassava as an anticancer agent have been proven in several cancer cells, but have not yet been widely studied regarding its potential in Raji cells. This study aimed to determine cytotoxic and antiproliferative activity and induction of p53 protein in Raji cells following treatment of linamarin from cassava leaves.

MATERIALS AND METHODS

Cassava leaves (*Manihot esculenta* Cranz) were obtained from the local market in Yogyakarta, Indonesia, then identified at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University. This research project was conducted from June 4, 2018 to December 4, 2018. Raji cells were obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University. This cell is a continuous cell line that grows floating, similar to lymphoblast cells (B lymphocytes) from Burkitt's lymphoma infected by Epstein-Barr Virus (EBV). Materials for growing Raji cells are RPMI solution (Sigma-Aldrich), Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich), HEPES, Fetal Bovine Serum (FBS)(Gibco), penicillin-streptomycin)(Gibco), DMSO (Sigma-Aldrich), tripan blue (E-Merck), and 3-(4-, 5 dimethylthiazol-2-yl) -2.5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich).

Linamarin isolation from cassava leaves: A 5 g batch of cassava leaves was cut into small pieces, then pounded in a mortar. The result was blended thoroughly with a total of 10 ml of 0.1M HCl solution. The mixture solution was centrifuged at 3500 rpm to obtain the supernatant. The supernatant liquid obtained was transferred to the Falcon tube. The supernatant liquid mixture with 0, 1 M HCl was linamarin extract of cassava leaf, which was then isolated. Finally, the linamarin extract was frozen at - 20°C[8].

Cytotoxic test on Raji cells: The cytotoxicity test was done colorimetrically using MTT reagents (Sigma-Aldrich, Darmstadt, Germany). Linamarin of 10 µL at various concentrations was added to Raji cell culture the day after transplantation. The concentration of linamarin used for treatment of Raji cells was 31.25, 62.5, 125, 250, 500, and 1000 µg/ml. Cells that were not treated were used as controls. On the third day, 20 µL of MTT reagent was added to approximately 5 mg/ml per well. After four hours of incubation, 100 µL of 0.1 N HCl-isopropanol was added to each well to dissolve the formazan crystals that had formed. Absorbance (A) was measured using a microplate reader at a wavelength of 595 nm. All steps were carried out three times.

Commented [WU2]: Please indicate both the manufacturer's name and location (including city, state, and country) for all specialized equipments, kits, software, incubators, instruments, pH meter, and reagents used in the experiment.

Antiproliferation test *(*doubling time) in Raji cells: Cells were fasted for 24 hours in culture media containing 0.5% of FBS. Afterwards, they were grown in a plate with a medium added, with linamarin at a non-lethal concentration of three series below the IC_{50} value. Then it was incubated in a 5% CO_2 incubator (Nuarie) at 37 °C for 24, 48, and 72 hours. Each well was calculated by the number of cells living using hemocytometrics (Neubauer).

Immuno-cytochemical test in Raji cells: In a microculture, 96 wells containing 100 µl of test cells, with a density of 2 x 10^4 cells/well, 100 µl of the test compound were added at concentrations of 10 µg/ ml. They were then incubated with 5% CO_2 flow at 37 °C for 24 hours. After being incubated overnight, 200 µl of cells from each well were taken and inserted in eppendorf tubes, then centrifuged to 1200rpm x 5 minutes. The supernatant liquid was removed, leaving the pellet, and then re-suspended. The cell suspension was extracted and placed on a glass object that had been coated with poly-lysine. The cells were fixated with acetone for 10 minutes. Later, they were washed with PBS (Phosphate Buffered Saline)(E.Merck) x 5 minutes and etched with hydrogen peroxidase 0, 1% for 10

minutes. After washing them with running water, they were rinsed with PBS for five minutes, dripped with 100 µl normal horse serum for 10 minutes, and cleaned without water. Finally, they were dripped with anti-p53 protein primary antibodies (Novocastra) and left for 24 hours.

The next day the suspension was:

- washed twice with PBS x 5 minutes each;
- dripped with biotinylated secondary antibodies (Novocastra) x 10 minutes;
- washed x 2 with PBS x 5 minutes each;
- dripped then incubated with Avidin Biotin reagent enzyme (Novocastra) x 10 minutes;
- washed x 2 with PBS x 5 minutes each;
- incubated with a peroxidase substrate (DAB) (Novocastra) x 10 minutes or until the coloring appeared;
- washed with running water;
- counterstained with hematoxylin for 10 to 20 seconds, then washed with running water; and
- Dehydrated using 95% ethanol and xylen x 10 minutes each.
- The mounting media was dripped, and then covered with a glass deck.

The results were observed under a light microscope (Olympus, Japan) with 400x magnification. Cells positive for p53 protein showed nuclei or cytoplasm painted brown.

Data analysis: Raji cell cytotoxicity was analyzed using probit analysis to determine 50% Inhibition Concentration (IC_{50}). Probit analysis was obtained from the conversion of the percentage of inhibition to the probit value. Percentage of inhibition was calculated as follows:

$$\% \text{ Cell inhibition} = [(\sum A - \sum B) : \sum A] \times 100\%$$

∑ A: The number of living cells in untreated controls

∑ B: The number of living cells due to the treatment of compounds at various concentrations

The difference in percentages of cell inhibition between each treatment group was tested statistically using a one-way ANOVA test with 95% Confidence Interval. Analysis of doubling times was calculated by comparing the slope of the log graphs of the number of cells at different observation times. To find differences between groups, the average number of cells living at the various times was analyzed statistically using the one-way ANOVA test with a 95% confidence level. Expression of p53 protein was analyzed by observing its percentages as expressed in Raji cells after immuno-histochemical treatment. Cells that were positively stained with p53 protein showed nuclei or cytoplasm painted brown. The proportion of cells that were positively p53 protein was determined by calculating the presence of stained nuclei or cytoplasm per 100 cells.

RESULTS and DISCUSSION

Linamarin cytotoxic activity in Raji cells: Cytotoxic activity was tested to determine the toxicity of a linamarin compound on Raji cells. Raji cells are continuous cell lines that grow floating and unattached to the bases of flasks. The cell is similar to lymphoblast cells (B lymphocytes) from Burkitt's lymphoma infected by Epstein-Barr Virus (EBV). The cells are round and clustered. Living cells will appear bright under a contrast phase microscope while dead cells will appear dark.

The parameters used to express the potency of linamarin toxicity from cassava leaves are IC_{50} values. The results of calculating cell inhibition percentage of Raji cells after linamarin administration from cassava leaves are presented in Table 1. The Table 1. shows that at the highest linamarin concentration (1000 µg/ml), the percentage of Raji cell inhibition

was 97.550%, while at the lowest concentration (31.25µg/ml), the percentage was 27.194 percent.

The results of Kolmogorov-Smirnov's analysis showed that the average Raji cell inhibition was normally distributed (p = 0.135), while homogeneity test results were homogeneous (p = 0.088). The one-way ANOVA test was used to determine the differences in Raji cell inhibition between various linamarin treatments. The results of the one-way ANOVA analysis revealed significant differences between the Raji cell inhibition levels at various linamarin concentrations (p = 0.000).

Antiproliferation activity of linamarin in Raji cells: The concentration of the test compound used in the doubling time test was three concentrations below the IC_{50} value (15.63; 31.25; 62.50 µg/ml). Cell counts are carried out at 0, 24, 48, and 72 hours. Raji cells had been previously fasted (starved) for 24 hours using RPMI 1640 media containing FBS 0.5 %. Data of doubling time analysis of Raji cells after linamarin treatment and control (without treatment) can be seen in Table 2.

Data from Table 2 shows how the multiplication times of Raji cells after linamarin treatment, at concentrations below the IC_{50} value, run greater than the control times. Linamarin concentration of 62.50 µg/ml can delay the doubling times of Raji cells by ±2 x those of the Raji control cells.

From Fig. 1, it can be seen that at 30 minutes after the treatment of the test compound, there has been no inhibition of Raji cell growth, in contrast to observations at 24, 48, and 72 hours. One-way ANOVA analysis showed that there were significant differences (p = 0.023) in the average number of living Raji cells, dependent upon the elapsed time post-linamarin treatment (24, 48, and 72 hours).

Expression of p53 protein on Raji cells: The immunocytochemical test results showed that linamarin can increase the expression of p53 protein on Raji cells. Complete results of p53 protein expression tests are presented in Table 3.

According to those results, there is a tendency for greater p53 protein expression in the treatment group compared to the control group. Linamarin concentration of 62.5 µg/ml shows increased positive p53 protein expression in Raji cells by 77.5%, ± 3.07%, while linamarin concentration was 31.25 µg/ml at 40 ± 1.87%.. The one-way ANOVA test results showed a significant difference in the number of p53 protein expression in Raji cells at various linamarin concentrations (p = 0.000). Details pertaining to the expression of p53 protein in the nuclei and cytoplasm of Raji cells are presented in Table 4 and Fig. 1.

From Fig.1 it can be seen that in the Raji control cell there was a tendency to decrease the positive p53 protein expression, whereas in the Raji cells with linamarin, 32.5 and 62.5 µg/ml concentrations appeared to increase positive p53 protein expression, with most located in the nuclei (Table 4).

Linamarin cytotoxic activity in Raji cells: The cytotoxicity test determined the value of IC_{50}, which is a concentration capable of inhibiting cell growth, such as Raji cells, by up to 50 percent. The smaller the IC_{50} value, the more toxic the compound is. The potential for linamarin toxicity from cassava leaves (*Manihot esculenta* Cranz) to Raji cells is indicated by IC_{50} values of 71.865 ± 0.229 µg/ml. At its highest concentration (1000 µg/ml), the percentage of Raji cell growth inhibition was 97.550% ± 0.005%, while the lowest concentration of linamarine (31.25 µg/ml) was 27.194 ± 0.096% (Table 1). From Table 1, shows that at the higher the concentration of linamarin, the greater the percentage of Raji cell growth inhibition, with a significant statistical difference ($p < 0.05$). This proves that linamarin obtained from cassava leaves (*M. esculenta* Cranz) can suppress the growth of Raji cancer

cells. Linamarin is found in all parts of cassava plants (*M. esculenta* Cranz), but most abundantly at the roots, leaves, and root tuber skin. [5]

Yusuf *et al.*[7] found that linamarin from cassava leaves can inhibit the growth of Caov-3 cancer cells and Hela cells with IC_{50} values of 38 µg/ml and 57 µg/ml, respectively. Idibie [6] in his research, stated that IC_{50} values decreased when pure linamarin compounds and crude extracts of cassava tubers were given along with linamarase enzymes on MCF-7 cancer cells (adenocarcinoma breast cancer), HT-29 (adenocarcinoma colon), and HL-60 (cell line leukemia). Meanwhile, the IC_{50} values of crude extracts are higher than linamarin if not given along with the linamarase enzyme. Likewise, the results of Alfourjani's study [5] showed that the IC_{50} values of MCF cells after treatment with raw cassava leaf extract and boiled cassava leaves were 63.1 and 79.4 µg/ml, respectively.

Crude extracts are said to have strong potential as anticancer agents if the IC_{50} value is less than 30 µg/ml[19]. The results of this study showed IC_{50} value of Raji cells after linamarin administration to be greater than 30 µg/ml. In fact, they registered as high as 71.865 ± 0.229 µg/ml, meaning that the potency of linamarin toxicity in active Raji cells was weaker, or only moderately active ($30 \leq IC_{50} < 100$ µg/ml). This was presumably due to differences in the characteristics of cancer cells used in the study.

Raji cells are found in the Burkitt's lymphoma cell line in humans. Burkitt's lymphoma at the molecular level is characterized by synergistic Bcl-2 and c-myc expressions. C-myc is upregulation Bcl-2, so the increase in c-myc expression can also increase the expression of Bcl-2. As a result of this increase in expression, cells do not experience apoptosis[20,21]. Burkitt's lymphoma has chromosome translocation that activates c-myc. In some patients it also shows the occurrence of mutations in p53 which result in the inhibition of the apoptotic process in these cancer cells. Activating p16INK4a resulted in loss of CDK inhibitory function, diminishing loss of cell control of its growth. Changes

(mutations) also occur in the expression of pRb and p53, which are gene suppressor tumors, and in other genes, such as Bax, p73, and Bcl-6, which provide sufficient growth signals and inhibit apoptosis in cancer cells[22-24]. Mutations also occur in downstream Caspase-3 which causes Raji cells to be resistant to apoptosis[25,26].

The protein expression of the Epstein-Barr Nuclear Antigen 1 (EBNA1) in Burkitt's lymphoma, infected by Epstein- Barr Virus (EBV), can also inhibit the occurrence of apoptosis in cancer cells[27]. Through this mechanism, it is suspected that Raji cells can avoid the apoptotic mechanism triggered by linamarin compounds from cassava leaves. This is why the suspected cause of cassava leaf extract cytotoxicity against Raji cells is considered moderate.

Linamarin is said to be antineoplastic by its release of HCN during the process of hydrolysis. When HCN is released, the cancer cell is exposed to the lethal cyanide effect released by linamarin. Linamarin is broken down and cyanide is released only in the areas around the cancer cells. This causes gradual cancer cell death. Because normal cells do not have the linamarase gene, they will not be affected[5,6].

Inhibition of Raji cell growth is also due to ß-carotene content in cassava leaves. ß-carotene has an anticancer mechanism by its carcinogen-modulating metabolism and antioxidant activity, thus modulating the immune system, increasing cell differentiation, stimulating communication gap cell junctions to cells and affecting retinoid-dependent signals[28]. ß-Carotene is also directly related to inhibition of cell proliferation, increased apoptosis, induces cell cycle arrest[14]. In his research, Enger *et al.*[16] stated that ß-carotene is protective toward colon adenoma in the early stages of tumor formation. The same thing was determined by Gloria *et al.*,[14] who proved that carotenoids were able to increase breast cancer cell apoptosis.

Inhibition of Raji cell growth by linamarin can also be influenced by vitamin C. Cassava leaves contain vitamin C of 103 mg, higher than other green vegetables[16]. Vitamin C is known to act as an antioxidant in preventing infection, helps the absorption of iron and calcium, and is associated with the synthesis of collagen, carnitine, noradrenaline, and serotonin in the body[29-32]. Besides its function, vitamin C also plays an important role in activating genes involved in DNA repair, as well as modulating DNA damage in ROS-affected cells. The results of the Kontek *et al.*[17] study prove that vitamin C has a positive effect on the level of oxidative DNA damage. Vitamin C provides a protective effect for normal tissue to counteract the activity of toxic substances and their metabolites, thus affecting the extent of colon cancer cell inhibition[33,34].

Antiproliferative activity of Raji cells: Analysis of cell proliferation inhibition can be done by the doubling time test. Compounds that delay the multiplication times of cells can inhibit genes or proteins that regulate the cell cycle. The doubling time test is done by counting the number of cells treated in a time unit (e.g., 24 hours). Each sample is calculated by a hemocytometer, and then a curve with cell number versus incubation time is made. Differences in cells' doubling times can be determined from the slope of the curve or calculated by extrapolation[35]. Raji cells were previously fasted (starved) for 24 hours using RPMI 1640 media containing FBS 0.5 percent. Reducing this growth signal is necessary because it reduces the speed of cell growth, which causes the cell to be in the same initial start, or G0 phase. Without fasting when treated, the cells remain in different phases which makes it difficult to observe the inhibition properties of linamarin on cell cycle progression[36].

From Table 2 it can be seen that the doubling time value of Raji cells with linamarin treatment concentrations of 62.5 µg/ml is greater than the doubling time value of Raji cells

with linamarin treatments of 32.5 µg/ml and 15.63 µg/ml. This is supported by the linamarin curve slope value of 62.5 µg/ml, which is smaller than the linamarin slopecurve of the treatment with 32.5 µg/ml and 15.63 µg/ml. This means that linamarin 62.5 µg/ml has a better chance of postponing cell doubling time of Raji cells than linamarin 32.5 µg/ml and 15.63 µg/ml. It is suspected that the linamarin in cassava leaf extract can inhibit genes or proteins that regulate cell division. It may inhibit signal transduction through inhibition of growth signals or through inhibition of cell cycle progression by inhibiting proto-oncogenes such as CycD, cdk 4/6 and c-myc. Similarly, it may activate suppressor tumors such as caspase 3/8/9, p53, pRb, and Bcl2 inactivation[5,6].

The data in Table 2 shows that the doubling time value of Raji cells with linamarin treatment concentrations of 62.5 µg/ml is twice the doubling time value of Raji cells without treatment (control). This means that linamarin concentration of 62.5 µg/ml can cut the doubling time of Raji cells to half that of Raji cells doubling times without treatment (control). The price of doubling time for linamarin treatment is greater than that for control. This indicates that linamarin has the ability to inhibit Raji cell proliferation and possess cytotoxic activity. The higher the linamarin concentration, the longer the doubling time of Raji cells. A linamarin construction of 31.25 µg/ml can inhibit cell proliferation better than linamarin 15.63 µg/ml. This inhibition may occur in signal transduction through inhibition of growth signals or through inhibition of cell cycle progression by inhibiting proto-oncogenes such as CycD, cdk 4/6, and c-myc. Or, it may be able to activate suppressor tumors such as caspase 3/8/9, p53, pRb, and Bcl2 inactivation[37,38].

Expression of p53 protein in Raji cells with linamarine treatment: Immunocytochemical analysis is intended to determine the expression of p53 protein in Raji cells. In this study antibodies can be used to detect both wild and mutant type p53 proteins in cancer

cells. Positive expression of p53 protein is indicated by brown color in the cell nucleus or cytoplasm; wild or mutant types cannot be distinguished. The results showed that linamarin could increase the expression of p53 protein in Raji cell. Linamarin concentrations of 62.5 µg/ml can increase positive p53 protein expression (77.5 ± 3.07%) greater than linamarin 31.25 µg/ml (60% ± 1.87%) (Table 1). In Raji control cells or with linamarin treatment from cassava leaf extract, most p53 protein expressions are located in the cell nucleus, although some are located in the cytoplasmic part (Table 3). The control cells also shown have positive p53 protein expression but the amount was less than the treatment with linamarin concentrations of 31.25 µg/ml and 62.5 µg/ml (Fig.1). This shows that Raji cell death occurred through the mechanism of inhibition of Raji cell proliferation, by activating suppressor gene tumors such as p53. The presence of stress or DNA damage can spur the expression of p53 protein in Raji cells[39].

The increase in p53 protein expression in Raji cells after the linamarin treatment proved several possibilities: first, the increase was an increase in wild type p53 expression. P53 protein is encoded by p53 tumor suppressor genes and has an important role in cell regulation and proliferation[22]. The wild type of p53 protein is expressed very little in normal conditions, but there will be an increase in response to normal cells if there is DNA damage[40]. Increased expression of wild-type p53 will be activated through the p21 protein to stop DNA replication and cell division when DNA damage occurs. This happens because an increase in p53 protein will stimulate p21 gene transcription. The p21 protein is an inhibitor of CDK and has the ability to inhibit phosphorylation of pRB, thus blocking the release of E2F transcription factors and DNA replication. However, if DNA damage is too severe and cannot be repaired, p53 will induce apoptosis by stimulating Bax transcription, which will then inhibit the activity of the Bcl2 gene[41]. The Bcl2 gene functions to inhibit the response of apoptosis to various cell types caused by various stimulations related to apoptosis. Thus, p53

plays an important role in preventing the accumulation of cells with DNA abnormalities that can mutate into cancer cells[42].

If the p53 expression is the wild type, then DNA damage will cause a rapid rise in p53 protein expression, thus inducing a resting phase of the cell cycle during the G1 phase. Wild-type p53 will cause a cessation of growth in the G1 phase,[43] thus providing sufficient time for the DNA repair genes such as MLH, MSH_2, PMS_1, PMS_2, Mdm2, $BRCA_1$, and $BRCA_2$ [44]. If the DNA damage can be repaired, the cell will continue to divide into the S phase; if this improvement is not possible, then p53 will induce apoptosis[45].

The second possibility is that the increase in p53 expression is an accumulation of mutant type p53. P53 mutations will cause the protein to be more stable and have a longer half-life than the wild type. This causes the mutant type of p53 protein to be more easily detected immunocytochemically, although positive expression of p53 is not always associated with its gene mutation[46].

P53 mutation is the most common genetic lesion in neoplasms. P53 mutations are associated with increased cellular proliferation and transformation toward malignancy[47]. They will cause changes in the encoded protein products, so they cannot stimulate the transcription of p21 and Bax,[41] thus causing the accumulation of cells with DNA damage, which can turn into cancer cells[22].

The presence of positive p53 protein expression in the cytoplasm shows that inhibition of Raji cell growth occurs in the G1 phase of the cell cycle. Linamarin from cassava leaves can increase the expression of p53 protein in the cytoplasm compared to the control cells. Linamarin is thought to inhibit cell division in the G1 phase of the cell cycle by increasing the expression of p53 protein in the cytoplasm. According to Groeger,[48] most of the p53 genes act as 'the guardian of the genome': (1) p53 levels increase rapidly in response to DNA damage, (2) cause cell cycle inhibition during the G1 phase, (3) give cells time to repair DNA damage,

(4) if damage cannot be repaired, p53 will induce programmed cell death (apoptosis). Both wild type and mutant proteins migrate in the cell nucleus known as Nuclear Localization Signals (NLS) that are attached to their primary sequences [49]. According to Baker *et al.*[50] and Duler *et al.*,[51] p53 wild-type causes growth inhibition in the G1 phase, so that it can be interpreted that in order to enter S phase of the cell, p53 must be inactive.

Overall it can be concluded that linamarin from cassava leaves is toxic to Raji cells and can inhibit Raji cell proliferation through increased expression of p53 protein. The expression of p53 protein cannot be distinguished whether p53 is wild or mutant type but seeing the expression of p53 protein in the cytoplasm shows that inhibition of Raji cell proliferation is through cell cycle progression inhibition that occurs in the G1 phase. This provides an opportunity for genes that control DNA repair to restore DNA function. The limitation of this study is that it only observes the mechanism of Raji cell proliferation via p53 protein induction, so further research is necessary to discern the pathway(s) for proliferation inhibition through apoptosis induction, p21 expression, DNA repair pathways, and proliferative inhibition locations in the G1 phase of the cell cycle.

CONCLUSION

Linamarin isolated from cassava leaves (*M. esculenta* Cranz) has the potential to be developed as an anticancer agent**.**Linamarin from cassava leaves (*M. esculenta* Cranz) has cytotoxic activity on Raji cells with IC_{50}values of 71.865 ± 0.229 µg/ml, antiproliferation activity on Raji cells with a doubling time value of 40.723 hours on linamarin concentration of 62.5 µg/ml and can increase the expression of p53 protein in the nuclei and cytoplasm of Raji cells.

SIGNIFICANCE STATEMENT

Findings from this study could contribute to a better understanding of the mechanism of action of linamarin, which is derived from cassava leaves as an anticancer agent. Future efforts should be directed towards determining the specific cell signaling pathways involved in cancer cell toxicity. It also needs in vivo models in experimental animals and the development of an ideal anti-cancer drug formulation.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest or financial interest regarding the results of this research.

ACKNOWLEDGEMENTS

The authors would like to thank the Dean of Public Health Faculty of University of Diponegoro who has funded this study through APBN DIPA of Public Health Faculty of University of Diponegoro funding No. 106/UN7.5.9/HK/ 2018, dated May 31, 2018.

REFERENCES

Commented [WU3]: Author is advised to Provide DOI or URL of all listed references.

1. Akinpelu, A.O., Amamgbo, L.E.F., Olojede, A.O and Oyekale, A.S., 2011. Health implications of cassava production and consumption. J. Agric. Soc .Res.11:118-25. https://www.ajol.info/index.php/jasr/article/view/73684/64364
2. Adenle., A.A., Aworh, O.C., Akromah, R and Parayilet, G., 2012. Developing GM super cassava for improved health and food security: Future challenges in Africa. Agriculture and Food Security. 1:1-15. https://agricultureandfoodsecurity.biomedcentral.com/articles/10.1186/2048-7010-1-11
3. Ernesto, M., Cardoso, A.P., Nicala, D., Mirione, E and Massaza, F *et al.*, 2002. Persistent konzo and cyanide toxicity from cassava in northern Mozambique. Acta Tropica.82:357-362. http://biology-assets.anu.edu.au/hosted_sites/CCDN/papers/82_357_362_02.pdf
4. Sayre, R, Beeching, J.R., Cahoon, E.B., Eges, C and Fauquet, C*et al.*, 2011. The bio cassava plus program: Biofortification of cassava for sub-Saharan Africa. Annu.

Rev. Plant. Biol. 62:251-72. https://www.ncbi.nlm.nih.gov/pubmed/21526968. DOI: 10.1146/annurev-arplant-042110-103751.

5. Alfourjani, W.A., 2005. In vitro anticancer properties of linamarin controlled release from biodegradable poly-lactic co-glycolic acid nanoparticle. Master's Thesis, Universiti Putra Malaysia, Malaysia, pp: 87-90. http://psasir.upm.edu.my/id/eprint/5996/
6. Idibie, C.A., Davids, H and Iyuke, S.E., 2007.Cytotoxicity of purified cassava linamarin to a selected cancer cell lines. Bioproc. Biosyst. Eng. 30: 261-69. https://www.ncbi.nlm.nih.gov/pubmed/17566787. DOI: 10.1007/s00449-007-0122-3
7. Yusuf, U.F., Ahmadun, F.R., Rosli, R., Iyuke, S.E and Billa, N*et al.*, 2006. An in vitro inhibition of human malignant cell growth of crude water extract of cassava (*Manihot esculenta* Crantz) and comercial linamarin. J. Sci. Tehnol.28:145-55. https://www.researchgate.net/publication/26469858_An_in_vitro_inhibition_of_human_malignant_cell_growth_of_crude_water_extract_of_cassava_Manihot_esculenta_Crantz_and_commercial_linamarin
8. Haque, M.R and Bradbury, J.H., 1999. Preparation of linamarase solution from cassava latex for use in the cassava cyanide kit. Food. Chem. 67: 305-9. https://eurekamag.com/research/003/239/003239769.php. DOI: 10.1016/s0308-8146(99)00117-x.
9. Girald, W., 2012. Toxicity and delivery methods for the linamarase/linamarin/glucose oxidase system, when used against human glioma tumors implanted in the brain of nude rats. Cancer. Lett. 313: 99-107. https://www.sciencedirect.com/science/article/pii/S030438351100526X?via%3Dihub. DOI:10.1016/j.canlet.2011.08.029,
10. Dorgan, J.F., Sowell, A., Potischman, N., Swanson, C and Miller, R*et al.*,1998. Relationship of serum carotenoids, retinol, α-tocopherol, and selenium with breast cancer risk: Results from a prospective study. Cancer. Causes. Control. 9:89-97. https://www.ncbi.nlm.nih.gov/pubmed/9486468. DOI: 10.1023/a:1008857521992
11. Cortes, M.L, Garcia-Escudero, V., Hughes, M and Izquierdo, M.,2002. Cyanide bystander effect of the linamarase/linamarin killer-suicide gene therapy system. J. Gene. Med. 4:407-14. https://www.ncbi.nlm.nih.gov/pubmed/12124983. DOI: 10.1002/jgm.280
12. Dominguez, Eduardo, R., Vazquez-Luna, A., Rodriquez-Landa, J.F and Diaz-Sobac ,R., 2013. Neurotoxic effect of linamarin in rats associated with cassava (*Manihot esculenta* Crantz) consumption. Food. Chem. Toxicol. 59:230-5. https://www.ncbi.nlm.nih.gov/pubmed/23778051 DOI: 10.1016/j.fct.2013.06.004
13. Duijnhoven, F.J.B., Buebo-De-Mesquita, H.B., Ferrari, P., Jenab, M and Boshuizen,H.C*et al.*, 2009. Fruit, vegetables and colorectal cancer risk: the European prospective investigation into cancer andnutrition. Am. J. Clin. Nutr. 89:1441-52. https://www.ncbi.nlm.nih.gov/pubmed/19339391. DOI: 10.3945/ajcn.2008.27120. Epub 2009 Apr 1.
14. Gloria, N.F., Soares, N., Brand, C., Oliveira, F.L and Borojevic, R*et al.*, 2014. Lycopene and beta-carotene induce cell-cycle arrest and apoptosis in human breast cancer cell lines. Anticancer. Res. 34: 1377-86. https://www.sciencedirect.com/science/article/pii/S030438351100526X?via%3Dihub. DOI: 10.1016/j.canlet.2011.08.029.
15. Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J and Wang, J.Y*et al.*,2000. The p53/p63/p73 family of transcription factors: Overlapping and distinct functions. J. Cell. Sci. 113: 1661-70. https://www.ncbi.nlm.nih.gov/pubmed/10769197

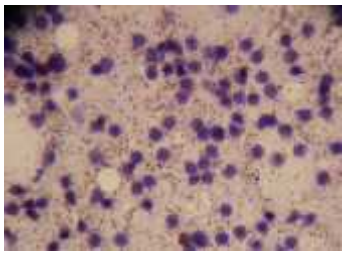
16. Enger, S.M., Longnecker, M.P., Chen, M.J., Lee, E.R and Frankl, H.D*et al.*,1996. Dietary intake of specific carotenoids and vitamins A, C, and E, and prevalence of colorectal adenomas. Cancer. Epidemiol. Biomarkers. Prev. 5: 147-53. https://pdfs.semanticscholar.org/cf7b/a52044641f18fae1d5320d3aef0e925a6f0b.pdf
17. Kontek, R., Kontek, B and Grzegorczyk, K., 2013. Vitamin C modulates DNA damage induced by hydrogen peroxide in human colorectal adenocarcinoma cell lines (HT29) estimated by comet assay in vitro. Arch. Med. Sci. 9: 1006-12. doi: 10.5114/aoms.2013.39791. https://www.ncbi.nlm.nih.gov/pubmed/24482643
18. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65: 55-63.
19. Itharat, A and Ooraikul, B., 2007. Research on Thai medical plants for cancer treatment. Adv. Med. Plant. Res. 37: 287-317. https://www.ncbi.nlm.nih.gov/pubmed/6606682 DOI10.1016/0022-1759(83)90303-4
20. He, Y., Zhu, Q., Chen, M., Huang, Q and Wang W *et al*., 2016. The changing 50% inhibitory concentration (IC_{50}) of cisplatin: a pilot study on the artifacts of the MTT assay and the precise measurement of density-dependent chemoresistance in ovarian cancer. Oncotarget. 7: 70803-21. https://www.ncbi.nlm.nih.gov/pubmed/27683123 DOI: 10.18632/oncotarget.12223.
21. Jorgensen, K., Morant, A.V., Morant, M., Jensen, N.B and Olsen, C.E., *et al*., 2011. Biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in cassava: isolation, biochemical characterization, and expression pattern of CYP71E7, the oxime-metabolizing cytochrome P450 enzyme. Plant. Physiol. 155: 282-92. https://www.ncbi.nlm.nih.gov/pubmed/21045121. DOI: 10.1104/pp.110.164053. Epub 2010 Nov 2.
22. Lane, D.P., Cheok, C.F and Lain, S., 2010. P53 based cancer therapy, Cold Spring Harbor. Perspect. Biol. 2: a001222. https://pubmed.ncbi.nlm.nih.gov/20463003-p53-based-cancer-therapy/. DOI: 10.1101/cshperspect.a001222
23. Afsar, T., Trembley, J.H., Salomon, C.E., Razak, S and Khan, M.R., 2016. Growth inhibition and apoptosis in cancer cells induced by polyphenolic compounds of *Acacia hydaspica*: Involvement of multiple signal transduction pathways. Sci. Rep. 6: 1-12. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4791679/ DOI: 10.1038/srep23077
24. Lehmann, B.D., Bauer, J.A., Chen, X., Sanders, M.E and Chakravanthy, A.B*et al.*,2011. Pietenpol JA. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J. Clin. Invest. 121: 2750-67. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3127435/ DOI: 10.1172/JCI45014
25. Khan, N., Afaq, F., Saleem, M., Ahmad, N and Mukhtar, H., 2006. Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. Cancer. Res. 66:2500-5. https://pubmed.ncbi.nlm.nih.gov/16510563-targeting-multiple-signaling-pathways-by-green-tea-polyphenol-epigallocatechin-3-gallate/ DOI: 10.1158/0008-5472.CAN-05-3636
26. Ghate, N.B., Hazra, B., Sarkar, R and Mandal N., 2014. Heartwood extract of *Acacia catechu* induces apoptosis in human breast carcinoma by altering bax/bcl-2 ratio. Pharmacogn. Mag.10:27-33 https://pubmed.ncbi.nlm.nih.gov/24695415-heartwood-extract-of-acacia-catechu-induces-apoptosis-in-human-breast-carcinoma-by-altering-baxbcl-2-ratio/. DOI: 10.4103/0973-1296.126654
27. Catz, S.D and Johnson, J.L., 2001. Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. Oncogene. 20: 7342-51.

https://pubmed.ncbi.nlm.nih.gov/11704864-transcriptional-regulation-of-bcl-2-by-nuclear-factor-kappa-b-and-its-significance-in-prostate-cancer/. DOI: 10.1038/sj.onc.1204926

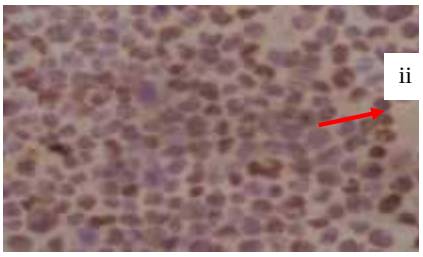
28. Bolhasasni, A., Khavari, A and Bathaie SZ., 2001. Saffron and natural carotenoids: biochemical activities and anti-tumor effects. Biochim. Biophys. Acta. 1845: 20-30. https://pubmed.ncbi.nlm.nih.gov/24269582-saffron-and-natural-carotenoids-biochemical-activities-and-anti-tumor-effects/. DOI: 10.1016/j.bbcan.2013.11.001
29. Duarte, T.L and Lunec, J., 2005. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free. Radic. Res. 39:671-86. https://pubmed.ncbi.nlm.nih.gov/16036346-review-when-is-an-antioxidant-not-an-antioxidant-a-review-of-novel-actions-and-reactions-of-vitamin-c/. DOI: 10.1080/10715760500104025
30. Verma, R.S., Mhta, A and Srivastava, N., 2007. In vivo chlorpyrifos induced oxidative stress: A enuation by antioxidant vitamins. Pestic. Biochem. Physiol. 88:191-6. https://www.sciencedirect.com/science/article/abs/pii/S0048357506001854. https://doi.org/10.1016/j.pestbp.2006.11.002
31. Szarka, A., Tomassovics, B and Bánheghyi, G., 2012. The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. Intern. J. Mol. Sci. 13:4458-83. https://pubmed.ncbi.nlm.nih.gov/22605990-the-ascorbate-glutathione-tocopherol-triad-in-abiotic-stress-response/. DOI: 10.3390/ijms13044458
32. Bindhumol, V., Chitra, K.C and Mathur, P.P., 2003. Bhisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicology. 188:117-24. https://pubmed.ncbi.nlm.nih.gov/12767684-bisphenol-a-induces-reactive-oxygen-species-generation-in-the-liver-of-male-rats/. DOI: 10.1016/s0300-483x(03)00056-8
33. Winkler, B.S., Orselli, S.M and Rex, T.S., 1994. The redox couple between glutathione and ascorbic acid: A chemical and physiological perspective. Free. Radic Biol. Med. 17: 333-49. https://pubmed.ncbi.nlm.nih.gov/8001837-the-redox-couple-between-glutathione-and-ascorbic-acid-a-chemical-and-physiological-perspective/. DOI: 10.1016/0891-5849(94)90019-1
34. Griffiths, H.R and Lunec, J., 2001. Ascorbic acid in the 21st century-more than a simple antioxidant. Environ. Toxicol. Pharm.10:173-82. https://pubmed.ncbi.nlm.nih.gov/21782574-ascorbic-acid-in-the-21st-century-more-than-a-simple-antioxidant/. DOI: 10.1016/s1382-6689(01)00081-3
35. Finlay, C.A., Hinds, PW and Levine, A.J., 1999. The p53 protooncogene can act as a suppressor of transformation. Cell. 57: 1083-93. https://pubmed.ncbi.nlm.nih.gov/2525423-the-p53-proto-oncogene-can-act-as-a-suppressor-of-transformation/. DOI: 10.1016/0092-8674(89)90045-7
36. Oraiopoulou, M.E., Tzamali, E., Tzedakis, G., Vakis, A., and Papamatheakis J, *et al*. 2017. In vitro/in silico study on the role of doubling time heterogeneity among primary glioblastoma cell lines. Biomed .Res. Int.1-12. https://www.hindawi.com/journals/bmri/2017/8569328/. https://doi.org/10.1155/2017/8569328
37. Atuegwu, N.C., Arlinghaus, L.R., Li, X., Chakravarthy, A.B and Abramson, V.G *et al*., 2013. Parameterizing the logistic model of tumor growth by DW-MRI and DCE-MRI data to predict treatment response and changes in breast cancer cellularity during neoadjuvant chemotherapy. Transl. Oncol. 6:256-64. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3660793/. DOI: 10.1593/tlo.13130

38. Bertuzzi, A., Gandol, A., Sinisgalli, C., Starace ,G and Ubezio, P., 1997. Cell loss and the concept of potential doubling time. Cytometry. 29:34-40. https://onlinelibrary.wiley.com/doi/abs/10.1002/%28SICI%291097-0320%2819970901%2929%3A1%3C34%3A%3AAID-CYTO3%3E3.0.CO%3B2-D. https://doi.org/10.1002/(SICI)1097-0320(19970901)29:1<34::AID-CYTO3>3.0.CO;2-D
39. Lowe, S.W., 1999. Activation of p53 by oncogenes. Endocr. Relat. Cancer. 6: 45-8. https://pubmed.ncbi.nlm.nih.gov/10732786-activation-of-p53-by-oncogenes/ DOI: 10.1677/erc.0.0060045
40. Rivlin, N., Ran, Brosh, R., Oren, M and Rotter, V., 2011. Mutations in the p53 tumor suppressor gene: Important milestones at the various steps of tumorigenesis. Genes. Cancer. 2: 466-74. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3135636/. DOI: 10.1177/1947601911408889
41. Sugermann, P.B and Savage, N.W., 1999. Current concepts in oral cancer. Aust.Dent. J. 44: 147-56. https://pubmed.ncbi.nlm.nih.gov/10592559-current-concepts-in-oral-cancer/. DOI: 10.1111/j.1834-7819.1999.tb00216.x
42. Petitjean, A., Mathe, E., Kato, S., Ishioka, C and Tavtigian, S.V*et al*., 2007. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum. Mutat. 28:622-9. https://pubmed.ncbi.nlm.nih.gov/17311302-impact-of-mutant-p53-functional-properties-on-tp53-mutation-patterns-and-tumor-phenotype-lessons-from-recent-developments-in-the-iarc-tp53-database/. DOI: 10.1002/humu.20495
43. Hainaut, P and Hollstein, M., 2000.P53 and human cancer: the first ten thousand mutations. Adv. Cancer. Res. 77:81-137. https://pubmed.ncbi.nlm.nih.gov/10549356-p53-and-human-cancer-the-first-ten-thousand-mutations/. DOI: 10.1016/s0065-230x(08)60785-x
44. Schlomm, T., Iwers, L., Kirstein, P., Jessen, B and Kollermann J*et al*., 2008. Clinical significance of p53 alterations in surgically treated prostate cancers. Mod. Pathol. 21:1371-8. https://pubmed.ncbi.nlm.nih.gov/18552821-clinical-significance-of-p53-alterations-in-surgically-treated-prostate-cancers. DOI: 10.1038/modpathol.2008.104
45. Macdonald, F and Ford, C.H.J., 1997.Molecular biology of cancer, Bios. Oxford: Scientific Publishers,pp: 53-60. https://archive.org/details/molecularbiology00fmac
46. Nozaki, M., Tada, M., Kobayashi, H., Zhang, C.L and Sawamura, Y *et al.*,1999. Roles of the functional loss of p53 and other genes in astrocytoma tumorigenesis and progression. Neuro. Oncol. 1:124-37. https://www.ncbi.nlm.nih.gov/pubmed/11550308. DOI: 10.1093/neuonc/1.2.124.
47. Oren, M and Rotter, V., 2010. Mutant p53 gain-of-function in cancer. Cold Spring Harb. Perspect. Biol. 2:a001107. https://www.ncbi.nlm.nih.gov/pubmed/20182618. DOI: 10.1101/cshperspect.a001107.
48. Groeger, A.M., Esposito, V., De Luca, A., Cassandro, R and Tonini, G *et al.*,2004. Prognostic value of immunohistochemical expression of p53, bax, Bcl-2 and Bcl-xL in resected non-small-cell lung cancers. Histopathology. 44:54-63. https://www.ncbi.nlm.nih.gov/pubmed/14717670. DOI: 10.1111/j.1365-2559.2004.01750.x
49. Shaulsky, G., Goldfinger, N., Tosky, M.S., Levine, A.J and Rotter, V., 1991. Nuclear localization is essential for the activity of p53 protein. Oncogene. 6: 2055-65. https://pubmed.ncbi.nlm.nih.gov/1719467-nuclear-localization-is-essential-for-the-activity-of-p53-protein/

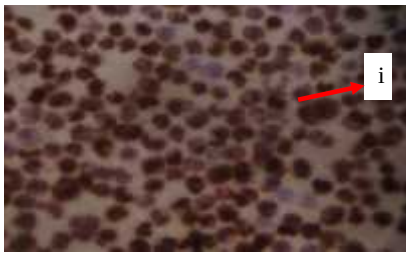
50. Burck, K.B., Liu, E. and Larick, J.W., 1988.Oncogenes: An introduction to the concept of cancer genes, New York: Springer-Verlag, pp: 87-99. ISBN 9781461237181 (online) 9780387964232 (print). DOI: 10.1007/978-1-4612-3718-1
51. McManus, E.J and Alessi, D.R., 2004. Cancer, oncogenes and signal transduction. Genome. Biol.5:332. https://genomebiology.biomedcentral.com/articles/10.1186/gb-2004-5-7-332



A. Raji control cell (without treatment)



B. Raji cells with linamarin 32.5µg/ml



C. Raji cells with linamarin 62.5µg/ml

Fig.1. Microscopic photo of Raji cells with treatment of linamarin 32.5 and 62.5 µg/ml and control (without treatment) with immunocytochemical staining (magnification 400x). Information: (i) positive cells with expression of p53 protein have brown nuclei or cytoplasm; (ii) cells that are negative for p53 protein expression have purple nuclei or cytoplasm.

Commented [WU4]: Author is advised to label the figure 1 correctly by which a reader can easily understand the results obtained in figure 1.

Table: 1. Average number of living cells vs. percentage of Raji cell inhibition after administration of various concentrations of linamarin

No	Linamarin concentration µg/ml	Absorbance				Average	% of Raji cell inhibition± SEM
		I	II	III	IV		
1	31.25	0.643	0.565	0.678	0.462	0.587	27.194 ± 0.096*
2	62.5	0.381	0.565	0.453	0.323	0.431	46.605 ± 0.104*
3	125	0.076	0.035	0.553	0.539	0.301	62.698 ± 0.284*
4	250	0.043	0.033	0.457	0.414	0.237	70.636 ± 0.230*
5	500	0.045	0.121	0.302	0.189	0.164	79.628 ± 0.109*
6	1000	0.021	0.026	0.019	0.013	0.020	97.550 ± 0.005*
7	Cell control	0.794	0.761	0.865	0.805	0.806	0.000 ± 0.043
8	Media control	0.043	0.033	0.031	0.033	0.035	0.000 ± 0.005

* $p < 0.05$ with one-way ANOVA test; SEM: Standard error of the mean

Table: 2. Doubling time of Raji cells after treatment with various concentrations of linamarin vs. control

Treatment	The hours of Raji cell lives				The equation between incubation time vs. number of living cells	Doubling time (hours)
	0	24	48	72		
Control	20.000	48.444	84.813	131.879	Y = 0.0113x + 4.345	22.749
Linamarin 62.50 µg/ml	20.000	31.482	47.458	63.491	Y = 0.0007x + 4.317	40.723
Linamarin 31.25 µg/ml	20.000	42.631	66.391	89.366	Y = 0.0089x + 4.354	27.804
Linamarin 15.63 µg/ml	20.000	51.046	69.236	89.500	Y = 0.0087x + 4.387	24.65

Table: 3. Percentage of p53 protein expression on Raji cell control and linamarin concentrations of 62.5 µg/ml and 31.25 µg/ml

Replication	Expression of p53 protein on Raji cells					
	Control		Linamarin 62.5µg/ml		Linamarin 31.25 µg/ml	
	Positive	Negative	Positive	Negative	Positive	Negative
I	12	88	86	14	40	60
II	8	92	73	27	45	55
III	14	86	73	27	39	61
IV	7	93	78	22	36	64
Total	41	359	310	90	160	240
Percentage (%) ± SEM	10.25 ± 1.65	89.75 ± 1.65	77.5 ± 3.07	22.5 ± 3.07	40 ± 1.87	60 ± 1.87

SEM: Standard error of the mean

Table: 4. Location of p53 protein expression of Raji cells control and linamarin concentrations of 62.5 µg/ml and 31.25 µg/ml

Treatment	Position of p53 protein expression of Raji cells									
	Nucleus					Cytoplasm				
	I	II	III	IV	Mean (%) ± SEM	I	II	III	IV	Mean (%) ± SEM
Control	9	7	8	9	80.49 ± 0.48	2	2	2	2	19.51 ± 0.00
Linamarin 62.5 µg/ml	76	65	67	70	89.68 ± 2.40	7	7	8	10	10.32 ± 0.71
Linamarin 32.25 µg/ml	33	26	30	33	76.25 ± 1.66	7	9	12	10	23.75 ± 1.04

SEM: Standard error of the mean

Gmail

adekutari pratiwi <adekutari25@gmail.com>

Fwd: Transaction confirmation

1 pesan

dwi sutiningsih <dwisuti98@gmail.com> 21 September 2020 09.29
Kepada: adekutari25@gmail.com

---------- Forwarded message ---------
Dari: **Science Alert** <no-reply@telr.com>
Date: Kam, 6 Feb 2020 21:26
Subject: Transaction confirmation
To: <dwisuti98@gmail.com>

Transaction confirmation

Science Alert

Transaction reference:	030024866355
Transaction type:	Sale
Amount:	$325.00
Description:	Publication / Processing Charges
Time:	6:26 PM on Thursday the 6th of February, 2020
Authorisation Code:	003470
Card:	Visa Credit ending 4319

Please retain this receipt for your records.

For more information, please visit http://sciencealert.ae/ or contact sarwarm@sciencealert.ae

Gmail **adekutari pratiwi <adekutari25@gmail.com>**

Fwd: 101171-IJCR-AJ - Request for Payment

1 pesan

dwi sutiningsih <dwisuti98@gmail.com> 21 September 2020 09.28
Kepada: adekutari25@gmail.com

---------- Forwarded message ---------
Dari: **Science Alert** <support@scialert.com>
Date: Kam, 6 Feb 2020 22:33
Subject: 101171-IJCR-AJ - Request for Payment
To: Dwi Sutiningsih <dwisuti98@gmail.com>

Dear Ms. Dwi Sutiningsih

This is with regard to your submitted manuscript, 101171-IJCR-AJ, titled Cytotoxic and Antiproliferative Activity and Induction of p53 Protein on Raji Cells after Treatment with Linamarin from Cassava Leaves (Manihot esculenta Cranz), submitted to International Journal of Cancer Research on 13 January, 2020 for consideration as a Research Article.

The above mentioned manuscript has been finally accepted by the Reviewer for publication in International Journal of Cancer Research as Research Article. You may download the final acceptance letter after log in to your account with User ID dwisuti98@gmail.com.

Before we can continue with final production, it is mandatory to pay Article Processing Charges. All articles published in our journals are open access and freely available online, immediately upon publication. This is made possible by an article-processing charge (APC) that covers the range of publishing services we provide. This includes provision of online tools for editors and authors, article production and hosting, liaison with abstracting and indexing services, and customer services. The APC, payable when your manuscript is editorially accepted and before publication, is charged to either you, or your funder, institution or employer.

You may download the invoice after log in to your account with User ID: dwisuti98@gmail.com.

If you have forgot your password, you may retrieve your password from the following link by providing your User ID dwisuti98@gmail.com.

http://scialert.com/forgot_password.php

Therefore, It is requested to please pay the Article Processing Charges urgently so that we may send your article to the production department for final publication.

We look forward to hearing from you.

Regard
Academic Editor
International Journal of Cancer Research

Gmail **adekutari pratiwi <adekutari25@gmail.com>**

Fwd: Status has been changed for your article No. 101171-IJCR-AJ

1 pesan

dwi sutiningsih <dwisuti98@gmail.com> 21 September 2020 09.27
Kepada: adekutari25@gmail.com

---------- Forwarded message ---------
Dari: **Science Alert** <no-reply@scialert.com>
Date: Sel, 14 Apr 2020 17:50
Subject: Status has been changed for your article No. 101171-IJCR-AJ
To: Dwi Sutiningsih <dwisuti98@gmail.com>

Dear Dwi Sutiningsih,

Status of your above mentioned manuscript has been changed. Current status of your manuscript is as under:

Published: Manuscript has been published in International Journal of Cancer Research

For further information, please logon the system at http://www.scialert.com/login.php with your user id and password.

Best Regards
Science Alert Support Team